A T to G mutation in the polypyrimidine tract of the second intron of the human β-globin gene reduces *in vitro* splicing efficiency: evidence for an increased hnRNP C interaction

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ABSTRACT

In a patient with a β-thalassemia intermedia, a mutation was identified in the second intron of the human β-globin gene. The U→G mutation is located within the polypyrimidine tract at position –8 upstream of the 3′ splice site. *In vivo*, this mutation leads to decreased levels of the hemoglobin protein. Because of the location of the mutation and the role of the polypyrimidine tract in the splicing process, we performed *in vitro* splicing assays on the pre-messenger RNA (pre-mRNA). We found that the splicing efficiency of the mutant pre-mRNA is reduced compared to the wild type and that no cryptic splice sites are activated. Analysis of splicing complex formation shows that the U→G mutation affects predominantly the progression of the H complex towards the pre-spliceosome complex. By cross-linking and immunoprecipitation assays, we show that the hnRNP C protein interacts more efficiently with the mutant precursor than with the wild-type. This stronger interaction could play a role, directly or indirectly, in the decreased splicing efficiency.

INTRODUCTION

Splicing of pre-mRNA introns occur within a multicomponent complex termed the spliceosome, which contains the U1, U2, U4/U6 and U5 small nuclear ribonucleoproteins (snRNPs) and a large number of non-snRNP protein factors (1–6). The accuracy of the splicing process involves the recognition of short conserved sequences within the pre-messenger RNA that delimit the exon/intron boundaries. These sequences include the 5′ splice site and three associated elements at the 3′ end, including the branch point sequence, the pyrimidine-rich tract and the 3′ splice site.

Genetic disorders, in which naturally occurring mutations affect the splicing process, have been a powerful tool for studying the functional role of *cis*-acting sequences on splicing (7). Recently, 101 cases of splicing mutations that lead to genetic diseases have been reported (8). Frequently, these mutations inactivate the correct splice sites and unmask cryptic sites, which are then simultaneously or even exclusively used, giving rise to abnormal messenger RNA. Most of these mutations involve the invariant dinucleotides 5′-GU and 3′-AG. However, mutations that occur in proximity to these invariant nucleotides also dramatically alter the splicing process. Only a few naturally occurring mutations have been described in the polypyrimidine tract. One is a C→G transversion at position –13 upstream of the 3′ splice site of the third exon of the steroid 21-hydroxylase gene; this mutation results in the activation of several cryptic sites (9). Two mutations found in the second intron of the β-globin gene lead to a mild β-thalassemia. The first is a C→G transversion at position –7 upstream of the 3′ splice site, but no functional study has been done (10). The second, described by one of us, is a U→G transversion at position –8 upstream of the 3′ splice site (11). The phenotypes induced by these mutations are as expected.

Numerous studies have shown that the pyrimidine tract plays an essential role in the splicing process. It acts at an early stage of spliceosome assembly and it is required for the first step of the catalytic process (12). The length of the pyrimidine tract and its composition are important in 3′ splice site recognition (12–14). Depending on the position, substitutions of uracil residues within the pyrimidine tract by purines can dramatically alter the splicing process (15). In addition, the polypyrimidine tract was suggested to play a role in alternative splicing (16–20). The function of the pyrimidine tract in the splicing process is mediated through sequential interactions with several proteins which include U2AF (21,22), IBP (23,24), PTB (13) and the hnRNP C proteins (28–31). U2AF is the major component found in the first pre-splicing E complex, and it is required for the U2 snRNP
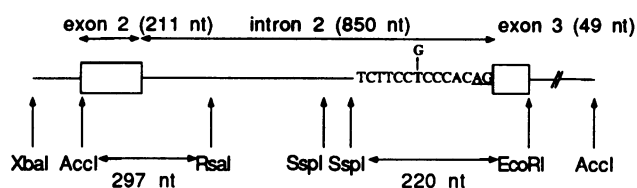
Figure 1. Schematic representation of the T7-generated β-globin transcripts; open boxes, exons; lines, intron. The location of restriction sites used for plasmid constructions is indicated.

branch point interaction (32,33). PTB was suggested to play a role in U2 snRNP branch site recognition (13). However, recent experiments seem to indicate that PTB is not an essential splicing factor (34). Immunodepletion experiments show that hnRNP C1 and C2 are important for splicing, but more recent studies suggest that direct binding of hnRNP C proteins to pre-mRNA may not be required for the splicing process (28,29,15).

