Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 737–741, February 1975

Nucleotide Sequence of an RNA Polymerase Binding Site from the DNA of Bacteriophage fd

(promoters/DNA sequencing/protein–DNA interaction)

HEINZ SCHALLER, CHRISTOPHER GRAY, AND KARIN HERRMANN

Max-Planck-Institut für Virusforschung, Tübingen and Lehrstuhl für Mikrobiologie der Universität Heidelberg, 6900 Heidelberg, Im Neuenheimer Feld 280, West Germany

Communicated by H. Gobind Khorana, December 6, 1974

ABSTRACT **The primary structure of a strong RNA polymerase binding site in the replicative form DNA of phage fd has been determined by direct DNA sequencing. It is:**

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5' T-G-C-T-T-C-T-G-A-C-T-A-T-A-A-T-A-G-A-C-A-G-G-G-T-A-A-
3' A-C-G-A-A-G-A-C-T-G-A-T-A-T-T-A-T-C-T-G-T-C-C-C-A-T-T-
   A-G-A-C-C-T-G-A-T-T-T-T-T-G-A 3'
   T-C-T-G-G-A-C-T-A-A-A         5'
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The molecule contains regions with 2-fold symmetry and sequence homologies to promoter regions from other DNAs. The startpoint of transcription is located in the center of the binding site.

Promoters contain, as an essential part, a site for the oriented and stable attachment of the enzyme RNA polymerase, thus ensuring the strand- and site-specific initiation of the mRNA chain. RNA polymerase binding sites can be isolated as DNA fragments protected by the polymerase against digestion with nuclease (1). From fdRF, the double-stranded replicative form DNA of bacteriophage fd, a single binding site has been characterized as a homogeneous DNA segment of about 40 nucleotide pairs (2). This promoter DNA fragment, pDNA-I, contains all the information necessary for maintaining the enzyme in the stable promoter complex (3), and for the specific initiation of transcription (4). In this paper we present its complete nucleotide sequence.

METHODS

pDNA was isolated as described (2). Pure pDNA-I was obtained by including into the isolation procedure an incubation with GTP, which results in a selective stabilization of the promoter polymerase–I complex by synthesis of an oligo(G) chain (5). The conditions for hybridizations and nucleotide incorporation reactions have been described (6).

5'-Terminal pDNA Fragments. Digestion with *Escherichia coli* exonuclease III [100 units/ml (25)] was carried out for 10 min at 40° in 10 mM Tris·HCl, pH 8.0, 0.3 mM $MgCl_2$, and 0.1 mM dithiothreitol. Alternatively fragments were obtained by the combined action of spleen endonuclease (a kind gift from Dr. G. Bernardi) and spleen phosphodiesterase (7). pDNA and calf-thymus DNA carrier (10 μg/ml) were treated with the endonuclease (10 μg/ml) in 50 mM ammonium acetate, pH 5.0, 0.1 mM EDTA for 10 min at 40°, and 5 min at 70°. Incubation was continued for 20 min at 40° in the presence of spleen phosphodiesterase at 20 μg/ml. Reaction mixtures were desalted on small columns of Sephadex G-50 (2 ml) in 10 mM ammonium carbonate, pH 8.6, and concentrated by lyophylization.

3'-Terminal pDNA Fragments. Digestion with lambda exonuclease (13) was carried out for 10 min at 40° in 50 mM glycine–KOH, pH 9.5, 2 mM $MgCl_2$ in the presence of 100 units/ml of enzyme.

Two-Dimensional Separations. Thin-layer chromatography on polyethyleneimine (PEI)-cellulose was employed. For 5'-terminal pDNA fragments the solvents used in the first dimension were 1.4 and 1.8 M lithium formate containing 7 M urea, pH 3.5. In the second dimension 0.7 M LiCl, 20 mM Tris·HCl, pH 8.0, 7 M urea was used (11). For pyrimidine tracts 2 M pyridine formate, pH 3.5, and 0.85 M LiCl were used (2,12). If only a few pyrimidine tracts were present, a mixture of [^{14}C]dT-labeled pyrimidine tracts was used as carrier to provide a background pattern of the thymine-containing pyrimidine tracts.

