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cis-Acting Elements and a *trans*-Acting Factor Affecting Alternative Splicing of Adenovirus L1 Transcripts

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Expression of the L1 region of adenovirus is temporally regulated by alternative splicing to yield two major RNAs encoding the 52- to 55-kilodalton (52-55K) and IIIa polypeptides. The distal acceptor site (IIIa) is utilized only during the late phase of infection, whereas the proximal site (52-55K) is used at both early and late times. Several parameters that might affect this alternative splicing were tested by using expression vectors carrying the L1 region or mutated versions of it. In the absence of a virus-encoded or -induced factor(s), only the 52-55K acceptor was used. Decreasing the distance between the donor and the IIIa acceptor had no effect. Removal of the 52-55K acceptor induced IIIa splicing slightly, implying competition between the two acceptors. Fusion of the IIIa exon to the 52-55K intron greatly enhanced splicing of the IIIa junction, suggesting that the IIIa exon does not contain sequences that inhibit splicing. Thus, the lack of splicing to the IIIa acceptor in the absence of a virus-encoded or -induced factor(s) is probably due to the absence of a favorable sequence and/or the presence of a negative element 5′ of the IIIa splice junction, or both. The presence of several adenovirus gene products, including VA RNAs, the E2A DNA-binding protein, and the products of E1A and E1B genes, did not facilitate use of the IIIa acceptor. In contrast, the simian virus 40 early proteins, probably large T antigen, induced IIIa splicing. This result, together with those of earlier studies, suggest that T antigen plays a role in modulation of alternative RNA splicing.

Most eucaryotic genes contain introns that are removed from the transcript during mRNA maturation. During processing of the precursor RNA of some of these genes, different combinations of splice donor and acceptor sites are joined to form different mRNAs. It is now clear that this alternative RNA splicing not only increases the complexity of products that are encoded by a given gene but also serves as an important regulatory mechanism. For example, both the rat fibroblast tropomyosin 1 and the skeletal muscle β-tropomyosin are expressed from a single rat gene via alternative RNA splicing (25). Many other examples of tissue-specific (9, 32) or developmentally regulated alternative splicing are known (7, 12; for reviews, see references 11 and 33).

Although many studies are being conducted to characterize alternative splicing, the mechanisms that govern this process are still ill defined. Specifically, it is not known how alternative splicing sites are chosen or even whether different signals are used during processing of transcripts that undergo simple versus complex patterns of splicing. Both simple and complex transcriptional units use more or less conserved consensus sequences identified at the donor and acceptor splice sites and the branch point (24, 36, 39). Moreover, sequence comparisons between splice junctions of constitutive and alternative exons have not revealed significant differences (11). Therefore, it is likely that regulation of alternative splice site usage requires other *cis*-acting elements. Furthermore, the results of a number of studies argue that *trans*-acting factors, which may interact with these *cis*-acting elements, are involved in differential splicing (9, 12, 20, 32). These putative *trans*-acting factors and the signals with which they interact have yet to be identified.

Human adenovirus is an extreme example of alternative splicing. The primary transcript(s) from each of five early regions (E1A, E1B, E2, E3, and E4) is alternatively spliced to, in general, yield several mRNAs encoding different proteins (8, 15; for reviews, see references 40 and 45). Similarly, during the late phase of adenovirus infection, the ~30-kilobase-long transcript initiated from the major late promoter (at nucleotide [nt] 6008 [42]) is processed by differential cleavage and polyadenylation into five families (L1 to L5) of RNAs. Alternative splicing of the tripartite leader (itself formed by splicing together of three exons encoded proximal to the promoter) to different acceptor sites within a family yields different family members that share the common 5′ tripartite leader and the same 3′ end (for reviews, see references 40, 45, and 53).

The adenovirus L1 late region is particularly intriguing. Although the L1 gene family is part of the major late transcriptional unit, it is expressed early as well as late in infection (2, 37, 46). During the early phase, the donor site (at nt 9723) is spliced to the proximal acceptor site (at nt 11040) to form a messenger encoding two related polypeptides (52 to 55 kilodaltons; designated 52-55K polypeptides) of unknown function. During the late phase, the distal acceptor site for the IIIa capsid protein mRNA as well as the 52-55K mRNA acceptor site are utilized (2, 37). Thus the alternative splicing of L1 is regulated in concert with the viral infectious cycle. We have chosen to study the adenovirus L1 family as a model of regulated alternative splicing to learn more about the *cis*-acting element(s) and *trans*-acting factor(s) involved in this regulation.

MATERIALS AND METHODS

Construction of L1 expression vectors. Most of the cloning steps were done in pGEM3*, a variant of the pGEM3 vector (Promega Biotec) that lacks the *Pvu*II site. Numbers in parentheses denote the nucleotide sequence number of the adenovirus serotype 2 (Ad2) genome (42).

The tripartite leader of late viral mRNAs and its flanking sequences including the splice donor site was constructed as follows. An Ad2 *Tha*I fragment (6029 to 6169) containing the

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first leader of the tripartite leaders was inserted in the unique *Sma*I site of pGEM3*, giving plasmid pT. An Ad2 *Sal*I fragment (9455 to 9832) carrying the third late leader was inserted in the unique *Sal*I site of pT, yielding plasmid pTS. pTS, carrying both inserts in the same orientation, was cleaved with *Pvu*II and *Xho*I, which cut in the Ad2 inserts at 6069 and 9689, respectively. A *Pvu*II-*Xho*I fragment, derived from the tripartite leader region of a cDNA copy of the fiber mRNA (pJAW [16]), was inserted into the *Pvu*II-*Xho*I-deleted pTS to give plasmid pTSC, which carries 11 base pairs (bp) of the genomic sequence in front of and 108 bp after the full-length Ad2 tripartite leader. pTSC contains the third-leader splice donor site. The *Bam*HI-*Sal*I fragment of pTSC was introduced between *Bam*HI and *Sal*I sites of pIC20R (34), yielding plasmid pICTSC. Another plasmid, pICXS, carrying only part of the Ad2 third leader, including its splice donor sites, was made by inserting the Ad2 *Xho*I-*Sal*I fragment (9689 to 9832) in pIC20R.

