

# One-dimensional diffusion of *Escherichia coli* DNA-dependent RNA polymerase: A mechanism to facilitate promoter location

(transcription/sliding/DNA-protein interaction)

M. RICCHETTI, W. METZGER, AND H. HEUMANN\*

Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany

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**ABSTRACT** The mechanism of promoter location by DNA-dependent RNA polymerase of *Escherichia coli* was investigated. The occupancies of DNA fragments carrying the A1 promoter of bacteriophage T7 were analyzed as a function of the length of flanking sequences adjacent to the promoter. Competition between the promoters on different fragments showed qualitatively that DNA sequences downstream of the promoter enhanced promoter occupancy, whereas upstream flanking sequences had little or no influence on occupancy. This was studied quantitatively by using a set of DNA fragments with four identical A1 promoters (I–IV) equidistant from each other, but with different lengths of flanking sequences upstream from promoter I and downstream from promoter IV. The relative occupancies of these promoters showed that downstream DNA sequences of up to 250 base pairs increased the occupancy of the adjacent promoter, whereas upstream sequences longer than 70 base pairs had little or no effect on occupancy. Promoter occupancies measured as a function of the length of the downstream flanking DNA sequences were fit by a published theory that takes into account an enhancement of signal-sequence location by linear diffusion.

Location of a DNA signal sequence by facilitated diffusion (1–4) has been proposed for a number of sequence-specific binding proteins. For example, there is good evidence in the cases of *lac* repressor (5, 6), *EcoRI* restriction endonuclease (7, 8), and DNA polymerase (9) for mechanisms that compress the three-dimensional diffusion volume. Such mechanisms involve formation of a nonspecific protein–DNA complex and subsequent diffusion of the protein within or along the DNA domain until either the signal sequence is located or dissociation occurs. Translocation within the DNA domain results in a positionally uncorrelated search pattern, where the protein either dissociates from the DNA microscopically and probes adjacent sites on the DNA (“hopping”) (4), or dissociates macroscopically and rebinds to a new site.

This paper addresses two questions: (i) to what extent do one-dimensional diffusion processes participate in the location of a promoter and (ii) is this process directional? Sliding as a means of accelerating the promoter-search process by DNA-dependent RNA polymerase (EC 2.7.7.6) was proposed in 1982 by von Hippel *et al.* (10). We have designed a system that allowed us to test this idea by means of competition experiments in which the occupancies of promoters with flanking DNA sequences of different lengths were compared. Our hypothesis was that nonspecific DNA sequences serve as “antennae” (10) along which the *Escherichia coli* RNA polymerase moves to the promoter. If this antenna effect exists, the occupancy of a promoter should be influenced by the length of the flanking sequences.

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## EXPERIMENTAL PROCEDURES

**Materials.** RNA polymerase of *E. coli* was purified according to Zillig *et al.* (11). DNA fragments were purified according to Heumann *et al.* (12). The concentration of RNA polymerase was determined by amido black staining with bovine serum albumin as a standard (13, 14). The activity of the RNA polymerase holoenzyme was  $80 \pm 10\%$  as judged by gel retardation (15), abortive initiation (16), and radioactive incorporation tests (17). The concentration of the DNA was determined by UV absorption (assuming an extinction coefficient at 260 nm of 20 cm<sup>2</sup>/mg).

**Methods.** Binding of RNA polymerase (3 μM) to promoter-carrying DNA fragments (3 μM) was assayed in 50 mM NaCl/6 mM MgCl<sub>2</sub>/8 mM Tris-HCl, pH 7.9/1 mM 2-mercaptoethanol, in a final volume of 20 μl. The mixture was incubated for 10 min at 37°C. Heparin was added to a concentration of 0.05 mg/ml. RNA synthesis was started adding ATP, UTP, GTP, and CTP (1 mM each). The enzyme/DNA ratio was <1. Run-off transcription was allowed to proceed for 10 min at 37°C, and the transcripts were analyzed by electrophoresis in an RNA sequencing gel (18). The radioactivity of the [ $\alpha$ -<sup>32</sup>P]UMP-labeled RNA was determined by scanning of autoradiograms or by cutting out the bands for scintillation counting. The two procedures gave comparable results when standard RNA fragments were used as reference. By determining the number of incorporated UMP molecules in each transcript synthesized by RNA polymerase starting from the various promoters, the occupancy of each promoter was calculated. The *Bam*HI–*Bam*HI fragment showed an inhomogeneity of the run-off transcripts, giving three products differing by about 5 bases. In this case, the radioactivity of all three products was added up.

