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# Synthesis and Maturation of Ribosomal Ribonucleic Acids in Isolated HeLa Cell Nuclei

## A TRACER STUDY ON THE TOPOLOGY OF THE 45S PRECURSOR OF RIBOSOMAL RIBONUCLEIC ACIDS

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The synthesis and processing of RNA by isolated HeLa cell nuclei was studied at low ionic strength in the presence of α-amanitin. The RNA polymerase reaction, with endogenous template and enzyme, rapidly reaches a plateau dependent on the amount of nuclei. Evidence is presented that incorporation of [$^3$H]UMP proceeds only in growing RNA chains, whereas initiation of new RNA chains is arrested. The product formed contains all the main components of the 45S pre-rRNA (precursor of rRNA) maturation pathway (45S, 32S and 20S pre-rRNA; 28S and 18S rRNA). Most of the labelled material is in the mature rRNA components and their immediate precursors, even at very short times of incubation (2min). Small, but definite, 5S and 4S RNA peaks are also observed. At shorter incubation times a substantial amount of [$^3$H]UMP is incorporated into RNA molecules in the 24S and 10–16S zones. This RNA material is considered to represent the non-conserved segments of 45S pre-rRNA in the process of nucleolytic degradation. A model for the tracer study of the topology of 45S pre-rRNA, on arrest of rRNA initiation, is discussed. The experimental evidence obtained supports the following structure of 45S pre-rRNA: 5′-end–28S rRNA unit–18S rRNA unit–non-conserved segment–3′-end.

The study of regulatory mechanisms in rRNA synthesis requires experimental systems capable of carrying out the correct transcription of rRNA genes.

A straightforward approach would be the transcription of isolated rRNA genes by purified RNA polymerase A. Transcription of isolated DNA by purified RNA polymerase A yields results showing that the enzyme initiates at nicks, ends or denatured regions of the template (Butterworth *et al.*, 1971, 1973; Meilhac & Chambon, 1973). Further, it was shown in two cases that purified RNA polymerase A does not transcribe isolated homologous chromatin (Butterworth *et al.*, 1971; Wintersberger *et al.*, 1973). On the other hand, transcription of isolated rRNA genes by purified *Micrococcus luteus* RNA polymerase has shown that with this template the enzyme initiates also at nicks acting as pseudo-promotors (Hecht & Birnstiel, 1972).

The study of transcription in isolated nuclei or nucleoli, with endogenous template and enzyme, may be an alternative system for studying rRNA synthesis. The transcription of rRNA genes by isolated nuclei has been shown by characterization of the product by nucleotide composition (Takahashi *et al.*, 1963; Widnell & Tata, 1964, 1966; Pogo *et al.*, 1967; Blatti *et al.*, 1970), nearest-neighbour nucleotide frequency (Widnell & Tata, 1966), 2′-*O*-methylation of nucleotides (Al-Arif & Sporn, 1972) and hybridization to isolated ribosomal DNA (Reeder & Roeder, 1972). The presence of $Mg^{2+}$ and low ionic strength appear to favour RNA polymerase A and transcription of rRNA genes (Widnell & Tata, 1966; Blatti *et al.*, 1970; Moulé, 1970; Smuckler & Hadjiolov, 1972).

Attempts at the molecular characterization of the product of rRNA synthesis in isolated nuclei have been made with nuclei from liver (Younger & Gelboin, 1970; Smith & Arnstein, 1972), HeLa cells (Zylber & Penman, 1971), mouse myeloma (Marzluff *et al.*, 1973) and *Rana pipiens* embryos (Caston & Jones, 1972), as well as with nucleoli of liver cells (Grummt & Lindigkeit, 1973). The results of these studies are variable, probably owing to interference by unspecific nucleolytic degradation, inadequate fractionation of the product or other unknown factors.

Here we report results on the synthesis and maturation of rRNA by isolated HeLa cell nuclei. The characterization by gel electrophoresis of the rRNA products made in our system revealed that they correspond to those formed *in vivo*. Hadjiolov (1967)

proposed that a tracer study on the topology of pre-rRNA* should be possible under some critical conditions. Since we have shown that in our system these conditions are reasonably met, the subject was studied further and a model is offered for the structure of HeLa cell 45S pre-rRNA and the respective transcription unit.

## Experimental

### *Isolation of nuclei*

Experiments were done with a hyperdiploid strain of HeLa cells grown as a monolayer culture. The cells were harvested in cold Hanks (1948) balanced salt medium in the exponential phase of growth (24h cultures) and sedimented for 2min at 600*g* at 4°C. All subsequent manipulations were carried out at 4°C with ice-cold media. The cells were suspended by gentle shaking in RSB medium (10mM-Tris–HCl, pH7.6 at 4°C, 10mM-KCl, 1.5mM-$MgCl_2$) and sedimented for 2min at 600*g*. The sediment (approx. $4\times10^7$ cells) was suspended in 40ml of RSB medium containing 2mM-dithiothreitol and swollen for 10min. The suspension was homogenized with 20 strokes in a hand-driven tight-fitting glass–Teflon Potter homogenizer and centrifuged for 2min at 600*g*. The supernatant was aspirated and the crude nuclear pellet was then suspended in 20ml of the same RSB medium plus dithiothreitol. The suspension was homogenized with 10 strokes, layered over 50ml of RSB medium containing 0.5M-sucrose and centrifuged for 3min at 800*g*. The supernatant was aspirated and nuclei were suspended in 50mM-Tris–HCl (pH7.9 at 30°C)–20% (v/v) glycerol–5mM-dithiothreitol. Nuclear counts were determined in a Burker haemocytometer and samples were taken for DNA (Burton, 1956) and protein (Lowry *et al.*, 1951) determinations. Calf thymus DNA and crystalline bovine serum albumin were used as standards. The nuclear suspension was used immediately.

