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Role of Polyadenylation in Nucleocytoplasmic Transport of mRNA

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To examine the role of polyadenylation in the nuclear export of mRNA, we have replaced the poly(A) signal in a Rev-responsive human immunodeficiency virus type 1-based reporter gene with a *cis*-acting hammerhead ribozyme. Transcripts from this gene thus acquire a 3′ terminus by *cis*-ribozyme cleavage rather than by polyadenylation. The nuclear and cytoplasmic distribution of transcripts was investigated using transient gene expression and quantitative RNase protection assays. In the absence of Rev, a basal level of polyadenylated unspliced mRNA transcribed from a poly(A) signal-containing control reporter gene was detected in the cytoplasm of transfected COS7 cells. However, cytoplasmic ribozyme-cleaved unspliced RNA was only barely detectable. The nuclear/cytoplasmic (n/c) ratio of polyadenylated RNAs was 3.8, while the n/c ratio for ribozyme *cis*-cleaved RNAs was 33. The cytoplasmic localization of the polyadenylated unspliced mRNA was enhanced about 10-fold in the presence of Rev and the Rev-responsive element. In marked contrast to this, ribozyme-cleaved RNA accumulated almost exclusively (n/c ratio of 28) in the nucleus in the presence of Rev. Actinomycin D time course analysis suggested that the low levels of the cytoplasmic ribozyme-cleaved RNAs in both the presence and absence of Rev were due to severe export deficiency of ribozyme-cleaved RNA. Finally, by inserting a 90-nucleotide poly(A) stretch directly upstream of the ribozyme cassette, we have demonstrated that a long stretch of poly(A) near the 3′ end of a ribozyme-cleaved transcript is not sufficient for directing mRNA export. Taken together, these results suggest that polyadenylation is required for the nucleocytoplasmic transport of mRNA and that Rev interaction with the Rev-responsive element cannot bypass this requirement.

Messenger RNA precursors (pre-mRNAs) are synthesized in the nucleus by RNA polymerase II and then subjected to a series of processing reactions which include the addition of a 7-methylguanosine cap at the 5′ end, the removal of introns by splicing, and the generation of mature 3′ ends. With the exception of histone genes, whose transcripts end in a highly conserved stem-loop structure (for reviews see references 2 and 36), the 3′ end is formed by cleavage and polyadenylation (recently reviewed; see reference 47). Mature RNA molecules are then translocated to the cytoplasm through the nuclear pore complex (see reference 12 for review). The mechanism of mRNA nuclear export is poorly understood, although this process is fundamental to eukaryotic gene expression. Recently, however, evidence has begun to accumulate that capping and subsequent processing of pre-mRNA to generate mature mRNA influence the efficiency of nuclear export (reviewed in references 12, 20, and 26).

Evidence that the 5′ monomethylguanosine cap structure may serve as a positive signal for mRNA nuclear export was derived from microinjection experiments carried out with *Xenopus* oocytes (9, 18, 21). It was shown that mRNAs carrying the normal 5′ cap structure were rapidly exported to the cytoplasm after being microinjected into oocyte nuclei, while mRNAs containing a different cap structure were delayed for export. On the other hand, studies of mRNA export in yeast and mammalian cells have implicated splicing components in the nuclear retention of pre-mRNA (5, 27). Mutations that allowed spliceosomes to form but that prevented splicing led to the retention of pre-mRNAs in the nucleus. Conversely, mutations that blocked the assembly of spliceosomes induced the cytoplasmic accumulation of unspliced mRNA. Therefore, removal of the splicing machinery from RNAs may be a prerequisite for export.

In contrast to the negative effects of spliceosomes on mRNA export, a positive link between 3′-end processing and the export of intronless mRNAs has been established (10). When an intronless, prokaryotic neomycin resistance gene was introduced into mammalian cells, cleavage/polyadenylation or histone 3′-end processing stimulated the egress of mRNA from the cell nucleus. However, when 3′ ends were formed by *cis*-acting ribozyme cleavage in vivo, nucleocytoplasmic transport did not occur. It was not clear from these studies, however, whether polyadenylation is of general importance for the export of mRNA, or whether a poly(A) sequence at or near the 3′ end is sufficient for promoting mRNA export.

Virus-encoded proteins involved in the regulation of viral gene expression have proven to be useful probes for cellular functions involved in nucleocytoplasmic mRNA export. Study of the function of the human immunodeficiency virus type 1 (HIV-1) Rev protein in the regulation of gene expression has provided especially useful insights. Rev facilitates the cytoplasmic transport of unspliced and partially spliced viral RNAs by binding to the Rev-responsive element (RRE) present in its target RNA molecules (5, 11, 13, 14, 34; for a review, see reference 7). Two major models have been proposed to explain how Rev controls mRNA export. The first suggests that Rev stimulates RNA export by displacing cellular splicing components from viral pre-mRNA (5, 24, 30). The second model suggests that Rev directly promotes export of pre-mRNA through a pathway distinct from that used for normal cellular mRNA (15, 16, 23, 33, 37, 45, 46). Of particular interest are results of a recent microinjection study performed with *Xenopus* oocytes (16). In that work it was reported that Rev can induce export of RRE-containing RNAs (including intron lari-

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ats) in the absence of polyadenylation. These data raised the possibility that the Rev function may bypass the possible requirement of polyadenylation for RNA export in *Xenopus* oocytes. Whether this is also the case for mRNA transport in mammalian cells has not yet been established.

We have been interested in the role of polyadenylation in mRNA nuclear export. Using a *cis*-acting ribozyme that uncouples 3′-end formation from the mammalian cellular polyadenylation machinery, we offer evidence here that polyadenylation is a prerequisite for the export of some HIV-related transcripts in mammalian cells. This hypothesis is further supported by the observations that Rev/RRE cannot stimulate the nuclear export of ribozyme *cis*-cleaved RNA as efficiently as it stimulates the export of polyadenylated mRNA and that a long stretch of poly(A) near the 3′ end is not sufficient for directing mRNA export, whether in the presence or absence of Rev/RRE. Finally, other results reported here reveal that Rev stabilizes ribozyme-cleaved, RRE-containing RNA in the nuclear compartment.

