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Bruno Charpentier, Christiane Branlant. The Escherichia coli gapA Gene Is Transcribed by the Vegetative RNA Polymerase Holoenzyme Esigma70 and by the Heat Shock the RNA Polymerase Esigma32 . Journal of Bacteriology, 1994, 176, pp.830 - 839. hal-01662827

HAL Id: hal-01662827

<https://hal.univ-lorraine.fr/hal-01662827>

Submitted on 13 Dec 2017

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The *Escherichia coli* *gapA* Gene Is Transcribed by the Vegetative RNA Polymerase Holoenzyme $E\sigma^{70}$ and by the Heat Shock RNA Polymerase $E\sigma^{32}$

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Received 9 August 1993/Accepted 15 November 1993

Escherichia coli D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is produced by the *gapA* gene and is structurally related to eukaryotic GAPDHs. These facts led to the proposal that the *gapA* gene originated by a horizontal transfer of genetic information. The yields and start sites of *gapA* mRNAs produced in various fermentation conditions and genetic contexts were analyzed by primer extension. The transcriptional regulatory region of the *gapA* gene was found to contain four promoter sequences, three recognized by the vegetative RNA polymerase $E\sigma^{70}$ and one recognized by the heat shock RNA polymerase $E\sigma^{32}$. Transcription of *gapA* by $E\sigma^{32}$ is activated in the logarithmic phase under conditions of starvation and of heat shock. Using a GAPDH⁻ strain, we found that GAPDH production has a positive effect on cell growth at 43°C. Thus, *E. coli* GAPDH displays some features of heat shock proteins. One of the *gapA* promoter sequences transcribed by $E\sigma^{70}$ is subject to catabolic repression. Another one has growth phase-dependent efficiency. This complex area of differentially regulated promoters allows the production of large amounts of *gapA* transcripts in a wide variety of environmental conditions. On the basis of these data, the present view of $E\sigma^{32}$ RNA polymerase function has to be enlarged, and the various hypotheses on *E. coli* *gapA* gene origin have to be reexamined.

The NAD⁺-dependent phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which converts D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate (22), is a key enzyme of the glycolytic and gluconeogenesis pathways. In eubacteria (5, 14, 17, 46) and archaeobacteria (4), the GAPDH gene (*gap*) belongs to a cluster of genes coding for several glycolytic enzymes: 3-phosphoglycerate kinase (*pgk*), class II fructose 1,6-bisphosphate aldolase (*fda*), and triosephosphate isomerase (*tpi*). A peculiar situation is observed in *Escherichia coli*, in which the *gapB* gene, located in this cluster, does not produce GAPDH activity (2, 25). The active *gapA* gene and the *pgk* gene are 22.6 min apart on the *E. coli* chromosome (7, 50). In addition, the *gapA* gene codes for a protein which has stronger homology with mammalian GAPDHs than with bacterial enzymes (6). A eukaryotic origin has thus been proposed for the *gapA* gene: enterobacteria would have acquired the *gapA* gene through a horizontal transmission of genetic information, concomitant with the loss of activity of the *gapB* gene (2, 16, 36). On the basis of their observation of a gene similar to the *E. coli* *gapA* gene in a cyanobacterium, Martin et al. (35) suggested an alternative explanation: the presence of both *gapA* and *gapB* genes in ancestor bacteria. In order to further address this problem, it was of interest to us which kind of transcriptional promoter controls the *gapA* gene. The exceptionally high expression of the cloned *gapA* gene (7) suggested that the *gapA* promoter was very efficient. Therefore, a study of the *gapA* promoter was also expected to yield some information on the structural parameters responsible for this high efficiency.

The majority of the *E. coli* transcriptional promoters are

recognized by the RNA polymerase holoenzyme $E\sigma^{70}$, which is formed by association of the holoenzyme with the major sigma factor, σ^{70} (24). A few subsets of genes that respond to various environmental or nutritional changes require specific recognition by RNA polymerase associated with the alternative sigma factor σ^{32} (21), σ^E (18, 52), σ^{54} (26), or σ^S (39). The heat shock response mediated by the $E\sigma^{32}$ RNA polymerase holoenzyme has been the subject of several studies (for reviews, see references 8 and 40). When *E. coli* cells growing at 30°C are shifted to 42°C, the rate of synthesis of the so-called heat shock proteins increases 5- to 20-fold, reaching a peak within 5 to 15 min; synthesis then declines until it reaches a new rate, characteristic of high temperatures. Seventeen proteins meeting these criteria were detected in *E. coli* (40). The *rpoH* gene, which codes for σ^{32} , is controlled by a multiple promoter system containing four different polymerase-binding sites (8). One, recognized by the $E\sigma^E$ holoenzyme, ensures heat inducibility; the three others are recognized by $E\sigma^{70}$ and are differentially regulated (8). A consensus sequence for recognition by the $E\sigma^{32}$ RNA polymerase has been proposed (15). An extensive analysis of heat shock transcriptional induction in *E. coli* revealed the presence of at least 36 genes whose expression is induced by heat shock (12).

In this paper, we demonstrate that the *gapA* gene of *E. coli* is controlled by a multiple promoter system consisting of four different RNA polymerase recognition sites; one of them has the characteristic features of $E\sigma^{32}$ promoters. Transcription initiation at this site was found to be increased when cells were subjected to heat shock. In this way, the transcription level of the *gapA* gene is preserved during heat shock. Using the GAPDH⁻ mutant strain DF221 (25), we showed that expression of a cloned GAPDH gene in this strain increases its capacity to grow at 43°C on succinate glycerol medium. Hence, in addition to genes whose transcription is increased upon heat shock, the heat shock regulon probably contains genes whose expression during heat shock is kept constant or at a minimal

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference
XL1-Blue	<i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA</i> (F' <i>proAB lacI^q lacZΔM15 Tn10</i>)	9
DF221	Derived from strain K10 (HfrC <i>tonA22 λ</i>)	25
M182	$\Delta(lacIPOZY)X74$ <i>galK galU strA</i>	11
M182Δ <i>crp</i>		10
TP2006	F ⁻ <i>xyl lacΔX74 Δcya glp-8306</i>	45

level by Eσ³² RNA polymerase transcription. These results are discussed in view of the present knowledge of the highly efficient *E. coli* *rrnB* promoter region (41), which has strong homology with the *gapA* promoter region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The relevant genotypes of the *E. coli* strains used in this study are listed in Table 1. These strains were transformed with the plasmid pBS::*EcogapA*. This plasmid was constructed by cutting out the *HindIII*-*Bam*HI 2,149-nucleotide fragment from phage M13mp9::*EcogapA* (38) and inserting it into plasmid BSIISK⁺ (Stratagene). The plasmid pBS::*EcogapA* contains the entire *gapA* gene with a 346-nucleotide upstream flanking region. *E. coli* DF221 was transformed with both plasmids pBR322 and pBR322::*EcoGAPDH* (7).

