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A small nuclear ribonucleoprotein is required for splicing of adenoviral early RNA sequences

(systemic lupus erythematosus antibodies/isolated nuclei/mercurated cytidine triphosphate/polyadenylylation)

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ABSTRACT **The size and structure of viral RNA species synthesized in nuclei isolated during the early phase of productive infection by adenovirus type 2 have been examined by electrophoresis in denaturing polyacrylamide cells and the nuclease S1 assay. The major products of transcription *in vitro* of early regions 1 and 2 in the adenoviral genome are processed RNA molecules that appear to be correctly spliced in isolated nuclei. Splicing of adenoviral RNA molecules is inhibited when nuclei are preincubated with antibodies from systemic lupus erythematosus patients that immunoprecipitate small nuclear ribonucleoprotein particles. The specificity of these antibodies suggests that ribonucleoprotein particles containing U1 RNA are required for splicing of the adenoviral RNA sequences we have examined.**

Most eukaryotic genes encoding mRNA species are interrupted by intervening sequences, as are the DNA sequences composing the genome of human adenovirus type 2 (Ad2). During the early phase of productive infection of human cells by Ad2, at least eight viral genes are expressed (1–5); mRNA species complementary to all but one early gene, that specifying polypeptide IX (6), are spliced (refs. 7–10; unpublished observations). Intervening sequences in these viral genes appear to lie within coding regions (11), and in this sense adenoviral early genes resemble the many eukaryotic genes whose coding sequences are also discontinuous (see, for example, references in ref. 12). Moreover, where sequence information is available (11, 13), the splice junctions in adenoviral early genes can be seen to resemble those of cellular genes. It therefore seems reasonable to infer that adenoviral early RNA sequences are spliced by the host cell's splicing machinery.

Little is known about the machinery and mechanisms of splicing, despite a rapid expansion of the catalogue of spliced eukaryotic mRNA species (e.g., ref. 12). Elegant studies of maturation *in vitro* of yeast tRNA species containing intervening sequences have shown that RNA secondary structure can be a critical parameter of the splicing reaction (14–16), promoting the close proximity of sequences to be ligated. It has recently been proposed that small nuclear RNA species may play an analogous role in the maturation of eukaryotic mRNA precursors: the 5′-terminal sequence of U1 RNA, which is present in small nuclear ribonucleoprotein particles (snRNPs) and can be precipitated by anti-ribonucleoprotein (RNP) and anti-Sm antibodies (Sm is a nuclear antigen) from certain patients with systemic lupus erythematosus (SLE) (17), exhibits extensive complementarity to a consensus nucleotide sequence derived from some 35 splice junctions (12). Other properties of this class of snRNPs are compatible with a splicing function (12). Similarly, Murray and Holliday (18) have considered a role for the small RNA species adenoviral VA-RNA$_I$ in the splicing of adenoviral late RNA sequences.

It has been reported recently that nuclei isolated during the late phase of Ad2 infection readily, and correctly, perform the splicing reactions necessary to synthesize the 5′-terminal leader segment (19, 20) and to join it to the body of late mRNA species (21, 22). Here, we show that mature adenoviral early RNA species can also be synthesized in isolated nuclei and exploit this system to investigate the role of snRNPs in splicing reactions.

MATERIALS AND METHODS

HeLa human cells, infected with Ad2 at 40 plaque-forming units per cell in the presence of cytosine arabinonucleoside at 20 μg/ml, were harvested 8 hr after adsorption of the virus and nuclei were isolated as described (23). After preincubation for 10 min at 4°C in the presence of IgG prepared from sera of SLE patients, nuclei were incubated at 25°C for 1 hr in a solution consisting of 30 mM Tris·HCl at pH 7.9, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 30 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 1 mM ATP, 0.25 mM CTP, 0.25 mM GTP, and [^{3}H]UTP at 200–250 μCi/ml (23) (1 Ci = 3.7×10^{10} becquerels). In some experiments, one-third to one-fifth of the CTP was replaced by Hg-CTP; mercurated RNA made *in vitro* was then purified from endogenous nucleic acids by chromatography on thiol-agarose under stringent conditions and subsequently demercurated (23).