MATERIALS AND METHODS

Restriction enzymes, T4 DNA ligase, and DNA polymerase I large fragment were from New England Biolabs and were used as suggested by the manufacturer. RNase T_1/T_2 was prepared as previously described (28). [α-^{32}P]UTP was from New England Nuclear. RQ1 DNase I and T3 and T7 RNA polymerases were from Promega. All recombinant plasmids were propagated in *Escherichia coli* JM83. Actinomycin D was from Sigma.

Cell culture and transfection. COS7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out with a modified $CaPO_4$ DNA coprecipitation method (4). Briefly, 4 μg of the indicated reporter plasmid along with 10 μg of the PyE (see below) expression vector was transfected into COS7 cells, with or without the cotransfection of Rev expression plasmid pRSV-Rev (obtained from Y. Luo and B. M. Peterlin). The molar ratio of the reporter plasmid and pRSV-Rev was 1:4. This ratio has previously been shown to provide optimal Rev effects (19). The total amount of DNA used for transfection per 15-cm plate was adjusted to 30 μg with pUC18.

Constructs. Plasmid pDM128 (19, 41, and Fig. 1) was obtained from Y. Luo and B. M. Peterlin. It derives from the HIV-1 Env region, contains a single HIV-1 intron which is flanked by the HIV-1 5′ and 3′ splice sites, and harbors the RRE. Transcription is driven by the simian virus 40 (SV40) early promoter/enhancer, and 3′ ends are formed by the HIV-1 poly(A) signal. For plasmid pDM128RZ, the *Eco*RI site within the intron of pDM128 was destroyed by partial *Eco*RI digestion followed by filling in ends using Klenow enzyme and religation. This rendered the *Eco*RI site proximal to the polyadenylation signal unique. This plasmid was digested with *Afl*II and *Eco*RI (removing the entire HIV-1 polyadenylation signal), and a 282-nucleotide (nt) *Dpn*I fragment comprising a ribozyme sequence from BS-RZ-2 (29) was inserted. Finally, a deletion from *Eco*RV to *Eco*RI (Fig. 1) was introduced to shorten the distance between the ribozyme cleavage site and the 3′ splice site to give pDM128RZ. To create the riboprobe used for all experiments, pDM128RZ was digested with *Bgl*II and the ends were made blunt and then digested with *Apa*I. The resulting *Apa*I to *Bgl*II fragment (1,758 nt) was then inserted into pBlueScript opened with *Apa*I and *Sma*I. The 818-nt riboprobe (Fig. 1) was synthesized with this plasmid as a DNA template for in vitro runoff transcription. Construct pDM128RZA was built by inserting a 90 bp-poly(dA/dT) fragment with *Eco*RI sites at both ends (43) directly upstream of the ribozyme cassette at the *Eco*RI site of pDM128RZ. Transcripts expressed from this vector possess a 90-nt poly(A) sequence near their 3′ ends, followed by a 31-nt non-A extension. Plasmid pRSV-Rev contains the Rev coding region from HIV-1 and is driven by the Rous sarcoma virus long terminal repeat promoter (6). PyE is the entire polyomavirus genome cloned into pUC18 at the *Bam*HI site. It expresses functional polyomavirus early mRNAs.

RNA preparation. RNAs were isolated 48 h after transfection by techniques as described previously (29). To prevent ribozyme cleavage in vitro, extreme care was taken during the preparation of RNA. Mg^{2+} ions, which are essential for ribozyme activity (8), were never present during either the isolation or the analysis procedures. In addition, cell fractionations were carried out on ice and the exposure of cells or cell nuclei to lysis buffer was minimized (less than 2 min on ice). Finally, in the preparation of nuclear, cytoplasmic, and total RNA we used solutions containing 4 M guanidinium thiocyanate, a strong secondary structure denaturant. For the preparation of cytoplasmic RNA, cells were rinsed free of media with ice-cold phosphate-buffered saline and were then disrupted with a modified Nonidet P-40 (NP40) lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.6, 10 mM NaCl, 3 mM $CaCl_2$, 0.5% NP40) on ice for 30 s. Cytoplasmic lysates were collected in new tubes, and appropriate amounts of guanidinium thiocyanate crystals were added to give a final concentration of 4 M. Cytoplasmic RNA was then purified through CsCl step gradients. For nuclear RNA, the above intact cell nuclei which were still attached to the plates were rinsed with ice-cold NP40 buffer twice, followed by lysis in 4 M guanidinium isothiocyanate–20 mM sodium acetate, pH 5.2–0.1 mM dithiothreitol–0.5% *N*-lauryl sarcosine. The RNA was then pelleted through cesium chloride as described above. To isolate total RNA, cells were rinsed free of media and lysed directly with the same guanidinium solution as for nuclear RNA. RNA was then pelleted through CsCl. To perform the actinomycin D time course analysis, cells were exposed to a 5-μg/ml final concentration of actinomycin D for 0, 60, or 120 min before RNA harvest. Forty-eight hours after transfection, RNAs were isolated by procedures described above.

RNase protection assays. Internally labeled RNA probes were made by in vitro transcription by T3 or T7 RNA polymerase in the presence of [α-^{32}P]UTP. DNA templates were removed by RQ1 DNase digestion followed by phenol-chloroform extraction. Internally labeled riboprobes were hybridized in a buffer lacking magnesium ions at 57°C overnight, as described previously (12). The hybridization products were digested with a T_1/T_2 mixture in a buffer lacking Mg^{2+} (28) at 37°C for 2 h, and the resulting samples were resolved on 6% denaturing polyacrylamide gels. Routinely, 10 μg of nuclear, cytoplasmic or total RNA samples were used for RNase protection, except that in the actinomycin D time course analysis of cytoplasmic RNAs, 30 μg of the RNA samples were used for protection. For cytoplasmic pDM128RZ RNAs, films were exposed three times longer.

Quantitation of RNase protection data. Protected bands were quantitated by using a Packard Instant Imager. Background was subtracted by using regions of identical sizes located immediately below each of the experimental bands. The nuclear/cytoplasmic (n/c) ratio of the internal control PyE spliced RNAs served as a useful way to accurately normalize experimental band results. In different experiments, the n/c ratio of spliced PyE mRNA varied between 0.50 and 0.55. We arbitrarily chose 0.50 as a standard ratio for normalization purposes. Thus, the experimental radioactive counts of spliced PyE mRNAs in Fig. 3B, lane 2, were reduced by 10%, to achieve a ratio of 0.50. This same 10% reduction was then applied to the count values for the corresponding experimental bands, Fig. 3A, lane 2. Similar corrections were performed for other RNase protection data.

