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Biochemistry

Site-specific cleavage by T1 RNase of U-1 RNA in U-1 ribonucleoprotein particles

(small nuclear RNA/RNA processing/systemic lupus erythematosus antibodies)

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ABSTRACT **The structures and functions of small nuclear ribonucleoprotein particles have become of interest because of their suggested role in processing heterogeneous nuclear RNA [Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. (1980) *Nature (London)* 283, 220–224]. To determine the conformation of U-1 RNA in U-1 ribonucleoprotein particles and whether proteins of these particles protect segments of U-1 RNA, intact particles and isolated U-1 RNA were digested with T1 RNase. The digested particles were immunoprecipitated with anti-Sm antibodies. A 5′-end fragment containing nucleotides 1–107 and 3′-end fragments containing nucleotides 108–165 and 108–153 were recovered in nearly quantitative yield from digestion of the particles, suggesting that position 107 is the principal cleavage site in them. At the same T1 RNase concentrations, deproteinized U-1 RNA was cleaved into many fragments. At low T1 RNase concentrations, a major cleavage site of deproteinized U-1 RNA was at nucleotide 69. Comparison of the cleavage sites of free U-1 RNA and of U-1 RNA in U-1 ribonucleoprotein particles suggested similar secondary structures. The resistance of the 5′ end of U-1 RNA to T1 RNase was unexpected inasmuch as this region has been implicated in hydrogen bonding with heterogeneous nuclear RNA splice junctions.**

U-1 RNA exists in the nucleus in small nuclear ribonucleoprotein (RNP, snRNP) particles (1–3). Suggestions that U-1 RNA is associated with heterogeneous nuclear RNA (hnRNA) (2–4) have led to studies that have shown that the 5′ end of U-1 RNA may base pair with intron–exon regions of hnRNA (3, 4). As an approach to the structure of U-1 snRNP particles and to identify regions of U-1 RNA that are bound to proteins, the present study was undertaken; it combines partial cleavage of the RNP with immunoprecipitation of the RNP particles.

This study showed that the 5′-end 107 nucleotides and most of the 3′ end of U-1 RNA are resistant to T1 RNase cleavage and are probably protected in several regions by the proteins of the U-1 RNP particle.

MATERIAL AND METHODS

Harvested Novikoff hepatoma ascites cells were incubated in Eagle's medium with [^{32}P]orthophosphate as described (5). The nuclei were isolated by homogenizing the cells in a Tissumizer in 10 mM NaCl/1.5 mM $MgCl_2$/0.5% Nonidet P-40/10 mM Tris buffer, pH 7.2, sedimented by centrifugation at 4000 × *g* for 10 min (6), and then sonicated in 100 mM NaCl/1 mM $MgCl_2$/10 mM Tris buffer, pH 8.5 until no intact nuclei were visible (2). The sonicates were centrifuged at 20,000 × *g* for 10 min, and the supernatant was used. The RNA concentrations of the supernatants were determined spectrophotometrically: one OD_{260} was equivalent to 50 μg. The usual range of the sonicate concentration was 200–300 μg/ml. The supernatant was incubated with T1 RNase at an enzyme/substrate ratio of 1:10 or 1:5 (wt/wt) or without enzyme for 30 min at 0°C. Preliminary experiments showed identical RNA patterns and yields after T1 RNase digestion when either antigen or antibody was used in excess. Immunoglobulin fractions of sera from patients with systemic lupus erythematosus previously demonstrated to precipitate U-1, U-2, U-4, U-5, and U-6 RNAs (anti-Sm sera) were then added at 2 mg/ml of sonicate, and the mixtures were incubated for 15 min on ice. The immune complexes were precipitated by addition of Pansorbin at 1 ml/2 mg of immunoglobulin (2), followed by 5 min of incubation on ice. The precipitates were washed five times with 150 mM NaCl/5 mM EDTA/0.05% Nonidet P-40/50 mM Tris buffer, pH 7.4.

The RNA was extracted from the Pansorbin pellets by using the hot phenol/NaDodSO_4 procedure, precipitated with 2 vol of ethanol, and then subjected to electrophoresis on 12% polyacrylamide/7 M urea, pH 8.3, gels. After the gels were subjected to autoradiography, the bands were excised and the RNA was extracted as described (7). Complete digestion of the RNA with T1 or pancreatic RNase and separation of the resultant oligonucleotides was carried out as described by Brownlee *et al.* (8). Polyethylenimine-cellulose sheets were used for homochromatography in the second dimension.

