The splicing regulatory SR protein, 9G8, has recently been proposed to function in mRNA export in conjunction with the export protein, Tap/NXF1. Tap interacts directly with the Mason-Pfizer monkey virus constitutive transport element (CTE), an element that enables export of unspliced, intron-containing mRNA. Based on our previous finding that Tap can promote polysome association and translation of CTE-RNA, we investigated the effect of 9G8 on cytoplasmic RNA fate. 9G8 was shown to enhance expression of unspliced RNA containing either the Mason-Pfizer monkey virus-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 hyperphosphorylated SR proteins remain stably associated with messenger ribonucleoprotein (mRNP) complexes during export and are released during translation initiation concomitant with increased phosphorylation. These results provide further evidence for crucial links between RNA splicing, export, and translation.

The serine/arginine (SR) proteins are a family of RNA binding proteins with well recognized roles in splicing regulation (1–3). These proteins all have C-terminal arginine- and serine-rich (RS) domains that can be phosphorylated and dephosphorylated on multiple serines by cellular kinases and phosphatases (2). The phosphorylation state is believed to play an important role in functional regulation (2, 4–6).

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 (9). 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 (10–12). Another study reported the presence of SR proteins in polyribosomes, suggesting a potential role in translational regulation (13). Specifically, it was reported that the SF2/ASF protein was able to promote translation of mRNAs from reporter constructs containing SR protein binding sites. Several recent studies have now provided additional support of a role for SR proteins in translation (14, 15).

In the case of most mammalian genes, the primary RNA transcript contains multiple introns that must be removed by splicing before the mRNA can exit the nucleus (16–18). Although it is not clear what restricts mRNA from export before splicing has been completed, the shuttling protein, Tap/NXF1 (hereafter referred to as Tap), is believed to play an important role in the export process of many mRNAs. It has been suggested that Tap is recruited to completely spliced mRNAs through multiple adaptor proteins (19, 20). In addition to the initially identified adaptor protein REF (21, 22), three shuttling SR proteins (9G8, SRp20, and SF2/ASF) have also been proposed to serve this function (10–12, 23). The SR proteins are recruited to the RNA during transcription in a phosphorylated form, and dephosphorylation may trigger the recruitment of Tap (12).

Although Tap has been suggested to be recruited to most cellular mRNA through protein-protein interactions, it was originally identified as an RNA-binding protein that interacted specifically with a constitutive transport element (CTE) (24) present in the genomic RNA of Mason-Pfizer monkey virus (MPMV) (25–27). Furthermore, we have recently demonstrated that the Tap gene, itself, also contains a functional Tap-binding CTE, which exists in a retained intron (28). 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 provides
further evidence that Tap regulates its own expression through a mechanism that involves direct RNA binding. This clearly demonstrates that Tap is capable of binding directly to cellular mRNA.

