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Nucleotide Sequence of the *xth* Gene of *Escherichia coli* K-12

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The *xth* gene of *Escherichia coli* K-12, which encodes exonuclease III, has been sequenced. Exonuclease III from a cloned copy of the *E. coli* K-12 gene has been purified and characterized. The molecular weight (30,921), the amino-terminal amino acid sequence, and the amino acid composition of the polypeptide predicted from the nucleotide sequence are in excellent agreement with those properties determined for the purified enzyme. The *xth* promoter was mapped by primer extension of in vivo transcripts. Inspection of the nucleotide sequence reveals that a region of dyad symmetry which could form a hairpin stem-loop structure in RNA characteristic of a ρ-dependent terminator lies immediately downstream from the *xth* gene.

The *xth* gene of *Escherichia coli* K-12 encodes the major AP (apurinic-apyrimidinic) endonuclease of *E. coli*, exonuclease III. The enzyme has five catalytic activities: (i) it is an AP endonuclease which cleaves phosphodiester bonds at AP sites to yield base-free deoxyribose 5′-phosphate end groups (42); (ii) it is a 3′-to-5′ exonuclease specific for bihelical DNA (42); (iii) it can remove a number of 3′ termini from duplex DNA, including 3′-phosphate (42), 3′-2,3-unsaturated deoxyribose (40), and 3′-phosphoglycolate (9, 15); (iv) it has an RNase H activity which preferentially degrades the RNA strand in an RNA-DNA hybrid duplex (42); and (v) it can act endonucleolytically at urea-*N*-glycosides in duplex DNA (18). The enzyme has a molecular weight of approximately 30,000 and is active as a monomer (41). Other AP endonucleases, endonuclease III and endonuclease IV, are also found in *E. coli*. Endonuclease III differs from exonuclease III in several respects. It cleaves on the 3′ side of AP sites via a β-elimination reaction (1); it has a glycosylase activity which releases ring-fragmented thymines (3, 4, 10, 16) and a cytosine UV photoproduct (11); and it incises damaged DNA at cytosines (11, 12, 14, 43, 44) and guanines (12). Endonuclease IV cleaves phosphodiester bonds at AP sites to yield base-free deoxyribose 5-phosphate end groups (9); it can remove 3′-phosphoglycolates, 3′-phosphates, and 3′-unsaturated deoxyribose from DNA (9); and it can cleave DNA at apyrimidinic sites formed by neocarzinostatin which are refractory to cleavage by exonuclease III (25). Endonuclease IV is induced by paraquat (methyl viologen), plumbagin, phenazine methosulfate, and menadione; the induction of the enzyme may be mediated via the production of superoxide radicals (5). Neither endonuclease III nor exonuclease III is induced by similar treatments (5).

To understand the structure, function, and regulation of these AP endonucleases in more detail, we have sequenced the genes for exonuclease III, endonuclease III (P. M. Wistort, H. Asahara, R. H. Bakerian, and R. P. Cunningham, manuscript in preparation), and endonuclease IV (S. M. Saporito and R. P. Cunningham, submitted for publication). In this paper we report the sequence of the exonuclease III gene, the identification of the structural gene, and the mapping of the promoter.

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MATERIALS AND METHODS

Bacterial strains and plasmids. All experiments were performed with *E. coli* RPC51 [*endA thi hsdR* Δ(*srlR-recA*)*306*]. All subcloning was done with plasmid pBR322. *E. coli* W was the host for pSGR3, which was used for exonuclease III overproduction.

Purification of exonuclease III. Exonuclease III was purified from *E. coli* W carrying the plasmid pSGR3 (27) as described by Rogers and Weiss (28). The purified enzyme was greater than 98% homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzymatic assay of exonuclease III. The exonuclease assay described by Rogers and Weiss (28) was used for monitoring the purification of exonuclease III and also for detecting enzyme overproduction from cells containing recombinant plasmids.

Polyacrylamide gel electrophoresis of denatured proteins. The method of Laemmli (21) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the determination of the molecular weight of denatured proteins, the following standard proteins were used for calibration: phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000, trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400). The R_f for each protein was determined, and the molecular weight of denatured exonuclease III was derived from a standard calibration curve.

