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Mutations in *rpoA* Affect Expression of Anaerobically Regulated Genes in *Salmonella typhimurium*

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***oxrB8*, a mutation that diminishes the anaerobic induction of *pepT* and other anaerobically regulated, *oxrA* (*fnr*)-dependent *Salmonella typhimurium* genes, is an allele of *rpoA*, the gene for the α subunit of RNA polymerase. Four additional *rpoA* mutations that affect anaerobic *pepT* expression have been isolated after localized mutagenesis of the *rpoA* region. All but one of these *rpoA* mutations appear to have relatively specific effects on genes that require the OxrA (FNR) protein, a positive transcriptional regulator of a family of anaerobically expressed genes. All of these mutations lead to amino acid substitutions in the C-terminal region of the α subunit. These results taken with a number of previous observations suggest a role for the α subunit in the interaction between RNA polymerase and positive transcriptional regulatory proteins. They also suggest that the C-terminal region of α is important for these interactions.**

pepT is a member of a group of *Salmonella typhimurium* genes (*oxd* genes) that are transcribed at higher levels under anaerobic growth conditions than in the presence of oxygen. Mutations that block the increased anaerobic expression of these genes map at two loci (41). One of these, *oxrA*, appears to be the *Salmonella* homolog of the *fnr* gene of *Escherichia coli* (19, 41). The product of *fnr* is necessary for the anaerobic induction of a variety of genes, most of which encode components of anaerobic respiratory pathways (for a review, see reference 39). The FNR protein is a positive transcriptional regulator of these genes (39). FNR shows significant sequence similarity to CRP (catabolite activator protein) and is believed to stimulate transcription initiation by a mechanism similar to that of CRP (39). Specific in vitro binding of FNR to the target site deduced from in vivo studies has recently been reported (13a). A second locus that leads to decreased anaerobic expression of this family of genes, identified only in *S. typhimurium*, is defined by the *oxrB8* mutation (41). This mutation has a phenotype similar to that of mutations at *oxrA* (decrease in anaerobic *pepT* expression with no effect on aerobic levels), but it is not linked to the *oxrA* locus. It also affects the anaerobic induction of other *oxd* genes (41).

Some of the properties of the *oxrB8* mutation are unusual. (i) On the basis of its effect on β-galactosidase production from a *pepT*::*lacZ* operon fusion, it appears to be partially dominant to the wild-type allele. (ii) The frequency with which mutations at *oxrB* are isolated seems to be very low compared with the frequency of *oxrA* mutations. When this work was begun, only one allele had been found. Attempts to isolate insertions in *oxrB* have been unsuccessful. (iii) The *oxrB8* phenotype is partially corrected by the presence of a plasmid carrying the *E. coli fnr* gene, suggesting that overproduction of the activator protein can compensate for the defect caused by *oxrB8*.

Although *oxrB8* was previously shown not to be linked either to *pepT* or to *oxrA*, its map position was not determined (41). The results of mapping crosses reported in this paper indicate that *oxrB* lies in the cluster of operons containing mainly ribosomal protein genes between 71 and 72 map units. One of these operons, the α operon, contains *rpoA*, the gene encoding the α subunit of RNA polymerase. Since *pepT* and the *oxd* genes are positively regulated at the transcriptional level, we suspected that the *oxrB8* mutation might affect *rpoA*, perhaps resulting in an RNA polymerase that is less efficiently activated by the product of *oxrA*. This hypothesis provides reasonable explanations for the properties of the *oxrB* mutation (see Discussion). We report here that *oxrB8* is indeed an allele of *rpoA*. In addition, we describe the isolation and characterization of other *rpoA* alleles that affect the anaerobic induction of *pepT*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and enzyme assays. The strains used in this work are described in Table 1. All bacterial strains are derivatives of *S. typhimurium* LT2 unless otherwise noted. Media and growth conditions have been described previously (41). Growth on alternative carbon sources was tested by streaking on NCE medium (9) supplemented with 0.4% carbon source. For some experiments, minimal medium was supplemented with 0.1% Casamino Acids (Difco). Growth rates were determined by monitoring the optical density at 600 nm of cultures grown aerobically in a shaking water bath. β-galactosidase activity was assayed according to the method of Miller (26) and expressed in Miller units.

Genetic methods. Standard methods were used for mutagenesis, P22 transduction, and conjugation (9). A tandem chromosomal duplication for testing dominance of *oxrB8* (*rpoA8*) was constructed as described by Anderson and Roth (1). Briefly, a strain carrying a Tn*10* insertion in a gene near *rpoA* (*argD1883*::Tn*10*) was used as the donor in a transduction cross with selection for prototrophic (Arg^+), tetracycline-resistant recombinants. These recombinants are formed from cells in the recipient population carrying tandem chromosomal duplications of the *argD* locus by recombination between the transducing DNA and one side of the duplication (1). Such recombinants are heterozygous dip-