MPMV, like all other retroviruses, produces an unspliced mRNA with a retained complete intron that has to be efficiently exported and translated in the cytoplasm. This mode of genetic organization and gene expression presents a general problem for retroviruses, because mRNAs usually remain in the nucleus until all introns are removed (30, 31). In complex retroviruses, such as HIV, the problem is solved by the expression of specific viral proteins (Rev in the case of HIV) that interact with cis-acting RNA elements in the viral genome (RRE in the case of HIV) (32–35). In HIV infection, the Rev-RRE RNA complex is exported with the help of the Crm1 cellular export receptor (36, 37). MPMV, however, does not encode any regulatory proteins. Instead, the CTE interacts directly with the Tap export protein and another cellular protein, NXT1/p15 (hereafter referred to as NXT1), to promote export (38–43). We have also shown that Tap/NXT1 enhances translation of CTE-containing RNA in the cytoplasm. Tap, but not NXT1, remained associated with polyribosomes (44). These results suggest that productive nuclear export of Tap-RNA complexes might be coupled to translation initiation. Here we show that moderate SRp20 or 9G8 overexpression 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 80 S 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.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cloning Procedures**—To facilitate identification, all of the plasmids used in this study were indexed as numbers in the form pHHRXXXX. The subgenomic HIV-1 reporter constructs, pCMVGagPol-CTE (pHR1361) (45), pCMVGag-Pol-TapCTE × 1 (pHR3405) (28), and pCMVGagPol-TapCTE × 2 (pHR3406) (28); a plasmid that expresses secreted alkaline phosphatase (SEAP), pCMVSEAP (pHR1831) (46); a plasmid that expresses a FLAG-tagged NXT1 protein, pcDNAFLAG-Tap (pHR2352) (44); and a plasmid that expresses a FLAG-tagged 9G8 protein, pcDNA9G8 (pHR2283) (47); and a plasmid that expresses a FLAG-tagged Tap protein, pcDNAFLAG-Tap (pHR2352) (44) have been described previously. Plasmids expressing T7-tagged SR proteins, pCGT7SRp20 (pHR2959) (48), were kind gifts from Dr. Adrian Krainer. pCMVSEAP (pHR1831) was constructed by inserting the BamHI/EcoRI and ligated into pGEX2T (AMRAD). pCMVGagPol-CTE (pHR1361) (45), pCMVGagPolTapCTE (pHR3405) (28), and pCMVGagPolTapCTE × 2 (pHR3406) (28); a plasmid that expresses secreted alkaline phosphatase (SEAP), pCMVSEAP (pHR1831) (46); a plasmid that expresses a FLAG-tagged NXT1 protein, pcDNAFLAG-Tap (pHR2352) (44); and a plasmid that expresses a FLAG-tagged 9G8 protein, pcDNA9G8 (pHR2283) (47); and a plasmid that expresses a FLAG-tagged Tap protein, pcDNAFLAG-Tap (pHR2352) (44) have been described previously. Plasmids expressing T7-tagged SR proteins, pCGT7SRp20 (pHR2959) (48), were kind gifts from Dr. Adrian Krainer. pCMVSEAP (pHR1831) was constructed by inserting the BamHI/SacI fragment of pCMVSEAP (pHR1831) into pGEM7Zf (Promega, Madison, WI). This plasmid was linearized with SacI to provide T7 polymerase a template for in vitro transcription. pGEXT2ZnK (pHR3035) was constructed by PCR amplification of pCGT79G8 (pHR2957) using oligonucleotide 5′-CGCCGGATCATCGAGATGCAGTACGAGGAC-3′ as the sense primer and oligonucleotide 5′-CGCGGAATTCCGTACGATGACATCATAAGGC-3′ as the antisense primer to obtain a fragment encoding the zinc knuckle domain of 9G8. This fragment was digested with BamHI/EcoRI and ligated into pGEXT2 (AMRAD).

**Cell Lines and Transient Transfections**—293T/17 cells (49) and B2.23 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% bovine calf serum. B2.23 cells are 293T/17 cells stably transfected with pCMVGagPolCTE and pCMVEnv-CTE. They are similar to the previously described B2.10 cells (44, 50). 293T/17 cells were transfected using a calcium phosphate transfection protocol (51).

**p24 Enzyme-linked Immunosorbent Assay and SEAP Quantitation**—Supernatants from transfected cells were collected at 65–72 h post-transfection and centrifuged briefly to remove residual cells and debris. Expression levels of p24 (HIV capsid protein) were determined by an enzyme-linked immunosorbent assay protocol using a p24 monoclonal antibody (183-H12–5C) and pooled human anti-HIV immunoglobulin G (52). The p24 antibody was obtained from the AIDS Research and Reference Reagent Program and was contributed by Bruce Chesebro (NIAID, National Institutes of Health-Rocky Mountain Laboratories). SEAP activity in the supernatants was measured with the Phospha-Light Chemiluminescent Reporter Kit (Tropix).

