The SR Protein 9G8 and the Wilms' Tumor Suppressor Protein WT1 Promote Translation of mRNAs with Retained Introns

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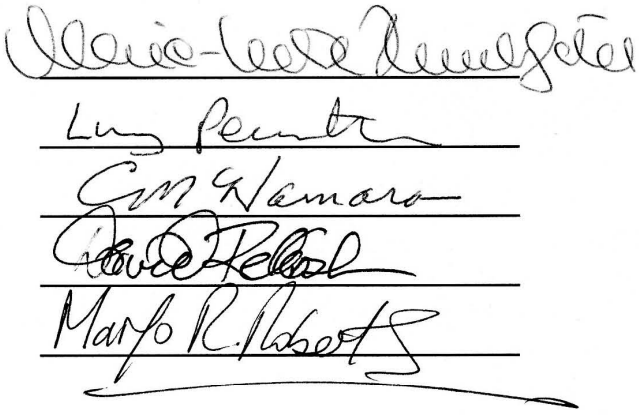

Abstract

Recent data indicate that the various steps in post-transcriptional gene expression are linked. In the studies presented in this thesis, we show further evidence of the interconnection between the multiple steps of mRNA metabolism. In one study, we show a link between splicing with nucleocytoplasmic export and translation. In another study, we show that the products of an alternatively spliced pre-mRNA have different roles in mRNA metabolism. Specifically, SR proteins, Tap, and WT1(+KTS) function similarly to link nuclear and cytoplasmic events in the expression of mRNAs with retained introns.

The first study examines the cytoplasmic role of the splicing regulatory SR protein, 9G8, which has recently been proposed to function in mRNA export in conjunction with the export protein, Tap/NXF1. We investigated the effect of 9G8 on cytoplasmic RNA fate. 9G8 was shown to enhance expression of unspliced RNA containing either the MPMV-CTE or the recently discovered Tap-CTE. 9G8 also enhanced polyribosome association of unspliced RNA containing a CTE. Hyperphosphorylated 9G8 was present in monosomes and small polyribosomes, whereas soluble fractions contained only hypophosphorylated protein. Our results are consistent with a model in which hypophosphorylated SR proteins remain stably associated with mRNP complexes during export and are released during translation initiation concomitant with increased phosphorylation.

The second study examines the Wilms' tumor 1 (WT1) gene, which plays an important role in mammalian urogenital development. Dysregulation of this gene is observed in many human cancers. Alternative splicing of WT1 RNA leads to the expression of two major protein isoforms, WT1(+KTS) and WT1(-KTS). Whereas WT1(-KTS) acts as a transcriptional regulator, no clear function has been ascribed to WT1(+KTS), despite the fact that this protein is crucial for normal development. Here we show that WT1(+KTS) functions to enhance expression from RNA possessing a retained intron and containing either a cellular or viral constitutive transport element (CTE). WT1(+KTS) expression increases the levels of unspliced RNA containing a CTE and specifically promotes the association of this RNA with polyribosomes. These studies provide further support for links between different steps in RNA metabolism and for the existence of post-transcriptional operons.

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CHAPTER 1

Introduction

The central dogma of genetic expression is that the information encoded on DNA is copied as individual messages of RNA through a process called transcription. The messenger RNA (mRNA) then takes that information to the protein-building machinery, which translates the message to synthesize the proteins required by the cell for structural and enzymatic functions. The mRNA undergoes multiple steps between its creation at transcription to its expression as a protein, and most of these steps have been shown to be interconnected (92).

In eukaryotic cells, DNA is transcribed into mRNA in the nucleus of the cell. This is carried out by multi-subunit complexes known as RNA polymerases, of which there are three types, RNA Polymerase (Pol) I, II, and III (117). Each polymerase synthesizes a different class of RNA. Pol I synthesizes the 45S pre-ribosomal RNA (rRNA) that eventually forms the major RNA sections of the ribosome upon maturation. Pol II synthesizes the pre-mRNAs and some small nuclear RNAs, including microRNAs and snRNAs. Pol III synthesizes tRNAs, the 5S rRNA, and some additional small RNAs found in both the nucleus and cytosol.

Metabolism of mRNA: Overview. The process of mRNA transcription consists of three stages: initiation, elongation, and termination (117). The promoter area is the site where transcription of a gene begins. Transcription factors bind to DNA sequence elements within the promoter, such as the TATA box, allowing RNA Pol II to assemble and initiate transcription. Upon initiation, the C-terminal domain (CTD) of Pol II becomes phosphorylated, which activates the elongation activity of the enzyme. Once the entire gene is transcribed, Pol II terminates the process, releasing the nascent mRNA and the DNA template. This pre-mRNA then undergoes several processing steps during its lifetime, which include: 5’ end capping, editing, splicing, 3’ end poly-adenylation, and ultimately, degradation. Each of these processing steps has functional relationships with other processing steps and been shown to be co-transcriptional, as well (145).

5' end capping. The 5' cap is an inverted methylated guanosine monophosphate, which protects the pre-mRNA from the actions of 5'-to-3' exonucleases (182, 186). 5' capping occurs co-transcriptionally as a result of the direct binding of the capping enzymes to the phosphorylated CTD of RNA Pol II. When the transcript reaches a length of 20-40 nt, the capping enzymes remove the 5' triphosphate and replace it with a guanosine monophosphate that is then methylated. The capping enzymes bind exclusively to the Pol II CTD, and therefore, Pol II transcripts are the only ones that are capped at their 5' ends. Beyond protecting the 5' ends of pre-mRNAs, the 5' cap has several other functions. In the nucleus, it binds the Cap Binding Complex (CBC) (122), which is comprised of Cap Binding Protein (CBP) 80 and CBP20. Binding of this complex occurs co-transcriptionally (200). The CBC has a role in the subsequent splicing of the first exon (35, 121, 123), promotes nucleocytoplasmic export of U snRNPs (65), and is involved in a "pioneer round" of translation in the cytoplasm (120). After the pioneer round, the CBC is replaced with eIF4E, which also binds directly to the cap (122), and which acts as the initiation factor for rapid, steady state translation (54, 86).

RNA editing. RNA editing is an RNA processing event that is less common than the other forms of RNA processing, but it has been observed in the RNAs coding for such important proteins as the glutamate receptor, the serotonin receptor, 5-HT2c, and the potassium channel Kv1.1 editing (145). There are several types of RNA editing, but in the context of co-transcriptional RNA processing, adenosine deamination is the type that is most well studied. The modification of adenine (A) to inosine (I) is catalyzed by enzymes called ADARs (adenosine deaminases) (6). Since inosine is recognized as a guanosine (G) by the splicing and translation machineries, adenine deamination is functionally equivalent to an A to G mutation, and this type of editing can both form new splice sites and change codons (6, 99, 130). Until recently, it was believed that RNA editing was the one form of RNA processing that did not occur co-transcriptionally. However, one of the ADARs, ADAR2, is auto-edited on intron 4 (170), and the CTD of Pol II was found to be necessary for efficient co-transcriptional ADAR2 auto-editing (114).

Splicing. The pre-mRNA that is a product of transcription includes introns that must be spliced before it can be used as a template for translation. In fact, in the case of most intron-containing mammalian

genes, all introns are removed by splicing before the mRNA can exit the nucleus (38, 45, 128). An exception to this is histone mRNAs, which are intronless. In splicing, introns are removed, and exons are ligated together. This is performed by a 60S ribonucleoprotein (RNP) particle, the spliceosome, which assembles at splice junctions. SR proteins, named for their RS domains rich in arginine and serine residues, bind to exonic splicing enhancers (ESEs) near the splice sites (15, 19). These SR proteins then recruit members of the spliceosome, specifically, the U1 small nuclear ribonucleoprotein (snRNP) to the 5' splice site and U2AF to the 3' splice site. U2 snRNP then enters to trigger a 2-step transesterification reaction of splicing (212). The promotion of splicing is antagonized by the presence of heterogeneous ribonucleoprotein particles (hnRNPs), which bind to exon splicing silencer (ESS) sequences and block the recruitment of splicing factors. SR proteins are involved in alternative splicing when splice sites are weak, and it is generally accepted that the choice of splice sites is determined by the amounts of individual SR proteins and hnRNPs available and their affinities for the various ESEs and ESSs near the splice sites. Splicing has been shown to occur both co-transcriptionally and post-transcriptionally, as observed in the Balbiani ring genes of *Chironomus tentans*, where nascent mRNAs are found lacking 5' introns, but still retaining the terminal introns (7, 205). Furthermore, the CTD of Pol II stimulates splicing both in human cells (50) and *in vitro* (78, 213).

3' end poly-adenylation. Except for replication-dependent histone genes, all eukaryotic RNAs that encode for protein are modified at their 3' ends by the addition of about 200 adenosine residues known as the poly(A) tail editing (159). Like the 5' cap, the poly(A) tail is important for stability and nucleocytoplasmic transport of the mature mRNA, as well as for translation (216). Before the tail can be added, the pre-mRNA must first be cleaved. The site of cleavage is usually a CA dinucleotide in between two *cis*-acting elements: the AAUAAA consensus poly(A) signal and the Down Stream Element (DSE) (159). Multi-subunit factors interact with the elements and perform the cleavage. The Cleavage and Polyadenylation Specificity Factor (CPSF) interacts with the AAUAAA element and interacts cooperatively with the Cleavage Stimulatory Factor (CstF) to bind the DSE (216). Cleavage Factor I (CFI) and CFII with the help of the Poly(A) Polymerase (PAP) cleaves the mRNA (202). Together, PAP and

CPSF then synthesize the poly(A) tail, and as the tail grows, it recruits the Poly(A) Binding Protein (PABP), which enhances processivity of the polymerase (202). Polyadenylation is intimately linked with the other steps of RNA processing. Transcription termination by RNA Pol II is dependent on the poly(A) signal and cleavage (12). The CBC enhances cleavage significantly by stabilizing the poly(A) complex with the mRNA (49). Alternative splicing can lead to the use of alternative poly(A) signals, which then yield different gene products (216). Conversely, polyadenylation can also affect splicing, specifically of the terminal intron. This is achieved through the interaction of PAP with $U2AF^{65}$ on the terminal exon (197).

Non-processing events: RNA folding. Before mRNA undergoes its ultimate processing step, degradation, it is involved with several non-processing interactions for various purposes, and most of these can be connected with each other or to the processing steps. These non-processing events include RNA folding, export and other sub-cellular localization, and translation. Single stranded mRNA has a relatively high free energy state, therefore it undergoes base pairing by folding back on itself to achieve its minimal energy state. Folding creates tertiary structures that recruit factors to the RNA beyond nucleotide sequence recognition alone. RNA folding is highly promiscuous, and proper folding is influenced by RNA chaperones, such as hnRNPs (Kim and Dreyfus, 2001), which are deposited onto the pre-mRNA during transcription, help to induce processing, and many remain bound after splicing.

Nucleocytoplasmic export. Since all RNA molecules are synthesized in the nucleus, and protein synthesis occurs in the cytoplasm, mRNA, tRNA, and rRNA molecules must undergo nucleocytoplasmic export before they can be used in translation (38, 154). Traffic between nucleus and cytoplasm occurs at nuclear pore complexes (NPCs). The size of an NPC limits passive diffusion through it to about 90 Å. Since most macromolecules, such as protein-laden RNA particles, are larger than 90 Å, passage of these molecules though the pores must be achieved actively. One well-studied mechanism providing the energy for this process is found by the switching of the small G protein, Ran, between its GTP-and GDP-bound states. GTPase-activating protein (GAP) hydrolyzes the bound GTP to GDP, while guanine nucleotide exchange factor (GEF) exchanges the bound GDP for a fresh GTP. Since GEF is predominantly nuclear-

localized, and GAP is cytoplasmic, nuclear-Ran is mainly found in its GTP-bound state, whereas Ran in the cytoplasm is bound by GDP, and this asymmetry provides directionality to nucleocytoplasmic transport. Ran binds to a class of molecules called transportins or karyopherins, which are receptors for "cargo" molecules that are the target of nucleocytoplasmic transport. Cargo molecules can be either protein or RNA. Transportins are subclassified into importins or exportins, depending on the direction through which they carry their cargo across the NPCs. Ran bound by GTP supports nucleocytoplasmic trafficking by either promoting the interaction between an exportin and its cargo or by stimulating the release of a cargo molecule from an importin. Likewise, Ran-GDP has the opposite effect, releasing exportins from their cargo while enhancing importin-cargo binding.

Most types of RNA export are facilitated by RanGTP and karyopherin-type receptors (38, 154). Importins and exportins recognize and bind to their cargo through nuclear localization signals (NLSs) and nuclear export signals (NESs), respectively. RNA-binding proteins harboring specific NESs mark RNAs for export and recruit exportins and Ran-GTP. The specific exportins and RNA-binding proteins vary for the different classes of RNAs. Crm1 is the best understood of the exportins. Crm1 was initially identified as a cofactor for the HIV-1 Rev protein, which is required for the export of unspliced and incompletely spliced HIV-1 mRNA (131). Crm1 function can be specifically inhibited by leptomycin B (LMB), and inhibition of export by LMB defines a class of molecules as Crm1-dependent. Several classes of RNAs (U snRNAs, both large and 5S rRNAs, HIV-1 mRNA, and a small subset of cellular mRNAs) and most shuttling proteins use Crm1 as their export receptor (52). For RNA export, Crm1 interacts with RNA-binding proteins that harbor leucine-rich type NESs (48, 52). Besides HIV-1 Rev, these include: PHAX (phosphorylated adaptor for RNA export) which binds to the nuclear cap binding complex proteins, CBP80 and CBP20, of U snRNAs (151); and Nmd3p, which is recruited to the 60S ribosomal subunit RNAs through Rp110p (79, 80). Another karyopherin-type receptor used for RNA export is exportin-t (Exp-t), which unlike Crm1, binds directly to tRNAs without the use of an adaptor protein (3, 106). Small non-coding RNAs, such as Y and VA RNAs and micro RNAs, seem to be exported in a RanGTP-dependent manner, as well, but export of these molecules is not dependent on Crm1 or Exp-t (69, 171).

Although Crm1 was discovered as the export receptor for incompletely spliced HIV-1 mRNA and exports some cellular mRNAs, the bulk of cellular mRNA export appears to be independent of karyopherins and Ran-GTP and remains poorly understood (52). The export of mRNA is highly selective in that only fully processed RNAs are exported. One mechanism to regulate mRNA export is that immature mRNAs may be bound by factors, such as those involved in splicing, which hold the RNA in the nucleus. For example, hnRNP C is an RNA-binding protein with a nuclear retention signal that is able to override the NESs of other mRNA-bound proteins (142). Bound hnRNP C prevents pre-mRNA from exiting the nucleus until its removal. Also, improperly processed mRNAs are removed from the nucleocytoplasmic pathway and are marked for degradation (208).

Retroviruses and export of intron-containing RNA. In spite of these restrictions, retroviruses are obligated to export unspliced viral RNAs, both as templates for synthesis of structural proteins and as RNA genomes to be packaged into progeny virions. As already mentioned, HIV-1 bypasses the intron-containing mRNA retention block through expression of the HIV-1-encoded Rev protein (71, 74, 131, 156). Rev is produced from a completely spliced transcript early in the course of HIV replication, and upon synthesis in the cytoplasm, it shuttles to the nucleus to bind to an RNA element (the Rev Responsive Element or RRE) found in the unspliced and partially spliced HIV transcripts. Rev then recruits Crm1 and RanGTP to the viral mRNAs, and export is achieved (52, 146). Unlike HIV, simple retroviruses do not encode a Rev-like *trans*-acting protein to mediate export, yet these viruses must also export intron-containing mRNAs. In the case of simian, type D retroviruses, such as Mason-Pfizer Monkey Virus (MPMV), a *cis*-acting element, known as the constitutive transport element (CTE), fulfills the function of the RRE (22, 47, 72, 73). Although the MPMV-CTE has a similar function to HIV-1 RRE/Rev in that they both promote the export of intron-containing mRNA, they are not completely equivalent. Through *Xenopus* oocyte injections and transfection experiments, it was shown that the CTE pathway is distinct from that used by RRE-Rev (which uses Crm1) and actually shares a pathway used by the cellular mRNAs (52). Furthermore, MPMV does not express a Rev-like protein to interact with the CTE. Therefore, the CTE must recruit cellular factors to promote the export function.

The export receptor, Tap. Using functional and impaired CTEs, a cellular factor was pulled out of HeLa nuclear extracts that bound directly and specifically to functional CTEs and promoted the export of CTE-containing mRNA in microinjected oocyte studies (67). This cellular factor was found to be a protein originally identified as interacting with the *Herpes saimiri* protein, Tip, therefore named Tip-associated protein (Tap) (210). It is thought that Tap is not only the nuclear export factor for CTE-containing mRNA, but that it has a role in the export of most cellular mRNA. This is based on a few studies. First, spliced Ad mRNA and DHFR mRNA can be blocked for export in microinjected *Xenopus* oocytes by saturating amounts of CTE RNA (153, 172). This block can be overcome by the addition of Tap (67). Second, the yeast protein, Mex67p, shows significant homology to the Tap protein. Mex67p is essential in yeast, and temperature-sensitive mutants accumulate mRNA in the nucleus rapidly upon incubation at the non-permissive temperature (180). Mex67p was shown to bind to both poly(A) mRNA and components of the nuclear pore complex, and the NPC interaction was stabilized through association with the Mtr2p protein (178). Subsequently, the Mex67p/Mtr2p heterodimeric complex is considered to be the key nuclear export factor of mRNA in yeast. Since Tap is the mammalian homologue of Mex67p, and Mex67p heterodimerizes with Mtr2p, an attempt was made to find a functional homologue of Mtr2p in mammalian cells. Through co-immunoprecipitation studies, a novel 15 kDa protein (p15) homologous to NTF2, a RanGDP-binding nuclear import factor, was identified (98). Although neither Tap nor p15 could complement Mex67p or Mtr2p null mutants respectively, co-expression of both Tap and p15 fully restored growth of either mutant and partially complemented the double mutant, establishing a functional relationship between Tap and p15. Tap was subsequently given the additional name of nuclear RNA export factor 1 (NXF1) for its role in mRNA export and is the founding member of the NXF family. p15 was independently discovered to be a RanGTP-binding protein through a database search for NTF2 homologues and given the alternate name NXT1 (13). “Tap” and “NXT1” will be used for the remainder of this dissertation.

Several other studies support Tap/NXT1 as being the mammalian equivalent of Mex67p/Mtr2p for mRNA export. Our own lab showed that NXT1 is an important cofactor in Tap-mediated export of intron-

containing RNA (68). In this study, fusion proteins of Tap and mutant forms of HIV-1 Rev (for which the RNA-binding function remains intact but are unable to export RRE-containing mRNAs) were able to complement Rev function, and addition of NXT1 significantly enhanced the ability of the fusion proteins to export the reporter mRNA to the cytoplasm. In other studies, Tap/NXT1 was shown to have the ability to bypass nuclear retention signals to stimulate mRNA export in oocyte injection experiments and to promote expression of inefficiently spliced mRNAs in CAT assays (20). Using siRNA to knock down Tap and/or NXT1, it was shown that these proteins are essential for both *Drosophila* (77) and *Caenorhabditis elegans* (195) nuclear export by observing nuclear retention of mRNA in the cells depleted of Tap or NXT1. Furthermore, NXT1 helps Tap effectively interact with components of the NPC (51, 206).

The Tap adaptor, REF/Aly. Although Tap is an RNA-binding protein (22, 47, 72, 125), it is widely accepted that Tap is recruited to cellular mRNAs through protein-protein interactions (164, 193). This is based on several observations, including: Tap binds to a specific sequence in the CTE, and most cellular mRNAs do not contain similar sequences; also, Tap has a relatively low affinity for cellular mRNA. For example, DHFR mRNA has three orders of magnitude less affinity for Tap than does the CTE (21). Also, even though CTE-containing mRNA and cellular mRNA seem to use the same export pathway via Tap/NXT1, the kinetics of the two groups seems to be different. This was determined based on several studies using saturating amount of various mRNAs or proteins to block export of specific mRNAs in oocyte injection experiments (90, 91, 153, 172). It was therefore postulated that the difference of kinetics between the two types of mRNAs could be attributed to the direct interaction between the CTE and Tap, bypassing several steps in the nuclear export pathway and overriding any nuclear retention mechanisms, such as the presence of introns (67). This implies there exists adaptor proteins that recruit Tap to properly processed cellular mRNA, and an effort was put forth to determine the identity of any such proteins.

Using tandem affinity purification, the yeast protein, Yra1p, which is an essential hnRNP-like protein, was pulled out of yeast extracts as a Mex67p-binding partner (192). This study demonstrated several lines of evidence that Yra1p is an adaptor protein for Mex67p/Tap. First, not only did Yra1p bind RNA and Mex67p, it was also found to bind directly to Tap (*i.e.*, in the presence of RNase). Expressing

Yra1p under the GAL1 promoter in yeast in the absence of galactose led to the accumulation of poly(A)$^+$ RNAs within the nucleus. In this same study, it was found that the murine protein, Aly, is highly similar to Yra1p, and along with several other similar and previously described proteins from multiple species, these were categorized as belonging to the REF-bp (RNA and export factor binding proteins) family. Aly, therefore, was also given the alternative name, REF1. Other studies showed that REF1/Aly enhances mRNA export from injected *Xenopus* nuclei (167, 217).

Exon junction complexes. REFs are also known to be a part of the exon junction complex (EJC), which is a group of proteins found only on spliced mRNAs. The EJCs are deposited on the mRNA in metazoans during the splicing process and are located at conserved positions 20-24 nucleotides upstream from each exon-exon junction (116). The core components of EJCs are Y14, Mogoh, REF1/Aly (165), MCN51, and eIF4AIII (196). Other proteins that associate with the core proteins are UAP56, Tap, SRm160, RNPS1, Upf3/3X, and Upf2 (60, 103, 115, 129). Upf2 is considered a component of the EJC even though it associates with the other proteins after nuclear export.

For many years, EJCs were thought to be important in mRNA export. This was based on a couple of observations. Firstly, mRNAs that had undergone splicing were expressed as protein more efficiently than were intronless mRNAs (126, 134, 149, 207). Also, more cytoplasmic mRNA was observed for the spliced mRNAs as compared to those that had not been spliced (115, 128, 217). Therefore, the studies indicating that Ref1/Aly recruits Tap to mRNAs for export were consistent with this model. However, it was later shown that splicing was not important for the export of most mRNAs (59, 126, 149). Instead, the EJCs have two other important and possibly interrelated post-export functions: enhancement of translation and mRNA degradation.

Cytoplasmic mRNAs that have undergone splicing have been shown to produce 2-3 times the protein per molecule than do similar mRNAs that do not have introns (149, 207). In one of these studies, intronless mRNAs could be made to translate as efficiently as spliced RNAs by tethering EJC core or Upf proteins to the open reading frames (ORFs) of the RNA (148). Interestingly, when the EJC components were tethered to the 3'-untranslated regions rather than the ORFs, instead of promoting mRNA translation,

the RNAs were degraded. This process is known as nonsense-mediated decay (NMD) reviewed in (2, 132). NMD works on mRNAs that contain premature termination codons (PTCs) derived from events such as intron retention, RNA editing, and point or frame-shift mutation. If these mRNAs were allowed to be expressed, they would give rise to truncated proteins that could have deleterious results on the cell. In fact, about thirty percent of inherited diseases are caused by the presence of nonsense codons, and the importance of NMD surveillance is demonstrated by the fact that removal of mutant allele-derived transcripts through NMD generally leads to a recessive mode of inheritance of these diseases (56, 81).

Translation: Pioneer round. Regardless of its ultimate fate, all EJC-containing mRNAs begin with a "pioneer" round of translation. In the case of those that undergo NMD, it is during pioneer translation that PTCs are detected (86). The substrate for pioneer round translation is the pioneer initiation complex: a newly exported mRNA bound at the 5' end by the CBC (CBP80/20 heterodimer), PABP2 at the 3' end, and EJCs at each exon-exon junction (34, 86). Although the process is not well understood, it is currently thought that pioneer translation is probably similar to steady-state translation, with some notable differences (34).

Initiation. Briefly, mRNA translation, like transcription, can be subdivided into three stages: initiation, elongation, and termination reviewed in (140, 158). Initiation can be cap-dependent or independent. Since NMD uses cap-dependent translation, only this type will be discussed. Translation initiation begins through the formation of the eukaryotic initiation factor 4F (eIF4F) complex, which binds to the 5' end of the mRNA: the cap-binding protein recruits eIF4A (an ATP-dependent RNA helicase), which in turn recruits eIF4G, a scaffolding protein. In the case of steady-state translation, the cap-binding protein is eIF4E, whereas in pioneer translation, the cap-binding protein is the CBC heterodimer. Also in the pioneer round, there is evidence that eIF4A may be substituted by eIF4AIII (34), a component of the EJC core with high similarity to eIF4A, but is mainly nuclear, whereas eIF4A is cytoplasmic. Separately, the 40S ribosomal subunit associates with eIF1, eIF1A, eIF2, and eIF3 to form the 43S pre-initiation complex (158, 188). eIF2 connects the 40S subunit to the initiator transfer RNA (tRNAi) carrying the

initiating amino acid, methionine (Met) in a GTP-bound state (eIF2-GTP/Met-tRNAi). The eIF4F complex promotes unwinding of the mRNA secondary structure so that the 43S pre-initiation complex can bind: eIF3 bridges eIF4G to the 40S ribosomal subunit, and eIF1 and eIF1A bind to the RNA. The poly(A) tail also plays a role in translation initiation in that eIF4G binds the poly(A)-binding protein (PABP), causing in a circularization of the mRNA (158). The resulting 48S initiation complex (eIF4F + 43S pre-initiation complex) translocates along the 5' untranslated region (UTR) to the start codon (AUG), to which the anticodon (CAU) of Met-tRNAi base pairs, stalling the ribosome. eIF5 promotes the hydrolysis of the eIF2-bound GTP, releasing both eIF2 and eIF3. The 60S ribosomal subunit is then recruited and joined to the 40S subunit by the action of eIF5B to form the complete 80S ribosome, with Met-tRNAi residing in the P-site of the ribosome, thus ending the initiation stage of RNA translation.

Elongation. Elongation begins with the arrival of the second aminoacyl-tRNA, with the aid of the elongation factor (EF), Tu, through base pairing with the next codon in the A-site of the ribosome (188). In a process that uses the hydrolysis of GTP, a peptide bond is formed between the amino acids in the P- and A-sites, releasing the initiating tRNA from Met and the P-site. The tRNA (now a peptidyl-tRNA) in the A-site is then translocated by EF-G to the P-site, and the process is repeated forming a growing polypeptide chain.

Termination. Elongation continues until a STOP codon (UGA, UAG, or UAA) occupies the ribosomal A site (188). Since no tRNA base pairs with termination codons, the ribosome stalls, leaving the A-site empty. Eukaryotic release factors (eRFs) are associated with the translating ribosome (97). eRF1 recognizes the STOP codons and facilitates the release of the polypeptide from the P-site. eRF3 hydrolyzes GTP and improves eRF1 function. With the release of the polypeptide, the ribosomal subunits become dissociated from the mRNA and each other and are recycled for a new round of translation initiation (173).

Nonsense-mediated decay. The ability of eRFs to recognize termination codons also plays a role in NMD. eRFs associate with translating ribosomes as part of the SURF complex, which is made up of the proteins Smg1, Upf1, and eRF1/3 (97). Upf1 (for up frameshift) is a RNA helicase that, when activated by two other Upf proteins, promotes degradation of mRNA by NMD reviewed in (9). Upf3 (or Upf3X) is

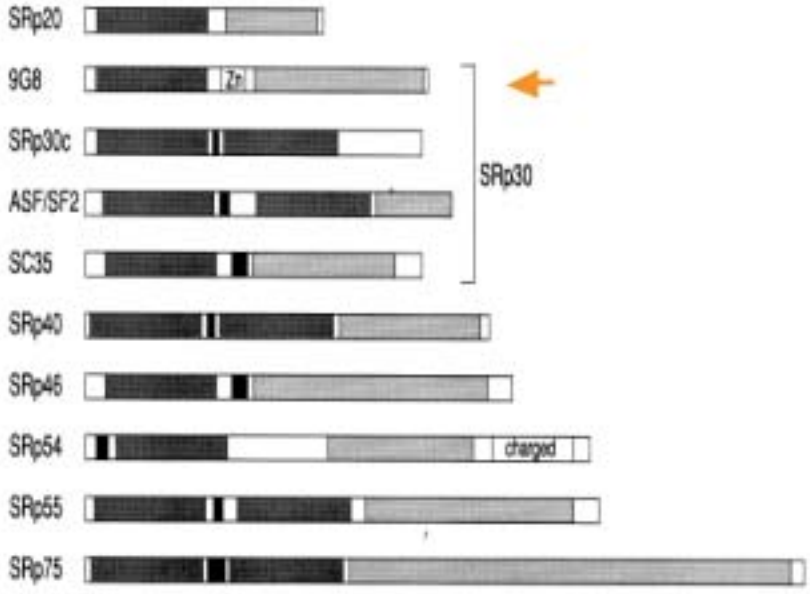
recruited to EJCs in the nucleus by binding to Y14 (97). Upon mRNP export into the cytoplasm, Upf2 is then brought into the EJC by binding to Ufp3 (97). Upf1 remains unassociated from Upf2/3 unless the ribosome encounters a PTC (97). A PTC is recognized as being a termination codon followed by an EJC at least 50-55 nucleotides downstream of the STOP codon (141). During the elongation stage of the pioneer round of translation, translocation of the ribosome strips away EJCs at exon-exon junctions (9). Since the ribosome stalls at termination codons, any EJCs that might exist downstream from a STOP codon (*i.e.*, a PTC) would not be removed from the mRNA. In this case, Upf1 in the SURF complex can associate with Upf2/3 in the downstream EJC, triggering phosphorylation of Upf1 by Smg1 (97). Phosphorylation of Upf1 allows Smg5, Smg6, and Smg7 to be recruited (150), which together, bring in PP2A, dephosphorylating Upf1. Both phosphorylation and the subsequent dephosphorylation of Upf1 are required for NMD to be triggered (9). Although the mechanism is not yet known, it is clear that these steps lead to changes at both ends of the mRNP: the cap is removed from the 5' end by the action of the decapping protein, DCP2, which exposes the 5' end to the activity of the 5'-to-3' exonuclease, XNR1; at the 3' end, the deadenylase, poly(A) ribonuclease (PARN), degrades the poly(A) tail, allowing access of 3'-to-5' exonucleases(132).

Remodeling and transition to steady-state translation. If, however, no PTC is detected, pioneer translation is terminated, and a remodeling of the mRNP occurs, during which the CBC is replaced with eIF4E, and the PABP2 is replaced with PABP1 (120, 132). Since EJCs are removed during the pioneer round, NMD is restricted to previously untranslated mRNAs. Upon remodeling, the mRNP is known as the steady-state initiation complex, as eIF4E initiates steady-state levels of mRNA translation. This is more efficient than the pioneer round of translation (33) and explains why more protein is produced from RNA that has been spliced, although how EJCs specifically help initiate the pioneer round remains unknown. What is known is that intronless mRNAs do not have EJCs and do not undergo the pioneer round and subsequent remodeling (120, 133). Intronless mRNAs are translated less efficiently than remodeled mRNAs but are also immune to NMD (133).

Therefore, the purpose of most EJC proteins remaining associated with the mRNA after splicing can be explained by their roles in the cytoplasm. However, since REFs are not associated with EJCs in the cytoplasm (104), it is unlikely they are involved in NMD or pioneer translation. Although the timing of EJC protein placement on the RNA corresponds with the observed kinetics for Tap recruitment, their importance in nuclear export has not been convincingly established. For example, REF1/Aly is dispensable for nuclear mRNA export in *Drosophila* cells, as are each of the EJC proteins (59). This does not rule out REFs as adaptors for mRNA export but implies that there are additional adapter proteins that recruit Tap to cellular mRNAs.

Other Tap adaptors: SR proteins. As an alternative to REF1/Aly, Serine/Arginine (SR) proteins were proposed as adaptor proteins for Tap. The SR proteins are a family of RNA binding proteins with well-recognized roles in splicing regulation (57, 66, 199). SR proteins are recognized by their structure [N-terminal RNA-Recognition Motif (RRM) and C-Terminal RS domain; Fig. 1] and their function (each individually will compliment a S-100 cytoplasmic extract deficient for splicing). 9G8 is unique among the SR proteins in that it contains a zinc knuckle (ZnK) domain between the RRM and RS domains (Figs. 1 and 3). The sizes of the ten identified SR proteins are conserved from Drosophila to man, and they can be purified from cellular extract to near homogenicity by a 2-step salt precipitation procedure (212). Based to a large extent on *in vitro* studies, SR proteins are major factors in alternative splicing since they bind to the ESEs to recruit the splicing machinery to specific splice sites on pre-mRNA (177, 194). SR proteins are redundant for constitutive splicing, but have different preferences for splice sites of RNAs that are alternatively spliced (66, 194). SR protein domains are modular in that their domains can be swapped between SR proteins and still retain proper constitutive splicing; however, alternative splicing tracks with the domains (24, 204). SR proteins are highly phosphorylated. The RS domain contains multiple serines, which are phosphorylated and dephosphorylated by cellular kinases and phosphatases, respectively (66). SR proteins share a phosphoepitope in this domain that is recognized by monoclonal antibody 104

Figure 1. Schematic representation of SR Proteins. The proteins are ordered according to their increasing molecular weight, except for SRp30 proteins, which are reshuffled in order to put 9G8 close to SRp20 and SRp30c close to ASF/SF2, because of the similarity between their respective RRMs. The RRMs, RS domains, and glycine-rich regions of the different SR proteins are in dark grey, light grey, and black, respectively. Other major domains, such as the zinc knuckle (Zn) of 9G8 and the charged domain of SRp54, are indicated.



Bourgeois, et al, (2004) *Prog. Nucleic Acid Res. Mol. Biol.* **78**, 37-88

(mAb104) (169, 212). The phosphorylation state is believed to play an important role in functional regulation and localization (28, 66, 138, 211).

The SR proteins were initially thought to have solely nuclear functions, but it was subsequently shown that several of these proteins shuttle between the nucleus and the cytoplasm (25). The functional importance of this remained unclear until studies by Steitz and colleagues demonstrated a potential role for two of the shuttling SR proteins (SRp20 and 9G8) in mRNA export (82, 83, 85). In addition, SF2/ASF has also been proposed to serve this function (110). Specifically, SR proteins were found to interact with Tap to promote the export of an intronless reporter mRNA (83). The SR proteins are recruited to the RNA during transcription in a semi-phosphorylated form, and dephosphorylation may trigger the recruitment of Tap (85, 110).

Potential cytoplasmic role for SR proteins. It was originally proposed that once the mRNP reaches the cytoplasm, the SR proteins rapidly become hyper-phosphorylated, losing their affinity for the mRNA and are recycled back to the nucleus (85). This was based on studies of the yeast protein, Npl3p, which is similar to SR proteins in that it binds mRNA and is involved in mRNA export (88). Sky1p is a cytoplasmic SR protein kinase that phosphorylates Npl3p, and *sky1* mutants accumulate Npl3p in the cytoplasm bound to poly(A)$^+$ RNA (63). However, another study reported the presence of SR proteins in polyribosomes, suggesting a potential role in the cytoplasm beyond export, specifically in translational regulation (176). In this study, it was reported that the SF2/ASF protein was able to promote translation of mRNAs from reporter constructs containing SR protein binding sites, and there was no difference in expression from the reporter mRNA regardless of whether or not it had undergone splicing. Several recent studies have now provided additional support for a role of SR proteins in translation (14, 175).

A dual role of export and translation is consistent for what we have observed in our own lab for Tap on intron-containing reporter mRNAs. While we did see an enhancement on export on an intron- and RRE-containing mRNA when expressed with the Tap-Rev mutant fusion protein in the presence of Nxt1 (68), when Tap and Nxt1 were co-expressed in 293T cells with an intron- and CTE-containing reporter, the

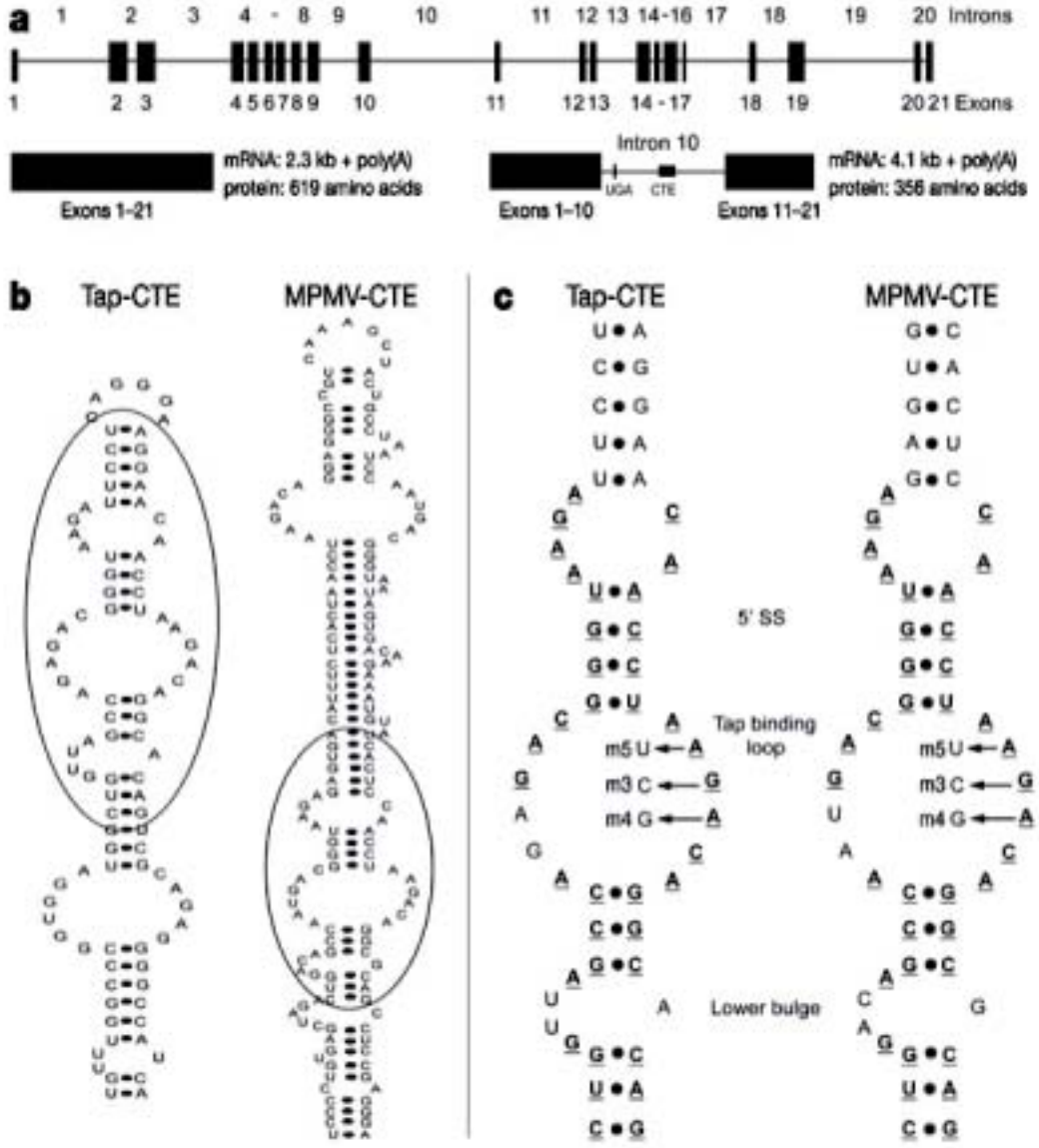
increase in protein expression observed was not explained by enhanced export (93). Looking further, we observed that Tap, but not NXT1, remained associated with polyribosomes throughout translation, and these proteins enhanced the association of the reporter mRNA with the polysomes (93). These results were the first to suggest that productive nuclear export of Tap-RNA complexes might be coupled to translation initiation.

Regulation of intron-retention. In addition, we have recently demonstrated that the *Tap* gene, itself, contains a functional Tap-binding CTE, which exists in a retained intron (125)(Fig. 2). An mRNA that retains this intron is exported to the cytoplasm and is translated into a small alternative protein isoform of Tap. Mutation of the Tap-binding loop of the Tap-CTE prevents expression from this mRNA. This demonstrates two things: Tap regulates its own expression, and it does so through binding directly to a cellular mRNA, *i.e.*, without the need for an adaptor protein. It also defies the commonly held belief that all cellular intron-containing mRNAs are degraded by NMD and are not expressed as protein.

Most of the SR proteins are also alternatively spliced, with intron-retaining isoforms being common. In the case of 9G8, Northern blot analysis shows that at least five mRNA isoforms are produced from alternatively spliced *9G8* RNA (119, 157). Intron 3 can be excised or retained, and there are two potential poly(A) signals in the 3'UTR of exon 8. In addition, an alternative 3' splice site within intron 3 produces a *9G8* isoform with a partially retained intron 3. Because of the presence of PTCs, the consequence of expression of either isoform retaining intron 3 (full or partial) would be the production of a truncated protein, containing the RRM and Zinc Knuckle domains, but lacking the RS domain (Fig. 3). The consequence of using one poly(A) signal over the other is an expanded 3' UTR or a shortened 3' UTR, but this would have no effect on the coding sequence of the mRNA. Like Tap, 9G8 influences its own expression but does so by promoting alternative splicing of its own RNA, although it is not known if either of the intron 3-containing isoforms actually produce protein (119).

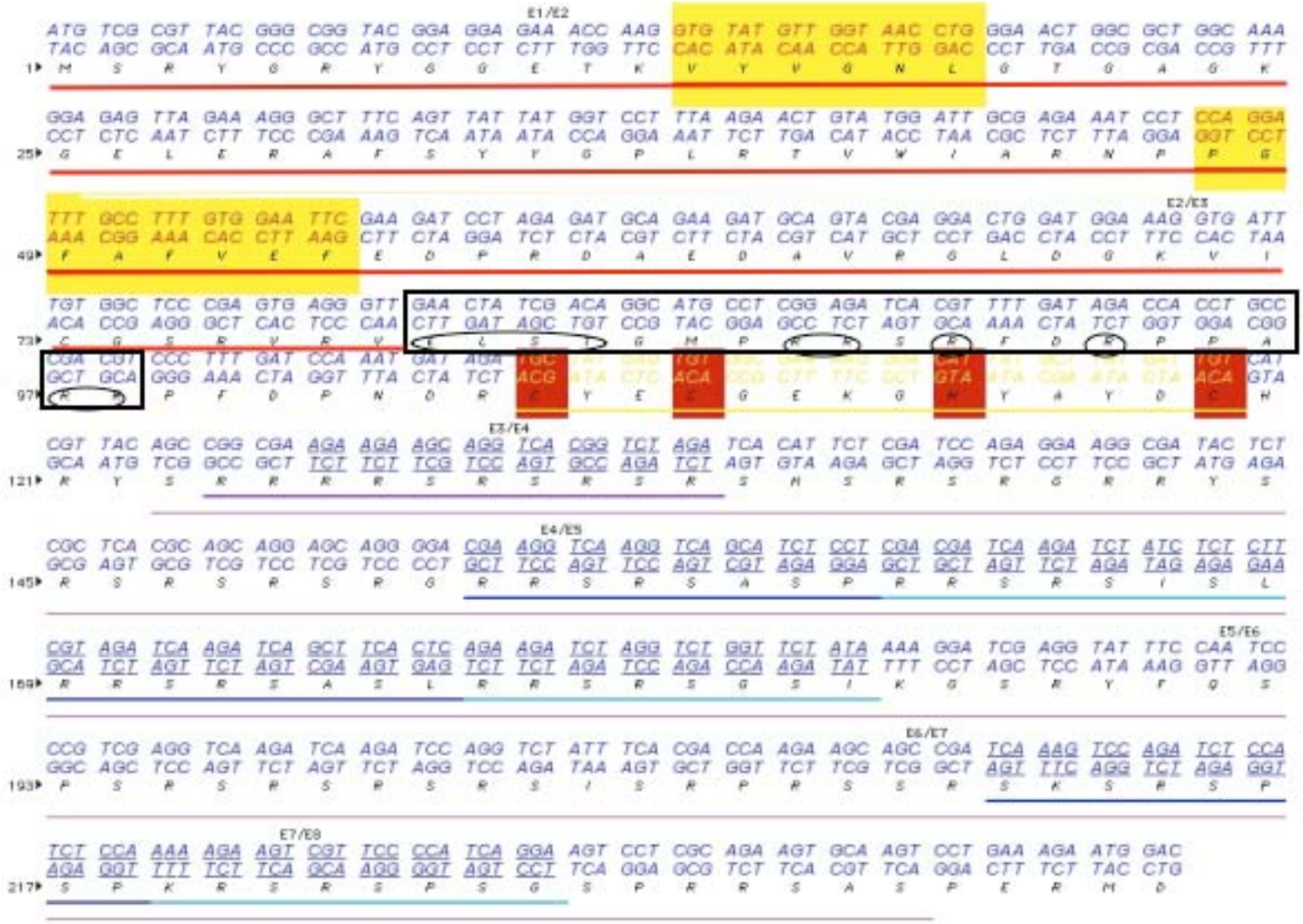
In the studies presented in this thesis, we show further evidence of the interconnection between the multiple steps of mRNA metabolism, in particular linking splicing with nucleocytoplasmic export and translation in one study and in another study showing that the products of an alternatively spliced pre-mRNA have different roles in mRNA metabolism. We show that the proteins and mRNAs involved may be components of one or more hypothetical post-transcriptional operons.

Figure 2. Genomic organization of the human *Tap* gene and comparison of the *Tap*-CTE and MPMV-CTE. *A*, Genomic organization of human *Tap*. Vertical bars represent exons. The UGA stop codon and putative CTE in intron 10 are indicated. *B*, Predicted secondary structure of the *Tap*-CTE and the MPMV-CTE structure. *C*, Close up of the CTE structures (Circled regions in *B*). Underlined bases indicate identity between the *Tap*-CTE and the MPMV-CTE. The m3, m4, and m5 mutations diminish Tap binding and CTE function in the context of the MPMV-CTE.



Li, et al, (2006) *Nature*. **443**, 234-237.

Figure 3. *9G8* cDNA and 9G8 protein sequences and various 9G8 domains. The cDNA sequence encoding the normally spliced 9G8 protein is given in *blue*. The protein sequence predicted for the cDNA is shown in *black italics* beneath the cDNA sequence. Exon-exon junctions are shown above the cDNA sequence (*e.g.*, *E1/E2* for the exon 1/exon 2 junction). The *solid yellow boxes* indicate the RNP consensus sequences of the RRM domain, which is underlined in *red*. The sequence encoding the zinc knuckle (ZnK) domain is shown in *yellow* and *underlined in yellow*, and the CCHC consensus residues and their codons are *boxed in solid red*. The RS domain is *underlined in magenta*, and six RS domain consensus sequences are shown by *underlining in alternating blue and teal*. The nuclear localization signal at the N-terminal part of this domain is *underlined in purple*. A flexible "linker" region, as determined by NMR, is indicated by the *black box* between the RRM and ZnK domains. Within this linker region is a region found by NMR to bind directly to RNA (the *ELST* sequence) and *argenine residues* found by immunoprecipitation to be important for binding the Tap protein (*circled*).


E1/E2
E2/E3
E3/E4
E4/E5
E5/E6
E6/E7
E7/E8

CHAPTER 2

Methods

EXPRESSION VECTORS

The following plasmids were used in these studies. A brief description for each plasmid is included and referenced, if described previously. Methods describing the construction of new plasmids are also included, and a list of any oligonucleotide primers used in their construction is found in the following section. The number before each plasmid name is the HamRek Archive Number.

#0016 pCMV

The previously described (190) pCMV is an empty vector used to ensue equivalent plasmid levels among cells that have been transfected with differing amounts of another CMV promoter plasmid. It uses the simian cytomegalovirus IE94 promoter/enhancer to drive expression.

#0030 pCMV-Rev

The previously described (124) pCMV-Rev expresses HIV-1 Rev using the simian cytomegalovirus IE94 promoter/enhancer.

#0163 pGEX-2T

This plasmid, from Amrad Corp. Ltd, was constructed to produce a fusion protein with the carboxyl terminus of glutathione S-transferase (*Schistosoma japonicum*), which allows for its purification from crude bacterial lysates using immobilized glutathione.

#0354 pCMV-GagPol-RRE-R

The previously described (190) pCMV-GagPol-RRE-R is a subgenomic HIV-1 reporter construct that encodes an intron-containing RNA, which results in the expression of the HIV P24 capsid protein only in the presence of HIV Rev.

#1361 pCMV-GagPol-MPMV

The previously described (190) pCMV-GagPol-MPMV is a subgenomic HIV-1 reporter construct that encodes an intron-containing RNA, in which the RRE has been replaced by the MPMV-CTE, that allows the reporter to become Rev resistant. This plasmid is more commonly referred to as pCMV-GagPol-CTE.

#1831 pCMV-SEAP

The previously described (10) pCMV-SEAP expresses secreted alkaline phosphatase from a fully spliced RNA message. The pCMV-SEAP was a gift from Michael Malim from the University of Pennsylvania.

#2128 pCMV-TAP(61-619)

The previously described (68) pCMV-Tap 61-619 encodes amino acids 61-619 of Tap. Tap is also known as NXF1.

#2208 pcDF-Sam68

The previously described (37) pcDF-Sam68 encodes the full-length mouse Sam68 with an amino-terminal, in-frame FLAG epitope tag. This plasmid was a gift from David Shalloway from Cornell University.

#2214 pcDNA3

The pcDNA3 plasmid is an empty vector used to ensue equivalent plasmid levels among cells that have been transfected with differing amounts of another CMV promoter plasmid. It uses the Cytomegalovirus enhancer-promoter for high-level expression. This plasmid was a gift from David Shalloway from Cornell University.

#2283 pFLAG-Nxt1

The previously described (13) pFLAG-Nxt1 expresses Nxt1, also known as P15, with an amino-terminal, in-frame FLAG epitope tag. This plasmid was a gift of the Bryce Pascal laboratory of the University of Virginia.

#2352 pFLAG-Tap

The previously described pFLAG-Tap plasmid expresses the full-length Tap protein (also known as NXF1) with an amino-terminal, in-frame FLAG epitope tag. This plasmid was a gift of the Bryce Pascal laboratory of the University of Virginia.

#2654 pGEMgagΔBstHII/PstI

The pGEMgagΔBstHIII/PstI plasmid was made by John Coyle and was generated by deleting the BssHII-PstI region of plasmid pHR0243 (HIV-1 gagpol) by digestion with these restriction enzymes, followed by T4 blunt end treatment and ligation. This plasmid is used as a template for *in vitro* transcription using T7 polymerase after AgeI digestion to produce *gag* RNAs of 267 nucleotides in length, which are then used as RNA purification recovery controls in Northern hybridization.

#2763 pCMV-GagPol-5A1

The pCMV-GagPol-5A1 plasmid was made by Yeou-cherng Bor (18) and was generated by inserting the 5A1 PCR product into pHR2739 (pCMV-gagpol-U2) restriction digested with BamHI and XhoI. 5A1 was pulled out of a COS cDNA library as a functional cellular CTE.

#2802 pcDNA3-FLAG-SRm160

This plasmid was a gift of Benjamin Blencowe of the University of Toronto. It expresses the full length SRm160 protein with an amino-terminal in-frame FLAG epitope tag.

#2907 pXJ42-FLAG-9G8-His

This plasmid was a gift of Joan Steitz of Yale University. It expresses the SR Protein, 9G8, in-frame with the FLAG epitope tag.

#2957 pCGT7-9G8

This plasmid was a gift of Adrian Krainer of Harvard University. It expresses the SR Protein, 9G8, in-frame with the T7 epitope tag.

#2959 pCGT7-SRp20

This plasmid was a gift of Adrian Krainer of Harvard University. It expresses the SR Protein, SRp20, in-frame with the T7 epitope tag.

#2974 pcDNA-Nxt1

The previously described pcDNA-Nxt1 encodes the Nxt1/P15 driven by the CMV promoter. It is not tagged.