**Theory.** The ratio of the occupancies ( $O_1/O_2$ ) of two competing promoters 1 and 2 with lengths  $L_1$  and  $L_2$  of the downstream flanking sequences (counted from the +1 position) can be expressed by Eq. 1 with the assumption that  $k_{on}^{sp}$ , the specific on-rate, is proportional to  $O$ . This assumption is discussed later. By using for  $k_{on}^{sp}$  an expression that regards rate enhancement by one-dimensional diffusion as described elsewhere (2, 4), the relative occupancies of promoters 1 and 2 may be given as

$$O_1/O_2 = [\ln(2L_2/b)/\ln(2L_1/b)][\tanh(L_1/l)/\tanh(L_2/l)], \quad [1]$$

where  $b$  is the interaction parameter of the RNA polymerase and the DNA and  $l$  is the stretch on the DNA that is used for sliding (1, 19).

$$l \approx (D_1/k_d^{nsp})^{1/2} \\ = \{[D_1 \ln(2L/b)][1000K^{nsp}/2\pi(D_P + D_D)sN]\}^{1/2}, \quad [2]$$

\*To whom reprint requests should be addressed.

where  $D_P$  and  $D_D$  are the three-dimensional diffusion coefficients of RNA polymerase and DNA, respectively,  $D_1$  is the one-dimensional diffusion coefficient of RNA polymerase,  $k_d^{nsp}$  is the nonspecific dissociation rate constant,  $K^{nsp}$  is the nonspecific binding constant,  $s$  is the distance between adjacent base pairs ( $s = 0.34$  nm), and  $N$  is Avogadro's number.

**RESULTS**

Each experiment was performed in two steps. First, A1 promoters (of the *E. coli* phage T7) with various lengths of flanking sequences were allowed to compete for RNA polymerase. Second, NTPs were added to produce run-off transcripts in order to determine the occupancies of the competing promoters.

To test our hypothesis that nonspecific DNA sequences flanking a promoter enhanced promoter occupancy due to one-dimensional diffusion of RNA polymerase along the DNA, we used conditions in which this effect was reaction-determining. These conditions were as follows. (i) The length  $L$  of the DNA fragments was  $< l$ , where  $l$  is the stretch of possible one-dimensional diffusion. Under these conditions the effective sliding stretch is limited to the contour length  $L$  of the DNA fragment. The length  $L$  was varied between 60 base pairs (bp) and 300 bp downstream of the initiation point and between 79 bp and 440 bp upstream. (ii) An enzyme/promoter ratio  $< 1$  was used, because effects due to competition disappear when the enzyme/promoter ratio approximates 1. (iii) The time of incubation of RNA polymerase with DNA was kept short (10 min) compared to the several-day half-life of the specific complex (20). Under these conditions the occupancy of a promoter is proportional to the apparent on-rate.

To quantify the occupancy of each of the promoters, run-off transcription was allowed to proceed. The size of the products allowed us to determine the fragment from which initiation took place. The amount of radioactivity incorporated in the products indicated the number of RNA chains initiated at each of the promoters. Reinitiation was suppressed by addition of heparin.

Fig. 1 shows the DNA fragments that were used for the experiments. The specificity of interaction of RNA polymerase with the A1 promoter was previously shown by gel retardation (15). Fig. 2 shows the run-off transcripts obtained with the various fragments, and Fig. 3 displays their relative occupancies calculated from the  $^{32}P$ -labeled NMPs incorporated. The influence of the upstream and the downstream flanking sequence on the occupancies of the promoters was studied in two separate experiments. Fig. 2A shows the transcripts from fragments that differ in the length of their downstream flanking sequences: 60 bp for the *Bam*HI-*Bam*HI fragment and 332 bp for the *Ava*I-*Sst*I fragment. The