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TABLE 1. Bacterial strains

Strain	Genotype[a]
TN1379	*leuBCD485*
TN1668	*pepT7*::Mud1(X)
TN2064	*leuBCD485 pepT7*::Mud1(X) *rpoA8* (*oxrB8*) *zhc-895*::Tn*5*
TN2224	*leuBCD485 zhc-895*::Tn*5*
TN2262	*leuBCD485 pepT7*::MudJ
TN2550	*leuBCD485 pepT7*::Mud1(X) DUP2001 [(*argD1883*::Tn*10*)*(*argD*$^+$)]
TN2551	*leuBCD485 pepT7*::Mud1(X) DUP2001 [(*argD1883*::Tn*10 rpoA*$^+$)*(*argD*$^+$ *rpoA8* (*oxrB8*) *zhc-895*::Tn*5*)]
TN2552	*leuBCD485 pepT7*::Mud1(X) *zhc-895*::Tn*5*
TN2555	*pepT7*::Mud1(X) DUP120[(*cysG1542*::Tn*5*)* (*cysG*$^+$)]
TN2649	*leuBCD485 pepT7*::MudJ/pCH21
TN2651	*leuBCD485 pepT7*::MudJ *rpoA8* (*oxrB8*) *zhb-3195*::Tn*10*Δ/pCH21
TN2757	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ
TN2758	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ *rpoA8* (*oxrB8*)
TN2770	*leuBCD485 pepT7*::Mud1(X) *rpoA8* (*oxrB8*) *rpsE102*
TN3557	*leuBCD485 zhb-1624*::Tn*10*Δ *rpoA8* (*oxrB8*)/ F'42 *finP301 lac*$^+$
TN3558	*leuBCD485 zhb-1624*::Tn*10*Δ/F'42 *finP301 lac*$^+$
TN3567	TN2262 *rpoA151*
TN3568	TN2262 *rpoA152*
TN3569	TN2262 *rpoA155*
TN3570	TN2262 *rpoA153*
TN3571	TN2262 *rpoA154*
TN3640	*leuBCD485 rpsE102 zce-850*::Tn*10*/F'42 *finP301 lac*$^+$
TN3641	*leuBCD485 rpsE102 zce-850*::Tn*10 rpoA151*/ F'42 *finP301 lac*$^+$
TN3642	*leuBCD485 rpsE102 zce-850*::Tn*10 rpoA155*/ F'42 *finP301 lac*$^+$
TN3643	*leuBCD485 rpsE102 zce-850*::Tn*10 rpoA153*/ F'42 *finP301 lac*$^+$
TN3644	*leuBCD485 rpsE102 zce-850*::Tn*10 rpoA154*/ F'42 *finP301 lac*$^+$
AK3195	*metE551 metA22 ilv-452 trpB2 hisC527 galE496 xyl-404 rpsL120 flaA66 hsdL6 hsdSA29 zhb-3195*::Tn*10*Δ

[a] The MudJ element is the mini-Mu MudI1734 of Castilho et al. (4). The *pepT7*::MudJ fusion was constructed by replacing the Mud1 of the *pepT7*::Mud1(X) mutation (41) with MudJ as described by Hughes and Roth (17). The fusions generated by these elements are operon fusions. Tn*10*Δ is the transposition-defective mini-tet element, Tn*10*Δ*16*Δ*17* (43). Insertions in unknown loci are designated by the "z.." nomenclature (36).

loids (in this case, *argD*$^+$/*argD1883*::Tn*10*) and frequently carry duplications of large chromosomal regions (up to 20% of the chromosome) (1). To construct a strain that is also heterozygous for the *oxrB* locus, one of the *argD* duplication strains (TN2550) was transduced to Kan^r (on MacConkey lactose kanamycin tetracycline plates) with phage grown on a strain carrying a Tn*5* insertion that is cotransducible with *oxrB8*. Many of the transductant colonies were small-center fisheye colonies, intermediate in appearance between *oxrB8* and *oxrB*$^+$. One such "small fisheye" strain was saved (TN2551) and shown to segregate both pink colonies and normal fisheye colonies (dark red centers clearly demarcated from a white periphery) after growth in nonselective medium (Luria-Bertani [LB]). This showed that the strain carries both *oxrB8* and *oxrB*$^+$, and the colony appearance suggests that the *oxrB8*/*oxrB*$^+$ strain shows reduced anaerobic expression of *pepT*.

Localized mutagenesis of the *rpoA* region of TN2757 was carried out either by mutagenizing P22 transducing lysates with hydroxylamine (16) or by preparing transducing lysates on diethyl sulfate-mutagenized cells. TN2757 carries a selectable tetracycline resistance marker (*zhb-1624*::Tn*10*Δ), which is 50% cotransducible with *rpoA*. These lysates were used to transduce TN2262 to tetracycline resistance on MacConkey lactose tetracycline agar. Transductants with altered appearance (white, pink, or small fisheye) were purified by two single-colony isolations. Transducing lysates prepared on these potential mutants were used to transduce TN2262 to tetracycline resistance on MacConkey lactose tetracycline plates to verify that the mutation responsible for the phenotype was linked to *zhb-1624*::Tn*10*Δ at the expected frequency.

Cloning and sequencing of the *S. typhimurium rpoA* locus. Chromosomal DNA from TN1379 (*oxrB*$^+$) or TN2758 (*oxrB8*) was amplified by polymerase chain reaction (PCR) with the following primers based on the sequences of the *E. coli* genes flanking *rpoA*: primer OxrB1 (from the 3' end of the *rpsD* gene), 5'GGTACCCACCTGATCGTCGAGCTT TA; and primer OxrB2 (from the 5' end of the *rplQ* gene), 5'GGTCTACACCACTCTTACGATGGCGAT. OxrB2 contains an *Xba*I site near its 5' end. Amplifications were carried out in 100 μl (total volume) containing 2 μg of chromosomal DNA (prepared according to the method of Maurer et al. [23]), 10 pmol of each primer, 16 μl of 1.25 mM dNTP, 10 μl of 10× PCR reaction buffer, and 0.5 μl of AmpliTaq polymerase (Perkin Elmer Cetus). DNA was denatured at 95°C for 1 min, hybridized at 60°C for 1 min, and extended at 72°C for 2 min for 25 cycles. Each reaction yielded a single 1-kb product, which was purified by extraction and precipitation. This DNA was treated with Klenow to repair the ends and cut with *Xba*I. *oxrB*$^+$ fragments were ligated into *Sma*I-*Xba*I-cut vector pSE380 (3), yielding plasmid pCM159. In a separate ligation reaction, *oxrB*$^+$ fragments were ligated into pSE380, which had been cut with *Nco*I, treated with S1 endonuclease, and cut with *Xba*I, yielding pCM173. *oxrB8* fragments were ligated into the modified pSE380 vector, and four independent transformants were saved as pCM174 through pCM177.

