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Enhanced expression of cro-β-galactosidase fusion proteins under the control of the P_R promoter of bacteriophage λ

Marc Zabeau and Keith K. Stanley

European Molecular Biology Laboratory, Postfach 102209, D-6900 Heidelberg, FRG

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Hybrid plasmids carrying *cro-lacZ* gene fusions have been constructed by joining DNA segments carrying the P_R promoter and the start of the *cro* gene of bacteriophage λ to the *lacZ* gene fragment carried by plasmid pLG400. Plasmids in which the translational reading frames of the *cro* and *lacZ* genes are joined in-register (type I) direct the synthesis of elevated levels of cro-β-galactosidase fusion protein amounting to 30% of the total cellular protein, while plasmids in which the genes are fused out-of-register (type II) produce a low level of β-galactosidase protein. Sequence rearrangements downstream of the *cro* initiator AUG were found to influence the efficiency of translation, and have been correlated with alterations in the RNA secondary structure of the ribosome-binding site. Plasmids which direct the synthesis of high levels of β-galactosidase are conditionally lethal and can only be propagated when the P_R promoter is repressed. Deletion of sequences downstream of the *lacZ* gene restored viability, indicating that this region of the plasmid encodes a function which inhibits the growth of the cells. The different applications of these plasmids for expression of cloned genes are discussed.

Key words: *cro* ribosome binding site/expression vector/fusion protein/β-galactosidase/gene fusion

Introduction

The ability to manufacture novel proteins by fusing together the coding sequences of different genes is a technique of growing importance in molecular genetics. For instance, various eucaryotic genes have been expressed in *Escherichia coli* by fusing the coding sequences of cloned genes to segments of well known *E. coli* genes. The *lacZ* gene coding for the enzyme β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) has been used extensively in operon and gene fusions, since earlier studies have shown that hybrid β-galactosidase proteins in which the first amino acids are replaced by the amino-terminal part of other proteins retain enzymatic activity (Muller-Hill and Kania, 1974; Silhavy *et al.*, 1976). This property was used to develop an *in vivo* selection procedure for isolating gene fusions encoding functional hybrid enzymes (Casadaban, 1976). Plasmids carrying truncated *lacZ* genes which encode enzymatically active carboxy-terminal fragments have recently been constructed, allowing any gene to be fused to the *lacZ* gene using *in vitro* recombination techniques (Casadaban *et al.*, 1980; Guarente *et al.*, 1980).

We have used one of these plasmids, pLG400 (Guarente *et al.*, 1980), to construct a series of plasmids carrying the beginning of the *cro* gene of bacteriophage λ fused to the *lacZ* gene, in which the expression of the hybrid gene is controlled by the P_R promoter of phage λ. Here we describe the properties of the *cro-lacZ* gene fusion plasmids which direct the synthesis of enhanced levels of β-galactosidase, and the construction of improved vectors for expression of cloned genes.

Results

Construction of cro-lacZ fusions

We have isolated a series of hybrid plasmids in which segments of variable length bearing the beginning of the *cro* gene of phage λ were fused to a fragment of the *lacZ* gene which encodes an enzymatically active carboxy-terminal fragment of β-galactosidase. The various steps in the construction are outlined in Figure 1. Plasmid pLK5 contains a 180-bp fragment of the phage λ chromosome bearing the P_R promoter and the first 96 bp of the *cro* gene. *Cro* gene segments of random length were generated by linearizing pLK5 at the *Bam*HI site 350 bp downstream of the *cro* gene and then resecting the linear DNA with the nuclease *Bal*31 (Gray *et al.*, 1975). Plasmid pLG400 carries a *lacZ* gene fragment derived from a *lacI-lacZ* hybrid gene (Muller-Hill and Kania, 1974), which lacks the promoter and the beginning of the *lac*I gene (Guarente *et al.*, 1980). The *lacZ* gene was exposed by linearizing the plasmid at the *Hin*dIII site preceding the *lacZ* fragment. The *cro* and *lacZ* genes were then fused by blunt-end ligation, during which the *Hin*dIII site was lost, but a unique *Bam*HI site next to the junction between the two genes was retained (Figure 2). The ligated DNA mixture was used to transform competent cells of the *lacZ*$^-$ strain 71-18. Since the parent plasmid pLG400 did not express β-galactosidase, Lac$^+$ transformants harbouring recombinant plasmids carrying *cro-lacZ* gene fusions were readily identified by plating the transformed cells on lactose MacConkey indicator plates supplemented with 25 μg ampicillin/ml, and scoring red colonies. Since some of the Lac$^+$ isolates were found to be unstable, we reisolated the *cro-lacZ* recombinant plasmids using conditions under which the expression of the hybrid genes was prevented by repressing the P_R promoter. For this we used the *lacZ*$^-$ strain LK111 harbouring the plasmid pcI857 which carries a temperature-sensitive λ repressor gene, and which is compatible with pBR322-derived plasmids (E. Remaut, H. Tsao, and W. Fiers, personal communication). After transformation, ampicillin-resistant colonies were selected at low temperature (30°C) and then repicked onto pairs of lactose MacConkey plates which were incubated at respectively low (30°C) and high (42°C) temperature.

