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Promoter for the *unc* Operon of *Escherichia coli*

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Fragments of DNA carrying possible promoters for the *unc* operon of *Escherichia coli* were cloned into a promoter detection plasmid (pRZ5255). Similar fragments were transcribed in vitro to produce transcripts whose sizes were used to determine the approximate start site for transcription. One strong promoter and at least two very much weaker ones were detected by these methods. The exact position of the strongest promoter, presumed to be the true *unc* promoter, was determined by S1 nuclease mapping and shown to lie 73 base pairs upstream from the open reading frame that precedes *uncB*. It therefore appears that this reading frame (*uncI*) is part of the *unc* operon. S1 mapping also revealed the presence of a third weak promoter 25 base pairs upstream of *uncI*. All of the weak promoters occur between the proposed *unc* promoter and *uncB*, but their role in vivo, if any, is unclear.

The eight genes that code for the eight known polypeptides that make up the proton-translocating ATPase of *Escherichia coli* are located at 83.5 min on the chromosomal map of *E. coli* (2). Genetic complementation studies (7), in vitro transcription-translation experiments (6, 8, 13), and DNA sequence data (9, 10, 16–18, 20, 22, 27, 29, 33) have established that the genes are arranged in the order BEFHAGDC coding for ATPase subunits a, c, b, δ, α, γ, β, and ε, respectively. The consecutive arrangement of the ATPase genes, as well as a study of polar mutations affecting their expression (11), suggest that they are coordinately transcribed and thus form an operon called the *unc* operon.

The sequence of nucleotides preceding the first known structural gene, *uncB*, has an open reading frame which could code for a hydrophobic protein of 14.2 kilodaltons (10). Although this reading frame is preceded by some promoter-like sequences, it also contains at least two possible promoters. It is therefore unclear where transcription of the *unc* operon begins and whether the open reading frame is part of the operon. On the basis of their DNA sequence data, Gay and Walker (10) originally proposed that a promoter 73 base pairs (bp) upstream of the open reading frame is the *unc* promoter. Some experimental support for this proposal has been provided by a study (32) of Tn*10* insertions in and around the open reading frame and by the in vitro transcription and DNase I footprinting of DNA from the start of the operon (16).

In this paper we show that a promoter 73 bp upstream of the open reading frame is strongly active both in vivo and in vitro; we conclude that this is the *unc* promoter, since all other promoter-like sequences in an approximately 1-kilobase region of DNA preceding *uncB* were found to be either inactive or only very weakly active. It follows that the open reading frame is part of the *unc* operon, and we rename it *uncI*.

MATERIALS AND METHODS

Plasmids, phage, and bacteria. The plasmid vectors pBR322 (4) and pRZ5255 were used as indicated below. Plasmid pAP55 carries the entire *unc* operon, including *uncI*, and has been described elsewhere (5). The transducing phage λ *asn* (25) carrying the wild-type *unc* operon was used for the initial cloning. This phage was obtained from *E. coli* KY7485, which is lysogenic for λ *asn* (19). The plasmid pRZ5255 and its derivatives were maintained in strain CSH26S (*lacZ rpsL*). All other plasmids were maintained in strain LE392.

Construction of plasmids. Methods for the restriction, endonuclease digestion, phosphatase treatment, ligation, analysis of restriction fragments, and transformation of *E. coli* cells have been described previously (14). Cells carrying derivatives of pRZ5255 were selected on medium containing kanamycin at 140 μg/ml.