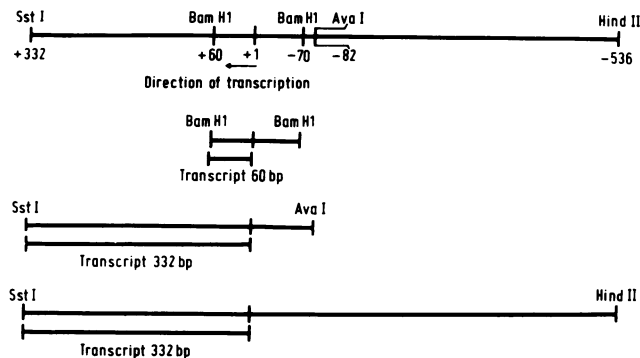


FIG. 1. Restriction maps of the DNA fragments with a single A1 promoter of the phage T7.

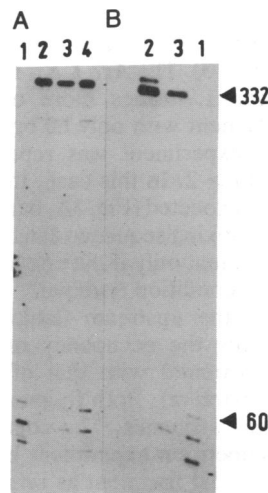


FIG. 2. Electrophoretic analysis of the run-off transcripts obtained from the DNA fragments shown in Fig. 1. (A) The products obtained from the *Bam*HI-*Bam*HI and *Ava*I-*Sst*I fragments, indicating the influence of the downstream flanking sequence. Lanes: 1, *Bam*HI-*Bam*HI fragment; 2, *Ava*I-*Sst*I fragment; 3, equimolar mixture of the two fragments; 4, as in lane 3 but with an enzyme/promoter ratio  $> 2$ . (B) Run-off products obtained from the *Bam*HI-*Bam*HI fragment and the *Hind*II-*Sst*I fragment. Lanes: 1, *Bam*HI-*Bam*HI fragment; 2, *Hind*II-*Sst*I fragment; 3, equimolar mixture of the two fragments. Arrowheads indicate positions of 332- and 60-bp-long transcripts.

two fragments have almost the same short upstream flanking sequence. For Fig. 2A, lanes 1-3, equimolar amounts of DNA fragments were used, and the polymerase/DNA molar ratio was 0.75. Lanes 1 and 2 display the products from the two DNA fragments in separate transcription assays. This control experiment shows that the  $^{32}P$  incorporated in each transcript divided by the known number of radioactively labeled nucleotides is a measure of the number of initiated polymerase molecules. Fig. 3 shows that this number is equal, as expected, since the same amount of enzyme was used in the

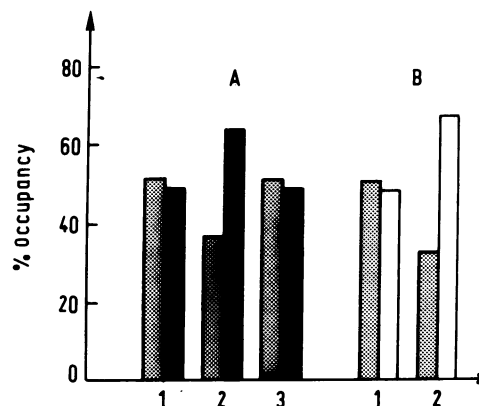


FIG. 3. Promoter occupancies calculated from the incorporated radioactivity of the products shown in Fig. 2. (A) Comparison of the occupancy of the *Bam*HI-*Bam*HI fragment (stippled bars) with that of the *Ava*I-*Sst*I fragment (solid bars) for the isolated fragments (bars 1; calculated from Fig. 2A, lanes 1 and 2); for the two fragments in a competition experiment (bars 2; calculated from Fig. 2A, lane 3), and for the two fragments in a competition experiment with an enzyme/DNA ratio  $> 2$  (bars 3; calculated from Fig. 2A, lane 4). (B) Comparison of the occupancy of the *Bam*HI-*Bam*HI fragment with that of the *Hind*II-*Sst*I fragment (open bars) for the isolated fragments (bars 1; calculated from Fig. 2B, lanes 1 and 2) and for the two fragments in a competition experiment (bars 2; calculated from Fig. 2B, lane 3).

two experiments. In the competition experiment, the fragment with the longer downstream flanking sequence had higher occupancy (Fig. 3). The *Ava* I–*Sst* I fragment with 332 bp downstream was 1.7 times more occupied than the *Bam*HI–*Bam*HI fragment with only 60 bp downstream.

