Physicochemical Studies on Interactions between DNA and RNA Polymerase. Isolation and Mapping of a T7 DNA Fragment Containing the Early Promoters for *Escherichia coli* RNA Polymerase†

Tao-shih Hsieh and James C. Wang*

ABSTRACT: The cleavage sites in the early promoter region of coliphage T7 have been mapped for four restriction enzymes. They are, from the left end in base pairs, 1100 and 740 for *Hinf;* 680, 620, 530, 240, 77, and 67 for *Hind II;* 620 and 530 for *Hpa II;* 790 for *Alu I.* The nucleotide sequence between the *Hind II* site at 680 base pairs from the left end and the *Hinf* site at 740 base pairs from the left end has been determined, from which the start point of the promoter A3 is located at 720 base pairs from the left end. The start points of the other two major promoters A1 and A2 are deduced to be at 460 and 580 base pairs from the left end, respectively, from the chain lengths of the in vitro transcripts off the 1100 base-pairs long *Hinf* fragment. Similar to the sequences of a p_L and p_R promoters of phage λ and a sequence in Simian Virus 40 used by *Escherichia coli* RNA polymerase as a promoter, the sequence of the A3 promoter of T7 also has a *Hind II* restriction site approximately 30 base pairs upstream to the start point of RNA synthesis. No such *Hind II* sites exist, however, for the A1 and A2 promoters. Experiments on the protection of some of the restriction sites on the 1100 base-pairs-long *Hinf* fragment by RNA polymerase binding support the electron microscopic observations of others that, in addition to the three sites A1, A2, and A3, there is at least a fourth site at which *E. coli* RNA polymerase can bind strongly. In addition to the *Hind II* site at 680 base pairs from the left end and the *Hinf* site at 740 base pairs from the left end, which are presumably protected by the binding of a single RNA polymerase at the A3 site, the *Hind II* site at 240 base pairs from the left end is also protected at a level of 5 polymerase molecules/DNA fragment. The possible existence of several minor promoter sites in the early promoter region, in addition to the three major promoter sites, is discussed.

The transcription of DNA by RNA polymerase has been studied extensively in the past decade and a number of reviews on this subject have appeared recently (Burgess, 1971; Losick, 1972; Bautz, 1972; Chamberlin, 1974). RNA polymerase can bind virtually to all sequences of a double-stranded DNA. Only certain unique sequences are used in vivo, however, as initiation sites for transcription. Under proper conditions, these in vivo promoters are also the major initiation sites in vitro with the purified enzyme.

Studies on the binding of RNA polymerase to the promoter sites have been hindered by two major factors. On the one hand, the presence of a large number of nonspecific binding sites complicates the measurements; on the other hand, few physicochemical methods are available which can be used to monitor interactions between two macromolecular species. Thus, one important approach is to isolate short DNA fragments containing the promoter sequences. Several groups have obtained such fragments by digesting exhaustively stable complexes formed between RNA polymerase and DNA with a nuclease (Heyden et al., 1972, 1975; LeTalaer et al., 1973; Schaller et al., 1975; Sugimoto et al., 1975; Walz and Pirrotta, 1975; Pribnow, 1975a,b). Typically, a DNA fragment approximately 40 base pairs long is protected by an *E. coli* RNA polymerase molecule against nuclease digestion. In several cases, the isolated polymerase–DNA fragment complex has been shown to be capable of one round of RNA synthesis upon the addition of ribonucleoside triphosphates, yielding a short messenger RNA fragmert (Walz and Pirrotta, 1975; Heyden et al., 1975; Pribnow, 1975a,b). The sequence of this short RNA fragment is the same as the beginning sequence of the RNA transcribed from the same promoter before digestion with the nuclease (Heyden et al., 1975; Pribnow, 1975a,b). It appears, however, that once the polymerase is dissociated from the DNA fragment, it cannot form the specific complex with the DNA fragment again (Walz and Pirrotta, 1975; Pribnow, 1975a,b; Heyden et al., 1975). Therefore, although such fragments have provided much information on the base sequences of the promoter sites, they are less useful for studying specific interactions between DNA and RNA polymerase.

With the availability of a large number of restriction enzymes, which cleave DNA at unique sequences, it became apparent that suitable DNA fragments could be obtained by the use of such enzymes. We have chosen the early promoter region of coliphage T7 DNA for our initial studies for a number of reasons. T7 DNA can be obtained in large amounts. It is also known that the early promoters, or initiation sites used by the host enzyme in the synthesis of early transcripts of the viral genome, are located close to one end of the viral DNA (Davis and Hyman, 1970; Delius et al., 1973; Dunn and Studier, 1973; Minkley and Pribnow, 1973). As we will describe later, these facts facilitate the mapping of the restriction sites and the isolation of fragments containing the early promoters in quantity. Furthermore, transcription from these promoters is probably not under the control of regulatory proteins. Thus, physicochemical studies with these promoters might be simpler and might lay the ground work for studies on the more complex promoters which are under the control of regulatory proteins. Finally, there are several interesting features of the early promoter region of T7 DNA. The high specificity of the early promoters in vitro, for the binding of *E. coli* RNA polymerase molecules, as well as for initiation of transcription, has been

† From the Chemistry Department, University of California, Berkeley, California 94720. *Received June 14, 1976.* This work was supported by Grant GM 14621 from the United States Public Health Service. This paper is the first of a series from this laboratory.

shown by a number of laboratories (Davis and Hyman, 1970; Dunn and Studier, 1973; Minkley and Pribnow, 1973; Chamberlin and Ring, 1972; Hinkle and Chamberlin, 1972; Bordier and Dubochet, 1974; Portmann et al., 1974; Dausse et al., 1976). Transcription studies and sequence analyses show that there are three independent and different promoters governing the synthesis of the early transcript (Dunn and Studier, 1973; Minkley and Pribnow, 1973). Results obtained by electron microscopy also indicate that there are at least three strong *E. coli* RNA polymerase binding sites in the early promoter region (Bordier and Dubochet, 1974; Portmann et al., 1974; Darlix and Dausse, 1975). The existence of a fourth strong binding site in this region has also been reported (Portmann et al., 1974). The presence of a multitude of sites with different structures adds an interesting dimension to studies on their interactions with the polymerase molecules.