Both strands of the inserts of the two independent *oxrB*$^+$ clones, pCM159 and pCM173, and one *oxrB8* clone, pCM174, were sequenced completely. The sequence change caused by the *oxrB8* mutation was confirmed by sequencing single-stranded template produced by PCR, as described below. Plasmid DNA was isolated by using anion-exchange columns (Qiagen) and prepared for sequencing by the method of Chen and Seeburg (6). Primers OxrB1 and OxrB2 as well as four additional primers internal to the *rpoA* gene, OxrB3 through OxrB6, were used in sequencing.

The nucleotide sequences of the additional mutant alleles *rpoA151* through *rpoA155* were determined directly from chromosomal DNA amplified by PCR. Both strands were sequenced for each mutant allele. Single colonies of mutant strains were suspended in 50 μl of water, 5 μl of which was added to a 100-μl amplification reaction mixture with primers OxrB1 and OxrB2 and amplified as described in the cloning procedure, with an initial 5 min of incubation at 95°C. This reaction mixture (2 μl) was then added to an asymmetric reaction mixture with 10 pmol of either primer OxrB1 or OxrB2 and amplified as described in the cloning procedure, with an additional 5 cycles. The single-stranded DNA generated was purified by 2 M NH_4OAc-isopropanol precipitation and sequenced. All DNA sequencing was carried out by the dideoxy nucleotide chain termination method

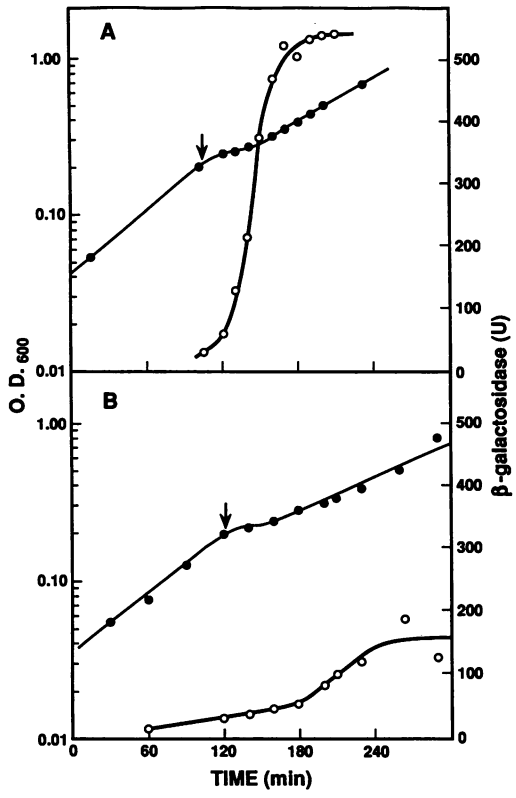

FIG. 1. Anaerobic induction of *pepT* in *rpoA*$^+$ and *oxrB8* (*rpoA8*) strains. Strains were grown in minimal glucose-leucine medium. Anaerobiosis was initiated by sparging the culture with N_2-CO_2 (95%:5%) at the time indicated by the arrow. (A) TN1668 *pepT7*::Mud1(X) *oxrB*$^+$ (*rpoA*$^+$); (B) TN2064 *pepT7*::Mud1(X) *oxrB8* (*rpoA8*). ●, optical density at 600 nm (OD_{600}); ○, β-galactosidase activity.

(37) with Sequenase (United States Biochemicals). Restriction digestion, ligation, transformation, and Klenow and S1 endonulease treatment were all carried out by standard procedures (35). All PCR reagents were obtained from Perkin Elmer Cetus. The Klenow fragment and *Xba*I and *Sma*I restriction enzymes were obtained from Bethesda Research Laboratories. S1 endonuclease was obtained from USB. Primers were synthesized at the Case Western Reserve University Molecular Biology Core Facility.

Nucleotide sequence accession number. The sequence of the *S. typhimurium rpoA* gene (see Fig. 3) has been designated STYRPOA and assigned GenBank accession number M77750.

RESULTS

Properties of *oxrB8*. A strain carrying a transcriptional fusion of *lacZ* to the *pepT* promoter (*pepT7*::MudJ) forms fisheye colonies on MacConkey lactose plates (41). This colony appearance presumably reflects the anaerobic induction of β-galactosidase in the cells growing in the center of the colony since mutations that block anaerobic induction (*oxrA* and *oxrB8*) form white to faintly pink colonies. The effect of the *oxrB8* mutation on anaerobic induction of β-galactosidase from a *pepT7*::MudJ fusion is clearly demonstrated in the induction curve shown in Fig. 1. The *oxrB*$^+$ strain responds rapidly to a shift to anaerobiosis and attains a steady-state level of gene product in less than a generation. Induction in an *oxrB8* strain is sluggish, however, and the steady-state level attained is approximately fourfold lower than that in an *oxrB*$^+$ strain. The residual induction seen in the *oxrB8* strain is completely dependent on the presence of a functional *oxrA* gene: an *oxrB8 oxrA1* strain shows no induction at all (data not shown).

