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THE EFFECT OF CYTOSOL FROM REGENERATING RAT LIVER ON THE IN VITRO RNA SYNTHESIS OF ISOLATED CELL NUCLEI FROM A MORRIS HEPATOMA; COMPARATIVE STUDIES ON MOLECULAR HYBRIDIZATION OF NUCLEAR RNA

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SUMMARY

In vitro RNA synthesis in isolated cell nuclei from Morris hepatoma 9121 is stimulated by cytosol from regenerating rat liver. Molecular hybridization to rat liver DNA shows that this stimulation is accompanied by an increased transcription of slightly repetitive and unique DNA sequences.

Isolated cell nuclei which preserve for some time their capability for synthesizing RNA (1, 2) are widely used to investigate transcriptional processes in vitro. Addition of cytoplasm to the incubation medium leads to a relatively high and prolonged rate of RNA synthesis (3), nucleo-cytoplasmic interactions apparently playing an important role in gene expression (4, 5). As a preceding paper indicates (6), cytosol from regenerating rat liver not only stimulates RNA synthesis in isolated cell nuclei from resting rat liver but also in cell nuclei from a minimum deviation rat hepatoma (Morris hepatoma 9121). Moreover it was shown by molecular hybridization (7) that in resting rat liver nuclei this stimulation was accompanied by a change in the transcription pattern. Therefore it should be investigated whether this effect is limited to normal rat liver nuclei. This paper reports on comparative studies on molecular hybridization of nuclear RNA synthesized in vitro by isolated hepatoma nuclei in the presence of homologous and heterologous cytosol.

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MATERIALS AND METHODS

Preparation of cell nuclei and cytoplasm

Nuclei were isolated from Morris hepatoma 9121 which had been transplanted to male ACI rats (inbred animals, weight about 250 g) into both upper thighs. 4 - 5 weeks after transplantation when the tumors had a diameter of about 4 cm cell nuclei and cytosol were prepared. For the isolation of nuclei in principle the method of Pogo et al. (8) was applied as described in a previous paper (6). Cytosol (= 105,000 g supernatant) from Morris hepatoma and regenerating rat liver was prepared as described previously (6) and dialyzed overnight against distilled water. Protein content was estimated according to (9). In the case of the regenerating rat liver the animals were killed 24 h after partial hepatectomy (10).

Synthesis and extraction of RNA

About $2 \cdot 10^7$ cell nuclei from Morris hepatoma were incubated in a total volume of 1.5 ml for 30 min. The incubation mixture (6) contained 2.5 m Ci [^{3}H]UTP and cytosol from hepatoma or regenerating rat liver. The reaction was stopped in an ice bath, the incubated nuclei were sedimented and the labeled nuclear RNA extracted as described previously (7). Sedimentation analysis of the labeled nuclear RNA was carried out by centrifugation in a Beckman SW 40 rotor for 17 hours at 25,000 rev./min. at 5°C on linear 15 - 30 % sucrose gradients containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 10 mM EDTA, 0.2 % sodium dodecyl sulphate. Sedimentation coefficients were calculated according to McEwen (11).

Isolation of rat liver DNA

Rat liver DNA was isolated by standard procedures (12) including a 3 hours treatment with 50 µg/ml heat-treated (100°C/10 min) RNase, followed by an overnight treatment with 200 µg/ml of self-digested (3 h/37°C) Proteinase K. After sonication with a Lehfeldt sonifier (6 x 10 s) DNA was dialyzed against 0.12 M sodium phosphate buffer. Sedimentation coefficients of DNA fragments were determined under denaturing conditions by standard techniques in a MSE analytical centrifuge (Mark II) and were found to be 4.59 ± 0.11 S. Hyperchromicity of all DNA preparations was about 30 %, optical density ratios 280/260 0.51 - 0.53.

