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Localization of *Escherichia coli* RNA Polymerase Binding Sites on Bacteriophage S13 and φX174 DNAs by Electron Microscopy

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Complexes between *Escherichia coli* RNA polymerase and bacteriophage S13 and phage φX174 replicative form III DNAs have been shown to form at specific locations on the phage genomes. The major locations on S13 have been mapped at 8 to 10 and 92 to 96% of the genome length, starting from the unique *Pst* I cleavage site. The locations correspond to the beginnings of genes D and B, respectively. Four minor locations map at 18 to 22, 28 to 32, 50 to 56, and 70 to 74% of the genome. The 70 to 74% site corresponds to the beginning of the A gene. The major locations on φX174 are at 8 to 10, 50 to 54, and 92 to 94% of the genome. The 50 to 54% site is at the start of the H gene and has an equivalent minor site on S13, but it is not a promoter site. Three minor sites on φX174, at 20 to 24, 26 to 32, and 68 to 74% of the genome, correspond to sites on S13. The data confirm the locations of sites identified by restriction fragment binding experiments (E. Rassart and J. H. Spencer, J. Virol. **27**:677-687, 1978) and the assignment of putative promoters at the starts of genes A, B, and D.

Locations of possible promoters on coliphage DNAs have been determined by three different methods. The first, binding of *Escherichia coli* RNA polymerase to restriction fragments generated by various restriction enzymes, provides a correlation of those fragments which bind polymerase under stringent conditions with the restriction map, thus localizing the binding sites on the restriction/genetic map (7, 17, 21, 22, 26). The second approach involves hybridization of mRNA's to the DNA template and to restriction fragments of the DNA. Correlation of the hybridized fragments to the restriction/genetic map locates the start sites of the various mRNA's, which are presumed to be the promoter sites (1, 12, 23). The third technique, electron microscopic visualization of RNA polymerase molecules bound to the DNA template (3, 9, 20, 27), has been made possible by recent modifications of the Kleinschmidt protein monolayer spreading technique (27) and development of alternate spreading methods for visualization of DNA-protein complexes in the electron microscope (3, 8, 14).

On bacteriophage S13 DNA, we have previously located three RNA polymerase binding sites as putative promoters by using the technique of binding polymerase to restriction fragments (21). The three promoters correspond to the beginnings of genes A and B and a site which includes gene D and the beginning of gene E.

A drawback to the restriction fragment localization method has been found with the filamentous DNA phages. Some restriction enzymes cleave at promoter sites, resulting in loss of promoter function (25); thus, not all RNA polymerase binding sites may be identified by this method. More recently, evidence has been presented that, apart from the RNA polymerase recognition sequence on the DNA, an area 5′ to the polymerase binding site may also be involved in promoter function (18).

The present report describes the location of *E. coli* RNA polymerase binding sites on bacteriophage S13 DNA as visualized by electron microscopy with a modification of the Kleinschmidt technique in which spreading is carried out in the presence of a detergent (27). As in the previous study, comparative experiments have been carried out with the closely related bacteriophage φX174 DNA (21).

MATERIALS AND METHODS

Bacteriophage S13 wild type was kindly supplied by I. Tessman and E. Tessman, and φX174 *am3* was supplied by D. T. Denhardt. Isolation of replicative form I (RFI) DNA has been described elsewhere (11). *E. coli* RNA polymerase was a generous gift of F. Grosveld, and its purification by the method of Bur-

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gess and Travers (4) and the details of its properties have been described previously (21). Restriction endonuclease *Pst* I, isolated from *Providencia stuartii* strain 164, was generously provided by B. Goodchild. Details of the isolation of the enzyme and the location of the single cleavage site in S13 RFI DNA have been described by Goodchild and Spencer (10).

Preparation of specimens for electron microscopy. Samples of S13 or φX174 RFI DNA were treated with endonuclease *Pst* I to convert the circular RFI DNA molecules to linear RFIII molecules (10). Binding of *E. coli* RNA polymerase to the appropriate DNA template was performed by incubating 8 μg of polymerase with 2 μg of S13 RFIII DNA or φX174 RFIII DNA in 250 μl of binding buffer (50 mM Tris-hydrochloride [pH 8] buffer containing 50 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM ATP, and 0.1 mM GTP). The mixture was incubated at 37°C for 10 min, and then 50-μl amounts were removed and diluted twofold with prewarmed (37°C) binding buffer to give a final DNA concentration of 4 μg/ml. Spreading on electron microscope grids was performed at 25°C, and electron microscopy was executed as described by Zollinger et al. (27). Electron micrographs were taken at magnifications of ×29,000.

