RNA binding specificity of hnRNP proteins: a subset bind to the 3' end of introns

Maurice S. Swanson and Gideon Dreyfuss

Department of Biochemistry, Molecular Biology and Cell Biology,
Northwestern University, Evanston, IL 60208, USA

Communicated by L. Philipson

The binding of hnRNP proteins to pre-mRNAs in nuclear extracts, and as isolated proteins, was studied by using monoclonal antibody immunopurifications of hnRNP proteins bound to RNase T1-generated fragments. Several major hnRNP proteins, A1, C and D, bind specifically to the 3' end of introns within a region containing the conserved polypyrimidine stretch between the branch site and the 3' splice site. Mutations which alter the conserved 3' splice site dinucleotide AG strongly impair or abolish the binding of the A1 protein as well as of an anti-Sm reactive component(s) to this region. The A1, C and D proteins do not bind efficiently to fragments of either bacterial RNA or the intronless spliced product (mRNA). The binding of these proteins at the 3' end of the intron does not require addition to the extract of exogenous ATP, but remains after ATP addition. These findings demonstrate that several hnRNP proteins have RNA binding specificities on pre-mRNA, and suggest a model for hnRNP particle structure and assembly.

Key words: hnRNP proteins/intron binding/monoclonal antibodies/RNA binding specificity

Introduction

In eukaryotic cells, mRNAs are generally formed by extensive processing of primary gene transcripts, the heterogeneous nuclear RNAs (hnRNAs) (Green, 1986; Padgett *et al.*, 1986; Maniatis and Reed, 1987; Steitz *et al.*, 1988). HnRNAs are found in cells in association with ~20 polypeptides in the mol. wt range of 34 000–120 000 daltons to form structures termed heterogeneous nuclear ribonucleoprotein (hnRNP) particles (Chung and Wooley, 1986; Dreyfuss, 1986; Dreyfuss *et al.*, 1988). These proteins have recently been characterized using monoclonal antibodies and two dimensional gel electrophoresis and designated hnRNP proteins A1 (mol. wt = 34 kd) through U (mol. wt = 120 kd), (Choi and Dreyfuss, 1984a,b; Piñol-Roma *et al.*, 1988). HnRNP particles are one of the major components of the nucleus, and since they are the structures within which hnRNAs exist, it is believed that the processing of pre-mRNA takes place within these ribonucleoprotein complexes. Indeed, using antibodies it was found that hnRNP proteins, particularly the C proteins, play a role in pre-mRNA splicing *in vitro* (Choi *et al.*, 1986; Sierakowska *et al.*, 1986).

Little is known about the structure of hnRNP particles other than that the bulk of these proteins are organized into monparticles—complexes that after limited cleavage of the

hnRNA with nuclease sediment at 30–40S and appear by electron microscopy as globular particles of ~200 Å (Samarina *et al.*, 1968; Pederson, 1974; Beyer *et al.*, 1977; Karn *et al.*, 1977; Martin *et al.*, 1973; Stevenin *et al.*, 1977). The prevailing model of the overall structure of hnRNP particles is that of beads-on-a-string motif with monparticles forming the beads and the hnRNA itself the string.

For a fundamental understanding of both the structure of hnRNP particles and the role of hnRNP proteins in pre-mRNA processing, it is necessary to know how hnRNP proteins are organized on pre-mRNAs, and in particular, if they localize to specific regions of the transcripts. It is widely believed that hnRNP proteins do not bind to RNA in a sequence-dependent manner, but instead monparticles simply 'coat' pre-mRNA, based largely on experiments in which fractions enriched in hnRNP proteins were incubated with a single type of single-stranded polynucleotide (RNA or DNA) to show that structures of characteristic morphology and sedimentation properties similar to those of hnRNP particles isolated from nucleoplasm were obtained (Pullman and Martin, 1983; Thomas *et al.*, 1983; Wilk *et al.*, 1983). In contrast, electron microscopic observations of spread chromatin preparations showed that protein particles of similar appearance to hnRNP monparticles are located at specific and unique positions on nascent pre-mRNAs (Beyer *et al.*, 1980; Osheim *et al.*, 1985), and a variety of biochemical analyses suggested a non-random arrangement of hnRNP proteins on specific regions of several pre-mRNAs (Augenlicht, 1979; Ohlsson *et al.*, 1982; Steitz and Kamen, 1981; Stevenin *et al.*, 1982; van Eekelen *et al.*, 1982). Recently, based on differential binding to RNA homopolymers, we found that several hnRNP proteins do, in fact, have RNA binding specificities (Swanson and Dreyfuss, 1988). Here we describe findings that indicate that hnRNP proteins in nuclear extracts possess RNA binding specificity for specific regions of pre-mRNAs. Surprisingly, the 3' end of introns is a preferred binding site for several of these proteins.

Results

hnRNP proteins bind to the 3' end of introns

Single-stranded polynucleotides become associated with hnRNP proteins upon addition to nuclear extract and can be immunopurified with antibodies to these proteins. This is not surprising considering the high free concentration of these RNA-binding proteins in this extract (see Discussion). To investigate whether hnRNP proteins bind preferentially to any specific sites on various pre-mRNAs we initially used the same experimental approach as that previously employed by Steitz and co-workers for the identification of the binding sites of snRNPs (Black *et al.*, 1985; Chabot *et al.*, 1985; Chabot and Steitz, 1987). High specific activity ³²P-labeled pre-mRNAs are produced by transcription *in vitro* with bacteriophage polymerase and incubated in nuclear extract

Fig. 1. Analysis of the binding of hnRNP proteins to the human β -globin pre-mRNA in nuclear extracts. (A) Precursor RNA was synthesized from the SP64-H β Δ 6 plasmid template and incubated in nuclear extract in the presence of 145 U of RNase T1 for 30 min on ice. RNA fragments bound to hnRNP proteins were purified by immunopurification with monoclonal antibodies while those fragments bound to anti-Sm determinant-containing proteins were immunopurified with the anti-Sm polyclonal antiserum. These RNA fragments were then analyzed on denaturing acrylamide gels. Lanes are: M, markers; total, non-immunopurified RNA; SP2/O, immunopurified with the mouse myeloma control ascites fluid; 4F4, immunopurification with the monoclonal antibody to the hnRNP C proteins; 4B10, monoclonal antibody to the hnRNP A1 proteins; 5B9, monoclonal antibody to the hnRNP D proteins; 4D11, monoclonal antibody to the hnRNP L proteins; 3G6, monoclonal antibody to the hnRNP U proteins; and Sm, polyclonal antiserum to the snRNP anti-Sm determinant. (B) RNA was synthesized from the human β -globin point mutant SP64-H β Δ 6-²⁸ in which the 3' splice site has been changed. Lanes are as described in (A) except for anti-U1, which is a monoclonal antibody against U1snRNP. (C) Structure map of SP64-H β Δ 6 and the RNase T1 fragments immunopurified (black boxes) with the indicated antibodies. The numbers refer to the designations of the fragments in (A) and (B). Exon 1 (E1) and exon 2 (E2) are shown as open boxes and plasmid RNA as a hatched box. The previously determined binding sites of U1 snRNP and the anti-Sm reactive component(s) are also illustrated. Conserved structural features of the intervening sequence (IVS) are also shown, including the branch point adenosine residue (A), the polypyrimidine stretch (Py) and the 3' splice site dinucleotide AG.

