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Nucleotide sequence of the operator-promoter region of the galactose operon of *Escherichia coli*

(regulation of transcription/DNA sequence)

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ABSTRACT **We have derived the nucleotide sequence of a segment of the operator-promoter region of the galactose operon of *E. coli*, by using a variety of DNA sequencing analyses. We have previously reported the sequence of the 5′ terminal portion of *gal* mRNA [Musso, R. E., de Crombrugghe, B., Pastan, I., Sklar, J., Yot, P. & Weissman, S. (1974) *Proc. Natl. Acad. Sci. USA* 71, 4940–4944] and of the 59 base pairs preceding the startpoint of *gal* transcription (J. Sklar, S. Weissman, R. Musso, R. Di Lauro, & B. de Crombrugghe, submitted). In conjunction with those results, the present data provide the sequence of the *gal* operator-promoter region. This sequence is compared with similar sequences in other promoters and operators. Tentative mechanisms for the regulation of the galactose operon are discussed.**

The galactose operon of *Escherichia coli* is subject to both positive and negative control. Adenosine 3′:5′-cyclic monophosphate (cAMP) and its receptor protein, CRP, enhance the rate of initiation of transcription from the *gal* promoter (1–3). The *gal* repressor, on the other hand, reduces *gal* transcription by interfering with formation of the preinitiation complex composed of RNA polymerase (RNA nucleotidyltransferase, nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6), CRP and cAMP at the *gal* promoter (4–10). An additional system of regulation appears to be mediated by the product of the *cap* R gene because mutations in the latter cause a moderate derepression of *gal* enzyme synthesis (11).

To help elucidate the molecular mechanisms involved in regulation of *gal* expression, we sought to determine the nucleotide sequence of the operator-promoter region for the *gal* operon. We previously reported the sequence of the 5′ terminal portion of *gal* mRNA (12), and the primary structure of 59 base pairs preceding this mRNA initiation site in the *gal* control region†.

Two lines of evidence have indicated that the sequence studies mentioned above do not define the entire *gal* control region. First, we have been unable to detect sequence alterations corresponding to several O^c mutations within the *gal* mRNA or the preceding 59 residues of the *gal* control region†. Second, we observed that CRP plus cAMP does not stimulate *gal* transcription from a restriction fragment extending to a *Hin*f cleavage site 59 residues upstream of the mRNA initiation site. However, CRP plus cAMP did stimulate transcription from a *Hha*-generated fragment which includes about 90 residues prior to the *gal* mRNA start site† (see Fig. 1). We therefore inferred that at least part of the CRP/cAMP recognition site and possibly the operator are located 60 to 90 residues prior to the *gal* mRNA initiation site. By using the restriction endonucleases *Hin*f and *Hha*, we have generated a DNA fragment corresponding to this portion of the *gal* regulatory region. The determination of the sequence of this fragment and some tentative proposals regarding the mechanisms of *gal* regulation are presented in this paper.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; CRP, adenosine 3′:5′-cyclic monophosphate receptor protein; *gal*, galactose operon; *lac*, lactose operon; *Hae* III, *Hap* II, *Hin*f, and *Hha*, restriction endonucleases isolated respectively from *Hemophilus aegyptius*, *Hemophilus aphrophilus*, *Hemophilus influenza* type f, and *Hemophilus haemolyticus*. R, unspecified purine nucleoside.

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† J. Sklar, S. Weissman, R. Musso, R. Di Lauro & B. de Crombrugghe, submitted for publication.

MATERIALS AND METHODS

Enzymes. Restriction endonuclease *Hae* III was prepared according to Roberts *et al.* (13); *Hha*, *Hin*f, and *Hap* II were prepared as described by Smith and Wilcox (14) except that for *Hap* II the crude extract was precipitated with 50% saturating ammonium sulfate and the resuspended pellet was applied to a column of Bio-Gel (A-0.5). Other enzymes and reagents were isolated or obtained from commercial sources as indicated†.

Preparation of Restriction Fragments. DNA extracted from the phage λp*gal*8 (15) was digested by *Hae* III and the *Hae*-9B fragment containing the *gal* regulatory region and proximal E cistron, was isolated as described†. Smaller subfragments of *Hae*-9B were obtained by digestion with the appropriate restriction enzymes and gel electrophoresis as reported†.

