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Accessibility of U1 RNA to base pairing with a single-stranded DNA fragment mimicking the intron extremities at the splice junction

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ABSTRACT

A DNA fragment containing a 16 nucleotide sequence mimicking the intron extremities of premessenger RNA aligned as proposed previously (1,2) in a model of splicing mechanism was prepared and used as a probe for accessibility of the 5' extremity of U1 RNA. Hybridization of U1 RNA to the probe under non denaturing conditions and digestion of the hybrid with RNase H revealed that the sequence of U1 RNA which is complementary to the extremities of introns is accessible to hybridization and to enzymes. Therefore, the configuration of isolated U1 RNA satisfies the criteria required for the alignment of introns and further enzymatic reactions of splicing.

INTRODUCTION

A 18 nucleotide long sequence close to the 5' end of U1 RNA was shown to be complementary to the nucleotides from intron transcripts adjacent to the splice point (1,2). From this observation, it was inferred that U1 RNA may insure the alignment of premessenger RNA sequences for correct cutting and splicing. By reexamining 69 intron sequences from various premessenger RNAs, we observed that no more than 8 nucleotides from U1 RNA could be reasonably expected to participate to the formation of an hybrid between the intron sequences and U1 RNA (3). These 8 nucleotides included a sequence UCCA which was complementary to the consensus sequence AG-GU from the two ends of the introns in all 69 cases. On the other hand, we studied the secondary structure of U1 RNA by different experimental approaches and we found that the 8 nucleotide sequence including UCCA was accessible to nucleases specific for single-stranded RNA, whereas most other flanking nucleotides on the 3' side were part of a stable hairpin (4). However, the diges-

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tion of the sequence by nucleases was moderate as compared to that of other single-stranded regions, suggesting that the conformation of the molecule may reduce the accessibility of the sequence. In order to determine directly whether this sequence of U1 RNA was available for hybridization, a single-stranded DNA fragment, including a 16 nucleotide long sequence complementary to the 5' end of U1 RNA was prepared and used as a probe. The formation of hybrids was assayed with RNase H which specifically cleaves RNA fragments hybridized to DNA.

METHODS

1. Isolation of U1 RNA. RNA was phenol-extracted from purified hen liver nuclei (5) and electrophoresed in polyacrylamide gels made up in Loening's buffer (6) in the absence of urea. Under these non denaturing conditions, U1 and U4 RNAs comigrated. The band containing the mixture of the two RNAs was eluted out for analysis.

2. Preparation of a single-stranded DNA fragment mimicking the intron sequence at the splice junction. The starting material was a HindIII fragment of the ovomucoid gene cloned in plasmid pBR322 (a generous gift of P. Gerlinger, M. LeMeur and A. Krust). The plasmid was extracted according to (7), linearized by SacI digestion and treated by HindIII (Fig. 1). The plasmid DNA, the HindIII-SacI and SacI-HindIII fragments were separated on a 5-20% linear sucrose gradient centrifuged for 24 h at 27,000 rev/min. The 1100 base pair SacI-HindIII fragment was collected

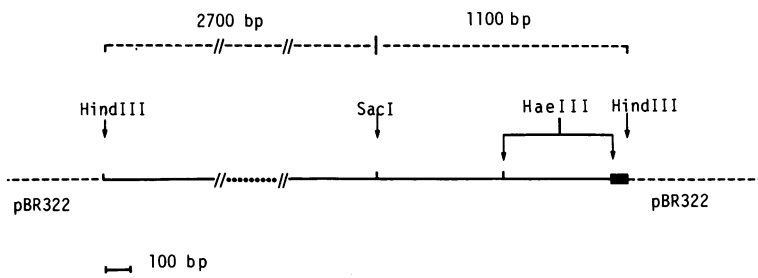

Fig. 1 : Preparation of the restriction fragment HaeIII-HindIII from the HindA fragment of the ovomucoid gene. The black box represents the fragment to be isolated.

and digested by HaeIII. After alkaline phosphatase treatment for 1 h at 65°C, the mixture was deproteinized and the 5' end of the DNA fragments were labeled with [^{32}P-γ] ATP and T4 polynucleotide kinase. The reaction was terminated by heating at 65°C for 5 min. The method of Maxam and Gilbert (8) was applied for DNA strand separation. Electrophoresis was in 12% polyacrylamide gels (acrylamide : bisacrylamide; 60:1). The large SacI-HaeIII and HaeIII-HaeIII fragments did not enter the gel or remained close to the top. After autoradiography of the gel, 3 bands corresponding to the undenaturated small HaeIII-HindIII fragment (black box in Fig. 1) and to its two strands were revealed (Fig. 2). The bands corresponding to the separated strands were eluted out of the gel and acrylamide was removed by chromatography on a small column of DE-52 cellulose.