#3029 pcDNA3-T7-9G8-1

This plasmid was constructed using plasmids pCDNA3 (#2214) for the backbone and pCGT7-9G8 (#2957) for the insert. Oligonucleotides 1115 and 1116 were designed to PCR-amplify the T7-9G8 region of the pCGT7-9G8 plasmid while incorporating HindIII and XhoI restriction sites into the 5' and 3' ends of the amplimer, respectively. The amplimer and the plasmid, pCDNA3, were each digested using restriction enzymes HindIII and XhoI and filter purified. The fragments were then ligated together to create pcDNA3-T7-9G8-1.

#3031 pcDNA3-T7-9G8-2

This plasmid was constructed using plasmids pCDNA3 (#2214) for the backbone and pCGT7-9G8 (#2957) for the insert. Oligonucleotides 1115 and 1117 were designed to PCR-amplify the 5' region of T7-9G8 through the region encoding the zinc knuckle domain while incorporating a HindIII restriction site into the 5' end of the amplimer. To the 3' end, sequence was added just after the exon 3-exon 4 junction to encode amino acids AENLRR and a stop codon (TGA) followed by an XhoI restriction site. The amplimer and the plasmid, pCDNA3, were each digested using restriction enzymes HindIII and XhoI and filter purified. The fragments were then ligated together to create pcDNA3-T7-9G8-2. Expression from this plasmid would mimic a 9G8 mRNA with retention of the entire intron 3. It is predicted to produce a protein that includes the RRM and the Zinc Knuckle domains of 9G8 but not the RS domain.

#3033 pcDNA3-T7-9G8-3

This plasmid was constructed using plasmids pCDNA3 (#2214) for the backbone and pCGT7-9G8 (#2957) for the insert. Oligonucleotides 1115 and 1118 were designed to PCR-amplify the 5' region of T7-9G8 through the region encoding the zinc knuckle domain while incorporating a HindIII restriction site into the 5' end of the amplimer. To the 3' end, sequence was added just after the exon 3-exon 4 junction to encode amino acids YLF and a stop codon (TAA) followed by an XhoI restriction site. The amplimer and the plasmid, pCDNA3, were each digested using restriction enzymes HindIII and XhoI and filter purified. The fragments were then ligated together to create pcDNA3-T7-9G8-3. Expression from this plasmid would mimic a 9G8 mRNA with retention of the 3' half of intron 3 that would result from the use of an alternative 5' splice site. It is predicted to produce a protein that includes the RRM and the Zinc Knuckle domains of 9G8 but not the RS domain.

#3035 pGEX-2T-Zn Knuckle

This plasmid was constructed using plasmids pGEX-2T (#0163) for the backbone and pCGT7-9G8 (#2957) for the insert. Oligonucleotides 1113 and 1114 were designed to PCR-amplify the region encoding the zinc knuckle domain of 9G8 and extending N-terminally 45 amino acids and C-terminally 3 amino acids from this domain. HindIII and XhoI restriction sites were also incorporated into the 5' and 3' ends, respectively. The amplimer and the plasmid, pGEX-2T, were each digested using restriction enzymes HindIII and XhoI and filter purified. The fragments were then ligated together to create pGEX-2T-Zn Knuckle.

#3056 pT7WT1(+exon5+KTS)

The T7WT1(+exon5+KTS) plasmid expresses the full-length mouse WT1 coding sequence It has a T7 tag at the N-Terminus and was made by Yeou-cherng Bor (18). It is also denoted as WT1(+/+).

#3057 pT7WT1(+exon5-KTS)

The T7WT1(+exon5-KTS) plasmid expresses the mouse WT1 coding sequence alternatively spliced to exclude the codons for "KTS" in exon 9. It has a T7 tag at the N-Terminus and was made by Yeou-cherng Bor (18). It is also denoted as WT1(+/-).

#3062 pEGR1

The EGR1 plasmid expresses the coding sequence for the early growth response 1 protein. It was made by Yeou-cherng Bor (18).

#3093 pCMV-GagPol-SIRT7

The pCMV-GagPol-SIRT7 plasmid was made by Yeou-cherng Bor (18) and was generated by cloning a COS cDNA library fragment into the BstXI site of plasmid pHR2016. The library was screened for cellular CTE activity, and SIRT7 was found to be positive. It was PCR amplified and cloned into the XhoI site of pHR2016.

#3100 pT7WT1(+AAA)

The T7WT1(+AAA) plasmid expresses the mouse WT1 coding sequence mutated so the sequence encoding the KTS is changed to AAA. It has a T7 tag at the N-Terminus and was made by Yeou-cherng Bor (18).

#3101 pT7WT1(-exon5+KTS)

The T7WT1(-exon5+KTS) plasmid expresses the mouse WT1 coding sequence alternatively spliced to exclude exon 5. It has a T7 tag at the N-Terminus and was made by Yeou-cherng Bor (18). It is also denoted as WT1(-/+).

#3164 pcDNA-FLAG-SIRT7

The pcDNA-FLAG-SIRT7 plasmid was made by Yeou-cherng Bor by isolating the 7 kb genomic sequence minus the promoter from a BAC and cloning it in-frame with the FLAG tag into a CMV vector.

#3405 pCMV-GagPol-1xTapCTE

The pCMV-GagPol-1xTapCTE plasmid was made by Yeou-cherng Bor (125) and was described previously. It encodes one copy of the cellular CTE found in intron 10 of the Tap gene.

#3406 pCMV-GagPol-2xTapCTE

The pCMV-GagPol-2xTapCTE plasmid was made by Yeou-cherng Bor (125) and was described previously. It encodes two copies of the cellular CTE found in intron 10 of the Tap gene.

OLIGONUCLEOTIDE PRIMERS

Oligonucleotides were synthesized by IDT. The number preceding each sequence is the HamRek oligonucleotide archive number.

1113 5'-CGCGGGATCCGAAGATGCAGTACGAGGAC-3'

1114 5'-CGCGGAATTCGTAACGATGACAATCATAAGC-3'

1115 5'-GATCGGATCCATGGCTAGCATGACTGGTGG-3'

1116 5'-GATCCTCGAGTCAGTCCATTCTTTCAGG-3'

1117 5'-GATCCTCGAGTCACCGTCTCAAATTTTCTGCCCTGCTTCTTCTTCGCC GGCT-3'

1118 5'-GATCCTCGAGTTAAAATAAATACCTGCTTCTTCTTCGCCGGCT-3'

1405 5'-TCACCCGTCGACTGTTTGGCCCCGGTGGATG-3'

1406 5'-GTTCTGGATCCCTCGAGCTCCTGATGGCCCCTCTGCG-3'

SMALL SCALE PLASMID PREPS

Small Scale Plasmid Preps were performed using a modified alkaline method. Small bacterial cultures (5 ml; usually DH5α) were grown overnight in LB Broth with antibiotic (usually 50 μg/ml Ampicillin). 1.5 ml of the culture was then pelleted, then resuspended in cold resuspension buffer (43.6 mM Tris-HCl, pH 8.0, 1 mg/ml lysozyme, 25 mM EDTA, 0.25 mg/ml RNase A). Samples were incubated on ice 25 minutes and lysed in freshly made lysis buffer (0.2 M sodium hydroxide, 1% SDS) on ice for 5 minutes. Samples were then neutralized in cold neutralization buffer (3 M potassium acetate, 2 M acetic acid) for 10 minutes on ice. Precipitated protein, membrane, and genomic DNA were pelleted (14k rpm, 4 C, 10 minutes), and the supernatant was extracted once with 500 μl phenol:Chisam (1:1). The aqueous phase was then precipitated in 550 ml isopropanol for 5 minutes at room temperature. The plasmid DNA was pelleted at room temperature (14k rpm, 5 minutes) and resuspended in 525 μl TE with 0.24 M Potassium acetate. Plasmid DNA was again precipitated with 1 ml ethanol for 30 minutes at –70 C and pelleted at 4 C for 10

minutes. The pellet was air-dried and resuspend in 50 µl TE and used for restriction analysis or DNA sequencing.

LARGE SCALE PLASMID PREPS

Plasmids were purified from bacterial cultures using the QIAGEN QIAfilter Plasmid Maxi Kit (cat #12263) using a modified version of the manufacturer's instructions. Briefly, 5 ml of LB medium containing antibiotic (usually Ampicillin) was inoculated with either a single colony from a plate of LB agar plus selection or a small amount (a loop-full) of archived bacterial glycerol stock. This was cultured for about 6 hours at 37 C plus shaking and then used to inoculate 250-500 ml of LB medium with selective antibiotic. This was grown at 37 C with shaking for 16 hours.

After cells had reached a density of about $3\text{-}4\times10^9$ cells per ml, the culture was centrifuged at 3000 rpm for 30 minutes at 4 C to pellet the bacterial cells. The supernatant was drained, and the cells were resuspended in 10 ml Buffer P1 plus RNase A with vortexing. The cells were lysed by the addition of 10 ml Buffer P2 with gentle mixing to avoid shearing of genomic DNA. When the lysate looked relatively clear (no more than 5 minutes) genomic DNA, proteins, membranes and other cellular debris were precipitated by the addition of 10 ml of Buffer P3 with gentle swirling. The precipitate was separated from the soluble fraction containing the plasmid DNA by transferring to centrifuge tubes and centrifuging at 10000 rpm at 4 C for 20 minutes. The cleared supernatant was further purified by passing it through a QIAfilter Cartridge. Plasmid DNA was recovered from the cleared supernatant by passing it through an equilibrated QIAGEN-tip 500, which bound the plasmid DNA, washing the tip with 2 x 30 ml Buffer QC, and eluting the DNA with 15 ml Buffer QF. The plasmid DNA was precipitated by the addition of 10.5 ml isopropanol and centrifuged at 13000 rpm for 30 minutes at 4 C. The DNA pellet was washed with 5 ml 70% ethanol, dried for 10 minutes, and redissolved in 1 ml dH_2O.

POLYMERASE CHAIN REACTION (PCR)

The following reagents were mixed together: 10 µl 10× thermopol buffer, 1.5 µl 10 mM dNTP mixture, 5 µl 100 ng/µl 5' oligonucleotide, 5 µl 100 ng/µl 3' oligonucleotide, 1 µl 10 ng/µl DNA template (plasmid), 76.5 µl dH20, 1 µl Deep Vent Polymerase. The following program was then run in a thermocycler: 95 C for 5 minutes; 35 cycles of 95 C for 30 seconds, 49 C for 45 seconds and 72 C for 1 minute; then 72 C for 7 minutes, and held at 4 C. Reactions were visualized by running through 1% agarose with 0.5 µg/ml ethidium bromide.

DNA SEQUENCING

For sequencing of plasmid DNA, 1 µg of DNA was mixed with 8 pmole oligonucleotide primer in 14 µl total volume and sent to the DNA Sequencing Core Facility at the University of Virginia for sequencing with an Applied Biosystems 377 Prism DNA Sequencer.

CELL LINES

293T/17 cells, although originally derived from the transfection of primary human embryonic kidney cells, are most likely neuronal, and not epithelial, in nature. Transfection with adenovirus type 5 sheared DNA gave rise to 293 cells, and further transformation with the SV40 Large T antigen yielded 293T/17 cells. B2.23 cells are a cell line derived from 293T cells by stable transfection of pCMVGag-Pol-CTE (pHR 1361) and pCMVEnv-CTE (pHR 1374). 293T/17 and B2.23 cells are maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% bovine calf serum, 1.5 mg/ml G418, and 0.5 mg/ml gentamycin at 37 C with 85% humidity and 5% carbon dioxide.

TRANSFECTIONS

Transfections were performed according to the calcium phosphate method of Graham and Van der Eb. Cells were plated the day before transfection so they had time to recover from the trypsinization process, but had not had a chance to grow significantly. For 293T cells, rather than counting the cells, which I

found to be inconsistent, I split from a confluent 15 cm plate to new 15 cm plates 1:4-1:5 (about 8.5×10^6 cells/plate), depending on how well the cells were growing, or from a 15 cm plate to 10 cm plates 1:12 (about 3×10^6 cells/plate). For me, this gave much more consistent results than counting the cells.

The following was for the transfection of 10 cm plates; for 15 cm plates, the volumes were doubled. Plasmid DNA was mixed with sterile water to 450 µl total. To this, 50 µl 2.5 M calcium chloride in 10 mM HEPES was added. This solution was then added drop-wise to 500 µl 2X HEBS (700 mM sodium chloride, 10 mM potassium chloride, 1.4 mM sodium phosphate, monobasic, 11.1 mM dextrose, 42 mM HEPES, pH 7.2) while vortexing. The solution was allowed to precipitate for 30-45 minutes then added drop-wise onto 293T cells in media. The cells were tilted briefly to distribute the DNA precipitate evenly over the cells, then left undisturbed at 37 C for 4-6 hours to allow the precipitate to settle. The media was then changed to fresh media to remove the calcium, and the cells were left to grow until the next phase of the experiment.

RNA PREPARATION FOR RT-PCR

RNA was isolated from 293T cells either using Tri Reagent (Molecular Research Center, Inc.) for total RNA or by hypotonic cell lysis for cytoplasmic RNA.

Whole Cell RNA Extraction

For whole cell lysis, the media was removed from the cells (which were not washed), and 1 ml Tri Reagent was added to each 10 cm plate. The lysate was pipetted up and down and transferred to a 1.5 ml microfuge tube to incubate at room temperature for 2-15 minutes. Lysate was centrifuged at 12000× g for 15 minutes at 4 C, the aqueous phase was transferred to a new tube, and 500 µl isopropanol was added to it to precipitate the RNA for 5-10 minutes at room temperature. The precipitate was pelleted at 12000× g for 8 minutes (4-25 C) and washed in 75% ethanol before air-drying 3-5 minutes. The pellet was resuspended in 50 µl DEPC-water with pipetting and incubation at 55-60 C for 10-15 minutes.

Cytoplasmic RNA Extraction

For cytoplasmic fractions, cells were washed in PBS and scraped into 1.5 ml microfuge tubes. Cells were pelleted at 4000 rpm for 2 minutes, and any residual PBS was aspirated. Cells were then resuspended in 250 µl RSB [10 mM Tris, pH 7.4, 10 mM sodium chloride, 1.5 mM magnesium chloride, 100 U RNasin Plus (40 U/µl; Promega)] and allowed to swell in the hypotonic solution. Then 250 µl lysis buffer (10 mM Tris, pH 7.4, 10 mM sodium chloride, 1.5 mM magnesium chloride, 1% Triton X-100, 1% deoxycholate, 2% Tween 20) was added, and the solution gently mixed and left to incubate on ice for 10 minutes. Nuclei were pelleted out by a brief 10-second spin, and the lysate was transferred to a new tube and centrifuged again at full speed for 10 minutes to remove any further cellular debris (membranes, mitochondria, etc.). The cleared lysate was then transferred to a new tube and extracted twice with 500 µl phenol/Chisam (1:1). The RNA was precipitated by adding 1/10 volume (~40-50 µl) sodium acetate and 2.5 volumes (~1100-1375 µl) cold ethanol and incubating at –70 C for 30 minutes. The precipitate was centrifuged for 15 minutes, air dried briefly, and resuspended in 50 µl DEPC-water.

DNase Treatment of RNA Preps for RT-PCR

To remove contaminating genomic and plasmid DNA, RNA preps were treated with RQ1 DNase. To each 50 µl RNA prep (whole cell or cytoplasmic), 6 µl 10× Reaction buffer (400 mM Tris-HCl, pH 8.0, 100 mM magnesium sulfate, 10 mM calcium chloride) and 100 U of RQ1 DNase were added. Reactions were incubated at 37 C for 1 hour and extracted once with phenol/chloroform and once with Chisam. RNA was precipitated in 1/10 volume sodium acetate and 2.5 volume cold ethanol at –70 C for 30 minutes, spun down, air dried, and resuspended in 50 ml DEPC-water. A 1/20 dilution was measured at OD_{260}, and RNA was used in RT-PCR. If the reverse transcriptase minus control gave positive results, the DNase treatment was repeated for those samples.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

This procedure was developed by Dr. Jack Schell in the Margo Roberts laboratory.

For the reverse transcriptase reaction, the following were mixed together in a 200 µl PCR tube: 10.75 µl DEPC-treated water, 1 µl 10 mM dNTP mixture, 1 µl 100 ng/µl random hexamers, 2 µl 100 ng/µl RNA. This was heated at 73 C for 5 minutes and snap-cooled on ice. To this was added 4 µl 5× RT buffer, 1 µl RNasin, and 0.25 µl Superscript II Reverse Transcriptase for a 20 µl total reaction volume (enough for 10 PCR reactions), which was heated to 42 C for 1 hour, then 95 C for 10 minutes, and held at 4 C.

For the amplification step, the following were mixed together for each reaction: 5 µl 10× PCR reaction buffer, 2 µl 50 mM magnesium chloride, 2.5 µl 100 ng/µl 5' oligonucleotide, 2.5 µl 100 ng/µl 3' oligonucleotide, 1 µl 10 mM dNTP mixture, 0.5 µl Taq polymerase, 2 µl RT reaction, and water to 50 µl (usually 34.5 µl). Then, the following program was run in a thermocycler: 95 C for 5 minutes; 35 cycles of 95 C for 30 seconds, 49 C for 45 seconds and 72 C for 1 minute; then 72 C for 7 minutes, and held at 4 C. Reactions were visualized by running through 1% agarose with 0.5 µg/ml ethidium bromide.

GENOMIC DNA PREPS

Adherent 293T cells (10 cm plate) were trypsinized and collected in a 1.5 ml microfuge tube. Cells were pelleted at 4000 rpm for 2 minutes, and washed 2× in cold PBS. Cells were resuspended in 300 µl digestion buffer (100 mM sodium chloride, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K). Cells were incubated at 50 C for 12-18 hours with shaking and then extracted with an equal volume of phenol/Chisam. The aqueous phase was transferred to a new tube and mixed with 1/2 volume 7.5 M ammonium acetate and 2 volume 100% ethanol. The DNA was precipitated at 1700×g for 2 minutes, washed in 70% ethanol and air-dried. The pellet was resuspended in TE and shaken at 60 C to facilitate solubilization.

P24 ASSAY

Seventy-two hours after transient transfection, supernatants from 293T cells were harvested and cleared of floating cells by centrifugation. Cleared supernatants were frozen at –70 C until assayed. P24 secreted into the supernatants from transiently transfected cells were assayed using an in-house P24 ELISA kit. The assay was performed as follows.

The day before the assay, 100 µl of the primary antibody [monoclonal IgG αP24, e.g., HamRek lab in-house supply; or NIH AIDS Research and Reference Reagents (catalogue #3537); or hybridoma 183-H12-5C (NIH catalogue #1513)] diluted to 5 µg/ml was added to the appropriate number of wells in a 96 well plate. The plate was sealed and incubated at 37 C overnight.

The next day, the each well was rinsed 4 times in PBS, and 250 µl blocking buffer [PBS, 5% bovine calf serum (BCS)] was added to each well. The plate was sealed and incubated at 37 C for 1 hour. The plate was then washed 7 times in PBS plus 0.5% Tween-20.

The p24 standard (soluble P24 particles, provided by Wes Sundquist, University of Utah, Salt Lake City, Utah) was serially diluted 2 fold in tissue culture media plus 10% serum with gentle mixing (not vortexing) to achieve a range of concentrations from 25-800 pg/ml. Sample supernatants were thawed and either left undiluted or diluted up to 1:100 in media plus serum. Except for one well for the blank, 10 µl lysis buffer (PBS, 10% Triton X-100, Trypan Blue) was added to each well followed by 100 µl standard or sample to each appropriate well. The plate was sealed and incubated at 37 C for 2 hours. The plate was then washed 7 times in PBS plus 0.5% Tween-20.

The secondary antibody [biotinylated human αHIV, polyclonal (NIH AIDS Research and Reference Reagents, #3957] was diluted to 1.25 µg/ml in assay buffer (PBS, 10% BCS, 0.5% Triton X-100), and 100 µl of this was added to each well, except the blank. The plate was sealed and incubated at 37 C for 1 hour. The plate was then washed 7 times in PBS plus 0.5% Tween-20.

Peroxidase-streptavidin (ICN Biomedicals #191394) was diluted 1:4000 in assay buffer, and 100 µl of this was added to each well, except the blank. The plate was sealed and incubated at 37 C for 30 minutes. The plate was then washed 7 times in PBS plus 0.5% Tween-20.

OPD tablets (Sigma #P-3804) were dissolved into substrate buffer (31.45 mM sodium phosphate, 15.19 mM citric acid, 1.25 % sodium stannate, 18.75 % hydrogen peroxide) (1 tablet/5 ml buffer), and 100 µl of this was added to each well, including the blank. The plate was incubated in the dark for 30 minutes, during which time an orange color developed with intensity commensurate to the amount of P24 in each sample. The reaction was stopped by the addition of 50 µl 2 M hydrogen sulfate to each well, and the optical density was read at 492 nm. The serially diluted P24 standards were used to generate a standard curve to determine the levels of P24 secreted from the transfected 293T cells.

SECRETED ALKALINE PHOSPHATASE (SEAP) ASSAY

Seventy-two hours after transient transfection, supernatants from 293T cells were harvested and cleared of floating cells by centrifugation. Cleared supernatants were frozen at –70 C until assayed. SEAP secreted into the supernatants from transiently transfected cells were assayed using the Tropix Phospha-Light Chemiluminescent Reporter kit (#BP100). The assay was performed as follows.

The Dilution Buffer was diluted from 5× to 1× with dH_2O. This was then used to dilute 10 µl of each sample with 30 µl of the Dilution Buffer. Samples were incubated at 65 C for 30 minutes. The heated samples were then mixed with equal volumes of Assay Buffer (usually 20 µl each) and incubated at room temperature for 5 minutes. Then in duplicate, 10 µl each sample was transferred to a 12 x 75 mm polystyrene luminometer tube containing 10 µl Reaction Buffer (CSPD Chemiluminescent substrate diluted 1:20 in Reaction Buffer Diluent). Samples were incubated at room temperature for 20 minutes, and then 5-second counts of chemiluminescent activity were measured using a Moonlight 2010 luminometer (Analytical Luminescence Laboratory).

SUBCELLULAR RNA FRACTIONATION FOR NORTHERN BLOT

Cytoplasmic RNA Isolation

For the isolation of cytoplasmic RNA for use in Northern Blot analysis, six 150 mm plates of 293T cells were transfected per condition by the calcium phosphate method. Sixty-five hours post-transfection, cells

were harvested by washing 2× in cold PBS and scraping the cells into 15 ml conical tubes, with pooling the cells from the six plates. The cells were pelleted at 1500 rpm and 4 C for 5 min (Sorvall RT-6000B), and any residual PBS was aspirated. The cells were resuspended in 3 ml cold RSB (10 mM Tris, pH 7.4, 10 mM sodium chloride, 1.5 mM magnesium chloride) and incubated on ice for 10 minutes. NP-40 was added drop-wise to the cell suspension to 0.1% final concentration (300 µl 1% solution) with gentle mixing. The nuclei were centrifuged out by two gentle spins, the first at 1600 rpm and 4 C for 5 minutes, and the second at 1200 rpm and 4 C for 5 minutes. To the supernatant, an equal volume (~3 ml) of 2× Proteinase K buffer (200 mM Tris, pH 7.5, 25 mM EDTA, 300 mM sodium chloride, 2% SDS, 400 µg/ml Proteinase K) was added left to react at 37 C for 30 minutes with shaking. The lysate was then extracted once with one volume of phenol:Chisam and twice with one volume Chisam. Sodium acetate was added to 300 mM (3 M, pH 5.2), and the RNA was precipitated by the addition of 2.5 volumes cold ethanol and left at –20 C overnight. The RNA was pelleted at 2500 rpm and 4 C for one hour (Baxter 6000) and resuspended in 10 mM Tris, pH 7.4, 10 mM EDTA, 0.2% SDS with heating at 65 C for 5 minutes. Sodium chloride was added to 0.5 M final concentration (630 µl 5 M NaCl/10 ml starting lysate). This material was then subjected to poly(A)$^+$ RNA selection.

Whole Cell RNA Isolation

For the isolation of cytoplasmic RNA for use in Northern Blot analysis, three 150 mm plates of 293T cells were transfected per condition by the calcium phosphate method. Sixty-five hours post-transfection, cells were harvested by washing 2× in cold PBS. Cold lysis buffer (0.2 M sodium chloride, 0.2 M Tris, pH 7.5, 1.5 mM magnesium chloride, 2% SDS, 20 µl/ml Proteinase K) was added to each plate (3 ml per plate) and swirled to distribute evenly until the cells were lysed and coming off the plates. The lysates were transferred to sterile 50 ml centrifuge tubes, pooling the three plates for each condition. The plates were rinsed with an additional ml of lysis buffer to collect any residual lysate and added to the pooled sample (10 ml total). Lysate was passed through a sterile plastic syringe with a 21 gauge needle to shear the genomic DNA until the viscosity resembled that of the lysis buffer and then incubated at 45 C for 1-2 hours in a

slow shaking water bath. Sodium chloride was added to 0.5 M final concentration (630 µl 5 M NaCl/10 ml starting lysate), and any precipitated DNA was further sheared using a 21-gauge needle. This material was then subjected to poly(A)$^+$ RNA selection.

Poly(A)$^+$ RNA Selection

For the poly(A)$^+$ selection of either cytoplasmic or whole cell RNA, 10 mg oligo dT cellulose (Promega) per 150 mm plate was used. The oligo dT cellulose was pre-equilibrated by hydration in 20 ml binding buffer (0.01 M Tris, pH 7.5, 0.5 M sodium chloride) for one hour at room temperature with shaking. The binding buffer was removed by centrifuging at 3000 rpm for 3 minutes and aspirated, and the pellet was resuspend in 20 ml 0.3 M sodium hydroxide and shaken for 30 minutes. The cellulose was again pelleted and washed two times with 10 ml DEPC-treated H_2O and then two times with binding buffer. The pellet was resuspended in binding buffer at a final concentration of 30 mg/ml. One ml of oligo dT cellulose was then added to each tube of lysate and tumbled at medium speed and room temperature for 60 minutes. The cellulose was pelleted at 3000 rpm and room temperature for 5-10 minutes and washed 4 times in binding buffer. After the last wash, the cellulose was resuspended in 500 µl binding buffer and transferred to a 0.45 µm filter cup (Millipore). The samples were spin-filtered at 12000 rpm for 15 seconds, and the flow-through was discarded. The RNA-bound oligo dT cellulose was washed in the insert cup with 400 µl binding buffer and repeated until the OD_{250} was less than 0.05 (usually one wash is sufficient). The poly(A)$^+$ RNA was eluted into a new tube using 200 µl elution buffer (0.01 M Tris, pH 7.5), waiting 2 minutes, spinning 10 seconds, adding an additional 150 µl elution buffer, waiting 2 minutes, and spinning 10 seconds. The eluted RNA was then precipitated by adding 2 µl 10 mg/ml tRNA stock solution, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold ethanol and stored at –70 C until ready to use. RNA yield and purity were determined by measuring the $OD_{260/280}$ ratio.

POLYRIBOSOME PURIFICATION

Seeding and Transfection

A very detailed description of this protocol was published online in Nature Protocols (17). Briefly, 293T cells were seeded (1:4 split; about 8.5×10^6 cells/plate) onto 15 cm culture plates the day before transfection. The cells were transfected the next day using the calcium phosphate protocol (Graham and van der Eb, 1973). The media was changed 6 hours after transfection. Twenty-four hours post-transfection, cells were split with trypsin 1:2 onto 15 cm plates. This step was found to be critical since when cells approach the stationary phase of growth, they tend to slow general protein translation, and polysomes extracted from these cells showed a shift from actively translating polysomes to a predominance of monosomes (a large 80S peak in the polysome profile).

Cycloheximide Treatment

Forty-eight hours post-transfection, 100 mg/ml cycloheximide was added to each plate (50 μg/ml final concentration) and incubated at 37 C for 30 min. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms and exerts its effect by interfering with peptidyl transferase on the 60S ribosome, thus blocking translational elongation. Addition of cycloheximide "freezes" the ribosomes to the mRNA, preventing them from completing translation and falling off the RNA during the polysome extraction procedure, thus giving a "snapshot" in time of translating polyribosomes. Also, after this step, all procedures were performed on ice, as the cold temperature helped slow down translation elongation and RNase activity.

Cell Harvest

To harvest polysomes, the growth medium was aspirated, and the cells were washed twice with 10 ml of ice-cold PBS containing 50 μg/ml of cycloheximide. The cells were scraped and transferred to a 1.5 ml microcentrifuge tube and centrifuged at 4000 rpm for 1 min. The residual PBS was aspirated. The cells were resuspended in 250 μl RSB + inhibitors [10 mM Tris, pH 7.4, 10 mM sodium chloride, 1.5 mM

magnesium chloride, 100 U RNasin Plus (40 U/µl; Promega), 1× Proteinase Inhibitor Cocktail (Sigma), 1× Phosphatase Inhibitor Cocktail I (Sigma), 1× Phosphatase Inhibitor Cocktail II (Sigma)] with vortexing. The cells were lysed by adding 250 µl of 2× polyribosome extraction buffer (10 mM Tris, pH 7.4, 10 mM sodium chloride, 1.5 mM magnesium chloride, 1% Triton X-100, 1% deoxycholate, 2% Tween 20), and mixed quickly by inversion. The solution was left to incubate on ice for 10 min, and then spun for 10 sec to pellet nuclei. The cytoplasmic extract was transferred to a new microcentrifuge tube and spun at 10,000× g for 10 min at 4 C to pellet any remaining cellular debris.

Sucrose Gradient

The cleared cytoplasmic lysate was layered onto the top of a 10-50% continuous sucrose gradient, which was prepared as follows: Sucrose solutions were prepared using Ultrapure sucrose (Invitrogen) as 10 and 50% concentrations in 75 mM potassium chloride, 10 mM Tris, pH 7.4, 1.5 mM magnesium chloride. The solutions were boiled for 10 minutes and then filtered through a 0.45 µm membrane. The 50% sucrose was used to underlay the 10% sucrose by gently inserting the tip of a blunt end stainless steel needle to the bottom of the tube (open top polyclear centrifuge tube, 14×89 mm, Seton Scientific, Los Gatos, CA) and slowly releasing 5.5 ml 50% sucrose solution from syringe to displace the 5.5 ml phase of 10% sucrose solution. Each tube was closed with a Biocomp cap and placed in the Biocomp gradient master (model 107ip, BioComp Instruments, Inc. New Brunswick, Canada, http://www.biocompinstruments.com, Fig. 4). The gradient parameter was set to long caps, 10-50%. When the gradient formation finished, the caps were carefully removed, and the tubes were transferred to an SW41Ti rotor basket. An additional 500 µl of 10% sucrose was carefully layered on top of gradient, and the cytoplasmic lysate was layered on top of this. Sometimes EDTA was used to disrupt the polysomes. For this, 30 µl of 250 mM EDTA was added to 470 µl of the cleared cytoplasmic extract (15 mM final EDTA concentration), before layering the extract on top of the gradient. SW41Ti rotor baskets were placed on the SW41Ti rotor and centrifuged in a Baxter ultracentrifuge at 36,000 rpm for 2 h at 4 C.

Figure 4. The BioComp Gradient Master (model 107ip) gradient maker. This gradient maker was used to create uniform sucrose gradients to resolve polysomes, monosomes, and ribosomal subunits for polysome analysis. Open-top polyclear 14×89 mm centrifuge tubes filled with 10% sucrose underplayed with 50% sucrose were capped with long caps and placed in the gradient maker. ml phase of 10% sucrose solution. Each tube was closed with a Biocomp cap and placed in the Biocomp gradient master. The gradient parameter was set to long caps, 10-50%, and the gradient makers followed a pre-set formula using parameters of angle of rotation, speed of rotation, and time of rotation to form the desired gradient.


GRADIENT
MASTER
BIOCOMP

Fractionation

When the centrifugation finished, the rotor baskets were removed and kept on ice until fractions from each tube were collected. From each gradient,19-20 fractions (20 seconds collections at a piston-lowering speed of 0.2 mm/sec) were collected into 1.5-ml microcentrifuge tubes on ice using the Piston gradient fractionator (BioComp Instruments, Inc. New Brunswick, Canada; Fig. 5) that was equipped with an UV monitor (Monitor UV-M II, GE Health, Amersham Biosciences, Piscataway, NJ), concomitantly measuring absorbance at 254 nm. The volume of each fraction was approximately 550 µl. Fractions were then analyzed for protein (using either immunoprecipitation for a specific protein or nonspecific isopropanol precipitation to concentrate the samples and to remove the sucrose for better loading and resolution on the SDS-PAGE) or RNA (next section). Protein analysis was performed immediately if protein phosphorylation was a factor in the analysis as freeze thawing of the samples caused extensive dephosphorylation. Immediately after fractionation, 10 ng of a 267-nucleotide long fragment of *in vitro* transcribed gag RNA was added to each fraction used in Northerns as a control for recovery. These were treated with Proteinase K (to each fraction, 60 µl of 10% SDS and 12 µl of 10 mg/ml Proteinase K were added and incubated for 30 min at 42 C), and then stored at –80 C until analysis.

RNA Isolation and Precipitation

Three hundred µl of each Proteinase K-treated fraction was transferred to a new microcentrifuge tube and on ice, extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol and once with an equal volume of chloroform/isoamyl alcohol. The supernatants were transferred to new microcentrifuge tubes, and RNA from each fraction was precipitated by the addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol with incubation at –80º C. The RNA was pelleted by centrifuging at 13000 rpm for 20 min at 4 C and washed with1 ml 75% ethanol. The supernatant was carefully aspirated from each tube, and the RNA pellet was air dried briefly.

Figure 5. The BioComp Piston Gradient Fractionator. This fractionator was used to uniformly take sample from the top of the polysome gradient while concomitantly detecting RNA by measuring absorbance at 254 nm using a UV monitor (Monitor UV-M II). Fractionation was performed manually by moving a rack of tubes at pre-determined intervals. For example, 20 second intervals usually gave 19 fractions of approximately 550 μl, and 30 sec intervals usually gave 13 fractions of approximately 800 μl.


PGE

NORTHERN BLOT ANALYSIS

Formaldehyde Gel Electrophoresis

The RNA pellets from sucrose gradient fractions were resuspended in 4 μl of DEPC-water and 15.5 μl of RS buffer. Alternatively, 4 μg of poly(A)$^+$ selected RNA was used. The samples were incubated at 55 C for 10 min, cooled on ice, and loaded onto a formaldehyde agarose gel [1% agarose, 0.02 M MOPS (3-[N-morpholino] propanesulfonic acid), pH 7.0, 5 mM sodium acetate, 0.1 mM EDTA, 16.2% formaldehyde]. The gel was run in MOPS Electrophoresis Buffer (0.02 M MOPS, pH 7.0, 5 mM sodium acetate, 0.1 mM EDTA, 8% formaldehyde) at 120 volts for 3 h with a peristaltic pump that recirculated the running buffer between the electrodes.

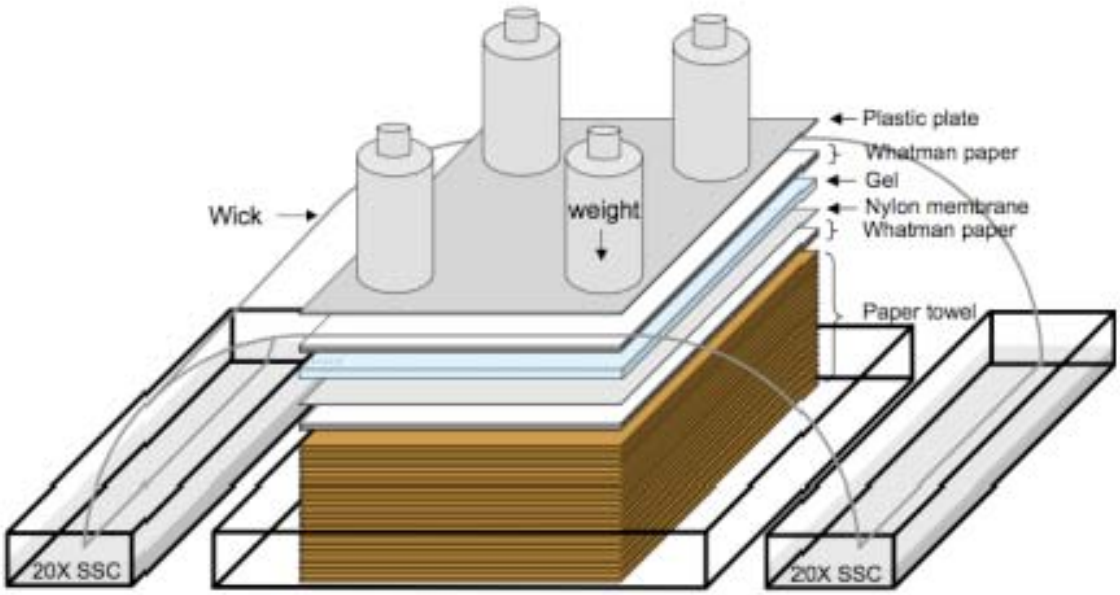
Preparation for Transfer

The gel was soaked in 250 ml of autoclaved water for 15 minutes, with 3 changes of the water and gentle rocking. The gel was denatured by complete immersion in 50 mM sodium hydroxide for 20 minutes, and then neutralized in 100 mM Tris-HCl (pH 7.0-7.6) for 20 minutes. The gel was then equilibrated in 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) for 15 minutes. Positively charged nylon membrane (BrightStar-Plus; Ambion) was cut to the size of the gel and soaked in 100 ml 10× SSC.

Transfer

Capillary transfer was performed by setting the up following (Fig. 6): 3 inches of cut-to-size paper towels were stacked in a Pyrex tray; 3 pieces of Whatman paper soaked in 10× SSC for 3 minutes were placed on top of the paper towels; the nylon membrane was placed on top of the Whatman paper; the gel with the top-side facing upward was placed on the membrane taking care that any bubbles between the gel and the membrane were removed; and 3 pieces of Whatman paper soaked in 10× SSC for 3 minutes were placed on top of the gel. 20× SSC (3.0 M sodium chloride, 0.3 M sodium citrate, pH 7.0) was added to two reservoirs and placed on each side of the Pyrex tray. A long piece of Whatman paper was soaked in 20× SSC for use as a wick. The wick was placed upon the 3 topmost pieces of Whatman paper with the wick

Figure 6. Schematic representation of transfer set-up. Filter paper acts as a wick to transfer the 20× SSC from the tray through the gel, carrying RNA to the membrane. Weights provide pressure to make transfer to the membrane uniform. Nylon membrane, gel, and Whatman paper are shown.



Bor, *et al.*, http://www.nature.protocols.com/2006/09/15/northern_blot_analysis_of_mrna.php

ends submerged in the reservoirs. A plastic or glass plate was placed on top of the wick, and the whole set-up was weighted with 4 plastic bottles, each containing 100 ml of water. The RNA was allowed to transfer overnight.

Post-transfer Processing

At the end of the transfer period, the nylon membrane was removed from the transfer set-up and rinsed briefly in 10× SSC. The nylon membrane was placed on a clean, dry piece of Whatman paper with the RNA side facing upwards, and the RNA was cross-linked to the nylon membrane using a UV cross-linker (Stratagene UV Stratalinker 2400) at120 mjoules/cm^2.

Blocking and Radioactive Probe Preparation

The membrane was placed in a heat-sealable bag (2.5 mm thick, 1 pint size; Kapak). Pre-hybridization solution (0.9 M sodium chloride, 0.06 M sodium phosphate monobasic, 0.006 M EDTA, 0.1% SDS, 0.0004% polyvinylpyrrolidone, 0.0004% Ficoll 400 and 0.0004% bovine serum albumin, pH 7.4) was added to the bag, and the bag was sealed. The membrane was incubated at 43 C for 1-4 hours in a slowly shaking water bath.

During prehybridization, DNA fragments [*Sac* I-*Bgl* II (nucleotides 682-2093) fragment of the HIV-1 BH10 clone and *Bam*H I fragment of the human SEAP cDNA (nucleotides 213-1698)] were labeled with [α-^{32}P]dCTP using the RediPrime II kit (GE Health, Amersham Biosciences) according to the manufacturer's instructions. The radiolabeled probe was purified from unincorporated nucleotides using the NICK column (GE Health, Amersham Biosciences) according to the manufacturer's instructions. One µl of probe was used to determine the labeling efficiency with a scintillation counter. The total incorporation of [a-^{32}P]dCTP and the specific radioactivity of the probe were calculated (1×10^9 cpm/mg of template was considered to be good).

Hybridization

The pre-hybridization solution in the heat-sealable bag was replaced with hybridization solution (50% formamide, 0.9 M sodium chloride, 0.06 M sodium phosphate monobasic, 0.006 M EDTA, 0.1% SDS, 0.002% polyvinylpyrrolidone, 0.002% Ficoll 400, 0.002% bovine serum albumin, and 0.1 mg/ml tRNA, pH 7.4). The probe was denatured by placing it in a boiling water bath for 5 minutes followed by snap-cooling on ice. The probe was briefly centrifuged, then added to the bag and sealed. Hybridization occurred overnight in a 43 C water bath with slow agitation.

Post-hybridization Washing

The nylon membrane was removed from the bag and rinsed briefly twice in a Rubbermaid container with 6×SSC (0.9 M sodium chloride, 0.09 M sodium citrate) plus 0.1% SDS. The membrane was washed three additional times for 15 minutes each at room temperature, then once at 43 C for 15 minutes. The membrane was then washed 2-3 more times with 0.1× SSC (0.015 M sodium chloride, 0.0015 M sodium citrate) plus 0.1% SDS at room temperature for 15 minutes each. The background of the membrane was checked using a Geiger Counter, and if the background was high, the blot was continued to be washed with 0.1×SSC plus 0.1% SDS at 43 C for 15 minutes per wash and repeated until the background was low using the Geiger Counter.

RNA Detection

The membrane was wrapped with Saran wrap and placed onto a PhosphorImager cassette against a PhosphorImager screen. Depending on hybrid yield and specific activity of the probe, the screen was left exposed to the membrane for 1-7 days, at which point the screen was scanned. The resulting data were analyzed with ImageQuant software.

CELL LYSIS FOR SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

For cell lysis, cells were washed 2× in cold PBS and scraped into 1.5 ml microfuge tubes. Cells were pelleted at 4000 rpm for 2 minutes, and any residual PBS was aspirated. The cells were then lysed in 1 ml cold lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 150 mM sodium chloride, 1% Triton X-100) with vortexing. Cellular debris (membranes, DNA, etc.) were pelleted by centrifugation at 14000 rpm for 15 minutes, and the cleared lysate was transferred to a new tube and used immediately since the phosphorylation observed on the 9G8 protein disappeared upon freeze/thawing.

ISOPROPANOL PRECIPITATION

In order to concentrate samples and to remove sucrose from polysome gradient fractions under circumstances where immunoprecipitation was not used, such as in the case of looking at endogenous proteins, isopropanol precipitation was used instead. For this, an equal volume of cold isopropanol was added to gradient fractions (200-600 μl each) and incubated at –20 C for 30 minutes. The precipitates were pelleted at 13000 rpm for 15 minutes and washed with cold acetone. The pellets were allowed to air dry and then resuspended in 1× SDS Sample Buffer (0.5% SDS, 12.5% glycerol, 62.5 mM Tris, 0.05% β-mercaptoethanol, small amount phenol red) plus ammonium hydroxide vapor, boiled for 15 minutes to aid in resuspension, and run on 15% SDS-PAGE.

IMMUNOPRECIPITATION

In order to concentrate samples, to remove sucrose from polysome gradient fractions, or to investigate the interaction of two proteins, immunoprecipitation was used. For this, cellular lysates or sucrose fractions were mixed with 20 μl of agarose-conjugated antibody slurry. TBS was added to each sample to either dilute the sucrose concentrations, which aided in pelleting the agarose for recovery of bound proteins, or to increase the volume in the 1.5 ml microfuge tube for better mixing of the samples during tumbling. Samples were tumbled at 4 C for 1-2 hours. For experiments in which immunoprecipitation was performed

to concentrate samples or to remove sucrose, samples were spun briefly to pellet agarose and bound proteins and were left unwashed. For experiments to determine protein interactions, samples were washed 4× in lysis buffer and 1× in TBS. Bound proteins were eluted by the addition of 1× SDS Sample Buffer (0.5% SDS, 12.5% glycerol, 62.5 mM Tris, 0.05% β-mercaptoethanol, small amount phenol red), boiled, and run on 15% SDS-PAGE.

RNASE A TREATMENT

In order to determine if the interaction between co-immunoprecipitated samples was direct or mediated by RNA, lysates were treated with 10 mg/ml RNase A, incubated on ice for 10 minutes, centrifuged at 13000 rpm at 4 C for 5 minutes, and supernatants were transferred to new tubes. Samples were mixed with 4× SDS Sample Buffer (2% SDS, 50% glycerol, 250 mM Tris, 0.2% β-mercaptoethanol, small amount phenol red) and run on 15% SDS-PAGE.

ALKALINE PHOSPHATASE REACTION

Sucrose gradient fractions (600 µl each) were either left untreated or treated with 1 µl of highly active recombinant calf intestinal alkaline phosphatase (140 U/µl; Roche Applied Science) for 30 minutes at 37 C. Fractions were immunoprecipitated using anti-T7-conjugated agarose or isopropanol precipitated and analyzed by Western blot.

PP1 PHOSPHATASE REACTION

Lysates (1 ml, 10 cm plate) from 293T cells transfected with T7-9G8 were divided into two parts. One half was treated with phosphatase inhibitors (Sigma, 1× final) and the other half was treated with 1 µl PP1 (New England Biolabs; 2.5 U/µl) at 30 C for 30 minutes. Samples were then analyzed by Western blot.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHOSESIS (SDS-PAGE)

SDS-PAGE was used to separate proteins based on their molecular mass. Proteins were either electrophoresed directly from cell lysates, or they were fractionated from a sucrose gradient and/or immunoprecipitated prior to electrophoresis. Fractionation and immunoprecipitation are described elsewhere. Cleared lysate (20-75 μl) was mixed with 4X SDS Sample Buffer (2% SDS, 50% glycerol, 250 mM Tris, 0.2% β-mercaptoethanol, small amount phenol red), boiled for 5-20 minutes, spun briefly, and loaded onto a SDS-15% polyacrylamide gel [resolving gel: 30:0.14 ratio of Acrylamide:Bis, 375 mM Tris, pH 8,6, 0.1 % SDS, 0.05 % ammonium persulfate, 0.1% TEMED; and the stacking gel: 5% acrylamide and 0.13% Bis (30:0.8 Acryl:Bis), 124 mM Tris, pH 6.8, 0.1 % SDS, 0.05% ammonium persulfate, 0.1 % TEMED]. Gels were electrophoresed at 50 volts (constant voltage) in SDS buffer (25 mM Tris, pH 8.5, 198 mM glycine, 0.1% SDS) until the phenol red dye front entered the resolving gel, at which point, the voltage was turned up to 78 volts and run overnight until the dye front ran off the bottom of the gel.

WESTERN BLOT

During the final 30 minutes of SDS-PAGE, Immobilon-P PVDF membrane (Millipore) was cut to the size of the gel and wetted in 100% methanol. The membrane was then soaked in Western transfer buffer (25 mM Tris-HCl, 200 mM glycine, 20% methanol) to equilibrate. Proteins were transferred from the gel to the membrane using the Genie Blotter transfer apparatus from Idea Scientific Company at 16 volts and 4 C for 45-60 minutes (longer transfer times for larger proteins of interest and SR Proteins, which are notorious for being difficult to transfer). After the transfer, the membrane was removed from the apparatus and blocked in either milk for regular blots (5% non-fat, powdered milk in PBS with 0.05% Tween 20) or BSA for phospho-blots (5% BSA in PBS, 0.05% Tween 20) for 1 hour at room temperature, or overnight at 4 C, with rocking.

ANTIBODIES

Anti-T7	Novagen (69522-3) mouse monoclonal IgG_{2b}	1:5000
Anti-FLAG M2 Affinity Gel	Sigma (A2220) mouse monoclonal	20 μl slurry/IP
Anti-T7 Agarose	Bethyl Laboratories, Inc. (S190-116) polyclonal goat	20 μl slurry/IP
Anti-T7	Novus (NB 600-371) polyclonal goat	1:1000
Anti-mouse IgG-HRP	Amersham Biosciences (NXA931) sheep	1:5000
Anti-rabbit IgG-HRP	Amersham Biosciences (NA934) donkey	1:5000
mAb105	ATCC hybidoma cell line (CRL-2067) mouse monoclonal IgM undiluted-1:100. Hybridoma cells were cultured by Bill Bailey at the Hybridoma Center of the University of Virginia.	
Anti-FLAG M2	Sigma (F3165) mouse monoclonal	1:500
Anti-Zinc Knuckle	BioSource Custom Antibodies rabbit polyclonal	1:250

GST-FUSION PROTEIN EXPRESSION AND PURIFICATION

The pGEX-T2-Zn Knuckle plasmid was retransformed into the *E. coli* strain, BL21(DE3) (Novagen), which carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. This allows expression of the GST-9G8 Zinc Knuckle fusion protein, which is under the control of the T7 promoter. A small (5 ml) culture of this transformation product was grown in the presence of Ampicillin overnight, 1 ml of which was then used to inoculate 100 ml of LB plus Ampicillin. This was grown at 37 C with shaking for 90 minutes, until the OD_{260} reached 0.6. Isopropyl-beta-D-thiogalactopyranoside (IPTG; 0.5 mM final) was then added to activate the *lacUV5* promoter, and the culture was allowed to continue to grow for another 3 hours with very vigorous shaking. The bacterial cells were then pelleted at 3000 rpm for 15 minutes, and the pellet was lysed in 4 ml B-PER (Pierce) with gentle shaking at room temperature for 15 minutes. The lysate was pelleted at 14000 rpm for 15 minutes. The pellet, containing the inclusion bodies, membranes and cellular walls, was washed 4× in B-PER diluted 1:10 plus lysozyme (40 ml each) with vortexing until the brownish bacterial cell wall was washed away leaving a white pellet containing virtually

pure GST-Zn Knuckle protein. The pellet was weighed and solubilized in Inclusion Bodies Solubilization Buffer (Pierce) at 3 mg/ml.

ANTIBODY PRODUCTION

Inclusion bodies purified from *E. coli* expressing the pGEX-T2-Zn Knuckle plasmid were solubilized in Inclusion Body Solubilization Buffer (Pierce, #78115) at 3 mg/ml. One and a half milliliters of this solution was sent to BioSource to immunize two New Zealand white rabbits (project 6893). At day 38 post-immunization, 2 ml bleeds were taken from each rabbit and sent back to us to test for immune reaction to the 9G8 protein. Both sera reacted in a Western blot, and BioSource was instructed to boost the rabbits and produce more sera. Two production bleeds of 25 ml each were produced from each rabbit followed by terminal bleeds of 75 ml. Sera contained 0.1% sodium azide. Each bleed was tested for reaction to 9G8 protein using Western blot. The sera were then mixed with glycerol (5% final concentration), aliquoted, and frozen at –20 C.

^{35}S LABELING AND IMMUNOPRECIPITATIONS

Transfected 293T cells were washed twice with methionine and cysteine-free Dulbecco's modified Eagle medium (Gibco/Invitrogen) and then incubated at 37°C in labeling medium containing 200 μCi of ^{35}S Trans-label (ICN) and 10% dialyzed bovine calf serum for 20min. "Chase" experiments and immunoprecipitations were performed as previously described (93).

