RIBOSOME PROCESSING IN HELA CELLS

Studies on Structural Aspects of Precursor and Mature Ribosomes

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ABSTRACT

Mature ribosomes, their subunits, and their ribonucleoprotein precursors were extracted from HeLa cells and prepared for electron microscopy by a method based on adsorption of nucleic acid to a charged grid. Images so obtained revealed the presence of two types of ribonuclease-sensitive fibrils of consistent morphology. One of these types of fibril is present on the 80S and 55S nucleolar ribonucleoprotein precursors and the mature 60S subunit. The other type is restricted to the 80S precursor. The morphology of the fibrils and their distribution in the various preparations suggest identification with known secondary structural features of isolated pre-rRNA.

Mature eukaryotic ribosomes are derived from large nucleolar precursors by a series of cleavage steps (13, 27). In HeLa cells, the primary transcript of the ribosomal RNA (rRNA) genes is part of a ribonucleoprotein (RNP) particle migrating at about 80S on sucrose gradients. The 80S precursor RNP is converted to a 55S particle, which is the immediate precursor to the large ribosome subunit (25). The cleavage steps and some properties of the RNA and RNP intermediates have been extensively characterized (13, 25–27).

These various processing steps suggest a gradual transfer of the ribosome from its site of synthesis in the nucleolus to its site of utilization in the cytoplasm, and raise the possibility of concomitant structural modifications en route. In an attempt to investigate some of these putative structural features, we examined purified precursors and subunits with an electron microscope technique based on absorption of nucleic acid to a charged grid (8, 10). Our results show that mature HeLa ribosome large subunits and their precursors possess identifiable RNase-sensitive projections or fibrils. The morphology and distribution of the fibrils among the ribosomal RNP fractions suggest that they may be identical with secondary structural features of deproteinized ribosomal precursor RNAs (pre-rRNA) as visualized by the modified Kleinschmidt technique developed by Wellauer and Dawid (28, 29).

MATERIALS AND METHODS

CELL CULTURE: Suspension cultures of HeLa cells (S3 strain) were maintained at between 3 and 6 × 10^5 cells/ml by daily dilutions with fresh Eagle's medium supplemented with 7% horse serum; and were prepared for cell fractionation as described earlier (13).

FRACTIONATION OF RIBOSOMES AND PRECURSORS: Cells were harvested by rapidly chilling the culture with frozen balanced salt solution, and were deposited by centrifugation at 1,000 *g* for 3 min. Isolation of nuclei, preparation of a nucleolar fraction, and resolution of the ribosomal precursors were done as described (13, 15), with minor modifications.

Ribosomes from the cytoplasmic fractions were concentrated by magnesium precipitation as described (26). The ribosomal pellet was resuspended in EDTA-con-

taining buffer (NEB:1.01 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.2) and the subunits were resolved on sucrose gradients made in NEB ("EDTA method") or low salt buffer containing magnesium (LSB: 0.01M KCl, 0.0015M $MgCl_2$, 0.01 M Tris-HCl, pH 7.2, "EDTA-Mg^{++} method"). Subunits were also prepared by treatment with puromycin in either of the following ways: (*a*) cells were grown in 100 μg/ml puromycin for 10 min at 37°C, were fractionated as above, and the ribosome fraction was resuspended in high salt buffer (HSB-1: 0.5M KCl, 0.02M Tris-HCl, pH 7.2, 0.005M $MgCl_2$) and fractionated on gradients made in HSB-1; or (*b*) a post-mitochondrial supernate was treated with 1 mM puromycin for 20 min at 37°C., with KCl and $MgCl_2$ added to the concentrations of HSB-1, and the subunits were fractionated on sucrose gradients made in 0.01M Tris-HCl, pH 7.2, 0.35M KCl, 0.005M $MgCl_2$ (HSB-2). These procedures gave ribosomal subunit populations which were indistinguishable morphologically. Gradients were collected with a continuously monitoring Gilford spectrophotometer set at 260 nm.

RNASE TREATMENT: Precursor and mature ribosomal particles were diluted to a concentration of 0.1 absorbance unit (260 nm) per milliliter, in buffers used for sucrose gradients, with sucrose omitted. Other buffers used are described in the text and figure legends. For ribonuclease treatment, 80 ng RNase A and 0.08 unit RNase T_1 were added to each milliliter of the diluted particles. Incubations were carried out for 1 h at 0°C or at room temperature. The reaction was terminated by addition of 1/4 volume of 25% glutaraldehyde neutralized with 1 M $NaHCO_3$. After 15-min fixation, particle suspensions were divided into aliquots and frozen.

