# NEAREST NEIGHBOUR BASE FREQUENCY OF THE RNA FORMED BY RAT LIVER DNA-DEPENDENT RNA POLYMERASE A and B WITH HOMOLOGOUS DNA

by

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SUMMARY

Four distinct peaks of DNA-dependent RNA polymerase activity were isolated from rat liver nuclei by DEAE-Sephadex chromatography. The product formed by the enzyme in the two major peaks (designated A and B) was subjected to nearest neighbour base frequency analysis. Using identical template (native, homologous DNA) and incubation conditions, it was shown that the product had different nearest neighbour frequencies, using $\alpha$-$^{32}$P labelled ATP or $\alpha$-$^{32}$P labelled CTP. The RNA product of enzyme A had sequences relatively richer in GMP and CMP than that formed by enzyme B. There was no evidence of ribonuclease activity in the isolated enzymic peaks, and products formed under identical conditions have similar sedimentation characteristics. These results suggest that the enzymic materials may have a recognition mechanism selecting areas for transcription.

Multiple eukaryotic DNA-dependent RNA polymerases have been described and may have specific intranuclear localizations (1). These enzymes have *in vitro* preference for template states, suggesting a different replication mechanism or recognition factor(s). There are no data to suggest a specific initiator (recognition) mechanism (2,3) but there is some evidence for specific product formation by the several enzymes derived from calf thymus (3). We set about to determine if different rat liver derived polymerases formed specific products, using the same homologous template. Nearest neighbour analyses of the product of nucleolar (designated A or I) (1,3) and nucleoplasmic (designated B or II) polymerases were different.

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## MATERIALS AND METHODS

Male Sprague-Dawley rats (100-120 grams body wt.) reared at the National Institute for Medical Research were used. Nuclei were prepared by a modification of the Blobel and Potter technique (4) and DNA-dependent RNA polymerases extracted by a modification of the method of Roeder and Rutter (1). DNA was isolated from rat liver nuclei by suspension of nuclei in 0.15 M NaCl and 0.015 M EDTA pH 7.4, digested with 1 mg. per ml. electrophoretically purified, boiled RNase, and 1 mgm. per ml. α-amylase. Digestion was carried out for 2 hours at 37°, 1 mg. per ml. of pronase was added, and the digestion continued for 1 hour. The DNA was extracted and purified by phenol extractions and chloroform-isoamyl alcohol treatment (5), dissolved in 0.015 M NaCl and 0.0015 M sodium EDTA pH 7.4, dialyzed and stored frozen. The hyperchromicity of the DNA so prepared when boiled for 20 minutes was in the order of 30%. Nearest neighbour analysis was carried out on RNA formed utilizing $\alpha P^{32}$ labelled ATP or CTP (obtained from the Radiochemical Center in Amersham, United Kingdom) with the nucleolar and nucleoplasmic enzymes and native rat liver DNA as template. The RNA formed in 1 hour was precipitated with perchloric acid to which 1 mg. per ml. of rat liver ribosomal RNA was added as a carrier. Precipitated materials were washed repeatedly, hydrolyzed in 0.5 M KOH for 16 hours at 37° C, and the nucleotides released were adsorbed to acid-washed, activated charcoal. The charcoal was washed with 0.025 M $HClO_4$, the nucleotides subsequently eluted and separated by column chromatography essentially by the method of Katz and Combs (6). The technique employed Dowex 50 x 8 and Dowex 2 x 8 resins prepared as chloride and formate salts respectively. Analysis for DNase and RNase in the polymerases utilized mouse ribosomal RNA, rat liver ribosomal RNA, $C^{14}$ labelled rat liver 28S RNA, $P^{32}$ labelled adenovirus DNA, polyadenylic acid, and tritium labelled polyadenylic acid. These substrates were added to the incubation mixtures identical to that used for polymerase assays. The release of acid-soluble 260 mμ absorbing material, the release of acid-soluble radioactivity, or analysis of the sedimentation of the $P^{32}$ adnovirus DNA or the $C^{14}$ rat liver 28S RNA was carried out in 5-30% sucrose gradients utilizing lithium dodecyl sulphate (7,8).