Plasmids containing the complete or the deleted Ad2 late L1 region were made as follows. An Ad2 *Bam*HI-*Kpn*I fragment (10680 to 14281), containing the entire L1 region, was isolated from a pGEM3*Ad2 *Bam*HI plasmid (10680 to 15403) and inserted in plasmid pICTSC, yielding plasmid pICTSCK1. The *Eco*RI fragment of pICTSCL1 was then inserted in the *Eco*RI site of the expression vector p91023B (28), giving plasmid pK1. pICTSCL1 was deleted of a *Bst*EII fragment (10708 to 11507), giving plasmid pICTSCL1B, whose *Eco*RI insert was moved to p91023B to give the pK2 vector. It contains a ~800-bp deletion that essentially removes the 52-55K acceptor site.

Construction of the pK3 vector, which contains the 3′ portion of the third leader upstream of a deleted and truncated L1 gene, was more complex. pGEM3*Ad2 *Bam*HI (10680 to 15403) was linearized with *Mlu*I, blunt ended with Klenow fragment (New England BioLabs, Inc.), and then religated in the presence of *Eco*RI linkers (New England BioLabs). The *Bam*HI-*Eco*RI fragment was isolated and inserted between *Bam*HI and *Eco*RI sites of pGEM3*. The resulting plasmid, pL1E, was cut by *Sac*II (11155) and *Pvu*II (12175), treated with Klenow fragment to obtain blunt ends, and religated to give pL1ESP. pL1ESP was further deleted between an *Ava*I site of the multicloning site of pGEM3* and the Ad2 *Ava*I site located at 10931 to give plasmid pL1ESPA. The *Bam*HI-*Kpn*I fragment containing the whole insert of pL1ESPA was introduced in homologous sites of pICXS to give plasmid pICXSL1ESPA. The *Eco*RI insert of this plasmid was transferred to the p91023B expression vector to give plasmid pK3 and to pUC119 (48) to verify this construct by sequencing (43).

To fuse the 52-55K intron sequence to the IIIa exon, pUC119XSL1ESPA was amplified in the *Escherichia coli* DNA methylation-minus strain JM110 (51) and cut at the *Bcl*I (12301) and *Nsi*I (11040) sites. The opened plasmid was incubated with S1 nuclease (5 U of S1 nuclease [Boehring Mannheim Biochemicals] per μg of DNA) at 30°C for 30 min and then phenol extracted, ethanol precipitated, and religated. *E. coli* MV1184 (48) was transformed with this DNA. Ampicillin-resistant colonies were grown and infected with M13K07, and the single-stranded DNA was isolated as described by Vieira et al. (48). Sequencing was done as described by Sanger et al. (43). Two clones, referred to as pUCBN1 and pUCBN2, were selected. In pUCBN1, 11039 (last nucleotide of the 52-55K intron) was fused to nt 12306 (12308 is the first nucleotide of the IIIa exon). This resulted in the duplication of AG at the splice junction, with the 5′ AG derived from the 52-55K intron and the 3′ AG derived from the IIIa intron. pUCBN2 has nt 11039 linked to nt 12311, again keeping the 52-55K intron intact but removing the first 3 nt of the IIIa exon. The *Pst*1 (12347)-*Ava*I (10931) fragment of these plasmids was inserted in homologous sites of pICTSCL1. The leader-L1-region insert was then moved into p91023B via *Eco*RI sites to give the pKBN1 and pKBN2 expression vectors. *Eco*RI-*Bam*HI fragments, which contain the L1 chimeric region, were isolated from plasmids pKBN1 and pKBN2 and inserted in homologous sites of pGEM3 to give the transcription vectors pGEM3BN1 and pGEM3BN2. These were used to generate 260-nt antisense RNA probes that cover the chimeric acceptor sites.

Cells and vectors. Human 293 and HeLa cells, originally obtained from J. Williams, were grown as monolayers in Dulbecco modified Eagle medium (Flow Laboratories, Inc.) supplemented with 10% calf serum, 100 μg of streptomycin per ml, 100 μg of penicillin per ml, and 2 mM L-glutamine. Cos-1 cells (21) were generously provided by Y. Gluzman and propagated as above except that calf serum was replaced by 5% fetal calf serum.

Plasmid p730 contains an Ad5 hr404 (31) *Bam*HI-*Eco*RI fragment (59 to 76 map units) cloned into pBR322. It carries and expresses the single-stranded-DNA-binding protein (DBP) gene under its own promoter and poly(A) signals. The vector, which we have termed pMK16/SV40, was kindly provided by Y. Gluzman (22). It contains the simian virus 40 (SV40) genome with a defective origin of replication. It was constructed as follow by Y. Gluzman. The SV40 genome was inserted in the *Bam*HI site of a *Bgl*I^r pMK16 plasmid and then cut with *Bgl*I, blunt ended with Klenow fragment, and religated.

