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Genetic Studies of the *lac* Repressor

V.† Repressors which Bind Operator More Tightly Generated by Suppression and Reversion of Nonsense Mutations

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Different amino acid substitutions were generated at positions 3 and 61 in the *lac* repressor polypeptide chain by the suppression and reversion of nonsense mutations. Tyrosine in place of proline at position 3 results in a repressor which binds *lac* operator 100 times more tightly than wild-type repressor. Binding to DNA not containing the *lac* operator is also increased. A similar effect is seen for the serine to leucine exchange at position 61, which recreates the repressor resulting from a previously characterized missense mutation, *X86* (Chamness & Willson, 1970; Jobe & Bourgeois, 1972; Appendix to this paper). Tyrosine in place of serine at position 61 also causes a tight binding effect, but not as strong as in the case of the leucine replacement. A repressor containing tyrosine at position 3 and leucine at position 61 resulted from the construction of the appropriate double mutant. This repressor binds both operator and non-operator DNA extremely tightly, the affinities being increased approximately 10,000-fold over wild-type. The binding to non-operator DNA (such as λ DNA) is sensitive to isopropyl-β, D-thiogalactoside.

1. Introduction

Altered repressor molecules which bind more tightly than normal to operator regions may provide important clues concerning how proteins recognize DNA sequences. New contacts may occur between the DNA and the repressor which can be detected by physical methods. Also, stretches of DNA resembling operators might be detected, since the negligible affinity of these regions for wild-type repressor may be greatly enhanced by the increased binding properties of the altered repressor. The similarities in structure between these pseudo-operators could then be analyzed.

A considerable amount of work has been carried out on the *lac* repressor of *Escherichia coli* (see reviews by Müller-Hill, 1975; Bourgeois & Pfahl, 1976). One tight binding repressor, from strains carrying the *X86* mutation, has been extensively characterized (Chamness & Willson, 1970; Bourgeois & Jobe, 1972; Pfahl, 1976). The *X86* mutation was originally detected in cells with a partially constitutive level of β-galactosidase, which is repressible at certain concentrations of inducer (Chamness & Willson, 1970). This mutation has now been shown to cause at least a 50-fold increased

† Paper IV in this series is Coulondre & Miller (1977*b*).

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affinity for *lac* operator by the altered repressor (Jobe & Bourgeois, 1972). The affinity for DNA not carrying the *lac* operator is also increased (Pfahl, 1976). Sequencing studies have shown that the *X86* mutation results in a serine to leucine replacement at position 61 in the repressor polypeptide chain (Appendix to this paper).

We have examined an extensive set of nonsense mutations in the *lacI* gene (Coulondre & Miller, 1977*a*) and found that one amber site, *A10*, results in a UCG to UAG change in the codon specifying position 61 (Appendix to this paper). This allows the exchange, by suppression, of several different amino acids at position 61. In this report we compare the properties of the repressors produced by nonsense suppression with those of the *X86* repressor. Moreover, we describe here an additional mutation which leads to tight-binding repressors as a result of a proline to tyrosine change at position 3 in the protein chain. This new mutation, termed *I12*, has been recombined with the amber site *A10*. The resulting double mutation allows the comparison of repressors containing a tyrosine residue at position 3 and different substitutions at position 61.

2. Materials and Methods

(a) *Bacterial strains*

The strains are described in Table 1.

(b) *Media and chemicals*

Rich medium (LB), minimal medium (A) and lactose MacConkey medium are as described by Miller (1972). Xgal† glucose medium contains 40 μg Xgal/ml (Bachem). When added to minimal medium, nalidixic acid (Sigma) was present at 40 μg/ml, streptomycin at 200 μg/ml, rifampicin (Sigma) at 100 μg/ml and phenyl-β,D-galactoside (Sigma) at 750 μg/ml. The preparation of these plates has been described by Miller (1972). [^{14}C]IPTG at 25 mCi/mmol was purchased from Schwarz/Mann, and chicken blood DNA was obtained from Calbiochem.

(c) *β,D-Galactosidase assays*

Assays and units are as described by Miller (1972).

(d) *Purification of repressor*

Wild-type repressor was purified as described by Platt *et al.* (1973). The I12‡and I12-X86 repressors were purified by the same technique, except that a gradient ranging from 0·12 M to 0·3 M-potassium phosphate was applied to the phosphocellulose column, since these repressors bind more tightly to phosphocellulose than wild-type repressor. The fractions containing the IPTG binding activity were pooled and concentrated by ammonium sulfate precipitation and after dialysis against buffer A (50 mM-Tris (pH 7·4 at 24°C), 3×10^{-4} M-dithiothreitol, and 10% glycerol) applied to a 20 ml DEAE-cellulose column (DE52, Whatman) equilibrated with the same buffer. The repressor was eluted with a linear gradient made of 30 ml of buffer A and 30 ml of buffer A containing 0·3 M-KCl. The fractions containing the IPTG binding material were again pooled and concentrated by ammonium sulfate precipitation. The pellet was dissolved in 1 M-Tris (pH 7·4 at 24°C), 3×10^{-4} M-dithiothreitol and 30% (v/v) glycerol and stored at −20°C. The purity of the repressor was 90% as judged by SDS/polyacrylamide gels (Laemmli, 1970).

† Abbreviations used: Xgal, 5-bromo-4-chloro-3-indolyl-β,D-galactoside; IPTG, isopropyl-β,D-thiogalactoside; SDS, sodium dodecyl sulfate.

‡ We refer to the repressors resulting from the *I12* and *X86* mutations as I12 and X86 repressors.

(e) *Determination of repressor concentration*

The concentration of purified repressor was calculated after equilibrium dialysis against [^{14}C]IPTG (Gilbert & Müller-Hill, 1966) using a binding constant (K_D) for IPTG of 10^{-6} M, which was determined by Scatchard plots as described by Miller (1972).

(f) *Protein sequencing*

The amino-terminal sequence analysis of purified I12 repressor followed the procedure of Weiner *et al.* (1972).

(g) *Isolation of* ^{32}*P-labeled* lac *operator-containing fragments*

Plasmid DNA was extracted from an *E. coli* strain carrying a pMB9 plasmid with an insertion containing 2 *lac* operators between 2 *Eco*RI restriction sites (Tanaka & Weisblum, 1975) and further purified by gel filtration over a Bio-Gel 5 m column. (The bacterial strain was kindly supplied by L. Johnsrud.) The purified plasmid was treated with the restriction endonuclease *Eco*RI to cut out the fragment containing the 2 operators (280 ± 6 nucleotides long) and the 5′ ends of the fragments radioactivity labeled with ^{32}P (Maxam & Gilbert, 1977). The restriction fragments were run on a 7% acrylamide, 0·24% *N*,*N*-methylenebisacrylamide slab gel (16 cm × 16 cm × 0·33 cm, well surface 3 cm × 0·33 cm) in 50 mM-Tris-borate (pH 8·3), 1 mM-EDTA. The position of the 280 base-pair fragment was determined by an autoradiograph of the gel on X-ray film (Kodak NS-5T). The gel slice containing the 280 base-pair fragment was crushed and the DNA eluted by diffusion into 5 times the volume of the gel slice of 0·5 M-ammonium acetate, 0·01 M-magnesium acetate, 0·1 mM-EDTA. The DNA was separated from the polyacrylamide by passing the solution through a syringe, whose outlet had been plugged with siliconized glass wool to retain the polyacrylamide, then concentrated and desalted by ethanol precipitations. The 280 base-pair fragment was now split into the 2 operator-containing fragments (115 ± 2 and 165 ± 4 base-pairs) by cleavage with the restriction endonuclease *Hae*III (New England BioLabs). The completeness of the reaction was controlled by running a portion of the digest again on a 7% polyacrylamide slab gel and subsequent autoradiography on X-ray film. The equimolar mixture of the 2 operator fragments was used for DNA binding studies.

(h) *DNA binding*

The DNA binding assays and preparation of ^{32}P-labeled DNA from heat-inducible phage were carried out as described by Riggs *et al.* (1970*a*,*b*). The ionic strength of the binding buffer was 0·05 M, if not otherwise stated. The binding buffers did not contain dimethylsulfoxide: 0·2 M binding buffer contains 0·16 M-KCl. Samples were incubated for 45 min at 24°C and 0·5 ml was filtered through Sartorius membrane filters (SM 11356, 25 cm). The counts retained on the filter after filtering only the DNA (<8% of the input) were not subtracted.

(i) *Preparation of partially purified repressor*

Partially purified repressor was prepared following the method described by Jobe & Bourgeois (1972).

(j) *Isolation of mutants*

Lac constitutive mutants were isolated on minimal medium containing 0·75 g phenyl-β, D-galactoside/l using strain GM1, as reported previously (Miller *et al.*, 1977). F′*lacpro* episomes were then transferred to a set of isogenic suppressor strains to detect *lacI* nonsense mutations. A complete description of this procedure has been given by Miller *et al.* (1977). The amber mutation *A10* was derived spontaneously, and the ochre site *Y1* was generated by treatment with ultraviolet light. Details of the u.v. mutagenesis are described elsewhere (Coulondre & Miller, 1977*a*). The starting F′*lacpro* episome carried the I^q allele (which causes a 10-fold overproduction of the repressor; Müller Hill *et al.*, 1968) and the *lacP* mutation *L8* (which results in a 16-fold reduction in the rate of synthesis of the *lac*

enzymes; Scaife & Beckwith, 1966). For the experiments reported in this paper, the *L8* mutation was crossed out and the wild-type *lacP* region restored. This was carried out in the following manner. Each episome was crossed into a strain deleted for the beginning of the *lacI* gene (X7955). Although recombination can restore the normal *lacP* region onto the episome, the *lacI* mutation cannot be crossed out, since the deletion covers the respective portion of the *I* gene. This strain was used as a donor to transfer the episome to a strain deleted for the entire *lacproB* region (P90CN). The mating mixture was plated on Xgal glucose medium with nalidixic acid, selecting for Pro^+, Nal^r. Strains receiving the episome carrying the P^+ allele were distinguished by their deep blue color (frequency = 1/800). These were purified and tested for the retention of the original *lacI* nonsense mutation by transferring the episome into different suppressor strains.