DNA/RNA hybridization

Hybridization reactions were carried out in excess DNA (13), DNA/RNA ratios being 10,000/1 and 20,000/1. Separation of single-stranded DNA and unhybridized RNA from double-stranded DNA and DNA-RNA hybrids was performed on hydroxyapatite columns using the urea-phosphate buffer system of Smith et al. (14). In this buffer unhybridized RNA and single-stranded DNA do not bind to hydroxyapatite at 40°C, whereas double-stranded DNA and DNA-RNA hybrids are bound. Hybridization mixtures containing DNA and total nuclear [^{3}H]RNA in 0.12 M or 0.48 M sodium phosphate buffer, 0.2 % sodium dodecylsulphate were sealed in capillary tubes, denatured by boiling for 7 min. and incubated at 69°C for various times. Equivalent $C_o t$ values were calculated according to (15). At the end of the incubation period the hybridization mixtures were diluted 100 fold with the urea-phosphate solution and passed over hydroxyapatite. The E 260 values of the fractions eluted at 40°C and 80°C were determined in order to estimate the amount of reassociated DNA. Each fraction was measured in "Unisolve" in a liquid scintillation counter in order to determine the content of hybridized [^{3}H]RNA. The chemicals used were purchased from E. Merck, Darmstadt, non-labeled nucleoside triphosphates from

Boehringer, Mannheim, [^{3}H]UTP from Amersham-Buchler, Braunschweig, hydroxyapatite (DNA-grade Bio-Gel HTP) from Bio-Rad, München.

RESULTS

Size distribution of the in-vitro synthesized nuclear RNA and specific activity

Sedimentation profiles of in-vitro synthesized [^{3}H]RNA on linear 15 - 30 % sucrose gradients are shown in Fig. 1. Total nuclear [^{3}H]RNA as used in hybridization reactions was synthesized in the presence of homologous as well as heterologous cytosol; size distribution was ranging from 5 S to 45 S with a slight shift to higher sedimentation coefficients in the case of [^{3}H]RNA synthesized in the presence of heterologous cytosol. Total nuclear RNA synthesized in a medium containing hepatoma cytosol had a specific activity of 16,650 cpm/µg whereas that of RNA synthesized in a medium containing cytosol from regenerating rat liver was 22,078 cpm/µg.

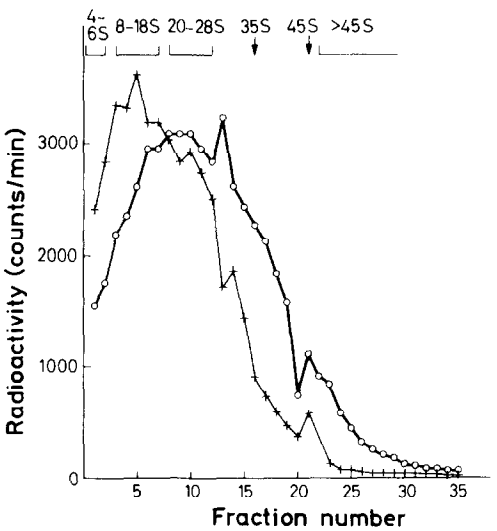
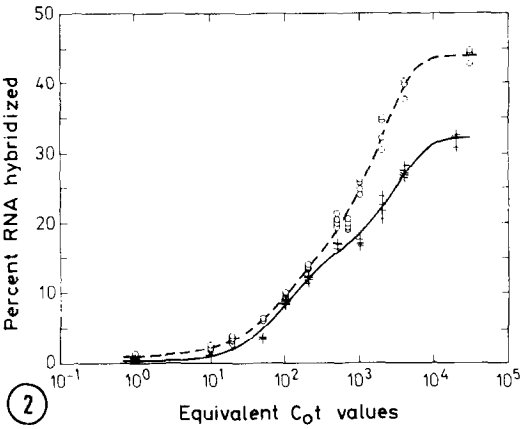


Fig. 1: Sedimentation analysis of nuclear [^{3}H]RNA synthesized in the presence of homologous (+) and heterologous (o) cytosol.



