Interaction of cyclic AMP receptor protein with the *ilvB* biosynthetic operon in *E. coli*

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Received 29 May 1984; Revised and Accepted 15 October 1984

ABSTRACT

DNase and restriction site protection studies show that cAMP and its receptor protein (CRP) bind to the promoter of the *ilvB* operon at approximately position -44 to -82. This region contains sequences that are homologous to those found in other CRP-dependent promoters. In vitro transcription from the *ilvB* promoter was markedly increased by the addition of cAMP and CRP. This stimulation was not found when the *ilvB* template lacked the proposed CRP binding site. cAMP-CRP did not alter the extent of transcription termination within the *ilvB* leader suggesting that this regulatory system may be independent of the attenuation mechanism involved in the negative control of this operon. The results of restriction enzyme site protection studies and experiments with altered promoter fragments indicate that the mechanism for CRP stimulation of the *ilvB* operon may be similar to a model recently proposed for *lac* (1).

INTRODUCTION

The *ilvB* operon of *Escherichia coli* K-12 contains the structural gene for acetohydroxy acid synthase I, an enzyme required for the biosynthesis of isoleucine, valine and leucine. Regulation of this operon is complex, involving negative control by attenuation (2,3) and positive control by a number of factors (4,5) including cAMP-CRP (6,2). The participation of cAMP-CRP in the regulation of a biosynthetic operon is very unusual since this complex is normally involved in the regulation of degradative operons (7). A recent report suggests that this control of *ilvB* may reflect a need to increase acetohydroxy acid synthase I when the flow of carbon, in the form of the substrates of the enzyme, is reduced (8). An examination of the DNA sequence of the *ilvB* promoter revealed structural features similar to the CRP-binding site consensus sequences proposed for other operons (2). It was therefore of interest to determine directly if CRP binds to the *ilvB* promoter and to further investigate the effect of cAMP-CRP on in vitro transcription in this operon. In addition, since this is the first operon shown to be controlled by attenuation and cAMP-CRP, it was feasible to investigate

possible interactions between these regulatory mechanisms.

We report here the results of DNase and restriction enzyme site protection studies which indicate that CRP binds to the ilvB promoter at approximately position -44 to -82. The addition of cAMP-CRP greatly increased transcription when restriction fragments containing the ilvB promoter were used as templates. However, cAMP-CRP had little or no effect on the percent of transcripts attenuated in vitro. In vitro transcription data and restriction enzyme site protection experiments indicate that a second RNA polymerase binding site may exist upstream of the binding site in the -35 region. This upstream site may play a major role in the cAMP-CRP stimulation of ilvB expression.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strain of E. coli K-12 used to isolate ilvB DNA was MF2356 which contains plasmid pTCN12(2). All other strains and plasmids are described in the text.

DNA preparation and agarose and polyacrylamide gel electrophoresis

Plasmid DNA was isolated from cells grown in L-broth (9) using the methods of Clewell and Helinski (10). Vertical agarose slab gels were used to analyze large DNA fragments using the procedures described by Childs et al. (11) except for the following changes. The buffer was 0.04 M Tris, 0.005 M sodium acetate, and 0.001 M disodium ethylenediamine-tetraacetate, pH 7.8. Gels were 0.7% agarose (W/V) dissolved in the buffer. Samples were run at 100 V for 2-3 h at room temperature. For restriction enzyme analysis of DNA fragments of less than 2,000 bp, vertical polyacrylamide slab gels were used (12). DNA fragments were isolated on preparative polyacrylamide gels according to the procedures of Maniatis et al. (12). The DNA bands were stained with ethidium bromide and visualized with long wave UV. Bands were cut out of the gel, placed in 1 ml pipet tips with attached dialysis tubing, and eluted overnight at 100 V.

In Vitro transcription. Conditions for in vitro transcription as described by Lee and Yanofsky (13) were modified as follows. The reaction mixture (total volume, 50 μ l) contained: Tris acetate (pH 7.9), 20 mM; KCl, 100 mM; EDTA, 100 μ M; dithiothreitol, 100 μ M; GTP, ATP, CTP, 125 μ M each; UTP, 12.5 μ M; 32 P-UTP, 5 μ ci (410 ci/mmol); 1 μ g RNA polymerase; and 0.5 pmol DNA. After incubation at 37°C for 10 min, rifampicin (10 μ g/ml) and Mg acetate (4 mM) were added. Reactions were incubated at 37°C for 20 min. RNA

was isolated (14) and fractionated by electrophoresis on 4.4 mm thick, 8% acrylamide gels containing 7M urea (12) and visualized by autoradiography. Readthrough transcription was measured using glyoxal gels (15).

