Volume 14 Number 6 1986 Nucleic Acids Research

Analysis and comparison of the internal promoter, pE, of the *ilvGMEDA* operons from *Escherichia coli* K-12 and *Salmonella typhimurium*

John M.Lopes and Robert P.Lawther*

Department of Biology, University of South Carolina, Columbia, SC 29208, USA

Received 26 November 1985; Accepted 19 February 1986

ABSTRACT

It was previously determined that the distal portion of the ilvGMEDA operon was expressed despite the insertion of transposons into ilvG and ilvE. This observation suggested the existence of internal promoters upstream of ilvE (pE) and ilvD (pD). The internal promoter pE, responsible for part of ilvEDA expression, has been analyzed both in vivo and in vitro. Our results indicate that: (1) pE exists in both E. coli K-12 and S. typhimurium; (2) pE is located in the distal end of the ilvM coding sequence; (3) the pE sequence is highly conserved in the two bacteria; (4) the amino acid sequence of the ilvM gene product is 93% homologous between the two bacteria; (5) transcription from pE can be demonstrated both in vivo and in vitro; (6) the efficiency of pE is essentially equivalent in the two bacteria.

INTRODUCTION

Our understanding of the expression of genes organized into an operon has evolved dramatically. Since gene expression is initiated as a unit from a single promoter, organization into an operon ensures the coordinate regulation of genes whose products are functionally related. It has become apparent that a variety of factors can result in the differential expression of genes within an operon. One of these factors is the internal promoter. While the presence of internal promoters has been documented genetically and biochemically, a complete understanding of their role in gene expression remains unestablished.

The initial evidence of internal sites for transcription initiation was the noncoordinate repression of genes within the tryptophan operon (1, 2, 3, 4). The presence of internal promoters in other operons was implied from the observation that portions of these operons continued to be expressed despite disruption of transcription from a primary promoter(s) (5, 6, 7, 8, 9). As more information has accumulated on a wider range of bacterial operons the prevalence of internal promoters has become increasingly evident, while the regulatory significance of many internal promoters remains to be elucidated. Acquiring an understanding of how these elements function and their contribution to the overall expression of their respective operons requires the

characterization of each promoter at the molecular level.

Five of the genes for the biosynthesis of isoleucine and valine form the ilvGMEDA operon (10, 11, 12). Genetic analysis with Tn5 (5) Tn10 (6, 7) and Mu (8) suggested the presence of internal promoters within this operon. In these studies, it was observed that despite the disruption of the operon the distal portion continued to be expressed. Biochemical analysis supports the presence of an internal promoter, pE, prior to ilvE. Both the binding of RNA polymerase to restriction fragments (13) and the protection of the SalI restriction site prior to ilvE by RNA polymerase (unpublished observations R.P. Lawther and G.W. Hatfield) indicate that pE is near the SalI site. Comparison of the DNA sequence near the SalI site to the consensus DNA sequence for promoters indicated that pE probably overlaps this restriction site (14). The more recent studies of Calhoun et al. (15) are consistent with this location.

Published studies on the expression and regulation of the ilvGMEDA operon have not presented a consistent pattern for the role of pE in the expression and regulation of this operon (6, 7, 8, 13, 16). In order to establish its role in the expression of this operon, pE from both Escherichia coli K-12 and Salmonella typhimurium has been characterized. The site of transcription initiation from pE was determined by transcription in vitro. Moreover, S1 analysis of cellular RNA suggested that the same initiation site is utilized in vivo. The DNA sequence of pE from S. typhimurium was determined to be 95% homologous to that of pE from E. coli K-12, and it also indicates that the ilvM product is 93% conserved between the two organisms.

MATERIALS AND METHODS

(a) Bacteria, Plasmids and Media

Three E. coli K-12 strains were used: M152 [galK2, recA3, rpsL200, IN (rrnD-rrnE)1], was obtained from the E. coli Genetic Stock Center, while FD1009 [rbs-302::Tn 5, ilvE12, $trpE_{am9829}$, $trpA_{am9761}$] and FD1022 [rbs-302::Tn 5, ΔilvGMEDA724::Tn 5-131, galK2, IN (rrnD-rrnE)1] were both constructed in this laboratory. The plasmids are described in Table 1 with reference to Fig. 1. Luria-Bertani (LB) broth and M63 minimal medium were prepared as described by Miller (17) and contained 100 μg/ml of ampicillin.

(b) Enzymes and Biochemicals

Restriction endonucleases, T4 DNA ligase, E. coli DNA-directed RNA polymerase, exonuclease III, and the large fragment of E. coli DNA polymerase I were obtained from New England Biolabs. S1 nuclease and [α-^{32}P]UTP were obtained from New England Nuclear Corporation. Calf alkaline phosphatase was

obtained from Boehringer Mannheim Biochemicals. T4 polynucleotide kinase and nucleoside triphosphates were obtained from P-L Biochemicals. [γ-^{32}P]ATP was obtained from ICN. Adenosine 5'-O-(3-thiotriphosphate) ([γ-S]ATP) and guanosine 5'-O-(3-thiotriphosphate) ([γ-S]GTP) were a gift of Dr. M.R. Stallcup. D-[1-^{14}C] galactose was obtained from Amersham Corporation. All other reagents were obtained from Sigma Chemical Company.

(c) Recombinant DNA Techniques

Plasmid DNA was isolated as previously described (18). Restriction endonuclease digestions were performed according to the specifications of the supplier. Other recombinant DNA techniques were as described by either Davis et al. (19) or Maniatis et al. (20). DNA fragments were sequenced as described by Maxam and Gilbert (21).

(d) Transcriptions *in vitro*

Transcription was carried out in a 20 µl volume as previously described by Lawther et al. (22). Unlabelled ATP, CTP, and GTP were present at final concentrations of 150 µM and [α-^{32}P]UTP (5mCi/µmole) was present at 50 µM. In experiments designed to determine the initiating nucleotide in vitro, either [γ-S]ATP or [γ-S]GTP was substituted for ATP or GTP respectively at a final concentration of 150 µM. Each reaction contained 1 µg of plasmid (based upon the absorbance at λ = 260 nm) and a 10-fold molar excess of RNA polymerase (RNAP). The template, RNAP, and buffer (20 mM Tris-acetate, 4 mM $Mg(OAc)_2$, 100 mM KCl, 0.1 mM EDTA and 10 mM β-mercaptoethanol) were incubated at 37°C for 5 min and 1 µl of 4 mg/ml heparin added. After an additional 5 min at 37°C, a solution of the nucleoside triphosphates (pre-warmed to 37°C) was added. The reaction was allowed to proceed for 15 min and then terminated by the addition of an equal volume of 2X TBE (100 mM Trizma base, 100 mM boric acid and 2 mM EDTA), 0.1% sodium dodecyl sulfate, 0.05% bromophenol blue, 0.05% xylene cyanole FF and 1.1 gm/ml of urea. The transcription products were heated at 95°C for 2 min, fractionated on a 7 M urea/6% polyacrylamide gel, and visualized by autoradiography. Transcripts generated using the thionucleoside triphosphates were purified from the reaction mixture and fractionated through a mercury-Sepharose column (Hg-Sepharose) as described by Zhang et al. (23). Bound transcripts were eluted (El) with TNES buffer (0.1 M Tris-hydrochloride, pH = 8.0, 1 M NaCl, 10 mM EDTA and 1% SDS). The flow through (Ft) as well as eluted fractions were collected and ethanol precipitated overnight at -20°C. The RNA fractions were dissolved and fractionated on a urea/polyacrylamide gel as described above. The transcription products were quantitated by densitometric scanning of the autoradiographs using a Beckman DU-8 spectrophotometer.

