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COMPARATIVE STUDIES ON MOLECULAR HYBRIDIZATION OF NUCLEAR RNA SYNTHESIZED BY ISOLATED RAT LIVER NUCLEI: EFFECT OF HOMOLOGOUS AND HETEROLOGOUS CYTOSOL

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SUMMARY

In vitro RNA synthesis in isolated nuclei from normal rat liver is stimulated by cytosol from regenerating rat liver. Molecular hybridization experiments indicate that this stimulation is accompanied by changes in the transcription pattern especially at slightly repetitive and unique DNA.

The capability of isolated cell nuclei to synthesize RNA in vitro (1,2) provides a system for investigating various effects on nuclear transcription. The extent of this RNA synthesis widely depends on the medium in which the nuclei are incubated. Apparently nucleo-cytoplasmic interactions play an important role in controlling gene expression of eukaryotic cells (3,4) because by adding cytoplasm to the medium (5) a relatively high and prolonged rate of RNA synthesis can be obtained. A preceding paper (6) contains a report that cytosol from regenerating rat liver stimulates the in vitro RNA synthesis of isolated rat liver nuclei as compared to the effect of homologous cytosol. These results led us to examine whether the stimulation is accompanied by changes in the transcription pattern. A suitable method is molecular hybridization of RNA with its template, hybridization in vast DNA excess also allowing conclusions with respect to the reiteration frequency of DNA sequences from which the RNA had been transcribed. This paper reports on comparative studies on molecular hybridi-

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zation of RNA synthesized in vitro in the presence of homologous and heterologous cytosol.

MATERIALS AND METHODS

Preparation of cell nuclei and cytoplasm

Nuclei were isolated from the liver of female Wistar-rats (weight 140-160 g). In principle the method of Pogo et al. (7) was applied as described in a previous paper (6). Cytosol (= 105,000 g supernatant) from normal and regenerating rat liver was prepared as described previously and dialyzed overnight against distilled water. Protein content was estimated according to (8). In the case of regenerating rat liver the animals were killed 24 h after partial hepatectomy (9).

Synthesis and extraction of RNA

1-2 x 10^8 rat liver nuclei were incubated in a total volume of 1.5 ml for 30 min. The previously described incubation mixture (6) contained 2.5 mCi [^{3}H]UTP and cytosol from resting or regenerating rat liver. The reaction was stopped in an ice bath, the incubated nuclei sedimented at 700 g and suspended in 3 ml of 50 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$. Then 3 ml of a buffer containing 1 % sodiumdodecyl sulphate, 0.01 M sodium acetate, 2 µg/ml polyvinyl sulphate, 0.15 M NaCl pH 5.2 were added and the mixture extracted twice at 60^{0}C with an equal volume of redistilled phenol saturated with 0.01 M acetate buffer pH 5.2 (10). The aqueous phase then was extracted with chloroform-isoamyl alcohol (24:1), layered onto a 100 x 2.5 cm Sephadex G-100 column and eluted with distilled H_2O in order to remove smaller molecules. Fractions containing labeled RNA were pooled and lyophilized, dissolved in distilled H_2O, containing 0.01 % sodiumdodecyl sulphate and stored at -20^{o}C. Sedimentation analysis was carried out by centrifugation in a Beckman SW 41 rotor on linear 15-30% sucrose gradients containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 10 mM EDTA, 0.2 % sodium deoxycholate. Sedimentation coefficients were calculated according to McEwen (11).

Isolation of rat liver DNA

Rat liver DNA was isolated by standard procedures (12,15) including a 3 hours treatment with 50 µg/ml heat-treated (100^{o}C/10 min.) RNase, followed by a further treatment with 400 µg/ml of self-digested (3 h/37^{o}C) Proteinase K of 3 hours. After sonication with a Lehfeldt sonifier (6 x 10s) 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 and were found to be 4.35 $\pm$ 0.18 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 ratio being 10,000/1. As shown by Smith et al. (14) unhybridized RNA and single-stranded DNA do not bind to hydroxyapatite at 40^{o}C in 8 M urea, 0.2 M phosphate buffer, 1 % sodiumdodecyl sulphate, whereas double-stranded DNA and RNA-DNA 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 % sodiumdodecyl sulphate were sealed in capillary tubes, denatured by boiling for 7 min. and incubated at 69^{o}C for various times. Equivalent C_0t 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 counted 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.

R E S U L T S

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 illustrated 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 about 45 S. Total nuclear RNA synthesized in a medium containing homologous cytosol had a specific activity of 37,850 cpm/µg whereas that of RNA synthesized in a medium containing cytosol from regenerating rat liver was 44,160 cpm/µg.

Hybridization of rat liver DNA with nuclear RNA synthesized in vitro

Second order reaction curves of nuclear [^{3}H]RNA hybridized with sheared rat liver DNA excess are shown in Fig. 2. The kinetics of reassociation of rat liver DNA which takes place in the same reaction are illustrated in Fig. 3. Curves were fitted by computer analysis assuming three reacting components. 25 % of 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.

