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TRANSCRIPTION OF REITERATED AND UNIQUE DNA SEQUENCES BY ENDOGENOUS RNA POLYMERASES IN RAT LIVER NUCLEOPLASM

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: A nucleoplasmic fraction isolated from rat liver nuclei was characterised with respect to *in vitro* transcription products by the use of mercurated CTP. At high salt concentrations which prevented initiation *in vitro* mercurated CTP was utilised as a substrate at about 50-60% of the efficiency of CTP. The difference was due to alteration of the elongation rates of the endogenous transcription complexes and not to selective inhibition of particular classes of enzymes. Hybridisation in vast DNA excess showed that more than 80% of the RNA could be driven into hybrid and that both reiterated and unique sequences were under transcription (mainly by RNA polymerase II) in the nucleoplasmic chromatin.

It has long been known that heterogeneous nuclear RNA, the mixture of molecules including messenger RNA precursors, consists of transcripts from both reiterated and unique DNA sequences in the eukaryotic genome (1). Most or all of this material is thought to be synthesised by RNA polymerase II (2). In an attempt to characterise the products of this enzyme in greater detail we have recently analysed the activity of an isolated nucleoplasmic fraction which continues to elongate RNA chains previously initiated *in vivo* under conditions whereby initiation cannot occur *in vitro* (3). Most of the incorporation was sensitive to low concentrations of α-amanitin and therefore due to RNA polymerase II, but hybridisation analysis of the transcripts was limited by the presence of endogenous RNA sequences preventing attainment of adequate DNA excess. In this paper we report the

Abbreviation. Hg-CTP, 5-mercuri-cytidine 5'-triphosphate.

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successful use of mercurated CTP (Hg-CTP) as an affinity label in the nucleoplasmic system as well as the efficient hybridisation of the transcripts.

MATERIALS AND METHODS

Materials. [^{3}H]UTP was from the Radiochemical Centre, England. ATP, GTP, CTP, UTP, deoxyribonuclease, ribonuclease and protease were from Sigma. Sephadex G-10, G-75 and Thiopropyl-sepharose 6B were from Pharmacia. Polyethyleneimine-cellulose thin-layer chromatography plates were from Camlab, England. α-Amanitin was a kind gift from Professor T. Weiland.

Hg-CTP was synthesised essentially as described by Dale et al (4) and purified by chromatography on G-10 and DEAE-cellulose prior to precipitation with acetone, filtration and storage at -20°.

Isolation of nucleoplasm and assay of RNA synthesis. These procedures were carried out as described elsewhere (3), assays being at high salt concentrations (0.3 M ammonium sulphate) to prevent initiation from occuring in vitro. Hg-CTP, where present, replaced CTP normally at 0.3 mM and [^{3}H]UTP was used at 0.05 mM.

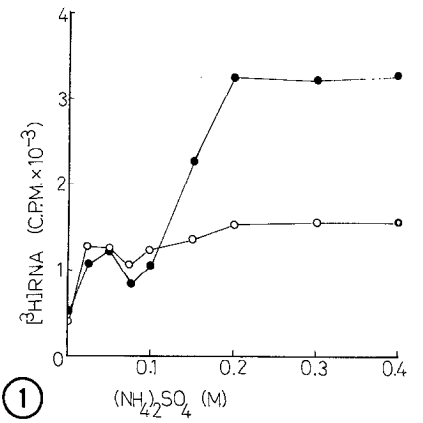
Numbers of active RNA polymerase molecules and elongation rates. Estimations were carried out by overnight hydrolysis of RNA labelled with [^{3}H]UTP in vitro followed by separation of [^{3}H]UMP and [^{3}H]-uridine on polyethyleneimine-cellulose (3,5). Numbers of RNA polymerase molecules were quantified from the amount of labelled uridine and expressed on a per-nucleus basis following DNA estimation (6). Elongation rates were calculated from the UMP:uridine ratios.

Purification of RNA. After incubations of nucleoplasm in vitro, RNA was isolated by digestion with deoxyribonuclease and protease, extracted with phenol and chromatographed on G-75 as described elsewhere (3). Following precipitation with ethanol, mercurated RNA was dissolved in 0.1 M sodium acetate pH 6, 0.25% (w/v) sodium lauryl sulphate and applied to a 1 x 6 cm column of thiopropyl-sepharose. After standing for 1 h at room temperature the column was washed with the loading buffer and the RNA eluted subsequently with buffer containing 0.1 M 2-mercaptoethanol. Demercuration of the RNA was then performed by standing in 3 M 2-mercaptoethanol overnight at room temperature following adjustment of the sodium lauryl sulphate to 1% (7). Finally the RNA was passed through a 1 x 10 cm column of Chelex-100, precipitated with ethanol and the pellet redissolved in a small volume of water for storage at -20°.