ELECTRON MICROSCOPY: Grids for the procedure referred to as the "charged-grid" technique were prepared as described previously (8). Particles at the above concentration were mounted on grids and stained with methanolic uranyl acetate as before (8). Routinely, particles were fixed as described above and frozen before use; a single freeze-thaw cycle had little effect on morphology. Electron microscopy was performed with a JEOL-100B or Siemens 101A, at magnifications of × 50,000 to 100,000, with a 30-μm objective aperture. Magnifications were calibrated with a diffraction grating replica (58,000 lines per inch). Anticontamination devices were always used.

RESULTS

Precursor Ribonucleoprotein Particles

The major features of ribosomal RNA maturation in HeLa cells, and isolation procedures for precursor particles, have been described previously (13, 25–27). Fractions of 80S and 55S precursors used for electron microscopy were homogeneous as defined by the presence of one major species of RNA, 45S, and 32S, respectively.

Figure 1*a* shows 80S precursor RNP particles as imaged by the charged-grid technique. The particles appear to consist of two components: a network of fine fibrillar material, containing many branches or intersections, and an amorphous, variably staining component with which the fibrils are intimately associated. In many particles (55%) the amorphous component is roughly divided into two (or sometimes more) regions, connected by a thinner or less dense area, or, in some cases, fibrils. The thickness of the fibrils is about 1 nm and is fairly uniform. In Figure 1*b*, particles diluted into NEB were treated with RNase before mounting on the grids. Under these conditions fibrils are completely abolished, and the images consist of a heterogeneous population of spots of varying density. The fibrils thus appear to consist of RNA or require RNA for their integrity. The amorphous component may correspond at least in part to the protein component of the precursors.

Inspection of a large number of 80S particles reveals two types of fibrillar projection which are consistent morphological features of these particles (Table 1). Type I, shaped like a Y or like two fibrils appearing to originate from the same point on the particle, can be recognized on 13% of the particles. Type II, of a more complex shape best represented diagrammatically (Figure 2*d*), is recognizable on 24% of the particles, of which the majority (80%) are divided into two or more regions. Figure 2*a–c* shows several examples of 80S particles showing both types of fibril. Such particles, which are always bi- or multipartite, are present at a frequency of 6.5%. The true frequencies of these fibrils are probably much higher since the method of preparation seems to influence their visibility (see below).

55S precursor RNP particles from the same preparation are shown in Fig. 3. Like 80S particles, they consist of amorphous and fibrillar components, but are of less morphological complexity in that fewer particles show division into regions (Table 1). RNase treatment of 55S particles yields images indistinguishable from those of Fig. 1*b*. Fibrils which are Type I, as judged by their morphology and dimensions, are present at a slightly higher frequency (16%) than on 80S particles. Type II fibrils, however, are virtually absent (0.8%).

Particle sizes are difficult to measure on charged-grid preparations. Negatively stained 80S and 55S precursors (stained with 0.5% aqueous uranyl acetate) were 678 ± 208 Å (u = 46) and

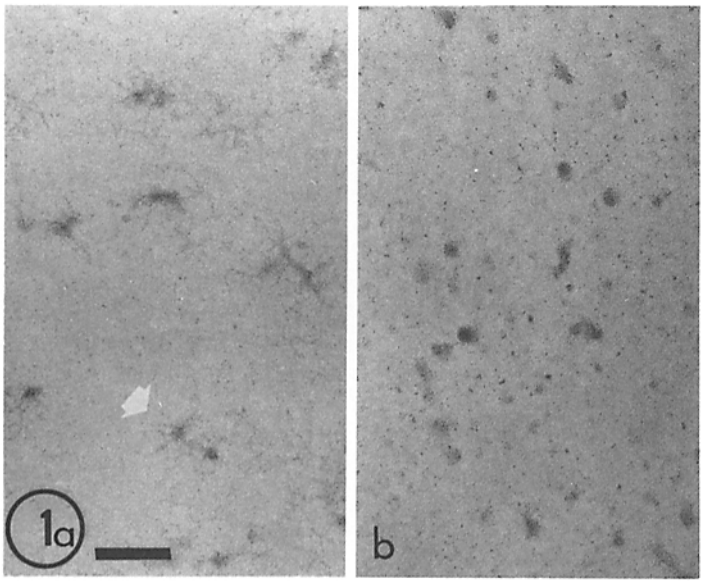

FIGURE 1 80S nucleolar ribonucleoprotein particles. (*a*) As isolated. Arrow indicates particle which would be scored as bi- or multi-partite. (*b*) Particles treated with RNase, 0°C., 60 min., in NEB buffer. × 100,000. Scale bar, 100 nm.