### *Incubation for measurement of RNA polymerase activity*

The standard incubation medium contained in 0.08ml: $10^6$ nuclei (about 100$\mu$g of protein and 15$\mu$g of DNA), 62mM-Tris–HCl (pH7.9 at 30°C), 10mM-KCl, 5mM-$MgCl_2$, 2.5mM-dithiothreitol, 10% (v/v) glycerol, 0.4mM each of ATP, GTP and CTP, 0.025mM-[$^3$H]UTP (1Ci/mmol) and 40$\mu$g/ml of $\alpha$-amanitin. Variations in the standard medium constituents are indicated in the text. Incubation was carried out at 30°C. The volume of the preparative incubation medium was 1.6ml (approx. $2\times10^7$ nuclei).

* Abbreviation: pre-rRNA, precursor of rRNA.

### *Extraction of nuclear RNA*

The incubation medium was mixed with an equal volume of phenol, saturated with 0.14M-NaCl (pH6.2) and containing 0.1% 8-hydroxyquinoline, and heated for 15min at 50°C with continuous stirring. The mixture was cooled for 15min to 4°C, 0.64ml of chloroform was added, and then the mixture was centrifuged for 15min at 4000*g* The water layer was aspirated and deproteinized with 1 vol. of phenol plus 0.64ml of chloroform for 15min at 4°C. The mixture was centrifuged for 10min at 4000*g* and RNA from the water layer was precipitated with 2.5vol. of 96% (v/v) ethanol for 4h at −20°C.

### *Incorporation of [$^3$H]UMP into acid-insoluble polynucleotides*

A portion (0.07ml) of the incubation mixture was pipetted on to Whatman no. 1 filter-paper discs (2.5cm diam.) and processed as described by Smuckler & Hadjiolov (1972). The dried discs were counted for radioactivity in a Packard Tri-Carb 3320 liquid-scintillation spectrometer with a toluene phosphor{toluene, 1 litre; PPO (2,5-diphenyloxazole), 5g; dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene], 250mg} at about 4% efficiency.

### *Purification of RNA*

The precipitate of nuclear RNA was dissolved in 1ml of 2mM-Tris–HCl (pH7.4) and a suspension of Dowex I (X8; formate form; 100–200mesh) was added (0.1ml containing about 50mg of resin). The mixture was stirred for 5min at 4°C, centrifuged for 5min at 4000*g* and then the supernatant was aspirated. The resin was washed once with 0.5ml of 2mM-Tris–HCl, and RNA from the combined supernatants was precipitated with 2.5vol. of 96% (v/v) ethanol plus 0.15ml of 1M-sodium acetate. The RNA precipitated overnight at −20°C.

### *Agar-gel electrophoresis of RNA*

This was done in 1.25% (w/v) agar as described by Tsanev & Staynov (1964). The separate RNA components are designated by their *s* values (Hadjiolov *et al.*, 1966). The agar-gel plates were dried and their $E_{260}$ measured. The dry film was cut into 1mm strips and the slices distributed in counting vials. Each slice was wetted with a drop of water, allowed to swell for 20min, and 0.3ml of a mixture of Protosol–toluene–water (9:10:1, by vol.) added. The vials were capped and heated for 90min at 60°C. After cooling, 5ml of a toluene–PPO–dimethyl-POPOP phosphor (as above) was added and the samples counted for radioactivity at about 12% efficiency.

*Chemicals*

Analytical-grade reagents were used throughout. α-Amanitin was a generous gift by Professor Th. Wieland, Heidelberg, Germany. ATP, GTP, CTP and UTP were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; dithiothreitol, PPO and dimethyl-POPOP were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; Protosol was from New England Nuclear Corp., Frankfurt/Main, Germany and heparin was from Spofa, Prague, Czechoslovakia. The [5,6-$^3$H]UTP (52Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Calf thymus DNA was from Sigma Chemical Co., St. Louis, Mo., U.S.A., and bovine serum albumin was from Pentex–Miles, Kankkaee, Ill., U.S.A.

## Results and Discussion

*Time-course of RNA synthesis: absence of initiation in isolated nuclei*

The synthesis of RNA by isolated nuclei is studied in the presence of α-amanitin, resulting in the complete inhibition of RNA polymerase B (see Chambon *et al.*, 1972). Addition of $(NH_4)_2SO_4$ or KCl causes a marked increase in α-amanitin-resistant RNA synthesis (Fig. 1). This is in line with observations by others (Younger & Gelboin, 1970; Grummt & Lindigkeit, 1973). However, it has been shown that a high ionic strength of the medium causes marked clumping of chromatin in isolated nuclei (Laval & Bouteille, 1973). Therefore our further experiments are performed at 10mM-KCl to minimize the damage to nuclear ultrastructure.