Preparation of DNA. Phage DNA was prepared as previously described (14) after induction of λ *asn* in KY7485 (19). Plasmid DNA was prepared by standard procedures (14). Fragments of DNA from restriction endonuclease digestions were purified from agarose gels after electrophoresis by a method similar to that of Vogelstein and Gillespie (30). Agarose containing DNA was dissolved in 5 volumes of sodium perchlorate solution (7 M), and glass beads were added in sufficient quantity to adsorb all of the DNA but not in great excess. (Glass beads [325-mesh silica] were prepared by taking a fraction that did not sediment

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during 1 h after stirring in water. This fraction was boiled in 50% [vol/vol] nitric acid and washed extensively in water. The final slurry [50% (vol/vol) H_2O] was tested empirically to determine the minimum amount required to bind a given quantity of DNA [approximately 10 μl/20 μg of DNA].) The mixture was turned gently, end over end, for at least 1 h at 4°C. The beads were centrifuged (2 min in an Eppendorf microfuge), washed twice in 200 μl of ice-cold $NaClO_4$ (7 M) and twice in ice-cold wash buffer (1:1 [vol/vol] ethanol:TE [TE, 10 mM Tris (pH 7.9), 1 mM EDTA]); they were resuspended in a convenient volume of buffer (TE) and incubated for 30 min at 37°C to elute the DNA. The suspension was centrifuged, and the beads were discarded.

Preparation of RNA. Total cellular RNA was prepared from strain LE392 by the method of von Gabain et al. (31). A second preparation, enriched for *unc* mRNA, was made from strain LE392 bearing the plasmid pAP55.

Assays of *lacZ* expression by pRZ5255 derivatives. Cultures of strain CSH26S carrying the plasmid of interest were grown, lysed, and assayed for β-galactosidase activity as described by Miller (26). The units of activity were calculated as described previously (26) and are proportional to the amount of substrate hydrolyzed per minute per bacterium.

In vitro transcription. In vitro transcription experiments were based on the method of Lee and Yanofsky (21). Each reaction mixture (25 μl) contained 20 mM Tris-acetate (pH 8.0), 4 mM magnesium acetate, 0.1 mM disodium EDTA, 1 mM dithiothreitol, 50 mM KCl, three unlabeled nucleoside triphosphates at 0.15 mM each, and 5 to 10 μCi of one labeled nucleoside triphosphate ([α-^{32}P]GTP; Amersham Corp.) at 0.02 mM. The DNA fragments were present at approximately 20 nM. Purified RNA polymerase was obtained from PL Biochemicals Inc. and was added to a concentration of approximately 35 μg/ml. The reactions were incubated at 37°C for 30 min, and the products were separated by electrophoresis on denaturing acrylamide gels: 5% acrylamide–7 M urea in TBE buffer (23).

S1 nuclease mapping. The procedure of Berk and Sharp (3) for mapping the 5′ end of in vivo mRNA was followed. The DNA fragment *Hin*dIII$_{1300}$ (Fig. 1) was

