Molecular analysis of the operon which encodes the RNA polymerase sigma factor σ^{54} of *Escherichia coli*

David H. A. Jones,[†] F. Christopher H. Franklin and
Christopher M. Thomas

Author for correspondence: Christopher M. Thomas. Tel: +44 21 414 5903. Fax: +44 21 414 5925.

School of Biological
Sciences, University of
Birmingham, PO Box 363,
Birmingham B15 2TT, UK

The *rpoN* gene (encoding the sigma factor σ^{54}) of *Escherichia coli* was cloned and its nucleotide sequence determined. Promoter probe analysis confirmed the presence of a promoter in a 350 bp fragment covering the start of *rpoN*. The likely promoter was identified. The nucleotide sequence of the region extending 2.1 kb downstream of *rpoN* was also determined. This region contained four open reading frames encoding potential polypeptides of 10750, 17959, 32492 and 9810 Da; maxicell and T7 promoter studies showed that four polypeptides of similar molecular masses were expressed from this region. The amino acid sequence of the 17959 Da polypeptide showed homology to the enzyme IIA domains of several proteins of the bacterial sugar phosphotransferase system (PTS), and the 9810 Da polypeptide showed homology to the HPr proteins of the bacterial PTS. The proteins encoded downstream of *rpoN* are known to negatively regulate σ^{54} activity. The homologies therefore suggest that this effect on σ^{54} may be mediated by sequential protein phosphorylation and suggest that there is a link between signal transduction and transcription of σ^{54} -dependent genes.

Keywords: *rpoN*, sigma factor, sugar phosphotransferase system, signal transduction, *Escherichia coli*

INTRODUCTION

The *Escherichia coli* *rpoN* gene encodes the novel RNA polymerase sigma factor σ^{54} (NtrA, GlnF). This sigma factor was first identified in enteric bacteria as being responsible for the transcription of nitrogen-regulated genes such as glutamine synthetase and the nitrogen fixation genes (Hirschman *et al.*, 1985; Hunt & Magasanik, 1985; de Bruijn & Ausubel, 1983). It has since been shown that a diverse range of genes are transcribed by core RNA polymerase associated with σ^{54} (reviewed in Kustu *et al.*, 1989). These include the *fdbF* gene of *E. coli* (Birkman *et al.*, 1987), the product of which is responsible for the anaerobic degradation of formate, the *xylABC* genes of *Pseudomonas putida* (Dixon, 1986) and the pilin gene of *P. aeruginosa* (Ishimoto & Lory, 1989). Most σ^{54} -dependent promoters so far described contain a conserved

motif of GG–10 bp–GC, and require an activator protein for transcriptional activity.

The *rpoN* gene has been cloned and sequenced from a number of bacteria including *Klebsiella pneumoniae* (Merrick & Gibbins, 1985), *Azotobacter vinelandii* (Merrick *et al.*, 1987), *Rhizobium meliloti* (Ronson *et al.*, 1987; van Slooten *et al.*, 1990), *Rhodobacter capsulatus* (Jones & Haselkorn, 1989), *Pseudomonas putida* (Inouye *et al.*, 1989; Kohler *et al.*, 1989), *Thiobacillus ferrooxidans* (Berger *et al.*, 1990), *Escherichia coli* (Sasse-Dwight & Gralla, 1990), *Bradyrhizobium japonicum* (Kullik *et al.*, 1991), *Salmonella typhimurium* (Popham *et al.*, 1991), *Alcaligenes eutrophus* (Warrelmann *et al.*, 1992), *Caulobacter crescentus* (Brun & Shapiro, 1992) and *Rhodobacter sphaeroides* (Meijer & Tabita, 1992). A homologue has also been cloned from *Bacillus subtilis* (Debarbouille *et al.*, 1991). All show a high degree of homology. The *E. coli* *rpoN* has been shown to lie between 3411 and 3413 kb on the *E. coli* physical map (Merrick *et al.*, 1993). In most cases the nucleotide sequences of the regions downstream of the *rpoN* ORF are also highly conserved between bacteria. Sequence data are most extensive for *K. pneumoniae* (Merrick & Coppard,

[†] **Present address:** Laboratory of Protein Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

Abbreviation: PTS, phosphotransferase system.

The GenBank accession number for the nucleotide sequence reported in this paper is Z27094.

putative promoter

10 -35 box 30 ORF stop -10 box
 GAACACGTTAAGCGTGTATACCTTGGGAAAGACTTCAGACTCTGATAGGGTGAAGTTTG
 E H V K R V Y L G E D F R L *

70 rbs 90 rpoN start 110
 CGACGTTTATGAGGAGTACGATTGTAACATGAAGCAAGGTTTGCACCTCAGGCTTA
 M K Q G L Q L R L S

130 150 170 Sal/HincII
 GCCAACACTGGCGATGACGCCACGCTCCACAGGCAATTCGCTGTTGCGATTGTCGA
 Q Q L A M T P Q L A L E S N P L L E Q I