(k) *Isolation of revertants from strains carrying* Y1

The ochre mutation *Y1* renders Su^- strains constitutive for the *lac* enzymes. The *lacpro* episome carrying *Y1* was transferred to strain T91, which carries the mutator gene *mutT*. An overnight culture of this strain was subcultured and mated with the recipient collector strain H3053 by mixing 0·5 ml of donor with 0·5 ml of recipient in a test-tube and placing on a roller drum at 20 revs/min for 60 min at 37°C. (The titer of recipients acquiring the episome was 4×10^7/ml.) H3053 contains a constitutive *lac* region in a *galE,recA* background (see Table 1). To select against the Arg^-, Str^s donor and to dilute out the β-galactosidase in the recipient, the mating mixture was grown for 10 generations in glucose minimal medium containing methionine, tryptophan, streptomycin, and 10^{-4} M-IPTG. IPTG was used since studies with the SuC derivatives of *Y1* showed that maximum repression occurred only in the presence of IPTG when tyrosine was inserted in response to the ochre codon. Because the UAA → UAC transversion is stimulated by *mutT* (Cox & Yanofsky, 1967; Miller *et al.*, 1977), tyrosine can appear at the position specified by the *Y1* ochre site by reversion with this mutagen. Dilutions were plated on glucose minimal medium containing methionine, tryptophan, 3×10^{-4} M-IPTG, and 0·5 g of phenyl-β, D-galactoside/l. In a $galE^-$ background, this medium prevents the growth of cells with a partial or full constitutive level of β-galactosidase (Davies & Jacob, 1968). In addition to revertants, both Z^- and GalK or GalT mutants will appear among the survivors. The *lacpro* episomes from surviving colonies were transferred into strain X7955 (i^-Z^+) to test for the ability to repress the *lac* operon in *trans* in the presence of 3×10^{-4} M-IPTG. Revertants with these properties appeared at a frequency of 10^{-6}. One of these, termed *I12*, was purified and extensively characterized.

(l) *Construction of double mutant carrying* A10 *and* I12

The heterodiploid depicted in Fig. 6 was constructed by transferring an F′*lacpro* episome carrying the *I12* mutation into a derivative of X7800 (see Table 1) carrying the *A10* mutation on the chromosome. This diploid was grown overnight, subcultured, and mated with P90CN by mixing 0·5 ml of donor with 0·5 ml of recipient and aerating on a roller drum at 20 revs/min for 60 min at 37°C. Dilutions were then plated on minimal medium containing 0·75 g phenyl-β,D-galactoside/l, nalidixic acid, and 2×10^{-4} M-IPTG. Only the P90N cells which became Pro^+, i^-Z^+ could grow on this medium. Because the episome carrying *I12* does not result in constitutive β-galactosidase synthesis in the presence of this concentration of IPTG, only cells receiving the episomes which had inherited the *A10* allele could grow. These were found at a frequency of 10^{-3}, several hundred-fold over the frequency of spontaneous i^- mutants. Approximately 300 colonies from the cross were purified, gridded onto master plates, and used as donors to transfer the recombinant episomes into different suppressor backgrounds (see Results).

(m) *Construction of heat-inducible prophage carrying* I12

The *lacpro* episome carrying *I12* was transferred to strain XA90N, which carries the wild-type *lac* region together with the I^Q mutation on a heat-inducible, lysis-deficient prophage. On lactose MacConkey indicator medium, *I12* (in the I^Q form) results in a Lac^- phenotype, even in the presence of a wild-type *I* gene. Therefore, such heterodiploids appear white on these plates. Because the homogenote *I12/I12* would also be

white on lactose MacConkey plates, it cannot be easily recognized. However, upon prolonged incubation every heterodiploid generates red sectors or papillae, which arise from the segregation of I^+/I^+ cells, and give the whole colony a slight rose-colored appearance. Homogenotes of the form *I12/I12* would not segregate such Lac$^+$ cells and would not, therefore, generate red papillae or sectors. For this reason we sought white colonies which, after prolonged incubation, remained white and did not throw off papillae. These were found at a frequency of 1 per 1000 colonies. After purification these colonies were used to transfer the *lacpro* episome to a strain deleted for the *lac* region to verify that it still carried the original mutations. The episome was, in addition, cured from the homogenotes with acridine orange, leaving a haploid strain carrying the *lac* region on a heat-inducible prophage. β-Galactosidase assays verified that the *I12* allele was present. This was further substantiated by sequence studies of repressor purified from this strain (see Results).

(n) *Preparation of strains carrying* I12 *and* A10 *on a heat-inducible prophage*

The haploid strain prepared in the preceding section (carrying *I12* on a prophage in the XA90N background) was used. The *lacpro* episome carrying both *I12* and *A10* was transferred to this strain. Homogenotes of the form *I12,A10/I12,A10* were selected from the *I12,+/I12,A10* heterogenote by plating on Xgal glucose indicator plates containing 10^{-4} M-IPTG. On these plates the desired homogenote will be blue, since the *A10/A10* combination renders the cell constitutive in an Su$^-$ strain. The starting heterodiploid appears white or pale blue due to the presence of the I12 repressor. Deep blue colonies were detected at a frequency of 1 per 200. These were purified and tested. The presence of the prophage was verified by heat sensitivity, and the *A10/A10* constitution was substantiated by the β-galactosidase induction profile, which showed constitutivity at all concentrations of IPTG tested.

Su6$^+$ derivatives of this strain were constructed by P1 transduction. Lysates of P1*vir* were made on XA106C (see Table 1) and transduction was carried out at 34°C selecting for Arg$^+$. Because the XA106C strain carries the same *argE* amber mutation as the XA90N derivative with *I12/A10* on the prophage, only transduction of the *sup* locus will result in Arg$^+$ transductants. Arg$^+$ colonies appeared at a 100-fold greater frequency than the control without P1. Several purified transductants were tested for heat sensitivity. To test for the presence of Su6, the prophage was cured by heat induction, and the episome was cured with acridine orange (Miller, 1972). F′*lacpro* episomes carry special *lacI* nonsense mutations which are suppressed only by specific suppressors (Coulondre & Miller, 1977*a*,*b*). In all of 5 cases tested, Su6 was easily identified as being the only suppressor present. The corresponding transductants (before prophage curing) were saved for biochemical analysis.

(o) *Mapping* Y1, A10 *and* I12

Both *Y1* and *A10* are nonsense mutations which result in the i$^-$ phenotype. These were tested for recombination with a set of *galE* strains carrying different deletions of the *lacI* region (see Schmeissner *et al.*, 1977). Based on the sequencing results reported in this paper and in the Appendix, the mapping results allow further correlations of the genetic and physical map. The *I12* mutation was mapped against a set of early deletions (*27*, *B80* and *196*) which had been converted to Gal$^+$ derivatives (details of this strain construction will be reported elsewhere). This enabled Lac$^+$ recombinants to be selected from the Lac$^-$ heterodiploids, as described previously (Miller *et al.*, 1968), since *I12* confers a Lac$^-$ phenotype on strains carrying it. Diploids were plated on lactose minimal medium and incubated at 37°C for 36 h. After replication onto the same medium, these plates were replicated onto Xgal glucose plates with and without IPTG. Lac$^+$ colonies which were inducible by IPTG were scored. Although somewhat leaky, the *I12* mutation can be mapped by this procedure. However, the reversion rate provides a limitation to the resolution of this method. Inducible revertants appeared at 2×10^{-7}. Although the *Y1* ochre site clearly maps in the interval between *B80* and *196*, the *I12* mutation can only be located in the larger interval between deletions *27* and *196*, since it fails to give recombinants over the reversion rate (which is 10^{-7}) with deletion *B80*. In any case, the

mapping of *112* demonstrates that the mutation responsible for the i^s property lies in the very beginning of the gene.

(p) *Attempted separation of a second mutation from* Y1

In the presence of SuC, *Y1* results in an i^r or i^s-like repressor and a Lac$^-$ phenotype. An experiment was designed to determine whether a second mutation in the *I* gene was responsible for the i^s property. The *lacpro* episome carrying *Y1* was transferred to strain X7996, which carries deletion *196* (Schmeissner *et al.*, 1977). This deletion fails to recombine with *Y1* (which has been shown by sequencing results reported in this paper to affect the codon for position 3) but does recombine with mutations in the codon specifying residue 5 in the repressor. This heterodiploid was used to transfer the *lacpro* episome to strain XA100C, which is deleted for the *lacproB* region and carries SuC (see Table 1). The object of this experiment was to detect recombinants which have lost a hypothetical second mutation located past the point specifying residue 5, provided this mutation causes the i^s property seen in SuC derivatives of *Y1* (acting either independently or in concert with *Y1*). Such recombinants would be Lac$^+$. Therefore, selection was on lactose minimal medium containing methionine and nalidixic acid (to counterselect against the donor). A control cross was done using a donor carrying the *Y1* mutation on the episome, but with no other *lac* region on the chromosome. Lac$^+$ colonies were gridded and replicated on Xgal glucose plates with and without IPTG to score for inducible colonies. These occurred at a frequency of 4×10^{-7} on plates for both the control cross and the cross against deletion *196*. The failure to detect Lac$^+$, i$^+$ recombinants above the reversion frequency strongly suggests that the mutation responsible for the i^s character of *Y1* in SuC lies before the point specifying residue 5, since the deletion *196* recombines with mutations specifying this residue with frequencies significantly higher than 4×10^{-7} (Schmeissner *et al.*, 1977).

(q) *Construction of a strain carrying* X86 *on a heat-inducible prophage in combination with* I^Q

An F′*lac* episome carrying *X86* (kindly supplied by Dr G. Chamness) was introduced into strain P90. Xgal glucose plates were used to detect recombinants which had higher constitutive levels of β-galactosidase, due to the presence of the *X86* allele on both the chromosome and the episome. These were separated into two classes based on their reaction with lactose tetrazolium plates. The strains appearing deeper red (more Lac$^-$) also had lower constitutive levels of β-galactosidase. These were assumed to carry the I^Q allele on the chromosome in combination with X86, which was verified by measuring repressor levels after induction of the prophage (Jobe & Bourgeois, 1972). By curing the prophage and examining the remaining F′*lac* episome, the I^+,*X86* constitution of the episome was ascertained. The I^Q,*X86* mutations were put onto an F′*lacpro* episome by transferring the episome from GM3 into the lysogen described above and detecting Lac$^-$ colonies on lactose MacConkey medium (which still carried the F′*lacpro* factor) after curing of the prophage at 42°C. In the absence of the chromosomal *lac* region, only those strains carrying I^Q,*X86* on the episome will appear Lac$^-$ and still be Pro$^+$. β-Galactosidase assays at different concentrations of IPTG verified the genotype of the episome.