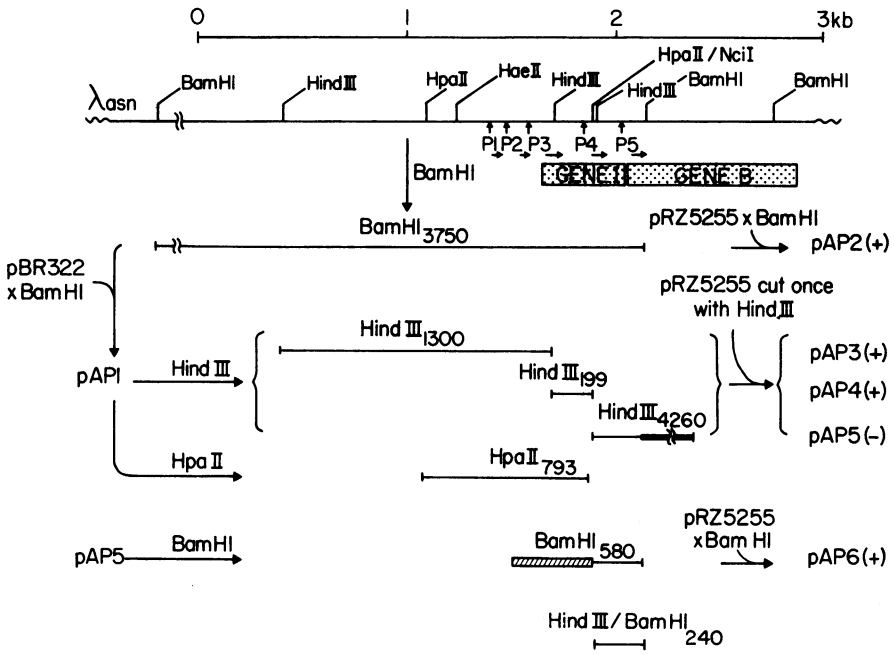

FIG. 1. Restriction map of the *uncI*-containing region of the chromosome showing restriction fragments of interest and cloning strategies. All sites for the enzymes *Bam*HI and *Hin*dIII are shown, but only two for *Hpa*II and one for *Hae*II are included. No *Hpa*II sites occur between the two that are shown, and the *Hae*II site shown is unique in the fragment *Hin*dIII$_{1300}$. A site for *Nci*I that is unique in the *Hin*dIII$_{199}$ fragment coincides with one of the *Hpa*II sites. Published DNA sequence data include all the DNA to the right of the lefthand *Hpa*II site shown but none to the left. The terminal purine residues for each of the promoter-like sequences P1 through P5 (Fig. 2) are located as shown by upward-pointing arrows; small horizontal arrows indicate the direction in which, if active, these sequences would promote transcription. Restriction fragments are named according to the enzyme(s) used to generate them, with numerical subscripts indicating their size in bp. DNA from pBR322 is represented by a heavy line; a hatched line represents pRZ5255 DNA, and the remaining lines represent DNA from λ *asn*. Fragments of DNA cloned into pRZ5255 were oriented with the promoter-like sequences directed towards (+) or away from (−) *lacZ*. It was possible to determine the orientation of inserts in the pRZ5255 derivatives by performing the following digestions: pAP2 with *Hin*dIII, pAP3 with *Hae*II, pAP4 with *Nci*I, pAP5 with *Bam*HI, and pAP6 with *Hin*dIII. In each case a restriction fragment of a size that could only derive from a plasmid with the indicated orientation was detected. kb, Kilobases.

labeled at its 5′ ends by the use of T4 polynucleotide kinase (BRL Inc.) and [γ-^{32}P]ATP (Amersham Corp; 4,000 Ci/mmol) and then digested with restriction endonuclease *Hae*II. The two resulting fragments were separated by agarose gel electrophoresis, and the smallest fragment (*Hae*II/*Hin*dIII$_{471}$) was isolated from the gel and analyzed further. Five portions (0.1 to 0.2 μg each) were subjected to five of the partial cleavage reactions of Maxam and Gilbert (24). Another portion (about 50 ng) of the same DNA was mixed with RNA (20 μg) prepared from strain LE392. An identical portion of DNA was mixed with RNA (20 μg) that was enriched for *unc* mRNA (i.e., RNA prepared from a strain bearing plasmid pAP55). Both RNA-DNA mixtures were precipitated with ethanol, lyophilized, and dissolved in 30 μl of hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(α-ethanesulfonic acid); pH 6.4], 0.4 M NaCl, 1 mM EDTA, and 80% [vol/vol] deionized formamide); they were then heated at 80°C for 15 min, rapidly shifted to 55°C, and incubated for 3 h before 0.3 ml of ice-cold S1 buffer (280 mM NaCl, 30 mM sodium acetate, 4.5 mM zinc acetate) containing 1,000 U of S1 nuclease (Boehringer Mannheim Corp.) was added. After the mixtures had been rapidly chilled in ice water, they were incubated at 37°C for 2 h and made 0.3 M in sodium acetate. The DNA was precipitated by the addition of 2 volumes of ethanol and lyophilized. This protected DNA was electrophoresed next to the five partially degraded samples of DNA on an 8% acrylamide sequencing gel (24). The relative amounts of protected DNA fragments represented by the bands in one lane of an autoradiogram were estimated by scanning the bands with a Joyce-Loebel densitometer and comparing peak sizes.