RESULTS

Synthesis of Mature Adenoviral Early RNA Species Is Inhibited by Anti-RNP and Anti-Sm Antibodies. Nuclei isolated from HeLa cells harvested during the early phase of Ad2 infection were incubated for 10 min at 4°C in the absence or presence of antibodies prepared from characterized SLE sera (17); previous work of Bolden *et al.* (24) and our observation that some 30% of the RNA labeled during incubation of nuclei at 25°C for 1 hr is released nonspecifically indicated that antibody molecules should penetrate nuclei. After this preincubation, nuclei were incubated at 25°C for 1 hr in the presence of [^{3}H]UTP under the conditions described in *Materials and Methods*. The size of labeled RNA products complementary to early regions 1 and 2 in the adenoviral genome was then determined as described in the legend to Fig. 1.

As illustrated in the top left-hand panel of Fig. 1, *Bal* fragment I (0.8–6.0 units) can hybridize to all mRNA species com-

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Abbreviations: Ad2, adenovirus type 2; RNP, ribonucleoprotein; snRNP, small nuclear RNP; SLE, systemic lupus erythematosus.

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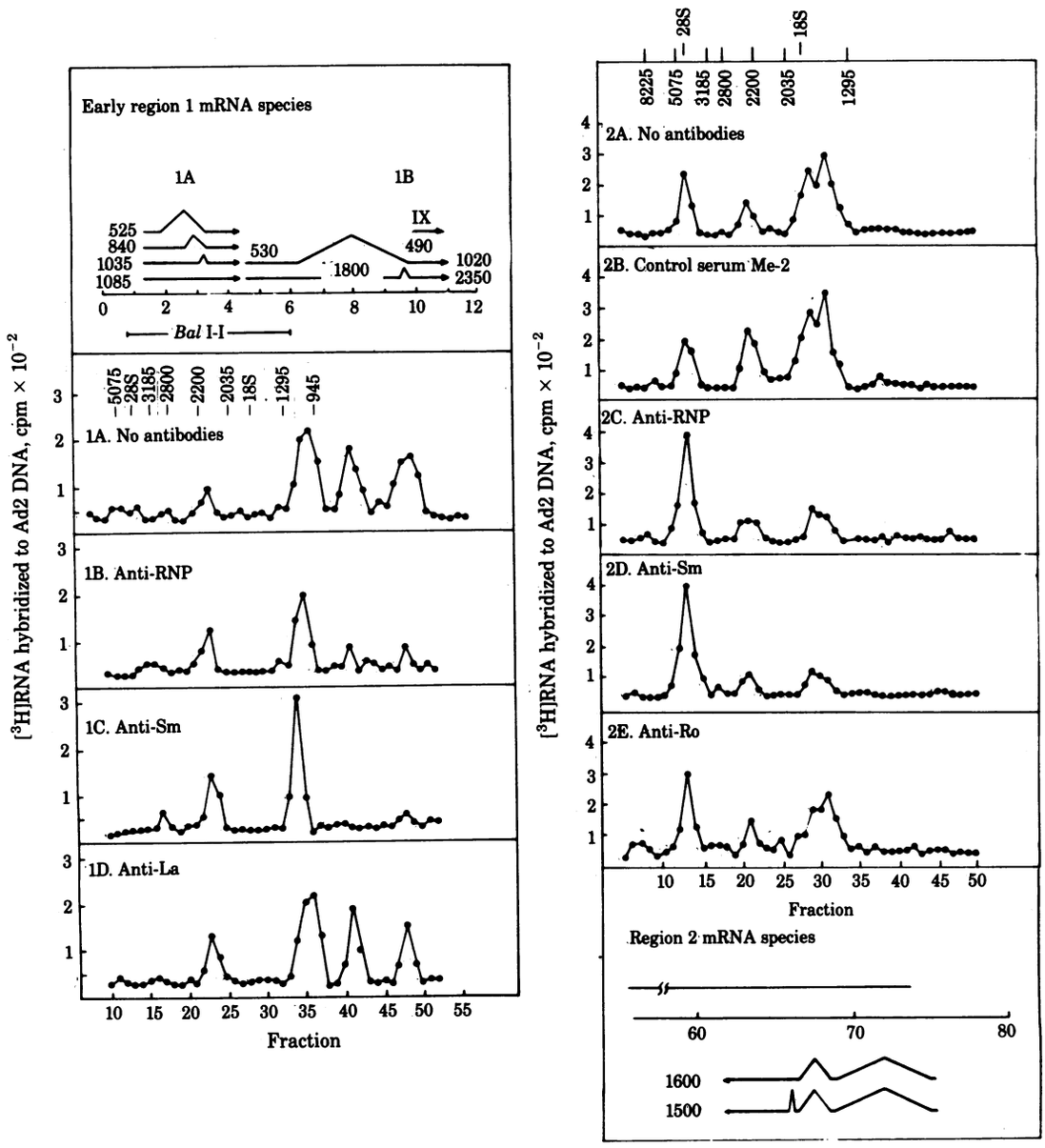

