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Synthesis of Ribosomal 5S RNA by Isolated Nuclei from HeLa Cells in Vitro†

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ABSTRACT: The fidelity of 5S ribosomal RNA transcription in isolated HeLa cell nuclei has been studied by molecular hybridization using an *E. coli* hybrid plasmid which contained *Xenopus* 5S DNA as a probe. As a prerequisite, the incubation conditions were optimized for the synthesis of a specific gene product in nuclei. The synthesis of 5S RNA was dependent on the presence of Mg^{2+}, while increasing quantities of Mn^{2+} progressively inhibited its formation. The most dramatic effect on the amount of 5S RNA synthesized was exerted by the ionic strength of the medium. An optimum was observed at 50 mM NH_4Cl while a significant depression occurred at higher ionic strengths and only 20% or less of the maximal 5S RNA synthesis occurred at 150 to 200 mM for monovalent ions, respectively. At these latter concentrations, bulk RNA synthesis was still very active, indicating a clear dissociation of 5S and bulk RNA syntheses. The synthesis of hybridizable 5S RNA sequences is sensitive to high concentrations of amanitin, demonstrating that RNA polymerase C is responsible for their synthesis. It was shown, however, that conditions for maximal activity of enzyme C in isolated nuclei do not warrant an optimal production of ribosomal 5S RNA, reemphasizing the necessity of specific assay systems for the analysis of defined transcription products. Under optimized incubation conditions, 0.059 pmol of ribosomal 5S RNA was synthesized per 2×10^6 nuclei during 40 min of incubation. This corresponds to 18 000 molecules per nucleus, demonstrating an efficient reinitiation of 5S RNA synthesis. The fidelity of 5S rRNA transcription was assayed by subjecting the in vitro products to a combination of size fractionation and molecular hybridization. Two low-molecular-weight products were identified by gel electrophoresis representing 5S RNA and a compound which is probably the 4.5S precursor to tRNA. Hybridization of the individual gel fractions to 5 S containing plasmid DNA showed that only the component which comigrated with mature in vivo 5S RNA hybridized. Moreover, it has been observed by Sephadex G-100 gel filtration that there are no hybridizable 5S sequences in RNA of high molecular weight. Hybridization of in vitro 5S RNA is completed to essentially 0% by the addition of a great excess of cold in vivo 5S RNA. Chromatography of T1 ribonuclease digests of 5S RNA synthesized in vitro on diethylaminoethyl-Sephadex in the presence of 7 M urea revealed a similar oligonucleotide pattern obtained from in vivo 5S ribosomal RNA. Transcription of antisense RNA is therefore unlikely and, from these results, we conclude that isolated nuclei synthesize 5S rRNA accurately with respect to sequence and size.

The synthesis of low-molecular-weight RNA species in isolated cell nuclei has been studied by several investigators under different experimental conditions (Price and Penman, 1972; Reeder and Roeder, 1972; Marzluff et al., 1974; Weinmann and Roeder, 1974; Udvardy and Seifart, 1976; Weil and Blatti, 1976; Sarma et al., 1976). In view of the lack of a specific assay system, none of them have, however, optimized the conditions for the synthesis of a specific gene product. Thusfar the criteria of optimization were either maximal incorporation of labeled precursors into bulk RNA or the linearity of this process during a defined incubation period, and, since total RNA synthesis and the production of a specific class of RNA are not necessarily correlated, this question was investigated. In addition we attempted to resolve the question whether the 5S-sized product formed in nuclei was homologous in sequence to ribosomal 5S RNA and, alternatively, whether sequences detectable by molecular hybridization were necessarily of 5S size. It was, therefore, analyzed whether high-molecular-weight RNA products contained sequences for 5S RNA, possibly indicating a read-through of transcription or, alternatively, whether 5S rRNA sequences were detectable in low-molecular-weight fragments resulting from incorrect initiation or premature termination of RNA polymerase. It was hitherto impossible to obtain sufficient amounts of a complementary probe required for quantitative studies of this kind in isolated nuclei because of the presence of large amounts of endogenous RNA. Current advances in the construction of bacterial plasmids containing foreign DNA (Cohen et al., 1973) enabled us to employ this chimeric DNA as a probe for specific DNA–RNA hybridization analyses. We have chosen to study the synthesis of 5S RNA because of a number of significant

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advantages. Apart from few, if any, base substitutions, the primary sequence of ribosomal 5S RNA is known to be almost identical in all higher eukaryotic cells so far studied (Forget and Weissman, 1969; Williamson and Brownlee, 1969; Averner and Pace, 1972; Wegnez et al., 1972; Ford and Southern, 1973; Takai et al., 1975; Ford and Brown, 1976). The low-molecular-weight (120 bases long) and comparative abundance in cells, together with the availability of bacterial plasmids harboring purified and well characterized *Xenopus* 5S DNA coding for its synthesis (Carroll and Brown, 1976), rendered this system extremely advantageous for a detailed analysis of specific transcription. Using this plasmid DNA as a probe for molecular hybridization, we have studied the synthesis of ribosomal 5S RNA in isolated HeLa cell nuclei.

Materials and Methods

E. coli Strains. E. coli strains, harboring the hybrid plasmid Col E1-pSC101 *Xenopus laevis* (or *Xenopus mulleri*) 5S DNA, were constructed in the laboratory of Dr. D. D. Brown (Carnegie Institution, Baltimore, Md.) and kindly provided to us by Dr. R. G. Roeder (Washington University, St. Louis, Mo.).

Growth Conditions of Cells and Extraction of DNA. E. coli cells were grown in 2-L Erlenmeyer flasks containing 1200 mL of modified H medium (Gussin, 1966; 2.4 g of NH_4Cl, 6.0 g vitamin-free casamino acids, 6.0 g of NaCl, 6.0 g of glucose, 3.0 mL of glycerol, 1.26 g of Tris,[1] 0.055 g of $K_2HPO_4 \cdot 3H_2O$ adjusted to pH 7.3 with HCl. After autoclaving this medium, 2.5 mL each of sterile 1 M $CaCl_2$, 1 M $MgSO_4$, 2.5% thiamine hydrochloride, and 1% tetracycline hydrochloride were added). Ten flasks were handled simultaneously. The culture was grown in a gyrotory shaker at 37 °C and 200 rpm until an A_{590} of 0.5–0.7 was reached, at which time 250 mg of solid chloramphenicol was added per flask. Incubation was subsequently continued for 12 h to allow the selective replication of plasmid DNA (Hershfield et al., 1974). The cells were harvested at 6500*g* for 5 min and resuspended in 200 mL of 25% sucrose in 0.05 M Tris-HCl, pH 8.0. The suspension was then treated by the consecutive addition of lysozyme (40 mL, 5 mg/mL) and EDTA (80 mL, 0.25 M) at 0 °C (Guerry et al., 1973). Cell lysis was accomplished by the addition of 16 mL of 20% sodium dodecyl sulfate. After cellular lysis, 84 mL of 5 M NaCl was added to the viscous solution with gentle mixing by a glass rod. The lysates were kept at 4 °C for 20 h and subsequently centrifuged at 17 000*g* for 30–40 min at 4 °C. The resulting viscous solution of yellowish color is referred to as crude supernatant. It contains little, if any, detectable high-molecular-weight *E. coli* chrosomal DNA as evidenced by agarose gel electrophoresis.