TABLE 1

Fibril Dimensions and Distribution

Origin and types of ribosomal particles	Bi-(multi-) partite	Type I fibril		Type II fibril	
		Particles	Length	Particles	Length
	%	%	Å (SD)	%	Å (SD)
Nucleolar					
80S	55	13	‡746 (102) n = 25	24	‡1451 (213) n = 16
55S	17	16	797 (87) n = 29	0.8	–
Cytoplasmic					
60S	0	70*	784 (86) n = 22	0	–
40S	0	1	–	0	–

* Measured on puromycin subunits (cf. Results).

‡ See Figure 2*d* for portions of fibrils actually measured.

398 ± 62 Å (u = 51) in their largest dimension, respectively (results not shown).

Mature Ribosome Subunits

Preparation of the precursor RNPs with EDTA is essential since in the presence of divalent cations they aggregate in a nonspecific fashion (14). Since homogeneous preparations of large and small mature ribosome subunits can be made in both the presence and absence of Mg^{++}, it was of interest to determine whether the fibrillar projections were artifacts produced by the chelation of Mg^{++}. Subunits were prepared from ribosomes by three methods (cf. Materials and Methods): the EDTA method, the EDTA-Mg^{++} method, and the puromycin procedures. The results are shown in Fig. 4. Large subunits from all three methods showed fibrillar projections of which the most prominent is Type I as judged by morphology and dimensions (Table 1). Small subunits, photographed under conditions in which fibrils are clearly demonstrable, occasionally showed fibrillar extensions; however, Type I fibrils were rare and probably may be ascribed to contaminating 60S subunits (Fig. 4*d*). Type II fibrils were totally absent from both large and small subunits.

Although the different isolation procedures have no effect on the presence of rRNA in the

