Crosslinking of hnRNP proteins to pre-mRNA requires U1 and U2 snRNPs

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Received February 3, 1990; Revised and Accepted April 25, 1990

ABSTRACT

Proteins interacting with pre-mRNAs during early stages of spliceosome formation in a HeLa nuclear extract were investigated by photochemical RNA-protein crosslinking. The level of protein crosslinking to a β -globin pre-mRNA was positively correlated with the presence of an intron. Proteins of 110,000, 59,000 and 39,000 mol. wt. were crosslinked to the β -globin pre-mRNA, the latter of which was identified as the A1 hnRNP protein. Comparable experiments with an adenovirus pre-mRNA revealed crosslinked proteins of 110,000, 56,000 and 45,000 mol. wt., with the latter identified as belonging to the C group hnRNP proteins. Crosslinking of hnRNP proteins to both the β -globin and adenovirus pre-mRNAs was eliminated by oligodeoxynucleotide-directed RNase H excision of an internal region (nt 28–42) of U2 RNA, but was not affected by oligo/RNase H cleavage of the 5'-terminal 15 nucleotides of U2 RNA. Cleavage of the 5'-terminal 15 nucleotides of U1 RNA preferentially eliminated crosslinking of the hnRNP A1 protein to both pre-mRNAs. The requirement of intact U1 snRNP for A1 protein crosslinking was further demonstrated by the fact that although micrococcal nuclease-treated extracts did not support crosslinking of A1 hnRNP protein to β -globin pre-mRNA, crosslinking was restored by addition of a U1 snRNP-enriched fraction.

INTRODUCTION

An *in vitro* system (1) used to elucidate the pathway of mRNA splicing (2–5) has also facilitated the identification of components of the splicing apparatus, including small nuclear RNPs (5) and hnRNP proteins (6–8). The hnRNP proteins were first defined in nuclear ribonucleoprotein particles that contain pre-mRNA (9–15). However, the mode by which these proteins bind to pre-mRNA is unknown. Here we have investigated the interaction of hnRNP proteins with human β -globin and adenovirus pre-mRNAs, employing RNA-protein crosslinking procedures we have previously developed (16–19). We find that the level of crosslinking of hnRNP proteins to pre-mRNA is dependent on the presence of the intron and two splicing cofactors, the U1 and U2 snRNPs. These results point to interactions of hnRNP proteins with the U1 and U2 snRNPs during the early stages of spliceosome assembly.

MATERIALS AND METHODS

Transcripts

β -globin pre-mRNAs were generated by SP6 RNA polymerase transcription of SP62-H β A Δ 44 or SP64-H β Δ 6 (1) (provided by Adrian Krainer and Tom Maniatis, Harvard University) after linearization with BamHI. An intron-lacking β -globin RNA was transcribed from a cDNA clone, SP62-H β -IVS1,2 (provided by Douglas Melton, Harvard University) after linearization with either BamHI or EcoRI. (This unpublished cDNA clone is distinct from the plasmid SP64-H β Δ 6-IVS1,2 described in ref. 1). Human histone H4 RNA was transcribed from pSPH4+ (provided by Kimberly Mowry and Joan Steitz, Yale University School of Medicine) after linearization with HindIII. The adenovirus-2 pre-mRNA was transcribed from BglI-cleaved pRSP-1- Δ IVS or from Sau3A-cleaved pBSAd10 (both provided by Maria Konarska and Phillip Sharp, MIT). All RNAs were transcribed in the presence of 0.5 mM 7-methylguanosine (5') triphospho-(5') guanosine (7-meGpppG) (20) and were labeled to high specific activity by reducing the concentrations of CTP, GTP and UTP to one-tenth of that prescribed (21) and including [α -³²P]-CTP, [α -³²P]-GTP, and [α -³²P]-UTP at a concentration of 0.8 mCi of each per ml of transcription reaction. Reaction volumes were typically 25 μ l.

RNA-protein crosslinking in splicing extracts

Formulation of nuclear extracts, splicing reactions and RNA-protein crosslinking were as detailed previously (1,7,16,19,22). After incubation of ³²P-RNA in nuclear extract (60 mM KCl) for 30 min. (30°C), aliquots were prepared for crosslinking by chilling on ice and centrifuging 3 min. at 12,000 $\times$ g (4°C). Approximately 10% of the radioactivity pelleted. The supernatants were removed and the KCl concentration was adjusted to 200 mM. After crosslinking (4000 μ W/cm², 15 min., see ref. 16), samples were diluted with one volume of buffer D (22), made 2 mM in CaCl₂, and digested with micrococcal nuclease and RNase A as previously described (18,19). Measurement of trichloroacetic acid-insoluble ³²P radioactivity revealed that approximately 90% of the RNA was digested under the conditions employed. Very similar patterns of crosslinked proteins were observed when 5- or 10-fold higher nuclease concentrations were used, indicating that these are limit digests with respect to the protein-crosslinked (oligo)nucleotides. After nuclease digestion, proteins were acetone-precipitated (23) and analyzed by electrophoresis in 11% polyacrylamide gels containing 0.1% SDS followed by autoradiography.

Immunoselection

Crosslinked samples were immunoselected either before or after nuclease digestion as above. Samples were incubated with antibody for 60 min. on ice, followed by selection of antigen-antibody complexes with protein A-Sepharose (23). Four murine monoclonal antibodies were used in this investigation. 4B10 reacts with the hnRNP A1 protein (24), 4F4 reacts with the hnRNP C1 and C2 proteins (25), and iD-2 reacts with all six hnRNP core proteins (26). The RNP monoclonal antibody reacts specifically with the U1 small nuclear RNP particle (27). Non-immune mouse IgG was used for antibody specificity controls.

Competition experiments

³²P-labeled β -globin pre-mRNA was incubated in nuclear extracts as detailed above in the presence of an increasing molar excess of the same RNA, or of the intron-lacking β -globin transcript. (The ³²P-labeled and competitor RNAs were pre-mixed and then nuclear extract was added). The competitor RNAs were labeled to low specific activity with ³H-UTP to facilitate setting the competitor:substrate RNA ratios.

Oligodeoxynucleotide-mediated cleavage of U-snRNPs

Oligodeoxynucleotides complementary to nucleotides 2–14 of U1, U2 or U5 RNAs (counting the cap as nucleotide 0), to nucleotides 28–42 of U2 RNA, or to nucleotides 88–102 of U6 RNA were incubated at 200 μ g/ml for 30 min. (30°C) in a nuclear extract (500 μ M ATP, 20 mM creatine phosphate, 3.2 mM MgCl₂, 1,000 U/ml RNasin). Pre-mRNA was then added and the incubation reaction was continued for an additional 30 min.