190 210 230
 CGCTGGAACTTCAGCAGAGCTACAGCGGCGTGGAGAGTAATCCGCTGCTTGAGCAAA
 L E L Q Q E L Q Q A L E S N P L L E Q I

250 270 290
 TCGACACTCATGAAGAATCGACACCGGAAACGCAAGACAGTGAACCGTGGACCG
 D T H E E I D T R E T Q D S E T L D T A

310 330 350
 CGACGCGCTCGAACAAGAGATCGCGAAGAGCTCGCGCTCGATGCCATTGGGACA
 D A L E Q K E M P E E L P L D A S W D T

370 390 410
 CCATTACACGCTGGTACACCATCGGCAACCGGCTGACTACATGACGACGAGCTGC
 I Y T A G T P S G T S G D Y I D D E L P

430 450 470
 CGGTCTACAGGCGGAAACGACGACGCTGACGAGTATCTGATGTCGAGGTTGAGC
 V Y Q G E T T Q T L Q D Y L M W Q V E L

490 510 530
 TGACACGTTTTCCGACACTGACCGCTATTGCTACCTCTATCGTGCATGCCGTTGATG
 T F F S D T D R A I A T S I V D A V D E

550 570 590
 AAACGCGTTATCTGACTGTCCCGCTGAAGATATCTCGAAAGTATAGCGGATGAAGAGA
 T G Y L T V P L E D I L E S I G D E E I

610 630 BamHI 650
 TCGACATCGCAAGATGGAAGCGCTCTTAAGCGGATCGACGCTGATCGGCTCGGTG
 D I D E V E A V L K R I Q R F D P V G V

670 690 710
 TGGCGGCAAAAGATCTGCTGACTGTCTGCTGATCCAACTCTCCCAATTCGATAAGACCA
 A A K D L R D C L L I Q L S Q F D K T T

730 750 770
 CGCGCTGGCTGGAAGGCGCAGCTGATCATTAGCGATCATCTCGATCTGTTAGCCAATC
 P W L E E A R L I I S D H L D L L A N H

790 810 830
 AGCACTTCGCGCATTTAATGCGCGTCAACGCTGTGAAAGAGATGTGCTGAAGAAAGCGC
 D F R T L M R V T R L K E D V L K E A V

850 870 890
 TCAATCTGATCGCTGCTGATCGCGCGCGCGGCGAGTGCATCGACATGGCGAAGCTG
 N L I Q S L D P R P G Q S I Q T G E P E

910 930 950
 AGTATGTCATTCCAGATGTGCTGGTGAAGCATAACGGTCACTGGACGGTGAAGATCA
 Y V I P D V L V R K H N G H W T V E L N

970 990 1010
 ACAGTGCAGCACTCCGCGTCTGCAATCAACGACCTACGCTGATGTGCAATTAAGC
 S D S I P R L Q I N Q H Y A S M C N N A

1030 1050 PstI 1070
 CGCGCAACAGTGTGACAGCGATTTATCCGACGATTCGAGGATGCCAATGGTGA
 R N D G D S Q F I R S N L Q D A K W L I

1090 1110 1130
 TCAAGAGTCTGGAAGCGCTAACGATACGCTACTCGCGTGAAGTGTATCGTTGAAC
 K S L E S R N D T L L R V S R C I V E Q

1150 1170 1190 EcoRV
 AGCAGCAAGCTCTTTGAGCAAGGTGAAGATATATGAACCGATGGTACTGGCGGATA
 Q Q A F F E Q G E E Y N K P M V L A D I

1210 1230 EcoRV 1250
 TCGCCAGGCTGTGAAATGCATGAATCGATATCTCGCGTGAAGTGTGCAAAATACC
 A Q A V E H M E S T I S R V T T Q K Y L

1270 1290 1310
 TGCATAGTCCACAGGCAATTTTGAACGTAAGTATTTCTTCCAGTCACTCAATACCG
 H S P R G I F E L K Y F F S S H V N T E

1330 1350 1370
 AGGCGCGCGCGGAAAGCTTCTCCCAAGCGGATTCGCGCTGGTGAAGAAATTAATCGCG
 G G G E A S S T A I R A L V K K L I A A

1390 1410 1430 HincII
 CGGAAACCCAGGAAACCGTTGAGCGACAGCAAGTTAACTCTTTCGCTGCGGAACAG
 E N P A K P L S D S K L T S L S E Q G

1450 1470 1490
 GTATCATGCTGGCAGCGCAGCTGTTCGGAAGTACCGAGAGCTTTATCCATTCCGCGCT
 I M V A R R T V A K Y R E S L S I P P S

1510 rpoN stop 1530 rbs ORF1 start
 CAAACCGGTAAACAACCTGTTGACCCCAACCGATTAAGGAAGACACTATGCAGCTCAAC
 N Q R K Q L V * M Q L N

1570 1590 1610
 ATTACCGGAATAACGTCAGATCACCGAGGCACTGCGGCAATTTGTTACAGCAAAATTT
 I T G N N V E I T E A L R E F V T A K F

1630 1650 1670
 GCCAACTTGAGCAATATTTGACCGAATCAACAGGCTCTATGTTGCTGAAAGTGGAG
 A K L E Q Y F D R I N Q V Y V L K V E

1690 1710 1730
 AAAGTCAACCACTCAGATCAACACTGCAATGAACGGTGGTGAATTCATGCCAGC
 K V T H T S D A T L H V N G G E I H A S

1750 1770 1790