CHAPTER 3

The Splicing Regulatory SR Protein, 9G8, Plays a Role in Translation

Introduction

9G8 and *Tap* are both alternatively spliced and can be expressed as isoforms with retained introns. In addition, 9G8 and Tap each regulate their own expression and are thought to interact with each other to promote mRNA export. Tap also promotes translation of intron- and CTE-containing transcripts. It is an attractive thought that the proteins that determine which splice sites to use might also play a role in expression of those spliced products. In light of these observations, we set out to determine if SR proteins could enhance expression from a reporter mRNA containing an intron and a CTE. The consequence of intron 3 retention of 9G8 was also explored. Here we show that moderate SRp20 or 9G8 over-expression significantly stimulates translation from unspliced RNA containing either a viral or a cellular CTE. This enhancement can be inhibited in a dominant negative manner by a 9G8 protein lacking the RS domain. Using 9G8, we show that RNA export is not affected, but that SR protein expression enhances polyribosome association of RNA containing the CTE. Furthermore, we show that both over-expressed and endogenous 9G8 are present in 80S monosomes and small polyribosomes and that hyperphosphorylated forms of this protein are significantly enriched in these fractions. Phosphorylation of 9G8 coincides with Nxt1 dissociation from Tap and monosomes, and inhibition of 9G8 RS domain phosphorylation blocks 9G8 polysome release. These results suggest further links between RNA export and translation and highlight the complexity of post-transcriptional gene regulation. Much of this work has been published (194). In the course leading to these important findings, we had several technical obstacles, and this chapter also describes the details and the rationale for the refinement of the “polysome assay” (published as a Nature Protocol; 17) and the development of a 9G8-specific antibody that was necessary to perform several of the experiments. Credit is given in the figure legends to those who helped obtain the data presented.

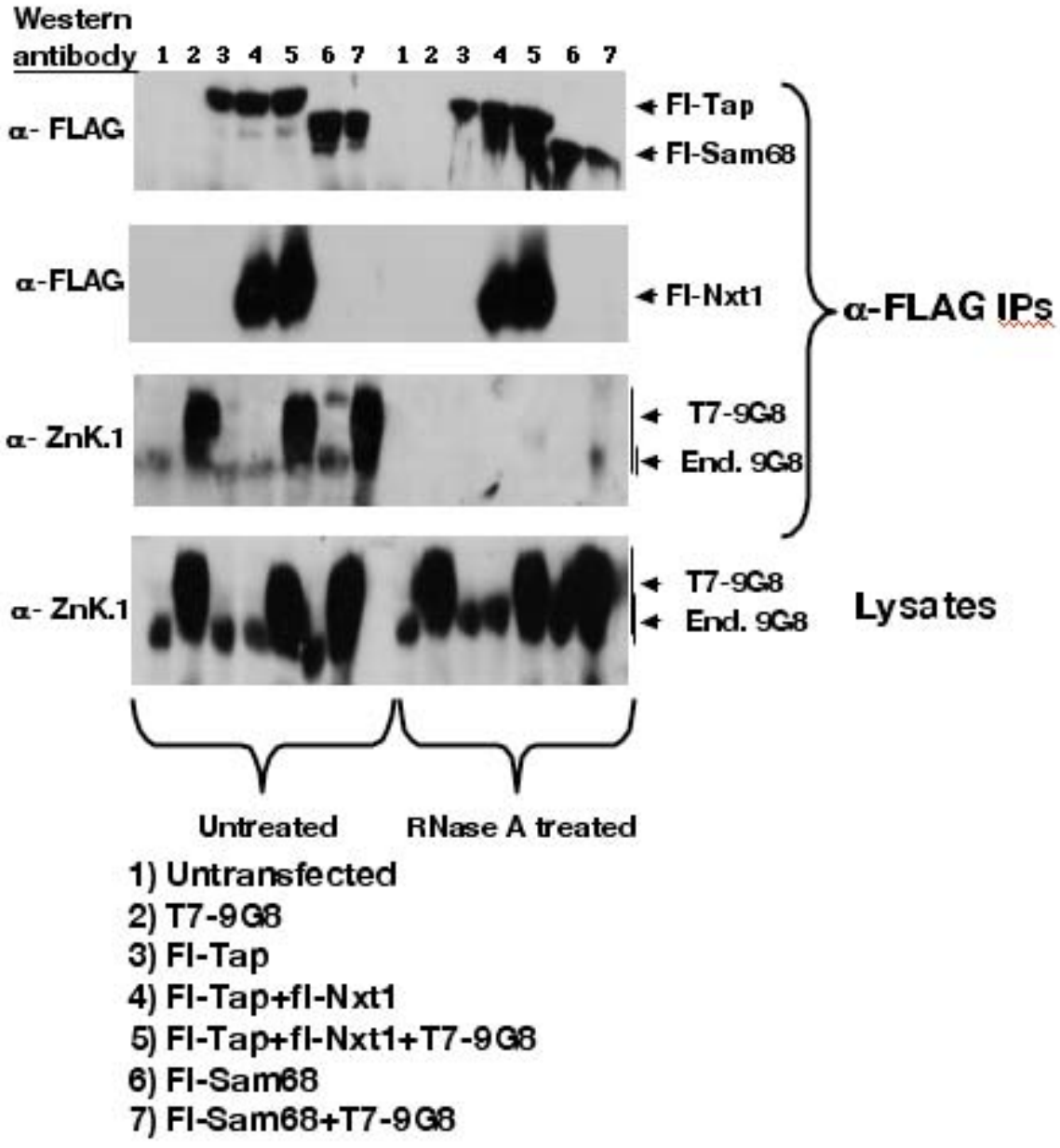
Results

9G8 and Tap do not co-immunoprecipitate in the presence of RNase. It has been previously reported that Tap and 9G8 interact directly to promote export of intronless RNA (82-85). In the reports, it was found that 9G8 had affinity for Tap only when 9G8 was in a hypophosphorylated state (85). Although one experiment was shown using cellular lysates (82), it was poorly controlled, and most of the evidence for a direct 9G8-Tap interaction was obtained from *in vitro* studies. Since we wanted to use this result to base an entire new project, we thought it was important to confirm the interaction *in vivo* and have it be properly controlled.

293T cells were transfected with plasmids expressing Tap and Nxt1 that were both FLAG-tagged as well as a T7-tagged form of 9G8. Fresh lysates were made and divided into two parts: one that remained untreated, and the other was treated with RNase A. RNase A was used in order to prevent the pull-down of one RNA-binding protein by another, mediated by an RNA to which both proteins are attached, even though the proteins may actually have no direct interaction with each other. Half of each lysate was combined with FLAG-conjugated M2 agarose beads, and the other half was reserved for running directly on the SDS-PAGE. The beads were washed and eluted with SDS Sample Buffer and loaded onto two identical gels for Western Blot analysis. The first blot was probed with anti-FLAG antibody to show that the immunoprecipition of Tap and Nxt1 was successful (Fig. 7, top two panels). The second blot was probed with an anti-9G8 Zinc Knuckle antibody (described later) to show co-immunoprecipition, if any, of 9G8 (Fig. 7, third panel). A third blot contained the non-immunoprecipitated lysates to show that the 9G8 protein had been expressed and that RNase A treatment had not affected the protein, itself (Fig. 7, bottom panel). Included in this experiment is Sam68, which had also been reported to interact with 9G8 (lanes 6 and 7) (43).

Although in the RNase A-untreated samples (Fig. 7, left set), 9G8 did come down with Tap and Nxt1 (lane 5; also with Sam68, lane 7), it also came down in the sample that had no FLAG-tagged protein whatsoever (lane 2). Since there appeared to be just as much 9G8 coming down in this condition as with

Figure 7. Tap/9G8 co-immunoprecipitation. 293T cells were transfected as indicated. Cells were lysed, divided into two parts, one of which was left untreated, and the other was treated with RNase A. Samples were then either run directly on SDS-PAGE (*lower panel*) or immunoprecipitated using α-FLAG antibody conjugated to agarose beads prior to SDS-PAGE (*upper three panels*). Western blots were either probed with α-FLAG antibody (*upper two panels*) to detect Tap, Nxt1, or Sam68 or with α-ZnK antibody (*lower two panels*) to detect transfected or endogenous 9G8.

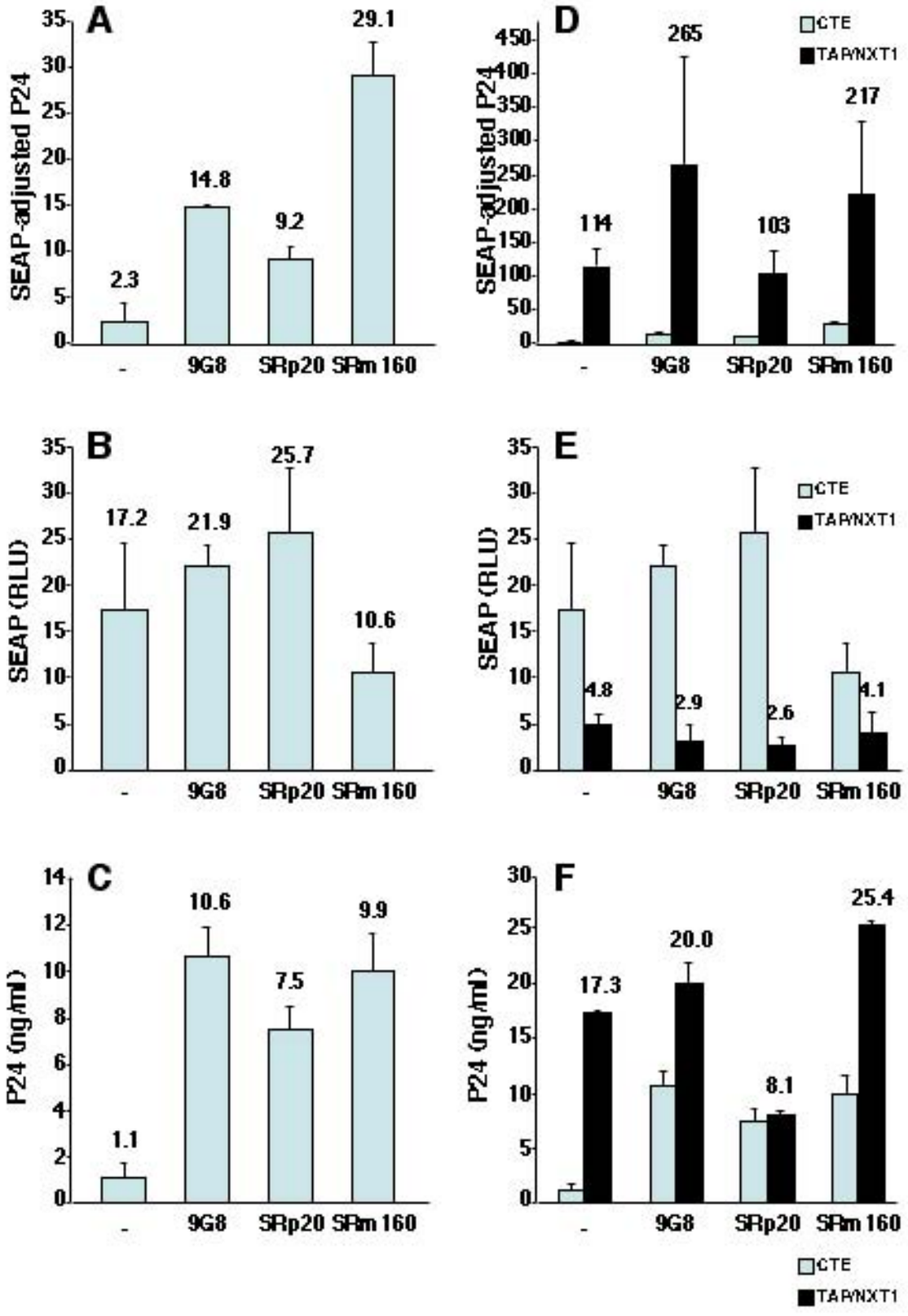

Western
antibody
1 2 3 4 5 6 7 1 2 3 4 5 6 7
α- FLAG
Fl-Tap
Fl-Sam68
α-FLAG
Fl-Nxt1
α-FLAG IPs
α- ZnK.1
T7-9G8
End. 9G8
α- ZnK.1
T7-9G8
End. 9G8
Lysates
Untreated
RNase A treated
1) Untransfected
2) T7-9G8
3) Fl-Tap
4) Fl-Tap+fl-Nxt1
5) Fl-Tap+fl-Nxt1+T7-9G8
6) Fl-Sam68
7) Fl-Sam68+T7-9G8

Tap/Nxt1 or Sam68, we must conclude that this was a non-specific pull-down. Furthermore, treatment with RNase A (Fig. 7, right) completely prevented the immunoprecipitation of 9G8 either specifically with Tap/Nxt1 (lane 5) or nonspecifically (lane 2). Since RNase did not seem to affect the protein (bottom panel, compare right with left), all the proteins were expressed at high levels, and the immunoprecipitation of Tap and Nxt1 was efficient, we conclude that under our conditions, 9G8 does not co-immunoprecipitate with Tap. This experiment was also repeated in the presence of phosphatase inhibitors or calf intestinal alkaline phosphase (CIAP) with similar results (data not shown).

Two shuttling SR proteins and an EJC protein enhance expression from unspliced RNA containing the MPMV-CTE. SRp20 and 9G8, two of the shuttling SR proteins, have been reported to bind to Tap in the nucleus and contribute to Tap-mediated RNA export (82, 83, 85, 110). Since Tap binds to the MPMV-CTE, and we have previously demonstrated that moderate over-expression of Tap and NXT1 increases translation from an RNA containing the CTE in 293T cells (93), we decided to test the effect of these SR proteins on CTE-mediated expression. In addition, we included a member of the exon junction complex (EJC), SRm160, which is an SR-like protein that binds mRNA, and like SR proteins, it has an RS domain. In these studies, we utilized a CTE reporter construct (pCMVGagPol-CTE) that encodes the HIV Gag and GagPol proteins from an mRNA that remains unspliced. This mRNA is efficiently exported because of the presence of the CTE, but it is inefficiently translated in 293T cells. Expression of the Gag and GagPol proteins from the mRNA results in the release of virus-like particles into the medium of transfected cells. This can be quantified by an ELISA assay measuring the HIV p24 protein. In these assays, cells are also transfected with a plasmid expressing SEAP (pCMVSEAP) from a spliced RNA. SEAP activity is measured in the supernatant and serves as a control for transfection efficiency, potential promoter competition and other "non-specific" effects. We have utilized this assay in many previous studies of CTE function (68, 93, 125).

Figure 8 shows that expression of 9G8, SRp20, or SRm160 (left panels) in conjunction with the CTE reporter in 293T cells resulted in a significant enhancement of p24 expression (Fig. 8C) but had no

Figure 8. Effects of SR and SR-like proteins without (*A-C*) or with (*D-F*) Tap/Nxt1 on p24 expression from a GagPol-CTE reporter construct in 293T cells. 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP without or with pCGT79G8, pCGT7SRp20 or SRM160. At 72 h post transfection, supernatants were collected and analyzed for p24 levels (*C and F*) and SEAP activity (*B and E*). p24 activity was adjusted to SEAP activity (*A and D*). The data represent the average of two independent transfections. [p24 assay performed by Yeou-cherng Bor (YCB)]


A
SEAP-adjusted P24
35
30
25
20
15
10
5
0
2.3
14.8
9.2
29.1
-
9G8
SRp20
SRm 160
D
SEAP-adjusted P24
450
400
350
300
250
200
150
100
50
0
114
265
103
217
CTE
TAP/NXT1
-
9G8
SRp20
SRm 160
B
SEAP (RLU)
35
30
25
20
15
10
5
0
17.2
21.9
25.7
10.6
-
9G8
SRp20
SRm 160
E
SEAP (RLU)
35
30
25
20
15
10
5
0
4.8
2.9
2.6
4.1
CTE
TAP/NXT1
-
9G8
SRp20
SRm 160
C
P24 (ng/ml)
14
12
10
8
6
4
2
0
1.1
10.6
7.5
9.9
-
9G8
SRp20
SRm 160
F
P24 (ng/ml)
30
25
20
15
10
5
0
17.3
20.0
8.1
25.4
-
9G8
SRp20
SRm 160
CTE
TAP/NXT1

significant effects on SEAP levels (Fig. 8B). Levels of p24 adjusted to SEAP are shown in Fig. 8A. In the experiment shown, 1 μg of plasmids expressing the SR or SR-like proteins were used together with 5 μg and 0.5 μg of the CTE and SEAP reporter plasmids, respectively, to transfect 3×10^6 cells. Quantitative Western blot analysis using 9G8 specific antibodies indicated that this led to about 10-fold over-expression of 9G8 (data not shown). However, a similar enhancement was seen with as little as 100 ng of the SR plasmids (Fig. 10). Thus, moderate over-expression of either 9G8 or SRp20 specifically increased CTE function without significant effects on SEAP expression.

For comparison, p24 and SEAP expression is also shown in the presence of Tap and Nxt1 (right panels). The data for the SR proteins is identical on the left and the right, with the scales expanded in Figs. 8D and F to include the Tap/Nxt1 data. As previously shown, Tap/Nxt1 enhances p24 expression from GagPolCTE (Fig 8F). In addition, Tap/Nxt1 has moderate additive effects when combined with 9G8 and SRm160. SRp20 actually seems to counter, somewhat, the Tap/Nxt1 enhancement. Unlike the SR proteins, Tap/Nxt1 does have an effect on SEAP expression (Fig. 8E) by inhibiting it about 60-90%. Recent data in the lab indicates that it is Nxt1 rather than Tap that negatively affects SEAP expression (data not shown). This affects the SEAP-adjusted p24 values (Fig. 8D) so that the combination of Tap/Nxt1 with 9G8 or SRm160 changes from an additive effect to one that is synergistic, and the negative effect shown by SRp20 is nullified. Thus, co-expression of Tap/Nxt1 with 9G8 or SRm160 have either an additive or a synergistic effect of CTE function, depending on whether or not the effect on SEAP expression is taken into account.

To demonstrate that the effect of the SR proteins was not limited to expression in transient transfections, we repeated the experiments using B2.23 cells, a 293T cell line that is constitutively expressing p24 from an integrated CMVGagPol-CTE plasmid (similar to B4.14 cells described in (189). We chose to perform the experiments with one of the SR proteins, 9G8, because it was the SR proteins, not EJC proteins, that had been reported to interact with Tap. Furthermore, although in the absence of exogenous Tap/Nxt1, our results showed that the effects of SRp20 and 9G8 on CTE expression were very similar, in the presence of transfected Tap/Nxt1, SRp20 had a negative or no additional effect. Even though 9G8 and SRp20 have overlapping functions, in the context of Tap function, there may be differences.

Transfection of B2.23 cells with the plasmid expressing 9G8 resulted in a significant (almost 10-fold) enhancement of p24 expression (Fig. 9A). As before, the cells were co-transfected with the SEAP-expressing plasmid (Fig. 9B). 9G8 had no significant effect on SEAP-expression, confirming the results from the original experiments.

We have found that the transfected amount of plasmids expressing certain proteins can have profound effects on the level of expression from the GagPol reporter plasmids. At very low plasmid amounts, not enough protein is made to produce an effect. As more plasmid is titrated into the cells, more protein is produced from the plasmid, and higher levels of p24 enhancement are observed. Usually, however, there is a maximal level of protein that is beneficial for reporter expression, and additional protein will either produce no additional enhancement or even have a negative effect. For this reason, it is important to transfect a range of plasmid amounts to determine the optimum to use in subsequent experiments. To determine the optimal amount of 9G8 plasmid to use in GagPolCTE expression, 293T cells (3×10^6) were transfected with 5 μg CMVGagPolCTE plasmid, 0.5 μg SEAP plasmid, and 0, 0.05, 0.1, 0.2, 0.5, 1, or 5 μg 9G8 plasmid. For comparison, 1 μg Sam68 plasmid, which has previously been shown to enhance expression from the CTE reporter in 293T cells (37), was also transfected along with the reporter plasmids. Figure 10 shows that as little as 0.1 μg was sufficient to enhance p24 expression. Enhancement increased commensurate with plasmid amount until 1 μg where enhancement leveled off. Over 1 μg of plasmid resulted in decreasing levels of enhancement of unadjusted p24 levels. The SEAP-adjusted p24 levels still seemed to be increasing, but this is misleading since the greater quantity of plasmid also had a negative effect on SEAP expression. Therefore, the optimal amount of 9G8 plasmid to use to enhance p24 expression from the GagPolCTE reporter plasmid in 3×10^6 293T cells is between 0.1 and 1 μg.

Figure 9. Transfection of a plasmid expressing 9G8 enhances p24 expression in B2.23 cells, a 293T cell line in which the CMVGagPol-CTE reporter was stably integrated. Transfections were performed and analyzed as described in *Fig 8*. 293T cells were transfected with pCMVSEAP in the absence or presence of pCGT79G8.

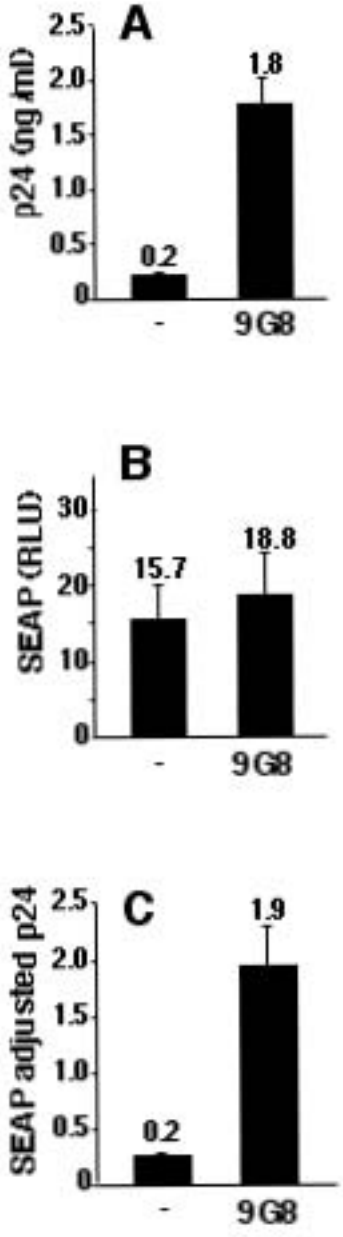
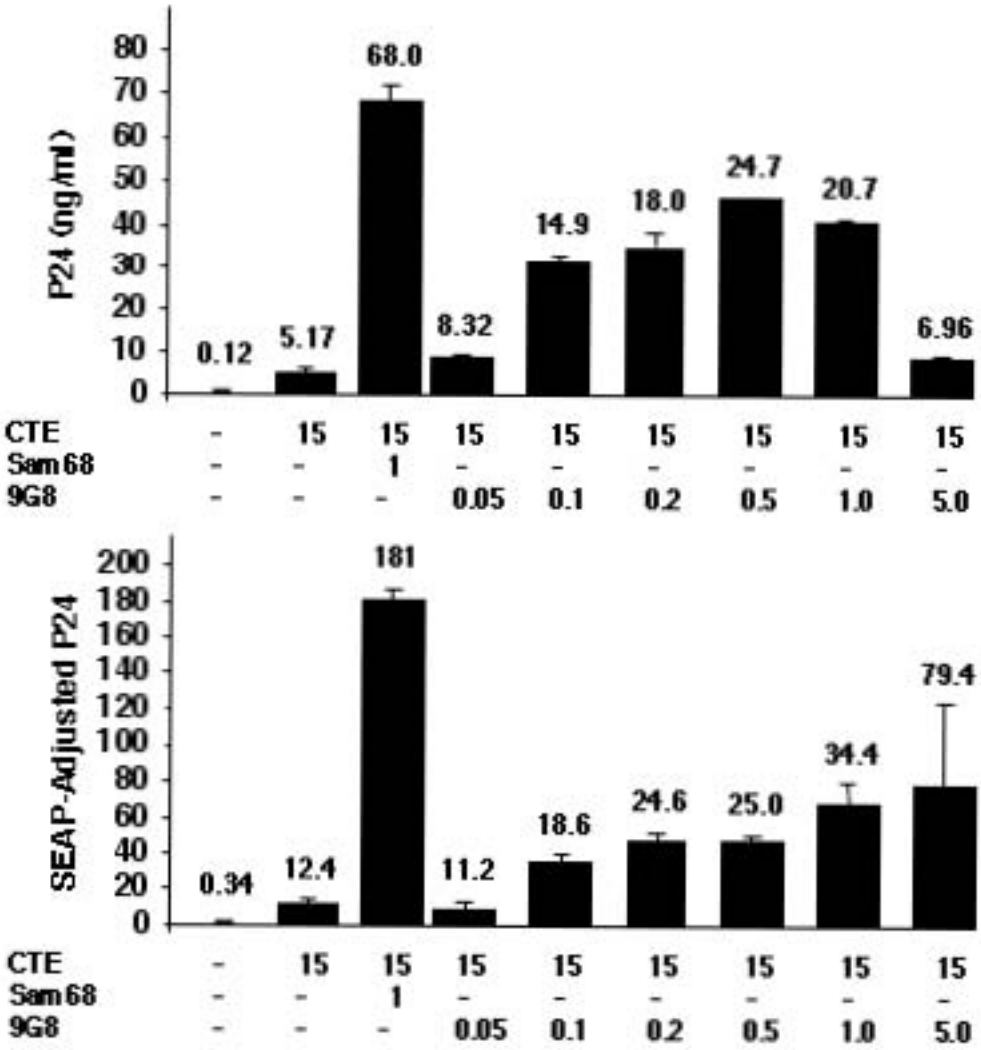

A
p24 (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
1.8
0.2
-
9G8
B
SEAP (RLU)
30
20
10
0
15.7
18.8
-
9G8
C
SEAP adjusted p24
2.5
2.0
1.5
1.0
0.5
0
1.9
0.2
-
9G8

Figure 10. 9G8 plasmid titration. Transfections were performed and analyzed as described in *Fig 8*. 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP in the absence or presence of Sam68 or a range of amounts of the pCGT79G8 plasmid.


P24 (ng/ml)
80
70
60
50
40
30
20
10
0
0.12
5.17
68.0
8.32
14.9
18.0
24.7
20.7
6.96
CTE - 15 15 15 15 15 15 15 15
Sam68 - - 1 - - - - - -
9G8 - - - 0.05 0.1 0.2 0.5 1.0 5.0
SEAP-Adjusted P24
200
180
160
140
120
100
80
60
40
20
0
0.34
12.4
181
11.2
18.6
24.6
25.0
34.4
79.4
CTE - 15 15 15 15 15 15 15 15
Sam68 - - 1 - - - - - -
9G8 - - - 0.05 0.1 0.2 0.5 1.0 5.0

9G8 enhances expression from a cellular CTE. We also performed an experiment to determine whether 9G8 would enhance expression mediated by the cellular CTE that we recently identified in the *Tap* gene (125). To this end, we transfected cells with the HIV-GagPol reporter containing one or two copies of the Tap CTE. The GagPol reporter containing the MPMV-CTE is shown for comparison. We performed co-transfections with plasmids expressing Tap/NXT1 or 9G8. The results of this experiment (Fig. 11) demonstrated that 9G8 was able to enhance p24 expression from the Tap-CTE several fold. The enhancement was even greater than what was obtained with Tap/NXT1, indicating that 9G8 can function efficiently in conjunction with the cellular CTE element.

Expression of 9G8 does not increase levels of cytoplasmic GagPol RNA. To evaluate whether the enhancement from a GagPol reporter was a consequence of increased RNA export, we next performed a Northern blot analysis on total and cytoplasmic RNA from cells transfected with pCMV GagPol-CTE either with only pSEAP (Fig. 12, lanes 1 and 8) or also co-transfected with the plasmid expressing 9G8 (lanes 4, 5, 11, and 12). Tap/Nxt1, previously shown to have no effect on export of GagPolCTE RNA (93) was included as a negative control (lanes 2 and 9). As a positive control, GagPolRRE without (lanes 6 and 13) and with HIV Rev (lanes 7 and 14) were also included. The Northern blot analysis shows that co-expression of 9G8 in this experiment increased the total GagPol-CTE RNA levels less than 2-fold (relative to the SEAP control) and had no effect on the cytoplasmic levels of this RNA. SRm160 was also tested in this experiment and had no effect on GagPol mRNA export (lanes 3, 5, 10, and 12). Thus, the observed increase in GagPol protein expression after co-expression of 9G8 or SRm160 cannot be explained by increased RNA export.

9G8 promotes polysome association of CTE-containing RNA. We have previously shown that expression of Tap and NXT1 in 293T cells promotes polyribosome association of the GagPol-CTE mRNA once it reaches the cytoplasm (93). To analyze whether the increase in p24 expression that was seen with shuttling SR proteins could be similarly explained by an increased polyribosome association, we subjected

Figure 11. Effects of 9G8 on p24 expression from TapCTE-containing reporter constructs in 293T cells. 293T cells were transfected with pCMVGagPol-TapCTE×1 or pCMVGagPol-TapCTE×2 and pCMVSEAP without or with pCGT79G8 or pcDNAFLAG-Tap and pcDNANXT1. Analyses were performed as described for *Fig. 8.*

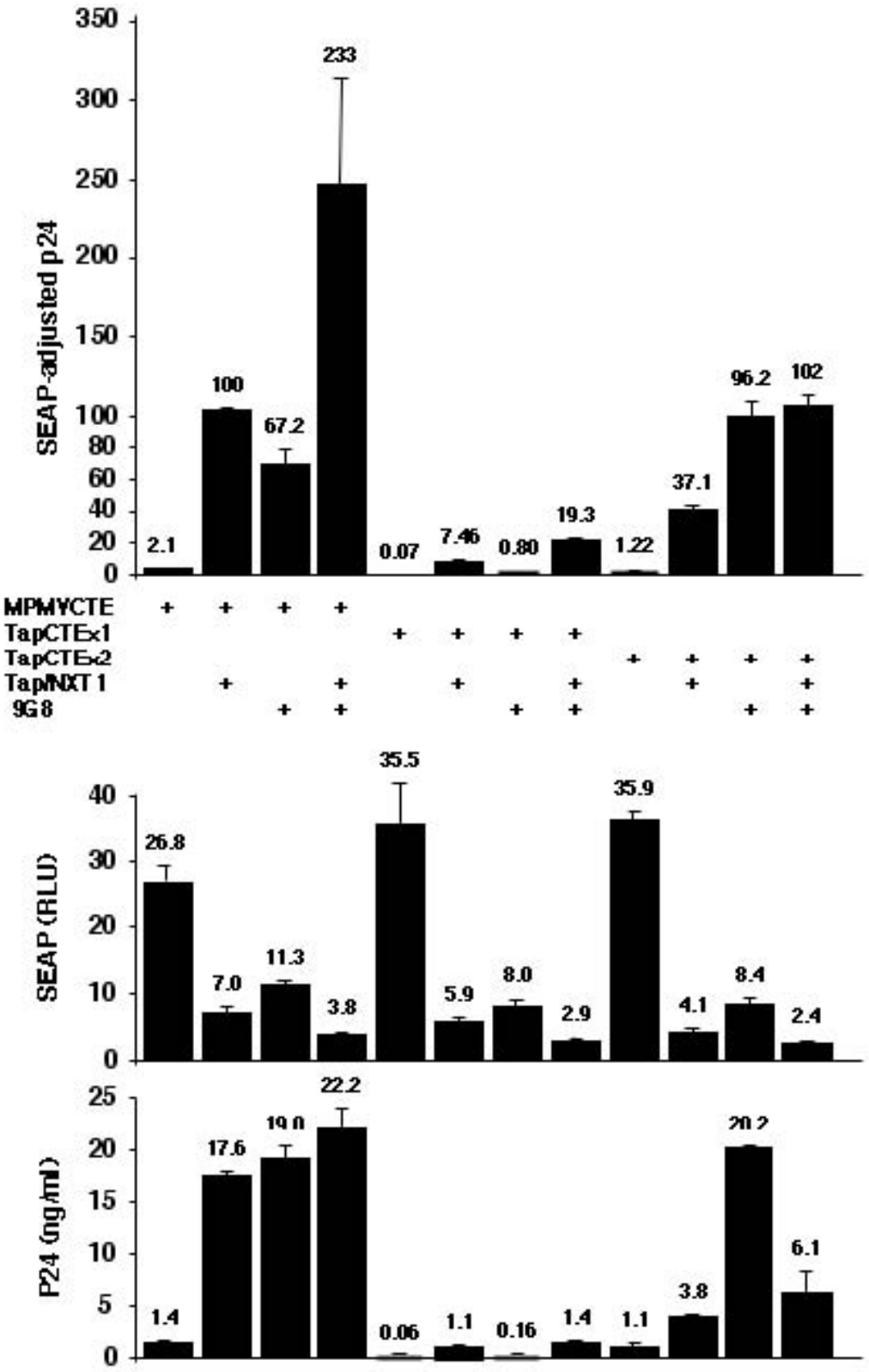

SEAP-adjusted p24
350
300
250
200
150
100
80
60
40
20
0
2.1
100
67.2
233
0.07
7.46
0.80
19.3
1.22
37.1
96.2
102
MPMVCTE + + + +
TapCTEx1 + + + +
TapCTEx2 + + + +
TapNXT1 + + + + + +
9G8 + + + + + +
SEAP (RLU)
40
30
20
10
0
26.8
7.0
11.3
3.8
35.5
5.9
8.0
2.9
35.9
4.1
8.4
2.4
P24 (ng/ml)
25
20
15
10
5
0
1.4
17.6
19.0
22.2
0.06
1.1
0.16
1.4
1.1
3.8
20.2
6.1

Figure 12. Northern blot analyses were performed on cytoplasmic and total poly(A)+ RNA isolated from the 293T cells 65 h after transfection. *GagPol* and *SEAP* mRNAs were detected using specific radiolabeled DNA probes. The blots were analyzed using a Molecular Dynamics PhosphorImager and ImageQuant software. The numbers under each lane indicate the fold differences in the levels of the *GagPol-CTE* RNA bands compared to CTE alone after normalization for variations in SEAP mRNA levels. (Northern blot performed by Jin Li)

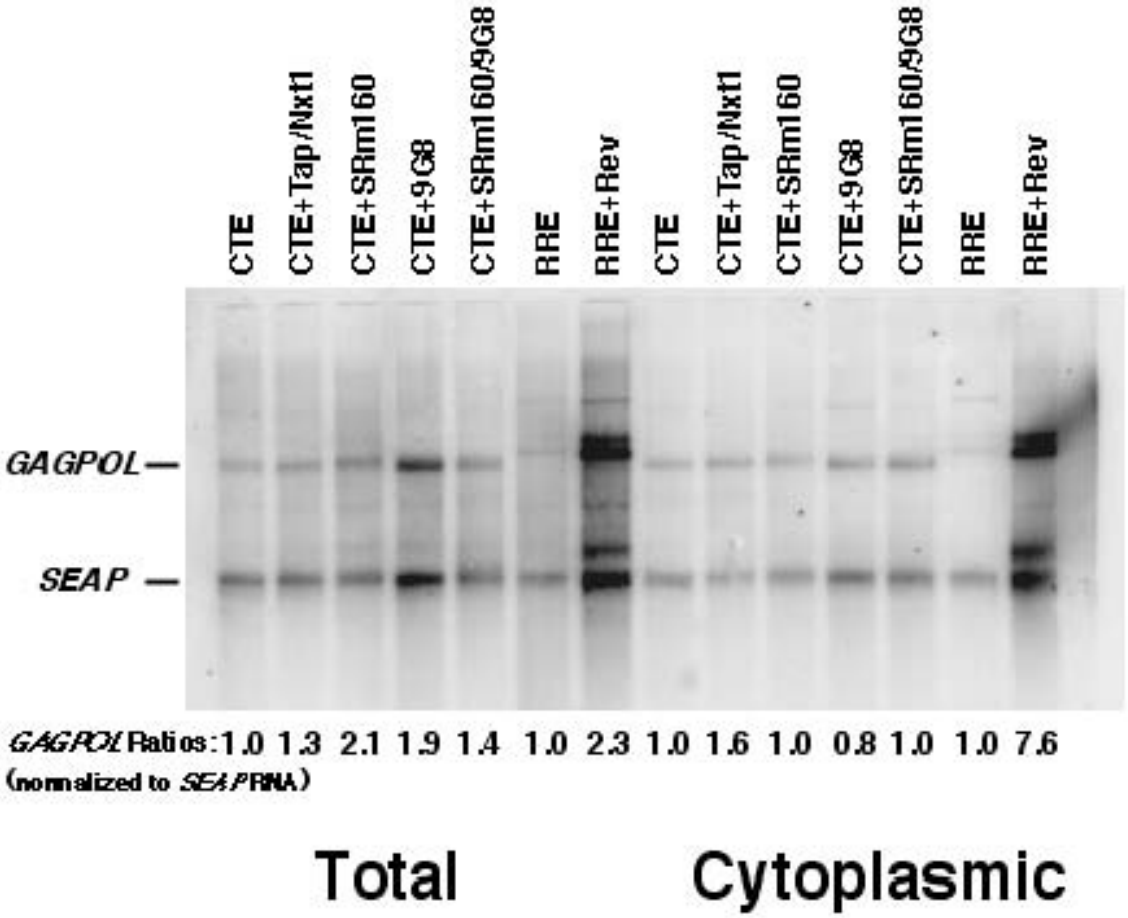
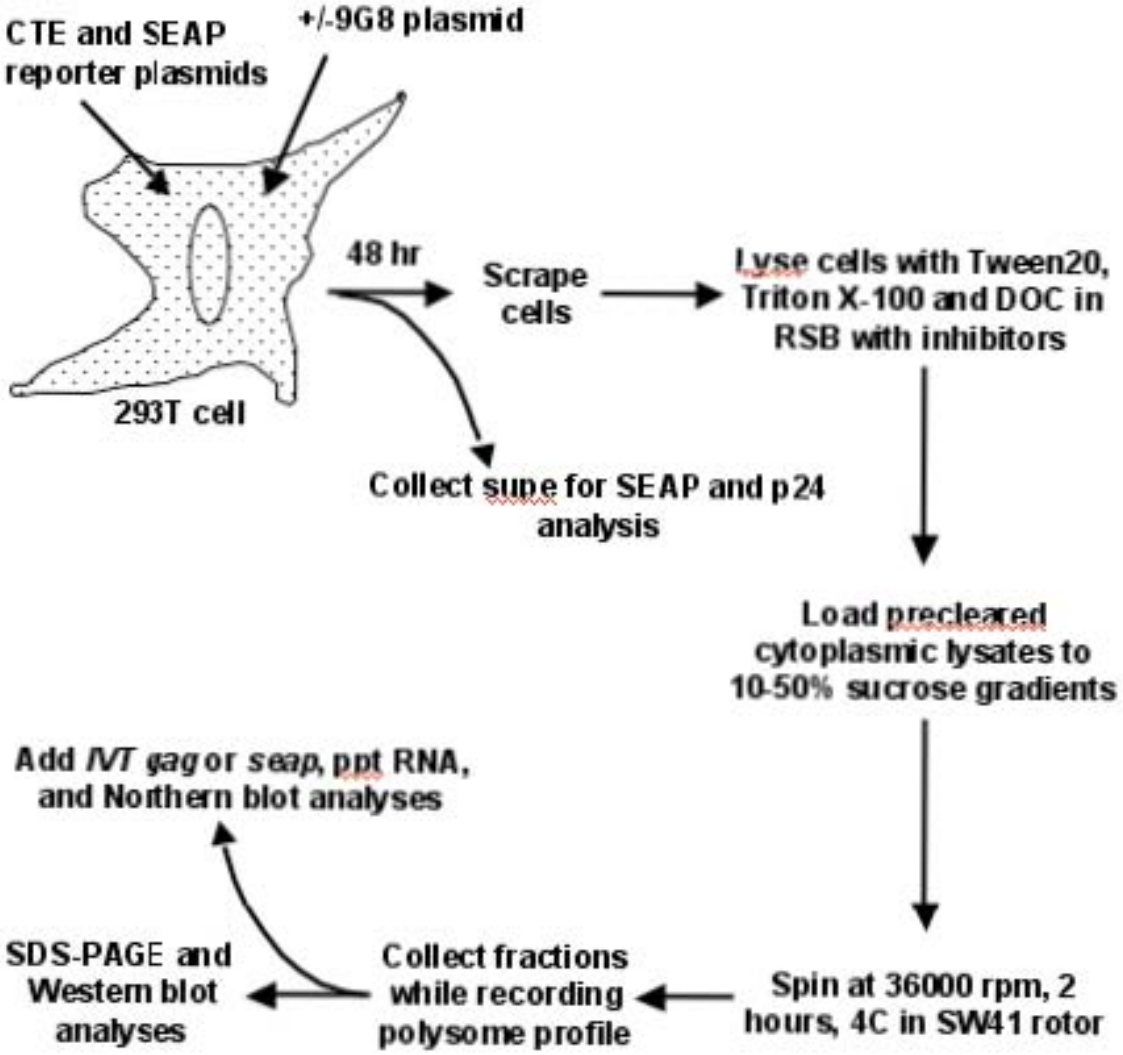

CTE
CTE+Tap/Nxt1
CTE+SRm160
CTE+9G8
CTE+SRm160/9G8
RRE
RRE+Rev
CTE
CTE+Tap/Nxt1
CTE+SRm160
CTE+9G8
CTE+SRm160/9G8
RRE
RRE+Rev
GAGPOL
SEAP
GAGPOL Ratios: 1.0 1.3 2.1 1.9 1.4 1.0 2.3 1.0 1.6 1.0 0.8 1.0 1.0 7.6
(normalized to SEAP RNA)
Total
Cytoplasmic

Figure 13. Polysome experimental design (original). 293T cells were transfected with CTE and SEAP reporter plasmids in the absence or presence of the pCGT79G8 plasmid. Forty-eight hours post-transfection, supernatants were collected for SEAP and p24 analysis, and cells were harvested for polysome analysis. Cells were lysed in RSB with three detergents (Tween 20, Triton X-100, Deoxycholate) and protease, phosphatase, and RNase inhibitors. Lysates were cleared of nuclei and cellular debris and loaded onto 10-50% sucrose gradients and centrifuged at 36,000 rpm for two hours in the cold. Gradients were then fractionated with concomitant RNA detection. Fractions were subdivided for protein or RNA analysis. For protein analysis, samples were immuno- or isopropanol precipitated and run on SDS-PAGE for Western blot analysis. For RNA analysis, *in vitro* transcribed *gag* or *seap* RNA was added to each sample, and the RNA was recovered and analyzed by Northern blot.


CTE and SEAP reporter plasmids
+/-9G8 plasmid
293T cell
48 hr
Scrape cells
Lyse cells with Tween20, Triton X-100 and DOC in RSB with inhibitors
Collect supe for SEAP and p24 analysis
Load precleared cytoplasmic lysates to 10-50% sucrose gradients
Spin at 36000 rpm, 2 hours, 4C in SW41 rotor
Collect fractions while recording polysome profile
SDS-PAGE and Western blot analyses
Add IVT gag or seap, ppt RNA, and Northern blot analyses

cytoplasmic extracts from cells (10^7) transfected with pCMVGagPol-CTE (15 μg) in the presence or absence of 9G8 (1 μg) to sucrose gradient analysis, as depicted in the schematic in Fig. 13. As before, the cells were also transfected with pCMVSEAP (0.5 μg) to serve as a control. Sucrose gradient centrifugation separated the translation machinery components according to size, where the 40S and 60S subunits remained near the top of the gradient, the 80S ribosomes (monosomes) migrated a short distance into the gradient, and the translating polyribosomes (polysomes) migrating the furthest. Bands corresponding to each fraction could be readily visualized in the centrifuge tube (Fig. 14), and increasing numbers of ribosomes on the mRNAs gave a laddering effect in the polysome section of the gradient. Gradients were fractioned from the top using the BioComp piston gradient fractionator (Fig. 5) into 20 fractions. Figures 15A and B show the polysome profiles for cells transfected with pCMVGagPol-CTE and pCMVSEAP alone and with T7-9G8, respectively. The 40S and 60S subunit, the 80S monosome, and the polysome peaks are clearly depicted.

After fractionation of the gradients, a small amount of *in vitro* synthesized *seap* RNA was added to each fraction as a recovery control (Figs. 15C and D, lower panels). RNA was then prepared from each fraction, and Northern blot analyses were performed using Gag- and SEAP-specific probes (Figs. 15C and D, upper and middle panels). Figure 15C shows that in cells transfected with pCMVGagPol-CTE and pCMVSEAP alone, most of the *gagpol-cte* RNA was present in the fractions containing the 80S ribosome complex and very small polyribosomes. In contrast, most of the *seap* RNA was detected throughout the polyribosome fractions. This confirms our previously published results that demonstrated that the *gagpol-cte* mRNA is poorly translated in 293T cells and is absent from fractions representing heavy polyribosomes (93). In the co-transfections that included 9G8 (Fig. 15D), a significantly larger fraction (40% *vs*. 4%) of the *gagpol-cte* mRNA was detected in the fractions representing polyribosomes while similar amounts of *seap* mRNA was found in these fractions. These differences are depicted in bar graph form in Figs. 15E and F.

However, Figs. 15C and D also show there was a large difference in the total amount of *gagpol-cte* RNA recovered from the gradients between the two conditions. This is in contrast to the straight

Figure 14. Polysome gradient. After the cytoplasmic lysate has been centrifuged through a 10-50% sucrose gradient for 2 hours at 36,000 rmp, the ribosomal subunits, monosomes and polyribosomes are resolved and can be detected within the gradient as separate bands. Polysomes can be resolved further into multiple bands indicating increasing numbers of ribosomes associated with the mRNA.

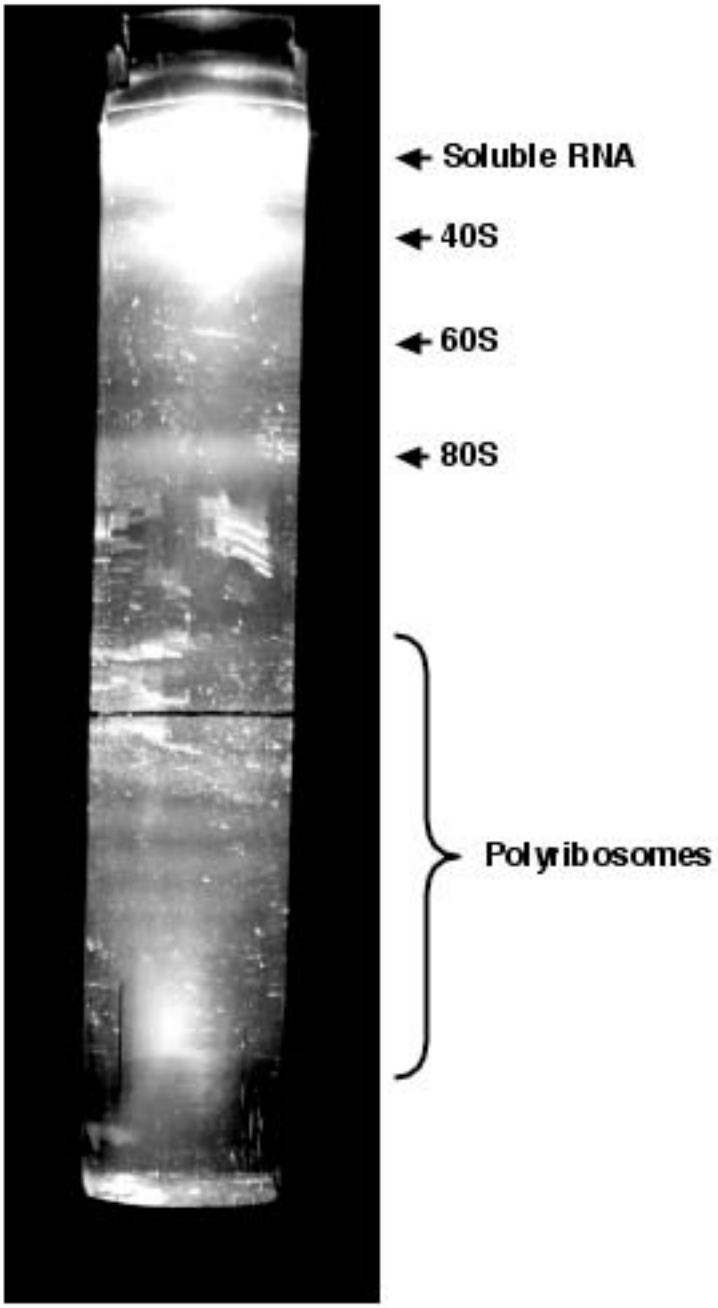
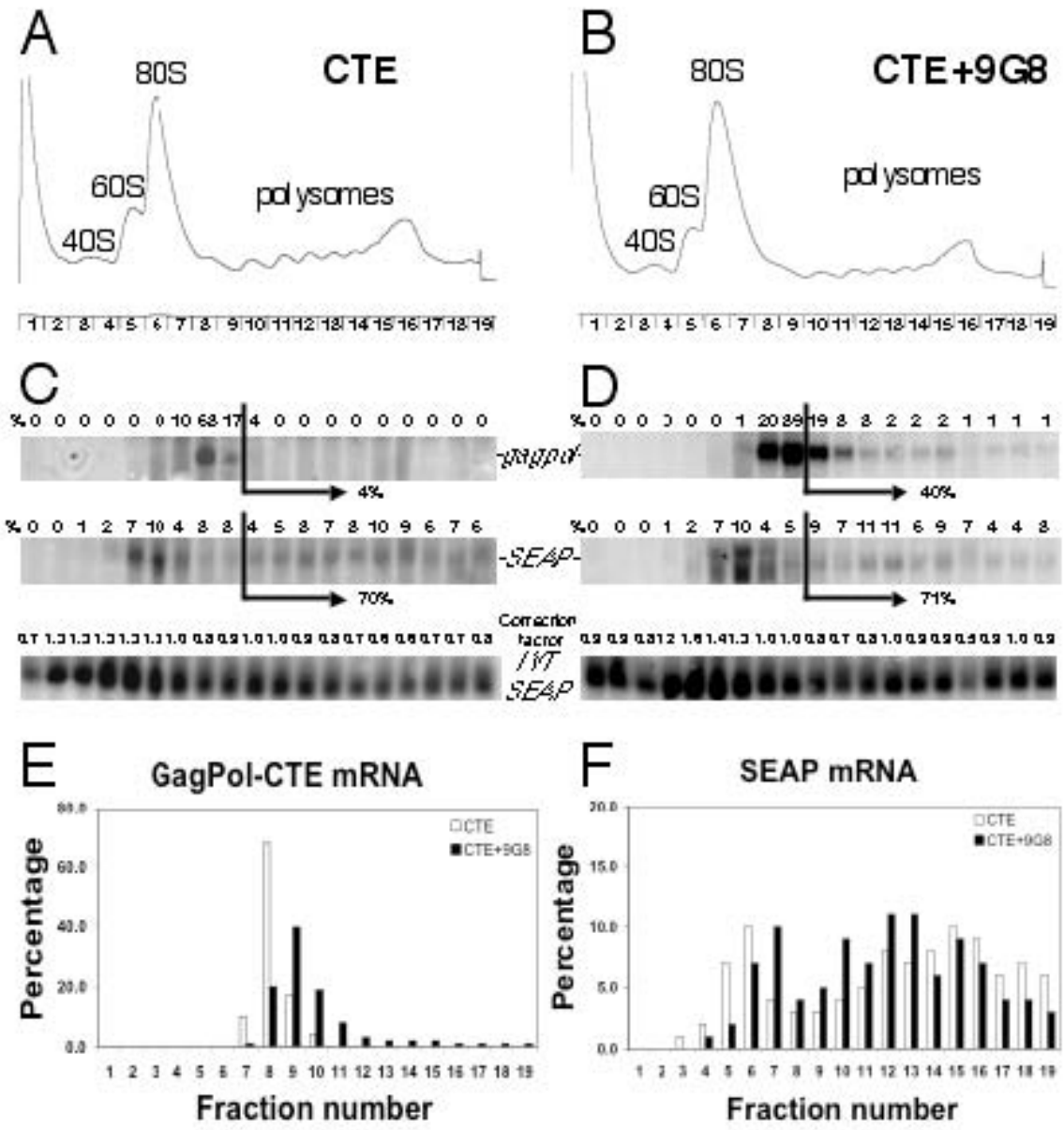

Soluble RNA
40S
60S
80S
Polyribosomes

Figure 15. Polyribosome profile analysis of GagPol-CTE mRNA in transfected 293T cells by sucrose gradient centrifugation. *A-B*, 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP in the absence (*A*) or presence (*B*) of pCGT79G8. At 48 h post transfection, cytoplasmic extracts were made and subjected to sucrose gradient centrifugation, as described in Experimental Procedures. The gradients were fractionated, and the absorbance across the gradient was monitored at 254 nm. After addition of *in vitro* transcribed *seap* RNA (*ivt seap*) for normalization purposes, RNA was isolated from each fraction and subjected to Northern blot analysis using Gag- and SEAP-specific probes (*C* and *D*). Bands corresponding to *gagpolcte* (*top panels*), *seap* (*middle panels*) and *ivt seap* (*lower panels*) were quantitated using a PhosphorImager and ImageQuant software. The measured intensity of each GagPol-CTE and SEAP band was corrected for recovery using the *ivt seap* band in each fraction. The number above each lane indicates the percentage of the total *gagpolcte* and *seap* mRNA found in the individual fractions after recovery-normalization using the *ivt seap* signal. *E-F*, The graphs show the distribution of *gagpolcte* (*E*) or *seap* mRNA (*F*) in the CTE (*white bars*) and CTE + 9G8 gradients (*black bars*). (Polysome preparation and fractionation performed by JES; Northern blots performed by YCB)


A
CTE
80S
60S
40S
polysomes
B
CTE+9G8
80S
60S
40S
polysomes
C
-gagpol-
4%
-SEAP-
70%
Correction factor
/ IVT SEAP
D
40%
71%
E
GagPol-CTE mRNA
Percentage
CTE
CTE+9G8
Fraction number
F
SEAP mRNA
Percentage
CTE
CTE+9G8
Fraction number

cytoplasmic Northern analysis (Fig. 12) in which similar levels of RNA were recovered. Although it appeared that 9G8 promoted a specific shift of this RNA into translating polysomes, it is possible that even in the absence of 9G8, *gagpol-cte* RNA was in these fractions, too, but was undetectable because of the lower levels of RNA recovered. The methods used to recover the mRNA were quite different between the two experiments (straight Northern versus polysome Northern). It is possible that 9G8 promoted a stabilization of the *gagpol-cte* RNA that would otherwise have been degraded during the extensive polysome analysis process, an instability that would not be apparent during the relatively quick straight Northern. In order to convince ourselves that 9G8 was truly promoting the polysome association of *gagpol-cte* RNA, we determined that we needed to improve the recovery of this RNA from the polysome gradients.

