A U1 Small Nuclear Ribonucleoprotein Particle with Altered Specificity Induces Alternative Splicing of an Adenovirus E1A mRNA Precursor

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We have altered the specificity of U1 small nuclear RNA by replacing its 5' splice site recognition sequence (nucleotides 3 to 11) with sequences complementary to other regions of either the adenovirus E1A or the rabbit β -globin mRNA precursor. We then used a HeLa cell transient expression assay to test whether such altered U1 small nuclear ribonucleoprotein particles (snRNPs) could interfere with splicing of the targeted mRNA precursors. The altered U1 snRNPs were able to cause novel splicing of the E1A mRNA precursor, minor changes in the ratio of E1A 12 to 13S mRNAs, and modest nuclear accumulation of β -globin mRNA precursors with either one of the two introns removed. Most of the altered U1 snRNPs did not affect the level of mature cytoplasmic mRNA significantly, but in one case an altered U1 snRNP (α 1) whose intended target was located downstream from the adenovirus E1A 12S 5' splice site was able to reduce the level of cytoplasmic 12S mRNA by approximately 60% and that of 13S mRNA by 90%. This α 1 snRNP induced an additional E1A splice, resulting in the appearance of 10 and 11S E1A mRNAs normally found only late in adenovirus infection. Thus, a *trans*-acting factor can induce alternative splicing. Surprisingly, the effects of α 1 on E1A splicing were not abolished by deleting the intended target sequence on the mRNA precursor.

The splicing of mRNA precursors requires four distinct small nuclear ribonucleoprotein particles (snRNPs). The U1, U2, and U5 snRNPs each contain a single RNA species, whereas the U4/U6 snRNP contains the U4 and U6 small nuclear RNAs (snRNAs) base paired to each other (7, 15). The protein components of the U snRNPs can be divided into two classes, those common to all U snRNPs (B', B, D, D', E, F, and G) and those unique to a single snRNP (e.g., the 70K, A, and C proteins of the U1 snRNP) (24). One primary function of the U snRNPs in splicing is to recognize specific sequences on the mRNA precursor; the U1 and U2 snRNPs bind to the 5' splice site and the branchpoint sequence, respectively, and the U5 snRNP may help to recognize the 3' splice site (reviewed in references 27, 37, and 39). In addition, interaction between the U snRNPs (and possibly between the U snRNAs themselves) are believed to bring the distant splice sites together by assembling the mRNA precursor into a large multicomponent complex called the spliceosome in which the cleavage and ligation reactions take place (8, 10, 12).

The hypothesis that the 5' end of U1 snRNA recognizes the 5' splice site of mRNA precursors through conventional base pairing was first proposed by Lerner et al. (22) and by Rogers and Wall (35) on the basis of complementarity between the 5' splice site consensus sequence and nucleotides 3 to 11 in rat U1 snRNA. The results of subsequent biochemical experiments were consistent with this hypothesis (19, 31), but direct evidence for the postulated base pairing came from genetic experiments showing that some 5' splice site mutations can be efficiently suppressed by a compensatory base change in the 5' end of human (45) or yeast U1 snRNA (36, 38). In both the human and yeast systems, however, there were 5' splice site mutations that could not be suppressed. Thus, base pairing between U1 and

the 5' splice site is necessary but not sufficient for mRNA splicing.

Selective inhibition of gene expression using antisense RNA is a new tool for understanding the normal function of a specific gene (43). Antisense RNA has been particularly useful in higher eucaryotes, in which genetic analysis is much more difficult than in bacteria and in yeasts. The effectiveness of antisense RNA inhibition is determined by a variety of factors, including the structure, stability, and concentration of the antisense RNA, access of the antisense RNA to its target, and the stability of the desired hybrids in the presence of RNA-unwinding activities (3). Naturally occurring antisense RNAs, which regulate the expression of certain genes, have been found in procaryotes (13, 17).

Here we have explored the possibility of using altered U1 snRNPs to deliver antisense RNA to mammalian cells. To do this, we attempted to change the specificity of the U1 snRNP by replacing nucleotides 3 to 11 of U1 snRNA, which normally bind to the 5' splice site, with sequences complementary to other regions of mRNA precursors. Our rationale was that (i) the strong U1 gene promoter and the ability of the resulting transcript to associate with snRNP proteins would assure the abundance and stability of the antisense RNA; (ii) the nuclear location of altered U1 snRNPs would enable the ribonucleoproteins to intercept the target mRNA precursor or mRNA or both before they reached the cytoplasm; (iii) the protein components of the altered U1 snRNP might assist the antisense RNA in searching nuclear RNA for complementary sequences; and (iv) the altered U1 snRNPs could be targeted to sequences near essential splice sites in the mRNA precursor, thus increasing the likelihood that the target RNA sequence would be accessible in the heterogeneous nuclear ribonucleoprotein structure.

