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Cloning and Characterization of the *gsk* Gene Encoding Guanosine Kinase of *Escherichia coli*

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The *Escherichia coli gsk* gene encoding guanosine kinase was cloned from the Kohara gene library by complementation of the *E. coli gsk-1* mutant allele. The cloned DNA fragment was sequenced and shown to encode a putative polypeptide of 433 amino acids with a molecular mass of 48,113 Da. Minicell analysis established the subunit M_r as 43,500. Primer extension analysis indicated the presence of an adequate Pribnow box and suggested that the transcript contained a 110-base leader sequence. Strains harboring the *gsk* gene on multicopy plasmids overexpressed both guanosine and inosine kinase activities. N-terminal sequence and amino acid composition analyses of the 43,500-M_r polypeptide band confirmed the correct reading frame assignment and the identity of this band as the *gsk* gene product. Comparison of the amino acid sequence with the protein database revealed similarity to regions of other mononucleotide-utilizing enzymes.

Eukaryotic and prokaryotic cells in which ribonucleoside kinase activity has been investigated usually contain two enzymatic activities, one specific for adenosine and the other specific for uridine and cytidine. *Escherichia coli* and *Salmonella typhimurium* are exceptions to this because they contain guanosine-inosine kinase (ATP:guanosine 5′-phosphotransferase [EC 2.7.1.73]) instead of adenosine kinase (24). Guanosine-specific ribonucleoside kinase activity has been demonstrated with certainty in only a few organisms, including *E. coli*, *S. typhimurium* (24), and *Trichomonas vaginalis* (20). It is also found in the mitochondria of plants and mammals (8, 33). The *E. coli* enzyme is encoded by the *gsk* gene, which has been mapped to 11 min on the linkage map (2, 14).

The utilization of exogenously supplied or endogenously synthesized guanosine and inosine for nucleotide biosynthesis in *E. coli* and *S. typhimurium* occurs through two pathways. The major pathway involves phosphorolysis of guanosine or inosine to the corresponding nucleobase and ribose 1-phosphate. This reaction is catalyzed by purine nucleoside phosphorylase, which is encoded in *E. coli* by the *deoD* gene. Guanine and hypoxanthine are subsequently phosphoribosylated to their corresponding nucleoside monophosphates by guanine and hypoxanthine phosphoribosyltransferases (*gpt* and *hpt*, respectively). The minor pathway involves direct phosphorylation of guanosine or inosine to the corresponding nucleoside 5′-monophosphate solely through the action of guanosine kinase. The guanosine-inosine kinase pathway can be studied unambiguously in vivo only in *deoD* mutant strains where the function of guanosine kinase has been demonstrated to be essential for guanosine salvage (11, 14, 15).

Methods. The *E. coli* K-12 strains used in this study are shown in Table 1. The growth medium was either NZY broth (13) or AB minimal medium (7) supplemented with glucose (0.2%) and thiamine (0.5 mg/liter). When necessary, methionine or histidine was added to 40 mg/liter. Hypoxanthine, adenine, or guanine was added to 15 mg/liter, and guanosine was added to 30 mg/liter. Ampicillin was used at a concentration of 100 mg/liter, and tetracycline was used at 10 mg/liter. The cloning vectors used were pBR322 (6), pUHE23.2 (provided by H. Bujard, University of Heidelberg, Heidelberg, Germany), pBluescriptKS$^+$, and pBluescriptKS$^-$ (Stratagene Cloning Systems). In addition, pKB800 was used to provide a plasmid-borne bacteriophage λ*c*I857 allele (3). Procedures for transduction by bacteriophage P1 (19) and bacteriophage λ (31), as well as transformation with plasmid DNA (18), have been previously described.

Plasmid DNA and bacteriophage λ DNA were isolated by standard techniques (5, 9). Restriction endonuclease digestions, removal of cohesive ends of DNA fragments with the large fragment of *E. coli* DNA polymerase I, dephosphorylation of DNA with calf intestinal alkaline phosphatase, and ligation of DNA fragments with T4 DNA ligase were performed as recommended by the suppliers of the enzymes. Nucleotide sequences were determined by the chain termination method with dideoxyribonucleotides and single-stranded phagemid DNA derived from pBluescriptKS$^+$ and pBluescriptKS$^-$ as the templates (28, 29). Polymerization was achieved with Sequenase T7 DNA polymerase as recommended by the supplier (United States Biochemical Corp.). Sequence ladders were established by electrophoresis of sequencing reactions in buffer gradient gels containing 8 M urea and 6% polyacrylamide followed by autoradiography of the dried gels as previously described (28).