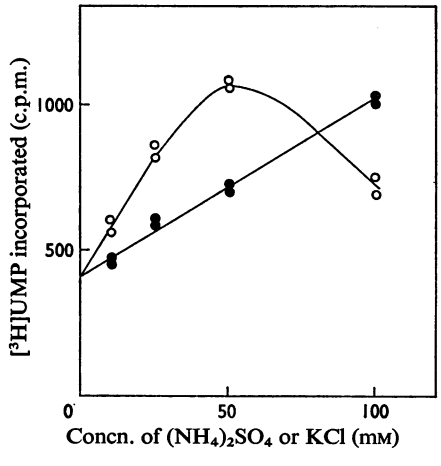



Fig. 1. *Dependence of α-amanitin-resistant RNA polymerase activity of HeLa cell nuclei on $(NH_4)_2SO_4$ and KCl concentration*

Isolated HeLa cell nuclei were incubated for 20min in the standard assay medium (see the Experimental section) in the presence of 40μg of α-amanitin/ml and various concentrations of $(NH_4)_2SO_4$ (○) or KCl (●),

The kinetics of the endogenous RNA polymerase reaction shows a rapid initial rate in the first 10–20min and reaches a plateau in about 20–40min (Fig. 2*a*). The attained plateau value is stable for at least 40min, thus indicating the absence of substantial nuclease degradation of the polynucleotide product. Further, both the initial rate and the plateau value show a linear dependence on the amount of nuclei (Fig. 2*b*). The plateau value shows only a slight and irregular dependence on added nucleotides, [$^3$H]UTP in particular. It is reached at about 1% of [$^3$H]UTP incorporated into acid-insoluble material. Therefore we considered the possibility that exhaustion of the endogenous template or inactivation of RNA polymerases may account for the rapid attainment of the plateau by the reaction.

Addition of nuclei after the plateau is reached results in further polymerization proceeding at a rate and to a plateau identical with the initial ones (Fig. 3*a*). Addition of nucleotides, including [$^3$H]UTP, does not cause additional RNA synthesis. Both results show that the plateau reached by endogenous RNA polymerase is not due to substrate exhaustion. Preincubation of nuclei for 40 or 60min in the absence of nucleotides shows that endogenous RNA polymerase is only slightly affected (Fig. 3*b*) and inactivation of the enzyme cannot account for the rapid attainment of the plateau. On the other hand, preincubation of nuclei in the presence of the four unlabelled nucleotides, followed by addition of [$^3$H]UTP, shows that endogenous polymerization is almost completely exhausted in about 40min.

The above results indicate that endogenous RNA polymerase A catalyses the elongation of pre-existing RNA chains, but cannot initiate new RNA chains. This conclusion is further confirmed by the absence of inhibition by added heparin (Table 1), a known inhibitor of initiation for both bacterial (Fuchs *et al.*, 1967) and animal, form B (Cox, 1973), RNA polymerases.

From the amount of [$^3$H]UMP incorporated into polynucleotide chains at the plateau value, we attempted to calculate the amount of rRNA formed in the absence of initiation. Considering that the HeLa cell nucleus has 1100 rRNA-transcription units (Jeanteur & Attardi, 1969) and a 'christmas-tree' distribution of 100 growing rRNA chains/transcription unit (Miller & Bakken, 1972) it is estimated that incorporation of [$^3$H]UMP proceeds up to about 10% of the maximum value. Since we do not know the extent of [$^3$H]UTP dilution by endogenous nuclear UTP, the above estimates are likely to be at the lower margin. They suggest that in isolated nuclei a substantial amount of rRNA-transcription units

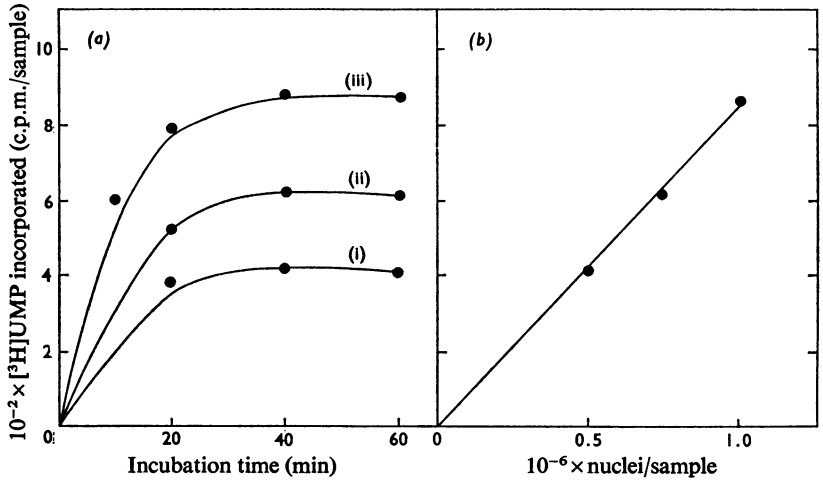



Fig. 2. *Dependence of α-amanitin-resistant RNA polymerase activity on the amount of nuclei*

The standard RNA polymerase reaction (see the Experimental section) was done in the presence of $0.5\times10^6$ (i), $0.75\times10^6$ (ii) and $1.0\times10^6$ (iii) nuclei/sample. (*a*) Time-course of the RNA polymerase reaction. (*b*) Dependence of the plateau value of the reaction on the concentration of nuclei in the assay medium.