3. Results

(a) *Substitutions at position 61*

A set of closely isogenic strains carrying different nonsense suppressors has been prepared during the course of this work. We have examined in these strains the induction of β-galactosidase over a series of IPTG concentrations in the presence of episomes carrying amber site *A10*. This mutation results in chain termination at the site normally specifying serine in position 61 of the *lac* repressor. In response to the suppressors Su1, Su2, Su3 and Su6, the respective amino acids serine, glutamine, tyrosine and leucine will occupy position 61 in the repressor polypeptide chain.

TABLE 1

Bacterial strains

Strain	Sex	Genetic markers
P90CN	F⁻	*ara Δ(lacpro) nalA*
T91	F⁻	*ara Δ(lacpro) mutT metB argE-am rif*
S90C	F⁻	*ara Δ(lacpro) strA*
X7026r	F⁻	*Δ(lacpro) supE recA*
XA100	F⁻	*ara Δ(lacpro) nalA metB argE-am rif*
XA101	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supD*
XA102	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supE*
XA103	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supF*
XA105	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supG*
XA106	F⁻	*ara Δ(lacpro) nalA metB argE-am rif sup6*
XA10B	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supB*
XA10C	F⁻	*ara Δ(lacpro) nalA metB argE-am rif supC*
XA90	F⁻	*ara Δlacpro nalA argE-am rif*
XA91,2,3,5, 6, B and C	F⁻	XA90 with *supD,E,F,G,6,B*, and *C*, respectively
X7800	F⁻	*ara Δ(lacpro) galE strA* (*ϕ*80d*lac*) *val*ʳ
X7800-1,2, etc.	F⁻	Derivatives of X7800 with *tonB* deletions extending into *lacI* on the *ϕ*80d*lac*; thus, X7955 carries *Δ155*, and X7996 carries *Δ196*.
XA90N	F⁻	*ara Δ(lacpro) nalA argE-am rif* (*λcI857St68*h80d*lac*); the *lac* region on the phage carries the I^Q mutation
P90	F⁻	*ara Δ(lacpro)* (*λcI857St68*h80d*lac*); the *lac* region on the phage carries the I^Q mutation
H3053	F⁻	*ara val*ʳ *Δ(lacpro) galE strA recA nalA supF metB argE-am rif* (*ϕ*80d*lac*) *ΔtonB-trpA-lacI*
GM1	F′*lacpro*†	*ara Δ(lacpro)*
GM3	F′*lacpro*†	*ara Δ(lacpro) mutT metB rif argE-am strA*
—	F′*lac*‡	$\Delta(lac)_{RV}$
XAN1	F⁻	Identical to XA90N, but *lacI* region on phage carries *A10*
XAN2	F⁻	*sup6* derivative of XAN1
XAN3	F′*lacpro*(I^Q,*I12*)	Same as XA90N, but both the phage and episome carry *I12*
XAN4	F⁻	Episome cured derivative of XAN3
XAN5	F′*lacpro*(I^Q,*I12*,*A10*)	Same as XA90N but both phage and episome carry *I12* and *A10*
XAN6	F′*lacpro*(I^Q,*I12*,*A10*)	Same as XAN5 but also *sup6*
PA1	F′*lac*(*X86*)‡	*ara Δ(lacpro)* (*λcI857St68*h80d*lac*); phage carries I^Q,*X86*
GM300	F′*lacpro*(I^Q,P^+)	*ara Δ(lacpro)*
GM301	F′*lacpro*(I^Q,*A10*)	*ara Δ(lacpro)*
GM302	F′*lacpro*(I^Q,*Y1*)	*ara Δ(lacpro)*
GM303	F′*lacpro*(I^Q,*I12*)	*ara Δ(lacpro)*
GM304	F′*lacpro*(I^Q,*I12*,*A10*)	*ara Δ(lacpro)*
GM305	F′*lacpro*(I^Q,*X86*)	*ara Δ(lacpro)*
GM306	F′*lac*(*X86*)‡	*ara Δ(lacpro)*

All strains presumably carry the *thi* marker.

† *lac* region on the episome carries the I^Q mutation (Müller-Hill *et al.*, 1968) and the P^- mutation, *L8* (Scaife & Beckwith, 1966).

‡ *lac* region on the episome carries the *X86* mutation (Chamness & Willson, 1970).

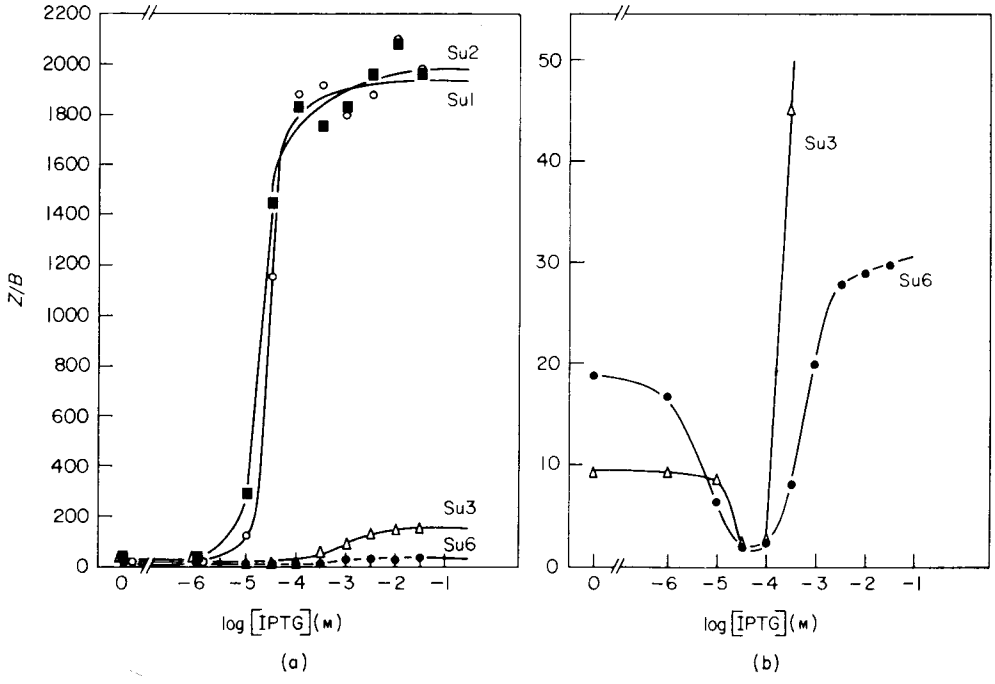

FIG. 1. (a) The β-galactosidase induction curve at 37°C is shown as a function of increasing IPTG concentration resulting from *A10* in the presence of different amber suppressors. Units are as given by Miller, 1972. (b) An expansion of the region at the lower part of the activity scale. The *A10* mutation is carried on an F'*lacpro* episome in combination with I^q. The suppressor strains are given in Table 1. (○) Su1; (■) Su2; (△) Su3; (●) Su6.

Figure 1(a) shows the induction curves for this set of strains. Whereas both serine (the wild-type amino acid at this point) and glutamine result in a normal induction profile, both tyrosine and leucine lead to repressors which are not fully induced, even at high concentrations of IPTG (3×10^{-2} M). A closer examination of these latter two curves (Fig. 1(b)) indicates that low concentrations of IPTG increase repression (maximum repression occurring near 3×10^{-5} M-IPTG), while higher concentrations result in a small but incomplete induction. This is the reported behavior for strains carrying *X86* (Chamness & Willson, 1970), a missense mutation resulting in a serine to leucine change at position 61 (Appendix to this paper). Figure 2 compares the effects on the induction curve of the serine to leucine change at residue 61 produced by nonsense suppression (*A10* in Su6) or by missense mutation (*X86*). Both mutations are shown in combination with promoters of different strengths (see legend to Fig. 2) resulting in different levels of repressor being synthesized. This gives a family of curves with minima centered around 3×10^{-5} M-IPTG. Different curves for strains carrying *X86* have already been reported at two different repressor concentrations (Jobe & Bourgeois, 1972). This extends these observations to five different concentrations, and shows the strict dependence of these curves on repressor level. It can be seen that for levels of synthesis of repressor which are nearly equivalent, the Su6 derivative of *A10* gives the same induction profile as strains carrying *X86*.

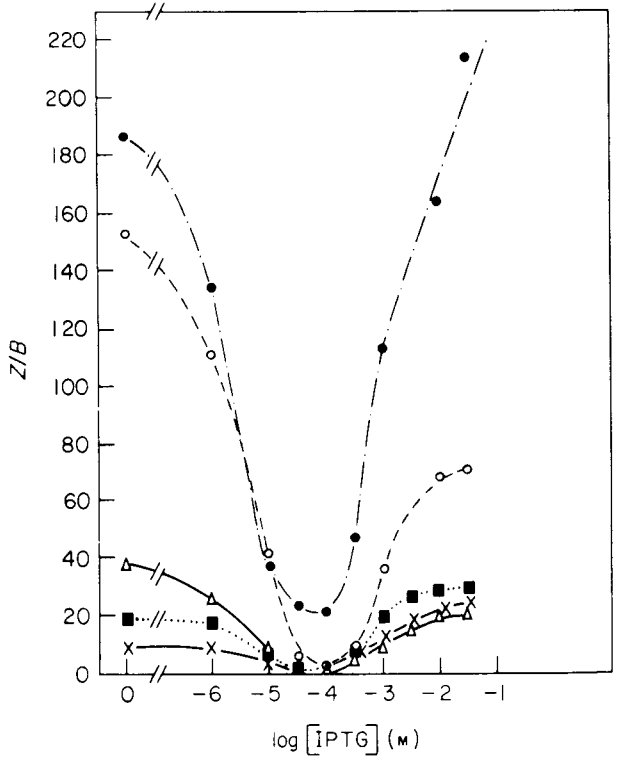

FIG. 2. β-Galactosidase induction curves are shown as in Fig. 1. All assays were carried out at 37°C, except for the P90 heterodiploid, which was assayed at 30°C. The I^Q mutation results in a 10-fold overproduction of repressor relative to wild type (Müller-Hill *et al.*, 1968) due to a single base change in the *I* promoter (Calos, 1978). The Su6 suppressor in XA106C results in 70% suppression of *A10*. (●) F′*lacpro*(I^+,*A10*)/Su6; (■) F′*lacpro*(I^Q,*A10*)/Su6; (○) F′*lac*(I^+ *X86*)/*RV*; (×) F′*lacpro*(I^Q,*X86*)/P90CN; (△) F′*lac*(I^+ *X86*)/*P90*(I^Q,*X86*).