FIG. 1. Effect of SLE antibodies on synthesis in vitro of adenoviral early RNA species. HeLa cell nuclei, isolated from 4×10^7 cells during the early phase of Ad2 infection and suspended in transcription buffer, were preincubated for 10 min at 4°C in the absence or presence of IgG (17) isolated from the different sera listed in the figure (and described in the text). The amount of antibody used in each experiment was based on its effectiveness in immunoprecipitating snRNP from a known number of nuclei (17). RNA synthesized in such preincubated nuclei during a 1-hr reaction at 25°C under the optimal conditions given in *Materials and Methods* was purified by the method of Seeburg *et al.* (25), deproteinized, and precipitated with 2 vol of ethanol at −20°C. Aliquots of each sample containing equal amounts of labeled RNA (2.5×10^6 cpm, about 20 μg) were collected by centrifugation, dissolved in 20 μl of 0.2 × TBE (1 × TBE is 9 mM Tris borate, pH 8.3/2.5 mM EDTA) containing 8 M urea, heated at 68°C for 10 min, and immediately subjected to electrophoresis in 5% polyacrylamide slab gels cast in TBE containing 7 M urea as described by Maniatis *et al.* (26). Electrophoresis at 50–70 V was continued until a xylene cyanol FF marker (26) had migrated *at least* 15 cm. One track of each gel contained Ad2 DNA digested with *Hin*dIII and denatured as described by Maniatis *et al.* (26), while a second contained ribosomal RNA denatured as described above. The positions to which these size markers migrated are indicated in *1A* and *2A*, with the numbers of nucleotides indicated. Under these conditions of electrophoresis, a plot of the logarithms of the molecular weights of the single-stranded nucleic acids against their mobilities gave a straight line, except for molecules larger than 5075 nucleotides (*Hin*dIII fragment B). The nature of the nucleic acid does not affect its mobility (see also ref. 26). This behavior is illustrated in the figure for markers greater than 950 nucleotides in length and was established for smaller chains in initial calibration experiments (data not shown) that included smaller DNA fragments, such as *Hpa* I fragment G of Ad2 DNA, 525 nucleotides, as well as 7S and 5S RNA. Each gel track was excised and cut into 2-mm slices. The RNA was eluted in about 2 ml of 0.01 M Tris·HCl, pH 7.4, containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% sodium dodecyl sulfate, and 1 mM EDTA. After centrifugation to remove polyacrylamide, each eluate was ethanol precipitated and hybridized to pBR322-derived plasmids, generous gifts of S. M. Berget and J. Sambrook, containing *Bal* fragment I and *Hin*dIII fragment A in experiments 1 and 2, respectively. The eluted [^{3}H]RNA was hybridized to 0.5 μg of DNA for at least 24 hr at 61–62°C under the conditions described by Casey and Davidson (27). Shown in each panel are the RNase-resistant ^{3}H cpm. Also indicated for each region analyzed are the locations of the DNA probes used and of the complementary adenoviral early mRNA species (9, 10); the numbers to the side of each RNA species illustrated give its total length in nucleotides whereas those drawn above discrete segments of a spliced mRNA give the length, in nucleotides, of that segment. Locations are given in units (1% of the genome; 1 unit ≈ 350 nucleotides).