Gel filtration of native proteins. Sephadex G-75 superfine was equilibrated with a solution of 0.1 M KCl–0.05 M potassium phosphate (pH 7.5)–10^{-4} M dithiothreitol. The column (1.5 by 45 cm) was operated at a flow rate of 2 ml cm^{-2} h^{-1}. Protein mixtures were applied in a volume of 0.7 ml. Exonuclease III was detected by enzymatic assay; albumin (M_r 67,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,000), and RNase A (M_r 13,700) were detected by A_{280}. The void volume of the column was determined by measuring the elution volume of dextran blue 2000. K_{av} for each protein was determined, and the molecular weight of exonuclease III was derived from a standard calibration curve.

Protein sequence and amino acid composition determina-

tions. The N terminus sequence analysis was performed by using an Applied Biosystems INC470A gas-phase protein sequenator which was connected on-line to an ABI120 (high-pressure liquid chromatography) phenylthiohydantoin analyzer. Data were collected and yields were analyzed on a Nelson analytical 3000 Series chromatography system. Amino acid composition was determined by analysis of a 6 N HCl hydrolysate on a Dionex 500 ion-exchange high-pressure liquid chromatography system.

Subcloning of the *xth* gene. The *xth* gene is located on plasmid pLC10-4 (29). A 3.2-kilobase-pair (kb) fragment from pLC10-4 was cloned into the *Eco*RV site of pBR322. Deletions were made with the exonuclease BAL 31 (Promega Biotec) from either the *Hin*dIII or the *Bam*HI site of pBR322. Synthetic *Hin*dIII or *Bam*HI linkers (Pharmacia) were added to the ends of the treated fragments. Positive clones were selected by their ability to overexpress exonuclease III as measured by an enzymatic assay (28). In this manner, the *xth* gene has been isolated on a 1.4-kb *Hin*dIII-*Bam*HI restriction fragment on pRPC156.

DNA sequence analysis. Restriction fragments of the *xth* gene and its deletion derivatives were subcloned into M13 cloning vectors mp18, mp19, um20, or um21. The nucleotide sequence was determined by the dideoxy chain termination method (32, 36) with M13 universal sequencing primers and either the Klenow fragment of *E. coli* polymerase I or Sequenase (U.S. Biochemical Corp.).

Isolation of cellular RNA. Cellular RNA used for mapping the *xth* transcriptional start site was prepared from a 40-ml exponentially growing culture of RPC51(pRPC156) (A_{650}, 0.3) grown in K medium (31) at 37°C. Cells were quick-chilled, collected by centrifugation, and washed with 8 ml of ice-cold 10 mM Tris hydrochloride (pH 7.5). They were resuspended, transferred to a microcentrifuge tube, pelleted, and lysed by the lysozyme freeze-thaw method (24) in lysis buffer (150 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 10 mM dithiothreitol, 45 U of RNase inhibitor [Promega Biotec], 0.25 mg of lysozyme [Sigma Chemical Co.] ml^{-1}).

The contaminating DNA was removed by adding RNase-free DNase I (40 $\mu g\ ml^{-1}$ [Boehringer Mannheim Biochemicals]) in the presence of 20 mM magnesium acetate and 6 U of RNase inhibitor (Promega Biotec) for 45 min on ice and then for 10 min at 30°C, with additional RNase-free DNase I (8 $\mu g\ ml^{-1}$) added. The sample was extracted in phenol once and in chloroform-isoamyl alcohol (24:1) three times in the presence of 10 mM acetic acid and 0.5% sodium dodecyl sulfate.

The RNA was precipitated with 10 mM magnesium acetate–300 mM sodium acetate (pH 5.2)–2.5 volumes of 95% cold ethanol for 2 h at −20°C. The RNA pellet was suspended in 100 μl of diethylpyrocarbonate-treated water and stored at −80°C.