DNA transfection. HeLa and 293 cells were transfected by a modified calcium phosphate precipitation method (20). Precipitated DNA (30 μg) was added to 100-mm-diameter dishes of cells in a dropwise manner directly through the culture medium. The medium was removed 4 h later, and cells were shocked with a 10% glycerol solution in Tris-buffered saline for 1.5 min. Cos cells were transfected by using DEAE-dextran as described by Kaufman (28). Cells were washed with phosphate-buffered saline and exposed to 1 ml of DEAE-dextran (500 μg/ml) in Tris-buffered saline containing 30 μg of plasmid DNA.

RNA isolation and analysis. RNA was extracted between 40 and 48 h posttransfection. Cytoplasmic RNA was prepared as described by Yen et al. (52). Characterization of the RNA was performed primarily by S1 nuclease analysis as described by Yen et al. (52), with the following modifications. A 15-μg sample of total cytoplasmic RNA was hybridized to 5′-end-labeled probes and then incubated for 30 min at 45°C with 50 U of S1 nuclease. The S1-resistant fragments were resolved on a 4% acrylamide–7 M urea electrophoresis gel. RNase protection analysis was done as described by Melton et al. (35).

RESULTS

To define some of the factors that govern the alternative splicing of the L1-region transcript, several different expression vectors were constructed (Fig. 1). The intact or mutated L1 region together with all or a portion of the tripartite leader, including the donor splice site of the third leader, were introduced in vector p91023B (28), expression of which is under the control of the Ad2 major late promoter and the SV40 polyadenylation signals. These vectors were transiently expressed in human HeLa or 293 cells or in simian Cos-1 cells (21). The products resulting from splicing be-

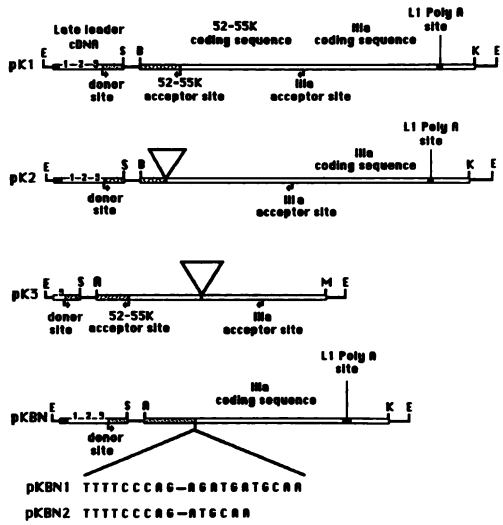

FIG. 1. Structures of normal or altered L1 genes. Construction of the plasmids is described in Materials and Methods. Symbols: ——, bacterial plasmid sequences; □, adenoviral sequences; ▩, the 52-55K intron; ▽, deletion of the 52-55K splice acceptor region in pK2, deletion of a large portion of the IIIa intron in pK3, and deletion of the 52-55K-coding sequence in the pKBN plasmids. Numbers 1, 2, and 3 refer to the Ad2 late tripartite leader. Sequences shown below pKBN represent the 3′ end of the 52-55K intron, separated by a hyphen from the 5′ modified end of IIIa exon on the right. A, B, E, K, M, and S correspond to the *Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Mlu*I, and *Sal*I restriction enzyme sites, respectively.

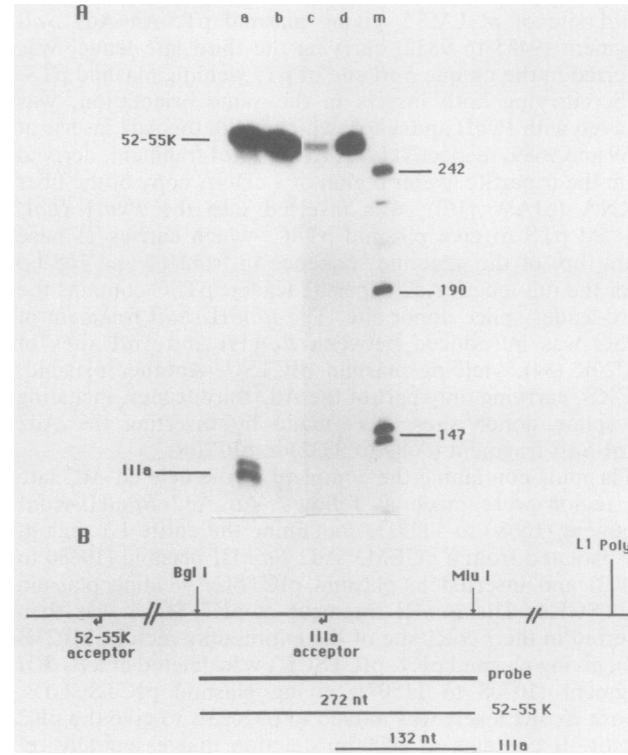

FIG. 2. Alternative splicing of the L1 gene transcript. (A) Total cytoplasmic RNAs of human 293 cells were extracted 24 (lane b) or 48 (lane a) h after Ad2 infection or 48 h after transfection with plasmid pK1 (lane d). Total cytoplasmic RNA from mock-transfected 293 cells was used as a control (lane c). RNA (15 μg) was hybridized to the 5′-end-labeled *Bgl*I-*Mlu*I probe (B). After S1 nuclease treatment, protected fragments were analyzed on a 4% acrylamide–7 M urea electrophoresis gel. The size markers shown in lane m were 5′-end-labeled fragments of plasmid pIBI20 (IBI, Inc.) cleaved with *Hpa*II. (B) The *Bgl*I-*Mlu*I 5′ probe is shown as a heavy line below the appropriate section of plasmid pK1 (Fig. 1). The 52-55K mRNA protects the entire 272-nt probe, whereas the IIIa mRNA covers only the 5′ 132 nt.

tween the donor of the third leader and either the 52-55K acceptor or the IIIa acceptor were analyzed by S1 nuclease mapping.