Hybridisation in vast DNA excess. This was carried out essentially as described elsewhere (3) under optimal conditions determined experimentally and involving incubations at 58° in the presence of 0.12 M sodium phosphate pH 6.8 and 0.5% sodium lauryl sulphate.

RESULTS AND DISCUSSION

Nucleoplasm actively synthesised RNA in the presence of Hg-CTP, but at the high salt concentrations required to stimulate the endogenous RNA polymerases maximally and prevent initiation in



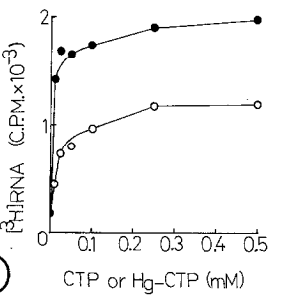


Figure 1: Dependence of mercurated RNA synthesis on salt concentration. Nucleoplasm from 500 mg liver was used in standard assays for 10 minutes at 37°. ● = With 0.3 mM CTP; ○ = With 0.3 mM Hg-CTP.

Figure 2: Dependence of RNA synthesis on substrate concentration. Assays contained nucleoplasm from 250 mg liver and were with either CTP (●) or Hg-CTP (○) and 0.3 M ammonium sulphate.

vitro it was less effective as a substrate than CTP (Figure 1). The biphasic response to salt is characteristic of nucleoplasm and nuclei from rat liver (3,8).

In figure 2 the effects of various CTP and Hg-CTP concentrations on [^{3}H]UMP incorporation by nucleoplasm at 0.3 M ammonium sulphate are shown. It is clear that the maximal velocity of the reaction was reduced by substituting Hg-CTP for CTP, in accord with previous studies of intact nucleoli (7). It was important to determine whether this reduction of activity was caused by a general effect on the elongation rates of all the RNA polymerases or whether a particular sub-fraction of the polymerase population was selectively inhibited by the analogue. Thus an attempt was made to measure numbers of active RNA polymerases and elongation rates directly and the results are presented in table 1. The analysis strongly suggests that similar numbers of RNA polymerases were synthesising RNA in the

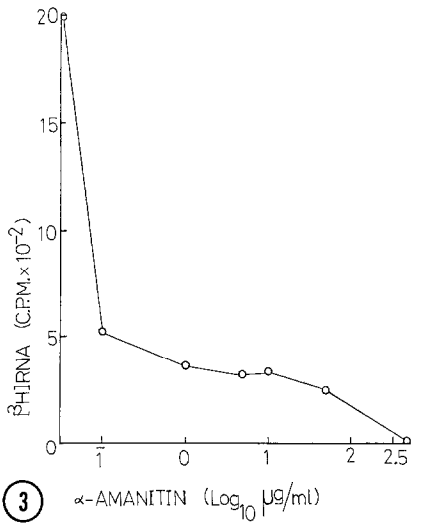
Table 1. Estimation of RNA polymerase numbers and elongation rates.

Substrate	Incubation time (min)	[3H]Uridine		[3H]UMP	
		pmoles/mg n/plasmic DNA	No. RNA polymerase mols/nuc.	pmoles/ mg DNA	Elongation rate (nucs/sec)
CTP	1	1.82	1×10^4	15.0	0.25
	2	1.82	1×10^4	27.8	0.23
Hg-CTP	1	1.72	9.4×10^3	4.7	0.08
	2	1.79	9.8×10^3	8.5	0.07

Nucleoplasm was incubated to allow RNA synthesis as described elsewhere (3) with [3H]UTP at 10 Ci/mmole. After hydrolysis of RNA in 0.3 M KOH, [3H]UMP and [3H]Uridine residues were separated on polyethyleneimine -cellulose and quantified. Allowance was made for 0.4% conversion of UMP to uridine during hydrolysis and for 50% recovery of total nuclear DNA in the nucleoplasmic fraction.

presence of both CTP and Hg-CTP, and that a non-specific effect on elongation rates was responsible for the reduced activity with Hg-CTP. Controls described elsewhere (3) have established that the UMP and uridine residues were derived entirely from alkaline hydrolysis of the RNA with negligible artefacts arising from endogenous UTP pools and nuclease and phosphatase contamination. Thus it seems likely that the RNA molecules labelled *in vitro* in the presence of Hg-CTP were representative of the sequences previously initiated *in vivo* at least to the same extent as those elongated with CTP.

