The EMBO Journal vol.13 no.17 pp.4105–4112, 1994

Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA

Utz Fischer[1,3], Sylvie Meyer[2,3], Michael Teufel[2], Corinne Heckel[2], Reinhard Lührmann[1,4] and Guy Rautmann[2]

[1]Institut für Molekularbiologie und Tumorforschung, Emil-Mannkopff-Straße 2, D-35037 Marburg, Germany and [2]Marion Merrell Dow Research Institute, Strasbourg Research Center, 16 rue d'Ankara, 67080 Strasbourg cedex, France

[3]Both authors contributed equally to this work

[4]Corresponding author

Communicated by R.Lührmann

The Rev *trans*-activator of human immunodeficiency virus type 1 (HIV-1) is a protein that regulates the simultaneous appearance in the cytoplasm of both spliced and unspliced forms of viral mRNAs from the same viral transcripts by way of recognition of a target sequence termed the Rev-responsive element (RRE). Whether Rev acts directly on RNA export or by inhibition of splicing, or both, is still a matter of debate. We have addressed this issue in *Xenopus laevis* oocytes by microinjecting RNA molecules containing RRE along with purified recombinant Rev protein into the oocyte nuclei. Adenovirus pre-mRNA containing an RRE in the intron was spliced equally well in the absence and presence of Rev protein. Only in the presence of Rev was non-spliced pre-mRNA exported from the nucleus; more surprisingly, the excised intron lariat (containing RRE) was also exported. Furthermore, an RRE-containing mRNA molecule that lacked intron sequences was also efficiently exported from the nucleus in a Rev-dependent manner. Therefore our results demonstrate that Rev can act directly at the level of nuclear export, independent of any inhibitory effect that it may exert on the splicing of pre-mRNA. Finally, our finding that the Rev mutant M10, shown previously to be inactive in human lymphoid cells, was also unable to export RRE-containing RNA molecules from oocyte nuclei suggests that one or more cellular factors, evolutionarily conserved between humans and *Xenopus*, interact with Rev in both cell systems to promote nuclear RNA export.

Key words: HIV-1/mRNA splicing/nuclear RNA export/Rev protein/*Xenopus laevis* oocytes

Introduction

The pathogenic human immunodeficiency virus type 1 (HIV-1) is the aetiological agent of acquired immunodeficiency syndrome (AIDS). The genome of this virus encodes the Gag, Pol and Env proteins common to all retroviruses and, in addition, at least five other proteins [for a review see Cullen (1992)]. Among these, the two regulatory proteins Tat and Rev have been the subject of intensive studies, as they appear to be prototypes of novel genetic regulatory elements that involve specific RNA–protein interactions. A hallmark of HIV gene expression is the coordinated cytoplasmic production of both spliced and unspliced forms of viral messengers generated from the same primary transcript. This mechanism is controlled by the Rev protein acting in concert with a *cis*-acting regulatory element, the Rev-responsive element (RRE), possessed by all virally encoded primary transcripts (Feinberg *et al.*, 1986; Emerman *et al.*, 1989; Felber *et al.*, 1989; Malim *et al.*, 1989b; Chang and Sharp, 1990). The RRE has been mapped to a 243 nucleotide region that possesses a complex tertiary structure (Malim *et al.*, 1989b; Kjems *et al.*, 1991a). Physical binding of Rev by the RRE has been demonstrated *in vitro* (Zapp and Green, 1989; Kjems *et al.*, 1991a; Malim and Cullen, 1991).

Rev is a basic protein containing two essential functional domains. The N-terminal domain has been shown to be necessary and sufficient for RRE binding, multimerization of the protein and nuclear translocation (Malim *et al.*, 1989b; Malim and Cullen, 1991; Berger *et al.*, 1991). The C-terminal domain has been termed the activation or effector domain, and genetic studies *in vivo* suggest strongly that this domain interacts with one or more additional cellular factors required for Rev function. Mutations in this leucine-rich domain result in a *trans*-dominant negative phenotype (Malim *et al.*, 1989a, 1991; Mermer *et al.*, 1990; Venkatesh and Chinnadurai, 1990; Daly *et al.*, 1993). Despite much effort, the precise mechanism by which Rev brings about the export of unspliced and partially spliced viral pre-mRNA from the nucleus into the cytoplasm is still not fully understood.

Several hypotheses have been proposed to explain this regulation of gene expression. One of them is that Rev exerts a direct role in promoting the export of pre-mRNA from the nucleus into the cytoplasm (Emerman *et al.*, 1989; Felber *et al.*, 1989; Malim *et al.*, 1989a,b; Malim and Cullen, 1993). Alternatively, Rev might interfere with spliceosome formation on viral pre-mRNA by interacting with cellular spliceosomal factors, thus allowing nuclear export of incompletely spliced mRNA (Chang and Sharp, 1989, 1990; Kjems *et al.*, 1991b; Kjems and Sharp, 1993). On the other hand, Rev could mediate a subtle tuning at the level of both splicing and nuclear export. In addition, it has been reported that Rev might affect the utilization of viral mRNA at the level of translation (Arrigo and Chen, 1991; D'Agostino *et al.*, 1992).

In this study we have investigated the influence of Rev on the splicing and nuclear export of pre-mRNA in *Xenopus laevis* oocytes by nuclear microinjection of RNA molecules containing RRE along with purified recombinant Rev protein. We adduce evidence that Rev activates

directly the export of pre-mRNA molecules harbouring the RRE and does not interfere with pre-mRNA splicing. Moreover, the *trans*-dominant negative mutant of Rev identified previously, termed M10, is unable to induce nuclear export of RNA molecules harbouring the RRE in *Xenopus* oocytes. This indicates that the Rev protein requires additional cellular factor(s) evolutionarily conserved between amphibian and mammalian organisms to mediate the nuclear export of RRE-containing RNA molecules by way of specific interactions with the Rev effector domain.

Results

RNA molecules carrying the RRE bind specifically to recombinant Rev protein

Our experiments were designed to discover the fate of RNA molecules carrying RRE upon microinjection into the nuclei of *Xenopus* oocytes. Previous studies have shown that a truncated adenovirus 2 major late transcription unit consisting of the first (L1) and second (L2) leader exons and a portion of intron 1 was rapidly spliced in *Xenopus* oocytes (Hamm *et al.*, 1989; Hamm and Mattaj, 1990). Furthermore, all the cytoplasmic and nuclear splicing products could be displayed and identified by gel electrophoresis. To use this transcription unit as a model substrate for the investigation of Rev activity, we modified it by inserting a HIV-1 sub-genomic DNA fragment, which corresponded to the RRE sequence, into either the first leader exon or the intron of plasmid pBSAd (Frendeway and Keller, 1985), thereby generating plasmids pAd46 and pAd48, respectively. Plasmid pAd49 was obtained by inserting the RRE at the same location as in pAd48 but in the antisense orientation (Figure 1). For RNA synthesis, the various plasmids were linearized with appropriate restriction enzymes, as indicated in the figure legends, and then used as templates for the synthesis *in vitro* of ^{32}P-radiolabelled RNA with T7 RNA polymerase. To prevent premature degradation during microinjection, RNA molecules were capped co-transcriptionally by including either m^{7}GpppG, GpppG or ApppG cap dinucleotide in the transcription reaction (Melton *et al.*, 1984).