Terminal ^{32}P-Labeling of DNA Fragments. [γ-^{32}P]ATP (>1 Ci/μmol) was prepared by minor modifications in the procedure of Glynn and Chappell (16) and the 5′ ends of DNA fragments were labeled with ^{32}P by using T_4 polynucleotide kinase (polynucleotide 5′-hydroxyl-kinase, ATP:5′-dephosphopolynucleotide 5′-phosphotransferase, EC 2.7.1.78) as described by Maniatis *et al.* (17).

Partial Venom Exonuclease Digestion. The *gal* fragments with a 5′ ^{32}P label at one end were partially digested with snake venom phosphodiesterase (5′-exonuclease, oligonucleate 5′-nucleotidohydrolase, EC 3.1.4.1), and the products fractionated by electrophoresis on cellulose acetate (Cellogel) at pH 3.5 followed by either homochromatography on DEAE-cellulose thin-layer plates as described (17,†) or electrophoresis on DEAE-paper at pH 3.5.

Dimethyl Sulfate Analysis and Hydrazine Analysis. Fragments ^{32}P-labeled at one end were partially methylated with dimethyl sulfate or degraded by partial hydrazinolysis and the products further treated and analyzed by polyacrylamide gel electrophoresis according to the DNA sequencing method developed by Maxam and Gilbert (18 and *Proc. Natl. Acad. Sci. USA*, in press).

Pyrimidine Tract Analyses. The *Hha*/*Hin*f fragment was prepared from *Hae*-9B selectively labeled by [α-^{32}P]dTTP according to the "nick-translation" procedure (17). Pyrimidine tracts were prepared according to Burton (19) and fractionated in the two-dimensional homochromatography system as de-

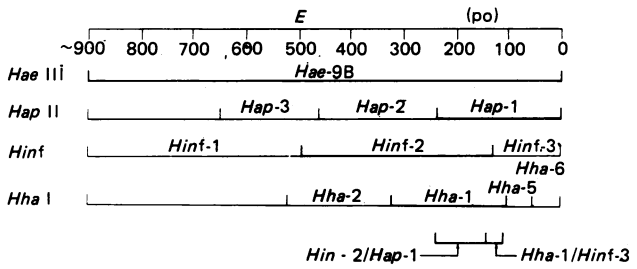

FIG. 1. Restriction map of the *gal* regulatory region. The *Hae*-9B fragment derived from λp*gal*8 is presented and pertinent sites for cleavage by other restriction endonucleases are shown. Subfragments are designated by the enzyme used to digest *Hae*-9B and a number in order of decreasing size among subfragments of that type. Fragments obtained by digesting *Hae*-9B with two enzymes are designated by the two single enzyme digestion products which overlap the mixed digestion fragment.

scribed by Ling (20). The composition of products was determined from their position in the two-dimensional fingerprint and by their electrophoretic mobility on DEAE paper at pH 3.5 (21,†).

RESULTS

Preparation of *Hha*-1/*Hinf*-3 for Sequence Analysis. We have isolated a fragment (*Hae*-9B) of about 900 base pairs which contains the *gal* regulatory region from an *Hae* III digest of λp*gal*8 DNA. The pertinent cleavage sites for other restriction enzymes within this *Hae* fragment are presented in Fig. 1. The nucleotide sequence of the *Hinf*-2/*Hap*-1 subfragment which extends 59 residues prior to the transcription initiation site has been reported†. The adjacent fragment, *Hinf*-3, is cleaved twice by *Hha* and the three subfragments are resolved by gel electrophoresis. The 5′ end labeling of *Hinf*-3, before and after digestion by *Hha*-1, located subfragment *Hha*-1/*Hinf*-3 at one end of *Hinf*-3. Placement of *Hha*-1/*Hinf*-3 adjacent to *Hinf*-2/*Hap*-1 was established by sequential digestion of *Hap*-1 with *Hha* and *Hinf*. As expected, *Hha*-1/*Hinf*-3 was also isolated as a terminal subfragment by digestion of *Hha*1 with *Hinf*.