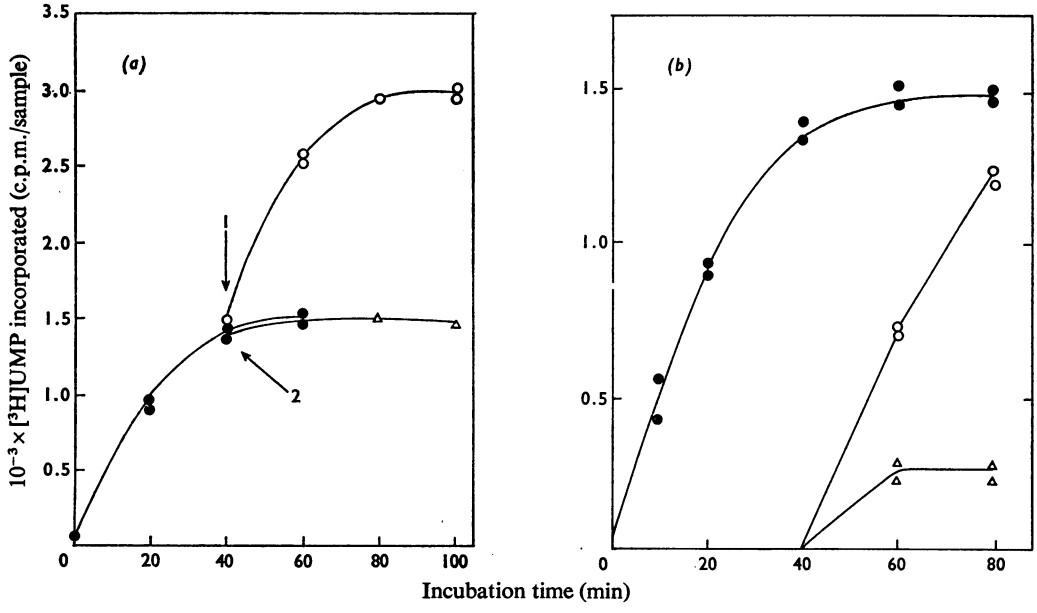



Fig. 3. *Evidence for the limiting role of the chromatin template in the α-amanitin-resistant RNA polymerase reaction of isolated HeLa cell nuclei*

(*a*) Effect of addition of nuclei (○) or substrate nucleotides (△) at plateau value of the RNA polymerase reaction (●). Incubation was in the standard assay mixture for different time-intervals. The arrows indicate the addition of: (1) $1\times10^6$ nuclei and (2) nucleotides [0.4mM each of ATP, GTP and CTP plus [$^3$H]UTP (2Ci/mmol) to 0.025mM]. (*b*) Effect of preincubation of nuclei in the standard incubation medium: controls (●) and nuclei preincubated at 30°C for 40min in the absence of substrate nucleotides and [$^3$H]UTP (○). Preincubation was in the presence of standard concentrations of ATP, GTP and CTP plus 0.025mM-UTP. After 40min at 30°C, 2$\mu$Ci of [$^3$H]UTP (sp. radioactivity 52Ci/mmol) was added (△).

Table 1. *Effect of heparin on RNA polymerase activity of isolated HeLa cell nuclei*

Isolated nuclei were incubated in the standard incubation medium with 40 $\mu$g of $\alpha$-amanitin/ml (see the Experimental section). Heparin was added to the nuclear suspension before addition of substrate nucleotides.

| Concn. of heparin ($\mu$g/ml) | Incorporation of [$^3$H]UMP (c.p.m./$10^6$ nuclei) | |
|---|---|---|
| | 10min incubation | 20min incubation |
| 0 | 410 | 590 |
| 20 | 455 | 500 |
| 40 | 450 | 580 |
| 60 | 440 | 565 |
| 100 | 390 | 520 |

is active in elongation. The reason for the observed arrest of rRNA initiation may be looked for either in a critical damage to the chromatin template or in a constant need for the supply of cytoplasmic protein factors.

*Characterization of the RNA product formed by endogenous RNA polymerase A*

The RNA isolated from HeLa nuclei incubated under conditions favourable to RNA polymerase A was analysed by agar-gel electrophoresis. As shown in Figs. 4 and 5, the 28S and 18S rRNA u.v. peaks are clearly delineated in all RNA preparations of nuclei incubated *in vitro* for at least up to 20min. The intactness of the 28S rRNA peak indicates the

