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ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · OCTOBER 1988

Impact Factor: 4.33 · DOI: 10.1016/0022-2836(88)90088-5

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J. Mol. Biol. (1988) **203**, 29–37

Mutations in *rpoD*, the Gene Encoding the σ^{70} Subunit of *Escherichia coli* RNA Polymerase, that Increase Expression of the *lac* Operon in the Absence of CAP–cAMP

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(Received 11 August 1987, and in revised form 21 December 1987)

We have isolated a new class of mutations in *rpoD*, the gene encoding the σ^{70} subunit of *Escherichia coli* RNA polymerase, that alter the transcription initiation properties of RNA polymerase holoenzyme. The *rpoD*(Lac) mutations increase expression of the *lac* operon in the absence of CAP–cAMP, allowing a strain lacking adenyl cyclase to grow on lactose. Four of the six alleles isolated have three- to fivefold increases in the amount of *lac* mRNA and β-galactosidase per cell. We show that these four mutations increase transcription initiation from the same promoter used by wild-type RNA polymerase. The mutations were mapped and sequenced. One mutation occurs in the codon for amino acid 389 of the σ^{70} polypeptide. The remaining five mutations are clustered, affecting residues 570, 571 and 575. These five mutations are within or near a proposed helix-turn-helix motif in the C terminus of σ^{70}.

1. Introduction

The σ subunits of bacterial RNA polymerases are required for specific initiation of transcription (Burgess *et al.*, 1969; Burgess & Travers, 1970). The particular σ factor bound to core RNA polymerase determines the promoter specificity of RNA polymerase holoenzyme both *in vivo* and *in vitro* (for a review, see Reznikoff *et al.*, 1985). In addition to the major σ factor responsible for transcription of most genes during growth, bacteria have other σ factors that specify the transcription of groups of genes with diverse biological functions. Alternative σ factors were first identified in *Bacillus subtilis* and have been implicated in the temporal regulation of development in that organism (for a review, see Doi & Wang, 1986). The additional σ factors identified in *Escherichia coli* have a role in cellular responses to environmental stimuli such as heat shock (Grossman *et al.*, 1984) or nitrogen starvation (Hirschman *et al.*, 1985; Hunt & Magasanik, 1985).

The major σ factor in *E. coli* is σ^{70}, encoded by the *rpoD* gene. Many promoters transcribed by holoenzyme containing σ^{70}, $E\sigma^{70}$, have been identified and a consensus sequence for promoters recognized by $E\sigma^{70}$ has been determined (Hawley & McClure, 1983). In general, strong promoters contain consensus bases at many positions in the promoter, while promoters with fewer matches to the consensus sequence are weaker promoters (Youderian *et al.*, 1982; Mulligan *et al.*, 1984).

Exactly how σ factors confer promoter specificity is unclear. σ factors may contain all or part of the specificity determinants required for recognizing promoters (Losick & Pero, 1981). Isolation of mutant σs that alter the promoter recognition properties of RNA polymerase holoenzyme should lead to the identification of regions involved in specificity.

We have looked for mutations in *rpoD* that alter promoter recognition by selecting for increased expression of a gene transcribed from a weak promoter. Efficient transcription of the *lac* promoter requires activation by the CAP–cAMP complex both *in vivo* and *in vitro* (Perlman & Pastan, 1969; Schwartz & Beckwith, 1969; Zubay *et al.*, 1970). In the absence of this positive regulatory system, the *lac* promoter is very weak. Mutations in either *crp* (encoding CAP) or *cya* (encoding adenyl cyclase) reduce transcription to 2 to 5% of the wild-type level and prevent growth on lactose. We report the isolation, mapping, sequencing and characterization of *rpoD* mutations that enable a strain lacking adenyl cyclase (Δ*cya*) to grow on lactose. We call these *rpoD*(Lac) mutations.

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0022-2836/88/170029-09 $03.00/0

2. Materials and Methods

(a) *Bacteria, phage and media*

The bacterial strains used in this work are listed in Table 1. λpJH62 is a phasmid derivative of Charon 25 that contains the *rpoD* operon (Hu & Gross, 1988). *λimm21red3* was obtained from M. Feiss (University of Iowa). LB and M9 plates and liquid media, and MacConkey agar medium were made as described by Miller (1972). M9 plates and media were supplemented with glucose (0·2%, w/v), lactose (0·2%, w/v), or succinate (0·5%, w/v), vitamins, and 20 amino acids (20 μg/ml). NZY broth and plates were prepared as described by Maniatis *et al.* (1982). Kanamycin (Kan, 30 μg/ml), ampicillin (Amp, 100 μg/ml) and tetracycline (Tet, 20 μg/ml) were added sterilely where indicated.

Table 1.
Strains

Strain	Genotype	Source
KD1067	*mutD5 arg⁻ his⁻ su⁺*	M. Howe
CAG67	HfrH *thi-1, relA1, spoT1*	C.G.S.C.
CAG7085	MG1655 *gal-3 Δcya*	This work
CAG7189	MG1665 *gal-3 Δcya* (*λimm21red3*)	This work
CAG7190	HfrH *Δcya* (*λimm21red3*)	This work
CAG7227	MG1655 *gal-3* λpJH62*rpoD⁺*	This work
CAG7231	MG1655 *gal-3 Δcya* (*λimm21red3*) λpJH62*rpoD⁺*	This work
CAG7270	CAG7190*rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD⁺*	This work
CAG7272	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD903*	This work
CAG7327	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD901*	This work
CAG7328	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD904*	This work
CAG7329	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD911*	This work
CAG7330	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD912*	This work
CAG7331	CAG7190 *rpoD40*(Am) *zgh*::Tn*10* λpJH62*rpoD920*	This work
CAG7420	MG1655 *gal-3 Δcya rpoD⁺ zgh*::Tn*10*Kan Δ(*pro lac*)XIII *zaj*::Tn*10* F′*pro lac⁺*	This work
CAG7426	CAG7420 *rpoD901* F′*pro lacUV5*	This work
CAG7432	CAG7420 *rpoD904* F′*pro lacUV5*	This work
CAG7438	CAG7420 *rpoD911* F′*pro lacUV5*	This work
CAG7444	CAG7420 *rpoD912* F′*pro lacUV5*	This work
CAG7631	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10* (*λimm21red3*) λpJH62*rpoD⁺*	This work
CAG7632	CAG7431 λpJH42 *rpoD903*	This work
CAG7633	CAG7631 λpJH42 *rpoD920*	This work
CAG7634	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10* *rpoD⁺ zgh*::Tn*10*Kan	This work
CAG7635	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10 rpoD901 zgh*::Tn*10*Kan	This work
CAG7636	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10 rpoD904 zgh*::Tn*10*Kan	This work
CAG7637	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10 rpoD911 zgh*::Tn*10*Kan	This work
CAG7638	MG1655 *gal-3 Δcya Δcrp zhe*::Tn*10 rpoD912 zgh*::Tn*10*Kan	This work
CAG4274	*araBA*::MudX4064 *Δcya araD139 ΔlacU169 rpsL relA flbB malT*p7 (*λimm21*)	Hu & Gross (1988)
CAG17170	CAG4274 λpJH62*rpoD⁺*	This work
CAG17171	CAG4274 λpJH62*rpoD901*	This work
CAG17172	CAG4274 λpJH62*rpoD903*	This work
CAG17173	CAG4274 λpJH62*rpoD904*	This work
CAG17174	CAG4274 λpJH62*rpoD911*	This work
CAG17175	CAG4274 λpJH62*rpoD912*	This work
CAG17176	CAG4274 λpJH62*rpoD920*	This work

