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NOTES

Localization of *Escherichia coli* RNA Polymerase-Binding Sites on Bacteriophage S13 Replicative Form I DNA by Protection of Restriction Enzyme Cleavage Sites

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Protection of restriction endonuclease cleavage sites by *Escherichia coli* RNA polymerase bound to the replicative form I of bacteriophage S13 DNA has been used to identify a number of regions of RNA polymerase binding. Digestion with *Hin*cII, *Alu*I, *Hin*fI, or *Hae*III, under conditions optimized for "open" complex formation, revealed 12 regions of RNA polymerase binding. Based on differential salt sensitivities, five of the regions were classified as strong or tight binding sites. These were located before genes *A* (two sites), *B*, and *D* and at the 5′ end of gene *F*. The seven regions which exhibited weaker binding were located at the 5′ end of gene *C* (two sites), in the middle of gene *D*, just before and at the 3′ end of gene *F*, at the 5′ end of gene *G*, and in the middle of gene *H*. The sites before genes *B* and *D* coincide with sites previously identified as promoters in bacteriophage φX174. One of the sites before gene *A*, that at nucleotides 5175-5211, represents a new putative promoter site in bacteriophages S13 and φX174 located before the previously identified *A* gene promoter at nucleotides 10-45.

Many of the regulatory events that occur at the transcriptional level involve RNA polymerase-DNA interactions. The promoters recognized by *Escherichia coli* RNA polymerase holoenzyme comprise two domains upstream of the start of transcription: a −35 region with the consensus sequence TTGACA and a −10 region, the Pribnow box, with the consensus sequence TATAAT (11, 23, 28). Despite the extensive sequence similarity of all *E. coli* RNA polymerase promoters investigated so far, the differences in the sequences do not explain adequately the enormous variations in promoter efficiencies, nor the affinity of RNA polymerase for other sites on DNA molecules that are not part of transcription initiation locations. The closely related icosahedral bacteriophages S13 and φX174 are amenable to the study of these problems. Their relatively small circular genomes (5,386 nucleotides) differ by only 2.06% (16, 25). Both phages code for nine identified genes (8) and one predicted gene, *K* (16, 26). Six of the genes located within the nonstructural domain of the genome (Fig. 1) are overlapping: genes *A*/*A**, *B*, *K*, *C*, *D*, and *E* (16, 24).

In vitro and in vivo transcriptional studies in φX174 have established three major regulatory regions in broad areas before the *A*, *B*, and *D* genes (3, 12, 29). Sequence analyses of the 5′ termini of in vitro φX174 transcripts (30) and subsequent comparison with the DNA sequence of the phage (24) clearly identified the *A* gene start at position 3962 (45 in S13). Although A and G residue starts were known for transcripts initiated before genes *B* and *D*, respectively (2), insufficient 5′-terminal sequences were available to unambiguously position the exact start sites (30). Binding of *E. coli* RNA polymerase to restriction fragments of S13 and φX174 (7, 20) localized very broad areas of template-polymerase interaction before the *B* and *D* genes; and in the structural domain in the middle of the *F* gene, before the *H* gene, and from the end of the *H* gene, through the intergenic *H*/*A* region, to the beginning of the *A* gene. Visualization of polymerase-replicative form I (RFI) complexes in the electron microscope revealed similar broad areas of interaction in approximately the same regions (7, 21, 34). RNA polymerase has been shown to protect at least 16 restriction enzyme cleavage sites on φX174 RFI molecules, revealing, in addition to sites coinciding with those previously mentioned, regions at the beginning of the *F* and *G* genes (27).

Mutational studies with S13 have identified a promoter site broadly before the *A* gene (33). In contrast, a report by Pollock et al. (18) on the UV sensitivity (radiological mapping) of in vivo transcripts of S13 and φX174 came to the conclusion that the transcripts of the *A* and *B* genes were initiated 2,000 bases upstream of the genes. To clarify these complexities and contradictions, we have undertaken to determine more accurately the location of the transcriptional regulatory regions in S13, using φX174 for comparison. Regions of DNA tightly associated with bound RNA polymerase are protected from the action of restriction endonucleases, thus allowing precise mapping of the binding sites.