In this paper, we analyze the first natural mutation isolated in the polypyrimidine tract. It is a transversion, T→G, located in the second intron of the β-globin gene at position –8 upstream of the 3' splice site (+843 of IVS 2). This defect was detected in a patient affected by a thalassemia intermedia with mild anaemia [Hb : 9g/dl; (11)]. We demonstrate by *in vitro* splicing assays that: the U→G mutation decreases the splicing efficiency of the pre-mRNA compared to the wild-type; this decrease is correlated with a reduction of spliceosome formation and that hnRNP C interacts more strongly with the mutated sequence than with the wild-type RNA.

MATERIALS AND METHODS

Plasmid constructions

The Klenow-filled *Acc*I (+292)–*Acc*I (+2088) fragment of the β-globin gene was cloned into the *Sma*I site of an M13-derivative plasmid called MICE 11, which contains a T7 late promoter. The *Acc*I–*Acc*I fragment is 1800 nucleotides (nt) long and contains 211 nt of exon 2, the whole of intron 2 (850 nt) and exon 3 (127 nt), and 600 nt of the 3' part of the β-globin gene. To construct the plasmid which contains a fragment of the β-globin gene with a shorter intron 2, the β-globin gene fragment in the MICE 11 plasmid was digested with *Xba*I and *Eco*RI: this fragment (1100 bp) was then digested by *Rsa*I and *Ssp*I, and the fragments of *Xba*I–*Rsa*I (297 bp) and of *Ssp*I–*Eco*RI (220 bp) were inserted into the PGEM 4Z vector (Fig. 1). The shorter construction obtained contains 211 nt of exon 2, an intron of 257 nt and 49 nt of exon 3.

RNA transcription and splicing

Template DNA was cleaved with *Eco*RI, whose site is located 49 nt from the 5' end of exon 3, and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP. The splicing reactions were incubated at 30°C in a 25 μl reaction mixture containing 0.01 pmol ^{32}P-labeled pre-mRNA (2.13 × 10^6 c.p.m./pmol), 60 mM KCl, 1 mM MgCl$_2$, 20 mM creatine phosphate, 0.5 mM ATP, 3% Polyvinyl Alcohol (PVA), 0.85 U RNasin and 60% (v/v) nuclear extract (7). HeLa cell nuclear extract was prepared as described (35,36). Splicing reactions were stopped by adding 20 μg

of proteinase K, and incubating for 30 min at 30°C. RNA was phenol–chloroform extracted and ethanol precipitated. The splicing products were separated on a 7% polyacrylamide–7 M urea gel. Splicing complexes were separated by electrophoresis on a 4% non-denaturing polyacrylamide gel (37). Quantification was done with a PhosphorImager (Molecular Dynamics). The splicing efficiency was determined as the ratio of mRNA over mRNA plus precursor RNA. A correction factor of 6.25 for the large transcript and 2.43 for the short one was used for the mRNA to take into account the loss of radioactivity after the loss of the lariat. The precursor corresponds to the amount of radioactivity that remains after incubation in splicing conditions.

UV cross-linking

β-globin pre-mRNA (0.07 pmol) uniformly labeled with [α-^{32}P]UTP and [α-^{32}P]CTP (3.93 × 10^6 c.p.m./pmol) was incubated under splicing conditions without PVA. Ten μl of the reaction mixture was then transferred to a microtiter plate on ice and irradiated with UV light (254 nm wavelength, 3 mW/cm^2) in a Stratalinker (Stratagene) for 15 min. For the competition assays, unlabeled pre-mRNA (2–10-fold molar excess over uniformly labeled RNA) was pre-incubated with nuclear extracts under splicing conditions for 10 min. Labeled pre-mRNA was then added and the incubation was continued for either 10 or 20 min. After irradiation, RNase A was added to final concentration of 1 μg/μl and the samples were incubated at 30°C for 20 min. After addition of SDS–PAGE loading buffer, the samples were boiled for 3 min and loaded on 12.5% SDS–polyacrylamide gels (38). ^{32}P-Labeled cross-linked proteins were detected by autoradiography and quantified with a PhosphorImager. The percentage of binding was expressed as a ratio of the amount of radioactivity of the cross-linked protein over the total radioactivity on each lane.

