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NASCENT AND TOTAL NUCLEAR RNA FROM RAT LIVER ARE OF SIMILAR SEQUENCE COMPLEXITIES

Trevor J.C. Beebee and Denrick S. Carty

Department of Biochemistry, University of Sussex, Falmer, Brighton BN1 9QG, U.K.

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SUMMARY: We have investigated the proportion of the rat liver genome under transcription by studying the sequence complexity of nascent RNA molecules initiated *in vivo* and permitted to elongate *in vitro* in the presence of mercurated CTP. The mercurated RNA was purified by affinity chromatography on thiopropyl sepharose, and by employing this material as a driver with trace amounts of labelled single-copy DNA in hybridisation reactions it has been possible to show that nascent RNA is not significantly more complex than the steady-state nuclear RNA population normally isolated.

We were interested in establishing whether nuclear RNAs with high rates of turnover are under-represented in samples extracted by usual methods, and thus the possibility that previous studies may have given rise to underestimates of the proportion of genomes under transcription in various cells and tissues. Under conditions of RNA excess and with the single-copy fraction of DNA which can be isolated by hydroxyapatite chromatography, nucleic acid hybridisation techniques have been used to estimate nuclear RNA sequence complexities in a number of different cell types over the last few years; in the case of rat liver, probably between 6.0-7.0% of genomic single-copy DNA can be driven into hybrid by nuclear RNA (1-3) though these figures need to be interpreted with some caution due to possible methodological problems (4).

Nuclei isolated from rat liver are able to elongate *in vitro* nascent RNA molecules previously initiated *in vivo*, and at high ionic strength in the presence of mercurated CTP there is essentially negligible reinitiation and nuclease degredation; such molecules can be readily isolated by affinity chromatography (5). We have used RNA prepared in this way as a driver in RNA excess hybridisation reactions to determine whether the sequence complexity of this population differs from that of total nuclear RNA.

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MATERIALS AND METHODS

Materials. (^{3}H)dCTP and γ(^{32}P)ATP were from the Radiochemical Centre, England. dTTP, dATP, dGTP, deoxyribonuclease I, ribonuclease A, protease V, alkaline phosphatase, S1 nuclease, ATP, GTP, CTP and UTP were from Sigma. E. coli DNA polymerase I and polynucleotide kinase were from Boehringer. Sephadex G-10, G-75 and thiopropyl sepharose 6B were from Pharmacia. GF/C and DE-81 filters were from Whatman, England.

Hydroxyapatite was a kind gift from Dr. A. MacGillivray. Mercurated CTP was synthesised as described elsewhere (6,7) and stored at -20°. For some experiments total nuclear RNA was mercurated directly under conditions of very low ionic strength (6), repurified and used to drive hybridisation reactions.

Isolation of nascent RNA. Nuclei were prepared from rat liver as previously described (8) and incubated in the presence of 0.3 M ammonium sulphate, 0.25 mM mercurated CTP and other components listed elsewhere (8) except that (^{3}H)UTP was replaced by unlabelled UTP, for 30 minutes at 37°. Nascent RNA was then isolated by protease digestion, phenol extraction, gel filtration on Sephadex G-75 and two rounds of thiopropyl sepharose chromatography; nuclei from 150 g of liver routinely yielded 10-20 µg nascent RNA which has been estimated to be at least 98% pure (5).

Total nuclear RNA for some experiments was isolated as described previously (8).

Preparation of DNA. High molecular weight DNA was obtained from liver nuclei by the method of Gross-Bellard et al (9) and either labelled directly by nick-translation (10) in the presence of trace amounts of deoxyribonuclease I or sonicated to fragment lengths of less than 1000 base-pairs followed by denaturation, renaturation to a Cot of 250, and passage through hydroxyapatite at 60°. The latter procedure was repeated once more, and material still single-stranded reannealed to Cot 30,000 prior to nick-translation in the absence of deoxyribonuclease. Finally, radioactive DNA of both types was centrifuged through alkaline sucrose gradients (11) and material in the size range 100-500 nucleotides (normally more than 80% of the total) isolated and used in experiments.

For some experiments single-copy DNA fractionated on hydroxyapatite as described above was end-labelled after the method of Maxam and Gilbert (12) using alkaline phosphatase, polynucleotide kinase and γ(^{32}P)ATP. Both methods yielded DNA labelled to between 10^6-10^7 counts/min./µg.