Several authors have described the use of mobility-shift assays to monitor the binding of Rev to RRE sequences *in vitro*. Rev and M10 Rev mutant proteins (Malim *et al.*, 1989a) were obtained from *Escherichia coli* by overexpression of synthetic genes corresponding to the HIV-1 Rev, as described in Materials and methods. To ascertain whether our recombinant Rev proteins were able to form specific RNA–protein complexes with adenovirus RNA molecules displaying RRE sequences, we performed RNA gel retardation assays as described previously (Kjems *et al.*, 1991a). ^{32}P-radiolabelled T7 polymerase transcription products corresponding to plasmids pAd48 and pAd49 were incubated in the presence of increasing amounts (75–600 ng) of either Rev or M10 Rev recombinant proteins to allow the binding of RNA to protein. The complexes were subsequently fractionated on non-denaturing polyacrylamide gels. Labelled RNA molecules corresponding to plasmid pAd48 generated bandshifts in the presence of both Rev (Figure 2A) and M10 Rev (Figure 2C). The mobility in the gel of the RNA–protein complexes decreased when the molar ratio of Rev or M10 Rev over RNA was increased from 3- to 24-fold (Figure 2, lanes 2–5 and 7–10, respectively). This effect is most likely due to the binding of multiple Rev proteins to one RRE-containing RNA template, consistent with previous reports (Kjems *et al.*, 1991a,b; Malim and Cullen, 1991). Saturation of all binding sites on the RRE was obtained by incubation with a 10- to 20-fold molar excess of recombinant protein. As expected, RNA molecules corresponding to pAd49, possessing the complementary strand of the RRE, did not show any decrease in mobility with either Rev (Figure 2B) or M10 Rev (Figure 2D). Thus, cloning RRE sequences into the adenovirus transcription unit neither precluded the proper folding of RRE nor prevented its subsequent binding to recombinant Rev protein *in vitro*. A similar result was also obtained with RNA molecules corresponding to plasmid pAd46 (data not shown). From these results we conclude that both the radiolabelled transcripts and the recombinant proteins are

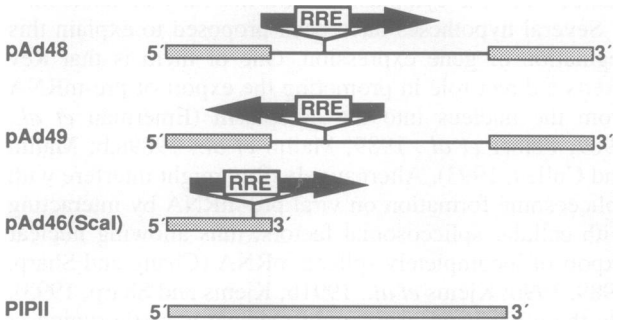

Fig. 1. Transcription units used to monitor the biological function of Rev. Schematic drawing of the transcription units used in this study. Shaded regions represent leader or exon sequences, lines are introns. The black arrow represents the RRE (nucleotides 7362–7595; Muesing *et al.*, 1985) inserted in either the sense or antisense orientation. The 3′ termini of pAd48, pAd49 and pAd46ScaI corresponding to a *Bam*HI (see Materials and methods) or a *Sca*I restriction site, respectively (Frendeway and Keller, 1985). PIPII RNA contains, as control RNA, neither an intron nor the RRE. Drawings are not to scale.

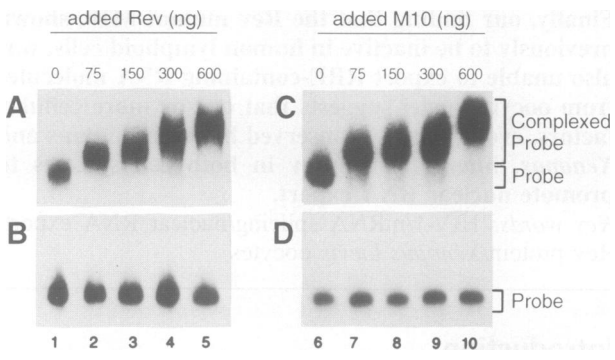

Fig. 2. Monitoring *in vitro* Rev binding to RRE using gel mobility shift assays. Reaction mixtures containing 25 ng of uniformly labelled RNA carrying the RRE in the sense pAd48 (**A** and **C**) or antisense pAd49 (**B** and **D**) orientation were incubated with the indicated amount (ng) of recombinant Rev (lanes 1–5) or M10 (lanes 6–10) proteins. Complexes were fractionated by native PAGE. Electrophoretic mobility of free or complexed probe is as indicated.

suitable tools to proceed with for our *in vivo* studies on *Xenopus* oocytes.

Rev facilitates nuclear export of RRE-containing RNA molecules in X.laevis oocytes

In the first experiments, pAd48 and pAd49 pre-mRNA were injected into the nuclei of oocytes to check whether the presence of additional sequences in the transcription unit was deleterious to correct splicing. As an internal control to monitor whether microinjection occurred only into the nucleus, we co-injected U6 RNA. This RNA is, unlike the other spliceosomal snRNAs, not exported to the cytoplasm and therefore stays exclusively in the nucleus after injection (Hamm and Mattaj, 1989; Terns *et al.*, 1993). Ninety minutes after microinjection, single oocytes were dissected into nuclear and cytoplasmic fractions and the RNAs from the two cellular compartments were analysed by denaturing RNA–gel electrophoresis. Both pre-mRNAs were efficiently spliced in the oocyte (Figure 3A) to produce the spliced product and the excised intron lariat. The mRNA precursor and the excised intron lariat were found only in the nucleus, whereas ~60–80% of the spliced mRNA was found in the cytoplasm (Figure 3A). These observations are in good agreement with previous reports (Hamm *et al.*, 1989; Hamm and Mattaj, 1990), and they indicate that the introduction of the RRE sequence into the adenovirus transcription unit does not preclude normal splicing and nuclear export. We then co-injected pAd48 pre-mRNA along with a 10-fold molar excess of recombinant Rev protein into the oocyte nuclei. Even under these conditions splicing could still occur, since significant amounts of intron lariat and spliced mRNA product were generated (Figure 3A). However, after 90 min the distribution of the pre-mRNA and the excised intron lariat between nucleus and cytoplasm were dramatically different from those found in the absence of Rev. In the presence of Rev, not only the pre-mRNA but, surprisingly, also the excised intron lariat were completely exported; both molecules contain the RRE. In contrast, the distribution of spliced mRNA product remained unaltered (Figure 3A, compare