Guanosine kinase was partially purified from strain HO340 harboring pHA460 by precipitation with ammonium sulfate at 40 to 50% saturation, followed by chromatography on Poros 50 HQ (Perseptive Biosystems) strong anion-exchange medium with elution from 0 to 1 M NaCl over a period of 30 min. These two steps resulted in approximately 40-fold purification. The material was then subjected to electrophoresis as previously described (17). Four lanes of different amounts of partially purified guanosine kinase were electrophoresed (2 × 4.8 μg and 2 × 9.6 μg of total protein). Prestained molecular weight markers (Bio-Rad) were loaded on the same gel, and after separation, the region of the gel between the markers corre-

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TABLE 1. Bacterial strains

Strain	Sex	Genotype	Source or reference
CAG12164	F^-	*malF3089*::Tn*10*	C. Gross; 32
HO340	F^-	*araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF*	23
HO644	F^-	*minB thi rpsL his lac mtl man mal xyl tonA*	12
HO1070	F^-	*supF relA spoT rpsL purE deoD gsk-1 lamB malF*::Tn*10*	P1(CAG12164) × SØ445 Tet^r
HO1071	F^-	*supF relA spoT rpsL purE deoD gsk-1*	P1(HO340) × HO1070 Mal^+
NM522	F′	*supE thi* Δ(*lac-proAB*) Δ(*hsdMS*)/F *lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$	10
SØ445	F^-	*supF relA spoT rpsL purE deoD gsk-1 lamB*	15

sponding to M_rs of 50,000 and 35,100 was electroblotted to Immobilon P polyvinylidene difluoride membrane (Millipore) by using a semidry electroblotter and stained as previously described (26). The band corresponding to guanosine kinase (M_r, 43,500) was excised from each lane. A single band was used for automated N-terminal sequence analysis employing an Applied Biosystems 477A Protein Sequencer with on-line phenylthiohydantoin-amino acid analysis and utilizing the blot cartridge. Sequencing protocols were those recommended by the manufacturer. Bands from three lanes were used for amino acid composition analysis (about 3.5 μg of protein). The bands were treated with 6 M HCl for 24 h (26), and hydrolysates were analyzed as previously described (4).

Cloning of the *gsk* gene. The *purE gsk-1 deoD* strains HO1070, HO1071, and SØ445 (Table 1) require purines for growth. This requirement can be satisfied by the purine base hypoxanthine or by the combined addition of the purine bases adenine and guanine to the growth medium. In contrast, adenine and guanosine, in the presence of histidine, cannot satisfy the purine requirement because of the strains' lack of guanosine kinase and purine nucleoside phosphorylase activities (15). In the presence of histidine, the histidine biosynthetic pathway is repressed and the byproduct of this pathway, 5′-phosphoribosyl-5-amino-4-imidazole carboxamide, also a precursor in de novo purine biosynthesis, is not available to allow IMP synthesis. As a consequence of this, adenine cannot be converted to IMP. Lack of IMP prohibits the formation of guanine nucleotides through IMP dehydrogenase and GMP synthetase, and guanosine cannot be directly converted to guanine nucleotides because of the lack of guanosine kinase. Thus, the strains lack the means of producing a guanine nucleotide pool under these conditions. Complementation with a functional *gsk* allele, however, allows guanosine to serve as a guanine nucleotide source in the presence of histidine.

Complementation of *gsk-1* by the virulent λ phages of the Kohara gene bank was achieved as follows. The bacteriophage λ-sensitive strain HO1071 was transformed with pKB800 DNA. The resulting strain, HO1071/pKB800, was then infected by bacteriophage λ and the cells were plated at 32°C on glucose minimal medium supplemented with adenine and guanosine as the purine sources with histidine present. Complementation of *gsk-1* resulted in growth on this medium. The presence of pKB800 served to repress lytic growth of the virulent recombinant phages. Complementation by plasmid-borne *gsk* alleles was achieved by plating transformed *gsk* cells on medium containing adenine and guanosine as the purine source and with histidine and the required antibiotic present.