**Western Blot Analysis and Antibodies**—Proteins were separated by SDS-15% PAGE (acrylamide/bisacrylamide ratio: 30/0.14). Western blot analysis was performed essentially as previously described (33). Briefly, proteins were transferred to an Immobilon-P membrane (Millipore), and the membrane was blocked in 5% milk (or 5% bovine serum albumin for phospho-blots) and probed with antibody. For detection of T7G9G and T7SRp20, blots were probed with anti-T7 antibody (1:1000, Novagen). For detection of phospho-SR proteins, blots were probed with monoclonal antibody 104 (mAb104, 1:25, ATCC) (54). To detect endogenous 9G8, blots were probed with a polyclonal 9G8 antibody raised in rabbits (1:250, BioSource) against a GST-9G8 zinc knuckle domain (amino acids 61–122) fusion protein produced from the pGEX2TznK vector (pHR3035). After washing, blots were incubated with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000, Amersham Biosciences), and proteins were visualized using ECL (Amersham Biosciences). Quantitation was performed with ImageQuaNT analysis software.

**Polyribosome Analysis**—Untransfected or transfected (48 h post-transfection) cells (4 × 10⁷) were exposed to 50 µg/ml cycloheximide at 37 °C for 30 min (RNA analysis) or 5 min (protein analysis), washed twice with cold phosphate-buffered saline containing 50 µg/ml cycloheximide, and harvested by scraping from the plate. Pelleted cells were resuspended in 250 µl of cold RSB (10 mM Tris-HCl at pH 7.4, 10 mM NaCl, 3 mM MgCl₂, protease and phosphatase inhibitor cocktails (Sigma), and 250 units of RNasin (Promega)) and lysed by the addition of an equal volume of 2× lysis buffer (RSB containing 1% Triton X-100, 1% deoxycholate, and 2% Tween 20) with incubation for 10 min on ice. Lysates were centrifuged for 10 min at 10,000 × g, and supernatants were loaded onto gradients of 10% to 50% (w/v) sucrose in 10 mM Tris-HCl (pH 7.6), 75 mM KCl, and 3 mM MgCl₂, and the samples were centrifuged for 3 h at 30,000 r.p.m. to sediment ribosomes. Fractions were collected, and fractions containing 80 S monosomes and small polyribosomes were pooled for analysis (53).
A Role of 9G8 in Translation

MgCl₂. In some experiments, before loading onto the gradient, EDTA was added to the lysate at a final concentration of 15 mM to disrupt the polysomes (55, 56). After centrifugation at 36,000 rpm for 2 h at 4 °C in an SW41Ti rotor, the gradient was collected from the top using a Piston Gradient Fractionator (Bio-comp) into 13 or 20 fractions, with the A₂₅₄ continuously measured using a UV-M II monitor (Amerham Biosciences) during collection. Fractions were either isopropanol-precipitated or immunoprecipitated prior to SDS-PAGE, or they were purified for RNA. For the immunoprecipitations, an equal volume of ice-cold isopropanol was added to each fraction and incubated on ice 30 min. The precipitate was pelleted at 13,000 rpm for 15 min and washed in cold acetone. To purify RNA from the sucrose, 10 ng of precipitate was pelleted at 13,000 rpm for 15 min and washed in

**Northern blot analysis**—We also performed Northern blot analysis to determine whether the enhancement 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 alone or co-transfected with the plasmid expressing 9G8 (Fig. 1E). The Northern blot analysis shows that co-expression of 9G8 in this experiment increased the total GagPol-CTE RNA levels <2-fold (relative to the SEAP control) and had no effect on the cytoplasmic levels of this RNA. Thus, the observed increase in GagPol protein expression after co-expression of 9G8 cannot be explained by increased RNA export.