The nature of the RNA polymerase activities responsible for the incorporation *in vitro* was established by the use of α-amanitin (Figure 3). Clearly more than 80% of the synthesis was sensitive to low α-amanitin concentrations and therefore catalysed by RNA polymerase II. The residual activity was inhibited over the range 10-200 μg/ml and was therefore due



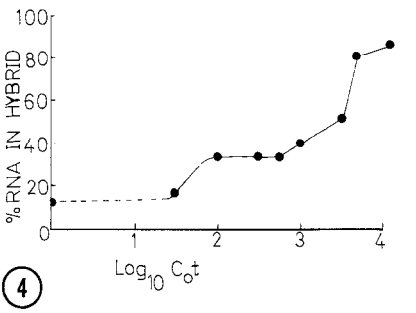


Figure 3: Effects of α-amanitin on mercurated RNA synthesis. Assays contained nucleoplasm from 250 mg liver.

Figure 4: Hybridisation in vast DNA excess. Hybridisation assays were in final volumes of 30-50 µl with DNA at 7.5-12.5 mg/ml and other components as described in methods. Each assay contained 4,000-6,000 c.p.m. of (^{3}H) RNA and DNA:RNA mass ratios were about 400,000:1.

to RNA polymerase III. This was very similar to the pattern observed with CTP (3) and further confirmed that there was no selection against either of the predominant RNA polymerase species in nucleoplasm due to the use of Hg-CTP.

Hybridisation of RNA synthesised by nucleoplasm in the presence of Hg-CTP and purified as outlined in Methods is shown in figure 4. As much as 85% of the RNA could be driven into hybrid in a reaction which exhibited distinctly biphasic kinetics. About 10% of the RNA was resistant to ribonuclease from the outset, perhaps due to intramolecular base-pairing (3,9). A further 25% annealed with a $C_o t_{\frac{1}{2}}$ of about 50, characteristic of transcripts from sequences reiterated several hundred times in the genome (1,10). The remaining 45-50% hybridised over the $C_o t$ range 10^3-10^4, suggesting that transcription from sequences

present only once or a few times per genome was the major source of activity in the nucleoplasm. These observations underlined the benefits of using mercurated probes to purify RNA synthesised in vitro, since comparative studies with RNA unseparated from endogenous sequences failed to drive more than 40% of the molecules into hybrid (3,11). Although the kinetic patterns were quite similar it was not possible to be certain that they were representative of the total transcript population when 60% of the RNA remained single-stranded. It is now clear that the hybridisation of RNA molecules initiated in vivo and briefly elongated in nucleoplasm in vitro follows similar kinetics to heterogeneous nuclear RNA and is quite dissimilar from nucleolar transcripts which anneal only at low C_0t (1,7).

Hg-CTP will undoubtedly continue to prove useful in studies of chromatin transcription. We have also carried out experiments with mercurated UTP and brominated UTP; neither of these proved as successful as Hg-CTP, the former because it had a greater inhibitory effect on overall activity and the latter because discrete separation from unbrominated RNA could not be achieved by the methods attempted (caesium gradient centrifugation). However, it is worth pointing out that some problems have been encountered with the use of Hg-CTP. In particular, the sizes of the transcripts after purification seem to be smaller than those made with CTP and care is needed to ensure that they do not degrade below the size necessary for efficient hybridisation.

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REFERENCES

1. Melli, M., Whitfield, C., Rao, K.V., Richardson, M. and Bishop, J.O. (1971) Nature New Biol. 231, 8-12.

2. Beebee, T.J.C. and Butterworth, P.H.W. (1977) Biochem. Soc. Symp. 42, 75-98.
3. Beebee, T.J.C. (1978) Biochem. J. 176, 715-725.
4. Dale, R.M.K., Martin, E., Livingston, D.C. and Ward, D.C. (1975) Biochemistry 14, 2447-2457.
5. Coupar, B.E.H. and Chesterton, C.J. (1977) Eur. J. Biochem. 79, 525-533.
6. Burton, K. (1956) Biochem. J. 62, 315-324.
7. Beebee, T.J.C. and Butterworth, P.H.W. (1976) Eur. J. Biochem. 66, 543-550.
8. Johnson, J., Jant, B., Sokoloff, L. and Kaufman, S. (1969) Biochim. Biophys. Acta 179, 526-532.
9. Fedoroff, N., Wellauer, P.K. and Wall, R. (1977) Cell 10, 597-610.
10. Britten, R.J. and Kohne, D.E. (1968) Science 161, 529-532.
11. Bastian, C. (1978) Biochem. Biophys. Res. Commun. 83, 893-900.