Non-denaturing gel electrophoresis of RNP complexes

Adenovirus pre-mRNA, transcribed from Sau3A-linearized pBSAd10 DNA, was incubated at 30°C in a nuclear extract (20 mM creatine phosphate, 2.2 mM MgCl₂, 0.4 mM ATP, 33% nuclear extract (vol/vol), and ~0.16 μ g/ml pre-mRNA). At 30 min. 1/10 volume of 20 mg/ml heparin was added and incubation was continued for 10 min. 10 μ l aliquots were subjected to electrophoresis in a 4% polyacrylamide-0.5% agarose composite gel in 50 mM Tris-glycine buffer (pH 8.3) for 2–3 hours at 200V.

snRNP fractionation by CsCl centrifugation

1 ml of nuclear extract was layered on 40% CsCl (wt/vol) (in 15 mM MgCl₂, 20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT) and centrifuged for 65 hours at 32,000 rpm at 4°C in a Beckman SW50.1 rotor. 500 μ l fractions were collected and dialyzed against buffer D containing 3.2 mM MgCl₂. An aliquot of each fraction was extracted with phenol:chloroform and ethanol precipitated. The RNA was analyzed on a 8.3M urea-10% polyacrylamide gel, and then stained with ethidium bromide. The remaining fractions were frozen at –80°C until needed.

Complementation experiments

Nuclear extract was treated with 830 units of micrococcal nuclease per ml in the presence of 1 mM CaCl₂ for 30 min. at 30°C. CaCl₂ was then chelated with a 3-fold molar excess of EGTA. The treated extract was made splicing permissive by the addition of 500 μ M ATP, 20 mM creatine phosphate, and 3.2 mM MgCl₂. An equal volume of CsCl-purified snRNPs (15 μ l of the desired CsCl fraction, 5 μ l H₂O, 1 μ l 10 mM ATP,

Figure 1. Proteins crosslinked to β -globin pre-mRNA and intron-lacking RNAs. Equimolar concentrations of ³²P-labeled β -globin pre-mRNA (transcribed from BamHI-cleaved SP62-H β A Δ 44 DNA), an intron-lacking β -globin RNA (transcribed from BamHI-cleaved SP62-H β -IVS1,2) or a human histone H4 RNA (transcribed from Hind III-cleaved pSPH4+) were incubated in nuclear extract for 30°C, crosslinked, immunoselected with an antibody (iD-2) specific for all six hnRNP core proteins (26), followed by nuclease digestion and electrophoresis. Lane 1: β -globin pre-mRNA; lane 2: intron-lacking β -globin RNA; lane 3: histone H4 RNA. Lane 4 shows the results of crosslinking with β -globin pre-mRNA using mouse non-immune IgG in place of the hnRNP protein antibody. The numbers to the left of lane 1 denote the positions of C¹⁴-methylated protein molecular weight markers: 92,000-phosphorylase b, 69,000-bovine serum albumin, 46,000-ovalbumin, 30,000-carbonic anhydrase.

1 μ l 80 mM MgCl₂, 1 μ l 0.5 M creatine phosphate and 1 μ l RNasin) was added to the nuclease-treated extract, followed by addition of ³²P-labeled β -globin pre-mRNA. Incubation was for 30 min. at 30°C, followed by crosslinking and electrophoretic analysis of proteins as described above.

RESULTS

Specificity of β -globin pre-mRNA-protein crosslinking

Figure 1, lane 1 shows the results of protein-RNA crosslinking and immunoselection after incubation of β -globin pre-mRNA in nuclear extract for 30 min. at 30°C. The immunoselection was performed with a monoclonal antibody (iD-2) that reacts with all six of the hnRNP core proteins (C2, C1, B2, B1, A2, A1). It can be seen in lane 1 that this antibody selected β -globin pre-mRNA complexes containing crosslinked proteins of 59,000 and 39,000 mol. wt. A 110,000 mol. wt. protein can also be seen

as a faint band and was more evident in a longer autoradiographic exposure (not shown). When a β -globin transcript lacking the intron was used, a much lower level of these crosslinked proteins was observed (Figure 1, lane 2). This was also the case with an intron-lacking human histone H4 transcript (Figure 1, lane 3). Deliberate over-exposure of this gel revealed that there was a finite, albeit low, level of crosslinkable protein in the intron-lacking complexes (data not shown). Quantitative densitometry of films from several experiments revealed a 5- to 10-fold difference in the level of protein crosslinking to the intron-containing vs. intron-lacking β -globin transcripts.

Several possibilities exist for the different levels of protein crosslinking observed with the intron-containing vs. intron-lacking RNAs, of which the most obvious is the presence or absence of the intron itself (28,29). A second possibility is the length difference of the intron-containing versus intron-lacking β -globin RNAs, which are 567 and 437 nucleotides long, respectively. Perhaps there is a minimal length of RNA that can bind the 59,000 and 39,000 mol. wt. proteins in a crosslinkable form. However, this possibility is indirectly contradicted by the low level of protein crosslinking observed with the histone H4 transcript, which is approximately 700 nt long. To directly examine this point, we compared a pair of β -globin RNAs that were generated from templates cut so as to equalize the transcripts' lengths. SP64-H β Δ 6 was cut with BamHI to generate a 497 nt intron-containing transcript, and the β -globin cDNA clone, SP62-H β -IVS1,2, was cut with EcoRI to generate a 501 nt intron-lacking transcript. Despite their very similar lengths, the level of crosslinked 59,000 and 39,000 mol. wt. proteins was 5-fold higher with the intron-containing transcript (Figure 2).

A related possibility is that protein crosslinking to β -globin pre-mRNA depends not on the intron *per se*, but on the pyrimidine-rich tract 3' of the lariat branch point, since pyrimidines are known to be favorable RNA-protein crosslinking sites (30). However, this is contradicted by the fact that the histone H4 transcript contains several tracts of 7–9 uninterrupted pyrimidines (31), and yet displays a low level of crosslinking (Figure 1). This points to the possible involvement of other factors in the high level of crosslinking to the β -globin pre-mRNA.