## RESULTS

Four peaks of RNA polymerase were reproducibly isolated from extracts of rat liver nuclei upon chromatography on DEAE Sephadex (Fig. 1). The first small peak (Pre-A) eluted during the 0.1 M ammonium sulphate wash. This material is α-amanitin insensitive, but on rechromatography yields peaks A and B. The second peak (the nucleolar, I or A enzyme) elutes at 0.12-0.15 M $(NH_4)_2SO_4$. A very small peak is eluted before the last major peak of activity (designated B or II, the nucleoplasmic) which elutes at 0.28 M ammonium sulfate. The first three

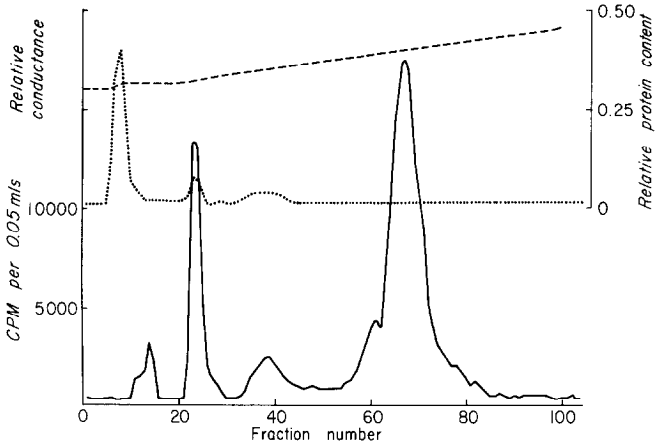


FIGURE I

Elution profiles of DNA-dependent RNA polymerase from rat liver nuclei. The enzymes were isolated, chromatographed on DEAE-Sephadex with increasing concentration of $(NH_4)_2SO_4$, as indicated (1). 0.7 ml. fractions were collected and 50 µl samples assayed in a total volume of 0.08 mls. containing 0.8 mM ATP, GTP, CTP, 100 µg gm./ml. denatured calf thymus DNA, 86 mM Tris pH 8.3, 0.029 mM EDTA $Na_2$, 0.14 mM DTT, 57 mM KCl, 0.29 mM mercaptoethanol, 2 mM $MnCl_2$, glycerol 7% and 0.7 µm UTP-$H^3$ (Sp. Ac. 1 mCi/m mole). Incubation was carried out for 30 min. at 37° C., the material pipetted to filter paper discs and processed for radioactive analysis. Protein was measured by the method of Bramhall (9). The two major peaks of activity are A and B which were used for nearest neighbour analysis, while the less active peaks are Pre A and Pre B and were not further studied.

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peaks, Pre-A, A, and Pre-B, are not sensitive to α-amanitin. B is inhibited to 90% in the presence of 2 micrograms of α-amanitin. Native DNA is utilized more readily by enzyme A, whereas enzyme B utilizes native or denatured template, denatured DNA more effectively (about 15% in these experiments).

Nearest neighbour analyses were determined on the RNA product of the enzymes A and B using $\alpha P^{32}$-ATP and CTP substrates, with native rat liver DNA as template. There were significant differences in product formed by two polymerases measured by nearest neighbour base technique (Table 1). The product of nucleolar enzyme (A) was more ribosomal-like than the product obtained from nucleoplasmic enzyme (B), which was more DNA-like. Analysis of the product showed both enzymes produced an RNA peaking at about 4-6 S in sucrose gradients and with a leading edge down as far as 18S (Fig. 2). No release of acid soluble radioactivity or of 260 mµ absorbing material, nor of altered sedimentation properties of ribosomal RNA was found when the enzymes were incubated with RNA as substrate for RNase activity. $P^{32}$ labelled DNA from adenovirus showed an altered sedimentation upon incubation with peak Pre-A (Fig. 3).

TABLE I

Nearest Neighbour Analysis of RNA Formed by Isolated DNA-Dependent RNA.
% of Total $^{32}P$ of RNA Hydrolyzate Recovered.

| Label | Nucleoside | Enzyme Added to Incubation A (amanitin resistant) | B (amanitin sensitive) |
|---|---|---|---|
| [α-32P] ATP | U | 32.12 ± 2.37 | 28.64 ± 1.89 |
| | G | 28.66 ± 1.25 | 21.64 ± 0.69 |
| | C | 16.22 ± 1.88 | 23.67 ± 1.10 |
| | A | 22.99 ± 2.00 | 25.93 ± 1.13 |
| [α-32P]CTP | U | 33.97 ± 0.10 | 37.06 ± 2.95 |
| | G | 29.44 ± 1.97 | 20.33 ± 2.16 |
| | C | 18.03 ± 0.59 | 19.26 ± 1.35 |
| | A | 18.56 ± 1.48 | 23.10 ± 0.54 |