in the presence of ATP and creatine phosphate for 30 min on ice to allow binding of nuclear extract components without concomitant RNA splicing. The samples are treated with

high levels of RNase T1 to completely digest the pre-mRNA except in regions protected either by RNA secondary structure or by snRNP binding. It should be noted that there

Table I.

pre-mRNA	Source/size (nt)	3' intron T1 fragments/size (nt) ^a
pRSP-1-ΔIVS	adenovirus(Ad-2) major late transcript/454	ccuuuuuuuuccacag/17 (1) ^c uccuuuuuuuuccacag/18 ^b (2) cuuuuuuuuuccacagCUCG/19 (3)
pSP64-HβΔ6	human β-globin wt/497	auuuuuccacccuuag/16 (1) auuuuuccacccuuagG/17 (2) ucuauuuuccacccuuag/19 ^b (3) ucuauuuuccacccuuagG/20 (4)
-HβΔ6 ^{ag} -gg	human β-globin 3' ss mutant/497	ucuauuuuccacccuuag/18 ^d
-HβΔ6 _{Δ3'}	human β-globin 3' polypyrimidine deletion/472	None
p5Eco0109	adenovirus(Ad-2) major late transcript/209	ccuuuuuuuuccacag/16 (1) ccuuuuuuuuccacag/17 ^b (2) gccuuuuuuuuccacag/18 (3)
-0109 ^{ag} -ug	adenovirus(Ad-2) major late transcript/196	ccuuuuuuuuccacag/17 ^d
-0109 ^{ag} -au	adenovirus(Ad-2) major late transcript/196	ccuuuuuuuuccacauCUCG/21 ^d

^aT1 fragments immunopurified with anti-hnRNP protein monoclonal antibodies were eluted from 15% denaturing polyacrylamide gels and subjected to secondary RNase digestions and complete T1 RNA fingerprinting. Fragments longer than the complete T1 digestion product are partial fragments cleaved at an upstream or downstream G residue while the shorter fragments are generated at 30°C by a minor contaminating nuclease.

^bExpected complete T1 fragment from this region.

^cNumbers in parentheses designate fragments referred to in Figures 2(C), 3(C) and 4(B).

^dOnly the expected complete T1 fragments are listed for the mutants.

is no evidence that individual proteins can protect the pre-mRNA under these conditions. Finally, RNA fragments bound to hnRNP proteins are immunopurified using monoclonal antibodies against individual hnRNP proteins, and purified fragments are analyzed by electrophoresis on denaturing polyacrylamide gels. The binding of hnRNP proteins to pre-mRNA is thus studied in the presence of a large excess of endogenous unlabeled hnRNA (~1–2 ng labeled RNA/1.5 μg endogenous hnRNA) in the nuclear extract which serves as a competitor of the radioactively labeled input pre-mRNA. All nuclear extracts used in this study efficiently spliced competent pre-mRNAs. To identify the immunopurified fragments unambiguously, the indicated fragments were eluted from the gel and secondary RNase analyses, including complete two-dimensional RNA fingerprinting, were carried out (Brownlee, 1972). The hnRNP proteins used in this study (A1, C, D, L and U) have recently been characterized (Piñol-Roma *et al.*, 1988) and are all abundant in the nuclear extract. The amount of monoclonal antibody required to completely immunopurify each of these hnRNP proteins from the nuclear extract was determined and an excess over this amount was used to ensure that differences in the immunopurification of proteins bound to RNA fragments was not due to limiting antibody concentration.

Figure 1 shows the results of such an analysis of the binding of hnRNP proteins to a human β-globin 497 nt transcript, pSP64-HβΔ6, which contains the first exon and intron and a part of the second exon (Krainer *et al.*, 1984). Monoclonal antibodies to the C1 and C2 proteins (Figure 1, Globin wt, lane 4F4), (Choi and Dreyfuss, 1984b), the A1 protein (lane 4B10) (Piñol-Roma *et al.*, 1988) and the D proteins (lane 5B9) (Piñol-Roma *et al.*, 1988; Piñol-Roma and Dreyfuss, manuscript in preparation), efficiently immunopurify the 19mer RNase T1 fragment which encompasses the polypyrimidine stretch between the branch

adenosine residue and the 3' splice site, as well as a 20mer which contains an extra G residue (Table I, pSP64-HβΔ6, fragments 3 and 4)—in fact, the 20mer is more efficiently immunopurified than the 19mer. Monoclonal antibodies to the L (lane 4D11) (Piñol-Roma and Dreyfuss, manuscript in preparation) and U (lane 3G6) (Dreyfuss *et al.*, 1984b) hnRNP proteins, which are also RNA-binding proteins (Piñol-Roma *et al.*, 1988), immunopurify these fragments much less efficiently although under these conditions L consistently binds this region better than U. The amount of the L and U proteins in the nuclear extract, and the affinity of the respective antibodies for them are not sufficiently different from the corresponding parameters for A1, C and D to account for the observed difference. Furthermore, antibodies to the C proteins immunopurify 2- to 3-times fewer counts of this transcript but not of the adenovirus transcript (see below) than either A1 or D. None of the fragments are immunopurified with the non-immune mouse immunoglobulin control, SP2/0. In agreement with previous results (Chabot *et al.*, 1985; Chabot and Steitz, 1987), a polyclonal antiserum against the snRNP Sm determinant immunopurifies the same 19- and 20mer fragments as well as a protected 15mer corresponding to the U1 snRNP binding site at the 5' end of the intron (cf. Figure 1, AG→GG, lane U1) (Mount *et al.*, 1983). It should be noted that since the anti-Sm antibody is polyclonal antiserum, whereas the anti-hnRNP antibodies are monoclonals, the lower signals in the anti-Sm lanes do not necessarily reflect lower binding efficiency of the Sm-reactive component(s).