To determine the action of T1 RNase on deproteinized U-1 RNA, gel-extracted ^{32}P-labeled U-1 RNA was added to unlabeled nuclear sonicates prepared as described above. The sonicates were then incubated with T1 RNase at enzyme/substrate ratios ranging from 1:3125 to 1:5 for 5 min at 0°C. The RNA was then extracted, subjected to gel electrophoresis, and identified as described above.

The T1 and pancreatic RNases and Pansorbin were obtained from Calbiochem. The polyethylenimine-cellulose sheets were obtained from Brinkmann.

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Abbreviations: RNP, ribonucleoprotein; snRNP, small nuclear ribonucleoprotein; hnRNA, heterogeneous nuclear RNA.

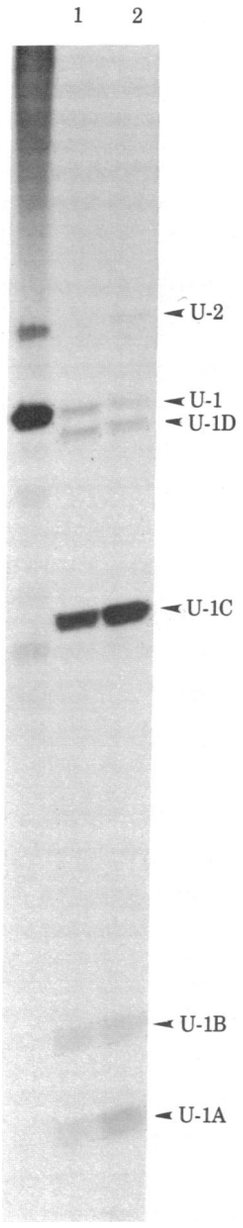

FIG. 1. Autoradiograph of RNA extracted from Pansorbin pellets and subjected to electrophoresis on a 12% polyacrylamide/7 M urea, pH 8.3, gel. Nuclear sonicates were incubated with T1 RNase at enzyme/substrate ratios (wt/wt) of 1:10 (lane 1) or 1:5 (lane 2) and anti-Sm antibody as described in *Materials and Methods*.

RESULTS

An autoradiograph of a 12% polyacrylamide gel on which RNA from the Pansorbin-treated pellets was separated is shown in Fig. 1. Each slot of the gel contained the products obtained from an aliquot of the nuclear sonicate and an equal amount of each immunoglobulin fraction. Autoradiographs of the complete T1 RNase fingerprints of U-1 RNA and U-1 RNA fragments A, B, C, and D are shown in Fig. 2. The average molar yields of these fragments (considering 100% as the ^{32}P nucleotide of the U-1 RNA precipitated when T1 RNase was not added) were 77%, 54%, 37%, and 9%, for U-1C, U-1A, U-1B, and U-1D, respectively. Under these conditions, less than 5% of the U-1 RNA was undigested.

The T1 RNase fingerprint of the major fragment (U-1C) (see Fig. 2*B*) contained spot 21, which is the 5′ end of U-1 RNA (9). Fragment U-1C was also digested with nuclease P1 and subjected to electrophoresis on DEAE paper at pH 3.5 (not shown); it contained the "cap." Therefore fragment U-1C contained nucleotide 1 of U-1 RNA. Fragment U-1C also contained oligonucleotide T-22 (see Fig. 5), which is composed of nucleotides 95–107 of U-1 RNA. Inasmuch as fragment U-1C contained all the T1 RNase fragments from the 5′ end of U-1 RNA, including spot 22 (nucleotides 95–107), and the next guanosine phosphate (at position 109) was found in fragments U-1A and U-1B in a combined molar yield of 91% (see below), fragment U-1C extended from nucleotide 1 to nucleotide 107 of U-1 RNA.

The T1 RNase fingerprint of fragment U-1B (see Fig. 2*C*) contained spot 17, the 3′-end fragment. This fragment did not contain spot 22, nucleotides 95–107 of U-1 RNA, but did include spot 13, nucleotides 112–118. In addition, the complete pancreatic RNase fingerprint of fragment U-1B included the oligonucleotide G-G-G-A-A-A-Cp, which suggests that this fragment extends in the 5′ direction to nucleotide 108. Therefore, fragment U-1B contained nucleotides 108–165 and thus represents the portion of the U-1 RNA structure not included in fragment U-1C.

