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CHARACTERISATION OF HEPARIN-RESISTANT COMPLEX FORMATION AND RNA SYNTHESIS BY WHEAT GERM RNA POLYMERASES I, II AND III, IN VITRO ON CAULIFLOWER MOSAIC VIRUS DNA

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SUMMARY.

Wheat germ RNA polymerases I, II and III form stable, heparin-resistant binary complexes on CaMV DNA in vitro. The proportion of stable complexes is increased by preincubation with ATP and GTP. RNA chains are initiated with a greater frequency in GTP than in ATP, this preference for GTP being particularly marked for enzyme II. RNA synthesised has an average length of 100-200 nucleotides for enzymes I and III and 550 nucleotides for enzyme II. This RNA hybridises to all regions of the CaMV genome, although one region is overrepresented in transcripts with enzyme II. Our results are compatible with an initiation of RNA synthesis at single-strand nicks, in which RNA polymerase II Initiates synthesis more efficiently at a specifically-located single-strand interruption on the CaMV genome.

INTRODUCTION.

It has been shown that Escherichia coli RNA polymerase holoenzyme is capable of forming specific initiation complexes on homologous DNA in vitro at sites at which transcription is initiated in vivo (1). These complexes are resistant to polyanions, such as heparin (2, 3), which greatly reduce non-specific complex formation (4, 5). Among eucaryotic RNA polymerases, heparin-resistant synthesis has been demonstrated for class III enzymes (6, 7). Wheat germ RNA polymerase II, in contrast to its animal counterparts, forms relatively stable binary complexes on superhelical SV40 DNA (8) and on Adenovirus DNA (9) in the presence of polyanions. In the latter case, the localisation by electron microscopy of the sites of formation of some of the binary complexes at positions which could correspond to in vivo promoters leads the authors to conclude that these complexes may functionally correspond to those formed by the procaryotic holoenzyme.

More recently, however, several reports have raised the possibility that the heparin-resistant RNA synthesis observed by SEIDMAN et al. (9) may be preferentially initiated at single-strand nicks (10, 11, 12). As the RNAs transcribed on Adenovirus DNA have not been characterised, the correspondence of complexes

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formed, in this case, by wheat germ RNA polymerase II to those formed by the procaryotic holoenzyme has not been demonstrated.

The combination of wheat germ RNA polymerases and cauliflower mosaic virus (CaMV) DNA provides a more homologous system for the study of in vitro transcription. CaMV DNA is double-stranded, has a molecular weight of about $5x10^6$ daltons (13) and possesses three single-strand interruptions at specific sites (14). We have previously shown that wheat germ RNA polymerases I, II and III are capable of heparin-resistant RNA synthesis on this template in the presence of Mn^{2+} (15). In this study we have investigated the stability of complexes formed by the 3 enzymes on CaMV DNA and characterised heparin-resistant transcripts by labelling of the 5' end of the RNA chain, determination of the average chain length and hybridisation of the transcripts to restriction fragments of CaMV DNA.

MATERIALS AND METHODS.

Propagation of CaMV and extraction of virion DNA have previously been described (16, 17). Integrity of the DNA was estimated by densitometry of photographic negatives of denatured DNA separated on agarose gels (10). We estimate that our DNA preparations contain less than 0.2 single-strand nicks per molecule in addition to the 3 specifically localised interruptions.

DNA was digested 16 h with restriction endonuclease Bgl II (a gift from G. ROIZES, MONTPELLIER) in 6mM Tris-HCl, pH 7.5, 6mM $MgCl_2$, 6mM β-mercaptoethanol, followed by Eco RI (Boehringer-Mannheim) as described by the suppliers. Restriction fragments were separated on neutral agarose gels (18) and transferred to nitrocellulose filters (Millipore HAWP, ref. 19).

Wheat germ RNA polymerases I, II and III were prepared as described (20, 21). Specific activities were 48, 128 and 83 units/mg respectively. One unit corresponds to the incorporation of 1 nmole UMP in 30 minutes at 35° on incubation of enzymes with 15 µg native calf thymus DNA in a standard buffer: 39mM Tris-HCl, pH 7.8, 1.5mM NaF, 1.1mMdithiothreitol, 1.25mM $MnSO_4$, 5mM $MgCl_2$, 100mM $(NH_4)_2SO_4$, 1.25% glycerol, supplemented with 0.5mM each of ATP, GTP and CTP and 0.04mM [^{3}H]UTP (specific activity 150 cpm/pmole). Conditions of synthesis for individual experiments are given in figure legends.