A third possibility is that the lower level of protein crosslinking to the intron-lacking RNAs might reflect their preferential degradation during incubation in the nuclear extract. In several experiments where the level of protein crosslinking differed by a factor of 10, the stability of the two β -globin transcripts was found to differ by only 25% (the intron-containing RNA being the more stable). In another nuclear extract we found that both transcripts were completely stable over 30 min. of incubation (Figure 3A), and yet the level of protein crosslinking again differed by 5-fold (Figure 3B).

To further explore the binding specificities of hnRNP proteins to pre-mRNA we carried out competition experiments. 32 P-labeled intron-containing β -globin transcript was incubated in nuclear extract in the presence of an increasing molar excess of competitors, consisting of either the same RNA or the intron-lacking β -globin RNA (Figure 4). Crosslinking of the 59,000 and 39,000 mol. wt. proteins (but not of the 110,000 mol. wt. protein) was partially competed by a 5-fold excess of the intron-containing RNA (Figure 4, lane 2) and almost completely by a 10-fold excess (lane 3). In contrast, a larger excess of the intron-lacking RNA was required to achieve a comparable level of competition (compare lanes 5 and 6 with lanes 2 and 3). Note in lane 7 that the 59,000 mol. wt. protein and also a 45,000 mol.

Figure 2. Proteins crosslinked to β -globin transcripts of equivalent length. A 497 nucleotide-long β -globin pre-mRNA was transcribed from BamHI-cleaved SP64-H β Δ 6 DNA and a 501 nt intron-lacking β -globin RNA was transcribed from EcoRI-cleaved SP62-H β -IVS1,2 DNA. Equimolar concentrations of the 32 P-labeled transcripts were incubated in nuclear extract (30°C, 30 min.), crosslinked, immunoselected with antibody iD-2 and nuclease digested. Lane 1: proteins crosslinked to the 497 nt β -globin pre-mRNA; lane 2: proteins crosslinked to the 501 nt intron-lacking β -globin RNA.

wt. protein are not completely competed by a 20-fold excess of the intron-lacking RNA. These results provide further evidence that the crosslinking level of the 59,000 and 39,000 mol. wt. proteins is intron-related.

Identification of A1 and C hnRNP proteins crosslinked to pre-mRNA

To identify the crosslinked proteins, we used monoclonal antibodies specific for either the A1 or C hnRNP proteins. The A1 hnRNP protein antibody (24) selected β -globin pre-mRNA complexes containing crosslinked proteins of 110,000, 59,000 and 39,000 mol. wt. (Figure 5, lane 1), a pattern similar to that selected by antibody iD-2 (Figure 1, lane 1). When these crosslinked complexes were digested with nucleases prior to immunoselection with the A1 hnRNP protein antibody, only a 39,000 mol. wt. protein was selected (Figure 5, lane 2). These results show that these β -globin pre-mRNA complexes contain the A1 hnRNP protein and that this protein, and also ones of

Figure 3. Control experiment addressing RNA stability. Equimolar concentrations of 497 nt ^{32}P -labeled β -globin pre-mRNA (transcribed from BamHI-cleaved SP64-H β Δ 6) or a 437 nt intron-lacking β -globin RNA (transcribed from BamHI-linearized SP62-H β -IVS1,2 DNA) were incubated in nuclear extract at 30°C. *A*: At 0 and 30 min., aliquots were removed from each reaction, and the RNA was deproteinized, ethanol-precipitated, denatured (80% formamide, 90°C, 1 min.) and displayed by electrophoresis in a 10% polyacrylamide gel containing 8.3 M urea. Lane 1: β -globin pre-mRNA at 0 min; lane 2: β -globin pre-mRNA at 30 min; lane 3: intron-lacking β -globin RNA at 0 min; lane 4: intron-lacking β -globin RNA at 30 min. *B*: At 30 min., aliquots were removed, crosslinked and nuclease digested. (No immunoselection step was used in this experiment). Lane 1: proteins crosslinked to β -globin pre-mRNA; lane 2: proteins crosslinked to intron-lacking β -globin RNA.

110,000 and 59,000 mol. wt., are in direct contact with the pre-mRNA. Comparable experiments with an antibody for the hnRNP C proteins (25) revealed that although these proteins are present in the β -globin pre-mRNA:protein complexes, they are much less crosslinkable to the pre-mRNA than the A1 protein, and thus difficult to resolve (data not shown).

We carried out similar experiments with an adenovirus pre-mRNA. The C protein antibody selected adenovirus pre-mRNA complexes containing crosslinked proteins of 110,000, 56,000 and 45,000 mol. wt. (Figure 5, lane 3). The A1 hnRNP protein antibody selected crosslinked complexes with a similar protein pattern (data not shown). When these crosslinked complexes were nuclease digested prior to immunoselection with the C protein antibody, only the 45,000 mol. wt. protein was observed (Figure 5, lane 4). Thus, hnRNP C proteins are present in the adenovirus pre-mRNA complexes and, in addition, they are readily crosslinkable to the RNA (unlike the case with β -globin complexes).

The U1 snRNP is a component of the crosslinked β -globin pre-mRNA:hnRNP protein complexes

The U1 small nuclear ribonucleoprotein particle (U1 snRNP) binds to the 5' splice site very early in the mRNA splicing

pathway (32–41). We therefore wanted to authenticate the crosslinked pre-mRNA:hnRNP protein complexes by the criterion of U1 snRNP's presence, defined by reactivity with a U1 snRNP-specific monoclonal antibody (27). As shown in Figure 6, this antibody selected complexes containing crosslinked 59,000 and 39,000 mol. wt. proteins when the intron-containing β -globin transcript was used (lane 1), but not with the intron-lacking transcript (lane 2). This experiment was carried out at 60 mM KCl. At 200 mM KCl it is known that the U1 snRNP particle dissociates from hnRNP particles (42) and from spliceosomes (36,38). As expected, the U1 snRNP antibody did not react with the intron-containing β -globin complexes at this ionic strength (Figure 6, lane 3).

hnRNP protein crosslinking to pre-mRNA requires U1 and U2 snRNPs

We next inquired whether the higher level of protein crosslinking observed with β -globin pre-mRNA in the splicing extract might be related to factors that contribute to spliceosome assembly and function. We considered the U1 and U2 snRNPs prime candidates and therefore pretreated the nuclear extract with oligodeoxynucleotides complementary to regions of U1 or U2 snRNPs that had previously been shown to be essential for their