Because the region 60 to 90 base pairs preceding the *gal* mRNA start site is defined by two different restriction enzymes,

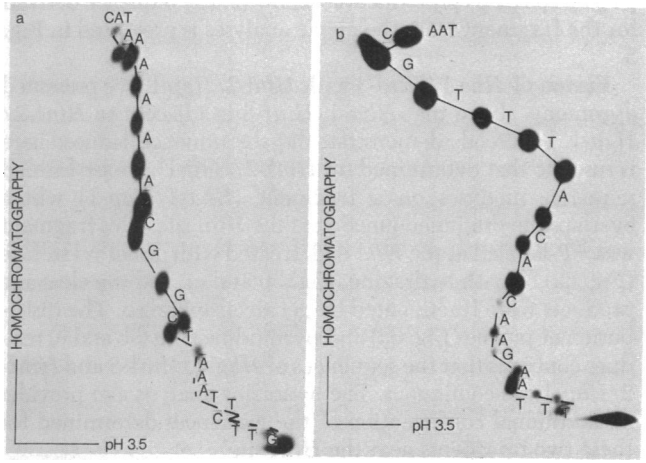

FIG. 2. Partial venom analyses of *Hha*-1/*Hinf*-3. The *Hha*-1/*Hinf*-3 fragment labeled at the *Hha* end (a) or the *Hinf* end (b) was partially digested with venom exonuclease plus pancreatic DNase. The products were fractionated by electrophoresis on cellulose acetate (left to right) followed by homochromatography (bottom to top). The sequence derived from the mobility shifts can be read top to bottom (5′ → 3′).

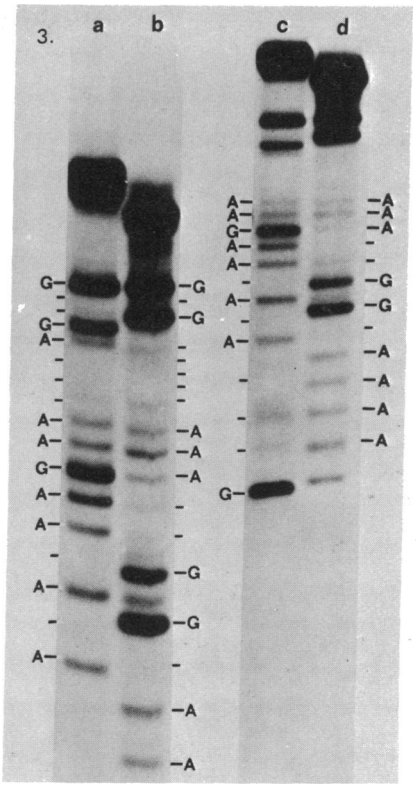

FIG. 3. Dimethyl sulfate analysis of *Hha*-1/*Hinf*-3. The degradation products from methylated, end-labeled *Hha*-1/*Hinf*-3 were fractionated by electrophoresis through a 20% polyacrylamide slab gel in 7 M urea and detected by autoradiography. Electrophoresis was at 800 V for 12 hr in (a) ^{32}P at *Hinf* end and (b) ^{32}P at *Hha* end or for 6 hr in (c) ^{32}P at *Hinf* end and (d) ^{32}P at *Hha* end. The purine residues corresponding to the degradation products are aligned.

the corresponding small fragment, *Hha*-1/*Hinf*-3, is ideally suited for direct DNA sequence analyses. We followed the strategy of Maniatis *et al.* (17), and prepared *Hha*-1/*Hinf*-3 selectively labeled with ^{32}P at both ends by the use of T_4 polynucleotide kinase. The end-labeled *Hha*-1/*Hinf*-3 fragment was isolated by gel electrophoresis and then analyzed as described below.

Partial Venom Exonuclease Digests. Two-dimensional fingerprints of partial snake venom exonuclease digests of *Hha*-1/*Hinf*-3 labeled at either end are presented in Fig. 2. Based on the mobility shifts between successively larger products (17, 21, 22) tentative sequences were proposed for about 20 residues from the 5′ ^{32}P-terminus of each DNA strand. Alternate analyses were used to determine the first few residues at the 5′ ends of each DNA strand. These involved two-dimensional fractionation of the venom digests by electrophoresis on cellulose acetate at pH 3.5 followed by electrophoresis on DEAE paper at pH 3.5. The identity of products up to four residues long was confirmed by their electrophoretic mobilities on DEAE paper at pH 3.5 and pH 1.7. Oligonucleotides of known composition (purchased from Collaborative Research) were used as standards for these analyses.