Preferential use of the 52-55K acceptor in transfected human cells. There are several possibilities that could account for the lack of splicing to the IIIa acceptor during the early phase of infection. A viral protein or virus-induced host factor might inhibit use of the IIIa splice site or favor selection of the 52-55K site. This protein or factor would be lost or inactivated during the late phase, thus allowing splicing to the IIIa acceptor. Alternatively, the 52-55K site may be the preferred acceptor under most cellular conditions, but during the late phase of infection the nuclear environment or splicing apparatus is altered to enhance IIIa or inhibit 52-55K acceptor utilization. To distinguish between these two general possibilities, the pK1 expression vector (Fig. 1) was transfected into HeLa or 293 cells (a human embryonic kidney cell line transformed with the E1A and E1B region of Ad5 [23]).

Cytoplasmic RNA extracted 48 h posttransfection was analyzed by S1 nuclease mapping, using a 5′-end-labeled, 272-bp *Bgl*I-*Mlu*I fragment that covers the IIIa acceptor site (Fig. 2B). The 52-55K mRNA hybridized to the entire probe, whereas the IIIa mRNA protected only the 5′ 132 nt. In transfected HeLa (data not shown) or 293 cells (Fig. 2A, lane d), only the 52-55K mRNA (272-nt band) was present. In contrast to transfection with the vector, during infection by Ad2, processing of the viral L1 transcript resulted in increasing amounts of IIIa mRNA (132-nt band) during the late phase (Fig. 2A, lanes a and b). The lack of IIIa mRNA in transfected HeLa or 293 cells suggests that normally the 52-55K site is strongly preferred but that during the late phase of infection a newly made or activated factor(s) enhances IIIa or blocks 52-55K acceptor site usage.

Shortening of the distance between donor and acceptor site had no effect. To define L1 sequences involved in the preferred usage of the 52-55K acceptor or necessary for the switch during infection, a series of altered L1 genes was constructed and tested by transient expression in 293 cells. Studies were done with 293 rather than HeLa cells because of the higher efficiency of transfection of the former. The distance between the donor and acceptor can be an important determinant in splice site selection (18, 20). To determine the importance of this factor, a large deletion was introduced into the 52-55K coding region, which reduced the distance of the IIIa acceptor to the donor of the third leader from 1765 to 493 nt. In this vector (pK3; Fig. 1), the 52-55K acceptor is separated from the IIIa acceptor by 247 nt (115 nt 3′ of the 52-55K site and 132 nt 5′ of the IIIa acceptor site). S1 analysis with a 5′-end-labeled, ~490-bp *Bam*HI-*Eco*RI probe covering both the 52-55K and IIIa acceptors (Fig. 3B) detected only the 52-55K mRNA in pK3-transfected 293 cells (~370-nt band; Fig. 3A, lanes a and b). Decreasing the distance between the two acceptors by more than fivefold had no effect on IIIa splicing, suggesting that distance from the donor site was not a major determinant of L1 acceptor site usage.

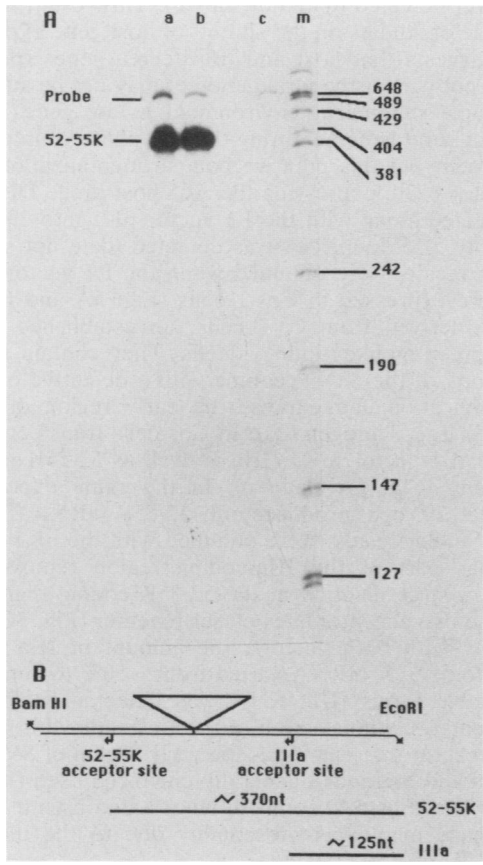

FIG. 3. Effect on IIIa splicing of shortening the donor-to-acceptor distance. (A) The S1 mapping analysis of cytoplasmic RNA from 293 cells transfected with the pK3 vector (lanes a and b; experiment done in duplicate) or from mock-transfected 293 cells (lane c) was similar to that described in the legend to Fig. 2. (B) The *Bam*HI-*Eco*RI 5′-end-labeled probe covers both the 52-55K and IIIa splice acceptor sites. The 52-55K and IIIa mRNAs anneal to and protect ~370 and ~125 nt, respectively, of this ~490-bp probe.

