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Antibodies to hnRNP core proteins inhibit *in vitro* splicing of human β-globin pre-mRNA

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ABSTRACT

In vitro splicing of human ß-globin pre-mRNA can be fully inhibited by treatment of the splicing extract with polyclonal antibodies against hnRNP core proteins prior to the addition of pre-mRNA. Inhibition of the first step in the splicing pathway, cleavage at the 5' splice site and lariat formation, requires more antibodies than inhibition of the second step, cleavage at the 3' splice site and exon ligation. The anti-hnRNP antibodies can also inhibit the splicing reaction after the formation of the active nucleoprotein splicing complex which is known to occur during the initial lag period. Thus, hnRNP core proteins appear to be present in the complex that performs pre-mRNA splicing.

INTRODUCTION

In eukaryotes, pre-mRNA in the nucleus is found in ribonucleoprotein particles (hnRNP, 1). These particles contain a set of about 9 interrelated and evolutionarily conserved major proteins, termed hnRNP core proteins (Mr 32-42,000), and a number of minor protein components (2-4). The core proteins form particles immediately after transcription, but their specific function is unknown except for their ability to protect the primary transcript from degradation by ribonucleases (1). Since pre-mRNAs undergo a series of RNA processing reactions in the nucleus, it seems likely that these hnRNP particles, not the "naked" pre-mRNA, serve as substrate for subsequent RNA processing steps. This is also suggested by the requirement of a large 50S nucleoprotein complex during *in vitro* splicing (5-7) and by the recent observation that hnRNPs bind to splice junctions in certain Drosophila transcripts (8). Since hnRNP core proteins have a high affinity for RNA and will form particles with it

in vitro (9-13), it seems plausible that these proteins may be present in the splicing complex and involved in its formation in the *in vitro* splicing reaction.

A useful way of identifying the factors involved in RNA processing is to inhibit one or more of the processing steps in an *in vitro* reaction with antibodies directed against particular nuclear components. This approach has been taken using antibodies to small nuclear RNPs (snRNPs; U1, U2, etc.) and has shown convincingly that U1-RNP is a necessary splicing factor (6,7, 14-16). In similar experiments, snRNPs have been implicated in pre-mRNA polyadenylation (17). We report here analogous observations using antibodies raised against purified proteins isolated from hnRNP particles (18). These antibodies are added to an *in vitro* splicing reaction with truncated human ß-globin pre-mRNA transcripts as substrate. Our results show that these antibodies can prevent the pre-mRNA transcripts from undergoing any step in the splicing reaction. In addition, under certain conditions, the antibodies can inhibit the production of the final spliced product without blocking the first step in the splicing reaction, i.e. cleavage at the 5' splice site and lariat formation.

MATERIALS AND METHODS

DNA templates were prepared by linearizing the plasmid pSP64HbΔ6 (19) with either restriction enzyme Bam HI or Acc I which both cleave within the second exon of the human ß-globin gene (Fig. 1A). Transcription and capping of the substrate RNA was carried out essentially as described by Konarska et al. (20). *In vitro* splicing was carried out with nuclear extract from HeLa cells (19,21) and the products analyzed on polyacrylamide gels as previously described (22). Times of incubation at 30° are indicated in the Figure Legends.

Anti-hnRNP serum was obtained by Lahiri and Thomas (18) from chicken immunized with hnRNP proteins purified from bovine brain nuclei. 40S hnRNP particles were obtained by extraction of nuclei at pH 8, purified by sucrose density gradient centrifugation, and further fractionated by ammonium sulfate precipitation. This material was electrophoresed on a 12%

SDS-polyacrylamide gel (23). Bands containing proteins with molecular weight from 36-43 kDa were excised, emulsified with Freund's adjuvants, and used to immunize chickens (18). IgG was purified from chicken serum as described (24) and used in all experiments reported here. Immunoblots were carried out according to standard procedures (25). Biotinylated rabbit anti-chicken secondary antibodies (Cooper Biomedical, Malvern, PA) and avidin-biotin-horseradish peroxidase (Vectastain ABC kit, Vector Labs, Burlingame, CA) were used for detection according to manufacturer's protocols. Immunoprecipitations of [^{32}P]-labeled HeLa cell sonicates were carried out as described (26) using Pansorbin (Calbiochem, San Diego, CA) with an excess of rabbit anti-chicken sera as secondary antibody (Cooper Biomedical).