RNA was hybridised to DNA on nitrocellulose filters in 4xSSC, 40% formamide, 0.2% SDS for 24 h at 40°C. Filters were washed three times in 4xSSC, 0.2% SDS, dried, impregnated with 20% PPO in toluene and fluorographed at - 70°C using Kodak RP Royal X-Omat X-ray film.

RESULTS AND DISCUSSION.

Formation of stable, heparin-resistant complexes. The stability of complexes formed between wheat germ RNA polymerases I, II and III and CaMV DNA was measured by the method of SARAGOSTI et al. (8) by observing the residual heparin-resistant RNA synthesis after various times of incubation in the presence of the polyanion at 0°C. Fig. 1 shows that the dissociation curves for all three enzymes are biphasic, with a fraction of the complexes dissociating in less than 2 minutes

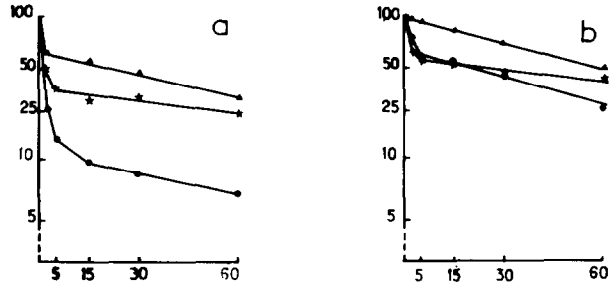

Figure 1.- Stability of heparin-resistant complexes between wheat germ RNA polymerases and CaMV DNA in the absence and presence of ATP and GTP.

Enzymes and DNA were incubated in standard buffer at 37°C for 10 mn in the absence (a) or presence (b) of 0.6mM ATP and GTP. Heparin was added to a final concentration of 100 µg/ml and the incubation continued at 0°C. At the times indicated CTP was added to the medium, with ATP and GTP if necessary, to 0.5mM each and [^{3}H] UTP (specific activity 1500 cpm pmole^{-1}) to 0.04mM. TCA-insoluble radioactivity was measured after 30 min. synthesis at 37°C.The divalent cation was Mn^{2+} at 2mM final concentration. Enzyme : DNA ratioswere 4 for enzymes I and III and 7.4 for enzyme II.

The figure shows the results for polymerases I (●), II (▲) and III (★) in the absence (a) and presence (b) of ATP + GTP.

The stable fraction represents 14%, 63% and 35% of complexes formed for enzymes I, II and III respectively. The average half-lives of these complexes are about one hour for enzymes I and II and two hours for enzyme III. For enzyme II this stability is comparable with that reported by SAGAROSTI et al. (8)using superhelical SV 40 DNA and SEIDMAN et al. (9) with Adenovirus 2 DNA. These results demonstrate, in addition, that wheat germ RNA polymerases I and III form complexes with similar kinetic properties on this template in the presence of Mn^{2+}. A comparison of the absolute levels of synthesis at time t = 0 shows that RNA polymerase III forms the greatest number of stable complexes with CaMV DNA, enzyme I forming only a small number of such complexes.

In the presence of ATP and GTP in the preincubation medium the proportion of stable complexes formed increases for the three enzymes. These complexes now represent 60%, 100% and 54% of all complexes for polymerases I, II and III respectively.

RNA synthesis initiation with ATP and GTP and length of RNA chains synthesised. Despite the observed similarities, measurement of the ratio of initiations with GTP and ATP shows clear differences in the initiating nucleotide for the three enzymes.

The 5'-terminal nucleotide of in vitro synthesised RNA was labelled using [β-^{32}P] ATP and [β-^{32}P] GTP as precursors. In the same experiment internal residues of the RNA were labelled with [^{3}H] UMP allowing us to calculate the average length of the RNAs synthesised.