Figure 4. Competition of protein crosslinking to β -globin pre-mRNA. ^{32}P - β -globin pre-mRNA and the indicated molar excess of either β -globin pre-mRNA or intron-lacking β -globin RNA were pre-mixed on ice. Nuclear extract was then added to produce a final ^{32}P - β -globin pre-mRNA concentration of 10 ng/ μm . After incubation (30°C, 30 min.), the reactions were crosslinked, nuclease digested and the proteins displayed (no immunoselection step). Lane 1: standard reaction with no competitor RNA; lanes 2–4, competition with intron-containing β -globin RNA; lanes 5–7, competition with intron-lacking β -globin RNA. The numbers at the bottom of each lane denote the molar excess at which the competitor RNA was present relative to the ^{32}P - β -globin pre-mRNA. The +’s and –’s indicate the presence or absence of the intron in the competitor RNA.

ability to serve as splicing cofactors (32,34,43). When oligodeoxynucleotides complementary to nucleotides 2–14 of U1 or U2 RNAs were incubated with nuclear extracts, the resulting DNA-RNA hybrids were attacked by endogenous RNase H producing RNAs shortened to the extent expected (lanes 2 and 3 of Figure 7 respectively). An oligo complementary to nucleotides 28–42 of U2 RNA, a region required for splicing (34,40,44), produced the expected RNase H cleavage product (see * in lane 4 of Figure 7, representing nucleotides 43–188 of U2 RNA). The 5’ terminal U1 and U2 oligo-dependent RNase H cleavages were 75–90% complete (Figure 7, and additional data not shown). In contrast, the internal U2 oligo (nt 28–42) usually resulted in only about 50% cleavage (Figure 7, lane 4), which remained the case even when a 3-fold higher concentration of this oligo was used (data not shown). The high sequence specificities of the oligo-directed cleavages are evident from the lack of perceptible cleavage of any RNAs in the gel other than the one against which the tested oligo was directed. An oligo complementary to nucleotides 2–14 of U5 RNA did not induce RNase H cleavage (Figure 7, lane 5), presumably due to the involvement of this region of U5 RNA in its secondary structure (45–47).

To verify that these oligo-directed cleavages were of functional impact, we analyzed the formation of spliceosomes in oligo-treated extracts by non-denaturing gel electrophoresis of RNP complexes. Figure 8 shows that RNP complexes with mobilities

Figure 5. Crosslinking of proteins to β -globin and adenovirus pre-mRNAs. ^{32}P -labeled β -globin or adenovirus pre-mRNAs (transcribed from BamHI-cleaved SP64-H β Δ 6 or BglII-cleaved pRSP-1- Δ IVS DNAs, respectively) were incubated in nuclear extract for 30 min. at 30°C. After crosslinking, each reaction was divided in half. One aliquot from each pre-mRNA reaction was immunoselected, subjected to nuclease digestion, and the proteins displayed by electrophoresis and autoradiography. The other aliquot from each pre-mRNA reaction was nuclease digested first, followed by immunoselection. Lane 1: β -globin pre-mRNA, immunoselection with antibody 4B10 specific for the A1 protein (24), followed by nuclease digestion; lane 2: β -globin pre-mRNA, nuclease digestion followed by immunoselection with 4B10; lane 3: adenovirus pre-mRNA, immunoselection with antibody 4F4 specific for the C1 and C2 proteins (25), followed by nuclease digestion; lane 4: adenovirus pre-mRNA, nuclease digestion followed by immunoselection with 4F4.

characteristic of previously described adenovirus pre-mRNA spliceosome complexes (40,48,49) were assembled in the control and U5 oligo-treated extracts (lanes 2 and 6), but did not form in extracts that had been incubated with the U1 or U2 oligos (Figure 8, lanes 3–5). On longer exposures A complexes, but not B complexes, can be identified in U1 oligo-treated extract (data not shown).

As shown in Figure 9 (lanes 3 and 6), when β -globin or adenovirus pre-mRNAs were incubated in nuclear extracts that had been pretreated with the oligo complementary to nucleotides 28–42 of U2 RNA, the crosslinking of all proteins was reduced to a very low level. In contrast, when the 5’ end of U1 RNA was cleaved, the crosslinking of the A1 hnRNP protein to β -globin pre-mRNA was preferentially eliminated (Figure 9, lane 2). A protein of electrophoretic mobility identical to the β -globin

Figure 6. U1 snRNP antibody selection of crosslinked β -globin pre-mRNA:protein complexes. Equimolar concentrations of the 32 P-labeled β -globin pre-mRNA or intron-lacking β -globin transcripts used in Figure 1 were incubated in nuclear extract (30°C, 30 min.). One half of each reaction was crosslinked at 60 mM KCl, and the other half was adjusted to 200 mM KCl before crosslinking. The crosslinked samples were then immunoselected with RNP monoclonal antibody (27), nuclease digested and the proteins were displayed by electrophoresis. Lane 1: β -globin pre-mRNA crosslinked at 60 mM KCl; lane 2: intron-lacking β -globin RNA crosslinked at 60 mM KCl; lane 3: β -globin pre-mRNA crosslinked at 200 mM KCl; lane 4: intron-lacking β -globin RNA crosslinked at 200 mM KCl.

pre-mRNA crosslinked A1 protein was also detected with the adenovirus pre-mRNA (lane 4). Its crosslinking to the adenovirus pre-mRNA was likewise completely eliminated by U1 RNA 5' end cleavage (lane 5). In contrast, the crosslinking of the C hnRNP protein to the adenovirus pre-mRNA was unaffected by U1 RNA cleavage (lane 5).