DNase I protection. DNA fragments were isolated and labeled as described above. The protection procedure of Galas and Schmitz was followed (16). Approximately 0.07 pmole of DNA was incubated in 100 μ l of 20 mM Tris-HCl pH 7.5, 80 mM KCl, 5 mM MgCl₂, 0.1 mM DTT, 5 mM CaCl₂ plus cAMP and CRP at 37°C for 20 min. 60 ng DNase I was added and the reaction stopped after 2 min by the addition of 25 μ l 3 M Na-acetate, 0.25 M EDTA and 100 μ g/ml tRNA. Each sample was phenol extracted, ethanol precipitated and washed. Samples were resuspended in urea-dye mix and run on 4 mm 7 M urea-8% polyacrylamide gels.

Restriction site protection. The procedure of Deeley and Yanofsky (17) was followed. Approximately 50 ng of ³²P end-labelled DNA plus various proteins were incubated in 50 μ l of transcription buffer (see above) for 15 min at 37°C. One unit of the appropriate restriction enzyme was added and the reaction allowed to proceed for 15 min. Reactions were stopped by the addition of 200 μ l of 0.3 M Na-acetate, 100 μ l phenol and 3 μ l 10 mg/ml tRNA. After extraction, the samples were processed and run on gels as described (17).

Materials. Polyacrylamide was purchased from Serva. DNase I and RNase A were purchased from Worthington. Restriction enzymes were obtained from New England Biolabs or Bethesda Research Labs. RNA polymerase and Klenow enzyme (large fragment *E. coli* DNA polymerase I) were purchased from Bethesda Research Labs. Radioactive compounds were obtained from Amersham. Most other chemicals were purchased from Sigma. Lipoprotein DNA was a gift from M. Inouye. CRP was kindly donated by J. Krakow.

RESULTS

In vitro transcription

Studies utilizing the *ilvB* promoter-regulatory region (Fig. 1) in an *in vitro* transcription system were carried out to confirm and extend our previous *in vivo* (6) and *in vitro* (2) observations on cAMP activation of *ilvB* expression. The addition of cAMP and CRP to the *in vitro* reaction resulted in a 3 to 10- fold stimulation in the amount of *ilvB* leader RNA synthesized (Fig. 2). This activation of transcription was dependent on the addition of both cAMP and CRP and on the concentration of CRP used (data not shown). Most of the RNA made in this system is terminated within the leader region and little or no run-off transcripts can be detected (2). However, when the RNA was more

Fig. 1. DNA sequence of the *ilvB* promoter-regulatory region. The nucleotides are numbered from the proposed start of transcription at position 1 essentially as described previously (2).

completely denatured by treatment with glyoxal and run on a 5% polyacrylamide gel with a sodium phosphate buffer (15), a band corresponding to a run-off transcript that had read through the terminator was observed (Fig. 3). This allowed us to test the possibility that the stimulatory effect of cAMP-CRP on

Fig. 2. Effect of cyclic AMP-CRP on *in vitro* transcription of the *ilvB* leader region. Lanes: a, transcription of the *EcoRI-HaeIII* fragment (see Fig. 1) in the presence of cAMP (5mM) and CRP (6 μ g/ml); b, transcription without cAMP-CRP. L denotes the *ilvB* leader RNA band. Lpp denotes the lipoprotein mRNA band synthesized from an *S.marcescens* *lpp* gene DNA fragment. The *lpp* DNA is included as an internal control. The RNA bands were cut out and counted in a liquid scintillation counter. A 7-fold increase in *ilvB* leader RNA was found with added cAMP-CRP. This increase fluctuated from experiment to experiment and varied from 3 to 10 fold.

Fig. 3. Effect of cAMP-CRP on transcriptional read through at the *ilvB* attenuator. Lanes: a, transcription products of the EcoRI-HaeIII fragment; b, plus cAMP (5mM) and CRP (6 μ g/ml). RT corresponds to the read through RNA. L is the terminated *ilvB* leader RNA. Samples were treated with glyoxal prior to loading on the gel (see Materials and Methods). The gel was 5% polyacrylamide with a 10mM sodium phosphate buffer. The gel buffer was recirculated to prevent the pH from rising. The RNA bands were cut out and counted in a liquid scintillation counter. The results in cpm were as follows: No additions; terminated RNA (2136), read through RNA (890). CAMP-CRP added; terminated RNA (7450), read through RNA (1665).

ilvB expression could be in part due to a reduction in transcriptional termination within the leader region. The addition of cAMP-CRP to the *in vitro* transcription system did not increase the number of transcripts that readthrough the *ilvB* terminator (Fig. 3). These data suggest that, as in other systems, the major effect of cAMP-CRP is to influence transcription initiation (18).

