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Interaction of *Escherichia coli* RNA polymerase with promoters of several coliphage and plasmid DNAs

(complex formation/complex stability/promoter strength)

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ABSTRACT The interaction of *Escherichia coli* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) with restriction fragments obtained from various *E. coli* related DNAs was studied *in vitro*. The DNAs investigated included several coliphage genomes (T5, λ, T7, fd) and plasmid DNAs (pML 21, pSC101). By using the nitrocellulose filter binding of the enzyme–DNA complexes, fragment-specific relative rates of complex formation as well as complex stabilities were determined. Promoter-specific relative rates of polymerase binding were derived from fragment-specific rates by taking into account the number of major binding sites for RNA polymerase within several DNAs. Estimates of the stability of complexes formed between some major binding sites and the enzyme were obtained by studying the rate of complex decay. Both characteristics—rate of complex formation and rate of decay—varied widely and independently of each other. The promoters reacting most efficiently with *E. coli* RNA polymerase were found in the early region of coliphage T5 whereas some promoters in pML 21, or for example, the λ promoter P_I, belong to signals binding the enzyme most slowly. Based on the second-order rate constant determined for the interaction of *E. coli* RNA polymerase with promoters of phage fd, the fastest promoters characterized so far reacted with rates in the order of 10^8 $M^{-1}s^{-1}$. The hierarchy of promoters established here is of interest from the viewpoint that promoter strength correlates with the rate of polymerase binding. Among the promoters studied here this rate spans a range of 2 orders of magnitude.

Studies in two coliphage systems have shown that the *in vitro* rate of complex formation between RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) and promoters, rather than the stability of the complex, reflects their *in vitro* and *in vivo* activities, provided that no further positively or negatively acting elements are involved in the control (1, 2).

In coliphage fd the second-order rate constants for complex formation differ by an order of magnitude between the "slowest" and the "fastest" promoter (1), and within the hierarchy of T5 promoters 6- to 7-fold differences in the relative rates of complex formation have been observed (2). Thus, changes in the recognition sequence of a promoter can apparently modulate promoter activity over a wide range and it has been reported that, in the *Escherichia coli lac* operon, a single base pair change within the nucleotide sequence of the promoter drastically influences the transcriptional activity (ref. 3; J. Gralla, personal communication).

Because of the correlation between promoter strength and the rate of polymerase binding *in vitro*, it appeared of interest to us to study the range over which promoters can differ in their binding characteristics for RNA polymerase. Furthermore, by identifying promoters with extreme properties, one could obtain candidates for the nucleotide sequence work required for the understanding of the structural features specifying a promoter.

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In studying the RNA polymerase binding to DNA of several coliphages including T5, T7, λ, and fd as well as DNA of plasmids pML21 and pSC101, large differences in the efficiency of polymerase binding were observed. Similarly, the complex stabilities varied widely, although the question of whether some of the extremely stable complexes found have promoter function must await further investigation.

MATERIAL AND METHODS

The sources of most of the DNAs used and their extraction, fragmentation by restriction enzymes, and electrophoretic analysis have been described (2). *E. coli* strains carrying pSC101 (4) and pML21 (5) were obtained from S. N. Cohen (Stanford) and H. Boyer (University of California, San Francisco) respectively. Individual restriction fragments were isolated according to Blin *et al.* (6). The isolation and characterization of *E. coli* polymerase as well as the synthesis of initial transcripts and their analysis according to Southern (7) were as described (2).

Filter binding experiments for the determination of relative rates of polymerase binding by promoters ("on-rates") and of the half-lives of the enzyme–promoter complexes as well as their quantitative evaluation were performed as reported (2). In a typical on-rate experiment, DNA was incubated in 10–50 μl of binding buffer [20 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/0.1 mM EDTA/0.1 mM dithiothreitol/5% (vol/vol) glycerol/120 mM KCl] with varying amounts of RNA polymerase. The assay contained 100–350 μg of DNA per ml to which 0.03–3.5 pmol of RNA polymerase was added in order to achieve polymerase-to-promoter ratios between 0.01 and 0.9. After incubation for 5 min at 37°C, single-stranded competitor DNA (usually 10 μg of fd DNA) was added in prewarmed binding buffer without KCl such that the assay volume was doubled and the KCl concentration was decreased to 60 mM. After another 3 min, the reaction mixture was passed through a nitrocellulose filter (0.45 μm pore, Sartorius, Goettingen, Germany) mounted on a stainless steel support and equilibrated at 37°C. The filter was washed with an equal volume of warm binding buffer containing no KCl before it was immersed in 30–50 μl of elution buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA, 0.1% sodium dodecyl sulfate) and kept at 4°C for 2 hr. In a typical experiment in which the decay of RNA polymerase–promoter complexes was monitored, the complex formation was allowed to take place as described above at polymerase-to-promoter ratios between 0.1 and 0.2.