 CGGGAAGGTGAGGACATGACGCTGCAATGATGGCTTAATGATAAGCTGGCAGCGGAC
 A E G Q D M Y A A I D G L I D K L A R Q

1810 1830 ORF1 stop 1850
 TTGACCAACATAAAGATAAATGAAACCAACTAATGTCGCGGCAATTAGCATGTGCA
 L T K H K D K L K Q H * hair-pin

1870 1890 1910
 TGGCGGTCTGTTGTGCGGCACACGCGGCATTGTACGTTAATGCTCCGAGCCTGTTCC

1930 rbs 1950 ORF11 start 1970
 ACTGTTTGGATGGGCAAGTTCTTACGTGAATAATGATACAAATATGATCAACTCTACAG
 M T N N D T T L Q

1990 2010 2030
 CTAGCAGTGTCTTAACAGGAAATGATCGGAAGCGCGCTCAGCTGTCAAGCAAAAAA
 L S S V L N R E C T R S R V H C Q S K K

2050 2070 2090
 CGCGCCCTGGAAATCATCAGCGAGCTGGCGGCAAACTTAGCCTGCGGCTCAGGTTG
 R A L E I I S E L A A K Q L S L P P Q V

2110 2130 2150
 GTTTTGAAGCTATCTGACCGGGGAAAAATGGCAGTACCGGTATCGCAATGGTATT
 V F E A I L T R E K M G S T G I G N G I

2170 2190 2210
 GCCATCTCCCATGGCAAACTGGGAAGATACTTGGCGGCGCTTGGGCTTTTCGTTTCAG
 A I P H G K L E E D T L R A V G V F V Q

2230 2250 2270
 CTCGAACCGCTATAGCTTTCGATGCCATGACAAACCGGTGGATCTACTTTTGGCC
 L E T P I A F D A I D N Q P V D L S F A K

2290 2310 2330
 CTGCTGGTGGCGAGCAAACTAAACCGCACTGCATCTCTGCTGGTGGCGGAA
 L L V P A D Q T K T H L H T C T G L S L V A K

2350 2370 2390
 CGTCTGGCGGCAAAACCATCTGCGCGCTTACCGCGAGCCAGCGATGAAGAGCTG
 R L A D K T I C R R L R A A Q S D E E L

2410 2430 ORF11 stop 2450
 TATCAATCATTCAGATACCGAAGCTACTCCGATGAAGCGTATGTTTCGTTAATGTC
 Y Q I I T D E T G T P D E A *

2470 rbs ORF11 start 2510
 TCTTTTAGACGTTGTGAGGAAACAGTACATCTGATGATCGTCAAGCGGACGTTCA
 M V L M I V S G R S

2530 2550 2570
 GGTTCAGTAAATCTGTCGCGCTGCGTGGAGATATGGGTTTTTACTCGCTGGAT
 G S G K S V A L R A L E D M G F Y C V D

2590 2610 2630
 AACCTTCCGCTAGTGTGTTACCGGATCTGGCTGCAACCTGGCGATCGGAGATTCTT
 N L P V V L L P D L A R T L A D R E I S

2650 2670 2690
 GCGCGCTGAGCATGTGTCGTAATATGCCGAGTACCAGAAATATTCGAAAGCGG
 A A V S I D V R N M P S E I F E Q F A

2710 2730 2750
 ATGAGTAACCTGCGCTGACGCTTTCTACCGCACTCTCTGCTGGATGCGGACCGTAAT
 H S N L P D A F S P Q L F L D A D R N

2770 2790 2810
 ACCTTAATCTGCTGTTACAGTGACACGCGCGATGATCGCTTCCAGCAAAACCTG
 T L I R R Y S D T R R L H P L S S K N L

2830 2850 2870
 TCGCTGGAAGTGTCTATCGCAAAAGAGCGATTGCTGGAGCTTGGCTGGCGAGCG
 S L E S A I D K E S D L L E P L R S R A

2890 2910 2930
 GATCTGATGTGATACCTCAGAAATGTCGCTTACGAGCTGGCTGAATGCTGCGTACC
 D L I V D T S E M S V H E L A E M L R T

2950 2970 2990
 CGTCTGCTGGTAAAGCTGAACGCACTGACCATCTGTTGAGCTTTTCGCGTTCAAA
 R L L G K R E R E L T M V F S E F G F K

3010 3030 3050
 CACGCTATCCCTATCGATGAGATACGTTTACGCTGCGCTTCTTGGCAACCGCAC
 H G I P T D A D Y V F D V R F L P N P H

3070 3090 3110 BamHI
 TGGATTCGAAACCTGCTCAATGACAGCTCTGATAAATCTGCGGCGCTTCTCGAC
 W D P K L R P M T G L D K F V A A F L D

3130 3150 3170
 CGCCACAGAAATACAAATTTATCTACAGACGCGAAGCTATCTTGAGCTATGGTTA
 R H T E V H N F I Y Q T R S Y L E L W L

3190 3210 3230
 CCTATGCTGGAACCAACCGTAGCTACTGACGTGGCATGCTGTTGTTACCGCGG
 P M L E T N N R S Y L T V A I G C T G G

3250 3270 3290
 AAGCACGCTGGTGTATATTGACAGACACTGGCAGCTACTTCCGCTGCGGCTGAAA
 K H R S V Y I A E Q L A D Y F R S R G K

3310 3330 rbs ORF11 start/ORF11 stop
 AACGTCAGTCAAGCATCTGATCGCTGGAAGAAACGTAACACGCTCAAGCAAACTG
 N V Q S R H R T L E K R K P M * T V K T V