(b) *Plasmids*

Col3A was obtained from W. Reznikoff (University of Wisconsin, Madison, WI). Col3A contains a *lac Hin*cII fragment that extends from the *Hin*cII site at nucleotide 936 in *lac*I to the *Hin*cII site at nucleotide 1725 in *lacZ* (Silhavy *et al.*, 1984).

(c) *Mutagenesis and selection*

λpJH62 was mutagenized by growing on KD1067, which contains the *mutD5* mutator allele (Fowler *et al.*, 1974). Mutator activity was induced by adding 10 μg thymidine/ml to cells growing exponentially in NZY broth upon phasmid infection. The frequency of clear plaque-forming mutants in the mutagenized lysates was between 1 and 3%. Overnight cultures of CAG7189 and CAG7190, grown in NZY broth containing 0·2% (w/v) maltose and 5 mM-cAMP, were infected with mutagenized and control phasmid lysates at a multiplicity of infection of 0·3. Phage were absorbed for 20 min, mixed with 1 ml of NZY, and incubated for 40 min at 37 °C to allow expression of kanamycin resistance. Infected cells were pelletted by centrifugation, resuspended in M9 salts, and plated on LB Kan plates to determine the number of Kan^r lysogens and on M9 lactose Kan plates to select Lac$^+$Kan^r colonies. Plates were incubated at 30, 37 or 42 °C. Lac$^+$ candidates were colony-purified on MacConkey lactose Kan plates. Phage recovered from the Lac$^+$ isolates were tested for the ability to transfer the Lac$^+$ phenotype by infecting fresh cultures of either CAG7189 or CAG7190, selecting Kan^r lysogens and scoring the Lac phenotype.

(d) *Strain constructions*

Transductions using P1*vir* were done as described by Miller (1972). Transductions to *rpoD40*(Am) were done by cotransduction with a Tn*10* that is 90% linked to *rpoD*. The presence of *rpoD40*(Am) in the phasmid-containing strains was confirmed by P1*vir* transduction into a strain containing *supF*(Ts), a temperature-sensitive amber suppressor. The *rpoD40*(Am) mutation could not be transduced into the non-phasmid-containing parental strain, CAG7190.

The *rpoD*(Lac) mutations were transferred from λpJH62 to the chromosome by a modification of the method used by Hu & Gross (1988). P1*vir* was grown on strains carrying an *rpoD*(Lac) mutation on the phasmid and with *rpoD40*(Am) *zgh*::Tn*10* on the chromosome. (The *zgh*::Tn*10* insertion is 90% linked to *rpoD*.) These lysates were used to transduce CAG7085 to Tet^r. Tet^r colonies were selected on LB Tet plates containing 1 mM-IPTG† and 50 μg X-gal/ml, and the *rpoD*(Lac) alleles detected by screening for blue colonies. Lac$^+$ colonies were 1 to 3% of the total Tet^r transductants.

(e) *β-Galactosidase assays*

β-Galactosidase activity was measured in log-phase cultures growing in M9 minimal medium containing 1 mM-IPTG, to induce *lac* operon expression, and

† Abbreviation used: IPTG, isopropyl-1-thio-β-D-galactoside.

kanamycin (for phasmid-containing strains). When used, cAMP was at 5 mM. The carbon source was either glucose (0·2%) or succinate (0·5%). Assays were done essentially as described by Miller (1972), using 0·1% (w/v) SDS and $CHCl_3$ to lyse the cells.

(f) *Purification of plasmid and phasmid DNA*

Plasmids were amplified (Maniatis *et al.*, 1982) and plasmid DNA prepared by polyethylene glycol precipitation as described by Humphreys *et al.* (1975). Phasmid DNA was prepared from 10-ml lysates grown in NZY broth. Phage were pelleted by centrifugation for 1 h at 40,000 revs/min in a SW40 rotor. Phage pellets were allowed to resuspend overnight at 4°C in 0·5 ml of 10 mM-Tris·HCl (pH 7·5), 10 mM-$MgSO_4$. One-fifth volume of 0·2 M-EDTA, 1% SDS was added, DNA was extracted twice with phenol/chloroform, precipitated 3 times with ethanol and resuspended in 100 μl of 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA.

(g) *Mapping and DNA sequencing*

Mutations on the phasmid were mapped as described by Hu & Gross (1988), except that MacConkey lactose Amp Kan plates were used to detect wild-type recombinants. Mutant alleles were subcloned by *Eco*RI cleavage and religation of phasmid DNA (Hu & Gross, 1988). Restriction fragments were 5′ end-labeled using phage T4 polynucleotide kinase as described by Maxam & Gilbert (1980), except that calf intestinal alkaline phosphatase was used, and recut with a second restriction enzyme. Gel purified fragments were sequenced by the chemical cleavage method of Maxam & Gilbert (1980).

