Agrobacterium tumefaciens RNA Polymerase: A New Purification Procedure and a Study of the Stable Binding Sites on Homologous Deoxyribonucleic Acid†

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ABSTRACT: RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) of *Agrobacterium tumefaciens* has been purified according to a fast and efficient procedure. The method involves only two chromatographic steps and yields a highly active enzyme. The RNA polymerase was studied with respect to the ability to bind its homologous genome. *A. tumefaciens* deoxyribonucleic acid (DNA) binds the enzyme even when fragmented at undergenic size (300 base pairs). The general binding is unspecific and very labile at low concentrations of heparin (0.66 μg/mL). The number and distribution of the stable binding sites, class A sites [Hinkle, D., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157–185], have been calculated from the heparin-induced dissociation kinetics of binary complexes formed between the enzyme and DNA fragments of various sizes. A total of 3.5×10^3 class A sites (forming binary complexes with a half-life of 16.6 min) are present on *A. tumefaciens* genome, a large number of which show a distribution of 800–1000 base pairs. The rest have a more widely spaced distribution. The interactions between *Escherichia coli* RNA polymerase and the *A. tumefaciens* template have also been examined, and it has been observed that *E. coli* holoenzyme forms stable complexes with a shorter half-life and recognizes a lower number of class A sites on *A. tumefaciens* genome.

Most dicotyledonous plants develop crown-gall tumors after infection by *Agrobacterium tumefaciens* at the level of wound sites (Schilperoort et al., 1980; Schell & Van Montagu, 1980). [All virulent strains harbor at least one large plasmid (95–160 megadaltons) (Zaenen et al., 1974).] Recently, it has been shown that a relatively small part of this plasmid, called T-DNA, is transferred and integrated in the plant genome (Drummond et al., 1977; Chilton et al., 1977). This fragment of foreign DNA[1] is conserved in tumor cell cultures (Thomashow et al., 1980; Merlo et al., 1980). The presence of transcripts in an octopine-type tumor cell line has been shown, and translation products have been found in the wheat germ in vitro translation system (Gurley et al., 1979a; McPherson et al., 1980). To our knowledge a study of the interaction between *A. tumefaciens* RNA polymerase and its homologous genome has not been carried out, although the enzyme has already been purified and characterized (Knopf, 1974), and the possibility that RNA polymerase from infected plants might recognize promoter sites on the bacterial plasmid has not been examined. In order to carry out a detailed analysis of the interactions between RNA polymerase and template in this unique prokaryotic–eukaryotic system, we have given priority to the characterization of the DNA binding properties of *A. tumefaciens* RNA polymerase on *A. tumefaciens* DNA. For this purpose RNA polymerase holoenzyme has been purified; a new simple method that involves only two chromatographic steps and yields a pure highly active enzyme was developed. Subsequently, we have used the purified RNA polymerase to examine the DNA class A sites (Hinkle & Chamberlin, 1972) and to study their distribution on the bacterial genome. Investigations of this kind have been carried out on viral (Schäfer et al., 1973a,b) bacterial (Venetianer et al., 1976; Pedone et al., 1978), and eukaryotic genomes (Di Mauro et al., 1980a,b; Witney et al., 1980). In these reports binary complexes belonging to different classes of stability have been described and characterized. Strong evidence has been presented that in bacterial systems a correspondence exists between the genetically identified promoters and the stable binding sites for RNA polymerase in vitro (Chamberlin, 1976).

Materials and Methods

Heparin and calf thymus DNA, grade I, were purchased from Sigma Chemical Co. DEAE-Sephadex A-25 and Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Nucleoside triphosphates were obtained from Koch Light Laboratories. [^{3}H]Thymidine (48 Ci/mmol) and [^{3}H]uridine triphosphate (10 Ci/mmol) were from Radiochemical Center (Amersham, United Kingdom), *Escherichia coli* RNA polymerase was purchased from Miles Laboratories; its specific activity was 10^3 units/mg. *A. tumefaciens* was grown in a complete medium TY (Beringer, 1974), and the cells were harvested at the late log phase and stored at −80 °C. The buffers were as follows: buffer A, 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 12 mM 2-mercaptoethanol, 25% glycerol, 0.1% Triton X-100, and 0.2 mg/mL phenylmethanesulfonyl fluoride; buffer B, 50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM 2-mercaptoethanol, 5% glycerol, 235 mM NaCl, 0.025 mg/mL phenylmethanesulfonyl fluoride; buffer C, 10 mM Tris-HCl, pH 7.9, 2 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, and 200 mM NaCl; buffer D, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 50 mM NaCl.