RESULTS

Cloning possible promoters into a promoter detection plasmid. Five sequences that were considered as possible *unc* promoters are compared in Fig. 2. The positions of these sequences with respect to various chromosomal restriction sites and the genes *uncB* and *uncI* are shown in Fig. 1, which also shows the fragments of DNA that were studied and the cloning strategy. Four plasmids, pAP2, pAP3, pAP4, andpAP6, were constructed by using pRZ5255 (Fig. 3) as a vector and were designed to test for promoter activity by the fragments *Bam*HI$_{3750}$, *Hin*dIII$_{1300}$, *Hin*dIII$_{199}$, and *Hin*dIII/*Bam*HI$_{240}$, respectively. The ability of these plasmids to promote the expression of *lacZ* was measured as described in Materials and Methods. The results (Table 1) show that *Bam*HI$_{3750}$ and *Hin*dIII$_{1300}$ promote transcription of *lacZ* well, whereas *Hin*dIII$_{199}$ and *Hin*dIII/*Bam*HI$_{240}$ carry only weak promoter activity. Since the former two fragments both carried P1, P2, and P3, it seemed likely that one of these three sequences was the *unc* promoter.

In vitro transcription of DNA carrying possible promoters. Three fragments of DNA, *Bam*HI$_{3750}$, *Hin*dIII$_{1300}$, and *Hpa*II$_{793}$, were purified and transcribed in vitro. For each fragment of DNA used as a template, a single major transcript was observed. The sizes of these transcripts were estimated by electrophoresis through denaturing polyacrylamide gels and autoradiography (Fig. 4) and are included in Table 2, in which they are compared with the sizes that would be expected if any one of the sequences P1 through P5 were an active promoter. The comparison strongly favors P3 as an active promoter, suggesting that it is responsible for β-galactosidase production by pAP2 and pAP3. The 120- and 290-base transcripts produced by fragments *Hin*dIII$_{1300}$ and *Hpa*II$_{793}$,

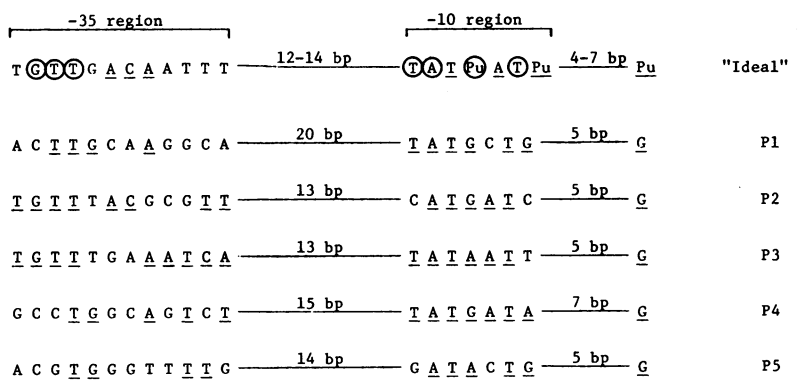

FIG. 2. Comparison of five promoter-like sequences (P1 through P5) that precede *uncB* with an "ideal" promoter sequence (28). Residues in this sequence that are conserved in greater than 75% (circled) or 50% (underlined) of 46 previously characterized promoters are indicated. Two consensus sequences (the −10 and −35 regions) precede the terminal purine (Pu) residue at which transcription is initiated. Sequence data for P1 through P5 were taken from reference 33; underlined residues are those that agree with the ideal sequence. The sequence P3 was originally proposed by Gay and Walker (10) as the *unc* promoter. Kanazawa et al. (17) noted the sequence P4 as a possible promoter. The other possible promoters have not been mentioned previously. The positions of the terminal guanine residues of P1 through P5 are shown in Fig. 1.

