The gal operon regulatory region contains two overlapping promoters, P1 and P2, regulated by cyclic AMP and the cyclic AMP receptor protein (cAMP-CRP). Starting with a mutation that eliminated P1, the promoter that is usually dependent on cAMP-CRP, we constructed and characterized increasing amounts of DNA sequence from upstream of P2, the promoter that usually functions in the absence of cAMP-CRP. Expression from P2 *in vivo* was halved by deletions that replace the −35 region with unrelated sequences, showing that the −35 sequence participates in promoter function, but is not essential. *In vitro* studies show that replacement of the −35 sequence increases the time for open complex formation at P2, but does not alter the transcription start point.

We examined the effects of the same deletions at the wild type gal promoter region: again, the deletion that replaces the −35 region halves expression *in vivo*. However, in this case, in the absence of cAMP-CRP, the deletion switches expression from the P2 promoter to P1, the promoter that is usually dependent on cAMP-CRP. Moreover, although the deletion also removes the specific cAMP-CRP binding site, this P1 activity is sharply inhibited in a crp+ background. We argue that this is due to a direct contact between CRP and RNA polymerase bound at the P1 Pribnow box, and we discuss the role of the −35 sequence at these and other promoters.

It is generally accepted that the crucial sequences required for the function of promoters in *Escherichia coli* are located about 35 and 10 bp upstream from the transcription start point. The evidence for this is 3-fold: (a) comparison of the DNA sequence at different *E. coli* promoters reveals homologies just in these zones (1), (b) most promoter mutations that reduce transcription initiation fall in either of these sequences (2), and (c) RNA polymerase protects these zones on promoter DNA from attack with enzymic or chemical probes (3). Hence, the dogma that, to initiate transcription, RNA polymerase holoenzyme must form a complex involving contacts with specific sequences at both −10 and −35, often referred to as the “Pribnow box” and “−35 region” sequences (reviewed in Ref. 4).

In a previous study, we found some expression *in vivo* from the E. coli gal operon regulatory region even when the −35 region sequences were removed by deletion (5). We have now characterized this transcription and we show that expression from either of the two overlapping gal operon promoters is possible in the absence of the wild type sequence around −35.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Host Strains**—By convention, the sequence of the gal regulatory region is numbered with the start point for the cAMP-CRP-dependent promoter, P1, as +1; upstream and downstream sequences are labeled with −" and +" prefixes, respectively (6). The gal regulatory region, either with or without the p14 promoter mutation, was cloned on a 144-bp EcoRI-HindIII fragment in the lac expression vector, pAA187 (7, 8) (Fig. 1).

Unidirectional deletions starting at the upstream EcoRI site were made as previously described (5). These stretched from the EcoRI site to positions in the gal promoter region. In the course of the constructions, a short linker was placed between the EcoRI site and the truncated gal promoter region (5): manipulation of the restriction sites in this linker allowed alterations in the sequence upstream of the promoters, but outside of the gal sequence. Standard methods were used for preparing plasmids and for the isolation, manipulation, and sequencing of DNA fragments (9).

As host for the various pAA187 derivatives, we used a Δcrp derivative of the Δlac strain, M182 (5). Δ-Galactosidase levels in this host carrying different plasmids were determined as previously described (7). In experiments to determine the effects of cAMP-CRP on promoter activity, the plasmid pRKCRP was also present: this is the broad host-range low copy number plasmid, pRK2501 (10), into which the crp gene was cloned.

**Transcription in Vitro and in Vivo**—*In vitro* transcription assays and the experiments to determine the time of open complex formation were performed on PstI-HindIII fragments isolated from pAA187 and derivatives, exactly as described by Spassky et al. (11) using 150 nM RNA polymerase holoenzyme and 2 nM DNA fragments.

The 5′ end of the gal message was determined using the primer extension or S1 nuclease method as described in Refs. 12 and 13, respectively. Total RNA was extracted from cells containing pAA187 or derivatives. For primer extension, the RNA was hybridized at 45 °C in buffer containing 20 mM Hepes, pH 6.5, 0.4 M NaCl, and 80% formamide to a labeled probe covering the sequence between 45 and 105 bp downstream of the gal transcription start point, and the mixture was subsequently incubated with reverse transcriptase and deoxynucleoside triphosphates. The length of the extension to the probe, deduced after gel electrophoresis, indicates the position of the start of the gal transcript. With the S1 nuclease method, the RNA was hybridized at 52 °C in buffer containing 40 mM Pipes, pH 6.4, 0.4 M NaCl, and 80% formamide to the EcoRI-HindIII gal promoter fragment from pAA187 labeled at the HindIII end. After subsequent treatment with S1 nuclease (final concentration 200 units/ml) for 1 h at 25 °C, the size of the DNA fragments protected by the RNA was measured on a sequencing gel.