Media. The rich medium used was L broth (34). The defined medium was M63 (19) supplemented with thiamine (1 μg/ml) and Casamino Acids (0.1%, wt/vol). The specific carbon source was either a mixture of succinate (0.4%, wt/vol) and glycerol (0.08%, vol/vol) or glycerol (0.2%, vol/vol). Strains containing plasmids were grown in the presence of ampicillin (100 μg/ml). The effect of cyclic AMP (cAMP) in the medium was tested with cAMP at 1 mM.

Cell cultivation and viability experiments. In routine experiments cells were grown aerobically at 37°C. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). For temperature shift experiments, cultures grown at 30°C in a gyratory water bath were split in two in the middle of the exponential growth phase (OD₆₀₀ = 0.5). One half was cultivated at 30°C for 10 min, and the other half was cultivated at 42°C for 10 min. A gyratory water bath was used in both cases. To test for the effect of cAMP in the medium, cells were grown at 37°C, to an OD₆₀₀ of 0.2, in supplemented M63 minimal medium with 0.2% glycerol as the carbon source. The culture was split in two, and one half was supplemented with cAMP at a concentration of 1 mM. For both cultures, growth was continued at 37°C to an OD₆₀₀ of 0.4.

To test for the effect of temperature on cell growth, cells were grown with shaking at 30°C to an OD₆₀₀ of 0.2 or 0.3; 100-ml fractions of the culture were then placed in a gyratory water bath at 30, 43, or 53°C, and growth was monitored by OD₆₀₀ measurements at various times following the temperature shift. The 53°C culture was also used for cell viability experiments. Aliquots were periodically plated on M63-succinate-glycerol agar plus ampicillin and incubated at 37°C for 24 h.

RNA isolation. Total RNA was isolated by the hot-phenol extraction procedure (1); extractions were always performed with the same amount of cells, corresponding to an OD₆₀₀ of 6. These cells were collected at various times during the culture or after temperature shifts or tests of cAMP effect. In the latter cases, RNA was also extracted from control cells which were not subjected to the temperature shift or incubated in the

presence of cAMP. The total amount of extracted RNA was determined by OD₂₆₀ measurement.

In vitro transcription. In vitro transcription assays were performed with the commercial *E. coli* Eσ⁷⁰ RNA polymerase holoenzyme (Boehringer) with the supercoiled plasmid pBS::*EcogapA* as template. The reaction mixture contained 40 mM Tris hydrochloride (pH 8), 30 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 200 μM (each) ATP, CTP, GTP, and UTP, 2 to 3 nM DNA, and 50 nM RNA polymerase. Incubation was for 25 min at 37°C. The reaction was stopped by phenol-chloroform extraction, which was followed by precipitation with alcohol. The precipitated RNA was analyzed by primer extension, as described below.

Primer extension of in vitro-synthesized RNA transcripts and mRNAs. Two synthetic oligonucleotides, OG1 (5' TTTCT GAGCAGCACGGAA 3') and OG2 (5' ATAGTCATATGT TCCTCCA 3'), complementary to nucleotide positions 52 to 68 and 8 to -11 of the *gapA* gene, respectively (6), were 5' end labeled with [γ-³²P]ATP (3,000 Ci/mmol) (Amersham) and T4 polynucleotide kinase (Boehringer) and used as primers. In standard experiments, 2 μg of RNA isolated from bacterial strains containing plasmid pBS::*EcogapA*, 20 μg of RNA isolated from bacterial strains without plasmid, or all transcripts obtained in an in vitro transcription assay were annealed with 5 ng of 5'-end-labeled primer. An exception was the experiment to study the evolution of *gapA* mRNA composition in the course of a fermentation. In this case, each primer extension was performed with an amount of total RNA corresponding to 6 × 10⁸ cells. For annealing, the RNA and the labeled oligonucleotide were heated to 70°C for 5 min in 10 μl of 50 mM Tris hydrochloride (pH 8.3)–40 mM KCl–6 mM MgCl₂ and then cooled slowly to room temperature (20 min). cDNA synthesis was performed in the same solution for 30 min at 45°C in the presence of the nonradioactive deoxyribonucleoside triphosphates (dNTPs) at a concentration of 225 μM each and 1 U of Rav-2 reverse transcriptase (Amersham). The reaction was stopped by addition of 100 μl of 0.3 M sodium acetate and 500 μl of ethanol, and precipitation was done at -80°C. The precipitated material was dissolved in a formamide dye solution (34). The sample was electrophoresed on a 7% polyacrylamide–8 M urea sequencing gel and compared with dideoxy sequencing reaction products (48) obtained with the same primer and the single-stranded form of phage M13mp9::*EcogapA* as the template.

Reverse transcript quantification. Reverse transcript quantification was achieved with a Molecular Dynamics Phosphorimager 410 system. The sensitivity and linearity of the Molecular Dynamics signal was demonstrated on model systems (32). For quantification the reverse transcripts were fractionated on a 7% polyacrylamide–8 M urea sequencing gel. The gel was scanned for radioactivity for 10 min to 12 h, depending on the amount of radioactivity loaded. The results obtained were quantified by using the Molecular Dynamics Imagequant software. We proceeded as follows. A rectangular surface was designed so that it could include any of the bands to be quantified. It was then successively fitted with each of the bands, and the grey levels in the fitted rectangular surfaces were registered. The values obtained are expressed in Phosphorimager signal units. The background was automatically subtracted by this software.

mRNA sequencing with reverse transcriptase. Direct sequencing of mRNA was done as described by Hu Qu et al. (29) with oligonucleotide OG1 as the primer. Annealing between the *gapA* mRNAs and the 5'-end-labeled oligonucleotide used as the primer was performed as described above, with 25 ng of primer and 10 μg of RNA, except for the presence of

2',3'-dideoxyribonucleoside triphosphates (ddNTPs) at a dNTP/ddNTP ratio of 1:5.