TABLE 1. PLASMIDS

PLASMID	DESCRIPTION	REFERENCE
pBR322	cloning vector	(54)
pKO4	galK fusion vector	(28)
pDR720	galK fusion vector	(55)
mp9	lacZ fusion vector	(56)
pRL5	4.5 Kb HindIII fragment, that includes the promoter proximal portion of the ilvGMEDA operon (of Escherichia coli K-12), into ilvE, inserted into pBR322	(22, 53)
pRL103	5.5 Kb HindIII fragment, that includes the promoter proximal portion of the ilvGMEDA operon (of Salmonella typhimurium), into ilvE, inserted into pBR322	(57)
pRL113	6.8 Kb KpnI fragment, that includes ilvMEDA (of Escherichia coli K-12) inserted into the KpnI site of pRL5 so as to reconstitute the ilvGMEDA operon	This lab
LP019	1,160 bp PvuII/HindIII fragment, from pRL5 (denoted in Fig. 1), inserted into the SmaI/HindIII sites of mp9	This study
pRL149	165 bp Sau3A/RsaI fragment, from pRL5 (contains ilvGMEDA attenuator/terminator), inserted into the BamHI/SmaI sites of pDR720	This lab
pJL206	1,160 bp EcoRI/HindIII fragment, from LP019 (RF), inserted into the EcoRI/HindIII sites of pKO4	This study
pJL209	2.5 Kb PvuII fragment, from pRL113 (denoted in Fig. 1, contains entire coding sequence for ilvME), inserted into the PvuII site of pBR322 such that ilvME is oriented towards the Tet^r gene	This study
pJL211	Similar to pJL209 except that ilvME is oriented towards the Amp^r gene	This study
pJL230	630 bp AluI fragment (backfilled) from pJL206 (denoted in Fig. 1), inserted into the EcoRI/BamHI (backfilled) sites of pKO4 regenerating the EcoRI and BamHI sites	This study

TABLE 1. PLASMIDS (cont.)

PLASMID	DESCRIPTION	REFERENCE
pJL231	950 bp AluI fragment (backfilled), from pRL103 (denoted in Fig. 1), inserted into the EcoRI/BamHI (backfilled) sites of pKO4 regenerating the EcoRI and BamHI sites	This study
pJL239	550 bp Sau3A fragment, from pRL149 (contains ilv attenuator/terminator), inserted into the BamHI site of pJL230	This study
pJL240	550 bp Sau3A fragment, from pRL149 (contains ilv attenuator/terminator), inserted into the BamHI site of pJL231	This study

The peak areas were converted to moles of transcript by comparison to a known standard.

(e) S1 Mapping Analysis

RNA was extracted using the procedure described by Jones et al. (24). The RNA concentration was determined by measuring the absorbance at λ = 260 nm. Restriction fragments were 5' end-labelled with T4 polynucleotide kinase and treated with 4000 units of exonuclease III for 30 min at 37°C in order to generate single stranded probes (25). Approximately 5 µg of probe, plus 100 µg of RNA, was precipitated by the addition of ethanol. The pellet was dried and dissolved in 50 µl of hybridization buffer (100 mM Tris-hydrochloride, pH = 8.0, 300 mM NaCl and 2.5 mM EDTA). Hybridization mixtures were sealed in microcapillary pipettes and incubated 16 hrs at 65°C. The DNA:RNA hybrids were transferred to a 1.5 ml Eppendorf tube and digested one hour at 20°C in 30 mM NaOAc, pH = 4.5, 125 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, 10 µg/ml sonicated calf thymus DNA and 50 units S1 nuclease. The digestion was terminated by precipitation with ethanol. The nucleic acids were dissolved in 20 µl of sequencing dye (21), fractionated on either an 8 M urea 8% polyacrylamide or an 8 M urea/20% polyacrylamide gel, and the S1 digestion products were visualized by autoradiography.

(f) Enzyme Assays

Transaminase B (ilvE) was assayed as described by Lawther and Hatfield (26). Galactokinase was assayed by a method similar to that previously described (27, 28). A 10 ml bacterial culture (3 x 10^8 cells per ml) was chilled, collected by centrifugation (3,000 x g for 10 min), and the pellet resuspended in 2.0 ml of galactokinase sonication buffer (20 mM Tris-hydrochloride, pH = 7.2, 8 mM $MgCl_2$ and 2.2 mM dithiothreitol). One ml of this sample was

sonically disrupted using three 5s pulses at a microtip setting of 2 (Heat Systems-Ultrasonics, Inc., W-220F Sonicator). The supernatant was separated from cell debris by centrifugation (15,000 x g for 10 min) and then transferred to another 1.5 ml Eppendorf tube. Each 50 µl reaction for the determination of galactokinase was prepared from stock solutions as described (27, 28) and contained 100 mM Tris-hydrochloride, pH = 7.2, 4 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol, 3.2 mM NaF and 1.6 mM [1-^{14}C]galactose (2 µCi/µmole). The reaction was initiated by the addition of extract and 10 µl aliquots were removed at 5, 10 and 15 min. The aliquots were spotted onto 2.4 cm Whatman DE81 filters and plunged immediately into 1 liter of ice-cold distilled water. The filters were washed 5X with 20 ml distilled water per filter; 2X with 95% ethanol and dried prior to quantitation by liquid scintillation counting. Protein was determined by the method of Bradford (29).