This screening procedure revealed that the Lac$^+$ derivatives of strain LK111 (pcI857) could be divided into two classes which exhibited clearly distinct growth phenotypes (see Table I). The first class of Lac$^+$ isolates was conditionally lethal in that they were not viable at 42°C, while all class II isolates grew normally at 42°C. In addition, the class I clones formed red colonies on lactose MacConkey plates at 30°C, in contrast to the class II isolates which formed white colonies at this temperature. The class I isolates thus synthesized a detectable level of β-galactosidase in the presence of *c*I repressor.

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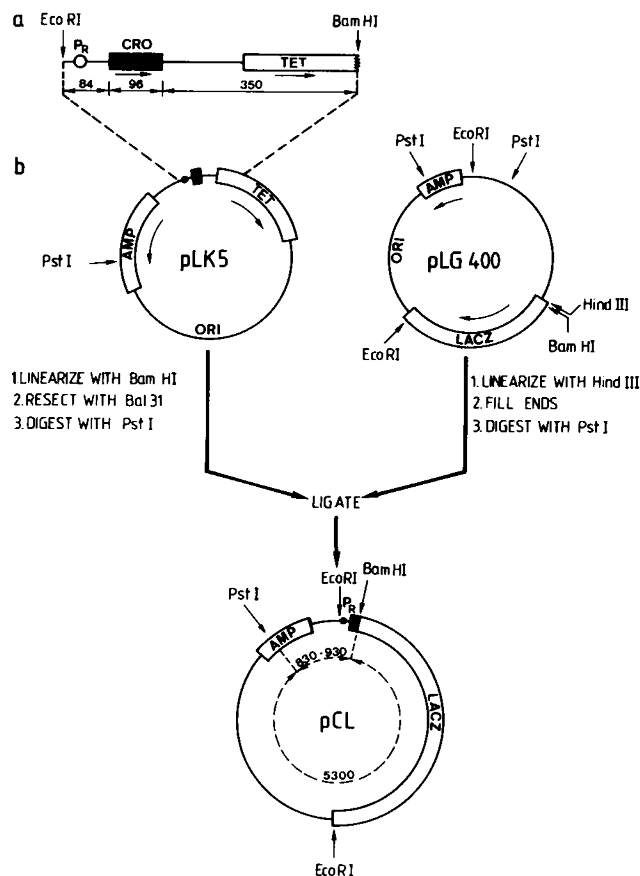

Fig. 1. Schematic representation of the construction of plasmids carrying *cro-lacZ* gene fusions. Arrows indicate the approximate positions of relevant restriction enzyme cleavage sites. Open boxes show the relative positions of the gene coding for β-lactamase (*amp*), tetracycline resistance (*tet*), and β-galactosidase (*lacZ*), and the orientation of the genes is indicated by arrows. *ori* represents the region containing the origin of replication. Fragment sizes are indicated in base pairs (bp). **(a)** Detailed map of *Eco*RI-*Bam*HI region of pLK5 bearing the P_R promoter, the beginning of the *cro* gene, and the beginning of the Tc^R gene. **(b)** Construction of the *cro-lacZ* fusion plasmids (see text). Recombinant plasmids, carrying *cro-lacZ* fusions, designated pCL, resulted from ligation of the 830–930-bp *cro*-fragment of pLK5 and the 5.3-kb *lacZ* fragment of pLG400.

