Specific Regulation of mRNA Splicing In Vitro by a Peptide from HIV-1 Rev

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Summary

The Rev protein of HIV-1 regulates the synthesis of partially spliced forms of cytoplasmic viral mRNA by binding to a cis-acting RNA sequence, the Rev response element (RRE). We have investigated the regulation of splicing in vitro and have shown that Rev specifically inhibits splicing of pre-mRNAs containing an RRE by 3- to 4-fold. A synthetic peptide of 17 amino acids containing the RNA-binding domain of Rev is highly functional and specifically inhibits splicing by up to 30-fold. Other peptides that bind to the RRE with high affinity, but with low specificity, do not specifically inhibit splicing. Six repeated monomeric binding sites for the peptide can substitute for the RRE, indicating that regulation by Rev requires interactions with multiple sites. The peptide acts at a step in the assembly of splicing complexes, suggesting that one of the functions of the basic region of Rev is to prevent formation of a functional spliceosome.

Introduction

Accumulating data suggest that the expression of many cellular and viral genes is regulated at a posttranscriptional level. RNA splicing appears to be a common target of this regulation, including intron inclusion, exon exclusion, and use of alternative 5' or 3' splice sites (reviewed by Andreadis et al., 1987). Alternative splicing is likely to be common to many genes, but only a few have been shown to be specifically regulated (reviewed by Maniatis, 1991). Little is known about regulatory mechanisms of splicing. Some regulatory proteins may specifically interact with pre-mRNAs to either block or enhance splice site usage. Alternatively, specific factors may interact with the splicing machinery and modulate its specificity for different splice sites.

A small number of proteins that have an active role in regulation of RNA splicing have been identified. In Drosophila, several factors regulate RNA splicing in a cascade of reactions that determines the sex (Baker, 1989). The sex-determining gene product, Sex lethal (Sxl), binds directly to a polypyrimidine tract of the transformer (tra) gene transcript and apparently blocks the use of the adjacent male-specific 3' splice site (Frueh et al., 1990; Gajewska et al., 1989). Sxl protein also regulates its own splicing through positive autoregulation (Bell et al., 1991). In conjunction with transformer-2 (tra-2), the tra gene product regulates the splicing of the double sex (dsx) gene transcript to produce either male- or female-specific products (Burtis and Baker, 1989; Nagoshi et al., 1988). Tra-2 binds specifically to a repeated sequence within the dsx pre-mRNA and enhances the usage of an upstream female-specific 3' splice site (Hedley and Maniatis, 1991). The regulatory functions of Sxl and Tra-2 require that the target sequences be located at restricted positions within the regulated pre-mRNA.

Splice site selection has also been shown to depend on the concentration of constitutively expressed splicing factors. SF2, which is an activity necessary for 5' splice site cleavage in vitro, may play a regulatory role in some systems. For example, high concentrations of SF2 promote the use of the 5' splice site proximal to the 3' splice site in vitro (Krainer et al., 1990). Studies of alternative splicing of pre-mRNA encoding SV40 large T (t) or small t (T) antigens show that the relative usage of the two alternative 5' splice sites can be modulated by a cellular factor named ASF (Ge and Manley, 1990), which is identical to SF2.

Retroviruses use alternative splicing to generate multiple mRNAs from the same gene (Varmus, 1988). For these viruses, it is essential to maintain a balance between spliced and unspliced mRNA in the cytoplasm. This balance may be achieved by using suboptimal RNA processing signals and efficient transport of unspliced mRNA (Katz and Skalka, 1990; Fu et al., 1991). For most retroviruses the ratio of spliced to unspliced mRNAs remains fairly constant during the viral life cycle. However, in a subgroup of retroviruses, the Lenti viruses, the ratio of spliced to unspliced mRNAs in the cytoplasm is critical for viability of the virus (Kim et al., 1989). Human immunodeficiency virus type 1 (HIV-1), a Lenti virus implicated in the pathogenesis of acquired immunodeficiency syndrome (AIDS); Barre-Sinoussi et al., 1983; Gallo et al., 1984), encodes a small protein, Rev, which regulates the cytoplasmic appearance of unspliced or singly spliced mRNAs (Feinberg et al., 1986; Malim et al., 1988; Rosen et al., 1988; Emerman et al., 1989; Hadzopoulou-Cladaras et al., 1989). The unspliced and singly spliced mRNAs encode primarily the structural proteins gag, pol, and env; doubly spliced mRNAs primarily encode the viral regulatory proteins Rev and Tat (reviewed by Cullen and Green, 1989). The mechanism of Rev regulation is unknown, but it has been suggested that Rev functions directly at the level of RNA splicing (Chang and Sharp, 1989; Lu et al., 1990) or through activation of a transport mechanism to the cytoplasm (Malim et al., 1989a; Felber et al., 1989; Emerman et al., 1989; Hammarskjöld et al., 1989). These two models are not mutually exclusive and may operate in conjunction with one another (Chang and Sharp, 1990).