Gel Filtration. Columns of Sephadex G-50 or G-75 (1 cm^2, 50 cm) equilibrated in 20 mM ammonium carbonate, pH 8.6, were standardized using [5'-^{32}P]oligo(dT) with chain-lengths of 6, 10, and 13 as reference compounds (10). Samples were applied in 0.05–0.5 ml, and 0.5 or 1 ml of fractions were collected and assayed for radioactivity. In analytical runs [^{3}H]-poly(dA-dT) and tritiated H_2O were included as standards.

Pyrimidine Tracts. ^{32}P-Labeled pDNA and 10 μg of calf-thymus DNA were incubated with 30 μl of 66% formic acid and 2% diphenylamine for 15 hr at 30°, or for 10 hr at 37° (8). Samples were processed essentially as described in ref. 9.

Partial Micrococcal Nuclease digestion was carried out for 10 min at 37° in 40 mM Tris, pH 9.0, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ in the presence of 20 μg/ml of enzyme and 50 μg/ml of calf-thymus DNA as carrier.

Nucleotide Analysis. Terminal phosphate was removed from oligopyrimidines by treatment with alkaline phosphatase (50 μg/ml) for 30 min at 40° in 50 mM Tris·HCl, pH 8.5, 5 mM $MgCl_2$. 3'-Mononucleotides were obtained by digestion for 60 min at 37° with spleen phosphodiesterase (Worthington, 50 μg/ml) in 50 mM ammonium acetate, pH 5.0, and 1 mM EDTA, and 5'-mononucleotides by digestion with venom phosphodiesterase (Worthington, 10 μg/ml) for 10 min at 37° in 10 mM Tris, pH 9, 2 mM MgCl and 0.1 mM 5'-dTMP. Nucleotides were separated by 2-step chroma-

Abbreviations: RF, replicative form DNA; pDNA-I, RNA polymerase binding site (polymerase-protected DNA) from fd promoter-I.

TABLE 1. *Sequences and yields of pyrimidine tracts from pDNA-I template (minus) strand and 5'-terminal fragments*

	Chainlength (n)	38	38	38	38	26	23	21	19	15	9	4
Pyrimidine tract	Labeled nucleotide	T(C)	T, C	G	A(C)				A (C)			
pT-C-T-T-Tp (A)		1.1	1.0	—	1.0	1.0	1.0	1.0	1.0	1.0	—	—
pC-C-C-Tp (G)		1.1	1.0	1.0	(0.8)	(0.8)	(0.7)	(0.8)	—	—	—	—
pT-C-Tp (A)		1.0	0.9	—	0.9	0.7	0.4	—	—	—	—	—
pT-Tp (A)		1.0	0.9	—	0.9	0.7	0.1	—	—	—	—	—
pT-Cp (A)		2.1	1.8	—	2.2	1.1	1.2	1.1	1.4	1.0	1.0	—
pTp (A)		1.0	0.9	—	1.1	0.2	—	—	—	—	—	—
pCp (A)		(0.7)	0.8	—	(0.7)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
P_i		—	—	5.1	4.6	4.2	5.1	4.5	4.2	4.1	4.9	1

fd RF DNA was labeled with α-^{32}P nucleotides (and with [^{3}H]dCMP) in its minus strand, and pDNA-I and 5'-terminal fragments were isolated. The material was analyzed for its constituting pyrimidine tracts (see Fig. 2 and *Methods*). The figures listed give the relative molar yields obtained. Figures in parentheses were based on [^{3}H]dCMP-labeled material. The nearest neighbor base is given in parentheses after the pyrimidine tract.

tography on PEI-cellulose (11) using 1 M acetic acid and 0.85 M LiCl as solvents.

RESULTS

pDNA-I is a double-stranded DNA molecule of about 40 base pairs. It is 66% A+T and has a melting temperature, T_m, in 0.15 M NaCl of 68° (2, 5). Partial information on its primary structure had been obtained by pyrimidine tract analysis, which indicated that pDNA-I contained eight to nine pyrimidine tracts per DNA strand (2, 5). To obtain the complete nucleotide sequence, the different pyrimidine tracts of each strand were sequenced and then ordered relative to each other in both strands.