Preparation of A. tumefaciens [^{3}H]DNA Fragments. Cultures (100 mL) of *A. tumefaciens*, strain C58 (pTi B6),

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[1] Abbreviations used: PEI, poly(ethylenimine); NaDOC, sodium deoxycholate; RNA polymerase, RNA nucleotidyltransferase (EC 2.7.7.6); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene; $NaDodSO_4$, sodium dodecyl sulfate; UMP, uridine 5′-phosphate.

0006-2960/81/0420-6097$01.25/0

Table I: Purification of *A. tumefaciens* RNA Polymerase[a]

fraction	step	$(NH_4)_2SO_4$ (M)	volume (mL)	protein (mg/mL)	sp act. (units/mg)
crude extract	I	0.10	80	0.7	60
DEAE-Sephadex A-25 eluate	II	0.20	65	0.2	600
heparin-Sepharose 4B eluate	III	0.32	3.5	1.0	2000

[a] The purification was carried out on 60 g of cells. The enzyme activity was assayed as previously reported (Di Mauro et al., 1978). Protein concentration was measured by the method of Lowry et al. (1951). One unit of enzyme corresponds to the amount of protein that incorporates 1 nmol of UMP into Cl_3CCOOH-insoluble material in 10 min at 37 °C under the standard assay conditions (Di Mauro et al., 1978).

were grown in medium TY containing 1 mCi of [^{3}H]thymidine (48 Ci/mmol) to late log phase. Bacteria were collected by centrifugation at 12000*g* for 15 min, and the pellet was resuspended in 40 mL of SSC (150 mM NaCl and 15 mM sodium citrate) and then lysed by addition of sodium dodecyl sulfate to the final concentration of 2% (w/v). DNA was extracted twice with chloroform–isoamyl alcohol (24:1 v/v), precipitated with cold ethanol, and purified by centrifugation in CsCl (ρ = 1.4000 g/cm^3) for 64 h at 34 × 10^3 rpm in an SW 42.1 Beckman rotor at 18 °C (Buongiorno-Nardelli et al., 1977). DNA was dialyzed exhaustively against 0.1 M NaCl. The DNA was made 0.3 M in sodium acetate, pH 5, precipitated with 2 volumes of cold ethanol, and then centrifuged at 30000*g* for 20 min. The DNA was resuspended in SSC at a final concentration of 1.25 mg/mL. The A_{260}/A_{280} ratio was 1.95.

Fragments of *A. tumefaciens* DNA were obtained by hand-shearing through a syringe (needle gauge 0.45 mm, 5–50 strokes) and by sonication (50 s to 10 min) with a MSE sonifier (10-mm probe at 20 kHz). Fragments of various molecular weights were separated by sedimentation through a 5–25% linear sucrose density gradient in 1 mM EDTA, 10 mM Tris-HCl, pH 8, and 1 M NaCl in a SW41 Beckman rotor for 7 h at 36 × 10^3 rpm at 4 °C. The fragment size was estimated according to Studier's interpolations (Studier, 1965). The specific activity of the labeled DNA was 3 × 10^4 dpm/μg.

Millipore Binding Assay. The assays were performed according to the method of Jones & Berg (1966) as modified by Hinkle & Chamberlin (1972). Binding mixture (300 μL) containing RNA polymerase, [^{3}H]DNA, and buffer D (for details see legends to the figures) was incubated for 10 min at 37 °C. The reaction was stopped by addition of 1 mL of buffer D, and the samples were immediately filtered through millipore filters (GSWPO, pore size 0.22 μm). The filters were dried and counted in 5 mL of toluene–PPO–POPOP (5 g/L and 0.2 g/L, respectively). In the heparin-induced dissociation kinetics the drug was added after 10 min of preincubation, and 300-μL samples were withdrawn at the indicated times, diluted to 1 mL of buffer D, and immediately filtered.