3370 3390 3410
 TTGAAATCAAAACAGCTGGGCGATGATGCCGCGCTGCAATGAAGCTGTTGAATTA
 E I T N K L G H H A R P A M K L F E L M

3430 3450 3470
 TGCAGGGTTTTCAGCTGAAGTCTTACGTAATGACGAAGGACCGAGGCTGAAGCCA
 Q G F D A E V L L R N D E G T E A E A N

3490 3510 3530
 ACAGCGTTATGCGCTGATGATGGAATCTGCAAAAGGACCGGCAATGAAGTTGAAG
 S V I A L L M L D S A K G R Q I E V E A

3550 3570 3590
 CGACCGTCCACGAGGAGGAGGACGCTGGCGCGCTTATCGGCTCTTTAATCTGGT
 T G P Q E E E A L A V I A L F N S G G F

ORF11 stop 3630 3650
 TTGATGAAGATTAACTTCATCACTTTCATCACTACCTCTTCAAACTCCCCCGACAA
 D E D * potential rho-dependent transcriptional terminator

TAAGA

Fig. 1. Nucleotide sequence and amino acid translations of the *rpoN* operon. Potential ribosome-binding sites (rbs) are indicated, as are restriction sites which were used for subcloning parts of the operon.

1989) and *E. coli* (Imaishi *et al.*, 1993). Studies in *K. pneumoniae* indicated that there are at least three ORFs cotranscribed with *rpoN*, and that they negatively regulate the activity of σ^{54} (Merrick & Coppard, 1989). Sequence similarities led to the suggestion that these downstream genes may provide a means of sensing nutritional state and regulating σ^{54} activity by phosphorylation (Reizer *et al.*, 1992; Imaishi *et al.*, 1993).

In this report we describe the cloning and sequencing of what appears to be the complete *E. coli rpoN* operon. The sequence of the downstream ORFs which have not been previously analysed strengthens current ideas about how the downstream-encoded polypeptides may regulate σ^{54} .

METHODS

Bacterial strains and plasmids. *E. coli* strain JM83 (Yanisch-Perron *et al.*, 1985) was used for all cloning and manipulations. *E. coli* strain Q359 (Karn *et al.*, 1980) was used to generate the genomic library, and bacteriophage λ EMBL3 (Frischauf *et al.*, 1983) was used as the library vector. *E. coli* strains CSR603 (Sancar *et al.*, 1978) and JM109 (Yanisch-Perron *et al.*, 1985) were used for maxicell expression and T7 promoter expression respectively. *E. coli* C600K⁻ (McKenny *et al.*, 1981) was used for promoter probe studies. Cultures were grown at 37 °C in L-broth or M9 (Kahn *et al.*, 1979) media, and penicillin was added to 100 µg ml⁻¹ (liquid media) or 300 µg ml⁻¹ (solid media) as required.

Plasmid pUC18 (Yanisch-Perron *et al.*, 1985) was used as the standard cloning vector. Plasmids pKO1 and pKO6 (McKenny *et al.*, 1981) were used as promoter probe vectors. Plasmids pGBT30 (*tacp*; Jagura-Burdzy *et al.*, 1991) and pGEM3Z (T7p; Promega) were used as expression vectors. Plasmid pMM17 (Merrick & Stewart, 1985), which contains the *K. pneumoniae rpoN*, was kindly given by Dr M. Merrick, University of Sussex, UK. Plasmids constructed during the course of this work are described in the appropriate figure.

DNA manipulations. Plasmid DNA was isolated by the alkaline-SDS method of Birnboim & Doly (1979), and whole-cell DNA was prepared according to Frey *et al.* (1983). Bacteriophage DNA was prepared as described in Sambrook *et al.* (1989). Enzymes were used according to the manufacturer's instructions and DNA was purified using Gene-clean (Bio101). Other techniques were performed as described in Sambrook *et al.* (1989).

DNA sequence analysis. DNA sequencing by the chain-termination method (Sanger *et al.*, 1977) was carried out on double-stranded plasmid DNA template using the Sequenase kit (United States Biochemical). Template DNA was purified by CsCl/ethidium bromide density-gradient centrifugation. Primers were either sequencing or reverse sequencing primers from USB, or 16mer oligonucleotides synthesized by Alta Bioscience. Nucleotide and amino acid sequences were analysed using the uwgCG programs (Devereux *et al.*, 1984).

Construction of the gene library and screening for *rpoN*. Total DNA of *E. coli* Q359 was partially digested with *Sau*3A and size-fractionated on a sucrose gradient (Frey *et al.*, 1983). DNA fragments of approximately 15–22 kb were collected and ligated into the *Bam*HI site of bacteriophage λ EMBL3. The resulting plaques were screened with the 1.8 kb *Cla*I fragment of pMM17 (the *K. pneumoniae rpoN* gene) which had been labelled with [α -³²P]dATP (Sambrook *et al.*, 1989). Southern hybridization of DNA fragments separated by gel electrophoresis was performed as described in Sambrook *et al.* (1989).

Maxicell analysis of polypeptides. *E. coli* strain CSR603 harbouring the required plasmid was treated essentially as described by Shingler & Thomas (1984). Labelled polypeptides were analysed by SDS-PAGE (Laemmli, 1970) followed by fluorography.