In our attempts to improve the recovery of *gagpol-cte* RNA from polysome gradients, we ultimately made several changes to the polysome protocol outlined in Fig. 13. The first change that helped in recovery was to ensure that the cells were in an actively growing state at the time of cell harvest. We found that when the cells became confluent, the rate at which they grew slowed. Slower cell growth corresponded with a lower rate of general translation, presumably yielding less RNA in these fractions. This could be visualized by the polysome profiles obtained from the fractionation of the sucrose gradients (compare polysome profiles in Figs. 15A and 17A). Cells that had slower cell growth had a high 80S peak and a small area under the polysome peaks, indicating low levels of translation. In this case, the RNA was likely bound to single ribosomes and poised for translation but was stalled awaiting a signal for translation. Cells with higher rates of growth had a relatively low 80S peak and a large area under the polysome peaks, indicating high levels of translation. We found that we could create polysome profiles of the second class by controlling the confluency of the cells throughout the course of the experiment. This was done by optimizing the number of cells plated prior to transfection as well as splitting the cells on the day between transfection and cell harvest.

The second change made to the polysome protocol was to attempt to more closely mimic the RNA extraction procedure from the straight Northern experiment. In the original polysome RNA extraction procedure, we simply treated the gradient fractions with phenol and chloroform, followed by ethanol

precipitation. The straight Northern also included a Proteinase K and SDS treatment prior to the phenol/chloroform extraction. Since RNA in polysomes is complexed with numerous proteins, it is possible that without the Proteinase K treatment, much of the RNA had been drawn into the interface with the protein during the phenol/chloroform step. For this reason, we included an incubation of the gradient fractions with SDS and Proteinase K at 55 C before phenol/chloroform extraction.

Although the previous modifications to the polysome profile somewhat improved the amount of *gagpol* RNA we were able to recover from the gradients, we still weren't getting similar levels without and with 9G8. Since these levels were similar in the straight Northern, this suggested that the prolonged time from cell lysis to RNA extraction between the two procedures (at least 3 hours longer for the polysome protocol) allowed for a specific loss of the *gagpol* RNA, which was rescued by the inclusion of exogenous 9G8. Stalled ribosomes could lead to the ribosomes falling off the mRNA, leaving it susceptible to degradation. Cycloheximide is known to lock ribosomes onto mRNA, which would promote greater stability. Cycloheximide has been shown to be an inhibitor of nonsense-mediated decay (NMD) (132) and to inhibit the formation of P bodies, which are sites of RNA degradation (183). To test if cycloheximide would help us recover more *gagpol* RNA from polysome gradients, we treated transfected 293T cells for 30 minutes with 50 μg/ml cycloheximide just prior to cell harvest for polysome analysis. Addition of cycloheximide to the cells did greatly improve *gagpol* RNA recovery (Fig. 17), and this step was added to the protocol. The final protocol is outlined in Fig. 16, with the changes to the original shown in color.

Figure 17 shows the results from the improved polysome protocol. Once again, Figs. 17A and B show the polysome profiles for cells transfected with pCMVGagPol-CTE and pCMVSEAP alone and with T7-9G8, respectively. Below the profiles are panels of Northern blots showing probes to *gagpol, seap, and in vitro* synthesized *gag* RNA (in this experiment, a small amount of *ivt gag* RNA was added to each fraction as a recovery control rather than the *ivt seap* used in the previous experiment). This time, it is clear that in cells transfected with pCMVGagPol-CTE and pCMVSEAP alone, most of the *gagpol-cte* RNA was only present in the fractions containing the 80S ribosome complex and very small polyribosomes, and a significantly larger fraction of the *gagpol-cte* RNA was detected in the fractions representing

Figure 16. Polysome experimental design (modified). After much trial and error, the polysome assay was redesigned to improve RNA recovery from the sucrose fractions. The following changes were made to the protocol outlined in *Fig. 13*. First, cell confluency was optimized by plating cells at about $0.8\text{-}1.0\times10^7$ cells per 15 cm plate prior to transfection, followed by the addition of splitting the cells 1:2 (or 1:3 if the cells were very confluent) on the day between cell transfection and harvest. Second, on the day of cell harvest, the cells were treated with cycloheximide for 30 minutes prior to cell lysis. Third, RNA recovery from the sucrose fractions was modified to be more similar to the standard Northern Blot RNA preparation by the addition of SDS and Proteinase K to disrupt RNP complexes prior to phenol/chloroform extraction.

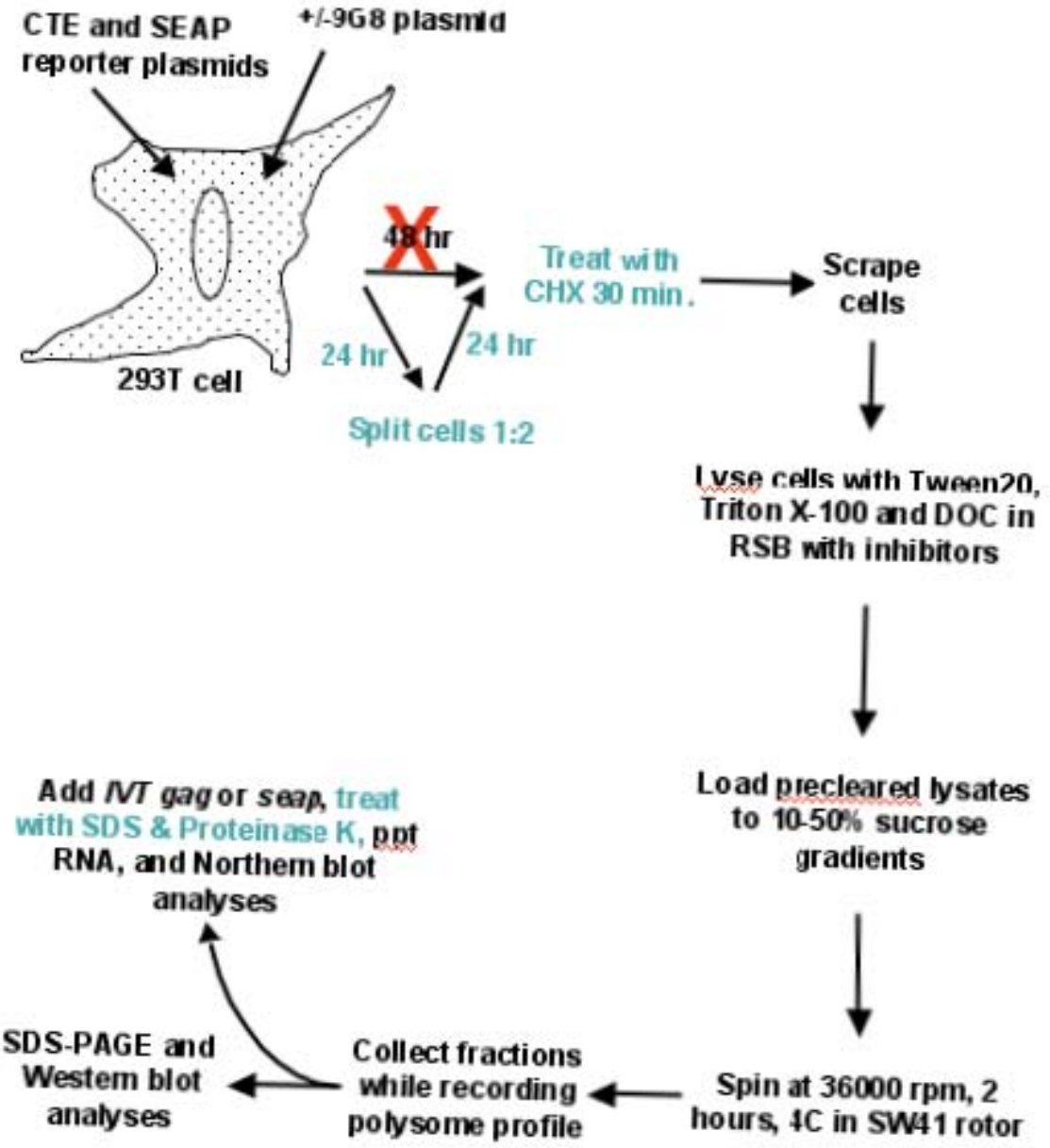
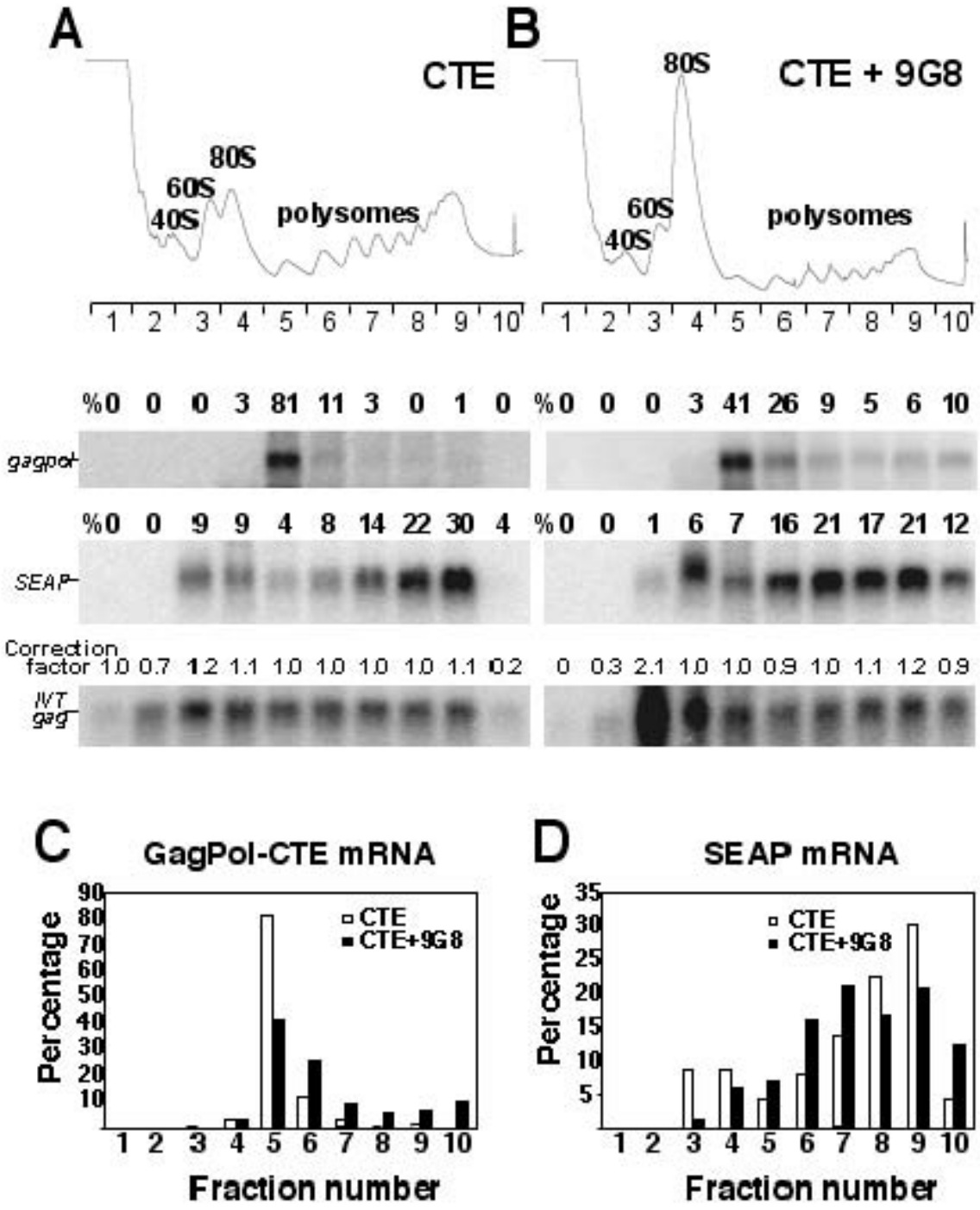

CTE and SEAP reporter plasmids
+/-9G8 plasmid
293T cell
48 hr
24 hr
24 hr
Split cells 1:2
Treat with CHX 30 min.
Scrape cells
Lyse cells with Tween20, Triton X-100 and DOC in RSB with inhibitors
Load precleared lysates to 10-50% sucrose gradients
Spin at 36000 rpm, 2 hours, 4C in SW41 rotor
Collect fractions while recording polysome profile
SDS-PAGE and Western blot analyses
Add IVT gag or seap, treat with SDS & Proteinase K, ppt RNA, and Northern blot analyses

Figure 17. Polyribosome profile analysis of GagPol-CTE mRNA in transfected 293T cells by sucrose gradient centrifugation. *A-B*, 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP in the absence (*A*) or presence (*B*) of pCGT79G8. At 48 h post transfection, cytoplasmic extracts were made and subjected to sucrose gradient centrifugation, as described in Experimental Procedures. The gradients were fractionated, and the absorbance across the gradient was monitored at 254 nm (*top panels*). After addition of *in vitro* transcribed *gag* RNA (*ivt gag*) for normalization purposes, RNA was isolated from each fraction and subjected to Northern blot analysis using Gag- and SEAP-specific probes (*lower panels*). Bands corresponding to *gagpolcte*, *ivt gag* and *seap* were quantitated using a PhosphorImager and ImageQuant software. The measured intensity of each GagPol-CTE and SEAP band was corrected for recovery using the *ivt gag* band in each fraction. The number above each lane indicates the percentage of the total *gagpolcte* and *seap* mRNA found in the individual fractions after recovery-normalization using the *ivt gag* signal. *C-D*, The graphs show the distribution of *gagpolcte* (*C*) or *seap* mRNA (*D*) in the CTE (*white bars*) and CTE + 9G8 gradients (*black bars*). (Polysome preparation and fractionation performed by JES; Northern blots performed by YCB)


A
CTE
80S
60S
40S
polysomes
1 2 3 4 5 6 7 8 9 10
B
CTE + 9G8
80S
60S
40S
polysomes
1 2 3 4 5 6 7 8 9 10
%0 0 0 3 81 11 3 0 1 0
gagpol
%0 0 0 3 41 26 9 5 6 10
%0 0 9 9 4 8 14 22 30 4
SEAP
%0 0 1 6 7 16 21 17 21 12
Correction factor 1.0 0.7 1.2 1.1 1.0 1.0 1.0 1.0 1.1 0.2
IVT gag
0 0.3 2.1 1.0 1.0 0.9 1.0 1.1 1.2 0.9
C
GagPol-CTE mRNA
Percentage
CTE
CTE+9G8
Fraction number
D
SEAP mRNA
Percentage
CTE
CTE+9G8
Fraction number

polyribosomes. The *ivt gag* RNA bands were quantitated and given a correction factor value, which was then applied to the *gagpol* and *seap* bands. The percentages of the CTE and SEAP mRNAs that were present in each fraction was then calculated and are shown above each lane. The percentages are summarized in panels C and D. This analysis shows that the GagPol-CTE mRNA is shifted to heavier fractions in the co-transfection with 9G8, whereas no significant shift is observed in the case of the SEAP mRNA, indicating that 9G8 specifically promotes translation of the CTE-containing RNA.

A significant amount of cytoplasmic 9G8 is found in polyribosomal fractions and is phosphorylated in these fractions. Since 9G8 promoted polyribosome association of the GagPol-CTE mRNA, we decided to analyze sucrose gradient fractions for presence of the 9G8 protein. For this experiment, cells were transfected with a lower amount of the 9G8-expressing plasmid (0.33 μg per 10^7 cells). Since the transfected plasmid expresses a 9G8 protein that contains a T7-epitope tag, immunoprecipitation (IP) using anti-T7 antibody-conjugated agarose was performed on each gradient fraction to specifically precipitate the exogenously expressed 9G8. The immunoprecipitated samples were then analyzed on Western blots using either the T7-specific antibody (Fig. 18B) or mAb104 (Fig. 18C), which specifically detects phospho-epitopes within the RS domain of SR proteins. Even though one of the criteria to define a protein as being an SR protein is its ability to be recognized by mAb104, and even though those in the SR protein field recognizes 9G8 as a *bona fide* SR protein, it is commonly believed that mAb104 does not recognize 9G8. The evidence for this seems to be anecdotal, and may stem from the fact that 9G8 is similar in size to several other SR proteins (SRp30c, ASF/SF2, SC35; Fig. 1) and is expressed endogenously at relatively lower levels. To directly demonstrate that mAb104 can detect phosphorylated 9G8, we performed a Western blot experiment with this antibody using whole cell lysates from untransfected and transfected cells (Fig. 19). This blot clearly demonstrated that mAb104 is able to detect the 9G8 protein expressed from the transfected plasmid in addition to multiple endogenous SR proteins.

Figure 18. T79G8 protein is mainly present in monosomes and small polyribosomes. 293T cells were transfected with pCGT79G8. *A*, The polyribosome profile analysis was performed as described for *Fig. 16*. *B*, Fractions were immunoprecipitated with anti-T7 antibody, separated by SDS-15% PAGE and subjected to Western blot analysis using the anti-T7 antibody. Cumulative percentages were calculated by adding the percent present in each fraction to the total percent lower in the gradient, and this value is shown below each lane. *C*, The Western blot shown in (*B*) was also probed using mAb104.

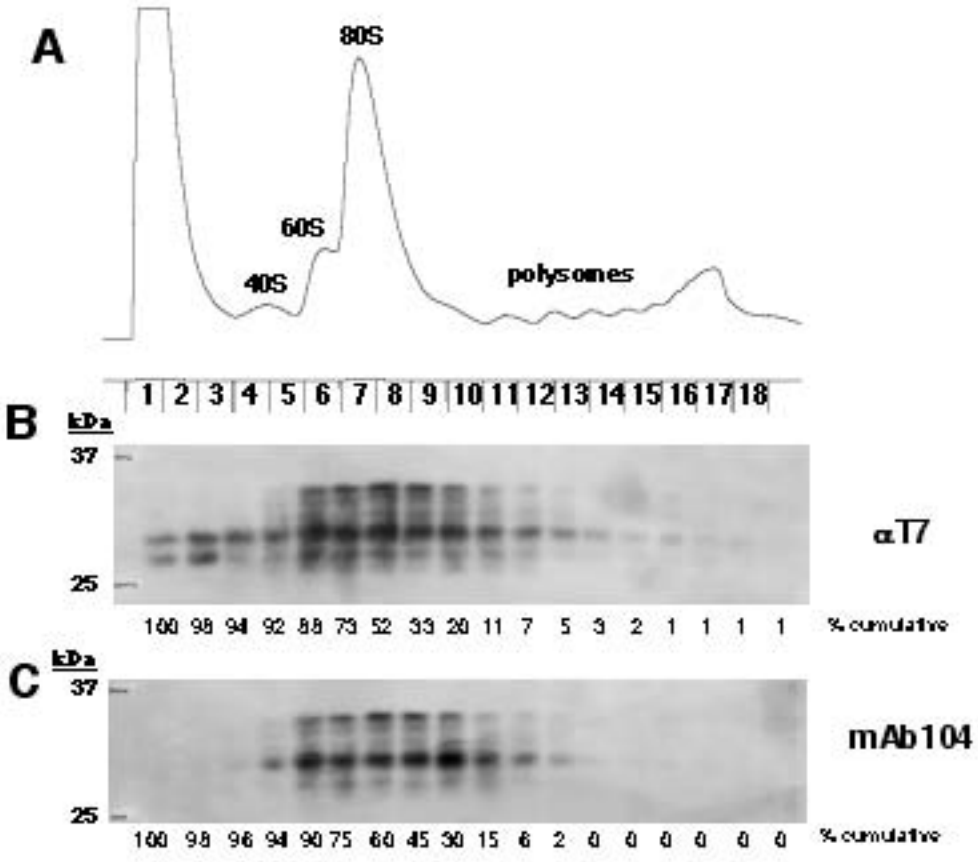
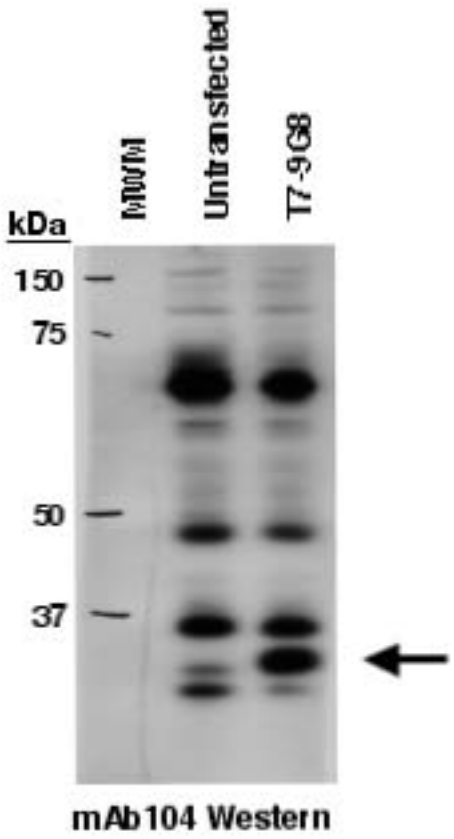

A
80S
60S
40S
polysomes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
kDa
37
25
αT7
100 98 94 92 88 73 52 33 20 11 7 5 3 2 1 1 1 1
% cumulative
C
kDa
37
25
mAb104
100 98 96 94 90 75 60 45 30 15 6 2 0 0 0 0 0 0
% cumulative

Figure 19. The mAb104 antibody recognizes T79G8. 293T cells were left untransfected or transfected with pCGT79G8. Lysates were analyzed by Western blot using mAb104. The *arrow* points to the band corresponding to T79G8. (Western blot performed by Yukiko Masawa)


kDa
MWM
Untransfected
T7-9G8
150
75
50
37
mAb104 Western

The IP/Western blot analysis of the sucrose gradient fractions revealed that both the T7 and mAb104 antibodies detected 9G8 proteins of several different molecular weights (Figs. 18B and C). The higher molecular weight bands were strongest in the fractions representing 60 and 80S complexes and small polysomes (fractions 5-10). Although the pattern seen with the two antibodies was fairly similar in these fractions, the T7 antibodies also detected two bands in fractions 2-4 that were not detected with the phospho-specific antibody. These results suggested that the fractions representing "soluble" non-ribosomal complexes contained hypophosphorylated 9G8 proteins, whereas heavier fractions contained phosphorylated protein that migrated slower because of the modifications. To confirm this, we performed an experiment in which sucrose fractions from transfected cell lysates were treated with calf intestinal alkaline phosphatase (CIAP) before Western blotting with the anti-T7 antibody (Fig. 20). As expected, this treatment “collapsed” the different 9G8 bands into a single band that still reacted strongly with the anti-T7 antibody. This band has lower apparent molecular weight than any of the bands detected with the T7 antibody in the absence of CIAP treatment (compare Figs. 18B and 20B) and was no longer detectable with mAb104 (data not shown), demonstrating that 9G8 resolves into multiple species due to differential phosphorylation.

CIAP is a non-specific serine/threonine/tyrosine phosphatase, and there is no evidence that it acts on SR proteins in the cell. This role has been attributed the serine/threonine phosphatases, protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) (31). To determine if PP1 would have a similar effect to CIAP on the 9G8 protein, 9G8-transfected 293T cellular lysate was either left untreated or treated with PP1 prior to Western blot analysis. Figure 20C shows that PP1 could also promote the mobility of 9G8 in an acrylamide gel, suggesting that the 9G8 protein had become hypophosphorylated upon PP1 treatment.

The presence of 9G8 in 80S and polysomal fractions represents a true association with translation complexes. To verify the association of 9G8 with the translation machinery, we performed additional sucrose gradient experiments in the presence or absence of 15 mM EDTA. This concentration of EDTA has been shown to efficiently disrupt mono- and polyribosomes, but not mRNP complexes or individual

Figure 20. Western blot analysis of T79G8 protein expression after phosphatase treatment. After sucrose gradient analysis (*A*), each fraction was treated with 140 U of calf intestinal alkaline phosphatase (CIAP). The fractions were then subjected to immunoprecipitation and Western blot analysis using anti-T7 antibodies (*B*). *C*, Protein phosphatase 1 (PP1) was also used in the treatment of 293T lysates expressing T79G8.

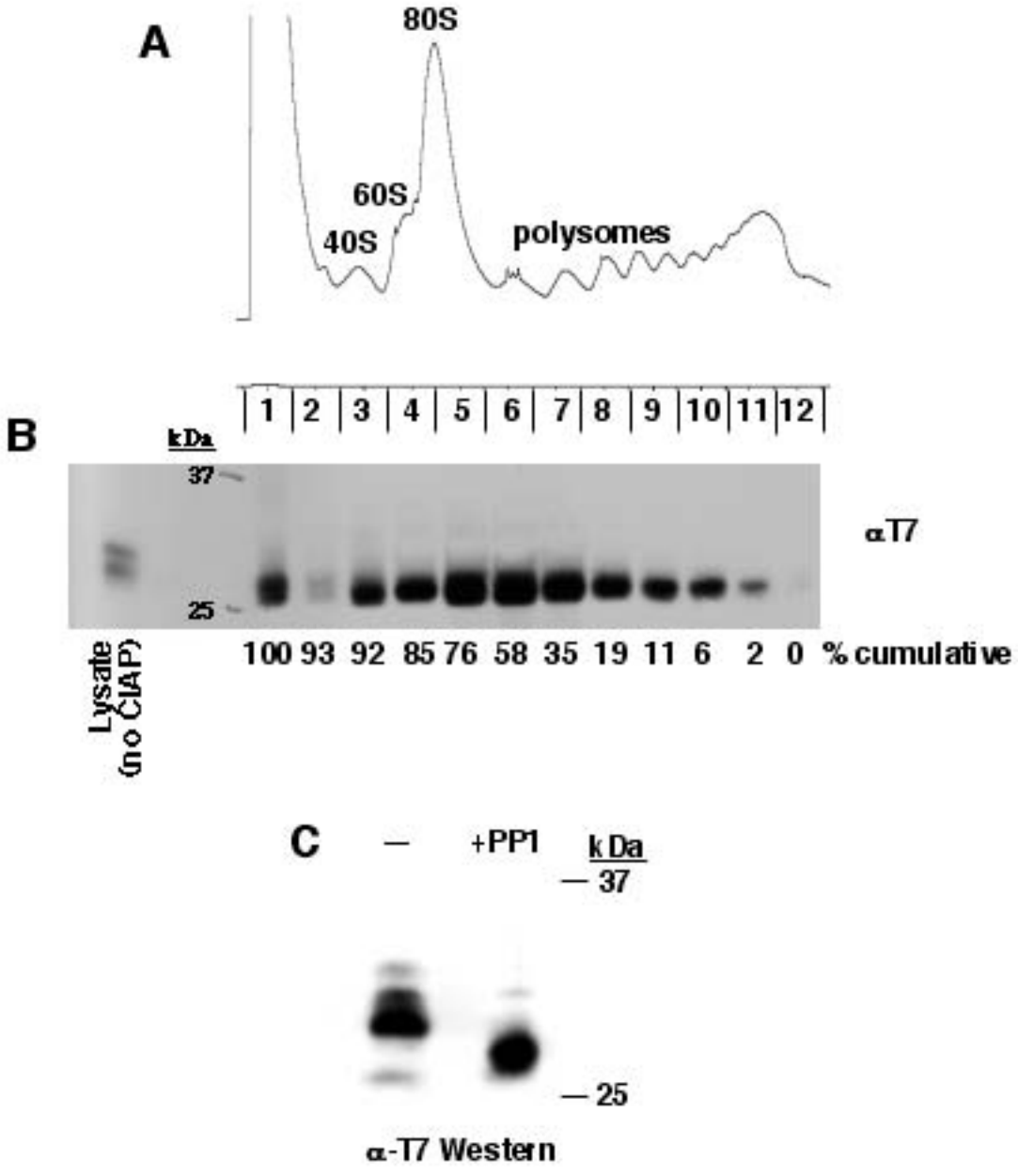
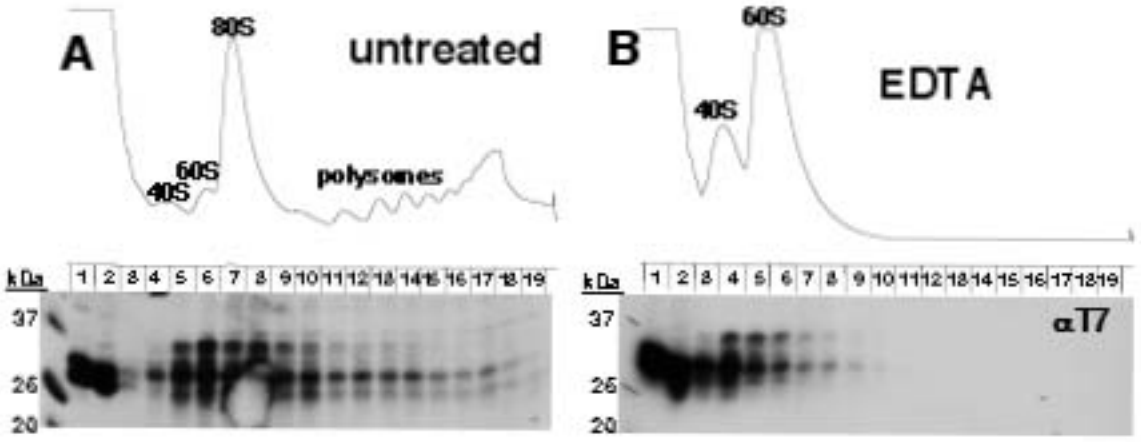

A
80S
60S
40S
polysomes
B
1 2 3 4 5 6 7 8 9 10 11 12
kDa
37
25
αT7
Lysate (no CIAP)
100 93 92 85 76 58 35 19 11 6 2 0 % cumulative
C
—
+PP1
kDa
— 37
— 25
α-T7 Western

Figure 21. Polysome gradient analyses of cells transfected with pCGT79G8 without (*A*) or with (*B*) 15 mM EDTA added to the lysate before loading onto the sucrose gradient. Polyribosome profiles and Western blot analyses were performed as described for *Fig. 16*.

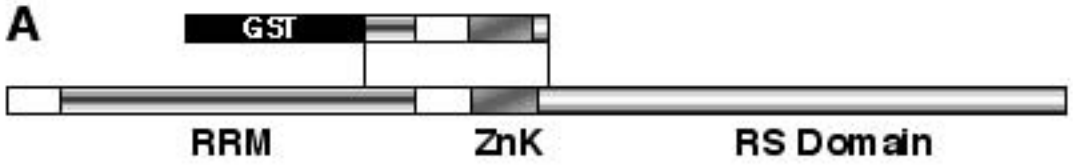

A
untreated
80S
60S
40S
polysomes
B
EDTA
60S
40S
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
37
26
20
αT7

ribosomal subunits (27, 94). These experiments were performed on cells transfected with the plasmid expressing the full-length 9G8 protein (Fig. 21). In the presence of EDTA (Fig. 21B), there was a significant shift, with most of the protein being present in fractions 1-6. These results indicate that 9G8 is normally a part of active polyribosome complexes and can be disrupted with EDTA. However, the data do not allow us to distinguish whether 9G8 is part of hnRNP complexes that sediment in the same position as the ribosomal subunits or whether it is associating with one or both subunits. In the absence of EDTA (Fig. 21A), we again observed several bands for the full-length protein, with hyperphosphorylated forms in the same fractions as before.

Development of a 9G8-specific antibody. Although the T7-tagged version of 9G8 enabled us to specifically detect transfected 9G8, lack of quality commercially available 9G8-specific antibodies prevented us from analyzing endogenous 9G8. A non-commercially available antibody, developed in the Stevénin laboratory and originally used to discover and characterize the 9G8 protein (30), exists, and small amounts of this antibody can be obtained. However, this antibody recognizes the C-terminal RS domain of the protein, which can be significantly phosphorylated, and it is not known if the phosphorylation state alters the ability of this antibody to recognize the protein. It was in our interest to be able to identify 9G8 in all of its phosphorylation states. Furthermore, we wanted to have the ability to recognize a 9G8 protein lacking the RS domain. For these reasons, we developed our own 9G8-specific antibodies using a GST-9G8 fusion protein that contained the unique zinc knuckle (ZnK) domain of 9G8 as the immunogen (see Fig. 22A).

GST was fused to the 9G8 fragment in order to use it to purify the peptide from bacterial cell lysate. However, this peptide was found almost exclusively in inclusion bodies and was extremely insoluble, even at high concentrations of salt or urea (Fig. 22B). As an alternative to GST-purification, a bacterial lysis reagent, B-PER (Pierce), designed to purify recombinant mammalian protein from induced *E. coli* cells, was used. B-PER has the ability to solubilize even the most difficult proteins, and those that B-PER is unable to solubilize exist in inclusion bodies practically free from bacterial protein

Figure 22. Development of 9G8-specific antibodies. *A*, Schematic diagram of the 9G8 protein is shown with its RNA recognition motif (RRM), the zinc knuckle domain (ZnK) and the RS domain. The region of 9G8 cDNA encoding the unique zinc knuckle that was fused to the GST coding sequence and expressed in *E. coli* is also shown. *B*, Coomassie stain of acrylamide gel of attempt to purify bacterially expressed GST-ZnK protein.


A
GST
RRM
ZnK
RS Domain

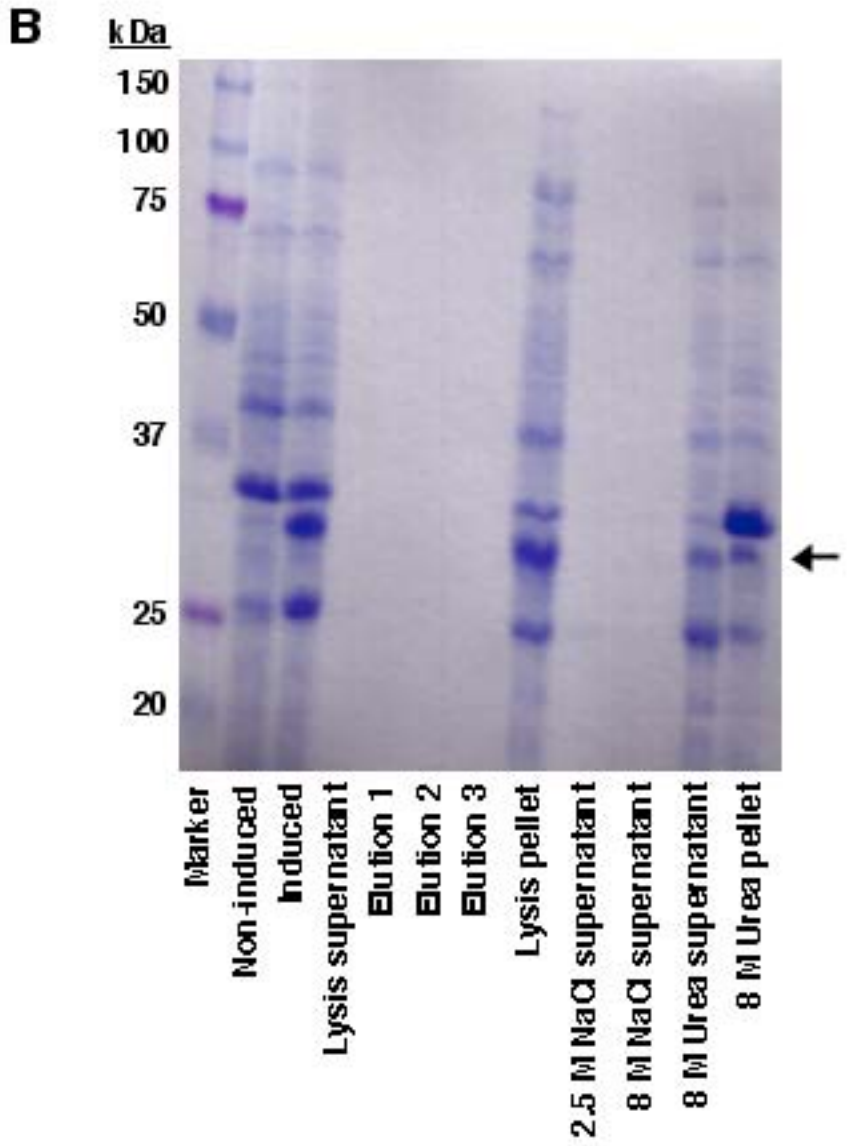
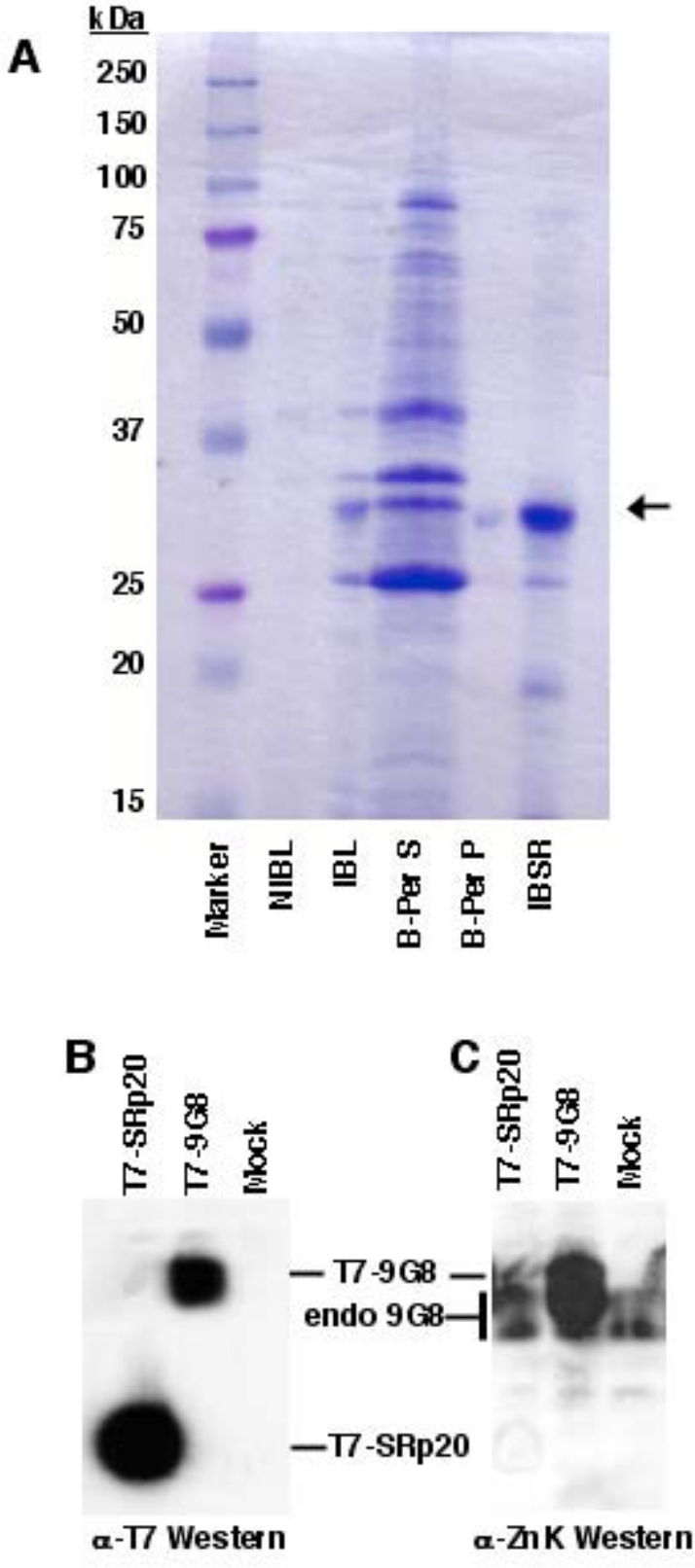

B
kDa
150
100
75
50
37
25
20
Marker
Non-induced
Induced
Lysis supernatant
Elution 1
Elution 2
Elution 3
Lysis pellet
2.5 M NaCl supernatant
8 M NaCl supernatant
8 M Urea supernatant
8 M Urea pellet

Figure 23. Development of 9G8-specific antibodies, continued. *A*, Coomassie stained acrylamide gel of B-Per purification of bacterially expressed GST-ZnK protein. Purified fusion protein was used to produce polyclonal antibodies in rabbits. *B-C*, Detection of T79G8 and T7SRp20 using an anti-T7 antibody and an anti-ZnK (9G8) antibody. 293T cells were transfected with pCGT7SRp20, pCGT79G8, or pCMV and harvested 48 h post transfection. Proteins were separated by SDS-PAGE, and subjected to Western blot analysis.


A
kDa
250
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75
50
37
25
20
15
Marker
NIBL
IBL
B-Per S
B-Per P
IBSR
B
T7-SRp20
T7-9G8
Mock
T7-9G8
endo 9G8
T7-SRp20
α-T7 Western
C
T7-SRp20
T7-9G8
Mock
α-ZnK Western

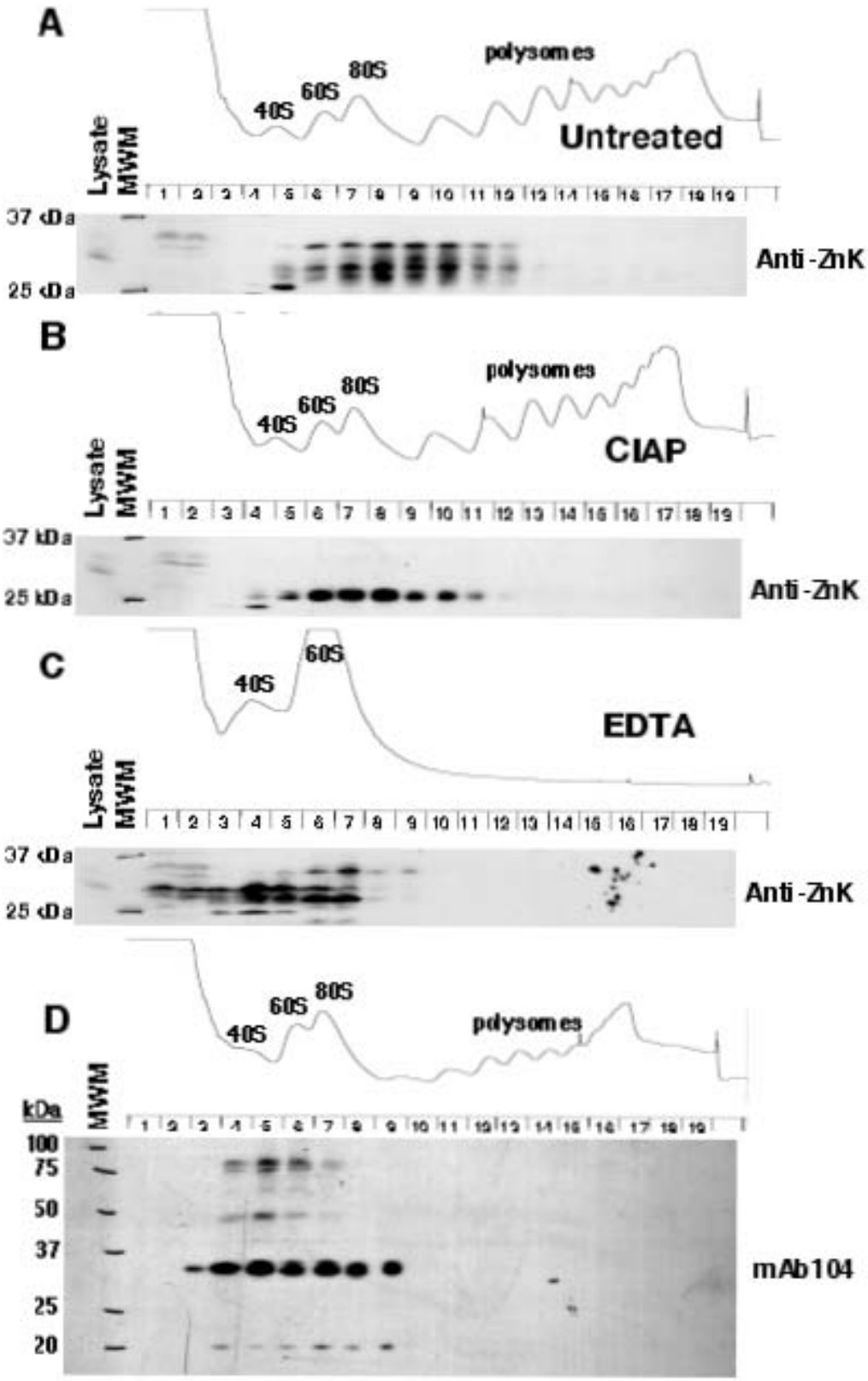
contamination, rendering GST-purification unnecessary. GST-9G8-ZnK was found to be highly insoluble even in B-PER with most of the protein remaining in the pellet from the B-PER extraction (Fig. 23A). The pellet was resuspended in Inclusion Body Solubilization Reagent (IBSR, Pierce). Since the protein at this state was very near homogenicity as determined by Coomassie staining, it was sent to BioSource to be inoculated directly into two New Zealand rabbits. The rabbits were boosted twice with the protein over the length of a month, and we were given four bleeds total from each rabbit (Fig. 28).

Serum from a rabbit inoculated with the 9G8-ZnK protein was shown to react specifically with the 9G8 protein, but had no cross-reactivity with SRp20 (compare Figs. 23B and C). The antibody also detected bands migrating slightly faster than T7-9G8 in both untransfected cells and cells transfected with T7-SRp20 (Fig. 23C), suggesting that these bands represent the endogenously expressed 9G8 protein.

Polyribosome analysis of endogenous 9G8. Using the 9G8 specific antibodies, we next performed an experiment to examine the distribution of endogenous 9G8 after sucrose gradient centrifugation in the presence or absence of EDTA or CIAP (Fig. 24). The results of the analysis in the absence of EDTA or CIAP (Fig. 24A) demonstrated that endogenous 9G8 was present in the fractions containing monosomes and light polyribosomes with a distribution similar to that seen for T7-9G8 expressed after transfection. As before, slower migrating forms of 9G8 were detected in the 60S, 80S and small polyribosomal fractions (6-11). After CIAP treatment (Fig. 24B), all of these bands disappeared, and a single band of around 25 kD was detected. Fractions 1-2 did not appear to contain any observable 9G8 protein (with or without CIAP treatment), whereas fractions 3-5 contained lower molecular weight bands. Although some larger bands were also observed in these fractions, none of these changed position after CIAP treatment, and these are most likely non-9G8 proteins reacting non-specifically with the unpurified polyclonal ZnK antibody.

In the presence of EDTA (Fig. 24C), no 9G8 proteins were detected beyond the 60S peak. Most of the 9G8 protein was found in the fractions containing 40 and 60S ribosomal subunits, and these fractions contained all the different species of 9G8. Taken together, these results suggest that the 9G8 protein is phosphorylated in the context of the translation machinery.

Figure 24. Polyribosome analysis of endogenous9G8. *A-D,* Cytoplasmic extracts from untransfected 293T cells were subjected to sucrose gradient analysis. The absorbance across the gradient was monitored at 254 nm (*top panels*). Western blot analysis (*lower panels*) was performed on isopropanol precipitated fractions using an anti-ZnK (9G8) antibody (*A-C*) or mAb104 (*D*). Fractions were either left untreated (*A* and *D*) or treated with 140 U CIAP (*B*), or prior to layering on the gradient, the lysate was treated with 15 mM EDTA (*C*).


A
40S
60S
80S
polysomes
Untreated
Lysate
MWM
37 kDa
25 kDa
Anti-ZnK
B
40S
60S
80S
polysomes
CIAP
Lysate
MWM
37 kDa
25 kDa
Anti-ZnK
C
40S
60S
EDTA
Lysate
MWM
37 kDa
25 kDa
Anti-ZnK
D
40S
60S
80S
polysomes
MWM
kDa
100
75
50
37
25
20
mAb104

For comparison, polysome gradient fractions from untransfected cells were also analyzed by Western blot for all endogenous SR proteins using mAb104 (Fig. 24D). Several bands of varying sizes are apparent, presumably representing several of the shuttling SR proteins. The bands observed at 20 and 35 kDa, for example, are most likely SRp20 and ASF/SF2, respectively. The doublet at 35 kDa may also include SRp30c since the other SR proteins of this molecular weight are SC35 and 9G8. SC35 does not shuttle, so it should not be present in the cytoplasm (nor the polysomes). 9G8, even at its most highly phosphorylated, is probably a little too small to be at this position (see Fig. 24A) and may not be represented on this blot. This would not be surprising, since as already mentioned, endogenous 9G8 expression is relatively low compared to the other SR proteins, such that it is generally accepted that mAb104 does not recognize 9G8. However, we have repeatedly shown that this antibody does recognize 9G8 produced from a transfected plasmid (Figs. 18 and 19). Several larger SR proteins are also detected in fractions containing the monosomes and subunits on this blot. However, unlike 9G8, none of these endogenous proteins are detected in anything larger than the very lightest of the polysomes. This is in contrast to the Sanford studies showing transfected ASF/SF2 in heavy polysomes (175, 176). It may be that over-expression of this SR protein drove the protein into larger polysomes than it usually exists (as can happen with 9G8; compare Figs. 21A and 24A). Another possibility is that in our experiment, the ASF protein in these fractions is hypophosphorylated (175), and is, therefore, undetectable by mAb104.