RESULTS

I. Expression of Transaminase B (*ilvE*) from pE

Genetic analysis indicated the presence of an internal promoter (pE) upstream of ilvE in E. coli K-12 (5-8). DNA sequence analysis (14) and protection of a SalI restriction site (within ilvM) from digestion with HincII by RNA polymerase (unpublished observation, R.P. Lawther and G.W. Hatfield) implied that pE was close to or included this SalI site. The SalI site and ilvE are both included on a 2.5 kilobase pair (Kb) PvuII restriction fragment that starts within ilvG and ends within ilvD (Fig. 1). Plasmids pJL209 and pJL211 were constructed by insertion of this fragment into the PvuII site of pBR322. The plasmid pJL211 has the PvuII fragment inserted in the same orientation as that of tet gene transcription, while pJL209 has ilvE inserted in the opposite orientation. Both plasmids complement the ilvE12 mutation in FD1009. The specific activity of transaminase B resulting from each of these plasmids is presented in Table 2. The data indicate that transaminase B is expressed at an equivalent level from each of these plasmids independent of the orientation of the PvuII fragment. Furthermore the PvuII fragment includes the distal portion of ilvG, which contains a strong transcriptional polar site (14, 26). This site would reduce or preclude any expression into ilvE originating from a pBR322-encoded promoter. Therefore, ilvE expression must originate from within the PvuII fragment.

Published experiments designed to investigate the regulation of pE in E. coli K-12 (8, 13) and S. typhimurium (6, 7, 16) suggested that expression of ilvEDA from this promoter may be modulated by the presence of isoleucine (ile),

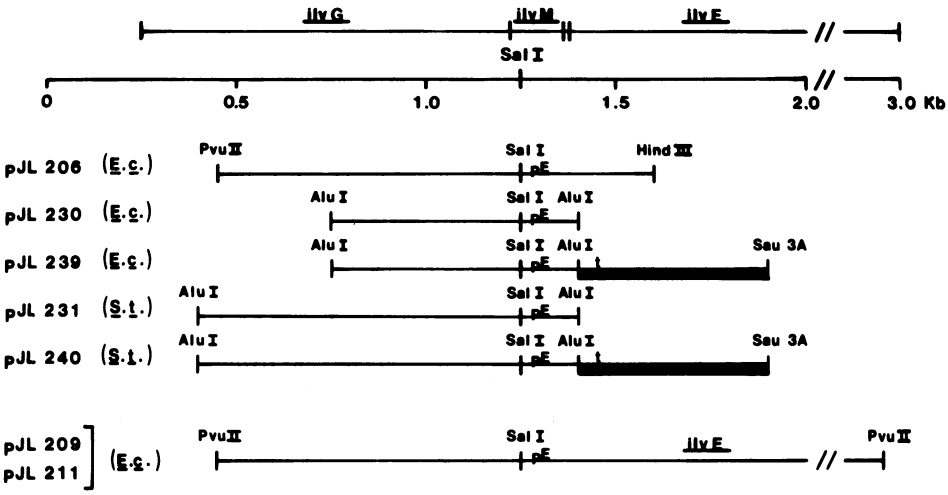

Figure 1 - Restriction fragment inserts used to construct galK fusions to pE and pBR322 ilvE derivatives. The predicted location of pE is indicated for each construction. The source of DNA for each construction is indicated in parentheses next to the plasmid name (E. c. = Escherichia coli K-12, S. t. = Salmonella typhimurium). The blackened box in pJL239 and pJL240 indicates the Sau3A fragment from pRL149 containing the ilvGMEDA attenuator with the transcription termination site denoted as t (see also Table 1).

leucine (leu) and valine (val). Furthermore, expression of the ilvGMEDA operon is almost completely repressed when cells are grown in LB-broth (13, 30, 31). This observation implies that expression from the primary promoter as well as pE may be regulated. Bacteria containing pJL209 and pJL211 were grown under repressing conditions and assayed for transaminase B activity. Consistent with

TABLE 2. SPECIFIC ACTIVITY OF TRANSAMINASE B (ilvE) IN FD1009 HARBORING PLASMIDS WITH INSERTS FROM THE ilvGMEDA OPERON of E. coli K-12

Plasmid/Strain	Media	Transaminase B (ilvE) Specific Activity[a]
none/FD1009	M63	0.0
pJL209/FD1009	M63	66.8
pJL211/FD1009	M63	68.8
pJL209/FD1009	M63, ile, leu, val[b]	41.5
pJL211/FD1009	M63, ile, leu, val	30.4
pJL209/FD1009	LB-broth	1.9
pJL211/FD1009	LB-broth	3.5

[a] nmoles of α-ketoisovalerate/minute/mg of protein
[b] The concentrations of isoleucine, leucine and valine were 0.5, 0.5 and 1.0 mM, respectively.

TABLE 3. GALACTOKINASE ASSAY (galK)

Plasmid/Strain	Galactokinase (galK) Specific Activity[a]
none/M152	0.0
pKO4/M152	7.6
pJL206/M152	21.8
pJL230/M152	17.8
pJL231/M152	25.8

[a] nmoles of galactose-1-phosphate/minute/mg of protein

previous observations (8), the expression of ilvE (Table 2) decreased approximately 40 to 60% in the presence of 0.5 mM ile, 0.5 mM leu, and 1.0 mM val, and 95% when cells were grown in LB-broth.

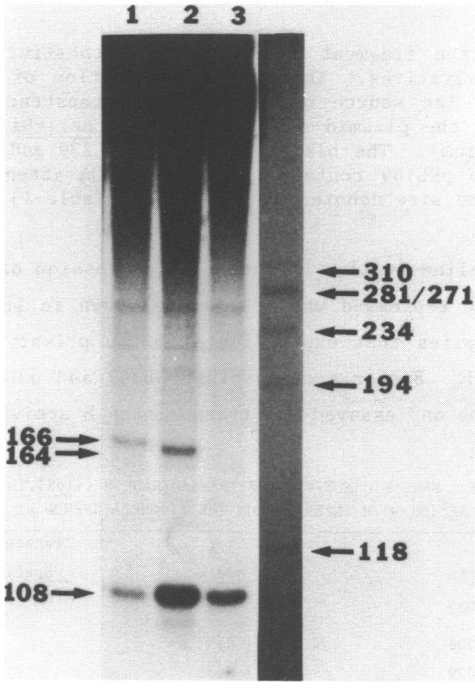

Figure 2 - Autoradiograph of the products of transcription in vitro of pJL239 (lane 1), pJL240 (lane 2), and pJL230 (lane 3). The plasmids were transcribed as described in Materials and Methods and the transcripts fractionated on a 7M urea/6% polyacrylamide gel. The size of the relevant transcripts are indicated in number of nucleotides: ColE1 (108), pE from pJL239 (166), pE from pJL240 (164). The last lane contains a size standard of φX174 DNA cut with HaeIII.