The T1 RNase fingerprint of fragment U-1A (see Fig. 2*D*) was identical to that shown in Fig. 2*C* except that it lacked spot 17. Fingerprint analysis of this fragment after complete pancreatic RNase digestion indicated that it also included the oligonucleotide G-G-G-A-A-A-Cp. Therefore, fragment U-1A extends from nucleotide 108 to nucleotide 153 (or 155).

The T1 RNase fingerprint of fragment U-1D (see Fig. 2*E*) contained all of the spots of the T1 RNase fingerprint of whole U-1 RNA (see Fig. 2*A*) except spot 17 at the 3′ end of U-1 RNA. Therefore, fragment U-1D included nucleotides 1–153 (or 155).

Other experiments (not shown) with pancreatic RNase have produced several additional fragments, four of which were obtained in yields of >20%. The fragment in highest yield (69% molar yield) extended from nucleotides 109 to the 3′ terminus of U-1 RNA. Two fragments were products of cleavages within oligonucleotide T-21 (see Fig. 4), the first 11 nucleotides of U-1 RNA, and both extended in the 3′ direction to nucleotide 108. Their combined molar yield was 51%. The other fragment, obtained in 21% molar yield, was cleaved in oligonucleotide T-12 at nucleotide 31, 32, or 34 and extended to the 3′ end of U-1 RNA.

Other minor fragments were obtained in yields of 10% or less but sufficient for fingerprint analysis. Two obtained by T1 RNase digestion were products of a single cleavage at nucleotide 70 and contained nucleotides 1–69 and 70–165. Of the four obtained by pancreatic RNase digestion, two were products of the cleavages described above but in different combinations; one fragment contained nucleotides 110–165, the other was cleaved in oligonucleotide T-12 and contained nucleotides 32 (or 33 or 35)–108. The other two suggested two different sites of pancreatic RNase attack, nucleotide 102 and within oligonucleotide T-11 (see Fig. 4), and contained nucleotides 103–165 and 109–150 (or 151 or 152) (in oligonucleotide T-11).

The cleavage patterns obtained from digestion of deproteinized U-1 RNA under varying T1 RNase concentrations, including that shown in Fig. 1, are shown in Fig. 3. Fragments larger than 20 nucleotides accounted for 12%, 20%, 33%, 54%, and 60% of the U-1 RNA recovered from sonicates at enzyme/substrate ratios of 1:5, 1:25, 1:125, 1:625, and 1:3125, respectively. When isolated U-1 RNA was digested under the same conditions but in the absence of added sonicate, similar results were obtained. Fingerprint analysis of the major fragments showed that fragments 1–4 and fragment 6 were cleaved at Gp 69. Fragment 1 contained nucleotides 70–165, fragment 2 contained