Materials and Methods

Chemicals. Rifampicin (Rifampin) was from Calbiochem. Nucleoside triphosphates were from P-L Biochemicals. [γ-^{32}P]ATP[1] was prepared according to Glynn and Chappell (1964). [α-^{32}P]ATP was obtained from New England Nuclear. Reagents for the preparation of polyacrylamide gel were purchased from Bio-Rad.

DNA and Enzymes. Wild-type phage T7 was grown on *E. coli* B. The cell lysate was processed according to Yamamoto et al. (1970), except that the CsCl density gradient centrifugation step was done at 35 000 rpm for 24 h. The phage band was collected, dialyzed against 5 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.25 M NaCl, and extracted with buffer-saturated phenol, which had been distilled under nitrogen. The DNA was dialyzed into 10 mM Tris-HCl (pH 8), 50 mM KCl, 0.1 mM Na_3 EDTA for storage.

E. coli RNA polymerase was purified according to the procedures of Burgess and Travers (1971) with some modifications. After the Agarose A-5m gel filtration step, the holoenzyme was fractionated by chromatography on a phosphocellulose column in the presence of 50% glycerol (Gonzales and Chamberlin, personal communication). Fractions containing the holoenzyme were pooled and concentrated by a small DE-52 DEAE-cellulose column. The enzyme was stored in 55% glycerol, 10 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.2 M KCl at −20 °C. Sodium dodecyl sulfate gel electrophoresis was performed according to Maizel (1969). The homogeneity of holoenzyme was established by the following two criteria: (1) no polypeptide band, other than β, β', σ, and α, was present within our detection limit; (2) the relative intensities of the Coomasie blue stained bands gave the expected stoichiometry for the holoenzyme.

Restriction endonucleases *Hind* and *Hinf* were purified, respectively, from *Haemophilus influenzae* serotype d and *Haemophilus influenzae* serotype f. The published procedure of Smith (1974) was followed in these purifications. *Hpa I, Hpa II, Alu I,* and many other restriction enzymes used in the beginning stage of this work were kindly provided to us by Dr. M. Botchan. *Hind III* was a gift of Dr. J. Mertz and Mr. S. Goff. *Eco* RI, *E. coli* alkaline phosphatase, and T4 polynucleotide kinase were generous gifts of Dr. P. Modrich. T4 DNA polymerase was kindly provided by Dr. I. R. Lehman.

Terminal Labeling of DNA. The 3′-terminal nucleotide of a DNA fragment can be specifically labeled by the exchange reaction catalyzed by T4 DNA polymerase (Englund, 1971) or *E. coli* DNA polymerase I (Donelson and Wu, 1972). A typical terminal labeling experiment using T4 DNA polymerase is as follows. A 200-μl reaction mixture, containing 70 mM Tris-HCl (pH 8), 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 59 nmol of each of the four deoxyribonucleoside triphosphates (with dATP ^{3}H-labeled at a specific activity of 13 Ci/mmol), and 33 μg of T7 DNA, was incubated with 40 units of T4 DNA polymerase at 11 °C for 80 min. The reaction mixture was then phenol extracted and dialyzed into a medium containing 10 mM Tris-HCl (pH 8), 20 mM NaCl, and 7 mM $MgCl_2$.

The 5′ termini of a DNA fragment can be labeled with ^{32}P by successive treatments with bacterial alkaline phosphatase and T5 polynucleotide kinase (Weiss et al., 1968). Terminal phosphate groups were first removed by incubating 20 μg of T7 DNA with 5 μg of bacterial alkaline phosphatase in 1 ml of solution containing 10 mM Tris-HCl (pH 8), 50 mM KCl, and 0.1 mM Na_3EDTA (TKE). After 40-min incubation at 37 °C, the sample was phenol extracted and then ether extracted. Residual ether was blown away by a jet of N_2. The solution was then made to be in TKE plus 10 mM $MgCl_2$ plus 8 mM dithiothreitol plus 5% glycerol, and was incubated at 37 °C for 20 min with 0.6 nmol of [γ-^{32}P]ATP and 2 units of T4 polynucleotide kinase. The reaction mixture was phenol extracted and dialyzed into TKE.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in a slab-gel apparatus using either Tris-acetate–EDTA buffer (Loening, 1967) or Tris-borate–EDTA buffer (Peacock and Dingman, 1969). The dimensions of the slab gel and the percentage of acrylamide were selected depending upon the amount and the molecular-weight range of the DNA sample. To resolve DNA fragments with chain lengths ranging between 100 and 1000 base-pairs long, 4% polyacrylamide gel was used. The *Hind* digests of SV 40 DNA (Danna and Nathans, 1971) and λ DNA (Maniatis et al., 1975) were used as molecular weights standards. After electrophoresis, the gel was immersed in the electrophoresis buffer containing 0.6 μg/ml of ethidium bromide at 4 °C for 30 min. The gel was placed on top of a short-wavelength UV light source (Ultraviolet Products, Inc., Model C51) and photographed on Polaroid type 105 film through an orange filter. For the gel with ^{32}P-labeled samples, the gel was wrapped in a thin polyethylene sheet (Glad Wrap, Union Carbide) and radioautographed at 4 °C using Kodak No-Screen x-ray film. To detect ^{3}H-labeled DNA, the gel was cut into 1.5-mm thick slices and each slice was incubated with a tissue solubilizer NCS (Amersham/Searle) and counted according to the procedures recommended by the manufacturer of the solubilizer.

Isolation of DNA Fragment by Gel Electrophoresis. To scale up the amount of DNA that could be run on the slab gel electrophoresis for preparative purpose, a slab gel apparatus which could accommodate a 40 × 22 × 0.6 cm gel was constructed. After the polyacrylamide was polymerized in the slab, it was preelectrophoresed at 1 V/cm for 6 h without a sample loaded on. The electrophoresis buffer in the anode chamber was drained and replenished with the fresh solution. The cathode and anode chambers were then connected by tubings through a pump so that the electrophoresis buffer could be circulated.

For isolating the DNA fragment (*Hinf* T7)$_{1100}$ (see text), a *Hinf* digest containing as much as 5 mg of DNA could be loaded on this gel in a single electrophoresis run. The electrophoresis was performed at room temperature and 1 V/cm for

[1] Abbreviations used are: DEAE, diethylaminolethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATP, UTP, CTP, GTP, adenosine, uridine, cytidine, and guanosine triphosphates.