The specificity for Rev activity is mediated by a 240 nucleotide RNA element, the Rev response element (RRE), located within the env gene (Malim et al., 1989a;
Figure 1. Structure of Substrates

(A) Templates derived from PAL4, which contains the second intron from the rabbit β-globin gene. PAL S was also used as recipient plasmid in some experiments (see Experimental Procedures).
(B) Templates derived from PIR 7A, which contains a modified version of the first intron in the major late transcript of adenovirus (see Experimental Procedures). +RRE indicates the insertion of a 240 bp MboII-Sau3AI fragment from HIV-1 (Malim et al., 1989a), which contains the RRE; +ClO indicates the insertion of a 220 bp HindIII-SacI control fragment from the second intron of the rabbit β-globin gene (Padgett et al., 1983); +1 x IIB indicates the insertion of a 38 bp fragment containing a single Rev-binding site; +6 x IIR indicates the insertion of six tandem repeats of Rev-binding sites. +Globin indicates the insertion of a tandem 65 nucleotide repeat suggesting that several Rev molecules bind specifically to the RRE in vitro (Zapp and Green, 1989). It has also been shown, using a HIV-1 env expression system, that Rev responsiveness required the presence of a 5’ splice site and that interaction with the U1 small nuclear ribonucleotide protein particle (snRNP) was essential (Lu et al., 1990). In this report we have investigated the effect of Rev on RNA splicing in vitro.

Results

Rev Specifically Inhibits RNA Splicing In Vitro

RNA splicing in vitro can be monitored by the appearance of splicing products, the lariat intron and ligated exons, and the intermediate products, lariat introns (two main upper bands), and intermediate lariat composed of intron and 3’ exon (appear as a weak band just above each lariat product band and marked with an asterisk). Ligated exons and 5’ exons migrated near the bottom of the gel and are not shown. Owing to topological constraints, lariat products migrate more slowly than the pre-mRNAs on high percentage gels. The ligated exons migrate faster than the pre-mRNA and in a region that contains a substantial amount of degraded RNA. It is therefore convenient to use the yield of lariat as a measure of splicing efficiency. The splicing of the RRE-containing substrate was inhibited 4 fold as compared with the internal control substrate at 0.15 μg/ml Rev protein.

In addition to the RRE, cis-acting repressive sequences in the mRNA are important for the Rev response (Rosen et al., 1988). These sequences probably stimulate retention of the mRNA in the nucleus in the absence of Rev. Although the precise nature of the cis-acting repressive sequences has not been determined, sequences containing a single splice site can act as a cis-acting repressive sequence (Chang and Sharp, 1989). For example, a pre-mRNA containing a β-globin intron can become responsive to Rev if the RRE is present in the mRNA and if either of the 5’ or 3’ splice sites is attenuated by mutation (Chang and Sharp, 1989). It has also been shown, using a HIV-1 env expression system, that Rev responsiveness required the presence of a 5’ splice site and that interaction with the U1 small nuclear ribonucleotide protein particle (snRNP) was essential (Lu et al., 1990). In this report we have investigated the effect of Rev on RNA splicing in vitro.

Rev Specifically Inhibits RNA Splicing In Vitro

Figure 2. Specific Inhibition of Splicing of β-globin Pre-mRNA Derivatives by Intact Rev

PALN1/RRE (containing the RRE) and PAL4 (internal control) pre-mRNAs were incubated together under splicing conditions in the presence of the indicated amount of intact Rev protein. The splicing products were fractionated on a 6% polyacrylamide gel containing 8 M urea, 100 mM Tris-borate (pH 8.3). The identities of the products are indicated schematically; RRE is indicated by a black box; open boxes and thin lines correspond to the exons and intron, respectively. The splicing products include intact pre-mRNAs (two lower bands), lariat introns (two main upper bands), and intermediate lariat composed of intron and 3’ exon (appear as a weak band just above each lariat product band and marked with an asterisk). Ligated exons and 5’ exons migrated near the bottom of the gel and are not shown. Owing to topological constraints, lariat products migrate more slowly than the pre-mRNAs on high percentage gels. The ligated exons migrate faster than the pre-mRNA and in a region that contains a substantial amount of degraded RNA. It is therefore convenient to use the yield of lariat as a measure of splicing efficiency. The splicing of the RRE-containing substrate was inhibited 4 fold as compared with the internal control substrate at 0.15 μg/ml Rev protein.

