DNA-DEPENDENT RNA POLYMERASE C FROM *XENOPUS LAEVIS* OVARIES: FORMATION OF STABLE HEPARIN-RESISTANT DNA-BINDING COMPLEXES

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1. Introduction

Despite accumulation of extensive information concerning the properties of the different eukaryotic RNA polymerase classes [1], it is not yet clear what kind of molecular interactions are involved in the recognition of a specific gene by these enzymes.

The polyanion heparin, as a DNA competitor, efficiently inhibits initiation but not elongation of transcription by either prokaryotic or eukaryotic RNA polymerases [2,3]. *Escherichia coli* RNA polymerase, upon preincubation with DNA in the absence of nucleotide precursors, is able to form DNA-binding complexes from which RNA synthesis can be initiated in presence of heparin with the addition of ribonucleotides [4]. It has also been shown that heparin eliminates all artificial starts in vitro and that only 8 stable resistant complexes are formed on T7 DNA [5]. In contrast to the prokaryotic enzyme, purified RNA polymerase A from rat liver does not form heparin-resistant complexes after incubation with various DNA templates [6]. To our knowledge, no attempt to form heparin-resistant complexes with eukaryotic RNA polymerase B has been reported.

In this report we present evidence for the formation of stable heparin-resistant DNA-binding complexes by RNA polymerase C from *Xenopus laevis* ovaries. This process is temperature-dependent, does not require the presence of a divalent cation, and is independent of nicks in the DNA template.

2. Materials and methods

Heparin (151 units/mg) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio 44128. Other chemicals were purchased as described [7].

RNA polymerase C from *Xenopus laevis* ovaries was prepared as described [7]. The form C_{II} obtained after chromatography on DEAE–Sephadex was used for the experiments described in this paper. The assay of heparin-resistant DNA-binding complex was as follows. The RNA polymerase was preincubated at 30°C for 10 min in a volume of 95 μl with 20 mM Tris–HCl (pH 7.9), 0.1 mM dithiothreitol, 2 mM $MnCl_2$, 0.25 mg/ml RNAase-free bovine serum albumin [7], ammonium sulfate and DNA as indicated in the figure legends. Heparin was the added. After the time interval indicated the four ribonucleotides were added (0.5 mM of each ATP, GTP and CTP, 0.005 mM of [^{3}H]UTP at the specific activity indicated) and the sample was further incubated at 30°C. The final volume was 110 μl. Samples were precipitated and counted as described [8]. In each experiment, a sample containing all the components was precipitated at 0°C and the counts were substracted from the values obtained with incubated samples. One unit of enzyme catalyses the incorporation of 1 pmol of UTP into acid-precipitable material in 15 min under the standard assay conditions [8].

Xenopus bulk DNA, high molecular weight *Xenopus* DNA and sonicated *Xenopus* DNA were prepared as described [7,9]. Some of the high molecular weight DNA was sheared in a Virtis 60 homogenizer at 2500 rev./min, –7°C in 67% glycerol for 30 min, ethanol-precipitated and collected as described by Davidson et al. [10]. All DNA preparations were extensively dialysed against 10 mM Tris–HCl (pH 7.9), 10 mM NaCl and 0.5 mM EDTA before use. High molecular weight DNA was always used within two weeks.

3. Results and discussion

RNA polymerase C_{II} from *Xenopus laevis* ovaries is inhibited by heparin (fig.1). 50% inhibition is reached at about 2 μg/ml or 0.35 units/ml which is very close to the value of 0.33 units/ml reported for 50% inhibition of rat liver RNA polymerase A [6]. In an attempt to form heparin-resistant DNA-binding complexes using *Xenopus* ovarian RNA polymerase C_{II}, two different heparin concentrations were added after preincubation of the enzyme with DNA template (table 1). One was the minimal concentration required for full inhibition (22 μg/ml) and the other was 45 times that value (1 mg/ml). After preincubation at 30°C, significant heparin-resistant RNA synthesis took place in both cases. After preincubation at 0°C, heparin-resistant RNA synthesis was still observed with 22 μg/ml heparin, whereas it was practically reduced to background levels with 1 mg/ml heparin. It is therefore necessary to add an excess of heparin in order to eliminate the binding complexes that can form at 0°C. True DNA-binding complexes able to resist high concentrations of heparin are formed only after preincubation at 30°C. Such a temperature-dependence has also been observed with *E.coli* RNA polymerase [2].

