Secondary Structure Maps of RNA: Processing of HeLa Ribosomal RNA
(electron microscopy/45S RNA/exonuclease/nucleolar RNA)

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ABSTRACT Ribosomal RNA precursors and mature 28S ribosomal RNA from HeLa cells display a highly reproducible secondary structure of hairpin loops after they are spread and examined in an electron microscope. This structure was used to map the linear arrangement of these molecules. Partial digestion with 3'-exonuclease from ascites cell nuclei established that the 28S RNA region is located at the 5'-end of the 45S precursor. A spacer sequence follows the 28S region. This is followed by the 18S RNA region, and by another spacer sequence at the 3'-end. The 41S RNA contains both 18S and 28S regions. The 32S RNA contains only 28S RNA plus spacer, and the 20S RNA contains only 18S RNA plus spacer. Several minor nucleolar RNA components were also mapped with respect to the 45S RNA. These experiments lead to a more detailed processing pathway for HeLa ribosomal RNA which is in good agreement with earlier work.

During length measurements of various RNA molecules in an electron microscope we noticed that HeLa 28S ribosomal RNA (rRNA) displayed a characteristic secondary structure, apparently involving hairpin loops reproducibly located along the length of each molecule. We report here a "secondary-structure map" of HeLa rRNA and its precursors. This study locates and orients 18S and 28S rRNA molecules within the 45S RNA precursor and confirms and extends the previously deduced pathway of rRNA processing in HeLa cells (1–3). In addition, the study demonstrates the usefulness of secondary-structure mapping of RNA.

MATERIALS AND METHODS

Nucleolar RNA from HeLa cells was prepared, separated on sucrose gradients, and analyzed on polyacrylamide gels as described by Weinberg et al. (1).

RNA was prepared for electron microscopy by a modified Kleinschmidt technique. The single components of the spreading solution were mixed together exactly as described by Robberson et al. (4). The concentrations in the final solution at pH 8.5 were: 0.5–1.0 μg/ml of RNA, 30 μg/ml of cytochrome c (Type VI, Sigma), 4 M urea, 30 mM Tris buffer, 1 mM EDTA, and 80% formamide (v/v, certified, Fisher). The hypophase was distilled water. In all spreadings with formaldehyde-denatured RNA, Tris was replaced by 30 mM EDTA adjusted to pH 8.5 with NaOH. After they were spread, samples were adsorbed onto pure carbon films, stained with uranyl acetate, and shadowed with Pt–Pd.

RESULTS

Mapping Secondary Structure in rRNA. All HeLa rRNA molecules, with the exception of 18S RNA, showed regions of secondary structure which we interpret as hairpin loops (Fig. 1). The position and length of the loops and their particular morphology were highly reproducible. In measuring the regions of secondary structure we assumed that they were hairpin loops and measured their length twice ("up and down"). The thick lines in the secondary-structure maps (Figs. 2–4) represent the single-strand lengths of RNA segments involved in these double-stranded loops. Vertical lines across looped regions separate consecutive multiple loops in any one region, e.g., the double loop at 0.5–0.8 μm of the 28S RNA is symbolized by a thick line separated into two parts by one vertical bar. For practical reasons, the total length of each multiple-loop region was measured and then divided into equal subsections, which symbolize the small individual loops. Thus, the total length and the number of loops are accurately represented, but the length of each "sub-loop" is not. In constructing the maps, the molecules of each class of RNA were adjusted photographically to the same average length. The actual variation in measured lengths is given in Table 1.

Secondary-structure maps for HeLa rRNA and ribosomal precursor RNAs are presented in Fig. 2. Both the left-to-right orientation of the molecules and the location of smaller molecules within their precursors can be determined unambiguously by comparison of these maps. The assignment of 5’-to-3’ polarity is based on experiments with exonuclease described below. The 28S rRNA (Figs. 1f and 2) corresponds to the 5’-end of the 45S RNA and to no other region of that molecule. The 18S rRNA shows no secondary structure loops when spread under the present conditions (Fig. 1i). The only fully extended region in the 45S RNA of sufficient length to accommodate the 18S molecule is between 2.6 and 3.3 μm from the 5’ end, and we therefore assign it to that region. This conclusion is supported by analysis of intermediates.