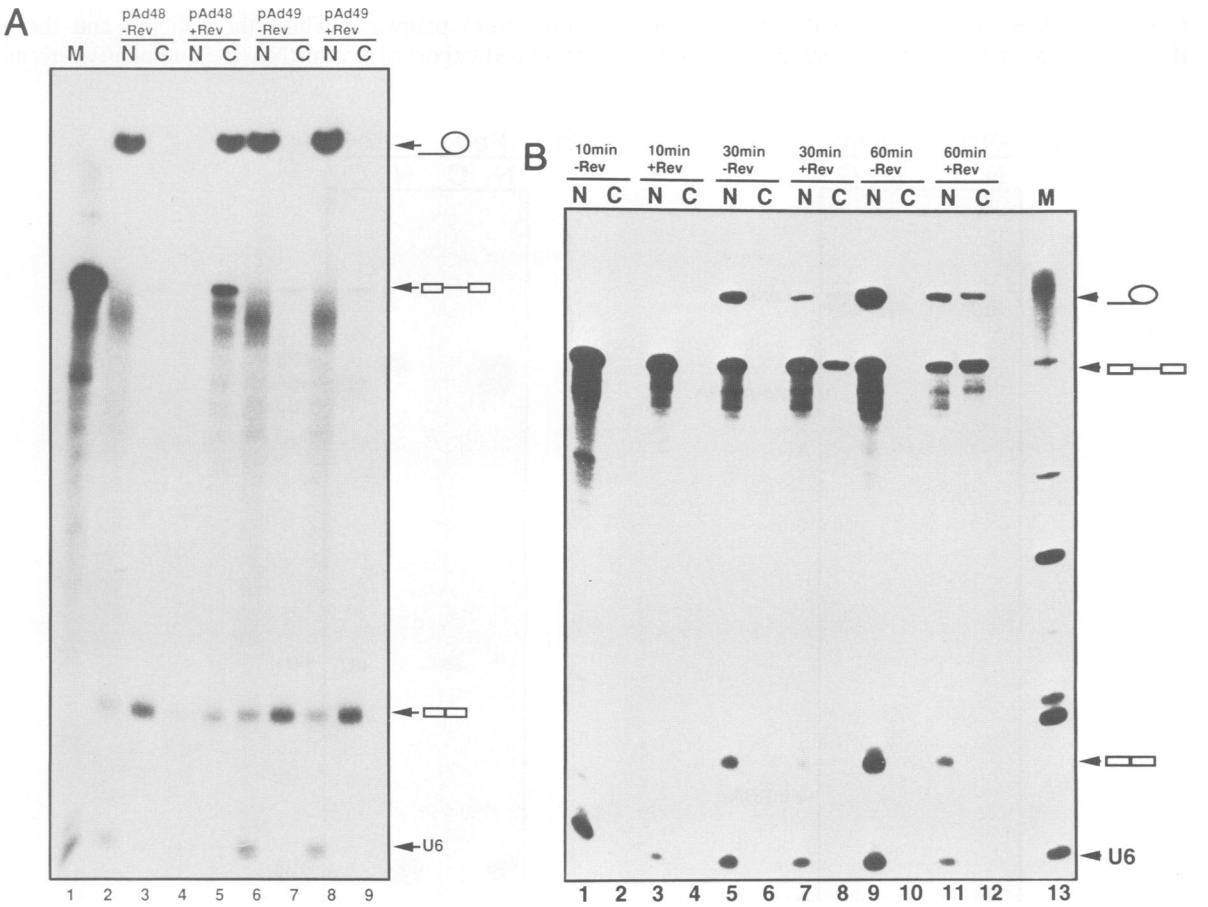

Fig. 3. *X.laevis* oocytes as the model system to monitor *in vivo* Rev biological activity. (**A**) 15 nl of ^{32}P-labelled, m^{7}G-capped pAd48 or pAd49 RNA (20 fmol at 1–2×10^6 c.p.m./pmol) were injected into the nucleus of *X.laevis* oocytes either alone (−Rev) or along with a 10-fold molar excess of Rev protein (+Rev). As an internal control to monitor the site of injection, ^{32}P-labelled, GpppG-capped U6 RNA transcribed *in vitro* was co-injected in each experiment. Oocytes were incubated for 90 min at 20°C before being dissected into nuclear (N) and cytoplasmic (C) fractions. RNA was extracted and separated by electrophoresis on denaturing polyacrylamide gels. ^{32}P-labelled RNA molecules were detected by autoradiography. Lane 1 shows an RNA size marker. (**B**) Time course of splicing and of the Rev-mediated nuclear export of pre-mRNA and excised intron lariat. 15 nl (20 fmol) of ^{32}P-labelled pAd48 RNA was injected into the nucleus of *X.laevis* oocytes, either alone (−Rev) or after preincubation with a 10-fold molar excess of Rev protein (+Rev). ^{32}P-labelled U6 RNA was co-injected in each experiment. Oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions after 10, 30 and 60 min (lanes 1–4, 5–8 and 9–12, respectively) and analysed as described above. Lane 13 shows an RNA size marker. We note that the majority of the pre-mRNA molecules injected are being spliced in the time period between 60 and 90 min (see A for comparison).

lanes 2 and 3 with 4 and 5). Thus, the two RNAs that harbour RRE were exported efficiently, but only in the presence of Rev. Co-injection of purified Rev with pAd49 pre-mRNA containing the RRE in the antisense orientation did not affect the cellular distribution of the excised lariat and the unspliced pre-mRNA (Figure 3A, compare lanes 6 and 7 with 8 and 9). The binding of Rev to the RRE structure is therefore a prerequisite for the cytoplasmic appearance of RNA molecules that harbour RRE upon microinjection into oocyte nuclei. Generally, we observe after 90 min in the presence of Rev that ~50% of the pre-mRNA is exported to the cytoplasm as unspliced RNA, the other 50% being spliced in the nuclear compartment. It has been proposed that Rev may stabilize HIV-1 pre-mRNA in the nucleus (Schwartz *et al.*, 1992; Malim and Cullen, 1993). However, Rev does not stabilize significantly in the oocyte the RRE-containing RNA molecules used in this study. This is most probably due to the intrinsically stable adenovirus pre-mRNA molecules in the oocyte (only ~20–30% of the injected RNAs are degraded after 90 min).