9G8 Enhances Expression from a Cellular CTE—We also performed an experiment to determine whether 9G8 would

**RESULTS**

Two Shuttling SR Proteins 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 (10–12,23). Because Tap binds to the MPMV-CTE, and we have previously demonstrated that moderate overexpression of Tap and NXT1 increases translation from an RNA con-
enhance expression mediated by the cellular CTE that we recently identified in the Tap gene (28). The Tap-CTE contains only one Tap-binding loop, in contrast to the MPMV-CTE, which contains two Tap-binding loops. We have previously demonstrated that in the context of the GagPol reporter, two Tap-binding loops are important for efficient expression (28). For this reason, we transfected cells with the HIV-GagPol reporter containing either one or two copies of the Tap-CTE alone or in co-transfections with plasmids expressing Tap/NXT1 or 9G8. The results of this experiment (Fig. 2) demonstrated that 9G8 enhanced p24 expression from the Tap-CTE severalfold. As expected, two copies of the CTE were required for high levels of p24 expression. With the TapCTE×2 plasmid, the enhancement with 9G8 was even greater than what was obtained with Tap/NXT1, indicating that 9G8 can function efficiently in conjunction with the cellular CTE element.

In addition, 9G8 significantly enhanced p24 expression from the TapCTE×1 plasmid in co-transfections with Tap/NXT1.

**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 (44). 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 cytoplasmic extracts from 10⁷ cells transfected with 15 μg of pCMVGagPol-CTE in the presence or absence of 1 μg of 9G8 to sucrose gradient analysis. As before, the cells were also transfected with 0.5 μg of pCMVSEAP to serve as a control.

After fractionation of the gradients, a small amount of in vitro synthesized seap RNA was added to each fraction as a recovery control. RNA was then prepared from each fraction, and a Northern blot analysis was performed using Gag- and SEAP-specific probes. Fig. 3A shows that, in cells transfected with pCMVGagPol-CTE and pCMVSEAP alone, most of the CTE RNA was present in the fractions containing the 80 S 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 (44). In the co-transfections that included 9G8 (Fig. 3B), a significantly larger fraction of the CTE RNA was detected in the fractions representing polyribosomes. The percentage of the CTE and SEAP mRNAs that were present in each fraction is shown above each lane, and the percentages are summarized in panels C and D. Without transfection of 9G8 (Fig. 3B), a significantly larger fraction of the CTE RNA was detected in the fractions representing polyribosomes. The percentage of the CTE and SEAP mRNAs that were present in each fraction is shown above each lane, and the percentages are summarized in panels C and D. Without transfection of 9G8, only 4% of the CTE RNA is in polyribosomes larger than dimers. With transfection of 9G8, 40% of CTE RNA is in large polyribosomes. 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—Because 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). Because the transfected plasmid expresses a 9G8 protein that contains a T7-epitope tag, immunoprecipitation 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. 4B) or mAb104 (Fig. 4C), which specifically detects phospho-epitopes within the RS domain of SR proteins. To directly demonstrate that mAb104 can detect phosphorylated 9G8, we also performed a Western blot experiment with this antibody using whole cell lysates from untransfected and transfected cells (Fig. 4D). This blot clearly demonstrated that mAb104 is able to detect the 9G8 protein expressed from the transfected plasmid. As expected, this antibody also detected several endogenous SR proteins in both untransfected and transfected cells.

FIGURE 3. Polyribosome profile analysis of GagPol-CTE mRNA in transfected 293T cells by sucrose gradient centrifugation. A and 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 under “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 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 (lower panels). Bands corresponding to gagpol, seap, and ivt 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 seap band in each fraction. The number above each lane indicates the percentage of the total gagpol and seap mRNA found in the individual fractions after recovery-normalization using the ivt seap signal. C and D, the graphs show the distribution of gagpol (C) or seap mRNA (D) in the CTE (white bars) and CTE plus 9G8 gradients (black bars).
The immunoprecipitation/Western blot analysis of the sucrose gradient fractions revealed that both the T7 and mAb104 antibodies detected 9G8 proteins of several different molecular weights (Fig. 4, B and C). The higher molecular weight bands were strongest in the fractions representing 60 and 80 S 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. 4F). 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 Fig. 4, B and F) and was no longer detectable with mAb104 (data not shown), demonstrating that 9G8 resolves into multiple species due to differential phosphorylation.