(h) *S_1 mapping*

An end-labeled probe for S_1 mapping was prepared from plasmid Col3A. Plasmid DNA was digested with *Pvu*II, reacted with 5 units of the Klenow fragment of DNA polymerase I in 6 mM-Tris·HCl (pH 7·5), 6 mM-$MgCl_2$, 50 mM-NaCl for 30 min at room temperature to create a 5′ overhang (Z. Burton, personal communication), dephosphorylated using calf intestinal alkaline phosphatase and labeled at the 5′ end with [γ-^{32}P]ATP using phage T4 polynucleotide kinase. The end-labeled probe was electroeluted from a polyacrylamide gel, then recut with *Hha*I. The full-length probe is 226 base-pairs. RNA was prepared by the method of Salser *et al.* (1967) from cells growing exponentially in M9 glucose medium containing 1 mM-IPTG, kanamycin and, where indicated, 5 mM-cAMP. Buffers for RNA–DNA hybridization and S_1 digestion were those of Berk & Sharp (1978). RNA samples (adjusted to 50 μg of RNA/sample with tRNA) were hybridized to 0·015 μg of purified probe for ≥4 h at 50°C, then treated with S_1 nuclease (Boehringer-Mannheim, Indianapolis, IN; 150 units/reaction) for 1 h at 37°C. Protected fragments were run on an 8% polyacrylamide gel containing 50% (w/v) urea (Maxam & Gilbert, 1980). Bands were located by autoradiography, excised and quantified by measuring radioactivity using a scintillation counter.

3. Results

(a) *Selection of* rpoD *mutants with altered expression of the* lac *operon*

We looked for mutations in *rpoD* that increase expression of the *lac* operon in the absence of CAP–cAMP. Such mutations should allow a Δ*cya* strain to grow on minimal lactose plates. We directed mutagenesis to a cloned copy of the *rpoD* operon carried in the Kan^r phasmid λpJH62, described in the accompanying paper (Hu & Gross, 1988), to selectively increase the frequency of Lac$^+$ mutations in *rpoD*. Cells will have a wild-type *rpoD* gene in addition to the mutated copy, alleviating the potential problem that *rpoD* mutations severely altering the promoter recognition properties of RNA polymerase could be lethal for the cell. The presence of wild-type σ^{70} requires that the mutant phenotype be codominant.

We infected a Δ*cya* host with mutagenized λpJH62 and selected Kan^r lysogens able to grow on lactose. The frequency of Kan^r Lac$^+$ colonies was between 10^{-4} and 10^{-5} (Table 2). In parallel infections with unmutagenized λpJH62, the frequency of Lac$^+$ colonies was 10 to 100-fold lower, and none of the Lac$^+$ colonies was due to mutations on the phasmid. Lac$^+$ mutants were selected in CAG7189 at 37 or 42°C and in CAG7190 at 30°C. The selections were done at different temperatures in the two strain backgrounds for technical reasons. In CAG7189, recovery of Lac$^+$ colonies following mutagenesis was 50 to 100-fold higher at 37 to 42°C than at 30°C. In strain CAG7190, the frequency of spontaneous Lac$^+$ colonies was too high at 37 or 42°C successfully to recover primarily *rpoD* mutations (Table 2). Although isolated in two different strain backgrounds at different temperatures, each of the *rpoD*(Lac) mutations leads to the same Lac$^+$ phenotype in both strains, at 37°C. Ten Lac$^+$ *rpoD* mutants were kept from each strain. We call these mutations *rpoD*(Lac) mutations.

(b) *Map position and sequence changes of the* rpoD*(Lac) mutations*

Mutations were localized within *rpoD* by recombination between the phasmid and a set of ampicillin-resistant *rpoD* deletion plasmids as described in the accompanying paper (Hu & Gross, 1988). The 20 mutations mapped to two clusters within *rpoD* (Table 3). Six mutations mapped to interval 1, between amino acid residues 368 and 434. The remaining 14 mutations mapped to

Table 2
Selection of Lac$^+$ pseudorevertants of Δcya

Δ*cya* host	Phasmid mutagenesis	Lac$^+$ Kan^r colonies/total Kan^r lysogens 30°C	37°C	42°C
CAG7189	None	$3·8 \times 10^{-8}$	$2·7 \times 10^{-7}$	$1·2 \times 10^{-7}$
	mutD5	$2·5 \times 10^{-6}$	$6·2 \times 10^{-5}$	$2·5 \times 10^{-4}$
CAG7190	None	3×10^{-7}	$1·5 \times 10^{-5}$	$1·5 \times 10^{-5}$
	mutD5	$4·5 \times 10^{-5}$	$1·4 \times 10^{-4}$	$1·4 \times 10^{-4}$

λpJH62 was mutagenized and infections done as described in Materials and Methods.

Table 3
In-vivo *phenotypes of the* rpoD*(Lac) mutants in a* Δcya *strain*

Representative *rpoD* allele	Sequence change	Map interval	No. of isolates	β-Gal units	β-Gal units *rpoD*(Lac)/*rpoD*$^+$	RNA (cts/min) *rpoD*(Lac)/*rpoD*$^+$
rpoD$^+$				88	1·0	1·0
rpoD901	SF389	1	5	306	3·5±0·4	4·6±0·1
rpoD920	DG570	2	2	114	1·2±0·2	0·8±0·3
rpoD912	DN570	2	1	335	3·8±0·2	4·5±0·1
rpoD904	YC571	2	1	275	3·1±0·2	3·9±0·2
rpoD903	EG575	2	1	150	1·6±0·2	1·4±0·3
rpoD911	EK575	2	2	415	4·7±0·2	4·5±0·0

The sequence change for each allele is shown as the 1-letter code for the wild-type amino acid, the mutant amino acid and the position in the σ^{70} polypeptide. β-Galactosidase activity and *lac* mRNA levels were measured in strains where only mutant σ^{70} was present. β-Galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units. The data shown are the average of at least 2 determinations. The level of *in-vivo lac* mRNA was measured by quantitative S_1 mapping as described in Materials and Methods; the data shown are the average of 2 experiments.

interval 2, which extends from amino acid residue 565 to the end of the gene.

