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Isolation and characterization of transducing phage coding for σ subunit of *Escherichia coli* RNA polymerase

(*rpoD* and *dnaG* genes/immunoprecipitation/UV irradiation/restriction analysis/cloning)

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ABSTRACT **A transducing phage has been isolated which codes for the σ subunit of *Escherichia coli* RNA polymerase. Transducing phage were selected from *E. coli* shotgun collections of *Hin*dIII or *Sac* I fragments cloned into Charon 25, a new bacteriophage λ vector that is capable of forming lysogens at high temperature. Transduction of an *E. coli* strain carrying a temperature-sensitive mutation in the σ gene was used for the selection. The positions of restriction sites for *Sac* I, *Hin*dIII, *Xho* I, *Bgl* II, and *Kpn* I in the cloned bacterial DNA segments were determined. Phage containing the *Hin*dIII fragment complement both primase (*dna*G) and σ (*rpo*D) whereas those containing the *Sac* I fragment complement only σ. Results of analyses of the proteins made both *in vivo* after infection of UV-irradiated cells and *in vitro* in a coupled transcription/translation system suggest that a *Sac* I site separates the promoter for σ from the σ structural gene. The direction of transcription of σ was determined to be clockwise with respect to the *E. coli* genetic map.**

Escherichia coli RNA polymerase is a multisubunit enzyme composed of α, β, β′, and σ subunits. The enzyme is found in two forms: as holoenzyme ($\alpha_2\beta\beta'\sigma$), capable of selective DNA binding and initiation; and as core polymerase ($\alpha_2\beta\beta'$), capable of RNA chain elongation but not selectivity (1). Transducing phage that code for each of the subunits of *E. coli* RNA polymerase except σ have been isolated (2–4). The characterization of these transducing phage has proved to be a powerful way of analyzing detailed features of operon structure and regulation.

The σ subunit is necessary for selective DNA binding and initiation and may play a pivotal role in the regulation of transcription initiation in *E. coli* (5). The gene for σ, termed *rpo*D, is located at 66 min on the *E. coli* chromosome (6–8), very near to and clockwise from the *dna*G gene (8–10). Several σ mutants have been reported (8–11). We have characterized a new temperature-sensitive mutation in the σ gene of *E. coli*, termed *rpo*D800, which reverts infrequently ($<10^{-8}$) and results in inability to grow at 42°C (12). An *E. coli* strain containing this mutation was used to select transducing phage containing segments of *E. coli* DNA that code for the σ subunit of *E. coli* RNA polymerase. Transducing phage carrying the σ gene on either a *Hin*dIII or a *Sac* I fragment were obtained. The *Hin*dIII fragment that contains the σ gene also includes at least part of the *dna*G gene coding for primase (13), the enzyme responsible for catalyzing the polymerization of RNA primers for Okazaki fragments of the growing DNA chain. We have located the promoter and the structural gene for σ in the cloned fragments and determined the direction of transcription of the σ gene relative to the *E. coli* genome.

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MATERIALS AND METHODS

Bacteria and Phage. For selection of the transducing phage containing the σ gene, *E. coli* strain PB1*rpo*D800*mal*$^+$ containing a temperature-sensitive σ allele was used. For a complementation test for *dna*G, we used *E. coli* strain PC314 (14) containing a *dna*G^{ts} marker (obtained from Phil Carl). For UV experiments, we used *E. coli* S159 (15), a UV-sensitive strain obtained from Masayasu Nomura and a derivative of S159 lysogenic for λ*imm*21. For growth of large-scale phage stocks we used *E. coli* strain K802 (16). Growth and purification of phage and purification of phage DNA were as described (16). DNA fragments were isolated as described (17).