RFI preparations of S13 and φX174 (16) were digested with *Alu*I, *Hae*III, *Hin*cII, and *Hin*fI since their cumulative cleavage sites span the entire genomes (Fig. 1). In addition, the recognition sequence of *Hin*cII, GTPyPuAC, has extensive sequence homology to the consensus sequence TTGACA of the −35 region of the *E. coli* promoter region. Reaction mixtures (50 μl) contained 0.15 pmol of S13 RFI, S13 RFIII (linearized with a single-site restriction enzyme), or φX174 RFI DNA, incubated at 37°C with various molar

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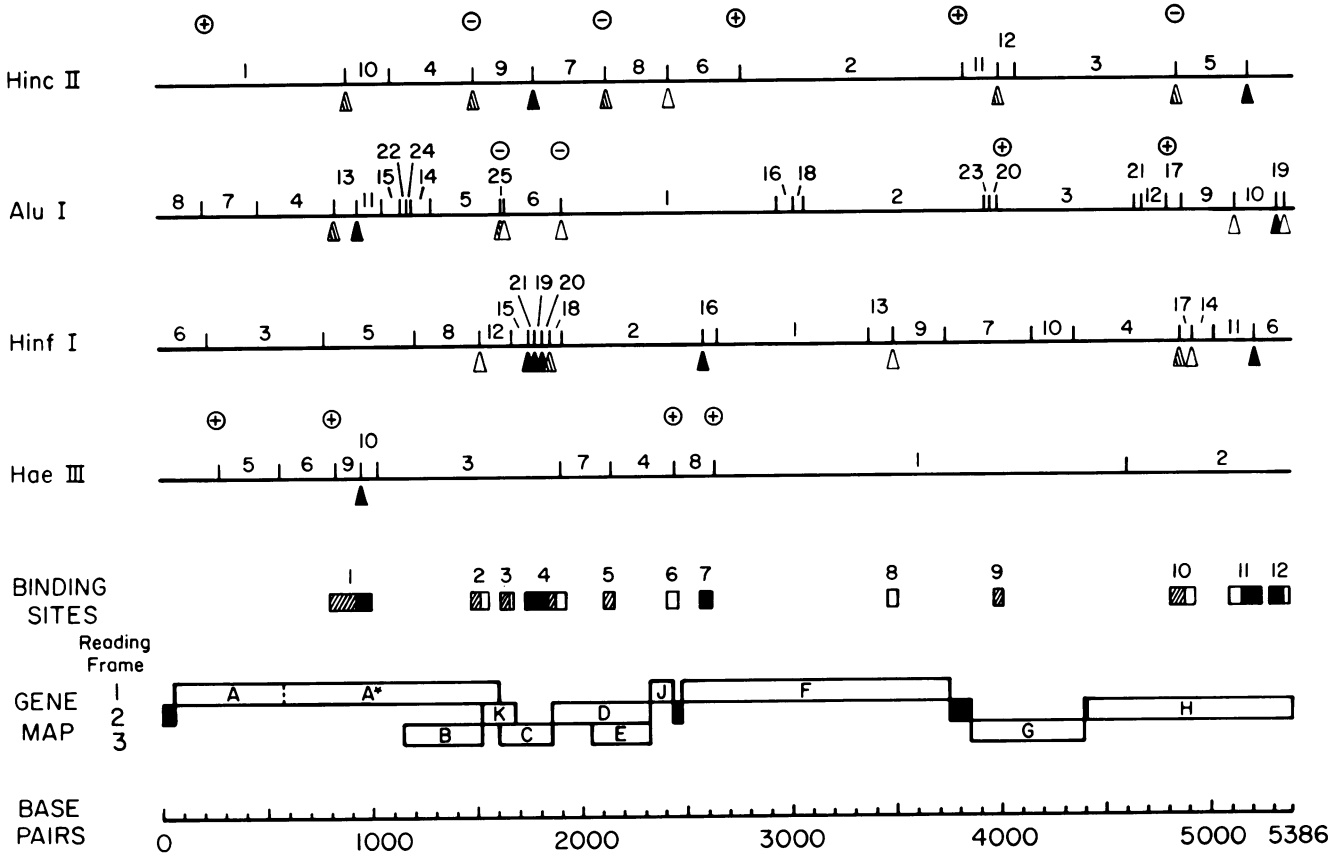

FIG. 1. Restriction enzyme sites in bacteriophage S13 RFI DNA protected from hydrolysis by bound *E. coli* RNA polymerase. The restriction maps were derived from computer searches of the sequence of S13. Triangles identify the individual sites protected by (△) weak, (◮) moderate, and (▲) strong binding. The regions of binding identified by the rectangles relative to the genetic map are the cumulative restriction site data. Circles above sites represent differences between the ϕX174 data of Shemyakin and Shumilov (27) and the present S13 data. ⊕ indicates a protected site in ϕX174 (27) which is not protected in S13. ⊖ indicates a protected site in S13 which was not found to be protected in ϕX174 (27).