Our results show that U1 snRNPs with altered specificities can indeed interfere with the splicing of target mRNA precursors, but the effects were generally too weak to inhibit

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the level of mature cytoplasmic mRNA. In one special case, however, an altered U1 snRNP reduced the cytoplasmic level of 12S mRNA by approximately 60% and that of 13S mRNA by 90%. This $\alpha 1$ snRNP was targeted to a sequence downstream of the adenovirus E1A 12S 5' splice site and induced an additional splice to yield 10 and 11S E1A mRNAs which normally appear only late in adenovirus infection. Although we do not understand why the effects of $\alpha 1$ are not abolished by deletion of its target sequence on the E1A mRNA precursor, our data clearly show that a *trans*-acting factor can induce alternative splicing.

MATERIALS AND METHODS

Mutant constructions. An mp8 clone containing the U1-4u gene in a 681-base-pair *Hae*III fragment fitted with *Bam*HI linkers (45) was used as a template for site-directed mutagenesis. For each U1 mutant, a 29-mer (or 38-mer for $\alpha 2$) oligodeoxynucleotide was synthesized to be complementary to the U1 gene sequence from position -8 in the 5'-flanking region to position 21 in the coding region, except for positions 3 to 11, which were changed as desired. Site-directed mutagenesis was performed by the method of Kunkel (20). The desired U1 mutants were identified by dideoxy sequencing of the single-stranded recombinant bacteriophage DNAs, using a primer complementary to positions 121 to 142 of U1. The mutant U1 genes were then recloned into a pUC13 vector as a *Hind*III-*Eco*RI fragment and used for transfection.

E1A mutant d979/1008 was constructed by replacing the *Cla*I-*Sma*I fragment (positions 918 to 1008 of adenovirus 2) in the plasmid pSVE1A (45) with the *Cla*I-*Mbo*II fragment (positions 918 to 978 of adenovirus 2), also isolated from pSVE1A. The *Cla*I ends were ligated first, and the *Mbo*II end was then polished with Klenow fragment before being ligated with the *Sma*I end. The identity of the resulting construct was verified by restriction mapping and sequencing.

Transfection and RNA preparation. Transfections were performed as described previously (16), using 6 μ g of U1 gene and 6 μ g of E1A or β -globin gene per 6-cm plate. The plasmid pSVE1A was used as the source of the E1A gene (45), and plasmid pSK⁺ was used as the source of the rabbit β -globin gene (2, 26). About 48 h after transfection, cytoplasmic RNA was extracted from the postnuclear supernatant of cells lysed with Nonidet P-40 (16). To prepare nuclear RNA, the nuclei were treated with ~450 μ g of RNase-free DNase I per ml (Bethesda Research Laboratories, Inc.) in 50 μ l of digestion buffer (50 mM Tris hydrochloride [pH 7.6], 10 mM MgCl₂, 5 mM dithiothreitol, 1 mM EDTA, 2 U of RNasin per μ l) at 37°C for 20 min. The reaction was brought to 250 μ l with water and stopped by adding 250 μ l of 7 M urea-350 mM NaCl-10 mM Tris hydrochloride (pH 7.6)-10 mM EDTA-1% sodium dodecyl sulfate. After phenol-chloroform extractions, RNA was precipitated with ethanol. For one experiment (see Fig. 2B), HeLa cells were cotransfected with four U1 mutants ($\alpha 1$, $\alpha 4$, $\alpha 5$, and $\alpha 6$), using 2 μ g of each construct.

RNase and S1 nuclease protection analysis. RNase mapping was performed essentially as described previously (28). RNase digestion was at 30°C for 30 min and stopped with 2% sodium dodecyl sulfate. The samples were then treated with 400 μ g of proteinase K per ml at 37°C for 15 min, extracted with phenol-chloroform, ethanol precipitated, and fractionated on a denaturing polyacrylamide gel. S1 mapping was performed essentially as described previously (16), except

FIG. 1. Target sites on the adenovirus E1A and rabbit β -globin mRNA precursors for U1 snRNPs with altered specificities. Brackets indicate the approximate locations of complementarity between the altered U1 snRNPs and the target sites (not drawn to scale). Numbers in parentheses indicate the positions of the target sites with respect to the nearest 5' or 3' splice site. Altered U1 snRNAs of the α and β series target the E1A and β -globin mRNA precursors, respectively. The target site for $\alpha 8$ is not shown but lies at +9, 1 nucleotide upstream from the $\alpha 1$ target site.

that the hybridization was at 42°C and the digestion with 200 U of S1 nuclease (Pharmacia, Inc.) was at 30°C for 1 h.