DNase I protection

In order to determine the site of interaction between *ilvB* DNA and cAMP-CRP we did a DNase protection experiment (16). An EcoRI-HaeIII fragment containing the *ilvB* promoter region (Fig. 1) was end-labeled with 32 P and cut

Fig. 4. DNase I footprinting of cAMP-CRP in the *ilvB* promoter region. Lanes: a, DNase I digestion pattern of the *ilvB* fragment without added proteins; b, DNase I digestion pattern with cAMP (0.25 mM) and CRP (30 µg/ml); c, DNase I digestion pattern with cAMP (0.055 mM) and CRP (70 µg/ml); d, G+A Maxam and Gilbert sequencing reaction.

with *Ava*II to yield a 200 bp singly end-labeled DNA fragment (extending from bp -172 to +27). This fragment was used to locate the region of DNA protected from DNase I digestion by cAMP-CRP. The results of these experiments are shown in Fig. 4. Lane a shows the DNase I digestion pattern of the *ilvB* fragment without added proteins. Lanes b and c show the DNase I digestion pattern with cAMP-CRP added. Lane d shows a G+A Maxam and Gilbert sequencing reaction to identify the protected bases. The region from approximately bp -44 to -82 has an altered DNase I pattern (in comparison to the control) presumably due to the presence of cAMP-CRP on the DNA. This protection was dependent on the addition of both cAMP and CRP (data not shown). Bands with reduced intensity indicate phosphate bonds protected from DNase I cleavage by the added protein. Bands with increased intensity represent bonds with enhanced cleavage, possibly indicating changes in DNA structure (19). The

Fig. 5. Restriction site protection of the *RsaI* site within the *ilvB* CRP binding site. Lanes: a, control digestion of the ^{32}P end-labeled *EcoRI-HaeIII* fragment with no added proteins; b, digestion in the presence of cAMP (5 mM) and CRP (50 $\mu\text{g/ml}$); c, digestion in the presence of RNA polymerase (20 $\mu\text{g/ml}$); d, RNA polymerase added to 40 $\mu\text{g/ml}$; e, digestion in the presence of cAMP, CRP and RNA polymerase (20 $\mu\text{g/ml}$); f, control digestion with BSA added (50 $\mu\text{g/ml}$); g, undigested DNA. Arrows: (1) undigested DNA; (2 and 3) products of *RsaI* digestion.

significance of the increased intensity of some bands upstream of -100 is not known. This upstream area does not appear to be involved in CRP activation at the *ilvB* promoter since cutting at the *MspI* site at -107 does not effect cAMP-CRP stimulation of *ilvB* transcription (data not shown).

Restriction enzyme site protection

In order to confirm the results of the DNase I protection experiments, protection of the DNA by cAMP-CRP against cutting by the restriction enzymes

Fig. 6. Restriction site protection of the Sau3A site within the ilvB CRP binding site. Lanes: a, digestion of the 32 P end-labeled EcoRI-HaeIII fragment with no proteins added; b, digestion in the presence of cAMP (5mM) and CRP (50 μ g/ml); c, same as b but with CRP at 5 μ g/ml; d, digestion in the presence of RNA polymerase (20 μ g/ml); e, same as d but with RNA polymerase at 40 μ g/ml; f, digestion with cAMP (5mM), CRP (50 μ g/ml) and RNA polymerase (20 μ g/ml) present; g, undigested DNA. Arrows: (1) undigested DNA; (2 and 3) products of Sau3A digestion.