ratios of *E. coli* RNA polymerase holoenzyme (4) shown to have 1 mol of σ subunit per 2 mol of α subunit by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15, 17). Restriction enzyme buffers, adjusted according to the manufacturers' instructions to make the salt concentrations more comparable, included: for *Alu*I, 10 mM Tris hydrochloride (pH 7.6), 6 mM $MgCl_2$, and 6 mM 2-mercaptoethanol; for *Hae*III, 50 mM Tris hydrochloride (pH 7.9), 5 mM $MgCl_2$, and 0.5 mM dithiothreitol; for *Hin*cII; 10 mM Tris hydrochloride (pH 7.9), 6.6 mM $MgCl_2$, and 1.0 mM dithiothreitol; for *Hin*fI, 6 mM Tris hydrochloride (pH 7.6), 6 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. Final NaCl concentrations varied from 20 to 170 mM. After preincubation at 37°C for 30 min, the appropriate restriction endonuclease was added (3 U/μg of DNA), and digestion was allowed to proceed at 37°C for 2 to 24 h. Reactions were stopped by phenol extraction and ethanol precipitation.

Determination of the optimum RNA polymerase-to-DNA ratio for protection studies of *Hin*cII sites revealed that new bands appeared and some original bands diminished or disappeared as the ratio increased to 70:1. Subsequent experiments were carried out at a ratio of 90:1 to assure an excess of RNA polymerase (14).

The protection of *Hin*cII sites by *E. coli* RNA polymerase and the effect of increasing salt concentrations as a measure of binding strength are shown in Fig. 2. At 20 mM NaCl there was complete disappearance of bands R1, R3, R5, R7, and R9 and attenuation of R4, R8, R10, and R12 (Fig. 2, lane 2). Twenty new bands (prefix N) of various intensities appeared. Comparison of the sizes in base pairs (bp) of the bands which had disappeared with those that appeared revealed that N20 approximates the sum of bands R11 and R12 and arose due to protection of the *Hin*cII 11/12 site located at the 5′ end of gene *G* (Fig. 1). Shemyakin and Shumilov (27) observed protection of the *Hin*cII 1/9 and 9/10 sites in ϕX174 (*Hin*cII 2/11 [position 3818] and 11/12 [3980], respectively, in S13) and of the *Alu*I 15a/3 site (*Alu*I 20/3 [position 3913] in S13). However, only the *Hin*cII 1/9 site was protected in a *Hin*cII/*Alu*I double digest of ϕX174 (27). The *Alu*I sites in this region were not protected in S13 (Fig. 1 and see below), and there are no sequence differences between S13 and ϕX174 in these regions to account for the different results with the two phages.

The appearance of N17 and N19 resulted from protection of the *Hin*cII 4/9 and 9/7 sites, respectively, locating binding sites before the *C* and *D* genes. *Hin*cII 4/9 was not protected in ϕX174 (27), but support for the S13 data was seen with *Hin*fI. We examined *Hin*cII site protection in ϕX174 and confirmed that there was no protection at *Hin*cII 5/7b (*Hin*cII 4/9 [position 1497] in S13). The sequences in the two phages are the same in this region; thus, there is no obvious explanation for the differences observed.