Hybridisation conditions. Labelled DNA was mixed with an appropriate excess of either unlabelled DNA or RNA and lyophilised overnight. The nucleic acids were then redissolved usually in 1-2 µl of hybridisation buffer containing 50% (v/v) formamide (10,13), transferred to siliconised glass capillaries which were then sealed, heated for 5 minutes at 70° and finally transferred to a 43° incubator where they remained for the appropriate times. Extent of annealing was estimated by measuring resistance to S1 nuclease digestion using precipitation on GF/C discs or adsorption onto DE-81 filters (14) unless otherwise stated.

RESULTS AND DISCUSSION

The results of allowing labelled total or single-copy DNA to reanneal to an excess of sonicated unlabelled rat DNA are shown in figure 1. The rather high background nuclease resistance in this experiment was a feature of the assay method (involving filtration on GF/C discs). Nevertheless it was evident that the radioactive DNA was in both instances capable of hybridisation up to at least 70% and that the fractionation on hydroxyapatite removed sequences of intermediate reiteration which otherwise anneal at low Cot values (15).

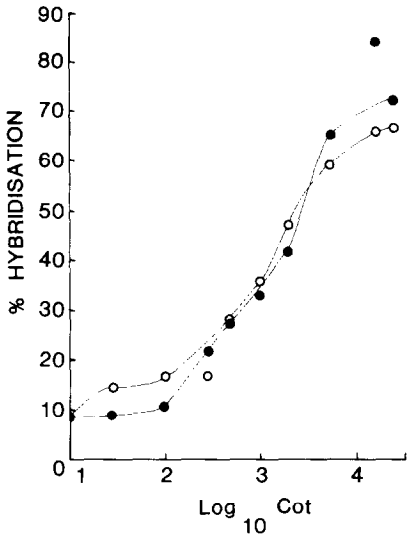

Figure 1: Reannealing of labelled DNAs. Assays contained 0.5 ng (^{3}H)DNA (4000 counts/min) and 30 µg unlabelled sonicated DNA in final volumes of 2 µl. Extent of annealing was estimated by resistance to S1 nuclease digestion. ● , single-copy (^{3}H)DNA; ○ , unfractionated (^{3}H)DNA.

Hybridisation of the radioactive DNAs to nuclear RNA is shown in figure 2. In this instance about 5.2% of the total (unfractionated) DNA was driven into hybrid; adjustment based on 70% hybridisability yields a true value of 7.4% complementarity, uncompensated for transcriptional asymmetry, which is within the range anticipated on the basis of previous studies using single-copy DNA (1-3). However, the procedure used by us to prepare single-copy DNA consistantly yielded material of which about 12% could be driven into hybrid. This enrichment of hybridisable sequences may be due to the use of rather large fragments of DNA in the reannealing and hydroxyapatite fractionations; it is not a result of the S1 nuclease assay procedure (see table 1). Presumably because of this large fragment size only about 35% of the input DNA was recovered in our single-copy fraction, which we chose to use on account of its high hybridisability and therefore resolving power for detecting differences between RNA populations. Clearly, however, it would be unsafe to determine in any absolute way the proportion of the genome under transcription using data obtained with this DNA fraction and only relative variations in hybridisation

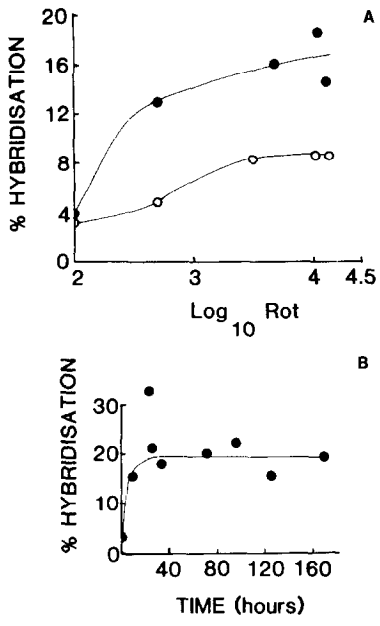

Figure 2: Hybridisation of (^{3}H)DNA to nuclear RNA. A: Total nuclear RNA, in this instance at 11.5 mg/ml, with either single-copy (^{3}H) DNA (●) or unfractionated (^{3}H)DNA (○). B: Excess mercurated nuclear RNA with (^{3}H)single-copy DNA. Duplicate samples were assayed for hybrid formation using S1 nuclease digestion and adsorption onto DE-81 discs.

have been considered valuable. The mechanism underlying this selection for hybridisable sequences remains obscure, especially since previous studies have shown messenger-coding sequences to be generally interspersed with and close to repetitive ones (16).