Purification of RNA Polymerase. The purification of *A. tumefaciens* RNA polymerase was attained through a simplified version of the method reported by Ballario et al. (1980). All the operations were carried out at 4 °C if not otherwise specified.

Frozen cells of *A. tumefaciens* strain C58 (pTI B6) (60 g) were thawed, suspended in 200 mL of buffer B, and homogenized in Waring Blendor for 3 min at full power. After 20 min of incubation, NaDOC was added to the final concentration of 0.05% (w/v), and the incubation was continued for 10 min at 15 °C. To the very viscous clear lysate was added 280 mL of buffer C, and the DNA was sheared with a Vibromixer (Chemap, Switzerland) for 45 s at full speed. The cell extract was centrifuged for 25 min at 30000*g*. A pH 7 buffered PEI solution was added dropwise to a final concentration of 1% (v/v) while stirring. Stirring was then continued for 30 min, and the precipitated nucleic acids were spun for 25 min at 30000*g*. Solid $(NH_4)_2SO_4$ to 65% saturation was added to the supernatant under stirring, and after 30 min the solution was centrifuged for 25 min at 30000*g*. The crude RNA polymerase pellets were carefully drained and resuspended in buffer A. The conductivity was measured and the protein solution was diluted with the same buffer until the concentration of the $(NH_4)_2SO_4$ reached 100 mM, corresponding to a conductivity (16.5 × 10^3 $\mu\Omega^{-1}$). The sample was then loaded onto a DEAE-Sephadex A-25 column (45 × 1.5 cm i.d.), preequilibrated with 100 mM $(NH_4)_2SO_4$ in buffer A. The column was washed with 100 mL of the same buffer at a flow rate of 100 mL/h. The enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ in buffer A (2 × 350 mL, 100–350 mM), 12-mL fractions were collected, and aliquots were assayed for RNA polymerase activity (Hinkle & Chamberlin, 1972). A single sharp activity peak was found at a concentration of 200 mM $(NH_4)_2SO_4$ (33.0 × 10^3 $\mu\Omega^{-1}$). The fractions exhibiting polymerase activity were pooled, diluted with buffer A to 100 mM $(NH_4)_2SO_4$, and loaded onto a heparin–Sepharose 4B column (20 × 1 cm i.d.) at a flow rate of 56 mL/h. The column was washed with 70 mL of 10 mM $(NH_4)_2SO_4$ in buffer A and eluted with a linear gradient of $(NH_4)_2SO_4$ (2 × 150 mL, 100–500 mM). Fractions (2.5 mL) were collected and assayed. The RNA polymerase was eluted in one sharp peak at 320 mM $(NH_4)_2SO_4$ (45.0 × 10^3 $\mu\Omega^{-1}$, average conductivity of the pooled fractions). The enzyme was concentrated by applying it to a microcolumn of heparin–Sepharose 4B (1.2 × 1 cm) and eluted in a stepwise manner with 400 mM $(NH_4)_2SO_4$. The RNA polymerase was stored frozen in small aliquots at −80 °C with no detectable loss of activity for several months. In Table I the purification procedure of the enzyme is reported. The purity of the enzyme was checked by NaDodSO$_4$–polyacrylamide gel electrophoresis (Laemmli, 1970) (Figure 1). The transcriptional activity of the purified fraction was completely DNA dependent.

Results

Formation of RNA Polymerase·DNA Binary Complexes and Retention on Nitrocellulose Filters. It is known that RNA polymerase has a high affinity for the DNA and polyanions in general (Hinkle & Chamberlin, 1972). One molecule of enzyme is able to retain on a nitrocellulose filter DNA fragments of various sizes. The retention of these complexes has been observed in viral genomes, such as T7 DNA (Schäfer et al., 1973b), in prokaryotes (Pedone et al., 1978), and in eukaryotes (Di Mauro et al., 1980a,b). It has been also pointed out that this general binding takes place at both specific and nonspecific sites and it is so extensive that the DNA may be completely covered by the enzyme, further binding being prevented only by steric hindrance caused by the RNA polymerase molecules. Such a generic binding occurs between *A. tumefaciens* RNA polymerase and its ho-