RsaI and Sau3A was examined. These restriction enzyme cleavage sites are located within the proposed cAMP-CRP binding site centered at bp -78 and -67 respectively. The results of protection against RsaI digestion are shown in Fig. 5. Comparison of the control with no added protein (lane a), to the lane containing cAMP-CRP (lane b), shows that the addition of these components completely inhibited cutting of the DNA by RsaI. A control with BSA added (lane f), shows that the observed enzyme inhibition is not due to the presence of added protein. Protection against Sau3A digestion gave identical results

Fig. 7. Restriction site protection of the AluI site within the 1lvB RNA polymerase binding site. Lanes: a, undigested EcoRI-HaeIII DNA fragment; b, control digestion with no added proteins; c, digestion in the presence of cAMP (5 mM) and CRP (50 μ g/ml); d, digestion with RNA polymerase (20 μ g/ml) added; e, same as d but with RNA polymerase at 40 μ g/ml; f, digestion with cAMP, CRP and RNA polymerase (20 μ g/ml) added. Arrows: (1) undigested DNA; (2 and 3) products of AluI digestion.

(Fig. 6). The inability of cAMP-CRP to inhibit digestion by AluI at a site centered around bp -12 (Fig. 7, lane c) suggests that CRP protection of restriction enzyme sites is specific for the proposed CRP binding site.

When RNA polymerase was used in the protection experiments, some unexpected results were obtained. RNA polymerase alone inhibited cutting at the RsaI and Sau3A sites (Fig. 5), lanes c and d and Fig. 6, lanes d and e), but not at the AluI site which is located within the proposed polymerase binding site (Fig. 7, lanes d and e). When both cAMP-CRP and RNA polymerase were present, protection of both the AluI and RsaI sites was observed (Fig. 5, lane e and Fig. 7, lane f). These data suggest that RNA polymerase binds

Fig. 8. RNA polymerase protection of the AluI site after altering the putative upstream (P_2) promoter with Sau3A. The EcoRI-HaeIII fragment was digested at -65 with Sau3A and the Sau3A-HaeIII fragment was isolated and end-labeled. A fragment of equivalent size (ca 275 bp) was isolated by cutting the EcoRI-HaeIII fragment with MspI at -108 and +167. The Sau-3A-HaeIII fragment should contain an altered ilvB P_2 site and a normal ilvB P_1 site. The P_1 and P_2 promoters should be normal in the MspI-MspI fragment. Lanes: a, digestion of the MspI-MspI fragment; b, digestion in the presence of RNA polymerase, (20 $\mu\text{g/ml}$); c, digestion in the presence of RNA polymerase (40 $\mu\text{g/ml}$); d, digestion of the Sau3A-HaeIII fragment; e, digestion with polymerase (40 $\mu\text{g/ml}$). Arrows: (1) undigested DNA; (2), (3) products of AluI digestion.

poorly at the "normal" -35 region in the absence of cAMP-CRP. Under these conditions polymerase appears to bind at a site upstream from the -35 region, which encompasses the RsaI and Sau3A sites, close to or including the region for CRP binding. We have located a possible upstream polymerase binding site, CTCAAT, at position -59, which is nearly identical to an upstream promoter recently found in lac at -59 (1,20). We attempted to alter binding at this site by cutting the ilvB promoter with Sau3A. In sharp contrast to the results obtained with the uncut promoter, RNA polymerase strongly protected the Sau3A fragment from digestion at the AluI site centered around bp -12

Fig. 9. Effect of altering the putative upstream (P_2) promoter on *ilvB* *in vitro* transcription. The MspI-MspI fragment (P_1+P_2) and the Sau3A-HaeIII fragment (P_1) were used as templates in an *in vitro* transcription assay. Lanes: a-e, transcription for 2, 3, 4, 5 and 10 min; f-j, transcription in the presence of cAMP (5 mM) and CRP (6 μ g/ml) for 2, 3, 4, 5 and 10 min. Arrows: (1) Sau3A-HaeIII fragment (P_1) used as template; (2) MspI-MspI fragment (P_1+P_2) used as template. The RNA bands were cut out and counted (see Fig. 10).

(Fig. 8). These data suggest that RNA polymerase binding in the -35 region was strongly enhanced in the Sau3A fragment as compared to the uncut promoter.

Effect of altering the putative upstream promoter on *ilvB* expression

We examined next if the apparent changes in RNA polymerase binding caused by cutting with Sau3A were reflected in alterations in *ilvB* promoter expression. In these experiments the amount of *ilvB* leader RNA synthesized *in vitro* was compared using the normal template and the DNA fragment obtained by Sau3A digestion. The data in Figs. 9 and 10 show that the amount of leader RNA produced from the Sau3A fragment was approximately 3-times that made from the uncut template. The addition of cAMP-CRP to the reaction mixture sharply increased transcription from the normal template but these compounds had no positive effect when the Sau3A fragment was used. These data are consistent with those obtained in the AluI protection experiments and suggest that