T7 promoter analysis. An overnight culture of *E. coli* JM109 containing the test plasmid was diluted 1 in 20 and grown to OD₆₅₀ 0.7 in M9 medium containing 0.4% glucose and 1 mM thiamin.HCl. Duplicate samples were taken; IPTG was added to one set to 0.5 mM and these were incubated for a further 1 h. Rifampicin was then added to 200 µg ml⁻¹ (prepared immediately before use in ethanol) and the culture was shaken at 37 °C for 80 min. Then 75 ml [¹⁴C]protein hydrolysate (Amersham cat. no. CFB25) was added to each 1 ml sample, and this was left at 37 °C for 10 min. Cells were then harvested and a sample of each analysed by SDS-PAGE (Laemmli, 1970) followed by fluorography.

Assay for galactokinase. Assays were performed as described by McKenny *et al.* (1981).

RESULTS

Cloning and nucleotide sequence determination of *rpoN* and flanking regions

The gene library constructed in λ EMBL3 was screened by plaque hybridization using the 1.8 kb *Cla*I fragment of pMM17 (which contains the *rpoN* homologue of *K. pneumoniae*; Merrick & Stewart, 1985). From approximately 5000 plaques, eight hybridization-positive plaques were identified and bacteriophage DNA was isolated from these. Restriction mapping showed three related classes of clone. One clone was digested with a variety of restriction enzymes. Southern blotting showed that none gave a single hybridization-positive band, but digestion with *Bgl*II produced two positive bands, of 4 and 6 kb. These fragments were cloned into the *Bam*HI site of pUC18 to give pRPO4 and pRPO6 respectively. In this way large flanking regions of DNA could be studied, and as the gene would not be intact, any toxic effect of having *rpoN* at a high copy number could be eliminated.

The nucleotide sequence of the cloned *rpoN* was determined on both strands (its location assigned by homology to *rpoN* genes from other bacterial species), and the sequence of upstream and downstream flanking regions was also determined. To ensure that the two *Bgl*II fragments were contiguous, the region around the *Bgl*II site of pMM18 (a plasmid containing the intact *rpoN* of *E. coli*, kindly provided by Dr M. Merrick) was also sequenced.

The nucleotide sequence is shown in Fig. 1. Downstream of *rpoN* there are four putative ORFs which are shown in Fig. 1 (designated ORFs II–V). Details of the proteins (designated by P followed by the number of constituent amino acids) are shown in Table 1. The protein products of ORFs II and III show high levels of homology to the equivalent proteins of other bacteria. P95 shows 87% identity to that of the *K. pneumoniae* homologue, dropping to 22% identity to the *R. meliloti* homologue. P163 also shows 87% homology to the *K. pneumoniae* ORFIII homologue.

The potential ribosome-binding sites are indicated in

Table 1. Features of predicted products of ORFs in the *rpoN* operon

	Molecular mass (Da) (SDS-PAGE)	Calculated molecular mass (Da)	Calculated pI
σ^{54} (<i>rpoN</i>)	—	53902	4.46
P95 (ORFII)	8000	10750	7.09
P163 (ORFIII)	19000	17959	5.71
P284 (ORFIV)	30000	32492	7.26
P90 (ORFV)	11000	9810	4.12

Plasmid in <i>E. coli</i> C600K ⁻	Galactokinase units
pKO1	0.1
pKO6	0.5
pRPO10	5.7
pRPO11	0.4

Fig. 2. Construction of plasmids for promoter probe analysis. The table shows the results of assaying galactokinase in *E. coli* C600K⁻ carrying the promoter probe plasmids.

Fig. 1, as is a potential σ^{70} -dependent promoter located upstream of *rpoN* (see next Results section). A hairpin structure is also indicated which overlaps the stop codon of ORFII and may act to stabilize the mRNA. Downstream of the last ORF is a cytosine-rich region followed by an AT-rich region (bases 3635–3663). This may be indicative of a rho-dependent transcriptional terminator (Alifano *et al.*, 1991). Polypeptide analysis described below indicates that ORFV is the last ORF in this operon.

The sequence of *rpoN* reported here differs from that reported by Sasse-Dwight & Gralla (1990). Our sequence has an additional base at position 386 (in Fig. 1) and one less at position 402. This has the effect of producing a frame-shift of five amino acids in the polypeptide. Fig. 1

Fig. 3. Construction of plasmids to place ORFs II–V under the control of *tacp* and T7p to allow visualization of polypeptides in maxicells or in whole cells in the presence of rifampicin.

also shows the end of a potential reading frame upstream of *rpoN*. Preliminary data (sequence from one strand only) indicates that this upstream ORF extends at least 550 bp upstream of the indicated stop codon, consistent with analysis reported by Imaishi *et al.* (1993).

Promoter activity upstream of *rpoN*

The start codon for *rpoN* is contained within a 350 bp *HincII* restriction fragment such that 250 bp of DNA upstream of *rpoN* are present in this fragment. This fragment was cloned into the probe vectors pKO1 and pKO6 (McKenny *et al.*, 1981) as shown in Fig. 2, allowing promoter activity in both directions to be determined. The assay results (Fig. 2) provide evidence for a promoter upstream of *rpoN*.

A potential $E\sigma^{54}$ promoter is indicated in Fig. 1. The sequence of the –35 and –10 boxes and the intervening sequence is absolutely conserved between *E. coli* and *K. pneumoniae* and *S. typhimurium*. Outside this region the homology falls between species. It would therefore seem likely that this is the *rpoN* promoter.