Because nascent RNA necessarily contains mercurated nucleotides which attempts to remove can cause problems with subsequent hybridisability (7), it was important to determine the effect of mercuration on the extent of hybridisation. To do this some nuclear RNA was mercurated directly and used in hybridisation reactions; because mercuration caused a marked decrease in RNA solubility there were difficulties in accurate concentration determination and consequently the hybridisation was recorded as a function of time rather than Rot (figure 2B). A small but consistant increase in the production of nuclease-resistant DNA was observed, such that a net of about 16% was apparently driven into hybrid. The reasons for this effect have not been investigated.

Table 1. Hybridisation of single-copy DNA to nascent and total nuclear RNA.

A: S1 nuclease assays

µg RNA in assay	(^{3}H)DNA (counts/min)		%Hybridisation	
	-S1	+S1	Total	After 0-RNA subtraction
0	800(33)	36(0)	4.5	
2.5 total nascent	1115(117)	250(6)	22.4	17.9
5.0 total nascent	1001(321)	202(39)	20.2	15.7
2.0 mixed RNA	1125(86)	186(13)	16.5	12.0
2.5 nascent+amanit.	814(115)	172(11)	20.5	16.0
5.0 nascent+amanit.	803(3)	133(30)	16.5	12.0

B: Hydroxyapatite chromatography assays

µg nuclear RNA in assay	(^{32}P)DNA (Counts/min)		%Hybridisation		
	Total	Retained on hydroxyapatite	Total	After 0-RNA subtraction	After RNAase
0	1214	43	3.5		
6	2925	492	16.8	13.3	3.1
12	4134	686	16.6	13.1	1.8

Nascent RNA was isolated as described in methods; α-amanitin, when present, was at 1 µg/ml in the *in vitro* incubations. The mixed RNA sample was from a single passage through thiopropyl sepharose (see text). RNA concentrations in the hybridisation assays were around 2 mg/ml with 0.5 ng labelled DNA in final volumes of 1-2 µl. In all instances reactions were continued to Rots in excess of 10^4. Standard deviations are given in brackets for A; data in B are from single determinations. Ribonuclease digestion of hybrids was carried out in 50 mM phosphate buffer pH 6.8 with 20 µg/ml enzyme for 1 hour at 37° prior to phenol extraction and chromatography on hydroxyapatite in B.

Table 1A shows the results of experiments using nascent RNA in hybridisations; the use of two different amounts confirmed that the RNA was in excess. An average of 16.8% of the DNA was driven into hybrid by total nascent RNA, essentially the same as for total (mercurated) nuclear RNA. The inclusion of α-amanitin in the *in vitro* incubation reduced the yield of nascent RNA by more than 50%, which is comparable with its effect on incorporation of (^{3}H)nucleotides (5). However, little effect was apparent on the complexity of sequences under transcription with 14% of labelled DNA still being driven into duplex. Since it is generally thought that RNA

polymerase II (sensitive to α-amanitin) is responsible for structural gene transcription in eukaryotic cells this was a perplexing observation. It may be that some of the nascent RNA being synthesised by this enzyme was elongated a short distance prior to inhibition, such that one or more mercurated nucleotides were still incorporated into the growing chains.

A mixed population of nascent and total nuclear RNA (1:4) is obtained after a single passage through thiopropyl sepharose (5). When this was used to drive hybridisations the final level of S1 nuclease resistance was similar to that of total nuclear RNA, inferring that the two populations were essentially homologous in terms of sequence complexity. Significant heterology should have led to an overall increase in DNA hybridised.

Many previous studies of single-copy DNA hybridisation have used hydroxyapatite chromatography rather than nuclease digestion to measure duplex formation. Although the latter is more rapid and convenient, and has been shown to yield similar results to the former and perhaps to be more desirable in principle (4,14) it was important to compare the situation with our 35% DNA fraction. The results of table 1B confirmed that the extent of hybridisation of (^{32}P)DNA against nuclear RNA was virtually identical on hydroxyapatite to that seen using (^{3}H)DNA and S1 nuclease. Controls in table 1 also confirmed that duplex formation was negligible in the absence of added RNA and sensitive to ribonuclease digestion at low ionic strength.

Taken together these results suggest that in terms of sequence complexity total nuclear RNA accurately reflects the transcriptionally active part of the rat liver genome since nascent RNA appears to be essentially identical. The use of nuclear RNA and cloned gene fragment hybridisations as a method for identifying promoter regions of eukaryotic genes also receives support from our results, which make unlikely the existence of rapidly degraded long leader sequences that might disguise the true start points for transcription in such analyses.

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