Alterations in the Polypyrimidine Sequence Affect the in Vitro Splicing Reactions Catalyzed by HeLa Cell-free Preparations*

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The polypyrimidine tract, located at the 3’ end of intron 1 of the adenovirus major late transcript, was studied for its role in splicing using cell-free preparations isolated from HeLa cells. A plasmid (plz) was constructed in which seven purine bases were substituted for pyrimidine bases within the 14-nucleotide polypyrimidine sequence. Runoff transcripts extending to the middle of intron 2 were tested for their ability to support in vitro splicing. The efficiency of these reactions was compared with pre-mRNA transcripts made from the wild-type nonmutated plasmid (plz-2). Neither spliced products nor splicing intermediates were detected in reactions with the plz pre-mRNA. The formation of the nucleoprotein complexes involved in splicing was examined with this altered pre-mRNA. No 5S splicing complex was detected and only low levels of the 30S presplicing complex formed (30-fold less than with wild-type pre-mRNA). However, when a longer runoff transcript was prepared from the polypyrimidine mutated plasmid plz, spliced RNA was formed. This activity required specific downstream sequences, since transcripts produced from plz which contained substituted downstream sequences were not spliced.

Although intron 2 of the adenovirus major late transcript does not contain a discernible 3’ polypyrimidine sequence, pre-mRNA (p2-3) containing this intron was efficiently spliced. However, when the 3’ region of intron 2 was substituted for the polypyrimidine sequence of intron 1, the resulting pre-mRNA did not support efficient splicing in vitro. However, when the polypyrimidine sequence of intron 1 was substituted for the sequence at the 3’ end of intron 2, efficient splicing occurred, and the rate of formation of splicing intermediates and the accumulation of nucleoprotein complexes was greater than with the wild-type pre-mRNA (p2-3).

The consensus sequence of the 5’ and 3’ splice sites of pre-mRNAs has been determined from a large number of known intron sequences (1–3). These compilations identified the 5’ consensus sequence (A)AG/GU(AG)AGU, whereas the 3’ consensus sequence contains a run of pyrimidines followed by a nonconserved sequence, a pyrimidine, and then the invariant AG dinucleotide at the intron-exon border ((U/C)n(N(C/U)AG/G)).

Alterations of the 3’ conserved sequence and their effects on splicing have been studied both in vivo and in vitro. In vivo studies have shown that deletion of the 3’ AG dinucleotide partially reduced the efficiency of the first step in splicing (formation of the 5’ exon and intron-exon lariat), removal of the polypyrimidine region markedly reduced this step (7, 8). When the 3’ AG dinucleotide was changed to AC, splicing was abolished and reduced levels of 5’ exon and intron-exon lariat were detected (9). Thus, changes in the 3’ intron sequence markedly affect both steps in the splicing reaction. Interestingly, Fu et al. (10) have shown that changes in the polypyrimidine sequence of the SV40 viral early region altered the selection of the small tumor antigen and large tumor antigen 5’ splice sites without affecting the level of spliced products formed. These results suggest that the polypyrimidine site should be recognized by a protein component of the overall splicing and indeed a small nuclear ribonucleoprotein-associated protein with this property has been detected (11–13). Alterations in the polypyrimidine sequence reduced the binding of this factor (11). However, this factor has not been purified or further characterized.

A number of introns lack a polypyrimidine sequence at their 3’ ends (2). In several cases, such introns are involved in alternative splicing reactions (14–16). This is the case with intron 2 of the adenovirus major late transcript which separates exons 2 and 3. In the middle of intron 2 an alternatively spliced exon is present, which has been called the “i leader.” This exon is found only in late mRNAs whose main nucleotide sequences lie just downstream of the tripartite leader, such as the 52- and 55-kDa mRNAs (17, 18). However, most late mRNAs of adenovirus do not contain the i leader.