Primer extension. The 5' end of synthetic oligodeoxynucleotide primers was labeled with [γ -³²P]ATP and T4 polynucleotide kinase. Primer extension on U1 RNA was performed with the Klenow fragment of DNA polymerase I at 37°C for 20 min in the presence of 38 μ M each dTTP, dCTP, and dGTP (45). Primer extension on E1A RNA was performed as follows. RNA was mixed with the labeled primer in 12 μ l of 125 mM KCl-83 mM Tris hydrochloride (pH 7.6), heated to 65°C for 10 min, incubated at 37°C for 10 min, and allowed to cool to room temperature for 30 min. The mixture was made 3 mM in MgCl₂, 10 mM in dithiothreitol, 50 μ g/ml in actinomycin D, and 0.5 mM in each deoxynucleoside triphosphate, and 1 μ l of M-MLV (200 U/ μ l) reverse transcriptase (Bethesda Research Laboratories) was added per 20- μ l reaction mixture. The reaction mixture was incubated at 42°C for 30 min. Another 0.5 μ l of reverse transcriptase was added, and incubation was continued at 42°C for 30 min. The reaction was stopped with 25 mM EDTA, ethanol precipitated, and fractionated on a denaturing polyacrylamide gel.

Immunoprecipitation. Sonicated cell extracts and immunoprecipitation with anti-Sm monoclonal antibody (21) and anti-(U1)RNP serum (33) were performed essentially as described previously (29).

RESULTS

We used site-directed mutagenesis to make 12 different human U1 snRNA mutants in which the 9-nucleotide U1 sequence (positions 3 to 11) that recognizes the 5' splice site of mRNA precursors was replaced by a new 9-nucleotide sequence (or an 18-nucleotide sequence for mutant $\alpha 2$) complementary to either the adenovirus E1A or rabbit β -globin mRNA precursor (Fig. 1 and Table 1). Target sites on the mRNA precursors were chosen to include sequences adjacent to the 5' splice site, the branchpoint sequence, and the 3' splice site. Altered U1 snRNAs of the α series target the E1A mRNA precursor; altered U1 snRNAs of the β series target the β -globin mRNA precursor. The effect of the altered U1 snRNAs on splicing of the target mRNA precursor was determined by using a transient expression assay in

TABLE 1. Expected 5' terminal sequences of U1 snRNAs with altered specificities^a

Sequence	snRNA
cap AUACUU <u>ACCUG</u>	Normal U1
cap AUUAACACAAA	$\alpha 8$
cap AUCUAACACAA	$\alpha 1$
cap AUCCACAUA <u>AUCUAACACAA</u>	$\alpha 2$
cap AUCACCCUCUU	$\alpha 3$
cap AUUCUUUAAAC	$\alpha 4$
cap AUUUUAAAAA	$\alpha 5$
cap AUAUUUUUCAC	$\alpha 6$
cap AUCACCCACUG	$\alpha 7$
cap AUGGAUACCAA	$\beta 1$
cap AUCUGCCAGG	$\beta 2$
cap AUAGGUCCCC	$\beta 3$
cap AUAAGGUCCC	$\beta 4$

^a For comparison, the normal human U1 sequence is shown without ribose methylations or base modifications (3). The 5' splice site recognition sequence (nucleotides 3 to 11) in the normal U1 sequence is underlined. The $\alpha 8$ target site is shifted 1 nucleotide upstream from the $\alpha 1$ target site. Compared with $\alpha 1$, $\alpha 2$ could in principle form nine additional base pairs with the target site. The $\beta 3$ target site is shifted 1 nucleotide upstream from the $\beta 4$ target site. The adenovirus 2 E1A sequence is from Gingeras et al. (11), and the rabbit β -globin sequence is from Hardison et al. (14).

HeLa cells cotransfected with genes encoding the altered U1 RNA and the target mRNA precursor.

U1 snRNAs with altered specificities are efficiently synthesized and assembled into snRNPs. To determine whether U1 snRNAs with altered specificities are efficiently synthesized and stable, we used a primer extension assay to measure the fraction of mutant U1 RNA in transfected cells. A synthetic oligodeoxynucleotide primer complementary to nucleotides 17 to 46 of U1 was extended with the Klenow fragment of DNA polymerase I in the presence of dTTP, dCTP, and dGTP. Klenow polymerase gives more faithful transcription than reverse transcriptase when one deoxynucleoside triphosphate is omitted from the reaction (45). In the absence of dATP, the primer extension products derived from the endogenous (normal) and mutant U1 RNAs are different because of sequence differences between the snRNA templates at positions 3 to 11. Most U1 snRNAs with altered specificities were relatively abundant compared with endogenous U1 (Fig. 2A). Assuming a typical transfection efficiency of about 30%, we estimate that the most abundant mutant U1 RNAs account for 25 to 30% of the total U1 snRNA in transfected cells. We do not understand why some mutant U1 RNAs are more abundant than others, but the effect cannot be due to variation in transfection efficiency or RNA preparation since it is not eliminated when the mutant U1 genes are introduced into the same cells by cotransfection (Fig. 2B and data not shown). The extension products for the $\beta 2$, $\beta 3$, and $\beta 4$ RNAs were shorter than expected, but this may be a gel electrophoresis compression artifact because all three extension products have a 5'-terminal oligo(G) tract (Table 1).