RESULTS

Identification of the *gapA* gene transcription start sites in vivo and in vitro. We previously observed two putative overlapping RNA polymerase recognition sites in the upstream noncoding region of the *gapA* gene (6). They are denoted P1 and P'1 in Fig. 1A. To identify the transcriptional start sites used in vivo, *E. coli* XL1-Blue with or without the pBS::*EcogapA* overproducing plasmid was grown in L broth at 37°C, and total RNA was isolated from exponentially growing cells (OD₆₀₀ of 0.5). The *gapA* mRNAs were analyzed by primer extension with oligonucleotide OG1 or OG2 (Fig. 1A) as the primer. To get quantitative results, reverse transcriptase and all substrates, except the template mRNA, were used in excess. Under these conditions, the amount of reverse transcript obtained was found to be proportional to the amount of template transcript (43a). Similar patterns of fractionation of the reverse transcripts were obtained for the host strain and the transformed strain, no matter which oligonucleotide was used as the primer (Fig. 2). A major reverse transcript (band P1), whose 3' end maps to the expected initiation site of the putative P1 promoter (Fig. 1), was always present, as were other reverse transcripts. None of them corresponded to an initiation at the putative P'1 promoter. The minor products, with high electrophoretic mobilities, were probably due either to degradation of mRNA initiating from P1 or to incomplete reverse transcription of P1 transcripts. The more likely explanation for the observation of reverse transcripts P2, P3, and P4, which had low electrophoretic mobilities, is the presence of at least one upstream promoter. These reverse transcripts were not generated by a nonspecific hybridization of the primer oligonucleotide with other mRNAs, since two different primers gave the same result and since direct sequencing of the P2, P3, and P4 transcripts demonstrated that they are transcripts from the *gapA* gene (Fig. 3). To determine whether the four mRNAs observed in vivo can be produced by in vitro transcription, the supercoiled plasmid pBS::*EcogapA* was transcribed in vitro with the *E. coli* Eo⁷⁰ RNA polymerase. Only the P1 site and, to a very small extent, a new site denoted P5 were used under these conditions (Fig. 4).

To find an explanation for these results, the DNA sequences that precede the 5' ends of the various transcripts, detected in vivo and in vitro, were compared with the consensus promoter sequences of *E. coli* RNA polymerases Eo⁷⁰ (−35, TTGACA, and −10, TATAAT, separated by 17 bases) (23) and Eo³² (−35, CCCTTGAA, and −10, CCCCATnT, separated by 13 to 15 bases) (15) (Fig. 1B). We also looked for the presence of possible cAMP/cAMP receptor protein (CRP) binding sites on the basis of the consensus sequence AAATGTGATCTA GATCACATTT proposed for the most frequently observed cAMP-CRP binding sites (3) (Fig. 1C). Upstream from the P1 site, the sequences TTGACG and TAATTT, separated by 16 bases (Fig. 1A), were previously found as −35 and −10 potential promoter sequences, respectively, of the *E. coli* Eo⁷⁰ RNA polymerase (6). For P2, the sequences CCCTTTAA and CCCCATGT, separated by 15 bases (Fig. 1A and B), were found as potential promoter sequences recognized by the *E. coli* Eo³² RNA polymerase. This was surprising, since bacterial GAPDH had not been described as a heat shock protein previously. For site P3, the sequence TAAAAT was found as a potential −10 promoter sequence of the RNA polymerase Eo⁷⁰, and instead of a −35 sequence we observed the presence of a potential cAMP-CRP binding site (AAATCGTGAT

GAAAATCACATTT) (Fig. 1A). The organization of the P3 upstream sequence is very similar to that of the *gal* P1 promoter (Fig. 1C). For both sites P4 and P5, a potential −10 promoter sequence of Eo⁷⁰ was found (TAAACT and TATCGT, respectively), but no potential −35 promoter sequence was found. The results of this DNA comparison were in agreement with our experimental data. The presence of other potential promoters in addition to the potential P1 promoter was an explanation for the presence of mRNAs initiated at various sites. The fact that among the P1, P2, P3, P4, and P5 sites, only P1 was preceded by the −10 and −35 sequences required for in vitro transcription by the *E. coli* Eo⁷⁰ RNA polymerase explained the strong in vitro utilization of this promoter. The presence of very small amounts of transcripts initiated at the P5 sites in vitro may be an artifact or could correspond to the presence of an RNA polymerase binding site with very low transcriptional activity.

Effect of heat shock on *gapA* mRNA composition. To verify that initiation at the P2 site was generated by the Eo³² RNA polymerase, heat shock experiments were performed with *E. coli* XL1-Blue, with or without plasmid pBS::*EcogapA*, grown to mid-logarithmic phase at 30°C in L broth. We compared the relative abundances of *gapA* transcripts after 10 min of growth at 30°C and after 10 min of growth at 42°C. The *gapA* transcripts were analyzed by primer extension under conditions allowing their quantification (Fig. 5). After a 10-min shock at 42°C, either in the presence or in the absence of the overproducing plasmid, an increase in transcripts initiated at the P2 site, concomitant with a decrease in transcripts initiated at the P1 site, was observed (Fig. 5). This observation and the strong homology of the sequence upstream of the P2 site with heat shock promoters are strong indications that initiation at the P2 site is generated by the Eo³² RNA polymerase. We noticed stronger utilization of the P3 site at 37°C (Fig. 5C).

Effect of *gapA* gene expression on cell growth and viability at high temperatures. Several previously characterized *E. coli* heat shock proteins were found to increase growth and viability of cells at high temperatures (40, 53). To test whether GAPDH has the same effect, the growth of the GAPDH[−] *E. coli* strain DF221 transformed with plasmid pBR322 in M63-succinate-glycerol at 30, 43, and 53°C was compared with that of the same strain transformed with plasmid pBR::*EcoGAPDH*, which overproduces the GAPDH (Fig. 6). The growth of the two transformed strains is identical at 30°C. However, whereas cells transformed with plasmid pBR::*EcoGAPDH* continue to grow at the rate seen at 30°C for 4 h when the culture is shifted to 43°C, cells transformed with plasmid pBR322 grow at a slower rate after the temperature shift (Fig. 6B). Nevertheless, no significant positive effect of the expression of GAPDH on cell growth and viability at 53°C was observed (Fig. 6C and D). Thus, in *E. coli* DF221, the expression of the *gapA* gene has a positive effect on cell growth at 43°C.

Effect of cAMP and CRP on initiation at the P3 site. To determine whether utilization of the P3 site was subject to catabolic repression, *E. coli* TP2006 (which is deficient in cAMP production) and *E. coli* M182Δ*crp* (which is deficient in CRP production) and its isogenic strain M182 were transformed with plasmid pBS::*EcogapA* and grown at 37°C in M63-glycerol medium to mid-logarithmic phase. The *gapA* mRNAs were analyzed by primer extension (Fig. 7). No transcription took place at the P3 site in the absence of cAMP (Fig. 7A) or CRP (Fig. 7B). With strain TP2006, initiation at the P3 site was restored when the medium was supplemented with cAMP (Fig. 7A). Thus, utilization of the P3 site is subject to catabolic repression.