Because the *X86* repressor has been shown to have a greatly increased affinity for *lac* operator (Jobe & Bourgeois, 1972), DNA binding experiments were performed (Fig. 3, Table 2). It can be seen that both the *X86* repressor and that from the *A10*/Su6 strain give identical dissociation rates. In each case the half-life of the repressor–operator complex is increased 100-fold compared to wild type. Based on these data and the β-galactosidase induction curves mentioned above, we conclude that the entire phenotype resulting from *X86* can be attributed to the serine to leucine change at residue 61. Any additional mutations in strains carrying *X86* (acting either independently or in concert with *X86*) do not have a detectable effect on the properties of the repressor.

When tyrosine occupies position 61, the repressor also binds operator with increased affinity (Fig. 3, Table 2), although not as strongly as the leucine-substituted protein. As Table 2 indicates, a very slight but reproducible increase in the dissociation time is also seen with glutamine at position 61. However, this small change is clearly not sufficient to cause a detectable alteration in the induction profile (Fig. 1(a)).

TABLE 2

Half-life of repressor–operator complex

Mutations on F'*lacpro* factor	Suppressor background	Half-life of complex (min)
I^q, *A10*	Su1	16
I^q, *A10*	Su2	25
I^q, +	Su$^-$	17
I^q, +	Su1	15
I^q, +	Su2	17
I^q, +	Su3	20
I^q, +	Su6	20

The half-life of the repressor–operator complex was determined as described in the legend to Fig. 3, except that the binding buffer was 0·05 M. All strains were from the XA100 series, except the Su6 derivative, which was from the XA90 series and the Su$^-$ derivative, which was GM1 (see Table 1). The F'*lacpro* episome was from GM1, and carried the amber mutation *A10* in the first 2 entries in the Table. In strains carrying *A10*, Su1 restores wild-type repressor, whereas Su2 results in the replacement of serine by glutamine at position 61 in the repressor.

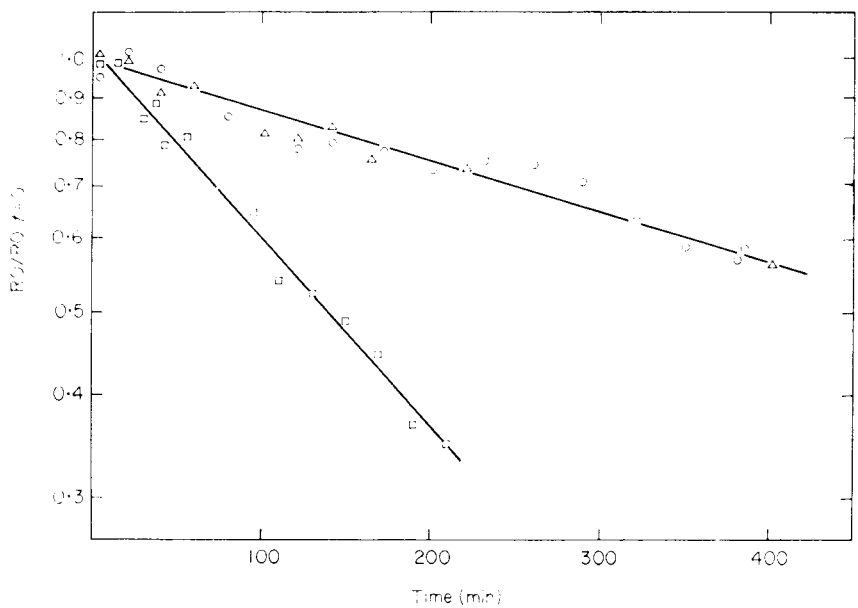

FIG. 3. Dissociation kinetics of the repressor–operator complex formed by repressor molecules with amino acid substitutions at position 61. Sufficient amounts of the partially purified repressors of the *X86* and the suppressed derivatives of the *A10*-carrying strains were added to a λ*plac*^{5} solution (5×10^{-12} M) in 0·2 M-binding buffer containing 7 μg chicken blood DNA/ml to give approx. 80% saturation of operator with repressor. After incubating for 45 min at 24°C, unlabeled λ*plac*^{5} DNA was added (100-fold excess) and, at the times indicated, triplicate 0·5 ml samples were filtered through Sartorius membrane filters at a rate of 0·6 ml/min per cm^2 and washed once with binding buffer (Riggs *et al.*, 1970*a*,*b*). Parallel samples in binding buffer with 5×10^{-3} M-IPTG were treated the same way to determine the background, which was subtracted for each point. Control experiments, where cold DNA instead of λ*plac*^{5} DNA was added showed no repressor–operator (RO) dissociation during the time-course of the experiments. Purified repressor from strains carrying *X86* (○), *A10*/Su3 (□), *A10*/Su6 (△). The ionic strength of the binding buffer was at 0·2 M.

(b) *Exchanges at position 3*

(i) *Isolation and sequencing*

One of the ochre (UAA) mutations detected in the *lacI* gene after u.v. mutagenesis maps between markers specifying amino acids 2 and 5 (Coulondre & Miller, 1977*a*). This ochre site, designated *Y1*, should therefore be derived from either the codon for proline 3 (CCX) or valine 4 (GUX). Although this conversion would require a change of at least the first two bases in each respective codon, such tandem double base changes are stimulated by ultraviolet irradiation (Coulondre & Miller, 1977*b*). In the presence of suppressors inserting glutamine or lysine (SuB and Su5, respectively), strains carrying *Y1* have normal induction curves for β-galactosidase. However, in the presence of the tyrosine-inserting ochre suppressor SuC, induction is not achieved by 10^{-4} to 10^{-3} M-IPTG. This property was exploited in selecting for revertants which synthesize active repressor in an Su^- strain in the presence of IPTG (see Materials and Methods). Revertants were selected after growth in a strain carrying *mutT*, a mutator gene which specifically stimulates the A·T → C·G transversion (Cox & Yanofsky, 1967; Miller *et al.*, 1977). As can be seen from Figure 4(a), this will result in one of three amino acids (glutamic acid, serine, or tyrosine) now appearing at the position originally specified by the ochre site. (From the suppression pattern described above, it was considered likely that only tyrosine-containing revertants would be found by the selection procedure used.)

A revertant, termed *I12*, was isolated and purified. The *I12* mutation was crossed

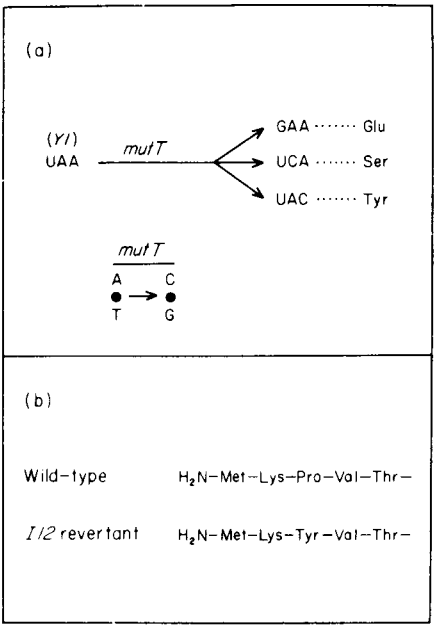

FIG. 4. (a) The amino acids expected to appear in response to *mutT*-stimulated reversion of an ochre codon, in this case *Y1*. Only the A·T → C·G transversion is induced by *mutT* (Cox & Yanofsky, 1967; Miller *et al.*, 1977). (b) The first 5 amino acids of both wild-type repressor and the I12 repressor. The *I12* mutant was derived by *mutT*-induced reversion of *Y1*. A tyrosine residue appears at position 3 of the I12 protein, in place of proline.

onto a heat-inducible prophage (see Materials and Methods), and the respective lysogen was used to prepare *lac* repressor. The *I12* repressor was purified by ammonium sulfate fractionation followed by phosphocellulose chromatography. The amino-terminal end was sequenced by Edman degradation and compared to that of wild-type repressor, which was purified and sequenced in parallel. As previously reported (Platt *et al.*, 1972; Adler *et al.*, 1972; Beyreuther *et al.*, 1973) the sequence of the first five residues of wild-type repressor was found to be H_2N-Met-Lys-Pro-Val-Thr-. However, the I12 repressor gave the sequence H_2N-Met-Lys-Tyr-Val-Thr-. Because the *I12* mutation represents a conversion to a sense codon from an ochre (UAA) triplet, this result demonstrates that the original ochre mutation arose from a CCX → UAA change at position 3 (where X is any of the four nucleotides), and suggests that the proline at position 3 is normally encoded by CCA. Steege (1977) and P. J. Farabaugh (unpublished results) have sequenced the initial portions of the *lacI* mRNA and DNA, respectively, and found that the CCA codon indeed specifies position 3 in the protein. We conclude, therefore, that ochre site *Y1* arises from the tandem double base change converting CCA → UAA, and that the suppression of *Y1* with SuC leads to the production of a repressor which, like the I12 repressor, has a tyrosine residue in place of proline at position 3.