Inhibition of the processing reaction was carried out by preincubating 24 µl of the reaction mixture (5mM ATP, 3.3mM $MgCl_2$, 20mM creatine phosphate, 2.6% polyvinyl alcohol, 15 µl nuclear extract) with an appropriate amount of the antibody (see Fig. 3) for 30 minutes at 0°. Subsequently, [^{32}P]-labeled RNA (10^5 cpm per sample) was added and the sample incubated for 90 minutes at 30°. In experiments involving inhibition of a splicing reaction already in progress (Fig. 4), a complete reaction mixture including [^{32}P]-labeled RNA was incubated for 30 minutes at 30° and then transferred on ice. One or 2 µl of the antibody was added, the incubation carried out for 30 minutes on ice, and then continued at 30°. The reaction products were analyzed as above and further quantitated by excision of appropriate RNA bands from polyacrylamide gels and scintillation counting.

RESULTS

Structure and properties of the pre-mRNAs

Two truncated human ß-globin transcripts were tested in the splicing assay (19,22). Transcript Bam HI (Fig. 1B) contains the 5' exon, the small intron, and most of the second exon. It is efficiently spliced in an *in vitro* system generating intermediates and products that have been characterized in detail (27, see Fig. 1B). Transcript Acc I (Fig. 1C), which is

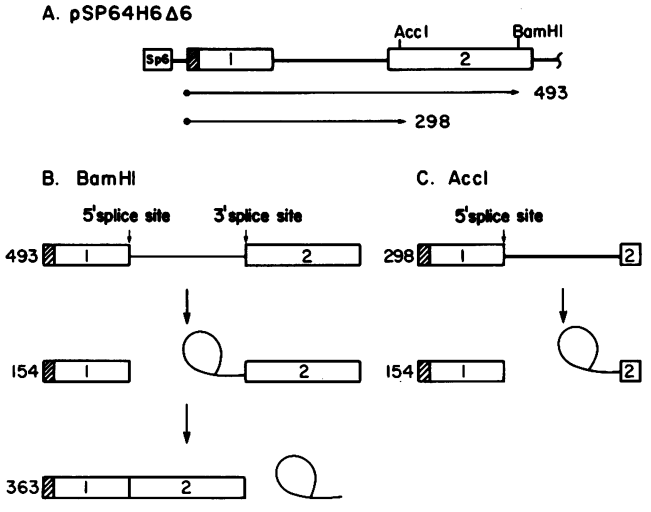


Figure 1. Structure of the DNA templates, pre-mRNA transcripts, and intermediates and products of the splicing reaction. A) The human ß-globin DNA template (plasmid pSP64HBΔ6 (19). Restriction sites of Bam HI and Acc I which cleave within the second exon of the gene are indicated. Open box marked SP6, SP6 polymerase promoter; open boxes, exons; thin line, intron; hatched area, transcribed sequences from the cloning vector. Arrows represent RNA transcripts; their length in nucleotides is indicated. B) Structure of transcript Bam HI, terminating at Bam HI site, and the intermediates and products of splicing. C) Structure of transcript Acc I, terminating at Acc I site, and the products of cleavage at the 5' splice site.

truncated within the second exon, 14 nucleotides downstream from the 3' splice site, is efficiently cleaved at the 5' splice site and forms a lariat at the correct branch point in the intron but is unable to undergo cleavage at the 3' splice site and exon ligation (22,28).

Specificity and purity of the antibodies

The anti-hnRNP antibodies were raised in chicken against gel purified core proteins isolated from hnRNP particles as described in Materials and Methods (see also 18). The molecular weight range of the core proteins is between 32-42 kDa which is in the range of some of the Sm- and U1-RNP antigens (revieved in 29 and 30). In addition, snRNPs have been shown to associate with hnRNPs (31). Thus it seemed possible that hnRNP core proteins used for immunization might