The *oxrB8* mutation affects several other *oxrA*-dependent, anaerobically regulated genes. The effect of *oxrB8* on the anaerobic expression of β-galactosidase in strains carrying various *oxd*::*lacZ* fusions (20, 41) was determined. All except two of these fusions (*oxd3* and *oxd14*) showed reduced anaerobic expression in strains carrying *oxrB8*. These two *oxd* fusions belong to the same map position class (20) and may be independent isolates of fusions to the same promoter. Two other fusions (*oxd5* and *oxd8*) showed small reductions (less than twofold), while all of the remaining fusions (*oxd2*, *oxd4*, *oxd6*, *oxd7*, *oxd11*, *oxd12*, and *oxd18*) showed effects (3- to 20-fold reduction in the anaerobic level) comparable to or greater than those seen with *pepT*.

The *oxrB8* mutation is partially dominant to the wild-type allele. Levels of β-galactosidase in a strain carrying a tandem chromosomal duplication heterozygous at *oxrB* [TN2551: *oxrB8*/*oxrB*$^+$ *pepT*::Mud1(*lac*)] were determined and compared with the levels in strains carrying (i) two copies of the wild-type allele (TN2555) or (ii) haploid strains carrying *oxrB8* (TN2552) or *oxrB*$^+$ (TN2553). The anaerobic level of the heterozygote (TN2551, 110 units) was substantially lower than that of either strain carrying only *oxrB*$^+$ (TN2555 and TN2553, both ~400 units) but somewhat higher than that of the *oxrB8* haploid strain (TN2552, 82 units). These results suggest that *oxrB8* is not a simple loss of function mutation and that it may lead to the formation of a gene product capable of interfering with the action of the wild-type product.

The presence of a high-copy-number plasmid carrying the *E. coli fnr* gene (pCH21) (19) partially corrects the *oxrB8* defect. A strain (TN2651) carrying *oxrB8 pepT*::Mud1(*lac*) and pCH21 forms fisheye colonies on MacConkey lactose and displays an anaerobic β-galactosidase level intermediate between that of an *oxrB8* strain without the plasmid and that of an *oxrB*$^+$ pCH21 strain (TN2649).

Map position of *oxrB8*. An insertion of Tn*10*Δ linked to (phage P22 cotransducible with) *oxrB8* was identified in the collection of Tn*10*Δ insertions described by Kukral et al. (20). This insertion (*zhb-3195*::Tn*10*Δ in strain TN2224) was used to target the formation of Hfrs with origins of transfer at the site of the insertion (8). Conjugation crosses with these Hfrs as donors and strains carrying auxotrophic mutations as recipients led to the conclusion that *oxrB8* is located between *argG* (69 map units) and *cysG* (73 map units). Several additional transposon insertions cotransducible with *oxrB8* were isolated, and P22 transduction crosses between these transposons and other markers in this interval were carried out. The results of these two-point transduction crosses (Fig. 2) showed that *oxrB8* lies between *aroE* (71 map units) and *argD* (72 map units) and that it is very close to *rpsE* (*spc*) at the *aroE* end of the large cluster of ribosomal protein genes at this location. These results do not unambiguously order *oxrB8* with respect to *rpsE*, however; so a three-point cross with *zhb-3195*::Tn*10*Δ, *oxrB8*, and *rpsE102* was carried out (Table 2). The results strongly suggested that *oxrB8* maps to the left of *rpsE*. This map position raised the possibility that *oxrB8* might affect the *rpoA* gene which encodes the α subunit of RNA polymerase and is part of the α operon located at precisely this position. The sequence of the

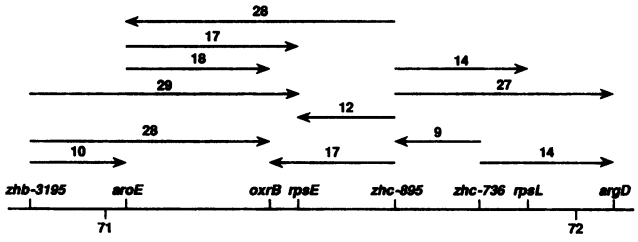

FIG. 2. Map positions of *oxrB8* and nearby transposons. Linkage data were obtained from P22 transduction crosses. The arrows point from the selected marker to the unselected marker and the number above the arrow is the distance (in kilobases) calculated as described by Sanderson and Roth (37). The markers used were *zhb-3195*::Tn*10*Δ (20), *aroE36*, *rpsE102* (*spc*), *argD1883*::Tn*10* (all from J. R. Roth), *oxrB8*, *zhc-895*::Tn*5* (41), and *rpsL120* (*strA*) (27).

corresponding region from *E. coli* has been determined, and *rpoA* is about 5 kb from *rpsE* (*spc*) (2, 5, 24, 28).

Complementation of *oxrB8* by a plasmid carrying the *E. coli rpoA* gene. A plasmid carrying the *E. coli rpoA* gene and essentially no additional bacterial DNA has been constructed by Matsuyama and Mizushima (22). This plasmid, pMAN225, was tested for its ability to complement the *oxrB8* mutation. Introduction of pMAN225 into an *oxrB8* strain (TN2758) led to colonies with an approximately wild-type appearance on MacConkey lactose (i.e., fisheyes) and restored the ability of the strain to induce β-galactosidase in response to anaerobiosis (data not shown). These observations strongly supported our hypothesis that the *oxrB8* mutation affects *rpoA*.