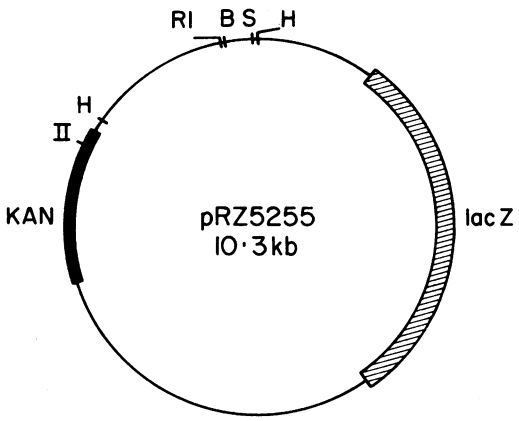

FIG. 3. Restriction map for pRZ5255 (data from L. Munson and W. Reznikoff, Biochemistry Department, University of Wisconsin, Madison; personal communication). Sites for the restriction enzymes *Bam*HI (B), *Bgl*II (II), *Eco*RI (RI), *Hin*dIII (H), and *Sal*I (S) are indicated. The positions of the gene (*lacZ*) for β-galactosidase and the gene responsible for resistance to kanamycin (KAN) are shown. The *Hin*dIII site closest to *lacZ* was used to clone *Hin*dIII fragments. This site is 342 bp away from the site for *Bam*HI. *Bam*HI/*Bgl*II double digests were performed on pAP3, pAP4, and pAP5 (Fig. 1) to check that the correct *Hin*dIII site had been used.

respectively, clearly indicate transcription from P3. Although the size (about 420 bases) of the transcript produced by $BamHI_{3750}$ is somewhat shorter than the length (560 bases) expected for promotion by P3, it could not have been produced by transcription from any of the other promoter-like sequences. Its short size may be the result of early termination of transcription in vitro. This presumably does not happen in vivo since no terminator-like sequences (28) can be found in the nucleotide sequence preceding *uncB* (10, 16, 17, 33). A large amount of labeled material of high molecular weight was synthesized from the $BamHI_{3750}$ template. Although this could correspond to the 749- or 670-base transcripts expected for transcription from P1 or P2, this seems unlikely since no evidence for transcription from these sequences appeared when other templates were used. A more likely explanation is that some large transcripts are made from another region of the $BamHI_{3750}$ template which contains the entire coding region for *gidB* and most of the coding region for *gidA* (32).

In addition to these major transcripts, several minor transcripts were made in vitro (Fig. 4). All of these were made in small quantities compared to the major transcripts, and most were of a size that did not coincide with any of the sizes predicted in Table 2. The most notable exception was a transcript from the fragment $BamHI_{3750}$, whose size (about 300 bases) suggested that it might result from transcriptional initiation at P4. However, this transcript, like many other minor transcripts, was not made reproducibly and was therefore ignored. None of the minor transcripts provided evidence to suggest transcriptional initiation at any of the promoter-like sequences P1, P2, P4, or P5. Furthermore, no set of three transcripts (one for each template) could be found with the appropriate sizes to support the possibility of transcription from any particular site other than P3.

S1 nuclease mapping of in vivo mRNA. A fragment of DNA ($HaeII/HindIII_{471}$) carrying the sequences P1, P2, and P3 was protected from S1 nuclease by two different preparations of RNA, one of them enriched for *unc* mRNA (i.e., prepared from a strain carrying the plasmid pAP55). A portion of the same DNA was sequenced by the method of Maxam and Gilbert (24), and the protected DNA fragments were electrophoresed on the same gel as the five Maxam-Gilbert reactions (Fig. 5). The same major protected DNA fragment was observed for both preparations of RNA, indicating that the transcriptional start site for the *unc* operon of pAP55 is the same as that for the chromosomal *unc* operon. As expected, the amount of DNA protected from degradation by S1 nuclease was greater when the RNA was enriched for *unc* mRNA than when it was not. The position of the major protected fragment suggests that transcription begins approximately two residues upstream of the guanine residue at which transcription would start if P3 were active. This two-base discrepancy may be accounted for, at least in part, by the differing action of S1 nuclease and