Shotgun collections of *E. coli* DNA were constructed by Bryan D. K. Biggers, Neil L. Drake, Tina M. Henkin, and Paula S. Henthorn as a project in the Student Prokaryotic Genetics Laboratory of the Department of Genetics (University of Wisconsin). They first constructed a new Charon phage (Ch25) containing an intact attachment site and the *c*I repressor gene of phage 21 (*imm*21). Details of construction and characterization will be reported elsewhere. *E. coli* DNA from strain DP50*sup*F (16) was digested to completion with *Hin*dIII or *Sac* I, mixed with Charon 25 DNA digested with the same restriction endonuclease, ligated overnight at 4°C with phage T4 DNA ligase, and packaged *in vitro* according to Blattner *et al.* (16, 18). The shotgun transducing phage culture was amplified by growth in liquid on *E. coli* K802. The use of this r^-m^+ strain was necessitated by the lack of K modification of DP50*sup*F DNA.

Restriction Endonuclease Digestion and Gel Electrophoresis of DNA. *Hin*dIII, *Sac* I, *Bgl* II, and *Kpn* I digestions were performed in 6 mM Tris·HCl, pH 7.4/6 mM $MgCl_2$/6 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml/20 mM NaCl. *Eco*RI and *Xho* I digestions were in the same buffer containing 150 mM NaCl. Fragments of DNA were separated on horizontal 0.5 or 1.0% agarose slab gels (19, 20) in 40 mM Tris base adjusted to pH 8.1 with acetic acid/20 mM sodium acetate/2 mM EDTA/0.2 μg of ethidium bromide per ml. This buffer was recirculated during electrophoresis.

Immunoprecipitation. Serum of rabbits immunized with *E. coli* RNA polymerase holoenzyme purified as described (21, 22) was the source of RNA polymerase antibody. Immunoprecipitations were performed in 0.6% sodium dodecyl sulfate ($NaDodSO_4$) so that each subunit could be precipitated independently of the others. Radioactive samples were incubated with enough antiserum to give complete precipitation and were kept on ice for 60–90 min to allow formation of the primary antigen–antibody complex. *Staphylococcus aureus* ghosts bearing protein A (Pansorbin, Calbiochem), which binds to the Fc portion of the antibody and precipitates the com-

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; kb, kilobase(s).

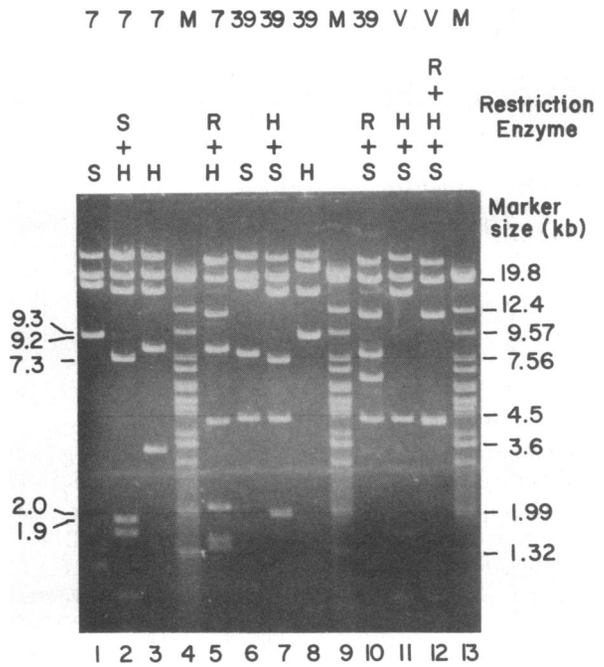

FIG. 1. Restriction enzyme digests of Ch25, Ch25sig-7S, and Ch25sig-39H DNA. DNA (0.2 μg) was digested with various restriction enzymes and run on 0.5% agarose gels at 1 V/cm for 12 hr to separate fragments. Fragment sizes [in kilobases (kb)] of cloned *E. coli* DNA are indicated at the left; fragment sizes of selected DNA markers are indicated at the right. The DNAs digested are indicated at the top: V, Ch25; 7, Ch25sig-7S; 39, Ch25sig-39H; M, DNA size marker mix (25). Restriction enzymes used: H, *Hin*dIII; S, *Sac* I; R, *Eco*RI.