The heparin-resistant RNA synthesis is only a fraction of the synthesis observed in the control incubated without heparin (table 1) for two reasons. Firstly, in the presence of heparin, RNA polymerase molecules cannot reinitiate transcription after completion of the first RNA chain. Secondly, a certain proportion of the RNA polymerase molecules is bound to nicks or sites which do not confer resistance to heparin. The ratio heparin-resistant RNA synthesis/control is influenced by several parameters: the ammonium sulfate concentration, the molecular weight of the DNA template and finally the ratio RNA polymerase/DNA. In the experiment reported in table 1 and carried out in DNA excess, the heparin-resistant RNA synthesis was 8.4% of the control. Values below 8% were obtained with limiting DNA

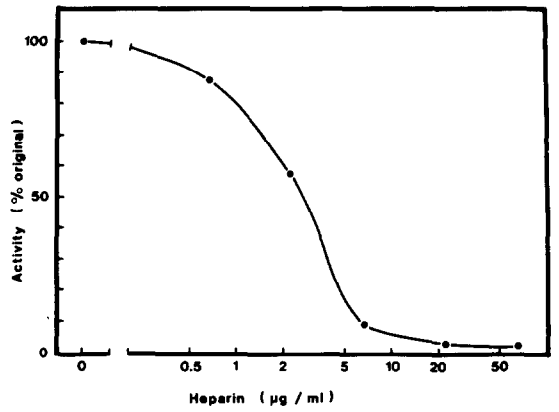

Fig.1. Inhibition of RNA polymerase C_{II} from *Xenopus laevis* ovaries by heparin. One unit of RNA polymerase C_{II} was incubated with increasing concentrations of heparin, 6.8 μg sonicated Xenopus bulk DNA and 80 mM ammonium sulfate. [^{3}H]UTP was at 870 cpm/pmol. The reaction was started by the addition of enzyme and was incubated for 15 min.

Table 1
Formation of heparin-resistant DNA-binding complexes by *Xenopus* ovarian RNA polymerase C_{II}

Preincubation temperature	Incorporation (% control)		
	No Heparin	Heparin, 0.022 mg/ml	Heparin, mg/ml
30°C	100	20	8.4
30°C, without $MnCl_2$	n.d.	n.d.	8.2
0°C	100	12	1.7

2 Units of RNA polymerase C_{II} were preincubated at the temperature indicated with 6.8 μg *Xenopus* bulk DNA and 40 mM ammonium sulfate in the preincubation mixture (Materials and methods). The ribonucleotides ([^{3}H]UTP at 870 cpm/pmol) were added 15 s after the heparin and the samples were further incubated for 10 min at 30°C. 2 mM of $MnCl_2$ was added just after the heparin to those samples preincubated without it. Incorporation is expressed as percentage of the control incubated without heparin.

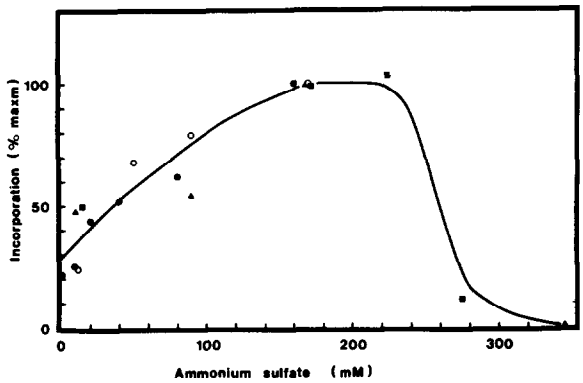

Fig.2. Formation of heparin-resistant DNA-binding complex by RNA polymerase C_{II} at different ammonium sulfate concentrations. After preincubation of RNA polymerase C_{II} with *Xenopus* bulk DNA at different ammonium sulfate concentrations in the preincubation mixture, heparin was added at 1 mg/ml. The four ribonucleotides were added 15 s later. After another 15 s all samples were adjusted to the highest ammonium sulfate concentration tested and were further incubated for 20 min. (■) 1 unit of RNA polymerase C_{II} with 3.4 μg DNA. (●) 2 units of RNA polymerase C_{II} (desalted to 0 mM ammonium sulfate by a 2 ml Sephadex G-50 column) with 6.8 μg DNA. (○) 2 units of RNA polymerase C_{II} (desalted to 100 mM ammonium sulfate) with 6.8 μg DNA. (▲) these samples were brought to 0.2 ml after the addition of ribonucleotides and adjusted to 165 mM ammonium sulfate. The contribution of ammonium sulfate by the RNA polymerase sample was taken in consideration. [^{3}H]UTP was at 4350 cpm/mol. Incorporation is expressed as percentage of the value obtained at 160 mM ammonium sulfate.