Primer extension. A 17-residue oligonucleotide was 5′ end labeled with 50 μCi of [γ-^{32}P]ATP (4,500 Ci $mmol^{-1}$; ICN) and 5 U of T4 polynucleotide kinase (International Biotechnology Inc.) in a 10-μl reaction mixture (100 mM Tris hydrochloride [pH 8], 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM spermidine) for 45 min at 37°C. The sample was heated for 3 min at 95°C, quick-chilled on ice, and extracted once with phenol, once with chloroform-isoamyl alcohol (24:1), and twice with ether. The 5′-end-labeled oligonucleotide was dried in vacuo, suspended in 20 μl of 1 mM EDTA, and stored at −20°C. The primer extension technique used is similar to that described by Belfort et al. (2).

In annealing buffer (50 mM Tris hydrochloride [pH 8], 60 mM NaCl, 10 mM dithiothreitol), 20 μg of RNA was annealed to 0.6 pmol of 5′-end-labeled primer for 3 min at 60°C. The annealing mixture was placed in a dry-ice–ethanol bath and then thawed on ice. The primer was extended with avian myeloblastosis virus reverse transcriptase (1 U; U.S. Biochemical Corp.) for 30 min at 48°C in annealing buffer containing 6 mM magnesium acetate, and 375 μM each deoxynucleotide.

The sequencing ladder was made by annealing the same 5′-end-labeled oligonucleotide to the M13mp19 recombinant template containing the *Pst*I-*Hin*dIII restriction fragment from pRPC156. The mixture was aliquoted into four microcentrifuge tubes, each containing each deoxynucleotide (375 mM) and one of the dideoxynucleotides (200 μM) in annealing buffer, 6 mM magnesium acetate, and avian myeloblastosis virus reverse transcriptase (1 U), for 30 min at 48°C.

The primer extension reaction and the sequencing reactions were stopped by adding 6 μl of sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and heating the mixture for 3 min at 95°C.

The samples were applied to an 8 M urea–8% acrylamide gel and electrophoresed at constant power for 2.5 h. The gel was exposed to Cronex X-ray film (Du Pont Co.), and the autoradiogram was developed by standard methods.

RESULTS

Subcloning of the *xth* gene. Cells which overproduced exonuclease III were originally found in a colony bank containing ColE1-*E. coli* hybrid plasmids (6). Two plasmids, pLC10-4 and pLC26-8, were found to carry the exonuclease III gene (29). The region of shared sequences suggested that the *xth* gene was to the right of the *Hin*dIII site at 3.9 kb on the restriction map of pLC10-4 (Fig. 1). A deletion derivative of pLC10-4 placed the gene either between the *Hin*dIII site at 3.9 kb and the *Bam*HI site at 4.8 kb or to the right of the *Bam*HI site at 8.3 kb (29). Initially we cloned a 1.6-kb *Bam*HI-*Bgl*II fragment from pLC10-4 into pBR322 to create pRPC112. This plasmid did not overproduce exonuclease III. This suggested that the *Bgl*II site at 10.9 kb was in the *xth* gene. Since exonuclease III is a small protein, it seemed unlikely that it would span both the *Bgl*II site at 10.9 kb and the *Nru*I site at 9.0 kb; therefore, we assumed that it would lie within the *Nru*I fragment which we used to create pRPC151. Plasmid pRPC151 overproduces exonuclease III to the same level as the parental plasmid pLC10-4 does. We used BAL 31 nuclease to create deletions to further localize the gene. Plasmids pRPC152 and pRPC154 were constructed, allowing us to place one end of the exonuclease III gene near the *E. coli*-ColE1 junction. A deletion extending rightward from the *Nru*I site of pRPC152 placed the other end of the gene very near the *Bgl*II site at 10.9 kb.

Nucleotide sequence of the *xth* gene. The sequence of most of the 1,400-base-pair fragment subcloned in pRPC156 was determined for both strands from overlapping DNA fragments. A detailed restriction map and the specific DNA fragments sequenced are shown in Fig. 2. The DNA sequence of 1,020 nucleotides including the exonuclease III gene is shown in Fig. 3. An open reading frame starting at nucleotide 139 with an ATG codon and ending at nucleotide 946 with a TAA codon was identified. The identified open reading frame has a codon usage consistent with the nonrandom codon usage identified for a number of *E. coli* genes.