The observation that a fraction of the pAd48 pre-mRNA injected into the nucleus was spliced while the rest was exported is consistent with the hypothesis that the Rev-mediated pre-mRNA nuclear export occurs independently of splicing and thus competes for the pool of common substrate pre-mRNA. To test this, we injected oocyte nuclei with ^{32}P-labelled pAd48 RNA, along with U6 RNA as an internal control, and examined the kinetics of its splicing and export, both in the presence and absence of a 10-fold molar excess of Rev protein (Figure 3B). Ten minutes after nuclear injection, neither splicing nor nuclear export of unspliced pre-mRNA had occurred to a significant level in any of the cells. After 30 min excised intron lariat, in addition to the pre-mRNA, became detectable in nuclei both with and without co-injected Rev. At the same time, cells in which Rev had been co-injected with the pAd48 RNA began to show pre-mRNA in the cytoplasm, while without Rev essentially all of the injected pre-mRNA was restricted to the nucleus. After 60 min the intron lariat also appeared in the cytoplasm of cells co-injected with Rev. The order of these events allows two conclusions to be drawn: (i) that without Rev the pre-mRNA and the excised lariat remained exclusively in the nucleus, and (ii) that in the presence of Rev the pre-mRNA was spliced and exported in two parallel (i.e. competing) pathways. Thus, the splicing and the Rev-mediated export of pre-mRNA are competitive events.

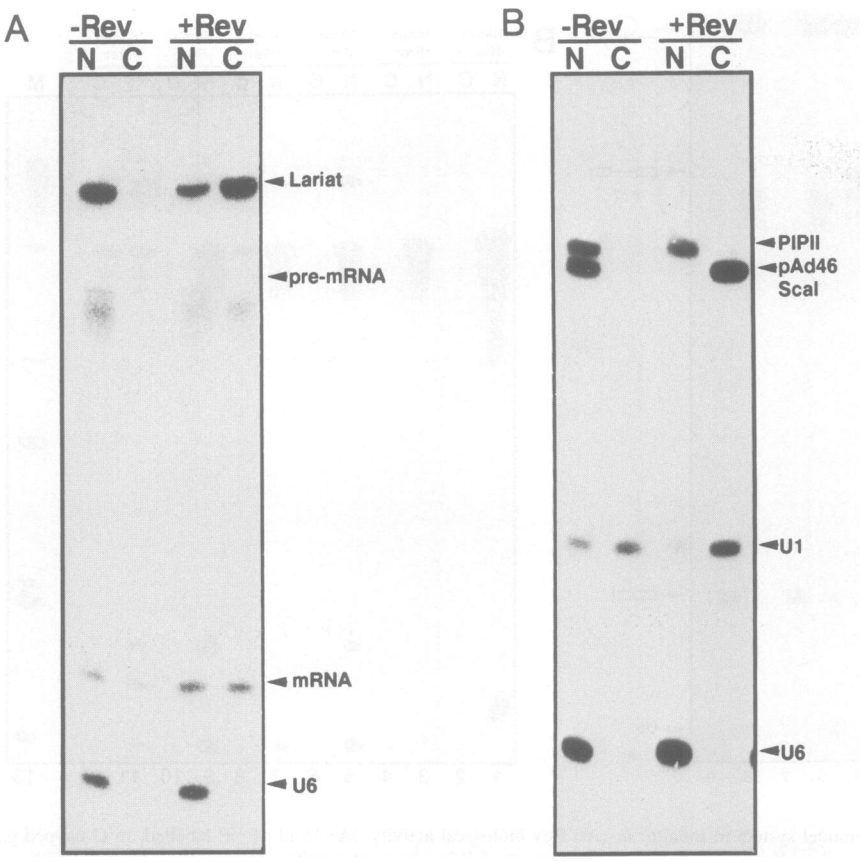

Fig. 4. Rev promotes nuclear export of non-spliceable RNA molecules harbouring the RRE. (**A**) ^{32}P-labelled pAd48 pre-mRNA was injected into the nuclei of oocytes along with ^{32}P-labelled control U6 RNA. After incubation for 90 min at 20°C, the same oocytes received a second nuclear injection of 15 nl of either Rev binding buffer (−Rev) or Rev protein (0.25 mg/ml in the same buffer) (+Rev). Oocytes were then incubated for 60 min at 20°C before dissection into nuclear (N) and cytoplasmic (C) fractions and analysis as described in Figure 3. (**B**) 15 nl (20 fmol) of ApppG-capped ^{32}P-labelled pAd46ScaI and PIPII RNAs were injected, along with ^{32}P-labelled GpppG-capped U6 and m^{7}G-capped U1 RNA as internal controls, either with (+Rev) or without (−Rev) a 10-fold molar excess of Rev protein into the nucleus of oocytes. The oocytes were incubated for 90 min at 20°C before being dissected into nuclear (N) and cytoplasmic (C) fractions. Analysis was carried out as described in Figure 3.

Rev-mediated export of non-spliceable RNA molecules

Next, we designed a double-injection experiment to investigate whether Rev function could occur independently of splicing. For this purpose, pAd48 pre-mRNA was injected first, along with U6 RNA, and the oocytes thus treated were incubated for 90 min so that splicing was almost 100% complete (see Figure 3A). We then injected Rev protein into the same oocytes and incubated them for a further 1 h. Thereafter, RNA extracted from the oocytes was analysed as performed previously (Figure 4A). The RRE-containing lariat was exported efficiently, while the U6 RNA co-injected as an internal control was strictly confined to the nucleus. This shows that nuclear export of excised intron lariat by Rev does not go hand in hand with splicing.

The experiments described above imply strongly that Rev acts directly at the level of nuclear export, rather than inhibiting splicing of pre-mRNA. If this is the case, one might hypothesize that Rev mediates the export of any RNA molecule that harbours an RRE, irrespective of whether it contains intronic sequences or not. To test this idea, we constructed an artificial RNA molecule (pAd46ScaI) that consisted of RRE fused to the first exon of adenovirus pre-mRNA (Figure 1). This 330 nucleotide-long RNA lacks an intron and, along with it, functional 5′ and 3′ splice sites. It should therefore be incapable of binding spliceosomal factors such as U snRNPs. pAd46-ScaI RNA was synthesized *in vitro* with an ApppG-cap at its 5′ end. The ApppG-cap was chosen instead of the physiological m^7G-cap for the reason that m^7G-capped RNA molecules are exported efficiently on their own; this is because the m^7G-cap is a nuclear export signal in its own right, governing the export of RNA molecules transcribed by polymerase II, such as mRNAs and U1 RNA (Hamm *et al.*, 1989; Hamm and Mattaj, 1990; Dargemont and Kühn, 1992; Terns *et al.*, 1993). The majority of ApppG-capped pAd46ScaI RNAs should thus be retained in the nucleus and not be exported (Hamm and Mattaj, 1990). As internal controls, we co-injected an ApppG-capped mRNA (PIPII-RNA, see Figure 1) that had neither RRE nor any introns (W.Kammerloher *et al.*, 1994), U6 RNA and also m^7G-capped U1 RNA. Of the control RNAs, only U1 RNA should be exported efficiently to the cytoplasm by itself. As expected, 90 min after injection of this RNA mixture into the nucleus, <10% of the two ApppG-capped mRNAs were exported, while >60% of U1 RNA was found in the cytoplasm. Co-injected U6 RNA remained entirely in the nucleus (Figure 4B). Upon co-injection of the same RNA mixture along with a 10-fold molar excess of Rev, however, the intracellular distribution of the pAd46ScaI RNA changed dramatically: after 90 min of incubation almost 95% of the RRE-containing pAd46ScaI RNA was found in the cytoplasm, while the distribution of U6 and PIPII RNA was unaltered (Figure 4B). In this particular experiment U1 RNA was exported to the cytoplasm slightly more efficiently in the presence of Rev. Such an effect of Rev on U1 RNA export, however, has not been observed reproducibly in other experiments. All in all, these experiments suggest strongly that Rev acts as a transport factor and facilitates the export of RNA as long as the RNA contains a functional RRE. Our data further show that the activity of Rev in exporting RNA does not necessarily require it to interfere with the cellular splicing machinery.