These results suggested that the class I and class II Lac^+ isolates differed primarily in the amounts of β-galactosidase directed by the recombinant plasmids. The levels of β-galactosidase enzyme activity measured after temperature induction shown in Table II, verified that the class I isolates produced a higher level of enzyme activity than the class II isolates. However, a far more pronounced difference was found when comparing the β-galactosidase protein visualized by SDS-polyacrylamide gel electrophoresis: all class I isolates produced a very intense band at the expected mobility (see Figure 6) while no such band was found in extracts of the class II derivatives. In the latter case the β-galactosidase protein was only visible after immunoprecipitation. From these results, we estimated that the class I clones produce 100–500 times more β-galactosidase protein than the class II derivatives. The discrepancy between the enzyme assay and the protein measurements will be discussed below.

Properties of the cro-lacZ gene fusion plasmids

Plasmid structure. Restriction enzyme analysis of the recombinant plasmids verified that both types of Lac^+ derivatives harboured similar pCL plasmids, exhibiting the expected structure shown in Figure 1. The precise position of the fusion point within the *cro* gene was determined by measuring the size of the small *Eco*RI-*Bam*HI fragment bearing the P_R promoter and the beginning of the *cro* gene (Figure 1). The results summarized in Figure 2 demonstrate that the two types of plasmids cannot be distinguished by the structure of the fused *cro* and *lacZ* genes. Indeed, the fusion points of the type I and II *cro-lacZ* fusions are not clustered together but are randomly distributed across the entire *cro* gene segment. The first clue to an interpretation of this result was given by the finding that the fusion point in one of the type II plasmids pCL77 was located upstream of the initiator ATG of the *cro* gene, within the 5′ non-translated region of the *cro* mRNA (Figure 2). In this plasmid the *lacZ* fragment was thus fused directly to the P_R promoter. Consequently, the β-galactosidase protein could only be synthesized starting from a secondary translation initiation site presumably located within the *lacZ* fragment itself (see below). Since all the type II strains produced comparable low levels of β-galactosidase activity (Table II), it seemed plausible that they all initiated enzyme synthesis in a similar manner. This would be the case when the translational reading frames of the *cro* and *lacZ* genes had been joined out-of-register. To verify this interpretation, we determined the nucleotide sequences around the fusion points in a subset of proximal fusions. The results summarized in Figure 2 demonstrate that in all six type I fusions the translational reading frames of the *cro* and *lacZ* gene segments were joined in-register, while the frames were out-of-phase in the six type II *cro-lacZ* fusions analyzed. The type I fusions thus represent genuine gene fusions producing high levels of a *cro-lacZ* hybrid protein while the type II fusions are P_R-*lacZ* fusions. The observed frequency of type I isolates (12/38) agreed well with the expected 1/2 ratio of in-register *versus* out-of-register fusions. Examination of the *cro* mRNA sequence shown in Figure 2 revealed two ATG triplets from which translation in the other reading frames could eventually be initiated. However, none of the type II fusions sequenced was joined in-register to either of these two ATGs. Translation of β-galactosidase must therefore be initiated from a secondary site within the *lacZ* gene fragment. The finding that the β-galactosidase protein produced by these fusions migrated slightly faster on SDS-polyacrylamide gels than the *cro-lacZ* fusion proteins further supported this conclusion.