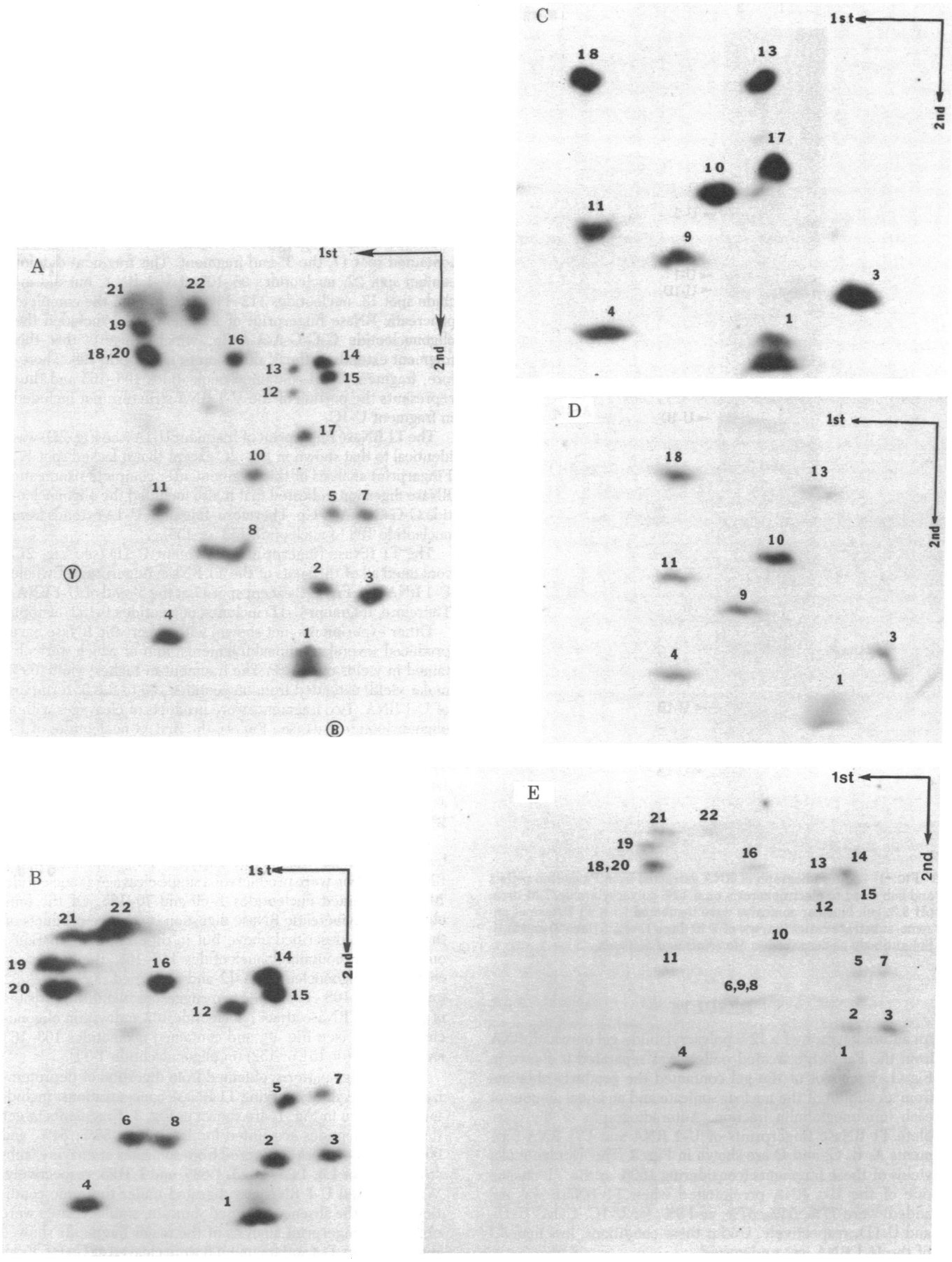

FIG. 2. *(Legend appears on following page.)*

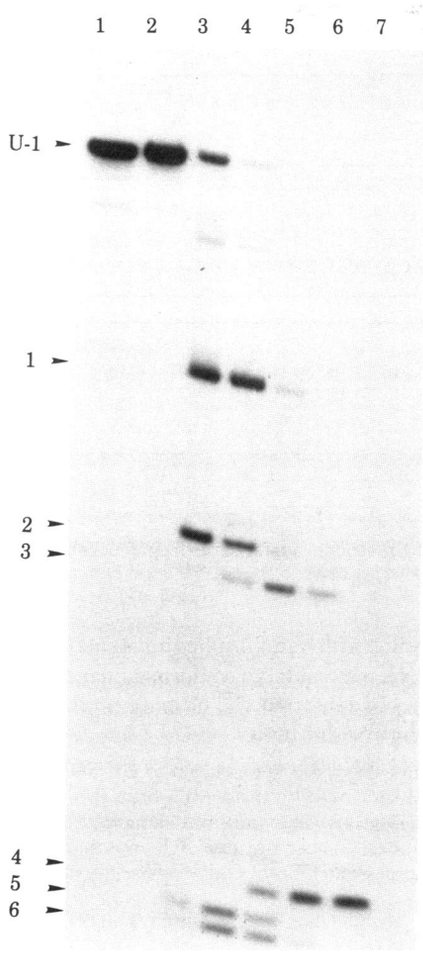

FIG. 3. Autoradiograph of products of T1 RNase digestion of isolated U-1 RNA, separated as described in Fig. 1. Lane 1, isolated U-1 RNA; lanes 2–7, fragments produced after incubation of isolated U-1 RNA in nuclear sonicates with T1 RNase at enzyme/substrate ratios of 0, 1:3125, 1:625, 1:125, 1:25, and 1:5 (wt/wt), respectively. At 1:5, there was marked fragmentation of the U-1 RNA by comparison with the result noted in Fig. 1.

nucleotides 1–69; fragment 3 contained nucleotides 70–131, fragment 4 contained nucleotides 70–107, and fragment 6 contained nucleotides 36–69. Fragment 5 was cleaved at Gp-35 and contained nucleotides 1–35.