Control experiments demonstrate that the effects shown in Figure 9 are due to site-specific cleavage of U1 and U2 snRNPs. First, pretreatment with an oligo complementary to nucleotides 2–14 of U5 RNA did not affect protein crosslinking to β -globin pre-mRNA (Figure 10, lane 'U5'). In addition, incubation of nuclear extract with an oligo complementary to nucleotides 88–102 of U6 RNA had no effect on protein crosslinking (data not shown). These experiments demonstrate that the effects on protein crosslinking shown in Figure 9 are not due to the presence of oligodeoxynucleotides *per se*. An even more intriguing result is that oligo-directed cleavage of the 5'-terminal 15 nucleotides of U2 RNA also did not affect the level of protein crosslinking (Figure 10, lane 'U2'), indicating that this region of the U2

Figure 7. Oligodeoxynucleotide-mediated RNase H cleavage of U1 and U2 RNAs. Oligodeoxynucleotides complementary to nucleotides 2–14 of U1, U2 or U5 RNAs, or to nucleotides 28–42 of U2 RNA, were incubated at concentrations of 200 μ g/ml in nuclear extract for 30 min. at 30°C. Aliquots were removed, and the RNA was deproteinized, denatured and analyzed by gel electrophoresis as in Figure 3. RNA bands were visualized by ethidium bromide staining, shown here by reverse-contrast photography. Intact U2, U1 and U5 RNAs are indicated, as are the cleaved molecules obtained with the oligos complementary to nucleotides 2–14 of U1 RNA (open circle, lane 2), nucleotides 2–14 of U2 RNA (filled circle, lane 3), and nucleotides 28–42 of U2 RNA (*, lane 4). Lane 5 shows the pattern of RNAs present in nuclear extract following incubation with an oligo complementary to nucleotides 2–14 of U5 RNA.

snRNP, unlike nucleotides 28–42, is not necessary for crosslinking of these proteins to β -globin pre-mRNA.

In other experiments oligo-treated extracts were incubated with DNase I under conditions known to bring about effective degradation of the oligo (43). In the case of the oligos complementary to nucleotides 2–14 of U1 RNA or 28–42 of U2 RNA, the same effects on protein crosslinking to β -globin pre-mRNA as shown in Figure 9 were observed when DNase I digestion was carried out prior to addition of 32 P-pre-mRNA (data not shown).

We also considered the possibility that the results shown in Figure 9 might reflect binding of oligodeoxynucleotides to the pre-mRNA, thereby blocking protein interaction or displacing bound protein. A related possibility is that the oligo might hybridize to a complementary site in the pre-mRNA leading to RNase H action and a decrease in protein crosslinking to the cleaved pre-mRNA. These possibilities were addressed in the

Figure 8. Formation of spliceosome complexes. ^{32}P -labeled adenovirus pre-mRNA (transcribed from Sau3A-cleaved pBSAd10 DNA) was incubated for 30 min. at 30°C in nuclear extracts that had been pretreated with oligodeoxynucleotides as in Figure 7. The samples were then analyzed by electrophoresis in 4% polyacrylamide-0.5% agarose composite gels as detailed in Materials and Methods. Lane 1: ^{32}P -labeled pre-mRNA not incubated in nuclear extract; lane 2: incubation of pre-mRNA in untreated nuclear extract; lane 3: incubation in nuclear extract pretreated with oligo complementary to nucleotides 2–14 of U1 RNA; lane 4: incubation in nuclear extract pretreated with oligo complementary to nucleotides 2–14 of U2 RNA; lane 5: incubation in nuclear extract pretreated with oligo complementary to nucleotides 28–42 of U2 RNA; lane 6: incubation in nuclear extract pretreated with oligo complementary to nucleotides 2–14 of U5 RNA. The positions of previously described A and B spliceosome complexes (40,48,49) are indicated. 'H' indicates non-specific complexes.

following experiment. It is known that the β -globin pre-mRNA contains a 9 nucleotide sequence, 5'-CTCACCTGG-3' (nucleotides 137–145 of exon 2), that is homologous to nucleotides 4–12 of U1 RNA (5'-CTTACCTGG-3'). Under the standard conditions used in the U1 oligo experiments (incubation of oligo in nuclear extract for 30 min. followed by addition of pre-mRNA and incubation for an additional 30 min.), we found that approximately 25% of the β -globin pre-mRNA was cleaved. To explore the possible relevance of this U1 oligo-dependent pre-mRNA cleavage to the reduced protein crosslinking (Figure 9), we deliberately pushed this cleavage reaction to its limit. ^{32}P -labeled β -globin pre-mRNA was incubated with or without the U1 oligo for 30 min. in the presence of *E. coli* RNase H (8 U/ml). As can be seen in Figure 11A, inclusion of the U1 oligo resulted in cleavage of the great majority of the β -globin pre-mRNA. Both

Figure 9. U1 and U2 snRNP dependence of protein crosslinking to pre-mRNA. The ^{32}P -labeled β -globin or adenovirus pre-mRNAs were incubated for 30 min. at 30°C in nuclear extracts that had been pretreated with oligodeoxynucleotides complementary to nucleotides 2–14 of U1 RNA or to 28–42 of U2 RNA, or had no prior oligo treatment. Crosslinking, nuclease digestion and electrophoresis were carried out as detailed in Materials and Methods, without any immunoselection step. Lane 1: proteins crosslinked to β -globin pre-mRNA in untreated nuclear extract; lane 2: proteins crosslinked to β -globin pre-mRNA in nuclear extract pretreated with U1 oligo; lane 3: proteins crosslinked to β -globin pre-mRNA in nuclear extract pretreated with U2 oligo; lanes 4–6: same as lanes 1–3 but with adenovirus pre-mRNA.

RNA samples (*i.e.* RNase H digested without or with the U1 oligo, Figure 11A) were then treated with DNase I, and nuclear extract was added followed by incubation and crosslinking as usual. It can be seen in Figure 11B that despite the deliberate and nearly complete cleavage of the pre-mRNA by the U1 oligo, the level of protein crosslinking to this pre-mRNA (lane 2) was nearly as efficient as with intact pre-mRNA (lane 1). (The slightly lower level of crosslinking seen with the cleaved pre-mRNA may reflect the fact that about 10% of the U1 RNA in the added nuclear extract was cleaved by a residual level of the U1 oligo that resists DNase digestion, unpublished results). This experiment establishes that the decrease in protein crosslinking to β -globin pre-mRNA following preincubation of nuclear extract with the U1 oligo (Figure 9) cannot be due to pre-mRNA cleavage, nor can it be due to occupation of pre-mRNA protein binding sites by this oligo.

We also investigated the possibility that the internal U2 oligo (nt 28–42) might induce RNase cleavage of the β -globin pre-mRNA, thereby possibly reducing or eliminating protein crosslinking (Figure 9). No cleavage of the pre-mRNA whatsoever was observed after incubation with *E. coli* RNase H and the U2 oligo under the same conditions as used in Figure 11A (data not shown).