Growth inhibition. Figure 3 illustrates the effect of the induction of β-galactosidase synthesis on cell growth in two cI^{ts} strains carrying type I or type II *cro-lacZ* fusion plasmids. While the strain carrying the type II plasmid continued to divide normally, the growth of the type I strain slowed down rapidly after the temperature shift. This result indicated that the expression of one or more genes under P_R promoter control in the type I plasmids inhibits cell growth. In Table III we have summarized the growth properties of cI^{ts} strains harbouring different type I *cro-lacZ* gene fusion plasmids. The extent of the inhibition of growth at the high temperature was apparently correlated with the amount of hybrid cro-β-galactosidase protein synthesized. Indeed, the pCL72 carrying strain which produced a 12-fold lower level of fusion protein (see below) grew normally, while the strains synthesizing very high levels of fusion protein were totally unable to form colonies.

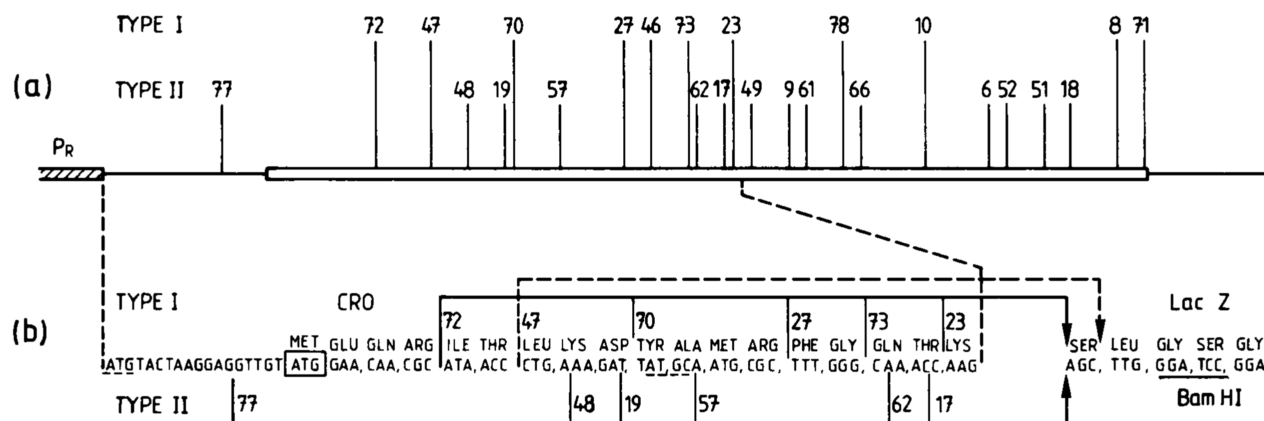

Fig. 2. Physical map and nucleotide sequences of the points of fusion in the type I and II *cro-lacZ* gene fusions. The open box represents the *cro* gene segment. (**a**) The positions of the fusion points were determined by measuring the sizes, by electrophoresis on 8% polyacrylamide gels, of the small *Eco*RI-*Bam*HI fragments (Figure 1) bearing the P_R promoter, the beginning of the *cro* gene, and 9 bp of the linker preceding the *Bam*HI site in the *lacZ* fragment. The calculated positions of the fusion points were subsequently corrected using the data from the nucleotide sequence analysis. (**b**) Nucleotide sequences of the proximal *cro-lacZ* gene fusions. The nucleotide sequences around the fusion points were determined using a modification of the chain terminator method described by Smith (1979). The first region shown represents the sequence of the 5' end of the *cro* mRNA including the untranslated region and the first 18 codons of the *cro* gene (Steege, 1977; Schwarz *et al.*, 1978). Alternative ATGs are indicated by dashed lines. The second region shows the first codons of the *lacZ* fragment from pLG400 (Guarente *et al.*, 1980). Arrows indicate how the *cro* and *lacZ* sequences are joined.

Table I. Phenotypes of the *cro-lacZ* gene fusions isolated in strain LK111 (pcI857)

Class	Number of isolates	Colour on lactose MacConkey at:	
		30°C	42°C
I	12	red	no growth
II	26	white	red

Table II. β-Galactosidase activity directed by the type I and type II *cro-lacZ* gene fusions [a]

Type I	β-Galactosidase units/bacterium	Type II	β-Galactosidase units/bacterium
CL8	524	CL6	101
CL10	405	CL9	135
CL23	250	CL17	83
CL27	1322	CL18	44
CL46	808	CL19	285
CL47	1333	CL48	44
CL70	1289	CL49	280
CL71	512	CL51	63
CL72	630	CL52	153
CL73	1006	CL57	131
CL78	569	CL61	132
		CL62	76
		CL66	256
		CL77	49

[a]β-Galactosidase activity was measured 60 min after temperature induction of cultures of strain LK111 (pcI857) harbouring the pCL plasmids as described in Materials and methods.