In the studies presented here, we have investigated the role of the polypyrimidine sequence in splicing, using base substitutions in this region rather than deletions. The plasmid p1-2 (previously named pKT.1) was used as the wild-type pre-mRNA because it is small (having an intron of 86 nucleotides) and well characterized (9, 19–22). It has been used extensively by our laboratory as a substrate for examining the effects of sequence changes in pre-mRNA on splicing (9, 19). In all of these mutational studies, no cryptic 3’ or 5’ splice sites were activated, and only a single alternative branch point has been detected (19). The experiments presented here focus on the introns of the adenovirus 2 tripartite leader of the major late transcription unit. Intron 1 contains a prominent polypyrimi-

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idine tract, whereas intron 2 lacks a discernible polypyrimidine sequence. The effects of alterations in the 3′ regions of these two introns were examined in pre-mRNAs containing both introns as well as pre-mRNAs containing only one intron.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis—The construction of plasmid pl-2 (previously known as pKT.1) has been described (22). A detailed description of the construction of other plasmids is presented in the Miniprint section at the end of this paper.

In Vitro Splicing Reactions—32P-Labeled transcripts were prepared by linearizing plasmids with the appropriate restriction enzyme (ScaI for cleavage in exon 2 and either PstI or HindIII for cleavage in exon 3), followed by incubation with SP6 RNA polymerase as described previously (22). The formation of spliced products and splicing complexes was measured in reaction mixtures (50 µl) containing 20 mM Hepes-KOH buffer (pH 7.6), 3 mM MgCl₂, 10 mM creatine phosphate, 0.4 mM ATP, 2% polyethylene glycol 8000, 2 mM dithiothreitol, 32P-labeled pre-mRNA (1-5 pmol, 2 × 10⁶ cpm/pmol), and nuclear extract (400 µg of protein) (23) incubated at 30 °C for the time indicated. Reactions were stopped by the addition of 7 volumes of a solution containing 1% sodium dodecyl sulfate, 0.3 M NaCl, and 2 mM EDTA. RNA was extracted with phenol and chloroform and ethanol-precipitated. The RNA was collected by centrifugation, resuspended in 3 µl of 80% formamide, heated to 80 °C for 2 min, and electrophoresed on polyacrylamide-urea gels using Tris-borate-EDTA as the running buffer. Splicing products and intermediates were quantitated by excising the appropriate bands from the dried gel following autoradiography and measuring their radioactive content by scintillation counting.

Formation and Analysis of Splicing Complexes—The partially purified fractions used for generating nucleoprotein complexes have been described previously (20). 32P-Labeled transcripts were incubated under the same conditions as described above for the standard splicing reaction, except that appropriate fractions (10 µl of fraction II (3-4 mg/ml) and fraction II plus 5 µl of fraction Ib (4 mg/ml)), isolated from nuclear extract of HeLa cells, were substituted for unfractonated nuclear extracts. Reactions were incubated at 30 °C for 2 h and then loaded directly onto 10-30% sucrose gradients, prepared, and centrifuged as described previously (20). Nucleoprotein complexes were also analyzed by neutral polyacrylamide-agarose composite gel electrophoresis (3.5% polyacrylamide:bisacrylamide 80:1 and 0.5% agarose using 1/4 Tris-borate-EDTA as the running buffer).

RESULTS

The Polypyrimidine Tract Is Required for Splicing—It has been shown previously that the polypyrimidine sequence located at the 3′ end of introns is required for efficient splicing in vitro (7, 8). These studies were carried out with pre-mRNAs in which all or part of this sequence was deleted. Such deletions resulted in a reduction in the level of spliced RNA in which all or part of this sequence was deleted. Such deletions were used. By this method, splicing was examined using pre-mRNAs in which base substitutions rather than deletions were used. By this method, the observed effects can be attributed to the perturbation of the sequence and not the positional changes caused by the deletion of nucleotides.