The first stage in the processing of 45S RNA leads to a 41S molecule, which still contains both the 28S and 18S sequences (1–3). RNA from the 41S region of sucrose gradients shows a secondary structure that matches that of the 45S molecules exactly from the 5’ end to a point close to the 3’ end of the 18S region (Figs. 1e and 2). The 41S molecule does not contain the 3’-terminal portion of the 45S molecule; this fragment was found as 24S RNA (see below).

The 41S rRNA is thought to be split into a 32S and a 20S RNA. The 32S RNA (Figs. 1e and 2) is easily identified as the 5’ end of the 45S molecule, containing the 28S region and an additional segment with several loops. The concentration of 20S RNA is low in the nucleolus of normal cells (1, 2). A fraction with the sedimentation and electrophoretic behavior of 20S RNA was purified by two cycles of centrifugation. It contained two types of molecules in about equal frequency. One type (Figs. 1a and 2) has the structure expected for the immediate precursor of 18S RNA: it contains a long extended
FIG. 1. Precursor rRNA and rRNA from HeLa cells after they were spread from 80% formamide and 4 M urea. Molecules are shown in reverse contrast. (a) 45S rRNA; (b) tracing of the molecule in a showing each of the secondary-structure regions as hairpin loops (28S and 18S rRNA regions are indicated); (c) 41S rRNA; (d) 24S rRNA; (e) 32S rRNA; (f) 28S rRNA; (g) 36S rRNA; (h) true intermediate 20S rRNA and '20S' rRNA (arrow); (i) 18S rRNA. Bar is 0.2 μm.
region, and two loops at its 5'-end. In terms of its structural map and in its absolute length (Table 1) it corresponds to the difference between the 41S and 32S RNA molecules. The other molecule in the 20S fraction (Figs. 1a and 2, 20S' RNA) originates from the 5' end of the 28S rRNA molecule. It cannot, therefore, be an intermediate of a processing pathway. Whether this molecule is formed in vivo or is an artifact of isolation is unknown.

Additional minor RNA components were observed. In the fraction containing mainly 41S RNA molecules we observed a class of molecules that we tentatively name 36S rRNA (36S RNA has been observed in HeLa cells and was considered not to be on the normal processing pathway; ref. 2). The 36S RNA molecule (Figs. 1g and 2) is derived from the 3' end of the 45S RNA; it is the expected product of a split that cleaves 45S RNA at the 3' end of the 28S RNA region. The proportion of 36S RNA in total nuclear RNA is very small. Another fraction was obtained by centrifugation of the light shoulder of the 28S RNA from these nuclei. The electrophoretic mobility of this RNA fraction suggested a molecular weight of 1.38 X 10^6. This RNA is probably the species named 24S RNA by Weinberg and Penman (2). In terms of its map and its length, it is identified as the 3' end of the 45S RNA molecule, which is cleaved in the processing step leading to 41S RNA (Figs. 1d and 2, and Table 1). Another molecule in this fraction, 24S RNA (Fig. 2), is derived from the 3' end of the 32S RNA. It contains at its 5' end those sequences of the 28S RNA that are missing in the 20S RNA molecule.

**Polarity of HeLa rRNA.** To assign 5'-to-3' polarity to these molecules, we partially digested 28S RNA with the 3'-exonuclease from ascites cell nuclei (6). Several molecules were photographed unselected and traced. The maps (Fig. 3) show clearly that these molecules lost various lengths from the multiple-loop region next to one end, while the other end, which contains the characteristic double loop, was left intact. Therefore, the end of 28S RNA without secondary structure is the 5' end and it is this end that forms the 5' end of the 45S RNA. A similar experiment was done with 45S RNA. Digestion products were found which had the 28S region intact and had lost various portions from the spacer* next to the 18S region, and from the 18S region itself.

**Processing Pathway of HeLa rRNA.** The maps derived here are static and cannot in themselves define a processing pathway, but they put constraints on any possible pathway. In conjunction with earlier work on kinetics of labeling, molecular weight based on gel electrophoresis, methylation patterns, and partial sequence analysis (1-3), these maps and the molecular weights listed in Table 1 define an unambiguous processing pathway (Fig. 4).