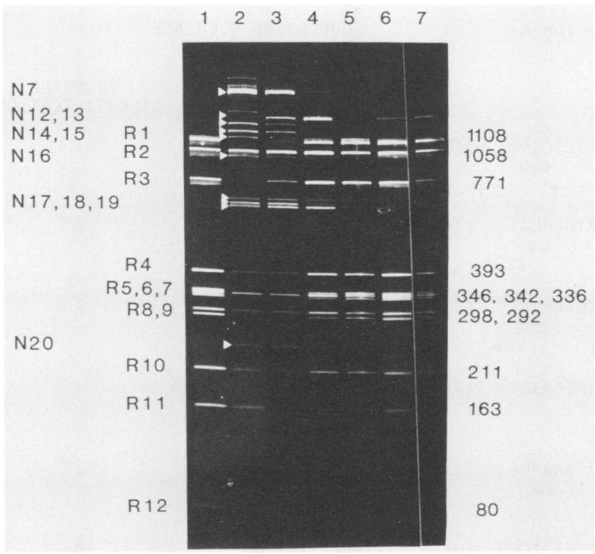

FIG. 2. Effect of salt concentration on the protection of *Hin*cII sites in bacteriophage S13 RFI DNA by *E. coli* RNA polymerase. A 0.15-pmol sample of S13 RFI DNA was preincubated at 37°C for 30 min with 13.5 pmol of *E. coli* RNA polymerase and various concentrations of NaCl, then digested with 3 U of *Hin*cII per μg of DNA for 2 h. The digests were separated on a 3.5 to 15% polyacrylamide gel. Lane 1, 70 mM NaCl, no polymerase; lane 2, 20 mM NaCl; lane 3, 70 mM NaCl; lane 4, 120 mM NaCl; lane 5, 170 mM NaCl; lane 6, 20 mM NaCl plus four NTPs; lane 7, 170 mM NaCl plus four NTPS. The NaCl concentration of the RNA polymerase storage buffer was 20 mM. R1 to R12, *Hin*cII restriction fragments; N7 to N20, new restriction fragments (see white triangles, lane 2) referred to in the text. The numbers on the right are the base pairs of the R fragments.

The equation of R9 and R7 with N19 is seen clearly in lane 4 of Fig. 2, and since the site was still protected at high salt concentration and R7 and R9 were completely absent at 20 and 70 mM NaCl, it is classified as a strong binding site. Because of the closeness in sizes between R7 plus R8 plus R6, it was impossible to determine which protected site resulted in N18. The size of N16 corresponds to a fragment possibly resulting from protection of both sites; hence, binding sites may exist within the *D* gene as well as at the 5′ end of the *F* gene. The equivalent *Hin*cII 7/8 site in φX174 (binding site 5, Fig. 1) was not protected (27). Again, there is no sequence difference between the two phages in this region, but the protection of *Hin*cII 7/8 in S13 is clear. The *Hin*cII 8/6 site is classified as a weak binding site because the R8 and R6 bands were present at all salt concentrations. In φX174 the same site (*Hin*cII 8/6 [2420]), and also the *Hae*III 4/8 (2447) site, were protected (region 6, Fig. 1) (27). This region partially overlaps with the J/F intergenic region from 2431 to 2469 and is the same as that suggested by van der Avoort et al. (32) for the location of a promoter, in φX174, which expressed the *F* and *G* genes when cloned into a plasmid chimera which did not contain any other viral or plasmid promoter sequences. There is a single but insignificant base change, C→T for S13→φX174, at position 2418 in S13.

A major area of protection occurred within the 3′ end of the *H* gene. Band N15 (Fig. 2, lane 2) probably resulted from protection of the *Hin*cII 3/5 site. The binding at *Hin*cII 3/5 (4829) but not at *Alu*I 12/17 (4779) is the reverse of that observed by Shemyakin and Shumilov in φX174 (Fig. 1) (27). There are two potential promoter sequences in this region (Fig. 3). That at position 4842 to 4876 (region 10b, Fig. 3) shows excellent homology with the consensus sequence, but there was no protection of *Alu*I 17/9 (4857). Band N12 (Fig. 2) resulted from protection of the *Hin*cII 5/1 (5174) site and was very salt sensitive, indicating a strong binding site. The importance of this binding site was accentuated by the *Hin*fI data (see below). The most intense new band, N7, approximates that of an R5-R1-R10-R4-R9-R7 complex, which could account for the low intensity of bands N15 and N12. Similarly, band N14 corresponds in size to an R10-R4-R9-R7 complex. Band N13 corresponds to protection of the *Hin*cII 1/10 site in the area before the *B* gene. The attenuation of R10 accords this region moderate binding status.

No differences were observed in the results in Fig. 2 when heparin was added just before the addition of restriction endonucleases, indicating no significant dissociation of RNA polymerase from the DNA after restriction endonuclease digestion was initiated. Lanes 6 and 7 of Fig. 2 were control experiments with nucleoside triphosphates (NTPs) demonstrating deprotection of all sites after the initiation of transcription. This was consistent with the notion that after a promoter is located and a tight binary "open" promoter complex is formed, addition of NTPs allows initiation of transcription to take place; the RNA polymerase moves along the DNA chain, and the previously protected sites are exposed to restriction enzyme cleavage once more (5, 28). The protected sites least sensitive to changes in salt concentration (strong binding sites) also had the greatest affinity for RNA polymerase at low enzyme-to-DNA ratios, consistent with RNA polymerase having different affinities for different DNA sequences.