Pyrimidine Tract Analysis. Analysis of pyrimidine tracts was facilitated by starting from *in vitro* fd RF DNA that carried label in only one strand (2). The oligopyrimidines obtained after depurination (7) were separated and identified by two-dimensional thin-layer chromatography (2, 7). Nucleotide sequences of short tracts were determined by analyzing for the constituting 3'- and 5'-mononucleotides (see *Methods*). Longer tracts were sequenced by partial endonucleolytic cleavage with micrococcal nuclease, which yielded mixtures of Y_np_n (Y = unspecified pyrimidine nucleoside) and of 5'-terminal Y_np_{n+1} fragments. The latter were resolved and identified as described above. In a typical experiment the oligonucleotide $T_3C_2p_6$ gave rise to the series of Cp_2, CTp_3, CT_2p_4, $C_2T_2p_5$, $C_2T_3p_6$, from which the sequence was deduced to be C-T-T-C-T. In addition to the pyrimidine sequences, purine nucleotides next to the 3'-terminus of pyrimidine tracts were determined by label transfer experiments. The results of pyrimidine tract analysis are summarized in the left-hand sections of Tables 1 and 2.

Sequential Arrangement of Pyrimidine Tracts. 5'-Terminal fragments of the pDNA strands were prepared by the combined action of spleen endo- and exonuclease (7) or by partial degradation of pDNA-I with *E. coli* exonuclease III. The resulting mixture of DNA chains of various lengths but identical 5'-ends were separated according to size and base composition in a two-dimensional thin-layer system (11). As shown in Fig. 1, chains containing up to 25 nucleotides could be resolved into individual spots. For longer chains a partial separation according to size was achieved by gel filtration on Sephadex G-75 (10). Spots from plates or fractions from gel filtration were analyzed for polypyrimidine tracts. In addition the material from each spot was also analyzed for its approximate chainlength by gel filtration on standardized Sephadex columns.

Results obtained for the pDNA-I minus strand are shown in Fig. 2 and Table 1, for the plus strand in Fig. 3 and Table 2. They show that with a decrease in chain-length, there are correspondingly fewer oligopyrimidine spots in the fingerprints. As a consequence, the order of most of the pyrimidine tracts can be deduced directly:

plus strand 5'—(T)—T_3C_2—CT—T—T—C—T—TC_2—T_5—3'
minus strand 5'—TC—T_4C—TC_3—T_2C—T_2—(T—TC—C)—3'.

Uncertainties remained with respect to the order of the short pyrimidine nucleotides in the 3'-terminal section of the minus

TABLE 2. *Sequences and yields of pyrimidine tracts from pDNA-I complementary (plus) strand and from 5'-terminal fragments*

	Chainlength (nucleotides)									
Pyrimidine tract	42	42	42	$\overline{32}$	$\overline{25}$	$\overline{20}$	18	14	12	8
pT-T-T-T-Tp (G)	1.0	1.0	0.9	0.3	—	—	—	—	—	—
pC-T-T-C-Tp	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pC-C-Tp	1.0	0.9	0.8	0.7	0.2	—	—	—	—	—
pC-Tp	1.0	1.1	1.1	1.0	1.2	1.2	1.2	0.9	1.2	—
pTp	4.6	4.0	4.1	3.2	2.8	2.2	2.4	1.0	—	—
pCp	1.1	1.1	1.2	1.2	1.1	0.8	—	—	—	—
P_i		14.2	12.7	11	10	4.8	3.5	1.0	1.4	0.7

pDNA-I labeled uniformly with ^{32}P in its plus strand was analyzed as described for the minus strand (see Table 1). Most of the pyrimidine fingerprints are shown in Fig. 3.