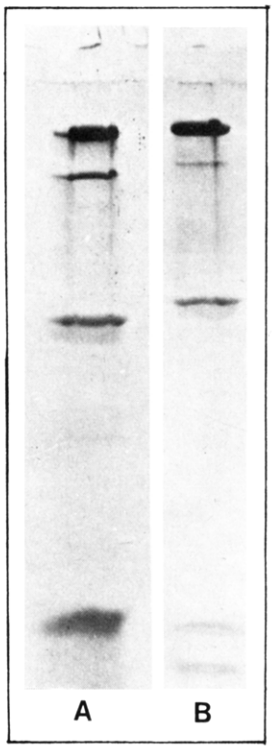

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of *A. tumefaciens* RNA polymerase. 12% $NaDodSO_4$–polyacrylamide slab gel electrophoresis (Laemmli, 1970) was performed for 12 h at 30 V. (Lane A) *E. coli* RNA polymerase (5 μg). (Lane B) *A. tumefaciens* RNA polymerase (5 μg). The molecular weights of the enzyme subunits in our preparation, calculated by plotting the molecular weights of the markers vs. their electrophoretic mobilities (Laemmli, 1970) on a semilog scale (not shown), are in good agreement with those obtained by Knopf (1974). As of now, it is impossible to state whether the lowest molecular weight band present in our preparation is part of the quaternary structure of the protein or, alternatively, represents a minor contaminant.

mologous template. In fact, the retention of the binary complexes depends only upon the amount of the enzyme used and not upon the size of the fragments. Apparently, small DNA fragments are retained less efficiently, but if the amount of enzyme is increased, 90% of the input 700 base pair fragments are retained on the filters (not shown). This indicates that genetically competent sequences are not required for general binding as also shown by Pedone et al. (1978).

Sensitivity of Binary Complexes to Heparin and Discrimination between Two Classes of Stability. The polyanion heparin is able to bind strongly free RNA polymerase and to displace holoenzyme molecules that are loosely bound to the DNA (Schäfer et al., 1973a; Giacomoni et al., 1977). Heparin has therefore been used as a tool to differentiate binary complexes formed at the level of promoter from those formed at nonpromoter sequences. Figure 2 shows the heparin-induced dissociation of binary complexes formed between *A. tumefaciens* RNA polymerase and 700 base pair DNA fragments. Most of the complexes are very unstable and are dissociated by low concentrations of the drug (0.7 μg/mL). From the data presented in the figure a fraction of the complexes appears to be resistant to 25-fold higher concentrations of heparin. These resistant complexes are analyzed in detail in the following experiments. The results reported in Figure 3 show the dissociation kinetics of binary complexes formed with the 700 base pair fragments at different concentrations of heparin. The dissociation rate of these stable complexes is a function of the concentration of heparin. The dissociation pattern of the stable bindings observed might be interpreted on the basis

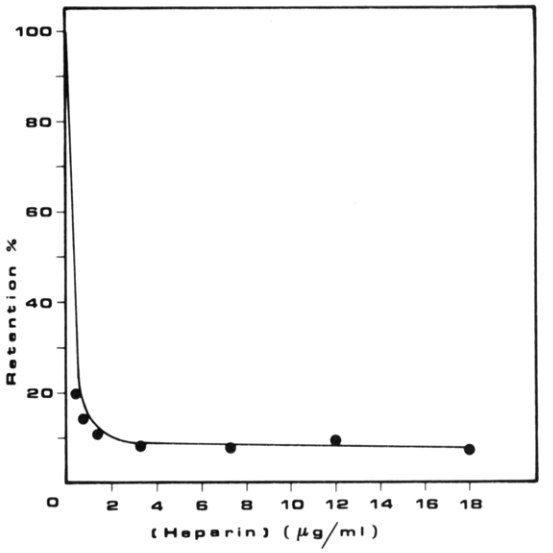

FIGURE 2: Effect of increasing concentrations of heparin on binary complexes 0.7 μg of *A. tumefaciens* RNA polymerase, 0.1 μg of [^{3}H]DNA (700 base pairs), and buffer D were incubated in a volume of 280 μL for 10 min at 37 °C. 20 μL of a solution of heparin in buffer D was added to obtain the final concentrations of drug indicated in the figure. The incubation was continued for 5 min at the same temperature, and the samples were diluted with 1 mL of ice-cold buffer D and immediately filtered. 100% represents the input DNA.