In analyses of *Alu*I digests (Fig. 4A), bands R4, R5, R10, R11, R13, and R19 disappeared almost completely at 20 mM NaCl, and there was clear attenuation of bands R2, R8, R9, R14, R15, and R16. Twenty-one new bands (prefix N) of various intensities appeared. Protection of the *Alu*I sites between bands R4, R13, and R11 (listed in map order) resulted in the creation of several new bands. Band N21 equates to the *Alu*I 13/11 site, N13 corresponds to the *Alu*I 4/13 site, and N11 corresponds to an R4-R13-R11 complex. Since R15 was visible (Fig. 4A, lane 2) and there was attenuation of R13 and R11 at higher salt concentrations, it is deduced that *Alu*I 13/11 (position 943) is in a strong binding area before the *B* gene (region 1b, Fig. 3). It is located within a DNA sequence of an expected φX174 *B* gene promoter (24). The *Alu*I 4/13 site is a moderate binding site since R4 was barely reduced in lanes 3 and 4 and the protection may be a reflection of steric hinderance. An open promoter complex may protect ~60 bp from nuclease digestion (28). Siebenlist et al. (28) have proposed that the polymerase interacts mainly with one face of the DNA, and thus other proteins may be able to interact with the DNA at "uncovered" areas. Conversely, a restriction endonuclease site near a tight RNA polymerase binding site may be partly or totally protected. A similar argument holds for the R9 and R8 bands flanking the strong binding site at *Alu*I 10/19 (position 5308) at the 3′ end of the *H* gene. Sequence 5301 and 5337 (region 12, Fig. 3) has a very good sequence homology to the promoter consensus sequence; however, the variant base in the 3′ position of the Pribnow box reduces the possibility of this site being a promoter (11, 28). Similar binding was observed in φX174 (27), and it correlates with the reported binding of polymerase to restriction fragments in both S13 and φX174 (7, 20).

A new band, N18, just above R5 (Fig. 4A) corresponds in size to an R5-R25 complex (band R25 is not visible on the