Lanes 1–3 and 5. The sizes and orientation of the inserted *Sac* I fragments were determined by digesting Ch25sig-7S with *Sac* I only, with *Sac* I and *Hin*dIII, or with *Hin*dIII only. When Ch25sig-7S was digested with *Sac* I, 9.2-kb and 1.1-kb pieces appeared (lane 1). The 9.2-kb piece is *E. coli* DNA, but the 1.1-kb piece most likely is the 1.1-kb *Sac* I piece from the Ch25 vector (Fig. 2*a*) because it contains a *Hin*dIII cut in the same position (data not shown). Upon digestion with both *Sac* I and *Hin*dIII, the 9.2-kb *Sac* I fragment disappeared and was replaced by a 7.3-kb and a 1.9-kb piece, indicating that the 9.2-kb *Sac* I fragment has an asymmetric *Hin*dIII cut (lane 2). An additional 1.5-kb piece observed in this digest arises from vector DNA (see Fig. 2*a*). Digestion with *Hin*dIII yielded an 8.2-kb and a 3.4-kb fragment (lane 3). The fact that the smallest piece observed in this digest is 3.4 kb enables us to orient both inserted pieces with respect to the left and right arms of the vector. Because the right arm of the vector contains a *Hin*dIII cut within 0.4 kb of the *Sac* I cut, neither inserted piece can contain a *Hin*dIII cut closer to the right arm than 3.0 kb. Because the 1.1-kb fragment contains a *Hin*dIII cut, it cannot adjoin the right arm of the vector because it would give a *Hin*dIII piece smaller than the 3.4-kb piece observed. We therefore place the 1.1-kb *Sac* I piece adjoining the left arm and the 9.2-kb *Sac* I piece adjoining the right arm. The fragment sizes observed after cutting Ch25sig-7S with *Hin*dIII and *Eco*RI (lane 5) are also consistent with the arrangement of fragments depicted in Fig. 2*b*.

Lanes 6–8 and 10. Digestion of DNA with *Hin*dIII only (lane 8) or with a combination of *Hin*dIII and *Sac* I (lane 7) enabled us to determine that the inserted piece, about 9.3 kb long, contained a *Sac* I site dividing the DNA fragment into 7.3-kb and 2.0-kb pieces. Upon digestion with *Sac* I only, we found a 4.4-kb vector piece and a 7.7-kb piece from the *Hin*dIII fragment (lane 6). The position of the *Sac* I cut is determined by reference to the *Sac* I cuts in the vector DNA remaining in the phage and is depicted in Fig. 2*c*.

plexes, were then added and incubated for an additional 15–30 min on ice (23). Samples were spun for 1 min in the Beckman Microfuge; the pellets were washed twice with 0.15 M NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$/0.01 M Tris·HCl, pH 7.2/1% Trasylol. $NaDodSO_4$ sample buffer (24) was added to the pellets, and the antibody and antigen were detached from protein A in the *S. aureus* ghosts by heating at