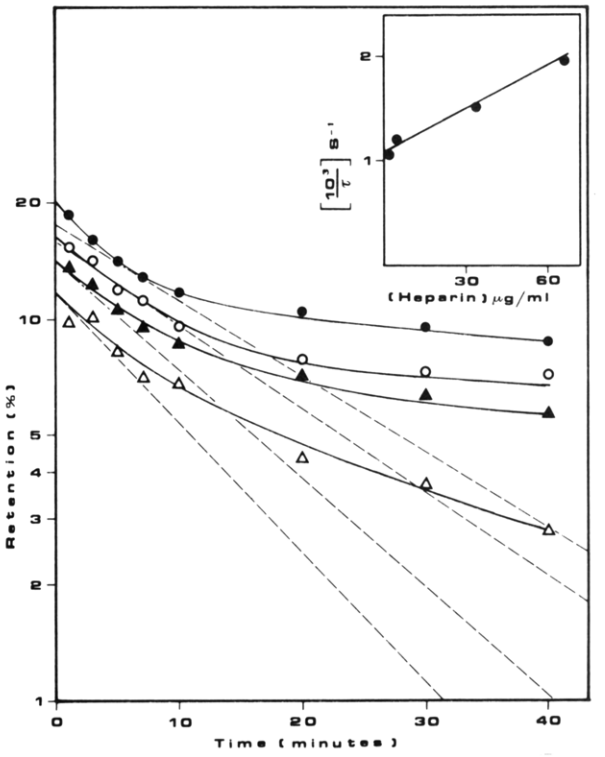

FIGURE 3: Heparin-induced dissociation kinetics of *A. tumefaciens* RNA polymerase [^{3}H]DNA homologous binary complexes. Appropriate amounts (see below) of *A. tumefaciens* RNA polymerase [^{3}H]DNA and buffer D were incubated for 10 min at 37 °C in a volume of 2.8 mL. 200 μL of heparin in buffer D was added to obtain the final concentration reported below. 300-μL samples (each containing 0.1 μg of [^{3}H]DNA, 0.7 μg of enzyme, and heparin) were withdrawn at the indicated times, diluted with 1 mL of buffer D, and immediately filtered. (●) 0.60, (○) 6.60, (▲) 33.30, and (△) 66.60 μg/mL heparin. (Insert) Plot of the rate constant $1/\tau$ vs. the heparin concentrations used in the experiment. $1/\tau = (T/2)^{-1}$ where $T/2$ is the time required to dissociate half of the complex formed at each heparin concentration.

of a different degree of stability of each promoter·RNA polymerase complex. As a matter of fact, *E. coli* RNA polym-