Electron microscopic measurements and calculations of results. For measurements of the DNA molecules, the micrographs were enlarged 25 times with a modified Keuffel and Esser microfilm projector and analyzed with an electronic pen. Each DNA molecule was measured twice, starting from each end, and the positions of the RNA polymerase molecules were calculated as percentages of the total length of the DNA molecule. From the plot of these results, a perfectly symmetrical histogram was obtained. To orient the molecules, each DNA molecule with an RNA polymerase at the same percent length position was plotted on another histogram, and the percentages at which the other polymerases occurred were noted. This was done for each RNA polymerase position on the symmetrical histogram. For each position, the other RNA polymerase molecules occurred either at identical positions, which were plotted, or at mirror image positions, which were not coincident and were discarded. When this orientation of molecules was completed for each position on the symmetrical histogram, a unique histogram was obtained showing the locations of the RNA polymerase binding sites.

RESULTS

Figure 1 contains some typical electron micrographs of *E. coli* RNA polymerase molecules bound to S13 and φX174 RFIII DNA molecules. The RNA polymerase molecules can be seen as dense spots bound to the DNA. Note that the ends of most DNA molecules are completely free of polymerase molecules; thus, background due to nonspecific binding to termini is not a problem with this methodology. These and other photomicrographs were used to construct the histograms in Fig. 2, which delineate the positions of the RNA polymerase molecules on the two phage DNAs oriented as described in Materials and Methods. To ensure that the spreading procedure did not alter the relative sizes of the S13 and φX174 molecules, the lengths of 54 S13 and 47 φX174 RFII DNA molecules were measured. Unit-length T7 DNA molecules were included in the DNA samples as internal length standards. The S13 and φX174 DNA molecules were approximately equal in size, and the lengths of 1.68 ± 0.02 and 1.69 ± 0.03 μm, respectively, normalized to a T7 length of 12.5 μm (37 molecules measured) (16, 27), were within the range of 1.64 ± 0.11 (13) to 1.89 ± 0.04 (6) μm reported previously for the two DNAs (24).

To construct the histogram in Fig. 2A, 118 S13 DNA molecules were measured. There was an average of four RNA polymerase molecules bound per S13 DNA molecule. For the φX174 DNA histogram (Fig. 2B), 102 DNA molecules were measured, and there were between four and five RNA polymerase molecules bound per DNA molecule. The DNA molecules measured were selected according to the following criteria: at least two enzyme molecules were bound per DNA molecule, and the entire length of the molecule could be followed unambiguously, and the DNA molecules were intact and unit length. More than 70% of all the molecules on the electron microscope grids fit these criteria for both the S13 and the φX174 DNAs. The results in Fig. 2 are presented in increments of 2% of the total length of each molecule. The automated tracing method gave higher resolution at the start of the measurement, and overall reproducibility was within ±1%. The 2% increment eliminated any discrepancy due to the arbitrary choice of measurement from one end of the molecule or the other.

The histograms in Fig. 2 show that the locations of the polymerase molecules on the two phage DNAs were almost identical. On S13 DNA (Fig. 2A) there were two sites which appeared to bind more strongly than others, one at 8 to 10% and the second at 92 to 96%. There were four other sites with more than 11 molecules bound per site. These were at 18 to 22, 28 to 32, 50 to 56, and 70 to 74%. There was one additional area, at 88 to 90%, with more than 11 molecules bound which was probably part of the strong site at 92 to 96%.

On φX174 DNA (Fig. 2B) there were three strong sites, at 8 to 10, 50 to 54, and 92 to 94%. Three other sites with more than 11 molecules bound per site were at 20 to 24, 26 to 32, and 68 to 74%.

