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# Binding of integration host factor (IHF) to the *ilvGp1* promoter of the *ilvGMEDA* operon of *Escherichia coli* K12

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ABSTRACT

Crude extracts of Escherichia coli K-12 were found to bind DNA restriction fragments containing ilvGp1. Our analysis using a series of restriction fragments and a BamHI linker mutation indicate that a factor binds to ilvGp1 or adjacent to it. Analysis with mutant strains of E. coli K-12 and purified IHF indicate that IHF binds to ilvGp1. Furthermore, both analysis in vivo and in vitro indicate that IHF precludes transcription from ilvGp1.

## INTRODUCTION

In Escherichia coli K-12, the genes for the biosynthesis of isoleucine and valine are organized into several transcriptional units (1). The largest of these, the ilvGMEDA operon, appears to be regulated by multiple factors. The most thoroughly established control is multivalent-regulation by the three amino acids isoleucine, leucine and valine (2-5). This regulation occurs in response to alterations in the aminoacylation of the cognate tRNAs via a leader attenuator. A second regulatory factor is ppGpp, or magic spot (6). In relA strains the specific activity of threonine deaminase (ilvA) was found to be reduced relative to an isogenic wild type strain. Several proteins have been implicated as regulators or effectors of ilvGMEDA expression. Bacterial strains containing altered integration host factor (IHF) were found to have reduced expression of the operon (7,8). Johnson and Somerville (9) have analyzed a series of deletions extending from the deo operon towards the thr operon. Their experiments indicate the presence of two genes, ilvR and ileR, that affect expression of the ilvGMEDA operon. The first is a positive regulator, while the second is a negative effector of gene expression. Finally, Gray et al. (10) identified a protein which is regulated in concert with the operon. The gene for this protein is approximately 2 kb upstream of the start of transcription of the operon.

Transcription in vitro of the ilvGMEDA regulatory region yields two RNA's, one of 186 nt and the other of 258 nt (3,11). These transcripts initiate from

two tandem promoters, ilvGp1 and ilvGp2 (Fig. 1) and terminate within the attenuator. A different pattern of transcription occurs in vivo, where initiation originates solely from ilvGp2 (11,12). This conclusion was established using RNA fingerprint analysis (11), S1 nuclease analysis (12) and fusion of ilvGp1 to galK, the gene for galactokinase (12). Ortuno and Lawther (12) proposed that transcription from ilvGp1 was undetectable because the binding of DNA dependent RNA polymerase was precluded by the binding of another protein to the DNA that includes ilvGp1.

In an effort to identify an ilvGp1 binding protein, several approaches have been pursued. This report describes the results of the analysis of protein binding in vitro to restriction fragments using polyacrylamide gel electrophoresis. Our studies indicate that a protein in extracts of E. coli K-12 binds to ilvGp1. The analysis of extracts of various mutant strains indicated that IHF binds to restriction fragments containing ilvGp1. Our analysis in vivo supports the DNA binding experiments. Galactokinase assays of extracts of strains defective for IHF demonstrate increased expression of galactokinase from the plasmid pMO164 in which galK is fused to ilvGp1. The results of primer extension experiments are consistent with the conclusion that the observed increase in galK expression in the IHF defective E. coli strains originates from ilvGp1. Furthermore, experiments in vitro demonstrate that purified IHF binds to restriction fragments containing ilvGp1 and that IHF precludes transcription from ilvGp1.

## MATERIALS AND METHODS

### Bacteria, Plasmids and Media

The E. coli K-12 strains and plasmids used in this study are presented in Table 1. Luria-Bertani (LB) broth and M63 minimal medium were prepared as described by Miller (14) and contained 100 μg/ml of ampicillin.

### Enzymes and Biochemicals

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, E. coli DNA-dependent RNA polymerase, and the large fragment of E. coli DNA polymerase I (Klenow fragment) were obtained from New England Biolabs. Cloned M-MLV reverse transcriptase was obtained from Bethesda Research Laboratories. [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]UTP were obtained from New England Nuclear Corp. Recombinant DNA linkers were obtained from Collaborative Research Inc. D-[1-$^{14}$C]galactose was obtained from Amersham Corporation. Nucleoside triphosphates were obtained from P-L Biochemicals. [$\gamma$-$^{32}$P]ATP was obtained from ICN. Purified IHF was generously supplied by Dr. Howard Nash and appears to be