To examine the possible involvement of functions encoded downstream of the *lacZ* gene we have constructed deletion derivatives of the *cro-lacZ* fusion plasmids. As diagrammed in Figure 4, we have transferred the P_R-*cro-lacZ* hybrid genes into an *Eco*RI-*Pvu*II segment of plasmid pBR322. The *lacZ* gene segment in these pCL△Y deletion derivatives ends at the *Hae*III site 9 bp after the three stop codons of the *lacZ* gene (Buchel *et al.*, 1980). As shown in Table III, strains carrying pCL△Y deletion derivatives were now able to form colonies at 42°C. The growth inhibition induced by the type I *cro-lacZ* fusion plasmids is thus caused by the enhanced expression of a function encoded downstream of the *lacZ* gene.

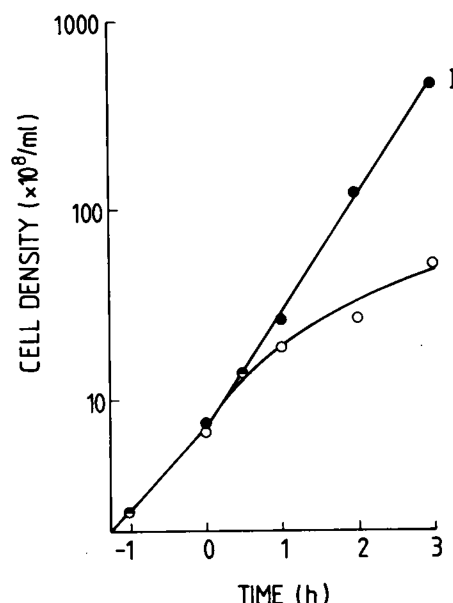

Fig. 3. Growth inhibition induced by type I pCL plasmids. Cultures of LK111 (pcI857) harbouring pCL19 (type I) and pCL27 (type II) were kept in exponential growth phase by diluting the cells so as to maintain a concentration between 2 x 10^7 and 2 x 10^8 cells/ml. At t = 0 the temperature was shifted from 30°C to 42°C. Cell densities were measured by following the OD_{600}.

Inhibition of plasmid replication. Figure 5a shows a comparison of the kinetics of β-galactosidase synthesis after temperature induction of the P_R promoter in strains carrying plasmid pCL47 and the deletion derivative pCL47△Y. The induction profiles in these two strains are similar in that the activity of β-galactosidase in the cells reaches a peak followed

by a rapid decline. Concomitantly with the unexpected drop in β-galactosidase activity we have measured a similar decrease in the level of β-lactamase in the cells (data not shown). Furthermore, analysis of the colonies obtained by plating aliquots of the cultures after different times of induction revealed that the drop in β-galactosidase activity was paralleled by the appearance of cells which were no longer resistant to ampicillin (Figure 5b). Taken together, these results show that the decrease in β-galactosidase and β-lactamase activities observed after induction of the P_R promoter was due to a rapid curing of the pCL plasmid. Since the direction of transcription originating from the strong P_R promoter is opposed to movement of the replication fork (see Figure 4), it seems likely that the efficient transcription of the region containing the origin of replication inhibits plasmid replication. It should be noted that the inhibition of plasmid replication does not markedly affect the growth of the colonies on selective plates (Table III).