3. Hybridization of U1 RNA to the single-stranded DNA fragment and cleavage by RNase H. The procedure was that described by Donis-Keller for the hybridization of RNA to oligodeoxynucleotides under conditions maintaining the secondary structure of the RNA (9). In order to avoid denaturation, the RNA was not preincubated at 50°C prior to hybridization. The hybrid was hydrolyzed with RNase H. The resulting RNA fragments were labeled with 5' [^{32}P] pCp and T4 RNA ligase (10), fractionated by polyacrylamide gel electrophoresis under denaturing condi-

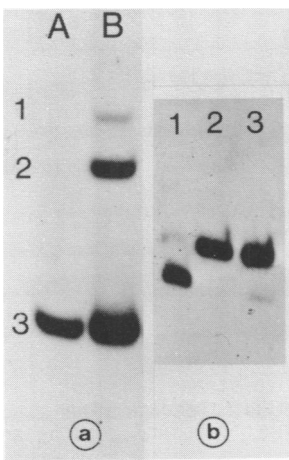

Fig. 2 : Isolation of the HaeIII-HindIII single-stranded DNA fragment. Panel(a) : The HaeIII digest of the SacI-HindIII fragment was 5' end labeled and electrophoresed in a 12% polyacrylamide slab gel without prior denaturation (A) or after heating of the sample at 90°C in 30% DMSO for 2 min.(B) Only the pertinent parts of the gels are shown. Panel(b) : Bands 1, 2 and 3 were eluted out from gels similar to that shown in panel (a) and reelectrophoresed in denaturing 10% polyacrylamide gels.

tions and sequenced by the method of Peattie (11).

RESULTS

1. Isolation and characteristics of the probe.

We looked for a DNA fragment containing a sequence complementary to that of the 5' end of U1 RNA. The HindIII A restriction fragment of the ovomucoid gene which was sequenced by Gerlinger, LeMeur and Krust (personal communication) satisfied this criterion. It contained a 16 nucleotide long sequence (Fig. 3 , positions 45-60) complementary to the sequence of nucleotides 4-19 of U1 RNA, with a single gap at position 50 of the DNA and 14 of the RNA.

The smallest piece of DNA containing this sequence was searched by studying the restriction sites of the HindIII A fragment. This led to the isolation of a HaeIII-HindIII fragment (Fig. 1) whose strands 1 and 2 were 80 and 85 nucleotides long, respectively (Fig. 3). The strands were separated electrophoretically in non denaturing conditions after 5' end labeling of the DNA (Fig. 2a). Band 2 was more intensely labeled than band1 by polynucleotide kinase. As strand 2 has a protruding 5' end, it was expected to be more easily labeled than strand 1 (Fig.3). Thus, bands 1 and 2 should correspond to strands 1 and 2, res-

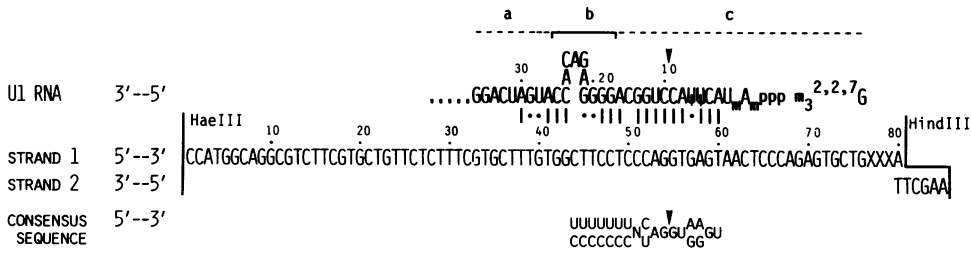

Fig. 3 : Complementarity between U1 RNA and the DNA probe (strand 1). Only the 5' part of U1 RNA is shown. The positions of a hairpin loop (a), a hairpin stem (b) and a free single-stranded region (c) according to the model of secondary structure proposed in (4) are indicated above the sequence. The nucleotide sequence of strand 2 which is complementary to strand 1 is not shown except for the protruding end obtained after HindIII cleavage. The consensus intron sequence is from (3). The splice point is indicated by an arrow-head.

pectively. However, strand 1 which is 5 nucleotides shorter than strand 2 should migrate more slowly, which was not the case. This might be related to differences of the secondary structure of the 2 DNA fragments. Indeed, after denaturation the mobility of the bands was reversed (Fig. 2b). Band 1 was therefore used as the DNA probe in further experiments.