We used anti-Sm and anti-(U1)RNP antibodies to determine whether U1 snRNAs with altered specificities are assembled into snRNPs. Cell sonic extracts prepared from HeLa cells cotransfected with the four U1 mutants $\alpha 1$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ were precipitated with anti-Sm, anti-(U1)RNP, or nonimmune sera. All four mutant U1 RNAs were immunoprecipitated by antistreptomycin and anti-(U1)RNP (Fig. 2B). Pulse-chase experiments would be required to determine what fraction of the mutant U1 RNA is assembled into snRNPs, but the abundance and stability of other mutants in the 5' end of U1 RNA (44a, 45) suggest that the α and β

FIG. 2. Expression and immunoprecipitation of mutant U1 RNAs with altered specificities. (A) HeLa cells cotransfected with various altered U1 genes and the corresponding target gene. Cytoplasmic RNA from transfected cells was hybridized to an oligodeoxynucleotide complementary to nucleotides 17 to 46 of U1. The resulting hybrids were extended with the Klenow fragment of DNA polymerase I in the presence of dTTP, dCTP, and dGTP and resolved on a 12% denaturing polyacrylamide gel. Cells were transfected with the pUC13 vector as a control (lane C). Primer extension assays for $\alpha 3$ and $\alpha 7$ are not shown because the resulting extension products are the same size as ($\alpha 7$) or 1 nucleotide shorter than ($\alpha 3$) that for wild-type U1. (B) HeLa cells cotransfected with the four U1 mutants $\alpha 1$, $\alpha 4$, $\alpha 5$, and $\alpha 6$. Transfected cells were sonicated and immunoprecipitated with anti-Sm (lanes Sm), anti-(U1)RNP (lanes U1), or nonimmune (lanes NI) sera. RNAs from precipitates (ppt) and supernatants (sup) were analyzed by primer extension as described for panel A.

series of mutants are likely to be efficiently assembled into snRNPs.

Except for $\alpha 1$, U1 snRNPs with altered specificities have little effect on E1A splicing. We used an RNase protection assay to test whether the α series of U1 mutants affected the splicing of E1A mRNA precursors. Cytoplasmic RNA from control cells transfected with the E1A gene alone protected three fragments corresponding to two 5' exons and the common 3' exon of the 12 and 13S mRNAs (Fig. 3, lanes 1, 8, and 11). The SP6 RNA probe used in this assay spans the *NaeI-XbaI* fragment of E1A gene (*NaeI* RNA probe; see Fig. 5D) and would not detect 9S mRNA if any were made.

Of all the U1 snRNPs with altered specificities, only $\alpha 1$ had a significant effect on E1A splicing (Fig. 3, lanes 2, 9, and 12), although some minor changes in the ratio of 13 to 12S RNAs were also observed in cells transfected with other altered U1s (lanes 3 through 7). Mutant $\alpha 1$, which is targeted

FIG. 3. Effect of the α series U1 mutants on splicing of E1A mRNA precursors. HeLa cells were transfected with the E1A gene and U1 mutants of the α series. Cytoplasmic RNA from transfected cells was hybridized to an SP6 antisense transcript of the E1A gene (*NaeI* RNA probe; see Fig. 5D). The resulting hybrids were then digested with T1 and pancreatic RNase, and the digestion products were resolved on a 5% denaturing polyacrylamide gel. Cells were transfected with the E1A gene and the pUC13 vector as a control (lanes C). The two novel protected fragments resulting from transfection with $\alpha 1$, $\alpha 2$, and $\alpha 8$ are indicated ($\rightarrow$).

to positions +10 to +18 downstream from the 13S 5' splice site, reduced 13S RNA by approximately 90% and 12S RNA by 60 to 70% and caused the appearance of two novel protected fragments (arrows). The target site of $\alpha 2$ (positions +10 to +27 downstream from the 12S 5' splice site) includes the $\alpha 1$ target site and 9 additional nucleotides (Fig. 1 and Table 1). However, $\alpha 2$ had less effect than $\alpha 1$; the synthesis of 13S RNA was almost normal, and the amount of one of the two novel protected fragments was greatly reduced (Fig. 3; compare lanes 8, 9, and 10). Since $\alpha 2$ RNA was as abundant as $\alpha 1$ (Fig. 2A) and is also assembled into a snRNP as efficiently as $\alpha 1$ (data not shown), these data suggest that the effect of $\alpha 1$ requires some aspect of U1 snRNP structure that is not compatible with a 9-nucleotide 5' extension on U1 RNA.