Figure 10. Protein crosslinking to β -globin pre-mRNA is independent of the 5'-end of U2 RNA. 32 P-labeled β -globin pre-mRNA was incubated in nuclear extract that had been pretreated with oligodeoxynucleotides complementary to nucleotides 2–14 of U1, U2 or U5 RNAs (30 min. at 30°C). The reactions were crosslinked, nuclease digested and the proteins displayed on an 11% polyacrylamide-SDS gel. Lane U5: proteins crosslinked to β -globin pre-mRNA in an extract pretreated with U5 oligo (which had not induced U5 RNA cleavage); lane U1: proteins crosslinked to β -globin pre-mRNA in an extract pretreated with U1 oligo; lane U2: proteins crosslinked to β -globin pre-mRNA in an extract pretreated with U2 oligo.

Crosslinking is eliminated by micrococcal nuclease pretreatment of nuclear extract and A1 hnRNP protein crosslinking is restored by addition of U1 snRNP

We sought to confirm the requirement of intact U1 and U2 snRNPs for hnRNP crosslinking by an alternative experimental approach to oligo-directed snRNA cleavage. We pretreated nuclear extracts with micrococcal nuclease, and then inactivated the nuclease by chelation of calcium. This permitted an analysis of protein crosslinking to subsequently added pre-mRNA. Figure 12A, lane 1, shows the extent of RNA digestion following incubation of nuclear extract with micrococcal nuclease. It can be seen that the great majority of both U1 and U2 RNAs is digested. Figure 12C, lane 2, shows that this pretreatment results in a nearly complete elimination of crosslinking of both the 59K and A1 hnRNP proteins to β -globin pre-mRNA. We noted (Figure 12C, lane 2) that nuclease treatment led to the crosslinking of a $\sim 70,000$ mol. wt. protein that is not seen with untreated extract. To determine if crosslinking of the 59K and A1 hnRNP

proteins could be restored, we isolated by CsCl gradient centrifugation a fraction enriched in U1 snRNP (Figure 12B, lane 1) as well as one enriched in both U2 and U1 snRNPs (Figure 12B, lane 2). Addition of either the U1-enriched fraction or the U2 and U1-enriched fraction to micrococcal nuclease pretreated nuclear extract resulted in a restoration of A1 hnRNP protein crosslinking to levels approximating that seen with untreated extract (Figure 12C, lanes 3 and 4 vs. lane 1). (Incubation of the pre-mRNA in the U1 and U2-enriched fractions alone did not restore A1 protein crosslinking.) These results show that crosslinking of the A1 hnRNP protein to β -globin pre-mRNA is dependent on at least U1 snRNP. However, crosslinking of the 59K protein was restored only when complete nuclear extract was added back to micrococcal nuclease-treated extract (Figure 12C, lane 5).

DISCUSSION

Crosslinking vs. binding

Numerous studies of ribosomes, hnRNP and mRNP particles, and RNA viruses have proven photocatalyzed RNA-protein crosslinking to be a very useful tool for investigating ribonucleoprotein structure. Yet, like any method, RNA-protein crosslinking has limitations. With 254 nanometer light (the wavelength we use), certain amino acids are especially photoreactive and pyrimidines are much more reactive than purines (30). Thus, it is possible for a protein to be very tightly bound to a nucleic acid and yet not be photo-crosslinkable. Our results must be interpreted with these limitations in mind. Thus, we have demonstrated that a crosslinkable aspect of the interaction of certain hnRNP proteins with pre-mRNA is intron-related and dependent on the functional integrity of U1 or U2 snRNPs.

The difference in hnRNP protein crosslinking observed with intron-containing vs. intron-lacking transcripts may not be due exclusively to a difference in binding. First, the A1 hnRNP protein antibody was observed to select the intron-lacking β -globin transcript after incubation of this RNA in the nuclear extract (unpublished results), even though this transcript displays a 5- to 10-fold lower level of protein crosslinking. While this result does not address the mass of A1 hnRNP protein bound to the intron-lacking transcript (in principle only one molecule of antigenically active A1 protein need be present per intron-lacking transcript), it does indicate that this protein can bind an intron-lacking RNA at some level. This is not unexpected, since the A1 protein is known to have a non-specific affinity for single-stranded nucleic acid (50,51). Second, the results of competition experiments (Figure 4, and additional results not shown) indicate that although the intron-lacking β -globin RNA is a less effective competitor of A1 and 59,000 mol. wt. hnRNP protein crosslinking to β -globin pre-mRNA than is the pre-mRNA itself, the magnitude of this difference in competition strength is not as large as the observed difference in protein crosslinking to the intron-containing vs. intron-lacking β -globin RNAs. While it is not straightforward to quantitatively compare the parameters of crosslinking on the one hand and an RNA's competition strength on the other, the foregoing evidence points out, once again, the possibility that crosslinking need not be an inevitable consequence of protein binding. *We therefore consider it probable that our results reflect the attainment of a RNA-crosslinkable state of hnRNP proteins as the spliceosome assembles.*

Figure 11. Oligodeoxynucleotide inhibition of protein crosslinking. *A.* ^{32}P - β -globin pre-mRNA was incubated with 200 $\mu\text{g}/\text{ml}$ oligodeoxynucleotide complementary to nucleotide 2–14 of U1 RNA in the presence of purified *E. coli* RNase H (8U/ml; Pharmacia), 500 μM ATP, 3.2 mM MgCl_2 , 20 mM creatine phosphate, and 1000 U/ml RNasin. After 30 min incubation at 30°C, the RNA was analyzed by gel electrophoresis. Lane 1: β -globin pre-mRNA incubated without oligo; lane 2: β -globin pre-mRNA incubated with oligo. *B.* The U1 oligo pretreated β -globin pre-mRNA and the untreated β -globin RNA were treated with DNase I at 1500 U/ml for 15 min. at 30°C. Nuclear extract was then added and incubation continued for 30 min. Crosslinking, protein sample preparation and electrophoresis were as described in Methods and Materials. Lane 1: proteins crosslinked to β -globin pre-mRNA; lane 2: proteins crosslinked to β -globin pre-mRNA pretreated with oligodeoxynucleotide complementary to nucleotides 2–14 of U1 RNA.

The crosslinkable proteins

The use of monoclonal antibodies in the present investigation made possible the identification of known hnRNP proteins among the crosslinked proteins. The presence of the A1 hnRNP protein in the crosslinked β -globin pre-mRNA:protein complexes was demonstrated by their immunoselection with the A1 monoclonal antibody (Figure 5). After nuclease digestion of crosslinked complexes, this antibody selected a protein of 39,000 mol. wt. that had crosslinked ^{32}P -nucleotides (Figure 5). The apparent molecular weight of the human A1 hnRNP protein in SDS-polyacrylamide gel electrophoresis is 32,000 (12), and the additional mass we observed (Figure 5) presumably reflects the contribution of crosslinked nucleotides.