In these experiments the salt concentration was shifted to 160 or 200 mM KCl at the time of the addition of the competitor DNA. The reaction mixture was kept at either 37 or 15°C, respectively. Samples were withdrawn at proper time intervals and filtered at 37°C as described above.

After elution from nitrocellulose filters, the DNA samples were analyzed by electrophoresis in 0.7 or 1.0% agarose gels as described (2).

RESULTS

Relative Rates of RNA Polymerase Binding to Various DNA Fragments. The principle of these experiments was as follows. DNAs of different origin, in intact form or after fragmentation by restriction enzymes, were mixed in nearly stoichiometric amounts and incubated with limiting amounts of RNA polymerase under standard assay conditions. After 3 min, single-stranded competitor DNA was added and the reaction mixture was analyzed by the nitrocellulose filter binding assay (8, 9) followed by gel electrophoresis (2). Under these conditions the fragments with the highest rate of complex formation are represented most strongly in the electrophoretic gel pattern, provided that the half-life of the complex clearly exceeds the time course of the experiment. Short-lived RNA polymerase–promoter complexes on the other hand can be trapped in reaction mixtures containing ribonucleotide triphosphates (GTP+ATP+CTP or GTP+ATP+UTP) because the fast-forming ternary complexes are highly stable (1, 10). Experiments performed under these conditions did not change the relative rates of polymerase binding to various fragments (data not shown), demonstrating a negligible contribution of these hypothetical binding sites.

Fig. 1 shows the result of an experiment in which a mixture of intact T7 DNA, the *Hin*dIII fragment of T5, and λ DNA as well as the *Bam*HI fragments of fd RF DNA were exposed simultaneously to limiting but increasing amounts of RNA polymerase. It can be seen from this experiment that the DNA fragments carrying the early promoters of T5 (2) compete most efficiently for the enzyme followed by the T5 fragments containing the promoters of the pre-early class (2). Only after the majority of T5 promoters have been titrated are T7, λ, and fd DNAs able to bind RNA polymerase in detectable amounts. Experiments of this type in which only two DNA species or their restriction fragments were compared, established, after quantitative evaluation, the relative rates of polymerase binding of the particular DNA fragments.