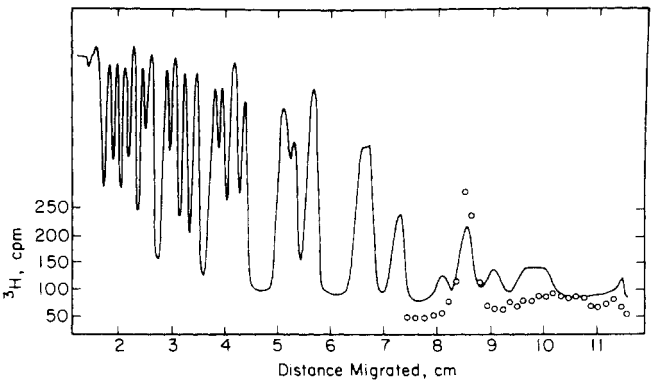

FIGURE 1: Electrophoretic pattern of [^{3}H]deoxyadenosine terminally labeled T7 DNA digested by *Hind II*. A limit digest of terminally labeled T7 DNA by a restriction endonuclease *Hind II* was run on a 4% polyacrylamide gel. The gel was stained, photographed, sliced, and counted as described under Materials and Methods. The curve shown is the tracing of the negative by a Joyce-Loebl microdensitometer. The circles give the ^{3}H counts of the slices. ^{3}H counts of slices from the upper part of the gel were not significantly above the background (ca. 45 cpm), and were not shown in the figure.

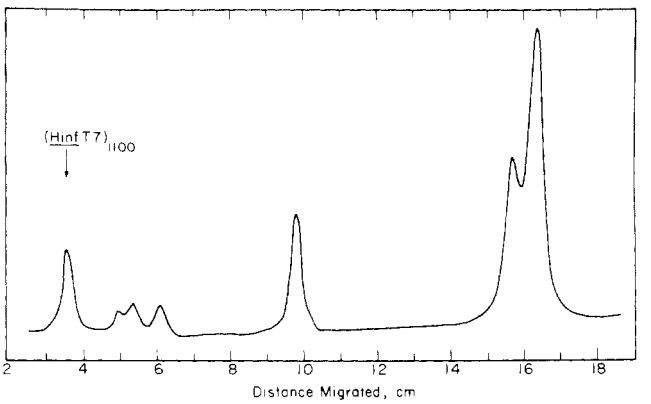

FIGURE 2:Mapping of the *Hind II* restriction sites on (*Hinf* T7)$_{1100}$, an 1100 base-pairs long *Hinf* restriction fragment containing the early promoter region of T7 DNA. One 5′ terminus of the fragment had been labeled with ^{32}P. A partial *Hind II* digest of this terminally labeled fragment was electrophoresed on a 4% polyacrylamide gel. The gel was radioautographed and the negative was traced with a Joyce-Loebl microdensitometer.

44 h. The gel was stained with ethidium as described in the previous section. The DNA bands were visualized by illuminating with a small UV light source (Ultraviolet Products, Inc., Model UVS 12). To minimize photochemical damage to the DNA, the bulk part of the gel was covered and only a thin strip of the gel was illuminated to allow visualization of the stained bands.

The slice of the gel containing the desired DNA band was homogenized with a Dounce homogenizer and extracted with an equal volume of 0.1 M sodium phosphate buffer (pH 6.8) three times. After spinning in a clinical centrifuge, the supernatant was loaded unto a hydroxlapatite column. After washing with 2 column volumes of 0.1 M sodium phosphate, the DNA was eluted with 0.5 M sodium phosphate (pH 6.8). The DNA thus purified was free from detectable amount of acrylamide or ethidium.

In Vitro Transcription from the DNA Fragments. In 50 μl of a medium containing 10 mM Tris-HCl (pH 8), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, 0.12 μg of (*Hinf* T7)$_{1100}$ was incubated with RNA polymerase at a molar ratio of 5 polymerase molecules/DNA fragment. After 10 min at 37 °C, 50 μl of a triphosphates stock solution was added to give a final concentration of 0.5 mM each of UTP, GTP, CTP, and 0.2 mM of [α-^{32}P]ATP (3 Ci/mmol). The reaction mixture was further incubated at 37 °C for 12 min. Transcription was terminated by the addition of 20 μl of a mixture containing 0.1 M Na_3EDTA, 8% sodium acetate, and 0.15 mg/ml of tRNA. The nucleic acids were then precipitated by adding 0.5 ml of absolute ethanol. After vortexing, the mixture was allowed to stand overnight at −20 °C. the mixture was vortexed again, centrifuged, and the supernatant was decanted. After washing once with 200 μl of 80% ethanol, the pellet was resuspended in 30 μl of 10 mM Tris-HCl, 5 mM NaCl, 7 M urea (Schwartz/Mann Ultrapure). Before loading on a 3% polyacrylamide slab gel (24 × 13 × 0.1 cm) in a buffer of 50 mM Tris-borate (pH 8.2), 1 mM Na_3EDTA, 7 M urea, the sample was heated in a boiling water bath for 1 min, and 6 μl of a solution containing 30% sucrose, 0.05% xylene cyanol, and 0.05% bromophenol blue was added. Electrophoresis was done at room temperature and 3 V/cm for 6 h. The gel was radioautographed at 4 °C, as described before. Satisfactory radioautograms could usually be obtained in 6 h. Broadening of bands by diffusion was insignificant for at least 1 week under these conditions.