To clone the *gsk* gene, we first compared the *proC-purE* region at 11 min of the *E. coli* linkage map (2, 14) with the physical map of Kohara et al. (16) around position 500 kbp. We analyzed bacteriophage λ clones λ8C4(151), λ12H5(152), λ4B10(153), and λ7C12(154) for the presence of the *gsk* gene by complementation of *gsk-1* and found that λ12H5(152) and λ4B10(153) were able to complement the *gsk-1* allele, whereas λ8C4(151) and λ7C12(154) were not. DNA was isolated from strain λ12H5(152), and a 4.9-kbp DNA fragment generated by restriction endonuclease *Eco*RV and *Bam*HI digestion was ligated to *Eco*RV-*Bam*HI-digested pBR322 DNA, resulting in the generation of pGSK2, which also complemented *gsk-1* (Fig. 1). The transformants harboring pGSK2 were able to utilize both guanosine and inosine. The *Bam*HI site (right-hand end of the bar showing pGSK2 in Fig. 1) was generated fortuitously

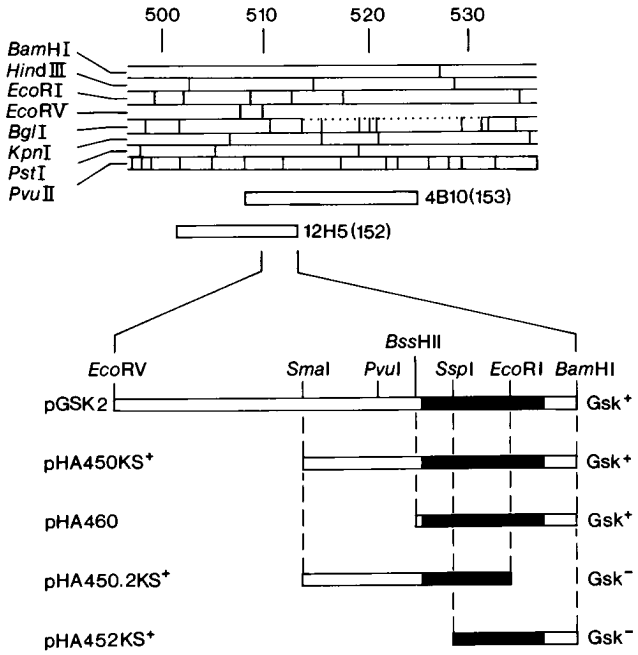

FIG. 1. Cloning, restriction, and complementation analyses of the *gsk* gene. The physical map of the 500- to 535-kbp region of the *E. coli* chromosome is shown at the top. A map of selected restriction endonuclease recognition sites is given, as well as the extent of the DNA fragments harbored in the 12H5(152) and 4B10(153) recombinant λ phages (redrawn from Kohara et al. [16]). Below the map of the λ phages, the 4.9-kbp *Eco*RV-*Bam*HI(*Sau*3AI) DNA fragment inserted in pGSK2 is shown. This insert DNA fragment was cloned in the *Eco*RI and *Bam*HI sites of pBR322. Below pGSK2 are shown the plasmids used for complementation and plasmid-specified polypeptide analyses. In pHA450KS$^+$, the 2.9-kbp *Sma*I-*Bam*HI DNA fragment of pGSK2 was inserted in the *Sma*I and *Bam*HI sites of pBluescriptKS$^+$. To construct pHA460, *Bss*HII-digested pHA450KS$^+$ DNA was filled in with dCTP and dGTP in the presence of the large fragment of *E. coli* DNA polymerase I and then digested with *Bam*HI. This DNA was ligated to pUHE23.2 DNA that had been digested with *Eco*RI, treated with S1 nuclease, and then digested with *Bam*HI. Plasmid pHA450.2KS$^+$ contains the 2.2-kbp *Sma*I-*Eco*RI DNA fragment of pGSK2 in pBluescriptKS$^+$, whereas in pHA452, the 1.3-kbp *Ssp*I-*Bam*HI DNA fragment of pGSK2 was ligated to the *Sma*I and *Bam*HI sites of pBluescriptKS$^+$. The solid bar indicates the open reading frame of *gsk*. To the right is indicated the phenotype that each plasmid confers on strain SØ445 *gsk-1* following transformation. The direction of transcription is rightward. Relevant restriction recognition sites are given.