**RESULTS**

**Deletion of the −35 Sequence of the gal P2 Promoter**—The gal operon regulatory region contains two overlapping pro-
moters controlled by the cAMP receptor protein, CRP (6). In the absence of the cAMP-CRP complex, gal transcription starts at the P2 promoter; in the presence of cAMP-CRP, the P2 promoter is blocked and transcription from the alternative promoter, P1, is triggered (13). The transcription start point S2, for the P2 promoter, is 5 bp upstream of the start point, S1, for the P1 promoter (Fig. 1). To study the P2 promoter in the absence of P1, we started with gal promoter DNA carrying the p14 mutation, a GC to AT transition at -14: we previously showed that this mutation inactivates P1 so that P2 is the only functional gal promoter both in vivo and in vitro (12). The gal operon regulatory region carrying this mutation was cloned between the EcoRI and HindIII sites of the lac expression vector, pAA187, (7) so that β-galactosidase expression was controlled by the gal P2 promoter (Fig. 1). Starting at the upstream EcoRI site, unidirectional deletions were created that removed progressively longer segments from upstream of the gal start sites.

The effects of these deletions were determined by measuring the β-galactosidase levels in cells carrying the pAA187 derivatives. Table 1 shows that β-galactosidase expression is not affected by the Δ234 deletion where the gal sequence upstream of -39 is replaced. Deletions that removed sequences as far as -29 decreased activity, but did not abolish it totally. To reduce the chance that we had accidentally replaced the -35 sequence with a related one, we constructed two such deletions, Δ420 and Δ421, which result in different sequences upstream of -29 (see Table I); the effect of the two deletions on gal promoter activity was identical, both reducing expression to just under one-half of the level found with the full length promoter. To show that the expression from the truncated gal promoters was due to the P2 Pribnow box, we combined the Δ420 deletion with a smaller 2-bp deletion at -14 and -15 that destroys the P2 Pribnow box. This reduced β-galactosidase expression to a very low level. The expression of β-galactosidase by the derivatives shown in Table 1 was unchanged when the medium contained the inducer, galactose, in addition to fructose (data not shown). This demonstrates that differences in expression are due to intrinsic alterations of promoter activity rather than modification of the interaction in the gal repressor: most likely, the gal repressor is titrated out by the multicycoplasmid.

Using a “run-off” transcription assay, we investigated the effects of these deletions on the transcription start point. Restriction fragments isolated from pAA187 and derivatives were incubated with E. coli RNA polymerase prior to the addition of 32P-labeled nucleoside triphosphates and heparin. RNA polymerase molecules that have formed an open complex “run” to the end of the fragment making a transcript: the origin of each transcript can be deduced from its length. Fig. 2A shows that with the PstI-HindIII fragments from pAA187 with the p14 mutation, transcription started at S2 and no initiation was found at S1. With the Δ234 and Δ420 deletions that replaced sequences upstream of -39 and -29, respectively, transcription also started at S2. However, in the case of the Δ420 deletion, the intensity of the band corresponding to the run-off transcript was less and a new band 18 bases shorter appeared. When the template contained both the Δ420 deletion and the second deletion at -14 and -15, neither the run-off transcript nor the shorter transcript was observed. From this, we conclude that RNA polymerase can initiate transcription at S2 in the absence of the normal -35 region sequence, but that the Pribnow box sequence is essential. To investigate the shorter transcript, we repeated the experiment after trimming the PstI-HindIII fragments by restriction with BstEI, which cuts 10 bp from the HindIII end (7). Fig. 2B shows that the run-off transcript starting at S2 is shortened by 10 bases, whereas the length of the shorter transcript is unchanged. Thus, the shorter transcript must be due to some termination event caused by the Δ420 deletion.

Deletions at the Wild Type gal Promoter—To investigate the role of the -35 region in the wild type gal promoter sequence, we repeated the experiments were repeated to create deletions in pAA187 without the p14 mutation; their effects on P2 activity were found by measuring β-galactosidase levels in M182 Δcrp cells carrying the various pAA187 derivatives (Table II). One of these contains the Δ420 deletion, the same as described in Table I, which replaces the -35 sequences. As with the p14 promoter, this deletion causes a reduction in promoter activity, but does not abolish it totally. In contrast, longer deletions that cut out the Pribnow box sequences reduce β-galactosidase expression to the background level.