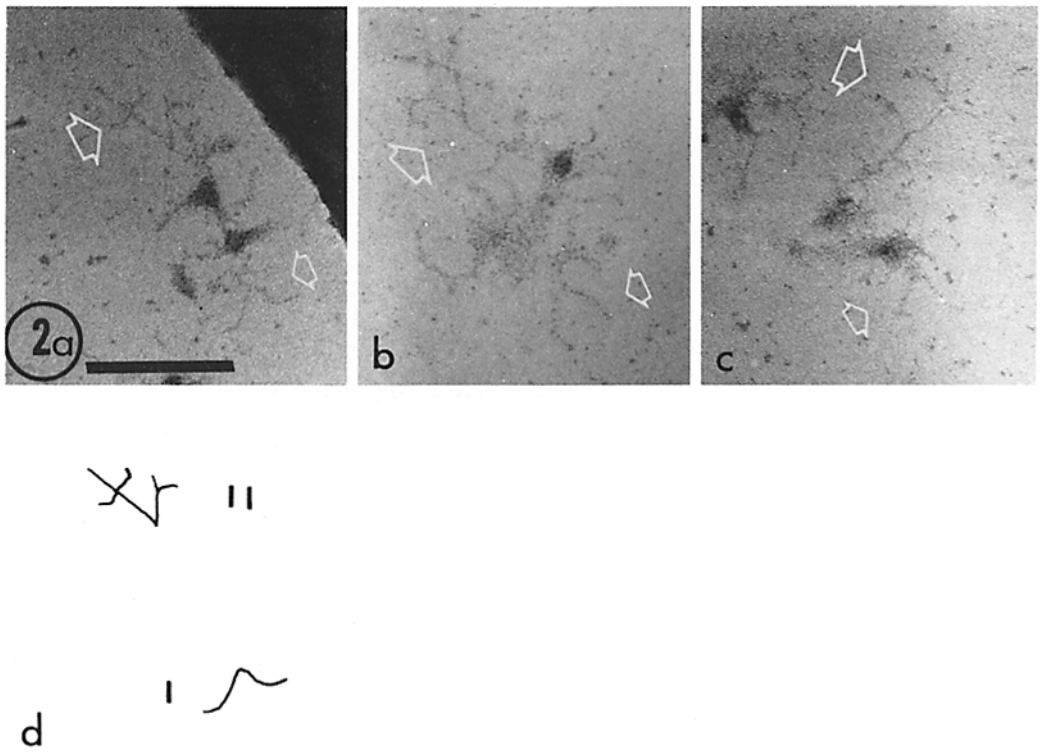

FIGURE 2 (*a*, *b*, and *c*) 80S nucleolar ribonucleoprotein particles showing both Type I (small arrows) and Type II (large arrows) fibrils. (*d*) Tracing of the fibrils in (*a*) shows portions measured dimensions of which appear in Table I. × 200,000. Bar, 100 nm.

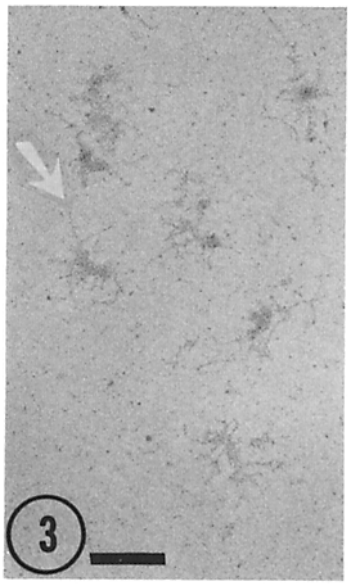

FIGURE 3 55S nucleolar ribonucleoprotein particles. Arrow indicates a Type I fibril. × 100,000. Bar, 100 nm.

subunits, the frequency of Type I fibrils in large subunits isolated on EDTA-containing gradients is lower than on subunits isolated in the presence of Mg^{++} (39% and 70% respectively). This may be due to EDTA-induced "unfolding" of the subunits (16) which could militate against proper scoring of the fibrils. In the case of the precursors, their much greater mass in combination with such unfolding may further reduce the chance that a fibril be oriented properly for unambiguous recognition.

The images obtained after RNase treatment of large subunits depend on the method of isolation and the digestion conditions. Subunits prepared with EDTA and digested with RNase in the presence of EDTA yield images similar to the ribonuclease-digested precursor particles, in that the fibrils are completely abolished (Fig. 5*a*). Puromycin subunits that are ribonuclease treated in HSB-1 buffer at 0°C are indistinguishable from undigested controls (Fig. 5*b*). If puromycin subunits are digested at room temperature in HSB-1, some subunits lose their attached fibrils while others

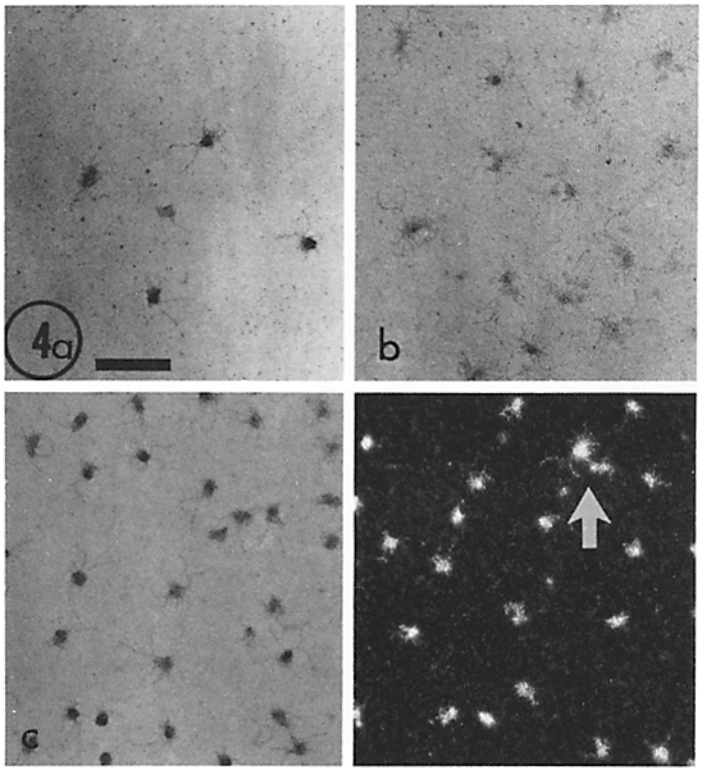

FIGURE 4 Mature ribosome subunits. (*a*) 60S, puromycin procedure. (*b*) 60S, EDTA procedure. (*c*) 60S, EDTA-Mg^{++} procedure. (*d*) 40S subunits, puromycin procedure, imaged by dark-field optics. Arrow indicates a particle with a Type I fibril, which is probably a contaminating 60S subunit (cf. Table I). × 100,000. Bar, 100 nm.