It has been previously demonstrated that a mutation at the 3' splice site which alters the AG dinucleotide to GG greatly reduces the binding of the Sm-determinant-containing factor(s) to the 19mer T1 fragment from this region (Chabot *et al.*, 1985), and Figure 1 (AG→GG) confirms this result (lane Sm) and shows that this is also the case for the hnRNP C (lane 4F4), A1 (lane 4B10), and D (lane 5B9) proteins,

Fig. 2. Partial RNase T1 analysis of the binding of hnRNP proteins to human β -globin wild-type and mutant transcripts. **(A)** Plasmids containing either the wild-type human β -globin gene (globin wt, pSP64H β Δ6), a point mutant in which the AG at the 3' splice site has been changed to a GG (AG → GG; H $\beta^{AG \rightarrow GG}$) or a thalassemia mutant, in which 25 nt is deleted between the branch adenosine residue and the 3' splice site and containing the polypyrimidine stretch (Δ3'; H $\beta^{\Delta 3'}$), were linearized with *Bam*HI and transcribed with SP6 polymerase. These precursor RNAs were incubated in the nuclear extract and cleaved with RNase T1 and immunoprecipitated with monoclonal antibodies or polyclonal antiserum as described in Materials and methods. **(B)** SP64-H β Δ6-IVS1,2, a plasmid construct in which the two introns of the human β -globin gene have been deleted, was linearized with *Bam*HI, transcribed and immunoprecipitated as described in (A). **(C)** Summary of the binding of hnRNP proteins to β -globin wild-type and mutant transcripts *in vitro*. The numbers refer to the RNA fragments indicated in the left hand margin of (A). The lengths of the black boxes are proportional to the sizes of the fragments.

although the binding of the D proteins is less affected than either A1 or C.

One potential difficulty with the above experiments is that since it is the largest fragment from the complete T1 digest which is immunopurified (the 19mer), the possibility cannot be ruled out that these fragments are simply more efficiently immunopurified because of their size. To address this issue, the incubation and nuclease digestion procedure was adjusted so as to maximize the yield of larger, partial, T1 fragments. This involved incubating the RNA transcript in nuclear extract without ATP or creatine phosphate (to prevent RNA

splicing) for 10 min, and then digesting with only 2 U of RNase T1 for 15 min at 30°C. If hnRNP proteins bind to any RNA fragments of 19 nt or larger independent of RNA sequence, then immunopurification should result in all of these larger fragments being immunopurified. However, as Figure 2A illustrates, only a discrete subpopulation of fragments larger than the 19/20mer bands is immunopurified. Under these conditions, the C proteins (Globin wt, lane 4F4) bind only to the fragments containing the polypyrimidine region near the 3' end of the intron (bands 3 and 4) weakly whereas the A1 (lane 4B10) and D (lane 5B9) proteins bind

fragments corresponding to this region much more effectively, including subfragments of the polypyrimidine 19mer generated at 30°C by the T1 nuclease preparation used in this study (bands 1 and 2). In addition, A1 and the D proteins bind other larger fragments (bands 5 and 6) corresponding to a region 5' to the polypyrimidine stretch which encompass the branch point residue. It is noteworthy that protection of the 5' splice site 15mer is not detectable under these partial RNase T1 experiments (Figure 2A, lane Sm and cf. Figure 1, lane U1). All of the above binding is to fragments derived from the intron. Consistent with these observations, Figure 2B shows that no specific binding sites for the A1 and C hnRNP proteins are detectable (this is also true for D, L, U and anti-Sm, data not shown) for the corresponding intronless splicing product, a 367 nt RNA prepared from SP64H β Δ 6-IVS1,2 from which the first IVS has been eliminated (Krainer *et al.*, 1984).

Mutations at the 3' splice site disrupt the binding of the hnRNP A1 protein

The 3' intron junction is one of the most highly conserved segments of the pre-mRNA and mutations in it impair RNA splicing (Green, 1986; Padgett *et al.*, 1986; Maniatis and Reed, 1987; Sharp, 1987; Steitz *et al.*, 1988). It was, therefore, of interest to determine if mutations in this part of the RNA have any effect on the binding of the hnRNP proteins to this region. As shown in Figures 1 and 2, when the conserved AG dinucleotide at the 3' splice site of the β -globin pre-mRNA is changed to a GG, the sizes of the T1 fragments from the polypyrimidine region decrease by one nucleotide (Table I) to an 18mer and a 19mer (Figure 2A, lanes AG $\rightarrow$ GG). Although binding of the C proteins to these fragments (lane 4F4) is not decreased (in fact, it increases 2-fold), the binding of A1 to these fragments is markedly reduced (>10-fold) and the binding to the subfragments of 16 and 15 nt is no longer detectable. The binding of A1 to the larger T1 fragments (fragments 5 and 6), however, is apparently independent of the binding to the 3' end of the intron as they are still immunopurified with the same efficiency as in the wild type. In contrast, the binding of the D proteins is much less sensitive to this point mutation in the 3' end of the intron region while binding near the branch point is unaffected. If the polypyrimidine stretch is entirely eliminated—as in the β -globin thalassemia mutant H β Δ 3' (Reed and Maniatis, 1985), which deletes a 25 nt segment from 13 nt 3' to the branch site to 2 nt 3' to the 3' splice site, then no binding of the C proteins is detected whereas the binding of A1 to the partial fragments from the branch site region is not affected (Figure 2A, lanes Δ 3', lane 4B10). Binding of the D proteins to the branch site is reduced, however, and the D proteins are now found to bind to very large fragments (lane 5B9).