Immunoprecipitation

4F4 antibodies (1 μl) was added to 2.5 mg of Protein A Sepharose (Pharmacia), which was pre-swollen in 500 μl NET-2 buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% NP-40) and allowed to react for 1 h at 4°C. After three washes with 1 ml of NET-2 buffer, 10 μl of cross-linked sample, incubated under splicing conditions (without ATP and PVA) and digested with RNase A, was added to 500 μl of NET-2 buffer containing the antibody bound to Protein A Sepharose. Incubation was with gentle stirring for 1 h at 4°C. The beads were washed three times with NET-2 buffer and the bound material was eluted from the agarose beads with the SDS–PAGE loading buffer. The samples were boiled for 3 min at 95°C and loaded on a 10% SDS–polyacrylamide gels.

RESULTS

The U→G mutation in the polypyrimidine tract reduces *in vitro* splicing efficiency

Based on the health of the patient and the fact that she could produce haemoglobin only with the β-globin allele containing the mutation in position –8 upstream of the acceptor site (the other allele being unproductive for β-globin protein), some normal splicing was expected. To investigate the mechanism by which the mutation interferes with the splicing process, *in vitro* splicing experiments using HeLa cell nuclear extracts were performed. The wild-type and the mutant precursors contain 211 nt of exon 2, the whole of intron 2 (850 nt) and 49 nt of exon 3 (Fig. 1).
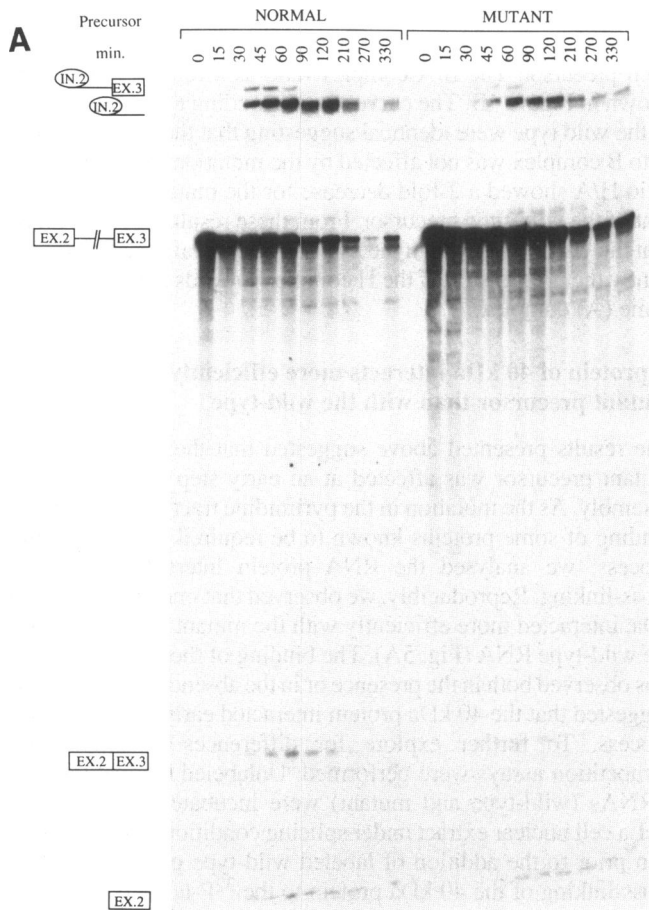
Figure 3. *In vitro* splicing reaction of the shorter wild-type and mutant transcripts (517 nt). Splicing was for the times indicated.

Figure 2. The U→G mutation in the pyrimidine tract decreases splicing efficiency. (**A**) *In vitro* splicing reaction of the wild-type and mutant transcripts (1100 nt). Times of reaction are indicated in minutes above each lane. Schematic representations of the intermediates and products are shown. (**B**) Quantification of the *in vitro* splicing reaction. The splicing efficiency is expressed as the ratio of mRNA over mRNA plus pre-mRNA as described in Materials and Methods and plotted as a function of time.