Because the sequences derived for the two DNA strands of *Hha*-1/*Hinf*-3 (Fig. 2) were complementary for the eight residues distal to the 5′ end, it was likely that the entire sequence of the fragment was represented in the venom analyses. To verify this and to confirm the sequences inferred from mobility shifts, we used the DNA sequencing techniques developed by Gilbert and Maxam (ref. 18 and personal communication).

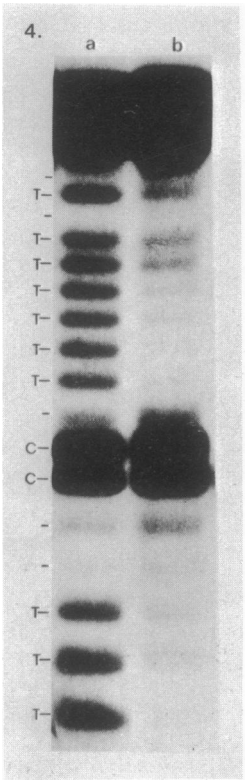

FIG. 4. Hydrazine analysis of *Hha*-1/*Hin*f-3. The degradation products from hydrazine treated *Hha*-1/*Hin*f-3 end-labeled at the *Hin*f end were fractionated on a 20% polyacrylamide–7 M urea slab gel. Hydrazinolysis was performed (a) in the absence of salt for pyrimidines and (b) in the presence of salt for cytosine residues, according to A. Maxam and W. Gilbert (*Proc. Natl. Acad. Sci. USA*, in press). Oligonucleotides shorter than 17 residues were run off the gel. Electrophoresis was at 600 V for 20 hr.

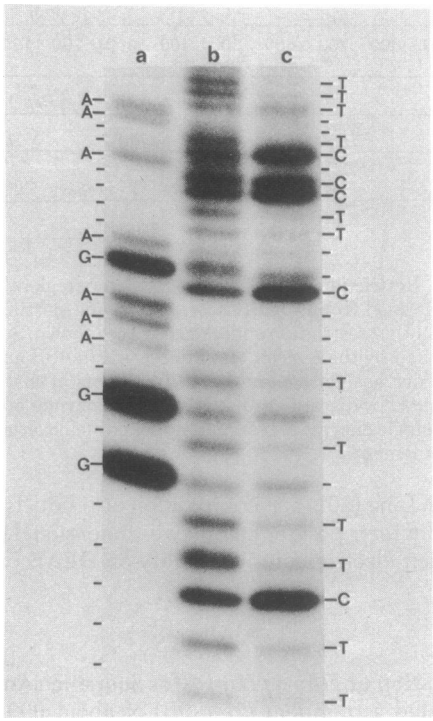

FIG. 6. Analysis of the *Hha*-1/*Hap*-1 fragment. The *Hha*-1/*Hap*-1 fragment with a terminal ^{32}P at the *Hha* end was subjected to dimethyl sulfate analysis for purine residues (a), and to hydrazinolysis in absence of salt for pyrimidines (b) or presence of salt for cytosine (c). The products were fractionated by polyacrylamide gel electrophoresis and detected by autoradiography. The purine and pyrimidine residues are aligned with the corresponding bands and the sequence can be read bottom to top (5′ → 3′) across the junction between the *Hha*-1/*Hin*f-3 and *Hin*f-3/*Hap*-1 fragments.

Dimethyl Sulfate and Hydrazine Analyses. *Hha*-1/*Hin*f-3, ^{32}P-labeled at either end, was partially methylated with dimethyl sulfate and then degraded by heat and alkali. Fig. 3 shows the series of products extending from the 5′ terminal ^{32}P to each successive purine after fractionation, according to size, by gel electrophoresis. Because guanine is methylated more readily than adenine, the predominant products can be correlated with G residues and the minor fragments with A residues in the tentative sequence derived above. The distribution of G and A residues in Fig. 3 does agree with the proposed sequence. In addition, the spacing between bands was consistent with the expected number of pyrimidine residues because the product mobilities were related to the logarithm of the chain lengths predicted by the proposed sequence. The latter analysis also proved useful to eliminate artifactual products which did not have a mobility consistent with an integral number of nucleotides. These artifact bands were also generally variable in yield upon repetition of the analysis.