The competition experiment was repeated with an enzyme/promoter ratio  $> 2$ . In this case, the promoters were equally occupied, as expected (Fig. 3A, bars 3). The influence of the downstream flanking sequence can be detected in such a competition experiment only if the enzyme/promoter ratio is  $< 1$ , according to condition *ii* above.

The influence of the upstream flanking sequence was studied by comparing the occupancy of the *Ava* I–*Sst* I fragment (82 bp upstream) with that of the *Hind*II–*Sst* I fragment (536 bp upstream). Both fragments have the same downstream flanking sequence. The comparison was done not by a direct competition experiment but by competition with the *Bam*HI–*Bam*HI fragment as reference. The experimental conditions were the same as for the previous experiment. Quantification of the run-off transcripts (Fig. 2B, lane 3) showed that the long fragment had twice the occupancy of the short one (Fig. 3B, bars 2). By using the *Bam*HI–*Bam*HI fragment as reference, the occupancies of the *Ava* I–*Sst* I and *Hind*II–*Sst* I fragments with short and long upstream flanking sequences could be compared (Fig. 3A, bars 2, and Fig. 3B, bars 2). This comparison indicates that the upstream flanking sequence has little or no influence on the promoter occupancy.

The result of these two sets of experiments encouraged us to study in a more quantitative way the influence of the flanking sequences. The quantification is more significant when the competing promoters are on the same fragment. Therefore, we constructed DNA fragments with four A1 promoters equally spaced and oriented (12). They were numbered I–IV with respect to the direction of transcription (Fig. 4). The upstream and downstream sequences of promoters I and IV, respectively, were varied. The flanking sequences of the middle promoters were left unchanged. Competition took place between these four promoters on a single DNA fragment. The advantages of this system are the following. (a) The need to apply in the competition experiment exactly determined amounts of promoters is fulfilled. (b) The promoters II and III have the same flanking sequences and can therefore serve as standards to normalize the occupancies measured on other fragments with different flanking sequences of promoters I and IV. (c) Corrections due to differences in the three-dimensional diffusion constants of DNA fragments of different sizes are avoided (see Eq. 1 and *Discussion*).

The quantification of the occupancies was performed in the same way as for the fragments carrying a single promoter. The only difference was that the run-off transcripts were synthesized from a single fragment in each experiment. The downstream promoter sequences, which had to be tran-

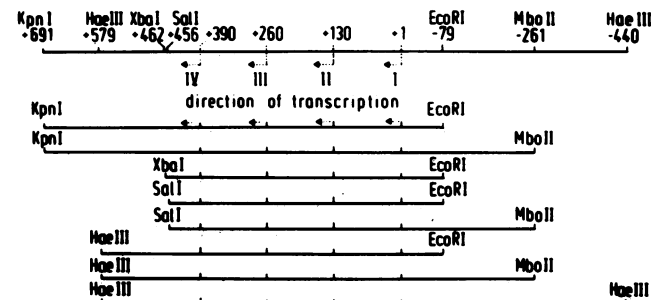


FIG. 4. Restriction maps of the DNA fragments carrying four promoters.

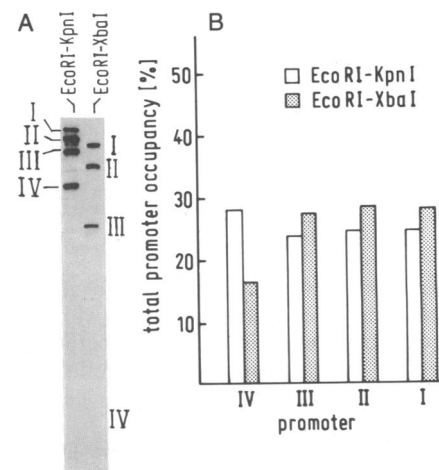


FIG. 5. Influence of the length of the downstream region on the occupancy of the adjacent promoter. (A) Electrophoretic analysis of the RNA products synthesized from the *Eco*RI–*Kpn* I fragment and the *Eco*RI–*Xba* I fragment. (B) Histogram of the occupancies of the four promoters as calculated from the incorporated radioactivity in each product synthesized by polymerase molecules starting from the four different promoters.

scribed, had no termination effect, as shown by product analysis after electrophoresis (Figs. 5A and 6A).