Five mutations that mapped to interval 1 were sequenced and all had the same mutational change, Ser389 to Phe. Seven mutations mapping to interval 2 were sequenced. Five different mutations affecting three codons were identified (Asp570 to Gly, Asp570 to Asn, Tyr571 to Cys, Glu575 to Gly and Glu575 to Lys). We chose one isolate of each of the mutations for further study (Table 3). The amino acid change and position rather than the allele number will be used to identify each *rpoD*(Lac) mutation, for example, the Ser389 to Phe change caused by *rpoD901* will be referred to as SF389.

(c) *The* rpoD*(Lac) alleles are viable in the absence of* rpoD$^+$

The *rpoD*(Lac) alleles were selected on a phasmid in the presence of a wild-type *rpoD* gene. To determine if the *rpoD*(Lac) alleles could support cell growth, we inactivated the chromosomal *rpoD*$^+$ gene. Cells with the mutant phasmids were viable when the *rpoD*$^+$ gene was replaced with the unsuppressed *rpoD40* amber mutation (see Materials and Methods). In addition, four of the alleles (SF389, DN570, YC571 and EK575) have been transferred from the phasmid to the chromosome, so that mutant σ^{70} is the only form of σ^{70} present in the cell (see Materials and Methods).

(d) *The* rpoD*(Lac) mutations increase transcription from* P_{lac}

We quantified the effect of the *rpoD*(Lac) mutations on *lac* operon expression by measuring the level of β-galactosidase. The results from these experiments are shown in Table 3. Four of the alleles (SF389, DN570, YC571 and EK575) increase β-galactosidase activity three- to fivefold. The other two alleles, DG570 and EG575, have small, but reproducible, increases in β-galactosidase activity. These small increases may be sufficient to allow growth on lactose in the absence of cAMP. Alternatively, when the mutants are growing on M9 lactose plates there may be a larger increase in *lac* expression than what was seen when the mutants were growing in M9 glucose medium in liquid culture. Similar increases in β-galactosidase activity were seen in Δ*crp* derivatives of the *rpoD*(Lac) mutants (data not shown), showing that increased expression is independent of both cAMP and CAP.

We measured the level of *lac* mRNA to determine if the increase in *lac* expression was due to increased transcription. The three- to fivefold increase in the levels of *lac* mRNA observed in SF389, DN570, YC571 and EK575 was comparable to the increases in β-galactosidase levels in these strains (Table 3), indicating that the effect of these mutations is at the transcriptional level. We could not detect reproducible increases in the level of *lac* mRNA with alleles DG570 and EG575, which show only small increases in β-galactosidase expression.

The *rpoD*(Lac) mutations could increase transcription of the *lac* operon by increasing the frequency of transcription initiation at the promoter normally used by RNA polymerase or by directing RNA polymerase to a new promoter. To distinguish between these two possibilities, we determined the 5′ end of *in-vivo lac* mRNA by S_1 mapping. Figure 1 shows that the 5′ end of the *lac* message is the same in *rpoD*$^+$ and *rpoD*(Lac) strains lacking cAMP (Δ*cya*). Figure 1 also shows that in cells containing wild-type σ^{70}, the 5′ end of the *lac* message is the same both in the presence and absence of cAMP (compare lanes 1 and 2 with lane 3). We conclude that RNA polymerase containing the mutant σ^{70} subunits uses the same promoter as RNA polymerase containing wild-type σ^{70}.

(e) *The effects of cAMP and* lac*UV5*

Transcription of the *lac* operon in strains containing mutant σ^{70} is still stimulated by the addition of cAMP. However, in strains containing alleles SF389, DM570, YC571 and EK575 the three- to fivefold increase in *lac* operon expression observed in the absence of cAMP disappears (Table 4A). The level of β-galactosidase is actually