Rev mutant M10 is unable to promote RNA export

Since *X.laevis* oocytes are not the natural host for HIV-1 infection, one is bound to ask whether Rev behaviour in oocytes can have any relevance to the biological function of Rev in HIV-1-infected cells. A different way of posing the same question would be to ask whether the factor(s) required for Rev to exert its biological activity in human lymphoid cells have been conserved in evolution between these cells and *X.laevis* oocytes. If there were similar interactions, Rev protein mutants should exhibit comparable phenotypes in both *Xenopus* oocytes and, for instance, COS cells, as the latter have been reported to support the biological function of Rev when transfected with appropriate reporter genes (Malim *et al.*, 1989a). To address this question, we tested the effect of a Rev mutant

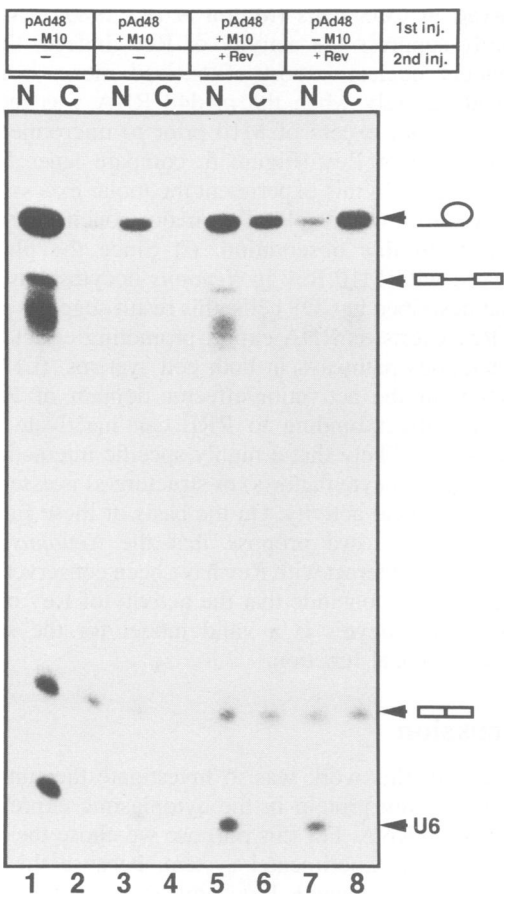

Fig. 5. The M10 mutant of Rev does not induce nuclear export of RNA molecules containing the RRE. ^{32}P-labelled, m^7G-capped pAd48 RNA was injected into the nuclei of oocytes, either alone (lanes 1–2 and 7–8) or after binding to a 10- (lanes 3–4) or 30-fold (lanes 5–6) molar excess of recombinant M10 Rev mutant. ^{32}P-labelled U6 RNA was co-injected in each experiment. Oocytes were incubated for 90 min at 20°C before they received a second nuclear injection of either Rev binding buffer (15) (lanes 1–4) or Rev protein (0.25 μg/μl) in the same buffer (lanes 5–8). Incubation was continued for 60 min before the oocytes were dissected; RNA from the nucleus (N) and cytoplasm (C) was then analysed as described in the legend to Figure 3. In the experiment shown in lanes 3 and 4, significantly less RNA was injected. Therefore, the bands from U6 and the spliced product are weak, and exposure of the X-ray film was prolonged to make them more clearly visible.

termed M10 upon the export of RRE-harbouring RNA molecules in *X.laevis* oocytes. M10 is a non-functional Rev mutant that carries a two amino acid substitution in its leucine-rich activation/effector domain. This mutation does not affect its binding to the RRE, yet renders the protein inactive in promoting the export of viral transcripts in COS cells (Malim *et al.*, 1989a). Our data have already shown that recombinant M10 Rev binds to pAd48 and pAd49 RNA molecules *in vitro* in a way similar to the binding of recombinant unmutated Rev (Figure 2). In our transport/splicing assay, a 10-fold molar excess of M10 affected neither the splicing nor the nuclear transport of ^{32}P-labelled pAd48 RNA when these were co-injected (Figure 5, lanes 3 and 4). In comparison, injection of the same molar excess of Rev protein along with pre-mRNA results in a quantitative export of excised intron lariat (see Figure 3A). Even larger amounts of M10 protein (up to a 30-fold molar excess) failed to facilitate the export of RRE-harbouring RNA molecules (data not shown). Moreover, as observed in COS cells (Malim *et al.*, 1989a), M10 can partially suppress the activity of Rev. Indeed, the Rev-dependent nuclear export of excised intron lariat was reduced severely when the pAd48 RNA was incubated with a 30-fold excess of M10 prior to microinjection in the presence of Rev (Figure 5, compare lanes 5 and 6 with 7 and 8). In this experiment the molar excess of M10 Rev over Rev was 3-fold. Two major conclusions can be drawn from this observation. (i) Since the phenotype observed with M10 Rev in *Xenopus* oocytes was similar to that described in COS cells, this result suggests strongly that Rev exerts its RNA export-promoting effect by way of analogous pathways in both cell systems. (ii) Since a mutation in the activation/effector domain of Rev that does not affect binding to RRE can inactivate Rev in oocytes, it is likely that a highly specific interaction with one or more oocyte factor(s) or structure(s) is essential for Rev's biological activity. On the basis of these functional assays *in vivo*, we propose that the *Xenopus* oocyte factor(s) that interact with Rev have been conserved during evolution. We conclude that the activity of Rev observed in *Xenopus* oocytes is a valid model for the study of Rev's biological function.

Discussion

The aim of this work was to investigate the function of the HIV-1 Rev protein in the cytoplasmic expression of unspliced mRNA. For this purpose we chose the *X.laevis* oocyte as an experimental system, because this system allows the simultaneous biochemical monitoring of splicing and nuclear export of pre-mRNA molecules synthesized *in vitro*. Thus the effects of Rev upon the splicing reaction and upon nuclear export could be investigated independently.