The Presence of 9G8 in 80 S 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 ribosomal subunits (55, 56). These experiments were performed on cells transfected with the plasmid expressing the full-length 9G8 protein (Fig. 5, A and B). In addition, we also analyzed cells transfected with a plasmid expressing a mutant form of 9G8 that lacks the RS domain (9G8/RS; Fig. 5, C and D). In the absence of EDTA, we again observed several bands for the full-length protein, with hyperphosphorylated forms in the same fractions as before. In the presence of EDTA, 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, these 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.

FIGURE 4. Hyperphosphorylated T79G8 protein is mainly present in monosomes and small polyribosomes. A–C, 293T cells were transfected with pCGT79G8. A, the polyribosome profile analysis was performed as described for Fig. 3, B, fractions were immunoprecipitated with anti-T7 antibody, separated by 5%–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. D, the mAb104 antibody recognizes T7G8. 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 T7G8. E and F, Western blot analysis of T79G8 protein expression after phosphatase treatment. After sucrose gradient analysis, each fraction was treated with 140 units of CIAP. The fractions were then subjected to immunoprecipitation and Western blot analysis using anti-T7 antibodies, as described above.
The 9G8 protein lacking the RS domain was also detected in fractions containing the ribosomal subunits, monosomes and polysomes. In fact, when compared with the full-length protein, 9G8ΔRS was found in relatively higher amounts in fractions representing heavy polysomes. Again, there was a significant shift to “lighter” fractions in the presence of EDTA, indicating a true association with polyribosomes. In addition, 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. As might be expected, expression of the 9G8ΔRS protein did not enhance p24 expression from the GagPol-CTE construct (data not shown).

Polyribosome Analysis of Endogenous 9G8—Although the T7-tagged version of 9G8 enabled us to specifically detect transfected 9G8, lack of 9G8-specific antibodies prevented us from analyzing endogenous 9G8. To be able to do this, we developed 9G8-specific antibodies using a GST-9G8 fusion protein that contained the unique zinc knuckle domain of 9G8 as the immunogen (Fig. 6). Serum from a rabbit inoculated with this protein was shown to react specifically with the 9G8 protein, but it had no cross-reactivity with SRp20 (compare Fig. 6, B and C). The antibody also detected bands migrating slightly faster than T7–9G8 in both untransfected cells and cells transfected with T7-SRp20 (Fig. 6C), suggesting that these bands represent the endogenously expressed 9G8 protein.

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. 7). It should be noted that this analysis was performed on cells that were not subjected to a transfection protocol. Thus the polyribosome profiles are reflective of more active protein synthesis, when compared with the profiles shown in the other figures.

The results of the analysis in the absence of EDTA or CIAP are shown in Fig. 7A. They demonstrate 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 60 S, 80 S, and small polyribosomal fractions (6–11). After CIAP treatment, all of these bands disappeared, and a single band of ~25 kDa was detected. Fractions 1 and 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 (Fig. 7B).

In the presence of EDTA (Fig. 7C), no 9G8 proteins were detected beyond the 60 S peak. Most of the 9G8 protein was found in the fractions containing 40 and 60 S ribosomal subunits, and these fractions contained all the different species of
Taken together, these results suggest that the 9G8 protein is phosphorylated in the context of the translation machinery.

**DISCUSSION**

In this study, we have shown that both SRp20 and 9G8 enhance expression from unspliced RNA containing CTEs and that 9G8 promotes the polyribosome association of the exported RNA. In addition, our results demonstrate that transfected or endogenous 9G8 undergoes phosphorylation of its RS domain in the context of the translation machinery.