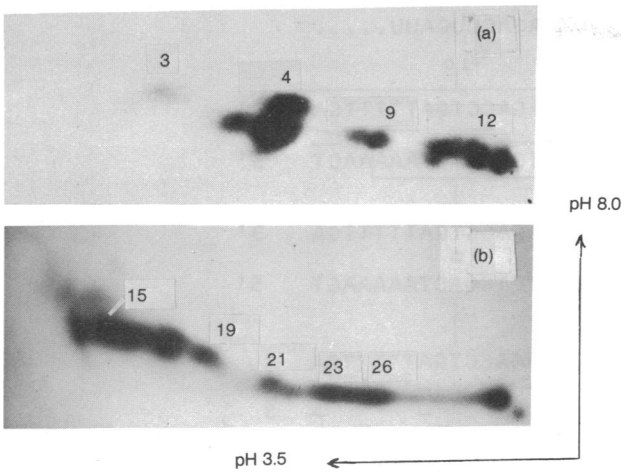


FIG. 1. Separation of 5′-terminal pDNA fragments. pDNA-I labeled with [^{32}P]dAMP and [^{3}H]dCMP in the minus strand was partially degraded with spleen endo- and exonuclease. The resulting fragments were separated into "long chains" ($n > 10$) and "short chains" ($n < 15$) by gel filtration on Sephadex G-50 and then separated further by thin-layer chromatography. The approximate chainlengths are indicated on the figures. (a) Short chains without phosphatase treatment; (b) long chains pretreated with phosphatase. For details see *Methods*.

strand (pyrimidine tracts in parentheses). This was due to the fact that the respective pDNA chains of about 25 to 40 nucleotide residues could not be resolved sufficiently well by gel filtration. The correct order was established by end group analysis (see below) and by 5′→3′ exonucleolytic degradation of pDNA with lambda exonuclease (13), which showed that the nucleotides pCp and pT-Cp were present in equimolar amounts in a series of 3′-terminal oligonucleotides. Endgroup analysis also indicated the presence of a pTp nucleotide at the 5′-terminus of the plus strand (see below), which was lost in the experiment described above during treatment with phosphatase prior to chromatography.

Terminal Nucleotide Sequences. 5′-Terminal nucleotides were identified after labeling with T_4 polynucleotide kinase (14) and were found to be pA (about 95%) for the minus strand, and pT (83%) and pG (15%) for the plus strand. Pyrimidine tract analysis of the terminally labeled product gave rise to pTp as the only labeled nucleotide, indicating a pT-R terminus for the plus strand (R = unspecified purine nucleotide). Further information on the 5′-terminal sequence was obtained by base analysis of short oligonucleotides. Thus, a trinucleotide from the plus strand gave equimolar amounts of pG and pC, and Tp and Gp, indicating a pT-G-C terminus for the plus strand. Similar experiments with short oligonucleotides from the minus strand (spots 3 and 4 from Fig. 1) indicated a 5′-terminal oligo(dA) sequence of at least three nucleotides.

Nucleotide sequences at the 3′-termini were selectively labeled using repair synthesis of double-stranded pDNA with *E. coli* DNA polymerase I. After limited reaction with a single nucleoside [α-^{32}P]triphosphate, the DNA strands were