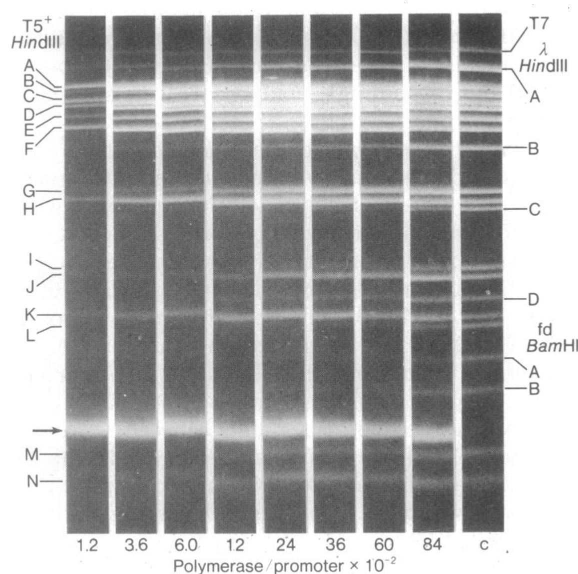

FIG. 1. Efficiency of polymerase binding by various DNAs. A DNA mixture containing T7 DNA, *Hin*dIII-fragmented T5 and λ DNA, and *Bam*HI-fragmented fd RF DNA was exposed to increasing but limiting amounts of RNA polymerase under standard assay conditions. The DNA isolated from the complexes formed was electrophoretically separated in 1% agarose gels. The positions of the various fragments are indicated at both sides of the figure. The arrow shows the single-stranded fd competitor DNA that was also used as an internal standard in the microdensitometric quantitation. The gels show, from left to right, which DNA molecules are preferentially complexed as polymerase concentration is increased in the indicated way. Lane c is the control gel containing the DNA mixture used. In quantitating these experiments, the masses of the individual fragments and their concentration in the mixture were taken into account. The early T5 fragments [A, C, F, H, K, and N (11)] compete most efficiently for the enzyme, followed by the pre-early fragments (D and E). The late T5 fragments (B, G, and J), T7 DNA λ fragment A, and fd fragment B follow next. Between these fragments and T5 fragment N is about a 10-fold difference in the rate of complex formation (Table 1).

The polymerase binding properties of plasmids pML21 and pSC101 are depicted in Fig. 2. The two DNAs were compared after dissection with *Hin*dIII and *Eco*RI, respectively. The following conclusions can be drawn. There are at least two polymerase binding sites in pML21 because both *Eco*RI fragments are retained on the filter. The DNA fragment carrying the information for kanamycin resistance binds the RNA polymerase distinctly slower than does the mini ColE1 part of the molecule (Fig. 2 *left*). When plasmid pML21 DNA was linearized, however, with a single *Hin*dIII cut, the rate of complex formation was practically determined by the binding properties of the mini ColE1 section of the molecule (data not shown).

An interesting feature was displayed by the DNA of pSC101 plasmid (Fig. 2 *middle* and *right*). The molecule bound RNA polymerase with clearly different rates depending on whether it was linearized with *Eco*RI or with *Hin*dIII. Because it is known that the *Hin*dIII site is located within the *tet* promoter (13), the observed difference in polymerase binding is due to the function of that promoter. It can be seen that after the destruction of the *tet* promoter the molecule binds RNA polymerase only with the efficiency of the mini ColE1 or PML 21 DNA, respectively.

Stability of the DNA·RNA Polymerase Complexes. As discussed above, the relative rates of complex formation between RNA polymerase and DNA can be determined by the technique used here only if the dissociation of the complexes formed is negligible within the time of the experiment. For this reason and also to learn about the range in which the stability of such complexes can vary, the half-lives of various polymerase·DNA complexes were determined. In such experiments, limiting amounts of RNA polymerase were mixed with DNA fragments in order to obtain only fast-forming complexes. After 3 min at 37°C, competitor DNA was added and the mixture was shifted to conditions accelerating complex decay (1, 10). At appropriate time intervals, aliquots were withdrawn and analyzed by filter binding and electrophoresis. For most RNA polymerase·promoter complexes, use of 200 mM KCl and 15°C ensured a dissociation within 0.1–20 hr. Half-lives of less stable complexes were monitored at 37°C after adjustment of the salt concentration to 160 mM KCl. These two conditions for complex decay were not compared in detail; however, the increase in ionic concentration to 0.2 M KCl and the lowering of the temperature to 15°C is estimated to reduce the half-life of the complexes to about 1/10th. Fig. 3 shows a decay experiment of complexes formed with pML21 DNA fragments, and the results of a number of such experiments are summarized in Table 1. The half-lives found for complexes formed with T7 and fd DNA agree well with published data (1, 9). Extreme values again were found for the complexes between RNA polymerase and some T5 sites. Even at 200 mM KCl and 15°C, *Hin*dIII fragment A has a half-life of 13 hr whereas no complex dissociation can be detected with fragment D over more than 48 hr (2).