In an attempt to understand why $\alpha 1$ strongly affected E1A splicing but $\alpha 3$ to $\alpha 7$ did not, we constructed a variant of $\alpha 1$ designated $\alpha 8$. The target site of $\alpha 8$ on the E1A mRNA precursor is almost identical to that of $\alpha 1$ but is displaced upstream by a single nucleotide; i.e., $\alpha 8$ is complementary to nucleotides +9 to +17 downstream of the 12S 5' splice site. $\alpha 8$ also had a weaker effect than $\alpha 1$ (Fig. 3; compare lanes 11, 12, and 13). Taken together, these data indicate that U1 snRNPs with altered specificities generally have little effect on splicing of the target mRNA precursors. The strong effect of $\alpha 1$ probably resulted from a fortuitous complementarity between $\alpha 1$ and a site in the E1A mRNA precursor other than the intended target sequence (see below).

$\beta 2$, $\beta 3$, and $\beta 4$ cause nuclear accumulation of incompletely processed β -globin mRNA precursors. We have also tested

four U1 snRNPs with altered specificities ($\beta 1$ to $\beta 4$; Fig. 1 and Table 1) targeted against the rabbit β -globin mRNA precursor, which has two introns and is not alternatively spliced. HeLa cells were cotransfected with the β -globin gene and various U1 snRNA genes, and cytoplasmic RNA was analyzed by an RNase protection assay (Fig. 4). In control cells transfected with the globin gene alone, four sets of protected fragments were observed (Fig. 4A, lane 2); three of these are apparently derived from the three globin exons, but the fourth set of fragments was unexpected and may be caused by mapping artifacts or by aberrant transcripts initiating in the 5'-flanking region.

No U1 snRNP with altered specificity had any obvious effect on either the variety or the level of cytoplasmic β -globin RNAs (Fig. 4A, lanes 3 to 6). However, when nuclear RNA from these transfected cells was analyzed, $\beta 3$ and $\beta 4$ produced an additional protected fragment of about 900 nucleotides (lanes 10 and 11, upper arrow), and $\beta 2$ produced an additional protected fragment of about 500 nucleotides (lane 9, lower arrow). The sizes of these protected fragments suggest that the 900-nucleotide fragment is derived from a β -globin mRNA precursor with only the first intron removed (the second exon, second intron, and third exon span 928 nucleotides) and that the 500-nucleotide fragment is derived from a β -globin mRNA precursor with only the second intron removed (the first exon, first intron, and second exon span 494 nucleotides) (Fig. 4C). An S1 nuclease protection assay on nuclear RNA from cells cotransfected with the globin and $\beta 3$ genes confirmed the existence of the β -globin mRNA precursor with only the first intron removed (Fig. 4B, lane 2). Thus, $\beta 3$ and $\beta 4$, which are targeted to sequences near the 5' splice site of the second intron, cause the accumulation in the nucleus of β -globin mRNA precursors with only the first intron removed. Similarly, $\beta 2$, which is targeted to a sequence near the 5' splice site of the first intron, causes accumulation of β -globin mRNA precursors with only the second intron removed. These data indicate that U1 snRNPs with altered specificities can indeed interfere with the use of a nearby 5' splice site, but the effect is generally too weak to reduce the level of mature cytoplasmic mRNA.

$\alpha 1$ induces a novel splice which normally occurs only late in adenovirus infection. We found earlier that cytoplasmic RNA from cells transfected with the E1A and $\alpha 1$ genes protected two novel fragments (Fig. 3, arrows). To identify the E1A transcripts responsible for these two protected fragments, we used both primer extension and RNase protection assays. When a 5'-end-labeled 16-mer complementary to the E1A 3' exon (Fig. 5D) was extended with reverse transcriptase, two novel E1A extension products, designated C1 and C2, were obtained; C2 predominated in cytoplasmic RNA (Fig. 5A, lanes 4 and 9), and C1 predominated in nuclear RNA (Fig. 5A, lane 8). Another E1A extension product, designated N1, was found exclusively in the nucleus (lane 8).