Purification of Plasmid DNA. The crude supernatant was diluted twice by the addition of an equal volume of double-distilled water. DNA was spooled from this solution onto a glass rod after the gentle addition of 0.6 volume of 2-propanol. Recovered DNA was resuspended in 0.2 × SET (1 × SET = 0.15 M NaCl–0.002 M EDTA–0.01 M Tris-HCl (pH 7.5)) with 1% $NaDodSO_4$ in one-quarter of the volume of the original crude supernatant. Protein was removed by shaking with an equal volume of water-saturated phenol for 1 h at room temperature. The aqueous phase was recovered by centrifugation and DNA was spooled onto a glass rod after the addition of 1.2 volumes of a mixture of ethanol and 1 M sodium acetate (90:10, v/v). At this stage, 40–60% of the A_{260} material corresponds to DNA. After resuspension in 0.2 × SET, $1/18$ volume of ethidium bromide (1 mg/mL) was added while stirring. Solid CsCl was then added to a final concentration of n_D^{20} 1.3930 (±0.0005) and the mixture was spun at 20 °C for 72–80 h at 33 000 rpm in the Beckman 60 Ti rotor. Banded DNA was recovered by aspiration, ethidium bromide was extracted with 2-propanol, and the DNA solution was extensively dialyzed against 0.2 × SET. Approximately 30–40 mg of plasmid DNA was recovered per centrifugal run and the overall yield was approximately 150 mg of plasmid DNA per 108 L of *E. coli* culture. It should be noted that this procedure yields mainly the relaxed form of plasmid DNA as judged by agarose gel electrophoresis.

Culture of HeLa Cells and Isolation of Nuclei. Culture conditions for HeLa S_3 and isolation of nuclei from 300 mL of suspension culture (approximately 8×10^7 cells) were as described previously (Udvardy and Seifart, 1976). Nuclei were resuspended at a concentration of 2×10^8/mL in buffer containing 10 mM $MgCl_2$, 50 mM Tris-Cl (pH 7.9), 5 mM dithiothreitol, 0.2 mM EDTA, and 25% glycerol.

Synthesis and Extraction of RNA. RNA was synthesized for 20 min at 25 °C in a final volume of 30 μL. RNA (5S) synthesis was optimized by individually changing the reaction components and specifically analyzing the product by hybridization. The final optimized conditions were as follows: 0.5 μg/mL α-amanitin, 50 mM NH_4Cl, 5 mM $MgCl_2$, 12.5% glycerol, 25 mM Tris-HCl (pH 7.9), 2.5 mM dithiothreitol, 0.1 mM EDTA, 1 mM each of ATP, GTP, CTP, 0.1 mM and 9–14 μCi of [^{3}H]UTP (Radiochemical Centre, Amersham). The reaction was terminated by the addition of 10 μL of a solution of *E. coli* tRNA (2.5 mg/mL in water) and 110 μL of DNase (Rnase-free, Worthington, 75 μg/mL in 3 mM $MgCl_2$). The incubation was subsequently prolonged for 10 min at 0 °C and DNase digestion was stopped by the addition of 150 μL of 1% $NaDodSO_4$. RNA was extracted with phenol and chloroform essentially as described by Udvardy and Seifart (1976) and subsequently washed four times by ethanol precipitation at −20 °C. It was dissolved in 0.5% $NaDodSO_4$ and employed as such for hybridization assays or polyacrylamide gel electrophoresis.

RNA–DNA Hybridization. Plasmid or *E. coli* DNA was dissolved in 0.02 × SET at a concentration of approximately 20 μg/mL and immobilized on nitrocellulose filters (14 cm diameter, type BA 85 from Schleicher and Schuell) after alkaline denaturation and neutralization. Disks of 9 mm were punched from these filters. The DNA content of individual disks was very constant for a given filter and was normally 25–30 μg per disk as determined by acid hydrolysis. Hybridization was conducted in 4 × SSC (1 × SSC = 0.15 M NaCl–0.015 M sodium citrate) and 0.2% $NaDodSO_4$ at 68 °C for 12 h with two plasmid and one *E. coli* DNA filter disks in a total volume of 0.25 mL. Filters were washed six times with 2 × SSC by mild gyrotation, treated with RNase (50 μg/mL pancreatic RNase and 50 units/mL RNase T_1 in 2 × SSC) for 1 h at room temperature and subsequently washed five times as before. The two plasmid DNA filters were counted separately and the radioactivity on the *E. coli* filter (routinely 8–19 cpm) was subtracted from the value obtained. Due to the large amount of cold endogenous RNA (approximately 0.16 μg per 2×10^6 nuclei; M. Yamamoto, unpublished), it was necessary to employ two separate filters as described.

Estimation of Hybridization Efficiency. To estimate the

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; 1 × SET, 0.15 M NaCl–0.002 M EDTA–0.01 M Tris-HCl (pH 7.5); 1 × SSC, 0.15 M NaCl–0.015 M sodium citrate.

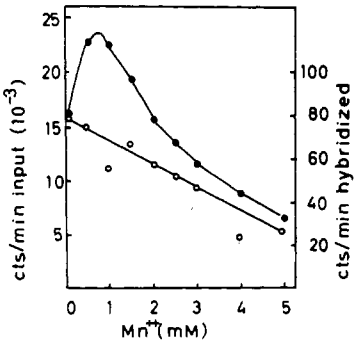

FIGURE 1: Effect of different Mn^{2+} concentrations on RNA synthesis of isolated HeLa cell nuclei. After 20 min of incubation at 25 °C, RNA was extracted as described. Aliquots were counted for the estimation of bulk incorporation and the remainder was employed for hybridization to plasmid DNA containing 5S DNA as described in Materials and Methods. The specific radioactivity was 3000 cpm/pmol of UMP and the efficiency of hybridization was 12%. Mg^{2+} (0.7 mM), derived from the nuclear pellet, was included in each assay since nuclei were not washed because of the tendency of aggregation in the complete absence of Mg^{2+}. (●) Input counts for the hybridization which also represents the bulk incorporation; (○) radioactivity hybridized.