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Figure 3. Specific Inhibition of Splicing of β-Globin Pre-mRNA Derivatives by Rev 34-50

(A) Splicing of PALN1/RRE (containing the RRE) and PAL4 (internal control) pre-mRNAs in the presence of the indicated amount of Rev 34-50.

(B) Splicing of the control PALN1/GLO pre-mRNA. The splicing products were fractionated on a 6% polyacrylamide gel containing 6 M urea, 75 mM Tris-borate (pH 8.3). The identities of the products are indicated schematically (see legend to Figure 2). Ligated exons and 5' exons migrated near the bottom of the gel and are not shown. NE indicates the absence of both Rev 34-50 and nuclear extract.

(C) Graphic representation of the inhibitory effect by Rev 34-50 on the splicing of β-globin-derived substrates. The level of specific inhibition of splicing was calculated as follows: for each concentration of Rev 34-50, the yield of intron lariat, containing the RRE or the control fragment, GLO, was divided by the yield of intron lariat from the control substrate without insert. The numbers were normalized to 1 in the absence of Rev 34-50, and plotted as a function of Rev 34-50 peptide concentration.

Variability in RNA recovery and splicing efficiency in the reactions was controlled by mixing the RRE-containing transcript with an identical transcript lacking the RRE.

Substrate RNA containing the wild-type 5' splice site was efficiently processed, whereas the substrate containing the mutated 5' splice site was processed at a significantly lower efficiency (data not shown). Titration of purified Rev protein into the reaction specifically inhibited splicing of the RRE-containing substrate 3- to 4-fold more than the control substrate lacking the RRE (Figure 2). Specific inhibition of splicing is generally measured by the ratio of the lariat produced from the pre-mRNA containing an insert and the lariat produced from the internal control pre-mRNA without insert and normalized to 1 in the absence of Rev. Maximum specific inhibition was observed at 0.1-0.2 µg/µl Rev in a reaction consisting of 25% nuclear extract (final concentration of nuclear protein = 5 mg/ml) and 1 mg/ml E. coli tRNA. The tRNA was added to control for nonspecific binding of the Rev protein. At higher concentrations of Rev (>0.2 µg/µl), nonspecific inhibition occurred and the processing of both transcripts was inhibited. The specific inhibition of RRE-containing mRNA splicing in vitro did not require a suboptimal 5' splice site, as observed for Rev responsiveness of the β-globin intron in vivo (Chang and Sharp, 1989). In fact, observation of specific inhibition was easier with RNA containing wild-type splice sites, owing to the overall higher level of splicing (data not shown). Thus, all subsequent experiments were performed using substrates containing wild-type splice sites.

A Peptide of Rev Specifically Inhibits RNA Splicing In Vitro

In a parallel study of the interaction of Rev and the RRE, we have shown that a 17 amino acid peptide (Rev 34-50) spanning the basic domain of Rev can specifically bind to multiple sites on the RRE (J. K., A. D. F., and P. A. S., submitted). The sites of Rev 34-50 binding were almost identical to the binding sites of intact Rev. Substitution of the Rev 34-50 peptide in the above protocol specifically inhibited splicing of the RRE-containing β-globin pre-mRNA by up to 15-fold, as compared with the control pre-mRNA lacking the RRE (Figures 3A and 3C). This level of specific inhibition by Rev 34-50 was significantly greater than that observed with intact Rev. Maximal specific inhibition occurred at 16-20 ng/µl Rev 34-50 in a reaction consisting of 33% nuclear extract (final concentration of nuclear protein = 7 mg/ml) and 0.5 mg/ml E. coli tRNA. As for intact Rev protein, the level of carrier tRNA was critical to obtain specific inhibition by Rev 34-50. To rule out the possibility that inhibition was due to a difference in lengths of the substrate RNAs, an unrelated control fragment of similar length to the RRE (220 bp) was inserted in the β-globin pre-mRNA at the same position as the RRE (see Figure 1A). Splicing of this control construct was only inhibited at high levels of Rev 34-50 (>20 ng/µl; Figures 3B and 3C).