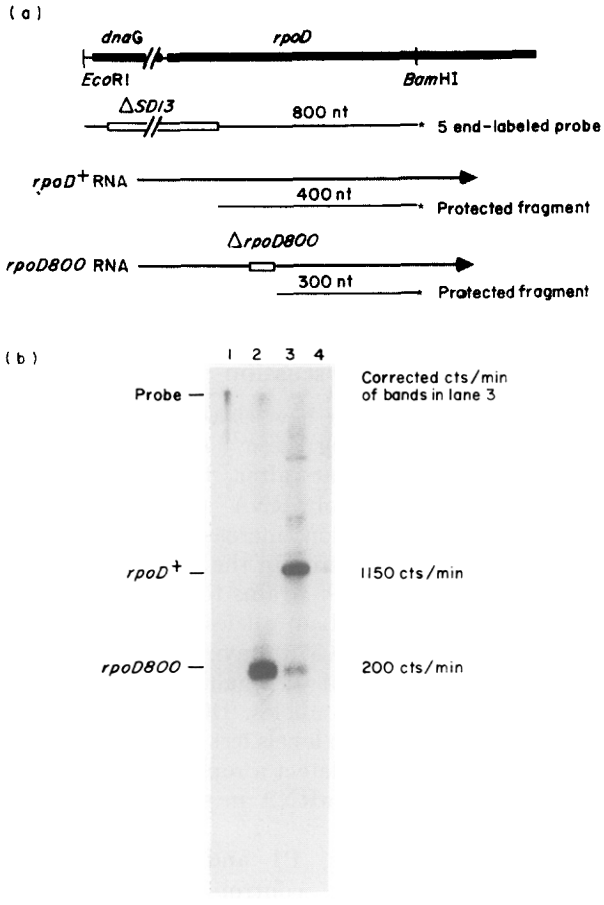

Figure 1. S_1 nuclease mapping the 5′ ends of the *in-vivo* *lac* transcript. The schematic shows the strategy for mapping the 5′ end of the *lac* transcript. The bottom portion is a restriction map of the *lac* promoter region. The P1 promoter (see Discussion) is drawn as an arrow to indicate the direction of transcription. Beneath the map the DNA fragment used for mapping, 5′-end-labeled at *Pvu*II (^{32}P indicated by a star) is shown along with the observed protection pattern. *lac* transcripts initiating from the P1 promoter should protect 145 nucleotides of the probe. *lac* transcripts initiation from the P2 promoter should protect 167 nucleotides of the probe. RNA isolated from phasmid-containing strains where the chromosomal *rpoD* gene was inactivated was hybridized to the 226 base-pair *Hpa*I–*Pvu*II fragment as described in Materials and Methods. The positions of full-length probe and the fragment protected by transcripts initiating at *lac* P1 are indicated. Lanes 1 and 2, 5 and 10 μg of RNA from CAG67 (*cya*$^+$ *rpoD*$^+$); lanes 3 and 11, 50 μg of RNA from CAG7270 (Δ*cya* *rpoD*$^+$); lane 4, 50 μg of RNA from CAG7272, Δ*cyaEG575*; lane 5, 50 μg of RNA from CAG7328, Δ*cyaYC571*; lane 6, 50 μg of RNA from CAG7329, Δ*cyaEK575*; lane 7, 50 μg of RNA from CAG7330, Δ*cyaDN570*; lane 8, 50 μg of RNA from CAG7331, Δ*cyaDG570*; lane 9, 50 μg of tRNA; lane 10, undigested probe; lane 12, 50 μg of RNA from CAG7327, Δ*cyaSF389*. DNA size markers were 5′-end-labeled *Hpa*II fragments from pBR322 (in base-pairs).

lower in strains containing *rpoD*(Lac) alleles DN570, YC571 and EK575 than in the *rpoD*$^+$ control strain in the presence of cAMP. There is only a 50% increase in the level of β-galactosidase when cells containing mutant allele SF389 are grown in the presence of cAMP. *rpoD*(Lac) alleles DG570 and EG575 cause only a small increase in β-galactosidase levels in the absence of cAMP (Tables 3 and 4A). When cells containing allele DG570 are grown in the presence of cAMP, the β-galactosidase levels are lower than in the *rpoD*$^+$ control strain. Mutant allele EG575 causes the same small increase in β-galactosidase levels both in the presence and absence of cAMP.

The *lac*UV5 mutation is a two base-pair change in the −10 region of P_{lac} that allows full expression of the *lac* operon in the absence of CAP–cAMP (Silverstone *et al.*, 1970; Reznikoff & Abelson, 1980). We examined the effects of four *rpoD*(Lac) alleles on transcription from this promoter. SF389, DN570, YC571 and EK575 do not increase transcription from *lacUV5* (Table 4B). In fact, the level of β-galactosidase is lower in strains containing these *rpoD*(Lac) mutations than in the *rpoD*$^+$ control strain. The increases in *lac* expression caused by SF389, DN570, YC571 and EK575 are not seen in the presence of cAMP or when transcription is from the *lacUV5* promoter.

(f) *The* rpoD*(Lac) mutations do not have general effects on catabolite-sensitive operons*

Guidi-Rontani *et al.* (1980) have isolated mutations in *rpoB*, the gene encoding the β subunit of RNA polymerase, that increase transcription from P_{lac} in the absence of CAP (Guidi-Rontani *et al.*, 1980; Guidi-Rontani & Spassky, 1985). These *rpoB* mutants also allow growth on arabinose and maltose, and increase expression of tryptophanase

Table 4
The effects of cAMP and lacUV5

A.		β-Galactosidase levels *rpoD*(Lac)/*rpoD*$^+$	
Strain	*rpoD* allele	−cAMP	+5 mM-cAMP
CAG7270	*rpoD*$^+$	1·0(89)	1·0(3525)
CAG7327	SF389	3·4±0·2	1·5±0·2
CAG7331	DG570	1·2±0·2	0·62±0·02
CAG7330	DN570	3·2±0·2	0·76±0·02
CAG7328	YC571	2·8±0·1	0·53±0·13
CAG7272	EG575	1·3±0·1	1·2±0·1
CAG7329	EK575	4·9±0·1	0·84±0·1

B.		β-Galactosidase levels *rpoD*(Lac)/*rpoD*$^+$	
Strain	*rpoD* allele	*lac*$^+$	*lacUV5*
CAG7420	*rpoD*$^+$	1·0(77)	1·0(4800)
CAG7426	SF389	3·2±0·2	0·8±0·09
CAG7444	DN570	3·3±0·10	0·8±0·03
CAG7432	YC571	4·0±0·5	0·6±0·01
CAG7438	EK575	3·3±0·2	0·6±0·03

β-Galactosidase assays were done as described in Materials and Methods. The assays were done using strains where only mutant σ^{70} was present. The *rpoD*(Lac)/*rpoD*$^+$ ratios are the average of at least 3 determinations. Values for *rpoD*$^+$ are arbitrarily set to 1·0. β-Galactosidase activity, expressed in units/cell O.D., is shown in parentheses for the *rpoD*$^+$ strains.

Table 5
Effect of the rpoD*(Lac) alleles on expression of the* araBAD *operon*

Strain	*rpoD* allele	β-Galactosidase levels *rpoD*(Lac)/*rpoD*$^+$
CAG17170	*rpoD*$^+$	1·0(36)
CAG17171	SF389	0·67±0·01
CAG17176	DG570	0·56±0·03
CAG17175	DN570	0·43±0·01
CAG17173	YC571	0·22±0·0
CAG17172	EG575	0·68±0·01
CAG17174	EK575	0·22±0·01

β-Galactosidase assays were done as described in Materials and Methods on log-phase cultures growing in M9 glucose containing 2% arabinose. The *rpoD*(Lac) mutations were carried on the phasmid, λpJH62, and *rpoD*$^+$ was present on the chromosome. The *rpoD*(Lac)/*rpoD*$^+$ ratios are the average of 2 determinations. Values for *rpoD*$^+$ are arbitrarily set to 1·0. β-Galactosidase activity, expressed in units/cell O.D., is shown in parentheses for the *rpoD*$^+$ strain.

in the absence of CAP. In contrast, the *rpoD*(Lac) mutations do not allow growth on arabinose or maltose, and do not increase expression of the *ara* or *mal* operons as judged by the phenotype on MacConkey indicator plates. We used a Mu dX *lacZ* fusion (Baker *et al.*, 1983) to measure expression from the *araBAD* promoter. None of the *rpoD*(Lac) alleles increases expression from P_{araBAD} (Table 5). In fact, in strains containing YC571 or EK575, expression of the *araBAD*::*lacZ* fusion is decreased fourfold. Thus, the *rpoD*(lac) alleles specifically increase expression from the *lac* promoter rather than causing a general increase in expression of catabolite-sensitive operons.