Upstream of this open reading frame, a −10 hexamer at nucleotides 98 to 103 was found. There was no hexamer at the appropriate distance away that showed significant homology to the consensus −35 sequence. Farther upstream, a

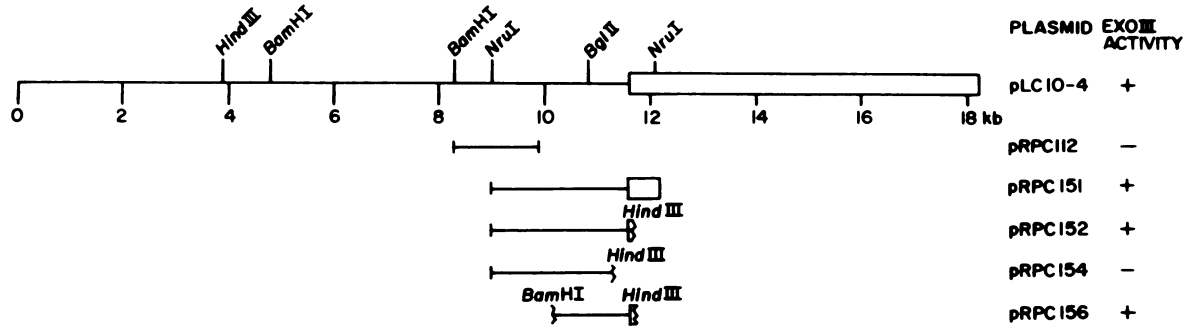

FIG. 1. Physical map of plasmid pLC10-4 and the sublones used to localize the *xth* gene. The circular map of pLC10-4 is linearized by cleavage through one end of the ColE1 DNA. Vertical lines represent restriction sites used for subcloning into pBR322; for plasmids pRPC152, pRPC154, and pRPC156, jagged vertical lines with new restriction sites indicate that *Hin*dIII or *Bam*HI linkers were added to the ends of fragments created by digestion with nuclease BAL 31. ▭, ColE1 sequences. The plasmids and their properties are tabulated on the right. The ability of plasmids to express exonuclease III (EXOIII) was determined by enzyme assay.

−10 hexamer at nucleotides 55 to 60 and an appropriately spaced −35 sequence at nucleotides 32 to 37 was found. A possible Shine-Dalgarno sequence (34) of ATGG was found at nucleotides 127 to 130 and was appropriately spaced from the start of translation. At 9 base pairs beyond the end of the *xth* gene we found a region of dyad symmetry with the potential to form a hairpin stem-loop structure if translated into RNA. This potential structure has a calculated free energy of −11 kcal mol^{-1} (−46 kJ mol^{-1}) (38) and may function as a rho-dependent terminator (30).

To determine whether the identified open reading frame is the structural gene for exonuclease III, we characterized purified exonuclease III. The enzyme was purified by published procedures (28) from a strain carrying a plasmid which overexpressed the cloned *xth* gene originally derived from pLC10-4 (27). We determined the native and denatured molecular weights of the protein as 32,000 and 25,500, respectively, which is in agreement with the values originally described for exonuclease III specified by the chromosomal gene of *E. coli* K-12 (41) and which is also in agreement with the value of 30,921 from our derived amino acid sequence. The purified enzyme preparation was used to determine the N-terminal amino acid sequence of exonuclease III. The sequence of the first 18 amino acids is in complete agreement with the predicted N-terminal sequence from the DNA sequence of the open reading frame (Fig. 3). The purified enzyme preparation was also used to determine the amino acid composition of exonuclease III. Table 1 shows the excellent agreement between the amino acid analysis and the composition predicted from the DNA sequence. These results establish the identified open reading frame as the structural gene for exonuclease III.