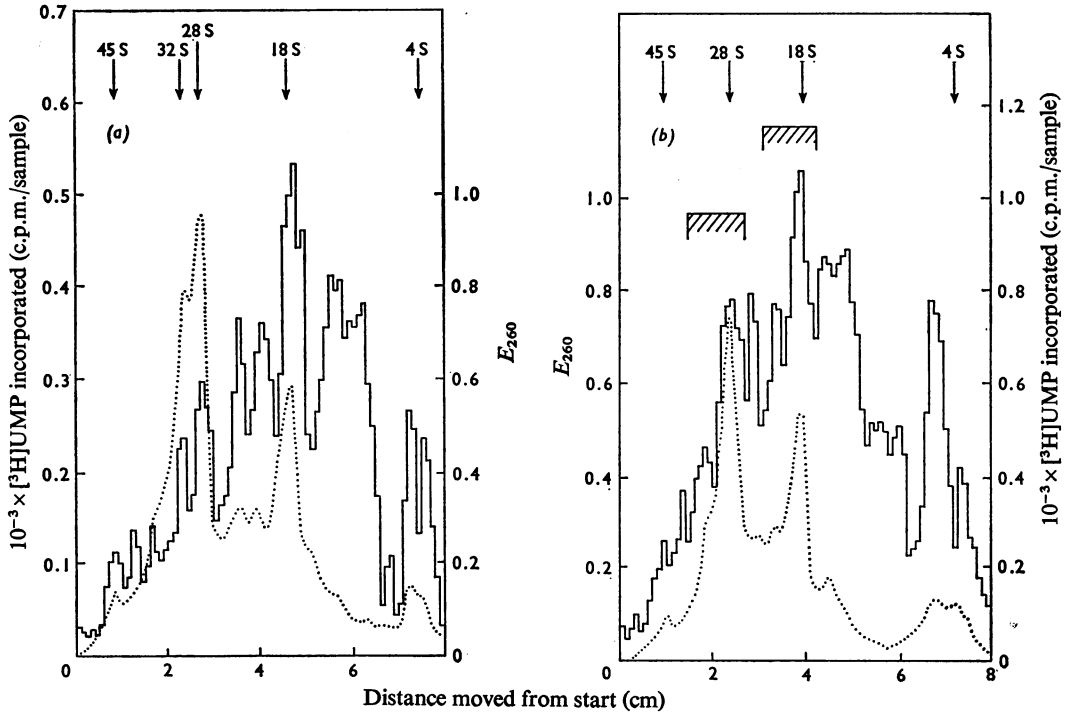



Fig. 4. *Agar-gel-electrophoresis analysis of the product of $\alpha$-amanitin-resistant RNA polymerase of isolated HeLa cell nuclei*

The nuclei were incubated in the large (1.6ml) standard incubation medium for 2min (*a*) and 10min (*b*). The sp. radioactivity of [$^3$H]UTP in the medium was twice as high (2Ci/mmol). The incubation was stopped, RNA extracted, purified and analysed as described in the Experimental section. The $E_{260}$ (·····) was recorded on the dried agar-gel films with a spectrodensitometer. The radioactivity (——) of 1mm slices of the dried agar gel was counted, after solubilization with a Protosol mixture and addition of a toluene–PPO–dimethyl-POPOP phosphor, in a liquid-scintillation spectrometer (see the Experimental section). The arrows indicate the position and the *s* value of marker HeLa cell nuclear RNA components. The shaded areas in Fig. 4(*b*) indicate the samples whose radioactivity was considered in the studies on the topology of 45S pre-rRNA (see below).

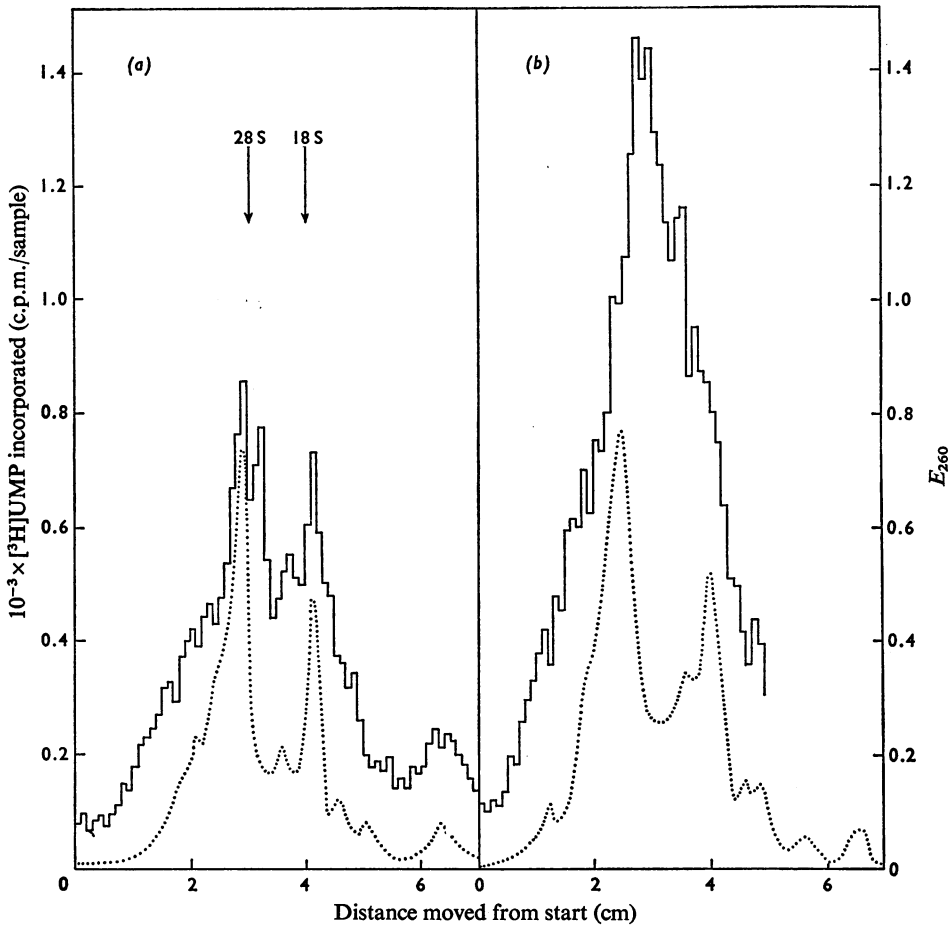



Fig. 5. *Product of α-amanitin-resistant RNA polymerase of isolated HeLa nuclei synthesized in the absence and presence of* $(NH_4)_2SO_4$

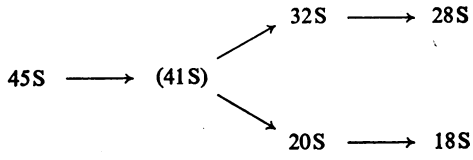
Incubation was in the large (1.6ml) standard incubation mixture (see the Experimental section) for 20min at 30°C in the presence of 10mM-KCl (*a*) or 50mM-$(NH_4)_2SO_4$ (*b*). All other procedures are as indicated in the legend to Fig. 4.

virtual absence of nuclease degradation (Venkov & Hadjiolov, 1967). Further, reproducible 45S, 32S and one or two other u.v. peaks located between 28S and 18S rRNA are observed in RNA preparations from non-incubated nuclei or nuclei incubated for 2min. The 45S and 32S u.v. peaks are decreased (or missing in some preparations) at longer incubation periods. This may reflect either degradation of some RNA species, or processing, or both.