The dimethyl sulfate analyses confirmed the overlap in sequences derived by partial venom digestion from both ends of the *Hha*-1/*Hin*f-3 fragment. Hydrazine treatment of *Hha*-1/*Hin*f-3 according to Maxam and Gilbert (*Proc. Natl. Acad. Sci. USA*, in press) further verified the correctness of the deduced sequence and, in particular, corroborated the existence of six successive A/T base pairs at the *Hha* end of the fragment (Fig. 4). Pyrimidine tract analysis of *Hha*-1/*Hin*f-3 selectively labeled by nick-translation with [α-^{32}P]dTTP also showed a pTpTpTpTpTpTp product (not shown). The sequence derived for the fragment from the above analyses is presented in Fig. 5.

Fusion of *Hha*-1/*Hin*f-3 with *Hin*f-2/*Hap*-1. We presented arguments above that *Hha*-1/*Hin*f-3 is adjacent to *Hin*f-2/*Hap*-1. To directly demonstrate that the sequence deduced here is fused to that determined for *Hin*f-2/*Hap*-1†, we performed sequence analyses on a fragment (*Hha*-1/*Hap*-1) which overlaps the presumed junction at the *Hin*f site. This fragment was ^{32}P-labeled at the *Hha* end, treated with dimethyl sulfate (Fig. 6a) or with hydrazine (Fig. 6b and c), and the cleavage products were fractionated by gel electrophoresis. The distribution of purine (Fig. 6a) and pyrimidine (Fig. 6b and c) residues confirms that the sequences of *Hha*-1/*Hin*f-3 and *Hin*f-2/*Hap*-1 are contiguous. The hydrazine analysis also provides an additional confirmation of the sequences determined for these two fragments near the *Hin*f site.

FIG. 5. The sequence for the *Hha*-1/*Hin*f-3 fragment is presented as shown in the above nucleotide series. The *gal* structural genes are located to the right of the fragment as presented.

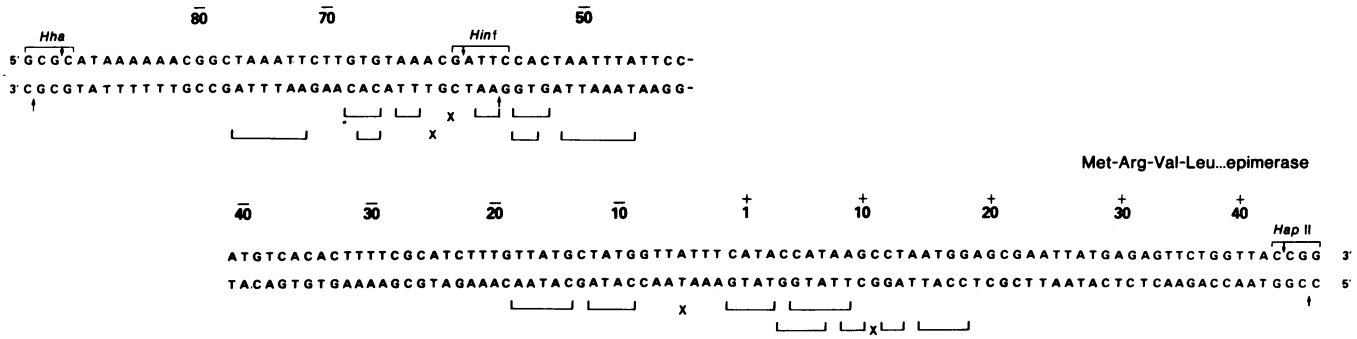

FIG. 7. Sequence of the *gal* regulatory region. Position +1 corresponds to the startpoint of cAMP/CRP dependent *gal* transcription. The sequence derived here for the *Hha*-1/*Hin*f-3 fragment corresponds to positions −92 to −57 and that of the *Hin*f-2/*Hap*-1 fragment † corresponds to positions −59 to +45. Regions of 2-fold rotational symmetry are indicated by lines under the sequence.

DISCUSSION

By using a combination of DNA sequencing methods, we have derived the nucleotide sequence of a DNA fragment that is part of the regulatory region for the *gal* operon. Restriction mapping and sequence analyses have shown that this fragment is adjacent to the *Hinf*/*Hap* fragment the sequence of which we have determined†. Thus, a sequence of 93 residues containing the *gal* regulatory region has been determined (Fig. 7).