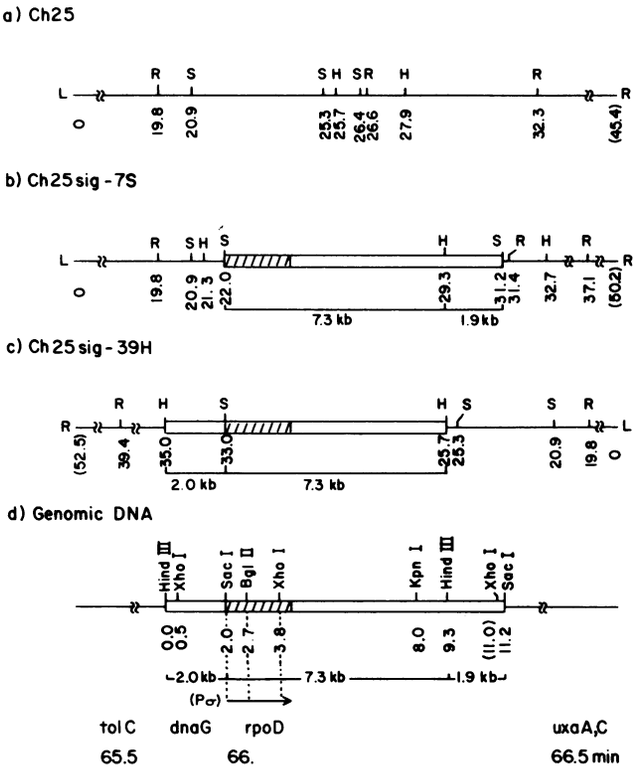

FIG. 2. Restriction maps. The open boxes indicate inserted *E. coli* fragments. The hatched region of each box indicates the proposed location of the σ structural gene. In *a*, *b*, and *c*, distance is indicated in kb from left end of Ch25. (*a*) The experiments used to construct the restriction map of Ch25 are to be described elsewhere. The distance between the R1 site at 32.3 and the right end of the vector is not firmly established and is based on previously determined distances in the phage used to construct Ch25. We suspect, based on the migration of R1 fragments in Fig. 1, that this distance is about 12 kb instead of 13.1. (*b*) Constructed from lanes 1–3 and 5 of Fig. 1. (*c*) Constructed from lanes 6–8 and 10 of Fig. 1. Note that the phage is presented backward so that the genomic DNA has the same orientation in *b*, *c*, and *d*. (*d*) Summary of all restriction sites we have determined in the genomic DNA. The fragments have been oriented so that left-to-right is equivalent to a clockwise direction on the *E. coli* map. The data presented in Figs. 3 and 4 enable us to place the σ promoter (Pσ) and structural gene as shown. The arrow indicates the length of the structural gene and direction of transcription. Scale is in kb starting from the left *Hin*dIII site. Neighboring *E. coli* genes are indicated along with approximate map positions in minutes (26).

90°C for 2 min. Samples were spun for 3 min in the Beckman Microfuge, and the supernatant was applied to a slab gel. For competition studies, the sample was treated identically except that antiserum was first preadsorbed for 30 min with unlabeled σ purified by the method of Lowe *et al.* (22).

RESULTS

Isolation of σ Transducing Phage. The bacterial strain PB1*rpo*D800, carrying a temperature-sensitive mutation in the σ gene, cannot grow at 42°C unless a wild-type σ gene is provided. Thus, transducing phage containing the wild-type σ gene and capable of forming lysogens at 42°C can be selected because they should enable PB1*rpo*D800 to grow at high temperature. PB1*rpo*D800 cells were infected at low multiplicity at 30°C with shotgun collections of the *E. coli* genome constructed by inserting either *Sac* I or *Hin*dIII fragments into the vector Ch25. Infected cells were spread at 42°C on plates seeded with 10^9 λ*imm*21*c*I$^-$ phage to provide a double selection; plating at 42°C selected for bacteria with a wild-type σ