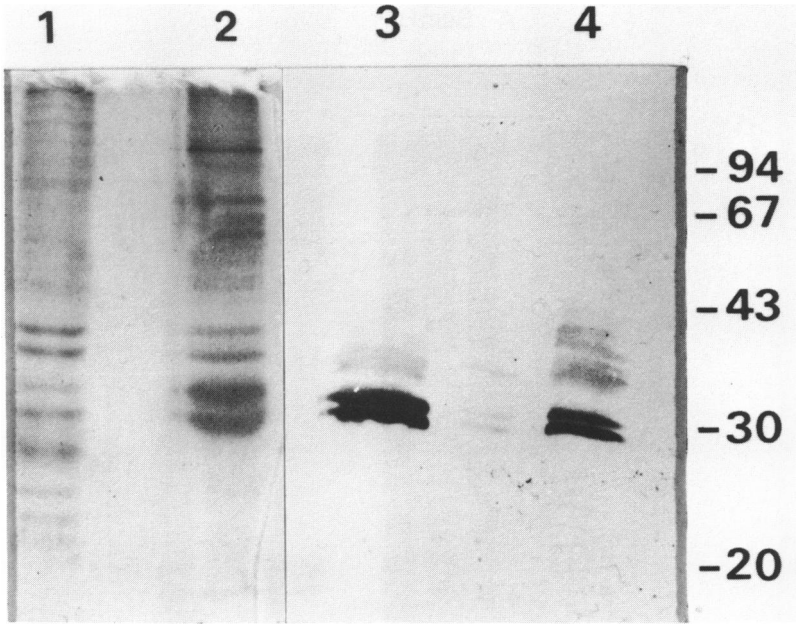
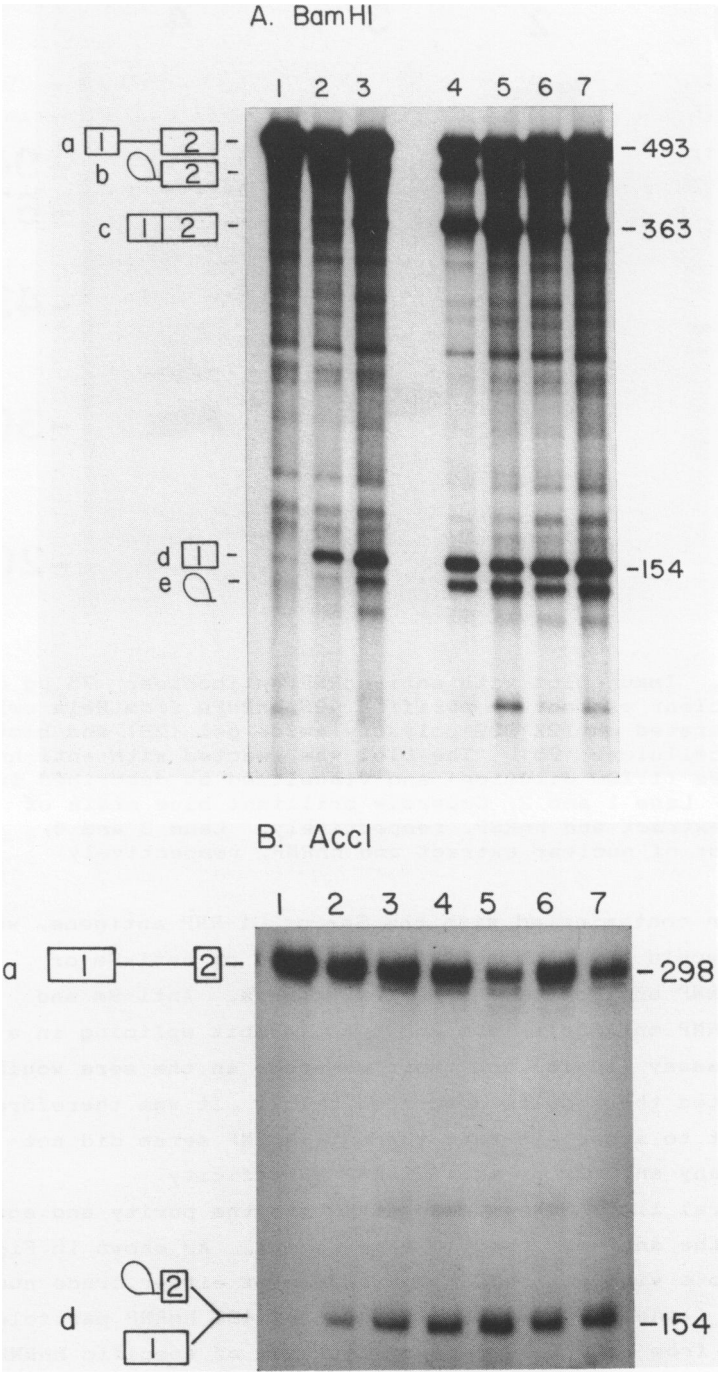