Figure 2A shows that the mutant precursor generates a reduced amount of both splicing intermediates and final products. The mutation affected predominantly the first step of the splicing process. T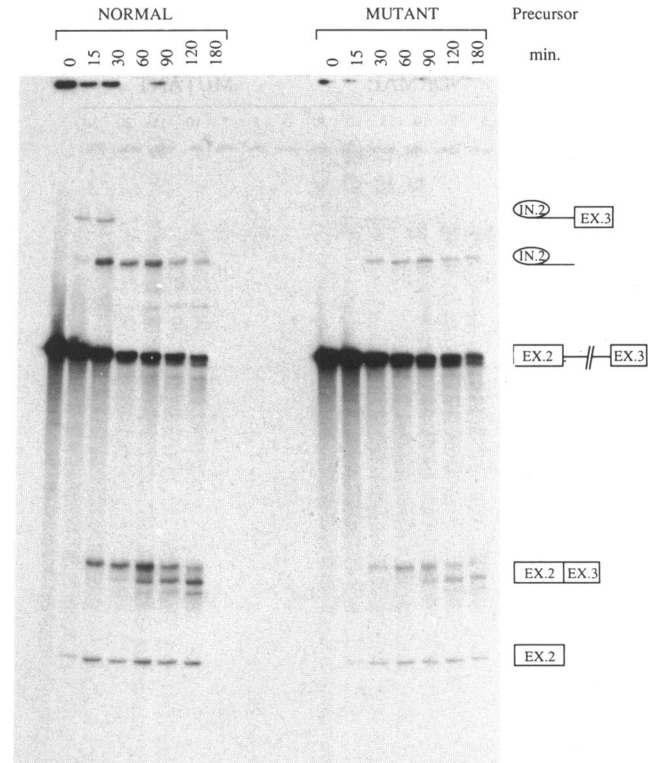he second step did not seem to be blocked as judged by the amount of the released final products (the intermediate lariat/final lariat ratio was the same for both precursors). No abnormal intermediates or products were detected, indicating that no cryptic sites were activated by the mutation. Quantitative data show that the splicing efficiency for the mutant precursor is between 2–3-fold lower relative to the wild-type (Fig. 2B).

The U→G mutation affects an early stage of spliceosome assembly

The ability of the mutant precursor to support spliceosome assembly was analyzed. Due to the large size of the transcript (1100 nt) and mainly the intron which is 850 nt long, the pre-spliceosome (A) and the spliceosome (B) could not be well resolved on a gel (data not shown). Glycerol gradients also did not separate the A and B splicing complexes (data not shown). To circumvent this problem, new constructs were made in which the length of the intron was reduced to 257 nt. The new versions of the wild-type and mutant precursors were 517 nt long. Figure 3 shows that decreasing the length of the intronic sequence does not suppress the mutation's effect. As was seen for the large precursors, both the intermediates and final products were reduced in the short mutant transcript compared to the wild-type. Splicing complex formation is shown in Figure 4A. Both the pre-spliceosome and spliceosome were formed with the mutant pre-mRNA. However, the assembled A and B complexes on the mutant transcript were 50% lower than those of the wild-type. These data were in agreement with the reduction level of spliced products. In summary, the results showed that the mutation in the pyrimidine tract induced a quantitative defect in splicing complex assembly.
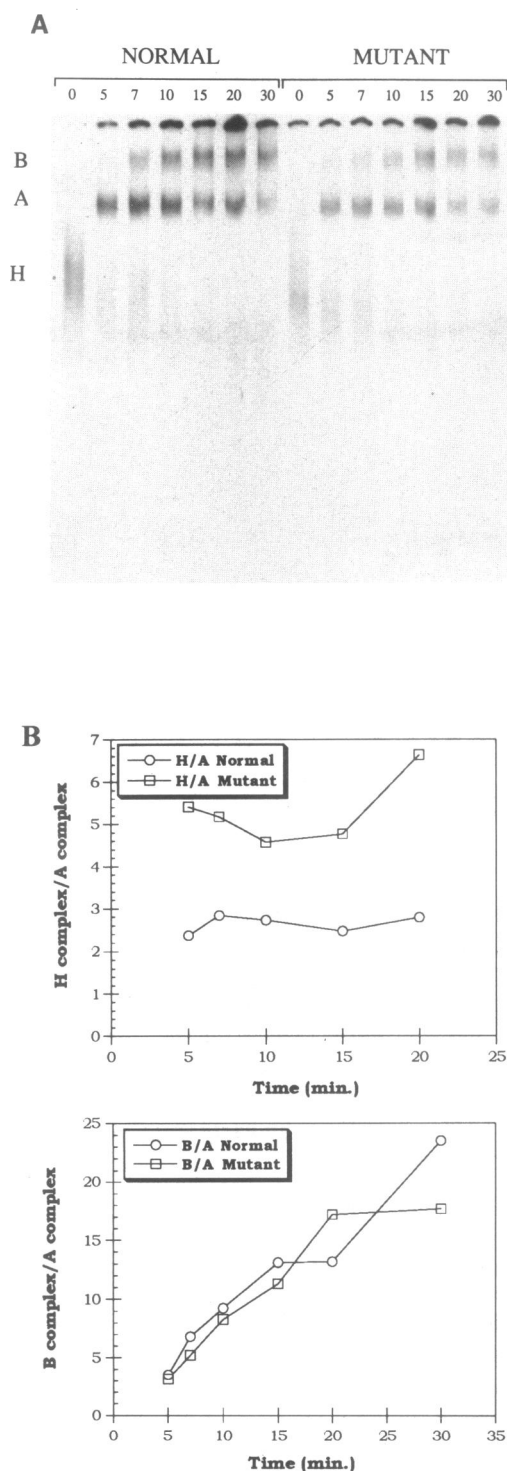
Figure 4. Effect of the U→G mutation on spliceosome assembly. (**A**) Time course of complex formation on the wild-type and mutant pre-mRNAs (short precursors). Splicing reactions were incubated under splicing conditions for the times indicated, treated with heparin (2 mg/ml) and loaded onto non-denaturing polyacrylamide gels. H, non specific complex; A, pre-spliceosome and B, spliceosome. (**B**) The U→G mutation affects the A complex assembly. The bands corresponding to H, A, B complexes were quantified. The ratios H/A complexes and B/A complexes were calculated at the different times of the kinetic.

