In Vitro Formation of a Lariat Structure Containing a G$^{2'-5'}$G Linkage*

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We have examined the effects of base changes at the lariat branch site of a modified adenovirus major late precursor mRNA (pre-mRNA). Replacement of the A residue at the lariat attachment site with a G residue was studied. Incubation of this altered pre-mRNA with nuclear extracts of HeLa cells yielded less spliced mRNA (10-fold) than similar reactions with the wild type pre-mRNA. The intron lariat formed during the reaction with the mutant transcript contained the predominant branch (2'–5' phosphodiester linkage) to an upstream A residue. In contrast, the intron/exon 2 lariat contained the predominant branch to the substituted G residue. These results indicated that detectable spliced RNA was formed when the lariat was attached at the A residue but not when the lariat was attached to the substituted G residue. A second mutation was introduced into the transcript by substituting an additional G residue at the alternative A branch site. When transcript derived from this plasmid was incubated with nuclear extract, cleavage occurred at the 5' splice site, and an intron/exon 2 lariat was produced, but spliced RNA was not detected. T1 RNase digestion and primer extension analyses of this intron/exon 2 lariat revealed that all of the lariat formed on the G residue at the normal attachment site.

Our current understanding of precursor mRNA splicing in mammalian cells has stemmed from the development of cell-free extracts capable of processing exogenously added pre-mRNA (1–3). Although the precise details of this reaction have yet to be resolved, a two-step mechanism has been proposed (4–6). In the first step of the reaction, cleavage occurs at the 5' splice site, and an intron/exon 2 lariat is generated; in such structures, the 5' phosphate residue from the 5' end of the intron forms a 2'–5' phosphodiester bond with an A residue 17–37 nucleotides from the 3' end of the intron (4, 6, 7). In the second step of the reaction, the 3' exon is cleaved from the intron/exon 2 lariat and covalently linked to the 5' exon yielding spliced RNA. The excised intron remains in a lariat configuration.

This two-step pathway, involving an obligatory formation of a lariat structure, has been deduced from the appearance of the RNA structures during the splicing of mRNA in vitro. Two recent observations support this pathway: first, discrete protein fractions which catalyzed the first step of the reaction were also required for the production of spliced RNA (8, 9), and second, the 5' exon and intron exon lariat were found in a macromolecular complex in which spliced RNA was produced (8, 10–12).

If the formation of a lariat structure were a crucial step in the production of spliced RNA, changes in the sequences comprising the lariat branch site should have a profound effect on the efficiency of the splicing reaction. Early studies, however, indicated that only the consensus splice junction sequences were important and that intron regions now recognized as necessary for lariat formation could be deleted (13). Recent studies have resolved this apparent paradox by showing that deletion or alteration of lariat branch site sequences resulted in the utilization of a cryptic lariat branch sites (14–16). In view of the limited consensus sequence found between various lariat branch site sequences (7, 17) and the promiscuous use of cryptic branch sites, at least two questions remain. Does the formation of the lariat require a specific branch site sequence, and are all lariat structures capable of participating in the formation of spliced RNA?

In this study we have examined the effect of changing the adenosine of the lariat branch site to a guanosine. Consistent with the observations of others, the synthesis of spliced RNA was decreased about 10-fold, and a cryptic lariat site was utilized (16). When the adenosine of the cryptic lariat site was also changed to a guanosine, the synthesis of spliced RNA was undetectable, and the first exon and a novel G$^{2'-5'}$G lariat structure were the only reaction products observed.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The plasmid pKT.1 contains the adenovirus major late mRNA leader 1 and leader 2 separated by a truncated intron of 86 nucleotides cloned into pSP65 (18). The base-substitution mutants were constructed using standard oligonucleotide-directed mutagenesis procedures (19). Single-stranded DNA used in this procedure was obtained by cloning the EcoRI–BamHI fragment of pKT.1 into M13mp18. The sequences of all mutants were checked by DNA sequence analysis (20) and T1 RNase digestion of RNA transcription products.