**Preliminary Experiments on the Basis of the Observed Secondary Structure.** The observed loops are likely to be due to complementary regions in the RNAs, and they appear to be related to high GC content. Several RNAs of lower GC content (18S RNA, rRNA from E. coli and from mitochondria, and two types of mRNA) did not show any secondary structure under these conditions. We attempted to eliminate the secondary structure by prior denaturation of the RNA in formaldehyde. RNA was heated in 3% or 10% formaldehyde in phosphate buffer for 10 min at 63° (7). The solutions were cooled, urea and formamide were added as before, and the samples were spread. This procedure reduced, but did not eliminate, the secondary structure (Fig. 5). After treatment with 10% formaldehyde, 28S RNA has very little structure left, mostly the double loop at about 0.5-0.8 μm. The two branches of the loop are now separated by a stretch of single-stranded RNA. When 45S RNA was treated with 10% formaldehyde most of its secondary structure was also lost, the most prominent resistant feature again being the double loop at 0.5-0.8 μm.

The lengths of the major nucleolar RNAs were measured with and without treatment with formaldehyde (Table 1).

* By “spacer” we denote regions of precursor molecules that are removed during maturation of 18S and 28S rRNA.
FIG. 2. Secondary-structure maps of HeLa precursor rRNA and rRNA. All maps are drawn to the same scale, which is measured in \( \mu \text{m} \). Thick lines represent the single-strand length of RNA within double-stranded hairpin loops. Thin lines represent regions of RNA without evident secondary structure. Vertical bars segment the looped regions to indicate the number of consecutive loops. Molecules placed under each other signify structure relations; note that the 3'-terminal components (36S and 20S RNAs) are slightly offset to the right of their respective “parent” molecules.

FIG. 3. Maps of 28S RNA molecules partially digested with 3'-exonuclease. All molecules on a set of photographs were traced. Only one of the 15 intact molecules is shown.

FIG. 4. See page 2831.

FIG. 5. Maps of 28S RNA after treatment for 10 min at 63° with 3% (a) or 10% (b) formaldehyde.
Lengths of molecules largely extended by formaldehyde treatment were the same as the lengths of the same molecules with secondary structure, provided that loops were measured twice ("up and down"). This fact supports our interpretation of the secondary structure as hairpin loops.

**DISCUSSION**

It has been observed that RNA spread for electron microscopy frequently contains collapsed regions. However, only one recent paper pointed out that these regions are at reproducible sites of RNA molecules (8). An observation closely related is that of Wu and Davidson (9) on the loops in single-stranded DNA at sites corresponding to tRNA genes. The secondary structure observed in the present work is likely to be related to the high GC content of the RNA molecules studied. It will be important to test whether changing the spreading conditions will reveal a regular secondary structure in RNAs of lower GC content.

Our secondary-structure maps and the independently measured lengths of the various HeLa rRNA molecules are in excellent agreement with one another, and they combine to confirm the previously derived processing pathway (1–3; Fig. 4). The first step is likely to be carried out by an endonuclease since the 3'-terminal fragment that is removed was found (24S RNA). We have no information whether the trimming of 32S and 20S RNAs proceeds endo- or exonucleolytically. If an exonucleolytic mechanism is involved, two different enzymes would be required, since 32S RNA (like 45S RNA) contains spacer sequences at its 3' end, while the 20S RNA is the only nucleolar molecule with spacer sequences at its 5' end.

None of the apparent endonucleolytic cleavage sites is located within a loop. It is not clear whether this fact is a coincidence or a feature of the cleavage mechanism.

The 28S region is at the 5' end of the 45S RNA. This conclusion agrees with the work of Choi and Busch (10) on rat 45S RNA, and with the preferred model of Perry and Kelley (11) for L-cell RNA. We also confirm the latter authors' conclusion that a spacer region is at the 3' end of the 45S and the 32S RNAs (11). In the frog *Xenopus laevis* Reeder and Brown (12; see also ref. 13) concluded that the 18S region is closer to the 5' end of the 40S rRNA precursor. The structure of this precursor molecule is presently under study by electron microscopy, but it is already clear that the arrangement of secondary structure regions in this molecule is very similar to that in HeLa 45S rRNA.

Secondary-structure mapping of RNA may be a generally applicable method for elucidation of structural relations. Even if the method should prove restricted to rRNA and its precursors, it will be valuable in delineation of the evolution of the arrangement of rRNA sequences.

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