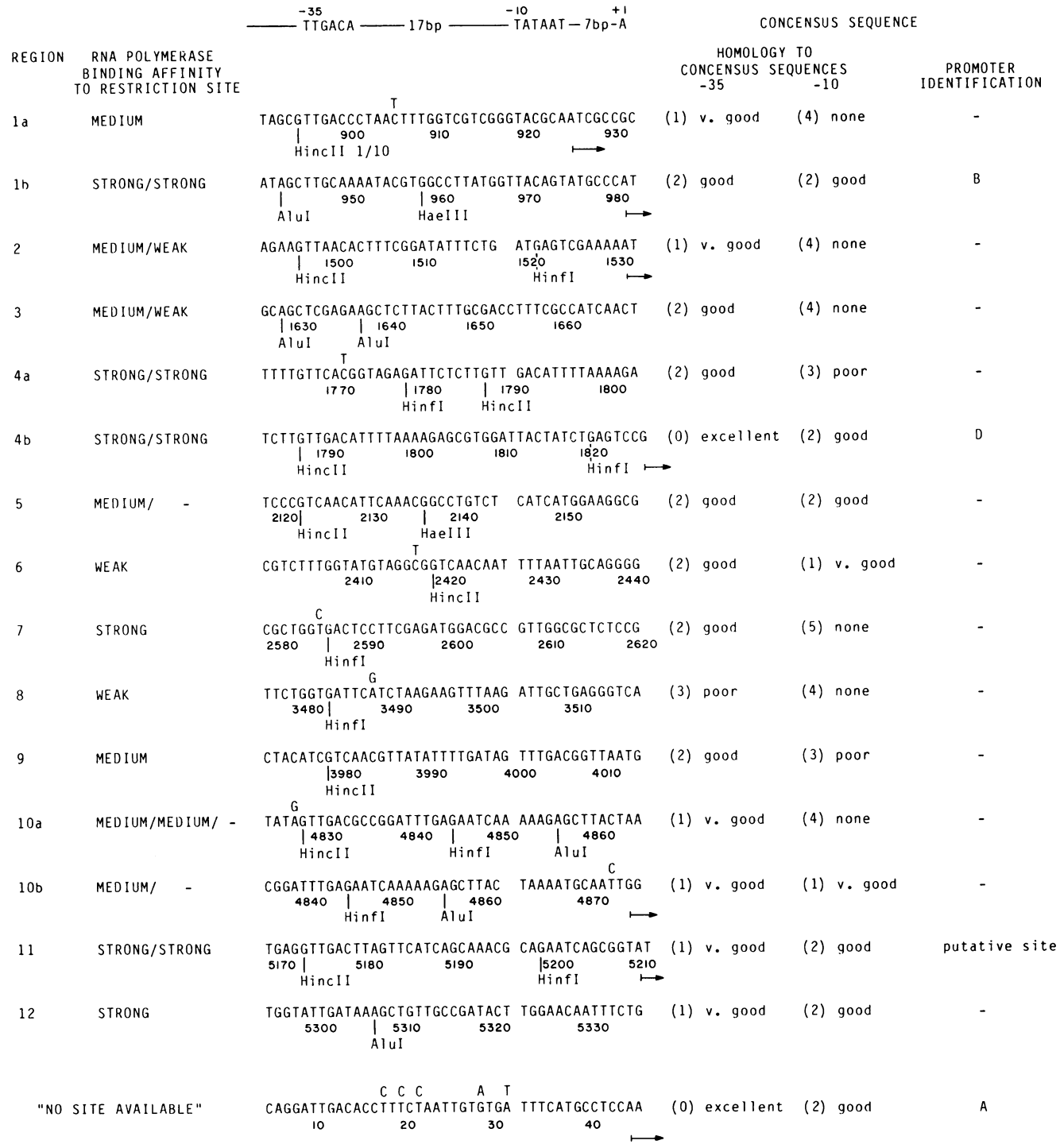

FIG. 3. S13 DNA sequences with homology to the consensus promoter sequences located within the regions protected by *E. coli* RNA polymerase. Binding affinity is based on salt sensitivity. Sequence homology ratings: no difference, excellent; one-base difference, very good; two-base difference, good; three-base difference, poor; four-base difference or more, no homology. The bases above the S13 sequence are those which differ in ϕX174.

gel). Since R5 was attenuated at 70 mM NaCl (Fig. 4A, lane 3), this region is accorded moderate binding strength. R6, which is adjacent to R25, was less protected than R5, consistent with the location of a protected site between R5 and R25. No binding of the same region in ϕX174 was observed (27).

Comparison of the *Alu*I digests of 24-h duration in Fig. 4A with the *Hinc*II digests of 30-min duration in Fig. 2 provides support for the existence of open promoter complexes at the strong binding sites. Even though protection was somewhat decreased after 24 h (comparative data not shown), it was still significant, reflecting half-lives consistent with the open

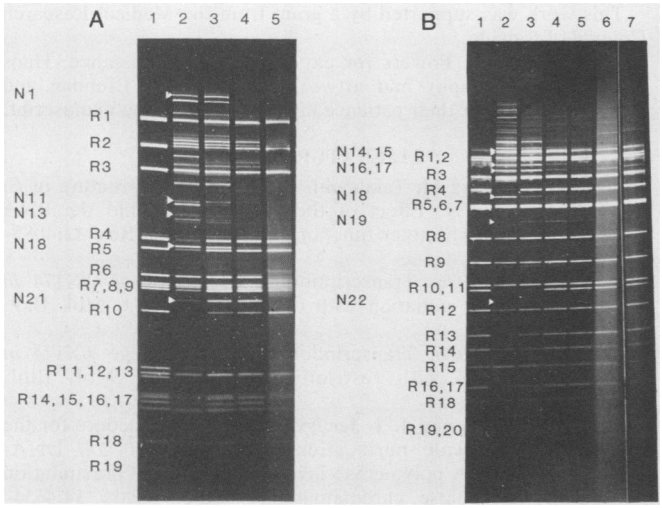

FIG. 4. Protection of *Alu*I and *Hin*fI sites in bacteriophage S13 RFI DNA by *E. coli* RNA polymerase. Conditions were as described in the legend of Fig. 2 except that for panel A digestion was for 24 h after the addition of *Alu*I. (A) *Alu*1. Lane 1, 70 mM NaCl, no polymerase; lane 2, 20 mM NaCl; lane 3, 70 mM NaCl; lane 4, 120 mM NaCl; lane 5, 70 mM NaCl plus 4 NTPs. Sizes (bp) of R1 to R19 *Alu*I restriction fragments: R1, 1,008; R2, 854; R3, 663; R4, 359; R5, 338; R6, 227; R7, 259; R8, 255; R9, 248; R10, 205; R11, 120; R12, 114; R13, 110; R14, 91; R15, 88; R16, 85; R17, 79; R18, 56; and R19, 43. N11 to N21 new restriction fragments (see white triangles, lane 2) are referred to in the text. (B) *Hin*fI. Lane 1, 70 mM NaCl, no polymerase; lane 2, 20 mM NaCl; lane 3, 70 mM NaCl; lane 4, 120 mM NaCl; lane 5, 170 mM NaCl; lane 6, 20 mM NaCl plus four NTPs; lane 7, 120 mM NaCl plus four NTPs. Sizes (bp) of R1 to R20 *Hin*fI restriction fragments: R1, 714; R2, 670; R3, 554; R4, 501; R5, 428; R6, 418; R7, 414; R8, 312; R9, 250; R10, 201; R11, 189; R12, 152; R13, 119; R14, 101; R15, 83; R16, 67; R17, 62; R18, 58; R19, 43; R20, 41: N14 to N22 new restriction fragments (see white triangles, lane 2) are referred to in the text.

promoter complexes described by Hinkle and Chamberlin (13).