In Vitro Splicing Reactions—$^{32}$P-labeled transcripts were synthesized using SP6 RNA polymerase and plasmid DNA linearized with Scal as previously described (18). Reaction mixtures (50 µl) containing 20 mM Hepes–KOH buffer (pH 7.6), 3 mM MgCl₂, 0.4 mM ATP, 10 mM creatine phosphate, 2 mM dithiothreitol, 2% polyethylene glycol 8000, $^{32}$P-labeled transcript (1.0 pmol, 2 × 10⁶ cpm/pmol), and nuclear extract (180 µg; Ref. 21) were incubated at 30 °C for the indicated time periods. Reaction mixtures used for isolating lariat structures were identical except that the RNA concentration was

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI, polyethyleneimine.
increased to 10 pmol/50 μl reaction mixture, and reactions were incubated for 60–75 min (with pKT.1 pre-mRNA) or 90-120 min (with pBY.1 and pUS.A pre-mRNA) at 30 °C.

Treatment of Putative Lariat Structures with Debranching Enzyme—Reaction mixture (20 μl) contained 10 mM Hepes-KOH buffer (pH 7.0), 3 mM MgCl₂, 0.25 mg/ml bovine serum albumin, RNA lariat structures (1-5 fmol), and purified debranching enzyme (100 units) and were incubated at 30 °C for 30 min. One unit of debranching enzyme is defined as the amount of enzyme required to convert 1 fmol of lariat RNA to linear RNA in 30 min at 30 °C. The enzyme was purified as described in the accompanying paper (22).

Enzymatic Analysis of RNA Branched Structures—Digestions with nuclease P1 were carried out in reaction mixtures (20 μl) containing 20 mM sodium acetate (pH 5.5), yeast tRNA (1 μg), nuclease, and P1 (1 μg) at 37 °C for 12 h. The reactions were then treated with phenol-CHCl₃ (1:1), the aqueous phase was extracted with chloroform, and the reactions separated were separated by PEI-cellulose thin layer chromatography using 0.5 M LiCl as the solvent.

Analysis with bacterial alkaline phosphatase was carried out in reaction mixture (20 μl) containing 10 mM Tris buffer (pH 10.5) and 0.1 unit of enzyme. The reaction was incubated at 37 °C for 3 h and then for 30 min at 70 °C. The products were separated by PEI-cellulose thin layer chromatography using 0.75 M potassium phosphate (pH 3.4) as the solvent.

T1 RNAse digestions were carried out in a reaction mixture of 5 μl containing 10 mM Tris-HCl buffer (pH 7.5), 10 μg of yeast tRNA, and 20 units of T1 RNAse. Reactions were incubated at 37 °C for 1 h followed by phenol-chloroform (1:1) and chloroform extraction as described above. The aqueous phase was collected and lyophilized to dryness. The residue was resuspended in 5 μl of 80% formamide, heated to 65 °C for 3 min, and then electrophoresed through 23% polyacrylamide-8 M urea gels.

Primed Extension Analysis—One pmol of 5'-32P-labeled oligonucleotide was hybridized to 9 μmol of purified intron/exon 2 lariat that had either been treated or mock treated with the debranching enzyme. The sequence of the oligonucleotide was 5' CGAAGAGTTTGTCCTCAACC 3' and was complementary to the exon 2 sequence beginning at nucleotide 645. The specific position of the A → G change in pBY.1 is indicated by the arrow. The speckled box represents the 15 nucleotides in the transcript that are derived from the SP6 vector. B, products from in vitro splicing of pKT.1 and pBY.1. Reaction mixtures containing either pKT.1 or pBY.1 pre-mRNAs were as described under "Materials and Methods".