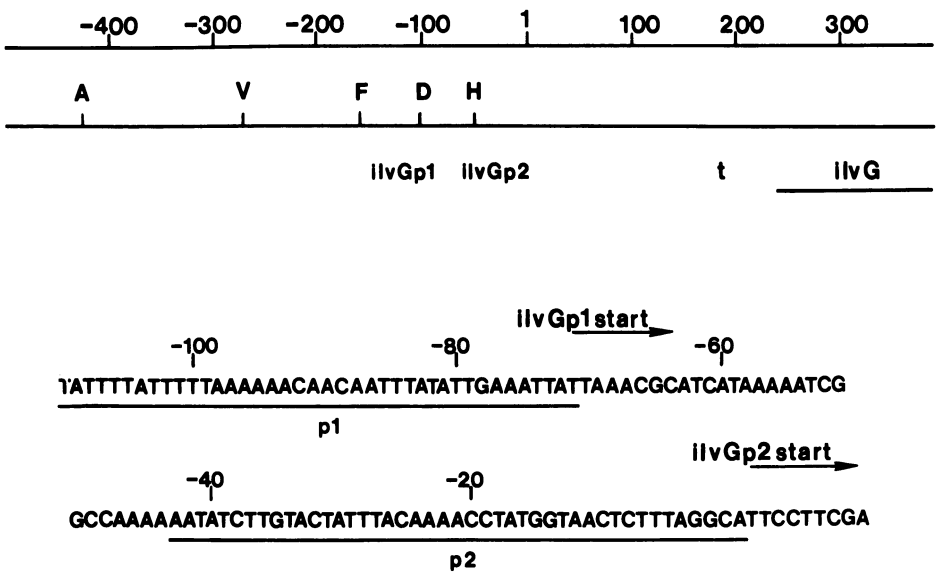


Figure 1. Representation of the promoter region of the ilvGMEDA operon. The figure is numbered relative to the transcription initiation site in vivo. The two promoters (ilvGp1 and ilvGp2) and the attenuator (t) are indicated. The restriction sites are designated as follows: A, AluI; D, DraI; F, FokI; H, HaeIII; and V, EcoRV.

homogeneous when analyzed by SDS polyacrylamide gel electrophoresis. All other reagents were obtained from Sigma Chemical Company.

Recombinant DNA Techniques

Plasmid DNA was isolated as previously described (15). Restriction endonuclease digestions were performed as suggested by New England Biolabs. The radionucleotide labelling of DNA and the isolation of restriction fragments were performed as described by Maxam and Gilbert (16). Other recombinant DNA techniques were as described by either Davis et al. (17) or Maniatis et al. (18).

Polyacrylamide Gel Electrophoresis DNA Binding Assay

A 10 ml culture of E. coli K-12 was grown to approximately $3X10^8$ cells/ml in LB broth. The bacteria were collected by centrifugation (3,000 xg for 10 min) and resuspended in 0.5 ml of extraction buffer (200 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 2.0 M NaCl, 2.5 mM EDTA, 4 mM dithiothreitol, 10% glycerol and 0.2 mg/ml phenylmethylsulfonylfluoride) and transferred to a 1.5 ml microcentrifuge tube. The cells were sonically disrupted using three 5s pulses at a microtip setting of 2.5 (Heat Systems-Ultrasonics, Inc., W-200 F Sonicator). The supernatant was resolved from cell debris by centrifugation (15,000 xg for 10

TABLE 1. Bacterial Strains and Plasmids

| Escherichia coli K-12 strains | | |
|---|---|---|
| Strain | Genotype | Source (reference) |
| FD1022 | galK2, rbs-302::Tn5, ΔilvGMEDA724::Tn5-131, IN(rrnD-rrnE)1 | This laboratory (15) |
| FD1054 | galK2, rbs-301::Tn10, ΔilvGMEDA723::Tn5, IN(rrnD-rrnE)1 | This laboratory (15) |
| M152 (N100)[a] | galK2, recA3, rpsL200, IN(rrnD-rrnE)1 | E. coli Genetic Stock Center (12) |
| REM776 (HN545) | galK2, rpsL200, himD157 (hip157), IN(rrnD-rrnE)1 | R.E. Musso |
| REM777 (HN428) | galK2, himA42, recB, rpsL200, IN(rrnD-rrnE)1 | R.E. Musso |
| REM778 (K5185) | galK2, ΔhimA82, rpsL200, IN(rrnD-rrnE)1 | R.E. Musso |
| 37-1 | Δ(deoD-trpR) | R. Somerville (9) |
| 61-1 | Δ(deoD-serB) | R. Somerville (9) |
| 122-1 | Δ(deoD-dye) | R. Somerville (9) |
| Plasmids | | |
| pAD1 | trp promoter-attenuator-rpoC terminator fusion plasmid. | A. Das (13) |
| pKO4 | galK fusion vector. | (12) |
| pMO139 | AluI-AluI, 1100 bp restriction fragment containing ilv promoter-leader-attenuator and the proximal portion of ilvG inserted into pKO4 using HindIII linkers. | (12) |
| pMO164 | AluI-HaeIII restriction fragment (Fig. 1) inserted using linkers EcoRI(AluI)-BamHI(HaeIII) into pKO4. | (12) |
| pMO173 | AluI-AluI, 1100 bp restriction fragment inserted into the EcoRI-HindIII sites of pKO4 using linkers. This plasmid contains a 10 bp BamHI linker inserted in the HaeIII site at -50 bp (Fig. 1 and 3C). | (12) |
| pMO177 | AluI-DraI restriction fragment (Fig. 1) inserted using linkers EcoRI(AluI)-BamHI(DraI) into pKO4. | This study |
| pMO186 | AluI-AluI, 1100 bp restriction fragment inserted into the EcoRI-HindIII sites of pKO4 using linkers. This plasmid contains a 10 bp BamHI linker inserted in the DraI site at -100 bp (Fig. 1 and 3C). | This study |
| pMO207 | rpoC terminator isolated on a 350 bp BamHI fragment from pAD1 inserted into the BamHI site of pMO164 (Fig. 8). | This study |

[a]Alternate strain designation

min) and the supernatant transferred to another 1.5 ml tube. Protein-DNA complexes were assayed on high ionic strength polyacrylamide gels as described by Pfeifer et al. (19). Binding assays were performed in a total volume of 10 µl containing: 3 µg of sonically disrupted calf thymus DNA; 1 µl of cell extract (3 µg protein); 10% glycerol and 25 ng [$^{32}$P]-DNA. After 15 min at 25°C the binding mixtures were loaded onto 4% polyacrylamide gels (acrylamide to bisacrylamide weight ratio of 40:1) in TBE buffer (90 mM Tris, 90 mM $H_3BO_3$, 2.5 mM EDTA). The gels were prerun for 2 hr at 20 mA and the DNA-protein complexes were resolved by electrophoresis at 25 mA.