RESULTS

Experimental strategy. To investigate the effect of polyadenylation on mRNA nucleocytoplasmic transport, we compared the export of a polyadenylated control reporter gene transcript with that of gene transcripts whose 3′ ends were generated by *cis*-acting ribozyme cleavage, in both the presence and absence of HIV-1 Rev. Constructs used are shown in Fig. 1. Plasmid pDM128 served as a positive control for nuclear and cytoplasmic localization and has been described previously (19). It derives from the Env region of HIV-1, contains a single HIV-1 intron which is flanked by HIV-1 5′ and 3′ splice sites and harbors the RRE. Transcription is driven by the SV40 early promoter/enhancer, and 3′ ends of the transcripts are processed by the HIV-1 polyadenylation signal. In pDM128RZ the HIV-1 poly(A) signal of pDM128 has been replaced by a *cis*-acting hammerhead ribozyme and a deletion has been made to shorten the distance between the ribozyme cleavage site and the 3′ splice site. This allowed us to detect both splicing and ribozyme cleavage with a single riboprobe. A consensus histone 3′ stem-loop sequence lacking the CAGA processing signal was inserted upstream of the ribozyme to stabilize the ribozyme-cleaved RNA should it be transported to the cytoplasm (10). Thus, construct pDM128RZ was expected to generate RNA transcripts which end in a histone 3′ stem-loop structure but which are not produced by the histone 3′-end processing mechanism. Finally, in an attempt to determine whether the poly(A) sequence at the 3′ end of mRNAs is sufficient for export, a stretch of 90 consecutive A residues was inserted directly upstream of the histone stem-loop structure and the hammerhead ribozyme to give pDM128RZA. Transcripts generated by this gene have poly(A) stretches near their 3′ ends, but this poly(A) is not produced by the polyadenylation machinery.

To assess nuclear export of mRNAs, the above indicated plasmids were each transfected into COS7 cells along with a plasmid expressing the PyE, with or without cotransfection of pRSV-Rev (6), which expresses the wild-type HIV-1 Rev pro-

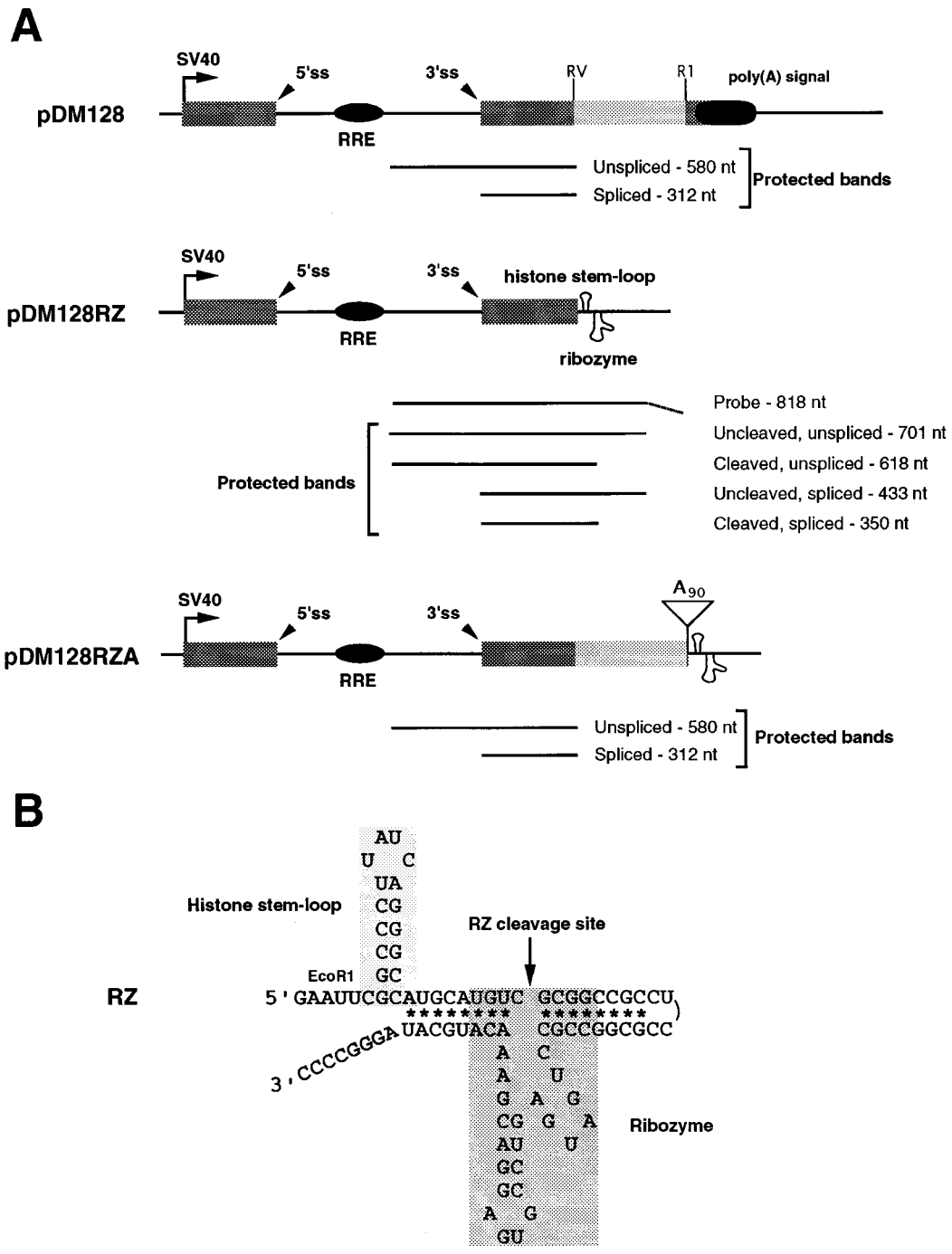

FIG. 1. Constructs used in this study. (A) Transcription from the three constructs is driven by the SV40 early promoter/enhancer, which is labeled by an arrowhead. Thin lines denote intron sequences, with the RRE indicated. Darkly stippled boxes represent exons, and the lightly stippled box depicts sequences missing in construct pDM128RZ. The HIV-1 poly(A) signal is marked by the solid ellipse. The 5′ and 3′ splice sites (5′ss and 3′ss) and two important restriction sites are also shown. RV, *Eco*RV; RI, *Eco*RI. In plasmid pDM128RZ, the *cis*-acting hammerhead ribozyme and the upstream histone 3′ stem-loop structure are indicated. In construct pDM128RZA, the 90-nt poly(A) insertion is marked by a triangle. The 818-nt riboprobe generated by HIV-RZ (see Materials and Methods) and the predicted protection fragments are shown. Constructs are not drawn to scale. (B) Schematic representation of the ribozyme cassette. The histone stem-loop and the hammerhead ribozyme portions are highlighted. The ribozyme cleavage site is 9-nt downstream of the histone stem-loop.