RESULTS

We have previously demonstrated that incubation of the adenovirus PTK.1 pre-mRNA (see Fig. 1A) with nuclear extracts of HeLa cells resulted in the formation of four ATP-dependent RNA species with electrophoretic mobilities on 15% polyacrylamide-8 M urea gels corresponding to nucleotide lengths of 430, 255, 94, and 58 (8). These species were identified as the intron/exon 2 lariat, the intron lariat, spliced RNA, and the 5' exon, respectively. In the following experiments, these reaction products were separated using 18% polyacrylamide-urea gel electrophoresis. This system provided a superior resolution of the intron/exon 2 and intron lariat species that migrated as molecules of 500 and 300 nucleotides in length, respectively.

The lariat branch site within this intron was originally identified as the adenosine residue 24 nucleotides from the 3' splice site (23). We have confirmed this location and have examined the role of the lariat branch site sequence by changing this adenosine to a guanosine. In the experiments described below, this single A→G mutant and the wild type will be referred to as pBY.1 and pKT.1, respectively (Fig. 1A).

The rate of splicing of pBY.1 pre-mRNA was compared with the rate of splicing of pKT.1 pre-mRNA after incubation with a nuclear extract (Fig. 1). The amount of spliced RNA produced was measured by excision of the gel fragment and determination of the radioactivity by liquid scintillation spectrometry. Following 30 min of incubation with PTK.1 pre-mRNA, 9% of pre-mRNA was converted to spliced RNA (Fig. 1B, lane 2), and this amount increased to 30 and 28% following incubation for 60 and 120 min, respectively (Fig. 1B, lanes 3 and 4). In contrast, when pBY.1 pre-mRNA was incubated with the nuclear extract for 30 min, spliced RNA was not detected (Fig. 1B, lane 6). However, after 60 and 120 min of incubation, 3 and 7% of pBY.1 pre-mRNA was converted to spliced RNA, respectively (Fig. 1B, lanes 7 and 8). These results demonstrated that the efficiency of splicing was reduced considerably when a guanosine residue was substituted for the adenosine residue at the lariat attachment site.

Incubation of pBY.1 pre-mRNA with the nuclear extract resulted in the time-dependent formation of an anomalously migrating species that corresponded in size to the intron/exon 2 lariat formed from pKT.1 pre-mRNA (500 nucleotides; Fig. 1B, lanes 2–4 and 6–8). A second anomalously migrating species presumed to be the intron lariat was detected. It possessed an apparent electrophoretic mobility of 240 nucleotides, distinctly different from the size of the intron lariat formed from pKT.1 pre-mRNA (300 nucleotides; Fig. 1B, compare lanes 2 and 7).

In order to verify that the anomalously migrating species formed during the incubation with pBY.1 were indeed RNA molecules possessing lariat configurations, these products were eluted from an 18% polyacrylamide-8 M urea gel and...
incubated with the lariat debranching enzyme. This enzyme is a phosphodiesterase specific for the 2'−5' phosphodiester bond at lariat branch sites (24) and has recently been purified in our laboratory (see accompanying paper (22)). Incubation of the anomalously migrating species formed from pKT.1 (Fig. 2, lanes 1 and 3) or from pBY.1 (Fig. 2, lanes 5 and 7) with the lariat debranching enzyme resulted in their conversion to RNAs with electrophoretic mobilities of 125 nucleotides (Fig. 2, lane 2), 86 nucleotides (Fig. 2, lane 4), 125 nucleotides (Fig. 2, lane 6), and 86 nucleotides (Fig. 2, lane 8). These are the expected sizes of the linear form of the intron/exon 2 lariat and the linear form of the intron lariat formed from both precursors confirming that these anomalously migrating species were RNA molecules with lariat configurations. These results also demonstrated that the linear intron lariats formed from pKT.1 and pBY.1 were the same length and therefore that the difference in the migration of the two structures was likely to be due to the use of an alternative branch point.