In an attempt to overcome the curing problem we have constructed derivatives of the *cro-lacZ* fusion plasmids carrying transcription termination signals downstream of the *lacZ* gene. The plasmid pBRT12 used in this construction carries two copies of the terminator of phage fd (Sugimoto *et al.*, 1977), inserted in tandem between the *Eco*RI and *Pvu*II sites of pBR322. Following a scheme similar to the one outlined in

Table III. Growth properties at 42°C of cI^{ts} strains carrying type I pCL plasmids and pCL△Y derivatives[a]

pCL plasmid	e.o.c.[a]	Colony type	pCL△Y plasmid	e.o.c.[a]	Colony type
pCL72	1	normal			
pCL47	0.3	micro	pCL47△Y	1	normal
pCL27[b]	10^{-4}	–	pCL27△Y	1	small
pCL23[b]	10^{-4}	–	pCL23△Y	1	small

[a]Freshly grown cultures of strain K-12△H1△*trp* were diluted and plated on ampicillin-selective plates which were incubated at 30°C or 42°C; e.o.c. efficiency of colony formation at 42°C relative to 30°C.
[b]All other type I pCL plasmids not listed exhibited identical growth properties.

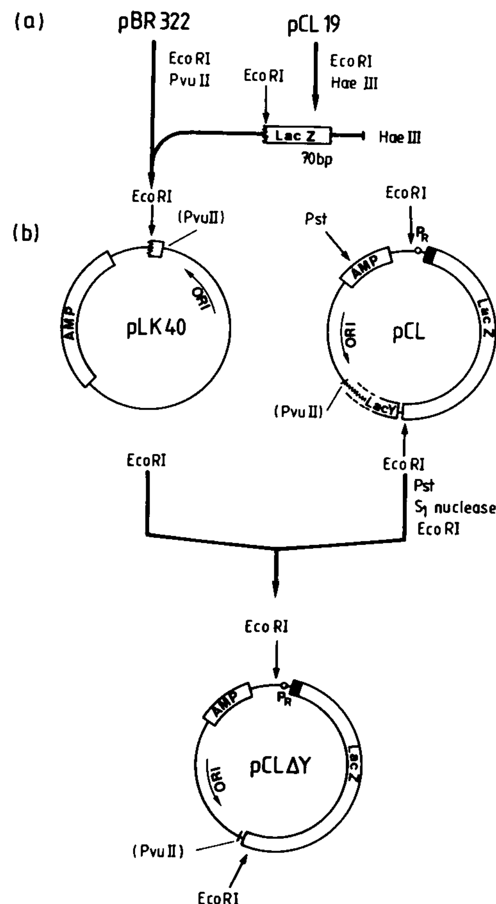

Fig. 4. Construction of deletion derivatives of the pCL plasmids. (**a**) A 70-bp *Eco*RI-*Hae*III fragment coding for the last 17 amino acids of β-galactosidase was inserted between the *Eco*RI and *Pvu*II sites in pBR322. The purified fragment was ligated to pBR322 cleaved with *Eco*RI and *Pvu*II. Bacterial clones carrying the desired recombinant plasmid, pLK40, were identified by scoring tetracycline-sensitive transformants. (**b**) *Eco*RI fragments from different pCL plasmids bearing the P_R promoter, and most of the hybrid *cro-lacZ* gene were inserted into the *Eco*RI site of pLK40. Lac^+ recombinants were scored after transformation into the $lacZ^-$ strain K-12△H1△*trp* on lactose MacConkey plates supplemented with ampicillin. A selection against the parental pCL plasmids was obtained by first cleaving the pCL plasmids with *Pst*I and digesting the single-stranded protrusions with nuclease S1.

Table IV. Synthesis of cro-β-galactosidase fusion protein

Cell cultures: *cro-lacZ* gene fusion	Protein % total	Activity units/bacterium	Specific activity µmol/min/mg cro-β-Gal	Lysate supernatants: Protein % total	Activity µmol/min/mg total protein	Specific activity µmol/min/mg cro-β-Gal
CL 72	2.6	2993	76	3.6	1.3	35
CL 47	15.9	5338	22	8.9	3.2	35
CL 70	30.3	924	2	4.0	1.2	31
CL 27	21.7	895	3	4.8	1.1	22
CL 73	17.3	1899	7	3.5	1.2	35
CL 23	19.9	644	2	3.4	0.8	22
K514 + IPTG	0.5	729	96	1.7	0.5	29