The wild-type pre-mRNA used in these studies was transcribed from plasmid pl-2 and contains intron 1 of the adenovirus major late pre-mRNA. This substrate, which was described previously as plasmid pKT.1 (18), is shown schematically in Fig. 1A. Plasmid plz was constructed so that

FIG. 1. Analysis of products and splicing complexes formed with pre-mRNAs pl-2 (wild-type) and plz (polypyrimidine-altered). A schematic summary of the structures of pre-mRNAs pl-2 and plz. In this figure, boxes represent exons, solid lines denote introns, and hatched boxes indicate splicing sequences. The sequences above and below the drawings are the nucleotides present in the polypyrimidine region of each transcript. The numbers below the line indicate the size of each exon or intron. B, the transcripts pl-2 and plz were synthesized from ScaI-digested plasmid DNA producing runoff products of 180 nucleotides. Following incubation with nuclear extract, as described under "Materials and Methods" for the indicated period, the RNA was isolated and the products separated on 18% polyacrylamide-urea gels. Lanes 1-4 show the products formed from pre-mRNA pl-2 after incubation for 0, 30, 60, or 120 min, respectively. Lanes 5-8 show the products produced by incubation of plz pre-mRNA with nuclear extract under splicing conditions for 0, 30, 60, or 120 min, respectively. The diagrams at the left of the gel represent the structures of the RNAs present in the adjacent gel bands. C, pre-mRNAs prepared from pl-2 and plz were incubated for 2 h with fractions derived from nuclear extract (fractions II and Ib (20)) as described under "Materials and Methods" and the complexes separated by electrophoresis on native acrylamide-agarose gels; gels were then dried and autoradiographed. The sedimentation values of the complexes determined by sucrose gradient centrifugation are shown on the left. The positions of complexes formed in the absence of ATP are also shown. The fractions used to generate the various complexes are indicated below each lane. Several of the pyrimidine residues within the polypyrimidine tract of the intron 1 were changed to purines (Fig. 1A). When transcripts prepared from plasmid plz were used as substrates in the in vitro splicing reaction, no intermediates or products were detected (Fig. 1B). The amount of spliced RNA formed after 2 h of incubation was at least 40-fold greater with the wild-type pre-mRNA (lane 4) than with the mutated pre-mRNA (lane 8).

In order to determine which reaction in the splicing process was affected by the alteration of the polypyrimidine sequence, wild-type and mutated transcripts were tested for their ability to support the formation of nucleoprotein complexes. Pl-2 and plz pre-mRNAs were incubated with separated fractions prepared from nuclear extracts. Earlier studies showed that incubation of wild-type pre-mRNA with these fractions resulted in the production of either a 30 S prespliceosome complex (fraction II alone) or the 55 S spliceosome complex (fractions II plus Ib) (20). Following incubation for 2 h at 30 °C, the reaction mixtures were loaded onto a nondenaturing polyacrylamide-agarose composite gel and the complexes separated by electrophoresis (Fig. 1C). Pre-mRNA plz sup-

2 Portions of this paper (including part of "Materials and Methods," and Fig. S1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

3 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

4 A, pl-2; B, plz; C, pl-2 plz
ported the synthesis of low levels of both 55 S splicing complex (Fig. 1C, lane 4) and 30 S presplicing complex compared to complexes formed with the wild-type pre-mRNA (approximately 30-fold; compare Fig. 1C, lanes 1 and 3). This result suggests that the polypyrimidine tract is required for efficient complex formation and indicates that it plays an important role in an early step in splicing.

Intron 2 Is Different Than Intron 1—Intron 2 of the adenovirus 2 major late mRNA lacks a discernible 3′ polypyrimidine sequence. The plasmid p2-3 was constructed in order to generate a pre-mRNA substrate containing this intron (Fig. 2A). When pre-mRNA p2-3 was incubated in the *in vitro* splicing reaction mixture, spliced RNA was produced (Fig. 2B). Quantitation of splicing products and intermediates formed in these reactions revealed that 20% of the pre-mRNA was spliced after 2 h at 30 °C.