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 (58). For example, mRNA that has undergone splicing is translated in the cytoplasm more efficiently than unspliced mRNA derived from cDNA (59, 60). This has been attributed to the presence of exon junction complex proteins that remain on the mRNA after splicing and export (61, 62). In this study we have analyzed the fate of an mRNA that contains an intron, but remains unspliced. Because this mRNA does not contain any exon junction complexes, our results suggest that shuttling SR proteins can function similarly to link nuclear and cytoplasmic events in the expression of mRNAs with retained introns.

Shuttling SR proteins have previously been suggested to promote export of completely spliced mRNA by serving as an adaptor for the Tap export protein (11, 63). In the models proposed, Tap is not envisaged to contact the RNA directly but merely to interact with the mRNP complex through protein-protein interactions. Yet, Tap is clearly an RNA-binding protein that has been shown to contain both RNP and leucine-rich region domains through which it interacts directly with CTEs (64).

It was previously proposed that CTEs are only present in viral RNAs and that they might serve to simply mimic cellular adaptor proteins. However, our recent discovery that Tap interacts directly with a CTE in an alternatively spliced Tap mRNA (28) clearly demonstrates that Tap is also capable of direct interactions with cellular mRNAs. This suggests that current models for how Tap functions are likely to be too simplistic. Further support for this view is the fact that 9G8 promotes function mediated by both the MPMV- and Tap-CTEs, although it would not be expected to function as an adapter protein in these cases. It also remains unclear why Tap, an RNA binding protein, would need an adaptor protein for recruitment to cellular mRNA.

However, it is possible that 9G8 serves to stabilize the RNA-Tap complex. The Tap-binding region of SRp20 and 9G8 lies between the RNA recognition motif and RS domains in these proteins and very near the RNA binding residues, as determined by NMR (65). Most mRNAs do not contain bona fide CTEs, but it is still possible that RNA-protein, as well as protein-protein interactions, could serve to generally promote the formation of stable Tap export complexes. In fact, preliminary results in our laboratory indicate that co-immunoprecipitation of Tap with 9G8 is RNA-dependent, in contrast to previously published results (11). An atomic resolution of a Tap-9G8-RNA trimeric complex would clearly be very helpful for further clarification of this issue.

**FIGURE 7. Polyribosome analysis of endogenous 9G8.** A–C, 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. Fractions were either left untreated (A) or treated with 140 units CIAP (B), or prior to layering on the gradient, the lysate was treated with 15 mM EDTA (C).

9G8. Taken together, these results suggest that the 9G8 protein is phosphorylated in the context of the translation machinery.
A Role of 9G8 in Translation

We found that 9G8 and SRp20, two shuttling SR proteins, can enhance expression from a non-canonical (intron and CTE-containing) mRNA through promoting polysome association. Other studies support the notion that shuttling SR proteins have a continued role beyond splicing and export. Sanford et al. (13) 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. Another study showed that SRp20 has the ability to promote expression from an RNA containing an internal ribosome entry site (66). In this case, SRp20 was shown to interact directly with the cellular internal ribosome entry site-binding PCBP2 protein.

We have previously shown that Tap and NXT1 also enhance translation of GagPol-CTE RNA and that Tap, but not NXT1, is associated with polyribosomes in 293T cells (44). Although NXT1 is absent from polyribosomes, it is present in gradient fractions containing the ribosomal subunits and monosomes. This has led us to speculate that a rearrangement of the export complex occurs in conjunction with translation initiation. The fact that 9G8 was previously reported to associate with Tap in mRNP export complexes (11, 12), coupled with our new data showing that it becomes phosphorylated in the subunit and monosome fractions, suggests that 9G8 protein phosphorylation might be a trigger for 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 (67), the exact location of this protein is not known. However, a recent study has reported that SRPK1 can be found associated with ribosomes (68). This would be consistent with our findings that SR proteins become hyperphosphorylated in conjunction with their association with ribosomal subunits. It should also be noted that the 9G8ΔRS protein showed increased association with heavy polyribosomes (Fig. 5, C and D). This might reflect a role for phosphorylation on the RS domain in mediating release from the polyribosomal complexes.