Figure 5B and C shows the data from an RNase protection assay using different SP6 RNA probes (Fig. 5D). Figure 5D also summarizes the results obtained with both the RNase protection and primer extension assays. Taken together, the data indicate that N1 RNA is an E1A mRNA precursor with a novel splice, most likely between the 9S 5' splice site and a novel 3' splice site at position 853 in the adenovirus 2 sequence (11); the sequences surrounding position 853 resemble the 3' splice site consensus, including the polypyrimidine tract and AG dinucleotide (30). C1 RNA is an E1A RNA with the novel splice and the 13S splice, whereas the

FIG. 4. Effect of the β series U1 mutants on splicing of rabbit β -globin mRNA precursors. (A) HeLa cells were transfected with the β -globin gene and different U1 mutants of the β series. Cytoplasmic and nuclear RNAs from transfected cells were hybridized to an SP6 antisense transcript of the β -globin gene (shown in panel C). The resulting hybrids were then digested with T1 and pancreatic RNase and resolved on a 5% denaturing polyacrylamide gel. Cells

C2 RNA is an E1A RNA with the novel splice and the 12S splice. This conclusion was confirmed by analysis of E1A RNA from cells transfected with $\alpha 1$ and an E1A gene having the pm1114 mutation at the 13S 5' splice site or an E1A gene having the pm975 and pm1114 mutations at both 12 and 13S 5' splice sites (data not shown; for discussion, see reference 45). After we had identified these transcripts, Stephens and Harlow (40) and Ulfendahl et al. (42) reported the characterization of two novel E1A mRNAs, the 10 and 11S RNAs, which appear late in adenovirus infection of HeLa cells and encode proteins with molecular masses of 30 and 35 kilodaltons. The C1 and C2 RNAs we detected in HeLa cells transfected with the E1A and $\alpha 1$ genes appear to be identical to the E1A 11 and 10S mRNAs, respectively. Thus, $\alpha 1$ induces a regulated E1A splice which normally occurs only late in the adenovirus life cycle.

Effects of $\alpha 1$ on E1A splicing not abolished by deletion of its target site. For two reasons, we suspected that the strong effect of $\alpha 1$ did not result from interaction of this altered snRNP with its intended target site; other U1 snRNPs with altered specificities generally had little effect on splicing of targeted mRNA precursors, and the effect of $\alpha 8$ was much weaker than that of $\alpha 1$ although the intended target sites were shifted by only 1 nucleotide (Fig. 3). We therefore constructed an E1A mutant (d979/1008) lacking the $\alpha 1$ target site (positions +10 to +18) by deleting sequences between positions +6 and +35 downstream of the 12S 5' splice site. We then tested whether $\alpha 1$ could affect the splicing of this mutant E1A d979/1008 mRNA precursor.

When cytoplasmic RNA from cells transfected with d979/1008 alone was analyzed by primer extension using a primer complementary to the E1A 3' exon (Fig. 5D), we were surprised to find that only one major E1A transcript was detected (Fig. 6, lane 3). This transcript was about 30 nucleotides shorter than 13S RNA, indicating that it represents 13S splicing of the E1A d979/1008 precursor. Since the 9-nucleotide sequence of the 12S 5' splice site is unchanged in d979/1008 (deletion of the G residue at +6 is restored by ligation to the G at +36), sequences immediately downstream of the E1A 12S 5' splice site appear to affect the efficiency of the 12S splicing. To our surprise, cotransfection of $\alpha 1$ with d979/1008 caused a new transcript to appear (Fig. 6, lane 5, arrow). This transcript is about 30 nucleotides shorter than the C1 RNA (Fig. 5D), suggesting that it has the same novel splice and 13S splice as C1. Thus, the effects of $\alpha 1$ on E1A splicing are not abolished by deletion of its target site on the mRNA precursor.

DISCUSSION

We have made a new class of mutant U1 snRNAs with altered specificities by replacing the 5' splice site recognition sequence of U1 (nucleotides 3 to 11) with sequences complementary to other regions of the adenovirus E1A or rabbit

were transfected with the E1A gene and a wild-type U1 gene as a control (lanes 2 and 7); denatured DNA size markers (in kilodaltons) were from an end-labeled *Hpa*II digest of pBR322 (lane M). The novel protected fragments from $\beta 2$, $\beta 3$, and $\beta 4$ ($\leftarrow$) and unexplained cytoplasmic and nuclear protected fragments (*) are indicated. (B) Nuclear RNA from cells transfected with the β -globin gene and U1 mutant $\beta 2$ or $\beta 3$ was hybridized to a 5'-end-labeled single-stranded DNA probe (see panel C), treated with nuclease S1, and resolved on a 5% denaturing polyacrylamide gel. Size markers (lane M) are as described for panel A. (C) SP6 and S1 probes used in panels A and B, respectively. Lengths of fragments (in nucleotides) are shown.