plementary to early region 1A (1.3–4.4 units) as well as to the two spliced mRNA species complementary to region 1B (4.6–11.2 units). The major products of the *in vitro* reaction performed in the absence of any antibodies that are detected with this adenoviral DNA fragment are RNA chains of some 550, 800, and 1000–1100 nucleotides (Fig. 1, *1A*). Of these, the chains of 550 and 800 nucleotides correspond in size to two mature (spliced) mRNA species complementary to early region 1A, whereas the 1000–1100 nucleotide long RNA product could include mature mRNA species complementary to both regions 1A and 1B, as well as unprocessed RNA transcribed from region 1A (see top left-hand panel of Fig. 1). Similarly, mature mRNA and unprocessed RNA chains complementary to early region 1B could be included in the minor species of 2150 nucleotides seen in panel *1A* of Fig. 1.

When RNA labeled in isolated nuclei was hybridized to *Hin*dIII fragment A (50.1–74.6 units), three RNA species of some 5000, 2300, and 1500–1600 nucleotides were observed (Fig. 1, *2A*). These exhibit the sizes predicted for the primary transcript of early region 2 (61.7–75.5 units), a processing intermediate in which 5′ terminal leader sequences at 75.5 units are joined to a second set of leader sequences at 68.8 units and the two fully processed RNA species depicted in the bottom right-hand panel of Fig. 1. The presence of labeled RNA species that exhibit the size of mRNA complementary to early regions 1 and 2 among the products of the *in vitro* reactions suggests that adenoviral early RNA sequences synthesized in isolated nuclei can be matured correctly *in vitro*. Consistent with this interpretation is our previous demonstration that adenoviral late RNA sequences are polyadenylylated and spliced correctly in isolated nuclei (21, 22).

When isolated nuclei are preincubated with anti-RNP or anti-Sm antibodies, the synthesis of both the 550- and the 800-nucleotide RNA chains that hybridize to *Bal* fragment I (Fig. 1, *1B* and *1C*) and the 1500- to 1600-nucleotide RNA complementary to early region 2 (Fig. 1, *2C* and *2D*) is substantially inhibited. It is also apparent that the 5000-nucleotide RNA transcribed from region 2 accumulates under these conditions (Fig. 1, *2C* and *2D*). A quantitative summary of the results of this kind of experiment is presented in Table 1. In calculating these data, it was assumed that saturation hybridization of the RNA recovered from each gel slice was achieved; analysis of unfractionated RNA purified from isolated nuclei (data not shown) indicates that this condition was met. In Table 1, we compare the relative amounts of individual RNA species synthesized with and without antibodies because the recoveries from the gels of RNA species of different size have not been estimated.

Table 1. Relative amounts of different species of ^{3}H-labeled RNA hybridized to Ad2 DNA fragments

	RNA hybridized, % of control				
	Region 1		Region 2		
Antibodies	800	600	5000	2300	1500–1600
None	100, 100	100, 100	100, 100	100, 100	100, 100
Me-2	106, ND	71, ND	99, ND	204, ND	110, ND
Anti-RNP	39, 14	56, 14	221, 173	79, 57	26, 20
Anti-Sm	41, 6	55, 13	265, ND	70, ND	23, ND
Anti-Ro	168, ND	79, ND	79, ND	100, ND	87, ND
Anti-La	102, 78	124, 74	135, 84	93, 119	78, 118

The amounts of [^{3}H]RNA hybridizing to Ad2 DNA fragments were estimated as the areas under peaks in experiments such as those presented in Fig. 1. Two sets of data, which represent the extremes of our observations, are presented as the percentage of the amount of RNA recovered from control reactions performed in the absence of antibodies. The RNA chains of about 550, 800, and 1500–1600 nucleotides are fully spliced products; that of 2300 nucleotides is a partially spliced intermediate, and that of about 5000 nucleotides is the region 2 precursor RNA (see Fig. 1 and text). ND, not determined.