The 80 nucleotide long strand 1 contains the 16 nucleotide segment complementary to the 5' end of U1 RNA. We showed by computer analysis that other fragments of strand 1 were complementary to U1 RNA. However, the largest of these fragments was only seven nucleotide long (positions 153-159 of U1 RNA) and almost all of them were complementary to regions of U1 RNA involved in base-pairing according to our model of secondary structure (4). The secondary structure of the probe may also influence its base pairing with U1 RNA. As shown by computer analysis, strand 1 DNA displays poor internal complementarity especially in the 16 nucleotide segment complementary to U1 RNA.

2. Hybridization of U1 RNA to the DNA probe and digestion with RNase H.

For our aim, it was essential that the native conformation of U1 RNA was preserved. Therefore, the RNA was fractionated by electrophoresis in the absence of urea. Under these non denaturing conditions, U4 RNA comigrated with U1 RNA and the mixture of the two RNA species was obtained after elution. Labeling with 5' [^{32}P]pCp and T4 RNA ligase and electrophoresis in denaturing conditions showed the presence of U1 and U4 RNAs and in addition of shorter RNA fragments (Fig. 4A). These fragments correspond to very small amounts of highly labeled material. Their 3' end is probably much more accessible to labeling by pCp than the 3' end of snRNA (4).

In order to maintain the secondary structure of U1 RNA, the hybridization and the digestion with RNase H were performed in the presence of 10 mM Mg^{++}, at a mild temperature (32°C). After 3' end labeling, the digestion products were electrophoresed under denaturing conditions (Fig. 4B) in parallel to untreated RNA (Fig. 4A). A decrease of the amount of labeled U1 RNA was observed together with the appearance of 4 new bands.

Fig 4 : Fractionation of the fragments resulting from digestion of the hybrid with RNase H. The mixture of U1 plus U4 RNAs was hybridized to the probe and digested with RNase H as described in Methods. The resulting products were 3'-end labeled and fractionated in a 15% polyacrylamide gel made up in Tris-borate 8M urea buffer (B). As a control the mixture of RNA which has been 3'-end labeled but neither hybridized nor hydrolysed was fractionated in parallel (A). An enlargement of the portion of the gel showing the separation of U1 RNA and band 1 is presented.

Band 1 migrated just after U1 RNA whereas bands 2, 3 and 4 corresponded to very small fragments. In other experiments, the presence of a fifth small band was observed, probably due to a variable extent of hydrolysis by RNase H. The amount of U4 RNA did not change suggesting that this RNA did not hybridize to the probe. The high labeling of the minor products found in undigested material decreased after the treatment suggesting that this RNA hybridized to the probe. After RNase H treatment these hybrids were probably reduced to very small pieces not detected on the gel autoradiographs.

3. Sequence analysis of the digestion products

The digestion products were sequenced by the chemical method described by Peattie (11). In the experiment of Fig.5, four bands of high electrophoretic mobilities (denoted 2 to 5) were analyzed. They correspond to the 5' end of the U1 RNA molecule and the results indicate that the cleavage by RNase H occured as follows :