Unlike most splicing regulatory sequences that are position specific, the RRE will affect the production of unspliced RNA in vivo when inserted at various positions within an intron or flanking exons. To examine the consequence of changes in the RRE position in vitro, the RRE and the β-globin control fragment were inserted into any one of four different positions in PiP7.A (see Figure 1B; M. Moore, unpublished data). This plasmid is a modified version of the first intron in the major late transcript of adenovirus (Konarska and Sharp, 1986; Garcia-Blanco et al., 1989). The RRE or the control fragment was inserted at two different positions within the intron (13 and 71 nucleotides from the 5' splice site) and within the 5' and 3' exons.
(see Figure 1B). The two pre-mRNAs containing the RRE within the intron exhibited strong specific inhibition of splicing by Rev 34-50. When the RRE was located 13 nucleotides downstream from the 5' splice site, splicing was inhibited up to 15-fold as compared with a PIP.7A RNA without any insert (Figures 4A and 4C) and up to 30-fold as compared with the control RNA containing the β-globin-derived insert (Figures 4B and 4C). Similar specific inhibition was observed when the RRE was located 71 nucleotides from the 5' splice site (Figure 4C).

Insertion of the RRE and the control fragment into the 5' exon or 3' exon, respectively, yielded a different result (see Figure 1B). Only about 2-fold specific inhibition of splicing was observed with the transcript containing the RRE in the 5' exon as compared with a control RNA of similar length, and no significant specific inhibition was observed with the RRE located in the 3' exon (data not shown). The lack of specific inhibition of pre-mRNA containing the RRE in the 3' exon may be due to an overall decrease in splicing efficiency of this construct (about 5% of the level of wild-type splicing; data not shown). The lack of specific inhibition of splicing of introns derived both from β-globin and adenovirus genes.

Specificity of Inhibition Depends on the Specificity of the Peptide/RRE Interaction

Rev 34-50, as compared with other basic peptides that bind RNA, was examined for specific inhibition of splicing using substrates derived from the β-globin gene (PAIL/...
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Table 1. Summary of Peptides Used and Their Functional Characteristics

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
<th>Binding to IIB RNA</th>
<th>Specificity for IIB RNA</th>
<th>Level of Splicing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev 34-50</td>
<td>TRQARRNRRRWRERQR</td>
<td>0.70</td>
<td>++</td>
<td>0.11</td>
</tr>
<tr>
<td>RPIA,</td>
<td>TRQARRNRRRWRERQR</td>
<td>0.78</td>
<td>+</td>
<td>0.39</td>
</tr>
<tr>
<td>RPIAD,</td>
<td>TRQARRNRRRWRERQR</td>
<td>0.09</td>
<td>nd</td>
<td>0.57</td>
</tr>
<tr>
<td>RP/Scram</td>
<td>RWRRERTRQRRNQRARR</td>
<td>0.07</td>
<td>nd</td>
<td>0.96</td>
</tr>
<tr>
<td>R1</td>
<td>YRRRRRRRRRRRRAAAA</td>
<td>0.59</td>
<td>-</td>
<td>0.82</td>
</tr>
<tr>
<td>R1*</td>
<td>YRRRRRRRRRRRAAAA</td>
<td>0.61</td>
<td>nd</td>
<td>0.78</td>
</tr>
<tr>
<td>R3</td>
<td>YRRRRRRRRRRRAAAA</td>
<td>nd</td>
<td>nd</td>
<td>0.62</td>
</tr>
<tr>
<td>K9</td>
<td>YKKKKKKKKKKKAAAA</td>
<td>nd</td>
<td>nd</td>
<td>1.14</td>
</tr>
</tbody>
</table>


† The estimate of peptide specificity is based on the relative binding efficiency to wild-type and mutated IIB RNA in the presence of 20 ng/μl carrier tRNA (J. K., A. D. F., and P. A. S., submitted); ++, high specificity, more than 10-fold difference; +, medium specificity, 2 to 10-fold difference; -, low specificity, less than 2-fold difference.

‡ Level of PAUN1/RRE RNA splicing relative to PAL4 RNA splicing measured in the presence of 25 ng/μl Rev 34-50 and normalized to 1.00 in the absence of peptide (see legend to Figure 3C).

nd, not determined. Abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; K, Lys; N, Asn; R, Arg; Y, Tyr; W, Trp.