As in the experiments with fragments carrying a single promoter, two sets of experiments were performed, one to study the influence of the downstream flanking sequences on the occupancy of the promoter IV (Fig. 5), and another to study the influence of the upstream flanking sequences on the occupancy of promoter I (Fig. 6). Fig. 5A shows the run-off transcripts from the tetrameric promoter arrangement, one with a long downstream flanking sequence (*Eco*RI–*Kpn* I fragment), and one with a short downstream flanking sequence of promoter IV (*Eco*RI–*Xba* I fragment).

The downstream flanking sequences clearly enhanced promoter occupancy (Fig. 5B): promoter IV of the *Eco*RI–*Xba* I fragment with 72 bp downstream showed a 35% lower occupancy than promoter II or III. In the *Eco*RI–*Kpn* I fragment the ratio was reversed. Promoter IV with 300 bp downstream had a 13% higher occupancy than promoter III or II. The most striking stimulation was demonstrated by comparing the occupancies of the promoters IV of the two

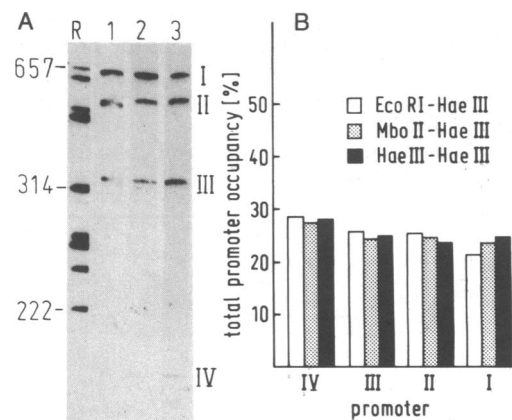


FIG. 6. Influence of the length of the upstream region on the occupancy of the adjacent promoter. (A) Electrophoretic analysis of the RNA products of the *Eco*RI–*Hae* III (lane 1), *Hae* III–*Hae* III (lane 2), and *Mbo* II–*Hae* III (lane 3) fragments. Lane R: reference (size markers). (B) Histogram of the occupancies of the promoters with different lengths of upstream sequence.

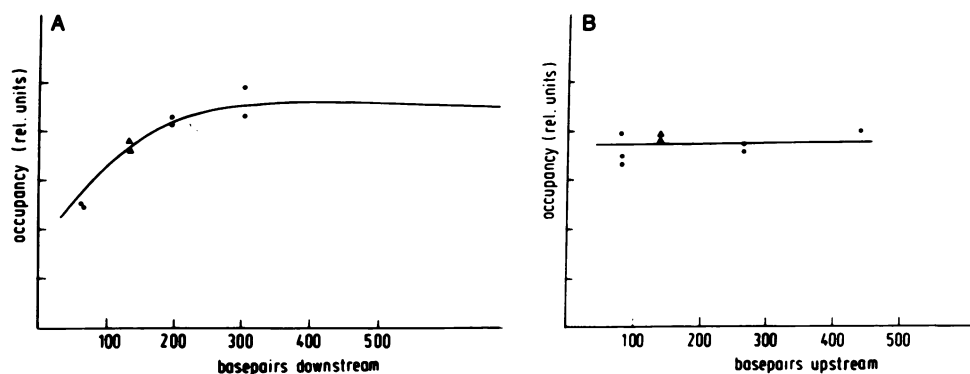


FIG. 7. Compilation of the occupancies (relative units) of promoters of all fragments. The occupancies of promoters II and III ( $\blacktriangle$ ) were used to normalize the other data. (A) Occupancies ( $\bullet$ ) of promoter IV with various lengths of downstream sequence when the length of the upstream region is kept constant. Curve shows occupancy calculated from Eq. 1. (B) Occupancies as a function of the length of the upstream sequence when the downstream sequence is kept constant.

fragments with 300 bp (*EcoRI-Kpn I*) and 72 bp (*EcoRI-Xba I*). The occupancies of promoter IV of these two fragments differ by 49%, when the promoters II and III are used as standards.