We investigated the effect of the Δ420 deletion on the wild type gal promoters in run-off transcription tests. Fig. 3A shows that with the full length gal promoter, as expected, most transcription starts at S2 with a trace initiating at S1 (11). However, with the Δ420 deletion, the gal promoter region was still active but, surprisingly, most transcription started from S1, the start point for the P1 promoter, which is usually only active in the presence of cAMP-CRP. We confirmed that it is the P1 promoter which is active in vivo in this case by extracting total RNA from growing cells and determining the gal transcription start point using the primer extension method. The results in Fig. 3B show that, in M182Δcrp cells, the wild type gal promoter region initiates transcription at S2, whereas the promoter carrying the Δ420 deletion initiates at S1.
TABLE I

Effects of deletions upstream of the p14 gal promoter

The table shows the β-galactosidase levels, measured as described in Ref. 7, made by various pAA187 derivatives containing the p14 mutation in growing M182acr cells. Each figure is the average of at least six independent experiments. The sequence of the upper strand of the gal promoter region is shown for each case in the right-hand half of the table. The triangles denote the positions of the upstream ends of gal sequence.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Upstream limit of gal promoter sequence</th>
<th>β-Galactosidase</th>
<th>Sequence of gal promoter region</th>
</tr>
</thead>
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<tr>
<td></td>
<td>units</td>
<td>%</td>
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</tr>
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<td>pAA187Δ420Δ14Δ15</td>
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<td>6</td>
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</tbody>
</table>

In a complementary experiment, we probed the gal transcription start point in vivo using the S1 nuclease method (Fig. 3C). RNA from cells carrying the gal promoter region with the Δ420 deletion was used to protect a labeled probe from S1 nuclease: gel analysis of the protected DNA shows a series of bands centered around the position corresponding to transcription initiation at the start point of the P1 promoter.

Kinetics of Open Complex Formation at Truncated gal Promoters—RNA polymerase was incubated for various times with fragments carrying different deletions and mutations prior to the addition of nucleoside triphosphates and heparin. After gel analysis of the transcription products (Fig. 4), the amount of run-off transcript from each gal promoter was measured in order to find the half-time for the formation of open complexes at each promoter in our conditions. With the wild type gal promoter region, open complexes form at P2 with a half-time of approximately 1 min (Fig. 4A). With the Δ420 deletion, the location of the complex is switched to P1,
TABLE II

The effect of deletions upstream of the wild type gal promoter on β-galactosidase expression by pAA187

β-Galactosidase levels were measured in growing M182 Arcp cells carrying pAA187 derivatives, as described in Ref. 7.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Upstream limit of gal promoter sequence</th>
<th>β-Galactosidase levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>units</td>
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<td>pAA189</td>
<td>No promoter</td>
<td>50</td>
</tr>
</tbody>
</table>

and the half-time for formation is approximately doubled (Fig. 4B). With the gal promoter region carrying the p14 mutation, complexes at P2 form with a half-time of just less than ½ min (Fig. 4C), whereas with the Δ420 deletion, the half-time is about 2 min, although P2 is still used (Fig. 4D).

gal Promoter Activity in a crp+ Background—The cAMP·CRP complex binds at the gal promoter region between -30 and -50 (14). Thus, the activities of truncated promoters were initially measured in a Δcrp background where effects due to changes in CRP binding would not arise. However, we repeated the measurements of lacZ expression from the different gal promoters in a crp+ background. To do this, the plasmid pRKCRP was introduced into M182Δcrp cells carrying pAA187 with the various gal promoters, and the β-galactosidase activity in these strains was measured (Table III). With the p14 mutation, either with or without deletions, β-galactosidase expression was similar in both the Δcrp and crp+ backgrounds. In contrast, with the wild type gal promoter, as previously reported (7), expression in a crp+ background was 2-fold higher due to cAMP·CRP stimulation of gal P1 transcription. In the presence of the Δ420 deletion, however, expression from the gal promoter region was sharply inhibited in the crp+ background in our growth conditions (Table III).

DISCUSSION

The control region of the gal operon contains two overlapping but discrete promoters, P1 and P2, that are regulated by the cAMP·CRP complex (Fig. 1). Our results show that, according to all criteria used, both promoters can function when the −35 region is replaced by unrelated sequences.