Evolution of *gapA* mRNA composition in the course of

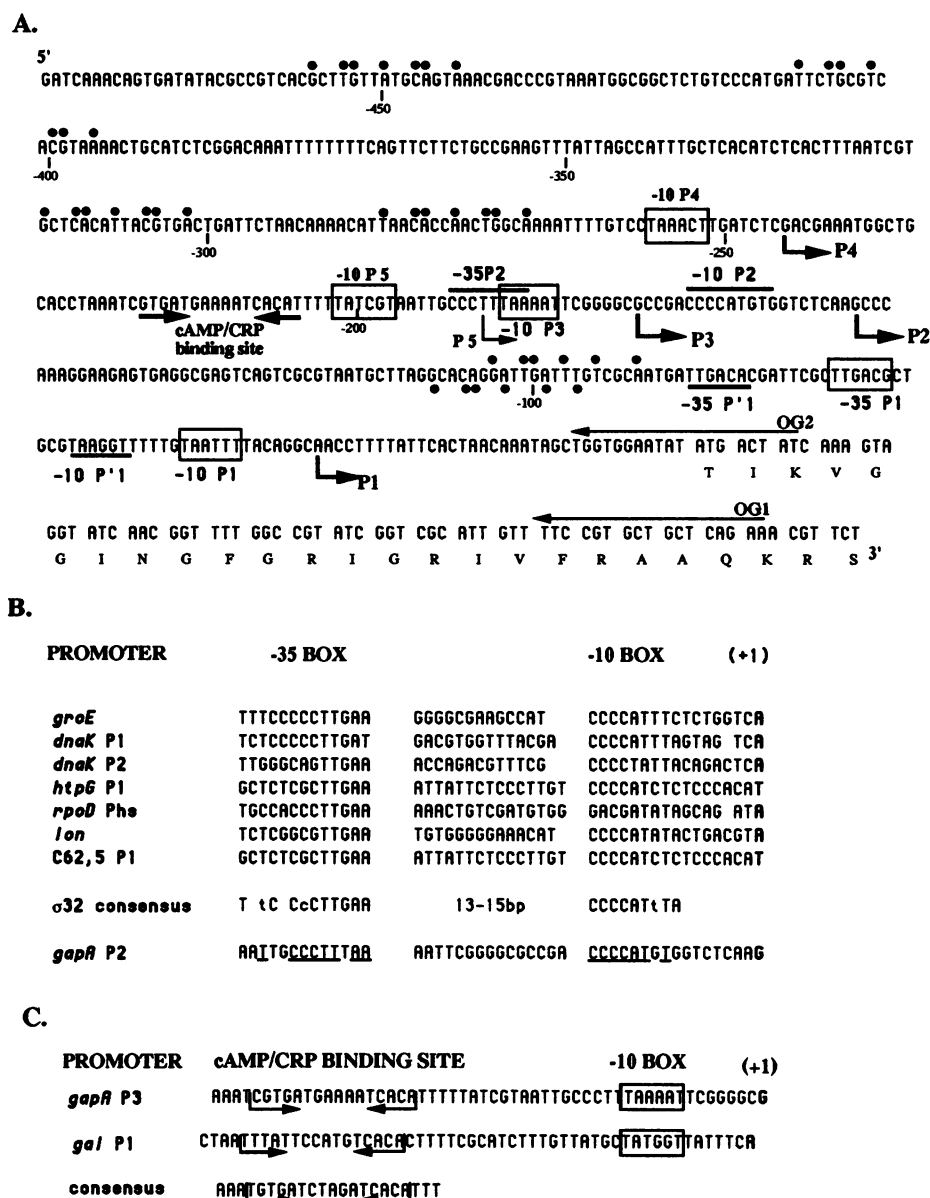


FIG. 1. Characteristic nucleotide sequence features of the *gapA* promoter region. (A) DNA sequence of the transcriptional regulatory region of the *E. coli gapA* gene. The nontemplate strand is shown. The sequence is from reference 6. The first nucleotide of the ATG initiation codon is numbered 1; the preceding one is numbered -1. The transcription initiation sites observed in vivo and in vitro are marked with numbered arrows. The -35 and -10 regions of the σ^{70} -controlled *gapA* promoters are boxed. The -35 and -10 regions of the σ^{32} -controlled P2 promoter are overlined. The -35 and -10 regions of the previously proposed P'1 promoter, which according to the results of this study are not used, are underlined. The cAMP-CRP binding site upstream of the P5 initiation start site is indicated by horizontal arrows in opposite directions. The horizontal arrows labeled OG1 and OG2 show the sequences which are complementary to the primer oligonucleotides. The putative binding sites for the FIS protein are indicated; positions in agreement with the FIS binding site consensus sequence G/T--YR--A/T--YR--C/A (27) are marked by filled circles. (B) Comparison of the DNA sequences of the *gapA* P2 promoter with those of other promoters which are recognized by the $E\sigma^{32}$ RNA polymerase (*groE*, *dnaK* P1, *dnaK* P2, *htpG* P1, *rpoD* Phe, *lon*, and *C62,5* P1) (40) and with the consensus sequence established for the σ^{32} -controlled promoter (15). Matches to the consensus sequence are underlined in the *gapA* P2 promoter. The initiation start sites are indicated by boldface. (C) Comparison of the DNA sequence of the *gapA* P3 promoter with that of the *gal* P1 promoter (33) and with the consensus sequence established for the cAMP-CRP binding sites (3). The -10 region is boxed, the cAMP-CRP binding sites are indicated by horizontal arrows in opposite directions, and the initiation start sites are boldfaced.

fermentation. To determine whether the relative amounts of the four *gapA* mRNAs were constant throughout the culture time or whether they varied, *E. coli* XL1-Blue transformed with plasmid pBS::EcogapA was grown at 37°C in 1 liter of L broth. Samples were collected at regular time intervals, and

mRNAs were analyzed by primer extension (Fig. 8). For quantitative comparison of the results obtained at different times during the culture, all primer extension assays were performed with a total RNA amount corresponding to the same number of cells. All of the reverse transcription reaction