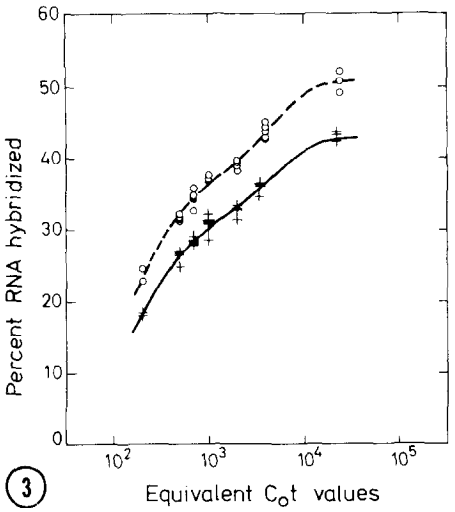


Fig. 2: Hybridization kinetics of nuclear [^{3}H]RNA synthesized in the presence of homologous (+) and heterologous (o) cytosol. C_0t = concentration of DNA (moles of nucleotides/litre) x seconds. DNA/RNA ratio 10,000/1.

Fig. 3: Hybridization kinetics of nuclear [^{3}H]RNA synthesized in the presence of homologous (+) and heterologous (o) cytosol. DNA/RNA ratio 20,000/1.

Hybridization of rat liver DNA with hepatoma nuclear RNA synthesized in-vitro

Second order reaction curves of hybridization of hepatoma nuclear [^{3}H]RNA with excess sheared total rat liver DNA are illustrated in Fig. 2 and 3. The kinetics of reassociation of rat liver DNA which takes place in the same reaction are shown in Fig. 4. Curves were fitted by computer analysis assuming three reacting components (7) including a least squares fit program described by Pearson et al. (16). 25 % of the sheared rat liver DNA anneal at C_0t values less than about 100. They represent highly repetitive and moderately repetitive DNA sequences. The remaining fraction consists of slightly repetitive and of unique DNA. Reiteration frequency (F) of the DNA sequences which hybridized with the nuclear [^{3}H]RNA was calculated by comparison with data given for E.coli RNA made in-vitro, using the relationship (13):

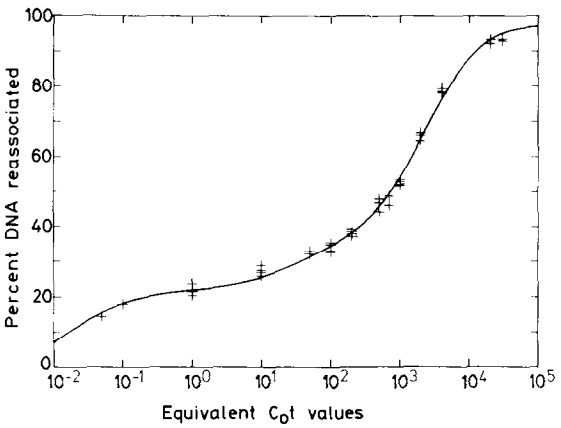


Fig. 4: Reassociation kinetics of sheared rat liver DNA.

$$F = \frac{C_0t_{\frac{1}{2}} \text{ (E.coli cRNA)}^*}{C_0t_{\frac{1}{2}} \text{ (rat nuclear RNA)}} \times \frac{G \text{ (rat)}}{G \text{ (E.coli)}}$$

$C_0t_{\frac{1}{2}}$ are the respective C_0t values at which half of each component of RNA hybridized. The data are comparable, since hybridization was carried out under corresponding conditions. G are the analytical complexities (total molecular weight of haploid DNA) that of rat being $1.8 \cdot 10^{12}$ and of E.coli $2.7 \cdot 10^{9}$. Fig. 2 shows the hybridization of [^{3}H]RNA to an excess of rat liver DNA at a DNA/RNA ratio of 10,000/1. About 2 % of the in-vitro synthesized [^{3}H]RNA hybridize with highly repetitive DNA sequences. 11 % of [^{3}H]RNA anneal with a $C_0t_{\frac{1}{2}}$ value of 74 when the [^{3}H]RNA has been synthesized in the presence of homologous and of 63 when having been synthesized in the presence of heterologous cytosol. From these values a reiteration frequency of 142 and 168 for the complementary DNA sequences is estimated. As the curve demonstrates the rate of hybridization is increased for RNA synthesized with heterologous cytosol. A third fraction of [^{3}H]RNA, 19 % when it was synthesized in a medium containing homologous, and 31 % when