Cloning and nucleotide sequence of wild-type and mutant *rpoA*. To further test this hypothesis, we cloned and sequenced the *rpoA* gene from both wild-type and *oxrB8* strains. Figure 3 shows the nucleotide sequence of the *S. typhimurium rpoA* gene. This sequence is very highly conserved compared with that of *E. coli*, with only 24 of 1,065 base pairs different between the two organisms. The predicted amino acid sequences are identical. The sequence of the gene from the *oxrB8* mutant differed from that of the wild type at only one nucleotide. The *oxrB8* (hereafter *rpoA8*) mutation is a G to A transition at nucleotide 966 (Fig. 3), resulting in an Arg for Gly substitution at amino acid 311 in the α protein.

Isolation of other *rpoA* alleles that affect *pepT* expression. Mutations at *rpoA* are rare among mutants identified in screens for reduced anaerobic expression of a *pepT*::Mud-J(*lacZ*) fusion. *rpoA8* is the only allele obtained from several screens, each of which produced many new *oxrA* mutations. The reason for this is now clear: *rpoA8* is a change-of-function mutation in a vital gene. To see whether other *rpoA* alleles that affect *pepT* regulation could be isolated, we screened for colonies with an altered colony appearance (white to pink or smaller-center fisheye) on MacConkey lactose agar after localized mutagenesis of the *rpoA* region. Five independent mutations were isolated by this procedure and designated *rpoA151* through *rpoA155*. Two mutations (*rpoA151* and *rpoA155*) were hydroxylamine induced, and the rest were isolated after mutagenesis with diethyl sulfate. The *rpoA* genes from these mutant strains were sequenced directly from genomic DNA with PCR-generated single-stranded templates. Each of these strains carried a single G · C to A · T transition mutation in *rpoA*. The nucleotide and amino acid changes caused by these mutations are shown in Fig. 3. Two independently isolated mutations, *rpoA151* and *rpoA152*, produced identical sequence changes. Both affect the same codon as *rpoA8* but change a different base and result in a distinctly different amino acid substitution: Gly-311 to Glu (*rpoA151* and *rpoA152*) rather than Gly-311 to Arg (*rpoA8*). The *rpoA153* mutation changes Arg-317 to His. Another mutation, *rpoA155*, converts Leu-289 to Phe. Surprisingly, *rpoA154* is a nonsense mutation that changes the UGG codon specifying Trp-321 to the chain-terminating UGA. This mutation should produce an α subunit that is nine amino acids shorter than the wild type. All of these mutations affect the region of the gene encoding

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                                                                CTCCAAGTAAAGCTTAGTACCAAAGAGAGGACACA      35
ATGCAGGGTTCTGTGACAGAGTTTCTAAAACCGCGCCTGGTAGATATCGAGCAAGTGAGTTCGACGCACGCCAAGGTGACCCTTGAGCCTTTAGAGCGTG    135
M  Q  G  S  V  T  E  F  L  K  P  R  L  V  D  I  E  Q  V  S  S  T  H  A  K  V  T  L  E  P  L  E  R  G   34
GCTTCGGCCATACTCTGGGTAACGCACTGCGCCGTATTCTGCTCTCATCGATGCCGGGTTGCGCGGTGACCGAGGTTGAGATTGATGGTGTACTACATGA    235
  F  G  H  T  L  G  N  A  L  R  R  I  L  L  S  S  M  P  G  C  A  V  T  E  V  E  I  D  G  V  L  H  E   67
GTACAGCACCAAAGAAGGCGTTCAGGAAGACATCCTGGAAATCCTGCTCAACCTGAAAGGGCTGGCGGTGAGAGTTCAGGGTAAAGATGAAGTTATTCTT    335
 Y  S  T  K  E  G  V  Q  E  D  I  L  E  I  L  L  N  L  K  G  L  A  V  R  V  Q  G  K  D  E  V  I  L   100
ACCTTGAATAAATCTGGCATTGGCCCTGTGACTGCAGCCGATATCACCCATGATGGGGATGTCGAAATCGTCAAGCCGCAGCACGTGATCTGCCACCTGA    435
T  L  N  K  S  G  I  G  P  V  T  A  A  D  I  T  H  D  G  D  V  E  I  V  K  P  Q  H  V  I  C  H  L  T  134
CCGATGAAAACGCGTCTATTAGTATGCGTATCAAAGTTCAGCGCGGTCGTGGTTATGTGCCGGCTTCTACCCGAATTCATTCGGAAGAAGATGAGCGCCC    535
  D  E  N  A  S  I  S  M  R  I  K  V  Q  R  G  R  G  Y  V  P  A  S  T  R  I  H  S  E  E  D  E  R  P   167
AATCGGCCGTCTGCTGGTCGACGCCTGCTACAGCCCTGTAGAGCGTATTGCCTACAATGTTGAAGCAGCGCGTGTAGAACAGCGTACCGACCTGGACAAG    635
 I  G  R  L  L  V  D  A  C  Y  S  P  V  E  R  I  A  Y  N  V  E  A  A  R  V  E  Q  R  T  D  L  D  K   200
CTGGTCATCGAAATGGAAACCAACGGCACAATCGATCCTGAAGAGGCGATTCGTCGTGCGGCAACCATCCTGGCTGAACAACTGGAAGCTTTCGTTGATT    735
L  V  I  E  M  E  T  N  G  T  I  D  P  E  E  A  I  R  R  A  A  T  I  L  A  E  Q  L  E  A  F  V  D  L  234
TACGTGATGTACGTCAACCGGAAGTGAAAGAAGAGAAACCAGAATTCGATCCGATCCTGCTGCGCCCTGTTGACGATCTGGAATTGACTGTCCGCTCTGC    835
  R  D  V  R  Q  P  E  V  K  E  E  K  P  E  F  D  P  I  L  L  R  P  V  D  D  L  E  L  T  V  R  S  A   267
                                                                T(rpoA155)
TAACTGCCTCAAGGCAGAAGCTATCCACTATATCGGTGATCTGGTACAGCGTACCGAGGTTGAGCTTCTTAAGACGCCTAACTTGGGTAAAAAATCTCTT    935
 N  C  L  K  A  E  A  I  H  Y  I  G  D  L  V  Q  R  T  E  V  E  L  L  K  T  P  N  L  G  K  K  S  L   300
                                                                F
                                  A(rpoA151,152)
                        (rpoA8)A                    A(rpoA153)   A(rpoA154)
ACCGAGATTAAAGACGTGCTGGCTTCCCGTGGACTGTCTCTGGGTATGCGCCTGGAAAACTGGCCACCGGCAAGCATCGCTGACGAGTAACCGGATCACA   1035
T  E  I  K  D  V  L  A  S  R  G  L  S  L  G  M  R  L  E  N  W  P  P  A  S  I  A  D  E  <              329
                        (rpoA8)R                    H           TER
                                  E(rpoA151,152)
GGTTAAGGTTTTACTGAGAAGGATAAGGTC                                                                        1065
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FIG. 3. Nucleotide and deduced amino acid sequence of the *S. typhimurium rpoA* gene. Nucleotides 1 through 10 are the last three codons and the termination codon of *rpsD*. The nucleotide sequence differs from that of *E. coli* (2) at 24 positions, all but one of which affect the third position in a codon. The base changes and predicted amino acid substitutions produced by the mutations described in this paper are indicated. The amino acid affected by the *E. coli rpoA109* mutation (Leu-290 to His) and the site of ADP ribosylation by phage T4 (Arg-265) are underlined. The *E. coli rpoA341* mutation (*phs*) is C terminal to Ile-159 (34).