Polypeptides encoded by the *rpoN* gene

To test whether the ORFs downstream of *rpoN* are cotranscribed with *rpoN* and actually encode proteins, this region was subjected to maxicell and T7 promoter analysis.

Fig. 4. Autofluorograph showing the result of maxicell analysis of pRPO200. +/– indicates induction with IPTG. Polypeptides of 8, 11, 19 and 30 kDa (arrows) are present in the induced pRPO200 sample, but not in the uninduced sample or the negative control.

Plasmid pRPO4 (which contains 0.8 kb of the *rpoN* ORF and 3.4 kb of downstream flanking DNA) was digested with *EcoRI* and *HindIII* as shown in Fig. 3. The 3.3 kb fragment was then treated with DNA polymerase to fill in the single-stranded ends and inserted into pGBT30 (Jagura-Burdzy *et al.*, 1991) or pGEM3Z (Promega) which had been digested with *SmaI*; the resulting plasmids (pRPO200 and pRPO100 respectively) were then transformed into the requisite strains. Plasmid pRPO200 was then subjected to maxicell analysis and pRPO100 to T7 promoter analysis. Both plasmids showed the same four polypeptide products of 8, 11, 19 and 30 kDa (Figs 4 and 5). A deletion was also made in pRPO100 as shown in Fig. 3. This removes the last 1.55 kb of inserted DNA such that ORFII and ORFIII remain intact and 0.5 kb of ORFIV remains intact. The result of analysis of this plasmid, pRPO101, is shown in Fig. 5. While the bands of 8 and 19 kDa remain the same, the 30 kDa protein is reduced in size to 24 kDa, and the 11 kDa protein disappears. ORFII and ORFIII encode predicted proteins

of 10750 and 17959 Da respectively, which are presumably the proteins of 8 and 19 kDa observed experimentally. The polypeptide of 30 kDa which becomes truncated to 24 kDa in pRPO101 is that encoded by ORFIV (encoding a polypeptide of 32492 Da), as this ORF is truncated to 0.5 kb in pRPO101, and would terminate in vector sequence. Finally the last ORF (encoding a protein of 9810 Da) must produce the polypeptide of 11 kDa, as this is lost in the deleted plasmid. It is of note that while ORFII produces a larger polypeptide than ORFV, upon electrophoresis it demonstrates a greater mobility. This is accounted for in that the product of ORFV is much more acidic (see isoelectric points in Table 1), so would bind less SDS and run more slowly on SDS-PAGE.

The polypeptide data indicate that the ORFs observed in the nucleotide sequence are cotranscribed with *rpoN*. In addition, no other polypeptide products were seen on any analysis even though pRPO100 and pRPO200 contain ample coding capacity for further ORFs. This would therefore indicate that ORFV is the last ORF of the *rpoN* operon, consistent with our identification of a likely rho-dependent terminator after ORFV (see above).

DISCUSSION

The work reported in this paper places a promoter to within 260 bp of the *rpoN* start codon and a probable promoter has been identified which is conserved in the *K. pneumoniae rpoN* gene (Merrick & Stewart, 1985). This is in agreement with work on the promoter of the *R. meliloti rpoN*, for which the 5' end of the mRNA was mapped to about 70 bp upstream of the start codon (Albright *et al.*, 1989). Therefore *rpoN* could be the first gene in the operon. However, previous studies have also demonstrated the presence of a conserved ORF of unknown function upstream of *rpoN* in several bacterial species, but with intervening lengths of DNA ranging from 12 bp in *T. ferrooxidans* (Berger *et al.*, 1990) to 180 bp in *R. meliloti* and *P. putida* (Albright *et al.*, 1989; Inouye *et al.*, 1989). Thus in *R. meliloti* the promoter lies in the region between the two genes but in *E. coli* the postulated promoter overlaps ORF0 and there is no transcriptional terminator to block transcription across ORF0 entering *rpoN*.

A potential ribosome-binding site for *rpoN* is shown in Fig. 1. While this sequence (AGGAGA) would appear to be a near-classical consensus motif, it is situated 14 bp from the start of *rpoN*. This may be a contributing factor in the low level of expression of *rpoN* (Castano & Bastarrachea, 1984).

The proposed *E. coli* σ^{54} is a protein of 53902 Da. This differs slightly from that reported by Sasse-Dwight & Gralla (1990) as a result of two frame-shift differences in the DNA sequences (see Results). Many motifs and homologies of σ^{54} proteins from other bacterial species have been described previously (Merrick *et al.*, 1987; Sasse-Dwight & Gralla, 1990) and will not be repeated here. P95 (the product of ORFII) shows 38% amino acid

Fig. 5. (a) Autofluorograph showing the result of polypeptide analysis using transcription from the T7p of pRPO100. +/– indicates induction of T7 RNA polymerase with IPTG. Polypeptides of 8, 11, 19 and 30 kDa (arrows) are present in the induced pRPO100 samples. (b) Comparison of polypeptides produced by pRPO100 (see a) with those produced by pRPO101 (the deletion derivative of pRPO100). Polypeptides of 8 and 19 kDa are still present in the pRPO101 sample, but the 11 kDa polypeptide is not present and the 30 kDa polypeptide is reduced in size to 24 kDa. These polypeptides are absent from the control pGEM3Z. Molecular mass markers correspond only to panel (a).

identity to the product of an ORF preceding *pheA* of *E. coli* (Hudson & Davidson, 1984); this similarity was previously reported for the *K. pneumoniae* homologue by Merrick & Coppard (1989).