Results

Selection of Restriction Enzymes and Preliminary Mapping of Restriction Sites in the Early Promoter Region of T7 DNA. As we have mentioned in the introductory section, the early promoters of T7 DNA are located in a region close to the left terminus. The restriction sites close to the left terminus can be mapped by the following method. If the left terminus of T7 DNA is labeled specifically, upon digestion of the DNA to completion with a restriction enzyme only the left terminal fragment is labeled. The size of the labeled fragment gives the position of the leftmost restriction site. If digestion is not carried out to completion, several sites close to the left terminus of the DNA can be mapped. Since the left 3′-terminal base of T7 DNA is an adenine and the right 3′-terminal base is a thymine (Weiss and Richardson, 1967; Englund, 1972), the left 3′ terminus can be labeled specifically by the T4 DNA polymerase catalyzed exchange reaction in the presence of labeled dATP and three other unlabeled deoxyribonucleoside triphosphates (Englund, 1971). Figure 1 depicts the result of a typical experiment. Intact T7 DNA was first labeled at the left 3′ end with [^{3}H]dATP and then digested to completion with a restriction enzyme *Hind II*. The digest was electrophoresed on acrylamide gel and stained with ethidium, and a fluorescence photograph of the stained gel was taken. The gel was then sliced into thin sections and each section was counted for ^{3}H. As shown in the figure, the position of the ^{3}H-labeled band corresponds to a fragment approximately 70 base-pairs long. Thus, the leftmost *Hind II* restriction site is approximately 70 base pairs from the left end of the DNA molecule. Similar experiments were done with several other restriction enzymes.

From these results, we chose to use the restriction enzyme *Hinf* for the isolation of a fragment containing the early promoter region. The leftmost restriction site for this enzyme is located at 740 base pairs from the left end of T7 DNA. If the left terminally labeled DNA is first incubated with RNA polymerase at a level of several polymerase molecules per T7 DNA, there is almost no cleavage at the 740 base pairs site by *Hinf*. Instead, radioactivity is found in an 1100 base-pairs long fragment. This 1100 base-pairs long fragment is the largest

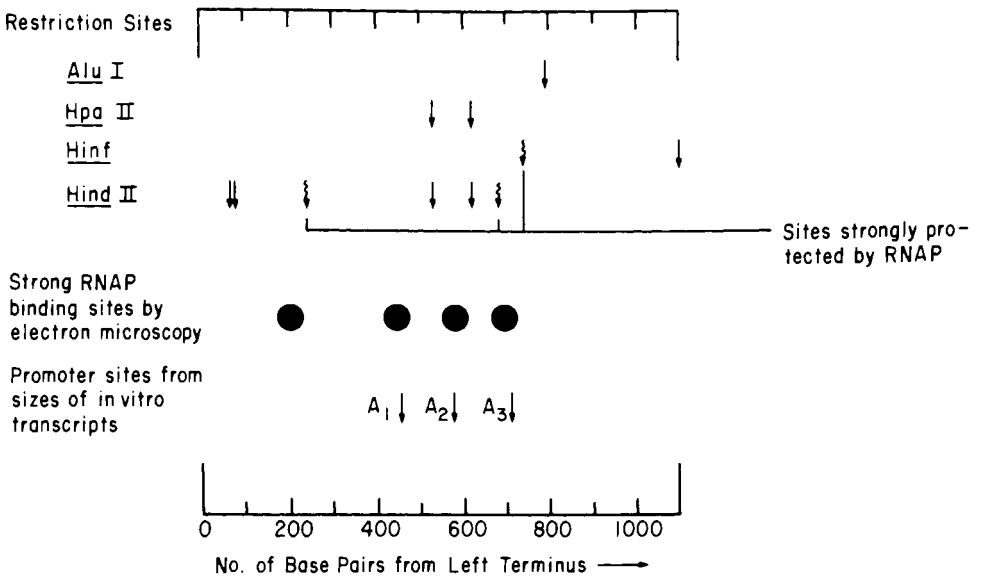

FIGURE 3: A summary of restriction sites, and positions of the major promoters and strong RNA polymerase binding sites in the early promoter region of phage T7 DNA. The positions of the strong RNA polymerase binding sites were measured by electron microscopy by J. Hirsch and R. Schleif (personal communication). All other results are from this work. See text for details.

one in the digest, and is well resolved from all other fragments upon gel electrophoresis. Therefore, this fragment, designated as (*Hinf* T7)$_{1100}$, can be isolated in quantity and was used in most of the subsequent work described in this communication.

Mapping of Restriction Sites of (Hinf T7)$_{1100}$. We have used a simple technique to give the positions of all cleavage sites by a restriction enzyme in a single experiment. Intact T7 DNA was first labeled at the 5′ ends with ^{32}P by a cycle of phosphatase and polynucleotide kinase treatments. During the kinase treatment, (cf. Materials and Methods) [γ-^{32}P] ATP of high specific activity (ca. 10^3 Ci/mmol) was used. The (*Hinf* T7)$_{1100}$ fragment was then obtained from the terminally labeled DNA, as described in the above section, and digested lightly with a second restriction enzyme. Figure 2 illustrates the results of such an experiment with *Hind II*. The *Hind II* digest of the (*Hinf* T7)$_{1100}$ fragment, which had the ^{32}P labeled at the left 5′ end, was electrophoresed on acrylamide gel and a radioautogram of the gel was taken. The sizes of the labeled fragments determined from the electrophoretic mobilities of these fragments give immediately the positions of the restriction sites of *Hind II*. From the results shown in Figure 2, there are six *Hind II* cleavage sites (*Hinf* T7)$_{1100}$, located at 67, 77, 240, 530, 620, and 680 base pairs from the left end of T7 DNA.

It appears that the six *Hind II* sites are not identical. For a fragment labeled at the left end, if the cleavage sites are numbered successively 1, 2, . . . i . . . from left to right and the probabilities for cleavage to occur at these sites are p_1, p_2, . . . p_i . . . , respectively, the probability of obtaining a fragment spanning the labeled end and the ith cleavage site is $p_i(1-p_1)(1-p_2)\ldots(1-p_{i-1})$, assuming that cleavage at any given site is an independent event. The relative areas under the bands shown in Figure 2 are inconsistent with all p_i's being equal. It also appears that the relative values of the p_i's are dependent on the conditions of digestion. A detailed analysis will be presented elsewhere.

Experiments identical to the one described above for *Hind II* were also done for several other restriction enzymes. There is no cleavage site on (*Hinf* T7)$_{1100}$ for *Eco RI* (at either high or low salt concentration), *Hpa I, Hind III, Mbo I, Mbo II,* and *Hae III*. The mapping results for all other restriction enzymes used are shown in Figure 3.