DISCUSSION

The sequences of U-1 RNA (9–11) and the major fragments recovered after digestion of U-1 RNA in U-1 RNP particles with T1 RNase are shown in Fig. 4. The fragment in highest (77%) molar yield (fragment U-1C) contained nucleotides 1–107. The remainder of the structure (fragment U-1B, containing nucleotides 108–165) was obtained in 37% molar yield. Fragment U-1A, containing nucleotides 108–153, was obtained in 54% molar yield. The longest fragment (U-1D), nucleotides 1–153, was obtained in 9% molar yield. These fragments account for 86–100% of the U-1 RNA precipitated by the antibody used in these experiments.

In contrast to the limited digests obtained when U-1 RNP particles were used as substrate, isolated U-1 RNA was more completely digested at much lower T1 RNase concentrations. At an enzyme/substrate ratio of 1:5, less than 15% of the original (undigested) radioactivity in isolated U-1 RNA was recovered as fragments having 20 or more nucleotides. In contrast, almost 90% of the original (undigested) radioactivity was recovered in four large fragments after digestion of U-1 RNPs under the same conditions. An equivalent degree of degradation with deproteinized U-1 RNA was not obtained, even when the enzyme/substrate ratio was reduced to 1:3125, indicating a difference in sensitivity of 500-fold or more.

The high resistance to cleavage of U-1 RNA in U-1 RNP particles suggests that this RNA is protected, probably by proteins. The identity and number of U-1 RNP proteins is uncertain at present. Earlier reports (1) suggested that 10 proteins are associated with U-1 and U-2 RNAs; more recent papers (2, 3) have reported that 7 proteins are precipitated with U-1 RNA by anti-RNP antibodies. The same seven proteins were also precipitated with U-1, U-2, U-4, U-5, and U-6 RNAs when anti-Sm antibodies were used. The sequences of U-1, U-2, U-4, U-5, and U-6 are being determined, and several general regions of homology exist (12). Such regions are potential protein-binding sites. Our preliminary data on U-2 RNA suggests that this RNA is also present as a T1 RNase-resistant form in snRNPs.

Comparison of the cleavage sites in isolated U-1 RNA and in U-1 RNP shows significant differences. The major site of T1 RNase cleavage of U-1 RNP particles was nucleotide 107, which accounted for almost 90% of the products. In contrast, only one of the six fragments obtained on digestion of isolated U-1 RNA was cleaved at this site and that only at the higher enzyme concentrations. The principal site of cleavage of isolated U-1 RNA was nucleotide 69. However, this position accounted for <5% of the U-1 RNP digestion products. This result suggests that the region of nucleotide 69 in U-1 RNA is shielded by protein binding in U-1 RNP particles. This also appears to be the case for nucleotides 35 and 131. Despite the differences in T1 RNase sensitivity indicated in this study, as well as the different sites of cleavage, it is notable that four of the five positions cleaved in isolated U-1 RNA are at or near the sites where pancreatic or T1 RNase cleaved U-1 RNA contained in U-1 RNP particles. This finding suggests that U-1 RNA maintains a similar structure in U-1 RNP.

Analysis of the partial fragments recovered suggested that only five sites or regions of U-1 RNP particles are attacked by ribonucleases. In searching for reasons for this specificity, we constructed several secondary structures for U-1 RNA by using the computer program of Korn *et al.* (13) and computed their stability numbers according to the method of Tinoco *et al.* (14). The structure having the highest stability number (+28) is shown in Fig. 5. It is notable that all of the cleavage sites obtained with U-1 RNP particles and isolated U-1 RNA occur either in the loops of this structure or in the non-hydrogen-bonded region at the 5′ end. This proposed structure is also compatible with products reported after partial nuclease S1 digestion of isolated U-1 RNA (11).

FIG. 2 (*on preceding page*). Autoradiographs of two-dimensional separations of the products obtained by complete T1 RNase digestion of complete U-1 RNA and fragments U-1A, U-1B, U-1C, and U-1D as described in *Materials and Methods*. (*A*) Complete U-1 RNA. (*B*) Fragment U-1C, which contains nucleotides 1–107. (*C*) Fragment U-1B, which contains nucleotides 108–165. (*D*) Fragment U-1A, which contains nucleotides 108–153 (or 155). (*E*) Fragment U-1D, which contains nucleotides 1–153 (or 155). Numbers next to the spots refer to the numbering of complete T1 fragments shown in Fig. 4. B, Bromphenol blue; Y, Pyramine Y.