Figure 2. Immunoblot with anti-hnRNP antibodies. 75 µg of crude nuclear extract or purified 40S hnRNPs from Hela cells were separated on 12% SDS-polyacrylamide gel (23) and blotted on nitrocellulose (25). The blot was reacted with anti-hnRNP antibodies (1:100 dilution) and visualized as described in Methods. Lane 1 and 2, Coomasie brilliant blue stain of nuclear extract and hnRNP, respectively. Lane 3 and 4, immunoblot of nuclear extract and hnRNP, respectively.

have been contaminated with the Sm- or U1-RNP antigens, which in turn would have led to the generation of anti-Sm or anti-U1-RNP antibodies in the immune sera. Anti-Sm and anti-U1-RNP antibodies are known to inhibit splicing in a similar assay (14-16) and their presence in the sera would have invalidated the results described below. It was therefore important to ascertain that the anti-hnRNP serum did not exhibit any anti-Sm or anti-U1-RNP specificity.

Several lines of evidence attest to the purity and specificity of the antisera used in this report. As shown in Fig. 2, immunoblots with anti-hnRNP antibodies of either crude nuclear extracts (lane 3) or partially purified 40S hnRNP particles (lane 4) from HeLa cells show reactivity of specific hnRNP


A. BamHI
1 2 3 4 5 6 7
a
b
c
d
e
-493
-363
-154
B. AccI
1 2 3 4 5 6 7
a
d
-298
-154

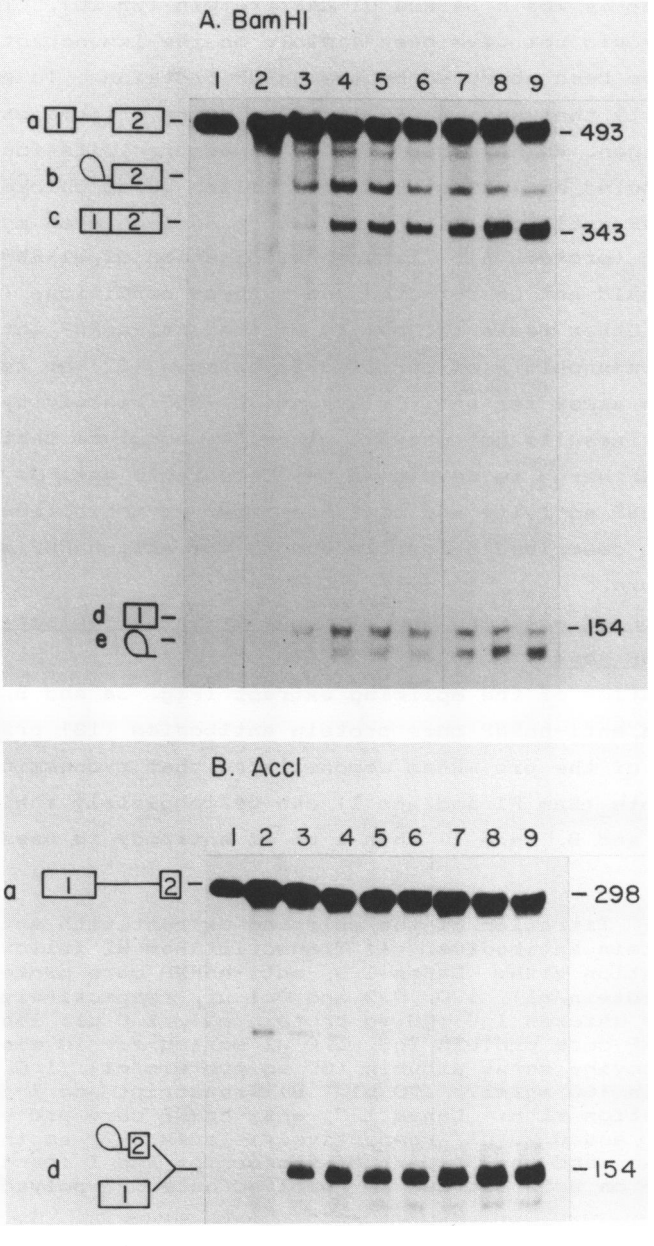
proteins (MW 32-42 KDa). No lower or higher protein bands could be detected in this experiment, suggesting that common Sm- or U1-RNP antigens (28 kDa, 16 kDa and 70 kDa) did not react with the antibodies. Another possible contaminating reactivity is for a 34 kDa U1-RNP protein (29,30). This protein would not have been visible on the immunoblot since it would have been obscured by the hnRNP proteins. To exclude the possibility that anti-hnRNP serum contains antibodies against this antigen, we have carried out immunoprecipitation of [^{32}P]-labeled sonicates from HeLa cells, using chicken anti-hnRNP antibody, secondary rabbit anti-chicken antibody and Pansorbin (protein A). Immunoprecipitation of U1-RNP or other snRNPs could not be detected under these conditions (not shown). Other tests for purity of the anti-hnRNP antibodies such as immunoblots of purified Sm antigen (32) or immunodiffusion assay for anti-Sm or anti-U1-RNP reactivity were also negative (results not shown). Thus, we conclude that the anti-hnRNP serum is devoid of any detectable anti-Sm or anti-U1-RNP activity and that the observed inhibition of splicing, described below, is due to the anti-hnRNP specificity of our sera.