To determine which step of spliceosome assembly was affected, we measured the B/A complex ratio and the H/A complex ratio for each precursor. The B/A complex ratio as a function of time is shown in Figure 4B. The curves corresponding to the mutant and to the wild type were identical suggesting that the progression of A to B complex was not affected by the mutation. In contrast, the ratio H/A showed a 2-fold decrease for the mutant compared to that of the wild-type precursor. From these results, we concluded that the U→G mutation in the pyrimidine tract affected predominantly the progression of the H complex towards the pre-spliceosome (A) complex.

A protein of 40 kDa interacts more efficiently with the mutant precursor than with the wild-type

The results presented above suggested that the splicing of the mutant precursor was affected at an early step in spliceosome assembly. As the mutation in the pyrimidine tract could affect the binding of some proteins known to be required for the splicing process, we analysed the RNA–protein interactions by UV cross-linking. Reproducibly, we observed that one protein of ~40 kDa, interacted more efficiently with the mutant RNA than with the wild-type RNA (Fig. 5A). The binding of the 40 kDa protein was observed both in the presence or in the absence of ATP, which suggested that the 40 kDa protein interacted early in the splicing process. To further explore for differences of interaction, competition assays were performed. Unlabeled competitor pre-mRNAs (wild-type and mutant) were incubated with 5% v/v HeLa cell nuclear extract under splicing conditions for 10 and 20 min prior to the addition of labeled wild-type pre-mRNA. The cross-linking of the 40 kDa protein to the ^{32}P-labeled wild-type precursor was efficiently reduced in the presence of the two competitors (Fig. 5B). However, the mutant precursor seemed to be a better competitor than the wild-type. In the presence of a 10-fold molar excess of unlabeled wild-type precursor, the level of the protein bound to the wild-type RNA was reduced to 50% while it was 80% reduced when the same molar excess of the mutant competitor was added (Fig. 5C). The reverse experiment with labeled mutant pre-mRNA gave the same result (data not shown). In addition, competition experiments performed with a small RNA molecule covering the 3' intronic region gave the same results (data not shown). These results confirmed that the 40 kDa protein interacted more strongly with the mutant precursor than with the wild-type.