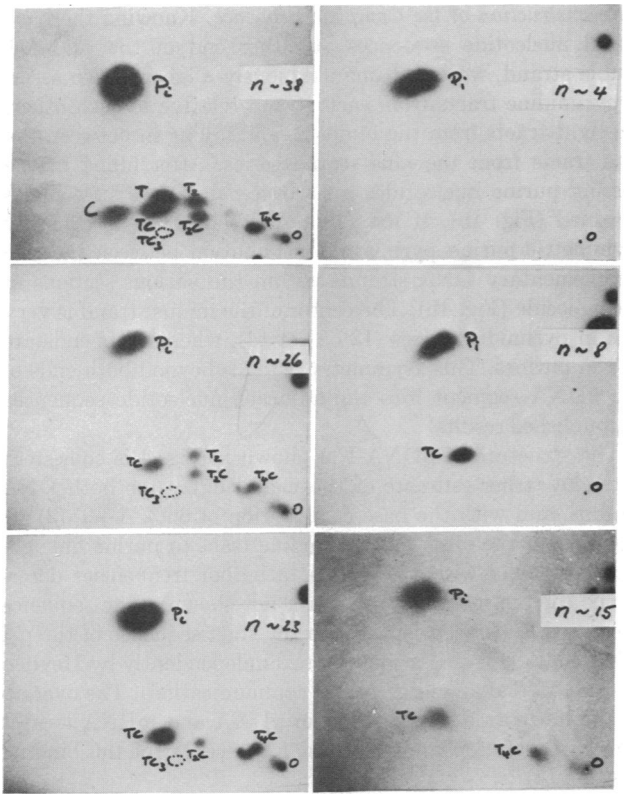


FIG. 2. Pyrimidine tract analysis of 5′-terminal fragments from pDNA-I minus strand. DNA fragments were prepared and separated as described in Fig. 1. Pyrimidine tracts from the individual spots were identified by two-dimensional thin-layer chromatography. Base composition of pyrimidine tracts and chainlength of the DNA fragments are indicated. Plate $n \sim 38$ was derived from full-length pDNA-I.

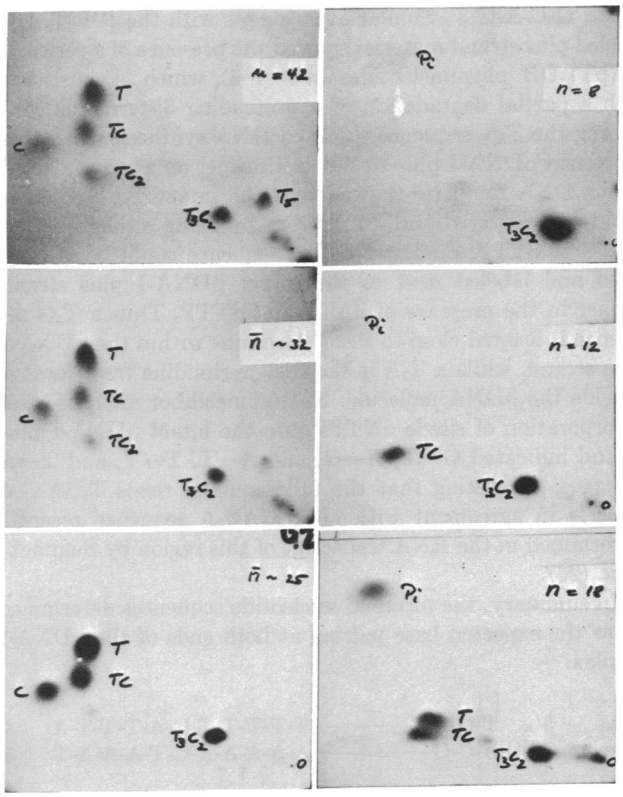


FIG. 3. Pyrimidine tracts from 5′-terminal fragments from p-I plus DNA. pDNA-I labeled with $^{32}PO_4$ in the plus strand was partially degraded with *E. coli* exonuclease III and the resulting DNA fragments were separated by two-dimensional thin-layer chromatography (plates n = 8, 12, and 18) or by gel filtration (plates $\bar{n} \sim 25$ and 32) and analyzed for their pyrimidine tracts. Plate n = 42 was derived from full-length pDNA-I.

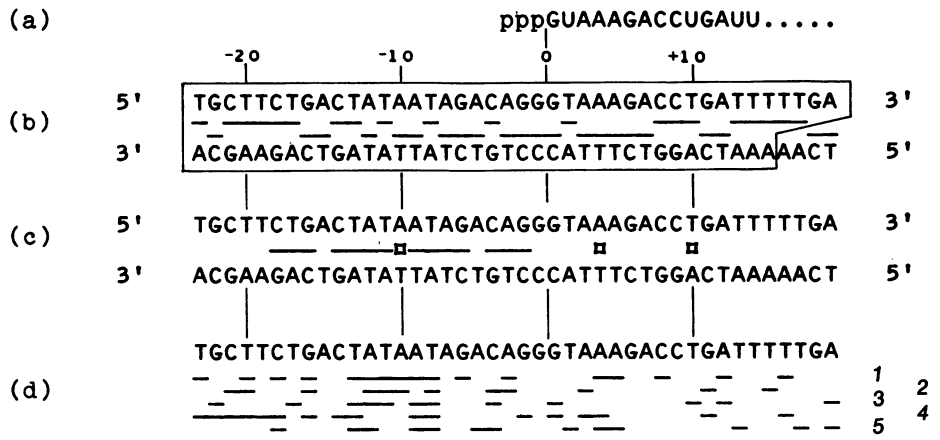
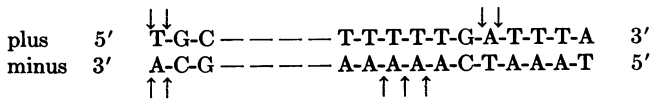