The cleavage sites of *Hpa II* at 530 and 620 base pairs from the left terminus are very close to two of the cleavage sites of *Hind II*, as shown in Figure 3. This is also confirmed by the following experiment. (*Hinf* T7)$_{1100}$ was digested separately with *Hpa II* and *Hind II* or successively with the two enzymes. Identical gel electrophoretic patterns were obtained for the sample digested with *Hind II* only, the sample digested successively with *Hpa II* and *Hind II*, and a mixture of the above two samples.

Positions of the Strong E. coli RNA Polymerase Binding Sites on (Hinf T7)$_{1100}$. Measurements by electron microscopy indicate that there are at least three strong *E. coli* RNA polymerase binding sites close to the left terminus of T7 DNA. Taking the total number of base pairs per T7 DNA as 38 000 (Freifelder, 1970; Schmid and Hearst, 1971) the positions of the binding sites in number of base pairs from the left terminus are calculated to be 440, 540, and 630 from the measurements of Bordier and Dubochet (1974). Nearly identical results have been obtained by Darlix and Dausse (1975). Portmann et al. (1974) have reported that there are at least four strong binding sites at 220, 440, 570, and 700 base pairs from the left end. Electron microscopic measurements of Hirsch and Schlief (personal communication) with the (*Hinf* T7)$_{1100}$ fragment prepared by us are in excellent agreement with the results of Portmann et al. (1974) obtained with whole T7 DNA. Four sites at 200, 470, 570, and 700 base pairs from one end of the fragment were observed, with an estimated error of ±40 base pairs.

The positions of two of the strong binding sites obtained by electron microscopy can also be deduced from the effects of *E. coli* RNA polymerase binding on cleavage by restriction enzymes. We have already mentioned that the *Hinf* site at 740 base pairs from the left terminus is strongly protected by *E. coli* RNA polymerase. The *Hind II* cleavage sites at 240 and 680 base pairs from the left terminus are also protected by RNA polymerase, as shown by the following experiments with unlabeled (*Hinf* T7)$_{1100}$.

The DNA fragment was first incubated for 10 min at 37 °C with *E. coli* RNA polymerase at an approximate ratio of 5

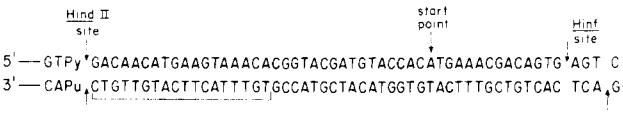

FIGURE 4: Sequence of the A3 promoter region of T7 DNA. The first three base pairs (from the left) are deduced from the known specificity of *Hind II*. The sequence underlined was obtained as described in the text. The rest of the sequence is taken from Pribnow (1975a,b).

polymerase molecules/DNA fragment, in a medium containing 10 mM Tris-HCl (pH 8), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. *Hind II* was then added and the mixture was incubated at 37 °C for 12 h. Gel electrophoresis of the digest was run after phenol extraction. Four fragments 480, 450, 90, and ~70 base-pairs long were obtained. The 480 and 450 base-pairs long fragments were isolated from the gel and digested again with *Hind II*. It was found that digestion of the 480 base pairs long fragment with *Hind II* gave two bands 60 and 420 base pairs long. This shows, by comparing with the *Hind II* sites shown in Figure 3, that the site at 680 base pairs from the left terminus is protected by RNA polymerase, as the 420 base-pair long fragment could only come from cleavage at this site. Similarly, digestion of the 450 base-pairs long fragment gave two bands 160 and 290 base-pairs long, which shows that the *Hind II* site at 240 base pairs from the left terminus is also protected by RNA polymerase. At the ratio of RNA polymerase to DNA used in the above experiments, no protection was observed at *Hind II* sites other than the sites at 240 and 680 base pairs from the left terminus. This is also supported by the observation that no protection was observed for the two *Hpa II* sites shown in Figure 3 under similar conditions.

The Location and the Nucleotide Sequence of the Promoter A3. Both in vivo and in vitro results show that there are three strong promoters, designated A1, A2, and A3, in a region close to the left terminus of T7 DNA (Dunn and Studier, 1973; Minkley and Pribnow, 1973). These promoters are spaced approximately 150 base pairs apart (Dunn and Studier, 1973; Minkley and Pribnow, 1973; Pribnow, 1975b). From the lengths of the transcripts it was deduced that the promoters A1, A2, and A3 were located approximately 200, 380, and 570 base pairs from the left terminus, respectively (Dunn and Studier, 1973).

Several lines of evidence clearly show that the initiation point in A3, i.e., the point at which RNA chain initiates at A3 promoter, is located at about 720 base pairs from the left terminus. Pribnow (1975a,b) has determined the nucleotide sequence of a fragment from the T7 A3 promoter site which is protected from DNase I digestion by *E. coli* RNA polymerase. Examination of the sequence shows the presence of the pentameric sequence 5'GAGTC3', located 13 base pairs downstream from the initiation point. Since this pentameric sequence is recognized by *Hinf* (R. Roberts, personal communication), the position of this sequence must coincide with the *Hinf* site at 740 base pairs from the left terminus. This places the initiation point in A3 at about 720 base pairs from the left terminus. The presence of the *Hinf* site within the region of A3 protected by RNA polymerase against digestion by DNase I is in complete accord with our observation that RNA polymerase binding prevents cleavage at the *Hinf* site at 740 base pairs from the left terminus.

We have also determined part of the nucleotide sequence to the right of the *Hind II* site at 680 base pairs from the left terminus. The fragment (*Hinf* T7)$_{1100}$ was first digested with *Hind II*, and the rightmost 420 base-pairs long fragment was isolated. This fragment was labeled with ^{32}P at the 5' ends by one cycle of alkaline phosphatase and polynucleotide kinase treatments, and then digested with *Hinf*. The smaller fragment of the *Hinf* digest, approximately 60 base-pairs long and ^{32}P labeled at the 5' terminus of the *Hind* generated end, was used in sequencing analysis according to the procedures of Maniatis et al. (1975). The fragment labeled at one end was digested partially with a combination of pancreatic DNase I and snake venom exonuclease, and the digest was fractionated by electrophoresis at pH 3.5 on cellulose acetate in the first dimension and by homochromatography on DEAE-cellulose in the second dimension. From the autoradiogram of the two-dimensional analysis the terminal sequence of the fragment was found to be 5'GACAACATGAAGTAAACA. The beginning 5' triplet is consistent with the fact that this end was generated by *Hind II*, which is known to cleave at 5'GTPyPuAC to give a 5' triplet of PuAC (Kelly and Smith, 1970). The right half of the sequence 5'GAAGTAAACA overlaps with the published sequence of the RNA polymerase protected fragment in the A3 region (Pribnow, 1975a,b).