Galactokinase Assays

Galactokinase was assayed as described previously (12). Bacterial cultures were grown in M63 minimal medium and subsequently sonically disrupted. Galactokinase activity was determined in a 50 µl reaction containing: 100 mM Tris-HCl pH 7.2, 4 mM $MgCl_2$, 5 mM ATP pH 7.0, 1 mM dithiothreitol, 3.2 mM NaF and 1.6 mM [1-$^{14}$C] galactose at a specific activity of 2 µCi/ µ mole. Relative plasmid copy number was assayed as described (12) and protein was determined by the method of Bradford (20) using reagent supplied by Bio-Rad Corporation.

Analysis of Cellular RNA by Primer Extension Using M-MLV Reverse Transcriptase

The appropriate bacterial strains containing the plasmid pMO164 were grown on M63 minimal medium (14). Whole cell RNA was extracted using the procedure described by Salser et al. (21). RNA concentration was quantitated by measuring the absorbance at 260 nm.

The oligonucleotide RL35, GCAGCAGAGGCGGATA (provided and analyzed, using a DuPont Zorbax HPLC-column, by R. York of the University of South Carolina Oligonucleotide Synthesis Laboratory), served as the primer and corresponds to the DNA sequence 137 bp upstream of galK. The primer was 5'-end labeled using [$\gamma$-$^{32}$P]ATP and polynucleotide kinase (16). Approximately 50 µg cellular RNA was hybridized for two hours at 42°C in reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol and 3 mM $MgCl_2$). Subsequently deoxyribonucleotide triphosphates (0.5 mM final concentration), and 1,000 units of M-MLV reverse transcriptase were added to the hybridization mixture (final volume 50 µl). This mixture was incubated at 37°C for one hour and the reaction terminated by the addition of EDTA to a final concentration of 2.0 mM. An equal volume of formamide dye mixture (16) was added to each sample. After heating at 95°C, the products of the primer extension reaction were resolved by loading one tenth of the above mixture onto a 10% polyacrylamide, 7.3 M urea, 20% formamide gel. The gel electrophoresis buffer consisted of 50 mM Tris, 50 mM $H_3BO_3$ and 2.5 mM EDTA.

Transcription *in vitro*.

Transcription in vitro was performed as described previously (12, 22). Unlabelled nucleoside triphosphates were present at a final concentration of 150 μM and [α-$^{32}$P]UTP was at 50 μM at a specific activity of 5 mCi/μmole. Each reaction contained 1 μg of plasmid (based upon the absorbance at 260 nm) and a 10-fold molar excess of RNA polymerase. The template plasmid, RNA polymerase, and buffer (20 mM Tris-Acetate pH 7.9, 100 mM KCl, 4 mM $Mg(OAc)_2$, 0.1 mM EDTA and 10 mM β-mercaptoethanol) were incubated at 37°C for 5 min and then 0.5 μl of 4 mg/ml heparin was added. After an additional 5 min at 37°C, the nucleotide triphosphates were added to the transcription mixture. The reaction was terminated after 15 min as described (22). The products of transcription were fractionated on a 7 M urea, 6% polyacrylamide gel and the transcripts were visualized by autoradiography.

## RESULTS

### 1. Characterization of an *ilvGP1* Binding Activity in Crude Extracts of *Escherichia coli K-12*.

Ortuno and Lawther (12) previously proposed that an unidentified ilvGp1 binding factor accounted for the observation that transcription could be initiated from ilvGp1 in vitro but not in vivo. An effective technique for the analysis for DNA binding factors is to assess whether the presence of a cellular extract results in retardation of a restriction fragment during migration through a polyacrylamide gel (23). Figure 2A presents an autoradiograph of the effect of a cellular extract on the migration of a restriction fragment that includes ilvGp1. The plasmid pMO164 when digested with the restriction endonucleases EcoRI and BamHI yields two restriction fragments. The two DNA molecules are the 400 bp ilv fragment corresponding to DNA from -450 bp to -50 bp, relative to the transcription initiation site of ilvGp2 (Fig. 1), and the parental vector pKO4 (3,700 bp). As can be seen by comparing lanes 1 and 2 of figure 2A, the presence of a crude cellular extract retards the migration of the purified 400 bp fragment from pMO164 containing ilvGp1.