About 6 % of nuclear RNA synthesized in homologous as well as in heterologous cytosol hybridizes with fast reassociating DNA se-

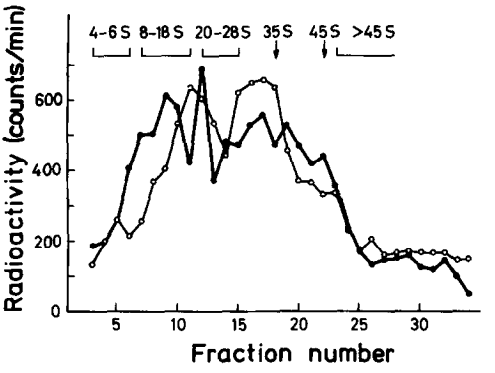
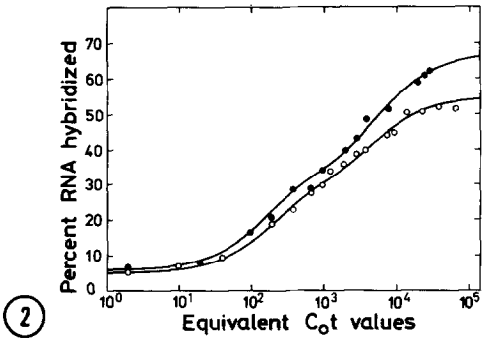


Fig. 1: Sedimentation analysis of nuclear [^{3}H]RNA synthesized in the presence of O homologous and ● heterologous cytosol on linear 15-30% sucrose gradients.



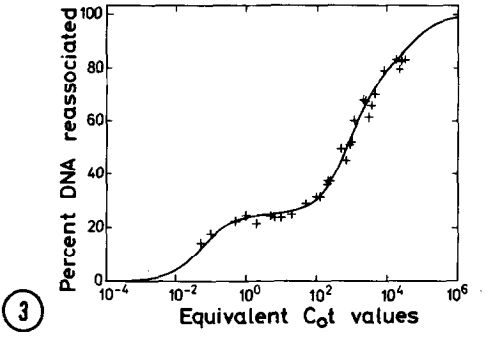


Fig. 2: Hybridization kinetics of nuclear [^{3}H]RNA synthesized in the presence of O homologous and ● heterologous cytosol. C_0t = concentration of DNA (moles of nucleotides/litre) x seconds. Mean values from 4 to 6 experiments. S.E.M. ranging from ± 0.49 to ± 1.35 % hybridized.

Fig. 3: Reassociation kinetics of sheared rat liver DNA. Mean values from 4 to 6 experiments. S.E.M. ranging from ± 0.78 to ± 2.46 % reassociated.

quences. A second component comprising about 20 % of the hybridizing RNAs is complementary to slightly repetitive DNA. Reiteration frequency of these DNA sequences estimated from hybridization with RNA synthesized in the presence of homologous cytosol is 71 and in heterologous cytosol 86. In the range of this compo-

nent the rate of hybridization is increased for the latter RNA. Reiteration frequency (F) was calculated by comparison with data given for E. coli RNA made in vitro, using the relationship (13):

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

$C_0t_{\frac{1}{2}}$ 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 nuclei being $1.8 \cdot 10^{12}$ and of E.coli $2.7 \cdot 10^{9}$. A third component of [^{3}H]RNA anneals with DNA at a $C_0t_{\frac{1}{2}}$ of 3000 when having been synthesized with homologous, and at $C_0t_{\frac{1}{2}}$ of about 3600 with heterologous cytosol. Using the above relationship, both RNAs should be complementary to DNA sequences reiterated about 3 times. Here again cytosol from hepatectomized rat livers leads to an increase in hybridization. Maximum hybridization of [^{3}H]RNA with sheared rat liver DNA is 51 or 62 percent, respectively.

DISCUSSION

The presented results indicate that cytosol from regenerating rat liver not only stimulates the in-vitro RNA synthesis of isolated rat liver nuclei as compared to cytosol from resting rat liver but in addition causes a change in the transcription pattern. Although nuclear RNA synthesized under both conditions has similar size distributions in sucrose density gradients the specific activity of the RNA increases due to the effect of cytosol derived from hepatectomized liver. Differences in hybridization kinetics, however, clearly demonstrate that cytoplasmic factors from regenerating rat liver enhance the transcription of certain sequences which are transcribed not at all - or only to a minor extent - in resting

* 15,9 Melli (13)

rat liver. The sequences concerned hybridize in the C_0t range of slightly repetitive and of unique DNA. Supposedly these sequences represent transcripts of genes coding for proteins involved in DNA replication. In regenerating rat liver DNA synthesis has its maximum 25 h after hepatectomy (16); this is about the time, when in our experiments hepatectomized rats were killed in order to obtain cytosol. The genes coding for rRNA are known to be redundant, values in the literature vary from 360 to 100 copies (17,18,19,20). Histone genes are redundant as well (21), in mouse and man they are found to be about 40 fold repeated (22,23). Although the increase in hybridization takes place in the range of slightly repetitive and single copy genes, the presented data allow general conclusions only on the effect of cytosol from a rapidly proliferating tissue on gene expression in cell nuclei of resting rat liver. For several reasons hybridization under the conditions of DNA excess cannot be complete. The extent of maximum hybridization obtainable depends on the DNA/RNA ratio. 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. By using highly labeled precursors we tried to overcome this problem achieving a DNA/RNA ratio of 10,000/1. The use of mercurated nucleotides as precursors in order to separate in vitro transcribed RNA from already present RNA cannot be considered to be very useful; as shown by Schäfer (24) these modified nucleotides cause an inhibition of RNA synthesis in isolated cell nuclei. Another reason for incomplete hybridization may be due to the fact that from certain late reassociating single copy genes a large number

of RNA-molecules is transcribed; so with respect to these special genes DNA cannot be in large excess. Complete hybridization as well as 100 % reassociation is also impaired by the necessity to use sheared DNA with random size distribution. Paired DNA fragments or hybridized DNA/RNA strands usually do not terminate at identical loci, instead they form double-stranded complexes with overlapping single-stranded ends.

Although hybridization in vast DNA excess in general remains incomplete, our results clearly demonstrate, that cytoplasmic factors from regenerating rat liver are able to enhance transcription of certain slightly repetitive and unique DNA sequences, probably in connection with processes for DNA replication. The type of genes actually involved cannot be identified as yet. Here competition hybridization might provide further information.

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