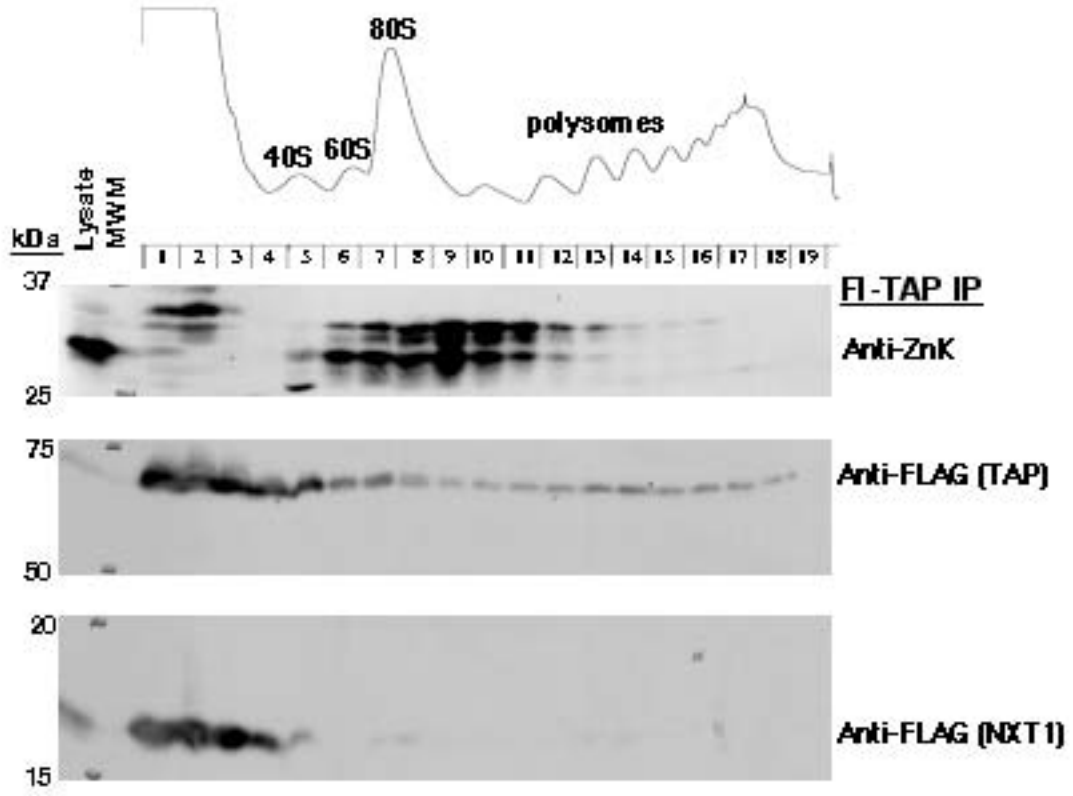
9G8 association and phosphorylation is concomitant with NXT1 release. Even though we were unable to demonstrate a direct interaction between 9G8 and Tap in the presence of RNase, our p24 data suggest the two proteins functionally interact, and the *in vitro* data published by other laboratories indicate that a direct interaction does exist but may be stabilized by RNA (76). Similarly to 9G8, we had previously shown that Tap/Nxt1 expression also enhances p24 production from the GagPolCTE reporter and shifts the mRNA to heavier polysomes (93). To determine if Tap and 9G8 interact in the context of the translation machinery, plasmids encoding FLAGGED versions of Tap and NXT were co-expressed in 293T cells, and lysates were run over sucrose gradients for polysome analysis. The FLAGGED proteins were immunoprecipitated using

M2 anti-FLAG-conjugated agarose beads and analyzed by Western blot (Fig. 25). As we previously showed, the Tap protein is found throughout the gradient (middle panel), whereas NXT1 is only found associated with ribosomal subunits and the "soluble" fractions (lower panel). The blot was also probed for endogenous 9G8 using the anti-ZnK antibody (top panel). Since RNase was not used in this experiment, we cannot say whether endogenous 9G8 was pulled down through direct binding to the tagged Tap protein or through non-specifically binding to the agarose beads mediated by RNA. We are, however, able to compare the localization of these proteins relative to each other and to the translation machinery. As was observed in the untransfected cells (Fig. 24), endogenous 9G8 is localized mainly with ribosomal subunits, monosomes and light polysomes and becomes progressively more hyperphosphorylated throughout the gradient. It is interesting to note that NXT1 becomes dissociated from Tap in the same fractions that 9G8 becomes associated and phosphorylated suggesting the possibility that the two events are related.

Phosphorylation of the RS domain releases 9G8 from the polysomes. The purpose of the phosphorylation of 9G8 within the polysomes remains unknown. However, since hyperphosphorylated SR proteins have a lower affinity for RNA, one of the consequences may be that this allows release of the SR protein from the RNA (64, 175). Supporting this notion, our results show 9G8 associated with the translation machinery exists with a concentration of hyperphosphorylated forms being found in the monosome and light polysome fractions, coinciding with translation initiation. In the following fractions, which represent a time point after initiation, hyperphosphorylated isoforms disappear from the gradient, leaving only hypophosphorylated forms (see, in particular, Figs. 18 and 21, where more hypophosphorylated protein is present than in the endogenous experiments, a possible artifact of over-expression).

To determine if the RS domain, which is the target of most of the phosphorylation of 9G8, is important for polysome release, we decided to analyze cells transfected with a plasmid expressing a mutant form of 9G8 that lacks the RS domain (9G8ΔRS). This plasmid is based on an intron 3-retaining mRNA isoform of 9G8 (157). 9G8 has been reported to be alternatively spliced, and it has been shown that the full-

Figure 25. Endogenous 9G8 co-immunoprecipitated with Tap/Nxt1. Cytoplasmic extracts from 293T cells transfected with FLAG-Tap and FLAG-Nxt1 were subjected to sucrose gradient analysis. The absorbance across the gradient was monitored at 254 nm (*top panel*). Western blot analysis (*lower panels*) was performed on α-FLAG immunoprecipitated fractions using an anti-ZnK (9G8) antibody (*upper panel*) or an anti-FLAG antibody (*lower panels*).


80S
40S
60S
polysomes
kDa
Lysate
MWM
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
37
25
Fl-TAP IP
Anti-ZnK
75
50
Anti-FLAG (TAP)
20
15
Anti-FLAG (NXT1)

length protein regulates alternative splicing of its own mRNA (119). However, the consequence of this self-regulation is not known. One of the alternative isoforms is an mRNA with intron 3 retained. If this mRNA were to be translated, it would produce a protein that is mimicked by that derived from the 9G8ΔRS plasmid (Fig. 26). It encodes a T7-tagged protein that retains the RRM and Zinc Knuckle domains and the first six of the ten amino acids of the nuclear localization signal (NLS). Six additional amino acids are encoded by the intron and are followed by a STOP codon, resulting in a protein lacking the RS domain (Fig. 27), the Serines, of which, are normally extensively phosphorylated. Although it has not been demonstrated that this truncated protein exists in nature, during the testing of the sera from the BioSource rabbits immunized with the GST-ZnK peptide, a band at the approximate expected size of the endogenous 9G8ΔRS was detected by bleeds 2-4 from rabbit 1 (Fig. 28). This band was slightly larger than that produced from the 9G8ΔRS plasmid (Fig. 27), but this could reflect differences in endogenous versus recombinant expression, including the possibility that additional alternatively-spliced isoforms exist other than the one upon which the 9G8ΔRS plasmid was based. Therefore, 9G8ΔRS protein may exist in nature, but more experiments must be done to demonstrate this.

Like the full-length protein (Fig 21A), the 9G8 protein lacking the RS domain was detected in fractions containing the ribosomal subunits, monosomes and polysomes (Fig. 29A). In fact, when compared to the full-length protein, 9G8ΔRS was found in relatively higher amounts in those fractions representing heavy polysomes. As expected, the protein lacking the RS domain migrated as a single band, supporting the notion that the multiple bands observed with the full-length protein represent modifications in the RS domain. This suggests that phosphorylation of the RS domain of 9G8 could release the protein from the translation complex. Again, there was a significant shift to "lighter" fractions in the presence of EDTA (Fig. 29B), indicating a true association with polyribosomes.

To provide further evidence that phosphorylation of the RS domain of 9G8 releases the protein from polysomes, lysates from both untransfected (Figs. 30C and D) or T7-9G8 transfected 293T cells (Figs. 30E and F) were either treated with phosphatase inhibitors (Figs. 30C and E) or CIAP (Figs. 30D and F) prior to running over the sucrose gradient. This is in contrast to the experiments shown in Figs. 20B and

Figure 26. Schematics of full-length 9G8 *vs*. 9G8ΔRS. *A*, The schematic of the full-length 9G8 protein is shown with the location of notable domains. RRM: RNA recognition motif, ZnK: zinc knuckle, RS: Argenine/Serine rich, RNP: RNA protein complex consensus sequence, RB: RNA binding, NLS: nuclear localization signal. *B*, The schematic of a protein that could be produced from a *9G8* mRNA isoform with retained intron 3. This protein would have the RRM, RB, Tap binding, and ZnK domains and part of the NLS but not the RS domain.

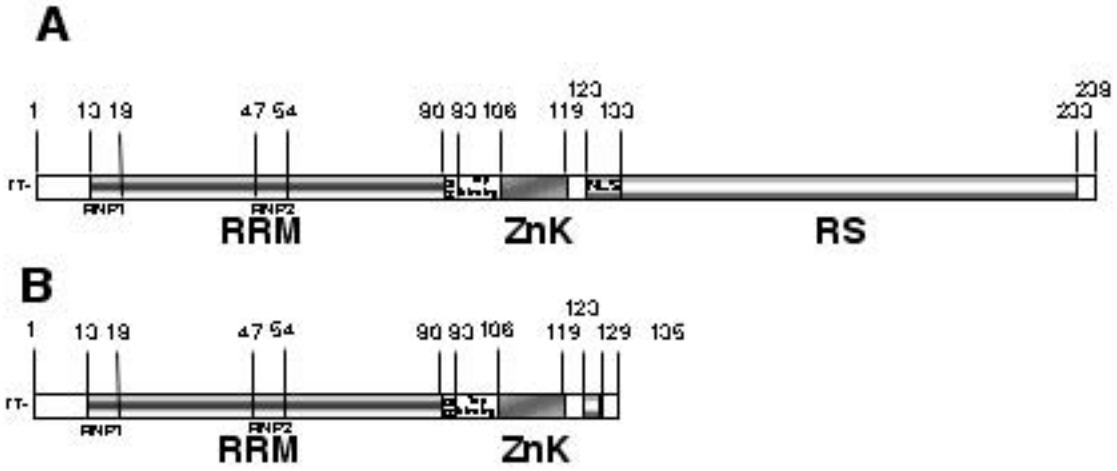
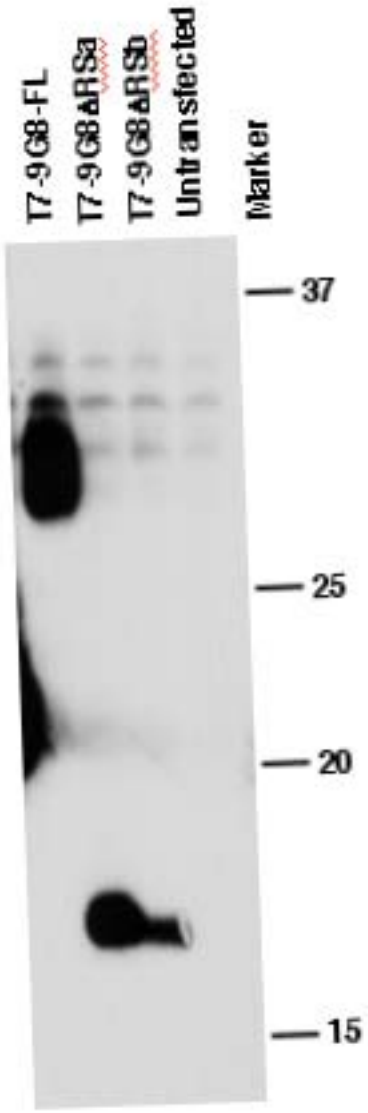

A
1
10
19
47
54
90
93
106
119
120
133
209
230
RT-
RNP1
RNP2
RRM
ZnK
RS
B
1
10
19
47
54
90
93
106
119
120
129
135
RT-
RNP1
RNP2
RRM
ZnK

Figure 27. Western of proteins expressed from T7-9G8ΔRS plasmids. 293T cells were transfected with plasmids expressing T7-tagged versions of the full-length 9G8 or 9G8 without the RS domain. Anti-T7 antibody was used to detect the relative levels and sizes of the proteins expressed from these plasmids.


T7-9G8-FL
T7-9G8ΔRSa
T7-9G8ΔRSb
Untransfected
Marker
37
25
20
15

α-T7 Western

Figure 28. Sera from rabbit 1 immunized against ZnK peptide may recognize endogenous 9G8ΔRS. 293T cells were left untransfected, transfected with empty plasmid, or co-transfected with plasmids expressing T7-9G8 and T7-SRp20. Lysates were run on SDS-PAGE and transferred to Immobilon P. The membrane was cut into strips, which were probed individually with sera from BioSource rabbits inoculated against a recombinant GST-9G8 ZnK protein. The ability of each antibody to detect transfected and endogenous 9G8 or cross-reaction to SRp20 was monitored, as was the potential to detect endogenous 9G8ΔRS proteins from alternatively spliced *9G8* isoforms.

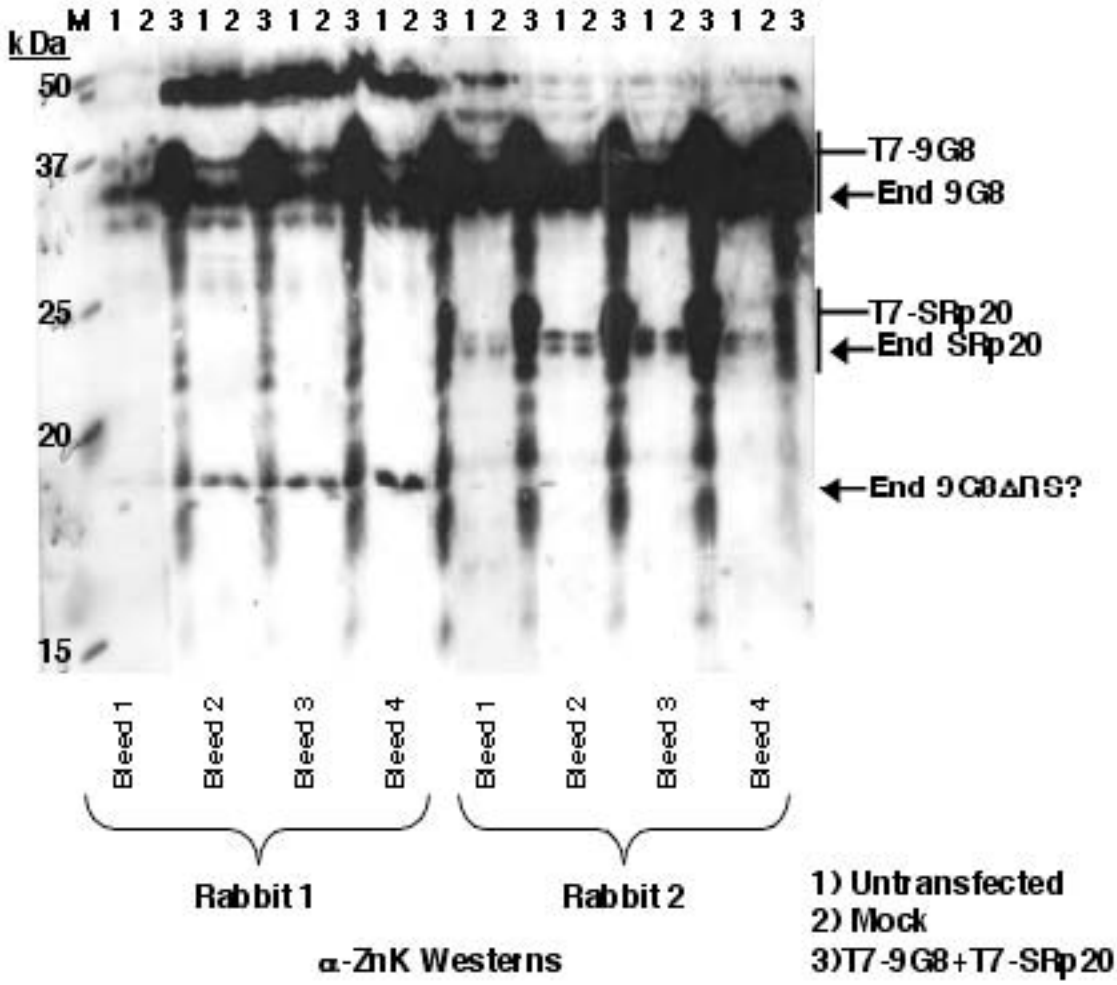

M 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
kDa
50
37
25
20
15
T7-9G8
End 9G8
T7-SRp20
End SRp20
End 9G8ΔRS?
Bleed 1
Bleed 2
Bleed 3
Bleed 4
Bleed 1
Bleed 2
Bleed 3
Bleed 4
Rabbit 1
Rabbit 2
α-ZnK Westerns
1) Untransfected
2) Mock
3)T7-9G8+T7-SRp20

Figure 29. Effect of EDTA on 9G8ΔRS in polysome gradient. Polysome gradient analyses of cells transfected with pCGT79G8ΔRS without (*A*) or with (*B*) 15 mM EDTA added to the lysate before loading onto the sucrose gradient. Polyribosome profiles and Western blot analyses were performed as described for *Fig. 16*.

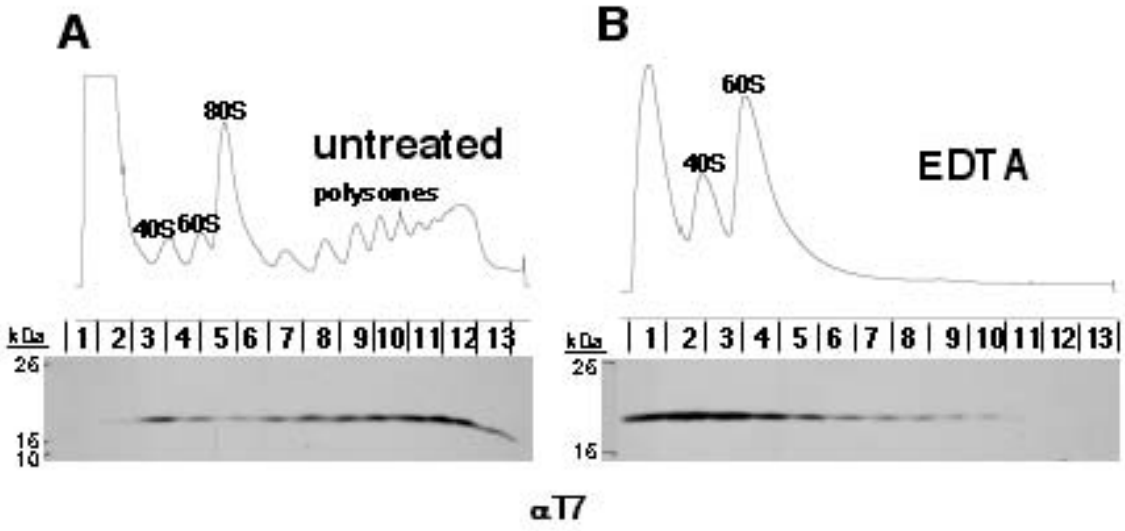
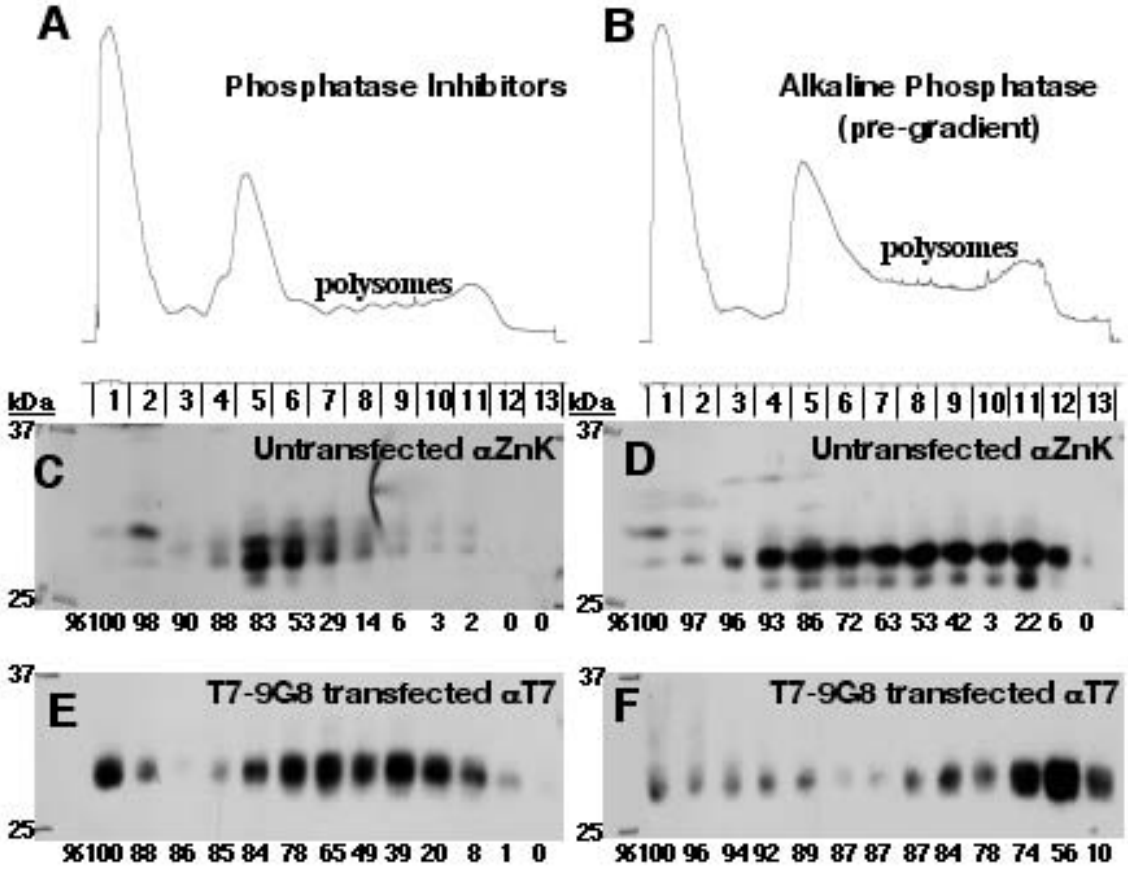

A
80S
untreated
polysomes
40S
60S
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
26
16
10
B
60S
40S
EDTA
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
26
16
αT7

Figure 30. Lysate treated with CIAP before loading onto gradient. Cytoplasmic lysates were made from untransfected or T79G8-transfected 293T cells. Unlike the experiment shown in *Fig. 20*, the lysates were treated with CIAP prior to layering atop the sucrose gradients (*B,D,F*)or were treated with phosphatase inhibitors (*A, C, E*). *A-B*, Representative polysome profiles. *C-D*, Western blots for polysome gradient fractions from untransfected cells probed with the α-ZnK antibody for detection of endogenous 9G8. *E-F*, Western blots for polysome gradient fractions from transfected cells probed with an α-T7 antibody for detection of over-expressed 9G8.


A
Phosphatase Inhibitors
polysomes
B
Alkaline Phosphatase
(pre-gradient)
polysomes
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
37
C
Untransfected αZnK
25
%100 98 90 88 83 53 29 14 6 3 2 0 0
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13
37
D
Untransfected αZnK
25
%100 97 96 93 86 72 63 53 42 3 22 6 0
37
E
T7-9G8 transfected αT7
25
%100 88 86 85 84 78 65 49 39 20 8 1 0
37
F
T7-9G8 transfected αT7
25
%100 96 94 92 89 87 87 87 84 78 74 56 10

24B where sucrose fractions were treated with CIAP after gradient centrifugation. Although the CIAP treatment had an interesting effect on the polysome profile (Fig. 30B), with a blurring of the distinct peaks, it did not seem to collapse the polysomes, such as EDTA or puromycin would, and the overall polysome shape was still present. This experiment was performed before conditions were optimized, and we had not yet learned how to resolve over-expressed 9G8 into its multiple isoforms. However, it is clear that treatment with the phosphatase not only collapsed the 9G8 isoforms to a faster migrating band, it also caused a dramatic shift in localization from monosomes and light polysomes toward the heavy polysome part of the gradient for both untransfected and over-expressed 9G8. This suggests that at the time of cell lysis, 9G8 is actually associated with the protein machinery all throughout the gradient, even with the heavier polysomes. By changing the phosphorylation of the RS domain, either by phosphatase activity or simply by removing the domain, release of 9G8 from the polysomes can be blocked. However, allowing the RS domain to remain phosphorylated results in a loss of the protein from the heavy polysomes over the course of the experiment. Precisely how phosphorylation of 9G8 releases the protein remains unknown, but our results suggest that since phosphorylation weakens the affinity of the SR protein for RNA, during the process of gradient centrifugation, the protein may simply fall off. In the cell, this may be actively performed by Transportin-SR, as has been suggested for the yeast homologues, Npl3p and Mtr10p (64, 181). More work needs to be done to determine if the yeast model is relevant for the mammalian system.

The 9G8ΔRS protein does not promote p24 expression from the GagPolCTE plasmid. Even though phosphorylation of the RS domain was shown to be important for the release of 9G8 from the polysomes, it was not known if this domain also had a role in the expression from the GagPolCTE reporter. It is conceivable that the role of 9G8 in translation is just to act as an adaptor for the recruitment of Tap, and if so, this could still be accomplished by the truncated 9G8 protein, which retains the Tap-binding domain (Fig. 26) (76). If, however, 9G8ΔRS is not able to promote p24 expression from the CTE reporter, this would suggest that phosphorylation of the RS domain may be important for 9G8 function in translation. In fact, 9G8ΔRS did not enhance p24 expression (Fig. 31) and acted in a dominant negative manner when co-

expressed with full-length protein (Figs. 32 and 33; same data presented in two representations). In light of the fact that 9G8 promotes alternative splicing of its own mRNA (119), and since the consequence of this splicing could result in a protein similar to that expressed by the 9G8ΔRS plasmid, this result opens the possibility of a very elegant mechanism of self-regulation by 9G8.

9G8 alternative splicing isoforms in whole cell lysate vs. cytoplasmic extracts. Very little is known about the role of alternatively spliced *9G8* transcripts. Northern blots of various tissues show at least 5 different isoforms are expressed (30, 157), and *in vitro* splicing assays demonstrate that the full-length protein influences splice site selection of its own transcript (119). We were interested in the role of the intron-containing transcripts, if any, and if different splicing patterns could be generated *in vivo*. Although 9G8 promoted the excision of intron 3 of its own mRNA *in vitro*, it is possible that different splicing patterns would emerge *in vivo*. Also, even though it is widely accepted that SR proteins only enhance splicing at their designated ESEs, at least one of the SR proteins can also have splicing inhibitory effects (16, 36, 184, 185). Furthermore, preliminary results in our laboratory indicate that 9G8, SRp20, and ASF/SF2 promote intron retention in SIRT7 mRNA, a gene that appears to contain a cellular CTE (Fig. 34, and Bor, *et al.*, unpublished results). We performed RT-PCR on RNA isolated from cytoplasmic and whole cell lysates of 293T cells to determine which isoforms are expressed and if they are exported. RT-PCR analysis was performed on untransfected cells or cells over-expressing full-length 9G8, 9G8ΔRS, or Tap/Nxt1. We found that both the full-length and partial intron 3 transcripts are produced in 293T cells and that these transcripts are exported into the cytoplasm. Splicing patterns seem to vary depending on which protein is over-expressed. Also, there is evidence indicating that additional alternatively spliced transcripts may exist. Since this work is preliminary, it is described in the appendix at the end of this dissertation.

Figure 31. P24 expression: 9G8ΔRS titration. Transfections were performed and analyzed as described in *Fig 8*. 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP in the absence or presence a range of amounts (0.1, 0.3, 1.0, and 3.0 μg) of the pcDNA3-T79G8-2 (ΔRS) plasmid.

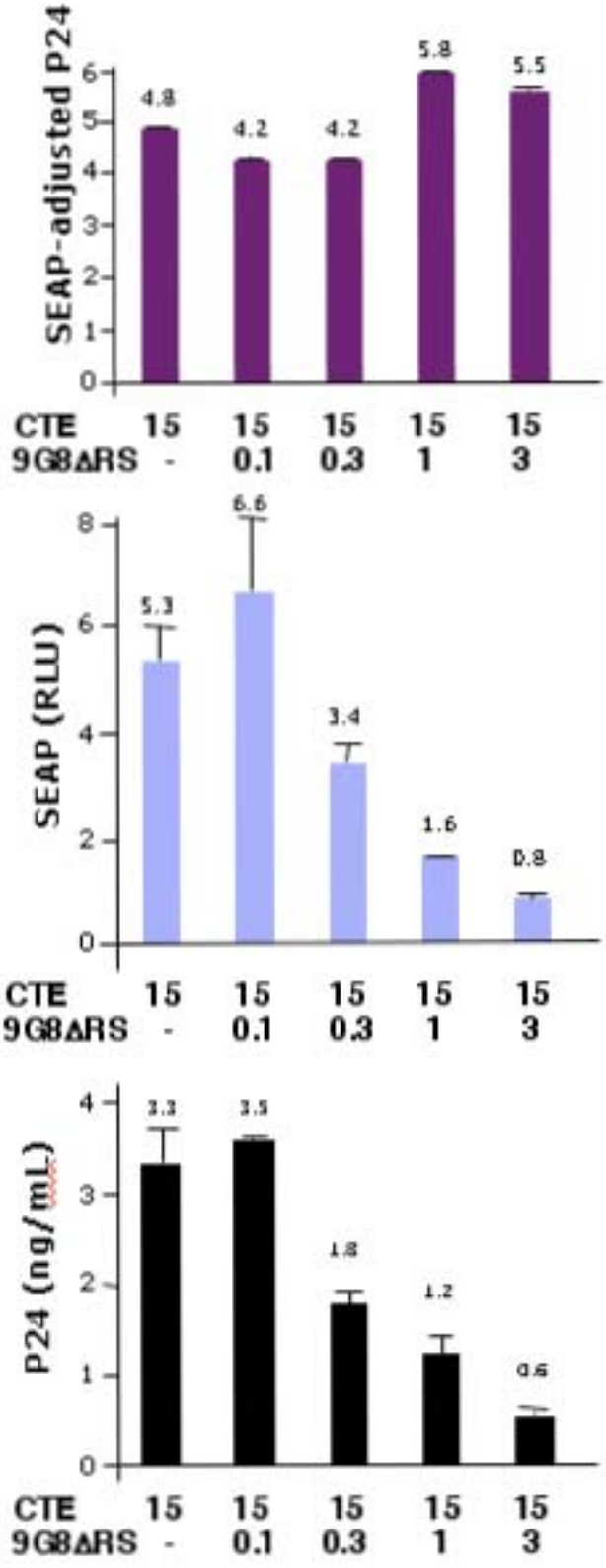

SEAP-adjusted P24
4.8
4.2
4.2
5.8
5.5
CTE 15 15 15 15 15
9G8ΔRS - 0.1 0.3 1 3
SEAP (RLU)
5.3
6.6
3.4
1.6
0.8
CTE 15 15 15 15 15
9G8ΔRS - 0.1 0.3 1 3
P24 (ng/mL)
3.3
3.5
1.8
1.2
0.6
CTE 15 15 15 15 15
9G8ΔRS - 0.1 0.3 1 3

Figure 32. P24 expression: 9G8ΔRS and 9G8 full-length titration matrix. Transfections were performed and analyzed as described in *Fig 8*. 293T cells were transfected with pCMVGagPol-CTE and pCMVSEAP. In addition, the pCGT79G8 plasmid was co-transfected over a range of plasmid amounts (0, 0.1, 0.3, 1.0, and 3.0 μg), and for each amount of pCGT79G8, a similar range of the pcDNA3-T79G8-2 (ΔRS) plasmid was also co-transfected.

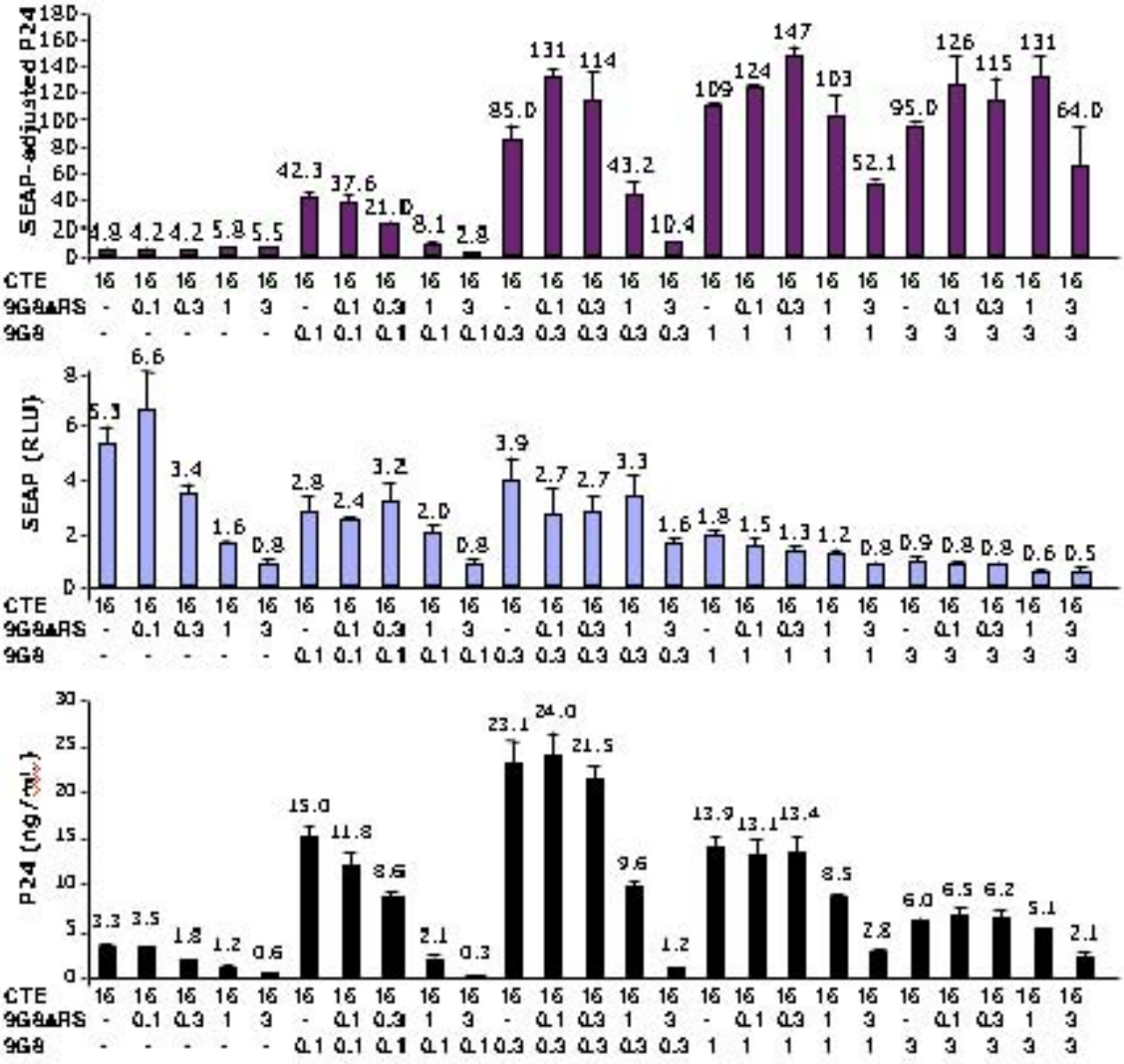
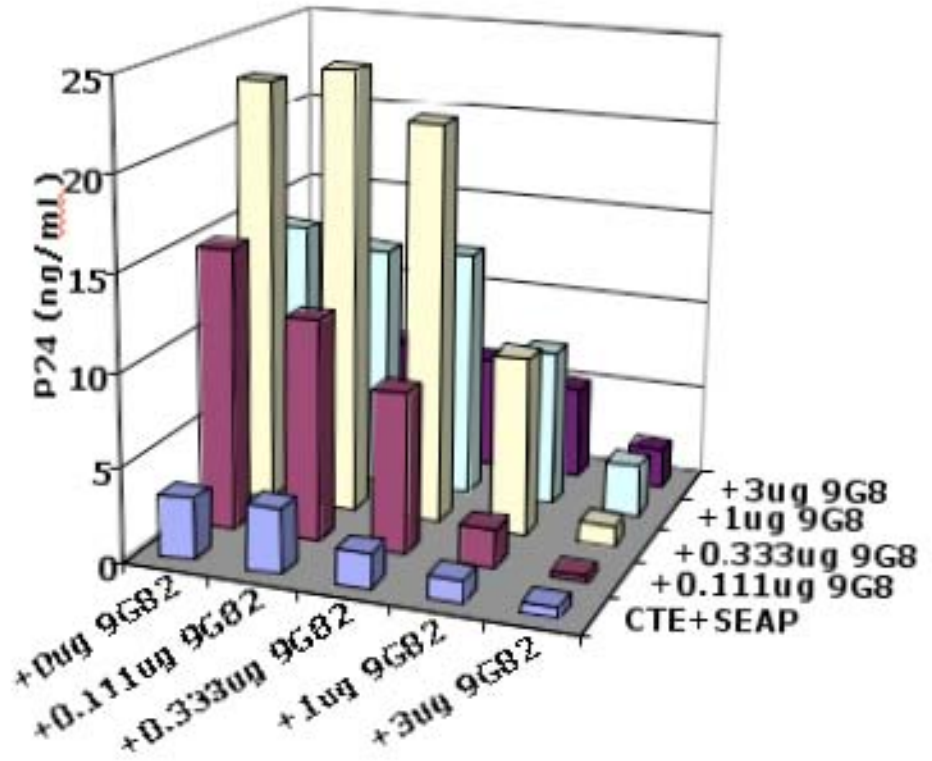

SEAP-adjusted P24
SEAP (RLU)
P24 (ng/ml)
CTE
9G8aRS
9G8

Figure 33. P24 expression: 9G8ΔRS titration with 9G8 full-length titration. 3D representation. The data depicted in *Fig. 32* shown in an alternate form.


P24 (ng/ml)
25
20
15
10
5
0
+0ug 9G82
+0.111ug 9G82
+0.333ug 9G82
+1ug 9G82
+3ug 9G82
+3ug 9G8
+1ug 9G8
+0.333ug 9G8
+0.111ug 9G8
CTE+SEAP

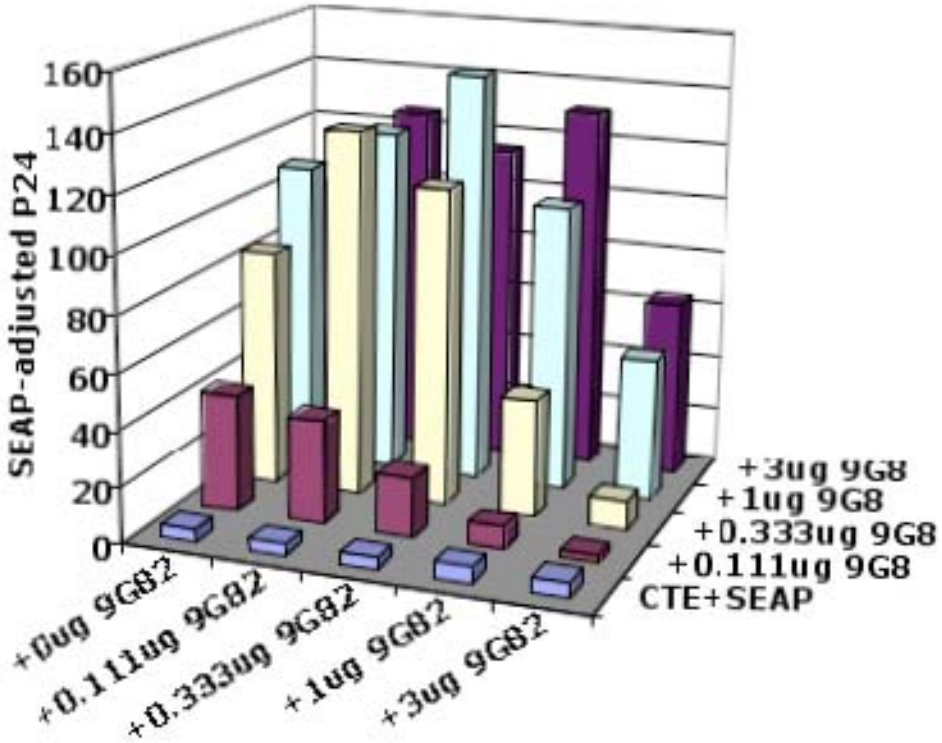
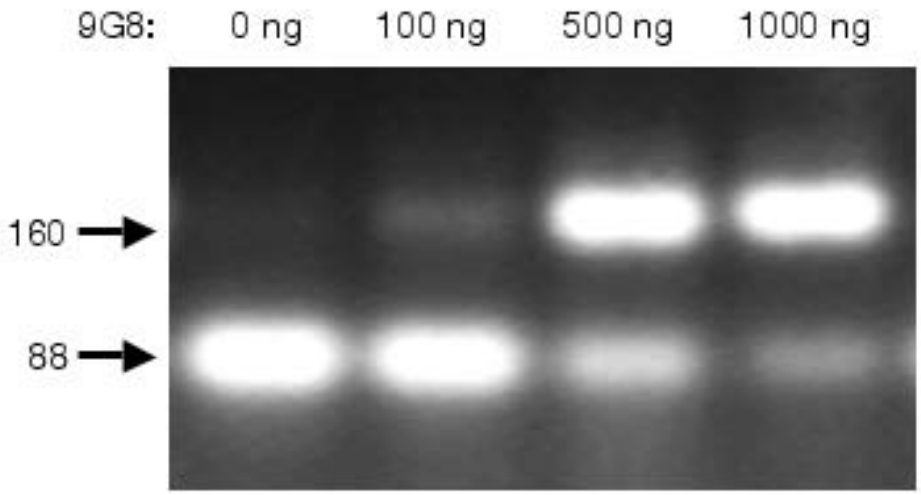
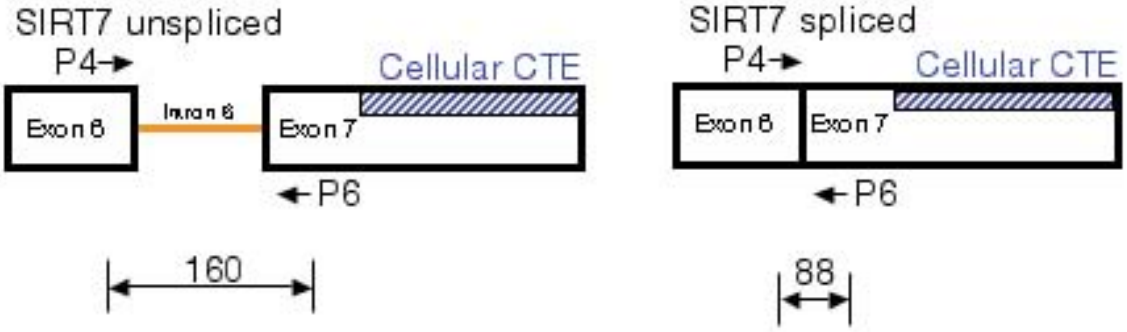

SEAP-adjusted P24
160
140
120
100
80
60
40
20
0
+0ug 9G82
+0.111ug 9G82
+0.333ug 9G82
+1ug 9G82
+3ug 9G82
+3ug 9G8
+1ug 9G8
+0.333ug 9G8
+0.111ug 9G8
CTE+SEAP

Figure 34. SIRT7 intron retention. Ten μg of the pcDNA-FLAG-SIRT7 plasmid encoding the genomic copy of the SIRT7 gene was transfected into 293T cells in the absence or increasing amounts of the 9G8 expression vector. At 48 hours post-transfection, cytoplasmic mRNA was isolated and analyzed by RT-PCR using the P4 and P6 oligos to amplify the region between exon 6 and exon 7 of SIRT7. A schematic representation depicting the predicted sizes of PCR products derived from unspliced or spliced transcripts is shown. (Experiment performed by YCB)


9G8:
0 ng
100 ng
500 ng
1000 ng
160
88


SIRT7 unspliced
P4
Cellular CTE
Exon 6
Intron 6
Exon 7
P6
160
SIRT7 spliced
P4
Cellular CTE
Exon 6
Exon 7
P6
88

Discussion

Here we show that moderate SRp20 or 9G8 over-expression significantly stimulates translation from unspliced RNA containing a CTE. Using 9G8, we show that RNA export is not affected, but that SR protein expression enhances polyribosome association of RNA containing the CTE. Furthermore, we show that 9G8 is present in 80S monosomes and small polyribosomes and that hyperphosphorylated forms of this protein are significantly enriched in these fractions. These results suggest further links between RNA export and translation and highlight the complexity of post-transcriptional gene regulation.

Shuttling SR proteins have previously been suggested to promote export of completely spliced mRNA by serving as an adaptor for the Tap export protein (82, 84). In the models proposed, Tap is not envisaged to contact the RNA directly, but merely interact with the mRNP complex through protein-protein interactions. Yet, Tap is clearly an RNA binding protein and has been shown to contain both an RNP and LRR domains through which it interacts directly with CTEs (21). It was previously proposed that CTEs are only present in viral RNAs and might serve to simply mimic cellular adaptor proteins. However, our recent discovery that shows that Tap interacts directly with a CTE in an alternatively spliced Tap mRNA (125) clearly demonstrates that Tap is capable of direct interactions with cellular mRNAs. This shows that the current models for how Tap functions are likely to be too simplistic. Also, it remains unclear why Tap, an RNA binding protein, would need an adaptor protein for recruitment to cellular mRNA. In fact, our laboratory has shown that co-immunoprecipitation of Tap with 9G8 is RNA-dependent, in contrast to the published data (82).

Since we were unable to reproduce published results, it seems appropriate to postulate possible reasons why this may be. In the Huang, *et al.,* experiment, three conditions were compared: 293T cells were transfected with plasmids expressing FLAG-hnRNPA1, FLAG alone, or FLAG-Tap. Lysates from each condition were immunoprecipitated using an anti-FLAG antibody, and Western blots were probed using an anti-9G8 antibody to detect endogenous 9G8 co-immunoprecipitating with the FLAG protein. In this experiment, 9G8 came down more efficiently with the FLAG-Tap protein, whereas only a small amount came down with the FLAG-hnRNPA1 protein and none with FLAG alone. One possible cause for

this outcome may be that since Nxt1 was not co-expressed with Tap, very little Tap protein was actually expressed when compared to hnRNPA1. Since FLAG-hnRNPA1 and FLAG alone were expressed at high levels, it may be that these proteins were preferentially adsorbed to the FLAG-beads, blocking non-specific binding of RNA (which is quite sticky) and a subsequent pull-down of endogenous 9G8. In contrast, since there was very little FLAG-Tap protein present, this may have allowed some RNA to be adsorbed, resulting in the appearance of a direct Tap-9G8 interaction. A second point is that their experiment differs from ours in that they never showed what happens to 9G8 when there is no FLAG protein present. Furthermore, although they used RNase in their experiment, the source of their RNase was from an in-house preparation. It is possible that this RNase wasn't as highly reactive as was the commercial RNase that we used. Although no endogenous hnRNPA1 co-immunoprecipitated with FLAG-Tap in the presence of RNase in their experiment, since a small amount of endogenous 9G8 did come down with FLAG-hnRNPA1, unless hnRNPA1 and 9G8 interact directly, the pull-down was probably mediated by RNA, indicating that their RNase may not have been reactive enough for this experiment. These two points could explain why we see a difference between their *in vivo* immunoprecipitation experiments and ours. However, their *in vitro* data looks very convincing and was confirmed by Hargous, *et al*. In fact, Hargous was able to determine that the RNA- and Tap-binding sites are very near each other (three amino acids separates them) (76). Therefore, it is quite possible that RNA actually stabilizes the Tap-9G8 interaction in an *in vivo* setting, whereas *in vitro*, they can more readily interact directly by that fact that they are forced into near proximity at relatively high concentrations. Thus the atomic resolution of a Tap/9G8/RNA trimeric complex might be very helpful for further clarification of this issue.

Even though it is clear that most mRNAs do not contain *bona fide* CTEs, it is still possible that RNA-protein, as well as protein-protein interactions, serve to generally promote the formation of stable export complexes. It is furthermore intriguing that 9G8 promotes function mediated by both the MPMV- and Tap-CTEs since an adapter protein should not be necessary in these cases. Recent studies support the notion that shuttling SR proteins have a continued role beyond splicing and export. Sanford, *et al*., showed that the SF2/ASF–promoted association of an mRNA with the translation machinery occurred for both an

RNA in which introns had been spliced and for an unspliced, intronless RNA (176). Another study showed that SRp20 has the ability promote expression from an IRES-containing RNA (8). SRp20 was shown to interact directly with the cellular IRES-binding PCBP2 protein.

We found that shuttling SR proteins can enhance expression from a non-canonical (intron and CTE-containing) mRNA through promoting polysome association. A recent study has indicated that SR proteins may play a role in NMD (215). While it is not clear if our unspliced GagPol-RNA is a substrate for NMD, we find that over-expression of 9G8 appears to have a protective effect on the RNA. When the cells were not treated with cycloheximide (an NMD inhibitor) for thirty minutes prior to polysome isolation, it was very difficult to isolate detectable levels of GagPol mRNA from sucrose gradients in the absence of exogenous SR proteins. In the presence of SR proteins, however, GagPol mRNA was isolated at readily detectable levels, even without the use of cycloheximide. This suggests the possibility that the GagPol RNA is stabilized through SR protein-mediated association with the translation machinery.

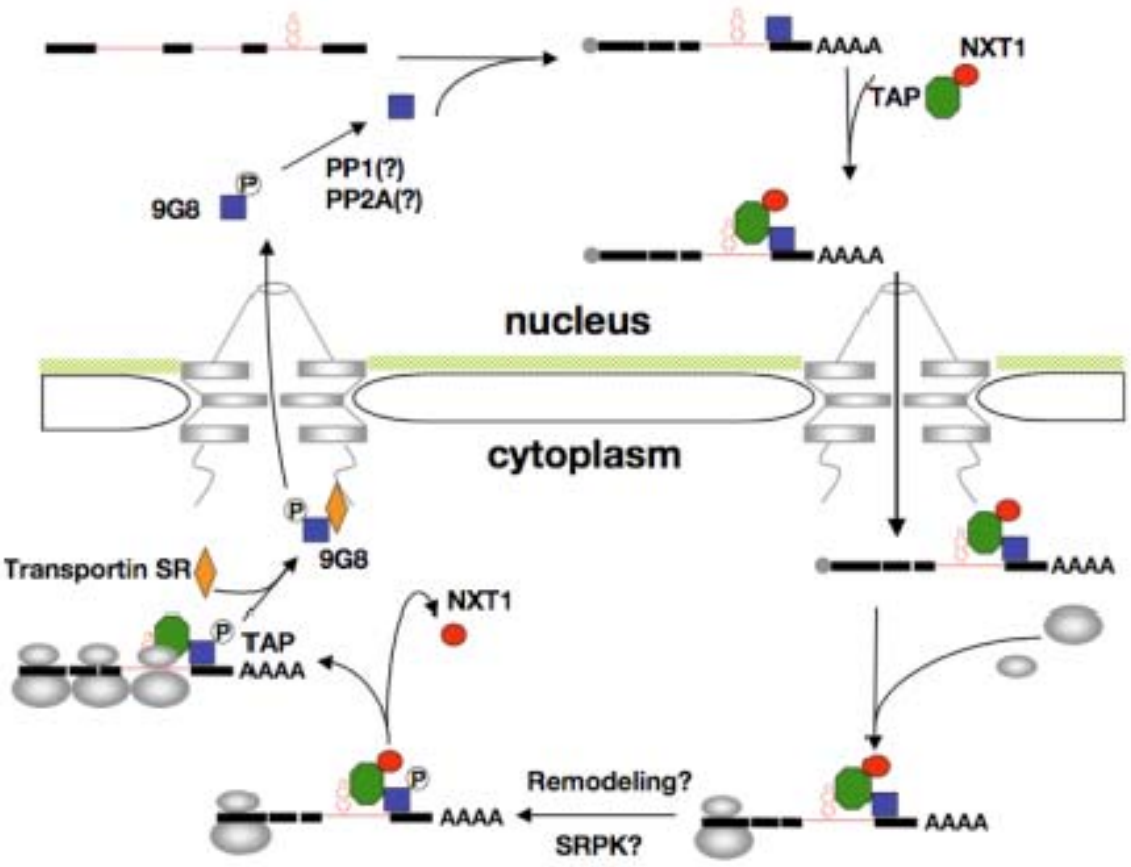
We have previously shown that TAP/NXT1 also enhances translation of the GagPol-CTE RNA and that TAP, but not NXT1, is associated with polyribosomes in 293T cells (93). Although absent from polyribosomes, NXT1 was shown to be present in gradient fractions containing the ribosomal subunits and monosomes leading us to speculate that a rearrangement of the export complex occurs in conjunction with translation initiation. The fact that we have now shown that 9G8, previously reported to associate with TAP in mRNP export complexes (82, 85), becomes phosphorylated in the subunit and monosome fractions suggests that SR protein phosphorylation might be the trigger for a remodeling of export complexes, which leads to a release of NXT1.