The first indication that Rev is involved directly in the export of unspliced mRNA came from the experiments with pAd48 pre-mRNA. It was observed that Rev induced not only the export of the unspliced mRNA, but also the export of the excised intron lariat. Both of these RNA species contain the RRE. In this connection, the observation that the intron lariat was exported is of particular importance for two reasons. First, it showed that pre-mRNA is not the only RNA species that, although never leaving the nucleus under normal physiological conditions, is exported in the presence of Rev. Secondly, this finding shows that the inhibition of splicing is not a prerequisite for the Rev-mediated export of an RNA, as the RRE-containing lariat is exported while the splicing reaction is proceeding in the same nucleus.

These experiments still failed to address the question of whether nuclear export by Rev requires the action of splicing factors, since the RRE-containing pre-mRNA and the intron lariat bind not only to Rev but also to UsnRNPs and to other, non-UsnRNP splicing factors. This was clarified by investigating the transport of an RRE-containing mRNA that contained no splicing sites and therefore could not bind splicing factors. Even this mRNA was exported from the nucleus, showing that the Rev-mediated nuclear export and the splicing of mRNA are two independent processes which take place in parallel in the nucleus and compete for substrate. Further support for this observation came from the finding that the co-injection of Rev and pre-mRNA resulted in a part of the pre-mRNA being spliced and the rest exported. On the basis of these experiments, we can conclude that Rev participates directly in the transport of RRE-containing mRNA out of the nucleus. This transport does not require any interaction between Rev and splicing factors or splicing sites on the pre-mRNA. Rev is thus best described as a sequence-specific RNA export factor.

Other workers have obtained results consistent with the above conclusion from an analysis of gene expression in cells transfected with HIV-1 expression vectors. It has been shown previously (Felber *et al.*, 1989; Malim *et al.*, 1989a; Malim and Cullen, 1993) that the transport of the various unspliced and partially spliced HIV-1 mRNAs was induced by Rev, while the splicing pattern of the HIV-1 mRNAs remained unaltered. However, these studies did not exclude the possibility that Rev may simply reverse a binding interaction between pre-mRNA and splicing factors and thus make the pre-mRNA available for export. This distinction can only be drawn in experiments that allow the splicing and transport processes to be separated.

It has been proposed that Rev may stabilize HIV-1 pre-mRNA in the nucleus (Schwartz *et al.*, 1992; Malim and Cullen, 1993). However, the data presented here suggest that this additional effect of Rev on viral pre-mRNA molecules does not play a crucial role in the export of RNA from the nucleus.

The significance of our results depends critically upon the extent to which Rev functions in infected lymphoid human cells, in the same way as in the *X.laevis* oocytes used in this work. We have shown here that the two cell types, in spite of the considerable evolutionary distance between them, are phenotypically very similar in respect of the Rev-mediated RNA export. An obvious similarity is that the nuclear export of RNA can only occur when the RNA bears an RRE; RNAs that lack this sequence or carry it in an inverse (antisense) orientation are not exported by Rev. However, the strongest argument that Rev acts in the same manner in these cell types comes from the experiments with the Rev mutant M10. Although this mutant binds to the RRE of mRNA, the presence of a mutation in the leucine-rich C-terminal region of Rev blocks its interaction with an as yet unidentified cellular factor. This leads to a *trans*-dominant negative phenotype

(Malim *et al.*, 1989a). Our observation that M10 Rev in *Xenopus* oocytes is functionally inactive and displays the *trans*-dominant negative phenotype, thus behaving identically in *Xenopus* and human cells, suggests strongly that Rev interacts with similar factors in the two cell types. It can further be inferred that the factors allowing Rev to induce nuclear transport in HIV-1-infected human cells are also present in the oocytes.

The effect of Rev upon the export and splicing of pre-mRNA in oocytes has been investigated recently by Ruhl and co-workers (Ruhl *et al.*, 1993). They injected the genes for the Rev protein and the RRE-containing pre-mRNA into oocyte nuclei. To obtain nuclear export of the RRE-mRNA, they needed to add the eukaryotic initiation factor eIF5A, a result that cannot immediately be reconciled with our observation that the oocytes contain all the cellular factors needed for nuclear export of RNA. The reasons for this discrepancy are not yet clear.

Studies by Kjems and Sharp *in vitro* have revealed that an RRE-binding peptide of the Rev protein blocks the association of the U4/U6.U5 tri-snRNP complex with the early spliceosome, thus inhibiting the splicing of RRE-containing pre-mRNA (Kjems *et al.*, 1991b; Kjems and Sharp, 1993). This was taken to indicate that the export of unspliced pre-mRNA was a result of the inhibition of the splicing process by the Rev protein. Our data support the alternative interpretation that neither intact Rev protein nor the M10 mutant inhibits the splicing reaction by interfering with splicing factors. Based on the *in vitro* bandshift data, both proteins are likely to saturate the Rev binding sites on the RREs also under the conditions in the oocyte. In addition, our data show that Rev also brings about the export of RNAs that fail to bind splicing factors; therefore, the export function of Rev does not necessarily require any interaction with these factors. The data presented here support the idea that the decisive element for the biological function of Rev is the activation/effector domain. This is also supported by reports of two studies in which it was shown that the RNA binding domain of Rev is not essential, as such, for the function of the protein (McDonald *et al.*, 1992; Venkatesan *et al.*, 1992). The RRE can be replaced by the RNA binding site from the coat protein of phage M2, when the Rev protein is directed to this binding site by fusing the phage coat protein to the Rev protein.

The exact mechanism by which Rev mediates the export of RRE-containing mRNA is at present unknown. In spite of the considerable evolutionary distance between human cells and frog oocytes, Rev causes the export of mRNA in both of these cell types, and this observation suggests strongly the existence of a general, rather than a virus-specific, pathway. Kinetic evidence has been adduced recently to show that different export pathways operate for mRNA, rRNA, tRNA and snRNA (Jarmolowski *et al.*, 1994). In principle, Rev could make the general m^7G-cap-dependent export pathway accessible for unspliced mRNA. However, this is not supported by our observations that both the uncapped intron lariat and ApppG-capped pAd46 RNA molecules are exported by Rev. One can thus imagine a model in which Rev opens the way for RRE RNA to join an export pathway that is not that of normal mRNA. It is interesting in this context that Rev is found in high concentrations in the nucleolus, from which ribosomal RNAs are exported (Felber *et al.*, 1989; Malim *et al.*, 1989a).

The availability of the *X.laevis* oocyte system to study the Rev-mediated nuclear export of mRNA can be expected to yield deeper insight into the mechanism by which Rev functions. This will include the identification of the cellular factor(s) that interact with Rev, as well as the relationship between the path of Rev-mediated nuclear RNA export and other pathways used by cellular RNA molecules.