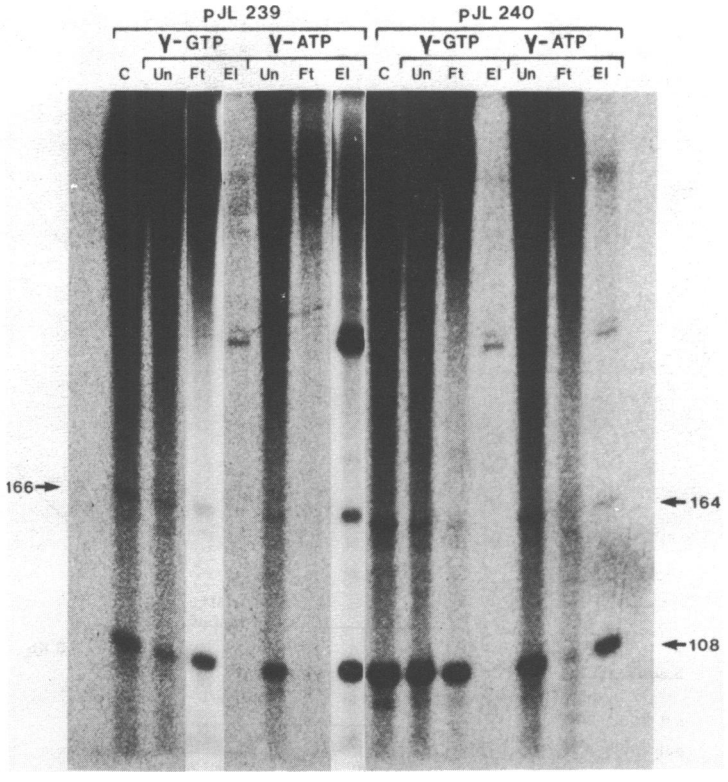

Figure 3 - Autoradiograph of the products of transcription in vitro of pJL239 and pJL240. The plasmids were transcribed as previously described using either [γ-S]GTP (lanes denoted by γ-GTP) or [γ-S]ATP (lanes denoted by γ-ATP) substituted for GTP and ATP respectively. The transcription products were fractionated on a 7M urea/6% polyacrylamide gel. The size of the relevant transcripts are indicated in number of nucleotides: ColE1 (108), pE from pJL239 (166), pE from pJL240 (164). The transcript sizes were determined by comparison to a ϕX174/HaeIII size standard (not shown). The products in lanes denoted by a C result from plasmids transcribed using GTP and ATP. Lanes denoted as Un, Ft and El contain the products of plasmids transcribed using the thionucleosides. Un = unfractionated. Ft = flow through (did not bind to Hg-Sepharose column). El = eluted (bound to Hg-Sepharose column).

II. Expression of Galactokinase (*galK*) from pE

To further delineate the location of pE, an 1100 base pair (bp) PvuII-HindIII fragment and a 630 bp AluI fragment (Fig. 1) were inserted into the galK expression vector pKO4 (28) to generate pJL206 and pJL230 respectively. Both of these plasmids conveyed the ability to express galK. Transfectants of M152 (galK2) exhibited a red phenotype on galactose MacConkey agar, and were also able to grow on minimal medium containing galactose as a sole

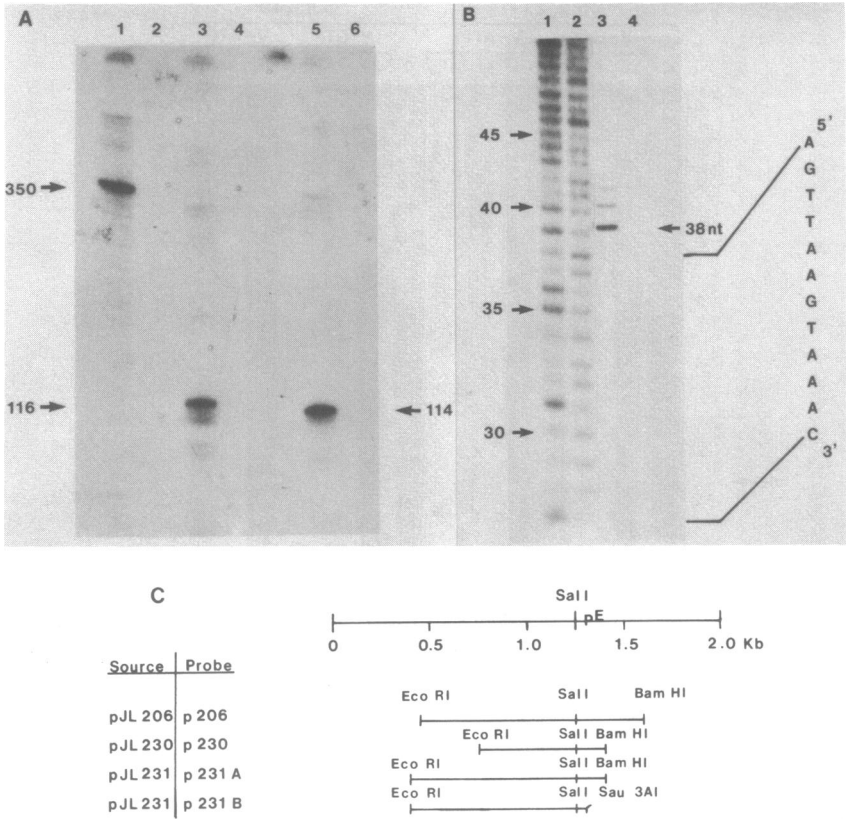

Figure 4A - Autoradiograph of the products of an S1 analysis of transcription in vivo from pE. RNA isolated from FD1022 transformed with pJL206 (lane 1), pJL230 (lane 3), pJL231 (lane 5), or untransformed (lanes 2, 4 and 6) was hybridized to p206 (lanes 1 and 2), or p230 (lanes 3 and 4), or p231A (lanes 5 and 6). Each probe was 5' end-labelled with [γ-^{32}P]ATP (using T4 polynucleotide kinase) and treated with exonuclease III, to generate a single stranded probe (25), prior to hybridization. The resulting DNA:RNA hybrids were digested with S1 nuclease and the products fractionated on an 8M urea/8% polyacrylamide gel. The sizes of the resulting products are indicated in nucleotides: lane 1 (350); lane 3 (116); lane 5 (114). The length of each product was determined by comparison to a ϕX174/HaeIII size standard (not shown). Figure 4B - Autoradiograph of the products of an S1 analysis of transcription in vivo from pE fractionated alongside a Maxam and Gilbert sequencing ladder (21). RNA extracted from pJL231 transfected FD1022 (lane 3) and FD1022 (lane 4) was hybridized to p231B. The probe was treated as described in Fig. 4A, prior to hybridization. The resulting DNA:RNA hybrids were digested with S1 nuclease and the products were fractionated on an 8M urea/20% polyacrylamide gel. Lanes 1 and 2 contain the products of A+G and C+T Maxam and Gilbert sequencing reactions performed on a 54 bp SalI-Sau3A fragment, from pJL231, 5' end-labelled at the Sau3A site. The distance from the Sau3A end is indicated in nucleotides to the left while the length of the S1 digested product is

indicated to the right (38 nt). The sequence for the non-coding strand beginning with the transcription initiating nucleotide is included for comparison. Figure 4C - Schematic representation of the probes used in the S1 analysis presented in Fig. 4A and 4B. Source indicates the plasmids from which the probes were prepared (see also Fig. 1 and Table 1). Probe establishes the name of the probe which will be utilized in the text (see section V).

carbon source. The level of galactokinase resulting from the presence of each of these plasmids was determined (Table 3). Both plasmids result in about a three-fold increase in galactokinase activity relative to pKO4.

