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IN VITRO TRANSCRIPTION OF POLYOMA DNA

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SUMMARY

In vitro transcription of polyoma DNA by E.Coli RNA polymerase is partially symmetric as shown by chromatographic analysis and RNAase digestion of polyoma cRNA. Visualisation of transcription complexes by Electron Microscopy shows long cRNA chains, sometimes more than three times as long as the template DNA, initiating from 5 sites and also provides evidence suggesting transcription from both DNA strands.

INTRODUCTION

Transcription of polyoma virus DNA in lytically infected cells appears to take place in two stages, before and after the onset of viral DNA synthesis, and gives rise to stable messenger RNA species which are accordingly described as early and late (1). Stable early mRNA is synthesised right throughout infection but stable late mRNA appears only to be synthesised after DNA synthesis has taken place. Stable early and late transcripts are synthesised from different strands of the coding template and each corresponds to about 50% of its length (2). Apart from the stable cytoplasmic mRNA species labile species are also synthesised in the nucleus in variable amounts from the complementary DNA strand both early and late during infection as indicated by the presence of a significant amount of nuclear double stranded mRNA isolated after short pulses of radioactive precursor (2,3); these labile species extend in length beyond the sizes of the stable transcripts and are thought to be processed by specific

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endonucleases to generate the mature cytoplasmic mRNAs.

The purpose of this report is to show that *in vitro* transcription of DNA from non-defective polyoma virus by Escherichia Coli RNA polymerase follows a pattern partly similar to that observed *in vivo* in that the cRNA is to a large extent double stranded, longer than the coding strand (4) and initiates from a limited number of sites.

MATERIALS AND METHODS

Supercoiled polyoma DNA (form 1) was prepared from non-defective virus, as described by Fried (5), and was found to be 100% sensitive to the restriction enzyme $EcoR_1$. RNA polymerase was extracted from Escherichia Coli MRE 600 according to the method of Burgess (6) and found to be more than 95% pure with an approximately equimolar amount of sigma subunit. Bacteriophage T4 Gene 32 protein was purified by the method of Alberts and Frey (7). The incubation mixture for synthesising RNA contained 20 µg/mℓ of polyoma DNA I, 75 µg/mℓ of E.Coli RNA polymerase (DNA/RNA polymerase molar ratio of 1:23), 50mM Tris-HCl pH:7.9, 10mM $MgCl_2$, 40-100mM KCl, 7mM mercaptoethanol, 1mM of ATP, CTP. GTP and UTP (or ^{3}H GTP to a final specific activity of 0.15 Ci/mMole). Incubation was carried out at 37°C.

^{3}H cRNA was extracted according to the method of Martin and Axelrod (8). Separation of single and double stranded RNA was performed using CF11 cellulose as described by Franklin (9). ^{3}H cRNA was extracted from the incubation mixture and dissolved in 0.05M Tris HCl pH: 6.98 containing 0.2M NaCl and 0.001M EDTA. Single stranded RNA has been shown to elute in 15% Ethanol and double stranded RNA in buffer alone (9).

Pancreatic RNAase digestion (20µg/ml) was performed for 1 hour at 37°C in 2 x SSC (SSC is 0.15M NaCl, 0.015M trisodium citrate, pH: 7.0).

DNAase digestion (40 µg/ml) was carried out at 37°C for 15 minutes.

Annealing of cRNA was performed at 65°C in 2 x SSC. ^{3}H cRNA was precipitated with cold 5% TCA, filtered through glass fibre discs (Whatman GF/C) and counted in a toluene based scintillator.