Number of Functional Binding Sites for RNA Polymerase within Various DNA Fragments. The number of major binding sites for RNA polymerase has been reported for DNA

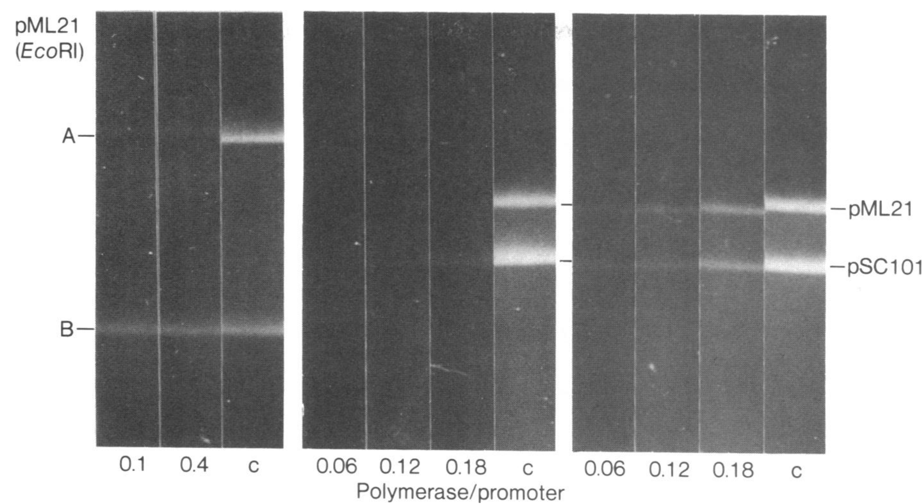

FIG. 2. Binding of RNA polymerase to linearized plasmids pML 21 and pSC101. The electrophoretic pattern was obtained as in Fig. 4. (*Left*) the mini colE1 part of the plasmid (*Eco*RI fragment B) competes more effectively (8-fold) for RNA polymerase than does *Eco*RI fragment A carrying part of the *kan* transposon. (*Middle*) If linearized by *Eco*RI, pSC101 DNA binds polymerase 2.5-fold more efficiently than does pML21 (linearized by *Hin*dIII). (*Right*) A reduction in the rate of binding is introduced by dissecting pSC101 with *Hin*dIII, so that pML21 and pSC101 now bind polymerase with about equal efficiency. *Hin*dIII is known to cut within the *tet* promoter (13). c, Control.

of phage T5, T7, and fd (1, 2, 9, 14, 15). In order to determine the sites at which the enzyme can form stable complexes and initiate RNA synthesis, initial transcripts of all DNAs under study were analyzed. These transcripts were obtained by allowing RNA polymerase to synthesize just one RNA chain of 150–300 nucleotides at each promoter. Assuming an identical rate of initiation at the various promoter sites, the amount of initial transcripts hybridizing to the proper DNA restriction fragments reflects the number of promoters per fragment. Fig. 4 shows the typical result of such an experiment, the principle of which has been described in detail (2). The promoter numbers determined in this way agree well with those obtained by different techniques (2, 15); they include both fast and slow binding sites because the complex formation takes place under conditions of excess RNA polymerase. Thus, seven to eight sites were identified in T7 DNA, which is known to contain three major and three or four minor promoters (17). The number of promoters determined for the DNAs under study here are summarized in Table 1.

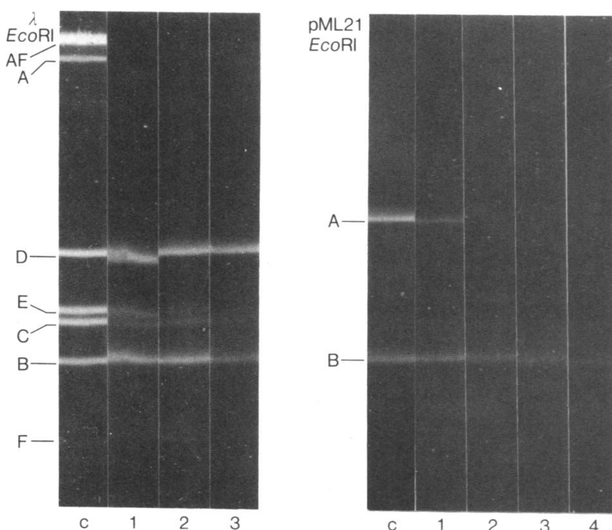

FIG. 3. Stability of RNA polymerase·DNA complexes. (*Left*) Electrophoretic pattern (1% agarose gels) of DNA isolated from complexes formed between *Eco*RI fragments of λ^+ DNA and limiting amounts of RNA polymerase. After shifting to complex-destabilizing conditions (200 mM KCl, 15°C) and addition of single-stranded competitor DNA, aliquots were taken at 0, 5, and 10 min (gels 1–3). The most stable complexes were with fragments D and C although it should be noted that C does not compete effectively for the enzyme. c, Fragment mixture used in the experiment. (*Right*) Corresponding experiment carried out with the two *Eco*RI fragments of pML21. In this case, destabilizing conditions were achieved by shifting the ion concentration to 0.160 mM KCl. Gels 1–4 depict the samples taken at 0, 30, 60, and 90 min after the shift to high-salt medium.