There is another difference in the phenotype of strains containing these two classes of RNA polymerase mutants. In strains containing the *rpoB* mutations, expression of β-galactosidase is high in succinate medium and low in glucose medium; the mutant strains still exhibit catabolite repression even though CAP protein is missing. To determine if strains with the *rpoD*(Lac) mutations also show this phenotype, we compared expression of the *lac* operon in Δ*crp* derivatives grown on glucose or succinate. Four of the six *rpoD*(Lac) alleles cause no or only a slight increase in β-galactosidase when glucose and succinate-grown cultures are compared (Table 6). Mutants SF389 and EK575 cause a 1·5-fold increase in β-galactosidase when cultures are grown on succinate, but this is lower than the fourfold increase seen in the *crp*$^+$ *rpoD*$^+$ control (Table 6). None of the *rpoD*(Lac) mutants shows wild-type levels of catabolite repression in the absence of CAP protein.

4. Discussion

We have described a new class of mutations in *rpoD*, the gene encoding the σ^{70} subunit of *E. coli* RNA polymerase, that alter the transcription initiation properties of RNA polymerase. The *rpoD*(Lac) mutants allow increased expression of genes in the lactose operon in the absence of cAMP or CAP, permitting such strains to grow on lactose. Four of the six *rpoD*(Lac) alleles isolated (SF389, DN570, YC571 and EK575) have three- to fivefold increases in the amount of β-galactosidase and *lac* mRNA. The other two alleles, DG570 and EG575, increase β-galactosidase levels less than twofold. We have not been able to detect a reproducible increase in the amount of *lac* mRNA in strains containing these two alleles.

Two promoter sites, P1 and P2, have been identified in the *lac* control region *in vitro* (Reznikoff *et al.*, 1982; Malan & McClure, 1984). However, P2 does not appear to be utilized *in vivo* (Peterson & Reznikoff, 1985). We have shown that *in vivo* the predominant *lac* transcripts initiate at P1 in both wild-type and *rpoD*(Lac) mutant strains in the absence of cAMP (Fig. 1). The mutant RNA polymerases use the same promoter as wild-type RNA polymerase, but mutant σ^{70} somehow allows that promoter to be utilized more efficiently.

We have compared the effects of both CAP–cAMP and the *lacUV5* mutation on transcription of the *lac* operon by RNA polymerase containing either wild-type σ^{70} or σ^{70} encoded by alleles SF389, DN570, YC571 or EK575. Transcription by the mutant RNA polymerases is stimulated by the

Table 6
The rpoD*(Lac) mutations do not restore catabolite repression*

Strain	Relevant genotype	β-Galactosidase activity in cultures grown in		
		M9 glucose	M9 succinate	Succinate/glucose
CAG7227	*crp*$^+$ λpJH62*rpoD*$^+$	936	3525	3·8
CAG7631	Δ*crp rpoD40*(Am) λpJH62*rpoD*$^+$	62	64	1·0
CAG7633	Δ*crp rpoD40*(Am) λpJH62-DN570	81	96	1·2
CAG7632	Δ*crp rpoD40*(Am) λpJH62-EG575	94	77	0·8
CAG7634	Δ*crp rpoD*$^+$	58	39	0·7
CAG7635	Δ*crp* SF389	203	313	1·5
CAG7638	Δ*crp* DN570	269	245	0·9
CAG7636	Δ*crp* YC571	246	210	0·8
CAG7637	Δ*crp* EK575	291	427	1·5

β-Galactosidase activity was measured as described in Materials and Methods and is expressed in units/cell O.D. (Miller, 1972).

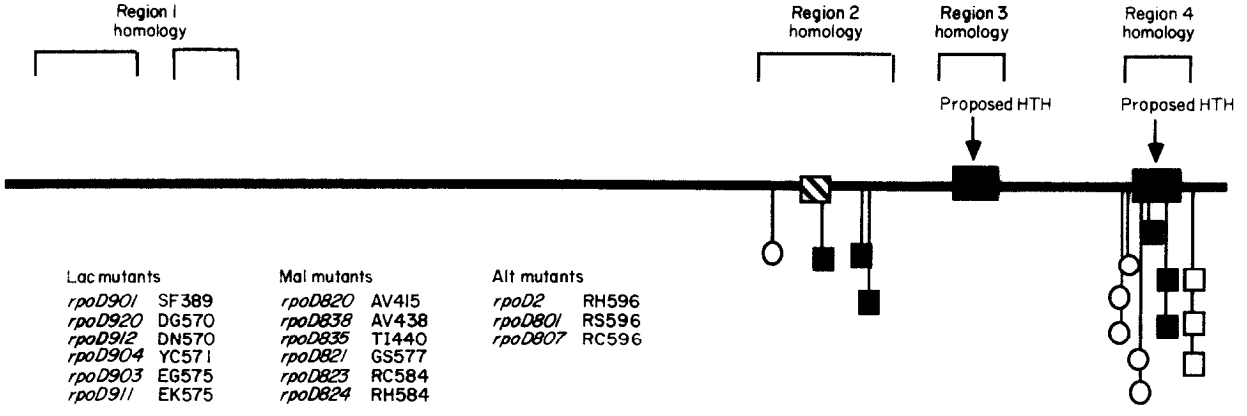

Figure 2. Summary of promoter recognition mutations in *rpoD*. A representation of the *rpoD* gene showing the positions of *rpoD*(Lac) (open circles), *rpoD*(Mal) (filled squares) and *rpoD*(Alt) (open squares) mutations. The key shows sequence changes for each allele as the 1-letter code for the wild-type amino acid, the mutant amino acid, and the position in the σ^{70} polypeptide. Regions homologous to other σ factors are indicated by brackets, using the nomenclature of Gribskov & Burgess (1986). Hatching indicates the 14 amino acid residues that are identical between σ^{70} and σ^{32}. The positions of 2 proposed helix-turn-helix (HTH) motifs are indicated by filled boxes.

addition of cAMP, but the three- to fivefold increase in β-galactosidase levels relative to *rpoD*$^+$, which is seen in the absence of cAMP, is not observed when cells are grown in the presence of cAMP (Table 4A). Similarly, these four *rpoD*(Lac) mutations do not increase the level of β-galactosidase when transcription is from the *lacUV5* promoter (Table 4B). In fact, cells containing σ^{70} encoded by three of these four alleles (DN570, YC571 and EK575) have lower levels of β-galactosidase when grown in the presence of cAMP or when transcription is from the *lacUV5* promoter than cells containing wild-type σ^{70}. The level of expression from *lacUV5* is also lower in cells containing allele SF389 than in *rpoD*$^+$ cells and, in the presence of cAMP, this allele causes only a 50% increase in the level of β-galactosidase relative to the *rpoD*$^+$ control.