The isolated enzymes were incubated with 0.8 mM UTP, GTP and either CTP or ATP depending on the radioactive precursor used, 100 µg of native rat liver DNA, 86 mM Tris, pH 8.3, 0.029 mM EDTA $Na_2$, 0.14 mM DTT, 57 mM KCl, 0.29 mM mercaptoethanol, 2 mM $MnCl_2$, glycerol 7%, 0.1 mM α-$^{32}P$ labelled ATP or CTP (Sp. ac. 200 mC/mM). $(NH_4)_2SO_4$ concentration was 30 mM for enzyme A and 70 mM for B, resulting in optimum incorporation. Following incubation at 37°C for 1 hour, 1 mg/ml rat liver ribosomal RNA was added as carrier, and the RNA precipitated with an equal volume of 0.5 M $HClO_4$ containing 1% sodium phosphate and 1% sodium pyrophosphate. The precipitate was resuspended and washed 5 times with 0.25 M $HClO_4$ containing phosphate and pyrophosphate, once with alcohol and ether (1:1) and dried with ether. The dried material was suspended in 1 ml. 0.3 M KOH and hydrolyzed for 18 hours at 37°C. Following hydrolysis an equivalence of 0.5 M $HClO_4$ was added, chilled, and the $KClO_4$ removed by centrifugation. The pH was adjusted to 2, and the nucleosides were absorbed on activated charcoal, the charcoal washed 3 times with 0.025 M $HClO_4$ containing phosphate and pyrophosphate, once with 0.025 M $HClO_4$ and the nucleosides eluted with $NH_3$:$H_2O$:$CH_3CH_2OH$ (2:48:50). The eluate was immediately lyophilized, redissolved in 0.05 M HCl, carrier nucleosides added (AMP, CMP, GMP, and UMP) and the nucleosides separated by Dowex 50 x 8 and Dowex 1 x 8 chromatography, as described by Katz and Comb (6). The radioactivity of the isolated nucleosides was measured with a liquid scintillation spectrometer, using Cherenkov radiation. The total recovery from each column was at least 94%, the total isolated radioactivity was used to calculate percentage radioactivity of each of the nucleosides. The results are presented as the means ± one standard deviation of three separate experiments using three different enzyme preparations.

## DISCUSSION

The separation of an initiation molecule (sigma factor) from E. coli DNA-

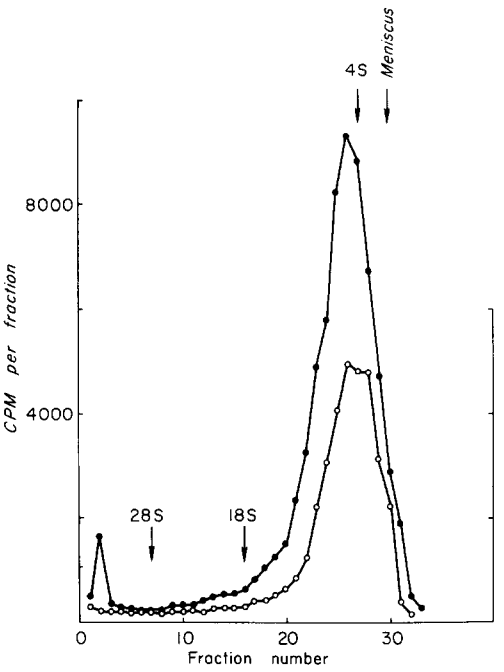


Fig. 2.

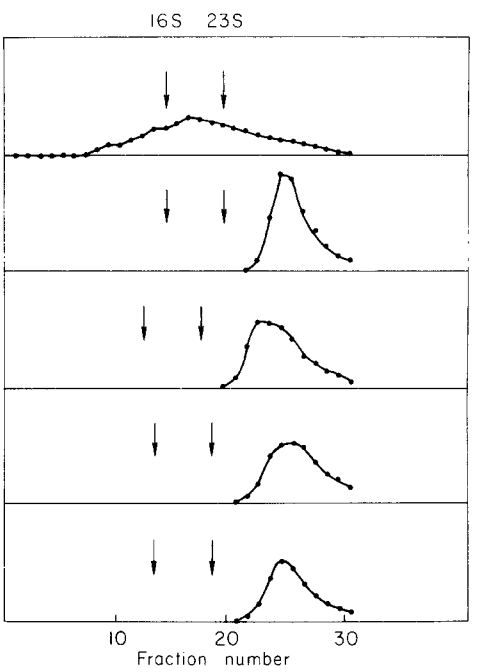


Fig. 3.