Rates of Complex Formation between RNA Polymerase and Individual Promoters. The rate at which a DNA fragment binds RNA polymerase depends primarily upon the contribution of the individual promoters within the fragment. If the promoter number of a fragment is known, average promoter-specific rates of polymerase binding can be derived from fragment-specific rates. Such average values, however, appear to be sensible only if DNAs with low and comparable numbers of promoters are analyzed. It also has to be kept in mind that the fastest binding site within a fragment will usually dominate the apparent rate of complex formation. We compared T5 *Hin*dIII fragments H, G, J, K, and N with intact T7 DNA, *Eco*RI and *Hin*dIII fragments of λ, and *Bam*HI fragments of fd RF DNA. All these DNA molecules carry two to four major promoters (Table 1). Experiments as shown in Fig. 1 demonstrated that fragments containing identical numbers of promoters bind RNA polymerase with greatly differing efficiencies (e.g., T5 *Hin*dIII fragments G, J, and K) and that the fragments originating from the early region of this phage compete most effectively for the enzyme (Table 1; ref. 2). Of special interest is the comparison of T5 *Hin*dIII fragment N with *Bam*HI fragment B of fd RF DNA (Fig. 5). Although this fd DNA fragment carries the four "fastest" promoters of the genome, of which two bind RNA polymerase fairly stably under our experimental conditions (1), it reacts at about 1/15th the rate of the T5 fragment which also contains only two major sites (15).

The numbers of major promoters in plasmid pML21 and pSC101 were determined by the analysis of initial transcripts (12) and by electron microscopy (D. Stüber, personal communication). Promoter-specific relative rates of polymerase binding can therefore be derived from the data given in Table 1. By comparing only DNA molecules carrying two to four major RNA polymerase binding sites, the result of our experiments (Table 1) show that the difference in the rate of poly-

Table 1. Size, promoter number, and polymerase binding properties of DNAs studied

DNA species	Fragment	Size, kb	No. of promoters	Rel. rates of complex formation	Half-life of complex, hr
	A	17.2	6–7	85	13
	B	15.2	2	25	1
	C	13.8	4	90	9
	D	12.9	3–4	55	>48
	E	11.6	3–4	50	9
T5	F	10.7	9–11	90	—
*Hin*dIII	G	6.7	2	35	1
	H	6.4	4	170	3
	J	6.5	2	—	<0.1
	K	3.9	2	260	0.3
	N	2.0	2	300	0.16
	A		2–3	—	—
	B	4.5	3–4	4.5	0.25
λ	C	5.7	1–2	—	0.5
*Eco*RI	D	7.2	4–6	7	2
	E	5.6	1	4.5	0.1
	F		1–2		—
fd	A	3.4	1	11	0.16
*Bam*HI	B	2.9	3–4	22	(intact RF)
T7	Intact	39.8	3(8)	20	?
pSC101	*Hin*dIII	8.9	3–5	2.0	0.2*
	*Eco*RI	8.9	4–6	4.7	
pML 21	Intact	11.2	3–4	2	0.5*
*Eco*RI	A	7.8	2	0.25	0.2*
	B	3.4	1–2	2	0.5*