In Fig. 3, the mapping of the RNA polymerase binding sites by electron microscopy is compared to data derived from the binding of *E. coli* polymerase to *Hin*d and *Hae* III restriction

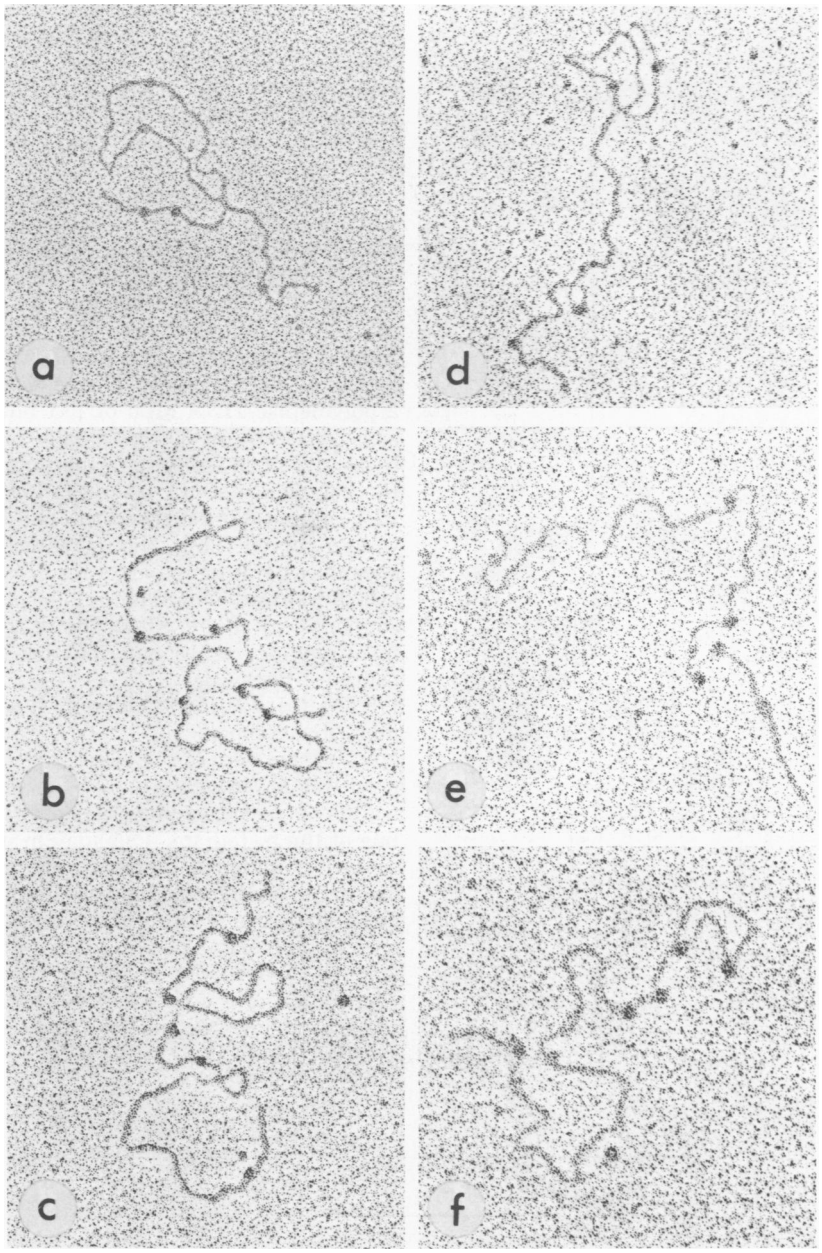

FIG. 1. *Electron micrographs of E. coli RNA polymerase molecules bound to S13 RFIII DNA (a through c) and φX174 RFIII DNA (d through f). Magnification, ×29,000.*

fragments (21). The maps have been aligned with the genetic map of the two phages by correlation with previous restriction fragment mapping alignments (11).