Inhibition of splicing and cleavage at the 5' splice site by anti-hnRNP sera

Titration of the splicing extract (Fig. 3A and B, lanes 1-3) with anti-hnRNP core protein antibodies (18) prior to the addition of the pre-mRNAs demonstrated that processing of both transcripts (Bam HI and Acc I) can be completely inhibited (Fig. 3A and B, lane 1) when 1 µl of antibody is used. At a

Figure 3. Titration of the splicing extract with anti-hnRNP core protein antibodies. A) Transcript Bam HI following preincubation with: Lanes 1-3, anti-hnRNP core protein IgG (60 mg protein/ml), 1.0, 0.3 and 0.1 µl, respectively; lane 4, nonimmune chicken IgG (60 mg protein/ml), 1.0 µl; lane 5, anti-hnRNP core protein IgG, 1.0 µl boiled for 10 minutes; lane 6, bovine serum albumin (60 mg protein/ml), 1.0 µl; lane 7, gelatin (60 mg/ml), 1.0 µl. B) Transcript Acc I following preincubation with: Lanes 1-3, anti-hnRNP core protein IgG 1.0, 0.5, and 0.25 µl, respectively; lanes 4-7, as in panel A. The 5' exon and the 3' part of transcript Acc I (band d) comigrate on a 5% but can be resolved on a 12% polyacrylamide gel (22).

lower level of anti-hnRNP antibodies (0.3 µl), the generation of the spliced product from transcript Bam HI was inhibited (Fig. 3A, lane 2, absence of bands c and e) although cleavage