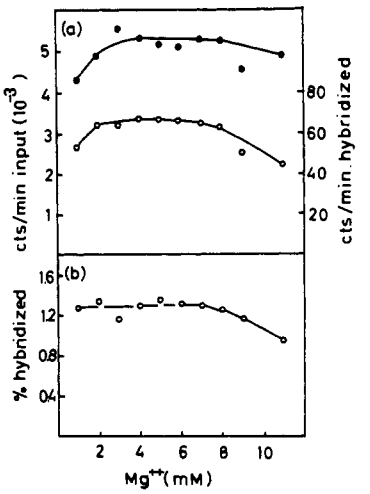

FIGURE 2: Effect of Mg^{2+} concentration on RNA synthesis in isolated nuclei. Nuclei were resuspended in Mg^{2+}-free buffer and incubated in the presence of different concentrations of Mg^{2+}. Mg^{2+} (1 mM) was derived from the nuclear pellet. After 20 min, RNA was extracted and hybridized. The specific radioactivity was 1800 cpm/pmol of UMP and the efficiency of hybridization was 24%. (a) (●) Input counts for the hybridization; (○) radioactivity hybridized. (b) Ratio of 5 S to total RNA synthesized in vitro.

hybridization efficiency, mock RNA synthesis was conducted without labeled UTP for every batch of nuclei. After terminating the reaction as described, a known amount of in vivo ^{3}H-labeled cytoplasmic 5S RNA was added, total RNA was subsequently extracted, handled, and hybridized as described. The reproducibility of the hybridization values obtained for in vivo 5S RNA was excellent for a given batch of nuclei, because the reaction was conducted with large quantities of endogenous 5S RNA (over 99.5% of the total 5S sequence) and with saturating quantities of probe DNA on the filter. Hybridization efficiencies of approximately 20% were routinely obtained as appropriately indicated in the figure legends. These values also agree well with those obtained by the internal standard procedure (Reeder and Roeder, 1972), which was also employed in some of the experiments.

Polyacrylamide Gel Electrophoresis. Low-molecular-weight RNA species were analyzed on polyacrylamide slab gels. Formamide gels were prepared as described by Pinder et al. (1974). The acrylamide concentration was 12% and ethylenediacrylate (0.25% (v/v) final concentration) instead of methylenebisacrylamide was used as a cross-linker.

Hybridization of Polyacrylamide Gel Fractions. Each polyacrylamide strip was sliced into 2-mm pieces and solubilized in 0.6 mL of 0.2% sodium dodecyl sulfate and 4 × SSC by incubating overnight at 68 °C. Aliquots (0.1 mL) were withdrawn and precipitated with Cl_3CCOOH for the estimation of input radioactivity. The remainder was used for the simultaneous hybridization with one plasmid DNA and one blank filter (9 mm in diameter, type BA85 from Schleicher and Schuell) at 68 °C overnight. The blank value (normally 2 to 15 cpm) was subtracted from the value obtained for each counterpart filter containing plasmid DNA.

Analysis of Oligonucleotides. [^{3}H]Uridine-labeled in vivo 5S rRNA was extracted from the cytoplasm of HeLa cells and purified by two consecutive passages through Sephadex G-100. In vitro 5S rRNA was purified by Sephadex G-100 filtration and subsequent hybridization of the 5S-sized fractions to plasmid DNA. RNA (5S) was then recovered from the DNA filters by incubation for 5 min at 95 °C in 0.1 × SSC. Either ^{3}H-labeled in vivo or in vitro RNA was digested with RNase T1 (500 units) with ^{32}P-labeled ribosomal 18 and 28S RNA and 200 μg of *E. coli* tRNA in 500 μL of 0.05 M Tris (pH 7.4) containing 0.002 M EDTA. The solution was incubated for 6 h at 37 °C and subsequently diluted ten-fold with buffer containing 0.02 M Tris (pH 7.9) and 7 M urea. Samples of in vitro and in vivo RNA were separately applied to columns of DEAE-Sephadex A-25 which were equilibrated in the same buffer and eluted with linear gradients of NaCl according to the procedure of Suzuki and Brown (1972). Aliquots of 0.5 mL were counted for radioactive assessment.

Results

(a) *Optimization of 5S RNA Synthesis.* (1) Effect of Mn^{2+} and Mg^{2+}. As has repeatedly been demonstrated in the past and is confirmed in Figure 1, Mn^{2+} stimulates total nuclear RNA synthesis at certain concentrations. The synthesis of 5S RNA is, however, progressively inhibited by increasing concentrations of Mn^{2+}. In a separate experiment, synthesis was conducted with rising Mn^{2+} concentrations (1–5 mM) in a system containing a constant amount of Mg^{2+} (5 mM). It was found (data not shown) that syntheses of total and 5S RNA were inhibited by rising Mn^{2+} concentrations in the presence of Mg^{2+}. The production of 5S RNA was, however, impaired much more severely and this cation was therefore eliminated from all subsequent experiments.

With Mg^{2+} as sole divalent metal cation, both total RNA and 5S RNA synthesis remained fairly constant up to 8 mM Mg^{2+} (Figure 2a). No apparent change was observed in the relative proportion of 5S to total RNA synthesized (Figure 2b), clearly recommending the future use of Mg^{2+} for these experiments.

(2) Effect of Salts. The presence of salt is known to stimulate nuclear RNA synthesis in vitro, although the species of ions and their respective concentration used by different investigators greatly vary. Figure 3 shows that bulk RNA synthesis is maximally stimulated at a monovalent ion concentration of 100–200 mM and remains active up to 400 mM. In contrast, hybridizable 5S RNA sequences were optimally synthesized at 50–75 mM NH_4Cl. They were significantly depressed at 150 mM and could no longer be detected beyond 300 mM NH_4Cl. Similar values were obtained for other monovalent salts (KCl, NaCl), whereas the optimal $(NH_4)_2SO_4$ concen-

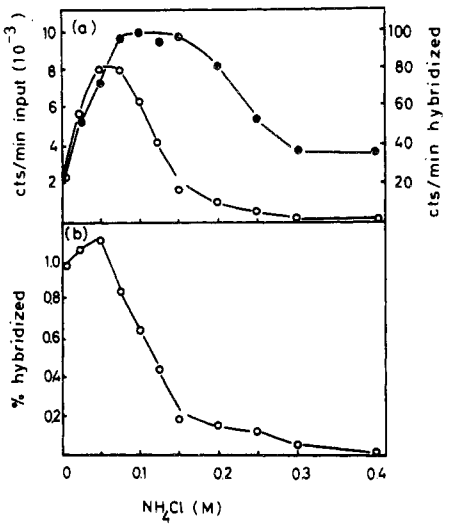

FIGURE 3: Effect of NH_4Cl concentration on RNA synthesis in isolated nuclei. RNA was synthesized in the presence of different concentrations of NH_4Cl. The concentration of Mg^{2+} was 5 mM and Mn^{2+} was omitted. After 20 min of incubation at 25 °C, RNA was extracted and hybridized to plasmid DNA as described. The specific radioactivity was 2000 cpm/pmol of UMP and the efficiency of hybridization was 17.6%. (a) (●) Input counts for the hybridization; (○) radioactivity hybridized. (b) Ratio of 5S to total RNA synthesized in vitro.