In vitro transcription of fragments from the *gal* operon indicated that this sequence defines the entire *gal* promoter†. In addition, we recently analyzed the sequence of fragments from several O^c mutants. Each of these mutations has been found to involve changes in the region 60–66 residues preceding the *gal* mRNA initiation site (R. Di Lauro, R. Musso, and B. de Crombrugghe, unpublished results). Thus, the sequence in Fig. 7 defines the entire operator-promoter region of the *gal* operon. Although further studies are required to precisely delineate the specific sites involved in operator and promoter function, tentative proposals can be made at this time.

RNA Polymerase Interaction Sites. Regions of hyphenated 2-fold rotational symmetry are centered at positions −5 and +11 (Fig. 7). Similar "palindromic" sequences have been found near the RNA initiation sites for other promoters. It remains uncertain whether these symmetries play a functional role in DNA transcription.

Pribnow (23) observed that several promoters have a sequence closely related to T-A-T-R-A-T-G preceding the transcription initiation site by six or seven residues. He proposed that this heptanucleotide sequence is important for formation of a stable RNA polymerase preinitiation complex with the DNA. A comparison of all known promoter sequences in this region reveals that the second (A), fourth (R), and sixth (T) residues of this sequence are indeed conserved while the other positions are more variable. Two related sequences are present in the *gal* regulatory region, each differing at positions 5 and 7 from the heptamer. One of these, T-A-T-G-C-T-T (−12 to −6), precedes the site for initiation of CRP dependent *gal* mRNA by six residues. The second, T-A-T-G-C-T-A (−17 to −11), is displaced by the same distance from position −5. We recently established the existence of a second initiation site for *gal* transcription located at this −5 position. Transcription initiation from this site is CRP independent (R. Musso, R. Di Lauro, and B. de Crombrugghe, manuscript in preparation).

Several observations indicate that RNA polymerase also interacts with a region 30–40 residues prior to the startpoint of transcription. Promoter mutations have been identified in this region of the *lac* (24) and lambda (25) promoters. Furthermore, others have shown that RNA polymerase will not form a stable complex with a promoter fragment containing 20–25 residues on each side of the transcription initiation site (23, 26, 27). The pentanucleotide T-G-T-T-G is present in two λ promoters and a simian virus 40-'promoter' (28) about 35 residues prior to the transcription initiation sites (25). We noted that the sequence A-C-A-C-T-T-T is present in *gal* at positions −36 to −30 and at a similar position in the *lac* (−33 to −27, ref. 24) and $tRNA_{III}^{tyr}$ (−37 to −31, ref. 29) promoters. Although no sequence is shared by all promoters, this region may still be important for RNA polymerase binding. Variations in the sequence of this region may reflect differences in the conditions or regulatory factors required for activation of transcription.

All promoters analyzed have sequences rich in A-T base pairs which may facilitate local denaturation of the DNA helix by RNA polymerase. In the *lac* promoter, an A-T rich region located about 30 residues before the startpoint of transcription is bounded on each side by G-C rich segments. Dickson *et al.* (24) postulated that CRP may overcome the stabilizing effects of the G-C rich segments to facilitate RNA polymerase binding

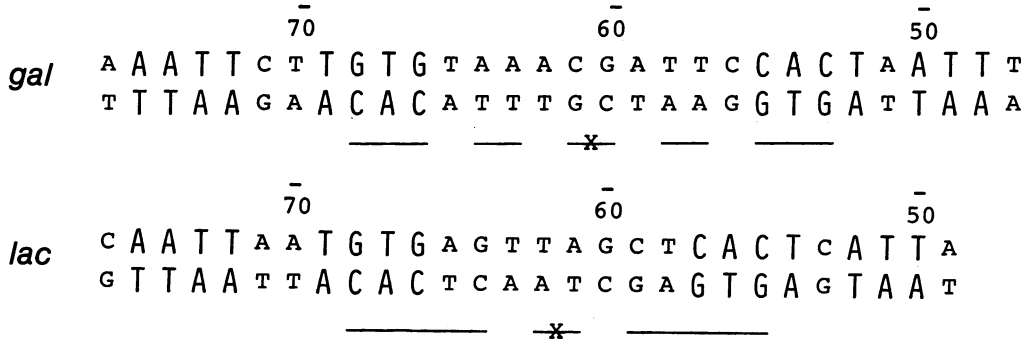

FIG. 8. Comparison of the DNA sequences around the CRP binding sites in *lac* and *gal*. Palindromes discussed in the *text* are indicated by lines under the sequences.

at this A-T rich region. Although the *gal* regulatory region is also very A-T rich, no G-C rich blocks analogous to those in *lac* are present.