(ii) *Properties or strains carrying* I12 *or* Y1

Figure 5(a) shows the β-galactosidase induction curve for strains carrying either the SuC-suppressed *Y1* mutation, or the *I12* mutation. Here we see somewhat different curves than those for strains carrying *X86*, in that maximum repression occurs at a higher concentration of IPTG (near 3×10^{-4} M as compared to 3×10^{-5} to 1×10^{-4} M for strains carrying *X86*). Moreover, very high concentrations of IPTG increase the enzyme levels only slightly (compare Figs 2 and 5(a)). The DNA binding curves are shown in Figure 5(b). Both the I12 repressor, and that from the SuC-suppressed *Y1* derivative bind operator approximately as tightly as the X86 repressor (see below). Therefore, equally tight binding molecules can be derived by certain amino acid substitutions at either position 3 or 61. Possible reasons for the differences in the induction curves are considered in a later section.

(c) *Construction of the double mutant containing both the* I12 *and the* A10 *mutations*

Do exchanges of amino acid residues at positions 3 and 61 increase the specific contacts between repressor and operator? If this is the case, or if each of these mutations acts independently by some other mechanism, then repressor molecules carrying substitutions at both positions might actually bind operator extremely tightly, the

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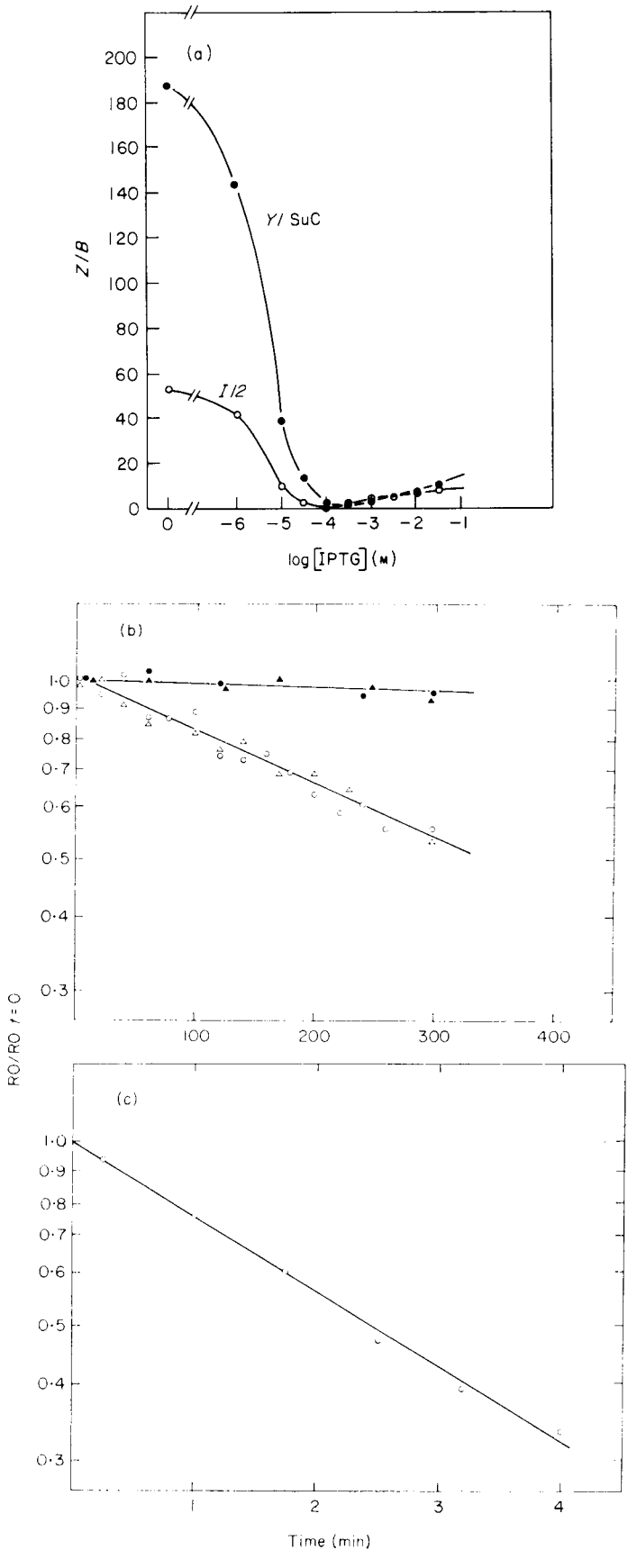
FIG. 5. (a) β-Galactosidase induction curves similar to those shown in Figs 1 and 2. F'*lacpro* episomes carrying either *Y1* (●) or *I12* (○) in combination with I^Q were used in either SuC (XA10C) or P90CN, respectively. (b) and (c) Dissociation kinetics of repressor–operator complex formed by partially purified repressor of strains carrying *Y1* and SuC, or *I12*, as given for (a). The experiments are described in the legend to Fig. 3. (b) Repressor purified from *Y1*/SuC (△); control experiment (competition with unlabeled λ DNA) (▲). Repressor from strains with *I12* (○); control experiment (●). (c) Dissociation kinetics of *I12* repressor–operator complex in the presence of 10^{-2} M-IPTG. This experiment was performed as described by Jobe & Bourgeois (1972).


(a)
Y/ SuC
I12
Z/B
log [IPTG] (M)
(b)
RO/RO t=0
(c)
Time (min)

affinity increase being the product of each respective increase. It was therefore of interest to construct the double mutations carrying both *I12* (resulting in a proline to tyrosine exchange at position 3) and *A10*, the amber mutation in the codon specifying position 61. Figure 6 depicts the diploid constructed for this purpose. A *lacpro* episome carrying the mutations I^Q, *I12* and *lacP-L8* was crossed into a strain carrying the amber mutation *A10* on the chromosome. This heterodiploid was used as a donor to transfer the episome into a strain carrying the *lacproB* deletion *X111*, and the mating mixture was plated on medium selecting for i^- cells (see Materials and Methods). As Figure 6 shows, four loci in each of two allelic states come into play in this construction, resulting in eight possible classes of recombinants. These are tabulated as four main groups, each being divided into two subgroups based on the segregation of the *lacP* mutation (Table 3). The frequency of each class will depend to a large degree on the relative sizes of the intervals between markers. The only selection in this cross is for i^-, this phenotype being generated by the recombination of the chromosomal *A10* mutation onto the episome. There is no selection bias involved as long as the "collector" strain is Su^-. All episomes inheriting the *A10* mutation will

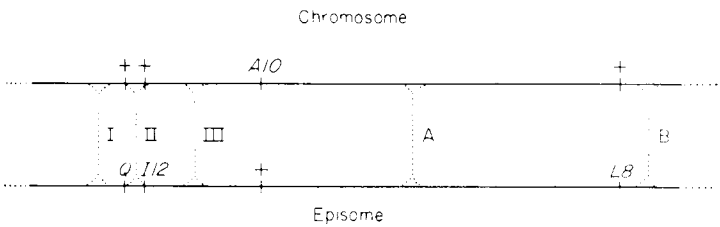


FIG. 6. A schematic representation of the cross producing the I^Q, *I12*, *A10* triple mutation is given. The F'*lacpro* episome carried I^Q, *I12* and *L8*, and the chromosome of the X7800 derivative carried *A10*. All episomes resulting in the selected phenotype (i^-) in the Su^- collector strain (see Results, section (c)) must inherit *A10 via* a cross-over on the left (at points I, II, or III) and also on the right (at A or B) of *A10* as shown. Each of these events produces different arrangements of the 4 sets of alleles which are involved. The results are presented in Table 3.

result in equally constitutive strains, regardless of the segregation of the other markers. Also, because the episome is transferred to a recipient strain deleted for the *lac* region, the genotype of the donor chromosome after the recombination event is unimportant.

Each class of recombinant can be easily recognized by transferring the recombinant episome into different suppressor strains. (The level in an Su^- strain enables us to distinguish P^+ from P^- strains.) By using Su1 derivatives we can differentiate between episomes carrying *A10*, and those carrying *A10* in combination with *I12*. In the former case, the insertion of serine recreates wild-type repressor, while in the latter case, the I12 repressor is generated. These can be recognized easily by their induction profiles, as has been shown in previous sections. The differentiation between I^Q and I^+ can be made by the level of β-galactosidase under conditions of maximal repression (see Fig. 2), and verified by direct repressor assays *in vitro* (Gilbert & Müller-Hill, 1966). The results of the cross are shown in Table 3.

(d) *Properties of the double mutants carrying* I12 *and* A10

Figure 7 shows the β-galactosidase induction curve for strains carrying the *I12-A10* recombinant episome in the presence of different amber suppressors. In Figure 7(a), Su1, Su2, Su3 and Su6 are shown. The Su1 strain inserts serine, recreating the *I12* mutant, and therefore has the same induction curve as the strains carrying *I12*. Su3 and Su6 result in slightly different curves, which are considered below.

Figure 7(b) and (c) shows logarithmic plots of the β-galactosidase levels against the IPTG concentrations used. In (b) the Su6 derivatives of both *A10* and *I12-A10* are shown, with the Su1 strain carrying *A10* and *I12* included for comparison. Because suppression of *A10* by Su6 recreates X86, the suppression of the *I12-/A10* derivative by Su6 will generate an I12-X86 repressor. This results in a curve that is clearly different from either "parent" curve. The levels of enzyme in the absence of inducer are much higher than for the *A10*/Su6 strain, although repressor assays *in vitro* show that similar levels of repressor are produced (data not shown). In fact, the constitutive level is 30 to 35% of the maximal level for this strain. Moreover, the addition of increasing amounts of IPTG continue to repress, even at very high IPTG concentrations. No detectable induction occurs, even at 3×10^{-2} M-IPTG.

A comparison of *I12-A10* in Su3 strains with the curves for *A10* alone in this background (see Fig. 7(c)) shows the same differences as found for the Su6 derivatives.