Size of DNAs and DNA fragments [in kilobases (kb)] is based on the molecular weight of phage ϕX174 DNA (16). The promoter numbers are estimates of the major RNA polymerase binding sites. The promoters of T5 DNA were determined by several independent methods (2) including electron microscopy of *in vitro* transcriptional complexes (15). Promoters of pML21 and pSC101 were characterized correspondingly (ref. 12; D. Stüber, personal communication). The major polymerase binding sites of phage fd DNA have been characterized (1, 14). *Bam*HI fragment B of the RF DNA spans in clockwise direction from *Hpa* II fragment G to E (ref. 14; H. Schaller, personal communication). The promoters shown for λ and T7 DNA are the result of initial transcript experiments. The data obtained in this way agree well with those published previously for λ (19) and T7 (18). It should be noted, however, that only three promoters of T7 DNA react rapidly with RNA polymerase (18) and therefore determine the kinetic characteristics of the T7 DNA·polymerase complex. The fragment-specific relative rates of complex formation shown in the fifth column all are related to either *Hin*dIII fragment N of T5 DNA or to *Bam*HI fragment B of fd RF DNA (Fig. 5); they remain unchanged over a 100-fold range of DNA concentration within the assay. The numerical values were derived by arbitrarily taking the rate of the T5 N fragment as 300. The half-lives of promoter DNA complexes were obtained after exposure of the corresponding DNA to limiting amounts of RNA polymerase. Therefore, they represent the stability of the rapidly forming complexes. The dissociation was monitored in 200 mM KCl at 15°C, except that values marked* were obtained in 160 mM KCl at 37°C.

merase binding for individual promoters spans at least 2 orders of magnitudes. The fastest promoters characterized so far are located in *Hin*dIII fragment N of T5 DNA, whereas very slow reacting binding sites are found in that section of pML21 that carries the information for kanamycin resistance.

If the relative forward rate of the most rapidly reacting fragment investigated here (T5 *Hin*dIII N) is related to the second-order rate constant determined for the fd promoter

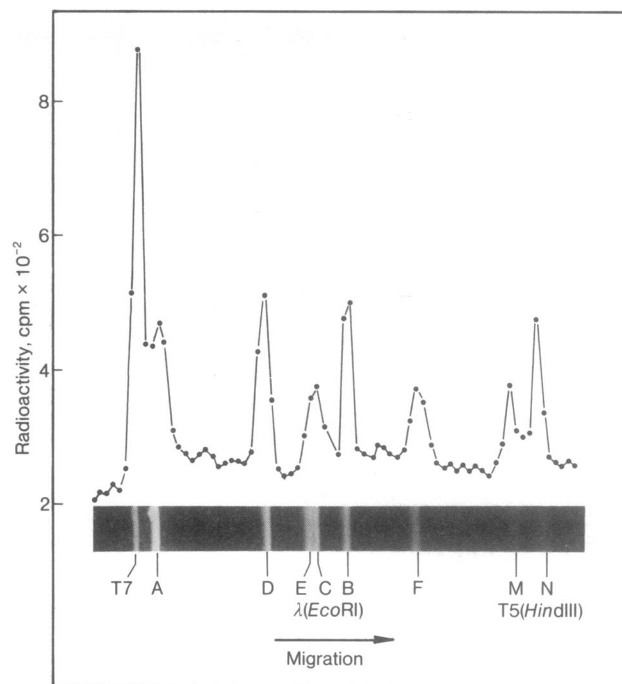

FIG. 4. Hybridization of initial transcripts to Southern blots. Lower part of figure shows the agarose gel used for the preparation of the nitrocellulose filter. The radioactivity plotted above the gel pattern reflects the [^{3}H]RNA hybridized to the different regions. In evaluating this particular experiment, T5 *Hin*dIII fragment N carrying two promoters was used as an internal standard. The following promoter numbers were determined: T7, 7–8; λ *Eco*RI fragment A, 2–3; D, 4; E+C, 2–3; B, 3–4; F, 2; T5 *Hin*dIII M, 1.

located in *Hpa* II fragment D (1), a value of $1\text{-}2 \times 10^8\ M^{-1}s^{-1}$ is found. A detailed analysis of the two promoters located in T5 *Hin*dIII fragment N, which can be separated by further dissection of the fragment (to be published elsewhere), supports this finding. Similarly, a rate of $\approx 10^6\ M^{-1}s^{-1}$ is calculated for one of the slowest reacting promoters found in *Eco*RI fragment A of pML21. This rate, however, applies to the linearized molecule, thus neglecting the effect of superhelicity on the binding of the polymerase (1).