TABLE 1. β-Galactosidase assays of strain CSH26S containing pRZ5255 or its derivatives

Plasmid	Cloned fragment of DNA	Promoter-like sequence(s) on fragment	β-Galactosidase activity (U)
pAP2	$BamHI_{3750}$	P1, P2, P3, P4, P5	710
pAP3	$HindIII_{1300}$	P1, P2, P3	340
pAP4	$HindIII_{199}$	P4	16
pAP6	$HindIII/BamHI_{240}$	P5	9
pRZ5255	None	None	3

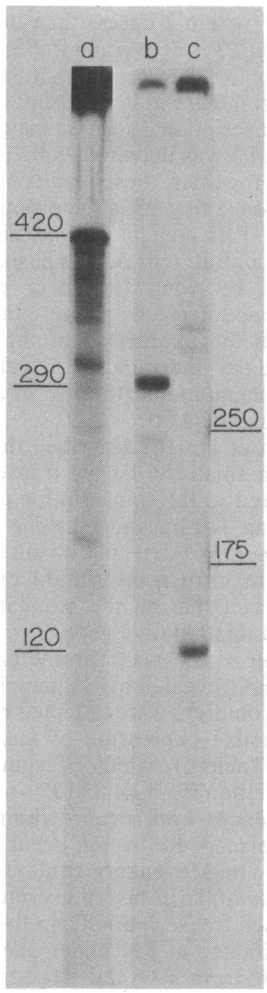

FIG. 4. Autoradiogram of ^{32}P-labeled RNA transcripts made in vitro and separated on a 5% acrylamide gel under denaturing conditions. The DNA fragments used as templates were *Bam*HI$_{3750}$ (a), *Hpa*II$_{793}$ (b), and *Hin*dIII$_{1300}$ (c). Three major transcripts of approximately 420, 290, and 120 bases were detected in lanes a, b, and c, respectively. Transcripts of known length (250 and 175 bases) were synthesized from a DNA template carrying the promoter for the *trp* operon and used as size markers.

the chemical reactions of Maxam and Gilbert. Thus, the 3'-terminal residue of a fragment generated chemically lacks a base but is phosphorylated, whereas that of a fragment treated with S1 nuclease has an intact base and a 3'-hydroxyl group (12, 15). Fragments generated by S1 nuclease therefore have a reduced electrophoretic mobility compared to corresponding fragments generated chemically. The comparison made in Fig. 2 between P3 and the "ideal" promoter sequence shows that the terminal guanine residue of P3 is a much more likely transcriptional start site than any of the few residues that precede it. We therefore conclude that P3 is active in vivo and results in transcriptional initiation at the predicted purine residue (Fig. 2) 73 bp upstream of *uncI*.

No other protected fragments of DNA larger than the major one were detected, indicating that neither P1 nor P2 is active in vivo. However, a minor protected fragment, 50 bases smaller than the major one, was detected reproducibly (not shown). Again, this fragment was protected better by RNA enriched for *unc* mRNA than by unenriched RNA. It therefore appears that a small amount of transcriptional initiation occurs at a guanine residue 25 bases upstream of *uncI*. Although none of the promoter-like sequences shown in Fig. 2 is located in this region, the guanine residue is preceded by −10 and −35 regions that contain some homology with the ideal sequence of Fig. 2 (not shown). By measuring the relative intensities on an autoradiogram of the major and minor protected fragments of DNA, the amount of transcription from the weak promoter was estimated to be 1/16 of that from P3.