To examine the influence of a polypyrimidine sequence at the 3′ end of intron 2, a new plasmid, pIIx, was constructed in which the sequence between the branch site and the 3′ splice junction of plasmid p2-3 was removed and replaced with the corresponding region of intron 1 (see Fig. 2A). In order to retain the branch point of intron 2 in modified pre-mRNA substrates the position of this site was determined. For this purpose, the intron-exon lariat formed from pre-mRNA p2-3 was isolated and the position of the 2′ to 5′ phosphodiester structure was determined by primer extension analysis. The branch point was mapped to a single adenine residue located 21 nucleotides upstream of the 3′ splice junction (data not shown; see Miniprint section for this sequence).

Pre-mRNA pIIx and pre-mRNA p2-3 were compared as substrates in splicing reactions (Fig. 2B). After 30 min of incubation, the level of spliced RNA formed with either pre-mRNA was approximately the same. However, the level of 5′ exon and intron-exon lariat formed was 6- to 10-fold greater with pIIx than with p2-3 transcript (Fig. 2B, lanes 2 and 6). After 2 h of incubation the level of final spliced product accumulated from pIIx pre-mRNA was only slightly higher (30%) than that found with p2-3 pre-mRNA (Fig. 2B, lanes 4 and 8). These results suggest that the presence of the polypyrimidine sequence in intron 2 markedly increased the rate of the first step of the splicing reaction but did not significantly increase the yield of the final product. This suggests that the second step may be the rate-limiting step in the overall splicing reaction.

The formation of nucleoprotein complexes was also examined with these RNA substrates. Pre-mRNAs from p2-3 and pIIx were incubated with fractions isolated from nuclear extracts, and the complexes were separated by sucrose gradient centrifugation. The closed circle symbols indicate the sedimentation profile observed when the pre-mRNA was incubated with fractions derived from nuclear extracts, and the products were separated by electrophoresis on 18% polyacrylamide-urea gels. Reaction mixtures incubated for 0, 30, 60, or 120 min with pre-mRNA p2-3 and pre-mRNA pIIx are shown in lanes 1-4 and 5-8, respectively. A diagram representing the structures of the RNA found in the adjacent gel bands is shown to the left of the figure. C, ribonucleoprotein complexes formed after incubation of p2-3 pre-mRNA or pIIx pre-mRNA with fractions derived from nuclear extracts as described under “Materials and Methods” separated by sucrose gradient centrifugation. The closed circle symbols indicate the sedimentation profile generated when the pre-mRNAs were incubated with fraction II alone, whereas the open circle symbols indicate the sedimentation profile observed when the pre-mRNA was incubated with fractions II plus Ia.

**Effects of the Presence of Intron 2 Sequence in Place of the Polypyrimidine Tract of Intron 1**—To determine whether the short uridine-rich region, . . . UUGUUGU . . ., present at the 3′ end of intron 2 functioned as a polypyrimidine tract, the plasmid ply was constructed from plasmid p1-2. Plasmid ply contains the sequence between the branch point and the 3′ splice junction of intron 2 inserted in place of the corresponding sequence of intron 1. This plasmid was used to generate transcripts which were then tested in the *in vitro* splicing reaction. As shown in Fig. 3B, low levels of spliced RNA were formed, indicating that this sequence can partially substitute for the polypyrimidine tract. The amount of spliced product formed after 2 h of incubation was considerably lower (8-fold) than the level of spliced RNA formed with the wild-type transcript p1-2 (Fig. 3B, lanes 4 and 8).