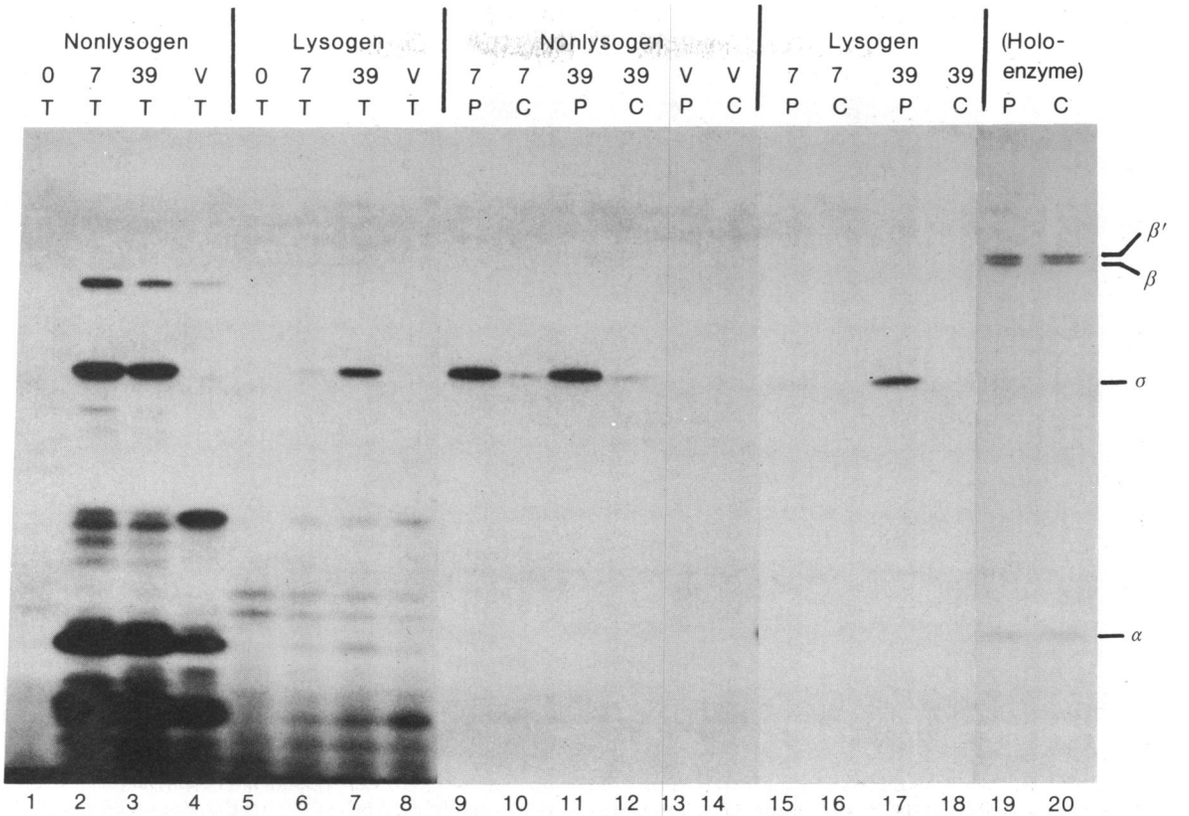

FIG. 3. Proteins produced after infection of heavily UV-irradiated lysogenic and nonlysogenic cells with Ch25sig-39H or Ch25sig-7S. Experiments were performed as described (15) except that S159 and S159λ*imm*21 were grown to 2×10^8/ml, concentrated 10-fold, and resuspended in M9 salts minus $MgSO_4$ for UV irradiation. Cells were irradiated for 5–6 min at 45 cm from a 15-W germicidal lamp and then infected with phage at a multiplicity of infection of 10. Cells were labeled with [^{35}S]methionine at 5 μCi/ml for 20 min (35–55 min after infection). At 55 min, cells were spun for 2 min in a Beckman Microfuge, resuspended in $NaDodSO_4$ sample buffer (24), and heated to 90°C for 2 min. A portion was immunoprecipitated and another portion immunoprecipitated after competition with purified σ. Samples were separated by electrophoresis on stacking Tris glycine/$NaDodSO_4$/7.5% polyacrylamide slab gels followed by fluorography (27). The strains infected were: nonlysogen, S159; lysogen, S159λ*imm*21. The infecting phage was: 0, none; 7, Ch25sig-7S; 39, Ch25sig-39H; V, Ch25. The treatments were: T, total proteins; P, immunoprecipitated proteins; C, proteins precipitated after competition with purified σ. Lanes 19 and 20 show ^{35}S-labeled RNA polymerase holoenzyme prepared by method of Gross *et al.* (28). Polymerase subunits are indicated at the right. Two minor proteins that migrated slightly ahead of α were present in the immunoprecipitates analyzed in lanes 9–12. These are unlikely to be related to σ because they were poorly precipitated and were not affected by competition with pure σ. They probably appear because of nonspecific binding to the antibody or *S. aureus* ghosts.

gene and plating in the presence of λ*imm*21*c*I$^-$ phage selected for lysogens of Ch25 phage. Twenty colonies were isolated and purified. Small liquid cultures were grown from each colony, and the spontaneously released phage from he supernatant of each culture were tested for the ability to transduce PB1*rpo*D800 to temperature-resistant growth. All candidates selected in the initial screening produced transducing phage able to complement the defective σ gene. One σ transducing phage (termed Ch25sig-7S) containing a *Sac* I fragment and one (termed (Ch25sig-39H) containing a *Hin*dIII fragment were chosen for further study.