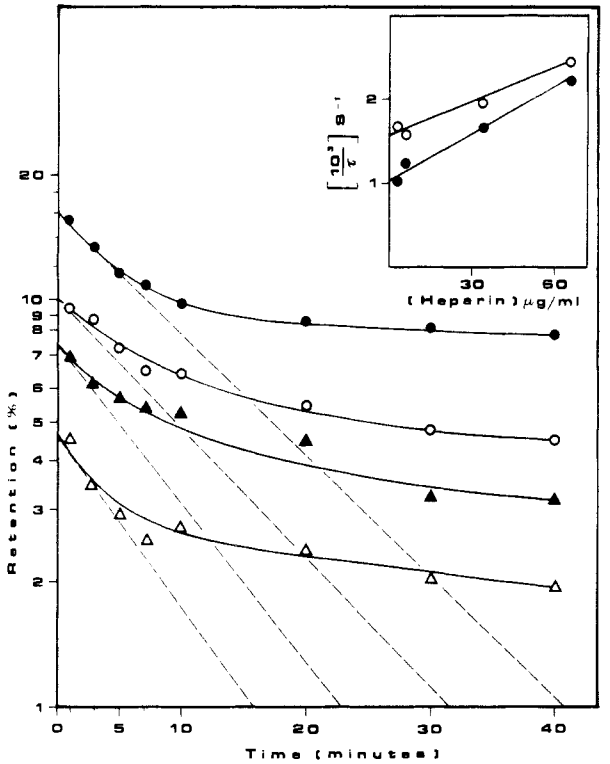

FIGURE 4: Heparin-induced dissociation kinetics of heterologous binary complexes formed between *E. coli* RNA polymerase and *A. tumefaciens* [^{3}H]DNA. Dissociation kinetics at different heparin concentrations were carried out under the same experimental conditions reported in Figure 3. Each sample contained 1.4 μg of *E. coli* RNA polymerase and 0.1 μg of *A. tumefaciens* [^{3}H]DNA (700 base pairs). The final concentrations of heparin were (●) 0.66, (○) 6.60, (▲) 33.30, and (△) 66.60 μg/mL. The amount of *E. coli* enzyme used retained 50% of the input DNA (0.1 μg, 700 base pairs). (Insert) Comparison of the plots of the rate constants $1/\tau$ vs. the heparin concentration of the heterologous binary complex (○) and the homologous binary complex (●). Data for the latter are taken from the insert of Figure 3.

erase has been reported to form complexes with bacteriophage *fd* promoters, each endowed of a characteristic stability (Seeburg, 1977). According to Hinkle & Chamberlin (1972), we define these stable binary complexes as class A complexes. From the extrapolations of the heparin-induced dissociation curves at zero concentration of drug (Figure 3, insert) the half-life of these complexes was estimated to be 16.6 min. A similar experimental approach has been previously used to calculate the half-life of class A complexes in *E. coli* and sea urchin (Pedone et al., 1978; Di Mauro et al., 1980a). We have examined the behavior of the heparin-resistant complexes also in a heterologous system with *A. tumefaciens* DNA and *E. coli* RNA polymerase. Heparin-induced dissociation kinetics have been carried out under the same experimental conditions reported in the legend of Figure 3. In Figure 4 the results of a typical dissociation experiment are reported. As expected, the behavior of the heterologous binary complexes does not seem to be qualitatively different from the homologous ones. In this case the estimated half-life of the stable class A complexes was 10.4 min. In the insert of Figure 4 the plot of the rate constant $1/\tau$ vs. the heparin concentration is shown for both the homologous and the heterologous system. The complexes formed between *E. coli* RNA polymerase and *A. tumefaciens* DNA seem to be less stable than the homologous ones. The different slope reflects a different response of the heterologous complex to the drug, and, in addition, it has been reported that the source of the enzyme might play some role (Di Mauro et al., 1980a).

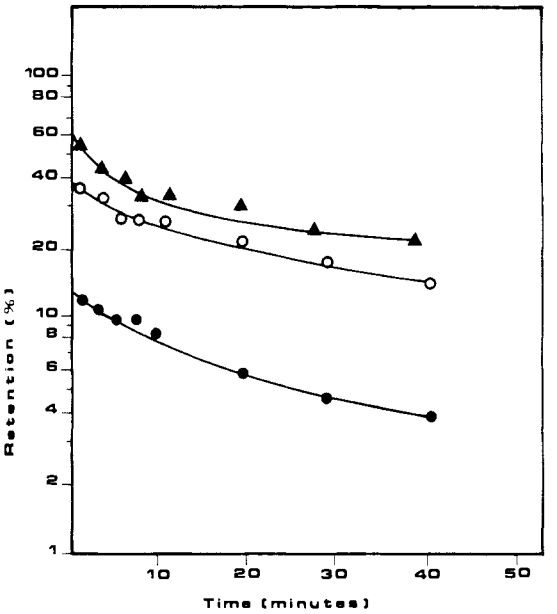

FIGURE 5: Heparin-induced dissociation kinetics of homologous binary complexes formed by DNA fragments of different sizes. Dissociation kinetics were performed as indicated in the legend of Figure 3, at a final concentration of 7 μg/mL. (●) 700 base pair [^{3}H]DNA; (○) 4000 base pair [^{3}H]DNA; (▲) 7000 base pair [^{3}H]DNA.