*15,9 Melli (13)

the medium contained heterologous cytosol hybridizes at a $C_o t_{\frac{1}{2}}$ value of 2200 and 1350 respectively. These values correspond to a reiteration frequency of 5 and 8. Here again heterologous cytosol leads to an increased hybridization. Maximum hybridization in these experiments reaches 32 % and 44 %, respectively. The kinetics of the hybridization of the nuclear [^{3}H]RNA to excess DNA at a DNA/RNA ratio of 20,000/1 are shown in Fig. 3. One portion of [^{3}H]RNA synthesized in the presence of homologous cytosol is annealed at a $C_o t_{\frac{1}{2}}$ value of 130 and in the presence of heterologous cytosol at a $C_o t_{\frac{1}{2}}$ of 114. From these values a reiteration frequency of 81 and 93 can be estimated. The other hybridizing fraction of [^{3}H]RNA is characterized by a $C_o t_{\frac{1}{2}}$ value of 3300 in the case of homologous and of 3060 in the case of heterologous cytosol corresponding to a reiteration frequency of the complementary DNA sequences of 3 to 5. As the curves show, cytosol from regenerating rat liver causes a significant increase in hybridization.

DISCUSSION

The presented results indicate, that cytosol from regenerating rat liver stimulates the in vitro RNA synthesis of hepatoma nuclei as compared to homologous cytosol. The size distribution of the nuclear [^{3}H]RNA on linear sucrose gradients shows a slight shift to higher sedimentation coefficients when synthesized in the presence of heterologous cytosol. So, in addition to a stimulation of nuclear transcription, the possibility of a reduced decay of newly synthesized RNA cannot be excluded. Hybridization to an excess of rat liver DNA clearly demonstrates that cytosol from regenerating rat liver effects gene expression in hepatoma nuclei in such a way that the portion of transcribed sequences

which are complementary to rat liver DNA is increased. Increase in hybridization is obtained in the experiments with a DNA to RNA ratio of 10,000/1 as well as in those with a DNA/RNA ratio of 20,000/1. The extent of maximum hybridization depends on the DNA/RNA ratio. Increasing the DNA/RNA ratio from 10,000/1 to 20,000/1 leads to a greater amount of hybrids formed at high $C_o t$ values. From a theoretical point of view 100 % hybridization should be achieved only at infinite DNA excess. In order to get an extreme, yet still finite DNA excess, RNA of very high specific activity would be required. Another difficulty arises from the fact, that in isolated nuclei the newly synthesized RNA is contaminated by already present unlabeled RNA. Mercurated nucleotides as precursors in order to separate in vitro transcribed RNA are apparently not useful in such experiments; as shown by Schäfer (17) they cause an inhibition of RNA synthesis in isolated cell nuclei. By using highly labeled precursors a DNA/RNA ratio of 10,000/1 and 20,000/1 could be achieved. Increase in hybridization takes place in the $C_o t$ range of slightly repetitive and unique DNA. The genes coding for histones are known to be redundant (18), those coding for rRNA as well (19, 20, 21, 22). Unique DNA contains sequences coding for proteins. Yet nuclear RNA is rather complex and the presented data allow general conclusions only with respect to the type of genes transcribed at an elevated level. Here, competition hybridization might provide further information.

Although hybridization to an excess of DNA in general remains incomplete, the presented results demonstrate, that cytosol from a normal rapidly proliferating tissue (regenerating rat liver) leads in hepatoma nuclei to an increased synthesis of RNA sequences complementary to slightly repetitive and single copy rat

liver DNA. The increase in hybridizing sequences takes place in the same $C_o t$ range as it is observed in nuclear RNA from resting rat liver nuclei when synthesized in the presence of cytosol from regenerating rat liver (7). In resting rat liver nuclei the additional sequences transcribed may play a role in processes connected with DNA replication. Whether the effect on gene expression in hepatoma nuclei is a similar one, might be clarified by competition and cross hybridization of the nuclear RNAs.

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