Since the effects of SF389, DN570, YC571 or EK575 and CAP–cAMP or *lacUV5* are not additive *in vivo*, they may all affect the same kinetic step during transcription initiation. For a given promoter, either binding or isomerization can be the rate-limiting step that determines the number of transcripts initiated per cell cycle. Transcription of P_{lac} appears to be limited by the binding of RNA polymerase. CAP–cAMP increases the binding affinity of RNA polymerase for P_{lac} on both linear and supercoiled templates *in vitro* (Malan *et al.*, 1984). The *lacUV5* promoter mutation increases the isomerization rate when transcription is from a linear template, but on a supercoiled template its major effect is to enhance binding of RNA polymerase (Malan *et al.*, 1984). These four *rpoD*(Lac) mutations may be increasing transcription of the *lac* operon by increasing the affinity of RNA polymerase for the *lac* promoter. Alternatively, the *rpoD*(Lac) mutations may not increase *lac* expression in the presence of CAP–cAMP or when transcription is from *lacUV5*, because transcription is already proceeding at the maximal rate. *In-vitro* kinetic experiments are needed to distinguish these two possibilities.

(a) *Position of mutations within* rpoD

Two other classes of *rpoD* mutations have been described that increase expression from catabolite-sensitive operons. The *rpoD*(Alt) mutants increase expression of the *ara* regulon (Silverstone *et al.*, 1972; Hu & Gross, 1985). The *rpoD*(Mal) mutations increase expression of the *mal* regulon (Hu & Gross, 1988). Together, these classes comprise most of the *rpoD* mutations known to affect gene expression.

We compared the positions of these 15 *rpoD* mutations within the σ^{70} polypeptide (Fig. 2) to regions of amino acid sequence homology between various phage and bacterial σ factors (Stragier *et al.*, 1985; Gribskov & Burgess, 1986). Eleven out of 15 *rpoD* mutations that were selected to alter the promoter recognition properties of RNA polymerase are in or near a conserved region proposed to be a helix-turn-helix motif (Landick *et al.*, 1984; Yura *et al.*, 1984; Gitt *et al.*, 1985; Stragier *et al.*, 1985; Gribskov & Burgess, 1986; Dodd & Egan, 1987) based on amino acid sequence similarity with the known helix-turn-helix DNA-binding motif of proteins such as λ repressor, λ cro and CAP (Pabo & Sauer, 1984). The amino acid sequence of this region and the position of the *rpoD* mutations is shown in Figure 3. Several of the mutations are at positions in the helix-turn-helix motif that are predicted to be solvent-exposed and available to contact DNA.

The four *rpoD* mutations that map further upstream also lie within a region of homology between σ factors. Several authors (Landick *et al.*, 1984; Stragier *et al.*, 1985; Gribskov & Burgess, 1986) have speculated that this region contains conserved structures involved in binding of σ factors to core RNA polymerase. The occurrence of promoter recognition mutations in this region does

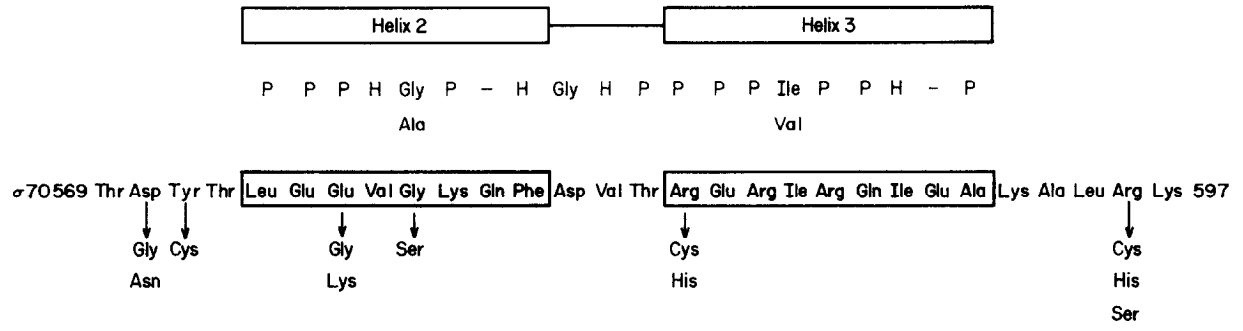

Figure 3. Amino acid sequence of a proposed DNA-binding domain in σ^{70}. The amino acid sequence of a proposed helix-turn-helix motif in σ^{70} is shown. The positions and amino acid substitutions of 11 *rpoD* mutations affecting promoter recognition are indicated. The residues conserved in other DNA-binding proteins (Pabo & Sauer, 1984; Dodd & Egan, 1987) are indicated above the sequence ((P, polar; H, hydrophobic).

not necessarily contradict these proposals. Even if the *rpoD* mutations in this region define direct contacts between σ^{70} and promoter DNA, this region of σ^{70} could also be involved in core binding. Several lines of evidence suggest that β and σ^{70} are near to one another and to the transcription startpoint (Stender *et al.*, 1975; Simpson, 1979; Chenchik *et al.*, 1981, 1982; Hanna & Meares, 1983; Ruetsch & Dennis, 1985; Bernhard & Meares, 1986). Alternatively, the effect of these *rpoD* mutations on promoter recognition could be indirect; the mutations could act by altering the conformation of σ^{70} or by altering contacts between the promoter and the core subunits.