To determine if the binding of hnRNP proteins to the 3' end of the intron of the β -globin pre-mRNA is a general phenomenon, we examined their binding to other pre-mRNAs. Analysis of the binding of hnRNP proteins to a 454 nt adenovirus (Ad-2) major late transcript (pRSP-1- Δ IVS) (Padgett *et al.*, 1984; Grabowski *et al.*, 1984) is shown in Figure 3. This transcript contains the first two leader exons, a shortened form of the first intron and a portion of the second intron, and has previously been shown to require hnRNP C proteins for splicing (Choi *et al.*, 1986). Again, under these experimental conditions, a

large number of partial RNase T1 fragments of up to 100 nt are generated (Figure 3A, lane total). The largest fragment generated by complete RNase T1 digestion is an 18mer (designated 2 in Figure 3) which encompasses the polypyrimidine stretch in the intron sequence and contains the AG dinucleotide of the 3' splice site (Figure 3C). None of the fragments are efficiently immunopurified with a control non-immune monoclonal antibody (lane SP2/0). In contrast, monoclonal antibodies to the hnRNP proteins C1 and C2 (lane 4F4), A1 (4B10) and D (lane 5B9) immunopurify only distinct subsets of the total fragments, further indicating that these hnRNP proteins have distinct RNA binding specificities. In addition to the protein-specific partial RNase T1 fragments, these three antibodies also immunopurify several common 17, 18 and 19 nt fragments (1, 2 and 3 in Figure 3A) with approximately equal efficiency. The D proteins (lane 5B9) bind to all of the fragments that are immunopurified with antibodies to the C and A1 proteins. There is also an intriguing similarity between partial T1 fragments immunopurified with the D proteins and those immunopurified with the autoimmune anti-snRNP antibody anti-Sm (lane 5B9 versus lane Sm). In contrast, antibodies to the L (lane 4D11) and U (lane 3G6) hnRNP proteins do not efficiently immunopurify any RNase T1 fragments of this transcript.

RNA fingerprinting demonstrated that the 18 nt fragment is the expected RNase T1 fragment from the 3' end of the intron, while the 17 and 19mer fragments are also derived from this region (Table I). The positions of the fragments on pRSP-1- Δ IVS are illustrated in Figure 3C. What is striking about the fragments that are bound to the hnRNP proteins C, A1 and D is that they are also exclusively in the intron. The small RNase T1 fragments (1, 2 and 3) encompass the polypyrimidine stretch and the larger RNase T1 partial fragments (4, 5 and 6) are derived from a region immediately 5' to this domain and include the branch site. Larger fragments (>60 nt), which encompass both the polypyrimidine and branch point regions, are under-represented in the total partial RNase T1 population because of the efficiency of RNase T1 cleavage near the polypyrimidine stretch (i.e. the large amount of fragments 1, 2 and 3) and therefore are not visibly immunopurified. Figure 3B shows the result of the same type of experiment using a bacterial RNA of similar size (pGEM, 494 nt) to pRSP-1- Δ IVS. In marked contrast to the results seen with pRSP-1- Δ IVS, antibodies to hnRNP proteins do not efficiently immunopurify any RNase T1 fragments from the bacterial RNA, further illustrating the specificity of binding of hnRNP proteins to the pre-mRNA fragments, although the A1 (lane 4B10) and D proteins (data not shown) do weakly immunopurify a 9mer fragment.

To further characterize the binding sites of hnRNP proteins on Ad-2 pre-mRNA, additional studies were performed using a set of Ad-2 3' splice site mutants. These were constructed from the Ad-2 major late transcription unit and generously provided by Walter Keller and co-workers (G.Lahr and W.Keller, unpublished data). The parent pre-mRNA for these experiments, p5Eco0109, is a truncated form of Ad-2 which produces a transcript of 209 nt containing shortened exons 1 and 2 and intron 1. As in the case for pRSP-1- Δ IVS and SP64-H β Δ 6 pre-mRNAs, the hnRNP proteins A1, C and D also bind to fragments derived from the polypyrimidine tract of this pre-mRNA (Figure 4A,

Fig. 3. Partial RNase T1 analysis of the binding of hnRNP proteins to the Ad-2 major late transcripts *in vitro*. **(A)** Precursor RNA, synthesized using SP6 polymerase from the Ad-2 major late transcription unit plasmid pRSP-1-ΔIVS1 (Padgett *et al.*, 1984) was incubated for 10 min at 30°C, in a nuclear extract under splicing conditions but without ATP or creatine phosphate, and subsequently digested with 2 U of RNase T1. RNA fragments specifically bound to hnRNP, or to snRNP, proteins were then immunoprecipitated with monoclonal antibodies and displayed on 15% sequencing gels. Lanes are as described in Figure 1(A). **(B)** RNA was synthesized from *Sph*I-linearized pGEM1, using T7 polymerase; immunoprecipitation and electrophoretic analysis was performed as in (A); **(C)** Summary of the binding of hnRNP proteins to the Ad-2 transcript. The binding sites of the A1, C and D proteins are designated by numbers which correspond to those shown on the left of (A), with the size of the solid boxes proportional to the size of the bound RNA fragments.

p5Eco0109, lanes 4F4, 4B10, and 5B9) and immunopurify 16, 17 and 18 nt fragments from this region (Figure 4B and Table 1). Unlike the larger pre-mRNAs, pRSP-1-ΔIVS and SP64-HβΔ6, larger partial RNase T1 fragments containing the branch site region are not detected. This is particularly

intriguing since the region between the branch site adenosine and the 3' splice site varies by only two nucleotides between pRSP-1-ΔIVS and p5Eco0109, AUCCGGGCCCCUUUUUUUCCACAG in p5Eco0109 while the underlined G residues are Us in pRSP-1-ΔIVS.

Fig. 4. Binding of hnRNP proteins to a truncated Ad-2 transcript and 3' splice site mutants. **(A)** A shortened version of the Ad-2 wild-type transcript, p5Eco0109, or two point mutants in which the 3' splice site has been altered to a UG or AU, p5Eco0109^{ag→tg} and p5Eco0109^{ag→at} were produced from *EcoRI* linearized plasmids and *in vitro* transcription with T3 polymerase. Nuclear extract incubations, immunoprecipitations with monoclonal antibodies and gel analysis were performed as in Figure 3. **(B)** Summary of the binding of hnRNP proteins to the truncated Ad-2 transcript.