Mapping the *xth* promoter. Analysis of the DNA sequence upstream of the *xth* structural gene revealed two potential promoters. To identify the in vivo site of transcriptional initiation, we mapped the *xth* promoter by primer extension mapping of *xth* transcripts. Cellular RNA from a strain bearing pRPC156 to enrich for *xth* transcripts was used as a source of mRNA. A synthetic primer 17 nucleotides long complementary to nucleotides 169 to 185 was 5′ ^{32}P labeled, annealed to cellular RNA, and extended with reverse transcriptase. This primer was also annealed to an M13 clone carrying a portion of the antisense strand of the *xth* gene, and a sequencing ladder was prepared by dideoxy sequencing. The DNA fragments extended by reverse transcriptase were displayed on a DNA sequencing gel. Figure 4 shows an autoradiogram of this sequencing gel. The fragment generated from reverse transcription of the primer annealed to RNA (lane 5) ends at T_{110} (lane 3) in the sequencing ladder. Since this is the sequence of the sense strand, transcription initiates at A_{110} in the sequence in Fig. 3. Thus, the promoter lacking a consensus −35 site is used in vivo under the conditions of cell growth used for transcript preparation.

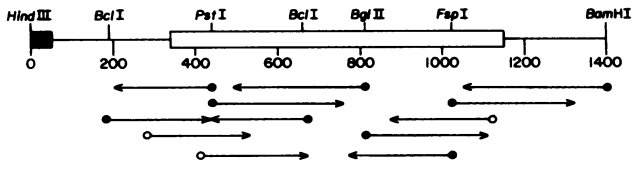

FIG. 2. Strategy and restriction sites used for sequencing the *xth* gene. The restriction fragment shown is a 1,400-base-pair fragment from pRPC156. Arrows indicate the direction and extent of each sequence determination. Symbols: ⟷, start sites corresponding to internal restriction sites; ○⟶, start sites created by digestion with nuclease BAL 31; ▭, *xth* structural gene; ▬, ColE1 sequences.

DISCUSSION

Exonuclease III is the major AP endonuclease in *E. coli* (23) under normal growth conditions. Endonuclease IV can be induced by treatment with paraquat to levels which are approximately equal to those of exonuclease III (5). Mutants deficient in exonuclease III exhibit a hyper-Rec phenotype (46), are sensitive to hydrogen peroxide (8), and are sensitive to methyl methanesulfonate and mitomycin C (7). Mutants simultaneously deficient in exonuclease III and dUTPase are inviable (37). Mutants deficient in both exonuclease III and endonuclease IV are sensitive to ionizing radiation and hypersensitive to hydrogen peroxide, methyl methanesulfonate, and mitomycin C (7). These results suggest that exonuclease III and endonuclease IV play major roles in repairing AP sites which arise in *E. coli* either spontaneously or by the action of glycosylases which recognize damaged or incorrect bases.

In this work we have sequenced the gene for exonuclease III. An open reading frame yielding a predicted polypeptide of approximately the molecular weight determined for exonuclease III was found. To verify that this open reading frame was the structural gene for exonuclease III, we purified exonuclease III and determined its N-terminal amino acid sequence and its amino acid composition. There is excellent agreement of the predicted N-terminal amino acid sequence and the predicted amino acid composition