The genes corresponding to ORFII and ORFIII downstream of *rpoN* are known to negatively regulate the transcription of σ^{54} -dependent genes (Merrick & Coppard, 1989). In view of possible polar effects and the possibility of interactions between products of other downstream genes we should not assume that only ORFII and ORFIII are involved in this regulation. The mode of action of this regulation is unknown. However, the polypeptides encoded by these genes show some interesting homologies which have led to the proposal that these genes sense nitrogen and carbon levels and regulate RpoN by phosphorylation (Reizer *et al.*, 1992). We find that not only the protein encoded by ORFIII (previously observed) but also that encoded by ORFV (not previously observed) show high levels of sequence similarity to domains of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) of bacteria. This system essentially comprises two general proteins, enzyme I and HPr, and the sugar-specific enzyme II complex (Saier & Reizer, 1992). A phosphoryl group is transferred from phosphoenolpyruvate to enzyme I, to HPr, and then to

the enzyme II complex. This complex is responsible for the uptake and concomitant phosphorylation of sugars. Enzyme II can consist of up to four distinct domains, though it commonly comprises a transmembrane domain (IIC) and two hydrophilic domains (IIA and IIB), each containing a sequentially-phosphorylated amino acid residue. Enzyme II may also have domains which serve as the general proteins enzyme I and HPr (Saier & Reizer, 1992).

P163 (the product of ORFIII) shows sequence similarity to the IIA domains of the fructose-specific enzyme II of *S. typhimurium* and *R. capsulatus* (Geerse *et al.*, 1989; Wu *et al.*, 1990), and the mannitol-specific enzyme II of *E. coli* and *Staphylococcus carnosus* (Lee & Saier, 1983; Fischer *et al.*, 1989). This observation has been previously reported by Reizer *et al.* (1992) and Imaishi *et al.* (1993).

We show here for the first time that P90 (the product of ORFV) shows similarity to the HPr proteins and HPr-like protein domains of the PTS. An alignment of all known HPr sequences with P90 is shown in Fig. 6. The percentage identities that P90 shows with members of this family range from 37% (*B. subtilis* HPr) to 25% (*E. coli* and *S. typhimurium* HPr) and 19% (*E. faecalis* HPr). The histidine indicated at position 16 in the P90 sequence is the

P90 1 MTKVQTVEITNKLGMHARPAMKLFELMQGFDAEVLRLN.D.EGTEAEA
 B. sub 1 MAQKTFK.VTADSGIHARPATVLVQTASKYDADVNLEY...NGKTVNL
 A. eut 1 MLQRDTT.IINKLGLHARASAKLTQLAGNFVSQVMSR...NGRQVDA
 R. cap 157 AQGIDVV.VTGAHGLHARPATTLVDLAKGFAAEIRIRN...GAKVANG
 S. t fru 285 VLSAEFV.VRNEHGLHARPATMLVNTIKQFNSEITVTNLDGTGKPAANG
 E. c fru 7 VLSAEFV.VRNEHGLHARPATMLVNTIKQFNSEITVTNLDGTGKPAANG
 S. aur 1 MEQNSYV.IIDETGHIHARPATMLVQTASKFDSIDQLEY...NGKKVNL
 S. car 1 MEQNSYV.IIDETGHIHARPATMLVQTASKFDSIDQLEY...NGKKVNL
 E. c 1 MFQOEVT.ITAPNGLHTRPAAQFVKEAKGFTSEITVTS...NGKSASA
 S. t 1 MFQOEVT.ITAPNGLHTRPAAQFVKEAKGFTSEITVTS...NGKSASA
 K. pne 1 MFQOEVT.ITAPNGLHTRPAAQFVKEAKGFTSEITVTS...NGKSASA
 E. fae 1 MEKKEFH.IVAETGHIHARPATLLVQTASKFNSDINLEY...KGSVNL
 S. sal 1 MASKDFH.IVAETGHIHARPATLLVQTASKFASDITLDY...KGAENVL

Consensus M.Q....I....GLHARPAT.LV.TA..F.S.I....LDGNGK.AN.

P90 47 NSVIALMLDLSAKGRQIEVEATGPOEEEAALAAVIALFNSGFDED
 B. sub 45 KSMGVMSLGIARGAETITISAGADENDALNALEETMKSE.GLGE
 A. eut 45 KSMGVMSLAAGIGSTVLTETDGPDEQAMDAALLALIANRFGGE
 R. cap 201 KSLISLNLGAQGAALRISAEAGDATAALAAIAAFAEA...GLEDE
 S. t fru 332 RSLMKVVALGVKKGHRLRFTAQGDADQALKAIGDAIAA...GLGEGA
 E. c fru 7 RSLMKVVALGVKKGHRLRFTAQGDADQALKAIGDAIAA...GLGEGA
 S. aur 45 KSMGVMSLGVGKDAEITTIYADGSDSDAIQAISDVLSKE.GLTE
 S. car 45 KSMGVMSLGVGKDAEITTIYADGSDSDAIQAISDVLSKE.GLTE
 E. c 45 KSLFKLTGLTGLTQGVTVTISAEGEDEQKAVEHLVKLMA.E..L.E
 S. t 45 KSLFKLTGLTGLTQGVTVTISAEGEDEQKAVEHLVKLMA.E..L.E
 K. pne 45 KSLFKLTGLTGLTQGVTVTISAEGEDEQKAVEHLVKLMA.E..L.E
 E. fae 45 KSMGVMSLGVGQGSDDVTITVDGADEAGMAAIVETLQKE.GLAEQ
 S. sal 45 KSMGVMSLGVGQGSDDVTITVDGADEAGMAAIVETLQKE.GLA