FIGURE II

The sedimentation behavior of $^{32}P$ labelled product of enzymes A and B was measured in LiDS sucrose gradients using 4, 18 and 28S rat liver RNA as marker. RNA was labelled with $\alpha$-$^{32}P$ labelled ATP and a broad band of radioactivity with a peak at 4-6S was found after sucrose density gradient centrifugation as described in the text. The curve with open circles (0) is RNA formed by enzyme A, closed circles (●) by B using native RNA template.

FIGURE III

Assay of DNase activity associated with the different RNA polymerases obtained after DEAE-Sephadex column chromatography. Adenovirus DNA labelled with $^{32}P$ was incubated with pooled material corresponding to each enzyme peak for 30 min. at 37° C., chilled, made 5% LiDS and 0.25 m Li acetate pH 5.0, and layered on a 5-30% sucrose gradient in LiDS. The gradients, following 16 hours centrifugation at 24,000 REV in a MSE 6 x 15 rotor were fractionated and each fraction's radioactivity measured. Bacterial 23 & 16S RNA were added as markers and detected by absorbance at 260 nm. The alteration in distribution of DNA with Pre A is identical to the effect of a KB cell endonuclease.

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dependent RNA polymerase provided a mechanism in prokaryotes for selective recognition of genetic sequences to be transcribed (10), although such a specific recognition and initiation role of the sigma factor does not seem to be universal (11). The identification of multiple forms of mammalian nuclear DNA-dependent RNA

polymerase (1) and the suggested formation of different classes of RNA by these enzymes (3) implies an intrinsic recognition mechanism or a differential topological localization of enzyme and specific template within the nuclear structure. A difference in the nature of products transcribed, using whole rat-liver nuclei (12) or calf thymus enzymes (3) implies both specific function and specific product formation by the multiple RNA polymerases. It was in order to test such a possibility of isolated rat-liver enzymes also forming different products, thus implying that these enzymes possessed inherent selection or recognition mechanisms, that RNA polymerases A and B were tested by nearest neighbour analysis of the RNA product formed using a homologous template.

Our evidence indicates that the isolated peaks of enzymic activity have different preferences for native and denatured DNA, using conditions of maximum activity. The RNA products transcribed by the two enzymes have different base sequences and perhaps partially correspond to those of the nucleolar and nucleoplasmic enzymes described by Chambon et al. (3) for calf thymus. The identical deproteinized template, the absence of significant deoxy- or ribonucleotidolytic activity, and the similar size of the product suggest that this selection is not due to preferential degradation of template or product but is a property of the enzyme preparation eluted from the DEAE-Sephadex column. Comparison of the results of our nearest neighbour analyses with those obtained with whole nuclei at low and high ionic strength (12) suggests that the enzyme A is preferentially, but not exclusively, transcribing ribosomal cistrons and enzyme B forming a relatively more DNA-like RNA. Further support for this notion comes from the amanitin sensitivity of enzyme B and the nucleolar location of A in agreement with the data presented by other workers (2, 3, 13). Although we were unable to show a rigorous discrimination of transcription in that the products of the two enzymes were not entirely rRNA-like or DNA-like, the consistent differences in nearest neighbour base distribution is at variance with the results described by Roeder, Reeder and Brown (14). These workers found that RNA polymerases I and II (corresponding to our enzymes A and B) extracted from early Xenopus embryos or from adult Xenopus liver gave identical products, as seen by DNA-RNA hybridization, irrespective of whether whole DNA or purified ribosomal DNA were used as template.

The mechanisms underlying the apparent selectivity for the site of DNA transcrip are not apparent. The complexity of material eluted under each peak (as revealed by SDS gel electrophoresis) may have within them one or more specific factors which could initiate, terminate or make accessible (unwind) native DNA segments. It is quite likely, as was also suggested by Roeder et al. (14), that the degree of purification of the polymerase may determine the extent to which the enzyme would selectively transcribe different genomic regions. The identifi-

cation of such mechanisms must therefore await more definite purification of all the components present in multiple RNA polymerase preparations.

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