Incubation and preparation of samples for visualisation of transcription complexes were carried out as described by Delius and Westphal (10). After preincubation in the absence of substrates and rifampicin for 3 minutes at 37°C nucleoside triphosphates and rifampicin (5µM) are added and incubation is carried out at 37°C for 15 minutes. Samples from the incubation mixture are diluted 10 times in 0.01M potassium phosphate (pH: 7.0), 0.002M EDTA containing 55µg/ml of gene 32 protein and incubated at 37°C for 3 minutes. Glutaraldehyde is then added to 10mM and incubation carried out for 15 minutes at 37°C for 3 minutes. The sample is spread for electron microscopy examination by the Kleinschmidt technique as described by Davis *et al* (11). Magnifications were calibrated and length measurements were made after five-fold photographic enlargement using a map-meter type of device.

All reagents were "ANALAR" grade from British Drug Houses (BDH). Glutaraldehyde was the special grade for Electron Microscopy. Enzymes were obtained from SIGMA.

RESULTS

cRNA synthesised after 60 minutes incubation was extracted and analysed by chromatography on CF11 cellulose which separates single stranded from partly or fully double stranded RNA, as described under Methods (9). Confirmation that there is a significant fraction of the cRNA which is at least partly double stranded, is provided by RNAase digestion under conditions which exclude the presence of DNA-RNA hybrids (Table 1). Polyoma cRNA was then further analysed by electron microscopy by visualisation of transcription complexes. Transcription complexes clearly showing long RNA chains were visualised by this technique are shown in Figure 1. Most DNA molecules observed carried RNA chains although the chains varied significantly in length, a finding which is likely to reflect asynchronous initiation. From the molecules shown in Figure 1, it is apparent that bacterial RNA polymerase when transcribing polyoma DNA is capable of moving past the initiation sequence(s) into a second and third round of synthesis producing transcripts of up to three times the length of the template strand. In the presence of rifampicin transcription is initiated from a small number of initiation sites (1-5 with 2-3 predominating) as shown in the histogram in Figure 2, and proceeds under ionic conditions which are suboptimal for overall synthesis at a rate which can be calculated to be of approximately 16 nucleotides per second, as calculated from data obtained by Delius & Westphal (10). When RNA chains on individual transcription complexes are lined up according to their length on "linearised" DNA molecules and measured two conclusions become apparent. Firstly it has been observed that of 32 complexes analysed (with one possible exception) the RNA chains could not have originated from a common initiation side either in the way suggested by Schaffer (12) or by escaping the inhibitory effect of rifampicin. The second conclusion is that under these conditions