Two Intron/Exon 2 Lariat Structures Are Formed by pBY.1 Pre-mRNA—In order to determine the site of lariat attachment within the pBY.1 intron, the lariat RNA species from pBY.1 and pKT.1 were eluted from 18% polyacrylamide-8 M urea gels and digested with T1 RNase (Fig. 3A). T1 RNase digestion of the pKT.1 lariat resulted in the formation of a 15-mer oligonucleotide (14*; Fig. 3A, lanes 2 and 3) that was not present among the T1 RNase products from the pKT.1 pre-mRNA (Fig. 3A, lane 1). The 14* oligonucleotide was derived by the addition of pGp to the 2'-OH group of the branch site adenosine contained within the 14-mer lariat forming the 14 oligonucleotide.

Similar analysis of the lariat species delivered from pBY.1 yielded a novel 14** oligonucleotide structure from the intron/exon 2 lariat and 14* and 9* oligonucleotides from the intron lariat (Fig. 3A, lanes 4 and 5). We will show below that the 14** oligonucleotide was formed from a lariat branch structure at the guanosine residue that was substituted for the adenosine residue in the pBY.1 mutant (Fig. 3B). Thus, the char-

![Fig. 2. Incubation of anomalously migrating species with the lariat debranching enzyme.](image-url)
characteristic size of the 14** species arises from the combined results of the addition of pGp and its resistance to T1 RNase that this addition confers. We will also show that the 9* structure resulted from the addition of pGp to the 2'-OH group of an adenosine residue within the 9-nucleotide T1 RNase fragment adjacent to the authentic branch site (Fig. 3B). The presence of a small amount of the 14** oligonucleotide in the intron lariat derived from pBY.1 resulted from the degradation of the intron/exon 2 lariat species.

**Structural Analysis of T1 Oligonucleotides 14** and 9**—**The structure of oligonucleotides 14** and 9** formed from T1 RNase digestion of PBY.1 RNA lariats was analyzed by treatment with nuclease P1. Each band was eluted from the gel, treated with nuclease P1, and the products were separated by PEI-cellulose thin layer chromatography in 0.5 M LiCl. Digestion of an unbranched oligonucleotide yielded only pG, whereas digestion of the 14* oligonucleotide derived from pKT.1 yielded pG and the P1-resistant compound pA<PGp (Fig. 4, lanes 1-3). Both 14** and 9* oligonucleotides yielded P1-resistant compounds and pG, indicative of the presence of a 2'−5' phosphodiester bond (4, 22). The P1-resistant compound (B) derived from the 14** oligonucleotide was more highly charged and contained twice the amount of radioactivity as pG (Fig. 4, lane 4). These properties were consistent with the presence of a pG<PGp linkage.

The presence of a pG<PGp linkage was unambiguously established by further analysis of the P1-resistant compound B. Treatment of compound B with bacterial alkaline phosphatase yielded [32P]Pi and a slower migrating labeled compound (Fig. 6, lane 4). These properties were consistent with the presence of a pG<PGp linkage.

**A. Analysis of the Lariat Structures**

The T1 RNase-generated oligonucleotides (14, 14*, 14**, and 9*) shown in Fig. 3A were isolated and treated with nuclease P1 as described under “Materials and Methods.” The digestion products were analyzed by PEI-cellulose thin layer chromatography using 0.5 M LiCl as the solvent. The linear 14 oligonucleotide derived from pKT.1 pre-mRNA is shown in lane 1. In lanes 2 and 3, the 14* oligonucleotide is derived from the pKT.1 intron/exon 2 and intron lariat structures, respectively. The 14** and 9* oligonucleotide derived from pBY.1 intron/exon 2 and intron lariat structures, respectively, are shown in lanes 4 and 5.