The ATP-dependent synthesis of the 30 and 55 S complexes with ply and p1-2 transcripts were examined by gel electrophoresis (Fig. 3C). The level of each complex formed with the non-polypyrimidine transcript (ply) was greatly reduced compared with the amount produced from the wild-type pre-
Fig. 3. Analysis of splicing reactions carried out with pre-mRNA ply (containing the 3' region of intron 2 inserted in place of the polypyrimidine tract of plasmid pl-2) or with pre-mRNA pl-2 (wild-type). The labeled pre-mRNAs were synthesized with SP6 RNA polymerase using ScaI linearized plasmid DNA as the template as described previously (20). A, a schematic summary of the ply and pl-2 pre-mRNAs is shown. The boxes indicate exons, solid lines introns, and hatched areas are plasmid sequences. The length of each exon and intron is indicated below each drawing. The sequences written above pl-2 and below ply indicate the differences between these two pre-mRNAs. The first A residue in each inserted sequence (reading left to right) is the site at which the branch structure is formed. B, pl-2 and ply pre-mRNAs were incubated with nuclear extract and the products separated by electrophoresis on 18% polyacrylamide-urea gel. Incubation of pl-2 pre-mRNA and ply pre-mRNA for 0, 30, 60, or 120 min are shown in lanes 1–4 and 5–8, respectively. The diagrams at the left of the figure indicate the structure of the RNA present in each adjacent gel band. C, nucleoprotein complexes, formed after incubating pl-2 or ply pre-mRNA with separated fractions as described under "Materials and Methods," were separated by electrophoresis on native polyacrylamide-agarose gels after which the gels were dried and autoradiographed. The sedimentation values of the complexes, determined by sucrose gradient sedimentation, are shown at the right of the autoradiograph. The position of ATP-independent complexes is also shown. The fractions used to generate the various complexes are indicated below each lane.

mRNA (20- and 30-fold less 30 and 55 S complex, respectively).

Downstream Sequences Influence the Requirement for a Polypyrimidine Tract—To examine the role of the polypyrimidine sequence on splicing of pre-mRNAs containing multiple introns, plasmids were constructed in order to generate the five different pre-mRNAs described in Fig. 4A. Each transcript was analyzed for its ability to support splicing. The results indicated that pre-mRNAs containing an altered polypyrimidine sequence of intron 1 (as shown with pre-mRNAs pIyIIx, pIyIIy, and pIzIIx), as well as a second intron, were efficiently spliced. This was in contrast to results obtained with pre-mRNAs containing a single mutated intron. A quantitative analysis of the products resolved by gel electrophoresis showed that the level of spliced RNA formed with pre-mRNA pIyIIy or pIyIIx was 90% of the level observed with the wild-type transcript synthesized from pl-2-3 (Fig. 4, lanes 9 and 12 compared with lane 3). This represented an 8-fold increase in the level of spliced RNA formed with the multiple intron pre-mRNA compared with the pre-mRNA ply which contained the single intron. In the case of the transcript, plzIIx, the level of spliced RNA was 40% of the spliced product formed from the wild-type pre-mRNA pl-2-3. In contrast, the single intron pre-mRNA plz yielded no spliced products. These results suggested that the efficiency of splicing of intron 1 containing an altered polypyrimidine site was influenced by downstream sequences.

5' Sequences from a Downstream Intron Can Overcome the Effects of an Upstream Polypyrimidine Mutant—To determine whether a complete second intron was required for the activation of an altered polypyrimidine sequence of intron 1, longer runoff transcripts from plasmids plz and pl-2 (referred to as plzHc and pl-2Hc, respectively) were prepared and
analyzed in the in vitro splicing reaction. These longer runoff transcripts contained a complete exon 2 and the first 27 nucleotides of intron 2. As shown in Fig. 5B, the plzHc transcript supported splicing at a level that was 40% of that observed with the wild-type transcript, pl-2Hc. The presence of this limited sequence added to plz (to form plzHc pre-mRNA) resulted in an increased level of excision of intron 1 as was found with the pre-mRNA pIzHx which contained two introns.

Examination of Fig. 5B, lanes 5 and 6, revealed that additional intron-exon lariat and intron lariat products were formed with pl-2Hc pre-mRNA (illustrated by arrows in Fig. 5B). Based on their altered gel migration, these products most likely represent a lariat formed at an alternative branch site located six nucleotides upstream of the normal branch site. This branch site was shown previously to be activated when the normal branch site of pl-2 was changed from an adenosine to a guanosine residue (19). Pre-mRNAs that formed a branch at this alternative adenosine site spliced normally (19).