N1/RRE and PAL4, Figure 1). Various synthetic peptides whose binding to a monomeric site within the RRE had been previously characterized (J. K., A. D. F., and P. A. S., submitted) were used (Table 1). The splicing of the substrate RNAs was examined in the presence of varying concentrations of mutant Rev 34-50 peptides, including the substitution of tryptophan 45 with alanine (RP/A45), arginines 42 and 43 with alanines (RP/A42/A43), and a scrambled sequence (RP/Scram; Figure 5A). In addition, several basic amino acid polymers including 15 arginines (R15), 12 arginines (R12), 9 arginines (R9), and 9 lysines (K9) were tested (data not shown). Each of these peptides inhibited splicing substantially less than Rev 34-50. The level of specific inhibition proceeding from high to low is as follows: Rev 34-50 > RP/A45 > RP/A42A43 > R9 = R12 = R15 > RP/Scram > K9 (Figure 5B). Mobility shift assays using these peptides had shown that RP/A45, R9, R12, and R15 all bind with approximately the same affinity to a single binding site of the RRE as compared with the binding of Rev 34-50 (J. K., A. D. F., and P. A. S., submitted; Table 1). However, the binding specificities of these peptides (comparison of the efficiency of binding with either a wild-type or mutant site) were all reduced as compared with wild-type Rev 34-50, with the relative order of binding specificity following that demonstrated for specific inhibition of splicing (J. K., A. D. F., and P. A. S., submitted; Table 1). Thus, the degree of specific splicing inhibition of the RRE-containing substrate correlates closely with the specificity of peptide binding, not the affinity of the peptide for the RRE.

Multiple Copies of a Defined Rev-Binding Site Can Substitute for the RRE RNA

The RRE specifically binds four to eight Rev molecules (Daly et al., 1989; Cock et al., 1991; Kjems et al., 1991; Malim and Cullen, 1991). To test whether this multiplicity of Rev binding is important for specific inhibition of splicing by Rev 34-50, templates containing either one or six copies of a high affinity Rev-binding site were inserted 13 nucleotides downstream from the 5′ splice site in Pip7.1 (see Figure 1B). One of these constructs (Pip/B1/1 x IIB, Figure 5A) was employed in splicing experiments (Figure 1B). One of these constructs (Pip/B1/1 x IIB, Figure 5A) was employed in splicing experiments (Figure 1B).
1B) contained a 39 nucleotide fragment (IIB, including position 43–79 of the RRE; J. K., A. D. F., and P. A. S., submitted), which has been shown to bind one molecule of Rev or Rev 34-50 (J. K., A. D. F., and P. A. S., submitted). Another construct (PIPB1/6 × IIB, Figure 1B) contained six tandem repeats of this binding site inserted at the same site. As estimated from mobility shift assay, these RNAs bind one and six Rev or Rev 34-50 molecules, respectively (J. K., unpublished data). To control for recovery and splicing efficiency of the RNA substrate, PIP7.A pre-mRNA was also included in the reaction. Even at high peptide concentrations no specific splicing inhibition of the substrate containing only one peptide binding site was observed (Figures 6A and 6C). In contrast, the splicing of the substrate containing six binding sites was specifically inhibited 20-fold, at 35 ng/ml peptide, as compared with the control transcript (Figures 6B and 6C). This specific inhibition is comparable with that observed with the wild-type RRE (Figure 6C), suggesting that the number of peptides bound, and not the structural integrity of the RRE, is important for specific inhibition of splicing by the Rev peptide.

**Rev 34-50 Interferes with Spliceosome Assembly In Vitro**

Pre-mRNA splicing is a stepwise process involving the assembly of the spliceosome. Approximately 20–25 min of incubation is required for the detection of splicing products of the β-globin pre-mRNA in vitro (Lamond et al., 1987). To determine the stage of spliceosome assembly at which the Rev 34-50 functions, peptide was added to a splicing reaction at different time points. In splicing reactions containing the β-globin transcripts PAL1/RRE and PAL4 as substrates (see Figure 1), Rev 34-50 was added 20 min before, or 5 s, 1 min, 5 min, or 15 min after addition of nuclear extract, and incubation was continued for an additional 2 hr (Figure 7). Preincubation of the substrate with the peptide for 20 min did not change the level of inhibition. In contrast, addition of the peptide 15 min after addition of the nuclear extract had no effect on splicing. This suggests that the peptide interferes with a spliceosome and that the peptide cannot dissociate preassembled spliceosomes.