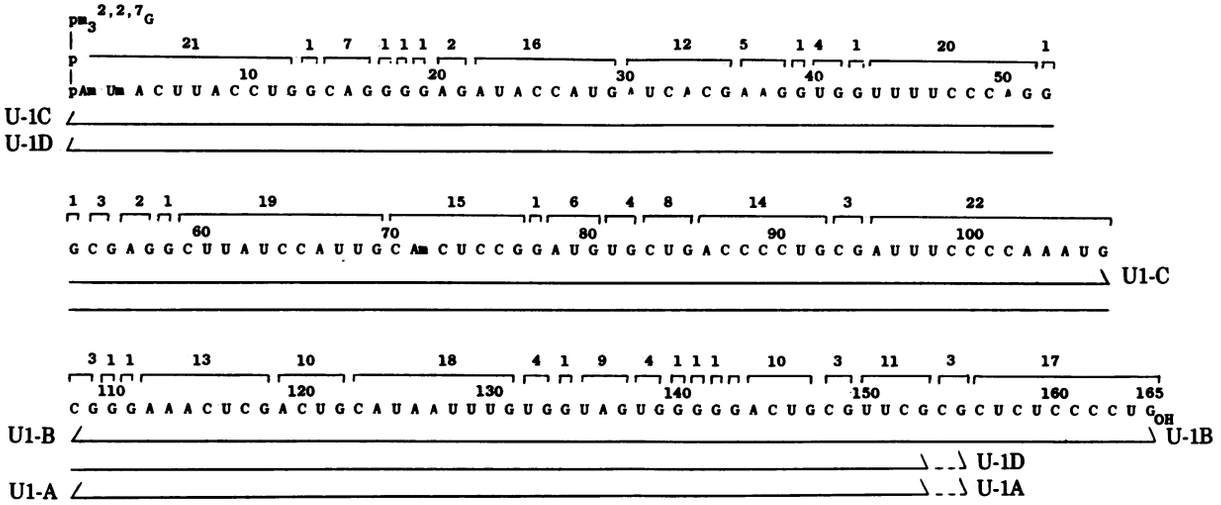

FIG. 4. Primary nucleotide sequence of Novikoff hepatoma U-1 RNA. Numbers above the sequence refer to the fragments shown in Fig. 2. Lines below the sequence refer to fragments U-1A, U-1B, U-1C, and U-1D.

Both the structure of the 5′ end of U-1 RNA and its resistance to T1 RNase would seem surprising in view of the hypothesis that the 5′-end 21 nucleotides of U-1 RNA are involved in hydrogen bonding with hnRNA splice junctions (2, 3). It is possible that U-1 RNA may undergo conformational changes or that U-1 RNP may separate from one or more proteins during association with hnRNA (or both).

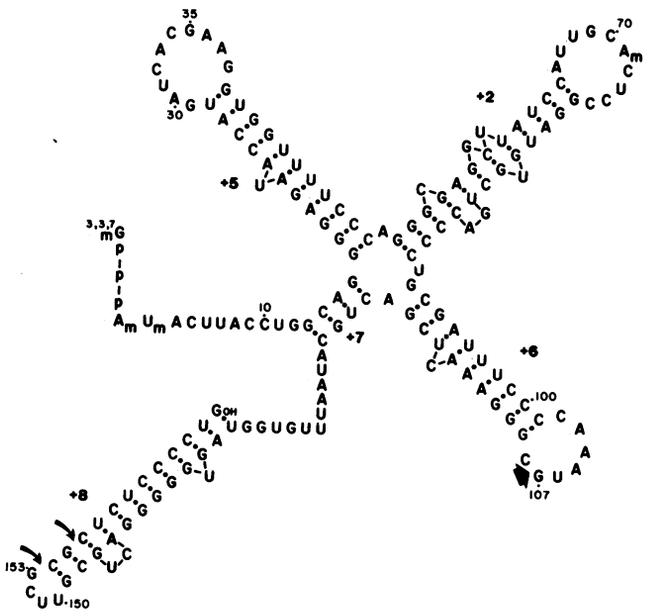

FIG. 5. A possible secondary structure of U-1 RNA. Arrow at nucleotide 107 indicates the major site of cleavage and arrows at nucleotides 153 and 155 indicate possible positions of the minor cleavage by T1 RNase in U-1 RNP. Numbers at each stem or stem and loop refer to the stability number of that region, as calculated by the method of Tinoco *et al.* (14). The stability number for the entire structure was +28.

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