To further localize the binding site(s), additional experiments were done by digesting the purified 400 bp EcoRI-BamHI restriction fragment with the restriction endonucleases EcoRV and FokI. Lanes 3, 4, 5 and 6 show that the binding factor interacts with the ilvGp1 end of the 400 bp restriction fragment. In figure 2A, lanes 3 and 4 show the effect of digesting the 400 bp fragment with the restriction endonuclease EcoRV (Fig. 2C). This yields a 170 bp fragment (from -450 bp to -280 bp) and a 230 bp fragment (from -280 to -50

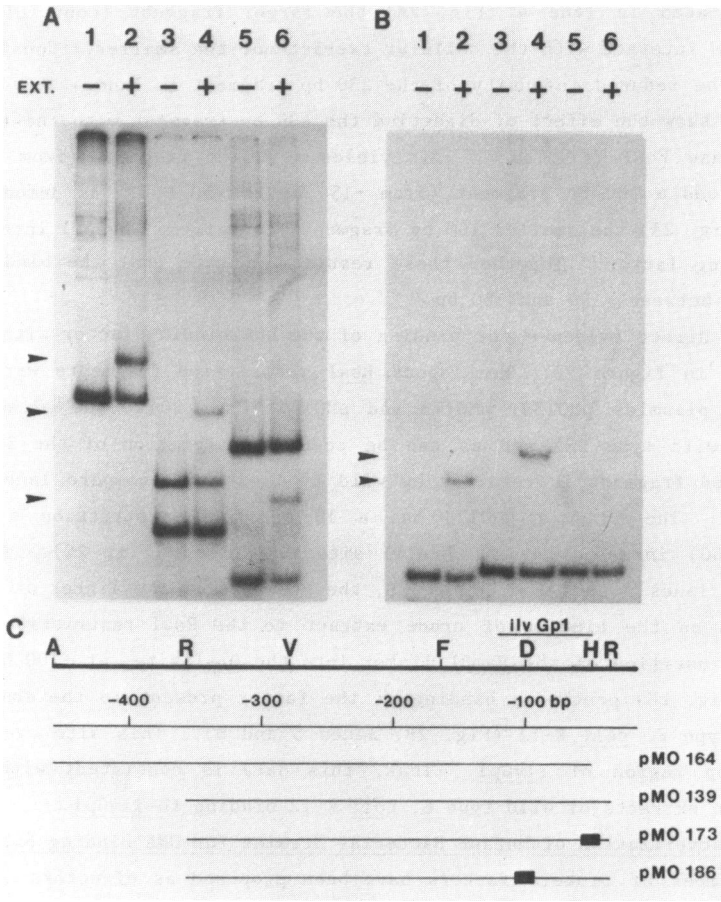


Figure 2. Analysis of the site of DNA binding of the factor present in crude extracts of E. coli K-12 using the polyacrylamide gel retardation assay. (A) Autoradiography of a restriction analysis of purified 400 bp restriction fragment containing ilvGp1 end labelled using [$\alpha$-$^{32}$P]dATP. Lanes 1 and 2 represent the undigested fragment in the absence (lane 1) and presence (lane 2) of the crude extract. Lanes 3 and 4 present the results of digestion of the fragment with EcoRV, while lanes 5 and 6 present the results after digestion with FokI. Arrows indicated the bands on the autoradiograph due to the decreased mobility of the bound DNA. (B) Autoradiograph of an analysis using the 340 bp RsaI restriction fragment from pMO139, pMO173 and pMO186. The RsaI fragments were kinased with [$\gamma$-$^{32}$P]ATP. Lanes 1 and 2 contain DNA from pMO139 in the absence (lane 1) or presence (lane 2) of E. coli extract. Lanes 3 and 4 contain DNA from pMO173, while lanes 5 and 6 contain DNA from pMO186. (C) Partial restriction site map and diagram of RsaI restriction fragments from pMO139, pMO173, and pMO186 with the 10 bp BamHI linker indicated by a rectangle. The restriction sites are indicated as follows: A, AluI; D, DraI; F, FokI; H, HaeIII; R, RsaI; and V, EcoRV.

bp). As seen in lane 4 (Fig. 2A) the larger fragment (containing ilvGp1) appears to interact with the cellular extract not the smaller. Consistent with this is the reduced intensity of the 230 bp fragment in lane 4. Lanes 5 and 6 (Fig. 2A) show the effect of digesting the 400 bp fragment with the restriction endonuclease FokI (Fig. 2C). This yields a 300 bp fragment (from -450 bp to -150 bp) and a 100 bp fragment (from -150 bp to -50 bp). As demonstrated in lane 6 (Fig. 2A) the smaller 100 bp fragment (containing ilvGp1) interacts with the binding factor. Together these results indicate that the binding factor interacts between -150 and -50 bp.

More direct evidence for binding of the DNA binding factor with ilvGp1 is presented in figure 2B. Homologous RsaI restriction fragments were isolated from the plasmids pMO139, pMO173 and pMO186 (Fig. 2C). The plasmid pMO139 contains wild type DNA and as can be seen the migration of the 340 bp RsaI restriction fragment is retarded by wild type extract (compare lanes 1 and 2, Fig. 2B). The plasmid pMO173 has a 10 bp BamHI restriction site linker (CCGGATCCGG) inserted at the HaeIII site at -50 bp (Fig. 2C). As seen by comparing lanes 3 and 4 of figure 2B, the presence of the linker at -50 bp has no effect on the binding of crude extract to the RsaI restriction fragment. However, insertion of the BamHI linker into the DraI site, at -100 bp (plasmid pMO186, Fig. 2C) precludes binding of the factor present in the crude extract of wild type E. coli K-12 (Fig. 2B, lanes 5 and 6). This site overlaps with the -35 bp region of ilvGp1. Thus, this data is consistent with a factor present in extracts of wild type E. coli K-12 binding to ilvGp1.