(e) *Properties of purified* I12 *repressor*

We measured the half-life of the I12 repressor–operator complex using λ p*lac*[5] DNA. The dissociation kinetics (Fig. 5(b)) show that partially purified repressor from strains *I12* and the SuC-suppressed *Y1* display a half-life of the repressor–operator complex of about 350 minutes in 0·2 M-binding buffer, which is similar to the 480 minutes measured for the X86 repressor (Fig. 3; Jobe & Bourgeois, 1972). The half-life of 150 seconds for the I12 repressor–operator complex in the presence

TABLE 3

Cross of X7800 (A10) × *F′* (I^q, I12, L8)

Recombinant class	Genotype of episome	Number found
IA	I^+, +, *A10*, P^+	262
IB	I^q, +, *A10*, P^-(*L8*)	14
IIA	I^q, +, *A10*, P^+	1
IIB	I^q, +, *A10*, P^-(*L8*)	2
IIIA	I^q, *I12*, *A10*, P^+	11
IIIB	I^q, *I12*, *A10*, P^-(*L8*)	8

The results of the cross illustrated in Fig. 6 are shown. The selection was for those episomes receiving the *A10* allele, which results in the i^- phenotype in the Su^- collector strain. The recombinant episomes carry either I^q or I^+, *I12* or +, and P^+ or P^-(*L8*), depending on which intervals the crossovers occurred in. The frequency of inheritance of each allele is in part dependent on the size of the respective intervals. A total of 299 i^- colonies resulting from this cross were examined. One colony arose from a spontaneous i^- mutation and was discarded.

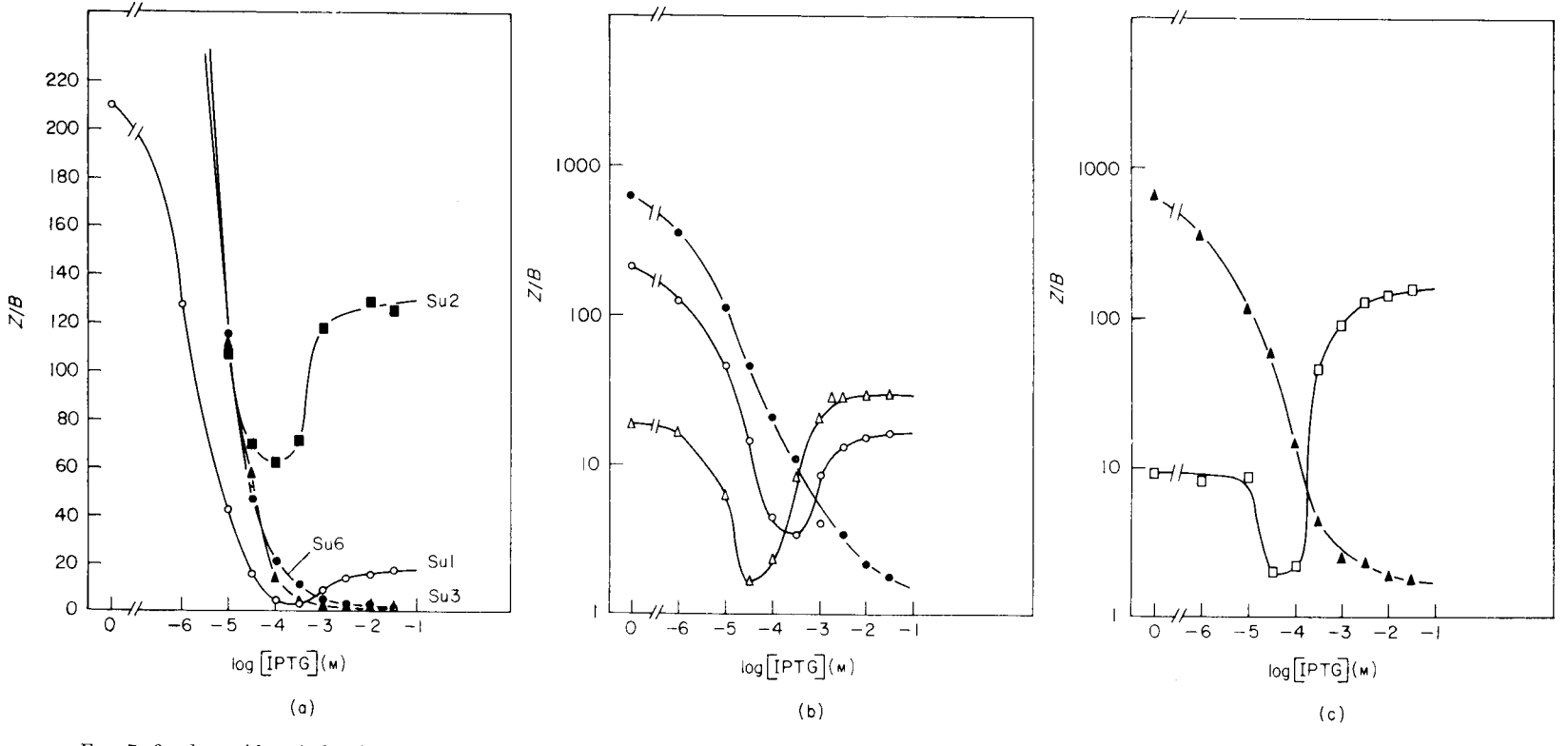


FIG. 7. β-galactosidase induction curves are shown, as in Figs 1 and 2. The F'*lacpro* episome carrying *I12* and *A10* in combination with I^Q was used together with different suppressors (a); see Table 1). (b) and (c) The β galactosidase activity is plotted logarithmically. The episome carrying only *A10* was used for comparison in (b) and (c). F'*I12,A10*/Su1 (○); F'*I12,A10*/Su2 (■); F'*I12,A10*/Su3 (▲); F'*I12,A10*/Su6 (●); F'*A10*/Su3 (□); F'*A10*/Su6 (△).

of 0·01 M-IPTG is also similar to the 180 seconds obtained using X86 repressor (Fig. 5(c); Jobe & Bourgeois, 1972).

The I12 repressor has concomitant with its increased affinity for operator a strong "non-operator" DNA binding compared to wild-type repressor (Fig. 8). The striking aspect of this binding is that it is partially IPTG-sensitive. This is a unique property of the I12 repressor, since wild-type repressor does not display IPTG-sensitive binding to λ DNA not containing the *lac* region (data not shown). Such binding for X86 repressor has not been reported.

The IPTG-insensitive binding to λ DNA is not the result of a reduced affinity for IPTG, since Scatchard plot determinations of the IPTG binding constant give values identical to wild type ($K_D = 10^{-6}$ M). (We cannot rule out the possibility that the increased IPTG-insensitive binding to λ DNA is due to a contaminent copurifying with the I12 repressor, or due to a decrease in the DNA binding activity in the wild-type repressor preparation used.)

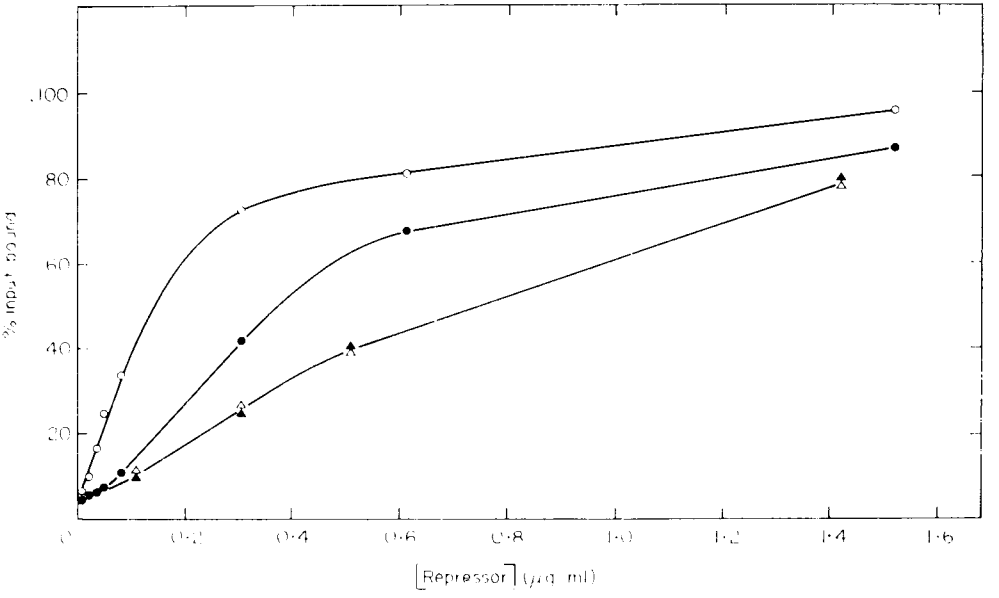


FIG. 8. Binding of wild-type and *I12* repressor to λ DNA. The DNA binding assays were done as described in Materials and Methods. The concentration of *λcI857S7* DNA was 4×10^{-12} M. Binding of purified *I12* repressor (○); with 5×10^{-3} M-IPTG (●). Binding of purified wild-type repressor (△); with 5×10^{-3} M-IPTG (▲).

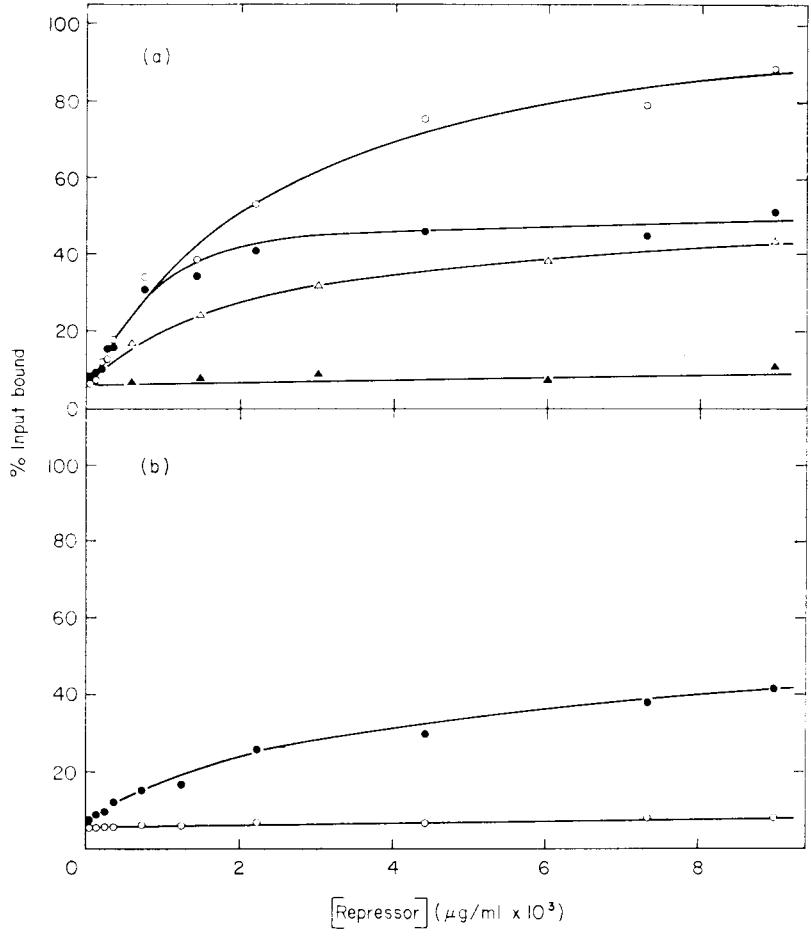
(f) *Properties of the purified I12-X86 repressor*