FIG. 6. Effects of $\alpha 1$ on E1A splicing not abolished by deletion of the $\alpha 1$ target site. Cytoplasmic RNA from HeLa cells transfected with the indicated genes was analyzed by primer extension as described for Fig. 5A. Lanes: 2, wild-type E1A alone; 4, wild-type E1A and U1 mutant $\alpha 1$; 3, E1A mutant d979/1008 alone; 5, E1A mutant d979/1008 and U1 mutant $\alpha 1$; M, size markers as described in the legend to Fig. 4. Novel E1A transcripts induced by $\alpha 1$ are identified on the right (C1, C2, and arrow).

β -globin mRNA precursor. Most of these U1 RNAs with altered specificities were efficiently synthesized and assembled into snRNPs in transfected cells (Fig. 2). We then used a HeLa cell transient expression assay to determine whether such altered U1 snRNPs could affect the splicing of target mRNA precursors and found that, with the exception of mutant $\alpha 1$ (discussed below), the effects were generally weak. We observed only minor changes in the ratio of E1A 12 to 13S RNAs (Fig. 3) or a slight nuclear accumulation of incompletely processed β -globin mRNA precursors (Fig. 4).

Mutant $\alpha 1$, whose intended target was a sequence downstream from the E1A 12S 5' splice site, was the only altered U1 with a significant effect on the targeted mRNA precursor. Mutant $\alpha 1$ reduced the amount of mature cytoplasmic 13S mRNA by approximately 90% and the amount of 12S mRNA

by 60% and induced a novel splice between the 9S 5' splice site and a 3' splice site located midway between the 9 and 12S 5' splice sites (Fig. 5D). Together with the 12 and 13S splices, this novel splice generates the 10 and 11S mRNAs that are normally induced only late in adenovirus infection (40, 42). $\alpha 1$, however, does not appear to induce a novel splice of the β -globin mRNA precursor (data not shown). Thus, our data demonstrate that a *trans*-acting ribonucleoprotein factor can induce alternative splicing of an mRNA precursor, perhaps by a mechanism resembling that of natural *trans*-acting factors responsible for alternative splicing (1, 4-6). Although variant U1 snRNAs have been found in mice (18, 23, 25), frogs (9), and sea urchins (44) and synthesis of these variants is known to be developmentally regulated in certain cases (9, 25), the 5' splice site recognition element (nucleotides 3 to 11) is absolutely conserved among all known variant U1 RNAs. Such conservation argues against the possibility that U1 snRNPs with altered specificities are one of the natural factors that regulate alternative splicing.

Our data indicate that U1 snRNPs with altered specificities are capable of binding to the target mRNA precursor (Fig. 4; see below). However, the finding that the $\alpha 1$ target sequence on the E1A mRNA precursor can be deleted without abolishing the novel splice induced by $\alpha 1$ (Fig. 6) suggests that $\alpha 1$ may also bind fortuitously to some other region(s) of the E1A mRNA precursor. A computer search revealed two sites in the sequence of the E1A precursor (11) which have the potential to form at least 6 base pairs with the first 11 nucleotides of $\alpha 1$: E1A positions 584 to 589 (just upstream of the 9S splice site at position 636) could form 6 base pairs with $\alpha 1$ nucleotides 3 to 8, and E1A positions 1067 to 1076 (between the 12 and 13S 5' splice sites at positions 973 and 1111, respectively) could form 8 base pairs with $\alpha 1$ nucleotides 2 to 11. Whether deletion of either of these potential $\alpha 1$ target sites from the E1A precursor would abolish the effect of $\alpha 1$ remains to be determined.

Three altered U1s (mutants $\beta 2$, $\beta 3$, and $\beta 4$) whose targets are near the two 5' splice sites of the β -globin mRNA precursor were able to modestly inhibit use of the nearest 5' splice site. This implies that altered U1s bind to the expected target sites, but we do not know whether the altered U1 snRNPs interfere with splicing by blocking the binding of the wild-type U1 snRNP or by promoting partial assembly of a nonfunctional spliceosome. In either case, the altered U1 snRNPs would have to compete with the wild-type U1 snRNP for binding to the mRNA precursor. Although the fraction of the altered U1 snRNAs in transfected cells may reach 25 to 30% (Fig. 2), it seems likely that this would not be sufficient to achieve a strong inhibitory effect, unless the altered snRNP binds to the mRNA precursor preferentially. Preferential binding might account for the efficient suppres-