Materials and methods

Plasmid constructions

Oligonucleotides needed for cloning purposes were synthesized chemically on an automated Applied Biosystems 381A DNA synthesizer. Oligonucleotide-directed mutagenesis was performed with the Amersham RPN1523 mutagenesis kit according to the manufacturer's instructions. For construction of pAd46, pAd48 and pAd49, the first step consisted of cloning a *PstI*–*Eco*RI restriction fragment of plasmid pBSAd1 (Frendeway and Keller, 1985) into phage M13TG131. The oligonucleotide 5′-AGGCCGACGGAATTCGGATCCAA-3′ was used to introduce a *Bam*HI and an *Eco*RI site, thus generating M13114. The 79 bp *Eco*RI fragment was deleted and a unique *BglII* restriction site was introduced into the intron of the transcription unit by using the oligonucleotide 5′-ACCAGATCTACGCGGCCA-3′. The *Eco*RI–*PstI* fragment of this clone was inserted into the vector pGEM4Z (Promega), generating p9043. The RRE sequence of HIV-1 (nucleotides 7362–7595; Malim *et al.*, 1989b) was amplified by PCR (Mullis and Fallona, 1987) using the primers 5′-AGCAGTAGATCTAGGAGCTTTGTTCCTT-3′ and 5′-ACCCCAAGGATCCAGGAGCTGTTGATCCT-3′, that included additional 5′ *BglII* and 3′ *Bam*HI flanking restriction sites. Cloning of the PCR-amplified fragment into the unique *BglII* site of clone p9043 generated two plasmids, pAd48 and pAd49, which possessed RRE sequences in the sense or antisense orientation, respectively. For construction of pAd46, a *BglII* restriction site was introduced into the first exon of the transcription unit by using oligonucleotide 5′-ACCCCAAGATCTGGCCCT-3′. The *Eco*RI–*PstI* fragment of this clone was inserted into the vector pGEM4Z (Promega), generating plasmid p9042. Subsequent cloning of the HIV-1 RRE PCR-amplified fragment into the unique *BglII* restriction site generated pAd46, which possessed the RRE sequence in the sense orientation. All constructs were sequenced with an Applied Biosystems Model 373A DNA automated sequencing system.

Expression and purification of Rev and M10 Rev

A synthetic gene encoding HIV-1 Rev protein was synthesized from oligonucleotides. The Rev M10 mutant gene was constructed by oligonucleotide-directed mutagenesis of the *rev* gene using the RPNII kit from Amersham. Rev genes were cloned into expression vector pMalCRI (New England Biolabs) by PCR using the Gene Amp kit (Perkin Elmer Cetus) leading to p9058 and p9059, respectively. The sequences of the clones were verified by automated sequencing using the Dyedeoxy sequencing kit (Applied Biosystems).

For routine protein production, *E.coli* NCM 522 was transformed with p9058 and p9059, respectively. Expression was induced with IPTG (1 mM final concentration) in the late exponential growth phase and incubated for another 3 h. After harvest (4000 r.p.m., 4°C, 20 min) cells were washed once with H_2O, resuspended in resuspension buffer (20 mM phosphate buffer pH 8.0, 0.5 M NaCl) and lysed by three passages through a French pressure cell (Amicon). Soluble proteins were recovered after centrifugation (28 000 r.p.m., 4°C, 30 min) and loaded onto a Chelating Sepharose column (XK50/20, Pharmacia) with immobilized Zn^{2+} ions. Bound protein was eluted by applying a pH gradient (20 mM phosphate buffer pH 8–3, 0.5 M NaCl) and fractions containing the fusion protein were pooled. Rev was liberated from the fusion protein after incubation with IgA protease (Boehringer Mannheim) and concentrated by ion-exchange chromatography on an S-Sepharose FastFlow column (XK16/50, Pharmacia) or MonoS HR 5/5, by a linear NaCl gradient (20 mM phosphate buffer, 0.5–2.0 M NaCl), respectively. Traces of uncleaved fusion protein were removed concomitantly by passage through a Chelating Sepharose Hitrap column (Pharmacia). Recombinant protein was characterized by microsequencing of the N-terminus and electrospray mass spectrometry.

Synthesis in vitro of RNA

Plasmid DNA was linearized with restriction endonuclease *Bam*HI (pAd48/49), *Sca*I (pAd46) or *Sty*I (pPIPII) and used as template for transcription *in vitro* with T7 RNA polymerase (Melton *et al.*, 1984). A typical reaction was carried out in 20 µl of T7 polymerase buffer (25 mM DTT, 0.1 µg/µl BSA, 0.1 µg/µl DNA template, GTP, ATP and UTP 250 µM each, 100 µM CTP, 80 µCi [α-^{32}P]CTP 3000 Ci/mmol; Amersham) with 1 µl of T7 RNA polymerase (1 U/µl; Promega). Capping of the transcripts was performed by adding m^7GpppG, GpppG or ApppG, as required, to give a concentration of 1 mM in the transcription reaction. Template DNA was eliminated from the reaction by a 15 min incubation at 37°C with 1 µl of DNase I (1 U/µl; Promega, RQ grade). Labelled RNA was extracted with phenol/$CHCl_3$, precipitated with ethanol and redissolved in Rev binding buffer (see below) immediately before use for microinjections.

Gel mobility shift assays

The protein concentrations of Rev or M10 were adjusted to 75 µg/ml by dilution of stock solutions with Rev storage buffer (1 M NaCl, 20 mM HEPES pH 7.9, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 1 mM DTT). Prior to the addition of renaturated RNA, 1–8 µl of either Rev or M10 protein were preincubated for 10 min on ice with 0.25 mg/ml *E.coli* tRNA (type XXI, Sigma) in a final volume of 12 µl Rev binding buffer (50 mM HEPES pH 7.9, 150 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 16% glycerol). 1.25 µg radiolabelled RNA was renaturated in 40 µl renaturation buffer (10 mM HEPES pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) by heating to 80°C for 10 min followed by a slow cooling to room temperature and subsequent chilling to 4°C. From this, 8 µl was taken and added to the protein sample. This solution was kept on ice for an additional 10 min to allow RNA–protein complexes to form before directly loading on native polyacrylamide gels (5% acrylamide/*N,N'*-methylene *bis*-acrylamide 39:1, 50 mM; Tris–glycine pH 8.9, containing 3% glycerol). Electrophoresis was performed at 20 V/cm for 90 min at room temperature.

Microinjection into oocytes and analysis of in vivo splicing products

Ovaries of *X.laevis* females were cut into small pieces and incubated for 3–5 h in modified Barth's solution (Hamm and Mattaj, 1990) containing 0.2% collagenase type II (Sigma). Defolliculated stage V and VI oocytes were collected, stored in small fractions and used for injection experiments within the following 2 days. Renatured ^{32}P-labelled pre-mRNA (0.5–1.0×10^6 c.p.m./µl, 0.7 µM) was incubated for 30 min on ice in Rev binding buffer (1 M NaCl, 20 mM HEPES–KOH pH 7.9, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol and 1 mM DTT) in the presence of a 10-fold excess of recombinant protein (7 mM) to allow formation of complexes. ^{32}P-labelled U6 RNA was added to all the incubation mixtures as an internal marker for monitoring the site of injection. A volume of 15 nl of solution was injected into each nucleus, and the oocytes were subsequently maintained for the indicated time at 20°C. The nucleus from each oocyte was dissected manually from the cytoplasm, and each fraction was homogenized separately in homo-buffer [10 mM Tris–HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1% SDS and 1 µg/µl proteinase K (Boehringer)]. After phenol/$CHCl_3$ extraction and ethanol precipitation, labelled RNA molecules were separated by electrophoresis on denaturating polyacrylamide gels (5% acrylamide/*N,N'*-methylene *bis*-acrylamide 19:1; 7 M urea) and visualized by autoradiography.