retain them (Fig. 5*c*). Puromycin subunits that are diluted into salt-free buffer before ribonuclease treatment at room temperature lose the fibrils completely, although the particles retain a compact structure different from that of particles digested in EDTA (Fig. 5*d*). Puromycin-derived subunits that were diluted into salt-free buffer and incubated without ribonuclease retain Type I fibrils (Figure 5*e*) as in untreated controls.

Some images of control and partially digested subunits suggest that the Type I fibril may have a substructure. Fig. 6 shows several images in which the stem of the fibril appears to be doubled, or in which a bridge seems to join the arms of the Y. It is possible, however, that the doubled stem is a negatively stained image of a single fibril.

Reassociated Monomers

It seemed possible that the Type I fibril might be involved in the binding of the two subunits together. To test this possibility, puromycin runoff monomeric ribosomes were prepared by growing cells in the presence of puromycin as described. Part of a post-mitochondrial supernate from those cells was layered on sucrose gradients made up in LSB-buffer in order to recover monomeric ribosomes (Procedure 1). The rest was dissociated into subunits in the presence of high salt (Procedure 2). The isolated subunits were recombined and dialyzed against LSB, and the reassociated monomers were purified on sucrose gradients made up in LSB-buffer as before. Electron micrographs of native and reassociated monomers are shown in Fig. 7. Type I fibrils are present in both preparations, indicating that they are not an artifact of dissociation. If the fibril is involved in subunit binding, there is no requirement for its association with the ribosome to be permanently altered at the level of resolution of this experiment.

A summary of the fibril dimensions and morphological features discussed is found in Table 1.