The influence of the upstream flanking sequences was studied by the same procedure, with the length of the flanking sequence upstream of promoter I varied between 79 and 440 bp (Fig. 4). From the transcription products of the fragments (*EcoRI-Hae III*, *Mbo II-Hae III*, and *Hae III-Hae III*) shown in Fig. 6A, the histogram in Fig. 6B was evaluated. The data show that there was little or no stimulation by upstream flanking sequences, in contrast to the effect of the downstream flanking sequences up to 250 bp. Fig. 7 summarizes the data on the occupancies of promoter IV and promoter I determined as a function of the length of the up- and downstream flanking sequence, with the promoters II and III as references. Fig. 7 includes additional data from fragments shown in Fig. 4.

The data in Fig. 7A were used to evaluate the one-dimensional diffusion coefficient  $D_1$  by fitting Eq. 1, which expresses the rate of promoter binding by assuming enlargement of the promoter sink due to one-dimensional diffusion of RNA polymerase along the DNA only from downstream. The following parameters were used:  $D_p = 3.1 \times 10^{-7}$  cm<sup>2</sup>/s;  $b = 5$  nm (24);  $K^{nsp} = 1 \times 10^{-6}$  M (27).  $D_p$  was obtained from the Svedberg equation by using a partial specific volume  $\bar{v} = 0.73$  cm<sup>3</sup>/g (21),  $s = 15$  S (22), and a molecular weight of 448,000 for RNA polymerase.  $D_1$  was estimated to be  $1.3 \times 10^{-9}$  cm<sup>2</sup>/s.

## DISCUSSION

There is at present no direct method available to prove rate enhancement in the location of a signal sequence by one-dimensional diffusion along the DNA. To elucidate the mechanism of the promoter-search process, an approach originally proposed by Richter and Eigen (1) was used to demonstrate the ability of the *lac* repressor to diffuse along the DNA. They proposed to measure the specific on-rates in relation to the length of the sequences flanking the operator. We compared occupancies of promoters that had flanking sequences of different lengths. The occupancy is proportional to the apparent specific on-rate, if the incubation time during which the polymerase is allowed to search for the promoter is short compared to the half-life of the specific complex. This condition is fulfilled in our system by using the A1 promoter. Additionally, no changes of the distribution of RNA products were observed when the incubation time was varied between 5 and 20 min. This assures us that we are dealing with a system in which the promoter binding can be

considered as irreversible on the time scale of the experiment. When ratios of occupancies are measured, it is not necessary to know anything about steric and electrostatic factors and the factors influencing competition by nonspecific binding. In the first competition experiment, we used DNA fragments carrying a single promoter. The results showed qualitatively that downstream flanking sequences enhance promoter location, whereas upstream flanking sequences contribute little or nothing. The use of a tetrameric promoter arrangement increased the significance of our data, especially since the occupancies of the middle promoters could be used to normalize the occupancies of promoters I and IV on the different fragments. The error of the occupancies is 2% when related to the occupancies of all four promoters within a fragment (Figs. 5B and 6B) and 10% when two promoters on different fragments are compared (Fig. 7). The use of fragments with the tetrameric promoter arrangement avoids the necessity to correct for the differences in the three-dimensional diffusion constants of DNA fragments of different sizes. The increase of the three-dimensional diffusion coefficient of a smaller DNA fragment counteracts the enhancement of signal-sequence location in larger DNA fragments. For a large protein like RNA polymerase from *E. coli*, which has a three-dimensional diffusion coefficient of  $3.1 \times 10^{-7}$  cm<sup>2</sup>/s, a 20% increase in the specific on-rate is expected when a 100-bp fragment is compared with an 800-bp fragment.