It is clear from the data presented in Table 1 that anti-RNP and anti-Sm antibodies inhibit the synthesis of the 550- and 800-nucleotide RNA species complementary to early region 1 DNA by 45–94% and the synthesis of early region 2 RNA of 1500–1600 nucleotides by 74–80%: the degree of inhibition observed in additional experiments (not shown) fell between these extremes. The results presented in Table 1 also reveal that anti-RNP and anti-Sm antibodies cannot be distinguished on the basis of the efficiency with which they inhibit synthesis of these adenoviral early RNA species. In contrast are the results observed with various control antibodies, Me-2 (a normal serum), anti-La [which recognizes a totally different class of nuclear RNPs (28)], and anti-Ro [which precipitates small cytoplasmic RNPs (28)]: none of these antibodies consistently altered the nature, or amounts, of the adenoviral RNA species synthesized in isolated nuclei (Fig. 1, Table 1). We may therefore conclude that anti-RNP and anti-Sm antibodies specifically inhibit the synthesis in isolated nuclei of RNA chains that correspond in size to fully matured mRNA species complementary to early regions 1 and 2 in the adenoviral genome. Because maturation of these RNA species includes splicing, this observation implies that anti-RNP and anti-Sm antibodies inhibit splicing in isolated nuclei.

Anti-RNP Antibodies Inhibit Splicing of Adenoviral Early RNA. To pinpoint the step at which certain SLE antibodies inhibit the appearance of mature early RNA species, the structure of adenoviral RNA molecules synthesized in isolated nuclei in the absence of antibodies or in the presence of anti-RNP or anti-Ro was determined by the nuclease S1/alkaline gel electrophoresis assay (8, 9). Isolated nuclei were preincubated as described in the previous section and incubated at 25°C in the presence of Hg-CTP under conditions that affect neither the gross properties of the transcription reaction nor the nature of the RNA products (22, 23). The mercurated RNA chains made *in vitro* were purified by chromatography on thiol-agarose columns, under stringent conditions that permit a complete separation of newly synthesized from endogenous RNA (22, 23), and then demercurated (23). In the experiment shown in Fig. 2, such RNA was hybridized to ^{32}P-labeled DNA of *Sma* I fragment E, 3.0–11.1 units, and the nuclease S1-resistant DNA products were analyzed in alkaline agarose gels. When the same DNA probe was hybridized to early cytoplasmic RNA prepared from Ad2-infected cells, three major colinear DNA segments of 1800, 560, and 440 nucleotides were observed (Fig. 2, track 4). DNA segments of this size have been described previously (8, 9, 29, 30) and are diagnostic of spliced mRNA species complementary to early region 1B (see top left-hand panel of Figure 1). These same DNA fragments, as well as an unique band of approximately 2100 nucleotides, (presumably unspliced RNA transcribed from region 1B) are generated when *Sma* I fragment E is hybridized to nuclear RNA synthesized in the absence of any antibodies or in the presence of anti-Ro (Fig. 2, tracks 1 and 3, respectively). Thus the adenoviral early RNA sequences synthesized in isolated nuclei appear to be spliced correctly. By contrast, when nuclei are preincubated with anti-RNP, the products diagnostic of spliced mRNA are either decreased significantly in concentration (the 1800-nucleotide RNA) or absent (the 560- and 440-nucleotide RNA species) (Fig. 2, track 2). Similar results have been obtained with other DNA fragments such as *Bam*HI fragment B (0–29 units), which includes early regions 1A and 1B, and *Sma* I fragment J (0–2.9 units), which is specific for early region 1A (data not shown). These obser-