The effect of 3' splice site mutations in this pre-mRNA on the binding of hnRNP proteins is also shown in Figure 4A. Both mutants, AG→TG and AG→AT, form reduced levels of splicing complexes *in vitro* (W.Keller, personal communication). As is the case for the β -globin 3' splice site mutant, the binding of A1 to the T1 fragment containing the 3' end of the intron and encompassing the poly-pyrimidine stretch is dramatically affected by a single nucleotide change of the 3' splice site from AG to UG (AG→TG, lane 4B10); the immunopurification of these fragments by 4B10 is abolished, indicating that A1 is not able to bind to this sequence even though the size of the

fragment is the same. The efficiency of the binding of the D proteins (AG→TG, lane 5B9) is also reduced and an increase in the immunopurification of a 14mer fragment occurs. The binding of the C proteins to the fragments terminating with UG is relatively less affected. The mutation of AG to AU results in a larger 3' splice site T1 fragment of 21 nt (Figure 4A, AG→AT), and the observed C protein binding is similar to wild type (AG→AT, lane 4F4). A1 binding is reduced for this mutant as well, although not as much as in the AG→TG mutant (lane 4B10). The D proteins still bind efficiently to this region and to the 14mer fragment which appears in the AG→TG mutant (lane 5B9). It is

Fig. 5. Binding of hnRNP proteins to the Ad-2 transcript in the presence of ATP. The pRSP-1- Δ IVS plasmid was linearized and RNA transcribed as described in Figure 3. Incubations in nuclear extract were as described previously with the exception that 1.5 mM ATP and 5 mM creatine phosphate were included.

interesting to note that snRNP proteins recognized by anti-Sm antibodies bind to similar fragments to the D proteins and respond to mutations similarly (lane 5B9 versus Sm); this was the case also for the other pre-mRNAs (cf. Figures 1, 2 and 3).

Thus, the studies with the 3' splice site mutants of both the Ad-2 and β -globin pre-mRNAs show that the binding of A1 to the 3' end of the intron is severely impaired by mutations at the 3' splice site. The binding of C and D is also visibly affected, although considerably less than that of A1.

The hnRNP proteins are also bound at the 3' end of introns in the presence of ATP

The preceding studies were performed without addition of exogenous ATP to the nuclear extract to prevent splicing complex formation and RNA splicing. The incubation of the pRSP-1- Δ IVS pre-mRNA under splicing conditions in the presence of ATP and creatine phosphate for 10 min at 30°C yields a different pattern of partial T1 fragments (Figure 5) than that generated in the absence of ATP (cf. Figure 3A). This altered pattern of nuclease fragmentation may be due to changes in the secondary structures of the RNA, or due

Fig. 6. Purified hnRNP proteins bind to the 3' end of the human β -globin pre-mRNA. hnRNP proteins were purified from 50 μ l of nuclear extract by incubation with hnRNP monoclonal antibodies, which had been previously bound to 25 μ l of Protein A-Sepharose, in the presence of 1% Empigen BB as described in Materials and methods. After re-equilibration of the purified proteins in RSB100/LPA, the proteins were incubated with RNase T1 partial fragments, produced from both SP64-H β Δ 6 (globin wt) and SP64-H β Δ 6-IVS1,2 as described in Figure 2 followed by phenol extraction and ethanol precipitation, in 50 μ l of RSB100/LPA for 30 min at 4°C, and bound fragments immunopurified by centrifugation and analyzed on 15% denaturing polyacrylamide gels. Lanes are as described in Figure 2(A).

to protein, and/or snRNP binding. Nevertheless, the binding of the hnRNP proteins to the fragments of the 3' end of the intron encompassing the polypyrimidine stretch and the splice site AG, remains under conditions where splicing is allowed to occur.

The binding specificity of the hnRNP proteins is an intrinsic property of these proteins

The binding of hnRNP proteins to pre-mRNAs in nuclear extracts may be influenced by a variety of other nuclear components such as other RNA-binding proteins and snRNPs. For example, the specific binding to the 3' end of introns could be the result of direction of the hnRNP proteins to this region by other factors in the nuclear extract or the result of exclusion from other regions of the pre-mRNA by the binding of other factors. To determine whether the specific binding of hnRNP proteins that is observed in nuclear extract is an intrinsic characteristic of these proteins, binding experiments were performed with purified proteins. To do so, labeled β -globin pre-mRNA (SP64H β Δ 6) and the corresponding intronless splicing product (SP64H β Δ 6-IVS1,2) were incubated in nuclear

extract, and RNase T1 was added to partially digest the RNA exactly as described in Figure 2. The proteins in the extract were then removed by extraction with phenol and the resulting RNA fragments were incubated with purified proteins. To purify hnRNP proteins, nuclear extracts were incubated with monoclonal antibodies to various hnRNP proteins in the presence of 1% Empigen BB under conditions which result in the immunopurification of the individual proteins (Choi and Dreyfuss, 1984a,b; Piñol-Roma *et al.*, 1988). These bead-bound proteins were washed extensively and incubated with a partial RNase T1 digest of the β -globin pre-mRNA, generated as described above. The results shown in Figure 6 when compared to Figure 2 demonstrate that the same RNase T1 fragments from the 3' end of the intron (20, 19 and 16mer) are immunopurified with monoclonal antibodies to the A1, C and D proteins, with the C proteins binding less well than A1 or D. In addition, no specific binding sites for these purified proteins exist on the intronless splicing product, pSP64H β Δ 6-IVS1,2, paralleling the binding of these proteins in nuclear extract (cf. Figure 2). However, the binding of hnRNP proteins to the branch point region is not observed with purified proteins indicating that their association with this region may either require other nuclear components or can only take place prior to digestion on the intact, uncleaved, RNA. Not unexpectedly, since there are no competing proteins, the level of non-specific binding exhibited by the purified proteins is higher than that seen in the nuclear extract. Nevertheless, the differences between the binding patterns of these proteins in the extract and as purified proteins are subtle and overall the purified proteins clearly do not bind indiscriminately to the RNA segments available to them, but rather closely resemble the binding seen in the nuclear extract.

Discussion

Two major conclusions of potential significance to both hnRNP packaging and processing can be drawn from the data described here: the first, and more generally important, is that several of the hnRNP proteins, A1, C and D, have RNA binding specificities, and second, that fragments of the introns, particularly those containing the 3' end of introns contain stable binding sites for these proteins. These conclusions are based on RNase T1 digestion/immunopurification experiments that have intrinsic limitations which need to be considered. These studies can identify only the minimal stable binding sites; additional such sites may exist which may be destroyed by the nuclease. It is therefore possible that other stable binding sites for the proteins exist on the wild-type pre-mRNA, on the mutant pre-mRNAs, and on the mRNA. An indication of this is that binding sites for the proteins within the intron upstream of the sites near the 3' splice site are destroyed by complete digestion with RNase T1 (cf. Figures 1 and 2). Another general limitation of the experimental approach that needs to be kept in mind is that since the binding studies involve fragmentation of the RNA, it cannot be concluded with certainty that these necessarily reflect the binding to the intact, uncleaved, RNA molecules. It is, however, reassuring that similar studies on the binding of snRNPs to pre-mRNA (Chabot *et al.*, 1985; Black *et al.*, 1985) have turned out to be correct by other independent criteria (e.g. Zhuang and Weiner, 1986). The conclusion that several of the hnRNP proteins have RNA

binding specificity is valid regardless of these limitations and it is also supported by binding assays to ribohomopolymers (Swanson and Dreyfuss, 1988). The term RNA binding specificity of hnRNP proteins is used here in the broadest sense; it can be the result of either primary and/or secondary structure of the RNA.