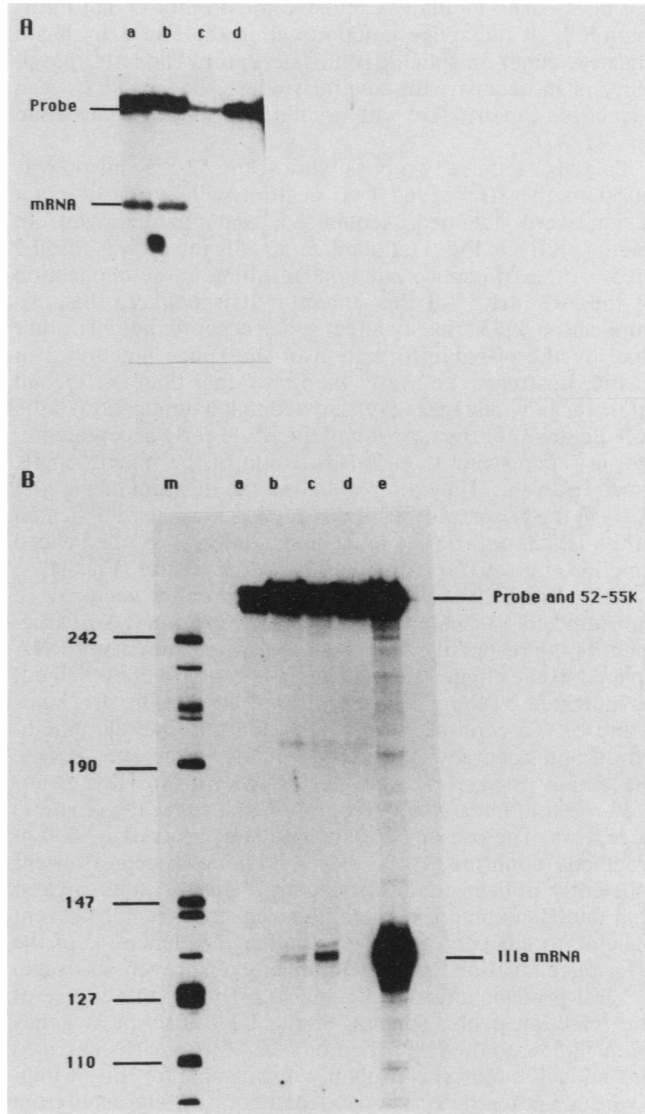

FIG. 4. Induction of IIIa RNA splicing by mutagenesis. (A) Effect on splicing of fusing the 52-55K intron to the IIIa exon. Cytoplasmic RNA from 293 cells transfected with the pKBN1 (lane a) or pKBN2 (lane b) vector was analyzed by S1 nuclease treatment, using a *Bam*HI-*Stu*I probe (940 bp) specific for the pKBN1 or pKBN2 sequence. As controls, the pKBN1 (lane c) and pKBN2 (lane d) probes were also incubated with RNA from mock-transfected 293 cells and S1 nuclease treated. Protected fragments were analyzed on a 3% acrylamide–7 M urea electrophoresis gel. (B) Effect on IIIa splicing of removal of the 52-55K splice acceptor site. S1 analysis was done on cytoplasmic RNA from 293 cells transfected with plasmid pK1 (lanes a and d; experiment done in duplicate) or pK2 (lanes b and c; experiment done in duplicate) or from 293 cells 48 h after infection with Ad2 (lane e). The 272-bp *Bgl*I-*Mlu*I fragment shown in Fig. 2B was used as the probe.

Partial activation of the IIIa acceptor by deletion of the proximal 52-55K acceptor. Competition between acceptor sites can lead to the underutilization of some sites (41). Removal or mutation of a normally used site can even result in activation of crytic splice sites (50). That such competition might be responsible in part or wholly for the lack of IIIa acceptor usage was suggested by comparison of the 52-55K and IIIa acceptor sequences. Computer analysis of many acceptor site sequences reveals a consensus sequence ($^{TT}_{CC}$T T^{TTTTTT}_{CCCCCC}NCAG [44]). Whereas the sequence just upstream of the splice acceptor junction of 52-55K is similar to this consensus sequence (see Fig. 1), that of IIIa exhibits little homology to it (GATGTTTCTGATCAG).

To test whether competition might be important, the 52-55K acceptor was removed by deletion of a *Bst*EII fragment from pK1 to generate the pK2 vector (Fig. 1). S1 mapping of RNA from pK2-transfected 293 cells by using the 272-bp 5′-end-labeled *Bgl*I-*Eco*RI probe (same probe used for the experiments shown in Fig. 2B) indicated that a very small amount of IIIa mRNA was synthesized (Fig. 4B, 132-nt band seen in lanes b and c). However, the failure to detect large amounts of IIIa mRNA suggested that competition was not primarily responsible for poor IIIa acceptor utilization. Thus, increased splicing to the IIIa acceptor during the late phase of infection probably involves an activation process that enhances use of the IIIa acceptor rather than simply represses 52-55K acceptor usage.

The IIIa exon does not repress splicing from a constitutive acceptor site. The poor utilization of the IIIa acceptor in the absence of viral infection could be due to sequences 5′ of the splice junction, for example, by virtue of their lack of homology to the consensus sequence. An alternative, but

not necessarily mutually exclusive, possibility is that information 3′ of the splice junction within the IIIa exon has a negative effect on splicing to this acceptor. The latter possibility is in accord with several studies showing that exon sequences can interfere with or influence splice site selection (26, 41, 47).