FIG. 5. Identification of E1A transcripts induced by $\alpha 1$. (A) Primer extension analysis. HeLa cells were transfected with E1A and U1 mutant $\alpha 1$. Cytoplasmic and nuclear RNAs from transfected cells were hybridized to a labeled oligonucleotide complementary to positions 1249 to 1265 within the E1A gene of adenovirus 2 (see panel D). The resulting hybrids were extended with reverse transcriptase, and the products were resolved on a 4% denaturing polyacrylamide gel. Control cells were mock transfected without DNA (lanes 2 and 6) or were transfected with the E1A gene and pUC13 vector (lanes 3 and 7). The novel E1A transcripts induced by $\alpha 1$ are indicated on the left (C1, C2, and N1). Size markers (lanes M) are as described in the legend to Fig. 4. (B) RNase protection analysis. Cytoplasmic RNA from transfected cells was hybridized to different SP6 antisense transcripts of the E1A gene (see panel D) and treated with T1 and pancreatic RNase, and the products were resolved on a 4% denaturing polyacrylamide gel. (C) RNase protection analysis. Nuclear RNA from transfected cells was analyzed as described for panel B. Additional bands not indicated in panel D are products of artifactual RNase cleavage at an oligo(U) tract located around position 1174 in the 13S intron. (D) Structures of the C1, C2, and N1 RNAs deduced from the analysis described above. The lengths of the cDNA products in the primer extension assay and the protected RNA fragments in RNase assay (in nucleotides) are indicated.

sion of the *hr440* 5' splice site mutation observed with U1-4u snRNA (45).

Although the failure of most antisense U1 snRNAs to interfere with splicing might reflect inefficient nuclear localization of the altered snRNPs, we think this is unlikely for two reasons. (i) Nuclear localization is caused by binding of the Sm antigen(s) to an internal site on the snRNA (reviewed in reference 24) and should not be affected by changing nucleotides 3 to 11 at the extreme 5' end of U1 snRNA. (ii) The sequences of nucleotides 3 to 11 in $\alpha 1$ and $\alpha 2$ are virtually identical and thus unlikely to affect nuclear localization, yet $\alpha 1$ affects splicing and $\alpha 8$ does not.

We attempted to make more-effective altered U1 snRNPs by stabilizing the binding of the snRNP to the target mRNA precursor. To do this, we increased the length of antisense in the altered U1 snRNA by replacing the 9-nucleotide 5' splice site recognition element of U1 snRNA with an 18-nucleotide sequence complementary to the E1A mRNA precursor ($\alpha 2$) or with a 94-nucleotide sequence complementary to the β -globin mRNA precursor (data not shown). We found that $\alpha 2$ has a much weaker inhibitory effect than $\alpha 1$, although $\alpha 2$ RNA is nearly as abundant as $\alpha 1$ (Fig. 2A), is assembled into snRNPs (data not shown), and has an 18-nucleotide target region that spans the entire $\alpha 1$ target site plus 9 additional nucleotides. How the 9-nucleotide extension at the 5' end of $\alpha 2$ prevents the fortuitous interaction responsible for the unique behavior of $\alpha 1$ is not clear. One possibility is that $\alpha 1$ requires the help of U1 snRNP proteins to scan RNA for complementary sequences or to stabilize the binding of the altered snRNP to the precursor; the 5' extension of $\alpha 2$ would then interfere with the structure of the altered U1 snRNP so that it could not perform these functions. One precedent for this would be the observation that insertion of one or two guanosine residues at position 11 in the U1-4u suppressor greatly reduces the efficiency of suppression (44a). Another possible precedent would be the failure of a sea urchin U7 RNA containing extra nucleotides at the 5' end to complement 3' processing of the sea urchin histone H3 mRNA precursor in *Xenopus* oocytes (41). We also found that the altered U1 snRNA with a 94-nucleotide 5' extension is unstable in transfected cells (data not shown).

Although our data demonstrate that U1 snRNPs with altered specificities can bind to target sequences in vivo, the target sequences are apparently not used as 5' splice sites. For example, the $\beta 1$ target sequence (UUGGUAUCC) shares four nucleotides (GGUA) with the 5' splice site consensus (CAG/GUAAGU) at positions -1 to +3 relative to the splice junction (indicated by the shill); however, this sequence was not used as a 5' splice site in the presence of $\beta 1$. Thus, U1 snRNPs with altered specificities are unable to inhibit the expression of target genes by generating aberrant spliced products that use the target sequences as a new 5' splice site. This conclusion is consistent with previous genetic experiments showing that some 5' splice site mutations can be suppressed by a compensatory change in U1, whereas others cannot (36, 38, 45). Evidently, simple base pairing between U1 and the mRNA precursor is not sufficient for splicing; specific residues within the 5' splice site or the 5' end of U1 snRNA are also required.

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