The finding of RNA binding specificity for hnRNP proteins appears contradictory to the widely held belief, based largely on reconstitution-type studies (e.g. Pullman and Martin, 1983; Thomas *et al.*, 1983; Wilk *et al.*, 1983), that hnRNP proteins do not possess RNA binding specificities, but they are general single-stranded nucleic acid-binding proteins which non-specifically bind any single-stranded RNA and DNA. How can these seemingly contradictory results be reconciled? Our interpretation follows similar lines to those introduced by Adam and Delbrück (1968), Richter and Eigen (1974), and later expanded upon and applied by von Hippel and colleagues (Berg *et al.*, 1981) to explain the fact that the binding of the *lac* repressor to operator DNA is much faster than predicted by a simple three-dimensional diffusion-controlled reaction mechanism. From the type of measurements reported here, the RNase T1 fragments that are isolated bound to hnRNP proteins represent relatively highly stable, and most likely also high affinity, binding sites. This makes it possible to retrieve specific RNA fragments that contain such a binding site after RNase T1 digestion, because the protein remains bound to them during the immunopurification procedure. We shall refer to such sites as high affinity binding sites. Without competitor RNAs, or in general, whenever there is an excess of hnRNP proteins over high affinity binding sites they will bind to a wide range of single-stranded RNA and DNA polynucleotides predominantly at low affinity binding sites. Once bound to low affinity binding sites, the hnRNP protein would continue to slide along, or associate and dissociate rapidly with, the single-stranded polynucleotide. If it encounters a high affinity binding site the residency time of the protein at that site is longer relative to its residency time at lower affinity binding sites. The advantage of having low affinity for single-stranded polynucleotides in general is that it reduces the dimensionality of diffusion, from three-dimensions to one-dimension—this accelerates the finding of high affinity binding sites, and furthermore may help melt secondary structures in the RNA.

Why do pre-mRNAs possess high affinity binding sites for hnRNP proteins? What is perhaps most intriguing in this regard is that these putative high affinity binding sites for at least three of the hnRNP proteins, A1, C and D, are within the intron, clustered near the 3' splice site. High affinity sites for the hnRNP U and L proteins, if such exist, are not detected by the assays used here on the pre-mRNAs we have tested. The binding specificity of A1, C and D, particularly that of A1, is remarkable; its sensitivity to mutations at the AG dinucleotide of the 3' splice site parallels the effect of such mutations on splicing complex formation. It is therefore possible that A1, in concert with additional proteins and snRNPs, is involved in 3' splice site recognition. Two other proteins have recently been reported to bind to the 3' end of the intron, the 70 kd (Gerke and Steitz, 1986) and the 100 kd (Tazi *et al.*, 1986) anti-Sm reactive proteins. The observations described here confirm and extend the previous reports on the binding sites and characteristics of the anti-Sm-reactive components, and their binding specificity for

the 3' end of introns seems to be similar to that of several of the hnRNP proteins, particularly A1. It is not, at this point, possible to conclude which of the candidates, A1, 70 kd or 100 kd, is in fact required for splicing but it may be that more than one 3' splice site binding factor exists, and that the factors do not all bind at the same time, or that they form a complex in which not all of the proteins are directly in contact with the RNA simultaneously. It would be interesting to determine whether any of the A1 proteins are part of the U2AF activity (Ruskin *et al.*, 1988). The insensitivity of the binding of the C proteins to mutations at the 3' end of the same fragments that A1 binds suggests that they bind immediately upstream of where A1 binds in the polypyrimidine segment. In the pre-mRNAs tested here, the polypyrimidine segment is uridine-rich and the observed binding to it of the C proteins is consistent with the finding that they are poly(U) binding proteins (Swanson and Dreyfuss, 1988). It is also interesting, in this vein, that the C proteins bind better, and can be photochemically cross-linked much more efficiently (M.S. Swanson and G. Dreyfuss, unpublished results), to the adenovirus-derived pre-mRNA than to the β -globin pre-mRNA, considering that in the former the polypyrimidine stretch is more uridine-rich than in the latter. Certain hnRNP proteins may therefore recognize discrete domains of hnRNAs with a gradient of affinities—we do not yet know what constitutes the highest affinity binding site for the A1 and D proteins. An interesting implication of the finding that high affinity binding sites for the hnRNP proteins A1, C and D are in the intron is that pre-mRNA splicing provides a mechanism for their removal from the pre-mRNA that is coupled to mRNA formation. The removal of the nucleus-specific hnRNP proteins from the pre-mRNA prior to, or coincident with, nucleocytoplasmic transport of the mRNA is likely to be an important aspect of the formation of cytoplasmic mRNA (Dreyfuss *et al.*, 1984a,b, 1988; Dreyfuss, 1986).

Most of the hnRNP proteins in the nucleoplasm of vertebrate cells are found in the form of very large particles where they are organized into 30–40S complexes called monoparticles. Monoparticles are associated with ~500–800 nt of hnRNA (Pullman and Martin, 1983; Wilk *et al.*, 1983; Piñol-Roma *et al.*, unpublished data), and it is therefore not likely that complete monoparticles form on the pre-mRNAs used here; these are shorter than 500 nt, and snRNPs would occupy a considerable proportion of their length. The hnRNP proteins that assemble on these pre-mRNAs probably form a specialized complex. The relationship between this hnRNP protein-containing complex and monoparticles is, at this point, unclear. It is possible that monoparticles form when there is sufficient length of hnRNA which does not contain the specific combination of sequences (e.g. 3' ends of introns) that would lead to formation of specialized complexes. In that case, provided that hnRNP proteins are in mass excess over high affinity binding sites (which, given the abundance of hnRNP proteins, is most likely the case), then the proteins may coalesce on the hnRNA to form monoparticles whose composition may reflect the relative abundance of the hnRNP proteins in the nucleus. This model considers a dual role for hnRNP proteins in pre-mRNA packaging, as monoparticles, and in pre-mRNA processing. Together with the observations described here, the considerable assortment of hnRNP proteins recently found (Piñol-Roma *et al.*, 1988) suggests that some of

these proteins may be involved in a variety of functions in mRNA formation.