tein. Expression of polyomavirus early mRNAs served as an internal control for transfection efficiency, nuclear and cytoplasmic fractionation of RNA (unspliced polyomavirus early transcripts are exclusively confined to the nucleus; data not shown, and see below), and RNA loading and as a standard for RNA stability assays. Use of this internal control thus made it possible to draw both qualitative and quantitative conclusions with respect to expression of transcripts in transfected cells. Forty-eight hours after transfection, nuclear and cytoplasmic RNAs were isolated and the intracellular distribution of RNAs was analyzed by quantitative RNase protection assays.

A hammerhead ribozyme is active in vivo. In previous stud-

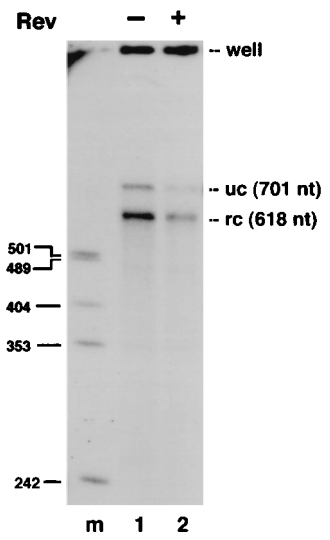

FIG. 2. A hammerhead ribozyme is active in vivo. Plasmid pDM128RZ was transfected into COS7 cells with (lane 2) or without (lane 1) cotransfection of pRSV-Rev. Total RNAs were prepared and were analyzed by RNase protection. uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved unspliced RNA; m: molecular size marker in nucleotides. The 818-nt riboprobe shown in Fig. 1 was used for RNase protection assays. The lower levels of RNA in lane 2 appear only because less RNA was loaded onto gel.

ies of polyomavirus gene expression, we exploited a *cis*-acting hammerhead ribozyme to uncouple 3′-end formation from the cellular polyadenylation machinery. It was found that viral RNAs whose 3′ ends were generated by ribozyme cleavage failed to accumulate in the cytoplasm (29 and unpublished data). Here we used the same ribozyme construct to investigate effects of polyadenylation on export of HIV-based gene transcripts.

Unambiguous interpretation of results obtained with constructs encoding a self-cleaving ribozyme are only possible if cleavage occurs in vivo and not during in vitro manipulation. Therefore, before approaching the issue of ribozyme-cleaved RNA nuclear export, we performed a series of control experiments. Figure 2 shows some of these results. To measure ribozyme cleavage in vivo and to examine possible effects of Rev on ribozyme activity, pDM128RZ was transiently transfected into COS7 cells with or without cotransfection of pRSV-Rev. Total RNAs were isolated and assayed by RNase protection using the 818-nt riboprobe capable of distinguishing between ribozyme-uncleaved unspliced, ribozyme-cleaved unspliced, ribozyme-uncleaved spliced, and ribozyme-cleaved spliced RNA species (Fig. 1 and 2). All experiments were carried out under conditions that prevent ribozyme cleavage in vitro (see Materials and Methods). For example, to extract total RNA, cells were lysed with 4 M guanidinium lysis buffer (low pH) followed by ultracentrifugation of the guanidinium lysate on CsCl step gradients to separate RNA from other cellular macromolecules. Particular care was taken to ensure that Mg^{2+}, which is crucial for ribozyme activity, was never present during RNA isolation and protection assays. As demonstrated in Fig. 2, the riboprobe protected two fragments. The 701-nt fragment represents ribozyme-uncleaved unspliced transcripts. The 618-nt fragment indicates ribozyme-cleaved unspliced RNA. Other fragments with the predicted mobilities for spliced products were not detected, indicating that splicing occurred extremely poorly, if at all, in the absence of polyadenylation (see Discussion). Quantitation of the two protected fragments (701 and 618 nt) by using a Packard Instant Imager and correcting for uridine content of the two species revealed that the efficiency of ribozyme cleavage in vivo is about 75%. Similar cleavage efficiencies in the presence and absence of Rev were observed (compare Fig. 2, lanes 1 and 2), indicating that Rev does not affect ribozyme activity in vivo.

RNAs whose 3′ ends are formed by ribozyme cleavage and not by polyadenylation are defective for nuclear export. To determine whether polyadenylation is required for nucleocytoplasmic transport of mRNA, pDM128 and pDM128RZ (which lacks a polyadenylation signal) were each transfected into COS7 cells along with plasmid PyE. Nuclear and cytoplasmic RNAs were isolated 48 h after transfection, and the intracellular localization of the transcripts was assessed by quantitative RNase protection assays. PyE RNAs served both as transfection controls to normalize RNA levels and as controls for efficient nuclear and cytoplasmic RNA fractionation. Unspliced PyE RNA is exclusively retained in the nucleus; its appearance in the cytoplasmic fraction is an indicator of leakage during subcellular fractionation. As before, all experiments were performed under conditions avoiding ribozyme cleavage in vitro (10 and Materials and Methods).