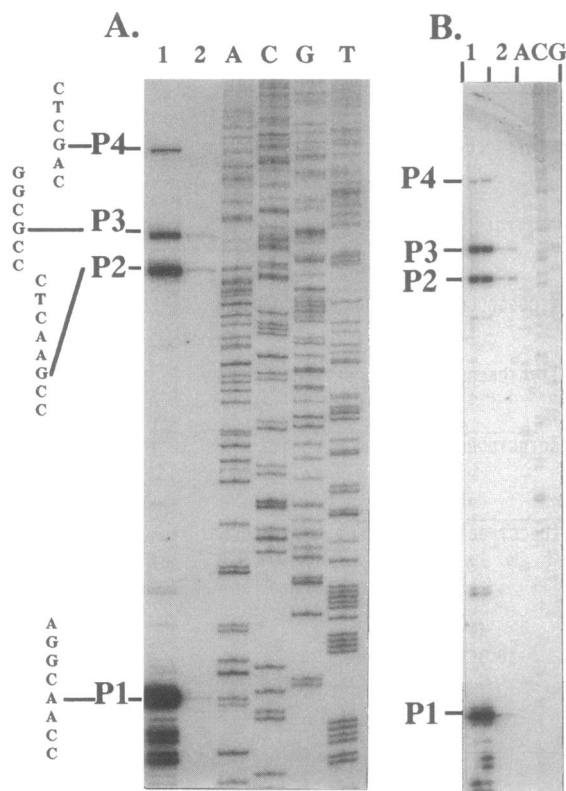


FIG. 2. Determination of the in vivo start sites of the *gapA* gene. Primer extension was performed with oligonucleotides OG1 (A) and OG2 (B) with total RNA extracted from *E. coli* XL1-Blue cells grown on L broth at 37°C to mid-log phase ($OD_{600} = 0.5$) in the presence (lane 1) or absence (lane 2) of plasmid pBS::EcogapA. Total RNA extraction and primer extension were performed as described in Materials and Methods. In this case 2 μ g of total RNA was used for both transformed and untransformed cells. Markers (lanes A, C, G, and T) were made by sequencing phage M13::EcogapA with both primers and T7 DNA polymerase. The positions of the four initiation sites P1, P2, P3, and P4 are indicated, and the nucleotide sequences around the different start sites are given on the left.

mixture was fractionated on a polyacrylamide gel, and the relative amounts of reverse transcripts were estimated with a Molecular Dynamics Phosphorimager system. The values obtained (Fig. 8C) reflect variations in the total amounts of transcript. Figure 8D is another representation of the results, showing the variations of the amount of each transcript in a given amount of total RNA extract during cultivation (relative abundance). Both the total amount and the relative abundance of transcripts initiated at the P1 site were very high during the exponential phase and decreased in the stationary phase. The relative abundance of transcripts initiated at the P2 site was high during the exponential phase, decreased abruptly at the beginning of the stationary phase, and increased again late in the stationary phase. The transcripts initiated at the P3 site peaked during the beginning of the stationary phase and were present in very small amounts during the late stationary phase. The transcripts initiated at the P4 site were found in very small amounts from the beginning of the culture to the late stationary phase, when they disappeared. Longer transcripts were detected in this late phase. The experiment was repeated several times and the same results were observed.

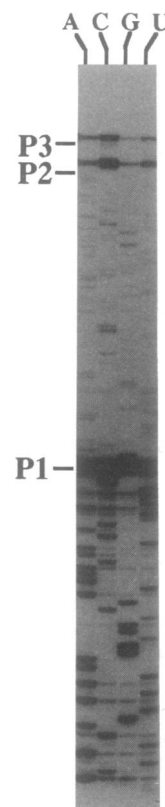


FIG. 3. Direct RNA sequencing of the *gapA* transcripts produced in vivo. The *gapA* mRNAs contained in 10 μ g of total RNA extract from *E. coli* XL1-Blue cells transformed with plasmid pBS::EcogapA and grown on L broth at 37°C to mid-log phase ($OD_{600} = 0.5$) were sequenced by the reverse transcriptase method as described in Materials and Methods, with oligonucleotide OG1 as the primer. The positions of the P1, P2, and P3 start sites are indicated.

DISCUSSION

On the basis of their homology with promoter sequences of the $E\sigma^{70}$ *E. coli* RNA polymerase, the existence of two putative overlapping promoter sequences upstream of the *gapA* coding region was previously reported (6). The promoter sequence denoted P1 in this paper (Fig. 1) is used in vitro as well as in vivo. The other one, P'1, was never found to be used in any of the experiments performed. This inefficiency is probably due to an unfavorable distance between the -10 and -35 sequences (19 bases) (Fig. 1A). The P1 promoter utilization is very strong at the beginning of the exponential phase and seems to depend upon growth phase (Fig. 8), a situation which is very similar to that found for promoters required for transcription of stable RNAs (42). The strong activation of the *rnnB* P1 promoter at the beginning of the exponential phase was found to be a positive regulation mediated by the binding of the FIS protein to an upstream DNA element (42, 44). The dependence of the *rnnB* P1 promoter upon growth rate is, in contrast, a negative regulation (20). The question is whether the *gapA* P1 promoter is subject to the same kind of regulation. In this respect, we observed several putative FIS binding sites in the upstream noncoding region of the *gapA* gene (Fig. 1).

In addition to the P1 promoter, we found that three other promoters were used in vivo. The P4 promoter was used to a very low extent under all fermentation conditions used and whatever the genetic context. The absence of a -35 promoter

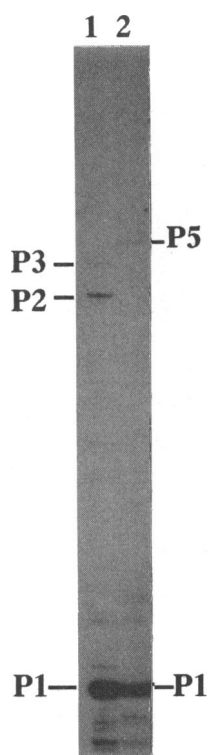


FIG. 4. Comparison of in vivo and in vitro *gapA* transcripts. The in vitro transcription reaction was performed under the conditions described in Materials and Methods, with the supercoiled plasmid pBS::EcogapA as the template. The holoenzyme used was the commercial *E. coli* E σ^{70} RNA polymerase holoenzyme. The in vitro transcription products (lane 2) and 2 μ g of total RNA (lane 1) extracted from mid-log-phase *E. coli* XL1-Blue cells transformed with plasmid pBS::EcogapA were analyzed by primer extension, with oligonucleotide OG1 as the primer. The P1, P2, and P3 sites used in vivo and the P1 and P5 sites used in vitro are indicated.

sequence and the inefficiency of this promoter in vitro suggest that either its utilization requires transcriptional activators or it represents a secondary RNA polymerase binding site. Indeed, secondary RNA polymerase binding sites, without efficient transcriptional activity and located upstream of the efficient promoters, have been observed in many *E. coli* genes, particularly those encoding stable RNA species or ribosomal proteins (51). It was proposed that their role is to recruit RNA polymerase and thus contribute to the efficiency of the strong downstream promoter (51). The P4 promoter, used to a low extent in vivo, as well as the P5 promoter, used to a low extent in vitro, may represent such secondary binding sites.