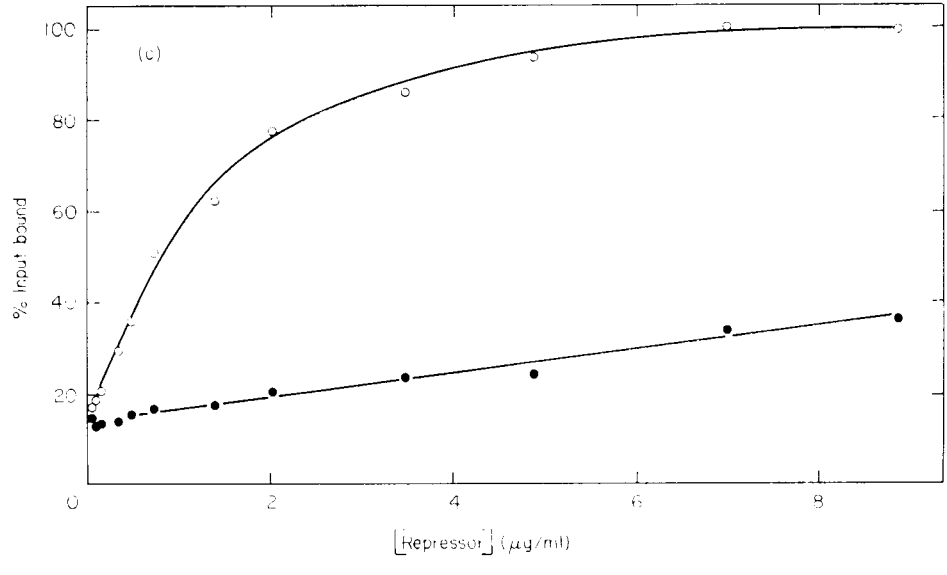
(i) *IPTG binding*

The IPTG binding constant of the I12-X86 repressor was determined by Scatchard plots and is the same as for wild-type repressor ($K_D = 10^{-6}$ M).

(ii) *Studies with λ plac*^{5} *DNA*

Figure 9(a) shows the binding of purified I12-X86 repressor to λ *plac*^{5} DNA. Some aspects of this binding are unusual. (1) In the absence of IPTG, 90% of the input


(a)
(b)
% Input bound
100
80
60
40
20
0
2
4
6
8
[Repressor] (μg/ml x 10³)


(c)
% Input bound
100
80
60
40
20
0
2
4
6
8
[Repressor] (μg/ml)

DNA is retained on the filters despite the low repressor concentrations used. With similar low repressor concentrations, wild-type repressor never retains more than 50% of the input DNA. (2) The binding curve in the *presence* of IPTG resembles the curve of operator binding of wild-type repressor obtained in the *absence* of IPTG.

Figure 9(b) depicts the same experiment as shown in Figure 9(a), but this time in the presence of an excess (330-fold) of chicken blood DNA over ^{32}P-labeled λ p*lac*^{5} DNA. In the absence of IPTG no binding of repressor to λp*lac*^{5} DNA is observed. However, with IPTG present, the repressor retains DNA on the filters. This type of excess of chicken blood DNA has a negligible effect on the binding of wild-type repressor to operator (Jobe *et al.*, 1972). The binding in the presence of IPTG displayed by the I12-X86 repressor is specific for operator, since unlabeled λp*lac*^{5} and not λ DNA competes for this binding (data not shown).

Taken together, these data allow us to draw the following conclusions. Like both the I12 repressor and the X86 repressor, the doubly altered repressor possesses an increased affinity for non-operator DNA. This is indicated by the fact that it binds to λp*lac*^{5} DNA at concentrations of repressor where no binding of wild-type repressor to $\lambda\phi$80 DNA can be detected (Riggs *et al.*, 1968), and non-operator DNA, like chicken blood DNA, competes for this binding. (We demonstrate this more directly in a subsequent section.) Also, IPTG either increases the operator affinity of the I12-X86 repressor, or else it decreases the operator affinity less than it does for non-operator DNA.

Is the operator binding of the I12-X86 repressor sensitive at all to IPTG? To answer this question the ionic strength of the binding buffer was increased to 1·04 M- (containing 1 M-KCl) to reduce the repressor–non-operator DNA interaction to such an extent that it would not be a factor in this filter binding assay. The results are shown in Figure 9(c). Since the increase of the ionic strength of the binding buffer also weakens the operator binding of the repressor (Riggs *et al.*, 1970*a*,*b*), high concentrations of I12-X86 repressor were necessary to retain operator DNA on the filter. The addition of excess chicken blood DNA does not interfere significantly with the binding of I12-X86 repressor to λp*lac*^{5} DNA under these conditions (data not shown).

(iii) *Binding to λ DNA*

The interaction of I12-X86 repressor with non-operator DNA could be shown directly by binding studies using λ DNA (Fig. 10). Up to 85% of the input DNA is bound to the filters in an IPTG -sensitive manner. Some binding is observed even in the presence of IPTG. The possible significance of these findings is considered in the Discussion.

(iv) *Binding to operator fragments*

^{32}P-labeled *lac* operator-containing fragments were isolated in order to characterize the I12-X86 repressor–operator interactions more precisely by minimizing the

FIG. 9. Binding of purified I12-X86 repressor to λp*lac*^{5} DNA. The DNA binding assays were performed as described in Materials and Methods. ^{32}P-labeled λp*lac*^{5} DNA was $2{\cdot}5 \times 10^{-12}$ M, and IPTG was 4×10^{-3} M in the reaction mixture. (a) DNA binding of I12-X86 repressor in 0·05 M-binding buffer; without IPTG (○); with IPTG (●). DNA binding of purified wild-type repressor with IPTG (▲); without IPTG (△). (b) DNA binding in the presence of 25 μg chicken blood DNA/ml, without IPTG (○); with IPTG (●). (c) DNA binding in 1·04 M-binding buffer (1 M-KCl), without IPTG (○); with IPTG (●).

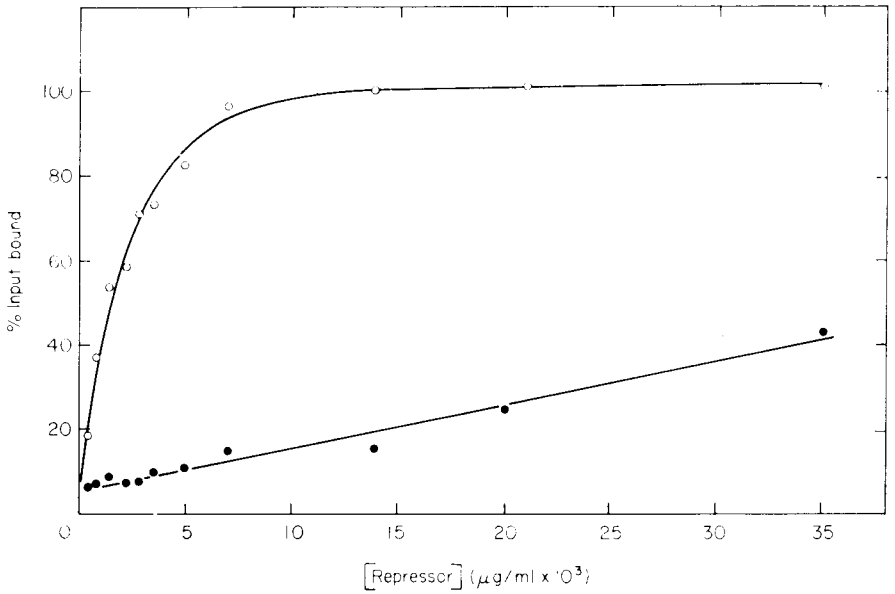


FIG. 10. Binding of purified I12-X86 repressor to λ DNA. The DNA binding assays were carried out as described in Materials and Methods. ^{32}P-labeled λ*cI857S7* DNA was $1{\cdot}6 \times 10^{-12}$ M, IPTG was 4×10^{-4} M in the reaction mixtures. DNA binding of I12-X86 repressor without IPTG (○); and with IPTG (●).

interference of high concentrations of non-operator DNA with respect to operator DNA (see Materials and Methods). The fragments were 115 ± 2 and 165±4 nucleotides long, and an equimolar mixture was used for the DNA binding studies. The decay of the I12-X86 repressor complex formed with the operator on these fragments was used as a criterion for the repressor–operator affinity. The residual non-operator DNA does not interfere with the operator interaction of the I12-X86 repressor (in contrast to the situation shown in Fig. 9(a), where whole λp*lac*^{5} DNA was used) and relevant data can now be obtained. Figure 11 illustrates the half-life of the repressor–operator fragment complex formed by I12-X86 and X86 repressor. The I12-X86 repressor does not dissociate to any detectable degree from the operator during three hours in 0·2 M-binding buffer. The X86 repressor–operator complex, which was used as a reference, has a half-life of 90 minutes under these conditions. These experiments were also performed in the presence of IPTG. In this situation the I12-X86 repressor–operator complex dissociates with a half-life of about 50 minutes, while the complex formed with X86 repressor decays too fast to be measured by this technique (<15 seconds).