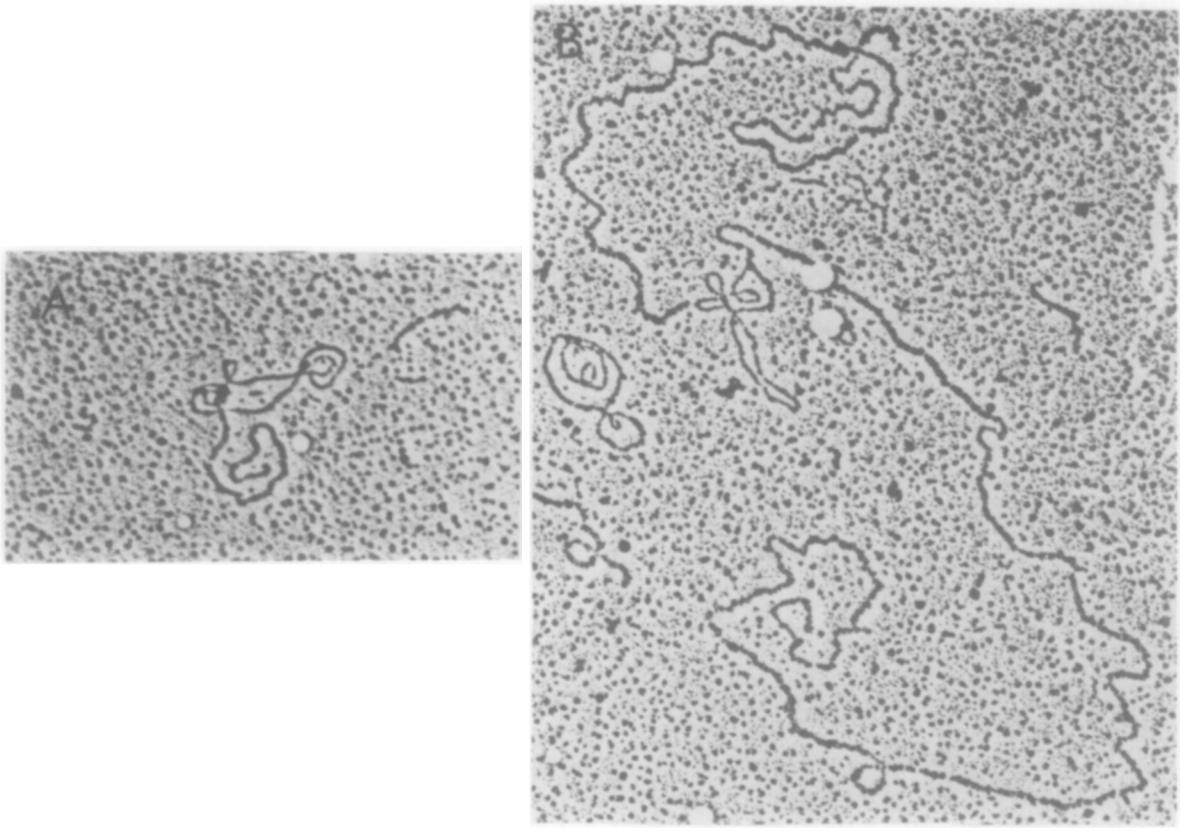

Figure 1. Visualisation of in vitro polyoma DNA transcription complexes

A shows one transcription complex with one RNA chain. B shows one transcription complex with two very long transcription chains.

both DNA strands are being transcribed as suggested by the finding that in some transcriptions complexes cRNA chains differ in length by more than a transcript of the entire DNA template. Measurements carried out in other transcription complexes are also irreconcilable with transcription from one strand alone. These findings are in agreement with our previous finding of a significant proportion of double stranded RNA (Table I). Another observation which might be of significance is the apparent "jamming" of molecules at certain points around the ring (complex D in Figure 1), again a possible consequence of symmetrical transcription occurring under these conditions.