**B. Treatment of Compound B with Bacterial Alkaline Phosphatase**

The reaction products were separated by PEI-cellulose thin layer chromatography using 0.75 M potassium phosphate (pH 3.4) as solvent and visualized by autoradiography. Treatment of pA<PGp with the purified debranching enzyme (Fig. 6, A and B). Treatment of pA<PGp yielded only [5'-32P]GMP, whereas compound B yielded 1 mol of [5'-32P]GMP and 1 mol of a 32P-labeled compound co-migrating with pGpU (compare Fig. 6, lanes 2 and 5). Successful removal of the 2'−5' phosphodiester bond was monitored by the formation of pGp after T1 RNase digestion (Fig. 6, lane 6). We thus conclude that oligonucleotide 14** contained G<PGp derived from a lariat branch structure. The position of this lariat branch structure can be unambiguously assigned to the guanosine residue 24 nucleotides from the 3' splice site (Fig. 3B) since only modification of this guanosine could result in a T1 RNase-resistant oligonucleotide migrating at the position expected by a 15-mer.

**Location of the Lariat Branch Structure in Oligonucleotide 9**—As described above, digestion of the 9* oligonucleotide with nuclease P1 yielded pG and a compound (labeled C) which co-migrated with pA<PGp (derived from the authentic branch site). Treatment with bacterial alkaline phosphatase revealed that the [32P]GMP present in the compound was in a phosphodiester structure (Fig. 5B). Both observations suggested that the addition of pGp had occurred at an adenosine residue.

In order to confirm the base composition and nature of compound C, pBY.1 pre-mRNAs labeled with either [α-32P]CTP or [α-32P]UTP were incubated with nuclear extracts, the lariat structures isolated, digested with T1 RNase, and separated by 23% polyacrylamide-8 M urea gel electrophoresis. The oligonucleotide 9* formed in each reaction was isolated from the gel, digested with nuclease P1, and the products were separated by PEI-cellulose thin layer chromatography (Fig. 7A). The [32P]CMP-labeled oligonucleotide 9*, digested with nuclease P1, produced only [5'-32P]CMP (Fig. 7A, lane 1). However, [32P]UMP-labeled oligonucleotide 9* yielded [5'-32P]UMP, 32Pα, and a nuclease P1-resistant compound (Fig. 7B).
The mutant pUS.A pre-mRNA yielded the first step inter-
mediate, the first exon, and an intron/exon 2 lariat species,
but at a reduced rate (10-fold less). The branch site of the
new plasmid pUS.A was constructed that is identical to pBY.l
except that the adenosine residue at the alternative branch
site was changed to a guanosine (Fig. 7A, lane 2). Pre-mRNAs
made from pUS.A and pKT.1 were incubated with nuclear extract
and the reaction products analyzed by polyacrylamide gel
electrophoresis (Fig. 8B). After 2 h, 15% of the pKT.1 pre-
mRNA had been converted to spliced RNA. In contrast, the
mutant pUS.A pre-mRNA produced no detectable spliced RNA
in the same time period. Quantitative analysis revealed that
the pKT.1 pre-mRNA produced greater than 40 times
more spliced RNA than the mutant pre-mRNA. Thus, we
concluded that the alteration of the alternative branch site
rendered the pre-mRNA incapable of being spliced.

The mutant pUS.A pre-mRNA yielded the first step inter-
mediates, the first exon, and an intron/exon 2 lariat species,
but at a reduced rate (10-fold less). The branch site of the
intron/exon 2 species produced from this mutant pre-mRNA
was mapped using the T1 RNase digestion analysis described
above. As already demonstrated, wild type intron/exon 2 lariat
produced a branched oligonucleotide (14*) after T1 digestion that was reduced to its original size (14 nucleotides) on pretreatment with debranching enzyme (Fig. 9A, lanes 5 and 6). The large size of this branched oligonucleotide indicated that the branch point must be to a guanosine residue (thereby blocking T1 RNase digestion) and can unambiguously be identified as the guanosine substituted for the adenosine of the authentic branch site, an oligonucleotide that was present if the lariat structure was pretreated with debranching enzyme prior to T1 RNase digestion (Fig. 3). Using this criterion, the intron/exon 2 lariat from pUS.A, digested with T1 RNase, are shown in lanes 1 and 4, respectively. Intron-exon lariats not treated with the debranching enzyme are in lanes 2 (pKT.1) and 5 (pUS.A); lariats linearized with debranching enzyme prior to T1 RNase digestion are presented in lanes 3 (pKT.1) and 6 (pUS.A). B, the structure of the branched oligonucleotide 11* deduced from T1 RNase analysis. The structure of the authentic branch point structure (14*) is indicated for comparison. C, the position of the large T1 RNase-generated oligonucleotides in pKT.1 pre-mRNA. The exon sequences are capitalized; the lowercase letters indicate the intron sequence. The slashed line represents the site of T1 RNase cleavage. The capitalized A, 24 residues from the splicing branch site, is the authentic branch site.