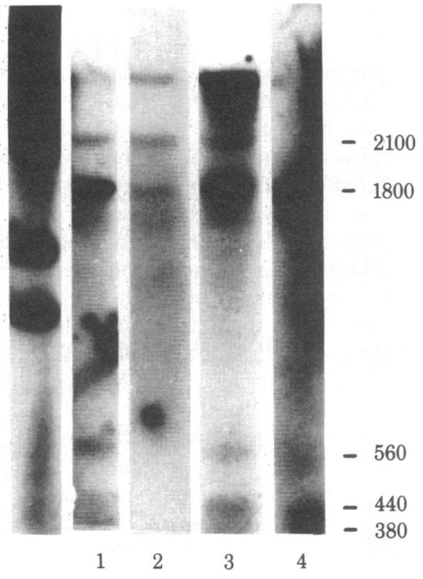

FIG. 2. Products of hybridization of RNA synthesized in isolated nuclei to *Sma* I fragment E (3.0–11.1 units). Mercurated RNA purified from isolated nuclei was demercurated (23) and coprecipitated with ^{32}P-labeled DNA of *Sma* I fragment E of Ad2 DNA (specific activity 1.5×10^6 cpm/μg). Hybridization was performed as described in the legend to Fig. 1 for 6–10 hr. After nuclease S1 digestion, the nucleic acids were precipitated with ethanol and subjected to electrophoresis in alkaline 1.4% agarose gels (8, 9). Hybridization of the DNA was to: RNA made *in vitro* in the absence of antibodies (track 1), RNA made *in vitro* in the presence of anti-RNP (track 2), RNA made *in vitro* in the presence of anti-Ro (track 3), and cytoplasmic RNA prepared from the infected cells from which the nuclei were isolated (track 4). These four tracks are from an experiment in which each RNA sample was hybridized with increasing concentrations of DNA and are the results observed with the highest DNA concentration. The left-hand track shows ^{32}P-labeled Ad2 DNA digested with *Hin*dIII. These fragments and *Hin*dIII fragment L, 250 nucleotides (not shown), provided markers for the size estimates shown in the figure. A DNA segment characteristic of region 1A RNA of approximately 380 nucleotides (9, 10) that should hybridize to *Sma* I fragment E appears just below the 440-nucleotide DNA segment, from which it is not well resolved.

vations therefore provide strong evidence that adenoviral early RNA molecules are spliced correctly in isolated nuclei and that anti-RNP inhibits the appearance of such spliced products.

Polyadenylylation Is Not Inhibited by SLE Antibodies. During the maturation of both early (31) and late (32) adenoviral RNA, polyadenylylation of 3′ termini appears to precede splicing. It is therefore possible that the inhibition of synthesis of spliced adenoviral RNA species observed in these experiments is a secondary consequence of a disruption in polyadenylylation. The results presented in Table 2 reveal that neither the overall polyadenylylation of RNA sequences labeled in isolated nuclei nor the addition of poly(A) to viral sequences is much affected by incubation of nuclei with anti-RNP antibodies. The concentration of viral RNA sequences that contain poly(A) was, for example, decreased by 9.5% when nuclei were preincubated with anti-RNP antibodies in the experiment reported in Table 2. A much more significant inhibition would be predicted were the primary effect of anti-RNP antibodies upon polyadenylylation: the viral early sequences we have examined in the experiments shown in Fig. 1 and 2—those complementary to early regions 1A, 1B, and 2—compose about half the adenoviral genetic information expressed during the early phase of productive infection (1, 2, 5, 10) and, more importantly, do not constitute a minor fraction of the total viral RNA sequences found in either the cytoplasm (10, 33) or the nucleus (29, 34) of infected cells. These facts, coupled with our observation of as much as 95% inhibition of splicing by certain SLE antibodies, render improbable the notion that the primary effect of these antibodies is upon polyadenylylation.

DISCUSSION

The results presented in this paper demonstrate that certain SLE antibodies, anti-RNP and anti-Sm, inhibit efficiently (up to 95%) the synthesis in isolated nuclei of RNA species the size of mature mRNA from RNA sequences transcribed from early regions 1 and 2 in the adenoviral genome (Fig. 1, Table 1). This inhibition appears to be the direct result of a disruption in the splicing of these viral RNA sequences: their polyadenylylation apparently proceeds normally in the presence of such antibodies (Table 2), yet spliced RNA chains are absent from, or much reduced among, the products of transcription reactions performed in the presence of these antibodies (Fig. 2).