The SalI site within ilvM is conserved in S. typhimurium (unpublished observation, J.M. Lopes and R.P. Lawther; 32). To test for the presence of pE in S. typhimurium a 950 bp AluI fragment, containing this SalI site, was inserted into pKO4 to yield pJL231. The phenotype of M152 transfected with this plasmid is indistinguishable from M152 transfected with either of the E. coli K-12 constructs. As indicated in Table 3, the level of expression of galK from pJL231 is similar to that for pJL206 and pJL230.

III. Transcription from pE *in vitro*

Transcription in vitro was used to determine the approximate location of pE. Experiments to demonstrate transcription from pE in vitro, using purified restriction fragments were unsuccessful. Transcription from a closed circular template required the insertion of a terminator (denoted as t in Fig. 1) downstream of pE. For this purpose, a Sau3A restriction fragment from pRL149, that contains the ilvGMEDA attenuator, was used. The terminating nucleotide (nt) within the attenuator is located 54 bp from one end of the Sau3A fragment. This fragment was inserted into the BamHI site of pJL230 and pJL231 to yield pJL239 and pJL240 respectively (Table 1, Fig. 1). Figure 2 presents an autoradiograph of a gel with the products of transcription in vitro of pJL239 and pJL240.

Transcription in vitro of pJL239 (E. coli K-12) yields a 166 nt RNA and the 108 nt ColE1 replication RNA (lane 1; 33), while transcription in vitro of pJL230 (E. coli K-12) does not yield the 166 nt RNA. This demonstrates that the 166 nt transcript from pJL239 terminates within the attenuator. Similarly, transcription in vitro of pJL240 (S. typhimurium) yields a 164 nt RNA and the 108 nt RNA. These data are consistent with the apparent conservation of this region of the ilv operon in E. coli K-12 and S. typhimurium (32), including pE, and indicates that the site of initiation of transcription in vitro is within 10 to 15 bp of the conserved SalI site.

IV. Transcription initiating nucleotide *in vitro*

Most E. coli K-12 transcripts are initiated with a purine nucleoside

triphosphate. The availability of [γ-S]ATP and [γ-S]GTP allows the direct determination of whether ATP or GTP is the initiating nucleotide of a transcript because the thio group specifically labels the 5' terminal nucleotide. The plasmids pJL239 and pJL240 were transcribed in vitro, with either [γ-S]ATP or [γ-S]GTP substituted for ATP or GTP respectively. The products of transcription were fractionated by chromatography through a mercury-Sepharose column (Hg-Sepharose). The transcripts initiated with the 5' thionucleotide bound to the column, while other transcripts passed through (Ft) the column. The bound transcripts were eluted (El) with TNES buffer (see Materials and Methods). Figure 3 presents an autoradiograph of a gel depicting the results of such an experiment using pJL239 and pJL240.

As a control both pJL239 and pJL240 were transcribed in vitro as described before (i.e., with ATP and GTP) and the products appear in the C lanes of Fig. 3. To ensure that the thionucleotides did not interfere with transcription, a portion of the products of each transcription reaction was characterized by electrophoresis without prior fractionation. These samples appear in the lanes designated Un. As shown in Fig. 3 the presence of this nucleoside does not alter the observed products of transcription in vitro from either pJL239 or pJL240.

Neither the 166 nt RNA (from pJL239) nor the 164 nt RNA (from pJL240) bound to the Hg-Sepharose column when transcription was carried out in the presence of [γ-S]GTP. These RNAs appeared in the unbound fraction (Ft) not in the eluted fraction (El). Both RNAs bound to the column when ATP was replaced by [γ-S]ATP, as indicated by their presence in the eluted fraction (El) and absence from the unbound fraction (Ft). The 108 nt ColE1 RNA follows a similar pattern. This transcript is known to be initiated with ATP (33). These observations indicate that the initiating nucleotide of a pE-directed transcript for both E. coli K-12 and S. typhimurium is ATP.

V. S1 analysis of cellular RNA

In order to determine if transcription in vivo from pE is identical to transcription in vitro, the transcripts formed in vivo from pE were analyzed using S1 nuclease. The strain FD1022, deleted for ilvGMEDA, was transformed with pJL206 (E. coli K-12), pJL230 (E. coli K-12) and pJL231 (S. typhimurium). This insured that products of the S1 nuclease analysis reflected plasmid-directed transcripts. EcoRI-BamHI restriction fragments (Fig. 4C) from each plasmid were used to generate probes for the analysis of the cellular transcripts. The results of this analysis are shown in the autoradiograph presented in Fig. 4A.

RNA was isolated from pJL206/FD1022, hybridized to p206 and treated with

S1 nuclease. A 350 nt protected fragment was observed, as shown in Fig. 4A (lane 1). The RNA from pJL230/FD1022 was analyzed for its ability to protect p230. This RNA protected a 116 nt fragment (lane 3). The RNA from pJL231/FD1022 was probed with p231A and a 114 nt S1 protected fragment was generated (lane 5). None of these products were observed when RNA from FD1022 was used, indicating that they are plasmid derived.

In order to determine the exact 5' terminus of the products generated in Fig. 4A, the products of an S1 nuclease analysis were fractionated alongside a Maxam and Gilbert sequencing ladder (21). The presence of a Sau3A site 54 bp downstream of the SalI site facilitated the analysis of transcription of pE from S. typhimurium. Figure 4B displays the results of such an experiment. The first two lanes contain Maxam and Gilbert A+G and C+T reactions (21). Lanes 3 and 4 show the products of an S1 nuclease treated RNA, from pJL231/FD1022 and FD1022 (respectively) and hybridized to p231B. The Sau3A restriction site lies 56 bp downstream of the SalI site. As shown in Fig. 4B, a number of products were generated (lane 3). These were not detected when RNA from FD1022 was used. This observation again indicates that these products are plasmid generated. The predominant product is 38 nt in length while the larger products probably result from incomplete S1 nuclease digestion. Alternatively, they might reflect multiple 5' message termini. Note that these S1 products migrate 1½ nts slower than their chemically cleaved counterparts. This is due to slight differences in structure of the 3' end of S1 products as compared to chemical cleavage products (34, 35, 36, 37).