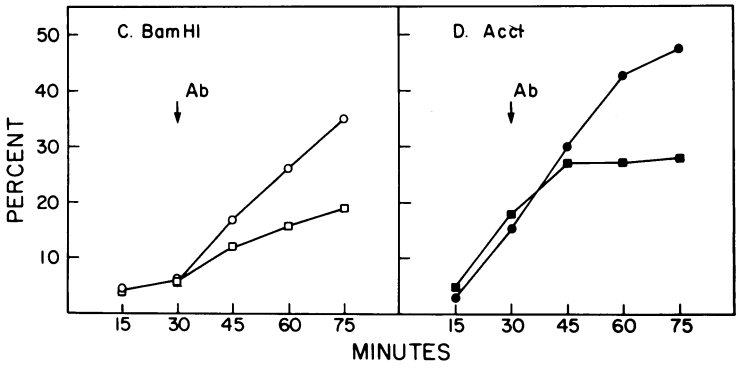


Figure 4. Effect of anti-hnRNP core protein antibodies on splicing after the lag period. A) Transcript Bam HI: Lane 1, input RNA; lanes 2 and 3, the complete reaction mixture (see Methods and Fig. 3) was incubated at 30° for 15 and 30 minutes, respectively; lanes 4-6, the complete reaction mixture was incubated for 30 minutes at 30°, kept on ice for 30 minutes with 1.0 µl of antibodies per 25 µl aliquot followed by incubation at 30° for 15, 30 and 45 minutes, respectively; lanes 7-9, as in lanes 4-6, except that the incubation on ice was with nonimmune IgG, 1.0 µl per 25 µl aliquot. B) Transcript Acc I: Lanes 1-9, as in A, except 2.0 µl of the anti-hnRNP antibodies and nonimmune IgG per 25 µl aliquot were used in lanes 4-6 and 7-9, respectively. C) Quantitation of the spliced product of transcript Bam HI. Bands from lanes 4-9, Fig. 4A were excised, their radioactivity determined, and the yields calculated by dividing the amount of radioactivity in the spliced product, band c, by the total amount of the radioactivity in all RNA bands. Yield of spliced product (time-course) with anti-hnRNP antibodies, []--[]; with nonimmune IgG, O--O. D) Quantitation of products of cleavage at the 5' splice site of transcript Acc I. Bands in lanes 4-9, Fig. 4B were analyzed as above and the yield calculated by dividing the amount of radioactivity in band d by the sum found in bands a and d; anti-hnRNP antibodies, ■--■; nonimmune IgG, ●--●.

at the 5' splice site and lariat formation still occurred (same lane, band b and d). Similarly, cleavage at the 5' splice site of transcript Acc I was only partially inhibited by 0.5 µl of the antibody and resistant to 0.25 µl (Fig. 3B, lanes 2 and 3, respectively). This shows that the first step in the splicing pathway, i.e. cleavage at the 5' splice site and lariat formation, is less sensitive to inhibition by anti-hnRNP antibodies than the subsequent step, cleavage at the 3' splice

site and exon ligation (see also below, Fig. 4). Specificity of inhibition by the antibodies is shown by the fact that nonimmune and boiled immune IgG as well as albumin and gelatin have no effect on the processing of both transcripts (Fig. 3A and 3B, lanes 4-7).

We have attempted to preabsorb the anti-hnRNP antibody with purified hnRNP particles or isolated core proteins to block inhibition of splicing by the antibodies. These attempts were unsuccessful, probably due to insufficient concentrations of purified antigens. Higher amounts of proteins and hnRNPs could not be used due to limitations of the volume that can be added to the reaction without affecting the splicing activity. Similar problems were encountered by Padgett et al. (14) in experiments testing anti-U1-RNP and anti-Sm sera in the splicing assay.

Inhibition of splicing and cleavage in the preformed splicing complex

In the experiments in Fig. 3, the nuclear extract was pre-treated with the antibodies before the addition of the pre-mRNAs. Under these conditions, antibodies presumably bind to free core proteins or to hnRNP particles and prevent them from participating in the formation of the active splicing complex which occurs during the initial 30 minute lag period of the splicing reaction (5-7, 19). To establish whether hnRNP core proteins are present in the complex itself, the following experiment was performed: The reaction mixture containing transcript Bam HI was incubated for 30 minutes at 30°C to form the splicing complex. The antibodies were added and the total mixture was incubated on ice for 30 minutes to stop the splicing reaction and allow the antibodies to react. After that time, the reaction was continued at 30°. A time course of the splicing reaction following the addition of the antibodies (Fig. 4A, lanes 4-6) or nonimmune IgG (Fig. 4A, lanes 7-9) shows that anti-hnRNP antibodies inhibit splicing only partially when the same amount is used (1 µl) which completely inhibits splicing during pretreatment of the extract (see Fig. 3A, lane 1). Quantitative comparison of the yields of the splicing reaction in the above experiment shows that splicing