In initial experiments, we worked with a simplified gal regulatory region carrying the p14 promoter mutation that stops expression from P1 so that P2 is the only functional gal promoter (12). A deletion that replaced the sequence upstream of −29 (i.e. 24 bp upstream of S2) reduced expression from P2 by only one-half, increasing the half-time for open complex formation in our in vitro conditions by approximately 4-fold.
In contrast, a deletion to −39 (i.e. 34 bp upstream of S2) had no effect on P2 expression. Hence, the sequence between −29 and −39 influences the speed of open complex formation at P2, but is not essential for P2 promoter activity. It is unlikely that our constructions had accidentally replaced the sequence upstream of the Pribnow box with another functional −35 sequence (e.g. as in Ref. 15) as both the Δ420 and Δ421 deletions have identical effects on P2 activity, although they place completely different sequences from 25–39 bp upstream of the P2 transcription start point (Table I). Additionally, we recently showed that large insertions in the −35 zone have similar effects on P2 activity (16). Hence, we conclude that, although RNA polymerase must make some contact in the −35 region (in agreement with footprint data (11, 17, 18)), the exact sequence in this zone is not essential for P2 promoter activity.

At the wild type gal promoter region (i.e. minus the p14 mutation), two promoters are potentially active, but normally RNA polymerase initiates transcription at S1 only in the presence of cAMP·CRP (6, 11, 13). Thus, it was surprising to find that transcription started at S1 rather than S2 in the absence of cAMP·CRP when the −35 sequences were replaced. The sequences downstream from −29 must be sufficient for P1 promoter activity and, in the absence of the −35 sequence, the wild type P1 Pribnow box sequence must present a stronger initiation signal than the P2 Pribnow box. With the full length wild type promoter sequence, in the absence of cAMP·CRP, RNA polymerase must be able to interact with both the P1 and P2 Pribnow box sequences, but a specific secondary contact with the −35 region must preferentially stabilize the interaction at P2. In the presence of cAMP·CRP, which binds between −30 and −50 (14), RNA polymerase must be prevented from making this contact and transcription switches to P1. However, for optimal expression from P1, RNA polymerase must then make new contacts in the −35 zone and must also interact directly with bound CRP (11, 16–18).
Surprisingly, at the Δ420 gal promoter, where the sequence upstream of −29 had been replaced, the expression from P1 was blocked in a crp* background (Table III). As there is no specific cAMP·CRP binding site downstream of −29 (14), we propose that the blockage is due to a direct contact between free cAMP·CRP and RNA polymerase bound at this truncated P1 promoter. We suggest that, although the cAMP·CRP cannot bind to its specific site on the DNA, it can still interact with RNA polymerase and that this contact blocks transcription. Interestingly, expression from the lac promoter carrying certain deletions of the CRP binding site is also lower in crp* cells (19).

The prima facie conclusion from our results is that the Pribnow box sequences alone provide a sufficient signal for the initiation of transcription in the gal promoter region. Contacts with the bases in the −35 region or with CRP bound to this zone appear secondary and merely modify this signal. In a similar study with some phage λ P2 mutant promoters, Rosenberg and colleagues have shown that the −35 sequence is also not essential (20). However, at most E. coli promoters, both the Pribnow box and −35 sequences must be essential, as in many instances, point mutations in the −35 regions abolish promoter activity (1, 2) and, in at least two cases (21, 22), deletions of the −35 region almost totally inactivate promoters. Drew et al. (23) have suggested that Pribnow box sequences can trigger changes in DNA structure necessary for transcription initiation. We argue that there must be something special about the gal and λ P2 Pribnow boxes that allows them to function without a specific −35 sequence. From their study with λ P2, Keilty and Rosenberg suggest that the sequence TG, 1 bp upstream of the Pribnow box hexamer, is sufficient to compensate for specific −35 region sequences. Our data corroborate this idea, as both gal P1 and P2 contain a TG motif upstream of the Pribnow box (Fig. 1), and mutational analysis shows that each motif is essential to the corresponding promoter (12, 16). Moreover, expression from gal P1 in the absence of the −35 sequences is drastically reduced by a 2-bp deletion that removes this TG sequence (Table III, lines 6 and 7).

Although more systematic studies may reveal other combinations that permit promoter activity in the absence of specific −35 region sequences, in some cases the activity may be "fragile" e.g. blocked by cAMP·CRP. Hence, caution is required in concluding that any sequence combination does not possess promoter activity, e.g. the effects of many point mutations in the −35 regions of promoters have been determined only in crp* backgrounds and it is possible that promoter activity would be unmasked in other backgrounds.

Acknowledgments—The initial idea for these experiments came from a discussion with Dr. Martin Rosenberg, whom we are happy to acknowledge. We thank Dr. Henri Buc for helpful comments on the manuscript.

REFERENCES

3 S. Keilty and M. Rosenberg, manuscript submitted for publication.