The 40 kDa protein is hnRNP C

Among the numerous proteins known to interact with the pyrimidine tract, hnRNP C seemed to be the best candidate. It has a molecular weight of 40 kDa. To test this possibility, splicing reactions were submitted to UV cross-linking and then immunoprecipitated using monoclonal antibodies 4F4 against hnRNP C (C1 and C2) proteins. Indeed, the 40 kDa protein was efficiently selected by the antibodies directed against hnRNP C1 and C2 (Fig. 6A). Reproducibly, we noticed that the level of the C protein selected by the antibodies was higher with the mutant than with the wild-type precursor. Immunoprecipitation after competition experiments with the antibodies against hnRNP C confirmed these results (Fig. 6B). By adding a 4-fold molar excess of wild-type competitor RNA, the hnRNP C protein bound to the wild-type transcript was almost unaffected. In contrast, the interaction of the hnRNP C was strongly reduced by adding the
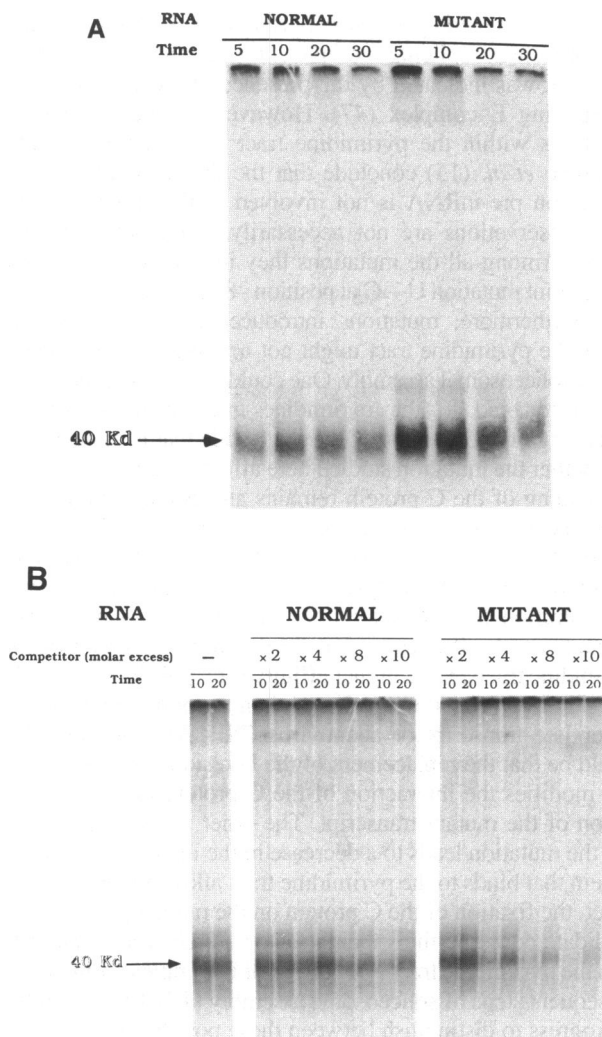
Figure 5. A protein of 40 kDa interacts more strongly with the mutant pre-mRNA. (**A**) Cross-linking of nuclear proteins to mutant and wild-type pre-mRNAs. ^{32}P labeled pre-mRNAs were incubated in HeLa cell nuclear extract and irradiated at 254 nm with UV light. Cross-linked products were resolved by SDS–PAGE. Times of incubation under splicing conditions are indicated, in minutes, above each lane. The arrow points to the 40 kDa protein. (**B**) Cross-linking of nuclear proteins to the labeled wild-type pre-mRNA in the presence of unlabeled competitor RNAs. Level of unlabeled competitor pre-mRNAs is indicated. (**C**) Quantification of the data shown in (B) was performed after 10 min of incubation under splicing condition. ^{32}P-RNA cross-linking efficiencies were plotted against the molar excess of unlabeled competitor RNA. The percentage of interaction was calculated relative to the interaction of the labeled precursor in the absence of competitor which was considered as 100%.

same molar excess of mutant RNA competitor (60%, data not shown). From these results, we concluded that the mutation affected either directly or indirectly the interaction of the hnRNP C protein with the polypyrimidine tract.