Table I shows that, independent of the nature of the cation, RNA polymerase II preferentially initiates RNA synthesis with GTP, as has been observed for the same enzyme using λ (10) or SV 40 DNA (12) as template. This preference for GTP is much less marked for enzymes I and III in the presence of Mn^{2+}. A shift towards GTP starts is observed for enzyme III using Mg^{2+}, although this figure may be over-estimated due to the low level of $[^{32}P]$ ATP incorporation.

The molar ratio of incorporation of $[^{3}H]$ UMP to incorporation of $[^{32}P]$ ATP and GTP provides an estimate of the average lengths of RNA chains synthesised, which again varies with the enzyme and divalent cation used. In all cases, these chains are very short. RNA polymerase II catalyses the synthesis of RNAs with an average length of about 550 nucleotides under these conditions, while enzymes I and III synthesis chains of less than 200 nucleotides on average, and less than 100 nucleotides for enzyme III in the presence of magnesium. It should be noted that, at the low triphosphate concentrations used in these experiments, the incorporation of $[^{3}H]$ UMP is reduced, particularly for RNA polymerase III, as compared with synthesis in standard conditions. This probably reflects a reduction in the number of initiations rather than a change in elongation. Indeed, for enzymes I and III the average size of the RNA synthesised in standard conditions measured by electrophoresis in denaturing conditions (results not shown) is similar to that estimated by the ratio $[^{3}H]$ / $[^{32}P]$ using low triphosphate concentrations. For enzyme II the average chain length measured by electrophoresis is lower than that estimated by the ratio $[^{3}H]$ / $[^{32}P]$. This could be due to the fact that this enzyme is able to perform some synthesis primed by the 3'hydroxyl end of DNA at nicks (12).

Regions of the CaMV genome transcribed. We have determined the regions of the CaMV genome transcribed by the three wheat germ RNA polymerases by hybridising in vitro synthesised RNA to the separated fragments of CaMV DNA obtained by double digestion with the restriction endonucleases Eco RI and Bgl II. Fig. 2 shows that the short RNA chains synthesised in vitro, in the presence of heparin, by the three enzymes hybridise to most, if not all, of the CaMV genome. The supplementary bands visible on the autoradiographs correspond to the products of partial digestion, which are present at very low levels in our digest. These results suggest that RNA chains are initiated at many dispersed sites on CaMV DNA. Evidence has recently been presented that wheat germ RNA polymerase II, in the presence of heparin, preferentially forms complexes and initiates at nicks rather than at specific double stranded regions of the DNA (10, 11, 12). The number of random nicks in our preparation is less than 0,2 nicks per molecule. CaMV DNA extracted form virions possesses 3 single-stranded interruptions localised at map positions 0 (strand 1), 20 (strand 2) and 52 (strand 2). Although the results pre-

Table I.- RNA synthesis initiation with ATP and GTP in vitro by wheat germ RNA polymerases on CaMV DNA and average length of RNA chains.

Enzyme and cation	pm [β-^{32}P] ATP or [β-^{32}P] GTP incorporated/µg ADN X 10^3	pm [^{3}H]-UMP incorporated µg ADN	G/A	Average length of RNA chains
I Mn	ATP 3,01 / GTP 4,01	0,286	1,33	163
II Mn	ATP 2,83 / GTP 5,45	0,995	2,96	546
II Mg	ATP 0,41 / GTP 1,17	0,225	2,83	566
III Mn	ATP 1,71 / GTP 2,60	0,195	1,51	181
III Mg	ATP 0,25 / GTP 1,39	0,039	5,55	96

Enzymes and DNA were preincubated for 10 min at 37°C in standard buffer containing 2mM Mn^{2+} (polymerases I, II and III) or 5mM Mg^{2+} (polymerases II and III; no significant RNA synthesis is detected using enzyme I in the presence of Mg^{2+} (15)). Either [β- ^{32}P] ATP or [β- ^{32}P] GTP, with the other unlabelled purine nucleoside triphosphate, were included in the preincubation medium. Heparin was added to 100µg/ml. After 15 min. at 0°C, CTP and [^{3}H] UTP were added and incubation continued for 30 min. at 37°C. To obtain a high specific activity for [^{32}P] purine nucleoside triphosphates (24,500 cpm pmole^{-1}), the concentration of ATP, GTP and CTP were 0.1mM each. [^{3}H] UTP (0.04mM) had a specific activity of 6,000 cpm pmole^{-1}.