proceeds at a lower rate in the presence of anti-hnRNP antibodies and is inhibited by about 50% (Fig. 4C). Doubling the amount of the antibodies in this experiment results in complete inhibition of splicing (not shown). In analogous experiments with transcript Acc I, treatment of the samples with an amount of anti-hnRNP antibodies that completely inhibits 5' cleavage when added to the extract prior to the addition of the transcript (1.0 µl, cf. Fig. 3B, lane 1) has no inhibitory effect on the reaction (not shown). By adding 2 µl of anti-hnRNP antibodies, cleavage at the 5' splice site is now inhibited compared to nonimmune controls (Fig. 4B, lanes 4-6 vs 7-9, respectively). Quantitation of this experiment (shown graphically in Fig. 4D) shows a drop in the rate of the 5' cleavage reaction during the first 15 minutes after the addition of the antibodies. At subsequent time points the reaction was completely inhibited. Therefore, transcript Acc I specifically shows that since higher amounts of antibodies are required to inhibit the reaction after the lag period, the core proteins are now less accessible to the antibodies.

It could be argued that anti-hnRNP antibodies may merely prevent the fraction of the pre-mRNA which is not in the splicing complex from entering it, without affecting the RNA already in the complex. Were this the case, the RNA which is in the complex after the lag period would be spliced regardless of the concentration of the antibody in the reaction. Since 2 µl of the antibody can completely inhibit splicing of transcript Bam HI after the lag period, the antibodies are also affecting the pre-mRNA already bound to the splicing factors in the complex. Therefore, hnRNP proteins are involved in splicing after the formation of the splicing complex.

DISCUSSION

In vivo, hnRNP particles form immediately after transcription of pre-mRNA (1). The hnRNP core proteins constitute the predominant protein component and provide the structural framework of these particles (33). *In vitro*, nucleoprotein particles that resemble native hnRNP can be reconstituted from core proteins and exogenous RNA (9-13). We

show here that hnRNP core proteins are a necessary component of splicing throughout the course of the reaction. The 5' splice site cleavage and lariat formation reaction appears to be more resistant to inhibition by anti-hnRNP antibodies than the subsequent steps in splicing. This differential effect, as well as the reduction in the degree of inhibition by the antibody after the lag period, argues that inhibition is not due to the removal of the transcript from the reaction mixture by precipitation of a pre-mRNA hnRNP core protein complex, but rather due to interference in splicing. Our results do not suggest that hnRNPs are unnecessary for the early splicing step. Instead, they imply that under conditions where the antibodies are totally inhibiting the formation of the spliced product, the components necessary for 5' cleavage are still functional.

The elegant work of Choi et al. (34) demonstrates that pretreatment of the splicing extract with monoclonal antibodies to the C hnRNP proteins completely inhibits in vitro splicing of an adenovirus-2 truncated transcript. It also shows by means of immunodepletion experiments that these proteins are necessary for the formation of the splicing complex. Our results obtained with a different splicing system and different antibodies complement their work and confirm the involvement of hnRNP proteins in the splicing phenomenon in general. Furthermore, we show in more detail that hnRNP proteins are needed for both steps in pre-mRNA splicing.

The overall accumulated evidence suggests that all pre-mRNA processing steps take place in a large multifunctional complex. SnRNPs, in particular U1 (14,15), U2 (35) and possibly U5 (36), were shown to be involved in splicing and appear to be a component of the splicing complex. Anti-U1-RNP and anti-La sera inhibit in vitro polyadenylation (17) suggesting involvement of snRNPs in this processing step. Similarly, our results and those of Choi et al. (34) suggest that hnRNPs function in splicing and Moore et al. has obtained evidence that monoclonal anti-hnRNP antibodies inhibit polyadenylation in vitro (C. Moore, MIT, personal communication). In addition, 5' cap structures, and presumably cap binding proteins seem to be

important for splicing (37), although not for polyadenylation (17). The processing complex appears to be independent of transcription since anti-Sm sera (14) and our anti-hnRNP antibodies (unpublished results) do not inhibit pre-mRNA transcription *in vitro*.

None of the data discussed above assigns a specific function for hnRNPs or snRNPs in RNA processing. Since purified hnRNP and snRNP particles are devoid of any known enzymatic activity (33,38,39), it seems unlikely that they are conducting any of the cleavage or ligation reactions themselves. They are most probably either acting as a foundation for the binding of the components that actually catalyze the splicing reactions or, in the possible case of self-splicing events (40), conforming the substrate into a structural environment that allows these reactions to occur under physiological conditions.

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