Materials and methods

Plasmids and RNA transcripts

RNA transcripts were synthesized using either [α - 32 P]UTP or [α - 32 P]GTP and 7 mGpppG and purified by 10% polyacrylamide–8 M urea gel electrophoresis as previously described (Choi *et al.*, 1986). Plasmid templates derived from the human β -globin gene were kindly provided by R. Reed and T. Maniatis (SP64-H β Δ 6, SP64-H β Δ 3', SP64-H β $^{98-99}$) and A. Krainer (SP64-H β Δ 6-IVS1,2), and linearized by *Bam*HI and transcribed with SP6 polymerase to generate pre-mRNAs of 497, 472, 497 and 367 nt, respectively. Plasmids derived from the Ad-2 major late promoter were pRSP-1- Δ IVS (Padgett *et al.*, 1984; Choi *et al.*, 1986) linearized with *Bgl*II and transcribed with SP6 polymerase to synthesize a transcript of 454 nt, and several truncated Ad-2 plasmids, p5Eco0109, p5Eco0109 $^{98-99}$ and p5Eco0109 $^{98-99}$ (kindly provided by G. Lahr and W. Keller) which were linearized with *Eco*RI and transcribed with T3 polymerase to generate RNA transcripts of 209, 196 and 196 nt, respectively. Bacterial RNA was synthesized from *Sph*I linearized pGEM1 (Promega Biotec) using T7 polymerase to produce a 494 nt RNA.

Nuclear extract incubations, RNase T1 digestions and monoclonal antibody immunopurifications

For complete RNase T1 digestions, the β -globin pre-mRNAs (250 000 c.p.m.) were incubated in the presence of ATP and creatine phosphate as previously described (Krainer *et al.*, 1984; Chabot *et al.*, 1985) for 30 min on ice with 145 U of RNase T1 (Calbiochem). Immunopurifications were performed by pre-binding 2 μ l of ascites fluid (monoclonal antibodies) or 5 μ l of polyclonal antiserum (anti-Sm) to 25 μ l (packed volume) of Protein A–Sepharose (Pharmacia) in 10 mM Tris–Cl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, 0.5% Triton X-100, 1 μ g/ml each of leupeptin and pepstatin and 0.5% aprotinin (Sigma Chem. Co) (RSB100/LPA) for 60 min at 4°C as described (Choi and Dreyfuss, 1984b). Beads were washed 3 times in RSB100/LPA, diluted to 0.5 ml with the same buffer and then added to the 25 μ l binding/RNase T1 incubation reactions. Following antibody binding for 10 min at 4°C, the beads were washed 5 $\times$ 1 ml and the RNA released by boiling for 3 min in 75 μ l of 1% SDS, 10 mM Tris–Cl, pH 7.4, 1 mM EDTA. After dilution of the SDS to 0.25% and phenol extraction and ethanol precipitation, immunoprecipitated fragments were analyzed by denaturing gel electrophoresis on 15% polyacrylamide–8 M urea gels.

For partial RNase T1 digestions of β -globin transcripts, the incubation with nuclear extract was similar except that ATP, creatine phosphate, and RNase T1 were omitted from the binding reactions which were incubated for 10 min at 30°C followed by the addition of 2 U RNase T1 and additional incubation at 30°C for 15 min. For partial RNase T1 analysis using the Ad-2 or pGEM transcripts, incubations were performed in 25 μ l, as described previously (Choi *et al.*, 1986) in 20 mM Hepes, pH 7.6, 2 mM MgCl₂, 11 μ l of HeLa cell nuclear extract (Dignam *et al.*, 1983) and 32 P-labeled precursor RNA (250 000 c.p.m.) with or without 5 mM creatine phosphate and 1.5 mM ATP, and incubation and T1 digestions done as above for the β -globin transcripts. Immunopurifications were performed as described above.

RNA analysis

Secondary RNase analysis of complete and partial T1 fragments was done by labeling the pre-mRNA with [32 P]GTP and by eluting specific fragments (labeled 1–6) from the 15% denaturing polyacrylamide gels. For secondary RNase A and U2 analyses, fragments were digested to completion and the sizes analyzed on 25% denaturing polyacrylamide gels. For two-dimensional RNA fingerprinting, eluted fragments were first digested to completion with 50 U RNase T1 for 30 min at 37°C and run in the first dimension on cellulose acetate strips (Schleicher & Schuell) in pyridine acetate (pH 3.5) and in the second dimension by homochromatography on PEI–cellulose (Brownlee, 1972).

Purification of hnRNP proteins

hnRNP proteins were purified from 50 μ l of nuclear extract by incubation in 0.5 ml 1% Empigen BB/1 mM EDTA/0.1 mM dithiothreitol in phosphate buffered saline with 25 μ l of antibody-bound protein A–Sepharose beads at 4°C. As previously described (Choi and Dreyfuss, 1984; Piñol-Roma, 1988), this procedure yields extremely pure preparations of hnRNP proteins. The bound proteins were then allowed to renature by washing and incubation.

tion for 60 min at 4°C in RSB 100/LPA. RNase T1 partial fragments, prepared in nuclear extracts as described above and subsequently purified away from protein by phenol extraction and ethanol precipitation, were added to the hnRNP protein-bound Sepharose beads in 50 µl of RSB100/LPA and incubated at 4°C for 30 min with constant mixing. The beads were then washed five times in the same buffer and the bound RNA fragments eluted by boiling in SDS, phenol extracted and ethanol precipitated as described above.

Acknowledgements

We thank B.Thimmapaya and M.Furtado for help with oligonucleotide fingerprinting; W.Keller, A.Krainer, G.Lahr, T.Maniatis and R.Reed for plasmids; The Center for Disease Control for human anti-Sm antiserum, S.Hoch for the anti-U1 monoclonal antibody, and R.Bandziulis, R.Lamb, D.Linzer, R.Morimoto and S.Piñol-Roma for comments on the manuscript. This research was supported by Public Health Service Grants GM-31888 and GM-37125 from the National Institutes of Health. G.D. is an Established Investigator of the American Heart Association.