The *dna*G gene maps close to the σ gene so these two might be contained on the same transducing phage. This was tested by determining if either phage could restore the ability of a *dna*G^{ts} strain, PC314, to grow at 42°C. We found that Ch25sig-39H transduced the *dna*G^{ts} strain PC314 to temperature-resistant growth but Ch25sig-7S did not. Thus, part or all of the *dna*G gene must be carried in that portion of Ch25sig-39H not included in Ch25sig-7S.

Size, Orientation, and Restriction Map of Genomic Fragments Carried by Ch25. The pattern of fragments obtained from various restriction enzyme digests of Ch25, Ch25sig-7S, and Ch25sig-39H are shown in Fig. 1 and interpreted in Fig. 2.

Only one fragment, the *Sac* I/*Hin*dIII fragment 7.3-kilobase (kb) pairs long, is shared by Ch25sig-7S and Ch25sig-39H and thus presumably codes for at least part of σ. The 2.0-kb fragment unique to Ch25sig-39H, by a process of elimination, must code for at least part of *dna*G. Because *dna*G is counterclockwise from σ on the *E. coli* map, we can conclude that the fragments are arranged on the *E. coli* genome in the order 2.0 kb–7.3 kb–1.9 kb going in a clockwise direction. Thus, the orientation of the inserted genomic piece is "n" in Ch25sig-7S (that is, the *E. coli* and λ coordinates increase in the same direction), whereas Ch25sig-39H is in the "u" or opposite orientation (16). The genomic map of this region, shown in Fig. 2*d*, also indicates the position of cuts made by *Bgl* II, *Kpn* I, and *Xho* I. No cuts are made in this region by *Xba* I or *Eco*RI.

Location of σ Structural Gene and Promoter. Results from analyses of proteins made *in vivo* and *in vitro* have been used to identify and map the σ gene and its promoter. When heavily UV-irradiated bacteria are infected with phage, the proteins synthesized derive from the phage template rather than from UV-damaged cellular nucleic acids. A strong protein band with molecular weight 90,000, corresponding to σ, is produced in this system with either Ch25sig-7S (lane 2) or with Ch25sig-39H (lane 3) (Fig. 3). This protein band was precipitated by antibody against RNA polymerase holoenzyme (lanes 9 and 11) and this