2. Characterization of Mutant Bacterial Strains for DNA Binding Factor.

A number of protein factors have been proposed as effectors of ilvGMEDA expression. These include IHF (6,7), ilvR and ileR gene products (9), and possibly a 15 kDa protein regulated in concert with the operon (10). Extracts of a series of mutant bacterial strains were analyzed for ilvGp1 binding activity (Fig. 3A). The plasmid pMO164 was digested with EcoRI and BamHI and then labelled with [$\alpha^{32}$P]dATP. Lane 1 of figure 3 shows the products of digestion of pMO164 resolved by polyacrylamide gel electrophoresis. Lane 2 demonstrates the effect of wild type extract (M152) on the products of digestion of pMO164. As before, the presence of wild type extract results in the retardation of migration of the 400 bp fragment of pMO164. Lanes 3 and 4 are extracts from two strains deleted between the rrnC and ilvGMEDA operons (15). The mutations in these strains (ilvGMEDA723::Tn5 and ilvGMEDA724Tn5-131) delete DNA (as indicated by the open bars in Fig. 3B) either within the ilvGMEDA operon (ilv723::Tn5) or the region between rrnC and ilvGMEDA (ilv724::Tn5-131).

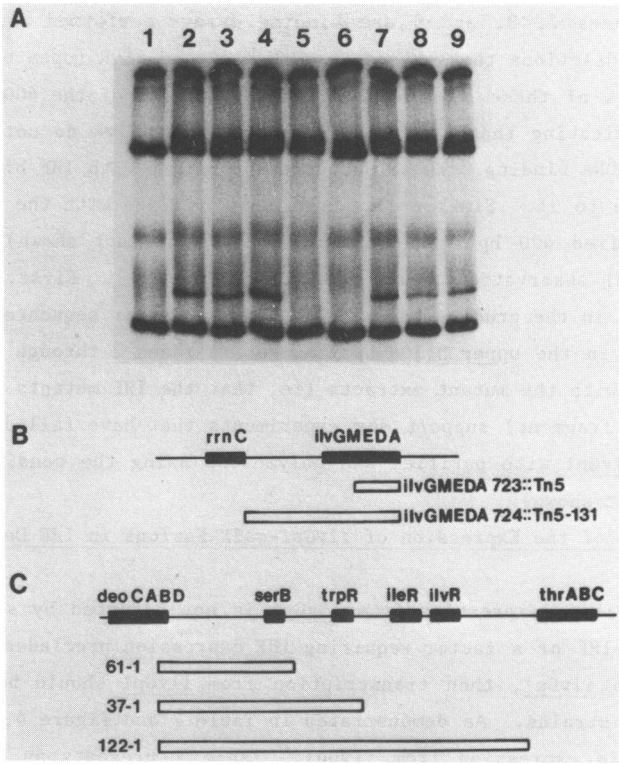


Figure 3. Analysis of mutant E. coli K-12 strains for DNA binding activity using the gel retardation assay. Lane 1 contains the products of digestion of pMO164 in the absence of any extract. Lane 2 demonstrates retardation of the 400 bp ilvGp1 containing fragment in the presence of a crude extract from M152. The remaining lanes contain the products of binding experiments with extracts from various mutant strains of E. coli. Lanes 3 and 4 contain extracts from the strains FD1054 and FD1022 containing the deletions ilv723::Tn5 (lane 3) and ilv724::Tn5-131 (lane 4). The open bars in part B of the figure indicate the extent of each of the deletions. Lanes 5 and 6 present the results using extracts of REM776, himD157 (hip157), and REM778, ΔhimA82. Lanes 7 through 9 analyze the role of ileR and ilvR. The extent of the three deletions 61-1 (lane 7), 37-1 (lane 8), and 122-1 (lane 9) are presented in part C of the figure.

Extracts of these two strains still retard migration of the 400 bp restriction fragment. Since the larger mutation deletes the gene for the 15 kDa protein described by Gray et al. (10), neither structural information within the ilvGMEDA operon nor the 15-kDa protein participates in the observed DNA binding. Lanes 5 and 6 are extracts of strains defective for IHF formation, REM776 (himD157) and REM778 (ΔhimA82) respectively. Extracts of neither of the two strains deficient for the subunits of IHF alter the migration of the 400 bp

fragment. Lanes 7, 8, and 9 are binding assays performed using extracts of strains with deletions that extend through ileR and ilvR (open boxes, Fig. 3C). Again extracts of these strains retard the migration of the 400 bp restriction fragment, indicating that the products of ileR and ilvR do not participate in the observed DNA binding. These data are consistent with IHF binding to ilvGp1 or very close to it. Similar data have been obtained with the mutant extracts and the purified 400 bp EcoRI-BamHI fragment (data not shown). Furthermore, two additional observations can be made from figure 3. First, factors appear to be present in the crude extracts that bind to vector sequences, as evidenced by the shift in the upper 3,700 bp fragment in lanes 2 through 6. Second, the observations with the mutant extracts (ie, that the IHF mutants do not bind the ilv specific fragment) support our experiments that have failed to demonstrate binding of ilvGp1 with purified RNA polymerase using the conditions described here (data not shown).