As shown in Fig. 3A, a basal level of unspliced mRNA generated from pDM128 accumulated in the cytoplasm of cells. Unspliced RNA (us, lanes 1 and 2) was distributed in a ratio of about 3.8:1 between the nuclear and cytoplasmic compartments (n/c ratio) while the spliced mRNA has an n/c ratio of 0.26:1, indicating more efficient export of spliced RNA. These observations are consistent with published data (5, 11, 13, 14, 34). In contrast, however, ribozyme-cleaved unspliced RNA (rc, lanes 3 and 4) was almost exclusively restricted to the nucleus with an n/c ratio of 33:1. Only after prolonged autoradiographic exposure was a small amount of rc RNA detected in the cytoplasm (data not shown). The 818-nt riboprobe again detected only two predominant RNA species (lanes 3 and 4): ribozyme-uncleaved unspliced (uc) and ribozyme-cleaved unspliced (rc) RNAs, similar to the results shown in Fig. 2 (see Discussion). Unspliced early-strand RNA transcribed from the control plasmid PyE was never found in the cytoplasm (n/c

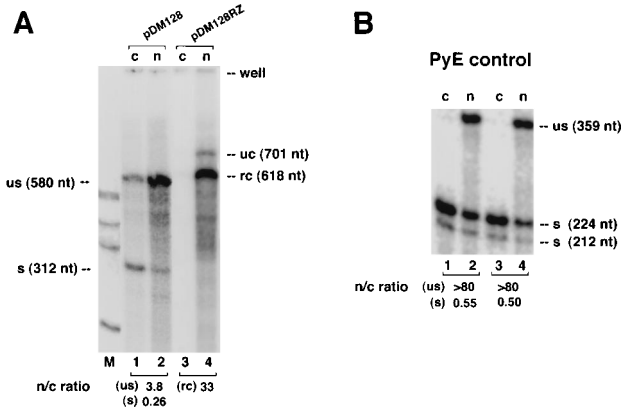

FIG. 3. Export deficiency of ribozyme-processed RNA. Plasmids pDM128 and pDM128RZ together with plasmid PyE were each transfected into COS7 cells. The subcellular distribution of RNAs was examined by quantitative RNase protection. (A) Expression of pDM128 and pDM128RZ by using the 818-nt riboprobe. uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved spliced RNA; c, cytoplasm; n, nucleus; m, molecular size marker (same as in Fig. 2). n/c, RNA distribution ratio between the nuclear and cytoplasmic compartments. The n/c ratios have been corrected by the levels of spliced PyE RNAs shown in panel B. (B) Expression of internal control plasmid PyE by using a polyomavirus early region-specific riboprobe.

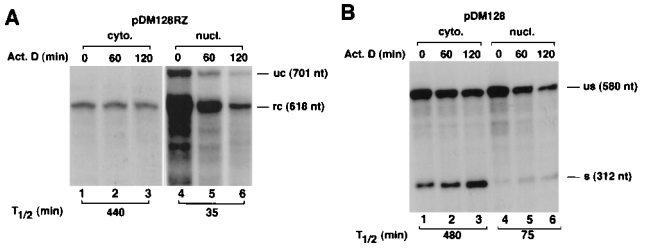

FIG. 4. Actinomycin D (Act. D) time course analysis of nuclear and cytoplasmic pDM128 and pDM128RZ RNAs. Plasmids pDM128RZ (panel A) and pDM128 (panel B) were each transfected into COS7 cells along with PyE. Nuclear and cytoplasmic RNAs were extracted 0, 60, and 120 min after treatment with a 5-μg/ml final concentration of actinomycin D and were analyzed by quantitative RNase protection. For cytoplasmic RNAs, three times more RNA samples were used in RNase protection. For pDM128RZ RNA, radioactive exposure was three times longer. The half-life ($T_{1/2}$) times given have been normalized as described in the text to the levels of the internal control PyE spliced mRNAs (data not shown). uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved unspliced RNA; us, unspliced mRNA; s, spliced mRNA; cyto., cytoplasm; nucl., nucleus.

ratio, >80), and there was minimal variation in the pattern of distribution of spliced PyE transcripts between the two cellular compartments (Fig. 3B). This indicates that clean subcellular fractionation was achieved and there was no detectable leakage of nuclear RNA to the cytoplasm.

The dramatic differences in distribution of ribozyme-cleaved unspliced RNA and unspliced RNA from pDM128 between the two cellular compartments suggests that mRNAs that are not polyadenylated are defective in nuclear export. To rule out the possibility that the low level of ribozyme-cleaved unspliced RNAs seen in the cytoplasm might result from rapid degradation, we measured half-life times of the two RNA species in both the nuclear and cytoplasmic compartments. Quantitative RNase protection assays following actinomycin D treatment of the transfected cells were performed. Nuclear and cytoplasmic RNAs were isolated from cells transfected with the indicated reporter plasmids together with plasmid PyE. As shown in Fig. 4, rc RNA is relatively stable, with a half-life time of 440 min, similar to that of us RNA from pDM128 (compare Fig. 4A, lanes 1 to 3, with Fig. 4B, lanes 1 to 3). However, the half-life time of rc RNA in the nucleus is only 35 min (Fig. 4A, lanes 4 to 6). These results showing different half-lives for rc species in the nuclear and cytoplasmic compartments support the idea that the small amount of rc RNA detected in the cytoplasm is due to poor export rather than to leakage. They also suggest little or no nuclear leakage. It is not clear, however, whether the cytoplasmic rc RNAs derived from true export of nuclear rc RNAs or from export of cryptically polyadenylated RNA followed by *cis*-ribozyme cleavage in the cytoplasm.

Interestingly, in Fig. 4B, lanes 1 to 3, right panel, the level of cytoplasmic s RNA increases during the assay period, in contrast to that of the us RNA. This suggests that the export rate of spliced RNA from the nucleus to the cytoplasm exceeds that of degradation in the cytoplasm. This also indicates that actinomycin D does not block nuclear export. A similar pattern is seen for the nuclear spliced RNA, possibly due to cytoplasmic contamination. On the basis of the above data, we conclude that polyadenylation is required for mRNA nuclear export.

Rev cannot enhance export of ribozyme-cleaved RNA as efficiently as it enhances export of polyadenylated mRNAs. It has been well established that Rev enhances nucleocytoplasmic transport of RRE-containing pre-mRNAs (5, 11, 13–16, 23, 33, 34, 37, 46). However, the mechanism of this action is not yet clear. Recently, evidence favoring the mode of direct action of Rev has begun to accumulate. First, in experiments with T lymphocytes (33) and *Xenopus* oocytes (16), it has been shown that an increased cytoplasmic localization of RRE-bearing mRNAs in the presence of Rev was not accompanied by a decreased level of fully spliced mRNAs in the cytoplasm. Second, Rev has been found to be a shuttling protein which may be associated with its target mRNAs during export (23, 37). Third, microinjection experiments (16) have shown that Rev promotes export of RRE-containing RNAs, including that of lariat introns, from the microinjected oocyte nuclei in the absence of polyadenylation. Finally, a number of cellular factors which interact with Rev have recently been identified (3, 15, 17, 45, 46), with some being components of or associated with nuclear pore complexes (3, 15, 17, 46).