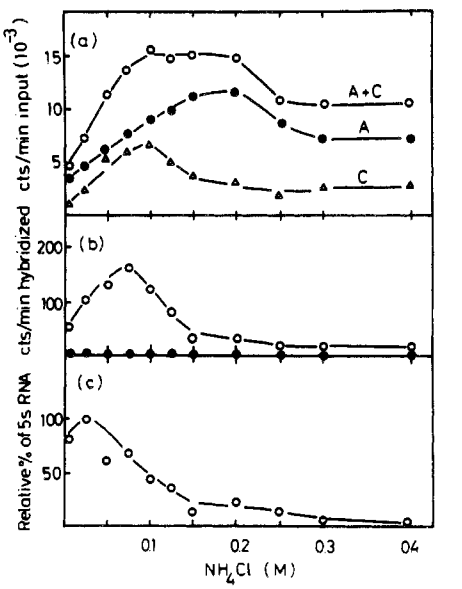

FIGURE 4: Effect of salt in combination with low and high concentrations of α-amanitin on nuclear RNA synthesis. RNA was synthesized in the presence of either low (0.5 μg/mL) or high (200 μg/mL) concentrations of α-amanitin. After 20 min of incubation, RNA was extracted and hybridized. (a) Bulk incorporation profile in the presence of low (○) and high (●) concentrations of α-amanitin. The value obtained at high α-amanitin concentration was subtracted from that obtained for the low concentration, to estimate the activity of RNA polymerase C at each salt concentration tested (△). (b) Hybridization of RNA synthesized in the presence of low (○) and high (●) concentrations of amanitin. (c) Relative ratio of 5 S to the RNA synthesized by enzyme C in vitro. The highest value was taken as 100%.

tration for the synthesis of 5S RNA was found to be 20–40 mM (data not shown). The clear dissociation of bulk and 5S RNA synthesis was observed for all salts tested and the most favorable ratio of 5S to total RNA synthesis was observed at 50 mM NH_4Cl or 20 mM $(NH_4)_2SO_4$.

As has repeatedly been shown (e.g., Weinmann and Roeder, 1974; Udvardy and Seifart, 1976), the activity of individual polymerase species can be dissociated by the use of α-amanitin in a mixed system like the intact nucleus. It was therefore analyzed whether the activity of a particular polymerase (enzyme C) in isolated nuclei reflects the rate of transcription of a particular gene. To determine this, RNA was synthesized in nuclei under conditions of various ionic strengths in the presence of low (0.5 μg/mL) and high (200 μg/mL) concentrations of amanitin, thus assessing the combined activity of RNA polymerase A+C and A, respectively. The numerical difference obtained between the two sets of conditions reflects the activity of RNA polymerase C (or III) alone. Analysis of the bulk incorporation values shows a maximum stimulation for enzyme A at 200 mM NH_4Cl (Figure 4a). In contrast, the calculated optimum for RNA polymerase C is clearly lower at around 100 mM NH_4Cl, although the enzyme remains partly active at very high concentrations of salt (Figure 4a). Hybridization of the corresponding samples to plasmid DNA (Figure 4b) revealed a monophasic stimulation curve with a maximum at 50–75 mM NH_4Cl and absence of hybridizable counts at high ionic strength as was previously shown in Figure 3. Figure 4c analyzes the ratio of hybridized 5S sequences (Figure 4b) to the activity of RNA polymerase C (Figure 4a) in HeLa cell nuclei. It shows that the ratio does not stay constant over the entire range of ionic strength investigated and, therefore, it is obvious that the extent of synthesis of 5S rRNA is not necessarily correlated to the activity of RNA polymerase C (or III) at high ionic strength.

(3) α-Amanitin Sensitivity of 5S RNA Synthesis. It was necessary to prove that RNA polymerase C was responsible for the synthesis of RNA sequences hybridized to 5S DNA. In addition it was required to rule out that UTP is added to preexisting 5S RNA molecules by a terminal transferase activity. Figure 4b shows that RNA synthesized in the presence of 200 μg/mL amanitin, known to eliminate RNA polymerase C from HeLa cells (Seifart and Benecke, 1975), does not hybridize to 5S DNA.

(4) Other Components. Previously published results (Marzluff et al., 1974; Sarma et al., 1976) had shown that the incubation temperature was of profound influence on the linearity and extent of total nuclear RNA synthesis. The influence of different temperatures at 20, 25, 30, and 37 °C on specific synthesis of 5S RNA was therefore investigated. The results indicated a flat optimum for 5S synthesis at 25–30 °C, although the effect of temperature was not very pronounced within the incubation time of 20 min. An incubation temperature of 25 °C was chosen for subsequent investigations because additional experiments (data not shown) indicated an excellent linearity of 5S RNA synthesis at that temperature within incubation times of up to 40 min.

The effect of substrate concentration was studied by varying the UTP concentration between 0.02 and 1.62 mM. RNA synthesis was dependent on the presence of all four nucleoside triphosphates and was depressed below concentrations of 0.1 mM UTP (80% residual activity at 0.02 mM). Within the substrate concentration outlined above, however, the ratio of 5S RNA sequences to total RNA synthesized remained constant irrespective of the precursor concentration studied (data not shown). Similar results were obtained for the influence of glycerol which depressed both bulk and 5S RNA synthesis at high concentrations. The relative proportion remained constant up to 12.5% glycerol, however, and, due to the beneficial effect on nuclear structure and homogeneity of the suspension, this glycerol concentration was maintained for future experiments.

TABLE I: Quantitation of 5S RNA Sequence Synthesized in Vitro under Optimized Conditions.[a]

Incubation time (min)	Input RNA (cpm)		Hybridized RNA (cpm)		Hybridization efficiency (%)	Calcd 5S RNA (pmol)	No. of 5S RNA (molecules/nucleus)
	^3H in vitro	^{32}P in vivo 5S	^3H	^{32}P			
20	10 577	1048	690	231	22	0.035	10 500
40	14 754	1012	1217	232	23	0.059	17 700

[a]RNA was synthesized under optimized conditions: i.e., 50 mM NH_4Cl, 5 mM Mg^{2+}, 1 mM each of ATP, GTP, and CTP, 0.12 mM and 12 μCi of UTP, 0.5 μg/mL α-aminitin. Incubation was conducted at 25 °C for 20 and 40 min. RNA was then extracted and hybridized. The specific radioactivity was 3333 cpm/pmol of UMP and the experimental values are given for 2 × 10^6 nuclei. ^{32}P-labeled in vivo 5S RNA was included within the sample RNA as internal standard for the estimation of the hybridization efficiency (Reeder and Roeder, 1972). The number of 5S RNA molecules was calculated assuming that one 5S RNA molecule contains 27 uridylic acid residues (Hatlen et al., 1969).