Jobe & Bourgeois (1972) measured the half-life of the X86 repressor–operator complex and obtained a value of 480 minutes using whole λ*φ*80d*lac* DNA in 0·2 M-binding buffer. They calculated a half-life of 1920 minutes in 0·05 M-binding buffer, assuming the same dependence on ionic strength of the dissociation rate of X86 and wild-type repressor–operator complex. IPTG decreases the half-life of the X86 repressor–operator complex in 0·05 M-binding buffer by a factor of 640 to a value of about three minutes (Jobe & Bourgeois, 1972). If this factor holds true for the interaction of the X86 repressor with the operator-containing fragments used in this study, then the half-life of the X86 repressor–operator complex in the presence of IPTG

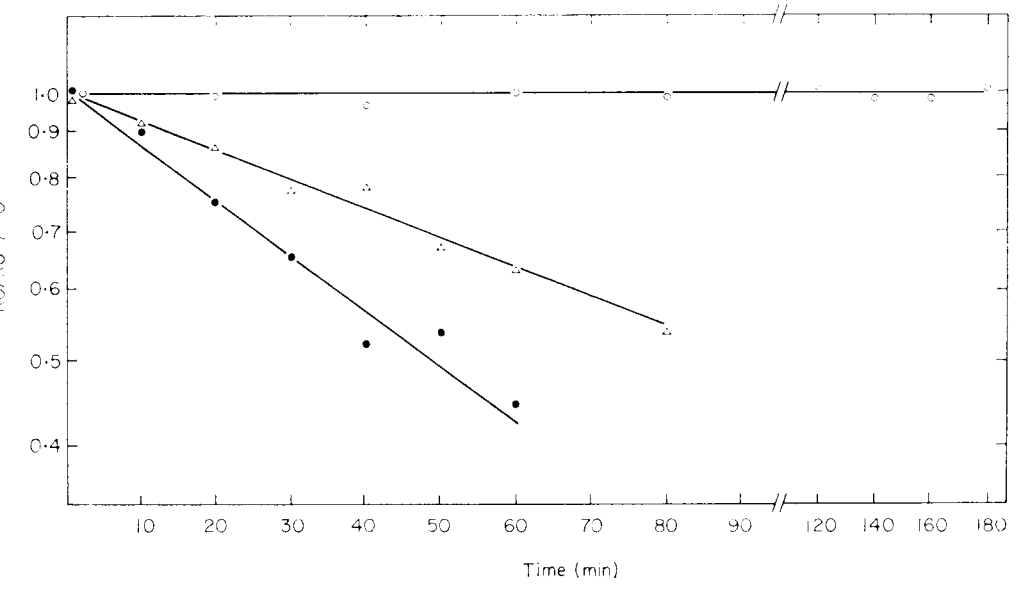


FIG. 11. Dissociation kinetics of repressor–operator fragment complex formed by purified X86 and I12-X86 repressor. The experiments were done as described in the legend to Fig. 3. In the case of the I12-X86 repressor, the counts retained on the filter when unlabeled λ*plac*^{5} DNA was added to the [^{32}P]DNA assay before the addition of repressor (and filtered) were subtracted from each point, since the I12-X86 repressor–operator complex did not dissociate completely in the presence of IPTG. Unlabeled λ*plac*^{5} DNA, but not λ DNA, competed for the binding of the I12-X86 repressor to the operator-containing fragments. The DNA fragments were isolated as described in Materials and Methods and were approx. 20 pM in 0·2 M-binding buffer. No chicken blood DNA was present. I12-X86 repressor (○); I12-X86 repressor in the presence of 4×10^{-3} M-IPTG (●); X86 repressor (△).

decreases to 8·5 seconds, and the operator affinity of the I12-X86 repressor in the presence of IPTG is increased by a factor of 350 compared to the X86 repressor (Fig. 11).

4. Discussion

lac repressors with greatly increased affinity for the *lac* operator are of considerable interest for the study of repressor–operator interactions. However, such altered molecules are rare, and only a few mutations resulting in tight binding repressors have been described (Jobe & Bourgeois, 1972; Betz & Sadler, 1976). The largest increase in binding (50 to 100-fold over wild-type) has been reported for the X86 repressor (Chamness & Willson, 1970; Jobe & Bourgeois, 1972), which leads to the serine to leucine change at position 61 of the repressor polypeptide chain (Appendix to this paper). We have utilized the suppression of nonsense mutations to generate altered *lac* repressor molecules (Miller *et al.*, 1975*a*,*b*). Amber site *A10* is derived from the codon for amino acid 61 (see Appendix) and we can therefore replace serine by either glutamine, tyrosine, leucine or lysine, simply by employing strains carrying the respective suppressors; Su2, Su3, Su6 and Su5. This permits a comparison of different replacements at the same position in the protein. Both tyrosine and leucine at position 61 result in tight binding repressors, and as described in Results, the

repressor produced by the suppression of *A10* by Su6 is identical in its properties to the X86 repressor.

A new tight binding repressor resulting from SuC-mediated suppression of the ochre mutation *Y1* is reported in this paper. *Y1* is derived from the codon normally specifying proline at position 3, and the replacement of proline by tyrosine at this point in the protein creates a molecule with similar properties to X86, operator binding being increased by 50 to 100-fold. Reversion of this ochre site also produced a tight binding repressor. This was induced by the action of *mutT* (Cox & Yanofsky, 1967; Miller *et al.*, 1977) and presumably generated the UAC codon. Sequence analysis established that the revertant, termed *I12*, synthesizes a repressor with tyrosine in place of proline at position 3. These manipulations underscore the flexibility provided by the use of nonsense mutations, since exchanging proline for tyrosine at a CCA codon would require three base changes by conventional mutagenesis.

We obtained a doubly altered repressor carrying a proline to tyrosine substitution at position 3 and a serine to leucine exchange at residue 61. This was achieved by using genetic recombination to construct a mutant carrying both *I12* and *A10*, and employing the leucine-inserting suppressor strain Su6. We used a method which permitted the detection of recombinant episomes carrying both *I12* and *A10* independent of the properties of the double mutant (see Fig. 6, and Results, section (c)). This eliminates any selection bias arising from the predicted properties of the desired double mutant.

In addition to its increased affinity for *lac* operator, the I12 repressor also shows increased binding to λ DNA, which is partly IPTG-sensitive (Fig. 8), and which is strong enough to be seen in equilibrium binding experiments. The I12-X86 repressor binds to both operator and non-operator DNA significantly tighter than either the X86 or I12 protein. Its binding to λ DNA can be calculated to be at least 280 times greater than wild type. This binding is sensitive to IPTG. In the presence of IPTG, the affinity of the doubly altered repressor is increased by a factor of 350 to *lac* operator over that displayed by the X86 repressor, and greater than 10,000-fold compared to wild-type repressor. Binding to operator in the absence of inducer is so strong for the doubly altered repressor that quantitative comparison with wild type or X86 is difficult. Even using operator fragments of 115 and 165 base-pairs, the I12-X86 repressor–operator complex does not dissociate under conditions where the X86 repressor–operator complex has a half-life of 90 minutes.

The induction behavior of the respective mutants can be interpreted in light of the increased binding and the explanation proposed by Pfahl (1976) for the *X86* mutant. The increase in non-operator DNA binding of the I12 repressor slows down the association rate of the I12 repressor and the *lac* operator, so that a fraction of the repressor molecules is trapped on the non-operator DNA in a growing cell culture, leading to constitutivity (von Hippel *et al.*, 1974). Low inducer concentrations increase the effective association rate of the repressor and operator by weakening the repressor–non-operator interactions and increasing the rate of dissociation from these sites. This leads to an increase in repression of β-galactosidase. Higher inducer concentrations decrease the operator binding of the repressor to such an extent that induction can now be observed. This induction is only partial, because the affinity of IPTG-saturated repressor for operator is still high enough to result in some repression. However, the induction profile of β-galactosidase in the *I12-X86* double mutant differs from that obtained for either *I12* or *X86* alone (Fig. 7) in the high level of enzyme seen in the

absence of IPTG and the gradual restoration of complete repression proportional to increasing IPTG concentration. No induction occurs, even at 3×10^{-2} M-IPTG. This reverse effect of IPTG can be demonstrated *in vitro*, since IPTG increases the binding of the I12-X86 repressor (5-fold over the background) to $\lambda plac^5$ in the presence of large amounts of DNA not containing the *lac* operator (in this case chicken blood DNA). This effect could not be shown as clearly in the case of the X86 repressor, since only a 30% increase was found when *E. coli* DNA was used as the source of non-operator DNA (Pfahl, 1976).

Experiments were started to determine whether the binding to regions of the DNA not containing the *lac* operator is sequence-specific, by measuring the IPTG-sensitive retention of *Hin*dII restriction endonuclease fragments of λ DNA on nitrocellulose filters. Preliminary results indicate that the binding of the I12-X86 repressor to these fragments does display sequence specificity. This repressor, therefore, may be a useful tool for isolating pseudo-operators. A comparison of the conserved sequence elements in a family of such pseudo-operators should contribute to a greater understanding of the repressor–operator interaction.

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APPENDIX:

Direct Identification of the Amino Acid Changes in two Mutant *lac* Repressors

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The sites of mutations in the *lacI* gene of *Escherichia coli* can be determined by direct identification of amino acid sequence changes in the mutant *lac* repressor gene product. The identification of these amino acid changes allows correlations to be made between the changes of function of the altered proteins and the particular amino acid residues involved. The specific proteolytic digestion of the amino terminus of the *lac* repressor polypeptide chain facilitates the identification of amino acid changes in this region of mutant repressor molecules. Tryptic digestion of *lac* repressor under native conditions specifically removes the first 59 amino acid residues, in five tryptic peptides, leaving a trypsin-resistant "core" molecule (Platt *et al.*, 1973). By the isolation and sequence analysis of these released peptides we have identified several mutations in this region of the *lac* repressor molecule (Weber *et al.*, 1972; Files *et al.*, 1974).

We have recently shown that the trypsin-resistant core has a unique amino-terminal sequence corresponding to amino acid residues 60 and beyond of the intact *lac* repressor polypeptide (Files & Weber, manuscript in preparation). Purified tryptic core can be sequenced directly, either by manual or by automated techniques, providing addition sequence data for *lac* repressor mutants. By sequencing tryptic core prepared from an Su3 (tyrosine-suppressed) derivative of *lacI* amber mutant *XA2*, we have recently identified the site of this mutation at the codon for residue 62 of wild-type repressor (Files *et al.*, 1975). We describe here the identification, by the