CRP Recognition Site. Two lines of evidence indicate that sites essential for RNA polymerase are located to the right of the *Hin*f cleavage site (Fig. 7) while at least part of the CRP recognition site is within the *Hha*-1/*Hin*f-3 fragment. First, a fragment extending to the right of the *Hin*f site is transcribed by RNA polymerase, but CRP plus cAMP does not stimulate this transcription. Transcription of the *Hha*-1 fragment, which extends 34 residues farther up the strand is, however, stimulated by CRP and cAMP†. Second, the *Hin*f site is protected against cleavage when a CRP/cAMP dependent preinitiation complex is formed at the *gal* promoter but not by a CRP/cAMP independent complex (unpublished results).

Comparison of the *gal* sequence near the *Hin*f site with the CRP recognition site in *lac* (24) reveals several similarities. Hyphenated 2-fold rotational symmetries are present in both the *gal* and *lac* sequences. Analyses of CRP site mutations (30) and protection against methylation by CRP (J. Majors and W. Gilbert, personal communication) indicate that one of the *lac* palindromes (centered at −62/−61) is important for CRP recognition. The segments G-T-G and C-A-C which define the outside of this *lac* palindrome also limit a palindrome centered at position −61/−60 in *gal*. The sequences between these two trinucleotides are quite different in *lac* and *gal* although both show symmetry. However, two segment of sequence homology between *gal* and *lac* do overlap these palindromes by the same G-T-G and C-A-C residues. The sequence A-A-T-T-C-T-T-G-T-G (−75 to −66) in *gal* differs at only two positions from the *lac* sequence A-A-T-T-A-A-T-G-T-G which is exactly the same distance preceding the transcription startpoint. On the other side of the palindromes, the *gal* sequence C-A-C-T-A-A-T-T (−55 to −48) resembles the *lac* sequence C-A-C-T-C-A-T-T (−57 to −50) (Fig. 8).

***gal* Operator.** Our previous analyses of O^c mutants failed to detect sequence changes to the right of the *Hin*f site†. Recently, we have observed that two O^c mutants are resistant to cleavage by *Hin*f and other mutations are located to the left of this site (R. Di Lauro, R. Musso, and B. de Crombrugghe, manuscript in preparation). Previous experiments demonstrated that these mutants were resistant to repression of *gal* transcription (9). Our present data, therefore, indicate that the *gal* repressor interacts in the region 60–66 base pairs preceding the transcription startpoint.

The *gal* operator is therefore overlapping or interdispersed with the CRP recognition site in the *gal* regulatory region. Interestingly, O^c mutations in ths region decrease the symmetry of the *gal* palindrome centered at position −61/−60 but do not grossly alter the stimulation of transcription by CRP (unpublished results).

This location of the *gal* operator is consistent with the observed interference between the repressor complex and the CRP dependent RNA polymerase complex (10). However, it is in striking contrast to the operator location in the *lac* (31), *trp* (32), and *lambda* (25) systems. In the latter cases, repressor binds next to the transcription initiation site and thereby prevents RNA polymerase from forming an initiation complex.

The location of the *gal* operator suggests that *gal* repressor interferes directly with CRP binding and thereby prevents CRP dependent transcription. RNA polymerase may still form a CRP-independent complex and initiate transcription from position −5. Thus, the existence of two initiation sites and the relative location of the operator would allow the basal level of *gal* expression required for biosynthesis of galactoside precursors of the cell wall. Further studies are certainly required to establish what fraction of the basal *gal* mRNA initiates at positions −5 and +1 and to determine how the *gal* repressor and possibly the *cap* R gene product influence transcription from these two sites.

We thank S. Adhya for many helpful discussions. We are very grateful to A. Maxam and W. Gilbert who generously provided us with detailed protocols for the dimethyl sulfate and hydrazine analyses. We also are indebted to R. Roberts who gave us access to his restriction enzyme bank. We thank Ray Steinberg for his help with the illustrations and Alana Muto for her diligence in typing the manuscript.

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