The distribution of labelled RNA species shows several discrete peaks, which correspond qualitatively to those observed on rRNA labelling *in vivo* (see Weinberg & Penman, 1970). Under our conditions, most of the labelled RNA is distributed in peaks coinciding with 28S and 18S rRNA, as well as with 32S and 20S pre-rRNA. The higher labelling of these rRNA species is independent of the time of incubation and is observed even in HeLa nuclei incubated for 2min (Fig. 4*a*). A small, but well-defined and reproducible, 45S pre-rRNA peak is found at shorter incubation times (2, 5 and 10min). We did not observe accumulation of labelled 45S pre-rRNA or other RNA species larger than 28S rRNA, as described by others (cf. Younger & Gelboin, 1970; Zylber & Penman, 1971). Further, one or two small peaks of labelled RNA located between 45S and 32S pre-rRNA are found, especially with shorter times of incubation. These may correspond to the 41S and 36S RNA components observed *in vivo* (Weinberg & Penman, 1970). In addition, a substantial amount of labelled RNA material is found as a distinct peak at about 24S and

as a broad peak (or peaks) in the zone between 10S and 16S (see Fig. 4). This labelled RNA material has no counterpart in labelling experiments *in vivo*. In our studies it is more pronounced at shorter times of incubation and decreases in nuclei incubated for 20min (Fig. 5*a*). In one experiment, where nuclei were incubated for 20min in the presence of 50mM-$(NH_4)_2SO_4$, most of the labelled RNA constituted a broad peak at about 24S overlapping with labelled 28S, 20S and 18S RNA (Fig. 5*b*). Finally, part of the label in nuclei incubated with α-amanitin is found as discrete peaks at about 4S and 5S RNA. This observation confirms the results of Price & Penman (1972) and shows that these two RNA species are probably synthesized by an α-amanitin-resistant RNA polymerase.

The results described in this section show that HeLa-cell nuclei are able to synthesize and process pre-rRNA molecules according to a pattern analogous to that found *in vivo* (see Weinberg & Penman, 1970; Maden *et al.*, 1972). In particular, the processing steps:




are obviously taking place rapidly in isolated nuclei. The absence of accumulation of 45S pre-rRNA reflects, most likely, the preservation in nuclei of active processing enzymes, while initiation of new 45S pre-rRNA is arrested. The substantial amount of labelled RNA in the 24S and 10–16S zones is more difficult to interpret. One possibility is that these are products of some α-amanitin-resistant RNA polymerase transcribing nucleoplasmic genes (Zylber & Penman, 1971). This RNA material could also originate from the non-conserved segments of 45S pre-rRNA whose processing is slowed down in isolated nuclei. We favour the latter explanation for the following reasons: (*a*) the size of the non-conserved segment in 45S pre-rRNA is about $1.2\times10^6$ daltons, which would yield an RNA in the 24S region: further endonucleolytic split could yield 10–16S pieces; (*b*) this labelled RNA material tends to decrease on longer incubation of nuclei, whereas it accumulates when nucleases are inhibited by higher $(NH_4)_2SO_4$ concentrations.

In summary, the investigation of the product of endogenous α-amanitin-resistant RNA polymerases shows that isolated HeLa-cell nuclei are able to synthesize and process 45S pre-rRNA molecules according to a pattern similar to that observed *in vivo*. However, since initiation of new pre-rRNA molecules is blocked, most of the rRNA synthesized *in vitro* is rapidly processed and accumulates as mature rRNA species.

*Topology of HeLa-cell 45S pre-rRNA*

Hadjiolov (1967) proposed that the 5′→3′ polarity of transcription should allow a tracer study of the topology of pre-rRNA. According to current evidence (see Maden, 1971) and our present results, the primary transcription product in HeLa cells is 45S pre-rRNA, which may be considered as constituted by three units: (*a*) 32S pre-rRNA (28S rRNA+non-conserved segment); (*b*) 20S pre-rRNA (18S rRNA+non-conserved segment); (*c*) large primary non-conserved segment (approx. $1.2\times10^6$ daltons, expected mobility 24S). The model for the sequential labelling of 45S pre-rRNA and the envisaged three constituent units is shown in Fig. 6. Since Maden *et al.* (1972) showed that both 45S and 41S pre-rRNA contain 28S+18S rRNA sequences, only the four possibilities for the arrangement of the three constituent units in 45S pre-rRNA shown in Fig. 6(*c*) (denoted A, B, C and D) may be envisaged. The model postulates a 'christmas-tree' random arrangement of presynthesized 45S pre-rRNA chains (see Miller & Bakken, 1972) growing with a 5′→3′ polarity. Fig. 6(*a*) shows that the first and second endonucleolytic splits of the 45S pre-rRNA molecule will occur at different sites along the polynucleotide chain depending on the postulated arrangement of the three constituent units. Consequently, different amounts of labelled precursor ([$^3$H]UMP in our case) will be found in the polynucleotide products of 45S pre-rRNA processing, reflecting the arrangement of the three constituent units along the 45S pre-rRNA chain.