DISCUSSION

Five of the genes for isoleucine/valine biosynthesis form the ilvGMEDA operon (10, 11, 12) which is transcribed in vivo from a site upstream of ilvG (8, 11, 31, 38). The presence of additional transcription start sites was initially suggested by Tn10 (6, 7), Tn5 (5) and Mu (8) insertions that were only partially polar on expression of the downstream genes. These mutants implied possible transcription starts upstream of ilvE (pE) and ilvD (pD). Moreover, a SalI site located in the coding sequence of ilvM has been demonstrated to be protected from HincII digestion by RNA polymerase (unpublished observation, R.P. Lawther and G.W. Hatfield). This latter observation taken with the fact that the SalI site is highly conserved among the Enterobacteriaceae (32), suggested that pE may be located in close proximity to the SalI site.

Blazey and Burns have presented data suggesting that pE is not present in

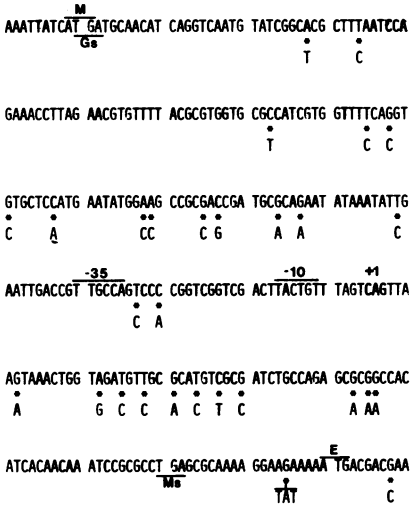

Figure 5 - DNA sequence of the region upstream of ilvE including the entire coding sequence of ilvM from Salmonella typhimurium. The asterisks (*) indicate those nucleotides which differ between S. typhimurium and E. coli K-12 (95% homology). M = translational start for ilvM. Gs = translational stop for ilvG. Ms = translational stop for ilvM. E = translational start for ilvE. The start of transcription from pE is indicated as +1 and promoter sequences are indicated as -10 and -35. Note that 5 nucleotides upstream of the start of ilvE (E) 3 nucleotides (TAT) in E. coli K-12 replace a single nucleotide (G) in S. typhimurium.

S. typhimurium (39). Their conclusion was based on two observations. First, fusion of a SalI-HindIII (1,200 bp downstream of the SalI site) fragment to galK yielded no detectable galactokinase activity. Second, subcloning a 5.9 Kb SalI fragment (containing the entire coding sequences for ilvEDA) into pBR322, in an orientation opposite that of tet gene transcription, resulted in a plasmid that did not express ilvEDA. The design of these constructions was based on the assumption that the SalI site was located within the distal portion of ilvG. However, heterologous DNA hybridization, using cloned portions of the ilvGMEDA operon from E. coli K-12 (32), and subcloning portions of the operon from S. typhimurium (unpublished observation, J.M. Lopes and R.P. Lawther) indicated that the SalI site is located within ilvM in an identical location to that in E. coli K-12.

In order to demonstrate that pE is present in both E. coli K-12 and S. typhimurium, a series of restriction fragments were analyzed for the presence of this promoter. A 2.5 Kb PvuII fragment from E. coli K-12 (containing the entire ilvE coding sequence and an additional 920 bp upstream) was subcloned

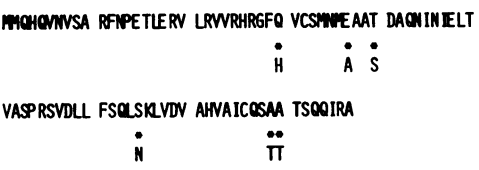

Figure 6 - Amino acid sequence of ilvM from S. typhimurium determined from the DNA sequence (Fig. 6). The asterisks (*) indicate those amino acids which differ between S. typhimurium and E. coli K-12. The NH_2-terminus of the ilvM protein (small subunit of acetolactate synthase isozyme II) from S. typhimurium has been determined by amino acid sequencing elsewhere to be M M Q H Q V N V S A R F N P E T L E R V L R V V R (40).

into pBR322 in both possible orientations. Both constructions yielded equivalent levels of transaminase B (ilvE) activity. To further localize pE, two restriction fragments from E. coli K-12 and one from S. typhimurium were fused to galK (28). All three fusions produced essentially equivalent levels of galactokinase activity.

Restriction fragments from S. typhimurium that include the SalI site (data not shown) were sequenced (Fig. 5). The nucleotide sequence of the region upstream of ilvE is 90% homologous to the corresponding region in E. coli K-12 (14, 22). Furthermore, the amino acid sequence of the ilvM protein (small subunit of acetolactate synthase isozyme II; Fig. 6) deduced from the DNA sequence (Fig. 5) is 93% homologous to that of ilvM from E. coli K-12. The deduced amino acid sequence agrees with both the NH_2-terminal sequence and the overall amino acid composition described by Schloss et al. (40). It is clear from this analysis that the location of the SalI site is entirely conserved within the ilvM coding sequence of these two bacteria, again demonstrating conservation of the location of pE.

Our results establish that the transcription initiating nucleotide in vitro (Fig. 2 and 3) is the same for both bacteria (denoted as +1 in Fig. 5; corresponding to nucleotide 2101 from the initiating nucleotide at the 5' end of the operon, ref. 14). While S1 analysis cannot categorically establish transcription initiation sites, the data presented suggests that the in vivo initiation site is the same as the in vitro site. The S1 analysis presented in Fig. 4A and 4B maps the 5' terminus to the same site as determined by the in vitro transcription experiments. Moreover, Wek and Hatfield (accompanying manuscript, 41) have performed S1 analyses on RNAs transcribed in vitro from pE as well as cellular RNAs, and their results are in complete agreement with those presented here.