A combination of these sequencing results is shown in Figure 4. From the nucleotide sequence, the fragment bracketed by the *Hind II* site at 680 base pairs from the left terminus and the *Hinf* site at 740 base pairs from the left terminus contains 48 base pairs plus 3 nucleotides at the *Hinf* end. The size of fragment based on the difference in the positions of these restriction sites is 60 base pairs, which is in reasonable agreement with the sequencing results, as the error in the difference between two large quantities is substantial.

The sequence shown in Figure 4 strongly suggests that the prevention of cleavages by *Hind II* at 680 base pairs from the left terminus and by *Hinf* at 740 base pairs from the left terminus by RNA polymerase binding is due to the binding of a single polymerase molecule at the A3 site.

Locations of Promoters A1 and A2. A radioautogram of the gel electrophoresis pattern of in vitro transcripts off (*Hinf* T7)$_{1100}$ is shown in Figure 5a. The lengths of the three major transcripts were calculated from their electrophoretic mobilities in an acrylamide–urea gel using *Hind II* and *Hinf* digested (*Hinf* T7)$_{1100}$ as length standards. From the plot shown in Figure 5b, the lengths of the three major transcripts are 645, 525, and 390 nucleotides.

Taking the starting point of the A3 transcript as 720 base pairs from the left terminus, the length of the transcript off (*Hinf* T7)$_{1100}$ is expected to be 1100–720 or 390 nucleotides, assuming that transcription terminates when the enzyme runs off the end of a DNA fragment (Pribnow, 1975a,b; Heyden et al., 1975; Walz and Pirrotta, 1975). This suggests that the 390 nucleotides-long major in vitro transcript observed is the A3 transcript. This assignment is supported by experiments similar to the one described in the legend to Figure 5a, except that the presynthesis incubation step was carried out at lower temperatures. Figure 5c illustrates the result of a typical experiment with the presynthesis incubation step carried out at 4 °C. The predominant transcript of (*Hinf* T7)$_{1100}$ at this low temperature is the 390 nucleotides long species. Therefore the assignment of the 390 nucleotides long species as the rightward A3 transcript is in agreement with the results of Dausse et al. (1976) that at low temperatures initiation of transcription occurs predominantly at A3.

Since the promoters A1 and A2 are known to be located approximately 300 and 150 base pairs, respectively, upstream from A3 (Dunn and Studier, 1973; Minkley and Pribnow, 1973; Pribnow, 1975a,b), it is reasonable to assign the 645 and 525 nucleotides long transcripts as the A1 and A2 transcripts

off (*Hinf* T7)$_{1100}$. These assignments are also supported by an experiment with γ-^{32}P-labeled ATP. The γ-^{32}P label was found only in the 645 and 390 nucleotides long transcripts (data not shown). It is known that the A1 and A3 transcripts start with an A, while the A2 transcript starts with a G (Dunn and Studier, 1973).

Taking the starting point of the A3 promoter as 720 base pairs from the left terminus, from the differences in the lengths of the three transcripts, we assign the positions of the starting points in A1 and A2 to be 460 and 580 base pairs from the left terminus, respectively. These positions are also shown in Figure 3.

Aside from the three major transcripts, a number of minor in vitro transcripts off (*Hinf* T7)$_{1100}$ can also be seen. Several of these are illustrated in the radioautograms shown in Figure 5a,c,d. Depending upon the incubation conditions, the amounts of some of the minor transcripts may be higher than the amounts of the A1 and A2 transcripts (see Figure 5d).

Discussion

By starting with (*Hinf* T7)$_{1100}$, an 1100 base-pairs long *Hinf* restriction fragment of T7 DNA containing the early promoter region, we have mapped the cleavage sites of a number of restriction enzymes and the locations of the three promoters. The results summarized in Figure 3 are self-consistent and, we believe, reliable. Our mapping results on the *Hind II* sites in this region do not agree with the recent results of Ludwig and Summers (1975). Aside from minor differences in the positions of the sites due to differences in length measurements of the restriction fragments, it appears that in their published data two of the six *Hind II* sites in this region were undetected and a third site was incorrectly assigned due to an error in ordering two of the *Hind II* fragments.

The positons of the promoters deduced from the lengths of the in vitro transcripts off (*Hind* T7)$_{1100}$ are in good agreement with the strong *E. coli* RNA polymerase binding sites mapped by electron microscopy (Bordier and Dubochet, 1974; Portmann et al., 1974; Darlix and Dausse, 1975). The agreement between our results and the data of Portmann et al. (1974) is particularly striking (see Figure 3). Portmann et al., however, assigned the strong binding sites at 220, 440, and 570 base pairs from the left terminus of T7 DNA as the promoters A1, A2, and A3, respectively, and attributed the strong binding site at 700 base pairs from the left terminus as a fourth promoter. Our results show that the fourth promoter site they proposed is actually A3.

The question is then on the nature of the binding site at about 220 base pairs from the left terminus. Our observation that the *Hind II* cleavage site at 240 base pairs from the left terminus is protected by RNA polymerase at a molar ratio of approximately 5 polymerase molecules per (*Hinf* T7)$_{1100}$ fragment strongly supports the microscopy results of others (Portmann et al., 1974; Darlix and Dausse, 1975; Hirsch and Schleif, personal communications) that there is a strong *E. coli* RNA polymerase binding site at this position. In vitro transcription studies with (*Hinf* T7)$_{1100}$ indicate that this site is *not* a major initiation site for transcription. We are uncertain on whether specific chain initiation can occur at this site. A radioautogram of the same gel shown in Figure 5a, after longer exposure, reveals several minor transcripts. A minor band with a chain length around 200 nucleotides can be seen. This minor band is also present among the transcripts if a complete *Hpa II* digest of (*Hinf* T7)$_{1100}$ is used as the template. It is therefore plausible that this minor band is the leftward transcript from the binding site at around 220 base pairs from the left terminus.