FIG. 4. Structure and characteristics of pDNA-I. All phosphodiester bonds have been omitted. Template and complementary strands (minus and plus strands) of the DNA duplex are represented by the lower and the upper sequences, respectively. The start sequence of mRNA from fd promoter-I (4) is included for comparison (a). Polymerase protected DNA sequences are boxed (b), and the distribution of pyrimidine bases is indicated by lines (b). A region of 2-fold symmetry and two additional symmetry axes (□) are indicated in (c). The sequence is compared to promoter sequences from (*1*) simian virus 40 DNA (22, 23), (*2*) lambda DNA (18), (*3*) tyrosine tRNA (19), (*4*) *lac* DNA (21), and (*5*) T7 DNA (D. Pribnow, personal communication) (*d*). These sequences were aligned for maximum sequence homology (− indicates identical residues). Startpoints of transcription are at positions +1 (fd, tyrosine tRNA, T7), −1 (simian virus 40), and +2 (*lac*).

separated and analyzed for nearest neighbor relationships. The results showed incorporation of T→T (5′→3′) into the plus strand and of A→C, C→G, G→A into the minus strand. Pyrimidine tract analysis showed that pCp was labeled after incorporation of [α-^{32}P]dATP or dCTP, indicating that the minus strand terminated with a sequence ... G-C-A 3′ or ... G-C-A-G 3′. Similar experiments with the [^{32}P]TMP-labeled plus strand material showed the presence of a series of pT(pT)$_n$OH oligonucleotides (n = 1–3), which is consistent with a partial degradation of a protruding 3′-terminus containing the T_5p_6 sequence. Limited DNA synthesis using this shortened pDNA-I plus strand as a primer on an extended fd minus DNA template showed that the intact T_5p_6 sequence could be recovered and ^{32}P labeled by using either [α-^{32}P]-dGTP or dTTP. Similarly a T_3p_4 sequence could be synthesized and labeled next to the intact pDNA-I plus strand primer in the presence of dATP and dTTP. Thus a T_5G sequence is located close to the 3′-terminus within the pDNA-I plus strand, while a T_3A is the first pyrimidine tract located outside the pDNA molecule. Nearest neighbor analysis after incorporation of single dNTPs onto the intact pDNA-I plus strand indicated G→T, A→G, and A→T, T→T, and T→A linkages, suggesting that the full sequence reads T_5GAT_3A. This is in agreement with an U_5GAU_3A sequence recently determined in the RNA transcript of this region by Sugimoto *et al.* (26).

In summary, the terminal nucleotide sequences determined show the expected base pairing at both ends of the pDNA-I duplex:



As indicated by the arrows, the nontranscribed (left) end of the binding site appears to be cut evenly by pancreatic DNase, whereas, the transcribed end is cut in a staggered way so that the template strand is shortened by three to four nucleotides. This asymmetry cannot be explained by the preference of pancreatic DNase for particular nucleotide sequences (15). It, therefore, most probably reflects the asymmetrical binding of the RNA polymerase molecule to the promoter DNA. A similar asymmetrical protection against DNase attack has been also observed with other RNA polymerase binding sites from fdRF DNA (2).

Reconstruction of the Complete Sequence. Knowing the base paired nucleotide sequences at either end of the pDNA-I double strand, we could unambiguously align the two series of pyrimidine tracts from each strand relative to each other, the eight tracts from the minus strand falling in between the nine tracts from the plus strand series. After filling in the missing purine nucleotides a 42 base-pair DNA molecule is obtained (Fig. 4b). It may be noted that there is a highly asymmetric purine–pyrimidine distribution between the two complementary DNA strands within the various sections of the molecule (Fig. 4b): The center of the minus strand is very rich in pyrimidine bases (12 out of 14), whereas, its ends are rich in purines. This asymmetry extends beyond both ends of the pDNA segment into the adjacent nucleotide sequences (unpublished results).