3. Analysis of the Expression of *ilvGp1-galK* Fusions in IHF Defective Strains

As described, expression from ilvGp1 is not detected by several criteria (11,12). If IHF or a factor requiring IHF expression precludes binding of RNA polymerase to ilvGp1, then transcription from ilvGp1 should be detectable in himA or himD strains. As demonstrated in Table 2 and figure 4, the absence of IHF results in expression from ilvGp1. Table 2 presents an analysis of the expression of galactokinase from pMO164 ( ilvGp1 fused to galK) and pMO177 (identical to pMO164 except ilvGp1 is deleted) in wild type, himA and himD strains. There is very little expression of galactokinase detected from pMO164 in the wild type strain M152 and a slightly lower level of expression is detected from pMO177 (deleted for ilvGp1). As can be seen a mutation in either himA (REM778) or himD (REM776) results in an approximately 10-fold increase in the expression of galK from pMO164, without a corresponding change in the expression of pMO177.

To support the conclusion that the increased expression of galactokinase in IHF defective strains was due to transcription initiating from ilvGp1, the 5' termini of cellular RNA was analyzed by primer extension (Fig. 4). Bacterial strains containing pMO164 were grown to approximately $2X10^8$ cells/ml in M63 minimal medium with glucose as the carbon source. RNA was extracted by standard techniques (21). As a control, pMO164 was transcribed in vitro to serve as a source of RNA initiated from ilvGp1. The 16 base oligonucleotide RL35 (which is complementary to a DNA sequence immediately upstream of galK) was hybridized to either RNA prepared in vitro or RNA extracted from cells con-

Table 2. Galactokinase Assays (galK) in IHF Defective Escherichia coli K-12 Strains

| Bacterial Strain | Relevant Genotype | Galactokinase[a] pMO164(ilvGp1) | pMO177(ΔilvGp1) |
|---|---|---|---|
| M152 | wild type | 0.74 | 0.53 |
| REM776 | himD157 | 8.5 | 0.48 |
| REM778 | ΔhimA82 | 6.2 | 0.44 |

[a]Activity in nmol/min/mg protein/fmol of plasmid

taining pMO164. The oligonucleotide served as the primer for reverse transcriptase generated DNA and the products of the reaction were resolved by electrophoresis on a urea/formamide polyacrylamide gel. Lane 1 of figure 4, demonstrates that RL35 primed DNA synthesis of RNA prepared by transcription in vitro yields the predicted 74 nucleotide product for transcripts originating at ilvGp1. Lane 2 contains a molecular weight standard consisting of HaeIII digested ØX174 kinased with [$\gamma$-$^{32}$P]ATP. The result presented in lane 3 is consistent with our previous analysis, indicating no expression from ilvGp1 in wild type E. coli K-12 (12), i.e. RNA extracted from M152 (wild type) containing pMO164 fails to yield a 74 nt product. Lanes 4, 5 and 6 all contain the 74 nt DNA molecule. Lane 4 contains the products of the analysis of RNA obtained from REM776 (himD157), lane 5 from REM777 (himA42) and lane 6 from REM778 (ΔhimA82). These results are consistent with transcription in vivo initiating from ilvGp1 in IHF defective strains.

4. Interaction of *ilvGp1* With Purified IHF

Our results are consistent with a protein binding to ilvGp1. The protein could be either IHF itself or a factor dependent upon the expression of IHF. To distinguish between these possibilities, the binding of purified IHF (24) to ilvGp1 containing DNA was analyzed (Fig. 5). Again the 340 bp and 350 bp RsaI fragments pMO139, pMO173 and pMO186 (Fig. 2C) served as substrates for the binding assay. As can be seen in figure 5, purified IHF binds (as evidenced by the altered migration of the restriction fragment) to the RsaI fragments from either wild type DNA (lanes 1 and 2) or to DNA with a BamHI linker inserted at -50 bp (lanes 3 and 4). However, insertion of the BamHI linker at -100 bp precludes binding of IHF to the RsaI fragment (lanes 5 and 6) as assayed by migration through a polyacrylamide gel. This result indicates that IHF is the protein that binds to the DNA segment that includes ilvGp1. This conclusion is further supported by a parallel analysis that demonstrates that the retardation