We thank Robert Glass, Richard Losick, and members of the Gross Laboratory for helpful comments on the manuscript. This research was supported by the College of Agriculture and Life Sciences of the University of Wisconsin–Madison and National Institutes of Health grant AI9635 (C.A.G.) C.A.G. was supported by a Research Career Development Award from NIH. D.A.S. was supported by an NIH Research Service Award.

References

Baker, T. A., Howe, M. M. & Gross, C. A. (1983). *J. Bacteriol.* **156**, 970–974.

Berk, A. J. & Sharp, P. A. (1978). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 3331–3335.

Bernhard, S. L. & Meares, C. F. (1986). *Biochemistry,* **25**, 5914–5919.

Burgess, R. R. & Travers, A. A. (1970). *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **29**, 1164–1169.

Burgess, R. R., Travers, A. A., Dunn, J. J. & Bautz, E. K. F. (1969). *Nature (London),* **221**, 43–44.

Chenchick, A., Beabealashvilli, R. & Mirzabekov, A. (1981). *FEBS Letters,* **128**, 46–50.

Chenchik, A. A., Bibilashvili, R. Sh., Mirzabekov, A. D. & Shik, V. V. (1982). *Molek. Biol.* **16**, 26–36.

Dodd, I. B. & Egan, J. B. (1987). *J. Mol. Biol.* **194**, 557–564.

Doi, R. H. & Wang, L.-F. (1986). *Microbiol. Rev.* **50**, 227–243.

Fowler, R. G., Degnen, G. E. & Cox, E. C. (1974). *Mol. Gen. Genet.* **133**, 179–191.

Gitt, M. A., Wang, L.-F. & Doi, R. H. (1985). *J. Biol. Chem.* **260**, 7178–7185.

Gribskov, M. & Burgess, R. R. (1986). *Nucl. Acids Res.* **14**, 6745–6763.

Grossman, A. D., Erickson, J. W. & Gross, C. A. (1984). *Cell,* **38**, 383–390.

Guidi-Rontani, C. & Spassky, A. (1985). *J. Mol. Biol.* **86**, 527–532.

Guidi-Rontani, C., Danchin, A. & Ullman, A. (1980). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 5799–5801.

Hanna, M. M. & Meares, C. F. (1983). *Biochemistry,* **22**, 3546–3551.

Hawley, D. K. & McClure, W. R. (1983). *Nucl. Acids Res.* **11**, 2237-2255.

Hirschman, J., Wong, P.-K., Sei, K., Keener, J. & Kustu, S. (1985). *Proc. Nat. Acad. Sci., U.S.A.* **82**, 7525–7529.

Hu, J. C. & Gross, C. A. (1985). *Mol. Gen. Genet.* **199**, 7–13.

Hu, J. C. & Gross, C. A. (1988). *J. Mol. Biol.* **203**, 15–27.

Humphreys, G. O., Willshaw, G. A. & Anderson, E. S. (1975). *Biochim. Biophys. Acta,* **383**, 457–463.

Hunt, T. P. & Magasanik, B. (1985). *Proc. Nat. Acad. Sci., U.S.A.* **82**, 8453–8457.

Landick, R., Vaughn, V., Lau, E. T., VanBogelen, R. A., Erickson, J. W. & Neidhardt, F. C. (1984). *Cell,* **38**, 175–182.

Losick, R. & Pero, J. (1981). *Cell,* **25**, 582–584.

Malan, T. P. & McClure, W. R. (1984). *Cell,* **39**, 173–180.

Malan, T. P., Kolb, A., Buc, H. & McClure, W. R. (1984). *J. Mol. Biol.* **180**, 881–909.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maxam, A. & Gilbert, W. (1980). *Methods Enzymol.* **65**, 499–560.

Miller, J. (1972). *Experiments in Molecular Genetics,* p. 466, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mulligan, M. E., Hawley, D. K., Entriken, R. & McClure, W. R. (1984). *Nucl. Acids Res.* **12**, 789–800.

Pabo, C. O. & Sauer, R. T. (1984). *Annu. Rev. Biochem.* **53**, 293–321.

Perlman, R. L. & Pastan, I. (1969). *Biochem. Biophys. Res. Commun.* **37**, 151–164.

Peterson, M. L. & Reznikoff, W. S. (1985). *J. Mol. Biol.* **185**, 535–543.

Reznikoff, W. S. & Abelson, J. N. (1980). In *The Operon* (Miller, J. H. & Reznikoff, W. S., eds), pp. 221–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Reznikoff, W. S., Maquat, L. E., Munson, L. M., Johnson, R. C. & Mandecki, W. (1982). In *Promoters: Structure and Function* (Rodriguez, R. L. & Chamberlin, M. J., eds), pp. 80–95, Praeger Publishers, New York.

Reznikoff, W. S., Siegele, D. A., Cowing, D. W. & Gross, C. A. (1985). *Annu. Rev. Genet.* **19**, 355–387.

Ruetsch, N. & Dennis, D. (1985). *J. Biol. Chem.* **260**, 16310–16315.

Salser, W., Gesteland, R. F. & Bolle, A. (1967). *Nature (London)*, **215**, 588–591.

Schwartz, D. O. & Beckwith, J. R. (1969). In *The* lac *Operon* (Zipser, D. & Beckwith, J., eds), pp. 417–422, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*, pp. 272–282, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Silverstone, A. E., Arditti, R. R. & Magasanik, B. (1970). *Proc. Nat. Acad. Sci., U.S.A.* **66**, 773–779.

Silverstone, A. E., Goman, M. & Scaife, J. C. (1972). *Mol. Gen. Genet.* **118**, 223–234.

Simpson, R. B. (1979). *Cell*, **18**, 277–285.

Stender, W., Stütz, A. A. & Scheit, K. H. (1975). *Eur. J. Biochem.* **56**, 129–136.

Stragier, P., Parsot, C. & Bouvier, J. (1985). *FEBS Letters*, **187**, 11–15.

Youderian, P., Bouvier, S. & Susskind, M. M. (1982). *Cell*, **30**, 843–853.

Yura, T., Takashi, Y., Tobe, T., Ito, K. & Osawa, T. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 6803–6807.

Zubay, G., Schwartz, D. & Beckwith, J. (1970). *Proc. Nat. Acad. Sci., U.S.A.* **66**, 104–110.

Edited by M. Gottesman