Fig. 10. Increased *ilvB* *in vitro* transcription after altering the putative upstream (P_2) promoter. The bands obtained in the *in vitro* transcription experiment described in Fig. 9 were cut out of the gel and counted in a liquid scintillation counter. The amount of radioactivity in counts per min (cpm) is plotted against the reaction time in min. Symbols: □, *MspI-MspI* fragment; ■, *MspI-MspI* fragment transcribed with added cyclic AMP-CRP; ○, *Sau3A-HaeIII* fragment; ●, *Sau3A-HaeIII* fragment transcribed with added cyclic AMP-CRP. For other conditions see Fig. 9.

sequences upstream of the -35 region negatively effect *ilvB* expression. These results also confirm our previous conclusion that the *Sau3A* site is within the CRP binding site.

DISCUSSION

Transcription of the *ilvB* operon is positively activated by cyclic AMP and its receptor protein, CRP. The addition of these compounds to an *in vitro* transcription system resulted in a 3 to 10-fold increase in *ilvB* leader RNA synthesis. As with other catabolite sensitive genes, this activation is associated with cAMP-CRP binding within the promoter region 5' to the start of transcription. DNase I footprinting and restriction site protection have tentatively defined this binding site as being between bp -44 and -82 in the *ilvB* promoter region.

Examination of the known CRP-dependent promoters reveals a striking variability in the location of the CRP binding sites. In some, such as gal (21), araC (22) and pBR-P4 (19), the CRP binding site overlaps the -35 region of the promoter. In others, such as lac (23), araBAD (22) and malT (24), CRP binds in a region outside of the RNA polymerase binding site (around bp -62, -90 and -70 respectively). The ilvB operon falls within the latter group with its CRP binding site centered at bp -63. Despite the lack of similarity in location, CRP binding sites do appear to have sequences in common. Based on comparison of the lac, gal and ara CRP binding sites, Taniguchi *et al* (21) derived the following 11 base consensus sequence for CRP binding: AAAGTGTGACA. This sequence (with four mismatches) appears within the ilvB CRP binding site between bp -75 and -65. The underlined portion of this sequence, which is thought to be most important for CRP-DNA interactions, appears without mismatches in the ilvB CRP site. Other consensus sequences for CRP binding have been suggested (18,19) and these sequences, differing by three nucleotides or less, appear in the proposed CRP binding region in the ilvB promoter.

It has been reported that cAMP-CRP, in addition to enhancing transcription initiation, can act as an anti-terminator to prevent premature termination of transcription (25). Because the ilvB operon is controlled by both attenuation and cAMP-CRP, a potential mechanism for cAMP-CRP regulation in ilvB would be to allow RNA polymerase to read through the termination site in the leader region, resulting in an increase in the amount of full length mRNA that is produced. This possibility was examined by measuring the ratio of terminated transcripts to readthrough transcripts synthesized *in vitro*, with and without cAMP-CRP. While the total amount of RNA made increased when cAMP-CRP were added there was little difference in the percent of transcripts that were terminated. Thus, the mechanism of cAMP-CRP activation of the ilvB operon does not appear to include antitermination within the leader region but, as in other systems, this complex seems to act primarily by enhancing transcription initiation (18). However, since cAMP-CRP was not tested in a coupled ilvB transcription-translation system, an effect on attenuation by this complex has not been ruled out.

With respect to the mechanism by which cAMP-CRP activates transcription some interesting results were obtained when RNA polymerase was used in the restriction site protection experiments. Unexpectedly, RNA polymerase inhibited cutting at the RsaI and Sau3A sites centered at bp -78 and -67 respectively. These data suggest that a second polymerase binding site may be