The possibility that a specific sequence was required for the activation of plz was also examined. For this purpose, plasmids were constructed in which pl-2 and plz DNAs were cut at the ScaI site in exon 2 and ligated to plasmid sequences. The sequences involved are indicated in the Miniprint section at the end of this paper. The resulting plasmids, pl-2X and plzX, were then linearized with HincII so that RNA runoff transcription placed at the 3' ends of plzHc and pl-2Hc pre-mRNAs. As shown in Fig. 5B, plzHc pre-mRNA did not support the synthesis of spliced RNA, whereas pl-2XHc pre-mRNA did. These results demonstrate that specific sequences are present in plzHc pre-mRNA that are responsible for the activation of this altered intron.

**DISCUSSION**

In this study we have described experiments that examined the role of the polypyrimidine sequence in pre-mRNA splicing in vitro. The polypyrimidine tract has been shown to be required in pre-mRNA splicing (4–8) and is bound by components of the splicing apparatus (11–13). Using a series of mutated pre-mRNAs we examined the function of the polypyrimidine region at various stages in the splicing process. These results show that although the polypyrimidine tract is important, it is not always essential for splicing.

The wild-type transcripts used in these studies, pre-mRNAs pl-2 and p2-3, contained introns 1 and 2 of the Ad2 major late tripartite leader sequence, respectively. Although pre-mRNA p2-3 lacks a discernable polypyrimidine sequence, both pre-mRNAs were spliced efficiently by nuclear extracts. Consistent with the findings of Ruskin and Green (7) and Frensdewey and Keller (8), we found that extensive alteration of the polypyrimidine tract of intron 1, as shown with pre-mRNA plz, blocked splicing. Neither spliced RNA nor splicing intermediates were formed when plz pre-mRNA was incubated with nuclear extract.

We have previously reported the use of fractions derived from nuclear extracts to study the formation of splicing complexes (20). Following incubation of pre-mRNA plz with these fractions, little 30 S presplicing complex or 55 S splicing complex formed. This finding indicates that the polypyrimidine tract affects an early step in the splicing process.

These results suggest a strong requirement for the presence of a polypyrimidine tract in splicing of intron 1 and an apparent lack of such a requirement for intron 2 of the Ad2 major late pre-mRNA. One possible explanation is that the 3' end of intron two contains a sequence that can function in place of the polypyrimidine sequence. To test this possibility, the plasmid ply was constructed, in which the sequence between the branch point and the 3' splice junction of intron 1 was placed at the end of this paper. The resulting plasmids, pl-2 and p2-3, contained introns 1 and 2 of the Ad2 major late pre-mRNA. One possible explanation is that the following section illustrates that the role of the polypyrimidine sequence in pre-mRNA splicing is essential for the high levels of splicing but is not absolutely
required. To show that this effect was due to specific sequences, we constructed a new plasmid, plzXHc, in which the 65 additional nucleotides present in pre-mRNA plzHc were replaced with 55 nucleotides of plasmid sequence. When this pre-mRNA was incubated in nuclear extract no spliced RNA formed. These same 55 nucleotides had no effect when added onto the 3′ end of pre-mRNA pl-2 (pre-mRNA pl-2XHc).

Splicing reactions carried out with pre-mRNA plzHc resulted in the formation of two different branched lariat products. It is possible that a second branch point located at the adenosine residue located six nucleotides upstream of the normal branch site was used with this pre-mRNA. We have described previously the activation of this branch site with pre-mRNAs in which a guanosine residue replaced adenosine at the normal branch point (19). This result suggests that the polypyrimidine tract may play a role in branch point selection.