TABLE 2. Three-point P22 transduction cross

Strain[a]		Selected marker	Recombinant class[b]	No. of recombinants	Order	Predicted rare class
Donor	Recipient					
AK3195	TN2770	Tet^r	OxrB$^+$ Spc^s	13	*zhb-3195 oxrB rpsE*	OxrB$^-$ RpsE$^+$ (Spc^s)
			OxrB$^+$ Spc^r	4	*zhb-3195 rpsE oxrB*	OxrB$^+$ RpsE$^-$ (Spc^r)
			OxrB$^-$ Spc^s	0		
			OxrB$^-$ Spc^r	473		

[a] AK3195: *zhb-3195*::Tn*10Δ oxrB*$^+$ *rpsE*$^+$ (Spc^s); TN2770: *pepT7*::Mud1(X) *oxrB8 rpsE102* (Spc^r).

[b] Recombinants were selected on MacConkey lactose tetracycline medium. The OxrB phenotype was scored by noting the colony appearance (fisheye [B$^+$] or white to pink [B$^-$]), and resistance or sensitivity to spectinomycin was determined by replica plating. The reciprocal cross was not carried out because *rpsE* (Spc^r) is recessive to the wild-type allele and a period of growth is required before plating in order for segregation to occur. As a result, transductants that arise may not be independent.

the C-terminal region of the protein, and four are localized in an 11-amino-acid segment (Gly-311 to Trp-321).

Phenotypes of *rpoA* mutations. All of the *oxrB*-type *rpoA* mutations were isolated by screening for colonies that had lost or reduced the size of the red center (fish eye) observed when a *pepT7*::MudJ strain was grown on MacConkey lactose agar. The effect of each of these mutations on anaerobic β-galactosidase expression from a *pepT7*::MudJ fusion correlates well with the appearance of colonies grown on MacConkey lactose agar (Table 3). The small-fisheye phenotype caused by *rpoA153*, *rpoA154*, and *rpoA155* is *oxrA* dependent. The presence of *oxrA2*::Tn*10* in strains carrying these mutations results in a white phenotype on MacConkey lactose agar. It is interesting that the anaerobic steady-state levels in *pepT7*::MudJ strains carrying *rpoA8* or *rpoA151*, which both form white to pink colonies, are higher than those of some of the *oxd* fusions which form distinct fisheye colonies (41). We believe this phenotypic difference is likely to be a reflection of the kinetics of induction. As shown in Fig. 1, the *rpoA8* mutation increases the time required to respond to the anaerobic shift. We hypothesize that this time is critical in determining the appearance of the colony on MacConkey lactose agar. The formation of a red center must require not only anaerobiosis to induce β-galactosidase production but also a period during which metabolism of lactose produces sufficient acid to generate the red form of the pH indicator dye. If cells in the center of the colony stop growing before colony development is complete, then the degree to which a fisheye can develop may be determined by the time interval between the response to anaerobiosis (β-galactosidase induction) and growth cessation.

Specificity of *rpoA* mutations. Mutations that affect an RNA polymerase subunit could potentially affect the transcription of many genes. To determine the extent to which our mutations affect the expression of genes other than *pepT*, we have characterized the growth properties of the *rpoA* mutations. Except *rpoA155*, none of these mutations has a significant effect on growth. The mutant strains (TN3567, TN3570, TN3571, and TN2758) grow in minimal glucose medium at approximately the same rate (50 ± 2 min) as isogenic *rpoA*$^+$ strains (TN2262 and TN2757); so, they are clearly not defective in the expression of any required biosynthetic pathway. Growth in minimal glucose medium supplemented with Casamino Acids (0.1%) is also not affected by the *rpoA* mutations (all strains grow at doubling times of 42 ± 2 min). The ability of strains carrying *rpoA* mutations to grow as single colonies on minimal media supplemented with various carbon sources was also tested. These experiments revealed no significant differences from isogenic *rpoA*$^+$ strains in colony size (after either 20 or 48 h of growth) with glucose, galactose, maltose, arabinose, or melibiose as carbon sources (all at 0.4%). Since *S. typhimurium* does not have a *lac* operon and is Lac$^-$, a wild-type *E. coli lac* operon was introduced on an F′ into strains carrying *rpoA* mutations. All of the resulting derivatives (TN strains 3557, 3641, 3642, 3643, and 3644) form dark red colonies of approximately the same size as those of isogenic *rpoA*$^+$ strains (TN3558 and TN3640) after 24 h of incubation on MacConkey lactose medium. All of these results suggest that *rpoA8*, *rpoA151*, *rpoA153*, and *rpoA154* do not have a seriously deleterious effect on transcription from most promoters.