To investigate other areas of the genome and further narrow the location of tight binding sites, analyses with restriction enzymes *Hin*fI (Fig. 4B) and *Hae*III (data not shown) were performed. In the *Hin*fI experiments, strong binding sites were located at *Hin*fI 2/16 (N15) at the 5′ end of gene *F* (Fig. 1). Shemyakin and Shumilov (27) identified strong binding at adjacent sites *Hae*III 8/4 and *Hin*cII 6b/1 in ϕX174 (*Hae*III 8/1 and *Hin*cII 6/2, respectively, in S13). They proposed that the region is a potential cyclic AMP-cyclic AMP receptor protein-regulated promoter sequence. In S13 there are five base changes within the GTG may account for the absence of binding at *Hin*cII 6/2. We consider it unlikely that this is a potential promoter region in S13. A strong binding site was located at *Hin*fI 11/6 (position 5200) (N16). The *Hin*cII 5/1 (5174) and *Alu*I 9/10 (5104) equivalent sites were also protected in ϕX174, confirming this region (region 11, Fig. 3) as a putative promoter site. Initiation of transcription at this putative promoter would give a transcript that would code for the translation of the *A′* protein (19) starting at position 37 (16). The Rho-independent termination site at 58-64 could be part of a type of attenuation control mechanism for the *A* gene involving this putative promoter (16).

Other strong binding sites were identified at *Hin*fI 15/21 (1755), 21/19 (1779), and 19/20 (1821) before the *D* gene. The binding at these sites may be accentuated by their proximity to each other and to *Hin*cII 9/7 (1788). The 4b region, 1784 to 1827 (Fig. 3), based on this binding and sequence homology, is identified as the *D* promoter. The +1 sequence is the start of a tetramer of 5′ mRNA sequence reported by Smith and Sinsheimer (30). Although Sanger et al. (24) identified this region in ϕX174 as the *D* promoter with the −10 region at position 1816, a later review placed it at position 1813 (10). Position 1813 is more likely (Fig. 3) since it has better sequence homology to the Pribnow box (11, 23, 28) and has the invariant T at the 3′ end. Also it is 9 bp from the +1 site, within the limits for activity shown by Aoyami and Takanami (1). Weak binding was observed at *Hin*fI 8/12 (N18) before the *C* gene, overlapping a moderate binding site at *Hin*cII 4/9, not seen in ϕX174. Regions 2, 3, and 4 (Fig. 1) are coincident with the binding of polymerase to *Hae*III 3 observed by Rassart and Spencer in S13 and ϕX174 (20) and by Chen et al. in ϕX174 (7).

Another weak site was at *Hin*fI 13/9 (N19), at the 3′ end of gene F. There are no *Alu*I, *Hae*III, or *Hin*cII sites closely adjacent to this site, but the binding in this region and region 9 (Fig. 1) correlates with the reported binding of polymerase to restriction fragments *Hae*III 1 and *Hin*cII 2 (1 in ϕX174) in S13 and ϕX174 (7, 20). Moderate binding sites *Hin*fI 4/17/14 (N17 and N22), coinciding with the middle of gene *H* (Fig. 1), overlapped the *Hin*cII 3/5 site.

Analysis with *Hae*III resulted in identification of a strong binding site at *Hae*III 9/10, very close to the *Alu*I 13/11 and *Hin*cII 1/10 sites. These are all in a region which includes two putative *B* gene promoter sites identified in ϕX174 by Sanger et al. (24) on the basis of sequence homology to five bases of 5′ mRNA sequence (30). Region I corresponds to an area of weak binding in S13 (21) (strong binding in ϕX174 [34]) and overlaps the *Hin*cII 1 fragment of S13 observed to bind *E. coli* RNA polymerase by Rassart and Spencer (20). Region 1a (Fig. 3), the site with the possible +1 start at position 927 (4843 in ϕX174), does not have the 5- to 7-bp separation of +1 from the −10 region, shown to be essential for promoter activity (1), and has a variant base at the 3′ end of the Pribnow box (11, 28). Region 1b is the *B* gene promoter. The conflicting data reporting no binding of polymerase and no hybridization of mRNA transcripts to restriction fragments from this region (3, 7, 20, 29, 31) were probably due to low amounts of binding of the smaller fragments to the nitrocellulose membranes under the conditions used in those experiments.