The effect of changing the length of the transcript on the requirement for the polypyrimidine tract in splicing indicates that the role of these sequences must be complex. Although the absence of a polypyrimidine tract in intron 2 suggests that this sequence is not required, the presence of this sequence increased the rate of the formation of 5′ exon and intron-exon lariats. The third exon of the Ad2 tripartite leader has a lariat structure did not lead to splicing, despite the fact that the polypyrimidine tract influences the selection of the 5′ donor site as observed for the SV40 early transcript (10).

The features governing splicing must include both specific sequence recognition and structural characteristics contained in ribonucleoprotein complexes. At present, only the two dinucleotide sequences at the 5′ and 3′ ends of introns are absolutely essential for splicing in higher eukaryotes. The sequence UACUAAC present in the small number of yeast introns is an essential third signal for splicing but only in that system. In view of the large number of introns present in higher eukaryotes and the added complexity of alternative splicing, it seems likely that additional structural features are required for the accuracy of the splicing reaction. It is evident that the formation of prespliceosome and spliceosome complexes, essential intermediates for splicing, do not guarantee that spliced RNA will ultimately form. We, and others, have shown that protein components (present in our fraction Ib) are required to further activate the spliceosome to generate the first covalent modification of the pre-mRNA (20, 24, 25). We have shown that formation of an intron-exon GpG (5′-2′) lariat structure rather than an intron-exon GpA (5′-2′) lariat structure did not lead to splicing, despite the fact that the branch site was formed at the normal distance from the 3′ splice site (19). Similar observations have been reported by Hornig et al. (26). How ribonucleoproteins interact with pre-mRNAs, juxtaposing and joining exons with the accuracy that is essential, remains to be elucidated.

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Role of Polypyrimidine Sequence in Splicing

Supplemental Material to:

Alterations in the Polypyrimidine Sequence Affect the In Vitro Splicing Reactions Catalyzed by RNA Cell-Free Preparations

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Construction of plasmids. In the description that follows, the numbers in parentheses refer to the nucleotide numbers of the restriction sites as they occur in the sequence of the adenovirus genome (1) and are shown in Fig. 1. Plasmid p1-2 was digested with Sal I and incubated with [32P]-dATP polymerase to produce the DNA with the sequence presented below. It contained the adenovirus 2 major late (ML) leader 1 and leader 2 (L1 and L2) (shown in upper case below) and the Sal I sites (underlined). The insert was deleted between the Sal I site (base 6491) and the Sal II site (base 7973). Sequences that arise from the vector pAR5/7 (4) are underlined. The transcription start site is indicated with an asterisk (*) while the bold A represents the brach site. The three nucleotides in parentheses located at the end of the sequence contains the Sal I site and are not present in the pre-ML DNA. Plasmids ply and pII were constructed by standard oligonucleotide-directed mutagenesis (2). The single-stranded DNA templates used for this procedure were obtained by cloning the p1-2 sequence into pAM9/1 (9).

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**Sequence of p1-2 pre-ML DNA**

Plasmid p1-2 carried a transplant that contained the last 5.8 nucleotides of the adenovirus 2 major late (ML) leader 2 and the first 5.8 nucleotides of leader 1 (shown in upper case letters), replaced by a transduced insert of 373 nucleotides (shown in lower case letters). The DNA sequences from the adenovirus 2 Sal I site (7115) and the Sal II site (8694) were cloned into the pAR5 (10) polylinker between the Sal I site and the Sal II site. The Sal II site (7023) was converted to a Sal I site by the addition of a linker, and joined to the Sal I site (5620) nearest in the 5' and of each 3', thus deleting the back of L2. The first 5.8 nucleotides of leader 1 and the last 5.8 nucleotides of leader 2 were then cloned with pML (5655) which cuts 5 nucleotides downstream of the Poly II site. Plasmid pII was constructed in the same way as p1-2, but the Sal I site fragment was derived from pII.

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**Fig 1. A map of eukaryotic 2 from nucleotides 6,000 to 10,000 spanning the major late transcription unit region.** The positions of the exons are indicated by boxes. The bracketed regions were deleted in the various cloned constructs. The critical restriction endonuclease sites and their nucleotide numbers are indicated.