In contrast, the *rpoA155* mutation clearly has several easily observed phenotypic consequences in addition to its effect on *pepT* expression. A strain carrying this mutation (TN3569) grows very poorly on minimal glucose medium, forming pinpoint colonies after several days of incubation. The mutant strain grows reasonably well when supplemented with either cysteine or methionine. Bacteriophage P22 forms clear plaques on an *rpoA155* strain (TN3569), indicating that this mutation interferes with lysogenization. As in the phage λ system, formation of a stable P22 lysogen requires positive transcriptional regulators (29), and it is possible that the *rpoA155* mutation leads to a polymerase defective in this interaction.

DISCUSSION

The major conclusion we draw from this work is that alterations in the α subunit of RNA polymerase can specif-

TABLE 3. Effect of *rpoA* mutations on anaerobic induction of *pepT*

Strain[a]	*rpoA* mutation	β-Galactosidase level[b]		Colony appearance
		+O_2	−O_2	
TN2262	*rpoA*$^+$	20 ± 3	520 ± 30	Fisheye
TN3567	*rpoA151*	18 ± 3	160 ± 10	White to pink
TN3569	*rpoA155*	22 ± 2	190 ± 15	Small fisheye
TN3570	*rpoA153*	21 ± 1	350 ± 30	Small fisheye
TN3571	*rpoA154*	22 ± 1	320 ± 15	Small fisheye
TN2757	*rpoA*$^+$	21 ± 2	510 ± 30	Fisheye
TN2758	*rpoA8*	20 ± 1	140 ± 2	White to pink

[a] All strains carry the *pepT7*::MudJ fusion, which places the *lacZ* gene under control of the *pepT* promoter.

[b] Cells were grown in minimal glucose leucine (0.4 mM) Casamino Acids (0.1%) medium to an optical density at 600 nm of 0.2 to 0.3. The cultures were inoculated from stationary-phase cultures, and anaerobic samples were continuously sparged with N_2 (95%)-CO_2 (5%). The average β-galactosidase activity (in Miller units) and standard deviation for duplicate assays of cultures from each of two single colonies are shown.

ically affect transcription. The evidence for this is that several *rpoA* mutations that do not interfere with normal growth substantially inhibit the transcription of certain anaerobically induced, *oxrA*-dependent genes. We believe that the simplest hypothesis to explain these results is that the α subunit is involved in the direct interaction of the polymerase with the OxrA activator protein. The results further suggest that it is the C-terminal region of the α subunit that is important for these interactions.

The hypothesis that *rpoA* mutations affect the interaction of RNA polymerase with OxrA provides reasonably satisfying explanations for the phenotypic properties of the *rpoA8* mutation. Mutations of the *rpoA8* type are rare relative to *oxrA* mutations because they alter the function of a vital gene, whereas any loss-of-function mutation in the dispensable *oxrA* locus will yield a similar phenotype. The *rpoA8* mutation is partially dominant because it affects a protein that is assembled into a multisubunit complex. Since the mutation affects a region of the α protein that appears not to be required for assembly (18), expression of both mutant and wild-type subunits should result in the production of a mixed population of wild-type, mutant, and hybrid polymerase forms. If, as a number of lines of evidence suggest, the two α subunits in the assembled polymerase are not equivalent (31, 32), an even more complex mixture of polymerase forms could be present. The *rpoA8* mutation is partially overcome by the presence of *fnr* (*oxrA*) on a multicopy plasmid. Since such plasmids also increase the anaerobic expression levels in *rpoA*$^+$ strains (19, 30), it appears that the level of the activator protein may be limiting. If *rpoA8* weakens but does not obliterate the interaction between the polymerase and OxrA, it seems reasonable that its effect could be partially suppressed by overexpression of FNR (OxrA).

The *rpoA* mutations described in this paper clearly have specific effects on gene expression. The most compelling argument for this conclusion is that all but one of them allow normal growth on minimal medium. The mutations must not have drastic effects on expression of any of the genes required for growth. In addition, all allow growth on a number of alternative carbon sources. Utilization of several of these carbon sources requires the action of CRP, a positive transcriptional regulator with similarities to FNR (39). The mutants clearly are not deficient in the expression of all positively regulated genes. The fact that *rpoA8* does not affect all of the *oxrA*-dependent *oxd* fusions to the same extent is not necessarily inconsistent with the proposal that *rpoA* mutations alter polymerase-OxrA interactions. The interaction between a transcriptional activator and RNA polymerase must be influenced by the sequence of the promoter with which they both interact. The spacing of the Fnr (OxrA)-binding site with respect to other elements of the promoter is an obvious variable that might be relevant. The *E. coli rpoA341* mutation has a much greater effect on *cysA* than on the other genes that are regulated by the positive regulator encoded by *cysB* (12). One class of pseudorevertants of *rpoA8* affects the anaerobic *pepT* promoter, creating a consensus −10 region from the wild-type nonconsensus sequence (21, 25). This mutant shows *oxrA*-dependent anaerobic induction of *pepT* even in the presence of *rpoA8*.