To determine whether polyadenylation is required for Rev to stimulate export of RRE-containing mRNA in a mammalian system, we performed the following experiments. pDM128 and pDM128RZ were each transfected into COS7 cells, together with the PyE plasmid and the Rev expression vector. Nuclear and cytoplasmic fractionation and RNase protection assays were carried out as described above. Results are presented in Fig. 5. As expected, Rev significantly increased the cytoplasmic localization of polyadenylated unspliced mRNA (us) (compare Fig. 5A, lanes 1 and 2, to Fig. 3A, lanes 1 and 2). The n/c ratio of us RNA switched from 3.8:1 in the absence of Rev to 0.4:1 in the presence of Rev. However, the n/c ratios of spliced pDM128 RNAs are similar in the both situations. This finding is in agreement with previously published results (5, 11, 13, 14, 34). Surprisingly, the nuclear and cytoplasmic distribution pattern of the ribozyme-cleaved unspliced RNA (rc) remains unchanged (compare Fig. 5A, lanes 3 and 4, to Fig. 3A, lanes 3 and 4). The simplest interpretation of these data is that Rev does not act independently of polyadenylation in this system. Should Rev stimulate export of mRNA independently of polyadenylation, a distribution pattern of rc RNA similar to that of us RNA from pDM128 in the presence of Rev should have been observed. However, this was not the case.

We next performed actinomycin D chase experiments to analyze the stability of rc RNAs in both cellular compartments. As shown in Fig. 6, lanes 1 to 3, cytoplasmic rc RNA has a half-life similar to that of polyadenylated unspliced pDM128 mRNA (Fig. 4A, lanes 1 to 3). This indicates that the low level

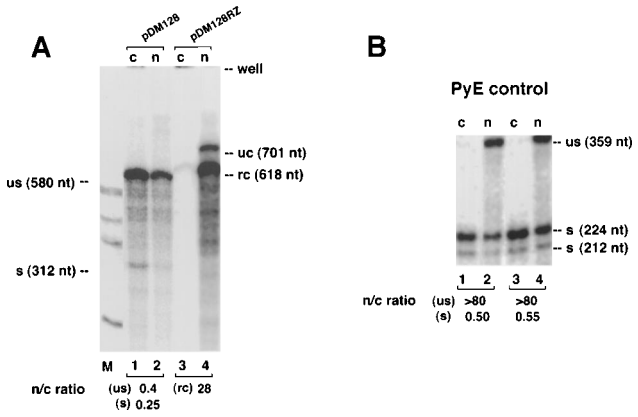

FIG. 5. Rev/RRE cannot bypass the requirement of polyadenylation for nuclear export of mRNAs. Plasmids pDM128 and pDM128RZ, together with plasmid PyE, were each transfected into COS7 cells, along with cotransfection of plasmid pRSV-Rev. Nuclear and cytoplasmic RNAs were isolated and quantitative RNase protection assays were performed. Labels are the same as in Fig. 3. (A) RNase protection assays of pDM128 and pDM128RZ; (B) RNase protection assays of the internal PyE control transcripts.

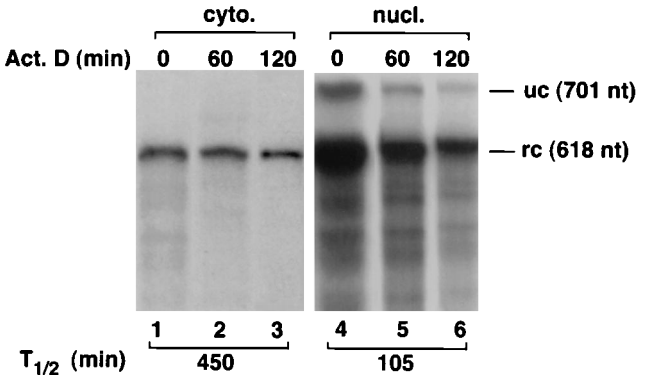

FIG. 6. Actinomycin D time course analysis of cytoplasmic and nuclear pDM128RZ RNAs. Plasmids pDM128RZ and PyE were together transfected into COS7 cells, with cotransfection of pRSV-Rev. Nuclear and cytoplasmic RNAs were prepared 0, 60, and 120 min after treatment with a 5-μg/ml final concentration of actinomycin D and were assayed by quantitative RNase protection. For cytoplasmic RNA, three times more RNA samples were used in RNase protection and exposure was three times longer. The half-life times have been normalized to the levels of the internal control PyE spliced mRNAs (data not shown). Labels are the same as in Fig. 4.

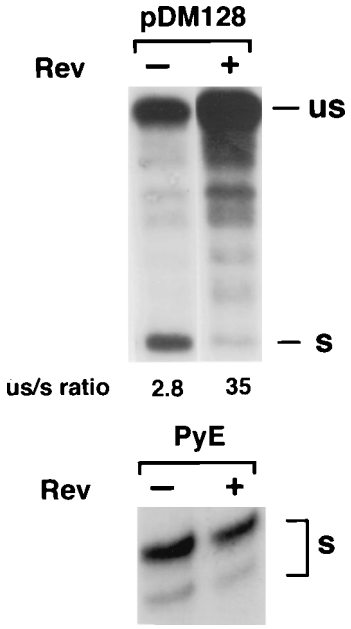

FIG. 7. Rev interferes with splicing. Plasmids pDM128 and PyE were transfected into COS7 cells, with or without cotransfection of pRSV-Rev. Total RNAs were extracted and assayed by quantitative RNase protection. Top panel, expression of pDM128. Bottom panel, expression of PyE. us, unspliced RNA; s, spliced RNA.

of rc RNA in the cytoplasm is not due to rapid degradation; rather, it is due to poor export. Unexpectedly, the half-life of nuclear rc RNA increased about threefold in the presence of Rev over that in the absence of Rev (compare Fig. 6, lanes 4 to 6, to Fig. 4A, lanes 4 to 6). This suggests that Rev stabilizes rc RNA in the nucleus. From the above results we suggest that polyadenylation is required for Rev to stimulate nuclear export of RRE-containing mRNAs.