DISCUSSION

The electron microscope technique reveals more RNA polymerase binding sites on both S13 and φX174 DNAs than were found in the previous study of the binding of *E. coli* RNA polymerase to *Hin*d and *Hae* III restriction fragments (21). The conditions used for binding in the present study differed from those in the restriction fragment binding experiments in a number of aspects. A lower salt concentration, 50 mM KCl, was used, because in electron mi-

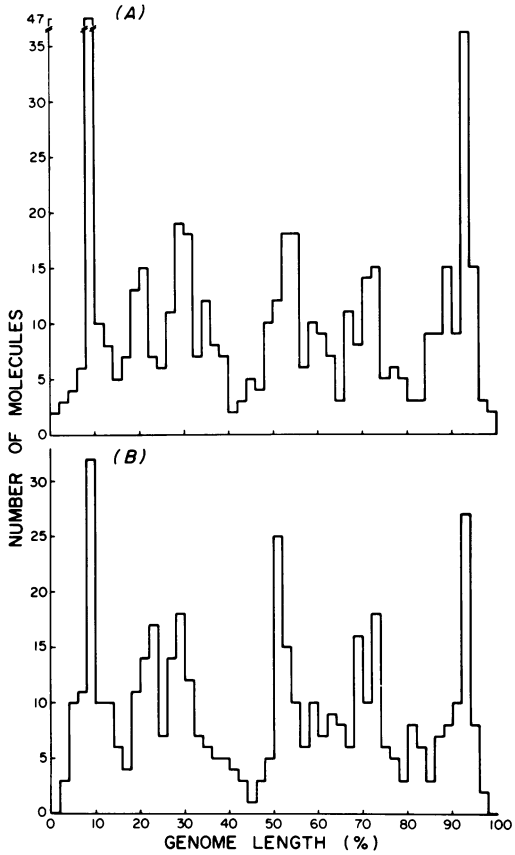

FIG. 2. *Histograms of the distributions of E. coli RNA polymerase molecules on S13 RFIII DNA (A) and φX174 RFIII DNA (B). The length increments are 2% of genome length, which corresponds to ~100 base pairs. As for the orientation of the viral strand, 5′ → 3′ is left to right, and the origin is the Pst I cleavage site.*

croscopy high salt concentrations, 0.2 to 0.3 M, interfere in surface spreading and result in crystallization of salt on the electron microscope grids. At lower salt concentrations, binding characteristics of the polymerase to the DNA are changed quite dramatically, and binding to sites of low affinity is enhanced (5).

The enzyme/DNA molar ratio in the present study was 30:1, whereas in the restriction fragment binding study it was 150:1. However, as previously reported, similar binding was observed to restriction fragments at a polymerase/DNA ratio of 50:1 (21). In a study of *E. coli* RNA polymerase binding to T7 DNA, Bordier and Dubochet (3) reported three binding sites at a polymerase/DNA ratio of 10:1 but two more sites at a ratio of 30:1. We have measured 20 molecules of S13 from an experiment in which the polymerase/DNA ratio was 15:1 in 50 mM KCl, and no differences in the number of sites were observed. Thus, the increased number of sites may be in large part a result of the lower salt concentration used for binding, a conclusion supported by the results of the restriction fragment binding studies undertaken at various salt concentrations (21).

The sites identified as binding sites are where more than 11 molecules of DNA were bound to any 2% increment of the DNA. The 11-molecule level was an arbitrary choice. In all cases, the widths of the peaks increase closer to the base line of the histogram. This may in part be due to loss of mapping precision, which is always lower, the smaller the number of molecules that are bound to any particular site. Some of the background may be due to weak binding at adenine-thymine-rich regions of the DNA (15), aggregation of the polymerase which is known to occur at low salt concentration (2), or dam-