DISCUSSION

Of the eight genes in the *unc* operon known to code for subunits of the proton-translocating ATPase, *uncB* is the first that is transcribed. The nucleotide sequence of 976 bp of DNA preceding *uncB* is known (10, 16, 17, 33) and includes the reading frame for *uncI* and 575 bp that precede it. Unless a long "leader sequence" (34) precedes the first gene of the operon, these 976 bp must include the promoter for the *unc* operon, assuming that either *uncB* or *uncI* is the first gene of the operon. The nucleotide sequence contains at least five promoter-like sequences, P1 through P5 (Fig. 2). Three techniques were used to establish which, if any, of these is the true *unc* promoter. The first, cloning into the

TABLE 2. Comparison of major transcripts made in vitro with those predicted for "runoff" transcription (Fig. 2)[a]

Fragment of DNA transcribed (Fig. 1)	Predicted size of transcripts (bases) if active promoter is:					Approx size of major transcript observed (Fig. 4)
	P1	P2	P3	P4	P5	
*Bam*HI$_{3750}$	749	670	560	300	119	420
*Hin*dIII$_{1300}$	310	231	121			120
*Hpa*II$_{793}$	480	401	291	31		290

[a] Sizes were predicted by using published DNA sequence data to determine the distances between the promoter-like sequences and the ends of the fragments to which these sequences are directed.

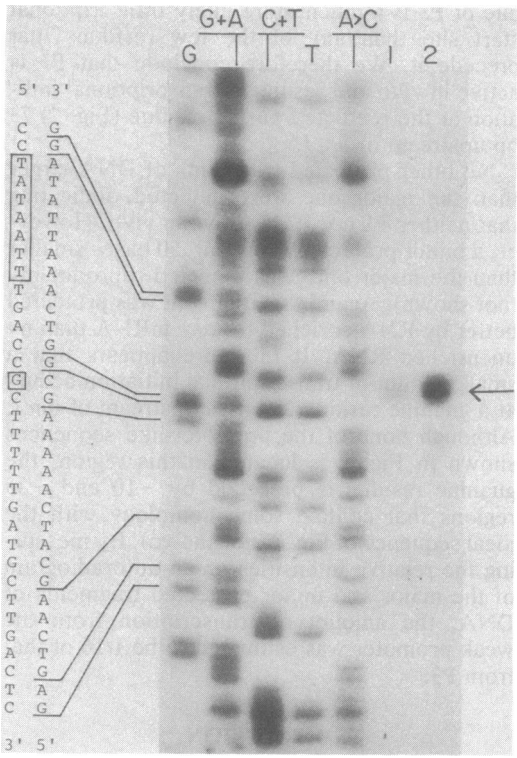

FIG. 5. S1 nuclease mapping of in vivo mRNA. A fragment of DNA (*Hae*II/*Hin*dIII$_{471}$) carrying the sequences P1, P2, and P3 was labeled at its 5′ end and divided into seven portions. Five portions were subjected to the partial chemical cleavage reactions of Maxam and Gilbert (24), whereas the remaining portions were hybridized to in vivo mRNA and treated with S1 nuclease. The five sequencing reactions (G, G+A, C+T, C and A>C) and the DNA protected with RNA from digestion by S1 nuclease (lanes 1 and 2) were analyzed on the same sequencing gel; an autoradiogram of this gel is shown. Two preparations of RNA were used, one (lane 1) from strain LE392 and the other (lane 2) from the same strain containing the plasmid pAP55. The pattern of DNA bands in the region in which the protected fragments migrated (arrow) is aligned with the published nucleotide sequence. Boxes surround the terminal purine residue and −10 region of P3.

promoter detection plasmid pRZ5255, showed that P4 and P5 were unlikely to be the *unc* promoter since fragments that carried these sequences caused only very weak expression of *lacZ* compared to the expression caused by a fragment of DNA which carried P1, P2, and P3. The second technique, in vivo transcription, indicated that P3 is an active promoter. No set of transcripts was found to indicate transcription from a particular site other than P3. Finally, S1 nuclease mapping showed, with a greater degree of precision (within 2 bases) than in vitro transcription, that P3 is active and that it is active in vivo. S1 mapping also showed that P1 and P2 are not active promoters in vivo, although the presence of a transcriptional start size about 16 times weaker than P3 was detected 25 bp upstream of *uncI*. Taken together, these results provide convincing evidence that P3 is the promoter for the *unc* operon. This conclusion was also reached by Kanazawa et al. (16) on the basis of in vitro experiments, by von Meyenberg et al. (32), whose evidence came from the effect of Tn*10* insertions on the expression of the ATPase c subunit, and by independent experiments involving S1 mapping and the use of a promoter detection plasmid (15a).