To address these two possibilities, the 52-55K intron was fused to the IIIa exon. Two vectors with a nearly exact fusion were obtained, sequenced, and characterized. In vector pKBN1 (Fig. 1), the intact 52-55K intron was fused 2 bp 5′ of the IIIa splice junction, resulting in the duplication of the AG just 5′ of this junction. It is unlikely that this duplication will seriously affect splice acceptor activity other than by the possible formation of the splice junction 2 nt further upstream (i.e., after the first rather than the second AG). Fu and colleagues (19) showed that a duplication of the AG did not alter the function of the SV40 early acceptor site. In the second vector, pKBN2, fusion of the intact 52-55K intron with the IIIa exon resulted in the deletion of the first ATG in the IIIa exon. Since this ATG is normally duplicated in the IIIa exon, the sequence immediately 3′ of the spliced junction (i.e., ATG) is maintained in this vector (Fig. 1).

RNAs from 293 cells transfected with either vector were subjected to S1 analysis, using a 940-bp *Bam*HI-*Stu*I fragment of the respective vectors. Substantial amounts of RNA spliced to the chimeric 52-55K–IIIa acceptor were obtained, as indicated by the ~580-nt protected bands (Fig. 4A, lanes a and b). To confirm these results and improve the quantitation and accuracy of mapping of the splice site, RNase protection assays (35) were performed with in vitro-generated ~260-nt antisense RNA probes that cover the chimeric acceptors. The presence of an abundant, protected ~130-nt fragment confirmed that these chimeric acceptors were efficiently utilized (data not shown). These results suggest that the IIIa exon does not contain sequences which prevent splicing to adjacent acceptors. Rather, sequences 5′ of the IIIa splice junction fail to facilitate or even prevent its usage.

Viral proteins affecting L1 splicing. During the course of the infection cycle, splicing of the L1 transcript switches from 52-55K to the IIIa acceptor site. Thus, a virus-encoded or -induced factor(s) is probably responsible for this switch. Two such potential virus-encoded factors are suggested from studies of the nature of the block(s) to human adenovirus growth in African green monkey kidney cells. The block in these abortive infections is complex, involving several steps in viral late gene expression. While transcription of the major late transcriptional unit is partially repressed (27), this alone does not account for the dramatic depression of synthesis of two late virion polypeptides, fiber and IIIa (3, 30). Very little IIIa mRNA is made from the L1 region (3, 27), whereas an altered pattern of ancillary leaders is found on the fiber mRNA (4) during the late phase of the abortive infection compared with that in a productive infection. The block to growth in monkey cells and the transcriptional and posttranscriptional aberrations are overcome by the presence of the SV40 T antigen (for a review, see reference 29) or a point mutation in the amino-terminal domain of the adenovirus-encoded, 72-kilodalton DBP (3, 13, 30). This mutation in DBP extends the host range of Ad2 or Ad5 to simian cells.

We initially tried to induce splicing to the IIIa acceptor of L1 transcripts encoded by the transfected vector by providing the DBP and other adenovirus-encoded or -induced proteins via infection with virions or cotransfection with genomic viral DNA. Whereas both 52-55K and IIIa splicing of L1 transcripts encoded by the viral genome were observed, IIIa-spliced transcripts from our transfected vectors were never detected (data not shown). This result, together with those of studies on the shutoff of host gene expression (5, 6), suggests that host and transfected genes (provided they are not part of the viral genome) may not be subjected to the same subnuclear environment as are genes on the replicating viral genome during the late phase of infection.

To circumvent this putative compartmentalization problem, vector p730, expressing the Ad5 host range DBP, was cotransfected along with the L1 vector pK1 into 293 cells. Again only 52-55K mRNA was detected (data not shown). Different results were obtained when the L1 vectors (e.g., pK1) were expressed in Cos-1 cells (Fig. 5A and C). Cos cells are derived from CV1 cells, an established line of African green monkey kidneys cells. They contain an integrated copy of the SV40 genome with a defective origin of DNA replication and express the early region of SV40, including large T antigen (21). In Cos cells transfected with the intact L1 vector, pK1, IIIa as well as 52-55K mRNAs were made (Fig. 5A, lane c); in the same experiment, transfected 293 cells produced only 52-55K mRNA (Fig. 5A, lane d). Similar results were obtained with the pK3 vector, which has part of the IIIa-coding region removed and contains a large deletion in the 52-55K-coding region that brings the two acceptor sites closer together (Fig. 5C, lanes c and d). With both vectors, the amount of IIIa mRNA relative to 52-55K mRNA varied from ~50% to sometimes undetectable levels (IIIa RNA was detected in 13 of 23 experiments). Cotransfection of the Cos cells with pMK16/SV40, a vector that expresses the early region of SV40 (see Materials and Methods), generally enhanced use of the IIIa acceptor site (Fig. 5A, compare lanes a and b with lane c). This enhancement was presumably due to the increased levels of T antigen.

Activation of the IIIa acceptor site in Cos but not HeLa or 293 cells could be due to species- or tissue-specific differences or to the presence of one or both of the SV40 early gene products, small t and large T antigens. To address this question, 293 cells were cotransfected with pK1 and pMK16/SV40. As before, transfection with the L1 vector alone failed to yield IIIa mRNA (Fig. 5B, lane a). However, cotransfection with the SV40 plasmid often led to synthesis of IIIa mRNA (Fig. 5B, lane b). As with the Cos cells, there was variability in the efficiency of IIIa splicing in cotransfected 293 cells (IIIa RNA was detected in three of seven experiments).

DISCUSSION

To gain a better understanding of the mechanism governing alternative splicing, we have studied the L1 region of adenovirus, whose expression is temporally regulated by alternative splicing (2, 37). Two major mRNAs are synthesized by splicing of the same donor site to different acceptor sites. The proximal acceptor site located at 11040 nt (1) is used during the early and late phases of infection to produce the 52-55K mRNA. In this study, the location of the distal site, which is utilized only during the late phase for generation of the IIIa mRNA, was mapped by S1 and RNase analyses to 12308 nt. This result directly confirmed the location previously inferred from sequence analysis (42).