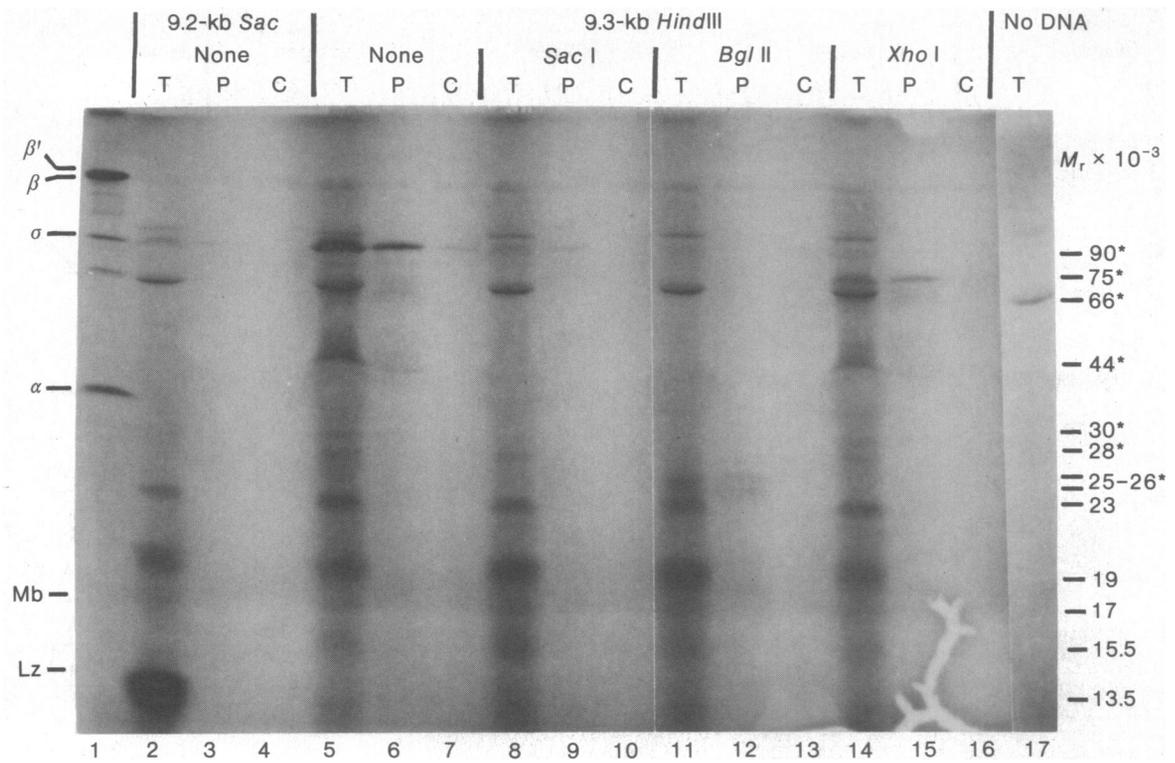

FIG. 4. *In vitro* protein synthesis directed by the 9.3-kb *Hin*dIII fragment and the 9.2-kb *Sac* I fragment. DNA-dependent *in vitro* protein synthesis was performed as described (29), except that each 40-μl assay mixture contained 16 μCi of [^{35}S]methionine and the reaction was for 90 min at 37°C. The reaction was terminated by heating with 2 vol of $NaDodSO_4$ sample buffer at 90°C for 4 min. Samples were analyzed on $NaDodSO_4$/10–18% gradient polyacrylamide slab gels. After staining with Coomassie brilliant blue, gels were prepared for fluorography by soaking for 1 hr successively in 7.5% acetic acid, Enhance (New England Nuclear), and water. Unlike the result of the fluorography method in ref. 27, the stained protein bands were still visible so the positions of molecular weight markers could be measured. The top of the figure is labeled to show the DNA fragment used as template, the restriction enzyme used for digestion, and the treatment (abbreviations as in Fig. 3). The positions of the β', β, σ, and α subunits and myoglobin (Mb) and lysozyme (Lz) are indicated at the left. The molecular weights of the proteins synthesized are displayed at the right; proteins immunologically related to σ are marked with asterisks. Many of these proteins are barely visible in Fig. 4 but show up on longer exposures. Lane 1 contains partially purified holoenzyme. Lane 17 shows the proteins labeled in a reaction containing no template DNA.

antibody reaction was inhibited by preadsorption of antibody with pure σ protein (lanes 10 and 12), confirming its identity as σ. We conclude that both Ch25sig-39H and Ch25sig-7S direct the synthesis of large amounts of σ after infection of a nonlysogenic cell, and thus both contain the complete structural gene for σ. Because the 7.3-kb fragment is the only one present in both phages, the gene must reside on this fragment.

When the UV-irradiated bacteria are lyosgenic and thus contain phage repressor, synthesis from most phage vector promoters is blocked so that most transcription must originate from bacterial promoters in the cloned segment. This principle can be used to detect the presence of such promoters. After infection of lysogenic bacteria, Ch25sig-39H still directed the synthesis of substantial amounts of the σ protein (lanes 7, 17, and 18) whereas Ch25sig-7S produced much less (lanes 6, 15, and 16). This finding strongly suggests that the Ch25sig-39H phage contains the bacterial promoter whereas the Ch25sig-7S phage does not. Because the only fragment present in Ch25sig-39H that is absent in Ch25sig-7S is the 2.0-kb fragment, it is tempting to assign the promoter for σ to that fragment reading rightward into the 7.3-kb fragment.