A close inspection of the data shown in Fig. 3 and 5 revealed that an increased level of unspliced cytoplasmic RNA produced by pDM128 was coincident with a lower level of cytoplasmic spliced RNA, and that these changes were associated with the presence of Rev. This suggested the possibility that Rev may interfere with splicing. In fact, if the sum of cytoplasmic and nuclear signals was considered, this result was also evident (data not shown). These results were observed in more than three independent experiments. To determine directly whether Rev interferes with splicing, total cellular RNA derived from cells transfected with pDM128 and PyE, with or without cotransfection of the Rev expression vector, was subjected to RNase protection analysis (Fig. 7). The ratios between unspliced and spliced pDM128 mRNAs are 2.8 and 35 in the absence and presence of Rev, respectively. In addition, the steady state level of spliced pDM128 mRNA (after being normalized for the level of PyE spliced RNA) is about fourfold higher in the absence of Rev than that in the presence of Rev. These results indicate that fewer RNAs were spliced in the presence of Rev (see Discussion).

A long poly(A) stretch near the 3′ end of an RNA molecule is not sufficient for export. To determine whether a poly(A) sequence near the 3′ end of mRNAs is sufficient for nucleocytoplasmic transport, we replaced the poly(A) signal in pDM128 with the hammerhead ribozyme cassette and then inserted a 90-bp poly d(A/T) stretch immediately upstream of the histone stem-loop to build pDM128RZA (Fig. 1). To analyze the subcellular localization of RNAs, pDM128RZA and PyE were transfected into COS7 cells with or without cotransfection of pRSV-Rev. Results of RNase protection assays are presented in Fig. 8. The 818-nt riboprobe could only distinguish between spliced and unspliced RNAs and could not distinguish between ribozyme-cleaved and uncleaved RNA species (Fig. 1). The transport phenotype displayed by unspliced transcripts (including ribozyme-cleaved and uncleaved RNAs), in both the presence and absence of Rev, is strikingly similar to that of pDM128RZ (compared with Fig. 3 and 5). Actinomycin D chase experiments (data not shown) revealed that the poly(A) stretch stabilized pDM128RZA transcripts to

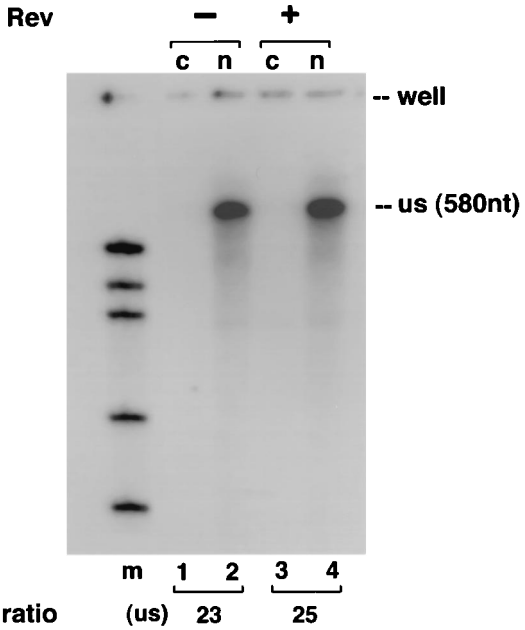

FIG. 8. A long poly(A) stretch is not sufficient for mRNA export. Plasmids pDM128RZA and PyE were transfected into COS7 cells, with or without cotransfection of pRSV-Rev. Nuclear and cytoplasmic RNAs were analyzed by RNase protection. us, unspliced pDM128RZA RNA; other labels are the same as in Fig. 3.

about the same extent as that seen by Rev on pDM128RZ transcripts (Fig. 6). In addition, Rev addition did not lead to further stabilization (data not shown). Taken together, these results suggest that a poly(A) stretch alone may not be sufficient for mRNA nuclear export. Rather, productive interaction of the pre-mRNA with the cellular polyadenylation machinery appears to be required. However, these results do not rule out the possibility that a poly(A) stretch at the very 3′ terminus of a transcript can stimulate export.

DISCUSSION

In this study we have employed a *cis*-acting hammerhead ribozyme to uncouple 3′-end formation of reporter gene transcripts from the cellular polyadenylation apparatus and have demonstrated that polyadenylation is required for nucleocytoplasmic transport of mRNA. The constructs used were derived from the HIV-1 genome, with the cytoplasmic localization of unspliced gene transcripts being regulated by Rev. In the absence of Rev, polyadenylated unspliced transcripts were readily detected in the cytoplasm of the transfected COS7 cells (Fig. 3, lanes 1 and 2). However, ribozyme-cleaved unspliced RNAs failed to appear in the cytoplasm. Instead, they accumulated to a high level in the nucleus (Fig. 3, lanes 3 and 4). Moreover, the intracellular distribution pattern of ribozyme-cleaved RNAs remained unchanged when Rev is present, whereas the level of cytoplasmic polyadenylated mRNAs was significantly increased (Fig. 5, compare lanes 1 and 2 with lanes 3 and 4). RNA stability measurements using actinomycin D suggested that the distinct distribution patterns of the two classes of transcripts within the cell were due to their differential nucleocytoplasmic transport. We therefore hypothesize that polyadenylation is required for mRNA nuclear export and this requirement cannot be bypassed by Rev/RRE. These findings are consistent with the observation that polyoma early region antisense RNAs produced by ribozyme cleavage are retained in the nucleus (29). However, these conclusions are inconsistent with results obtained from experiments which showed that in vitro synthesized, nonpolyadenylated mRNAs could be exported to the cytoplasm after microinjection into *Xenopus* oocyte nuclei (16, 18). Moreover, in one case an intron lariat containing the RRE sequence was found to be exported to the oocyte cytoplasm only in the presence of Rev (16). The discrepancies may be due to the different systems and techniques used. Alternatively, they may result from the different sizes of RNAs used (RNAs used for microinjection were less than 500 nt, while RNAs expressed from our vectors were around 4,000 nt). It is also possible that microinjected RNAs may enter an export pathway differently from endogenously expressed RNAs. Finally, it cannot be ruled out that microinjected RNAs underwent polyadenylation in vivo before being exported to the cytoplasm.