Several factors that might affect this alternative splicing were tested by using expression vectors carrying the L1 region or mutated versions of it. In transfected HeLa or 293 cells, only the 52-55K RNA was synthesized. This finding suggests that the 52-55K acceptor site is preferred in the absence of a virus-encoded or -induced factor(s), but during

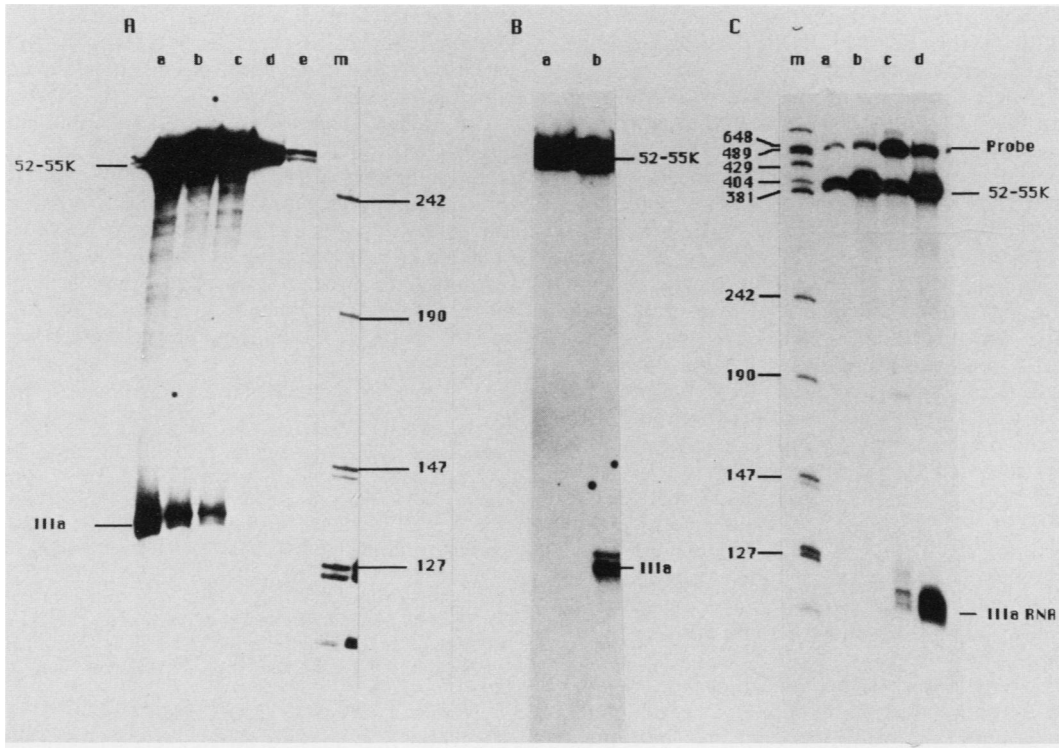

FIG. 5. Effects of SV40 early gene expression on alternative splicing of L1 transcripts. (A) Total cytoplasmic RNA was extracted from the following cells: mock-transfected 293 cells (lane e), 293 cells transfected with plasmid pK1 (lane d), Cos-1 cells transfected with plasmid pK1 (lane c), and Cos-1 cells cotransfected with plasmid pK1 and 10 (lane b) or 20 (lane a) μg of plasmid pMK16/SV40. S1 analysis was performed as described in the legend to Fig. 2 and used the same 272-bp *BglI-MluI* probe. (B) Total cytoplasmic RNAs from 293 cells transfected with plasmid pK1 (lane a) or cotransfected with plasmid pK1 and 30 μg of plasmid pMK16/SV40 (lane b) were analyzed by S1 nuclease mapping, using the 272-bp *BglI-MluI* probe and procedure described in the legend to Fig. 2. (C) Total cytoplasmic RNA was extracted from 293 (lanes a and b) or Cos-1 (lanes c and d) cells transfected with 15 (lanes a and c) or 30 (lanes b and d) μg of plasmid pK3. RNA was hybridized to the ~490-bp *Bam*HI-*Eco*RI probe shown in Fig. 3B.

the late phase of infection such a factor(s) either enhances IIIa acceptor site usage or partially blocks splicing to the 52-55K acceptor site. Decreasing the distance between the donor and the IIIa acceptor had no effect, but removal of the 52-55K acceptor resulted in synthesis of a small amount of IIIa mRNA. This result implies that competition between the two acceptors may be partly responsible for poor IIIa acceptor utilization. This could be due to the strong homology of the 52-55K, but not IIIa, acceptor with the acceptor site consensus sequence (36, 44). The greatly enhanced splicing to the IIIa exon junction when the 52-55K intron was fused to the IIIa exon is consistent with this possibility and moreover suggests that the IIIa exon does not contain sequences that inhibit splicing to adjacent acceptors. Thus, the failure to utilize the IIIa acceptor in the absence of a virus-encoded or -induced factor(s) is probably due to the absence of a favorable (positive) sequence and/or the presence of a negative element 5′ of the IIIa splice junction, or both. In this regard, it is noteworthy that the IIIa splice acceptor site contains ~50% purine residues and thus shows little homology to the pyrimidine-rich consensus sequence. Several groups have pointed out that pyrimidine-deficient splice acceptor sites are common in alternatively spliced transcripts and may require special factors for efficient utilization (10, 14, 19). If a 5′ negative element is responsible, it should lie within 132 nt of the splice junction or more than 1153 nt 5′ of it, since deletion of the region 133 to 1153 nt upstream of this junction in vector pK3 did not relieve the block to IIIa splicing.