Although it is well established that SRPK1, one of the major SR protein kinases, is localized to the cytoplasm (203), the exact location of this protein is not known. However, a recent study has reported that SRPK1 can be found associated with ribosomes (102) which would be consistent with our findings that SR proteins appear to get hyperphosphorylated in conjunction with association with ribosomal subunits.

Based on our results, we favor a model (Fig. 35) in which mRNP complexes associated with TAP/NXT1 and “shuttling” hypo-phosphorylated SR proteins associate with ribosomal subunits after

passage through the nuclear pore. In conjunction with this association, the SR proteins are phosphorylated, potentially resulting in a remodeling of the complex and the release of NXT1. After phosphorylation, the SR proteins could then be reimported into the nucleus by Transportin-SR, previously shown to preferentially interact with hyperphosphorylated SR proteins (109, 211). This model suggests that the effects of SR proteins on translation might be regulated by SR protein kinases as well as proteins involved in nuclear reimport. A recent study by Krebber and collaborators showed that the Npl3p protein associates with yeast polysomes (209). Based on genetic experiments, they proposed that Mtr10p, the import receptor for Npl3p, might be involved in a timely regulated release of Npl3p from the polysomes. In contrast, Sky1p, the SR-kinase, did not appear to be involved in this process. Since it has been proposed that Npl3p serves an analogous function to 9G8, it will be of clear interest to further examine the effect of the SRPKs and Transportin-SR2 on 9G8 function.

Figure 35. Schematic for the various roles of 9G8 as it cycles through the nucleus and cytoplasm. In this model, intron- and CTE-containing mRNP complexes associated with TAP/NXT1 and "shuttling" hypo-phosphorylated SR proteins are exported to the cytoplasm, where they associate with ribosomal subunits. In conjunction with this association, the SR proteins are phosphorylated, potentially resulting in a remodeling of the complex and the release of NXT1. After phosphorylation, the SR proteins are reimported into the nucleus by Transportin-SR. This model suggests that the effects of SR proteins on translation might be regulated by SR protein kinases as well as nuclear reimport.


AAAA
NXT1
TAP
PP1(?)
PP2A(?)
9G8
P
AAAA
nucleus
cytoplasm
P
9G8
Transportin SR
AAAA
P
TAP
AAAA
NXT1
P
AAAA
Remodeling?
SRPK?
AAAA

CHAPTER 4

The Wilms' Tumor 1 (WT1) Gene (+KTS) Isoform Functions With A CTE To Enhance Translation From An Unspliced RNA With A Retained Intron

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This chapter describes a project that I worked on with Yeou-cherng Bor. Although it was not my primary project, I was substantially involved so that I was made second author on the paper that resulted from the work (18). For context, I am including the entire paper, although much of the work in it was not done by me. I was primarily involved in the polysome analysis experiments for this project, which provided the key elements of the paper. Working with Dr. Bor, I performed most of the work preparing the polysome gradients and their fractionation. I also performed the Western blot analyses on the gradient fractions while Dr. Bor performed the Northern blot analyses. Each figure legend notates who performed the work to generate the figure. Besides the work presented here, Dr. Bor and I also worked out the protocols for the polysome assay during the generation of the data for this paper. This has been described in detail in chapters 2 and 3.

Introduction

The WT1 tumor suppressor gene was first identified and cloned based on a chromosome 11 deletion associated with WAGR syndrome (Wilms' tumor, aniridia, genitourinary malformations and mental retardation) (26, 62). WT1 encodes gene products that have been shown to play critical roles in the normal development of several organs in vertebrates, most notably the urogenital system (44). Mutations in the *WT1* gene have been detected in several human diseases including Wilms' tumors (nephroblastoma), Denys-Drash syndrome (typified by severe mesangial sclerosis), and Frasier syndrome (focal segmental glomerular sclerosis, pseudohermaphroditism and gonadoblastoma). We have recently used siRNA to show

that WT1 is required at specific stages of nephrogenesis in an *ex vivo* mouse kidney development system in which *WT1* silencing is associated with a block in nephrogenesis and abnormal levels of cell proliferation and apoptosis (40). In addition to its role in urogenital development, WT1 is also involved in hematopoiesis and is of great interest to clinicians due to its mis-expression in acute myeloid leukemia (168).

The WT1 gene encodes four Krüppel-type Cys_2-His_2 zinc fingers, consistent with a function as a transcriptional regulator. WT1's zinc fingers place it in the Early Growth Response (EGR) family of transcription factors, and several studies have documented that WT1 can function in this capacity, either as an activator, a repressor or a co-activator of a variety of growth associated genes (179). The *WT1* gene consists of ten exons and is alternatively spliced. Exon 5 can be skipped; its inclusion adds 17 amino acids in the central part of the protein. An alternative splice donor site at exon 9 inserts only three amino acids, lysine, threonine and serine (KTS), between the third and fourth zinc fingers (61, 70). A new class of mRNA transcripts has recently been described (*AWT1*), arising from the use of an alternative exon 1 present in intron 1. The use of this exon results in the use of an alternative, internal AUG start codon (39). In summary, alternative splicing, combined with upstream CUG alternative translation initiation codons and RNA editing, results in the potential expression of numerous *WT1* isoforms. Of these isoforms, the most conserved are the ones arising from the +/-KTS alternative splicing (101).

Whereas WT1(-KTS) has the properties of a typical DNA binding transcription factor (11, 161), no clear role has been established for the WT1(+KTS) protein, which binds DNA with reduced affinity (111, 214). The +/-KTS isoform ratio is tightly regulated, and disruption of the ratio has been implicated in the pathogenesis of Frasier syndrome in humans and in mice (4, 75, 105). Interestingly, a recent study has further highlighted the importance of the WT1(+KTS) isoform, by demonstrating that it is required for normal development of the olfactory system (201).

Several studies have suggested that WT1(+KTS) may function mainly at the post-transcriptional level. A potential connection between the +KTS isoforms and RNA processing was originally suggested by Larsson *et al*. (113), who demonstrated a preferential localization of WT1(+KTS) in nuclear speckles. Treatment with RNase, but not DNase, altered WT1(+KTS) nuclear localization and the protein co-

immunoprecipitated with snRNPs. Later work showed that WT1(+KTS) specifically associates with U2AF65, a key splicing factor (42), and WTAP, a putative splicing factor. The *Drosophila* homologue of WTAP, female-lethal (2)D, is involved in the alternative splicing sex determination pathway (152). Furthermore, WT1 is present in poly(A)(+) RNPs in expressing cell lines and fetal kidney extracts (108), and can shuttle between the nucleus and cytoplasm (198). The WT1 zinc fingers have been shown to bind to RNA, both *in vitro* and *in vivo*, with zinc finger 1 being particularly important in RNA binding (29, 107). Despite these numerous studies, a functional role for WT1(+KTS) in post-transcriptional gene regulation has not been established, and no definite *in vivo* RNA targets have been identified to date (5).

In previous studies, we have used HIV and other retroviral systems as models for elucidating mechanisms underlying the export and expression of mRNA possessing one or more retained introns. Retroviral RNAs contain special *cis*-acting elements that enable the export of viral RNA containing unspliced introns. In the absence of these elements, these RNAs are retained in the nucleus, where they are eventually degraded. The Rev Response Element (RRE) in HIV was the first such element to be identified. The RRE functions in conjunction with the HIV Rev protein (156).

Another specific viral element capable of facilitating the export of mRNAs with retained introns is derived from Mason-Pfizer Monkey Virus (MPMV), a type D retrovirus, and is known as the Constitutive Transport Element (CTE; reviewed in (72). This element is essential for the export of the genomic, unspliced RNA that is expressed in MPMV infected cells. The CTE has been shown to substitute for Rev and the RRE in export of HIV mRNA and to promote the export of cellular RNAs with retained intron (22, 47, 219). In contrast to the HIV RRE, which functions in conjunction with the viral Rev protein, the CTE interacts only with host-cell factors. It has been shown that the CTE binds specifically to Tap (NXF1), believed to play a major role as a cellular mRNA export receptor (67, 89). Tap forms a heterodimer with the cellular NXT1 protein (55), and both Tap and NXT1 have been shown to be important for CTE function (20, 68, 98). Although Tap and NXT1 function in mRNA export pathways, we have recently demonstrated that these proteins also promote translation of CTE-mRNA in the cytoplasm, with Tap, but not NXT1, associating with polyribosomes (93).

The fact that host-cell proteins interact directly with the MPMV-CTE to regulate post-transcriptional gene expression has led us to hypothesize the existence of cellular CTEs in the human transcriptome. These CTEs could function to enable cellular RNAs containing complete introns and other alternatively spliced RNAs to exit the nucleus and be translated in the cytoplasm. Although intron retention seems to be the least common form of alternative splicing in higher eukaryotes, several mammalian genes may be regulated in this way (58). To identify cellular CTEs, we developed an HIV vector based CTE trap system (to be described in detail elsewhere). This system was specifically designed to enable the cloning of elements that can substitute for Rev/RRE to export unspliced HIV RNA to the cytoplasm.

Here we describe the identification of a cellular CTE that shows strong homology to an RNA that co-immunoprecipitated with epitope tagged WT1(+KTS) protein. We demonstrate that this element can function as a CTE in HIV based reporter assays. Furthermore, we demonstrate that expression mediated by this element is enhanced by WT1(+KTS). Our studies also show that the WT1(+KTS) protein, in contrast to the WT1(-KTS) protein, is able to strongly enhance expression from reporter constructs containing the MPMV-CTE. We show that the expression of WT1(+KTS) promotes the polysome association of CTE-containing RNA, indicating a role for this protein in translational regulation of target mRNAs.

These results clearly demonstrate for the first time that WT1(+KTS) can function to promote gene expression at the post-transcriptional level.

Results

The WT1(+KTS) protein enhances expression from unspliced RNA containing either a cellular or viral CTE. In the course of selecting potential constitutive transport elements from a COS cell cDNA library using an HIV vector trap system, we isolated a 594 nt element (5A1) (Bor *et al.*, unpublished). A portion of this element is identical to an RNA that was previously found in a protocol aimed at identifying genes regulated by the WT1 protein at the post-transcriptional level (Ladomery and Hastie, unpublished). This RNA, named non-protein coding T12 mRNA (Genbank accession number 4375837) was isolated in a co-immunoprecipitation with epitope tagged WT1(+KTS) protein expressed in COS cells. As can be seen

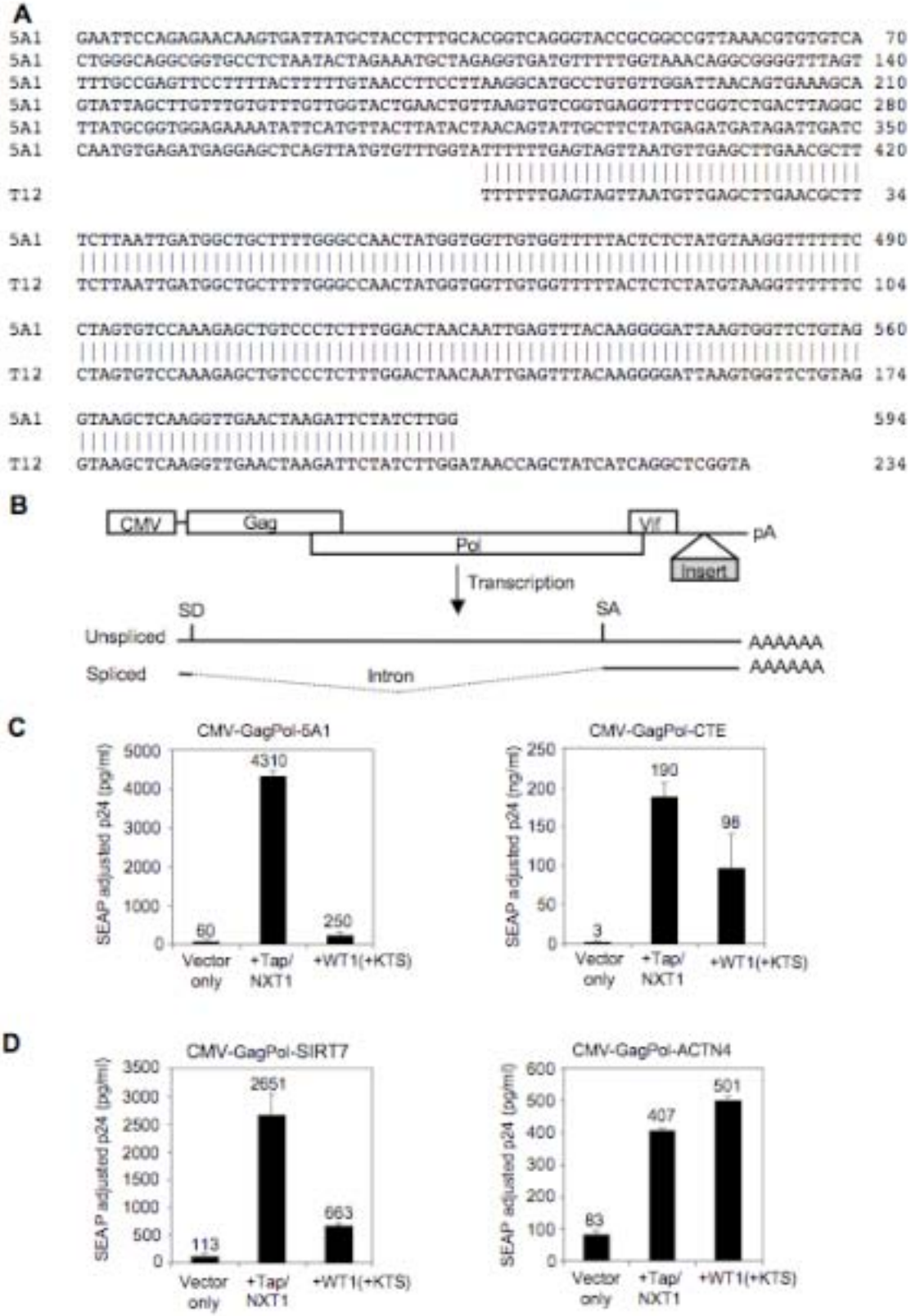
in Fig. 36A, the 234 nt T12 mRNA shows perfect homology with the 5A1 CTE in a 208 nt stretch at the 3' end of the 5A1 sequence.

Since WT1(+KTS) has been proposed to function in post-transcriptional regulation, we decided to test whether expression of WT1(+KTS) in transfected cells had any effect on expression from reporter constructs containing either the 5A1 sequence or the well-characterized CTE from MPMV. 293T cells were chosen for these experiments, since it has been well established that these cells do not express detectable amounts of WT 1 RNA or protein. Thus these cells provide an excellent "complementation" system for analysis of WT1 effects. In addition, we have previously demonstrated that expression of Sam68 and Tap/NXT can enhance expression from CTE containing RNAs in these cells (37, 93).

For these experiments we utilized a reporter assay based on the HIV-derived plasmid pCMVGagPol. A diagram of the important features of this plasmid is shown in Fig. 36B. Expression of GagPol proteins from this plasmid requires export and translation of an unspliced RNA that retains a complete intron. This only takes place if the unspliced RNA contains a *cis*-acting RNA element that can overcome a cellular restriction that normally prevents its export and expression. Expression of GagPol proteins from the reporter construct leads to expression and secretion of particles that contain HIV p24, which can be measured using a specific ELISA. As a control, cells are also transfected with a CMV vector expressing Secreted Alkaline Phosphatase (SEAP) (pHR1831) that is also secreted into the medium of the transfected cells. Since SEAP is expressed from a conventional spliced mRNA, normalization to SEAP values corrects for both transfection efficiency and generalized effects on "normal" mRNA metabolism. We have previously shown that insertion of the MPMV-CTE into the pCMVGagPol plasmid enables efficient export and expression of unspliced RNA (37, 93, 189).

293T cells were transfected with the 5A1 or MPMV-CTE-containing pCMVGagPol reporter plasmids alone or together with a construct expressing the WT1(+KTS) protein. In parallel, we transfected cells with plasmids expressing Tap and NXT1 (pHR2128 and pHR2283). We have previously shown that moderate over-expression of Tap/NXT1 is able to enhance expression from the pCMVGagPol-CTE plasmid in 293T cells (68, 93). Figure 40C demonstrates that both Tap/NXT1 and WT1(+KTS) were able

Figure 36. *A*, Nucleotide sequence alignment of a cellular element, 5A1, with the non-protein coding T12 mRNA sequence. Identical nucleotides are indicated by vertical lines. *B*, Schematic drawing of reporter constructs used in this study. The reporter plasmids contain the HIV-1 *gagpol* coding sequence with expression driven by the CMV promoter. The resulting unspliced transcript, containing a complete intron, is retained in the nucleus without the presence of a CTE. In the presence of a CTE, the unspliced mRNA is exported to the cytoplasm, allowing the expression of HIV p24 from the *gag* gene. SD, splice donor; SA, splice acceptor; pA, polyadenylation signal. The dotted line below the spliced message depicts the removed intron. *C*, Expression of p24 from pCMVGagPol-5A1 or pCMVGagPol-CTE is enhanced by co-transfection of plasmids expressing Tap/NXT1 or WT1(+KTS). 293T cells (3×10^6 cells in a 10 cm culture dish) were transfected with 15 μg of pCMVGagPol-5A1 or 5 μg of pCMVGagPol-CTE plasmids and 0.25 μg of pCMVSEAP alone or together with 2 μg of pCMVTap and 1 μg of pCDNA3FLAGNXT1 or 5 μg of WT1(+KTS) expression vectors. At 72 hours post-transfection, supernatants were collected and analyzed for p24 levels and SEAP activity. The p24 values were normalized to SEAP values that serve as a control for transfection efficiency. The values shown are averages of duplicate transfections. *D*, Expression of p24 from pCMVGagPol-SIRT7 or pCMVGagPol-ACTN4 is enhanced by co-transfection of plasmids expressing Tap/NXT1 or WT1(+KTS). Transfections were performed and analyzed as described in (*C*). (p24 experiments performed by YCB)


A
5A1 GAATTCCAGAGAACAAGTGATTATGCTACCTTTGCACGGTCAGGGTACCGCGGCCGTTAAACGTGTGTCA 70
5A1 CTGGGCAGGCGGTGCCTCTAATACTAGAAATGCTAGAGGTGATGTTTTTGGTAAACAGGCGGGGTTTAGT 140
5A1 TTTGCCGAGTTCCTTTTACTTTTTGTAACCTTCCTTAAGGCATGCCTGTGTTGGATTAACAGTGAAAGCA 210
5A1 GTATTAGCTTGTTTGTGTTTGTTGGTACTGAACTGTTAAGTGTCGGTGAGGTTTTCGGTCTGACTTAGGC 280
5A1 TTATGCGGTGGAGAAAATATTCATGTTACTTATACTAACAGTATTGCTTCTATGAGATGATAGATTGATC 350
5A1 CAATGTGAGATGAGGAGCTCAGTTATGTGTTTGGTATTTTTTGAGTAGTTAATGTTGAGCTTGAACGCTT 420
T12 TTTTTTGAGTAGTTAATGTTGAGCTTGAACGCTT 34
5A1 TCTTAATTGATGGCTGCTTTTGGGCCAACTATGGTGGTTGTGGTTTTTACTCTCTATGTAAGGTTTTTTC 490
T12 TCTTAATTGATGGCTGCTTTTGGGCCAACTATGGTGGTTGTGGTTTTTACTCTCTATGTAAGGTTTTTTC 104
5A1 CTAGTGTCCAAAGAGCTGTCCCTCTTTGGACTAACAATTGAGTTTACAAGGGGATTAAGTGGTTCTGTAG 560
T12 CTAGTGTCCAAAGAGCTGTCCCTCTTTGGACTAACAATTGAGTTTACAAGGGGATTAAGTGGTTCTGTAG 174
5A1 GTAAGCTCAAGGTTGAACTAAGATTCTATCTTGG 594
T12 GTAAGCTCAAGGTTGAACTAAGATTCTATCTTGGATAACCAGCTATCATCAGGCTCGGTA 234
B
CMV
Gag
Pol
Vif
pA
Insert
Transcription
SD
SA
Unspliced
AAAAAA
Spliced
Intron
AAAAAA
C
CMV-GagPol-5A1
SEAP adjusted p24 (pg/ml)
5000
4000
3000
2000
1000
0
4310
60
250
Vector only
+Tap/ NXT1
+WT1(+KTS)
CMV-GagPol-CTE
SEAP adjusted p24 (ng/ml)
250
200
150
100
50
0
190
98
3
Vector only
+Tap/ NXT1
+WT1(+KTS)
D
CMV-GagPol-SIRT7
SEAP adjusted p24 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
2651
663
113
Vector only
+Tap/ NXT1
+WT1(+KTS)
CMV-GagPol-ACTN4
SEAP adjusted p24 (pg/ml)
600
500
400
300
200
100
0
501
407
83
Vector only
+Tap/ NXT1
+WT1(+KTS)

to significantly enhance expression from the plasmid containing 5A1 as well as the plasmid containing the MPMV-CTE (In this experiment, SEAP values did not vary by more than 2 fold). Although much lower expression was obtained from the plasmid containing 5A1 (note the scale change), these experiments clearly support the notion that 5A1 constitutes a cellular equivalent of the CTE.

The HIV vector trap system that led to the identification of the 5A1 sequence also identified several other CTEs from additional cellular genes. Included among these are actinin4 (ACTN4) and SIRT7 (see supplemental data Table 1S for a description of these elements). ACTN4 is potentially particularly relevant to WT1 function, since preliminary experiments using a 3-hybrid system have shown that mRNA from a related family member *ACTN1* is a potential WT1 binding target (Ladomery et al., in preparation). ACTN1 and ACTN4 (non-muscle specific alpha-actinins) are closely related and coordinately expressed. Furthermore, ACTN4 is known to be important in kidney function and mutations in this gene cause focal segmental glomerular sclerosis (96). FSGS, a disease which is part of the triad seen in Frasier syndrome patients, is caused by altered WT1(+KTS)/WT1(-KTS) ratios (4, 75). The function of SIRT7 is less clear, but recently the gene has been identified to express an mRNA that retains an intron (58)

To test the effects of WT1(+KTS) expression on the CTEs from ACTN4 and SIRT7, we created pCMVGagPol reporter plasmids containing these elements and measured p24 expression as described above. The data (Fig. 36D) clearly demonstrate that WT1(+KTS) enhanced expression about 6 fold from these plasmids. Thus this protein is able to enhance the function of at least 3 different cellular CTEs.

The WT1(-KTS) protein inhibits WT1(+KTS) function in a dose-dependent manner. Since the viral CTE gave much higher p24 levels and is a well-characterized post-transcriptional element, we continued by analyzing the function of the WT1 protein with the GagPol-MPMV-CTE reporter construct. First, we wanted to determine whether the enhancement that we had observed was specific to WT1(+KTS). To this end, we performed experiments in which increasing amounts of plasmids expressing either WT1(+KTS) or (-KTS) proteins were cotransfected with pCMVGagPol-CTE (pHR1361; Figs. 37A-C). We also performed similar experiments using a plasmid expressing the EGR1 protein (Fig. 37D). Like WT1, the EGR1 protein

contains Cys_2-His_2 zinc fingers at the C-terminus. Zinc fingers 2, 3 and 4 of WT1 exhibit 60 per cent amino acid homology with three zinc fingers of EGR1 (160). However, in contrast to the WT1 proteins, this protein does not show significant binding to RNA.

As can be seen in Figs. 37A and B, the WT1(+KTS) protein enhanced CTE function in a dose-dependent manner, confirming and extending the previous results. At the highest concentration of WT1(+KTS) there was about a 45 fold increase in normalized p24 values (Fig. 37B). In contrast, WT1(-KTS) gave only a minimal increase in p24 expression (about 2.5 fold). However, the small increase that was observed can be clearly seen to be dose responsive (see data plotted on expanded scale in Fig. 37C) and is likely to be significant, since it was observed in repeated experiments (data not shown). In contrast, expression of EGR1 did not give any significant increase in p24 expression (Fig. 37D). Western blot analysis of the expressed WT1(+KTS), WT1(-KTS) and EGR1 proteins showed that they were all expressed at similar levels (Fig. 38).

Since the WT1(+KTS) and WT1(-KTS) isoforms have been shown to form heterodimers, we next examined the effects of co-expression of these proteins on expression from the CTE. To do this, we performed two experiments in which a fixed amount of either the plasmid expressing the +KTS isoform or the plasmid expressing the –KTS isoform was co-transfected with increasing amounts of the other plasmid. The results of these experiments, shown in Figs. 39A and B, clearly showed that expression of WT1(-KTS) inhibited the effects of WT1(+KTS) in a dose-dependent manner. This indicates that expression of the WT1(-KTS) protein shows a transdominant negative effect over the enhancement observed with the +KTS isoform. This observation may relate to the documented ability of WT1 to dimerize via the N-terminus (23, 46, 139, 162).

Exon 5 sequences and the amino acid sequence of the 3 amino acid insertion in WT1(+KTS) are not essential for promotion of CTE function. Alternative splicing gives rise to several different isoforms of WT1. One of the alternative splicing events is the skipping of exon 5. The original experiments that we had performed (shown in Figs. 36C and D, 37A and B, 39A and B), all used WT1 proteins that included this exon. To investigate whether exon 5 was essential for enhancement of CTE function, we analyzed the

Figure 37. WT1(+KTS), but not WT1(-KTS) or EGR1, specifically enhances p24 expression from pCMVGagPol-CTE reporter construct in 293T cells in a dose-dependent manner. Cells (3×10^6) were co-transfected with 5 μg of pCMVGagPol-CTE, 0.25 μg of pCMVSEAP, and increasing amounts of plasmids expressing either (*A*) WT1(+KTS) or WT1(-KTS), or (*D*) EGR1 proteins. *B* and *C*, The same set of data shown in (*A*) was plotted as "fold increase" on different scales. At 72 hours post-transfection, supernatants were harvested and analyzed for p24 levels and SEAP activity. The p24 values shown have been normalized for SEAP activity. The data represent the average of two independent transfections. (p24 experiments performed by YCB)

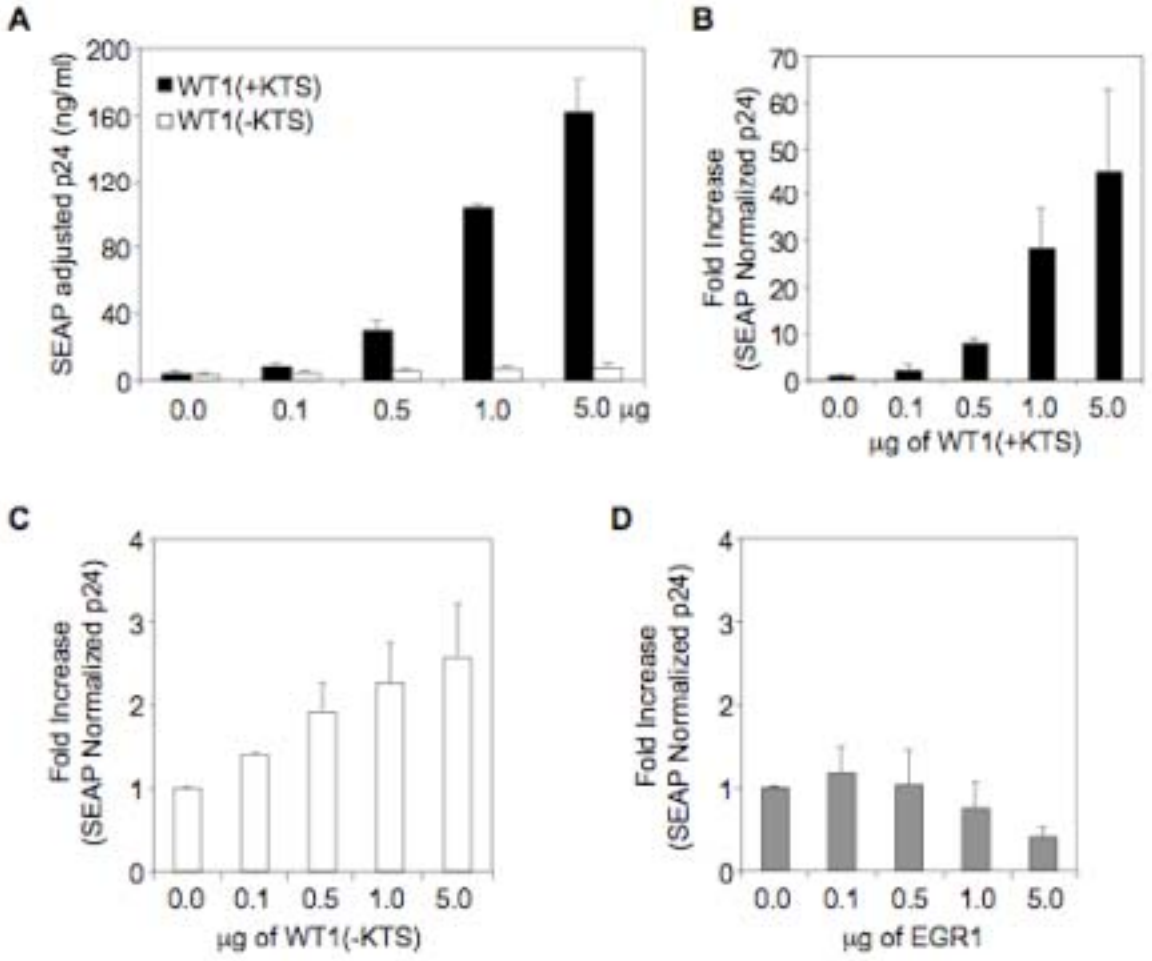

A
SEAP adjusted p24 (ng/ml)
200
160
120
80
40
0
WT1(+KTS)
WT1(-KTS)
0.0
0.1
0.5
1.0
5.0 µg
B
Fold Increase
(SEAP Normalized p24)
70
60
50
40
30
20
10
0
0.0
0.1
0.5
1.0
5.0
µg of WT1(+KTS)
C
Fold Increase
(SEAP Normalized p24)
4
3
2
1
0
0.0
0.1
0.5
1.0
5.0
µg of WT1(-KTS)
D
Fold Increase
(SEAP Normalized p24)
4
3
2
1
0
0.0
0.1
0.5
1.0
5.0
µg of EGR1

Figure 38. Western blots of expressed WT1 proteins and EGR1. 293T cells were transfected with 5 μg of plasmids expressing T7-tagged WT1(+KTS), WT1(-KTS), WT1(+AAA), or EGR1. Three days post transfection, cell lysates were prepared, and samples were analyzed by Western blotting with antibody to the T7 epitope. Results shown in lanes 1 and 2, and lanes 3 to 5 were obtained from two independent experiments. The molecular weight standards are indicated. (Western blots performed by JES)

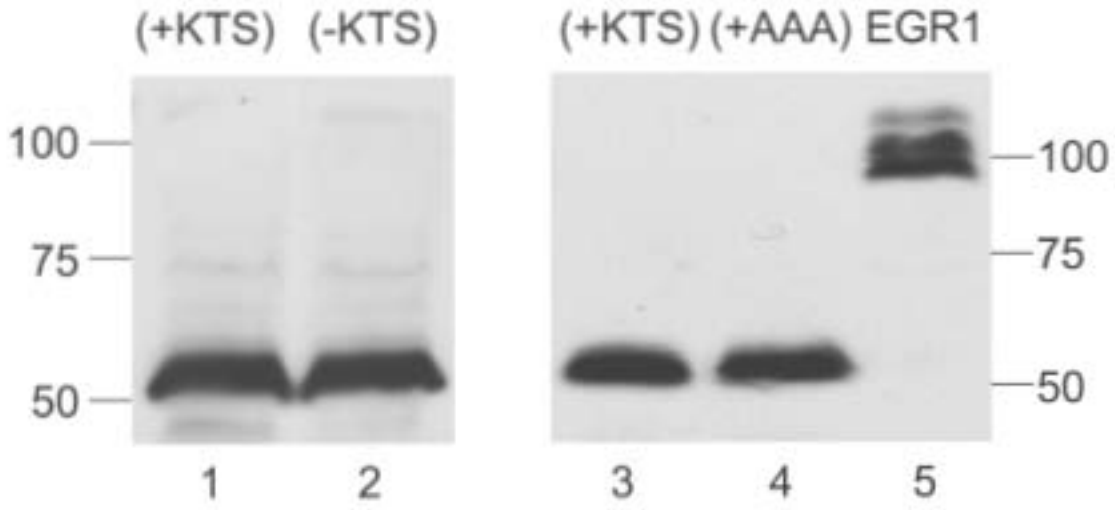
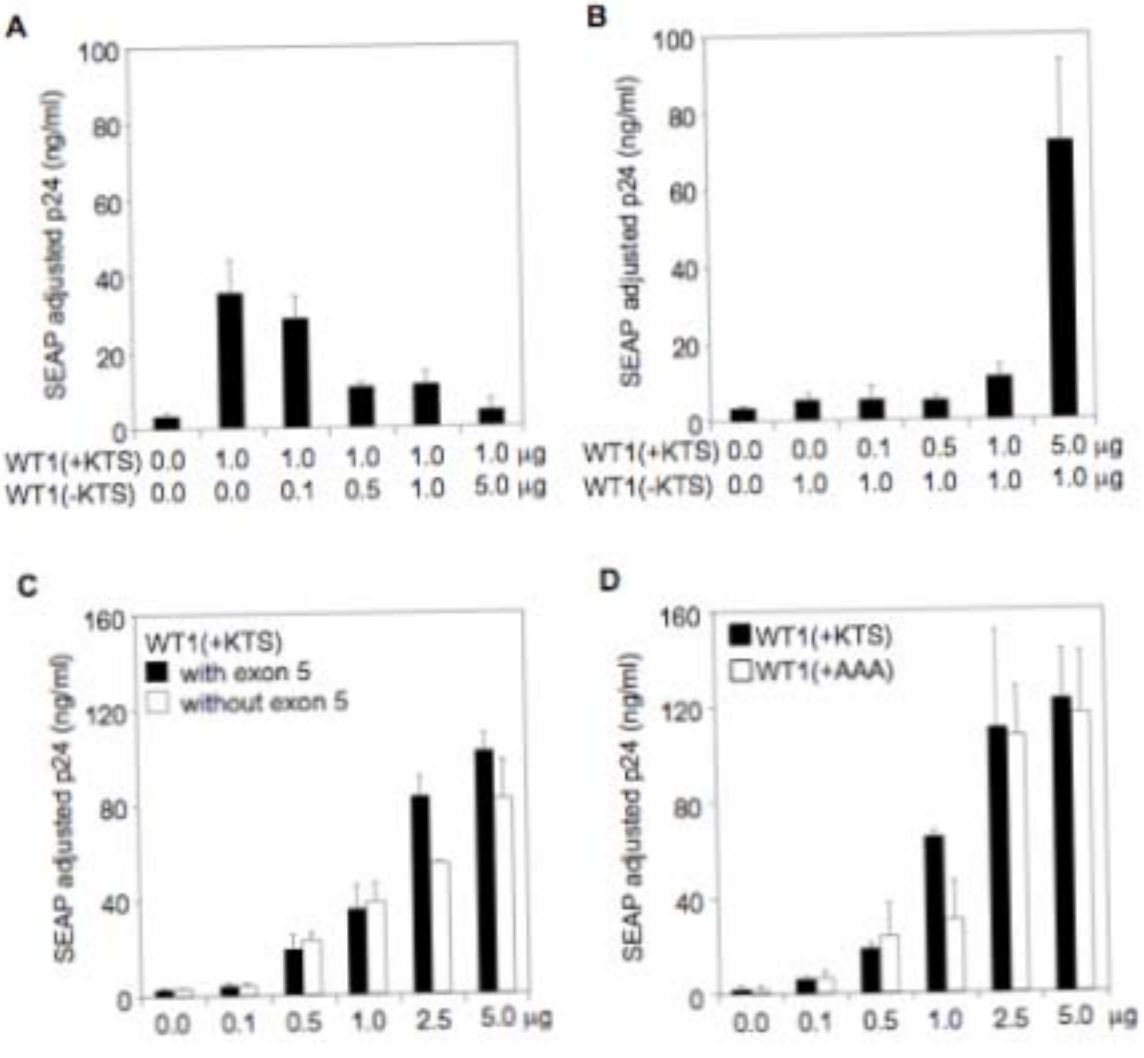

(+KTS)
(-KTS)
(+KTS)
(+AAA)
EGR1
100
75
50
100
75
50
1
2
3
4
5

Figure 39. Over-expression of WT1(-KTS) inhibits the enhancement effects of WT1(+KTS) in transfected 293T cells. *A* and *B,* Cells were co-transfected with 5 μg of pCMVGagPol-CTE, 0.25 μg of pCMVSEAP and increasing amounts of (*A*) WT1(-KTS) or (*B*) WT1(+KTS) while the amount of the plasmid expressing the other isoform was kept constant at 1 μg. *C* and *D*, Neither exon 5 nor the specific KTS amino acid sequence is important for the post-transcriptional function of WT1. Cells were co-transfected with 5 μg of pCMVGagPol-CTE, 0.25 μg of pCMVSEAP and increasing amounts of WT1 constructs, as indicated in the figure. In all cases, the overall amount of plasmid transfected was kept constant using an empty vector. Transfections and analyses were performed as described in the legend of *Fig. 37*. (p24 experiments performed by YCB)


A
SEAP adjusted p24 (ng/ml)
100
80
60
40
20
0
WT1(+KTS) 0.0 1.0 1.0 1.0 1.0 1.0 µg
WT1(-KTS) 0.0 0.0 0.1 0.5 1.0 5.0 µg
B
SEAP adjusted p24 (ng/ml)
100
80
60
40
20
0
WT1(+KTS) 0.0 0.0 0.1 0.5 1.0 5.0 µg
WT1(-KTS) 0.0 1.0 1.0 1.0 1.0 1.0 µg
C
SEAP adjusted p24 (ng/ml)
160
120
80
40
0
WT1(+KTS)
with exon 5
without exon 5
0.0 0.1 0.5 1.0 2.5 5.0 µg
D
SEAP adjusted p24 (ng/ml)
160
120
80
40
0
WT1(+KTS)
WT1(+AAA)
0.0 0.1 0.5 1.0 2.5 5.0 µg

effects of a WT1(+KTS) protein lacking this exon on CTE function. The results of these experiments, shown in Fig. 39C, clearly indicated that exon 5 is not essential for the observed effects.

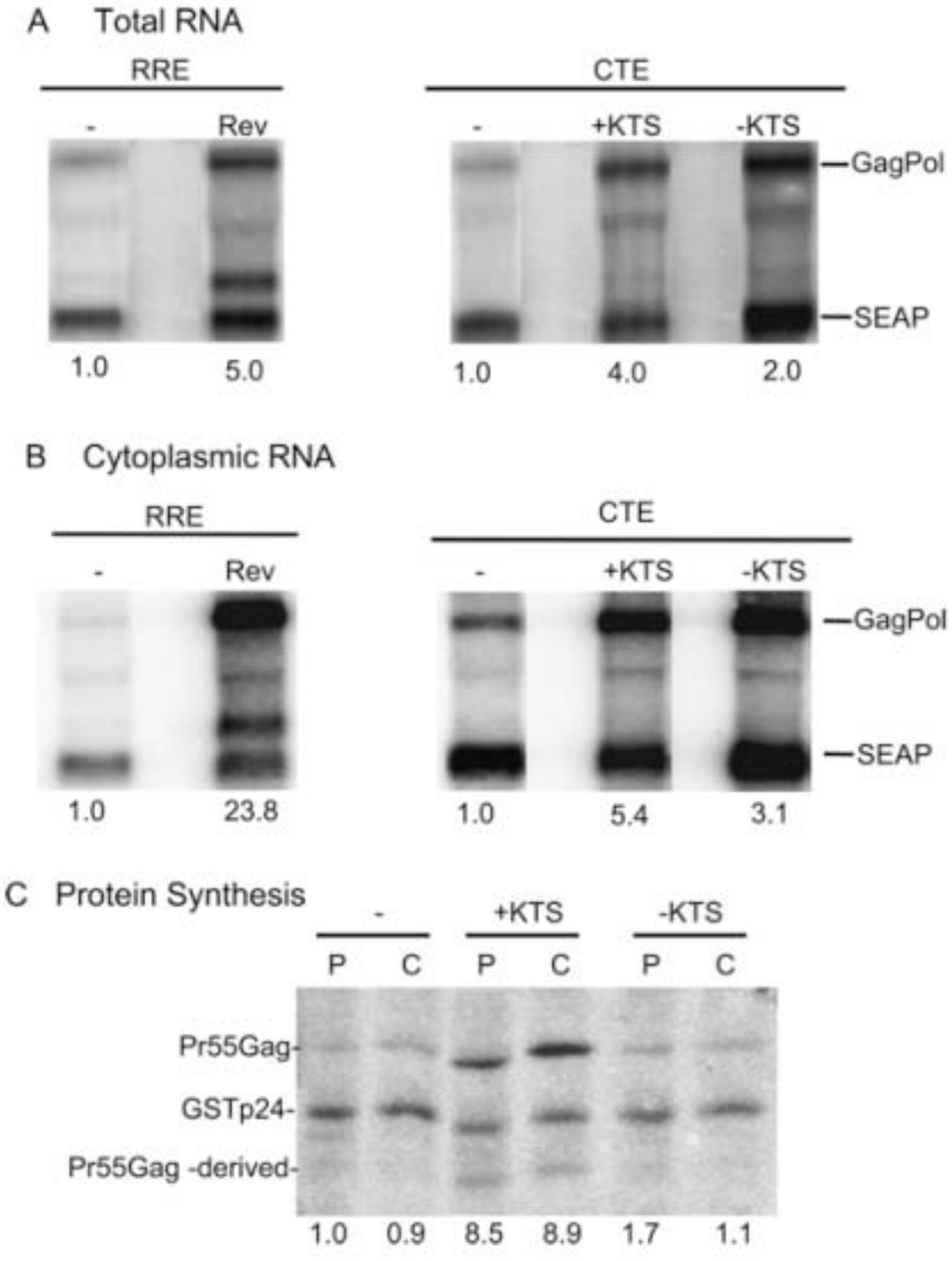
We also constructed a plasmid expressing a variant of WT1, in which the sequence encoding the KTS motif was mutated to encode three alanines (AAA). Western blot analysis of the protein produced from this plasmid showed that it was expressed at levels similar to the other WT1 proteins (Fig. 38). Transfection experiments using this construct showed that this protein was able to enhance CTE function as well as the +KTS protein (Fig. 39D). This demonstrates that the amino acid sequence of the KTS insertion is not essential for CTE enhancement. This supports the notion that the KTS insertion merely serves to structurally disrupt the zinc finger region of the WT1 protein and that the specific amino acid sequence is not important (41, 111).

Both WT1(+KTS) and WT1(-KTS) increase total as well as cytoplasmic levels of CTE-containing RNA. To analyze the potential mechanism for the observed effects of WT1 on CTE-mediated expression, we performed a Northern blot analysis of cytoplasmic as well as total RNA in cells transfected with WT1(+KTS) or WT1(-KTS) expressing plasmids. To control for fractionation, we also performed an analysis of cells transfected with the plasmid pCMVGagPol-RRE (pHR0354), in the absence or presence of Rev (pHR30). We and others have previously demonstrated that GagPol-RRE RNA is retained in the nucleus in the absence of Rev, but efficiently exported to the cytoplasm in its presence (37, 68, 93, 156).

The results of this experiment are shown in Fig. 40. The control experiment demonstrated that the levels of GagPol-RRE reporter RNA in the cytoplasm increased 24-fold in the presence of Rev, consistent with our previously published results (37, 68, 93). Total RNA levels were also increased, but only about 5-fold. This has been observed previously, and is likely due to an increased stability of the RNA, when it is exported to the cytoplasm and engaged by the translation machinery, compared to when it is retained in the nucleus.

In the transfections using the CTE construct, total RNA levels were increased about 2-fold in conjunction with WT1(-KTS), while the expression of WT(+KTS) gave a 4-fold increase. These results

Figure 40. Northern blot analyses of (*A*) total and (*B*) cytoplasmic mRNA, and (C) protein synthesis levels of Pr55Gag in transfected cells. *A* and *B*, 293T Cells (1×10^7 in 15 cm culture dish) were transfected with 15 μg of pCMVGagPol-CTE and 5 μg of pCMVSEAP in the absence or presence of 15 μg of WT1(+KTS) or WT1(-KTS) plasmids. As a control, cells were also transfected with 15 μg pCMVGagPol-RRE and 5 μg of pCMVSEAP in the presence or absence of 5 μg of pCMVRev. Sixty-five hours post-transfection, total and cytoplasmic poly(A)$^+$ mRNA was isolated from the transfected cells as described in Materials and Methods. The blot contains 5 μg of poly(A)$^+$ mRNA per lane and was hybridized with ^{32}P-labeled GagPol and SEAP probes. The values shown in the panels marked RRE, on the left side of each figure represents the fold difference in the levels of the GagPol RNA bands between transfections without and with Rev. The values underneath the CTE panels (the right side of the figure) represent the fold difference in the levels of the GagPol-CTE RNA in the presence of the indicated proteins compared to a transfection with pCMVGagPol-CTE alone. All values were normalized using the SEAP band. *C*, Pulse-chase analysis of Pr55Gag protein expressed from pCMVGagPol-CTE(myr- pro-) in transfected 293T cells. Cells (3×10^6) were transfected with 5 μg of pCMVGagPolCTE(myr- pro-) with or without 5 μg of WT1(+KTS), or 5 μg of WT1(-KTS). Thirty-six hours post-transfection, cells were pulsed with ^{35}S–translabel (methionine and cysteine) for 20 min and chased for 10 h. Lysates were made, ^{35}S-labeled GST-p24 was added as a recovery control, and immunoprecipitation was performed using an anti-p24 monoclonal antibody (183-H12-5C). The precipitates were analyzed on a 15% SDS-PAGE using a PhosphorImager. The lanes marked (P) contained the samples from the pulse and the lanes marked (C) contained the samples that were pulse-chased. The locations of the immunoprecipitated Pr55Gag band (and derivative) and the control GST-p24 protein are indicated. The relative normalized pixel intensity of the total Pr55Gag protein bands is shown under each lane. The data have been normalized using the intensity of the recovery control protein GST-p24 in each sample. (Northern blots and Pulse-chase analysis were performed by YCB)


A Total RNA
RRE
CTE
-
Rev
-
+KTS
-KTS
GagPol
SEAP
1.0
5.0
1.0
4.0
2.0
B Cytoplasmic RNA
RRE
CTE
-
Rev
-
+KTS
-KTS
GagPol
SEAP
1.0
23.8
1.0
5.4
3.1
C Protein Synthesis
-
+KTS
-KTS
P
C
P
C
P
C
Pr55Gag-
GSTp24-
Pr55Gag -derived-
1.0
0.9
8.5
8.9
1.7
1.1

again most likely reflect a stabilization of the RNA, rather than a transcriptional effect, since the SEAP mRNA is expressed from the same promoter as the CTE RNA, and the data is normalized using the SEAP mRNA. When cytoplasmic CTE-containing RNA was analyzed, WT1(-KTS) was shown to increase the RNA levels about 3-fold, whereas a 5-fold increase was observed in the presence of WT1(+KTS). These increases are only marginally different from what was observed in the total RNA preparation, suggesting that there was no significant specific increase in RNA export with either of these proteins. Clearly, since the slightly increased RNA levels observed with both WT1 isoforms cannot account for the differential increase in p24 protein levels seen with WT1(+KTS) compared to WT1(-KTS). This becomes clear when the data for cytoplasmic RNA from this experiment is used to normalize the data shown in Fig. 37. In that figure, transfection with 5 μg of WT1(+KTS) (the same amount used in this experiment) increased protein levels 42 fold, while transfection with WT(-KTS) increased p24 values only 2.6 fold. Thus, for WT(+KTS) there is over a 7.7 fold increase in protein levels (42/5.4) after normalization for RNA levels. On the other hand, for WT1(-KTS) the increase in RNA fully accounts for the increase in protein (2.6/3.1).

WT1(+KTS) increases the rate of synthesis of protein translated from GagPol-CTE RNA but WT1(-KTS) does not. To determine if the difference in p24 accumulation levels between cells transfected with pCMVGagPol-CTE and WT1(+KTS) versus WT1(-KTS) was a direct result of an increased rate of protein synthesis, we decided to directly measure the relative rates. The pCMVGagPol-CTE construct normally gives rises to polyproteins that are cleaved by the HIV protease and bud from transfected cells as virus-like particles. Because of this, it is difficult to directly visualize the primary translation product in transfected cells. This precludes a straightforward measurement of the rate of protein synthesis by examining the accumulating protein directly. To overcome this problem, we constructed a new plasmid, pCMVGagPol(myr^- pro^-)-CTE (pHR2900). This plasmid has point mutations in the HIV protease active site that makes the protease inactive (pro^-) and in the amino-terminus of Gag that destroys the myristoylation signal (myr-). Thus only non-myristoylated Pr55Gag and Pr160GagPol precursors, and a minor derivative (p41), likely to be derived by the use of internal initiation codon, are expected to be

produced from these vectors (187). Since myristoylation is required for virus particle budding, these proteins would be expected to remain inside the transfected cell in an uncleaved form. This was verified by showing that no p24 was detected in the medium supernatant (data not shown) and that only uncleaved Pr55Gag proteins accumulated in the cytoplasm of transfected cells.

To directly analyze the rate of protein synthesis and/or stability of the synthesized proteins, we performed a pulse-chase experiment on cells transfected with the modified (myr-, pro-) CTE construct. At 36 hours post transfection, cells were pulsed with ^{35}S-Translabel (methionine and cysteine) for 20 min. The cells were then washed twice with PBS and harvested (pulse), or re-fed with growth medium containing excess cold amino acids, and harvested 10 hours later (chase). Cell lysates were subjected to immunoprecipitation using a mouse anti-Gag (p24) antibody and analyzed for Pr55Gag and p41 by SDS-PAGE and autoradiography. To compensate for potential differences in immunoprecipitation efficiency between samples, *in vitro* purified GST-p24 protein was added to the cell lysates before the immunoprecipitation. A second immunoprecipitation was also performed on the supernatants after the initial immunoprecipitation to judge the efficiency of the first precipitation. Greater than 95% of each protein was pulled down in the first precipitation (data not shown).

The results of this pulse-chase experiment are shown in Fig. 40C. Only a very small amount of Pr55Gag was observed in the cells transfected with the GagPol-CTE plasmid alone. The amount of labeled protein did not decrease significantly after a 10-hour chase, indicating that the proteins produced were stable during this period. A significant increase in the intensity the of Pr55Gag band was observed in the presence of WT1(+KTS), but there was only a slight increase (if any) in the presence of WT1(-KTS). Again, the protein produced was stable during a 10-hour chase period. When the data are normalized for cytoplasmic RNA levels, using the values shown in Fig. 40B, the rate of Gag protein synthesis after transfection of WT(+KTS) was 2 fold higher than the rate observed with no added WT1 protein. However, the rate differences between the transfections with WT(+KTS) and WT(-KTS) were about 5 fold. This is the more important comparison, since the rate of synthesis might have been measured at a time when the effect of the WT1 proteins was not optimal. Thus this experiment clearly indicated that WT1(+KTS) was

able to specifically enhance translation from the GagPol-CTE RNA, as we previously observed for Tap/NXT1 (93).