cap $A_m U_m AC\psi\psi ACCUGGCA...$

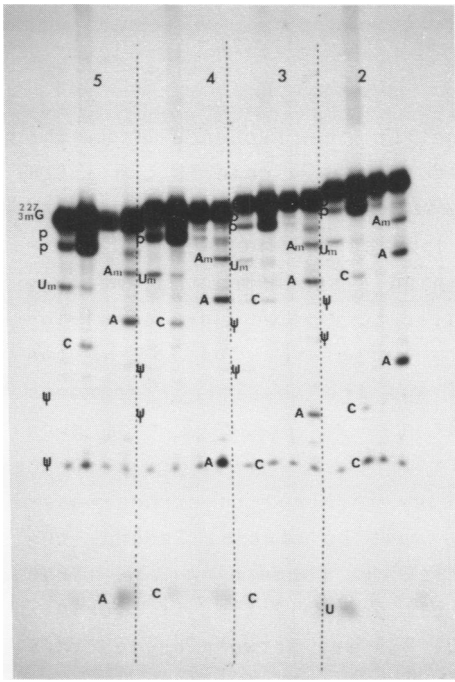

Fig. 5 : Sequence analysis of fragments 2,3,4 and 5. The 3'-end labeled fragments 2 to 5 were chemically digested according to Peattie (11) and the resulting products were fractionated on a 25% polyacrylamide gel. A compression of the material occurred between the first and the second nucleotide starting from the 3'-end. This was due to a slight difference of buffer concentration in the gel and in the tanks and explains the appearance of strong bands in all the columns at the level of the second nucleotide. Nevertheless the sequence of all the fragments can be read from the gel. As previously mentionned (5,12) ψ is hardly cleaved in these conditions, whereas the cap structure is cut by hydrazine.

As shown by sequence analysis, band 1 contains the mixture of the remaining 3' parts of the molecules which cannot be separated under the conditions required for the fractionation of fragments of less than 20 nucleotides.

Thus, the 5' end of U1 RNA, complementary to the DNA probe hybridized to the expected part of the probe. Only nucleotides A8 to U11 were detected as part of a hybrid in our experiment. As RNase H does not obligatorily digest all parts of the hybridized RNA to the same extent and as it requires at least 4 base-pairs as substrates (9), it is likely that nucleotides flanking positions 8-11 are also included in the hybrid. However, it seems unlikely that such hybrid would extend further than nucleotide A15 since a stable hairpin was shown to start at position 16 (4 and Fig. 3). Taking into account the secondary structure data (4), the stability of the putative hybrid between the 5' extremity of U1 RNA and the DNA probe (Fig. 3) and the observed hybridization of nucleotides 8-11, we may reasonably assume that the decanucleotide AC$\psi\psi$ACCUGG at position 4-13 of

U1 RNA hybridized to the probe.

DISCUSSION

In order to determine whether the sequence from the 5' end of U1 RNA which was proposed to play a role in the alignment of intron sequences of premessenger RNA (1-3) was indeed accessible to hybridization, we applied the method of Donis-Keller (9). A DNA fragment containing a sequence complementary to the U1 RNA sequence was hybridized to U1 RNA under conditions mild enough to maintain secondary structure. The RNA of the hybrid was digested with RNase H and the resulting fragments were sequenced. A positive result can be expected from such a method only if two criteria are fulfilled. First, the sequences to be hybridized must not be already involved in strong base-pairing and the hybrid to be made must be stable. Second, the hybrid must be accessible to RNase H. Concerning the first criterion, the most favourable of all possibilities of hybridization between the DNA probe and U1 RNA was between nucleotides 51 to 60 of the DNA and 4 to 13 of U1 RNA (which are in a single-stranded region, Fig. 3). Experimentally, hybridization was indeed observed in this region (nucleotides 8-11). This also indicated that the second criterion was fulfilled.As10 contiguous nucleotides (positions 4-13) may strongly base pair with the DNA fragment and as only 4 were displayed, we may assume that the 6 other nucleotides were less accessible to the enzyme, perhaps because of steric hindrance due to the conformation of the U1 RNA molecule or to specific unknown characteristics of the enzyme.

Our DNA probe mimicks the two extremities of introns aligned as proposed in the models of Lerner et al. (1), Rogers et al. (2) and Gallinaro et al. (3). The sequences 44-54 and 55-60 are representative of the 3' and the 5' extremities of introns, as shown by comparison with a consensus intron sequence (Fig. 3). Therefore, our results indicating that U1 RNA may hybridize to the DNA probe in this region also demonstrate that it is accessible for hydrogen binding with the sequences at the extremities of introns. It is particularly striking that the portion of the hybrid the most accessible to RNase H is

precisely at the splice junction (AC-CU of U1 RNA).

We conclude : 1) that the conformation of isolated U1 RNA is such that the alignment of intron sequences by hybridization to the sequence ACCU of its 5' extremity is possible and 2) that the conformation of the hybrid allows enzymatic reactions at the splice junction, possibly the cleavage and ligation required for the splicing reactions. Therefore, isolated U1 RNA satisfies the criteria required by the hypothesis already evoked (1-3). However, the results do not demonstrate that such events occur **in vivo** in the presence of the proteins associated with premessenger RNA or with U1 RNA.

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