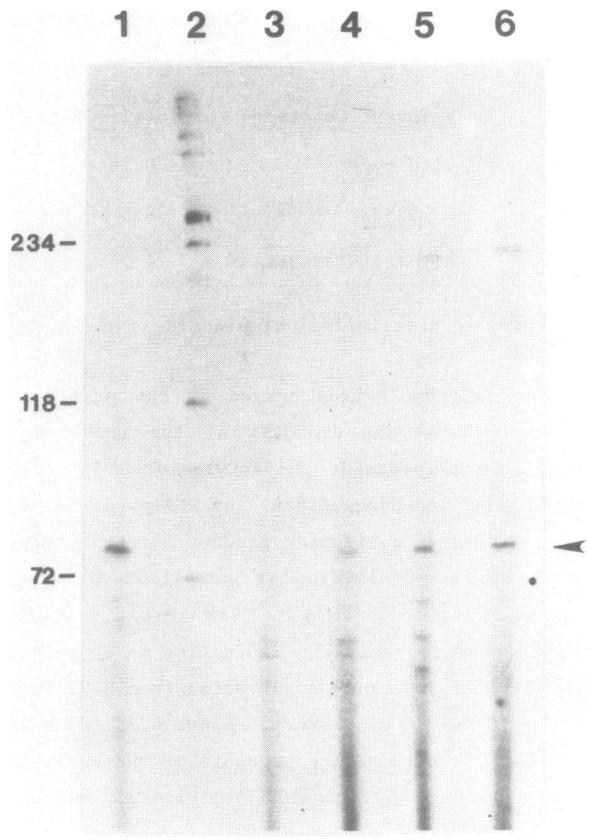


Figure 4. Autoradiograph of primer extension experiment to determine 5'-terminus of galK transcript in IHF-defective strains. The oligonucleotide RL35 (GCAGCAGAGGCGGATA) was end-labelled by kinasing and hybridized with RNA from: lane 1, transcription in vitro of pMO164; lane 3, M152; lane 4, REM776 (himD157); lane 5, REM777, himA42; and lane 6, REM778 ΔhimA82. Lane 2 contains [$^{32}$P]-labelled HaeIII digested ØX174 DNA as a molecular size marker. After hybridization, deoxynucleoside triphosphates and M-MLV reverse transcriptase were added. The products of the reaction were resolved by electrophoresis on a 7.3 M urea, 20% formamide, 10% polyacrylamide gel. The arrow indicates the 74 nt DNA product, which results from the primer extension reactions.

of migration of the 400 bp fragment is identical for both crude extracts and purified IHF (data not shown).

To further test the effect of IHF on expression from ilvGp1, transcription in vitro from ilvGp1 was analyzed in the presence or absence of IHF. Figure 6 presents an autoradiograph of such a transcription experiment. The plasmid pMO207 was utilized as a template. This plasmid is a derivative of pMO164 which was constructed by inserting the rpoC terminator (13) downstream of

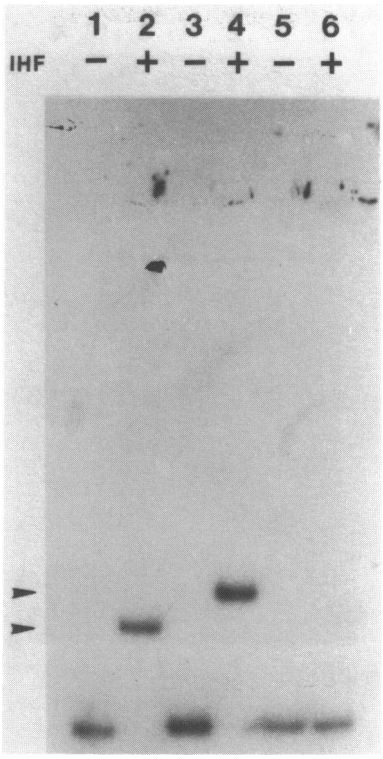


Figure 5. Autoradiograph of the binding of purified IHF to the 340 bp ilvGp1 containing RsaI restriction fragment from pMO139 (lanes 1 and 2); pMO173 (lanes 3 and 4); and pMO186 (lanes 5 and 6). Reactions 2, 4 and 6 contained 0.1 μg of IHF.

ilvGp1. As shown in lane 1 of figure 6, transcription in vitro of pMO207 yields transcripts of 108 nt and 184 nt. The shorter RNA originates from the ColE1 origin of replication, while the longer RNA corresponds to transcription originating at ilvGp1 and ending within the rpoC terminator. The remaining lanes indicate the effect of addition of purified IHF five minutes before the addition of RNA polymerase (lane 2), simultaneously with RNA polymerase (lane 3) or 12 minutes after the addition of RNA polymerase (lane 4). Either the addition of IHF prior to or together with RNA polymerase results in the total suppression of the formation of the 184 nt RNA. This result is consistent with the idea that IHF binds to pMO207 in a manner that precludes transcription from ilvGp1. The data present in lane 4 (IHF was added 12 min after the initiation of transcription) combined with the constant presence of the 108 nt RNA indicate that the absence of the 184 nt transcript is not due to RNase in the IHF

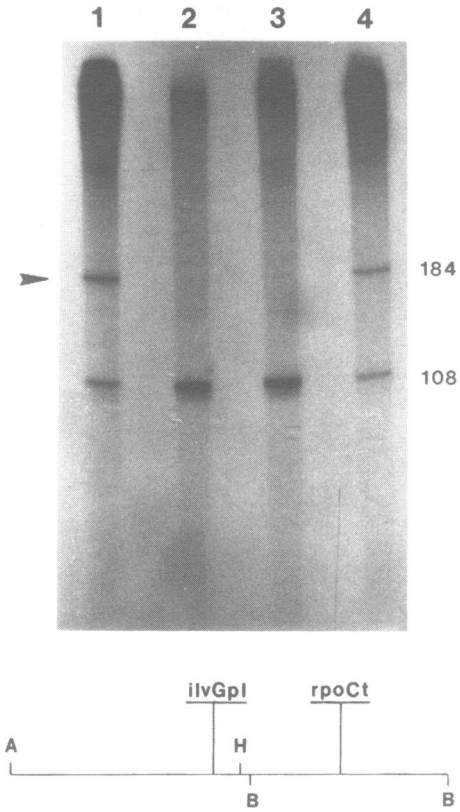


Figure 6. Autoradiography of transcription in vitro of pM0207 in the absence (lane 1) or presence (lanes 2-4) of purified IHF (0.1 μg). IHF was added either: 5 minutes prior to RNA polymerase (lane 2); simultaneously with RNA polymerase (lane 3); or 12 minutes after RNA polymerase. Fifteen minutes after the initiation of transcription with RNA polymerase, the reactions were terminated by the addition of a urea dye mixture as described (22).

preparation. An alternative explanation for the absence of the 184 nt RNA would be that IHF is acting as an antitermination factor. The consistent presence of the 108 nt RNA combined with our observations of other promoter-terminator combinations (data not shown) indicate that IHF is not acting as a general antitermination factor. Finally, it should be noted that the presence of IHF in lanes 2 and 3 appears to stimulate the formation of the 108 nt RNA. This implies that IHF can positively affect transcription from a promoter.