DISCUSSION

The data reported here characterize RNA polymerase binding sites of several *E. coli* phage and plasmid DNAs. These sites react with the enzyme very efficiently and form complexes stable enough to be identified via nitrocellulose filter binding assay (i.e., half-lives of the complexes >7 min at 60 mM KCl and 37°C). In a number of systems these fast reacting sites have been shown to function as promoters. Thus, the regions of the T5 genome carrying the "fastest" promoters are transcribed most intensely *in vivo* and *in vitro* (2). Similar correlations exist for phage fd (1), and in the phage T7 system the sites reacting most rapidly with *E. coli* RNA are the major early promoters (17, 19). There are two types of polymerase binding sites that are not considered in this study despite their possible biological significance: (*i*) sites that interact rapidly with RNA polymerase but form only short-lived complexes even in the presence of nucleoside triphosphates, and (*ii*) sites that in general become occupied only at extremely high polymerase concentrations (ratio of polymerase to fast binding promoter, >10).

The conclusions to be drawn from these studies, and which relate to the sites reacting rapidly with RNA polymerase, are the following.

(*i*) The polymerase binds to promoters of various origin at

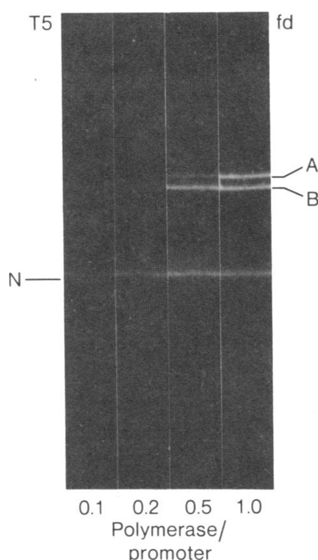

FIG. 5. Comparison of polymerase binding between *Bam*HI fragments of fd RF DNA and *Hin*dIII fragment N of T5 DNA. The experiment was carried out as described in Fig. 1 except that the electrophoretic analysis was performed in 6% acrylamide gels. Increasing polymerase concentrations are from left to right. From the difference in the appearance of *Hin*dIII fragment N of T5 DNA and fd *Bam*HI fragment B, a 15-fold difference in the relative binding of RNA polymerase was derived. *Bam*HI fragment B on the other hand bound the enzyme almost 3 times as efficiently as did fragment A. This is in agreement with the data of Seeburg *et al.* (1), according to which the fastest promoters in the fd genome are all located within *Bam*HI fragment B (see legend to Table 1).

specific rates. Consequently, the rate-limiting step in the complex formation is not diffusion controlled in the classical sense. [This is in agreement with earlier reports (1, 2).]

(*ii*) Within the systems investigated the relative rates of complex formation differ by at least 2 orders of magnitude. This indicates a wide range for transcriptional modulation because the rate of polymerase binding controls the transcriptional activity of a promoter (1, 2).

(*iii*) If the relative rates of complex formation between RNA polymerase and promoters reported here are related to the absolute rates described for the phage fd promoters (1), the range for the rates of polymerase binding lies between 10^6 and 10^8 $M^{-1}s^{-1}$.

In accepting these conclusions it has to be kept in mind that the promoter-specific rates of complex formation and decay were derived by analyzing DNA fragments containing more than one promoter and that the influence of the size differences of the various DNA molecules under study were assumed to be negligible. Thus, the promoter-specific rates of polymerase binding that can be derived from the data given in Table 1 are average values; however, they give a reliable estimate if DNA molecules such as *Hin*dIII fragments G, J, K, and N of T5, *Bam*HI fragment B of fd, and *Eco*RI fragment A of pML21 are compared with each other, all of which react with just two promoters under the conditions used.

The data presented here demonstrate a wide range in the polymerase binding properties of promoters within a selected sample of *E. coli* phage and plasmid DNAs. Up to 10-fold differences in the rate of complex formation and in complex stability were found not only within single DNA species (T5, pML 21)—as has been reported for RF DNA of phage fd (1)—but also between DNAs of different origins. In all systems investigated, the sites reacting rapidly with polymerase initiate *in vitro* RNA synthesis with high efficiency.

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