Kanazawa et al. (16) described the synthesis in vitro of a small (86 bases) transcript which they attributed to the transcription of a reading frame starting 340 bp and finishing 426 bp upstream of *uncI*. We detected no such transcript during our in vitro transcription experiments, despite the fact that all the templates that we used carried this putative gene.

In addition to the weak promoter 25 bp upstream of *uncI* revealed by S1 mapping, at least two weak promoters were detected by the small but reproducible expression of *lacZ* by pAP4 and pAP6 (Table 1), which presumably represent transcription from P4 and P5, respectively. These sequences both occur within *uncI* and probably correspond to the weak promoters reported by von Meyenberg et al. (32) based on their analysis of Tn*10* insertions into this gene. The combined transcription from these promoters was estimated at just under one-half of the transcription from P3 (32). The assays of β-galactosidase activity produced by derivatives of pRZ5255 can only be taken as a semiquantitative estimate of promoter activity on the cloned fragment of DNA. This is because variables such as the site into which the fragment is cloned and the size of the cloned fragment probably affect the amount of *lacZ* expressed by a given promoter. Nevertheless, a comparison of *lacZ* expression by pAP3, pAP4, and pAP5 (Table 1) suggests that the combined strength of P4 and P5 is 15 to 20 times smaller than the strength of P3, assuming that P3 is responsible for most of the expression of *lacZ* by pAP3. Furthermore, this is likely to be an overestimate since it has been shown that strong upstream promoters inhibit the transcription from weaker downstream promoters (1). On this basis, one would expect transcription from P3 to suppress transcription from P4 and P5. It is therefore difficult to account for the relatively large amount of transcriptional initiation from promoters within *uncI*, as reported by von Meyenberg et al. (32), unless some mechanism exists in vivo for reduc-

ing the amount of transcription from P3.

It is interesting to note that pAP2 produces approximately twice as much β-galactosidase activity as pAP3, despite the fact that P3 in the former plasmid is some 780 bp further away from the *lacZ* gene than it is in the latter. At first sight, this might suggest that P3 can act in concert with P4 and/or P5 to cause a greater amount of transcriptional initiation than could be caused by any one of these sequences alone. However, since transcription from P4 and P5 is probably repressed by transcription from P3 (see above), this effect is more likely to be the result of promoter activity upstream of P1 carried by $BamHI_{3750}$ but not by $HindIII_{1300}$.

Finally, one can speculate on the strength of the proposed *unc* promoter, P3. The derepressed promoter for the *lac* operon of *E. coli* produces 810 and 460 U of β-galactosidase activity when cloned into pRZ5255 at the *SalI* and *BamHI* sites, respectively (L. Munson and W. Reznikoff, personal communication). It therefore seems that the *unc* and *lac* promoters are of similar strengths.

As well as determining the position of the *unc* promoter, this work shows that *uncI* is transcribed as part of the *unc* operon. This conclusion is supported by the observation (15a) that a large transcript made in vivo hybridizes not only to DNA from the promoter-distal end of the *unc* operon, but also to DNA specifically from *uncI*. The *unc* operon therefore has a ninth gene, and, since the other eight genes of the operon have been designated *uncA* through *uncH*, we have named it *uncI*. We do not know the function of *uncI* but have shown in the accompanying paper (5) that it is expressed, producing a hydrophobic protein of the expected molecular weight.

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