Identification of the intron lariat splicing intermediate

For this purpose, nuclei of 10 oocytes were injected with ^{32}P-labelled pAd48 RNA. After 90 min incubation at 20°C the nuclei were isolated and RNA was extracted as described by Hamm and Mattaj (1990). Total nuclear RNA was divided subsequently into two equal portions. One was incubated for 30 min at 30°C in buffer (10 mM EDTA pH 8.0, 0.1 mg/ml BSA) supplemented with a 1/10 volume of nuclear extract enriched in debranching activity. The other half of the RNA was incubated with buffer only. The RNA was then phenol-extracted and ethanol-precipitated. Both samples were loaded on a denaturing polyacrylamide gel (5% acrylamide/*N,N'*-methylene *bis*-acrylamide, 19/1, 7 M urea) and run lane by lane.

Acknowledgements

We thank P.Woolley, C.Marshallsay and A.Atkinson for critically reading the manuscript. We would also like to thank A.Bindereif, W.Kammerloher and I.Mattaj for providing us with plasmids, and A.Lamond and H.Galinaro for the generous gift of nuclear extract enriched for debranching activity. We are grateful to P.Remy and J.M.Reichhart for transferring technologies to the Marion Merrell Dow Research Center in Strasbourg, and to C.Branlant for helpful discussions. We also thank our colleagues C.Danzin, M.Galvan, B.A.Harris and J.D.Fondacaro for continuous interest and support. This work was supported by the Marion Merrell Dow Research Institute and by grants from the Deutsche Forschungsgemeinschaft (to R.L. and U.F.) and the Fonds der Deutschen Chemischen Industrie (to R.L.). S.M. is a recipient of a CIFRE fellowship from the Association Nationale de la Recherche Technique.

References

Arrigo,S.J. and Chen,I.S.Y. (1991) *Genes Dev.*, **5**, 808–819.
Berger,J., Aepinus,C., Dobrovnik,M., Fleckenstein,B., Hauber,J. and Böhnlein,E. (1991) *Virology*, **183**, 630–635.
Chang,D.D. and Sharp,P.A. (1989) *Cell*, **59**, 789–795.
Chang,D.D. and Sharp,P.A. (1990) *Science*, **249**, 614–615.
Cullen,B.R. (1992) *Microbiol. Rev.*, **56**, 375–394.
D'Agostino,D.M., Felber,B.K., Harrison,J.E. and Pavlakis,G.N. (1992) *Mol. Cell. Biol.*, **12**, 1375–1386.
Daly,T.J. *et al.* (1993) *Biochemistry*, **32**, 8945–8953.
Dargemont,C. and Kühn,L.C. (1992) *J. Cell Biol.*, **118**, 1–9.
Emerman,M., Vazeux,R. and Peden,K. (1989) *Cell*, **57**, 1155–1165.
Feinberg,M.B., Jarrett,R.F., Aldovini,A., Gallo,R.C. and Wong Staal,F. (1986) *Cell*, **46**, 807–817.
Felber,B.K., Hadzopoulou-Cladaras,M., Cladaras,C., Copeland,T. and Pavlakis,G.N. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1495–1499.
Frendeway,D. and Keller,W. (1985) *Cell*, **42**, 355–367.
Hamm,J. and Mattaj,I.W. (1989) *EMBO J.*, **8**, 4179–4187.
Hamm,J. and Mattaj,I.W. (1990) *Cell*, **63**, 109–118.
Hamm,J., Dathan,N.A. and Mattaj,I.W. (1989) *Cell*, **59**, 159–169.
Jarmolowski,A., Boelens,W., Izaurralde,E. and Mattaj,I.W. (1994) *J. Cell Biol.*, **124**, 627–635.
Kammerloher,W., Fischer,U., Piechottka,G.P. and Schäffner,A.R. (1994) *Plant J.*, in press.
Kjems,J. and Sharp,P.A. (1993) *J. Virol.*, **67**, 4769–4776.
Kjems,J., Brown,M., Chang,D.D. and Sharp,P.A. (1991a) *Proc. Natl Acad. Sci. USA*, **88**, 683–687.
Kjems,J., Frankel,A.D. and Sharp,P.A. (1991b) *Cell*, **67**, 169–178.
Malim,M.H. and Cullen,B.R. (1991) *Cell*, **65**, 241–248.
Malim,M.H. and Cullen,B.R. (1993) *Mol. Cell. Biol.*, **13**, 6180–6189.
Malim,M.H., Böhnlein,S., Hauber,J. and Cullen,B.R. (1989a) *Cell*, **58**, 205–214.
Malim,M.H., Hauber,J., Le,S.Y., Maizel,J. and Cullen,B.R. (1989b) *Nature*, **338**, 254–257.
Malim,M.H., McCarn,D.F., Tiley,L.S. and Cullen,B.R. (1991) *J. Virol.*, **65**, 4248–4254.
McDonald,D., Hope,T.H. and Parslow,T.G. (1992) *J. Virol.*, **66**, 7232–7238.
Melton,D.A., Krieg,P.A., Rebagliahi,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Mermer,B., Felber,B.K., Campbell,M. and Pavlakis,G.N., (1990) *Nucleic Acids Res.*, **18**, 2037–2044.
Muesing,M.A., Smith,D.H., Cabradilla,C.D., Benton,C.V., Lasky,L.A. and Capon,D.J. (1985) *Nature*, **313**, 450–458.
Mullis,K. and Fallona,F.A. (1987) *Methods Enzymol.*, **155**, 335–359.
Ruhl,M. *et al.* (1993) *J. Cell Biol.*, **123**, 1309–1320.
Schwartz,S., Felber,B.K. and Pavlakis,G.N. (1992) *J. Virol.*, **66**, 150–159.
Terns,M.P., Dahlberg,J.E. and Lund,E. (1993) *Genes Dev.*, **7**, 1898–1907.
Venkatesan,S., Gerstberger,S.M., Park,H., Holland,S.M. and Nam,Y.-S. (1992) *J. Virol.*, **66**, 7469–7480.
Venkatesh,L.K. and Chinnadurai,G. (1990) *Virology*, **178**, 327–330.
Zapp,M.L. and Green,M.R. (1989) *Nature*, **342**, 714–716.

Received on March 3, 1994; revised on May 11, 1994