References

- Adam, G. and Delbrück, M. (1968) In Rich, A. and Davidson, N. (eds), *Structural Chemistry and Molecular Biology*. W.H. Freeman, San Francisco, pp. 198–215.
- Augenlicht, L.H. (1979) *Biochemistry*, **18**, 3780–3785.
- Berg, O.G., Winter, R.B. and von Hippel, P.H. (1981) *Biochemistry*, **20**, 6929–6948.
- Beyer, A.L., Christensen, M.E., Walker, B.W. and LeSturgeon, W.M. (1977) *Cell*, **11**, 127–138.
- Beyer, A.L., Miller, O.L., Jr and McKnight, S.L. (1980) *Cell*, **20**, 75–84.
- Black, D.L., Chabot, B. and Steitz, J.A. (1985) *Cell*, **42**, 737–750.
- Brownlee, G.G. (1972) In Work, T.S. and Work, E. (eds), *Techniques in Biochemistry and Molecular Biology*, Elsevier, New York, pp. 67–99.
- Chabot, B., Black, D.L., LeMaster, D.M. and Steitz, J.A. (1985) *Science*, **230**, 1344–1349.
- Chabot, B. and Steitz, J.A. (1987) *Mol. Cell. Biol.*, **7**, 281–293.
- Choi, Y.D. and Dreyfuss, G. (1984a) *Proc. Natl. Acad. Sci. USA*, **81**, 7471–7475.
- Choi, Y.D. and Dreyfuss, G. (1984b) *J. Cell Biol.*, **99**, 1997–2004.
- Choi, Y.D., Grabowski, P.J., Sharp, P.A. and Dreyfuss, G. (1986) *Science*, **231**, 1534–1539.
- Chung, S.Y. and Wooley, J. (1986) *Proteins*, **1**, 195–210.
- Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
- Dreyfuss, G., Adam, S.A. and Choi, Y.D. (1984a) *Mol. Cell. Biol.*, **4**, 415–423.
- Dreyfuss, G., Choi, Y.D. and Adam, S.A. (1984b) *Mol. Cell. Biol.*, **4**, 1104–1114.
- Dreyfuss, G. (1986) *Annu. Rev. Cell. Biol.*, **2**, 459–498.
- Dreyfuss, G., Swanson, M.S. and Piñol-Roma, S. (1988) *Trends Biochem. Sci.*, **13**, 86–91.
- Gerke, V. and Steitz, J.A. (1986) *Cell*, **47**, 973–984.
- Grabowski, P.J., Padgett, R.A. and Sharp, P.A. (1984) *Cell*, **37**, 415–427.
- Green, M.R. (1986) *Annu. Rev. Genet.*, **20**, 671–708.
- Karn, J., Vidali, G., Boffa, L.C. and Allfrey, V.G. (1977) *J. Biol. Chem.*, **252**, 7307–7322.
- Krainer, A.R., Maniatis, T., Ruskin, B. and Green, M.R. (1984) *Cell*, **36**, 993–1005.
- Maniatis, T. and Reed, R. (1987) *Nature*, **325**, 673–678.
- Martin, T.E., Billings, P., Levey, A., Ozarslan, S., Quinlan, T., Swift, H. and Urbas, L. (1973) *Cold Spring Harbor Symp. Quant. Biol.*, **38**, 921–932.
- Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A. and Steitz, J.A. (1983) *Cell*, **33**, 509–518.
- Ohlsson, R.I., Van Eekelen, C. and Philipson, L. (1982) *Nucleic Acids Res.*, **10**, 3053–3068.
- Osheim, Y.N., Miller, O.L. and Beyer, A.L. (1985) *Cell*, **43**, 143–151.
- Padgett, R.A., Konarska, M.M., Grabowski, P.J., Hardy, S.F. and Sharp, P.A. (1984) *Science*, **225**, 898–903.
- Padgett, R.A., Grabowski, P.J., Konarska, M.M., Seiler, S.R. and Sharp, P.A. (1986) *Annu. Rev. Biochem.*, **55**, 1119–1150.
- Pandolfo, M., Valentini, O., Biamonti, G., Rossi, P. and Riva, S. (1987) *Eur. J. Biochem.*, **162**, 213–220.
- Pederson, T. (1974) *J. Mol. Biol.*, **83**, 163–183.
- Piñol-Roma, S., Choi, Y.D., Matunis, M.J. and Dreyfuss, G. (1988) *Genes Dev.*, **2**, 215–227.
- Pullman, J.M. and Martin, T.E. (1983) *J. Cell Biol.*, **97**, 99–111.
- Reed, R. and Maniatis, T. (1985) *Cell*, **41**, 95–105.
- Richter, P.H. and Eigen, M. (1974) *Biophys. Chem.*, **2**, 255–263.
- Ruskin, B., Zamore, P.D. and Green, M.R. (1988) *Cell*, **52**, 207–219.
- Samarina, O.P., Lukanidin, E.M., Molnar, J. and Georgiev, G.P. (1968) *J. Mol. Biol.*, **33**, 251–263.
- Sharp, P.A. (1987) *Science*, **235**, 766–771.
- Sierakowska, H., Szer, W., Furdon, P.J. and Kole, R. (1986) *Nucleic Acids Res.*, **14**, 5241–5254.
- Steitz, J.A. and Kamen, R. (1981) *Mol. Cell Biol.*, **1**, 21–34.
- Steitz, J.A., Black, D.L., Gerke, V., Parker, K.A., Kramer, A., Frendewey, D. and Keller, W. (1988) In Birnstiel, M.L. (ed.), *Functions of the Abundant U-snRNPs. Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Berlin, pp. 115–154.
- Stevenin, J., Gallinaro-Matringe, H., Gattoni, R. and Jacob, M. (1977) *Eur. J. Biochem.*, **74**, 589–602.
- Stevenin, J., Gattoni, R., Keohavong, P. and Jacob, M. (1982) *J. Mol. Biol.*, **155**, 185–205.
- Swanson, M.S. and Dreyfuss, G. (1988) *Mol. Cell. Biol.*, **8**, 2237–2241.
- Swanson, M.S., Nakagawa, T.Y., LeVan, K. and Dreyfuss, G. (1987) *Mol. Cell. Biol.*, **7**, 1731–1739.
- Tazi, J., Alibert, C., Tamsamani, J., Reveillaud, I., Cathala, G., Brunel, C. and Jeanteur, P. (1986) *Cell*, **47**, 755–766.
- Thomas, J.O., Glowacka, S.K. and Szer, W. (1983) *J. Mol. Biol.*, **171**, 439–455.
- van Eekelen, C., Ohlsson, R., Philipson, L., Mariman, E., van Beek, R. and van Venrooij, W. (1982) *Nucleic Acids Res.*, **10**, 7115–7131.
- Wilk, H.-E., Angeli, G. and Schaefer, K.P. (1983) *Biochemistry*, **22**, 4592–4600.
- Zhuang, Y. and Weiner, A.M. (1986) *Cell*, **46**, 827–835.

Received on July 14, 1988