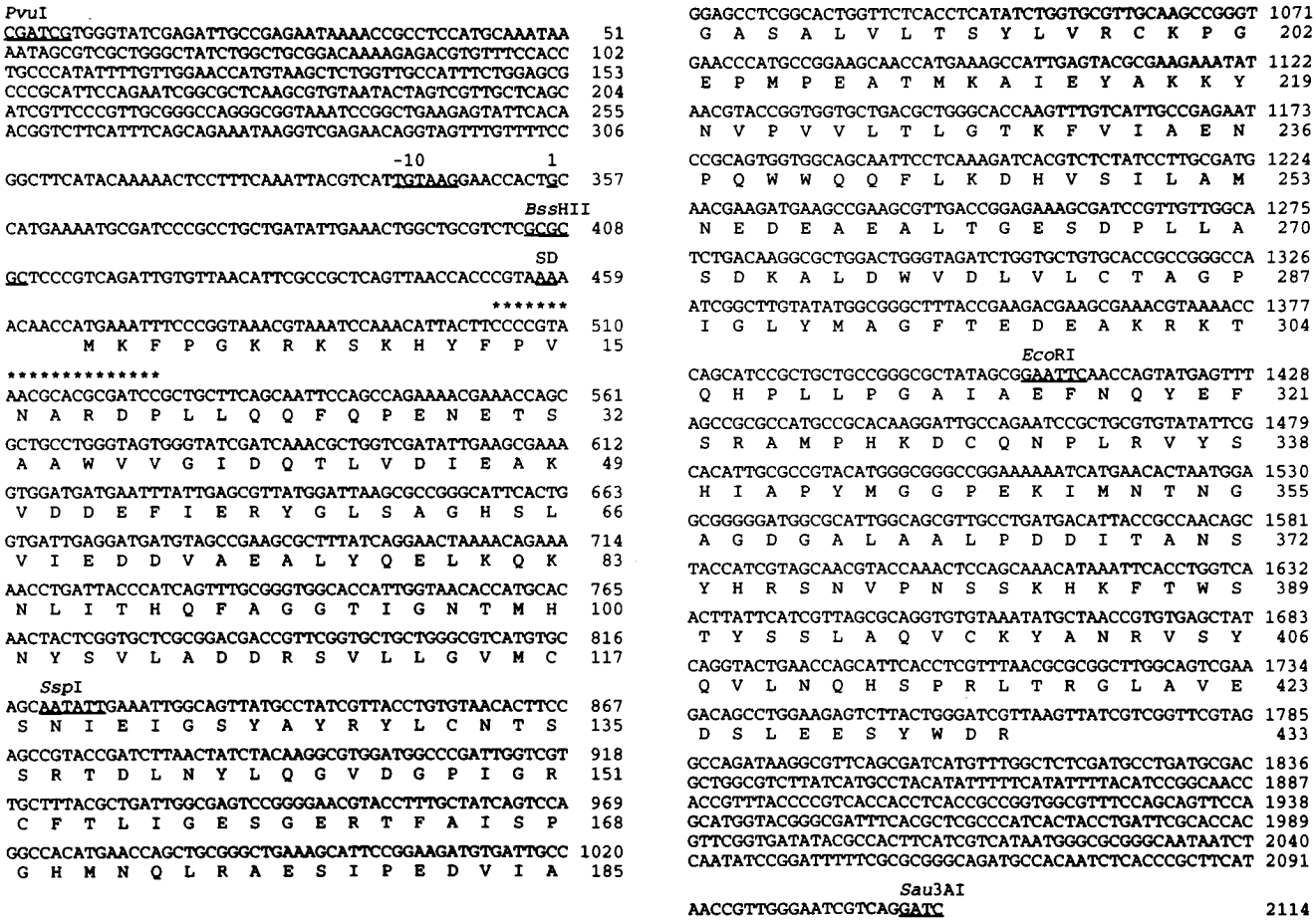

FIG. 2. Nucleotide sequence of the *gsk* gene. The sequence of the coding strand of the 2,114-bp *Pvu*I-*Sau*3AI DNA fragment is shown. The *Sau*3AI site at position 2,111 is part of a *Bam*HI site that was generated at the cloning junction of the insert and the cloning vector used by Kohara et al. (16). The number at the right of each line of nucleotides indicates the nucleotide position. The deduced amino acid sequence of guanosine kinase is shown below the nucleotide sequence. The number to the right of each line of amino acids indicates the amino acid position. Relevant features of the sequence are underlined and include restriction endonuclease recognition sites, a promoter sequence (−10), a transcription start site (indicated by the number 1 above nucleotide 356), and a putative Shine-Dalgarno sequence (SD) (30). The asterisks indicate nucleotides 504 to 524, which are complementary to the oligonucleotide used in primer extension analysis of the 5′ end of the *gsk*-specified mRNA.