The model would permit a reasonably accurate study of 45S pre-rRNA topology if the following basic conditions are met.

(*a*) Transcription of all pre-rRNA chains proceeds in the 5′→3′ direction. This has been invariably found in all known cases of transcription and revealed by the 'christmas-tree' pattern of active HeLa rRNA genes (Miller & Bakken, 1972).

(*b*) The synthesis of pre-rRNA molecules should be synchronized, or alternatively initiation of new pre-rRNA chains should be blocked. As shown above, the latter case is observed in isolated nuclei. Incorporation of [$^3$H]UMP proceeds mainly by elongation of pre-existing pre-rRNA chains.

(*c*) The processing of 45S pre-rRNA should yield quantitatively the products of the normal pre-rRNA maturation pathway (see Fig. 6*b*). In our experimental system (1–20min incubation) most of the label is in normal products of pre-rRNA processing, whereas only a small amount of label remains in 45S pre-rRNA. As discussed above, unspecific nuclease hydrolysis is not likely to interfere critically in our experimental system.

(*d*) The intranuclear pool of nucleotides should equilibrate rapidly with added substrates, in

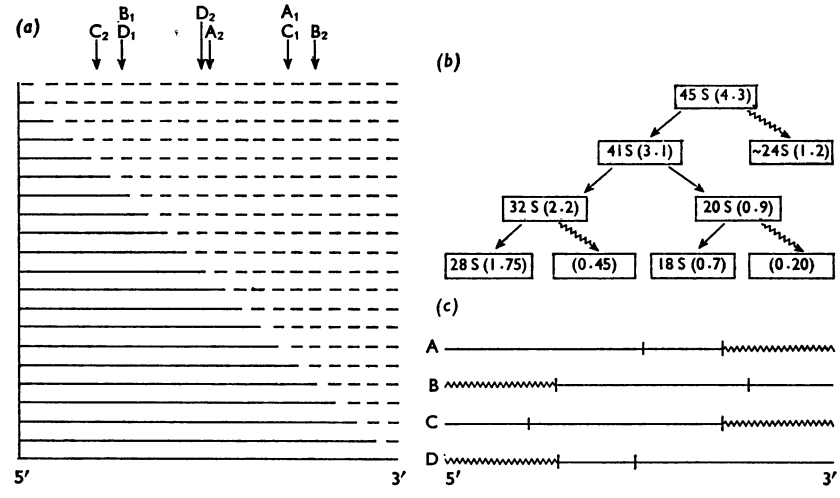



Fig. 6. *Model for the tracer study of the topology of HeLa cell 45 S pre-rRNA*

(*a*) Entrance of [$^3$H]UMP (----) into growing, pre-existing 45S pre-rRNA chains in the absence of initiation of new 45S pre-rRNA molecules. The model postulates a 'christmas-tree' random arrangement of presynthesized pre-rRNA chains (——) growing with a 5′→3′ polarity (see Miller & Bakken, 1972). The arrows indicate the position of the first and second endonucleolytic splits (designated by indexes) according to the four possible models (A, B, C or D) of the topology of 45S pre-rRNA. (*b*) Processing scheme for HeLa-cell 45S pre-rRNA used in the quantitative estimates of the model. The values in parentheses represent the molecular weights of pre-rRNA and rRNAspecies (Weinberg & Penman, 1970). (*c*) The four possible models for the arrangement of the 28S rRNA unit (32S pre-rRNA), the 18S rRNA unit (20S pre-rRNA) and the primary non-conserved segment (∼∼∼) in 45S pre-rRNA. The separate 45S pre-rRNA constituent units are delineated by vertical lines.

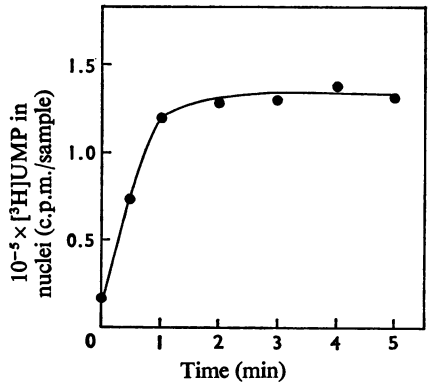



Fig. 7. *Entrance of added [$^3$H]UTP into HeLa cell nuclei*

Isolated HeLa cell nuclei were incubated in the standard incubation medium (0.08ml). At different times after the addition of nuclei, 0.05ml samples were layered on 2.1cm Millipore filters by water-pump suction, washed with 10ml of ethanol, dried and counted for radioactivity with a toluene–PPO–dimethyl-POPOP phosphor in a liquid-scintillation spectrometer (see the Experimental section).

particular with [$^3$H]UTP. This requirement was studied experimentally, and the results (Fig. 7) show that added [$^3$H]UTP equilibrates in about 60s with pre-existing intranuclear UTP.