Consensus KS.M.V..LG...G...TI.A.G.DE..A..AI.....EFGL.E

Fig. 6. Alignment of known HPr domain protein sequences including *E. coli* P90. A consensus is shown and invariant residues are underlined. Abbreviations and references are as follows: *B. sub*, *Bacillus subtilis* (Gonzy-Treboul *et al.*, 1989); *A. eut*, *Alcaligenes eutrophus* (Pries *et al.*, 1991); *R. cap*, *Rhodobacter capsulatus* (Wu *et al.*, 1990); *S. t fru*, *Salmonella typhimurium* (fructose) (Geerse *et al.*, 1989); *E. c fru*, *Escherichia coli* (fructose) (Orchard & Kornberg, 1990); *S. aur*, *Staphylococcus aureus* (Reizer *et al.*, 1988); *S. car*, *Staphylococcus carnosus* (Eisermann *et al.*, 1991); *E. c*, *Escherichia coli* (De Reuse *et al.*, 1985); *S. t*, *Salmonella typhimurium* (Byrne *et al.*, 1988); *K. pne*, *Klebsiella pneumoniae* (Titgemeyer *et al.*, 1990); *E. fae*, *Enterococcus faecalis* (Deutscher *et al.*, 1986); *S. sal*, *Streptococcus salivarius* (Gagnon *et al.*, 1992; Reizer *et al.*, 1993).

residue which becomes phosphorylated in the PTS pathway. In Gram-positive organisms the serine residue at position 46 in the *B. subtilis* sequence in Fig. 6 can also be phosphorylated (reviewed in Reizer *et al.*, 1993); however, the HPrs from Gram-positive species show a high degree of similarity around this serine which is not shared by the HPrs of the Gram-negative species (and this includes P90). The three-dimensional structure of HPr has been determined (Wittekind *et al.*, 1992; Chen *et al.*, 1993), and P90 will presumably have a near-identical structure. It is of note that one protein of the enzyme II complex of the fructose uptake system in *S. typhimurium* comprises enzyme IIA (to which P163 shows homology) at its N-terminus and HPr at its C-terminus. Thus P163 and P90 are homologous to the two ends of a single protein. Therefore by analogy to the bacterial PTS, P90 would be expected to phosphorylate P163, which itself acts as an intermediary in the transfer of a phosphate group between proteins, possibly to σ^{54} itself.

P284 contains a region with similarity to the glycine-rich phosphate-binding loop of many ATP- and GTP-binding proteins (Fig. 7; Walker *et al.*, 1982; Saraste *et al.*, 1990). It is possible that P284 is a kinase which could initiate a chain of phosphorylation steps linking σ^{54} to some sort of sensory apparatus. Consistent with this is limited sequence

Motif consensus	G X X X X G K TorS
P284 (residues 8–15)	G R S G S G K S
<i>E. coli</i> adenylate cyclase	G A P G C G K G T
Ras p21	G A G G V G K S
<i>E. coli</i> EF-Tu	G H V D H G K T
<i>E. coli</i> ATP synthase b-subunit	G G A G V G K T
Rat myosin heavy chain	G E S G A G K T
Chick tyrosine kinase	G P M F S G K S

Fig. 7. Sequence similarity of a region of P284 with proteins representing six of the major protein families known to contain a glycine-rich phosphate-binding loop. The consensus sequence for such a motif is also shown. All sequences are taken from Saraste *et al.* (1990).

similarity to a region of PgtA, an activator of the phosphoglycerate transport system of *S. typhimurium* (Yu & Hong, 1986), and to NarX, part of a two-component regulatory system which senses nitrate and nitrite concentration (Stewart *et al.*, 1989; data not shown).

The bacterial PTS controls more than just the translocation of sugars across the membrane; it is involved in the regulation of chemotaxis, carbohydrate permeases not otherwise associated with the PTS, catabolic enzymes, transcriptional antitermination and transcriptional regulation (reviewed in Saier, 1993, and accompanying articles). The additional similarities reported in this paper for ORFs IV and V strengthen the idea of a link between carbon metabolism and expression of σ^{54} -dependent genes, mediated via a phosphorylation pathway. An increasing number of kinases are being found in bacteria, and many responses are known to be mediated by protein phosphorylation. It may be that bacterial signal transduction is not dissimilar to that of eukaryotes, where extracellular signals can activate an elaborate network of intracellular signalling events. In eukaryotic cells a major mechanism of signal integration is that of protein phosphorylation and dephosphorylation, and the control of transcription by such pathways is well established (reviewed in Karin & Smeal, 1992). We predict that further analysis of this set of genes will reveal a similar system here.

ACKNOWLEDGEMENTS

We are very grateful to Mike Merrick for helpful suggestions and the gift of pMM17 and pMM18. D.H.A.J. was the recipient of an SERC Biotechnology Directorate CASE studentship.

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Received 15 September 1993; revised 12 November 1993; accepted 22 November 1993.