Although the β -globin pre-mRNA:protein complexes could be immunoselected with a monoclonal antibody for the C hnRNP

proteins, these proteins were not efficiently crosslinked to this RNA. In contrast, the C hnRNP proteins were very efficiently crosslinked to the adenovirus pre-mRNA (Figure 5). It is likely that this difference reflects the hyper-photoreactivity of pyrimidines (30), the known binding preference of the C proteins for homopolyuridylylate sequences (51), or both. Indeed the adenovirus pre-mRNA's intron contains a stretch of 14 uninterrupted pyrimidines in its polypyrimidine tract including 8 continuous U's (52). In contrast, the maximum uninterrupted pyrimidine run in the β -globin pre mRNA's polypyrimidine tract is only 8, and the longest continuous stretch of U's is only 4 (53). We also observed a 59,000 mol. wt. protein crosslinked to the β -globin pre-mRNA and a 56,000 mol. wt. protein crosslinked to the adenovirus pre-mRNA. Early studies of hnRNP particles isolated from mammalian cell nuclei revealed the presence of proteins in this molecular weight range (11,54), as has more recent work (24). A protein of 110,000 mol. wt. was crosslinked to both the β -globin and adenovirus pre-mRNAs. We considered the possibility that this was the reported 115,000 mol. wt. nuclear 7-methylguanosine cap-binding protein (55). However, crosslinking of the 110,000 mol. wt. protein to β -globin pre-mRNA was not affected by a 50-fold molar excess of 7-meGpppG (unpublished results).

hnRNP protein-snRNP collaboration in relation to spliceosome assembly and organization

In the present experiments, crosslinking of the A1 hnRNP and other proteins to pre-mRNA was assayed after incubation in nuclear extract at 30°C for 30 min., during which time spliceosomes assemble but little splicing occurs. Furthermore, we observed similarly efficient crosslinking of the A1 hnRNP protein and the 59,000 mol. wt. protein to β -globin pre-mRNA at 0°C, a condition in which the U1 snRNP binds to the 5' splice site (34,38), the 3' splice site becomes associated with the U5 snRNP (35,56), and U2AF binds the polypyrimidine tract/3' splice site (57). Thus, we conclude that the A1 and 59,000 mol. wt. hnRNP proteins bind to pre-mRNA in a crosslinkable state very early in the spliceosome assembly pathway. This conclusion is supported by our finding that the β -globin pre-mRNA complexes which contain crosslinkable A1 and 59,000 mol. wt. hnRNP proteins also contain U1 snRNP (Figure 6).

We previously predicted that hnRNP proteins bind to introns (29), but we had not anticipated that hnRNP protein crosslinking to pre-mRNA would be dependent on U1 or U2 snRNPs. Cleavage of an internal region of U2 RNA (nucleotides 28–42) completely eliminated crosslinking of all proteins to both the β -globin and adenovirus pre-mRNAs (Figure 9). This region of U2 RNA is known to be required for splicing (34,40,44) and for protection of the 5' splice site against RNase A digestion (58). Interestingly, we found that under conditions in which the oligo complementary to nucleotides 28–42 led to cleavage of only ~50% of the U2 RNA in the nuclear extract (Figure 7), spliceosome assembly and hnRNP protein crosslinking were nevertheless completely inhibited (Figures 8 and 9, respectively). Previous studies have reported greater than 95% cleavage of U2 RNA by this oligo (34,40), although these studies employed exogenous RNase H whereas in our experiments we used the endogenous RNase H activity present in the nuclear extract. Irrespective of this difference, our results indicate that both spliceosome assembly and hnRNP protein crosslinking to pre-

Figure 12. Proteins crosslinking after adding back snRNP fractions to micrococcal nuclease-treated extract. **A.** Nuclear extract was treated with 830 U/ml micrococcal nuclease at 1 mM CaCl₂ for 30 min. at 30°C, and EGTA was then added to 3mM. An aliquot of the reaction was prepared for RNA gel electrophoresis as described in Figure 3. Lane 1: RNA pattern after micrococcal nuclease digestion; lane 2: RNA profile of mock-treated nuclear extract. **B.** snRNPs were fractionated on CsCl gradient as detailed in Materials and Methods. Lane 1: CsCl gradient fraction 3, containing predominately U1 snRNP and some U5 snRNPs; lane 2: CsCl gradient fraction 5, containing U2 snRNP in addition to U1 and U5 snRNPs. **C.** Micrococcal nuclease-treated extract (see **A** above) was made 500 μ M ATP, 3.2 mM MgCl₂, 20 mM creatine phosphate and 1000 U/ml RNasin. An equal volume of buffer D, fraction 3, fraction 5, or untreated nuclear extract made 500 μ M ATP, 3.2 mM MgCl₂, 20 mM creatine phosphate, and 1000 U/ml RNasin was added to the micrococcal nuclease-treated nuclear extract. ³²P- β -globin pre-mRNA was then incubated in the complemented nuclear extracts for 30 min. at 30°C. Crosslinking, sample preparation and gel electrophoresis was as described in Figure 4. Lane 1: proteins crosslinked in a control nuclear extract (1mM CaCl₂, 3 mM EGTA); lane 2: proteins crosslinked in a micrococcal nuclease-treated nuclear extract + buffer D; lane 3: proteins crosslinked in a micrococcal nuclease-treated extract + fraction 3; lane 4: proteins crosslinked in a micrococcal nuclease-treated extract + untreated nuclear extract.

mRNA may be dependent on a distinct subpopulation of U2 snRNP. This is reminiscent of the earlier finding that oligo-directed 5' end cleavage of only 50% of the U1 snRNP in a HeLa nuclear extract nonetheless led to a complete inhibition of splicing (32).