Although all of the *rpoA* mutations isolated result in more or less specific transcription defects (even the *rpoA155* mutation allows nearly normal growth in medium supplemented with Casamino Acids), it seems to us unlikely that these mutations are absolutely specific in the sense that they affect only interactions with a single activator protein or a single promoter. It seems more likely that each has a spectrum of effects which may be revealed with further phenotypic characterization. Given the amino acid sequence conservation between the α subunits of *E. coli* and *S. typhimurium*, it is perhaps surprising that so many mutations that produce relatively subtle effects on transcription can be isolated.

Evidence from a number of other systems has suggested the importance of the C-terminal region of the α subunit in interactions with positive regulatory proteins. (i) The *E. coli* mutation *rpoA109* that interferes with late gene expression in phage P2 (42) and its satellite P4 (15) changes Leu-290 to His (7, 10). This mutation appears to block the action of the positive transcriptional activators of late gene promoters encoded by the *ogr* (P2) and δ (P4) genes (14, 15). (ii) The *rpoA341* (*phs*) mutation of *E. coli* causes a pleiotropic growth defect including a requirement for cysteine or methionine, an inability to grow on melibiose, and a substantially reduced rate of growth on arabinose (13, 34). The growth defects of *rpoA341* strains are apparently caused by diminished transcription of *cysA*, *melAB*, and the genes of the *ara* regulon (12). This diminished transcription has been attributed to an alteration in the ability of RNA polymerase containing the *rpoA341* mutant α subunit to interact properly with the positive regulators (CysB, MelR, and AraC) required for normal expression of *cysA*, *melAB*, and *araBAD* (12). The exact location of the *rpoA341* mutation is not known, but it is known to affect an amino acid C terminal to Ile-159 (34). The pleiotropic consequences of one of our mutations, *rpoA155*, include a requirement for cysteine or methionine. An *rpoA155* strain, when it is supplemented with methionine, grows on melibiose, however, so it appears that this mutation is phenotypically distinct from *rpoA341*. (iii) Two groups have isolated *E. coli rpoA* mutations that affect regulation of the expression of the OmpF and OmpC porin proteins. This expression is controlled by the products of *envZ* and *ompR*, now known to be the sensor and effector, respectively, of a two-component regulatory system (40). Matsuyama and Mizushima (22) characterized a mutation, *rpoA77*, that interferes with the suppression of *envZ11* by *ompR77*. They proposed that the mutant α subunit is altered in its ability to interact with the proteins required for regulation of OmpF and OmpC production. Garrett and Silhavy (11) isolated extragenic suppressors of the *envZ473* mutation. These suppressors (*sez* mutations) relieve the negative effects of *envZ473* on expression of *ompF*, *lamB*, and *phoA*. More recent work has shown that one of these mutations, as well as four newly isolated *rpoA* alleles that affect *envZ-ompR*-mediated regulation, result in amino acid substitutions in the C-terminal region of α. The mutant α subunit in these strains is apparently altered in its ability to interact with the OmpR regulatory protein (38). Recently, in vitro studies by Igarashi and Ishihama (18) have shown that mutant RNA polymerases containing truncated α subunits, while catalytically active, are unable to activate transcription from the CRP-dependent *lac* P1 promoter. These workers state that the simplest interpretation of these results is that the C-terminal region of the α subunit interacts with the CRP-cyclic AMP (cAMP) complex to allow activation of transcription, although indirect effects of the truncated α subunits on the function of other subunits of the polymerase have not been excluded.

Two additional lines of evidence are consistent with the idea that the α subunit interacts with transcriptional activators. (i) Riftina and coworkers have produced a monoclonal antibody directed toward an α epitope that is exposed in the assembled polymerase (32). This result indicates that a

region of the α subunit is available in the assembled RNA polymerase for interaction with other proteins. This same monoclonal antibody interferes with the interaction between the polymerase and the CRP-cAMP complex at the *lac* promoter, resulting in inhibition of transcription initiation (33). Although other interpretations have not been excluded, it is possible that binding of the antibody interferes with an interaction of the α subunit with CRP. (ii) Very soon after infection of *E. coli* by phage T4, Arg-265 of first one and then both α subunits is ADP ribosylated by phage-encoded enzymes (31). This phenomenon indicates that sites in the C-terminal region of the α subunit are sufficiently exposed to be accessible to enzymatic modification.

The screen we have used to isolate *rpoA* mutations takes advantage of the fisheye colony appearance on MacConkey lactose medium that is characteristic of *lacZ* fusions to *oxrA*-dependent promoters. Although the basis of this phenotype is not completely understood, it seems to be sensitive to subtle alterations in the regulation of these genes. This has allowed isolation of mutations that affect a vital gene whose product interacts in complex ways with other proteins and with DNA. Since it is easy to look for colonies that have regained the fisheye appearance, the system also provides ready access to revertants of *rpoA* mutations. Revertants of *rpoA8* have been isolated in this way, and, although not fully characterized, fall into at least three classes by map position: linked to *rpoA* itself (presumably true revertants or second-site suppressors), linked to *pepT* (the one characterized mutation creates a consensus −10 in the anaerobic promoter [21, 25]), and linked to *oxrA*.

The results described in this paper demonstrate the usefulness of this system for isolating mutations that produce interesting functional changes in the α subunit of RNA polymerase and the molecules with which it interacts. These mutations should contribute to our understanding of how the complex interactions between regulatory proteins, RNA polymerase, and promoters regulate transcription initiation.

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