The structure of the spliceosome, assembled in the absence and presence of Rev 34-50, was analyzed by fractionation of splicing reactions on sucrose gradients (Grabowski et al., 1985). The β-globin pre-mRNA, with and without RRE, was incubated with 25 ng/ml peptide for 2 hr under splicing conditions. Under these conditions a 15-fold specific inhibition of splicing of the RRE-containing RNA was observed (data not shown). The reactions were then fractionated on sucrose gradients. In the absence of Rev 34-50, both RRE- and non-RRE-containing transcripts formed 30–40S and 60S complexes (Figures 8A and 8B), consistent with previous results (Grabowski et al., 1985). The 30–40S peak is probably composed of pre-mRNA
Regulation of mRNA Splicing by Rev Peptide

Rev 34-50 was added to the splicing reaction containing a mixture of PAL/N1/RRE and PAL4 pre-mRNAs at the times (relative to the addition of nuclear extract) indicated, and the incubation was continued for 2 hr. - and + denote that the peptide was added before and after nuclear extract, respectively. Adding the peptide 15 min, or later, after addition of nuclear extract had no effect on the level of splicing. The identities of the products are indicated schematically.

Figure 7. Time Course for the Addition of Rev 34-50

Rev 34-50 was added to the splicing reaction containing a mixture of PAL/N1/RRE and PAL4 pre-mRNAs at the times (relative to the addition of nuclear extract) indicated, and the incubation was continued for 2 hr. - and + denote that the peptide was added before and after nuclear extract, respectively. Adding the peptide 15 min, or later, after addition of nuclear extract had no effect on the level of splicing. The identities of the products are indicated schematically.

bound to U1, U2, and a number of hnRNPs; the 60S peak contains fully assembled spliceosomes (Grabowski et al., 1985; Konarska and Sharp, 1986). In reactions containing the Rev 34-50 peptide, the profile of the RNA substrate lacking the RRE remains unchanged (Figure 8C), whereas the RNA substrate containing the RRE forms only one 50S complex (Figure 8D). The composition of this 50S peak remains to be determined. The lack of 60S spliceosomes formed on the RRE-containing pre-mRNA in the presence of Rev 34-50 may explain the decreased yield of splicing products.

Discussion

Regulatory mechanisms of alternative RNA splicing and of mRNA transport from the nucleus have not been extensively studied in vitro. Although several regulators of splicing have been identified in vivo, few of these factors have been purified and shown to regulate splicing in vitro. The finding that Rev or its 17 amino acid RNA-binding domain can specifically inhibit splicing of RRE-containing introns offers an opportunity to study the detailed mechanism of RNA splicing as well as Rev function. The specificity of inhibition of splicing by Rev-related peptides correlates closely with the specificity of the peptides binding to the RRE, suggesting that specific binding is the basis of the inhibition. Inhibition requires several binding sites, which can be provided by the wild-type RRE or by six repeated high affinity binding sites. The reduction in splicing efficiency is accompanied by an accumulation of splicing complexes that sediment slower than the complete 60S spliceosomes.

Previous experiments (Chang and Sharp, 1989) have shown that an intron from a wild-type β-globin pre-mRNA, containing the RRE, was not Rev responsive in vivo but that mutation of either of the splice sites rendered the intron Rev responsive. Since Rev regulates introns of HIV-1, which contain intact splice sites, it was proposed that the action of Rev is kinetically slower than that of the rapid splicing of some introns. In vitro, Rev and related proteins inhibit the splicing of the β-globin introns containing wild-type splice sites. This difference between the responsiveness of different forms of β-globin introns may reflect the fact that splicing in vitro, even of the most active introns, is highly inefficient as compared with that observed in vivo, perhaps allowing Rev to more easily compete with splicing factors. The observation that Rev 34-50 inhibits splicing when added 1–2 min after nuclear extract addition, but has no effect when added 15 min later, suggests that Rev 34-50 must bind the substrate RNA during spliceosome assembly and that preassembled spliceosomes are insensitive to the peptide. In a similar vein, preincubation of the substrate RNA and Rev peptide did not enhance the levels of specific inhibition, implying that the kinetics of peptide binding in vitro are more rapid than the kinetics of spliceosome assembly.