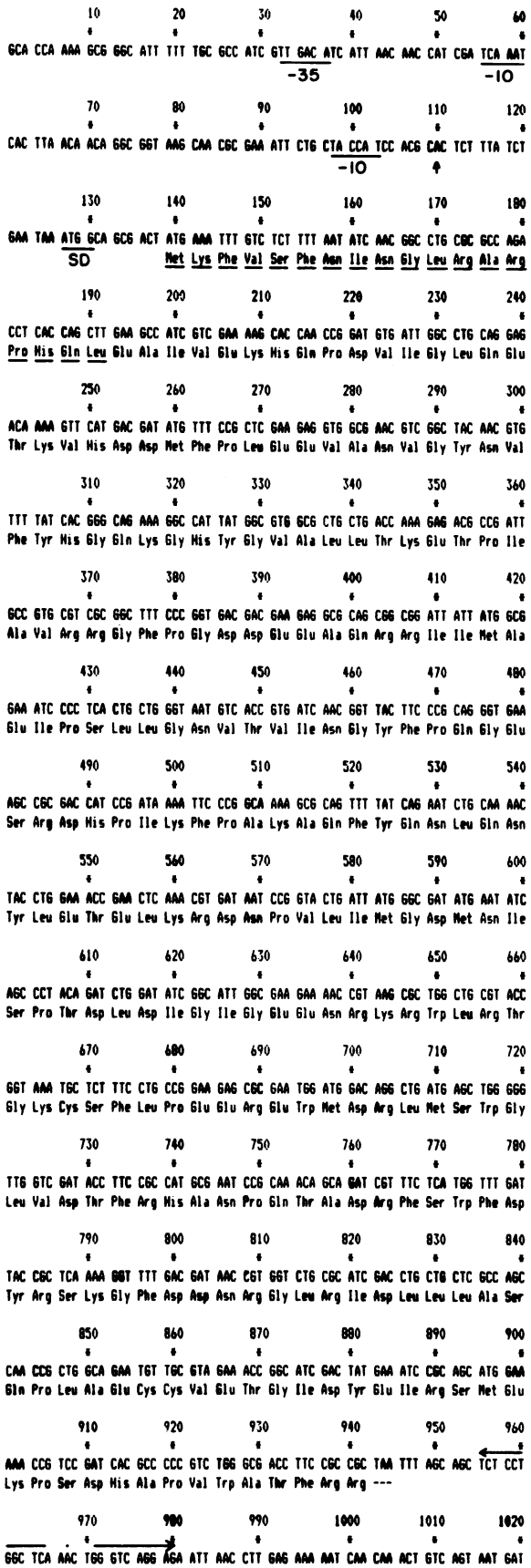

TABLE 1. Amino acid composition of *E. coli* exonuclease III

Amino acid	Exonuclease III composition[a] from:	
	Amino acid analysis[b]	DNA sequence
Ala	15	15
Arg	21	21
Asx	28	(32)[c]
Asn		13
Asp		19
Cys	ND[d]	3
Glx	31	(32)
Gln		11
Glu		21
Gly	18	19
His	8	8
Ile	16	16
Leu	24	22
Lys	13	12
Met	8	8
Phe	14	14
Pro	17	16
Ser	9	11
Thr	10	11
Trp	ND	5
Tyr	8	8
Val	13	15

[a] The molecular weight as determined from the DNA sequence was 30,921.
[b] Rounded to nearest integer.
[c] Parentheses indicate the sum of aspartic acid and asparagine or of glutamic acid and glutamine.
[d] ND, Not determined.

with the actual N-terminal amino acid sequence and the determined amino acid composition.

Examination of the sequence reveals potential regulatory signals for the gene. At 9 base pairs downstream from the end of the structural gene is a region of dyad symmetry capable of folding into a hairpin stem-loop structure if translated into RNA. On the basis of the free energy of this structure, we would not predict it to be a strong terminator. At 76 base pairs beyond the potential terminator structure is a region containing a promoter with a −35 and a −10 site, a Shine-Dalgarno site, and the beginning of an open reading frame (S. M. Saporito and R. P. Cunningham, unpublished results). These data suggest that exonuclease III is expressed monocistronically. We have found two potential promoters upstream of the structural gene. One has a perfect consensus −35 site separated by 17 base pairs from a −10 site with good homology to the consensus sequence. The other promoter has a −10 site but no consensus −35 site. We used primer extension of in vivo transcripts to map the start of transcription. The promoter lacking a −35 region is used in vivo. The absence of a −35 site suggests that the gene may be controlled by an activator protein (26). Further inspection of this promoter reveals that there is a T at −15 and a G at

FIG. 3. Nucleotide sequence of the *xth* gene and deduced amino acid sequence of exonuclease III. The DNA sequence of the antisense strand is shown; numbering is from the 5′ end. The proposed −10 and −35 hexamers of the *xth* promoter and the proposed Shine-Dalgarno site are underlined and labeled. The proposed terminator, a region of dyad and inverted symmetry, is overlined, with the center of symmetry indicated by a dot. The arrow below nucleotide 110 indicates the transcriptional initiation site. The underlined amino acids are those confirmed by protein sequence analysis of exonuclease III.