**DISCUSSION**

There is increasing evidence that the formation of an RNA lariat structure is an obligatory step in the formation of spliced mRNA (4, 5, 15, 16, 25). It is therefore important to establish the sequences within the pre-mRNA that are required for lariat formation. It is also critical to understand the sequence features of the lariat structure itself that are necessary for the formation of spliced RNA. Yeast pre-mRNAs contain an invariant sequence (UACUAAC) near the 3' splice site that encompasses the lariat branch site (26, 27). Changes in this sequence abolish both lariat formation and the production of spliced RNA (28). In contrast, studies of intron/exon 2 lariat structures derived from a number of metazoan pre-mRNAs have revealed only a weak consensus sequence (4, 7, 17).
Exon 2 lariats formed from pKT.1 and pUS.A pre-mRNA, annealing transcriptase. The primer-extended material was analyzed by electrophoresis on a 10% polyacrylamide-8 M urea gel. Treatment with debranching enzyme prior to primer extension analysis is indicated by +. Analysis of intron/exon 2 lariats from pKT.1 and pUS.A pre-mRNA are shown in lanes 1, 2 and 3, 4, respectively.

Moreover, appraisal of the importance of this sequence has been complicated by the utilization of cryptic branch sites (15, 16). In this study we have utilized a modified adenovirus major late pre-mRNA which contains a truncated 86-nucleotide intron (18). When an A→G change was made at the normal branch site, the rate of splicing was reduced 10-fold. This result is similar to an earlier study (16) with a mutant pre-mRNA that failed to produce any detectable spliced RNA and yielded only the 5′ exon species and the G2′-G intron/exon 2 lariat species formed at the guanosine of the original branch site. This result clearly indicates that there is only a finite number of cryptic lariat sites and that the residues in the intron involved in lariat formation are essential for the production of spliced RNA.

These studies support the requirement for the formation of a lariat structure containing the appropriate branch sequences; however, the actual function of this structure in the process of mRNA splicing remains unknown. It is likely that the branch point of the intron/exon 2 lariat structure is recognized by components of the splicesome, and this interaction is involved in directing the formation of spliced products. This is supported by our results that show that a lariat formed at the normal position but to an alternative nucleotide does not form spliced RNA (Fig. 8). However, the formation of a lariat structure with the correct sequence does not assure the production of spliced RNA. This was demonstrated with transcripts in which the branch point of the intron/exon 2 lariat structure is changed at the original site via a G2′-G linkage. This is the first demonstration that the lariat formation of this novel linkage strengthens the argument of the importance of this sequence has been complicated by the utilization of cryptic branch sites (15, 16). In this study we have utilized a modified adenovirus major late pre-mRNA which contains a truncated 86-nucleotide intron (18). When an A→G change was made at the normal branch site, the rate of splicing was reduced 10-fold. This result is similar to an earlier study (16) with a mutant pre-mRNA that failed to produce any detectable spliced RNA and yielded only the 5′ exon species and the G2′-G intron/exon 2 lariat species formed at the guanosine of the original branch site. This result clearly indicates that there is only a finite number of cryptic lariat sites and that the residues in the intron involved in lariat formation are essential for the production of spliced RNA.

It is evident that the nucleotide sequence as well as the structure of the pre-mRNA play important roles in splicing. The precise nature of these sequences, as well as the isolation and identification of the proteins and RNA components involved, must be clarified in order to understand how splicing takes place.

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