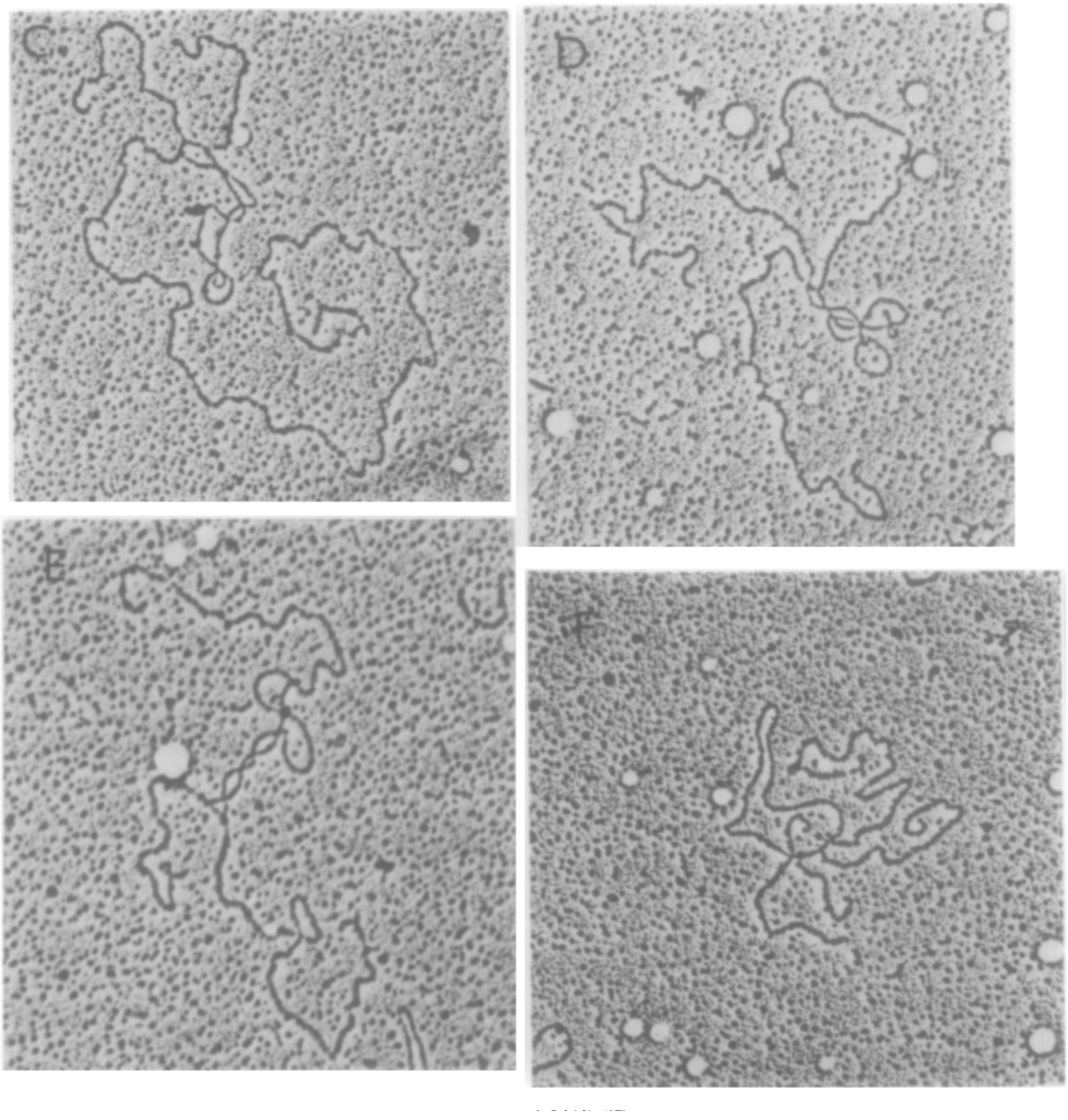

Fig. 1 cont.'d.

C-E are transcription complexes with three RNA chains. F shows a transcription complex with 5 RNA chains.

DISCUSSION

Our results show that supercoiled polyoma DNA from non-defective virus is a good template for transcription by E.Coli RNA polymerase.

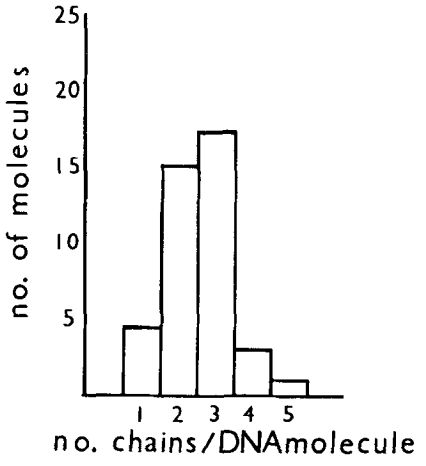

Figure 2. Histogram representing distribution of transcription complexes according to number of RNA chains present.

Analysis of polyoma cRNA by cellulose chromatography and RNAase digestion have shown that about 50% of the product is double stranded indicating that transcription is partially symmetric, i.e. from complementary DNA sequences. Visualisation of transcription complexes by EM has shown transcripts that are as long as three times the coding strand. From these long cRNA molecules a maximal rate of synthesis of 16 nucleotides per second was calculated under ionic conditions which are suboptimal. The EM studies have also shown that cRNA is transcribed in the presence of rifampicin, from a limited number of initiation sites (1-5 with 2-3 predominating) and strongly suggest transcription from both DNA strands. These findings agree with a previous report by Lescure et al (13) who also found a significant proportion of symmetric cRNA (47%) and 5 initiation sites for short nascent RNA chains and correlate well with the finding of 5 main A-T rich regions mapped on the polyoma genome after denaturation with bacteriophage T4 Gene 32 protein (14,15). Kamen et al (2) who were able to synthesise asymmetric polyoma cRNA using E.Coli RNA polymerase have clearly selected the "strongest" promotor under ionic conditions (0.5MKCℓ) that have been shown to