WT1(+KTS) increases association of CTE-containing RNA with polyribosomes. To test whether expression of WT1(+KTS) resulted in an increased polyribosome association of the GagPol-CTE RNA, we made cytoplasmic extracts from cells that were either transfected with the reporter plasmids alone or the reporter plasmids and WT1(+KTS). The extracts were then subjected to sucrose gradient centrifugation, fractionated, and each fraction was analyzed on a Northern blot with the same probes that were used in the experiment shown in Fig. 40. The results of this experiment are shown in Fig. 41. For the cells transfected with the SEAP and GagPol-CTE plasmids alone, it is striking that the heavier polyribosomal fractions were largely devoid of GagPol-CTE RNA (Fig. 41A, left panels), although the SEAP mRNA was found throughout the gradient. Specifically it was observed that a large percent of the CTE-RNA localized to the fractions corresponding to monosomes and very small polyribosomes. This is likely to indicate poor initiation rates for the CTE-RNA and is consistent with our previous results that demonstrated that GagPol-CTE RNA is poorly translated in 293T cells (93). Significantly, Fig. 41A right panels show that the addition of WT1(+KTS) caused the redistribution of a significant amount of the GagPol-CTE RNA to heavier polyribosomes. In contrast, the distribution of the SEAP mRNA remained largely unchanged. Figure 41B directly compares the distribution of CTE or SEAP RNA with or without WT1(+KTS) expression. Together, these results demonstrate that expression of WT1(+KTS) promotes polyribosomal association and translation of the GagPol-RNA containing the CTE, in addition to its slight effect on mRNA levels. In contrast, WT1(-KTS) had little effect on the polyribosomal association of GagPol-RNA, consistent with its lack of an effect on the rate of Gag protein synthesis (Fig. 42).

WT1(+KTS) associates with polyribosomes in 293T cells. A recent paper showed that both the WT1(+KTS) and WT1(-KTS) proteins shuttle between the nucleus and the cytoplasm in several different cell types. It was also reported that both WT1 isoforms associate with translating polyribosomes in transfected COS-7 cells (147).

Figure 41. Polyribosome profile analysis of GagPol-CTE mRNA in transfected 293T cells by sucrose gradient centrifugation. *A*, 293T Cells (8×10^6) were transfected with 15 μg of pCMVGagPol-CTE in the absence or presence of 15 μg of WT1(+KTS) plasmid. Forty-eight hours post-transfection, cells were harvested and cytoplasmic extracts were subjected to sucrose gradient centrifugation as described in Materials and Methods. The gradients were fractionated and the OD_{254} was measured using a continuous flow cell. An *in vitro* transcribed *gag* RNA (*IVTgag*) was then added into each fraction as a control for recovery of RNA before RNA was isolated from each fraction. The isolated RNA was then analyzed for GagPol-CTE, SEAP mRNA and *IVTgag* RNA using Northern blots. PhosphorImager analysis of the blot was used to quantitate the intensity of the bands. The measured intensity of each GagPol-CTE and SEAP band was then corrected for recovery, using the *IVTgag* RNA band in each fraction. The values shown for each fraction are the percentage of total GagPol-CTE or SEAP mRNA that was detected in that fraction. *B*, The graphs show the distribution of GagPol-CTE (left) and SEAP mRNA (right) in the CTE (*white bars*) and CTE +WT1(+KTS) gradients (*black bars*). (Polysome preparation and fractionation performed by JES; Northern blots performed by YCB)

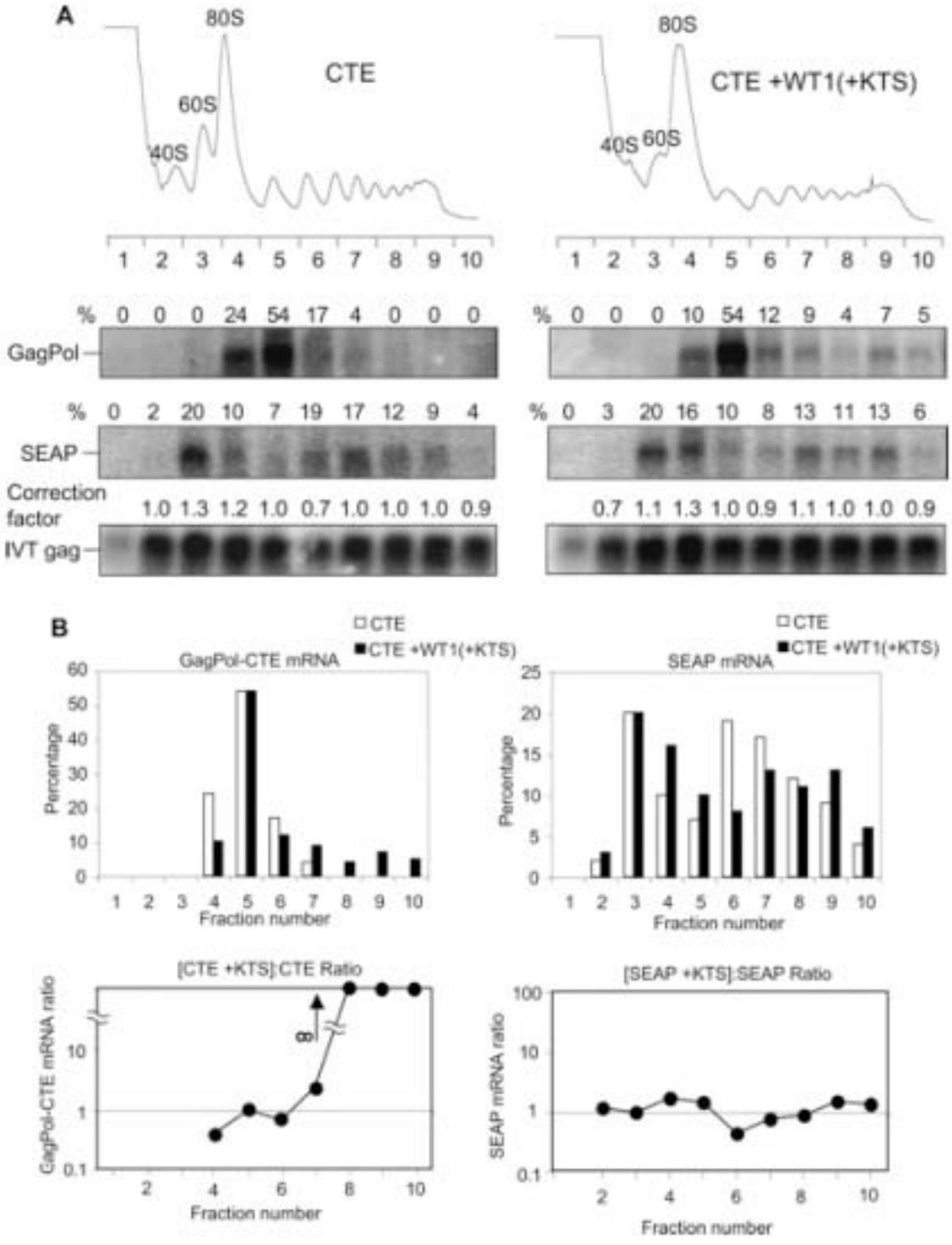
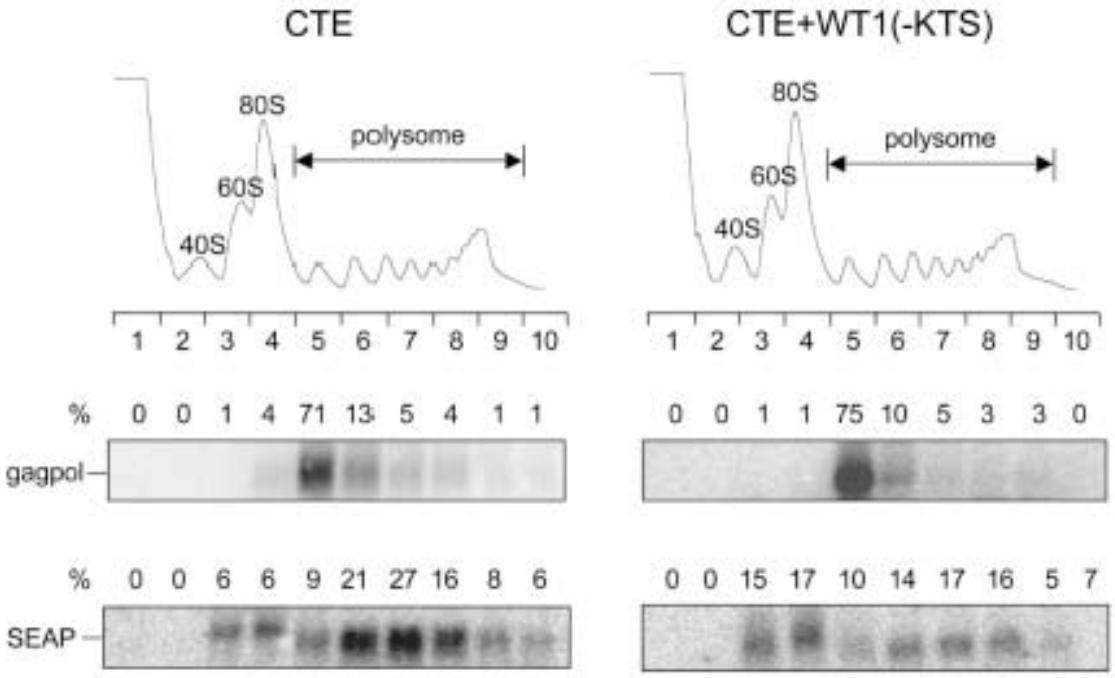

A
80S
60S
40S
CTE
1 2 3 4 5 6 7 8 9 10
% 0 0 0 24 54 17 4 0 0 0
GagPol
% 0 2 20 10 7 19 17 12 9 4
SEAP
Correction factor
1.0 1.3 1.2 1.0 0.7 1.0 1.0 1.0 0.9
IVT gag
80S
60S
40S
CTE +WT1(+KTS)
1 2 3 4 5 6 7 8 9 10
% 0 0 0 10 54 12 9 4 7 5
% 0 3 20 16 10 8 13 11 13 6
0.7 1.1 1.3 1.0 0.9 1.1 1.0 1.0 0.9
B
CTE
CTE +WT1(+KTS)
GagPol-CTE mRNA
Percentage
Fraction number
SEAP mRNA
[CTE +KTS]:CTE Ratio
GagPol-CTE mRNA ratio
8
[SEAP +KTS]:SEAP Ratio
SEAP mRNA ratio

Figure 42. WT1(-KTS) does not increase association of CTE-containing RNA with polyribosomes. 293T cells (8×10^6) were transfected with 15 μg of pCMVGagPol-CTE in the absence or presence of 15 μg of WT1(-KTS) plasmid. Forty-eight hours after transfection, cells were harvested and subjected to cytoplasmic fractionation and sucrose gradients centrifugation as described in Materials and Methods. The gradients were fractionated, and the OD_{254} of each fraction was measured. RNA was then isolated from each fraction and analyzed for GagPol or SEAP mRNA by Northern blots. An *in vitro* transcribed *gag* RNA (*IVTgag*) was added into each fraction as a control for recovery of RNA. The band intensity was analyzed using ImageQuant Software. The percentage of GagPol or SEAP mRNA found in that fraction is indicated above each band. (Polysome preparation and fractionation performed by JES; Northern blots performed by YCB)


CTE
CTE+WT1(-KTS)
80S
polysome
60S
40S
1 2 3 4 5 6 7 8 9 10
% 0 0 1 4 71 13 5 4 1 1
% 0 0 1 1 75 10 5 3 3 0
gagpol
% 0 0 6 6 9 21 27 16 8 6
% 0 0 15 17 10 14 17 16 5 7
SEAP

To investigate whether the WT1(+KTS) protein associated with polyribosomes in 293T cells, we transfected cells with pCMVGagPol-CTE and plasmids expressing either T7-tagged WT1(+KTS) and made cytoplasmic extracts. The extracts were subjected to sucrose gradient centrifugation, and each fraction was analyzed on a Western blot using an anti-T7 antibody. We also performed a similar analysis on fractions from gradients run with extracts treated with 15mM EDTA to specifically disrupt the polyribosomes prior to centrifugation.

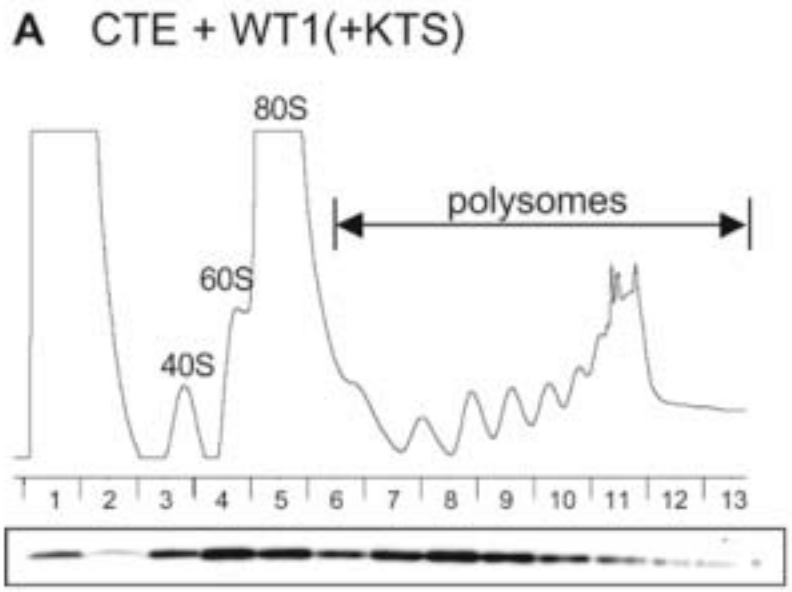
The Western blot analysis shown in Fig. 43A clearly demonstrated that WT1(+KTS) can be found throughout the gradient, including the polyribosomal fractions. This is consistent with the previous results reported in the COS-7 cells. The EDTA treatment (Fig. 43B) clearly collapsed the polyribosomes (compare the profiles of untreated and treated extracts) and shifted all of the WT1 protein to the fractions containing the ribosomal subunits. This strongly suggests that the WT1(+KTS) protein is associated with true polyribosomal complexes and that it may specifically associate with the ribosomal subunits.

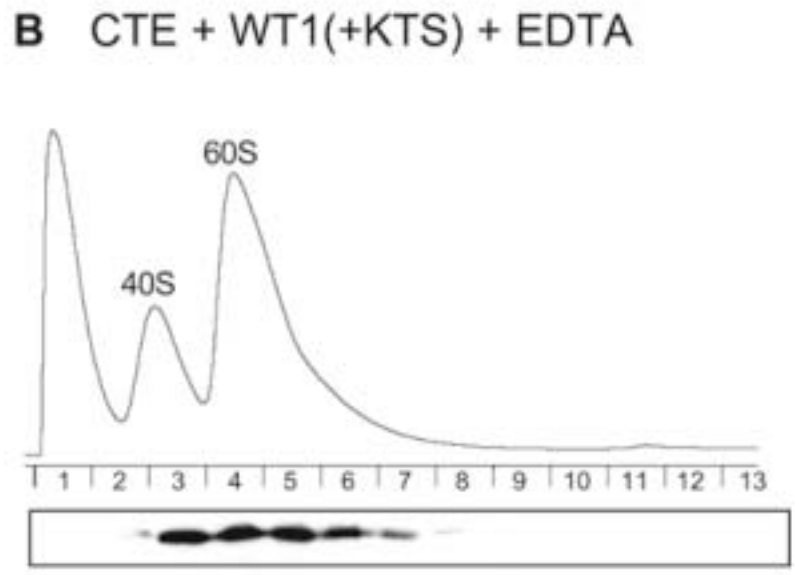
Discussion

The role of the WT1(-KTS) protein as an important transcription factor is well established. However, although a transcriptional role for a chimeric Ewing's Sarcoma gene (EWS)-WT1(+KTS) protein has been described (166), no clear function has been attributed to the native WT1(+KTS) protein, in spite of the fact that proper +KTS/-KTS ratios are crucial in the development of several organs (40, 75, 201). In this study, we have shown for the first time that WT1(+KTS) functions at the post-transcriptional level to regulate RNA expression and, specifically, that the +KTS protein can function in conjunction with a *cis*-acting CTE to promote translation of an unspliced RNA with a retained intron.

WT1(+KTS) has previously been proposed to be involved in post-transcriptional regulation because of the apparent preferential association of +KTS isoforms with nuclear speckles and splicing factors (107, 108, 113). This suggests that WT1(+KTS) might initially interact with the CTE-containing RNA in conjunction with the splicing machinery. Although the MPMV-CTE functions specifically at the post-transcriptional level to enable the export and translation of unspliced or incompletely spliced RNA, the

Figure 43. WT1(+KTS) is associated with polyribosomes in transfected 293T cells. *A*, Cells (8×10^6) were cotransfected with 15 μg of pCMVGagPol-CTE, 2.5 μg of pCMVSEAP and 15 μg of WT1(+KTS) plasmids and were subjected to polyribosome analysis as described in Materials and Methods. The gradient was fractionated and the OD_{254} of each fraction was measured. Proteins from each fraction were resolved by SDS-PAGE and analyzed by Western blotting using the anti-T7 antibody and ^{125}I-Protein A. Optical density profiles of the gradient fractions are shown at the top of the figure. *B*, The experiment was performed as (*A*), except that 15 mM EDTA was added to the lysate before loading on to sucrose gradients containing 15 mM EDTA. (Polysome preparation and Western blots performed by JES)


A CTE + WT1(+KTS)
80S
polysomes
60S
40S
1 2 3 4 5 6 7 8 9 10 11 12 13


B CTE + WT1(+KTS) + EDTA
60S
40S
1 2 3 4 5 6 7 8 9 10 11 12 13

CTE does not divert the RNA from the splicing machinery, but enables the RNA to circumvent a cellular restriction that appears to require intron removal before export (32, 73, 118). In fact, it has been well documented that RNAs which serve as substrates for CTE-mediated export initially interact with the splicing machinery even when they remain completely unspliced (73, 127). It has also been shown that U2AF65 can stimulate the nuclear export and expression of an unspliced reporter RNA retaining an intron and that exogenously expressed U2AF65 can recruit Tap to mRNP complexes (218). These results thus suggest a role for splicing factors in the formation of an export competent CTE-containing mRNP complex. Since WT1(+KTS) has been demonstrated to bind directly to U2AF65 (42), it will be of clear interest to determine whether this interaction plays a role in the ability of WT1(+KTS) to enhance CTE function.

The KTS insertion in WT1 protein is extremely well conserved. However, our results demonstrate that a protein with a substitution of AAA for the KTS sequence functions as well as the original WT1(+KTS) protein, supporting the notion that the sole function of the insertion is to disrupt the linker between the third and fourth zinc finger and thus the structure of the protein. It also supports the previously proposed idea that the conservation of the specific KTS sequence might be due to a conservation necessary at the RNA level for proper splicing and maintenance of the ratios between the two isoforms (41).

In contrast to the functional importance of the KTS insertion, previous studies examining the function of WT1 have not demonstrated a clear role for the mammalian- specific alternative exon 5, which includes or excludes 17 amino acids in the protein. Also, adult mice lacking this exon are viable and fertile (143). This exon is evidently dispensable for at least some of the post-transcriptional effects of WT1(+KTS), since a protein lacking this exon was fully functional in our system.

Although WT1(+KTS) clearly enhanced polyribosome association of CTE-containing RNA, this protein also slightly increased the overall levels of this RNA relative to the SEAP mRNA that was transcribed from the same promoter. This suggests that WT1(+KTS) might also directly affect some aspect of RNA metabolism. Effects of WT1(+KTS) at multiple levels would not be surprising, since many recent studies have highlighted links between different steps in RNA processing (84). In addition, Sam68, which

also functions to promote translation of CTE-containing RNA (37), has been reported to also effect alternative splicing, RNA stability and polyadenylation (136, 137).

Our experiments demonstrate that WT1(+KTS) can function to promote expression from an RNA with a retained intron. Although expression from unspliced and incompletely spliced RNA is commonly observed in viruses, and intron retention seems to be a common form of alternative splicing in plants (144), this form of alternative splicing seems to be relatively rare in higher organisms. In fact, there are only a few verified reports of protein expression from mammalian mRNAs with retained introns. One clearly documented example involves some of the members of the Id family of helix-loop-helix (HLH) transcription factors. Specifically, retention of the single small intron in the *Id3* gene after vascular injury results in expression of an alternative protein isoform, which appears to function as a modulator of the protein expressed from the spliced RNA (53, 135). This may be particularly relevant, since a recent study demonstrated that deletion of the WT1 (+KTS) isoform affected the expression of MASH1, an HLH transcription factor required for early neurogenesis (201). However, no effects on MASH1 transcription were observed, leading the authors to hypothesize a post-transcriptional effect. Since the *MASH1* gene, like the *Id* genes, contains a single small intron, it will be of interest to determine whether the effects of the +KTS protein on *MASH1* expression are related to intron retention.

WT1(-KTS), the "transcription factor" isoform of WT1, was shown to slightly increase RNA levels and protein expression from the CTE-containing RNA in the absence of WT1(+KTS). In contrast, expression of WT1(-KTS) clearly inhibited WT1(+KTS) enhancement of CTE function. This might be due to the previously demonstrated ability of the WT1 proteins to form heterodimers through their N-terminal domains (23, 46, 139, 162). In support of this hypothesis, preliminary experiments suggest that while amino-terminal deletion mutants of WT1(+KTS) can still enhance CTE function, expression is no longer inhibited by the WT1(-KTS) protein (Bor et al., unpublished). Consistent with this finding, co-expression of WT1(-KTS) in excess over WT1(+KTS) in *Xenopus* oocytes abrogated the localization of WT1(+KTS) in B-snurposomes, but only if the amino-terminus was present (107).

WT1(+KTS) associates with polyribosomes in transfected 293T cells, consistent with its ability to enhance mRNA translation. An association of this isoform with polyribosomes was also recently reported in monkey COS cells (147). However, these authors also found that the WT1(-KTS) isoform associated with polyribosomes. This raises a conundrum, since our data clearly demonstrate that only the WT1(+KTS) isoform enhances GagPol expression from a CTE-containing mRNA. Further experiments are necessary to address the potential role of the WT1(–KTS) protein in translational regulation. It may be that WT1(-KTS) has a different set of targets than WT1(+KTS) or that it functions as a translation repressor. In our experiments, the +KTS protein was found in fractions containing the ribosomal subunits after treatment with 15 mM EDTA, suggesting that WT1 might associate directly with ribosomal proteins during translation. Alternatively, the protein could simply be associated with mRNP complexes that sediment in these fractions when the polyribosomes are disrupted.

The fact that WT1(-KTS), a protein that has been shown to act as a transcriptional regulator, functions as an inhibitor of post-transcriptional effects of WT1(+KTS) suggests the intriguing possibility that alternative splicing of WT1 might serve to coordinate regulation of transcriptional and post-transcriptional events. One possibility is that genes that are regulated by WT1(-KTS) at the transcriptional level are also regulated post-transcriptionally by WT1(+KTS). Another possibility is that the two proteins serve to coordinately regulate expression of different genes at the transcriptional and post-transcriptional level. Elucidation of this will require the definitive identification of gene targets for WT1(+KTS). While the 5A1 sequence is clearly functioning as a cellular CTE and was identified in co-immunoprecipitations with WT1(+KTS), the physiological relevance of this remains unclear, since a coding mRNA containing this sequence has not been reported. More interesting is the fact that WT1(+KTS) also enhanced expression mediated by the recently discovered CTE in ACTN4. As mentioned above, the closely related ACTN1 has been identified in preliminary experiments as a potential WT1 target using a 3-hybrid system. These results coupled with the established role of ACTN4 in kidney function, suggest that WT1(+KTS) in conjunction with CTEs may regulate non-muscle alpha actinins in kidney cells.

The last several years have provided increasing evidence for the importance of gene regulation at the post-transcriptional level, especially in mammalian systems. The genome project has revealed that much of the complexity of higher organisms is not due to more genes, but to alternative splicing and other events at the post-transcriptional level. Keene and Tenenbaum originally proposed that untranslated sequence elements for regulation (USER codes) in mammalian mRNA could provide the signals for post-transcriptional operons, enabling the coordinated expression of certain subsets of mRNAs in response to signals during development and differentiation (100). The fact that WT1(+KTS) and Sam68, RNA binding proteins believed to play important but distinct roles in development and differentiation, can both interact with CTE-containing RNAs to enhance expression at the post-transcriptional level gives further credence to the validity of this hypothesis.

CHAPTER 5

Concluding Remarks and Future Directions

Through *in vitro* and *in vivo* studies in mammalian, yeast and oocyte systems, a model has emerged linking the various steps in post-transcriptional gene expression (174). For example, mRNA that has undergone splicing appears to be translated in the cytoplasm more efficiently than unspliced mRNA derived from cDNA (126, 149). This has been attributed to the presence of EJC proteins that remain on the mRNA after splicing and export (148, 207). In these studies we have analyzed the fate of an mRNA that contains an intron, but remains unspliced. Since this mRNA does not contain any EJC complexes, our results suggest that shuttling SR proteins, Tap, and WT1(+KTS) can function similarly to link nuclear and cytoplasmic events in the expression of mRNAs with retained introns.

Alternative splicing has become an important area of study in post-transcriptional regulation. Based on the number of genes in simpler organisms and the number of expressed sequence tags (ESTs), it was originally proposed that the human genome would contain between 100,000 and 150,000 genes. However, the human genome project has shown that the actual number is just over 20,000 (1, 155). It is estimated that at least 74% of all human genes are alternatively spliced (95), thus, the increased diversity is likely to be accounted for by alternative splicing. There are multiple types of alternative splicing: exon skipping, alternative 5' or 3' splice site usage, or intron retention, with the importance of intron retention being the most overlooked. This is because it is widely believed that intron retention is always a mistake, and mRNAs marked as harboring a retained intron through the presence of a PTC are immediately degraded through the NMD pathway. However, it is becoming more and more clear that intron retention exists and has important biological significance. This does not take away from the fact that mistakenly retained introns can be extremely detrimental to the cell, emphasizing the importance of careful regulation of appropriate intron retention, and these studies show how SR proteins may be important participants in this control.

It may seem puzzling that SR proteins would be involved in the promotion of intron-containing RNA expression, since SR proteins are generally thought of as factors that promote splicing. They bind to

ESEs near the splice sites and recruit the U2AF splicing factor. The promotion of splicing is antagonized by the presence of hnRNPs, which bind to exon splicing silencer (ESS) sequences and block the recruitment of splicing factors. SR proteins are involved in alternative splicing when splice sites are weak. It is generally accepted that the choice of splice sites is determined by the amounts of individual SR proteins and hnRNPs available and their affinities for the various ESEs and ESSs near the splice sites. However, it is likely that the effects of SR proteins on splicing are more complex than originally proposed since it has been previously reported that at least one of the SR proteins can also have splicing inhibitory effects (16, 36, 184, 185). Also, preliminary results in our laboratory indicate that 9G8 promotes intron retention in SIRT7 mRNA, a gene that appears to contain a cellular CTE (Fig. 34 and Bor, *et al.*, unpublished results). This suggests that promotion of intron-retention by 9G8 might be linked to a role for this protein in export and translation regulation to ensure proper expression of mRNA with retained introns. It is attractive to link splicing, export and translation regulation, particularly of non-canonical mRNAs, which may resemble aberrant messages, but which actually exist to increase diversity from a limited pool of genes. The alternative products might be expressed only at very specific times, such as during development or during periods of stress, or only in specific tissues and therefore be very tightly regulated. 9G8 involvement in alternative splicing, export and translation might be an example of this kind of control mechanism.

This type of regulation has been described as a post-transcriptional operon (100). As opposed to a bacterial operon, where multiple proteins are encoded as a polycistronic gene and regulated by a single *trans*-acting transcriptional factor, in a post-transcriptional operon, monocistronic genes are transcribed and are then regulated as subsets by a limited number of RNA-binding proteins. The model is that RNAs within a subset harbor Untranslated Sequence Elements for Regulation, otherwise known as USER codes, which are specifically recognized by one or more regulatory proteins. The RNAs with similar USER codes are thought to be functionally related and be part of the same biological process or pathway. In our studies, one such subset of mRNAs could be those that retain an intron and have a CTE as the USER code. SR proteins could be involved in promoting alternative splicing to retain the intron, and in conjunction with Tap, ensure

that the mRNA is expressed in the cytoplasm. It is interesting that many of the SR proteins direct the splicing of their own mRNA, and this may be one component of operon control. In fact, many RNA-binding proteins are alternatively spliced, and one initial step in a post-transcriptional operon may be to globally shift the splicing of the mRNAs that encode the proteins in a specific pathway.

The post-transcriptional operon model hypothesizes that the interaction of RNA-binding proteins and USER code-containing mRNAs of a pathway may be initiated through the action of a single "master" regulatory protein. One such protein may be Sam68. Sam68 has been shown to be involved in multiple steps in RNA metabolism. One study showed Sam68 to be a prototypic regulator of alternative splicing, the function of which depends on its phosphorylation in response to extracellular signals (136). Another study showed that a Sam68 homologue in rat, SLM-2, is also involved in alternative splicing and is associated with the SR protein, SRp30c (191). Sam68 was shown to have many mRNA binding targets, including the 9G8 transcript (87). Upon stress, Sam68 accumulates in bodies called stress-induced Sam68 nuclear bodies (SNBs) that contain pre-mRNA processing factors, including the SR proteins, SRp30c, ASF/SF2, and 9G8, and splicing patterns of Adenovirus E1A transcripts change upon stress induction (43). Sam68 was shown to cooperate with Tap to promote the expression of retroviral reporter constructs with either the RRE (in the absence of Rev) or the CTE (163). Our lab showed that similarly to Tap/Nxt1, 9G8, and WT1(+KTS), Sam68 enhances the cytoplasmic utilization of our intron- and CTE-containing reporter mRNA and is regulated by the nuclear kinase Sik/BRK (37) and unpublished data). All of this points to a role of Sam68 in the coordinated regulation of alternatively spliced mRNAs in response to cellular signaling. Whether Sam68 is a "master" controller or just another one of many RNA-binding proteins involved in the pathway has yet to be determined.

Based upon the studies described in this thesis, there is much yet to be discovered. We have somewhat of an inkling of some of the players involved in post-transcriptional mRNA regulation and some glimmerings of what roles they might have. Determining exactly the mechanisms by which they work and how they interact with each other will be fodder for many future graduate students. To get them started, I humbly submit some future directions.

The list of SR proteins that seem to have a role in translation thus far includes 9G8 and ASF/SF2. It would be interesting to determine if any or all of the remaining SR proteins have similar functions. It is possible that several of the other SR proteins could behave similarly as several have been found to shuttle continuously between the nucleus and the cytoplasm (25). As has been shown for 9G8, SRp20, and ASF/SF2, there is likely a purpose that these proteins exist in the cytoplasm. This could be to aid in export of mRNA, translation, or some other function. However, not all of the SR proteins shuttle. For instance, SC35 is not a cytoplasmic protein, and it would be of interest to determine if this protein differs from the shuttling SR proteins in their ability to promote expression from our CTE- and intron-containing reporter mRNA. It would also be useful to determine if the ability of a SR protein to promote translation of the reporter mRNA was based solely on its ability to shuttle or if other factors are involved.

The SR proteins have two basic domains, the RNA-binding RRM domain and the highly phosphorylated and protein-interacting RS domain. These domains have been found to be modular for the SR proteins in that they can be swapped amongst each other and retain splicing function. Specific alternative splicing tracks with the RRM domain. Once it has been established which SR proteins can promote expression from our reporter, the next step would be to determine if the SR protein domains are also modular for protein expression. For example, SC35 could possibly be made to shuttle by swapping domains with other SR proteins. Presuming SC35 would not function to promote mRNA expression, it would be of interest to determine if another SR protein domain is sufficient to give this function to SC35. It would be of added interest to determine if the simple addition of a NES to SC35 could change this protein functionally. If the other SR proteins are found to have differing functions, domain swapping between the proteins could help in determining the role of each domain.

One of the main questions arising from the 9G8 study is, what is the role of phosphorylation in regulation of translation? We have already proposed two possible reasons for this in Chapter 3: to induce a remodeling event allowing for the release of Nxt1; and to reduce the affinity for 9G8 for the mRNA allowing for its release and return to the nucleus. To explore these possibilities, several experiments could be performed. To determine if phosphorylation of 9G8 is important for Nxt1 release, the polysome analysis

experiment could be repeated in cells over-expressing Tap, Nxt1, and the 9G8ΔRS protein, which cannot be phosphorylated. We have shown that this truncated SR protein remains associated with the mRNA, even in the heavy polysome fractions, and therefore it may influence the release of Nxt1, as well. If Nxt1 release is important for translation initiation, this could explain why the ΔRS protein inhibits p24 expression and why over-expression of Nxt1 sometimes inhibits SEAP expression. To further understand the importance of phosphorylation on SR protein release from the polysomes, the experiments could be repeated in the presence of the SR protein kinase, SRPK1. Mutants of SRPK1 that are constitutively active or dominantly negative could also be used to determine if they have any effect on 9G8 release from the polysomes. If these proteins do affect 9G8 release, p24 assays could be performed to determine if a premature or an inhibited release of 9G8 alters expression from our reporter mRNA. Similar experiments could also be performed for over-expressed Transportin SR, which is the import receptor for the SR proteins. In yeast studies, it was found that Mtr10p (the yeast homologue of Transportin SR) was important for the release of Npl3p (the SR protein homologue) from polysome-associated mRNAs, but the Sky1p (the kinase homologue) was not important (209). It would be of interest to determine how closely the mammalian system mimics the yeast system.

Another intriguing avenue for further study concerns the purpose of the retention of introns in SR proteins. A recent study has shown that every member of the human SR protein family is alternatively spliced, and these alternative splicing events are either retained introns or alternative 3' UTR introns, targeting the resulting mRNAs to the NMD pathway (112). Furthermore, the mouse orthologues for these transcripts are similarly spliced, which is unusual since alternative splicing is not often conserved between mouse and human genes. This conservation implies that the alternative splicing of SR proteins is somehow important to the organism. The study showed that the alternatively spliced mRNAs are 2-14% of the spliced population for each gene under normal conditions. However, by blocking NMD through knocking down UPF1 or by treating the cells with cycloheximide, the population of the alternatively spliced mRNAs increases to 40-70% of the total, implying that a large portion of the SR transcripts is formed only to be quickly degraded. Although no protein product from an alternatively spliced SR protein transcript has ever

been shown, it is possible that the purpose of producing so many of these transcripts is so that in the event of NMD inhibition, the alternative mRNA isoforms could be expressed as protein. This would be an example of another type of post-transcription operon for which a single signal *(i.e.*, shutting off NMD) has profound downstream consequences (production of alternate SR proteins, which would change the splicing of subsequent transcripts for the entire genome and may affect mRNA export and translation, as well). This gives importance to discovering if the small band in Figure 28 is truly a truncated form of 9G8. One way to determine if this band is derived from an alternatively spliced transcript is to target the retained intron (intron 3) of 9G8 with a siRNA to knock down its expression. Completely spliced 9G8 transcripts would be immune from knock down and should still express the full-length 9G8 protein. Lysates from untransfected cells compared to the siRNA-treated cells would show if the small protein recognized by the ZnK antibody in Figure 28 disappears with the addition of the intron 3 siRNA. If so, this would be strong evidence that protein can be made from the transcript with a retained intron and indicate the possibility of a post-transcription operon involving NMD and alternative splicing.

The insertion of the KTS amino acids between the third and fourth zinc fingers of WT1 changes WT1 function from a transcription factor to an RNA regulator. Mutations that result in single amino acid changes within the zinc fingers have been isolated from patients with Denys-Drash syndrome. We have recently begun examining these mutations in both the +KTS and –KTS contexts in our p24 assay. Preliminary data indicates that changes in zinc finger 1 makes +KTS behave like a –KTS protein, and changes in zinc fingers 2 and 3 make –KTS behave more like +KTS protein. It will be of interest to determine if these patient-isolate mutations have similar effects on promoting the association of our reporter RNA with polysomes. Furthermore, since +KTS protein is associated with speckles and –KTS protein is localized with other transcription factors, it will also be of interest to determine if these mutations change localization of the protein.

In conclusion, it is clear there is much yet to be learned about RNA metabolism. Although little is known about the extent that regulation of RNA has on cell processes, the data presented here and elsewhere indicate that regulation of RNA metabolism could be as import as transcriptional control and protein

signaling. The concept of post-transcriptional operons continues to be an intriguing paradigm in this new and important field of cellular biology.

APPENDIX

I. 9G8 Alternative Splicing Isoforms in Whole Cell Lysate vs. Cytoplasmic Extracts

Introduction

Northerns of various tissues show at least 5 different *9G8* isoforms are expressed (30, 157), and *in vitro* splicing assays demonstrate that the full-length protein influences splice site selection of its own transcript (119). As mentioned at the end of Chapter 3, we were interested in the role of the intron-containing *9G8* transcripts, if any, and if different splicing patterns could be generated *in vivo*. Although 9G8 promoted the excision of intron 3 of its own mRNA *in vitro*, it is possible that different splicing patterns would emerge *in vivo*. Also, even though it is widely accepted that SR proteins only enhance splicing at their designated ESEs, at least one of the SR proteins can also have splicing inhibitory effects (16, 36, 184, 185). Furthermore, preliminary results in our laboratory indicate that 9G8, SRp20, and ASF/SF2 promote intron retention in SIRT7 mRNA, a gene that appears to contain a cellular CTE (Fig. 34, and Bor, *et al.*, unpublished results).

Results

In order to examine the effects of different proteins on the splicing patterns of *9G8*, primer pairs were designed to flank the relevant splice sites (both constitutive and alternative) around intron 3 (Fig. 44). As a control for an intron that should always be spliced, particularly in cytoplasmic fractions, additional primer pairs were chosen around intron 2. 293T cells were either mock transfected or transfected with Tap/Nxt1, full-length 9G8, or truncated 9G8 (no RS domain representing the product of an expressed intron-containing transcript). Cells were harvested, whole cell lysates or cytoplasmic extracts were made, and RNA was purified from each condition with DNase treatment. Each sample was subjected to random hexamer reverse transcriptase to generate cDNAs, and these were used with the various primer pairs in polymerase chain reactions. For positive controls, genomic and plasmid (full-length 9G8) DNA was also amplified. To check for DNA contamination, RNA samples that had not undergone the reverse

transcription step were used in the reaction, as well. A negative control with no template was also used to check for contamination of the reagents.

Two sets of reactions were performed around 9G8 intron 2, which has not been shown to be retained (Fig. 45). Oligos *a* and *w* (top panel) amplified exon 2 to exon 3, producing a 228 bp amplimer from a spliced mRNA (as shown by the plasmid DNA control) or a 538 bp amplimer for an mRNA with a retained intron (as shown by the genomic DNA control). For all the samples, the PCR product only showed fully spliced products, even for whole cell lysates that contain nuclear mRNA that may not have undergone complete splicing. Some of the samples from transfected cells showed product in the non-reverse transcribed (RT) reactions, indicating contaminating plasmid DNA. Since plasmid DNA does not have intronic sequences, the presence of contaminating plasmid DNA did not interfere with the analysis of the results. None of the samples seemed to have genomic DNA contamination. Oligos *a* and *v* (bottom panel) amplified from exon 2 into intron 2, producing a 412 bp amplimer as shown by the genomic DNA control. All the samples showed a product of the expected size, with the whole cell lysates showing stronger intensity, as would be expected. The cytoplasmic products may be the result of poor separation of the cytoplasmic and nuclear fractions, or could be the result of a retained intron. In this set, none of the RT negative samples showed any contaminating DNA, further indicating that the products seen for oligos *a* and *w* were from plasmid templates, which have no intronic sequences.

Analysis of intron 3 is more complicated since either the whole intron or the 3' half of the intron can be retained. This intron was analyzed from exon 3 to each of the possible isoforms (Fig. 46) or within the intron down to exon 4 (Fig. 47). Oligos complementary to exons 3 and 4 (*b* and *z,* middle panel) could potentially produce PCR products of three different sizes, depending on intron retention: fully spliced RNA produces a 149 bp product (as shown by the plasmid DNA control), a full intron retention produces a 1024 bp amplimer (as shown by the genomic DNA control), and a partial intron produces a 669 bp product, which cannot be mimicked by either control DNA. However, samples from the cells transfected with the truncated 9G8 protein do show products of this size, both for cytoplasmic and whole cell lysate at similar intensities, indicating that not only does this alternative splicing take place in the presence of 9G8ΔRS, the

resulting transcript is efficiently exported. Each of the conditions also produces fully spliced transcripts, and all but cells over-expressing the full-length 9G8 produce transcripts with a fully retained intron, none of which are exported. Again, a few of the transfected samples show products in the RT negative reactions, indicating plasmid DNA contamination, but no genomic contamination is evident.Oligos *b* and *x* (Fig. 37, top panel) amplify products (308 bp) only if the entire intron is retained. Each sample produces an amplimer for this primer set except for the cytoplasmic Tap/Nxt1 sample, the reaction of which may not have worked. Oligos *b* and *y* (bottom panel) amplify either potential intron 3 isoform, producing a 574 bp product if the entire intron is retained (as shown by the genomic DNA control) or a 219 bp product if the partial intron is retained (no example product). The 574 product is seen for each sample with varying intensities. Interestingly, the strongest intensity is for Tap/Nxt1 whole cell lysate, but the weakest is for cytoplasmic Tap/Nxt1. Also, there are two other bands present, which are not of an expected size. It is possible that alternative splicing of 9G8 is even more complicated than originally thought, and multiple isoforms exist. Another possibility is that the PCR reaction for this primer set does not have strict fidelity.

Oligos *d* and *z* (Fig. 47, top panel, 286 bp) amplify products of either intron retention event. Oligos *c* and *z* (middle panel, 713 bp) only amplify a product if the full intron is retained, as do oligos *c* and *y* (bottom panel, 263 bp). Oligo sets (*d* and *z*) and (*c* and *y*) show similar products for all the samples, although cells transfected with the truncated 9G8 do not seem to export this mRNA efficiently. Oligo set (*c* and *z*), like (*b* and *y*), shows multiple products, and may also indicate more complex splicing patterns or poor primer fidelity.

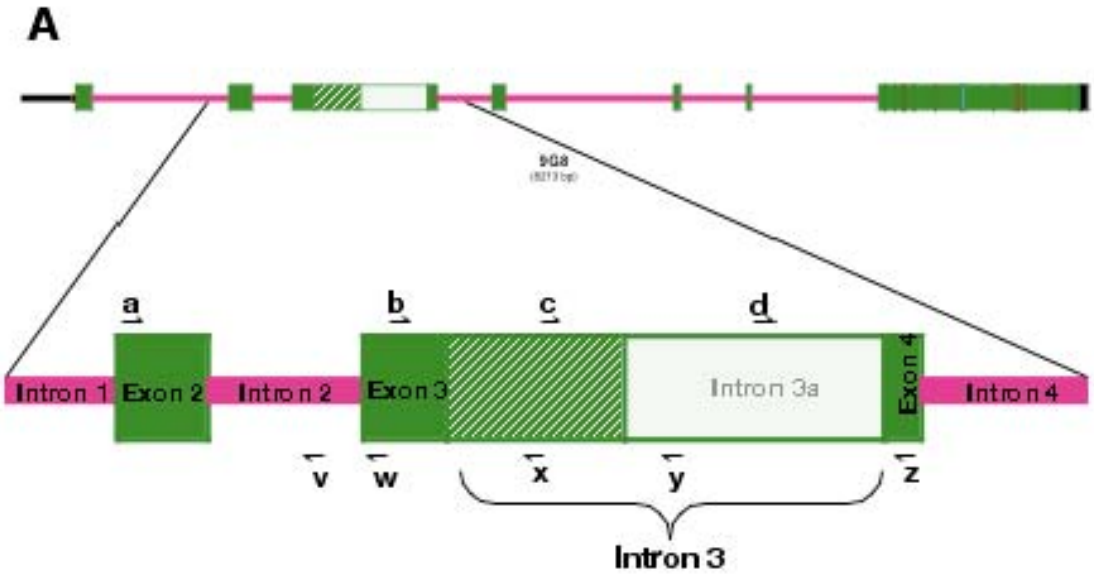
Discussion

Although the RT-PCR work is preliminary, some conclusions may be drawn for future work. Untransfected cells produce *9G8* mRNA with a retained full intron, but it seems to not be exported efficiently enough to compete with the smaller spliced form in the RT-PCR reaction. However, it can be seen in the cytoplasm by using primers within the intron. Tap/Nxt1, interestingly, did not seem to export the full-length retained intron, but maybe the reaction didn't work since the *cy* reaction gave a product. Cells over-expressing the

full-length 9G8 looked very much like the untransfected cells, except the entire intron was not seen in the *bz* primer pair. Cells over-expressing the truncated 9G8 protein do not efficiently export transcripts with the partial intron in the *dz* and *cy* primer pairs, but in the *bz* primer pair, the partial intron was retained rather than the entire intron. These results seem to contradict each other.

For primer pairs *by* and *cz*, I got multiple bands for all the samples. These may or may not be artifacts of the PCR reaction. However, assuming cryptic splice sites are being used producing multiple amplimers, it is of interest to compare the bands and speculate on influences generating them. For untransfected cells, three amplimers result for both primer set, with the largest being most abundant in the whole cell lysate. However, the smallest amplimer seems to have been exported more efficiently. Tap/Nxt1 resembles untransfected cells. Full-length 9G8 seems to make three amplimers of equal intensity with the lowest maybe exported more efficiently. Truncated 9G8 skews the amplimers to the smallest, and export reflects what was produced. To help narrow down whether the multiple bands are artifacts or cryptic splice site products, I compared the location of the primers with the outcome of the products. Oligos *bz* give amplimers of the expected sizes. Oligos *bx* give normal products, but *by* give extra bands, therefore an area of interest may lie between *x* and *y*. The *cy* primer set is also normal, further narrowing the region to the 43 bp region between and including *x* and *c*. Oligos *cz* also give extra bands, and since *dz* and *cy* are both normal, another area of interest (203 bp) may lie between *y* and *d*. It would be of interest to determine if other primer sets encompassing the same regions also give multiple amplimers or if cryptic splice sites are located in either of these regions.

Figure 44. *A*, Schematic of the unspliced *9G8* gene with an expanded schematic for the region encompassing exon 2 through exon 3. Exons are depicted as solid green blocks, and introns are depicted as pink lines. The alternatively spliced intron 3 is also shown as a green block, but with different shading or patterns to the constitutive exons. Since intron 3 can be retained in its entirety or only as the 3' half (intron 3a), this is represented by difference in shading within this intron. Also included are the primer locations along the gene used for RT-PCR experiments. Primers shown above the schematic (a, b, c, d) are 5' (or sense) primers, and primers shown below the schematic (v, w, x, y, z) are 3' (or antisense) primers. *B*, Key for the templates used in the PCRs shown in *Figs. 45-47.*



B

T0	Total RNA 293T cells untransfected
TT/N	Total RNA 293T cells tf'd w/ Tap&Nxt1
T1	Total RNA 293T cells tf'd w/ fl9G8
T2	Total RNA 293T cells tf'd w/ trunc 9G8
C0	Cyto RNA 293T cells untransfected
CT/N	Cyto RNA 293T cells tf'd w/ Tap&Nxt1
C1	Cyto RNA 293T cells tf'd w/ fl9G8
C2	Cyto RNA 293T cells tf'd w/ trunc 9G8
G	genomic DNA template
P	plasmid DNA template
NC	no template control

Figure 45. RT-PCR of exon 2 to exon 3 of *9G8* mRNA. 293T cells were left untransfected (0) or transfected with 2 μg Tap and 1 μl Nxt1 (T/N), 1 μg full-length 9G8 (1), or 5 μg 9G8ΔRS (2) plasmids. Twenty-four hours post-transfection, the cells were harvested to make either whole cell (T) or cytoplasmic lysates (C), and RNA was purified from each lysate and treated with DNase. Reverse transcription was performed using random hexamers (+RT). The reaction was also performed in the absence of reverse transcriptase (-RT) as a control for DNA contamination. The products of these reactions were used as templates for PCR reactions shown in this figure and *Figs. 46-47*. Genomic (G) and plasmid (P) DNA served as positive controls for the PCR. A negative control (NC) contained no template. For the PCR reaction shown in this figure, oligos complementary to sequences in exon 2 (oligo *a*) and exon 3 (oligo *w*) or intron 2 (oligo *v*) were used (see *Fig. 44* for oligo locations). The expected amplimer sizes for the *aw* primer pair are 536 base pairs (bp) for a retained intron and 228 bp for a spliced RNA. The expected amplimer size for the *av* primer pair is 412 bp for a retained intron and no product for a spliced RNA. The molecular weight marker (MWM) is a 100 bp ladder, of which, bands at 500 and 1000 bp are twice as intense as the other bands.

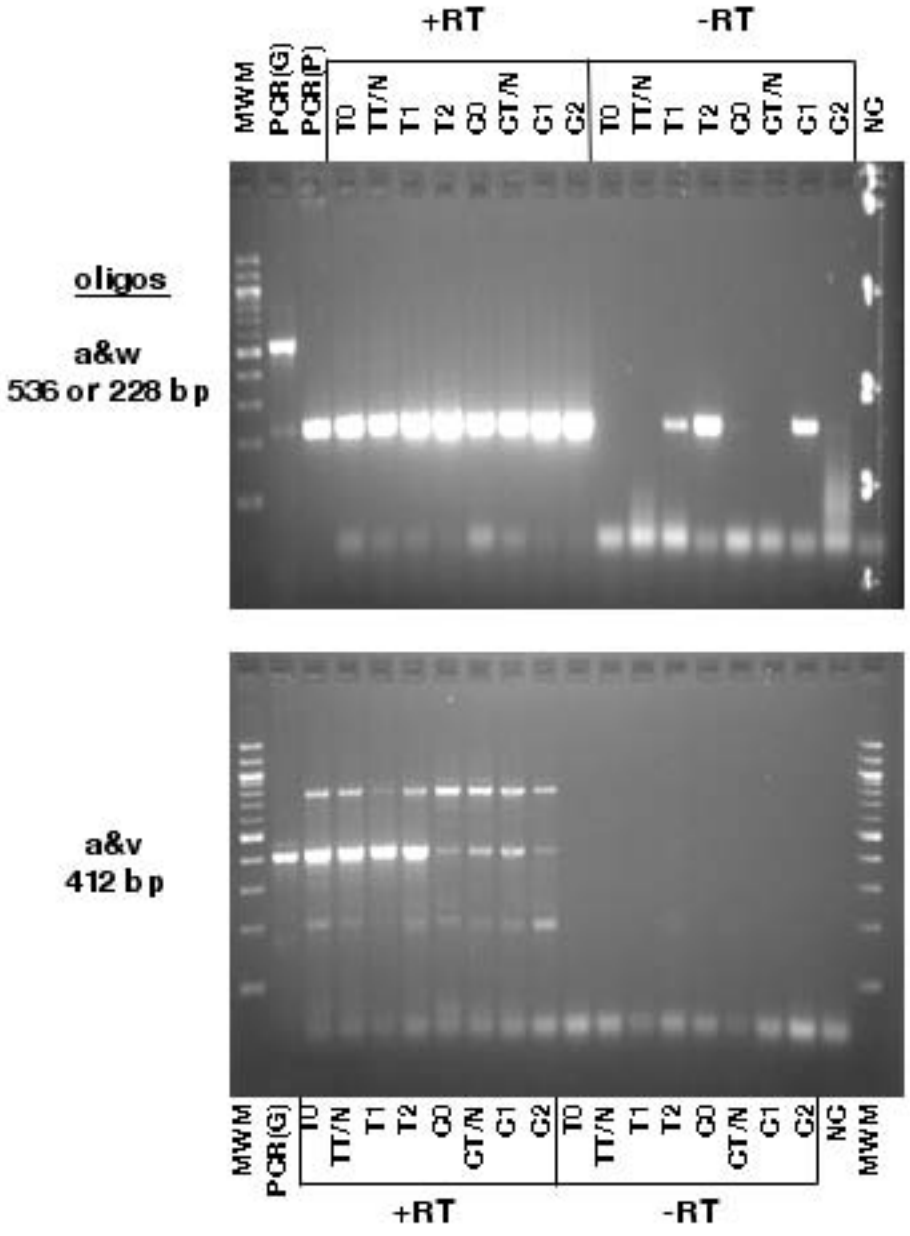

+RT
-RT
MWM
PCR(G)
PCR(P)
T0
TT/N
T1
T2
C0
CT/N
C1
C2
NC
oligos
a&w
536 or 228 bp
a&v
412 bp

Figure 46. RT-PCR of exon 3 to exon 4 of *9G8* mRNA. The templates described for *Fig. 45* were the same as used for this figure. For the PCR reaction shown in this figure, oligos complementary to sequences in exon 3 (5' oligo *b*) and exon 4 (3' oligo *z*) or intron 3 (3' oligo *x* or *y*) were used (see *Fig. 44* for oligo locations). The expected amplimer sizes for the *bx* primer pair are 308 base pairs (bp) for a retained intron and no product for a spliced RNA. The expected amplimer size for the *bz* primer pair is 1024 bp for a retained full intron, 669 bp for a partial retained intron, and 149 bp for a spliced RNA. The expected amplimer sizes for the *by* primer pair are 574 bp for a retained full intron, 219 bp for a partial retained intron, and no product for a spliced RNA.