Utilization of the P3 start site is subject to catabolic repression. This may seem surprising for an enzyme involved in glucose metabolism. But as we mentioned above, the activity of the P1 promoter is growth phase dependent. Under conditions causing an intermediate or low growth rate, cells may need GAPDH activity to metabolize glucose which has been synthesized by gluconeogenesis or which is produced by hydrolysis of another carbon source, for instance, lactose or saccharose. Under such conditions, the existence of a transcript induced by the cAMP-CRP complex may give a selective advantage to the cells. Another possibility is that transcripts initiated at the P3 site serve to produce GAPDH for gluconeogenesis. An illustration of the P3 site utilization was given by our study of the variation of *gapA* transcript composition in the course of an *E.*

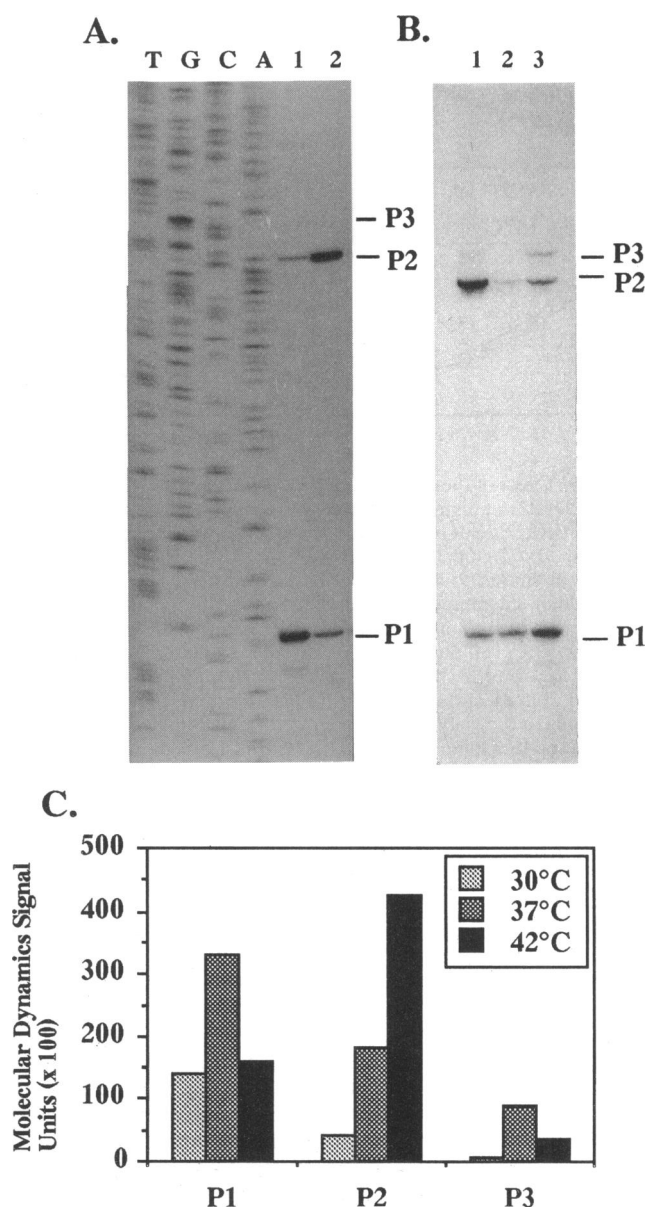


FIG. 5. Effect of temperature shift from 30 to 43°C on *gapA* mRNA composition. *E. coli* XL1-Blue cells with (A) or without (B) plasmid pBS::EcogapA were grown in L broth at 30°C. When the OD₆₀₀ reached 0.2, the cultures were divided; one half was then grown for 10 min at 30°C (lane 1 in panel A and lane 2 in panel B), and the other was grown for 10 min at 42°C (lane 2 in panel A and lane 1 in panel B). Lane 3 of panel B shows the culture of *E. coli* XL1-Blue, without plasmid, at 37°C without further treatment. Total RNA was extracted for each of the five samples and analyzed by primer extension (20 μ g for untransformed cells and 2 μ g for transformed cells), with oligonucleotide OG1 as the primer. The sequencing markers A, C, G, and T in panel A were made as described in the legend to Fig. 2. The relative amounts of the reverse transcripts shown in panel B were estimated by using a Molecular Dynamics Phosphorimager system, as described in Materials and Methods. The results obtained are represented in panel C; the values correspond to the grey levels in rectangles of identical surfaces fitted with each of the bands, expressed in Molecular Dynamics signal units.

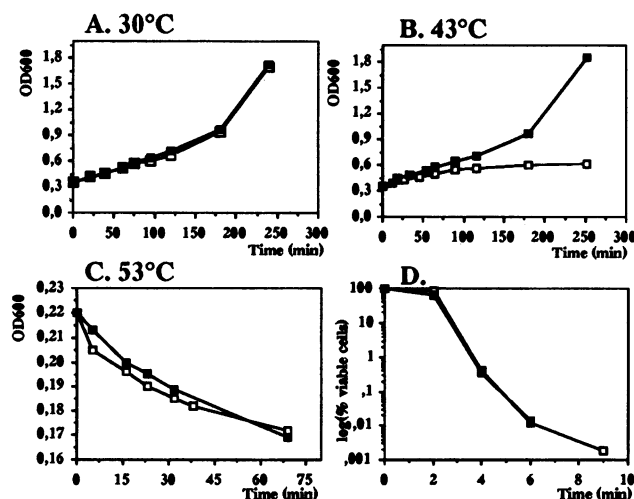


FIG. 6. Effect of the production of GAPDH on growth and survival of *E. coli* DF221 at high temperatures. *E. coli* DF221 transformed with plasmid pBR322::EcoGAPDH was grown in M63-succinate-glycerol at 30°C to an OD₆₀₀ of 0.3, and then the temperature was maintained at 30°C (A) or shifted to 43°C (panel B), or growth was to an OD₆₀₀ of 0.22, and then the temperature was shifted to 53°C (C and D). Growth was followed by measuring the OD₆₀₀. For determination of bacterial survival at 53°C, cell numbers were determined by plating aliquots of the culture grown at 53°C on agar-succinate-glycerol plates which were incubated for 24 h at 37°C (D). One hundred percent viability corresponds to the viable cell number determined immediately before heat treatment. Open squares, DF221 transformed with pBR322; closed squares, DF221 transformed with pBR322::EcoGAPDH.

coli XL1-Blue fermentation in L broth (Fig. 8). Between 4 and 10 h of incubation, the contribution of the P3 promoter to the production of *gapA* mRNAs was important. This is probably the result of a high concentration of cAMP-CRP complexes in the cells during this fermentation period.