Distribution and Number of Stable Binding Sites on A. tumefaciens Genome. To calculate the number of class A sites on the *A. tumefaciens* genome, we have essentially adopted the method previously reported (Pedone et al., 1978; Di Mauro et al., 1980a,b). Dissociation kinetics of binary complexes formed with DNA fragments of different length have been analyzed in order to estimate the number of class A sites. Figure 5 shows the dissociation curves of three different DNA fragments at 7 μg/mL heparin concentration. The efficiency of retention depends upon the size of the DNA fragments: large fragments are more likely to bear class A sites than the small ones, and complexes made with the former are therefore better retained than those made with the latter. By extrapolation at zero time of the curves shown in Figure 5, it has been observed that 12% of the 700 base pair, 38% of the 4000 base pair, and 58% of the 7000 base pair fragments carry class A sites. The same kind of experiments has been carried out by using a greater number of fragment sizes. The extrapolations at zero time of each experimental curve have been plotted vs. the size of the fragment used in this set of experiments, and the results are shown in Figure 6. The percentage of retention increases in a linear fashion until 1000 base pairs, meaning that this is the minimal distance at which two class A sites are found on the *A. tumefaciens* genome. As the DNA fragment size increases the slope decreases, reaching a plateau at a length of 6000 base pairs. According to De Ley (1975) the size of the *A. tumefaciens* DNA ranges from 3.1×10^9 to 3.6×10^9 daltons; therefore, if one assumes 3.35×10^9 as the average value, the total genome should be formed by 5.07×10^6 base pairs. From these data and from the results shown in Figure 6, it has been possible to calculate the total number of stable binding sites as in Pedone et al. (1978) and Di Mauro et al. (1980a,b). A total of 3.5×10^3 class A sites were estimated to be present on the *A. tumefaciens* genome. The same experimental approach has been used to determine the number and distribution of stable binding sites in the heterologous system where binary complexes have been formed between *E. coli* RNA polymerase and *A. tumefaciens* DNA. In Figure 7 are plotted the extrapolations at zero concentration of heparin-induced dissociation kinetics of complexes vs. the

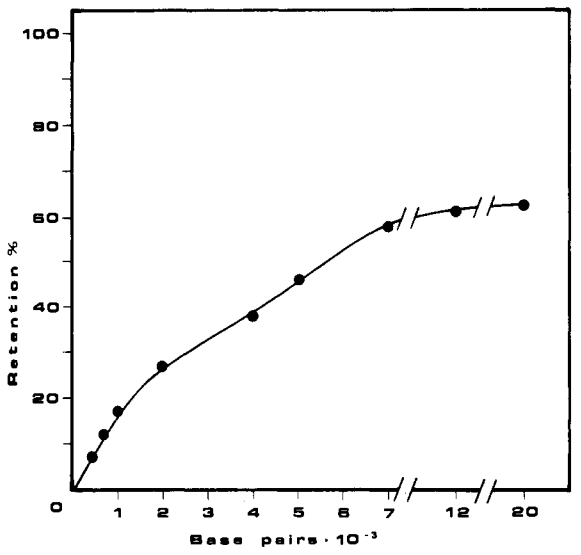

FIGURE 6: Homologous class A sites as a function of the base pair number of the DNA fragments. Plot of the extrapolation at zero time of nine experimental curves obtained as described in the legend of Figure 3.

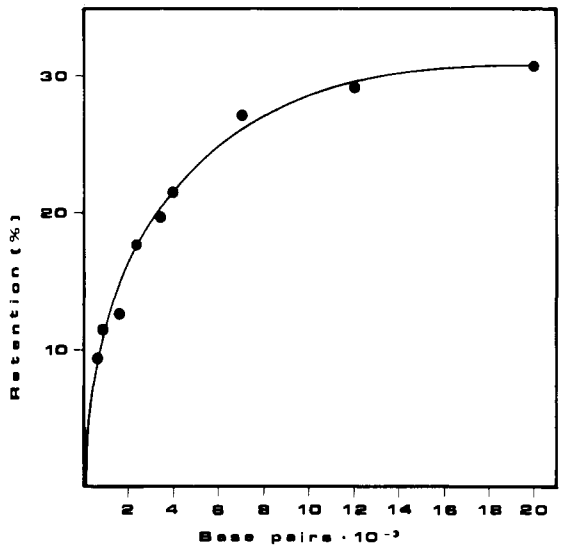

FIGURE 7: Heterologous class A sites recognized by *E. coli* RNA polymerase on *A. tumefaciens* genome. Dissociation kinetics of heterologous binary complexes were performed as reported in the legend of Figure 3 by using 7 μg/mL heparin (final concentration) and *A. tumefaciens* DNA fragments of various lengths (not shown). The extrapolations at zero time of the different dissociation curves were plotted vs. the size of nine different DNA fragments.