To differentiate between A start and G start promoter regions, ATP or GTP was added to reaction mixtures together with *E. coli* RNA polymerase. Ternary complexes were allowed to form before digestion of the template with *Hin*cII. Although open binary promoter complexes are extremely stable to salt, ternary complexes are even less sensitive to changes in salt concentration. Results (not shown) of the addition of ATP showed that the bands equivalent to N7 (R5 and R1) and N13 (R1 and R10) in Fig. 2 increased in intensity. These sites correspond to areas upstream of the *A* and *B* genes which, in ϕX174, have been shown to have A start promoters (2, 30). In contrast, addition of GTP increased slightly the intensity of bands equivalent to N16 and N17 (Fig. 2), which correspond to sites just upstream of gene *D*. In ϕX174, RNA initiated from the *D* gene promoter has GTP at the 5′ terminus (2).

In the single-stranded phages, negatively supercoiled RFI molecules are thought to be the templates for in vivo

transcription (9). However, the exact nature of the in vivo transcription complex has yet to be described. Supercoiled (RFI) and linearized (RFIII) S13 DNA templates were compared in protection experiments, but no visible differences in gel banding patterns were observed. Thus, in the presence of excess RNA polymerase, supercoiling does not appear to result in new or different polymerase binding sites even though the transcription rate is enhanced relative to linear relaxed templates. However, once a supercoiled molecule is cut it becomes an RFIII molecule, which complicates any such interpretation.

The protected restriction sites have been grouped into 12 regions on the S13 genome (Fig. 1 and 3). Five (1, 4, 7, 11, and 12) encompass strong binding regions; the other seven are weak binding regions, based on salt sensitivity. Within each region are sequences with some homology to the consensus sequence of *E. coli* promoters, particularly in the −35 region (Fig. 3). A number of the regions, 1a, 2, 4b, 5, 9, 10a, and 11 (Fig. 3), have *Hin*cII sites at the potential −35 regions which, as noted previously, have considerable sequence homology to the −35 consensus sequence. Regions 1b, 4b, 11, and 12 have very good homology with both domains of the consensus promoter sequence and strong binding characteristics. The first three have been designated as promoter sites.

The actual *A* gene promoter was not investigated in these studies, nor by Shemyakin and Shumilov in ϕX174 (27), because the restriction enzymes used have no sites close to the region 9 to 45 (Fig. 3) of the promoter. However, the sequence homology would be classified as excellent (−35) and good (−10).

The differences between the present study and that of ϕX174 were to be anticipated, not only because of the different templates, but also owing to the different conditions and restriction enzymes used. Binding studies cannot be equated directly with promoter sites. However, the correlation with the sequence shown in Fig. 3 does emphasize the role of DNA sequence in polymerase interaction. Unfortunately there are no sequence differences between the two phages in any of the 12 regions which could serve to explain any of the binding differences observed. The sequence data must be considered and coordinated with other data. For example, Richardson (22) has shown that some promoters may be in an inactive state. Half-lives of polymerase template complexes are another factor. Chamberlin et al. (6) have reported that the half-life of an *E. coli* RNA polymerase holoenzyme T7 A1 promoter complex is much more than 24 h under low salt conditions (15 mM NaCl, 10 mM $MgCl_2$), but is reduced to 5 min in 100 mM NaCl and 10 mM $MgCl_2$. This dissociation rate is consistent with that observed in our experiments with *Alu*I (Fig. 4A), where tight binding was still observed after 24 h in 20 mM NaCl and 10 mM $MgCl_2$ whereas in 70 mM NaCl there was little protection after 24 h. No attempts were made to measure half-lives, since the rate of inactivation of *E. coli* RNA polymerase is much greater than the rate of promoter-complex dissociation (6). Another factor which affects polymerase interaction is dimerization, which may play a role in situations where two restriction enzymes have adjacent sites.

This study has defined more precisely the location of the regions of polymerase/S13 DNA template interaction and has shown a correlation with many previous results from studies using restriction fragment binding and electron microscopy. The next stage requires a detailed examination of transcripts both in vitro and in vivo to determine which of the sites identified in the present study can actually be utilized.

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