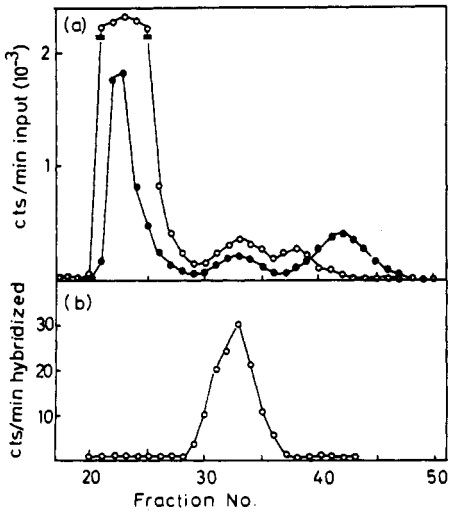

FIGURE 5: Size fractionation of RNA synthesized in vitro by gel filtration and hybridization of the obtained fractions. RNA was synthesized by isolated HeLa cell nuclei, purified, and loaded onto a column of Sephadex G-100 (1.5 × 60 cm). The column was eluted with 0.1 M sodium acetate (pH 5.5), 0.5% sodium dodecyl sulfate, and 4 μg/mL of poly(vinyl sulfate) with a hydrostatic pressure of 40 cm at room temperature. From fractions of 1.57 mL, 0.1 mL was withdrawn for the determination of acid-precipitable radioactive material and 0.5-mL aliquots were subjected to hybridization with 1 each of plasmid and *E. coli* DNA filters. ^{32}P-labeled in vivo high-molecular-weight 5S and tRNA isolated from the cytoplasm of HeLa cells were included as internal markers in the sample. (a) Elution profile from the column. (○) ^3H-labeled in vitro RNA; (●) ^{32}P-labeled in vivo RNA. The ^3H values also represent the input counts for the hybridization. (b) Hybridization profile of in vitro RNA.

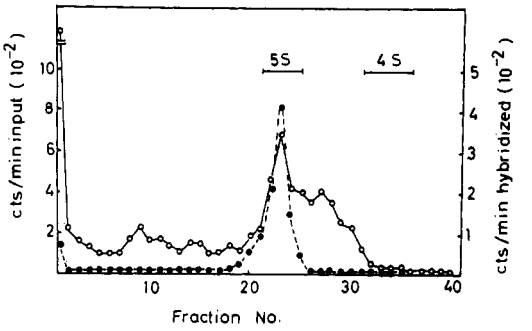

FIGURE 6: Analysis of low-molecular-weight RNA species on polyacrylamide gel electrophoresis and hybridization of the obtained fractions. RNA products synthesized in vitro by isolated nuclei were electrophoresed for 5 h on 12% polyacrylamide slab gels and hybridized to plasmid DNA as described. The radioactivity of each point in the electrophoretogram represents the input value employed for the hybridization assay. (○) Bulk RNA; (●) radioactivity hybridized; positions of ^{32}P-labeled marker 5S and tRNA are indicated by brackets.

(b) *Fidelity of the Product Synthesized.* 1. Quantitation of 5S RNA Synthesized. The extent of 5S RNA synthesis was determined under the optimized conditions described. As shown in Table I, approximately 0.035 and 0.059 pmol of 5S RNA sequences are synthesized per 2 × 10^6 nuclei in 20 and 40 min of incubation, respectively. This corresponds to approximately 10 000 and 18 000 molecules per nucleus for the respective time points.

2. Size Distribution of 5S RNA Sequences. Although the hybridization technique employed is specific for the primary base sequence of 5S RNA, the obtained results do not supply any information about the size of the RNA within which these sequences are contained. We have therefore undertaken experiments to hybridize in vitro products of defined molecular size to 5S DNA. Initially, assays were directed to the question whether 5S RNA sequences are contained within high-molecular-weight RNA species using Sephadex G-100 gel filtration chromatography. As show in Figure 5a, this column clearly separates ^{32}P-labeled, ribosomal high-molecular-weight, 5S, and mature tRNA employed as internal size-markers. The RNA synthesized by isolated nuclei in vitro is likewise separated into three detectable components, corresponding to high-molecular-weight, 5S RNA and pre-tRNA, respectively (Bernhardt and Darnell, 1969; Price and Penman, 1972). Hybridization of the column fractions to plasmid DNA resulted in a single peak corresponding to the position of 5S RNA. None of the other regions were associated with hybridized counts (Figure 5b), indicating that 5S RNA sequences are contained neither in high-molecular-weight RNA nor in fragments of lower molecular size than 5 S.

To achieve a better resolution of the low-molecular-weight RNA species, the samples were analyzed by polyacrylamide gel electrophoresis. Under these conditions, the low-molecular-weight RNA is separated into two main components (Figure 6) representing 5S and pre-tRNA species as previously shown in numerous reports (Price and Penman, 1972; Marzluff et al., 1974; Weinmann and Roeder, 1974; Udvardy and Seifart, 1976; Weil and Blatti, 1976; Sarma et al., 1976). Hybridization of each gel fraction to plasmid DNA yielded a sharp peak located in the 5S region with no counts detectable in regions of lower molecular weight (Figure 6). To exclude conceivable aggregation phenomena, in vitro RNA was coelectrophoresed with appropriate in vivo markers on formamide containing polyacrylamide gels (Figure 7). Hybridization of the fractions to plasmid DNA again revealed a single peak for in vivo and in vitro RNA at the 5S position.