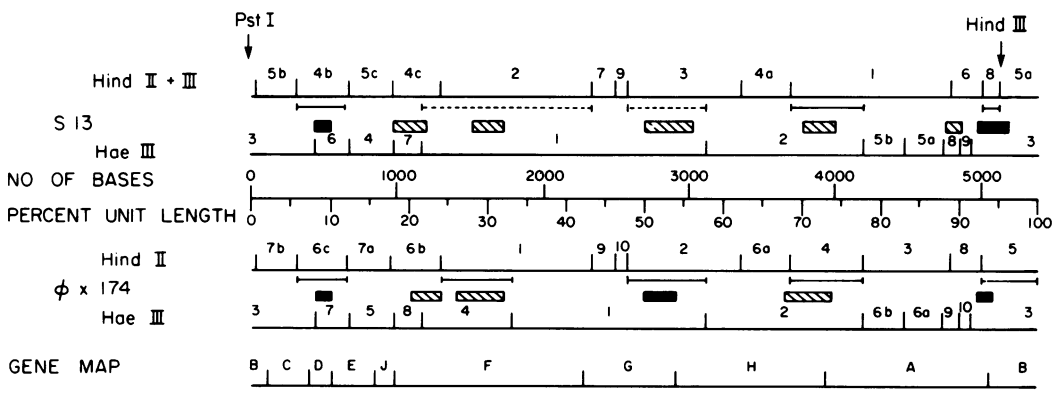

FIG. 3. *Schematic presentation of the E. coli RNA polymerase binding sites on S13 and φX174 RF DNAs in relation to the Hind and Hae III restriction endonuclease cleavage maps and the genetic map. Symbols: (⊢⊣) strong binding sites and (⊢---⊣) weak binding sites from restriction fragment binding data; (■) strong binding sites and (▨) weak binding sites from electron microscopy data (see Fig. 2). As for the orientation of the viral strand, 5′ → 3′ is left to right, and the origin is the Pst I cleavage site.*

aged holoenzyme in the polymerase preparation (9).

Figure 3 reveals that in bacteriophage S13 the two strong binding sites are located at the beginning of the D/E gene region and at the beginning of the B gene, corresponding to sites identified in the restriction fragment binding study (21). Of the four minor sites, one is located at the beginning of the A gene and also corresponds to a site located in the restriction fragment binding study. Two of the sites, one around 30% and one between 50 and 56%, are located in the *Hae* 1 and *Hin*d2 and 3 areas of S13 which showed higher-than-background binding of polymerase but which was not sufficient for them to be identified as RNA polymerase binding sites. The site between 50 and 56% overlaps the beginning of the H gene, but there is strong evidence that the F, G, and H genes are transcribed polycistronically in S13 (19); thus, it is doubtful that this location is a promoter site. The site around 20% does not overlap with any restriction fragment which bound RNA polymerase. These areas on S13 may have sequence characteristics which facilitate affinity for polymerase at low salt concentrations but not at 0.2 to 0.3 M KCl.

On bacteriophage φX174 DNA, three strong binding sites were observed. These are located at the beginnings of the D/E genes, the H gene, and the B gene and are almost in equivalent locations to sites found in S13 (21). Three other sites were observed on φX174 DNA; two correspond to equivalent sites on S13, at 70 to 74 and about 30%, and the remaining site, at 20 to 24%, does overlap with an S13 site in the 20% region but is shifted in the 5′ → 3′ direction and, thus, is not equivalent. It is this site which does not correlate with any restriction fragment polymerase binding site in S13 or in φX174. The other five sites in φX174 DNA all correspond exactly in location with the five polymerase binding sites observed in the restriction fragment binding study (21).

The similarities in the binding data between the two phages are closer than those found in the previous restriction fragment binding study. However, the site on S13 DNA at 88 to 90% has no equivalent in φX174 DNA. The extent of binding at the 50% region is apparently greater in φX174 than on S13 DNA, but if the total numbers of molecules bound in the equivalent areas are compared the observation is reversed.

The site on both phage DNAs at the beginning of the A gene (~70%), identified as a major site in the restriction fragment binding study, does not appear to be a strong binding site under the conditions used in the present study. The low salt concentration of the binding buffer in the present experiments was similar to that used by Chen et al. (7) in their study of RNA polymerase binding to restriction fragments of φX174 DNA in which approximately the same binding site was identified. Chen et al. (7) also identified sites at 50 and at ~6%. This latter site is close to the ~10% site but does not coincide with it. However, the ~10% site agrees with the transcription data of Axelrod (1). The discrepancy at this site between the binding data of Chen et al. (7) and transcript hybridization studies (1, 23) has been discussed elsewhere (21). Chen et al. (7) did not identify the minor sites at ~20 and 30%.

Transcriptional activity in the icosahedral phages is affected by the conformational state of the DNA. The present studies have used the linear relaxed RFIII form, and, although the differences between the previous study on restriction fragments (21) have been attributed to salt effects, there could be substantial differences between the present results and the status in the living cell, where the supercoiled RFI form is the template for transcription.

Although there are discrepancies between the data from electron microscope studies and restriction fragment binding studies (21), the three putative promoter sites identified on S13, at the beginnings of genes A and B and overlapping gene D and the start of E, are partially confirmed by the present study, and the D/E site is more precisely located at the start of gene D.

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