The important conclusion that hnRNP protein crosslinking to pre-mRNA is dependent on both U1 and U2 snRNPs comes not only from the oligo-directed RNase H cleavage experiments (Figure 9) but also from the fact that a U1 snRNP fraction can restore protein crosslinking to pre-mRNA in a micrococcal nuclease-treated nuclear extract (Figure 12). While the results of these two experimental approaches generally corroborate one another, two differences are noteworthy. First, since oligo cleavage of U2 RNA at nucleotides 28–42 so effectively eliminated A1 protein crosslinking, it was somewhat surprising that addition of a fraction containing predominantly U1 snRNP

and almost undetectable amounts of U2 snRNP was able to restore A1 protein crosslinking in a micrococcal nuclease-treated nuclear extract to an extent comparable to that observed when a fraction containing a much higher concentration of U2 snRNP (in addition to U1 snRNP) was added back (Figures 12B and 12C). Thus, from the add-back experiments we cannot conclude that U2 snRNP is capable of restoring efficient A1 protein crosslinking. The efficient crosslinking of the 59K protein was also not restored by adding back a U2 snRNP-containing fraction (Figure 12C, lane 4). It seems probable that another factor or factors, in addition to U2 snRNP itself, is required for efficient 59K protein crosslinking. Perhaps such a factor is bound loosely to U2 snRNP in nuclear extract but becomes dissociated during CsCl gradient centrifugation.

The fact that crosslinking of the A1 hnRNP protein is dependent on both the 5' end of U1 RNA and on nucleotides 28–42 of

U2 RNA (Figure 9) speaks to a collaborative mode of A1 protein interaction in the spliceosome, involving both U1 and U2 snRNPs. Previous studies have presented evidence for an interaction between the 5' splice site and the lariat branch point (e.g. 37,58,59), as well as hinting at a direct interaction of the U1 and U2 snRNPs themselves (60). Our results raise the possibility that the A1 hnRNP protein actually has a binding affinity for the U1 and U2 snRNPs (discussed further below).

Where do the hnRNP proteins fit in?

Previous work on the major hnRNP proteins (C2, C1, B2, B1, A2, A1) revealed little if any evidence for RNA sequence-specific binding (reviewed in ref. 29). Yet both the A1 and C group hnRNP proteins have recently been shown to be bound to intron sequences in and around the intron polypyrimidine tract of β -globin and adenovirus pre-mRNAs (61). Our present results provide complementary information, by showing that crosslinking of the A1 hnRNP protein and a 59,000 mol. wt. protein to β -globin pre-mRNA is intron-dependent. How are these results to be reconciled with the aforementioned apparent lack of RNA sequence specificity of hnRNP proteins?

It is highly likely that both the RNase T1-immunoselection assay (61) and the present crosslinking experiments address the most stable hnRNP protein:RNA interactions in these complexes. We previously proposed that the non-specific (low) affinity of hnRNP proteins for RNA might facilitate their binding to high-affinity sites, by analogy with principles established in thermodynamic studies of the non-specific vs. specific components of DNA-protein interactions (29, and references cited therein). Our crosslinking results and the previous RNase T1-immunoselection experiments (61) are entirely compatible with this view. However, our finding that crosslinking of the A1 and other hnRNP proteins to pre-mRNA is dependent on U1 and U2 snRNPs adds another, unexpected dimension to the specificity issue, and suggests that the crosslinkability of hnRNP proteins to pre-mRNA is an index of the early stages of spliceosome assembly. While we do not know whether the pre-mRNA sites at which hnRNP proteins become hyper-crosslinkable in our experiments necessarily are ones close to those initially occupied by the U1 and U2 snRNPs, it is clear that the U1 and U2 snRNPs contribute, either directly or indirectly, to the establishment of a spliceosome configuration in which hnRNP proteins become highly crosslinkable. There may be high affinity interactions between domains of hnRNP proteins and the U1 and U2 snRNPs themselves, in addition to contacts between other regions of these snRNP-interacting hnRNP proteins and the pre-mRNA. Either of the foregoing situations (or both) would explain why hnRNP proteins display no apparent RNA sequence specificity in simple solutions (purified hnRNP proteins and a single RNA species), but nonetheless interact with pre-mRNA in a more specific manner in nuclear extracts that contain snRNPs and all the other splicing cofactors.

At present, the only evidence that hnRNP proteins are functionally involved in pre-mRNA splicing consists of the inhibition of splicing by a monoclonal antibody for the C hnRNP proteins (6), and by a polyclonal antibody against hnRNP core proteins of 32,000–42,000 mol. wt. (8). Such results are suggestive, but they do not constitute definitive evidence for a role of these proteins in splicing. The subsequent demonstration that both the A1 and C hnRNP proteins bind to the lariat branch point/polypyrimidine tract (61), and our current findings that A1 hnRNP protein crosslinking to pre-mRNA is related to the

presence of an intron and also requires intact U1 and U2 snRNPs, provide additional but still indirect evidence for a role in splicing.

If the hnRNP proteins are indeed functionally involved in splicing, what might they do? In the case of the A1 hnRNP protein, it is possible that its non-specific, single-stranded nucleic acid-binding domain (50) destabilizes pre-mRNA secondary structure. It is noteworthy that two of the elements on which the splicing machinery must operate, the lariat branch site and the 3' splice site, are involved in thermodynamically favored secondary structure in the human β -globin pre-mRNA we have used here (62), suggesting the likely participation of splicing factors that locally melt these regions. This, we envision, would occur whenever, and wherever, the A1 protein binds to the pre-mRNA. Our present finding that the level of A1 protein crosslinking to an intron-containing pre-mRNA depends upon intact U1 and U2 snRNPs suggests that the on-RNA residence time of the A1 protein might be greater at pre-mRNA sites very near bound U1 and U2 snRNPs, due to a presumed A1 protein-snRNP interaction. Such a mechanism would tend to maximize the destabilization of pre-mRNA secondary structure in the spliceosome's reaction center (the juxtapositioned 5' splice site and lariat branch point), which can readily be envisioned as facilitating both the kinetics and the accuracy of splicing.

ACKNOWLEDGEMENTS

Supported by NIH grant GM-21595-15. We thank several investigators for generously providing cloned DNAs or antibodies: Gideon Dreyfuss, Sallie Hoch, Maria Konarska, Adrian Krainer, George Leser, Tom Maniatis, Terence Martin, Douglas Melton, Kimberly Mowry, Serafin Pinol-Roma, Phillip Sharp, and Joan Steitz. We also gratefully acknowledge John Goodchild for oligodeoxynucleotide synthesis in the Worcester Foundation's central facility (supported by NCI Cancer Center core grant P30-12708-19). We thank our colleagues Ann Kleinschmidt, Gary Kunkel, John Maddalena, Jeffrey Patton, Molly Rhoadhouse, Jamal Temsamani and Jin Wang for advice and assistance, and Rebecca Bell for highly competent secretarial skills.

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