3. Competition of Hybridization. *E. coli* plasmid DNA does not form a stable hybrid with eukaryotic RNAs (Morrow et al., 1974) and the spacer sequence of 5S DNA differs suffi-

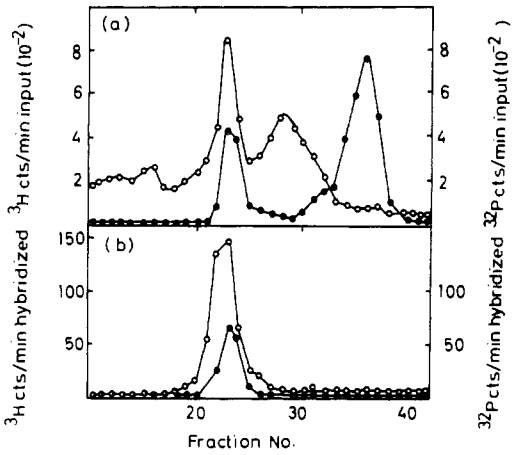

FIGURE 7: Analysis of low-molecular-weight RNA species on formamide-containing polyacrylamide gels. After ethanol precipitation, the RNA samples were analyzed by electrophoresis in 12% polyacrylamide gels polymerized in 99% formamide. Electrophoresis was for 9 h at room temperature. ^{32}P-labeled 5S and tRNA were added to the samples as internal markers. Obtained gel fractions were solubilized and hybridized to plasmid DNA. (a) Gel electrophoretogram. (○) ^{3}H-labeled in vitro RNA; (●) ^{32}P-labeled in vivo 5S and tRNA. (b) Hybridization profile. (○) ^{3}H; (●) ^{32}P.

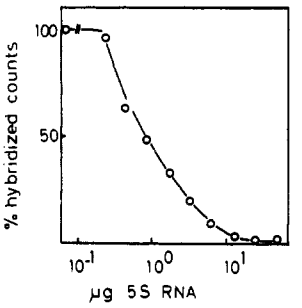

FIGURE 8: Hybridization-competition profile of in vitro synthesized RNA by unlabeled in vivo 5S rRNA. RNA was synthesized by isolated HeLa cell nuclei under standard conditions, except that the volume was increased tenfold (=300 μL final volume). Hybridization to plasmid DNA was performed in the presence of increasing amounts of cold cytoplasmic 5S RNA from rat liver. One hundred percent value represents 203 counts/min.

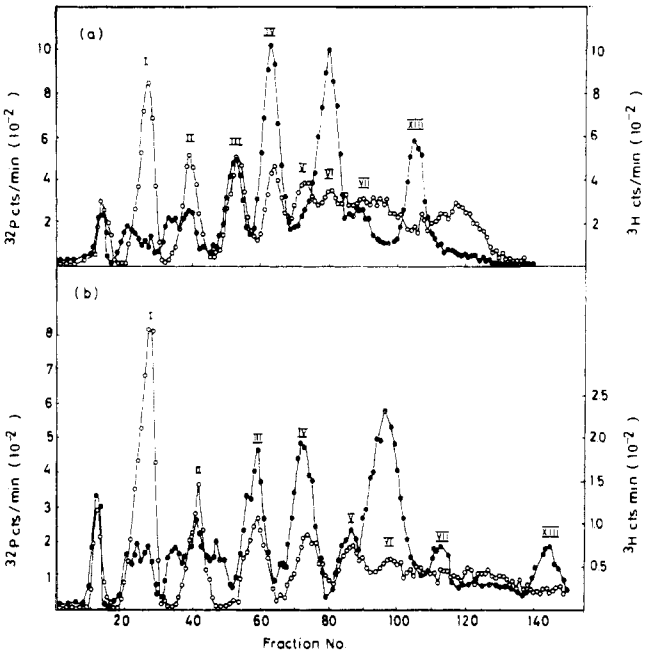

FIGURE 9: Analysis of ribonuclease T1 digests. ^{3}H-labeled 5S RNA was digested with RNase T1 in the presence of ^{32}P-labeled ribosomal 18 and 28S RNA as marker. RNA fragments were then applied onto columns of DEAE-Sephadex A-25 (0.6 × 23 cm) and chromatographed in the presence of 7 M urea as described in Materials and Methods. Linear gradients from 0.066 to 0.4 M NaCl (150 mL each) were employed. (a) In vivo 5S RNA; (b) in vitro 5S RNA. (●) ^{3}H-labeled 5S RNA; (○) ^{32}P-labeled high-molecular-weight ribosomal RNA used as internal marker.

ciently to prevent the formation of stable hybrids even between the two very closely related animals, *X. laevis* and *X. mulleri* (Brown and Sugimoto, 1973). Therefore, the hybridization technique which we have employed should permit the exclusive detection of 5S RNA gene transcripts from both sense and antisense strands. Hybridization competition experiments, conducted with cold mature 5S RNA from rat liver cytoplasm as competitor, have shown that essentially no hybridization occurred at high concentrations of competitor 5S RNA (Figure 8). As Reeder and Brown (1970) have shown, however, antisense strand transcripts could form RNA-RNA hybrids in the presence of a large amount of sense RNA and therefore this result by itself does not necessarily indicate the absence of antisense strand transcription. It was found, however, that virtually no hybridization of in vitro RNA occurred to plasmid filters which were prehybridized overnight with an excess of unlabeled cytoplasmic 5S RNA. Moreover the gel electrophoresis–hybridization data render unlikely that the antisense transcript would fortuitously be of exactly the same size as the sense transcript.

4. Analysis of Oligonucleotides. As shown in Figure 9, chromatography of the T1 ribonuclease digest of ^{32}P-labeled ribosomal RNA from HeLa cells on DEAE-Sephadex A-25 in the presence of 7 M urea yields the expected sequential elution pattern of mono- and oligonucleotides which were used as internal markers. The predominant mononucleotide peak is characteristic for high-molecular-weight ribosomal RNA. A comparison of the oligonucleotide pattern of the digest obtained from in vivo and in vitro 5S ribosomal RNA reveals a fair resemblance of the two RNA species in question. Since the two samples were run on separate columns (Figure 9), the elution of the respective oligonucleotides relative to their appropriate markers is more important than the absolute point of the column at which they were eluted. Peak V, representing the pentamer fragment of 5S RNA, is merely present as a shoulder in both in vivo and in vitro RNA. These RNA species were labeled with uridine and UTP, respectively, and since it can be compiled from the sequence of 5S RNA that the pentamer fragment should contain only one residue of uridylic acid, the experimental result is in good agreement with the expected value. The discrepancy in the shoulder observed between peaks II and III probably results from an ambiguity in termination, giving rise to a 3′-terminal fragment containing two additional residues of U as shall be discussed elsewhere (Yamamoto and Seifart, manuscript in preparation). The internal oligonucleotides obtained from in vivo and in vitro 5S RNA are, however, very similar (Figure 9).

Discussion

Optimization of 5S RNA Synthesis. In this paper we describe the systematic optimization of the conditions for the synthesis of 5S RNA in isolated nuclei from HeLa cells. Previous reports dealing with the synthesis of small-molecular-weight RNA species in similar systems (Price and Penman, 1972; Reeder and Roeder, 1972; Marzluff et al., 1974;

Weinmann and Roeder, 1974; Udvardy and Seifart, 1976; Weil and Blatti, 1976; Sarma et al., 1976) were merely based on the optimization of total nuclear RNA synthesis. It is obvious that this parameter is not necessarily correlated to the synthesis of a specific class of RNA. With the availability of a specific DNA probe which could be replicated in sufficient quantity in bacterial plasmids, it became possible to analyze in sufficient detail the conditions of incubation required for the optimal synthesis of 5S ribosomal RNA in isolated nuclei in vitro.