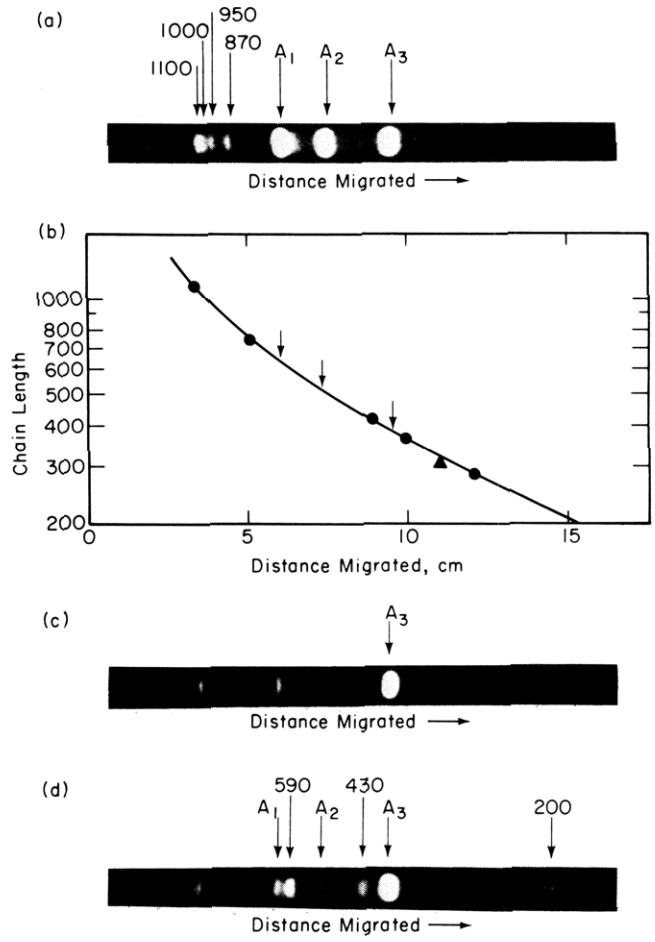

FIGURE 5: (a) A typical radioautogram of the in vitro transcripts off (*Hinf* T7)$_{1100}$ at 37 °C. RNA synthesis was carried out at a level of 5 enzyme molecules/DNA fragment, as described under Materials and Methods. The three major transcripts are from the promoters A1, A2, and A3 (see text). Four minor bands of higher molecular weights than the major transcripts can also be seen in the radioautogram. Their chain lengths are estimated to be 1100, 1000, 950, and 870 nucleotides, respectively. We have not identified these minor transcripts. From a densitometer tracing of the negative, the three major transcripts are estimated to account for about 80% of the total radioactivity. As described under Discussion, a longer exposure of the same gel also reveals a minor transcript with a chain length of about 200 nucleotides. The pattern of transcripts is not significantly different at higher enzyme to DNA ratios. If rifampicin was added to 10 μg/ml immediately before the addition of the triphosphates, the amount of synthesis was lowered by at least a factor of 2, but the pattern of the bands was not changed. (b) The calibration curve used to obtain chain lengths of transcripts from their electrophoretic mobilities in 7 M urea–3% polyacrylamide gel. DNA restriction fragments, terminally labeled with ^{32}P and heat denatured in the presence of 7 M urea, were used as standards. Filled and open circles represent data obtained from partial *Hinf* digestion and complete *Hind* digestion of (*Hinf* T7)$_{1100}$, respectively. The positions of the three major transcripts are indicated by arrows. The triangle represents the position of a marker dye xylene cyanol. (c) A radioautogram of the in vitro transcripts off (*Hinf* T7)$_{1100}$, when the enzyme and DNA mixture (molar ratio approximately 6:1) was first incubated at 4 °C for 30 min, and the temperature was shifted to 37 °C immediately *after* successive additions of triphosphates and rifampicin (to 0.5 mg/ml), as described by Dausse et al. (1976). RNA synthesis at 37 °C was terminated after 15 min and the sample was electrophoresed as described under Materials and Methods. RNA synthesis was undetectable after incubating for 1 h at 4 °C, in the absence of rifampicin. (d) A mixture of RNA polymerase and (*Hinf* T7)$_{1100}$ (molar ratio approximately 6:1) was incubated at 37 °C for 10 min, and then at 4 °C for 1 h. Triphosphates and rifampicin were added in succession, and the temperature was shifted to 37 °C for 15 min as described in c. Comparing with the pattern in c, two new bands, corresponding to chain lengths 430 and 590 nucleotides, appear between the transcripts from A2 and A3, and between the transcripts from A1 and A2. The short 200 nucleotides transcript can also be seen.

There are also longer minor transcripts off (*Hinf* T7)$_{1100}$ around 900 nucleotides long, which might be the rightward transcripts from this region. We have not attempted to identify and characterize these minor transcripts.

The nucleotide sequence of the A3 promoter shown in Figure 4 has an interesting feature in that there is a *Hind II* site 34 base pairs upstream from the initation point of the A3 transcript. The presence of a *Hind II* site approximately 30 base pairs upstream from the start of transcription has been previously noted for the p_L and p_R promoters of coliphage λ and a promoter in SV 40 DNA recognized by *E. coli* RNA polymerase (Maurer et al., 1974; Allet and Solem, 1974; Dhar et al., 1974; Maniatis et al., 1975). For the λ promoters, several promoter mutations are known to occur in the *Hind II* cleavage sites (Maurer et al., 1974). For several other promoters of known sequences, including *lac*, the p_{rm} promoter of λ, and the G2 and G3 promoters of coliphage fd, no *Hind II* cleavage site is present in the corresponding regions (Dickson et al., 1975; Sugimoto et al., 1975; Takanami et al., 1976; Kleid et al., 1976). Our results shown in Figure 3 also indicate that such a *Hind II* site is absent for the A1 and A2 promoters of T7.