DISCUSSION

In this paper, we have analysed a natural mutation within the polypyrimidine tract of intron 2 of human β-globin pre-mRNA (11). This mutation is a U→G tranversion at position −8 upstream

of the 3′ splice site. *In vivo*, this mutation coupled with a frameshift mutation on the other allele results in a mild β$^+$ thalassemia phenotype, suggesting that normally spliced β-globin mRNA is produced but at reduced level. The results we obtained *in vitro* are consistent with the *in vivo* observations. The mutant transcript shows a 2-fold decrease of spliced mRNA compared to the wild-type transcript. As the level of the globin mRNA in the patient's red blood cell precursors cannot be measured, a strict correlation between the *in vivo* and *in vitro* situation is difficult to establish. However, considering the hematologic features of the patient, one can assume that the low splicing defect observed *in vitro* is relevant to physiologic splicing.

Polypyrimidine tract and splicing process

The pyrimidine tract was shown to be essential for pre-mRNA splicing. In classical introns in which the pyrimidine tract is short, replacement of pyrimidine residues by purines either downstream of the branch site or upstream of the 3′ splice site blocks the splicing process prior to the first catalytic step (12). Recently, Roscigno *et al.* (15) reported an extensive mutational analysis within the pyrimidine tract. They showed that all the mutations that affect the splicing efficiency also prevent the formation of the A pre-splicing complex. The inhibition of the splicing process depends on both the position and the nature of the substitution within the pyrimidine tract. At a given position adenosine and guanosine residues are not equivalent; substitutions of pyrimidines by guanosine residues have a more dramatic effect on pre-mRNA splicing than substitutions by adenosine residues.

The results we obtained extend these studies by showing that a single point mutation within the pyrimidine tract interferes predominantly with the first step of the splicing process and mainly affects A complex formation. These data are consistent with previous work, showing that the polypyrimidine tract is involved at an early stage of the splicing process and that it is important for 5′ splice site cleavage and lariat formation (39). The mechanism by which the mutation acts negatively on the splicing process remains obscure. The decision for a pre-mRNA to enter into the splicing cycle is taken very early in the stepwise assembly pathway, and it has been suggested to occur at the level of the first pre-splicing E complex (40,41). The pyrimidine tract was shown to be involved in that step because substitution of the pyrimidine tract by an unrelated sequence prevents E complex formation (40). This suggests that the mutation perturbs the interaction of some proteins required to engage the pre-mRNA within the E complex. However, we cannot exclude the possibility that the mutation affects a later stage in the assembly pathway, such as the U2snRNP interaction at the branch site.