More precise information on the location of the σ structural gene and its promoter comes from analysis of proteins synthesized from DNA fragments by a DNA-dependent *in vitro* protein synthesizing system (Fig. 4). As expected, the 9.3-kb *Hin*dIII fragment of Ch25sig-39H directed synthesis of σ (lanes 5–7). When this fragment was cut by *Sac* I or the 9.2-kb *Sac* I fragment from Ch25sig-7S was used, full-length σ was produced but at a decreased rate (lanes 2–4 and 8–10). This is consistent with our notion that the promoter lies on the 2.0-kb fragment.

When the 9.3-kb *Hin*dIII fragment was cut with either *Bgl* II (lanes 11–13) or *Xho* I (lanes 14–16), truncated σ bands (capable of being specifically immunoprecipitated and affected by competition) are obtained. Because *Xho* I produces the longer of these and maps to the right of *Bgl* II, the amino terminus must be assigned to the left on the map (Fig. 2*d*). In fact, the molecular weights obtained (26,000‡ and 75,000) are completely consistent with the placement of the amino terminus near the left end of the 7.3-kb *Sac* I/*Hin*dIII fragment. Hence, the picture emerges that a *Sac* I site exists in genomic DNA between the promoter and the σ structural gene. DNA sequence studies will be required to determine the precise relationship of these elements to each other.

Other Proteins Coded for by the Cloned DNA. In addition to σ (and the related minor immunoprecipitable fragments thereof), we find five other proteins produced when the 9.2-kb *Sac* I fragment or the 9.3-kb *Hin*dIII fragment is used as template *in vitro*. These proteins are not related to σ immunologically, nor are they in the σ operon because cutting the template with *Sac* I does not diminish their rate of expression. Three of the proteins, molecular weights 17,000, 19,000, and 23,000, are

‡ Actually two proteins of molecular weight 25,000 and 26,000 are seen with the *Bgl* II-cut template. This is probably due to premature termination or degradation.

coded for by both fragments. Their genes must therefore be located in the 7.3-kb *Sac* I/*Hin*dIII fragment common to both transducing phage. A 15,500-dalton protein is produced only by the *Hin*dIII fragment. The gene for this protein must lie in the 2.0-kb fragment unique to Ch25sig-39H. Similarly, the abundant 13,500-dalton protein produced only by the *Sac* I fragment must be coded for by the 1.9-kb fragment unique to Ch25sig-7S. No protein of 60,000 daltons corresponding to *dna*G (13) has been found.

DISCUSSION

We have isolated two transducing phage for the *rpo*D gene which codes for the σ subunit of *E. coli* RNA polymerase. One of the phage, Ch25sig-39H, also carries at least part of the *dna*G locus which codes for primase. Immunoprecipitation studies have confirmed that σ is produced by these phage. We determined the physical and genetic map of the region and find that the σ gene is transcribed clockwise on the *E. coli* chromosome. A *Sac* I site separates the σ gene from it promoter.

Transduction of *dna*G occurs only when a 2.0-kb *Hin*dIII/*Sac* I fragment is present on the phage, although this piece of DNA is barely long enough to code for the primase gene. We have been unable to demonstrate any production of a primase-sized protein, but this failure may be for technical reasons. We do not have an antibody against primase that would enable us to detect it easily. Because it has been suggested that this protein is expressed at a low rate in *E. coli* cells (13), the same may be true for our phage. Alternatively, primase may be unstable in our stystem and be degraded to the 15,500-dalton protein that we do observe coded for by the 2.0-kb fragment. Finally, only a portion of the *dna*G gene, containing the *dna*G3 allele, may be cloned. In this case, the temperature-resistant colonies would arise from recombination within the lysogenized cell rather than complementation. We must await further studies to determine the location of *dna*G more precisely.

The phage vector system we have used was designed to permit stable lysogenization of clones at high temperature. In principle, this approach will be applicable to any gene for which a temperature-sensitive marker with a low reversion rate is available.

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