length of the DNA fragments used in these experiments. *E. coli* RNA polymerase seems to recognize a lower amount of class A sites of *A. tumefaciens* DNA; 9% of the 700 base pair, 13% of the 4000 base pair, and 27% of the 7000 base pair fragments bear sites that are stably bound by *E. coli* RNA polymerase, and the total number of class A sites recognized by the enzyme on the *A. tumefaciens* genome was estimated to be 1×10^3.

Discussion

A. tumefaciens has been studied for several years, and the interaction between this bacterium and the infected plants is a matter of intensive investigation (Schell & Van Montagu, 1980; Schilperoort et al., 1980). Part of the plasmid DNA has been shown to be stably integrated in the higher plant genome, and this has made *A. tumefaciens* a potential tool for the transformation of eukaryotic plant cells since its virulent plasmid could be exploited as a molecular vector of genetic information. Reports showing that *E. coli* RNA polymerase recognizes promoter sites on the tumoral DNA integrated into the plant genome have appeared (Gurley et al., 1979b). These studies have been carried out by using the nitrocellulose filter binding assay developed by Jones & Berg (1966), and the possibility that bacterial promoters function as eukaryotic promoter sequences in the plant has been suggested (Gurley et al., 1979b; Scherrer et al., 1978; Gannon et al., 1979).

In the light of these results it seems interesting to investigate in detail the interactions between *A. tumefaciens* genome and its homologous transcriptional enzyme. It is our aim to map the promoter sites on the virulent plasmid of *A. tumefaciens* and study whether these sequences are recognized by the plant RNA polymerase.

For this purpose the study of the DNA binding properties of *A. tumefaciens* RNA polymerase on its homologous genome is essential. Therefore, the bacterial transcriptional enzyme has been purified according to a new fast method that yields a pure holoenzyme. The RNA polymerase obtained by this procedure has a very high affinity for its template as also shown in other systems (Hinkle & Chamberlin, 1972; Chamberlin, 1976) since it can bind *A. tumefaciens* DNA even when fragmented at undergenic sizes (300 base pairs). Two classes of binary complexes are observed. The first [class B complexes as defined by Hinkle & Chamberlin (1972)] is unstable and heparin sensitive. The second one (class A complexes; Hinkle & Chamberlin, 1972) is stable and relatively resistant to heparin. The distribution of class A sites on the *A. tumefaciens* genome could be estimated from the heparin-induced dissociation of the stable binary complexes. These experiments have been performed according to the procedure previously reported, further proving that the interactions between RNA polymerase and genome obey general rules common to several biological systems (Pedone et al., 1978; Di Mauro et al., 1980a,b).

The differences between stability, amount, and distribution of stable binding sites for the homologous (*A. tumefaciens*) and the heterologous (*E. coli*) RNA polymerase on the *A. tumefaciens* genome are discussed in the text (see results of the experiments shown in Figures 4 and 7). A total of 3.5×10^3 stable binding sites seem to be recognized by RNA polymerase on its homologous template; this estimation has to be considered approximate since (1) the DNA fragment sizes were calculated according to Studier's interpolations and therefore refer to the predominant size of an heterogeneous population and (2) it has been shown (Pedone et al., 1978) that the experimental approach used in the work presented here does not allow the quantitation of all the genetically identified promoter sites. In fact, it has been reported that transcription can start at some promoters that do not seem to form stable complexes with RNA polymerase. In addition, open promoters seem to be sensitive to heparin (Stahl & Chamberlin, 1977).

Acknowledgments

We are indebted to Dr. R. Caneva for the computer elaboration of our experimental data. Thanks are also due to Dr. F. Pedone for helpful discussions and advice.

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