Since the above conditions are reasonably met in our system, we estimated the total amount of label in the products of 45S pre-rRNA processing corresponding to its constituent units as defined above. The amount of label in the 32S pre-RNA unit was estimated as the sum of the 32S and 28S RNA components, whereas the label in the 20S pre-rRNA unit is represented by the sum of the label in the 20S and 18S RNA components (see Fig. 4*b*). The labelling of the non-conserved segments in 32S and 20S pre-rRNA was neglected on the assumption that owing to their small size, lower content of UMP and comparable size, it would not critically change the estimated 32S/20S RNA labelling ratio. The expected labelling of the 45S pre-rRNA constituent units in dependence on their arrangement along the polynucleotide chain is given in Table 2. As can be seen wide variations in the labelling ratio 32S/20S RNA are expected depending on the four possible models of 45S pre-rRNA topology. In four independent

Table 2. *Expected total amount of label in the constituent segments of 45S precursor of ribosomal ribonucleic acids*

The four possible models for the topology of 45S pre-rRNA are given in Fig. 6(*c*). The total amount of label (arbitrary units) in the three constituent units of 45S pre-rRNA was calculated as the 'labelled' surface area of the rectangle corresponding to every constituent unit obtained after endonucleolytic splits corresponding to the four models and indicated by arrows (see Fig. 6*a*). 'UMP' denotes the total amount of expected counts in UMP of the 45S pre-rRNA constituent segments, calculated on the basis of data for the molar percentage of UMP in nuclear HeLa cell rRNA components: 45S, 17%; 32S and 28S, 16%; 20S, 20%; 18S, 21%; primary non-conserved segment, 10% (Willems *et al.*, 1968). Since in our experiments only UMP is labelled, these values more accurately represent the total amount of label in the separate 45S pre-rRNA constituent units. nc-RNA, primary non-conserved segment in 45S pre-rRNA.

| Model | Total amount of label in | | | | | | Ratio | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 32S RNA | | 20S RNA | | nc-RNA | | 32S/20S RNA | | (32S+20S/nc-RNA | |
| | Sum | UMP | Sum | UMP | Sum | UMP | Sum | UMP | Sum | UMP |
| A | 3872 | 619 | 3816 | 763 | 7104 | 710 | 1.02 | 0.81 | 1.08 | 1.95 |
| B | 8096 | 1295 | 5544 | 1109 | 1152 | 115 | 1.46 | 1.17 | 11.82 | 20.90 |
| C | 7040 | 1127 | 648 | 130 | 7104 | 710 | 10.86 | 8.66 | 1.08 | 1.77 |
| D | 11264 | 1800 | 2376 | 475 | 1152 | 115 | 4.75 | 3.79 | 11.82 | 19.75 |

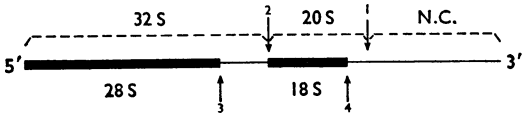



Fig. 8. *Proposed model for the topology of HeLa cell 45S pre-rRNA*

The position of the 28S and 18S rRNA sequences within the respective pre-rRNA constituent units is arbitrary (see evidence by Perry & Kelley, 1972). N.C., primary non-conserved segment in 45S pre-rRNA. The arrows indicate the location of subsequent (numbered 1 to 4) endonucleolytic splits in 45S pre-rRNA (or its respective products) to yield mature 28S and 18S rRNA.

experiments, with labelling times 10 or 20min, the labelling ratio 32S/20S RNA was found to be 0.97, 0.82, 0.77, 0.97 (average 0.88). These experimental results rule out models C and D of 45S pre-rRNA topology (see Fig. 6*c* and Table 2, column 9) and show that the 28S rRNA unit is closer than the 18S rRNA segment to the 5'-end of the 45S pre-rRNA chain. This ratio is also closer to the ratio expected from model A of 45S pre-rRNA topology although, owing to the experimental error inherent in the technique used, model B cannot be ruled out on the basis of these results only. The location of the primary non-conserved segment at the 3'-end of the 45S pre-rRNA molecule (model A) is further supported by the substantial amount of label in the zone of 24S and 10–16S RNA considered to originate from this segment (see above). An accurate estimate of the labelling ratio (32S+20S)/non-conserved segment is not possible, owing to the rapid degradation of the latter. However, this ratio is markedly below the value of 20.90 (see Table 2, column 11) requested from model B of 45S pre-rRNA topology.

Our experimental findings support the model shown in Fig. 8 for the topology of HeLa cell 45S pre-rRNA. The proposed arrangement of the constituent segments in 45S pre-rRNA in HeLa cells is in agreement with the conclusions on the topology of liver 45S pre-rRNA, reached by an independent approach (Choi & Busch, 1970; Quagliarotti *et al.*, 1970). It is also in agreement with a recent electron-microscopic study on the topology of 45S pre-rRNA (Wellauer & Dawid, 1973). The reverse arrangement of 28S and 18S rRNA segments in pre-rRNA has been proposed for HeLa cells (Siev *et al.*, 1969). *Xenopus laevis* (Reeder & Brown, 1970) and *Euglena gracilis* (Brown & Haselkorn, 1971). We consider that the approach used in the present work may be helpful in elucidating whether the 5'→3' arrangement of 28S rRNA unit, 18S rRNA unit and primary non-conserved segment is of general occurrence and reflects a common pattern in the structure of rRNA transcription units.

The fact that correct elongation, termination and processing of 45S pre-rRNA in isolated nuclei proceeds in the presence of α-amanitin further supports our view (Hadjiolov *et al.*, 1974) that the action of the drug *in vivo* is not due to a direct effect on rRNA synthesis and maturation.

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