sented in Fig. 2 indicate that there are no obvious qualitative differences between the RNAs synthesised by the three polymerases, scintillation counting of 1 mm bands cut from the nitrocellulose filters used in hybridisation shows that there are quantitative differences (Fig. 3). For enzymes I and III there is no overrepre sentation of the sequences near the precisely localised discontinuities among the in vitro transcripts, suggesting that these interruptions do not play a prominent role in the in vitro transcription by these two RNA polymerases. In the case of enzyme II a region of CaMV DNA localised near the interruption at 0 map units is overrepresented, suggesting that the complexes formed by this enzyme at this site initiate more efficiently. It has recently been shown that this interruption differs from those at 20 and 52 map units, being either a single-strand nick or a gap of very few nucleotides, while the other discontinuities consist of overlapping structures containing three DNA strands (22). It is interesting to note that a

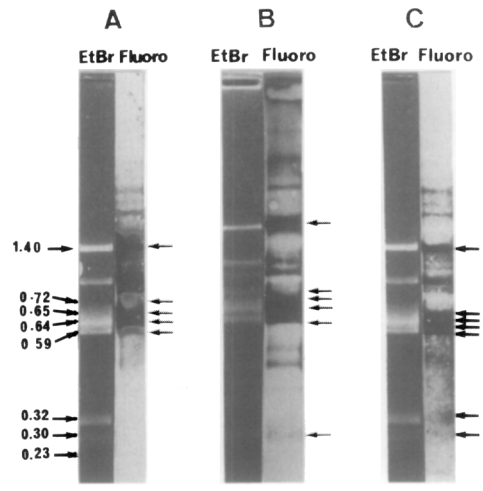

Figure 2.- Pattern of transcription of CaMV DNA by wheat germ RNA polymerases.

RNA was synthesised as described in the legend to Table I and purified according to Hossenlop et al. (7). Hybridisation conditions are described in Materials and Methods.

Ethidium bromide-stained gels (left, EtBr) are shown with fluorogrammes (right, fluoro) for RNA polymerases I (a), II(b) and III (c). Figures in (a) show molecular weights of fragments in daltons x 10^{-6}.

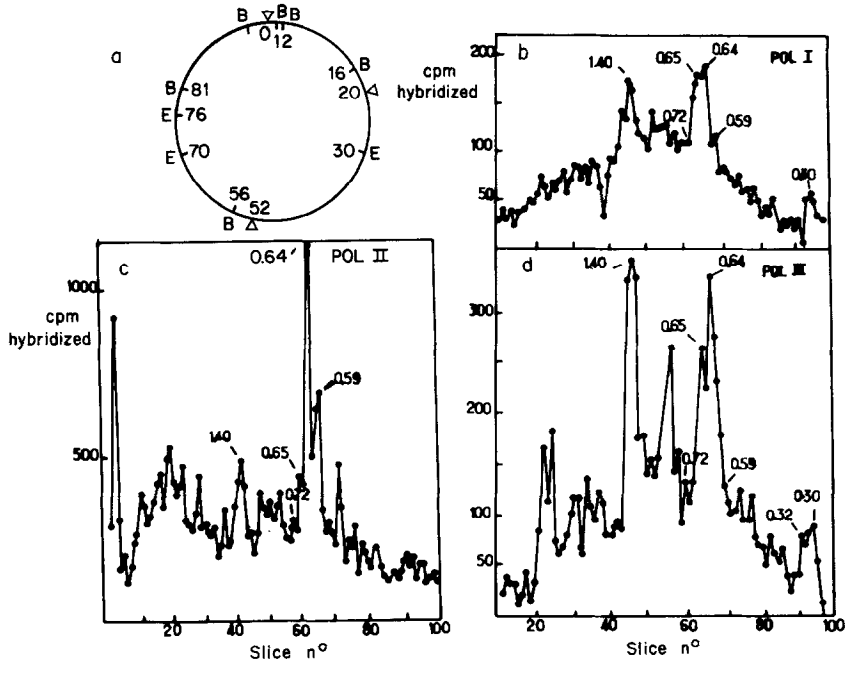

Figure 3.- Quantitation of hybridisation levels of in vitro synthesised RNA to different regions of the CaMV genome.