Mn^{2+} has been shown to be an activating agent for isolated class C (or III) enzymes from various tissues (Seifart et al., 1972; Wilhelm et al., 1974; Schwartz et al., 1974; Hossenlopp et al., 1975; Weil and Blatti, 1976) and it has also been shown to stimulate total nuclear RNA synthesis (Marzluff et al., 1973). The data reported here show, however, that Mn^{2+} inhibits the specific transcription of the genes for 5S rRNA and the use of Mg^{2+} is encouraged for future experiments of this kind (Figure 2). This is in agreement with findings reported for the specific transcription of the ribosomal genes in isolated systems from yeast (van Keulen et al., 1975; Holland et al., 1977).

Purified RNA polymerase C (or III) is known to remain transcriptionally active under conditions of fairly high ionic strength and is occasionally reported to display a biphasic ionic dependence when transcribing native DNA templates (Roeder, 1974; Schwartz et al., 1974; Weil and Blatti, 1976). Enzyme C in isolated nuclei likewise remains active up to fairly high salt concentrations as judged by differential syntheses obtained with high and low concentrations of α-amanitin (Figure 4a). Hybridization of the in vitro products to plasmid containing 5S DNA has shown, however, that the ionic conditions required for the synthesis of ribosomal 5S RNA sequences and bulk RNA are distinctly different. It was found (Figure 3) that, at monovalent ion concentrations exceeding 50 mM, 5S RNA is progressively inhibited, indicating that the efficiency of reinitiation for this class of RNA is impaired at high ionic strength. These results demonstrate that the extent of ribosomal 5S RNA production cannot be extrapolated from total nuclear RNA synthesis and, moreover, that higher activities measured for enzyme C in nuclei do not necessarily reflect a higher rate of 5S rRNA synthesis. Therefore, extreme care has to be exercised in future studies aimed at the synthesis of 5S RNA in in vitro systems reconstructed from transcriptional components, when employing conditions adapted from assays with isolated enzyme C (or III) in which only the bulk incorporation can be measured.

The results obtained show (Figure 4b) that the synthesis of RNA sequences hybridizable to ribosomal 5S DNA is completely suppressible by high concentrations of amanitin, proving that RNA polymerase C (III) catalyzes their synthesis. This conclusion was previously drawn for mouse myeloma (Weinmann and Roeder, 1974) and HeLa (Udvardy and Seifart, 1976; Weil and Blatti, 1976) cell nuclei on the basis of molecular weight analysis but had not been shown by sequence data.

Reinitiation of 5S RNA Synthesis in Vitro. The occurrence of repeated initiation events for small molecular weight RNA species has independently been suggested by several groups of investigators employing different methodological approaches (Price and Penman, 1972; Weinmann et al., 1976; McReynolds and Penman, 1974; Price and Penman, 1972; Marzluff et al., 1974; Udvardy and Seifart, 1976). Of these techniques, the hybridization method yields the most reliable estimates because all the other procedures are based on the size fractionation of the RNA as an absolute requirement. Since a contamination by low-molecular-weight RNA species of unknown nature but similar size cannot be excluded in most cases, an unequivocal interpretation of the data is often difficult. The elegant hybridization technique previously employed by Marzluff et al. (1974) unfortunately suffered from the limited quantity of the DNA probe available at that time which resulted in a low hybridization efficiency and prevented a systematic analysis of large numbers of samples. Although employing suboptimal incubation conditions for the synthesis of 5S RNA, these workers reported that 1000–4000 molecules were synthesized per haploid genome of the mouse myeloma cell containing approximately 500 copies of the 5S gene (Marzluff et al., 1975). Under the optimized conditions described, and employing the plasmid DNA as a hybridization probe, we found that one HeLa cell nucleus was able to synthesize 18 000 molecules of 5S RNA in 40 min of incubation. As shall be discussed subsequently, the sequences hybridizable to ribosomal 5S DNA are of distinct 5S size and do not represent fragments of homologous sequence. Using the hybridization-saturation technique, Hatlen and Attardi (1971) estimated that the number of 5S genes per exponentially growing HeLa cell was 7600. Even if it is assumed that all these genes are simultaneously active in vitro, the results in the present report show that multiple copies of 5S RNA are transcribed per gene. Similar conclusions are reached if it is assumed that there are approximately 2×10^4 molecules of RNA polymerase C per cell, of which only about one-third is retained in isolated nuclei (Weinmann et al., 1976). The 5S RNA molecules synthesized must therefore result from active recycling of the polymerase C molecules.

Fidelity of the 5S RNA Formed in Vitro. The experiments were conducted to analyze whether sequences detectable by DNA:RNA hybridization were of defined 5S size and, alternatively, whether the 5S-sized product formed in isolated nuclei from HeLa cells was homologous in sequence to ribosomal 5S RNA. The technique used in this study was a combination of size fractionation and subsequent hybridization of the RNA to an excess of 5S containing plasmid DNA which allows one to draw definitive conclusions concerning the above mentioned question.

The results demonstrate that hybridizable sequences are contained in discrete RNA species comigrating with mature 5S ribosomal RNA. This is in agreement with results found for 5S RNA synthesized in isolated nuclei of virus infected KB cells (Weinmann et al., 1976). It was found (Figure 5) that molecules of higher molecular weight do not contain such sequences showing that read-through of the polymerase into the spacer or other DNA regions normally not transcribed in vivo does not occur at a level detectable by the methodology employed. In addition it was found that hybridizable counts were not associated with RNA fragments of lower than 5S size (Figure 6), indicating that the probability of abberant initiations and/or terminations is very low in this system. This result was confirmed by electrophoresis in formamide containing polyacrylamide gels which should prevent aggregation of RNA fragments. Hybridization of the corresponding fractions reveals a symmetrical and well-defined peak at the 5S position. Since an excess amount of plasmid DNA over endogenous 5S RNA was used in our study, all species of complementary sequences to the inserted 5S gene region should have been detected. These results demonstrate that, with the hybridization technique described, it is possible to obtain fairly pure 5S RNA synthesized in vitro, which could be employed for further biochemical analyses without the danger of contamination by

pre-tRNA and other nonspecified small-molecular-weight RNA species.