The sequence of pE is highly conserved between the two bacteria, thus

TABLE 4. COMPILATION OF INTERNAL PROMOTERS

Operon	Promoter	-35[a]	Space	-10[a]	Homology Score[b]	Reference
------	Consensus	tcTTGACat	17	TAtAaT	100.0%	(42, 44, 45, 46)
ilv (E. c.)	pE	cgTTGCCag	18	TAcTgT	49.7%	This Study
ilv (S. t.)	pE	cgTTGCCag	18	TAcTgT	49.7%	This Study
trp (E. c.)	p2	ccGTGACat	15	TAcAaG	52.1%	(43)
his (E. c.)	Bp	tgTTTAAat	17	CAtTaT	47.9%	(58)
thr (E. c.)	Bp	cgGTGTCtt	16	TAcCcT	34.3%	(51)
deo (E. c.)	P3	taTCGCCgt	16	TAtAcT	51.5%	(47)
fts (E. c.)	pA	gaTTGCGcg	16	TAtGgT	40.2%	(48)
fts (E. c.)	pZ	agTTGGCtg	17	TTtTaT	53.3%	(48)
rpsU-dnaG-rpoD (E. c.)	Pa	cgCAGCTaa	17	TAtAcT	30.2%	(50)
rpsU-dnaG-rpoD (E. c.)	Pb	gtCTGACca	21	TAtCgT	35.5%	(50)

[a] Nucleotides denoted as capital letters indicate those found to be highly conserved among E. coli K-12 promoters, (Hawley and McClure, 44).
[b] Homology score was determined using TARGSEARCH (Mulligan et al., 42).

suggesting that the number of pE-directed transcripts should be equivalent in these two bacteria. Mulligan et al. (42) have devised a program (TARGSEARCH) that determines the extent of promoter homology to the consensus procaryotic promoter (expressed as Homology Score in Table 4). As shown in Table 4, pE from both bacteria share the same homology score (49.7%). Analysis of the in vitro pE-directed transcripts (Fig. 2), by densitometric scanning, demonstrates that the strength of pE is essentially equivalent in vitro. These observations are consistent with the results in vivo; where galactokinase assays (Table 3) show virtually equivalent levels of expression from pE of both organisms. This is not consistent with the proposal that pE in S. typhimurium is stronger than it is in E. coli K-12 (7, 13).

Previous studies indicated that pE may be constitutive (6, 7, 8) whereas others indicated that it may be regulated (16, 13). It is clear from Table 2 that pE-directed expression of ilvE is negligible when cells are grown in LB-broth. This is consistent with previous results that show literally no ilvGMEDA expression in vivo when cells are grown in LB-broth (13, 30, 31). There appears to be a 40-60% repression of pE in the presence of ile, leu and val, which is consistent with the results of Smith et al. (8). It should also be noted that the extent of repression observed with ile, leu and val is similar to that observed for the intact wild type operon (30, 31). However, our data may not accurately reflect the regulation of pE, since expression was measured from a multicopy plasmid. This is in contrast to the studies of an

internal promoter of the trp operon by Horowitz and Platt (43). Their analysis characterized the contribution of p2 to the expression of the trp operon of E. coli K-12 by directly measuring the percentage of p2-directed transcripts in vivo (quantitative S1 analysis). Their data indicate that transcription from p2 is unaltered regardless of the growth conditions (i.e., minimal media, minimal supplemented with tryptophan, or LB-broth). Moreover, their data indicate that p2 provides 3-5% of trpCBA expression under permissive conditions and 85-90% expression under repressed conditions. However, it is difficult to determine the contribution of pE in the overall expression of the ilvGMEDA operon, since the regulatory features of the operon are not yet totally understood.

The role of internal promoters remains unclear, while their prevalence and conservation within specific transcriptional units indicates their importance. Table 4 presents the homology scores for a number of internal promoters that have been described in the literature. It is clear from this compilation that these promoters all lie within a narrow range of homology scores and show relatively weak homology to the consensus procaryotic promoter (42, 44, 45, 46). While the fts and deo internal promoters are regulated by effector proteins to wide ranges of expressivity (47, 48, 49), others such as the rpsU-dnaG-rpoD internal promoters are constitutively expressed at very low levels (50). The presence of internal promoters in the fts and deo operons may serve to generate various stoichiometric proportions of the products of these operons required to meet the physiological demands of the cell in a given environment. The internal promoters of the amino acid biosynthetic operons all share a relatively weak homology to the consensus promoter. The thr operon's internal promoter (Bp) is weaker than the rest but its location has not been conclusively established (51). With the exception of pE, the internal promoters within amino acid biosynthetic operons are not repressed in the presence of the specific amino acids which their respective operons synthesize. This suggests that their role is to provide a basal level of the downstream gene products which may facilitate the cell's transition from a repressed state. The noted conservation of p2 in the trp operon among the Enterobacteriaceae (1, 2, 3, 4) and the suggestive evidence that pE in the ilv operon is also highly conserved (5, 6, 7, 8, 13, 14, 15, 16, 32), attests to the importance of these elements in the overall expression of their respective operons. Although pE is located in the coding sequence of ilvM, it seems improbable that its nucleotide sequence is conserved purely as a function of amino acid sequence conservation. The amino acids which span pE include valine, alanine, serine, and leucine all

of which can be encoded by either four or six codons. Thus, the nucleotide sequence could change dramatically without altering the protein sequence. In particular, a GCC encoded alanine and an AGT encoded serine, that span the -35 region, are rare codons for their respective amino acids as determined by codon utilization studies in E. coli K-12 (10). Moreover, a TTA encoded leucine, that spans the -10 region, is also a rare codon in both E. coli K-12 and S. typhimurium (personal communication B. Nichols).

The possibility exists for potential differences in the role of pE in E. coli K-12 and S. typhimurium as a result of a different genetic organization. In wild type E. coli K-12, ilvG is cryptic. A rho-dependent termination site (15, 26, 52) exists downstream of the site where the ilvG coding sequence is disrupted (14, 53). This may result in a major fraction of transcription terminating upstream of ilvE. Thus, it is conceivable that very little of the expression of ilvEDA originates from the ilvGMEDA promoter attenuator complex. Therefore, in E. coli, pE may provide a significant amount of ilvEDA expression. Since, in wild type S. typhimurium ilvG is not cryptic, pE may play a much less significant role in ilvEDA expression in this organism.

ACKNOWLEDGEMENTS

The authors wish to thank: E.A. Thompson and R.H. Showman for their advice and criticisms of this manuscript; R.C. Wek and G.W. Hatfield for their cooperation; W.R. McClure for sending us TARGSEARCH; and T.D. Parton-Lopes for typing this manuscript. This work was supported by Public Health Service grant GM28021 from the National Institute of General Medical Sciences.