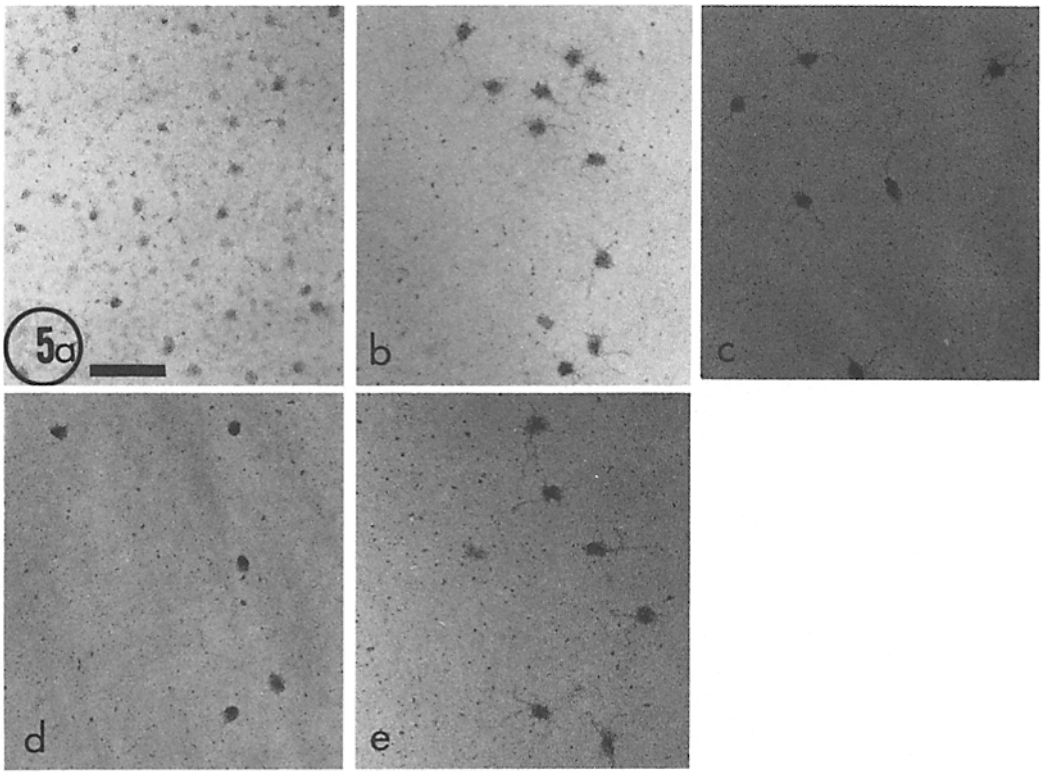

FIGURE 5 Ribonuclease treatment for 60S subunits. (*a*) EDTA method, 0°C., 60 min., in NEB buffer. (*b*) Puromycin procedure, 0°C., 60 min., in HSB-1. (*c*) Puromycin procedure, 22°C., 60 min., in HSB-1. (*d*) Puromycin procedure, 22°C., 60 min., in HSB-1 diluted 20-fold into 0.01 M Tris-HCl, pH 7.4. (*e*) As (*d*), but without RNase. × 100,000. Bar, 100 nm.

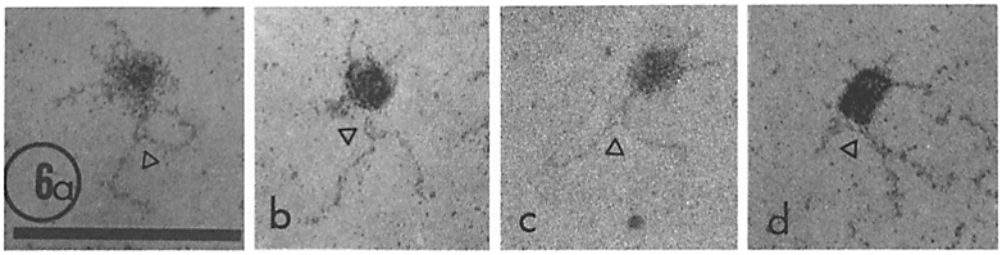

FIGURE 6 Possible substructure in Type I fibrils. (*a*, *b*, and *d*) Puromycin procedure. (*c*) EDTA-Mg^{++} method, RNase, 0°C., 60 min., in HSB-1. Points indicate bridge-like structures connecting distal arms of fibrils, or regions where the proximal stem appears doubled. × 300,000. Bar, 100 nm.

DISCUSSION

General

Other works have presented micrographs of isolated rRNP precursors (11, 18, 20, 23). Some have described fibrillar material tentatively identified as RNA (11, 18, 23); however, no morphologically consistent features of the fibrils were noted. Sedimentation studies on *Escherichia coli* ribosomes (7) and some electron microscopic studies on mature eukaryotic ribosomes (3, 6, 17) also suggest the presence of fibrillar or RNase-labile appendages. Meyer et al. (19) demonstrated a single RNase-labile 'tail' on the large subunit of rat liver ribosomes, and reported seeing similar structures on ribosomes from HeLa cells, rabbit

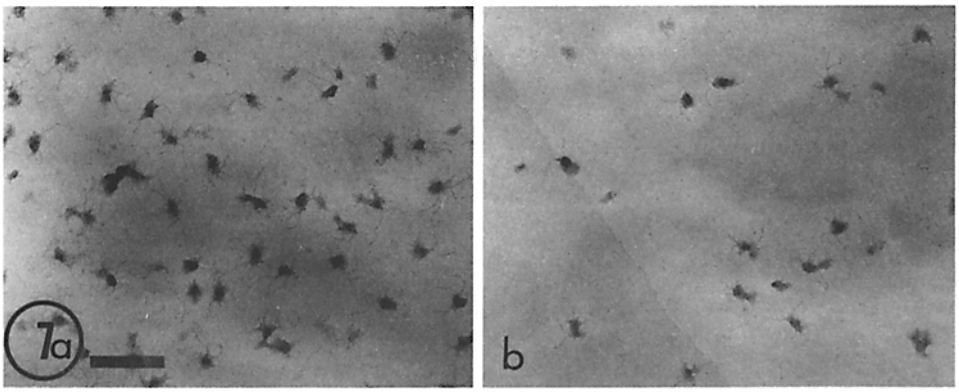

FIGURE 7 Native (*a*) and reassociated (*b*) monomers showing Type I fibrils. See Results. × 100,000. Bar, 100 nm.

reticulocytes, mouse brain, and chicken liver cells, but not on yeast or bacterial preparations. It is not clear whether the Type I fibril reported here corresponds to these observations.