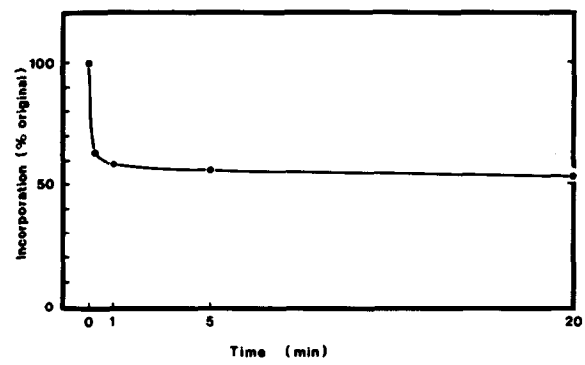

Fig.3. Stability of the DNA-binding complex in the presence of heparin. 5 Units of RNA polymerase C_{II} were preincubated with 7.5 μg *Xenopus* bulk DNA and 160 mM ammonium sulfate in 0.45 ml of preincubation mixture. After addition of heparin at 1 mg/ml, aliquots containing 1 unit of RNA polymerase C_{II} were removed at the time indicated and incubated with the four ribonucleotides ([^{3}H]UTP at 13 000 cpm/pmol) for 20 min. The 0-time aliquot was removed before addition of heparin and was added to a mixture of heparin and ribonucleotides. Incorporation is expressed as percentage of the 0-time value.

concentrations most likely because of a larger pool of free or loosely bound RNA polymerase molecules. As seen in table 1, heparin-resistant complexes are still formed if the cation Mn^{2+} is not added to the preincubation mixture. Mn^{2+} is therefore not necessary for their formation, while it is absolutely required for RNA synthesis [11]. The optimal ammonium sulfate concentration for the formation of the DNA-binding complexes is around 0.2 M (fig.2).

The stability of the DNA-binding complex in the presence of heparin is shown in fig.3. The kinetics of decay reveal the existence of two classes of complexes. The first has a very short half-life of less than 15 s and represents 45% of the complexes. The second is very stable with a half-life of more than 100 min and represents 55% of the complexes. In a similar experiment, the first class represented 35% of the complexes, the second 65%, and the kinetics were exactly the same. These two classes of complexes have also been observed with *E.coli* RNA polymerase on *E.coli* DNA [2,12] and T4, T5 and T7 DNA [4,5] and were called labile and stable initiation complexes. Experiments reported in table 1, fig.2 and table 2 of this paper concern only the stable class of DNA-binding complexes, since ribonucleotides were always added either 15 s or 1 min after the heparin. We prefer the term DNA-binding complex rather than initiation complex for the heparin-resistant complexes observed using *Xenopus* ovarian RNA polymerase C_{II} because initiation of transcription is still a vague concept in eukaryotes. We have found that the property involved in the formation of heparin-resistant DNA-binding complexes is also shared by RNA polymerase C_I (not shown), the other form of RNA polymerase C obtained after DEAE–Sephadex chromatography [7].

We have previously shown that RNA polymerase C from *Xenopus laevis* ovaries, besides being able to start RNA chains on nicks, is also able to transcribe an intact double-stranded DNA template [7]. However the sites on such an intact DNA where transcription can start are limited. In excess enzyme, the level of

Table 2
Formation of stable heparin-resistant complexes by RNA polymerase C_{II} with DNA templates of different molecular weights

DNA template	Incorporation (cpm)	
	Enzyme/DNA 2 units/μg	Enzyme/DNA 8 units/μg
High molecular weight DNA	701	871
Bulk DNA	616	n.d.
Sheared DNA	758	1048

1 unit of RNA polymerase C_{II} with 0.5 μg DNA (enzyme/DNA: 2 units/μg) and 2 units of RNA polymerase C_{II} with 0.25 μg DNA (enzyme/DNA: 8 units/μg) were preincubated with 160 mM ammonium sulfate in the preincubation mixture. Heparin was added at 1 mg/ml. The four ribonucleotides ([^{3}H]UTP at 13 000 cpm/pmol) were added 1 min later and the samples were further incubated for 20 min. The molecular weight of each DNA preparation was determined by sedimentation through 5–20% alkaline sucrose gradients [9] in a Beckman SW 41 rotor. The high molecular weight *Xenopus* DNA was sedimented at 8000 rev./min for 48 h. Its average molecular weight was 75×10^6 with its lower limit at 20×10^6. The *Xenopus* bulk DNA (10 000 rev./min for 40 h) was heterogenous in size with an average molecular weight of 10×10^6. The sheared DNA (16 000 rev./min for 20 h) had a molecular weight of 0.5×10^6. A [^{3}H]polyoma DNA probe served as marker in all the gradients.

transcription of a highly nicked DNA will therefore be higher than that of an intact DNA, because of the greater number of initiation sites [7]. We selected a ratio of RNA polymerase/DNA at which the level of transcription of a nicked DNA was twice that of a high molecular weight DNA, as well as a ratio four times greater, to test the formation of heparin-resistant complexes on these DNA templates of different molecular weights (table 2). No difference was found in the heparin-resistant RNA synthesis on the DNA templates that could be considered significant when compared to the difference found in the absence of heparin, namely two-fold at the enzyme/DNA ratio of 2 units/μg and even more at 8 units/μg. We conclude that the heparin-resistant DNA-binding complexes are not formed on nicks in the DNA template. The same conclusion has also been reached for *E.coli* RNA polymerase [5].

It will be of interest to investigate the nature of the sites in the *Xenopus* genome where these heparin-resistant DNA-binding complexes are formed.

Acknowledgement

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