The nitrocellulose filters used for fluorography shown in Fig. 2 were cut into 1mm strips which were counted in a toluene-based scintillation fluid. (a) Polymerase I, (b) polymerase II, (c) polymerase III.

Hind III fragment of CaMV containing this discontinuity is also preferentially transcribed in vitro by E. coli RNA polymerase (23).

Thus, our results suggest that the ability of wheat germ enzyme I, II, III to form relatively stable heparin resistant complexes on CaMV DNA does not reflect the capacity to initiate RNA synthesis at specific sites but rather initiations occuring essentially at random nicks on the template for enzyme I and III, a nick at a fixed position (0 map unit) being more efficiently recognised and transcribed by enzyme II. As with RNA polymerases of animal origin, one must therefore look for factor(s) which confer the ability to initiate transcription at iniation sites which are used in vivo.

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REFERENCES.

1. CHAMBERLIN, M.J. (1976) in RNA polymerase (LOSIK, R., and CHAMBERLIN, M., Eds.) pp. 159-191, Cold Spring Harbor Press, Cold Spring Harbor, NY.
2. PFEFFER, S.R., STAHL, S.J., and CHAMBERLIN, M.J. (1977) J. Biol. Chem. 252, 5403-5407.
3. MILLER, J.S., and BURGESS, R.R. (1978). Biochemistry 17, 2064-206 .
4. SCHAFER, R., KRAMER, R., ZILLIG, W., and CUDNY, H. (1973). Eur. J. Biochem. 40, 367-373.
5. REZNIKOFF, W.S. (1976) in RNA Polymerase (LOSICK, R. and CHAMBERLIN, M. Eds.) pp. 441-454. Cold Spring Harbor Press, Cold Spring Harbor, NY.
6. LONG, E., and CRIPPA, M. (1976) FEBS Lett. 72, 67-70.
7. HOSSENLOPP, P., SUMEGI, J. and CHAMBON, P. (1978). Eur. J. Biochem. 90, 615-631.
8. SARAGOSTI, S., LESCURE, B., and YANIV, M. (1979). Biochem. Biophys. Res. Comm. 88, 1077-1084.
9. SEIDMAN, S., SURZYCKI, S.J., DELORBE, W., and GUSSIN G.N. (1979). Biochemistry 18, 3363-3371.
10. DYNAN, W.S., and BURGESS, R.R. (1979). Biochemistry 18, 4581-4588.
11. CHANDLER, D.W., and GRALLA, J.(1980). Biochemistry 19, 1604-1612.
12. LEWIS, M.K., and BURGESS, R.R. (1980). J. Biol. Chem. 255, 4928-4936.
13. SHEPHERD, R.J. (1976) in Advances in Virus Research (LAUFFER, M.A. et al. Eds.)20, 305-339, Academic Press, NY.
14. VOLOVITCH, M., DRUGEON, G., and YOT, P. (1978) Nucleic Acid Res. 5,2913-2925.
15. TEISSERE, M., DURAND, R., RICARD, J., COOKE, R. and PENON, P.(1979). Biochem. Biophys. Res. Comm. 89, 526-533.
16. HULL, R., SHEPHERD, R.J., and HARVEY, J.D. (1976). J. Gen. Virol. 31, 93-100.
17. MEAGHER, R.B., SHEPHERD, R.J., and BOYER, H.W. (1977) Virology 80, 363-375.
18. Mc DONNELL, M.W., SIMON, M.N., and STUDIER, F.W. (1977) J. Mol. Biol. 110, 119-146.
19. SOUTHERN, E.M. (1975). J. Mol. Biol. 98, 503-517.
20. PENON, P., TEISSERE, M., AZOU, Y. and RICARD, J. (1977) in Nucleic Acids and Protein synthesis in Plants, pp. 195-199, Editions du CNRS, PARIS.
21. TEISSERE, M., PENON, P., AZOU, Y., and RICARD, J. (1977). FEBS Lett. 82,77-81.
22. FRANCK, A., GUILLEY, H., JONARD, G., RICHARDS, K., and HIRTH, L. (1980) Cell 21, 285-294.
23. VOLOVITCH, M., CHOUIKH, Y., KONDO, H., and YOT, P.(1980) FEBS Lett.116,257-260.