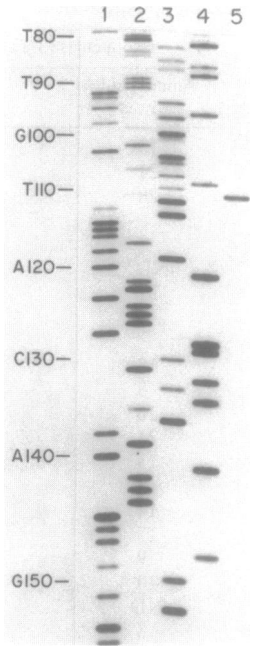

FIG. 4. Primer extension mapping of the *xth* promoter. The autoradiogram of a 8% polyacrylamide sequencing gel used to analyze a DNA primer extended by reverse transcriptase is shown. Equivalent amounts of a ^{32}P-end-labeled DNA primer were used for both primer extension and sequencing ladder reactions (see Materials and Methods). The sequencing ladder of the reverse strand was generated by dideoxy sequencing, A (lane 1), T (lane 2), G (lane 3), and C (lane 4), of the 440-base-pair *Pst*I-*Hin*dIII restriction fragment from pRPC156 with reverse transcriptase. For the primer extension reaction, 20 μg of total RNA isolated from RPC51 carrying pRPC156 was used (lane 5). The numbering of the nucleotide positions is in agreement with the complementary sequence shown in Fig. 3.

−14, which is characteristic of an extended −10 site (17). Whether this is sufficient to promote transcription without a consensus −35 site or an activator protein remains to be determined. It is possible that the other promoter is used under some growth conditions requiring a higher level of transcription. A potential ribosome-binding site (ATGG) is found 8 nucleotides from the start codon of the structural gene. There is a conserved A (19) three nucleotides upstream from the start codon, and the second codon (AAA) is a frequent second codon (35).

The codon usage for the *xth* gene was compared with the codon usage data assembled by Sharp and Li (33). The usage for *xth* was most closely related to the low-bias group, suggesting that the translation efficiency of the *xth* gene need not be high. A hydropathy profile for exonuclease III that was determined by the method of Kyte and Doolittle (20) was typical of a soluble protein with no long nonpolar stretches. The average hydropathy (−0.55) and the content of charged amino acids (Asp + Glu + Arg + Lys = 27.25 mol%) are also typical of a soluble protein.

We purified exonuclease III encoded by a cloned copy of the *E. coli* K-12 gene. We obtained a native molecular weight of 25,000 from Sephadex gel filtration and a molecular weight in the presence of sodium dodecyl sulfate of 32,000. Using the same techniques, Weiss reported values of 27,400 and 28,500, respectively, for exonuclease III purified from *E. coli* K-12 (41).

Verly and Rassart (39) purified the major AP endonuclease from *E. coli* B41, endonuclease VI, which has many of the properties of exonuclease III. Their enzyme has 3′-to-5′ exonuclease activity and 3′ DNA phosphatase activity, shows heat inactivation kinetics identical to those of exonuclease III, cleaves 5′ to AP sites, and has native and denatured molecular weights of 32,000 and 33,000, respectively (13, 39). Genetic data obtained by Yajko and Weiss (45) and by Ljungquist et al. (23) suggest that the two enzymes are the same protein (for a review of this point, see reference 42). We are unable to explain why the amino acid composition of endonuclease VI reported by Verly and Rassart (39) is unlike that determined for exonuclease III.

A computer search with the search algorithm of Lipman and Pearson (22) did not reveal any proteins with extensive similarity to exonuclease III in the National Biochemical Research Foundation protein sequence data library. This library included alkaline phosphatase, lambda exonuclease, staphylococcal nuclease, T4 endodeoxyribonuclease I, *E. coli* RNase H, pancreatic RNase, and *E. coli* DNA polymerase I, which all have activities similar to those of exonuclease III.

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