Polyadenylation is initiated by specific recognition of the highly conserved AAUAAA hexanucleotide sequence present on pre-mRNA substrates by polyadenylation factors, followed by endonucleolytic cleavage of the pre-mRNA 5 to 40 nt downstream of the hexanucleotide and the addition of 150 to 200 adenosine residues at the 3′ end (47). Some polyadenylated transcripts contain less than 100 adenosine residues (40). The poly(A) sequence itself has been implicated in regulating mRNA stability and translation in the cytoplasm (1, 22). The next question we asked, therefore, was whether a poly(A) stretch alone is sufficient for mRNA export. Results suggest that this is not the case and lead to the proposal that the process of polyadenylation is essential for mRNA export. The key to this hypothesis is that RNAs whose 3′ ends were formed by ribozyme cleavage and that possessed 90 adenosine residues near their 3′ ends failed to enter the cytoplasm, whether in the presence or in the absence of Rev (Fig. 8).

Poly(A) encoded in the above reporter gene differs from the normal poly(A) in that it has a 31-nt 3′ extension. One might argue that these non-A residues interfere with the cellular export machinery, or that the assembly of an export-competent complex requires a poly(A) stretch at the very 3′ end. Further experimentation is required to determine whether either of these models is true. However, it remains possible that binding of cellular proteins to the 90-nt-long A stretch is not itself sufficient for mRNA export, or that the binding of an important factor or factors which are essential for promoting mRNA export can occur only during the polyadenylation process. Further evidence supporting this comes from observations that a histone stem-loop structure alone was not sufficient for neomycin mRNA nuclear export; rather, the entire histone 3′ processing machinery was required for this event (10). Considering this with our results, we propose that the ability to link RNA 3′ processing and nucleocytoplasmic export of mRNAs may be a general, intrinsic property of 3′ processing signals. Finally, since it was shown that a 55- to 65-nt-long poly(A) stretch could promote mRNA export (25), we suggest that the 90-nt poly(A) stretch used here should also be sufficiently long for export.

cis elements present in our reporter transcripts are not responsible for the nuclear retention of these RNAs. First, the 2′,3′ cyclic phosphate terminus generated by ribozyme cleavage does not retain RNAs within the nucleus. This is supported by the observation that transcripts containing the histone coding region and a histone 3′ stem-loop generated by ribozyme cleavage were efficiently exported to the cytoplasm (10). Second, as normally processed histone mRNA can be efficiently transported to the cytoplasm, the same histone stem-loop present at the 3′ ends of our transcripts likewise cannot prevent the export of these RNAs. Finally, since unused 5′ and 3′ splice sites present in polyadenylated pDM128 transcripts do not retain RNAs in the nucleus in the presence of Rev, it is highly unlikely that the same splice sites retain ribozyme cleaved RNAs in the nucleus when Rev is present.

Another interesting finding of this study was that polyadenylation appeared to stabilize unspliced mRNA in the nucleus, although other differences in RNA structure might also have influenced nuclear lifetimes. Nuclear polyadenylated mRNAs were more stable than ribozyme-cleaved RNAs in the absence of Rev (Fig. 4). Rev was also found to enhance the stability of ribozyme-cleaved unspliced RNAs in the nucleus (compare Fig. 4 with Fig. 6). Whether these stabilization effects are manifestations of the same mechanism is unknown. It has been shown that the function of Rev is dosage dependent and requires Rev oligomerization (24, 35, 39, 48; unpublished results). We suggest, therefore, that binding of polyadenylation factors and/or poly(A)-binding proteins to the poly(A) sequence, binding of Rev to the RRE, and more importantly, productive protein-protein interactions between these proteins and other cellular factors, efficiently protect the substrate mRNAs from being degraded in the nucleus and thus ensure their availability for nuclear export.

Failure to see splicing in ribozyme-cleaved transcripts (Fig. 2, 3, 5, and 8) was quite surprising and interesting and may be the consequence of interference with "exon definition" during splice site selection and commitment (44). Terminal exon splicing may require recognition of both the poly(A) signal and the upstream 3′ splice site (31, 38, 44). In this model, a ribozyme would likely not be an acceptable "exon terminator"; therefore, terminal exon splicing could not occur.

Our studies also provide valuable insight into Rev function. In the presence of Rev, the steady-state level of the spliced mRNAs decreased compared with that in the absence of Rev, and this change was coincident with the increased expression of unspliced mRNAs (Fig. 3, 5, and 7). This indicates that Rev interferes with splicing. It is possible that this interference with splicing results from direct interaction of Rev with the cellular splicing machinery to free the unspliced mRNAs for nuclear export. Alternatively, it is possible that Rev binds to polyadenylated unspliced mRNAs and directly aids their rapid nuclear export. Export may be so rapid as to preclude access to the splicing machinery. Recent studies favor the second model, although the two are not necessarily mutually exclusive. In T lymphocytes, cytoplasmic levels of fully spliced mRNAs were independent of the presence of Rev (33). Moreover, Rev may shuttle between the nucleus and cytoplasm and direct RRE-containing mRNAs to the cytoplasm through its C-terminal activation domain (15, 23, 32, 37, 42). Finally, several nuclear proteins (including nuclear pore proteins) that interact with Rev have recently been identified (3, 17, 46), indicating that Rev may interact directly with the cellular export machinery. However, the reporter transcripts used in most of the above experiments were all polyadenylated (except in the case of *Xenopus* oocyte microinjection experiments [15]). Therefore, direct action of Rev in mRNA export does not exclude that polyadenylation is required for its function. Data from the microinjection experiments of Fischer et al. (15, 16) do not agree with our findings; possible explanations for these discrepancies have been discussed above.

Taking together the data we have presented here, one can envisage a model for the central importance of polyadenylation in general mRNA nuclear export. Polyadenylation may allow mRNAs to enter a pathway that leads to their efficient export. It is possible that only those that have undergone polyadenylation can be properly recognized by the nuclear pore complex and become accessible to the cellular export machinery. With regard to Rev's facilitation of mRNA export, binding of Rev to substrate mRNAs and its productive recruitment of cellular factors involved in export may occur only when mRNAs have been delivered to the appropriate intranuclear location by the action of the cellular polyadenylation machinery.

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