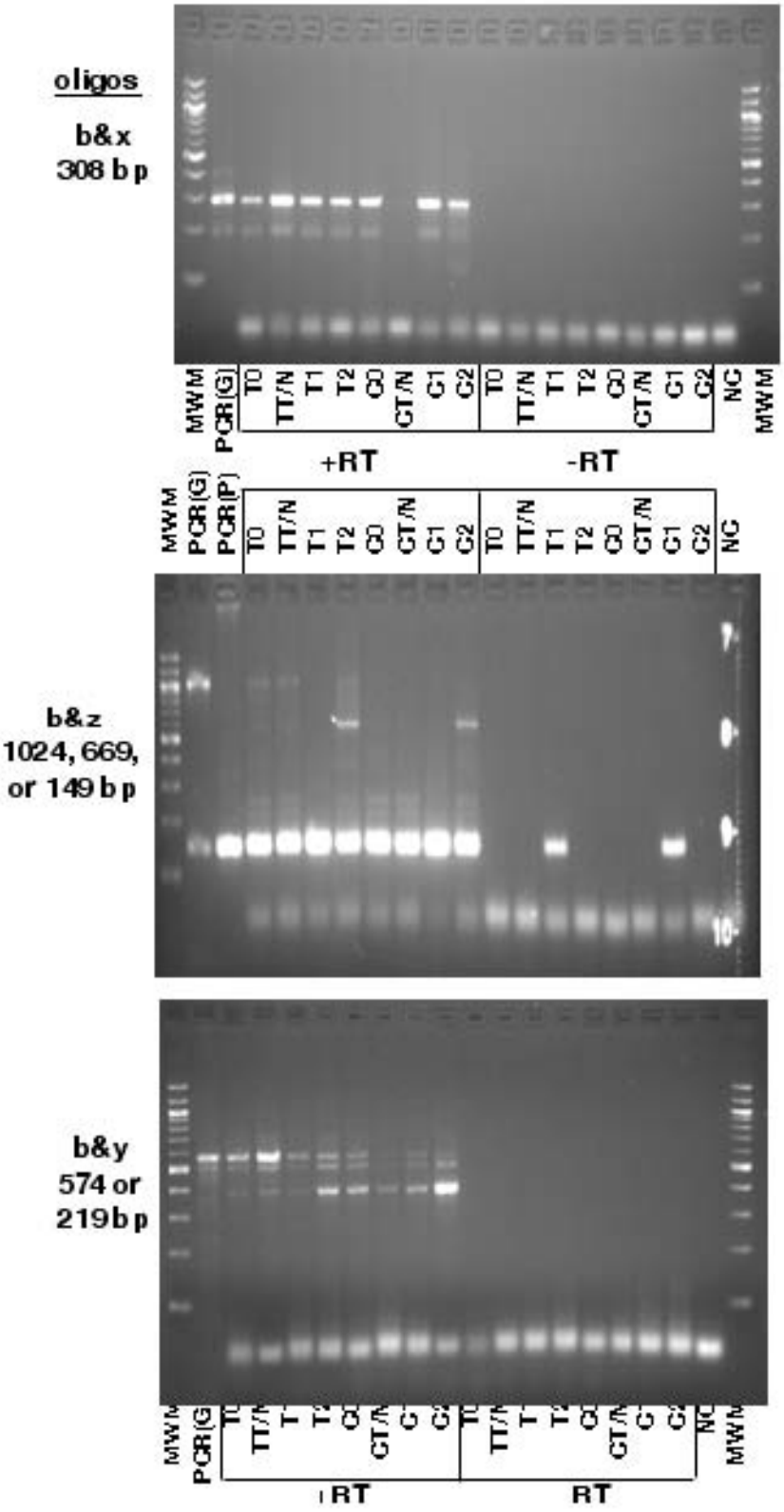
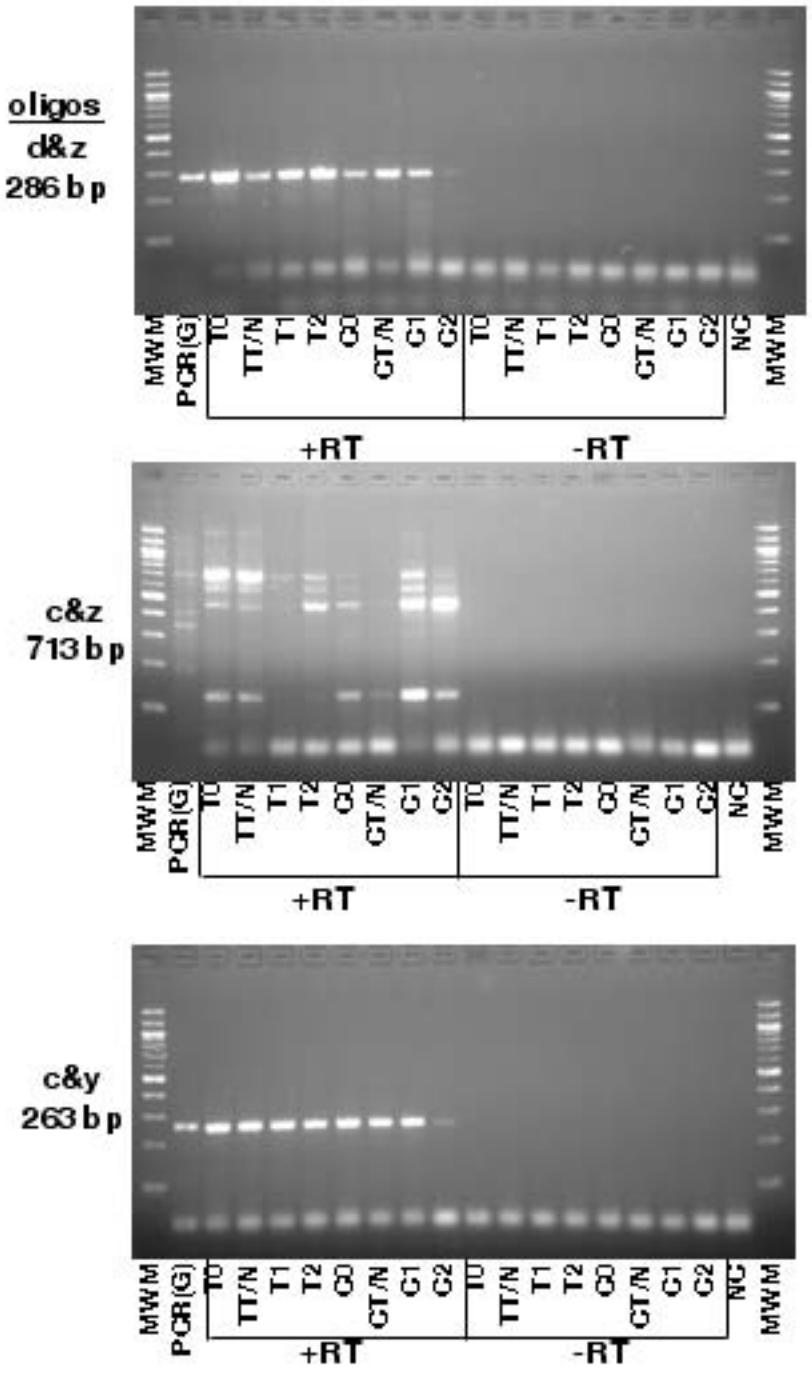

oligos
b&x
308 bp
MWM
PCR(G)
T0
TT/N
T1
T2
G0
CT/N
G1
G2
NC
+RT
-RT
PCR(P)
b&z
1024, 669,
or 149 bp
b&y
574 or
219 bp

Figure 47. RT-PCR of intron 3 to exon 4 of *9G8* mRNA. The templates described for *Fig. 45* were the same as used for this figure. For the PCR reaction shown in this figure, oligos complementary to sequences in intron 3 (5' oligo *c* or *d*) and exon 4 (3' oligo *z*) or intron 3 (3' oligo *y*) were used (see *Fig. 44* for oligo locations). The expected amplimer sizes for the *dz* primer pair are 286 base pairs (bp) for a retained intron and no product for a spliced RNA. The expected amplimer size for the *cz* primer pair is 713 bp for a retained full intron and no product for a partially retained intron or a spliced RNA. The expected amplimer sizes for the *cy* primer pair are 263 bp for a retained full intron and no product for a partially retained intron or for a spliced RNA.


oligos
d&z
286 bp
c&z
713 bp
c&y
263 bp
MWM
PCR(G)
TT/N
T1
T2
G0
CT/N
G1
G2
NC
+RT
-RT

REFERENCES

1. 2004. Finishing the euchromatic sequence of the human genome. Nature **431:**931-45.
2. **Amrani, N., M. S. Sachs, and A. Jacobson.** 2006. Early nonsense: mRNA decay solves a translational problem. Nat Rev Mol Cell Biol **7:**415-25.
3. **Arts, G. J., M. Fornerod, and I. W. Mattaj.** 1998. Identification of a nuclear export receptor for tRNA. Curr Biol **8:**305-14.
4. **Barbaux, S., P. Niaudet, M. C. Gubler, J. P. Grunfeld, F. Jaubert, F. Kuttenn, C. N. Fekete, N. Souleyreau-Therville, E. Thibaud, M. Fellous, and K. McElreavey.** 1997. Donor splice-site mutations in WT1 are responsible for Frasier syndrome. Nat Genet **17:**467-70.
5. **Bardeesy, N., and J. Pelletier.** 1998. Overlapping RNA and DNA binding domains of the wt1 tumor suppressor gene product. Nucleic Acids Res **26:**1784-92.
6. **Bass, B. L.** 2002. RNA editing by adenosine deaminases that act on RNA. Annu Rev Biochem **71:**817-46.
7. **Bauren, G., and L. Wieslander.** 1994. Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. Cell **76:**183-92.
8. **Bedard, K. M., S. Daijogo, and B. L. Semler.** 2007. A nucleo-cytoplasmic SR protein functions in viral IRES-mediated translation initiation. Embo J **26:**459-67.
9. **Behm-Ansmant, I., and E. Izaurralde.** 2006. Quality control of gene expression: a stepwise assembly pathway for the surveillance complex that triggers nonsense-mediated mRNA decay. Genes Dev **20:**391-8.
10. **Berger, E. A., T. R. Fuerst, and B. Moss.** 1988. A soluble recombinant polypeptide comprising the amino-terminal half of the extracellular region of the CD4 molecule contains an active binding site for human immunodeficiency virus. Proc Natl Acad Sci U S A **85:**2357-2361.
11. **Bickmore, W. A., K. Oghene, M. H. Little, A. Seawright, V. van Heyningen, and N. D. Hastie.** 1992. Modulation of DNA binding specificity by alternative splicing of the Wilms tumor wt1 gene transcript. Science **257:**235-7.
12. **Birse, C. E., L. Minvielle-Sebastia, B. A. Lee, W. Keller, and N. J. Proudfoot.** 1998. Coupling termination of transcription to messenger RNA maturation in yeast. Science **280:**298-301.
13. **Black, B. E., L. Levesque, J. M. Holaska, T. C. Wood, and B. M. Paschal.** 1999. Identification of an NTF2-related factor that binds Ran-GTP and regulates nuclear protein export. Mol Cell Biol **19:**8616-24.
14. **Blaustein, M., F. Pelisch, T. Tanos, M. J. Munoz, D. Wengier, L. Quadrana, J. R. Sanford, J. P. Muschietti, A. R. Kornblihtt, J. F. Caceres, O. A. Coso, and A. Srebrow.** 2005. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat Struct Mol Biol **12:**1037-44.
15. **Blencowe, B. J.** 2000. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. Trends Biochem Sci **25:**106-10.
16. **Blencowe, B. J.** 2003. Splicing regulation: the cell cycle connection. Curr Biol **13:**R149-51.
17. **Bor, Y. C., J. Swartz, Y. Li, J. H. Coyle, D. Rekosh, and M. L. Hammarskjoid.** 2006. Nothern Blot analysis of mRNA from mammalian polyribosomes. Nature Protocols.
18. **Bor, Y. C., J. Swartz, A. Morrison, D. Rekosh, M. Ladomery, and M. L. Hammarskjold.** 2006. The Wilms' tumor 1 (WT1) gene (+KTS isoform) functions with a CTE to enhance translation from an unspliced RNA with a retained intron. Genes Dev **20:**1597-608.
19. **Bourgeois, C. F., F. Lejeune, and J. Stevenin.** 2004. Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. Prog Nucleic Acid Res Mol Biol **78:**37-88.
20. **Braun, I. C., A. Herold, M. Rode, E. Conti, and E. Izaurralde.** 2001. Overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates nuclear mRNA export. J Biol Chem **276:**20536-43.

21. **Braun, I. C., E. Rohrbach, C. Schmitt, and E. Izaurralde.** 1999. TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus. Embo J **18:**1953-65.
22. **Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K. T. Jeang, D. Rekosh, and M. L. Hammarskjold.** 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev- independent. Proc Natl Acad Sci U S A **91:**1256-1260.
23. **Bruening, W., P. Moffett, S. Chia, G. Heinrich, and J. Pelletier.** 1996. Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product. FEBS Lett **393:**41-7.
24. **Caceres, J. F., T. Misteli, G. R. Screaton, D. L. Spector, and A. R. Krainer.** 1997. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J Cell Biol **138:**225-38.
25. **Caceres, J. F., G. R. Screaton, and A. R. Krainer.** 1998. A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev **12:**55-66.
26. **Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, W. H. Lewis, and et al.** 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell **60:**509-20.
27. **Calzone, F. J., R. C. Angerer, and M. A. Gorovsky.** 1982. Regulation of protein synthesis in Tetrahymena: isolation and characterization of polysomes by gel filtration and precipitation at pH 5.3. Nucleic Acids Res **10:**2145-61.
28. **Cao, W., S. F. Jamison, and M. A. Garcia-Blanco.** 1997. Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. Rna **3:**1456-67.
29. **Caricasole, A., A. Duarte, S. H. Larsson, N. D. Hastie, M. Little, G. Holmes, I. Todorov, and A. Ward.** 1996. RNA binding by the Wilms tumor suppressor zinc finger proteins. Proc Natl Acad Sci U S A **93:**7562-6.
30. **Cavaloc, Y., M. Popielarz, J. P. Fuchs, R. Gattoni, and J. Stevenin.** 1994. Characterization and cloning of the human splicing factor 9G8: a novel 35 kDa factor of the serine/arginine protein family. Embo J **13:**2639-49.
31. **Chalfant, C. E., B. Ogretmen, S. Galadari, B. J. Kroesen, B. J. Pettus, and Y. A. Hannun.** 2001. FAS activation induces dephosphorylation of SR proteins; dependence on the de novo generation of ceramide and activation of protein phosphatase 1. J Biol Chem **276:**44848-55.
32. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789-795.
33. **Chiu, I. M., P. R. Andersen, S. A. Aaronson, and S. R. Tronick.** 1983. Molecular cloning of the unintegrated squirrel monkey retrovirus genome: organization and distribution of related sequences in primate DNAs. J Virol **47:**434-441.
34. **Chiu, S. Y., F. Lejeune, A. C. Ranganathan, and L. E. Maquat.** 2004. The pioneer translation initiation complex is functionally distinct from but structurally overlaps with the steady-state translation initiation complex. Genes Dev **18:**745-54.
35. **Colot, H. V., F. Stutz, and M. Rosbash.** 1996. The yeast splicing factor Mud13p is a commitment complex component and corresponds to CBP20, the small subunit of the nuclear cap-binding complex. Genes Dev **10:**1699-708.
36. **Cowper, A. E., J. F. Caceres, A. Mayeda, and G. R. Screaton.** 2001. Serine-arginine (SR) protein-like factors that antagonize authentic SR proteins and regulate alternative splicing. J Biol Chem **276:**48908-14.
37. **Coyle, J. H., B. W. Guzik, Y. C. Bor, L. Jin, L. Eisner-Smerage, S. J. Taylor, D. Rekosh, and M. L. Hammarskjold.** 2003. Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK. Mol Cell Biol **23:**92-103.
38. **Cullen, B. R.** 2003. Nuclear RNA export. J Cell Sci **116:**587-97.

39. **Dallosso, A. R., A. L. Hancock, K. W. Brown, A. C. Williams, S. Jackson, and K. Malik.** 2004. Genomic imprinting at the WT1 gene involves a novel coding transcript (AWT1) that shows deregulation in Wilms' tumours. Hum Mol Genet **13:**405-15.
40. **Davies, J. A., M. Ladomery, P. Hohenstein, L. Michael, A. Shafe, L. Spraggon, and N. Hastie.** 2004. Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation. Hum Mol Genet **13:**235-46.
41. **Davies, R. C., E. Bratt, and N. D. Hastie.** 2000. Did nucleotides or amino acids drive evolutionary conservation of the WT1 +/-KTS alternative splice? Hum Mol Genet **9:**1177-83.
42. **Davies, R. C., C. Calvio, E. Bratt, S. H. Larsson, A. I. Lamond, and N. D. Hastie.** 1998. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. Genes Dev **12:**3217-25.
43. **Denegri, M., I. Chiodi, M. Corioni, F. Cobianchi, S. Riva, and G. Biamonti.** 2001. Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors. Mol Biol Cell **12:**3502-14.
44. **Discenza, M. T., D. Vaz, J. A. Hassell, and J. Pelletier.** 2004. Activation of the WT1 tumor suppressor gene promoter by Pea3. FEBS Lett **560:**183-91.
45. **Dreyfuss, G., V. N. Kim, and N. Kataoka.** 2002. Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol **3:**195-205.
46. **Englert, C., M. Vidal, S. Maheswaran, Y. Ge, R. M. Ezzell, K. J. Isselbacher, and D. A. Haber.** 1995. Truncated WT1 mutants alter the subnuclear localization of the wild-type protein. Proc Natl Acad Sci U S A **92:**11960-4.
47. **Ernst, R., M. Bray, D. Rekosh, and M.-L. Hammarskjöld.** 1997. A structured retroviral RNA element that mediates nucleocytoplasmic export of intron-containing RNA. Mol Cell Biol **17:**135-144.
48. **Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Luhrmann.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475-483.
49. **Flaherty, S. M., P. Fortes, E. Izaurralde, I. W. Mattaj, and G. M. Gilmartin.** 1997. Participation of the nuclear cap binding complex in pre-mRNA 3' processing. Proc Natl Acad Sci U S A **94:**11893-8.
50. **Fong, N., and D. L. Bentley.** 2001. Capping, splicing, and 3' processing are independently stimulated by RNA polymerase II: different functions for different segments of the CTD. Genes Dev **15:**1783-95.
51. **Forler, D., G. Rabut, F. D. Ciccarelli, A. Herold, T. Kocher, R. Niggeweg, P. Bork, J. Ellenberg, and E. Izaurralde.** 2004. RanBP2/Nup358 provides a major binding site for NXF1-p15 dimers at the nuclear pore complex and functions in nuclear mRNA export. Mol Cell Biol **24:**1155-67.
52. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051-1060.
53. **Forrest, S. T., K. G. Barringhaus, D. Perlegas, M. L. Hammarskjöld, and C. A. McNamara.** 2004. Intron retention generates a novel Id3 isoform that inhibits vascular lesion formation. J Biol Chem **279:**32897-903.
54. **Fortes, P., T. Inada, T. Preiss, M. W. Hentze, I. W. Mattaj, and A. B. Sachs.** 2000. The yeast nuclear cap binding complex can interact with translation factor eIF4G and mediate translation initiation. Mol Cell **6:**191-6.
55. **Fribourg, S., I. C. Braun, E. Izaurralde, and E. Conti.** 2001. Structural basis for the recognition of a nucleoporin FG repeat by the NTF2-like domain of the TAP/p15 mRNA nuclear export factor. Mol Cell **8:**645-56.
56. **Frischmeyer, P. A., and H. C. Dietz.** 1999. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet **8:**1893-900.
57. **Fu, X. D.** 1995. The superfamily of arginine/serine-rich splicing factors. Rna **1:**663-80.

58. **Galante, P. A., N. J. Sakabe, N. Kirschbaum-Slager, and S. J. de Souza.** 2004. Detection and evaluation of intron retention events in the human transcriptome. Rna **10:**757-65.
59. **Gatfield, D., and E. Izaurralde.** 2002. REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export. J Cell Biol **159:**579-88.
60. **Gatfield, D., H. Le Hir, C. Schmitt, I. C. Braun, T. Kocher, M. Wilm, and E. Izaurralde.** 2001. The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in Drosophila. Curr Biol **11:**1716-21.
61. **Gessler, M., A. Konig, and G. A. Bruns.** 1992. The genomic organization and expression of the WT1 gene. Genomics **12:**807-13.
62. **Gessler, M., A. Poustka, W. Cavenee, R. L. Neve, S. H. Orkin, and G. A. Bruns.** 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature **343:**774-8.
63. **Gilbert, C., F. Barabe, E. Rollet-Labelle, S. G. Bourgoin, S. R. McColl, B. B. Damaj, and P. H. Naccache.** 2001. Evidence for a role for SAM68 in the responses of human neutrophils to ligation of CD32 and to monosodium urate crystals. J Immunol **166:**4664-71.
64. **Gilbert, W., C. W. Siebel, and C. Guthrie.** 2001. Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation. Rna **7:**302-13.
65. **Gorlich, D., R. Kraft, S. Kostka, F. Vogel, E. Hartmann, R. A. Laskey, I. W. Mattaj, and E. Izaurralde.** 1996. Importin provides a link between nuclear protein import and U snRNA export. Cell **87:**21-32.
66. **Graveley, B. R.** 2000. Sorting out the complexity of SR protein functions. Rna **6:**1197-211.
67. **Gruter, P., C. Tabernero, K. C. von, C. Schmitt, C. Saavedra, A. Bachi, M. Wilm, B. K. Felber, and E. Izaurralde.** 1998. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. Mol Cell **1:**649-659.
68. **Guzik, B. W., L. Levesque, S. Prasad, Y. C. Bor, B. E. Black, B. M. Paschal, D. Rekosh, and M. L. Hammarskjold.** 2001. NXT1 (p15) is a crucial cellular cofactor in TAP-dependent export of intron-containing RNA in mammalian cells. Mol Cell Biol **21:**2545-54.
69. **Gwizdek, C., E. Bertrand, C. Dargemont, J. C. Lefebvre, J. M. Blanchard, R. H. Singer, and A. Doglio.** 2001. Terminal minihelix, a novel RNA motif that directs polymerase III transcripts to the cell cytoplasm. Terminal minihelix and RNA export. J Biol Chem **276:**25910-8.
70. **Haber, D. A., R. L. Sohn, A. J. Buckler, J. Pelletier, K. M. Call, and D. E. Housman.** 1991. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc Natl Acad Sci U S A **88:**9618-22.
71. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis.** 1989. The rev (trs/art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. J Virol **63:**1265-1274.
72. **Hammarskjold, M. L.** 2001. Constitutive transport element-mediated nuclear export. Curr Top Microbiol Immunol **259:**77-93.
73. **Hammarskjold, M. L.** 1997. Regulation of Retroviral RNA Export. Seminars in Cell & Developmental Biology **8:**83-90.
74. **Hammarskjold, M. L., J. Heimer, B. Hammarskjold, I. Sangwan, L. Albert, and D. Rekosh.** 1989. Regulation of human immunodeficiency virus env expression by the rev gene product. J Virol **63:**1959-66.
75. **Hammes, A., J. K. Guo, G. Lutsch, J. R. Leheste, D. Landrock, U. Ziegler, M. C. Gubler, and A. Schedl.** 2001. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell **106:**319-29.
76. **Hargous, Y., G. M. Hautbergue, A. M. Tintaru, L. Skrisovska, A. P. Golovanov, J. Stevenin, L. Y. Lian, S. A. Wilson, and F. H. Allain.** 2006. Molecular basis of RNA recognition and TAP binding by the SR proteins SRp20 and 9G8. Embo J **25:**5126-37.
77. **Herold, A., T. Klymenko, and E. Izaurralde.** 2001. NXF1/p15 heterodimers are essential for mRNA nuclear export in Drosophila. Rna **7:**1768-80.

78. **Hirose, Y., and J. L. Manley.** 1998. RNA polymerase II is an essential mRNA polyadenylation factor. Nature **395:**93-6.
79. **Ho, J. H., and A. W. Johnson.** 1999. NMD3 encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in Saccharomyces cerevisiae. Mol Cell Biol **19:**2389-99.
80. **Ho, J. H., G. Kallstrom, and A. W. Johnson.** 2000. Nmd3p is a Crm1p-dependent adapter protein for nuclear export of the large ribosomal subunit. J Cell Biol **151:**1057-66.
81. **Holbrook, J. A., G. Neu-Yilik, M. W. Hentze, and A. E. Kulozik.** 2004. Nonsense-mediated decay approaches the clinic. Nat Genet **36:**801-8.
82. **Huang, Y., R. Gattoni, J. Stevenin, and J. A. Steitz.** 2003. SR splicing factors serve as adapter proteins for TAP-dependent mRNA export. Mol Cell **11:**837-43.
83. **Huang, Y., and J. A. Steitz.** 2001. Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol Cell **7:**899-905.
84. **Huang, Y., and J. A. Steitz.** 2005. SRprises along a messenger's journey. Mol Cell **17:**613-5.
85. **Huang, Y., T. A. Yario, and J. A. Steitz.** 2004. A molecular link between SR protein dephosphorylation and mRNA export. Proc Natl Acad Sci U S A **101:**9666-70.
86. **Ishigaki, Y., X. Li, G. Serin, and L. E. Maquat.** 2001. Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell **106:**607-17.
87. **Itoh, M., I. Haga, Q. H. Li, and J. Fujisawa.** 2002. Identification of cellular mRNA targets for RNA-binding protein Sam68. Nucleic Acids Res **30:**5452-64.
88. **Izaurralde, E.** 2004. Directing mRNA export. Nat Struct Mol Biol **11:**210-2.
89. **Izaurralde, E.** 2002. Nuclear export of messenger RNA. Results Probl Cell Differ **35:**133-50.
90. **Izaurralde, E., U. Kutay, C. von Kobbe, I. W. Mattaj, and D. Gorlich.** 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. Embo J **16:**6535-47.
91. **Jarmolowski, A., W. C. Boelens, E. Izaurralde, and I. W. Mattaj.** 1994. Nuclear export of different classes of RNA is mediated by specific factors. J Cell Biol **124:**627-635.
92. **Jensen, T. H., K. Dower, D. Libri, and M. Rosbash.** 2003. Early formation of mRNP: license for export or quality control? Mol Cell **11:**1129-38.
93. **Jin, L., B. W. Guzik, Y. C. Bor, D. Rekosh, and M. L. Hammarskjold.** 2003. Tap and NXT promote translation of unspliced mRNA. Genes Dev **17:**3075-86.
94. **Johannes, G., and P. Sarnow.** 1998. Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. Rna **4:**1500-13.
95. **Johnson, J. M., J. Castle, P. Garrett-Engele, Z. Kan, P. M. Loerch, C. D. Armour, R. Santos, E. E. Schadt, R. Stoughton, and D. D. Shoemaker.** 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science **302:**2141-4.
96. **Kaplan, J. M., S. H. Kim, K. N. North, H. Rennke, L. A. Correia, H. Q. Tong, B. J. Mathis, J. C. Rodriguez-Perez, P. G. Allen, A. H. Beggs, and M. R. Pollak.** 2000. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet **24:**251-6.
97. **Kashima, I., A. Yamashita, N. Izumi, N. Kataoka, R. Morishita, S. Hoshino, M. Ohno, G. Dreyfuss, and S. Ohno.** 2006. Binding of a novel SMG-1-Upf1-eRF1-eRF3 complex (SURF) to the exon junction complex triggers Upf1 phosphorylation and nonsense-mediated mRNA decay. Genes Dev **20:**355-67.
98. **Katahira, J., K. Strasser, A. Podtelejnikov, M. Mann, J. U. Jung, and E. Hurt.** 1999. The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. Embo J **18:**2593-609.
99. **Keegan, L. P., A. Gallo, and M. A. O'Connell.** 2001. The many roles of an RNA editor. Nat Rev Genet **2:**869-78.
100. **Keene, J. D., and S. A. Tenenbaum.** 2002. Eukaryotic mRNPs may represent posttranscriptional operons. Mol Cell **9:**1161-7.

101. **Kent, J., A. M. Coriat, P. T. Sharpe, N. D. Hastie, and V. van Heyningen.** 1995. The evolution of WT1 sequence and expression pattern in the vertebrates. Oncogene **11:**1781-92.
102. **Khandjian, E. W., M. E. Huot, S. Tremblay, L. Davidovic, R. Mazroui, and B. Bardoni.** 2004. Biochemical evidence for the association of fragile X mental retardation protein with brain polyribosomal ribonucleoparticles. Proc Natl Acad Sci U S A **101:**13357-62.
103. **Kim, V. N., N. Kataoka, and G. Dreyfuss.** 2001. Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. Science **293:**1832-6.
104. **Kim, V. N., J. Yong, N. Kataoka, L. Abel, M. D. Diem, and G. Dreyfuss.** 2001. The Y14 protein communicates to the cytoplasm the position of exon-exon junctions. Embo J **20:**2062-8.
105. **Klamt, B., A. Koziell, F. Poulat, P. Wieacker, P. Scambler, P. Berta, and M. Gessler.** 1998. Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. Hum Mol Genet **7:**709-14.
106. **Kutay, U., G. Lipowsky, E. Izaurralde, F. R. Bischoff, P. Schwarzmaier, E. Hartmann, and D. Gorlich.** 1998. Identification of a tRNA-specific nuclear export receptor. Mol Cell **1:**359-69.
107. **Ladomery, M., J. Sommerville, S. Woolner, J. Slight, and N. Hastie.** 2003. Expression in Xenopus oocytes shows that WT1 binds transcripts in vivo, with a central role for zinc finger one. J Cell Sci **116:**1539-49.
108. **Ladomery, M. R., J. Slight, S. Mc Ghee, and N. D. Hastie.** 1999. Presence of WT1, the Wilm's tumor suppressor gene product, in nuclear poly(A)(+) ribonucleoprotein. J Biol Chem **274:**36520-6.
109. **Lai, M. C., R. I. Lin, and W. Y. Tarn.** 2001. Transportin-SR2 mediates nuclear import of phosphorylated SR proteins. Proc Natl Acad Sci U S A **98:**10154-9.
110. **Lai, M. C., and W. Y. Tarn.** 2004. Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. J Biol Chem **279:**31745-9.
111. **Laity, J. H., H. J. Dyson, and P. E. Wright.** 2000. Molecular basis for modulation of biological function by alternate splicing of the Wilms' tumor suppressor protein. Proc Natl Acad Sci U S A **97:**11932-5.
112. **Lareau, L. F., M. Inada, R. E. Green, J. C. Wengrod, and S. E. Brenner.** 2007. Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. Nature **446:**926-9.
113. **Larsson, S. H., J. P. Charlieu, K. Miyagawa, D. Engelkamp, M. Rassoulzadegan, A. Ross, F. Cuzin, V. van Heyningen, and N. D. Hastie.** 1995. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell **81:**391-401.
114. **Laurencikiene, J., A. M. Kallman, N. Fong, D. L. Bentley, and M. Ohman.** 2006. RNA editing and alternative splicing: the importance of co-transcriptional coordination. EMBO Rep **7:**303-7.
115. **Le Hir, H., D. Gatfield, E. Izaurralde, and M. J. Moore.** 2001. The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. Embo J **20:**4987-97.
116. **Le Hir, H., E. Izaurralde, L. E. Maquat, and M. J. Moore.** 2000. The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. Embo J **19:**6860-9.
117. **Lee, T. I., and R. A. Young.** 2000. Transcription of eukaryotic protein-coding genes. Annu Rev Genet **34:**77-137.
118. **Legrain, P., B. Seraphin, and M. Rosbash.** 1988. Early commitment of yeast pre-mRNA to the spliceosome pathway. Mol Cell Biol **8:**3755-60.
119. **Lejeune, F., Y. Cavaloc, and J. Stevenin.** 2001. Alternative splicing of intron 3 of the serine/arginine-rich protein 9G8 gene. Identification of flanking exonic splicing enhancers and involvement of 9G8 as a trans-acting factor. J Biol Chem **276:**7850-8.
120. **Lejeune, F., Y. Ishigaki, X. Li, and L. E. Maquat.** 2002. The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. Embo J **21:**3536-45.
121. **Lewis, J. D., D. Gorlich, and I. W. Mattaj.** 1996. A yeast cap binding protein complex (yCBC) acts at an early step in pre-mRNA splicing. Nucleic Acids Res **24:**3332-6.

122. **Lewis, J. D., and E. Izaurralde.** 1997. The role of the cap structure in RNA processing and nuclear export. Eur J Biochem **247:**461-9.
123. **Lewis, J. D., E. Izaurralde, A. Jarmolowski, C. McGuigan, and I. W. Mattaj.** 1996. A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site. Genes Dev **10:**1683-98.
124. **Lewis, N., J. Williams, D. Rekosh, and M. L. Hammarskjold.** 1990. Identification of a cis-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 rev and human T-cell leukemia virus types I and II rex proteins. J Virol **64:**1690-1697.
125. **Li, Y., Y. C. Bor, Y. Misawa, Y. Xue, D. Rekosh, and M. L. Hammarskjold.** 2006. An intron with a constitutive transport element is retained in a Tap messenger RNA. Nature **443:**234-7.
126. **Lu, S., and B. R. Cullen.** 2003. Analysis of the stimulatory effect of splicing on mRNA production and utilization in mammalian cells. Rna **9:**618-30.
127. **Lu, X. B., J. Heimer, D. Rekosh, and M. L. Hammarskjold.** 1990. U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus env mRNA that remains unspliced. Proc Natl Acad Sci U S A **87:**7598-7602.
128. **Luo, M. J., and R. Reed.** 1999. Splicing is required for rapid and efficient mRNA export in metazoans. Proc Natl Acad Sci U S A **96:**14937-42.
129. **Luo, M. L., Z. Zhou, K. Magni, C. Christoforides, J. Rappsilber, M. Mann, and R. Reed.** 2001. Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature **413:**644-7.
130. **Maas, S., A. Rich, and K. Nishikura.** 2003. A-to-I RNA editing: recent news and residual mysteries. J Biol Chem **278:**1391-4.
131. **Malim, M. H., J. Hauber, S. V. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338:**254-257.
132. **Maquat, L. E.** 2004. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol **5:**89-99.
133. **Maquat, L. E., and X. Li.** 2001. Mammalian heat shock p70 and histone H4 transcripts, which derive from naturally intronless genes, are immune to nonsense-mediated decay. Rna **7:**445-56.
134. **Matsumoto, K., K. M. Wassarman, and A. P. Wolffe.** 1998. Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. Embo J **17:**2107-21.
135. **Matsumura, M. E., F. Li, L. Berthoux, B. Wei, D. R. Lobe, C. Jeon, M. L. Hammarskjold, and C. A. McNamara.** 2001. Vascular injury induces posttranscriptional regulation of the Id3 gene: cloning of a novel Id3 isoform expressed during vascular lesion formation in rat and human atherosclerosis. Arterioscler Thromb Vasc Biol **21:**752-8.
136. **Matter, N., P. Herrlich, and H. Konig.** 2002. Signal-dependent regulation of splicing via phosphorylation of Sam68. Nature **420:**691-5.
137. **McLaren, M., K. Asai, and A. Cochrane.** 2004. A novel function for Sam68: enhancement of HIV-1 RNA 3' end processing. Rna **10:**1119-29.
138. **Mermoud, J. E., P. T. Cohen, and A. I. Lamond.** 1994. Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. Embo J **13:**5679-88.
139. **Moffett, P., W. Bruening, H. Nakagama, N. Bardeesy, D. Housman, D. E. Housman, and J. Pelletier.** 1995. Antagonism of WT1 activity by protein self-association. Proc Natl Acad Sci U S A **92:**11105-9.
140. **Morley, S. J., P. S. Curtis, and V. M. Pain.** 1997. eIF4G: translation's mystery factor begins to yield its secrets. Rna **3:**1085-104.
141. **Nagy, E., and L. E. Maquat.** 1998. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem Sci **23:**198-9.
142. **Nakielny, S., and G. Dreyfuss.** 1999. Transport of proteins and RNAs in and out of the nucleus. Cell **99:**677-90.

143. **Natoli, T. A., A. McDonald, J. A. Alberta, M. E. Taglienti, D. E. Housman, and J. A. Kreidberg.** 2002. A mammal-specific exon of WT1 is not required for development or fertility. Mol Cell Biol **22:**4433-8.
144. **Ner-Gaon, H., R. Halachmi, S. Savaldi-Goldstein, E. Rubin, R. Ophir, and R. Fluhr.** 2004. Intron retention is a major phenomenon in alternative splicing in Arabidopsis. Plant J **39:**877-85.
145. **Neugebauer, K. M.** 2002. On the importance of being co-transcriptional. J Cell Sci **115:**3865-71.
146. **Neville, M., F. Stutz, L. Lee, L. I. Davis, and M. Rosbash.** 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr Biol **7:**767-775.
147. **Niksic, M., J. Slight, J. R. Sanford, J. F. Caceres, and N. D. Hastie.** 2004. The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes. Hum Mol Genet **13:**463-71.
148. **Nott, A., H. Le Hir, and M. J. Moore.** 2004. Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. Genes Dev **18:**210-22.
149. **Nott, A., S. H. Meislin, and M. J. Moore.** 2003. A quantitative analysis of intron effects on mammalian gene expression. Rna **9:**607-17.
150. **Ohnishi, T., A. Yamashita, I. Kashima, T. Schell, K. R. Anders, A. Grimson, T. Hachiya, M. W. Hentze, P. Anderson, and S. Ohno.** 2003. Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. Mol Cell **12:**1187-200.
151. **Ohno, M., A. Segref, A. Bachi, M. Wilm, and I. W. Mattaj.** 2000. PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. Cell **101:**187-98.
152. **Ortega, A., M. Niksic, A. Bachi, M. Wilm, L. Sanchez, N. Hastie, and J. Valcarcel.** 2003. Biochemical function of female-lethal (2)D/Wilms' tumor suppressor-1-associated proteins in alternative pre-mRNA splicing. J Biol Chem **278:**3040-7.
153. **Pasquinelli, A. E., R. K. Ernst, E. Lund, C. Grimm, M. L. Zapp, D. Rekosh, M. L. Hammarskjold, and J. E. Dahlberg.** 1997. The constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV) accesses a cellular mRNA export pathway. Embo J **16:**7500-7510.
154. **Pemberton, L. F., and B. M. Paschal.** 2005. Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic **6:**187-98.
155. **Pennisi, E.** 2007. Genetics. Working the (gene count) numbers: finally, a firm answer? Science **316:**1113.
156. **Pollard, V. W., and M. H. Malim.** 1998. The HIV-1 Rev protein. Annu Rev Microbiol **52:**491-532.
157. **Popielarz, M., Y. Cavaloc, M. G. Mattei, R. Gattoni, and J. Stevenin.** 1995. The gene encoding human splicing factor 9G8. Structure, chromosomal localization, and expression of alternatively processed transcripts. J Biol Chem **270:**17830-5.
158. **Preiss, T., and W. H. M.** 2003. Starting the protein synthesis machine: eukaryotic translation initiation. Bioessays **25:**1201-11.
159. **Proudfoot, N. J., A. Furger, and M. J. Dye.** 2002. Integrating mRNA processing with transcription. Cell **108:**501-12.
160. **Rauscher, F. J., 3rd.** 1993. The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. Faseb J **7:**896-903.
161. **Rauscher, F. J., 3rd, J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran.** 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science **250:**1259-62.
162. **Reddy, J. C., J. C. Morris, J. Wang, M. A. English, D. A. Haber, Y. Shi, and J. D. Licht.** 1995. WT1-mediated transcriptional activation is inhibited by dominant negative mutant proteins. J Biol Chem **270:**10878-84.
163. **Reddy, T. R., H. Tang, W. Xu, and F. Wong-Staal.** 2000. Sam68, RNA helicase A and Tap cooperate in the post-transcriptional regulation of human immunodeficiency virus and type D retroviral mRNA. Oncogene **19:**3570-5.

164. **Reed, R., and E. Hurt.** 2002. A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell **108:**523-31.
165. **Reichert, V. L., H. Le Hir, M. S. Jurica, and M. J. Moore.** 2002. 5' exon interactions within the human spliceosome establish a framework for exon junction complex structure and assembly. Genes Dev **16:**2778-91.
166. **Reynolds, P. A., G. A. Smolen, R. E. Palmer, D. Sgroi, V. Yajnik, W. L. Gerald, and D. A. Haber.** 2003. Identification of a DNA-binding site and transcriptional target for the EWS-WT1(+KTS) oncoprotein. Genes Dev **17:**2094-107.
167. **Rodrigues, J. P., M. Rode, D. Gatfield, B. J. Blencowe, M. Carmo-Fonseca, and E. Izaurralde.** 2001. REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus. Proc Natl Acad Sci U S A **98:**1030-5.
168. **Rosenfeld, C., M. A. Cheever, and A. Gaiger.** 2003. WT1 in acute leukemia, chronic myelogenous leukemia and myelodysplastic syndrome: therapeutic potential of WT1 targeted therapies. Leukemia **17:**1301-12.
169. **Roth, M. B., C. Murphy, and J. G. Gall.** 1990. A monoclonal antibody that recognizes a phosphorylated epitope stains lampbrush chromosome loops and small granules in the amphibian germinal vesicle. J Cell Biol **111:**2217-23.
170. **Rueter, S. M., T. R. Dawson, and R. B. Emeson.** 1999. Regulation of alternative splicing by RNA editing. Nature **399:**75-80.
171. **Rutjes, S. A., E. Lund, A. van der Heijden, C. Grimm, W. J. van Venrooij, and G. J. Pruijn.** 2001. Identification of a novel cis-acting RNA element involved in nuclear export of hY RNAs. Rna **7:**741-52.
172. **Saavedra, C., B. Felber, and E. Izaurralde.** 1997. The simian retrovirus-1 constitutive transport element, unlike the HIV- 1 RRE, uses factors required for cellular mRNA export. Curr Biol **7:**619-628.
173. **Salas-Marco, J., and D. M. Bedwell.** 2004. GTP hydrolysis by eRF3 facilitates stop codon decoding during eukaryotic translation termination. Mol Cell Biol **24:**7769-78.
174. **Sanford, J. R., and J. F. Caceres.** 2004. Pre-mRNA splicing: life at the centre of the central dogma. J Cell Sci **117:**6261-3.
175. **Sanford, J. R., J. D. Ellis, D. Cazalla, and J. F. Caceres.** 2005. Reversible phosphorylation differentially affects nuclear and cytoplasmic functions of splicing factor 2/alternative splicing factor. Proc Natl Acad Sci U S A **102:**15042-7.
176. **Sanford, J. R., N. K. Gray, K. Beckmann, and J. F. Caceres.** 2004. A novel role for shuttling SR proteins in mRNA translation. Genes Dev **18:**755-68.
177. **Sanford, J. R., D. Longman, and J. F. Caceres.** 2003. Multiple roles of the SR protein family in splicing regulation. Prog Mol Subcell Biol **31:**33-58.
178. **Santos-Rosa, H., H. Moreno, G. Simos, A. Segref, B. Fahrenkrog, N. Pante, and E. Hurt.** 1998. Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores. Mol Cell Biol **18:**6826-38.
179. **Scharnhorst, V., A. J. van der Eb, and A. G. Jochemsen.** 2001. WT1 proteins: functions in growth and differentiation. Gene **273:**141-61.
180. **Segref, A., K. Sharma, V. Doye, A. Hellwig, J. Huber, R. Luhrmann, and E. Hurt.** 1997. Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. Embo J **16:**3256-71.
181. **Senger, B., G. Simos, F. R. Bischoff, A. Podtelejnikov, M. Mann, and E. Hurt.** 1998. Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. Embo J **17:**2196-207.
182. **Shatkin, A. J., and J. L. Manley.** 2000. The ends of the affair: capping and polyadenylation. Nat Struct Biol **7:**838-42.
183. **Sheth, U., and R. Parker.** 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science **300:**805-8.
184. **Shin, C., Y. Feng, and J. L. Manley.** 2004. Dephosphorylated SRp38 acts as a splicing repressor in response to heat shock. Nature **427:**553-8.

185. **Shin, C., and J. L. Manley.** 2002. The SR protein SRp38 represses splicing in M phase cells. Cell **111:**407-17.
186. **Shuman, S.** 2001. Structure, mechanism, and evolution of the mRNA capping apparatus. Prog Nucleic Acid Res Mol Biol **66:**1-40.
187. **Smith, A. J., N. Srinivasakumar, M. L. Hammarskjöld, and D. Rekosh.** 1993. Requirements for incorporation of Pr160gag-pol from human immunodeficiency virus type 1 into virus-like particles. J Virol **67:**2266-2275.
188. **Sonenberg, N., J. W. B. Hershey, M. B. Mathews, and editors.** 2000. Translational Control of Gene Expression. Cold Spring Harbor Laboratory Press.
189. **Srinivasakumar, N., N. Chazal, M. C. Helga, S. Prasad, M. L. Hammarskjold, and D. Rekosh.** 1997. The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type 1 vectors produced in stable packaging cell lines. J Virol **71:**5841-5848.
190. **Srinivasakumar, N., M. L. Hammarskjold, and D. Rekosh.** 1995. Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag- Pol precursor incorporation. J Virol **69:**6106-14.
191. **Stoss, O., M. Olbrich, A. M. Hartmann, H. Konig, J. Memmott, A. Andreadis, and S. Stamm.** 2001. The STAR/GSG family protein rSLM-2 regulates the selection of alternative splice sites. J Biol Chem **276:**8665-73.
192. **Stutz, F., A. Bachi, T. Doerks, I. C. Braun, B. Seraphin, M. Wilm, P. Bork, and E. Izaurralde.** 2000. REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. Rna **6:**638-50.
193. **Stutz, F., and E. Izaurralde.** 2003. The interplay of nuclear mRNP assembly, mRNA surveillance and export. Trends Cell Biol **13:**319-27.
194. **Tacke, R., and J. L. Manley.** 1999. Determinants of SR protein specificity. Curr Opin Cell Biol **11:**358-62.
195. **Tan, W., A. S. Zolotukhin, J. Bear, D. J. Patenaude, and B. K. Felber.** 2000. The mRNA export in Caenorhabditis elegans is mediated by Ce-NXF-1, an ortholog of human TAP/NXF and Saccharomyces cerevisiae Mex67p. Rna **6:**1762-72.
196. **Tange, T. O., T. Shibuya, M. S. Jurica, and M. J. Moore.** 2005. Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. Rna **11:**1869-83.
197. **Vagner, S., C. Vagner, and I. W. Mattaj.** 2000. The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3'-end processing and splicing. Genes Dev **14:**403-13.
198. **Vajjhala, P. R., E. Macmillan, T. Gonda, and M. Little.** 2003. The Wilms' tumour suppressor protein, WT1, undergoes CRM1-independent nucleocytoplasmic shuttling. FEBS Lett **554:**143-8.
199. **Valcarcel, J., and M. R. Green.** 1996. The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem Sci **21:**296-301.
200. **Visa, N., E. Izaurralde, J. Ferreira, B. Daneholt, and I. W. Mattaj.** 1996. A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export. J Cell Biol **133:**5-14.
201. **Wagner, N., K. D. Wagner, A. Hammes, K. M. Kirschner, V. P. Vidal, A. Schedl, and H. Scholz.** 2005. A splice variant of the Wilms' tumour suppressor Wt1 is required for normal development of the olfactory system. Development **132:**1327-36.
202. **Wahle, E., and U. Ruegsegger.** 1999. 3'-End processing of pre-mRNA in eukaryotes. FEMS Microbiol Rev **23:**277-95.
203. **Wang, H. Y., W. Lin, J. A. Dyck, J. M. Yeakley, Z. Songyang, L. C. Cantley, and X. D. Fu.** 1998. SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J Cell Biol **140:**737-50.
204. **Wang, J., S. H. Xiao, and J. L. Manley.** 1998. Genetic analysis of the SR protein ASF/SF2: interchangeability of RS domains and negative control of splicing. Genes Dev **12:**2222-33.

205. **Wetterberg, I., G. Bauren, and L. Wieslander.** 1996. The intranuclear site of excision of each intron in Balbiani ring 3 pre-mRNA is influenced by the time remaining to transcription termination and different excision efficiencies for the various introns. Rna **2:**641-51.
206. **Wiegand, H. L., G. A. Coburn, Y. Zeng, Y. Kang, H. P. Bogerd, and B. R. Cullen.** 2002. Formation of Tap/NXT1 heterodimers activates Tap-dependent nuclear mRNA export by enhancing recruitment to nuclear pore complexes. Mol Cell Biol **22:**245-56.
207. **Wiegand, H. L., S. Lu, and B. R. Cullen.** 2003. Exon junction complexes mediate the enhancing effect of splicing on mRNA expression. Proc Natl Acad Sci U S A **100:**11327-32.
208. **Wilusz, C. J., M. Wormington, and S. W. Peltz.** 2001. The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol **2:**237-46.
209. **Windgassen, M., D. Sturm, I. J. Cajigas, C. I. Gonzalez, M. Seedorf, H. Bastians, and H. Krebber.** 2004. Yeast shuttling SR proteins Npl3p, Gbp2p, and Hrb1p are part of the translating mRNPs, and Npl3p can function as a translational repressor. Mol Cell Biol **24:**10479-91.
210. **Yoon, D. W., H. Lee, W. Seol, M. DeMaria, M. Rosenzweig, and J. U. Jung.** 1997. Tap: a novel cellular protein that interacts with tip of herpesvirus saimiri and induces lymphocyte aggregation. Immunity **6:**571-82.
211. **Yun, C. Y., A. L. Velazquez-Dones, S. K. Lyman, and X. D. Fu.** 2003. Phosphorylation-dependent and -independent nuclear import of RS domain-containing splicing factors and regulators. J Biol Chem **278:**18050-5.
212. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev **6:**837-47.
213. **Zeng, C., and S. M. Berget.** 2000. Participation of the C-terminal domain of RNA polymerase II in exon definition during pre-mRNA splicing. Mol Cell Biol **20:**8290-301.
214. **Zhai, G., M. Iskandar, K. Barilla, and P. J. Romaniuk.** 2001. Characterization of RNA aptamer binding by the Wilms' tumor suppressor protein WT1. Biochemistry **40:**2032-40.
215. **Zhang, Z., and A. R. Krainer.** 2004. Involvement of SR proteins in mRNA surveillance. Mol Cell **16:**597-607.
216. **Zhao, J., L. Hyman, and C. Moore.** 1999. Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. Microbiol Mol Biol Rev **63:**405-45.
217. **Zhou, Z., M. J. Luo, K. Straesser, J. Katahira, E. Hurt, and R. Reed.** 2000. The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. Nature **407:**401-5.
218. **Zolotukhin, A. S., W. Tan, J. Bear, S. Smulevitch, and B. K. Felber.** 2002. U2AF participates in the binding of TAP (NXF1) to mRNA. J Biol Chem **277:**3935-42.
219. **Zolotukhin, A. S., A. Valentin, G. N. Pavlakis, and B. K. Felber.** 1994. Continuous propagation of RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones containing a cis-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. J Virol **68:**7944-7952.