The recognition of the P2 promoter by the heat shock RNA polymerase $E\sigma^{32}$ was unexpected. GAPDH was not found to be a protein overexpressed during heat shock (12, 40), and the transcription of the *gapA* gene was not found to be induced by heat shock (12, 13), probably because the sole effect of transcription of the P2 promoter by $E\sigma^{32}$ during heat shock is to maintain the *gapA* gene transcription level. The recent extensive study by Chuang and Blattner (12) of the *E. coli* genes which are induced by heat shock showed that at least 36 genes fit these criteria. It now appears that other genes, like the GAPDH gene, may be transcribed during heat shock by $E\sigma^{32}$ without an increase in their transcription yield and thus would not be detected by the method of Chuang and Blattner (12). The heat shock regulon of *E. coli* is considerably larger than is indicated by the number of *E. coli* heat shock proteins characterized on the basis of their increased synthesis upon heat shock (40). We have not tested whether the transcriptional activity of the *gapA* gene during heat shock resulted in GAPDH production. This is likely, however, and we observed an increased capacity of the *E. coli* GAPDH mutant DF221 transformed with a plasmid overproducing GAPDH to grow at 43°C. This observation indicates a positive effect of GAPDH on the growth of *E. coli* at high temperatures. In our experiments, the effect may have been reinforced by the fact that plasmid pBR322::EcoGAPDH overproduced GAPDH in very large quantities (7). An additional control will be to compare the abilities of the DF221 mutant and its isogenic wild-type strain to grow at high temperatures.

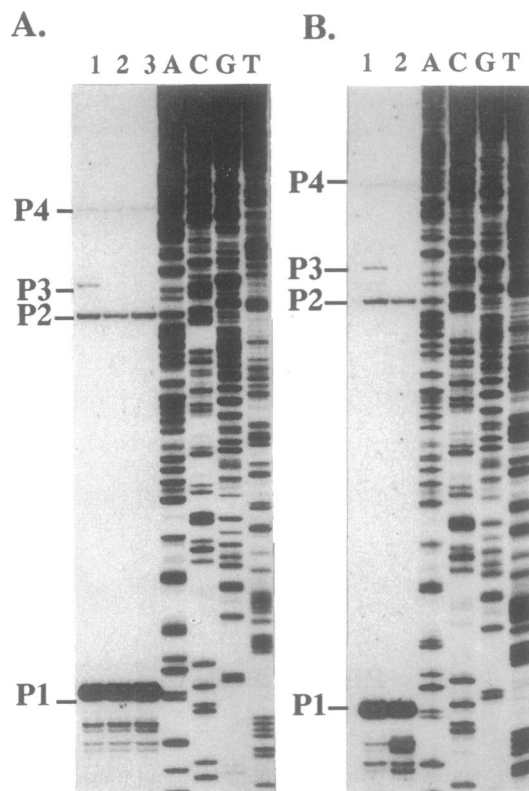


FIG. 7. Effect of the presence of cAMP and CRP on *gapA* mRNA composition. (A) Effect of the presence of cAMP. *E. coli* TP2006, transformed with plasmid pBS::Eco*gapA*, was grown in M63-glycerol medium at 37°C to an OD₆₀₀ of 0.2. The culture then was divided; cAMP at 1 mM was added to one half (lane 1), nothing was added to the second half (lane 2), and the two cultures were continued until an OD₆₀₀ of 0.4 was reached. A control culture was made without interruption for the same total time and in the absence of cAMP (lane 3). Total RNA was extracted from the three samples and analyzed by primer extension, with oligonucleotide OG1 as the primer. The sequence markers were made as described in the legend to Fig. 2. (B) Effect of the presence of CRP. *E. coli* M182Δ*crp* (lane 2) and the isogenic strain M182 (lane 1) transformed with plasmid pBS::Eco*gapA* were grown in L broth at 37°C to an OD₆₀₀ of 0.5. Total RNA was extracted and analyzed by primer extension, with oligonucleotide OG1 as the primer. The sequence markers were made as described in the legend to Fig. 2.

The production of 11 *E. coli* heat shock proteins is induced upon carbon starvation (31), and the level of σ^{32} was found to be increased upon starvation (30). Thus, our observation of a reactivation of the P2 promoter, late in the stationary phase when the medium is exhausted (Fig. 8), is likely explained by an increase of $E\sigma^{32}$ RNA polymerase activity late in the fermentation. This is a strong indication that the P2 promoter allows expression of the *gapA* gene, under conditions both of heat shock and of starvation, and together these observations suggest that in addition to glycolysis, GAPDH may have other biological roles in bacteria. In light of this hypothesis, it should be pointed out that several roles have been proposed for eukaryotic GAPDH (28, 37, 47).

An intriguing point in our observations is the strong utilization of the P2 site during the exponential phase of growth, with an abrupt decrease of utilization at the beginning of the stationary phase. Since the concentration of σ^{32} was not reported to be high in the exponential phase (8), one explana-