TABLE 1: CF11 Chromatography and RNAase digestion of Py cRNA

Exp.No,	cRNA cpm	CF11 15% Elu. cpm	CF11 0% Elu. cpm	RNAase sens. cpm	RNAase resist cpm	cRNA before chromatography
1	8030	4950 (62%)	3080 (38%)			Incubation 2 hrs/dmso grad/ RNA pooled precipitated
2	23125	12721 (55%)	10404 (45%)			Incubation 90 minutes/DNAase phenol extraction/precipitation
3	1905	777 (43%)	1128 (57%)	745 (45%)	959 (55%)	Incub. 2 hrs/DNAase/G 100/ annealled 67°C 2 x SSC 7 hrs
4	3659			1959 (53%)	1700 (47%)	As in Exp. 3 annealled 12 hrs

DNAase and RNAase digestion and cRNA annealing were carried out as described under Methods.

prevent even the binding of the enzyme to bacteriophage T7 DNA, one of its natural templates (16).

In vitro transcription by E.Coli RNA polymerase of polyoma DNA appear to have some features in common with the in vivo process. Although little is known about the number and mapping of the original transcription products "in vivo" the findings reported require the existence of at least two initiation sites on opposite strands (2). Finally the symmetry of the in vitro product which is clearly established in our experiments parallels the situation "in vivo" where a significant proportion of mRNA labelled with short pulses of radioactive precursors originates from complementary stretches of the DNA template (3,4). The double stranded cRNA molecules synthesised in vitro by the bacterial enzyme may also prove useful for the study of the putative endonuclease(s) which finally produce the functional messenger RNA molecules.

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REFERENCES

1. Hudson, J., Goldstein, D. and Weil, R. (1970) Proc.Nat. Acad. Sci., 65, 226-233.
2. Kamen, R., Lindstrom, D.M., Shure, H. and Old, R.W. (1974) Symposia on Quantitative Biology, Cold Spring Harbor, XXXIX, 187-197.
3. Aloni, Y. and Locker, H. (1973) Virology, 54, 495-505.
4. Acheson, N.H., Buetti, E., Scherrer, K. and Weil, R. (1971) Proc.Nat. Acad. Sci., 68, 2231-2235.
5. Fried, M. (1974) J. Virol., 13, 939-946.
6. Burgess, R.R. (1969) J. Biol. Chem., 244, 6160-6167.
7. Alberts, B. and Frey, L.J. (1969) Nature, 227, 1313-1317.
8. Martin, M. and Axelrod, D. (1969) Proc. Nat. Acad. Sci., 64, 1203-1210.
9. Franklin, R.M. (1966) Proc.Nat.Acad. Sci., 55, 1504-1511.
10. Delius, H., and Westphal, H. (1973) J. Mol. Biol., 74, 677-687.
11. Davis, R.W., Simon, M. and Davidson, N. (1971) Methods in Enzymology. 21D, 413-428.
12. Schaffer, R., Kramer, R., Zillig, W. and Ludny, H. (1973) Eur. J. Biochem., 40, 367-373.
13. Lescure, B., Oudet, P., Chambon, P. and Yaniv, M. (1976) J.Mol.Biol., 108, 83-97.

14. Monjardino, J. & Wilde, A. (1975) Nature (London) 255,249-252.
15. Lescure, B. & Yaniv, M. (1975) J.Virol. 16, 720-724.
16. Richardson, J.P. (1966) J.Mol.Biol., 21, 83-114.