during the preparation of the gene bank by ligation of a *Sau*3AI-generated DNA fragment to a *Bam*HI-generated vector DNA fragment. According to the physical map, this *Bam*HI site is not present in the chromosomal DNA. To further locate the *gsk* gene within the cloned DNA fragment, a number of subclones were constructed and their ability to complement *gsk-1* was analyzed (Fig. 1). The results of this analysis show that in addition to pGSK2, pHA450KS$^+$ and pHA460 complement *gsk-1*, whereas pHA450.2KS$^+$ and pHA452KS$^+$ do not. We therefore conclude that the coding sequence of *gsk* is located to the right of the *Bss*HII site and extends into the region between the *Eco*RI and *Bam*HI sites.

Nucleotide sequence and transcription of the *gsk* gene. Determination of the sequence of the *gsk* gene was performed by using a set of subclones covering the entire *Pvu*I-*Sau*3AI-(*Bam*HI) DNA fragment. The sequence of both strands with complete overlap of all cloning junctions was determined. The *Pvu*I-*Sau*3AI DNA fragment (Fig. 2) contains 2,114 bp and revealed an open reading frame which we assigned to the *gsk* gene on the basis of the complementation analysis described above (Fig. 1) and the minicell experiments described below. This open reading frame, which begins at adenylate residue 466 and covers 433 codons, encodes a putative polypeptide with a calculated molecular mass of 48,113 Da. The translation initiation codon of the open reading frame is preceded by a stretch of adenylate residues (SD in Fig. 2) with only poor homology to the consensus Shine-Dalgarno sequence (30).

To determine the transcription initiation point of the *gsk* gene, a primer extension analysis was performed on RNA templates isolated from strains harboring *gsk* on a multicopy plasmid, as well as from a haploid strain. The oligonucleotide used to prime reverse transcriptase-mediated polymerization annealed to a template sequence within the coding region of the *gsk* gene (Fig. 2). The results shown in Fig. 3 indicate that transcription is initiated at guanylate residue 356. Eight nucleotides upstream of the transcription start site is the hexanucleotide sequence TGTAAG, which we propose as a −10 region, as it is in reasonable agreement with the consensus Pribnow box TATAAT (27). From the nucleotide sequence, we were unable to identify a hexanucleotide sequence with proper spacing from the −10 region that exhibited significant homology to a consensus −35 region. As expected, the transcript found in the haploid strain showed the same start point as that from the *gsk* polyploid strain, but the *gsk*-specified mRNA in the haploid strain was less abundant. Inspection of the nucleotide sequence downstream of the *gsk* coding region failed to reveal

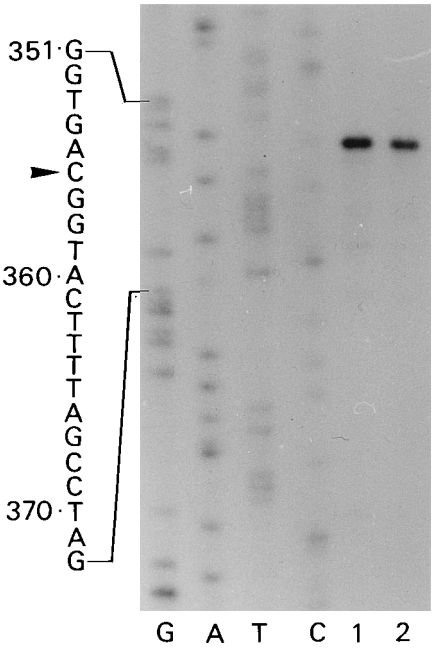

FIG. 3. Analysis of the *gsk*-specified mRNA by primer extension. An autoradiogram of a 6% denaturing polyacrylamide gel is shown. Primer extension was performed as previously described (13). Lanes G, A, T, and C are the sequence ladder. Lane 1 shows extension of the primer on an RNA template isolated from strain HO340/pGSK2 (i.e., a strain multiploid for the *gsk* gene). Lane 2 shows the extension of the primer on an RNA template isolated from strain HO340 (i.e., a haploid strain). The nucleotide sequence of the template strand is given to the left. The numbers on the left indicate nucleotide positions, and the arrowhead indicates the transcription initiation nucleotide.