The structure of pDNA-I as shown in Fig. 4 is consistent with the earlier estimate of the chainlengths for both DNA strands, and with the base composition of 66% A+T (2). It agrees with the observed pyrimidine tract to purine linkages (Table 2), and with the nearest neighbor frequencies determined for the minus strand (data not shown). The sequence presented is also consistent with the start sequence of the p-I RNA chain (Fig. 4a) as determined independently by Heyden (4) and M. Takanami (personal communication). The overlap of the nucleotide sequences from pDNA and mRNA locates the startpoint for transcription at the center of the binding site (Fig. 4).

DISCUSSION

We report here the sequence of those 42 nucleotides from promoter region-I in fd RF DNA that are protected against digestion with pancreatic DNase by the RNA polymerase molecule in the stable preinitiation complex. The structure of

the binding site displays several characteristics that may account for its highly specific interaction with the enzyme:

(*1*) pDNA-I is rich in A·T base pairs and shows an unequal distribution of pyrimidine and purine bases (Fig. 4b). Both properties are known to influence the secondary structure of the DNA double helix and to lower its stability (16, 17). Therefore, they may both play a role in primary promoter recognition, as well as in the temperature-dependent "opening" of the promoter DNA which is assumed to be essential for the formation of the stable preinitiation complex.

(*2*) pDNA-I contains three regions with 2-fold rotational symmetry. These are located around symmetry axes at positions +10, +4, and −10, and contain fractions of symmetrically arranged base pairs of 10/14, 8/14, and 14/16, respectively. Thus, the strongest symmetry is observed in the nontranscribed part of the binding site (Fig. 4c). Symmetries have also been detected in the analogous regions of other promoter sequences (18, 19), as well as in other DNA segments that are involved in site-specific DNA protein interaction (9, 20).

(*3*) pDNA-I shows sequence homology with analogous DNA segments from other promoter regions. As shown in Fig. 4d, homologies are present mostly in the nontranscribed section of these sites. A particular strong similarity is apparent in an A+T-rich hexanucleotide sequence around position −10, which is represented by at least four bases in all six sequences. Strong sequence homology is also observed between the adjacent terminal sequence of pDNA-I and the corresponding sequence from the *lac* promoter, as determined by R. C. Dickson, J. Abelson, W. Barnes, and W. S. Reznikoff (personal communication, and ref. 21). Such extensive homology is highly unlikely to occur at random in the DNAs from such a variety of sources. Therefore, these common nucleotide sequences seem to be essential for an RNA polymerase binding site. From the other features of pDNA-I that have been discussed above, none is shared by all six sites. It should be recalled, however, that several of these regions are also interacting specifically with regulatory proteins other than RNA polymerase. This may have resulted in a modification of the characteristics of a simple promoter sequence.

The lack of common stringent features for all sites can also be explained by the possibility that the isolated binding site no longer contains all of the sequence information needed for promoter function. As mentioned before, the pDNA–RNA polymerase complex as isolated after nuclease digestion behaves autonomously with respect to complex stability and RNA chain initiation (3, 4). It has, however, not been possible in this laboratory to reconstitute the specific promoter complex from the isolated binding site and the enzyme (unpublished results). A similar lack of "polymerase entry" has also been noticed with a DNA fragment from the lambda promoter that contained as many as 33 nucleotides of its nontranscribed nucleotide sequence (24). Furthermore, promoter mutations have been located in the *lac* operon around position −35 relative to the start nucleotide (21), i.e., approximately 15 base pairs outside of the polymerase-protected site as defined in this paper. Thus, it seems likely that an analysis of these presumed entry regions will reveal further characteristics common to all promoters.

Note Added in Proof. fd promoter-I has been mapped in map position 0.94 at the end of gene II (26).

We thank H. G. Khorana, W. Reznikoff, D. Pribnow, and M. Takanami for the exchange of unpublished information. This work was supported by the Deutsche Forschungsgemeinschaft.

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