The in vitro product can quantitatively be competed by cold cytoplasmic 5S rRNA in the hybridization reaction and, although this does not strictly exclude the possibility of antisense transcription, it is very unlikely that the size of a putative anti-5S RNA is fortuitously identical with that of in vivo 5S RNA. This is only conceivable if RNA polymerase C (or III) not only properly recognizes the complementary sequence of the termination signal as initiation site, but also terminates correctly at a sequence complementary to the normal initiation signal. Moreover, it was shown that virtually no hybridization occurred to DNA filters which were prehybridized to cold cytoplasmic 5S RNA. In addition it was found (Figure 7) that RNA synthesized in vitro, premixed, and separated in the same gel slot with purified in vivo 5S rRNA hybridizes with almost identical efficiency at the peak fraction. Therefore, antisense transcripts cannot contribute a significant portion to the total product, especially since they would hybridize with a much higher efficiency due to the absence of cold in vivo antisense 5S RNA.

The fidelity of transcription of ribosomal 5S RNA in isolated HeLa cell nuclei was additionally investigated by an analysis of T1 ribonuclease digests of the in vitro product on DEAE-Sephadex in the presence of 7 M urea (Figure 9). The comparative profiles, separately obtained for in vivo and in vitro 5S RNA, were shown to be very similar, although not identical in all the components. Discrepancies result from a slight ambiguity in termination, yielding 3′ termini containing up to two additional residues of U as outlined under Results. The profiles obtained for the internal oligonucleotides are, however, similar enough to exclude a large contribution of antisense transcription which should have completely distorted the oligonucleotide pattern. It was previously concluded by Marzluff et al. (1974) that antisense transcription was not detectable in isolated nuclei of mouse myeloma cells, using strand separated *X. laevis* 5S DNA as a probe. The interpretation of the data from their experiment, however, suffered from the presence of endogenous cold 5S RNA, vastly exceeding the quantity of available 5S DNA. Under these conditions, it is virtually impossible to detect any hybrid formation with antistrand RNA, because, even if present, these molecules will hybridize to the complementary DNA on the filter to a much lower degree in comparison with the very efficient RNA:RNA hybrid formation.

Collectively it was found in the course of this study that RNA polymerase C (or III), contained in isolated HeLa cell nuclei, efficiently synthesizes ribosomal 5S RNA of correct sequence and size. Random or antisense strand transcription of the 5S gene was not detected by the methodology employed. Conditions unfavorable for efficient synthesis of 5S rRNA resulted in a depression of the amount of product, without affecting the fidelity of its formation.

Acknowledgments

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Mutants of CHO Cells Resistant to the Protein Synthesis Inhibitors, Cryptopleurine and Tylocrebrine: Genetic and Biochemical Evidence for Common Site of Action of Emetine, Cryptopleurine, Tylocrebrine, and Tubulosine†

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ABSTRACT: Stable mutants resistant to the protein synthesis inhibitors cryptopleurine and tylocrebrine can be isolated in Chinese hamster ovary (CHO) cells, in a single step. The frequency of occurrence of cryptopleurine (Cry^R) and tylocrebrine (Tyl^R) resistant mutants in normal and mutagenized cell populations is similar to that observed for emetine resistant (Emt^R) mutants. The Cry^R, Tyl^R, and Emt^R mutants exhibit strikingly similar cross-resistance to the three drugs used for selection, to tubulosine and also to two emetine derivatives cephaeline and dehydroemetine, based both on assays of in vivo cytotoxicity and on assays of protein synthesis in cell-free extracts. The identity of cross-resistance patterns of the Cry^R, Tyl^R, and Emt^R mutants indicates that the resistance to all these compounds results from the same primary lesion, which in the case of Emt^R cells has been shown to affect the 40S ribosomal subunit. This conclusion is strongly supported by the failure of Emt^R, Tyl^R, and Cry^R mutants to complement each other in somatic cell hybrids. Based on these results it is suggested that the above group of compounds possesses common structural determinants which are responsible for their activity. The above mutants, however, do not show any cross-resistance to other inhibitors of protein synthesis such as cycloheximide, trichodermin, anisomycin, pactamycin, and sparsomycin, either in vivo or in vitro, indicating that the site of action of these inhibitors is different from that of the emetine-like compounds.

In bacteria, mutants resistant to various inhibitors of protein synthesis have been very useful in providing specific probes for the study of ribosome structure and function (Jaskunas et al., 1974). Unfortunately, studies on eukaryotic ribosomes have been hampered by the absence of similar appropriate mutations (Nomura et al., 1974). We have reported recently that stable mutants resistant to the protein synthesis inhibitor emetine can be isolated in a single step in Chinese hamster ovary (CHO)[1] cells (Gupta and Siminovitch, 1976). Protein synthesis in extracts of these mutant cells is resistant to the inhibitory action of emetine. In subsequent studies the emetine resistant (Emt^R) phenotype was shown to behave recessively in cell hybrids and was found to be due to an alteration in the 40S ribosomal subunit (Gupta and Siminovitch, 1977).

The successful isolation of Emt^R mutants indicated that it might be possible to obtain a spectrum of mutations involving a number of different ribosomal alterations. This approach was particularly attractive since a number of compounds are known which seem to act specifically as inhibitors of protein synthesis in yeast and mammalian cells (Pestka, 1971; Schindler and Davies, 1975; Battaner and Vazquez, 1971; Vazquez, 1974).

In this paper we report on the isolation of mutants of CHO cells resistant to the protein synthesis inhibitors cryptopleurine and tylocrebrine, two alkaloids of the phenanthrene group. However, detailed examination of the properties of these mutants as described here has revealed that these mutants are identical with the Emt^R mutants reported earlier (Gupta and Siminovitch, 1976, 1977) and that the protein synthesis inhibitors, emetine, tylocrebrine, cryptopleurine, and tubulosine, all act at the same site.

Experimental Procedures

Materials. Chemicals and antibiotics were obtained as follows: Emetine-HCl, cephaeline-HCl, and cycloheximide, Sigma Chemical Co., St. Louis, Mo.; cryptopleurine, Chemsea Manufacturing Pty, New South Wales, Australia; tylocrebrine and anisomycin, Dr. Nathan Belcher, Pfizer Inc., Groton, Conn.; pactamycin, Dr. C. P. Stanners, The Ontario Cancer Institute, Toronto, Canada; trichodermin, Dr. W. O. Godfredsen, Leo Pharmaceutical Products, Ballerup, Denmark; O-methylpsychotrine, Dr. H. T. Openshaw, Wellcome Research Laboratories, Kent, England; tubulosine, Dr. V. Deulofeu, Buenos Aires, Argentina; dehydroemetine, Roche Chemicals, Basel, Switzerland; sparsomycin, Dr. J. D. Douros, National Institutes of Health.

Cell Culture and Cell Lines. The techniques used in this laboratory for culturing CHO cells and for selecting various drug-resistance CHO lines have been described in detail earlier

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[1] Abbreviations used: CHO, Chinese hamster ovary cells; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.