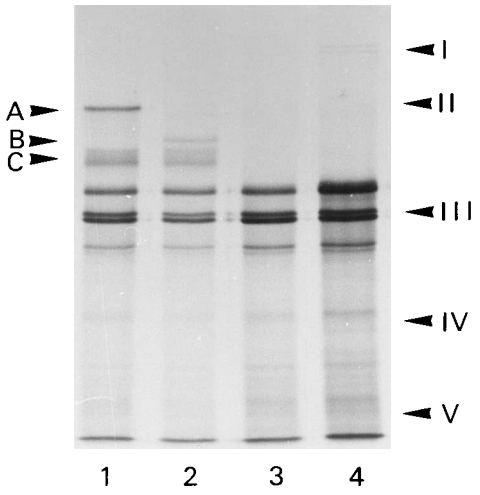

FIG. 4. Plasmid-specified polypeptides. An autoradiogram of a polyacrylamide gel is shown. Minicells containing various plasmids were isolated and analyzed by [^{35}S]methionine labelling and gel electrophoresis as previously described (12, 17). The minicells contained the following plasmids: lane 1, pHA450KS$^+$ (*gsk*$^+$); lane 2, pHA450.2KS$^+$ (Δ*gsk*); lane 3, pHA452KS$^+$ (Δ*gsk*); lane 4, pBluescriptKS$^+$. The following molecular weight markers were used: I, serum albumin (66,200); II, ovalbumin (45,000); III, carbonic anhydrase (31,000); IV, soybean trypsin inhibitor (21,500); V, lysozyme (14,400). The arrowheads at the left point to the bands discussed in the text, with M_rs of 43,500 (A, lane 1), 38,600 (B, lane 2), and 33,000 to 36,000 (C, lanes 1 and 2).

any transcription termination sequences resembling rho-independent terminators. Furthermore, an open reading frame begins at adenylate residue 1,823 and extends out of the sequenced DNA fragment. We do not have experimental evidence of translation of this open reading frame. The *gsk*-specified mRNA also appears to include a 110-nucleotide leader sequence 5′ to the translation start site shown in Fig. 2. The direction of transcription of the *gsk* gene is thus rightward in Fig. 1, i.e., towards higher nucleotide numbers on the physical map, and therefore clockwise on the genetic map.

Polypeptides encoded by *gsk*-containing plasmids. Expression of the *gsk* gene was analyzed in minicells. Figure 4 shows the results of an analysis of the polypeptides specified by the *gsk*-complementing plasmid pHA450KS$^+$, as well as the noncomplementing plasmids pHA450.2KS$^+$, pHA452KS$^+$, and vector pBluescriptKS$^+$. *gsk*-complementing plasmid pHA450 KS$^+$ (lane 1) produced a 43,500-M_r polypeptide which is absent from the three remaining lanes. This band was also visible in the Coomassie blue-stained gel. The noncomplementing plasmid pHA450.2KS$^+$ produced a polypeptide migrating as a faint band with an M_r of 38,600. This polypeptide represents a truncated version of the guanosine kinase subunit. Both pHA450KS$^+$ and pHA450.2KS$^+$ produced polypeptides that were derived from the upstream sequence and migrated as diffuse bands with M_rs of 33,000 to 36,000. We conclude that the polypeptide with an M_r of 43,500 represents the guanosine kinase subunit. This value is in rough agreement with the value of 48,113 Da obtained by calculation from the deduced amino acid sequence.