*To whom correspondence should be addressed

REFERENCES

1. Bauerle, R.H. and Margolin, P. (1967) J. Mol. Biol. 26, 423-436.
2. Jackson, E.N. and Yanofsky, C. (1972) J. Mol. Biol. 69, 307-313.
3. Campbell, A. (1979) in Biological Regulation and Development, Goldberger, R.F., Ed., pp. 19-55, Plenum Press, New York.
4. Largen, M. and Belser, W. (1973) Genetics 75, 19-22.
5. Berg, C.M., Shaw, K.J., Vender, J. and Borucka-Mankiewicz, M. (1979) Genetics 93, 309-319.
6. Berg, C.M. and Shaw, K.J. (1981) J. Bacteriol. 145, 984-989.
7. Blazey, D.L. and Burns, R.O. (1979) Molec. Gen. Genet. 177, 1-11.
8. Smith, J.M., Smith, F.J. and Umbarger, H.E. (1979) Molec. Gen. Genet. 169, 299-314.
9. Kleckner, N., Chan, R.K., Tye, B.-K. and Botstein, D. (1975) J. Mol. Biol. 97, 561-575.
10. Wek, R.C., Hauser, C.A. and Hatfield, G.W. (1985) Nucl. Acids Res. 13, 3995-4010.
11. Lawther, R.P. and Hatfield, G.W. (1980) Proc. Natl. Acad. Sci. USA 77,

1862-1866.
12. Nargang, F.E., Subrahmanyam, C.S. and Umbarger, H.E. (1980) Proc. Natl. Acad. Sci. USA 77, 1823-1827.
13. Subrahmanyam, C.S., Noti, J.D. and Umbarger, H.E. (1980) J. Bacteriol. 144, 279-290.
14. Lawther, R.P., Calhoun, D.H., Adams, C.W., Hauser, C.A., Gray, J. and Hatfield, G.W. (1981) Proc. Natl. Acad. Sci. USA 78, 922-925.
15. Calhoun, D.H., Wallen, J.W., Traub, L., Gray, J.E. and Kung, H.-F. (1985) J. Bacteriol. 161, 128-132.
16. Blazey, D.L., Kim, R. and Burns, R.O. (1981) J. Bacteriol. 147, 452-462.
17. Miller, J.H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory.
18. Driver, R.P. and Lawther, R.P. (1985) J. Bacteriol. 162, 598-606.
19. Davis, R.W., Botstein, D. and Roth, J.R. (1980) in Advanced Bacterial Genetics, Cold Spring Harbor Laboratory.
20. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning, Cold Spring Harbor Laboratory.
21. Maxam, A. and Gilbert, W. (1980) in Methods of Enzymology 65, 499-560.
22. Lawther, R.P., Nichols, B., Zurawski, G. and Hatfield, G.W. (1979) Nucl. Acids Res. 7, 2289-2301.
23. Zhang, Z.-Y., Thompson, E.A., and Stallcup, M.R. (1984) Nucl. Acids Res. 12, 8115-8128.
24. Jones, H.M., Brajkovich, C.M., and Gunsalus, R.P. (1983) J. Bacteriol. 155, 1279-1287.
25. Miller, K.G., and Söllner-Webb, B. (1981) Cell 27, 165-174.
26. Lawther, R.P. and Hatfield, G.W. (1978) Molec. Gen. Genet. 167, 227-234.
27. Adams, C.W. and Hatfield, G.W. (1984) J. Biol. Chem. 259, 7399-7403.
28. McKenney, K.H., Shimatake, H., Court, D., Schmeissner, U. and Rosenberg, M. (1981) in Gene Amplification and Analysis, Chirikijian, J. and Papos, T. Eds., Vol. II, pp. 383-415, Elsevier-North Holland Publishing, Co., New York.
29. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
30. Lawther, R.P. and Hatfield, G.W. (1977) J. Bacteriol. 130, 552-557.
31. Gayda, D.J., Leathers, T.D., Noti, J.D., Smith, F.J., Smith, J.M., Subrahmanyam, C.S. and Umbarger, H.E. (1980) J. Bacteriol. 142, 556-567.
32. Driver, R.P. and Lawther, R.P. (1985) J. Bacteriol. 162, 1317-1319.
33. Tomizawa, J.-I. (1984) Cell 38, 861-870.
34. Maxam, A. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
35. Söllner-Webb, B. and Reeder, R.H. (1979) Cell 18, 485-499.
36. Schmincke, C., Herman, K. and Hausen, P. (1976) Proc. Nat. Acad. Sci. USA 73, 1994-1998.
37. Tomizawa, J., Ohmori, H. and Bird, R. (1977) Proc. Natl. Acad. Sci. USA 74, 1865-1869.
38. Ortuno, J.M. and Lawther, R.P. Manuscript in Preparation.
39. Blazey, D.L. and Burns, R.O. (1982) Proc. Natl. Acad. Sci. USA 79, 5011-5015.
40. Schloss, J.V., Dyk, D.E.V., Vasta, J.F. and Kutny, R.M. (1985) Biochemistry 24, 4952-4968.
41. Wek, R.C. and Hatfield, G.W. (1986) Nucl. Acids Res. Accompanying manuscript.
42. Mulligan, M.E., Hawley, D.K., Entriken, R. and McClure, W.R. (1984) Nucl. Acids. Res. 12, 789-800.
43. Horowitz, H. and Platt, T. (1983) J. Biol. Chem. 258, 7890-7893.
44. Hawley, D.K. and McClure, W.R. (1983) Nucl. Acids. Res. 11, 2237-2255.
45. Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319-353.
46. Siebenlist, U., Simpson, R.B. and Gilbert, W. (1980) Cell 20, 269-281.

47. Valentin-Hansen, P., Hammer, K., Larsen, J.E.L. and Svendsen, I. (1984) Nucl. Acids. Res. 12, 5211-5224.
48. Yi, Q.-M., Rockenbach, S., Ward, J.E., Jr. and Lutkenhaus, J. (1985) J. Mol. Biol. 184, 399-412.
49. Lutkenhaus, J.F., Wolf-Watz, H. and Donachie, W.D. (1980) J. Bacteriol. 142, 615-620.
50. Taylor, W.E., Straus, D.B., Grossman, A.D., Burton, Z.F., Gross, C.A. and Burgess, R.R. (1984) Cell 38, 371-381.
51. Girons, I.S. and Margarita, D. (1985) J. Bacteriol. 161, 461-462.
52. Lawther, R.P. and Hatfield, G.W. (1978) J. Bacteriol. 136, 1201-1204.
53. Lawther, R.P., Calhoun, D.H., Gray, J., Adams, C.W., Hauser, C.A. and Hatfield, G.W. (1982) J. Bacteriol. 149, 294-298.
54. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H., Crosa, J.H. and Falkow, S. (1977) Gene 2, 95-113.
55. Russell, D.R., Auger, E.A., Vermersch, P.S. and Bennett, G.N. (1984) Gene 32, 337-348.
56. Messing, J. and Vieira, J. (1982) Gene 19, 269-276.
57. Taillon, M.P., Gotto, D.A. and Lawther, R.P. (1981) Nucl. Acids Res. 9, 3419-3432.
58. Grisolia, V., Riccio, A. and Bruni, C.B. (1983) J. Bacteriol. 155, 1288-1296.