## DISCUSSION

Previous studies (11,12) have shown a difference between the transcription patterns obtained *in vitro* and *in vivo* for the *ilvGMEDA* operon regulatory region. Analysis of transcription *in vitro* (3) indicated that the operon was preceded by the tandem promoters *ilvGp1* and *ilvGp2* (3,11). Examination of *ilvGMEDA* expression *in vivo* indicates that transcription originates solely from *ilvGp2* (11,12). To explain this difference, Ortuno and Lawther (12) proposed that an unidentified factor binds to the DNA upstream of *ilvGp2* precluding RNA polymerase from initiating transcription from *ilvGp1*. This study has utilized the observation that DNA protein complexes migrate at a reduced rate (relative to uncomplexed DNA) through polyacrylamide gels (23). Using this assay, extracts of wild type *E. coli* K-12 were found to contain a factor that retarded the migration of a restriction fragment that contains *ilvGp1* and identifies the factor as IHF. Because the transcription initiation site for *ilvGp1* is at -72 bp (Fig. 1), RNA polymerase must interact with DNA sequences that extend to -110 bp to bind to *ilvGp1*. Thus the binding of a factor, as indicated by the experiments presented here, to this region might preclude or compete with the binding of RNA polymerase.

The data presented both previously (12) and in this study lead to a simple model. First, the region upstream of the promoter utilized *in vivo*, *ilvGp2*, positively affects expression from that promoter. Second, although this upstream region (relative to *ilvGp2*) contains DNA sequences (*ilvGp1*) from which transcription initiates *in vitro*, expression from *ilvGp1* is precluded *in vivo* because of the binding of a protein, IHF (and possibly yet other unidentified factors). Third, the binding of IHF upstream of *ilvGp2* enhances expression from this promoter. Adams and Hatfield (25) published the first observations of a role for the region upstream of *ilvGp2*. They observed that the presence of these upstream DNA sequences enhanced expression from that promoter. Our own analysis further defined the region required for this enhancement as lying between -150 and -50 bp (12). The initial transcription *in vitro* experiments clearly demonstrated that two promoter-like sequences existed upstream of *ilvG* (3). Adams et al. (11) defined these two sites as *ilvGp1* and *ilvGp2* and determined the initiating nucleotide for each of the two transcripts. However, both their analysis and our own indicated that transcription *in vivo* could only be detected from the *ilvGp2* and not *ilvGp1* (11,12). The data presented in this report clearly indicates the basis of that observation, i.e., the presence of IHF in cells precludes the initiation of transcription from *ilvGp1*. This conclusion is supported by the polyacrylamide gel electrophoresis experiments

with crude extracts of wild type and IHF mutant strains of E. coli K-12 and by the experiments with purified IHF. Further experiments are necessary to define the exact site or sites to which IHF binds. Also, it remains to be established whether IHF precludes RNA polymerase from binding to ilvGp1, or if an inactive ternary complex between IHF, RNA polymerase and ilvGp1 is formed. The final aspect of this model (enhanced transcription due to IHF binding) remains to be rigorously tested. Our data, while certainly in concert with this hypothesis, do not prove it. However, the previous reports of Friden et al. (7) and Friedman et al. (8) that IHF defective strains have reduced levels of ilv gene expression are consistent with the model. Furthermore, as discussed previously (12), insertion of the BamHI linker into the DraI site eliminates the enhancement of expression from ilvGp2 by the upstream DNA sequences, and as described here, disrupts the binding of IHF (Fig. 2 and Fig. 5). Together, these observations strongly support the proposed model.

The actual or absolute physiological raison d'être for IHF participating in ilvGMEDA expression remains to be elucidated. This report does not address that important and intriguing question. Since mutations in himA and himD (hip) do not appear to be lethal, the genes are deemed to be nonessential. However, that conclusion may be premature considering the apparent requirement for compensatory mutations in the genes for DNA gyrase to isolate mutations in the gene for topoisomerase 1 (26). In part, understanding the role of IHF in ilv gene expression requires greater information about the physiological function of IHF and the regulatory mechanisms involved in maintaining cellular levels of IHF. For instance, one simplistic model is that IHF is a structural component of the E. coli genomic nucleoid. The ilvGMEDA operon is located adjacent to an IHF binding site and the ilv promoter may have fortuitously evolved to be dependent upon the upstream binding of IHF for optimal gene expression. Alternatively, IHF may be involved in an unrecognized general or universal cellular regulatory mechanism. Hence, establishing or understanding the physiological function of IHF on ilv gene expression may prove elusive. Experiments are in progress to investigate this and to define the molecular interactions of IHF with ilv DNA.

In summary, our data indicate that IHF binds to DNA sequences upstream of ilvGp2. Observations obtained by both analysis in vivo and in vitro indicate that IHF binds to or near ilvGp1 and, as a result, IHF precludes transcription from ilvGp1.

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