To confirm the reading frame assignment and identification of the 43,500-M_r band as the guanosine kinase subunit, N-terminal sequencing and amino acid composition analyses were performed from a blot of a partially purified enzyme preparation. The results of the amino acid composition analysis were within 10% of the values predicted for the amino acid composition derived from the nucleotide sequence (data not shown). The results of N-terminal analysis yielded the amino acid sequence Met-Lys-Phe-Pro-Gly-Lys-Arg-Lys-Ser-Lys with the expected mass yield based on amino acid analysis. This sequence is in perfect agreement with the N-terminal amino acid sequence predicted from the nucleotide sequence of the *gsk* gene. The presence of the initiating methionine residue in the sequence demonstrates that this residue is not cleaved from the synthesized guanosine kinase; there was no trace of lysine in the first cycle of the sequence.

Overexpression of the *gsk* gene. The amounts of guanosine and inosine kinase activities specified by some of the plasmids constructed during this work were measured as described before (14, 15). Strains harboring the plasmids which complement *gsk* (HO340/pHA450KS$^+$ and HO340/pHA460) contain high levels of activities, 250 and 578 nmol/min/mg of protein, respectively. This is 40 to 60 times the approximately 6 to 8 nmol/min/mg of protein found in the haploid strain (HO340/pBluescriptKS$^+$). Inosine kinase activities in strains HO340/pHA450KS$^+$ and HO340/pHA460 were 53 and 102 nmol/min/mg of protein, respectively, compared with less than 2 nmol/min/mg of protein in the haploid strain.

Sequence similarity. The protein sequence databanks were searched for homologous proteins by utilizing both the BLAST (1) and FASTA (25) algorithms. The databases employed were SwissProt (release 28.0), Pir (release 40.0), and GenPept (release 83.0). The BLASTP program was run three times with different percent accepted mutations (PAM) values of 40, 120, and 250 to increase the likelihood of finding significant matches with low identity. The PC-Gene suite (Intelligenetics, Inc.) of sequence analysis programs was used for protein primary sequence analysis.

Two entries exist for proteins with sequences nearly identical to ours. The first of these is identified as inosine-guanosine kinase with an unpublished 1,756-bp entry in the DNA database (22). Our derived amino acid sequence differs at several residues from this sequence and in the overall length of the polypeptide. The second sequence is a putative 34,600-Da polypeptide derived from the nucleotide sequence of the *visA* gene region of the *E. coli* chromosome (21). This region is very close to *gsk* on the chromosome, and the relevant nucleotide sequence, which encodes an open reading frame of 312 amino acids, appears to be a truncation of the *gsk*-specified open reading frame occurring from a frameshift error in the sequence determination. Further inspection of the results of the search revealed some interesting matches. Many of the proteins with known functions identified in the search exhibited similarity to two amino acid sequence regions within guanosine kinase. These two regions corresponded roughly to amino acids 206 to 276 and 330 to 410 in the guanosine kinase sequence. All except one of these proteins shared the property of utilizing mononucleotides. These proteins included IMP dehydrogenase, thymidine kinase, aspartyl and prolyl tRNA synthetases, and ribokinase. The one exception was the α-ketoglutarate dehydrogenase E1 component, which also showed homology to guanosine kinase in these regions. The finding of similarity between a number of mononucleotide-utilizing enzymes may suggest a common function for the areas of sequence similarity. One possible function of these two regions could be interaction with the ribose and/or phosphate portion of the mononucleotides. Especially interaction with the phosphate portion might explain why α-ketoglutarate dehydrogenase was also found to be similar to guanosine kinase in these regions. The E1 component of this enzyme binds thiamine; the phosphate moiety (or moieties) of the cofactor could interact with amino acids present in the two homologous regions.

Conclusion. We have cloned the *gsk* gene of *E. coli* and concluded that this gene is the structural gene for guanosine kinase: the cloned gene restores guanosine and inosine utilization to the *gsk-1* mutant strain, and cells harboring a plasmid-borne *gsk* gene contain elevated levels of guanosine kinase activity. Guanosine and inosine kinase activities were also increased in parallel. This work confirms that phosphorylation of both guanosine and inosine is catalyzed by the same enzyme, as has been previously shown by genetic and physiological studies (14, 15), and will facilitate the construction of Δ*gsk* strains that can be used to clone mutant *E. coli gsk* alleles, as well as heterologous guanosine kinase encoding genes.

Nucleotide sequence accession number. The nucleotide sequence of the 2,114-bp *Pvu*I-*Sau*3AI-generated DNA fragment discussed in this report has been submitted to GenBank and assigned accession number L35149.

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