Polypyrimidine tract and protein interaction

Numerous proteins are known to interact with the polypyrimidine tract. Among them are hnRNPs (13,25–31). From earlier reports it was thought that hnRNPs were simply abundant proteins associated with nascent transcripts and as such played no role in RNA processing. Several studies argue against this simple idea. It has been shown that pre-mRNAs interact with different subsets of hnRNP, suggesting that these proteins could be involved in the splicing process [see discussion in (42)]. In agreement with this observation, it has been proposed that hnRNP I (PTB) plays a negative role for some alternative splicing events (43,44). Moreover, hnRNP A1 was shown recently to be implicated both
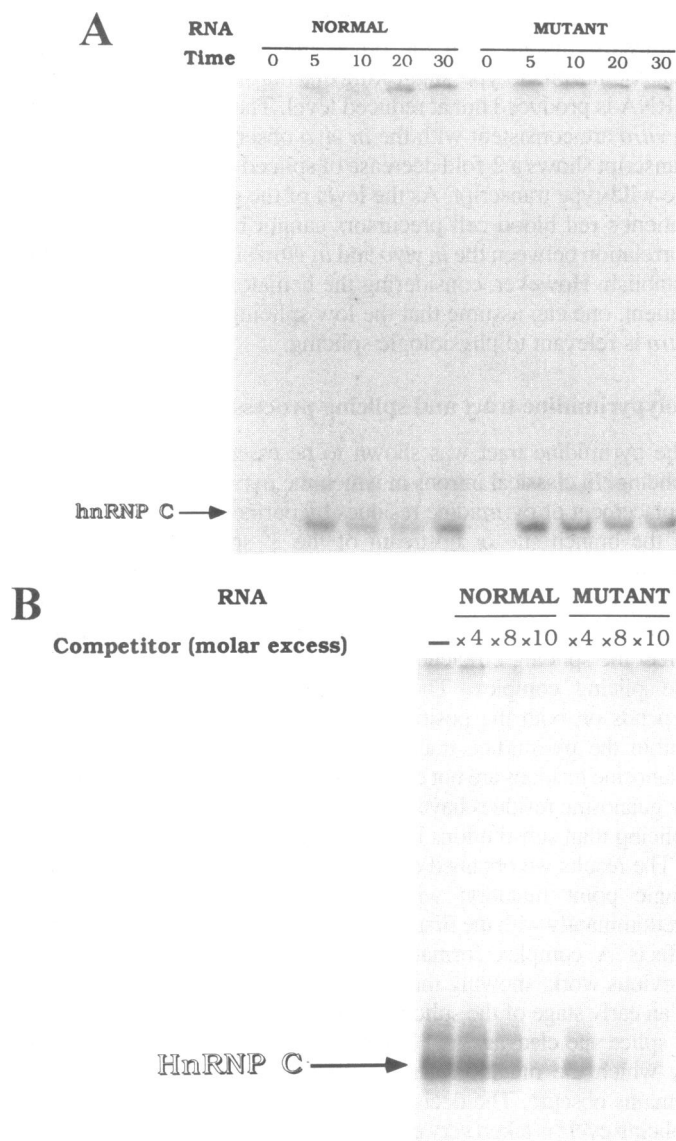
Figure 6. Identification of the 40 kDa protein. (A) Immunoprecipitation with the 4F4 monoclonal antibodies. ^{32}P-labeled precursors were incubated in splicing conditions for the indicated times, then UV irradiated. After RNase treatment, the cross-linked proteins were immunoprecipitated with the 4F4 monoclonal antibodies and analyzed by SDS–PAGE. (B) Immunoprecipitation assays of the hnRNP C protein with unlabeled competitor RNAs. The labeled wild-type pre-mRNA was incubated in splicing conditions for 10 min with increasing amounts of unlabeled wild-type or mutant transcripts. Immunoprecipitation experiments were in the same conditions as in (A).

in vivo and *in vitro* in the regulation of 5′ splice site choice (45,46). Using UV cross-linking experiments, we show that hnRNP C interacts more efficiently with the mutant pre-mRNA than with the wild-type. The difference in the binding level between the mutant and the wild-type precursors is low. However, in light of the splicing and complex assembly experiments, we did not anticipate a huge difference. The role of the C protein in the splicing process is unclear. Evidence for the importance of the C protein in RNA splicing came from earlier studies showing that

antibodies directed against the C protein blocked 5′ site cleavage and prevented splicing complex formation (28). A further argument was indicated by the presence of the C protein in the pre-splicing E complex (47). However, by analysing several mutations within the pyrimidine tract of adenovirus 2 intron, Roscigno *et al.* (15) conclude that the direct binding of the C protein on pre-mRNA is not involved in the splicing process. Their observations are not necessarily contradictory with our results. Among all the mutations they investigated, none was a single point mutation U→G at position –8 upstream of the 3′ splice site. Furthermore, mutations introduced at different positions within the pyrimidine tract might not necessarily affect the same step of spliceosomal assembly. One could suggest that each protein known to bind to the pyrimidine tract interacts differently depending on the position of the nucleotide that is mutated.

Whether the mutation studied here affects directly or indirectly the binding of the C protein remains an open question. In fact, experiments using ribonucleotide homopolymers have shown that the C proteins have a high affinity for poly(U), and they do not bind to poly(A), poly(C) or poly (G), except at low salt concentration for the latter (29). However, in a recent study Samuels *et al.* (48) report that the *Sxl* protein, which binds to pyrimidine tracts, has a higher affinity for a poly(U$_7$) in which an A residue was added to the 5′ end than for poly(U$_7$) itself or the major late transcript of adenovirus (PIP4Wt). One possibility would be that the replacement of the U residue at position –8 by a G modifies the interaction of the C protein in the 3′ intronic region of the mutant transcript. The other possibility would be that the mutation leads to a decrease in the interaction of another protein that binds to the pyrimidine tract allowing, by an indirect effect, the fixation of the C protein on the mutant precursor. One candidate could be the splicing factor U2AF whose interaction with the pyrimidine tract is required for E complex formation and subsequent steps of spliceosome assembly (47). Experiments are in progress to distinguish between these possibilities.

ACKNOWLEDGEMENTS

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