None of the antibodies employed inhibit transcriptional activity in isolated nuclei (not shown), and those with specificities distinct from anti-RNP or anti-Sm—for example, normal serum Me-2 and anti-Ro (28)—do not alter the splicing of adenoviral early RNA sequences (Table 1, Figs. 1 and 2). Most pertinent are the negative results of experiments in which nuclei were preincubated with anti-La (Table 1; Fig. 1, *1D*). This serum, unlike the other control antibodies employed, immunoprecipitates a class of small RNPs that are mostly nuclear, nearly as abundant as, but distinct from, those recognized by anti-RNP or anti-Sm (28). Nonetheless, anti-La antibodies do not impede splicing of adenoviral early RNA species in isolated nuclei (Table 1); this property is unique to anti-RNP or anti-Sm antibodies. Although it is conceivable that the patients' sera used here contain other antibody specificities, significant contamination seems unlikely because recent work with an anti-Sm hybridoma antibody shows precipitations of an identical constellation of RNA species and proteins (unpublished observations). We therefore conclude that an antigen(s) recognized by both anti-RNP and anti-Sm is necessary not only to the splicing reactions we have studied but almost certainly to those that mature cellular mRNA species (see Introduction).

Anti-Sm immunoprecipitates snRNPs containing five different HeLa cell snRNA species, U1, U2, and U4–U6, whereas anti-RNP recognizes only structures containing U1 RNA (12). A protein component of the snRNP appears to be recognized by anti-Sm, whereas anti-RNP is directed against a determinant that consists of both RNA and protein (35–37). The same set of polypeptides are immunoprecipitated by both antibodies (17). These facts argue that the component required for splicing that is recognized by both anti-RNP and anti-Sm must be snRNP containing U1 RNA.

It should come as no surprise that splicing of adenoviral RNA sequences complementary to early region 1A is mediated by

Table 2. Polyadenylylation of [^{3}H]RNA in isolated nuclei

	Poly(A)$^+$ RNA, %	
Antibodies	Total*	Hybridized to Ad2 DNA†
None	29.0	1.36
Anti-RNP	27.8	1.23

* The fraction of [^{3}H]RNA synthesized in isolated nuclei selected by chromatography on oligo(dT)-cellulose.

† Estimated by hybridization of poly(A)-containing [^{3}H]RNA to saturation with total Ad2 DNA.

cellular U1 snRNP, because these splice junctions closely resemble the consensus sequence discussed in the Introduction (11). Early region 1A also appears to be expressed at very early times during the infectious cycle, and one of its products is required for the accumulation of mRNA species complementary to other adenoviral early genes (29, 30). The mechanism of action of this region 1A gene product is not understood, but the results presented here reveal that it is not sufficient for splicing, that is, anti-RNP or anti-Sm antibodies inhibit the splicing of RNA sequences complementary to early region 1B (Fig. 2) and region 2 (Fig. 1). It should, however, be noted that these antibodies do not inhibit all the splicing events we have studied with equal efficiency. Thus, while production of mature RNA complementary to early region 2 is inhibited by 70–80%, synthesis of the 2300-nucleotide intermediate carrying only one splice (leader sequences at 75.5 joined to the second leader at 68.8 units) is decreased by only 20–40% (Table 1). Although there are a number of possible explanations for this observation, it does raise the intriguing possibility that the cellular splicing machinery becomes partially modified or wholly replaced by viral components as the infection progresses.

Our conclusion that antibodies to U1 snRNP inhibit splicing in isolated nuclei raises several important questions. Does this RNA–protein complex mediate splicing of all mRNA precursors in eukaryotic cells? Is the RNP itself the enzymatic machinery that achieves cutting and ligation as well as the scaffold upon which the correct substrate is built? The former question is difficult to address in the absence of additional sequence data and without specific antisera, analogous to anti-RNP, that recognize the individual snRNPs containing U2, U4, U5, or U6 RNA. A partial answer to the latter question may be provided by our observation that antibodies directed against U1 snRNP inhibit cutting, as revealed by the accumulation of precursor RNA species. Here, a clear precedent is provided by *Escherichia coli* RNase P, an RNA-processing enzyme that consists of a discrete small RNA molecule as well as protein components (38). Moreover, it should now be possible to investigate the role of snRNP directly by using reconstituted *in vitro* systems that include an appropriate precursor RNA and purified snRNPs.

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