The difference in the patterns of transcripts shown in Figure 5c,d is noteworthy. For the pattern shown in Figure 5c, the RNA polymerase–(*Hinf* T7)$_{1100}$ mixture was first incubated at 4 °C for 30 min. Triphosphates were then added and, 2 min later, followed by the addition of rifampicin. After the addition of rifampicin, the temperature was shifted to 37 °C for 15 min. Presumably, only those polymerase molecules which have initiated RNA synthesis at 4 °C can escape inhibition by rifampicin, and, upon incubation at 37 °C, extend the chains until the enzyme molecules run off the DNA templates. The pattern shown in Figure 5c therefore suggests that, at 4 °C, only RNA polymerase molecules bound at A3 can initiate reasonably efficiently. The experiment which gave the pattern shown in Figure 5d was identical to the one which gave the pattern shown in Figure 5c with an important exception that the enzyme–DNA mixture was first incubated at 37 °C for 15 min and then cooled to 4 °C. The difference between the patterns shown in Figure 5d,c indicates that incubation at 37 °C leads to the formation of enzyme–DNA complexes at sites in addition to A3. Such sites, at least after 1 h at 4 °C, are still capable of chain initiation when triphosphates are added. In other words, upon shifting the temperature from 37 to 4 °C, the complexes characteristic of the higher temperature do not seem to change rapidly to the complexes characteristic of the lower temperature. This kind of "hystersis" was not observed in measurements on the rate of dissociation of RNA polymerase–T7 DNA complexes (Hinkle and Chamberlin, 1972).

Acknowledgment

We are grateful to those who kindly provided us with enzymes and bacterial strains, and to Drs. D. Kleid and M. Ptashne for instructions on DNA sequencing techniques. We also thank Drs. M. Botchan, P. Sharp, and M. Chamberlin for helpful discussions, and Mr. U. Siebenlist and Dr. W. Gilbert for communicating to us their sequencing results prior to publication.

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Disruption of *Escherichia coli* Outer Membranes by EM 49. A New Membrane Active Peptide Antibiotic†

Ken S. Rosenthal,‡ Paul E. Swanson,‡ and Dan R. Storm*,§

ABSTRACT: A new peptide antibiotic, EM 49, is shown to disrupt the structure of *Escherichia coli* outer membranes and release outer membrane fragments into the surrounding media. Evidence supporting this conclusion includes EM 49 stimulated release of outer membrane phospholipids, lipopolysaccharide, and membrane fragments having a phospholipid and polypeptide composition similar to outer membranes. The density of the membrane fragments released by EM 49 was 1.22 g/cm^3, which was identical to isolated outer membranes. Approximately 10 to 15% of the *E. coli* lipopolysaccharide was released upon treatment with EM 49. Both scanning and transmission electron microscopy revealed that the antibiotic caused the formation of numerous protrusions or blebs on the surface of *E. coli*, with apparent release of membrane vesicles from the cells. Direct interaction between EM 49 and outer membranes was demonstrated using outer membranes labeled with the fluorescent dye diphenylhexatriene. Treatment of the fluorescent-labeled outer membranes with EM 49 increased fluorescence intensity and decreased polarization, indicating that the peptide perturbed outer-membrane structure. In addition, strong interactions between EM 49 and purified *E. coli* phospholipids were detected using the Hummel and Dreyer technique. Association constants between the peptide and phospholipids were approximately 10^5 M^{-1}. A model for the disruptive effect of EM 49 on outer-membrane structure is proposed in which the fatty acid chain of the antibiotic is inserted into the hydrophobic core of the membrane. This orientation would allow the polycationic, peptide portion of the antibiotic to disrupt the normal electrostatic interactions between divalent cations and components of the outer membrane. Evidence supporting this conclusion includes specific protection of *E. coli* from EM 49 by Mg^{2+} and Ca^{2+} and inhibition of EM 49 stimulated phospholipid release by these cations. Disruption of the outer membrane would allow the antibiotic to penetrate to the inner membrane, which is probably the primary killing site of EM 49.

EM 49 is a new broad spectrum antibiotic active against gram-positive and negative bacteria, as well as yeasts, fungi, and protozoa (Meyers et al., 1973a,b, 1974; Parker and Rathnum, 1973, 1975; Rosenthal and Storm, 1975). The antibiotic is a mixture of closely related peptides bearing structural resemblance to the polymyxins (Figure 1). Both families of peptides are cyclic peptide antibiotics containing a high percentage of 2,4-diaminobutyric acid with a fatty acid attached to the peptide through an amide bond. However, there are important structural differences between the polymyxins and EM 49. EM 49 is an octapeptide containing a C:10 or C:11 β-hydroxy fatty acid with no threonine residues, whereas the polymyxins are decapeptides containing a C:8 or C:9 fatty acid with 2 threonine residues/molecule. In addition, the biological properties of the polymyxins and EM 49 are significantly different. The antimicrobial spectrum of EM 49 is much broader than the polymyxins, and the antibiotics are not cross-resistant with respect to several polymyxin resistant *E. coli* strains (Meyers et al., 1974).

The mechanism for the antimicrobial activity of EM 49 has not been elucidated; however, preliminary electron microscopy studies have indicated that EM 49 caused the accumulation of numerous blebs on the outer surface of *E. coli* with release of membrane vesicles from the bacteria (Meyers et al., 1974). Data presented in this report establish that the antibiotic disrupts outer-membrane structure and releases outer-membrane fragments from *E. coli*. Furthermore, the antibiotic is shown to interact strongly with isolated *E. coli* phospholipids and outer-membrane preparations. A model for the disruptive effect of EM 49 on outer-membrane structure is proposed and discussed in terms of the antibiotic activity of these peptides against gram-negative bacteria.

Experimental Procedure

Materials

EM 49 (lot no. SQ 21, 286) as well as *E. coli* strains SC 9251, SC 9252, and SC 9253 were kindly supplied by the Squibb Institute for Medical Research. Ammonium 2-keto-3-deoxyoctanoate used for standards was supplied by Dr. H. E. Conrad. Polymyxin B sulfate was purchased from Sigma Chemical Co. Lysozyme, DNase, and RNase were obtained from Worthington. Enriched media consisted of 1% beef peptone, 0.5% NaCl, and 0.1% yeast extract at pH 7.0. Syn-

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois, 61801. *Received July 28, 1976.* This work was supported by Grant HEW PHS AI 12821 from the National Institute of Allergy and Infectious Diseases.
‡ National Institutes of Health Predoctorial Trainees.
§ Supported by Career Research Development Award AI00120-01.