The use of fragments carrying four promoters instead of the fragments carrying one promoter could have been a disadvantage if "sliding" through a promoter sequence occurred. This would obscure effects due to one-dimensional diffusion, since changes in the length of flanking sequence downstream of promoter IV would also influence the occupancy of the promoters III, II, and I in descending series. We did not find a dependence of the occupancies of promoters III and II on the length of flanking sequences of promoter IV. Additionally, the occupancies obtained with the fragments carrying a single promoter fit within experimental error the data obtained with the tetrameric construct (compare Fig. 5B and Fig. 3B, bars 2), indicating that sliding through promoters does not play a major role.

The experimental data in Fig. 7A are in reasonable agreement with a theoretical model that assumes an enlargement of the promoter sink according to Eq. 2 to  $l \approx 85$  nm due to one-dimensional diffusion of the polymerase. This agreement supports the linear-diffusion model, at least for the downstream region. The main uncertainty of the model concerns the value of the one-dimensional diffusion coefficient ( $D_1$ ). This parameter ( $D_1 = 1.3 \times 10^{-9}$  cm<sup>2</sup>/s) was obtained by fitting the experimental points. For comparison,  $D_1$  was

calculated by the hydrodynamic approach of Schurr (23), with the assumption that RNA polymerase moves along the groove of the DNA, where a rotation of the protein around the DNA axis is superimposed by the linear motion along the DNA. This approach has been used for the description of the linear diffusion of the *lac* repressor. The critical parameter is the distance  $b$  between the center of gravity of the protein and the DNA axis. From neutron-scattering studies,  $b$  was determined to be 5 nm in specific complex (24). This value should be a lower estimate for the radial distance of the polymerase from the DNA axis in the nonspecific complex if one assumes that the polymerase moves on an equipotential surface on the DNA (6). With  $b = 5$  nm, the one-dimensional diffusion coefficient is  $D_1 = 2.6 \times 10^{-9}$  cm<sup>2</sup>/s. This is a theoretical upper limit of  $D_1$ . The experimentally determined value of  $D_1$  is lower by a factor of 2, indicating that the pure hydrodynamic approach is not sufficient. There must be additional frictional forces between protein and DNA to explain this difference.

The finding that promoter occupancy was affected only by the downstream flanking region was unexpected and led us to question whether this apparent biased linear diffusion is an intrinsic effect of the polymerase-promoter interaction or is due to trapping of the polymerase at a sequence upstream of promoter I. If such tight binding sites (25, 26) exist in our fragments, they should be restricted to the region  $-1$  to  $-70$ . The experiments with the single-promoter fragments show qualitatively the same effect—i.e., no dependence of the promoter occupancy on the length of upstream flanking sequence—as the experiments with fragments carrying four promoters. Both systems have the same upstream region up to  $-70$  bp but differ further upstream, since the monomeric and the tetrameric arrangements were obtained from different plasmids. It is unlikely that tight binding sites upstream of the  $-70$  region are present on both fragments.

Trapping of RNA polymerase within the  $-1$  to  $-70$  region would have been detectable by gel retardation. Our previous studies (15) of DNA fragment carrying a single A1 promoter showed that RNA polymerase binds specifically at the promoter when the enzyme/promoter ratio is  $< 1$ . Therefore, the trivial explanation of the apparent one-way association by trapping of the polymerase upstream of the promoter is rather unlikely.

We do not know the mechanism of the apparent one-way association. However, we can rule out some trivial explanations and speculate about the mechanism. RNA polymerase (from *E. coli*) and the promoter are asymmetric structures. Only those molecules that are bound in the proper orientation with respect to the direction of transcription can successfully initiate transcription. This is one selection criterion. Another one has to take into account that only those polymerase molecules that approach the promoter from downstream are successful. Since a directionality of the linear diffusion is not possible, additional effects have to be taken into account. A potential barrier, working on the level of the DNA or the protein, might explain our findings. The potential barrier

could be caused by a set of base pairs upstream of the promoter that prevents the polymerase from sliding through. Such a mechanism would not conflict with the principle of detailed balance, if the time of the experiment is short compared to the half-life of the specific complex. Further studies are necessary to determine the mechanism of this biased linear diffusion.

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