located in the vicinity of the RsaI and Sau3A sites upstream from the normal site at -35. Examination of the DNA sequence in the region revealed the sequence CTCAAT, between bp -59 and -54. A second promoter, CTCACT, (termed lac P₂) has been recently found at position -59 to -54 in the lac transcriptional control region (1,20). This second promoter overlaps the CRP binding site and the polymerase site at -35 (termed P₁). Malan and McClure (1,20) have shown that RNA polymerase binds very strongly to lac P₂ but that transcription initiates very poorly from this promoter. They suggest that polymerase binding to lac P₂ prevents binding at lac P₁ resulting in minimal lac expression. cAMP-CRP activate transcription by preventing RNA polymerase from interacting at lac P₂ thus allowing polymerase to bind efficiently at lac P₁. Our results obtained in the AluI restriction site protection experiments are consistent with this model. RNA polymerase very poorly protected this site centered at bp -12. However, when cAMP-CRP were added in addition to RNA polymerase, strong protection of the AluI site was found. cAMP-CRP alone did not prevent cutting at this site. These data suggest that cAMP-CRP facilitates RNA polymerase binding at the proper ilvB promoter in the -35 region. In the absence of cAMP-CRP polymerase binds mainly upstream of the -35 region in the vicinity of the RsaI and Sau3A restriction sites. The Malan and McClure model predicts that other processes, in addition to cAMP-CRP, that decrease polymerase binding at the upstream P₂ site should enhance polymerase binding and subsequent transcription from P₁. We examined this possibility by cutting the ilvB promoter at the Sau3A site close to the putative upstream promoter (P₂) in an attempt to block polymerase binding at P₂. In contrast to our previous results with the uncut promoter, the AluI site at -12 was strongly protected by RNA polymerase alone against AluI digestion. In addition, this Sau3A fragment was 3 times more effective as a template for in vitro transcription than the complete ilvB promoter. These data suggest that upstream sequences reduce RNA polymerase binding and activity in the -35 region. A comparison of the lac P₂ site with the putative P₂ site in ilvB and with a similar region in the cAMP-CRP regulated tnaA (17) and xylA (26) promoters revealed striking similarities (Table 1). These include a 6 bp consensus sequence, CTCACT, within or partially overlapping the CRP binding site. This consensus sequence begins 24 bp upstream from the -35 region and is preceded by GTGA, which starts 10 bp upstream. None of the 4 promoters differ by more than 2 nucleotides from the proposed P₂ consensus (Table 1). Taken together the data suggest that the mechanism of cAMP-CRP activation in these operons may be similar and may include a promoter in proximity to the CRP binding site that can efficiently compete with the -35 region for RNA

Table 1. Comparison of lac P₂ and P₁ promoter sites with sites in ilvB, tnaA and xylA.^a

		P ₂	P ₁
<u>lac</u>	G T G A----5-----	⁻⁵⁹ C T C A C T----17-----	⁻³⁶ T T T A C A
<u>ilvB</u>	G T G A----7-----	⁻⁵⁹ C T C A A T----18-----	⁻³⁵ T T T C C A
<u>tnaA</u>	G T G A----5-----	⁻⁵⁹ T T C A C A----17-----	⁻³⁶ T A G A C A
<u>xylA</u>	G T G A----5-----	⁻⁶¹ C T C A A T----20-----	⁻³⁵ T T G A G C
Consensus	G T G A----6-----	⁻⁶⁰ C T C A C T----18-----	⁻³⁵ T T G A C A

^a The non-transcribed strand is shown in the 5' to 3' direction.

polymerase binding. Although this model suggests a mechanism for cAMP-CRP stimulation of these operons it does not fully account for the activation of lac by this complex (1). Malan and McClure (1,20) have found that in addition to preventing RNA polymerase from binding to a non-productive promoter, cAMP-CRP increases the binding constant of polymerase for lac P₁. We do not know if this mechanism is also involved in CRP activation of the ilvB operon. However, the enhancement of DNase I cleavage by cAMP-CRP at several positions in the ilvB promoter suggests additional CRP effects due to changes in DNA structure.

In contrast to lac and tnaA, ilvB transcription is not completely dependent in vivo (6) and in vitro (2; Fig. 2) on cAMP-CRP. In vivo this may be due to the presence of other activators, such as ppGpp and integration host factor, which stimulate ilvB expression (4,5). However, these activators are not normally added to the in vitro transcription system. Therefore, ilvB P₁ may be partially active without additional positive factors. Alternatively, an additional promoter, not dependent on CRP, may account for ilvB transcription in the absence of this protein. It is unlikely that significant transcription initiates from the putative upstream promoter (ilvB P₂) since we have not detected a transcript from this region (unpublished observations). However, we have noted that the ilvB transcript made in vitro consists of several distinct species closely related in size (2). These transcripts may originate from separate promoters. In this regard, the region we have designated as the -35 region of the ilvB promoter, P₁, (TTTCCA) is followed by a 6 bp sequence (TTGTCT) which Hauser and Hatfield (3) have suggested is the

ilvB promoter. Further work is necessary to decide if these constitute overlapping promoters, which, as has been found in the gal operon (27), respond differently to cAMP-CRP.

ACKNOWLEDGMENTS

This work was supported by Grant GM17152 from the National Institute of General Medical Sciences.

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