Numerous studies attest to the susceptibility of ribosome subunits to nucleases (2, 15, 24). The amount of nuclease-accessible rRNA ranges from 10 to 25% in various reports (2, 15). The Type I fibril, if assumed to be doubled-stranded, as suggested by the observation of substructure within it and by its response to RNase under varying conditions of salt and temperature, represents about 8% of the large subunit RNA of HeLa cells (assuming 3.3 Å interbase distance and a molecular weight of 1.9×10^6 for HeLa 28S rRNA [1]). This is likely to be an underestimate, since the stem portion of the fibril was not measured. There is also some reason to doubt the validity of assuming a 3.3 Å interbase distance for this type of preparation (see below).

Fibril Morphology

In 1973, Wellauer and Dawid (28) published a modification of the Kleinschmidt procedure (9) which, when applied to HeLa cell rRNA and its precursors, showed that certain regions of the RNA possessed consistent secondary structural features under conditions of partial denaturation. Two of the most prominent of these features were (*a*) a double loop in the central portion of the 28S rRNA molecule; and (*b*) a large branched loop in that portion of the transcribed spacer region immediately 5′ (in the revised polarity [4]) to the portion, of the 45S precursor containing 18S rRNA. We speculate that these structural features may be identical to Type I and Type II fibrils, respectively, on the basis of the following considerations: (*a*) The morphological resemblance is striking, particularly in the case of the Type II fibril (compare Figs. 2 and 8). (*b*) Given this identification, the distribution of the fibrils in the rRNP fractions is exactly that expected on the basis of the HeLa cells' processing pathway for rRNA (27, 28). (*c*) While the dimensions that we obtain for the fibrils are only about 60% of those obtained by Wellauer et al. (29) for their presumed counterparts in HeLa rRNA, the ratios of the lengths of Type II to Type I fibrils are nearly the same (1.9 for the present study, using the average value of all measured Type I fibrils; and 2.0 for their presumed counterparts, from data given and measurements made on micrographs in Wellauer et al. (29)). At present, we have no convincing explanation for the discrepancy in absolute dimensions. However, measurements of SV40 viral DNA adsorbed to similarly treated grids consistently give a greater mass per unit length than that predicted from Watson-Crick base pairing (T. Johnson, unpublished observations).

If the fibrils are the same as those seen in deproteinized pre-rRNA, several interesting points are raised. The possibility that these regions are not bound, or are loosely bound, to the main bulk of the ribosomal particles may be related to the evolutionary lability of these regions (22), particularly the transcribed spacer portion (Type II fibril). The regions of secondary structure in isolated rRNA are inferred to be double-stranded on the basis of their stability in the modified Kleinschmidt technique (28, 29), and their resistance to nuclease digestion (22); they consist of

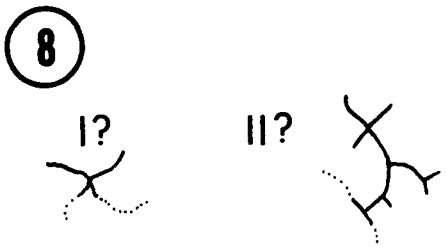

FIGURE 8 Possible counterparts of Type I and Type II fibrils in 45S precursor rRNA from HeLa cells, visualized by the modified Kleinschmidt procedure of Wellauer and Dawid (27). Redrawn from Plate III of Wellauer et al. (28). The dotted lines indicate the continuation of the single-stranded portion of the RNA strand.

about 80% rG.rC in nearly equimolar proportions (22). Our observations may thus suggest a negative correlation between extensive regions of secondary structure and protein binding, although the level of resolution as yet is crude.

Initial experiments with ribosomes from other sources (rat liver, BHK, and chick embryo) indicate that these ribosomes also possess structures probably analogous to Type I fibrils, supporting the suggestion of Meyer et al. (19) that RNase-labile appendages may be of widespread occurrence in eukaryotes. This might be expected if our assignment of Type I to the double loop in 28S rRNA is valid, since the double loop appears in many rRNAs (22). Experiments are in progress to permit better assessment of the validity of identifying these fibrils with the regions of rRNA secondary structure.

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