The range of Rev and Rev 34-50 concentrations required for specific inhibition of splicing is similar: 1–10 μM. This is 100- to 1000-fold higher than the Rev-RRE dissociation constant defined in the absence of nonspecific RNA (Daly et al., 1989; J. K., A. D. F., and P. A. S., submitted). However, in reactions containing 0.5–1.0 mg/ml tRNA, both Rev and Rev 34-50 bind to a single high
affinity site in the RRE with an apparent dissociation constant of 100–200 nM (J. K., A. D. F., and P. A. S., submitted) and with an apparent dissociation constant near 1 μM in the presence of 1 mg/ml tRNA and >7 mg/ml of nuclear protein (splicing conditions, J. K., unpublished data). The high concentration of Rev and Rev 34-50 needed to generate specific binding in the splicing reaction and to inhibit splicing in vitro reflects titration of the protein against the high concentration of nonspecific RNA present. The degree of change in the concentration of free Rev peptide available for binding during titration against this background of nonspecific binding is quite sharp. For example, nonspecific complexes with probe RNA were not observed in splicing reactions at concentrations less than 15 μM Rev or Rev 34-50, but at concentrations that are only 33% higher (20 μM), high molecular weight aggregates were formed (data not shown). This also suggests that the nonspecific inhibition of splicing by basic peptides that occurs at peptide concentrations of approximately 20 μM or greater is probably due to nonspecific aggregation of the substrate RNA.

Regulation of splicing in vitro by Rev 34-50 has many properties in common with Rev’s regulation of synthesis of unspliced RNA in vivo. First, both reactions are not intron specific. The Rev 34-50 peptide inhibits the splicing of introns derived from α-globin gene and from adenovirus genes. Second, regulation by Rev 34-50 in vitro requires the presence of an RRE or a number of high affinity Rev-binding sites in a cis position within the intron. The two processes are also similar in that the particular position of the RRE within the intron is not critical. However, unlike the reaction in vivo, where Rev can regulate introns from positions in the flanking 5’ and 3’ exons, effective specific regulation by Rev 34-50 in vitro is only observed when the RRE is present within the intron. It is not uncommon that reactions in vitro are less sensitive to the action of regulatory factors that bind at a distal site than the equivalent reaction in vivo. For example, transcription factors that bind to enhancer elements stimulate transcription at a promoter site separated by much longer lengths of DNA in vivo than in reactions in vitro. Consistent with the activity of Rev in vivo, the splicing reaction in vitro is inhibited before the first step in the reaction, i.e., cleavage at the 5’ splice site. Thus, Rev inhibition of splicing could generate unspliced pre-mRNA for transport to the cytoplasm.

How does a short peptide specifically inhibit splicing in vitro? The fact that addition of Rev 34-50 arrests splicing of pre-mRNA in the form of a 50S complex suggests that several cellular factors, and perhaps snRNPs, may be required for regulation. It is known that both Rev and Rev 34-50 binding induces a significant change in the structure of either the intact RRE or the IIB RNA containing a single binding site (Daly et al., 1990; J. K., A. D. F., and P. A. S., submitted). The conformational change probably consists of stabilization of a more extended duplex structure that forms the high affinity binding site of Rev or Rev 34-50. It is possible that this conformational change may form binding sites for cellular factors, e.g., splicing factors, which subsequently inhibit splicing or interfere with the binding of additional required factors. Alternatively, some part of the basic region of Rev might directly contact the hypothetical regulatory cellular factors, but only after binding to the RRE. Our result suggests that multiple peptides must interact specifically with the RRE to observe regulation, but that the general overall RRE structure is not crucial since a simple repeat of a single peptide-binding site is functional. The most direct evidence that the specific interactions of Rev 34-50 with the RRE are responsible for the specific inhibition of splicing in vitro is the lack of similar activity of a number of basic peptides that bind the RRE with a comparable affinity but nonspecifically. For example, a polyarginine peptide binds the RRE with a comparable affinity to the Rev 34-50 peptide, but does not specifically inhibit splicing of RRE-containing substrates.

Mutational studies suggest that the Rev-dependent in vitro splicing system described above cannot reflect the full range of Rev activities in vivo. This in vitro system is completely responsive to a Rev peptide that contains sequences from 34-50, while Rev activity in vivo is abolished by mutations outside this domain (Malim et al., 1989b; Olsen et al., 1990b; Hope et al., 1990). Change in sequences immediately flanking the Rev 34-50 region generate mutants that are defective for oligomerization of the Rev protein in vitro and Rev activity in vivo (Malim and Cullen, 1991; M. L. Zapp and M. R. Green, personal communication). This suggests that at effective intracellular concentrations, oligomerization of Rev is critical for activity, and mutants defective for this property, but active for specific binding, do not have Rev function. As discussed above, the specific inhibition by Rev 34-50 requires the binding of multiple peptides and thus may duplicate the oligomerization state essential in vivo. In vitro, oligomer binding can result from the addition of high concentrations of Rev and may not be dependent upon the presence of the particular residues important for oligomerization at intracellular concentrations. Additionally, mutations in a cluster of leucines near residue 80 inactivate Rev in vivo and also produce an intracellular product with a negative transdominant phenotype (Malim et al., 1989b). Thus, the short cluster of six amino acids flanking position 80 probably specifies a transactivation domain of Rev in vivo that is not required for inhibition of splicing in vitro. A possible integration of these mutational results with the specific inhibition of splicing observed with Rev 34-50 in vitro would be to suggest that Rev both regulates splicing of the premRNA through the binding domain and effects transport or release to the cytoplasm through the transactivation domain flanking position 80. The in vitro system would be expected to be responsive only to the splicing regulation step. Thus, it is likely that the inhibition of splicing observed in vitro upon addition of Rev reflects one step in a multistep process in vivo that regulates the synthesis of incompletely spliced cytoplasmic mRNA.