To define *trans*-acting factors, cells transfected with the L1 vectors were infected with adenovirus or cotransfected with the adenoviral genome or plasmids containing viral genes. Unexpectedly, infection or cotransfection with the viral gene did not facilitate IIIa splicing of the vector L1 transcripts, even though viral L1 transcripts were spliced to the IIIa acceptor. Perhaps during the late phase of infection, genes on the viral chromosome encounter a different subnuclear environment than do other genes in the nucleus. This view is consistent with the presence of viral DNA in distinct globular structures (49) and the preferential transport of viral compared with host RNAs from the nucleus to the cytoplasm during the late phase of infection (5, 6).

Defective splicing of the L1 transcripts (little or no IIIa mRNA) and fiber transcripts (altered patterns of ancillary leaders on the mRNA) in abortively infected monkey cells is overcome by host range mutations in the adenovirus DBP or the presence of the SV40 T antigen (3, 4, 27). These results imply that these two proteins can affect RNA splicing. However, when a DBP expression vector was cotransfected with the L1 vector into 293 cells, only 52-55K mRNA was made. This finding suggests that (i) DBP is not involved in bringing about the 52-55K-to-IIIa splice switch, (ii) not enough DBP is produced in transfection compared with the extremely large quantities made during infection to facilitate this switch, or (iii) other virus-encoded products directly or indirectly act in conjunction with DBP to facilitate IIIa splicing. Human 293 cells synthesize E1A and E1B gene products from an integrated copy of these genes, whereas

the L1 vector also contains and expresses the VA genes. These gene products, together with DBP, thus appear to be insufficient to facilitate IIIa splicing.

In contrast, expression of the SV40 small t and large T antigens in Cos cells or in 293 cells cotransfected with an SV40 plasmid permitted splicing to the IIIa acceptor (Fig. 5). Several explanations could account for enhanced IIIa splicing in the presence of SV40 early proteins. The L1 vectors contain the SV40 origin of replication and thus are likely to replicate in Cos cells and presumably also in 293 cells cotransfected with the SV40 plasmid. The increased template number might lead to enhanced L1 transcript levels that could affect the utilization of the IIIa acceptor site. However, several observations argue against this scenario. First, the variability from experiment to experiment in efficiency of IIIa splicing seen in Cos cells or cotransfected 293 cells did not correlate with levels of L1 transcripts. Second, the amounts of L1 transcripts were often similar in pK1-transfected 293 cells, in which only 52-55K mRNA was synthesized, versus pK1 and pMK16/SV40 cotransfected-293 cells, which made both L1 mRNAs. Finally, we found that the amount of L1 RNA from the vector could be enhanced in 293 cells by cotransfection with adenovirus genomic DNA, yet this did not facilitate IIIa splicing of the vector transcript (data not shown).

Alternatively, replication of the vector per se may be critical. Replication may alter the template in yet undefined ways that could affect transcription and the subsequent RNA-processing reactions. More likely, it may alter the location and subnuclear environment of the vector, which might influence the splicing reaction. However, our previous studies of the role of SV40 in helping adenovirus grow in monkey cells imply that this is not the mechanism responsible for the altered IIIa splicing. Normal adenovirus DNA replication occurs even in abortively infected cells, and this is not changed by T antigen in productive infections. Rather, the presence of T antigen results in altered splicing patterns of L1 and L5 (fiber) RNAs (3, 4, 27). These studies, together with genetic analysis showing that the carboxy-terminal part of large T antigen (which is not involved in DNA replication or origin of replication binding) carries the function necessary for overcoming the block to adenovirus multiplication in monkey cells (for a review, see reference 29), suggest that large T rather than small t antigen is responsible for utilization of the IIIa acceptor.

L1 transcripts from pK3, a vector containing large deletions both 5′ and 3′ of the IIIa splice junction, were spliced to the IIIa acceptor in the presence of T antigen. This result suggests that a *cis*-acting element(s) essential for T-antigen induction of IIIa acceptor usage is located within a region of 132 nt 5′ and 130 nt 3′ of the IIIa splice junction. The 5′ region contains the branch point which may be important for alternative splicing, as recently shown by Manley and colleagues (19, 38) for SV40 early-region splicing.

The ability of T antigen but not DBP to facilitate IIIa splicing in transfected cells suggests that these proteins may use different mechanisms to modulate alternative splicing. DBP may require other viral gene products, whereas T antigen can function in the absence of adenovirus-encoded or -induced factors. However, T antigen probably also acts indirectly through a host factor(s) to facilitate the IIIa splicing. It may do so by induction of the synthesis of the factor or modulation of its activity. The variability in the level of IIIa splicing in our transfection experiments may have been due to differences in responsiveness of the gene encoding the factor for T-antigen induction or to the concentration of the factor, which was modified by T antigen. For example, Forbes et al. (17) reported cell cycle-dependent variations in small nuclear ribonucleoprotein concentration, which could affect splicing.

Our earlier studies indicated that T antigen, as well as DBP, could affect RNA splicing in monkey cells (4, 27). Here we demonstrate that an SV40 early gene product (presumably large T antigen) alters L1 transcript splicing in human cells as well as in monkey cells. Moreover, the effect of T antigen on splicing is not dependent on adenoviral factors. This work, together with our earlier studies, provides evidence for an additional function of the SV40 T antigen, namely, modulating alternative RNA splicing.

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