It seems paradoxical that SR proteins would be involved in the promotion of intron-containing RNA expression, because SR proteins are generally thought of as factors that promote splicing. They are known to bind to exonic splicing enhancers near the splice sites and recruit the U2AF splicing factor. However, it is likely that the effects of SR proteins on splicing are more complex than originally proposed, because it has been previously reported that at least one of the SR proteins can also have splicing inhibitory effects (69–72). In this regard, preliminary results in our laboratory indicate that 9G8 promotes intron retention in SIRT7 mRNA, a gene that contains a cellular CTE.  

Our data suggest that promotion of intron retention by 9G8 might be linked to a role for this protein in export and translational regulation that ensures the proper expression of mRNA with retained introns. It is attractive to link splicing regulation, export, and translational regulation of these non-canonical mRNAs, because such RNAs resemble aberrant messages and may need to be very tightly regulated. These mRNAs may exist to increase diversity from a limited pool of genes, and the alternative products they encode might be expressed only at very specific times, such as during development, during periods of stress, or only in specific tissues. 9G8 involvement in alternative splicing, export, and translation might be an example of this kind of regulatory mechanism.

We favor a model in which complexes associated with Tap/ NXT1 and “shuttling” hypophosphorylated SR proteins associate with ribosomal subunits after passage through the nuclear pore. In conjunction with this association, the SR proteins are phosphorylated, 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 (6, 73). This model is consistent with our observation that the 9G8ΔRS protein, which cannot be phosphorylated on the RS domain, seems to remain associated with the polyribosomes.

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 Npl3 protein, a shuttling SR protein (74), associates with yeast polysomes (75). Based on genetic experiments, they proposed that Mtr10p, the import receptor for Npl3, might be involved in a timely regulated release of Npl3 from the polysomes. In contrast, Sky1p, the yeast SR-kinase, did not appear to be involved in this process. Because it has been proposed that Npl3 serves an analogous function to 9G8 (53), it will be of clear interest to further examine the effect of the SRPKs and Transportin-SR2 on 9G8 translational function.

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REFERENCES

1. Fu, X. D. (1995) RNA (N.Y.) 1, 663–680
2. Graveley, B. R. (2000) RNA (N. Y.) 6, 1197–1211
3. Valcarcel, J., and Green, M. R. (1996) Trends Biochem. Sci. 21, 296–301
4. Cao, W., Jamison, S. F., and Garcia-Blanco, M. A. (1997) RNA (N. Y.) 3, 1456–1467
5. Mermod, J. E., Cohen, P. T., and Lamond, A. I. (1994) EMBO J. 13, 5679–5688
6. Yun, C. Y., Velazquez-Dones, A. L., Lyman, S. K., and Fu, X. D. (2003) J. Biol. Chem. 278, 18050–18055
7. Sanford, J. R., Longman, D., and Caceres, J. F. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 33–38
8. Tacke, R., and Manley, J. L. (1999) Curr. Opin. Cell Biol. 11, 358–362
9. Caceres, J. F., Scretan, G. R., and Krainer, A. R. (1998) Genes Dev. 12, 55–66
10. Huang, Y., and Steitz, J. A. (2001) Mol. Cell 7, 899–905
11. Huang, Y., Gattoni, R., Stevenin, J., and Steitz, J. A. (2003) Mol Cell 11, 837–843
12. Huang, Y., Yario, T. A., and Steitz, J. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9666–9670
13. Sanford, J. R., Gray, N. K., Beckmann, K., and Caceres, J. F. (2004) Genes Dev. 18, 755–768
14. Blauwstein, M., Pelisch, F., Tanos, T., Munoz, M. J., Wenger, D., Quadrana, L., Sanford, J. R., Muschietti, J. P., Kornblitt, A. R., Caceres, J. F., Coso,