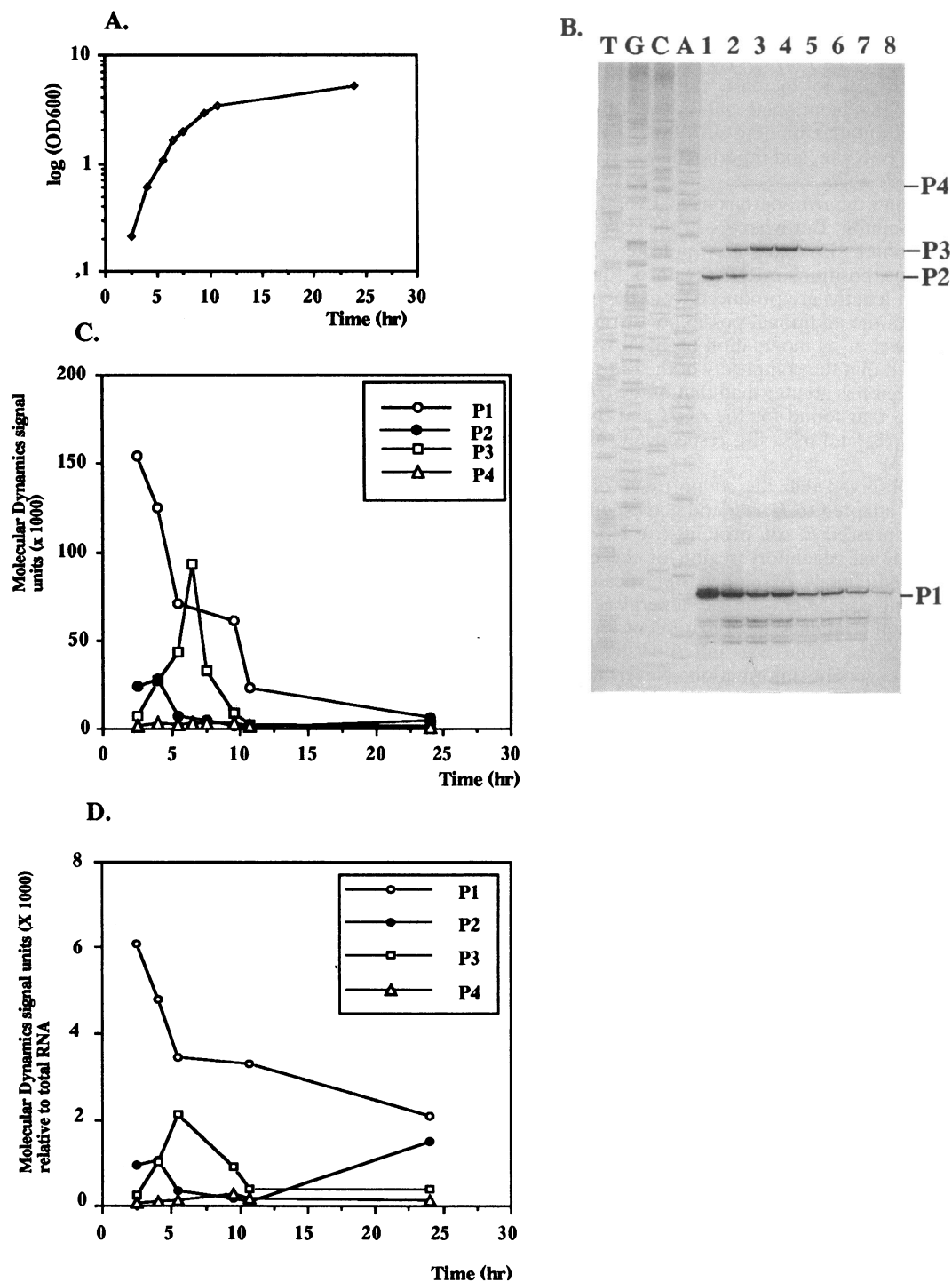


FIG. 8. Variation of *gapA* mRNA composition in the course of a fermentation. A 1-liter culture of *E. coli* XL1-Blue transformed with plasmid pBS::*EcogapA* was grown in L broth at 37°C. The growth curve is shown in panel A. Fractions corresponding to 6×10^8 cells were collected at regular time intervals, and total RNA was extracted and analyzed by primer extension, with oligonucleotide OG1 as the primer. All of the reverse transcription reaction mixture was fractionated on a polyacrylamide gel (B). Lanes: A, C, G, and T, sequencing markers; 1, 2 h; 2, 4 h; 3, 5.5 h; 4, 6.5 h; 5, 7.5 h; 6, 9.5 h; 7, 10.7 h; 8, 24 h. Products corresponding to initiation at the P1, P2, P3, and P4 sites are indicated. The amounts of the reverse transcripts were estimated with a Molecular Dynamics Phosphorimager system, and the results obtained are presented in panel C. For each reverse transcript, the value obtained by Phosphorimager analysis are plotted against the time at which the culture sample was collected. Panel D represents the variation of the quantities of transcripts P1, P2, P3, and P4 in a given amount of total RNA (1 μg) during the fermentation; for each reverse transcript the Molecular Dynamics signal units (C) were divided by the amount of total RNA recovered in the corresponding RNA extraction. The values obtained were plotted against the times at which the bacterial samples were collected.

tion could be the presence during this phase of protein factors reinforcing the utilization of the P2 promoter by $E\sigma^{32}$ RNA polymerase. The *rrnB* operon was recently shown to be transcribed by the *E. coli* RNA polymerase $E\sigma^{32}$ in vitro, and the FIS protein was found to increase the yield of in vitro transcription (41). As mentioned above, we observed the presence of several putative binding sites for the FIS protein upstream of the P4 start site, and experiments are under way to test for their possible roles.

The *gapA* gene and the *rrn* operons have several characteristic features in common. But whereas in the case of the *rrn* operons, transcription by $E\sigma^{70}$ and $E\sigma^{32}$ polymerases initiates at exactly the same position, mRNAs with 5' untranslated regions of different lengths are produced from the *gapA* gene. This situation offers the additional possibility of regulation at the translational level or by modulation of mRNA stability. It should also be noted that the complexity of the *gapA* transcriptional regulatory region is greater than that of the *rrn* operons. It is very similar to that found for the *rpoH* and *rpoD* genes, which produce σ^{32} (8) and σ^{70} (49), respectively, and for the *glnALG* operon (43).

We previously observed that the codon usage of the *gapA* gene was perfectly adapted to *E. coli* and corresponds to that found for highly expressed *E. coli* proteins (6). Now we show that the transcriptional regulatory region of the *gapA* gene belongs to the small family of highly sophisticated *E. coli* promoters which include several of the general *E. coli* transcriptional regulation strategies. This may seem to contradict the idea that the *gapA* gene was inherited by horizontal transmission of the genetic information. Nevertheless, very little is known about the rate at which promoters evolve, and an already optimized promoter region may have been fused to the eukaryotic GAPDH coding region. Thus, the hypothesis of horizontal transmission of the *gapA* gene cannot be excluded by our observation. Nevertheless, both the transcriptional characteristics of *gapA* and the recent observation of a *gapA*-like gene in a cyanobacterium (35) again raise the debate.

ACKNOWLEDGMENTS

This work was supported by the French CNRS and the Ministère de l'Enseignement Supérieur et de la Recherche. B. Charpentier was a fellow of the Ministère de l'Enseignement Supérieur et de la Recherche.

The constant interest and support of G. Branlant for this work is acknowledged. We thank A. Kolb for her helpful discussions and for providing us with mutant *E. coli* strains. We appreciate the technical assistance of J. Bayeul and E. Habermacher.

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