Experimental Procedures

Plasmid Constructions

PAL4 and PAL5 (D. D. Chang, unpublished data) contain the BamH-EcoRI insert of pBSAL4 and pBSAL5 (Lamond et al., 1987), respectively, cloned into the BamH-EcoRI sites of pGEM3 (Promega). PAL4 and PAL5, which are identical except for a GT to AT mutation at the
SPlice site of PAL5, contains 19 bp of the second exon, 357 bp of the second intron, and 54 bp of the third exon of the rabbit β-globin gene. PAL31/REV and PAL31/GLO were prepared by cloning a blunt-ended 253 bp KpnI–SacI fragment (REV) of pM7+ (Malim et al., 1988) or a 220 bp HincII–SpII fragment (GLO) from the second intron of wild-type β-globin (Padgett et al., 1985), respectively, into a blunt-ended Ncol site of PAL4.

PIP7A (M. Moore, unpublished data) is a modified version of PIP3 (García-Blanco et al., 1988). It contains a 55 bp 5 exon, a 125 bp intron, and a 56 bp 3′ exon. The PIP7A derivatives were prepared by cloning either the RRE or GLO DNA fragments into either blunt-ended EcoRI, BamHI, Sall, or PstI sites as shown in Figure 1.

**Construction of PIP/B1/b IIB and PIP/B1/b IIB**

Two overlapping oligonucleotides, IIB (5′-CGTATGCCGCACGCCCTCTGAGCGCACTGACGTCATCAG-3′; corresponding to a single Rev-binding site; J. K., A. D. F., and P. A. S., submitted) and IIB (5′-CGTATGCCGCACGCCCTCTGAGCGCACTGACGTCATCAG-3′; overlapping IIB) were synthesized. One microgram of each oligonucleotide was phosphorylated using T4 polynucleotide kinase (NEB), annealed by heating to 100°C for 3 min and slow cooling to 37°C, and ligated using T4 DNA ligase (NEB). The ligated oligonucleotides were partially cleaved with 1 U of HpaII (NEB) for 30 min, fractionated on a 5% nondenaturing polyacrylamide gel, and fragments, corresponding to one and six copies of IIB, were purified. Both fragments were blunt ended and cloned into the Smal site of PBS+ (Stratagene) to produce the plasmids PIP7.A (M. Moore, unpublished data) is a modified version of PIP3. Two overlapping oligonucleotides, IIB (5′-GGTATGGGCGCAGCGCAG-3′; corresponding to a single Rev-binding site of PIP7.A derivatives were prepared by cloning either the RRE or GLO DNA fragments into either blunt-ended EcoRI, BamHI, Sall, or PstI sites as shown in Figure 1.

**Preparation of Rev Protein, Peptides, and RNA Transcripts**

Rev was prepared as described previously (Kiem et al., 1991, 1991b), and the peptides were prepared as described by Calnan et al. (1991a, 1991b). Transcripts of uniformly labeled, GpppG-capped pre-mRNAs were prepared by run-off transcription in vitro using standard conditions (Lamond et al., 1987). [α-32P]UTP (NEN, 3000 Ci/mmol) was incorporated into the splicing reaction. Some variation in the level of Rev−peptide binding was observed between individual preparations of nuclear extract. Gels were quantitated using a Molecular Dynamics Phospho Imager and Image Quant™ software v.5.0.

**Sucrose Gradients**

Splicing reactions (60 µl) were incubated in the presence of 25 ng/µl Rev 34-50 under standard conditions. Some variations were made to allow for convenient specific inhibition. The remaining 50 µl was loaded onto 5 ml of 10% to 30% sucrose gradients as described by Agrin et al. (1989). About 25 fractions were collected from the bottom of the tube and 3P content was measured by densitometry. RNA was isolated from pooled peak fractions and RNA content was analyzed by densitometry and gel electrophoresis.

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