Exponentially growing cultures of strain K-12△H1△*trp* harbouring type I pCL plasmids were induced at 42°C for 90 min and β-galactosidase activity was measured in cell cultures and in lysate supernatants as described. Protein concentrations were estimated from SDS-polyacrylamide gels (see Figure 6). β-Galactosidase activity was converted from units/bacterium to µmol/min/mg using the conversion factor given by Miller (1972). For comparison, enzyme and protein levels were measured in the $lacZ^+$ strain K514 grown in the presence of 20 µg/ml IPTG. The different specific activity of wild-type β-galactosidase probably result from the different methods of breaking the cells.

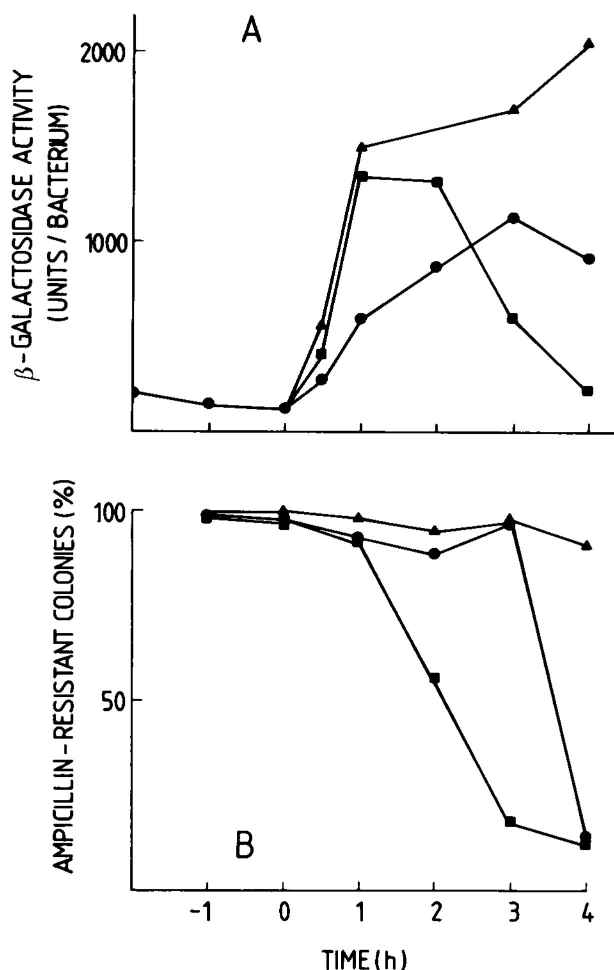

Fig. 5. (**a**) Kinetics of β-galactosidase synthesis and (**b**) curing of pCL plasmids after growth at 42°C. (**a**) The synthesis of β-galactosidase after temperature induction was measured as described in Materials and methods. Cultures of strain LKIII (pcI857) harbouring pCL47 and its derivatives were induced at $t = 0$; ●——● pCL47; ■——■ pCL47△Y; ▲——▲ pCL47△Y-T. (**b**) Samples of the cultures at each time point were diluted and plated onto non-selective plates. 100 colonies from each plate were picked onto plates supplemented with ampicillin and the number of ampicillin-resistant colonies was scored.

Figure 4, the P_R-*cro-lacZ* fragment was inserted into pBRT12, in front of the terminator signals. Upon temperature induction of the P_R promoter in these pCL△Y-T plasmids, we no longer observed a decrease in β-galactosidase synthesis (Figure 5a), and the curing of these plasmids was also considerably reduced (Figure 5b). These results demonstrate that the inhibition of plasmid replication caused by the transcription originating from a strong promoter can be overcome by inserting transcription termination signals before the origin of replication.

Analysis of the cro-β-galactosidase fusion proteins

Table IV compares the amounts of cro-β-galactosidase fusion protein and the β-galactosidase enzyme activity directed by the type I pCL plasmids after temperature induction in the cI^{ts} strain K12△H1△*trp*. The quantitations of the amounts of protein synthesized were based on the SDS-polyacrylamide gel shown in Figure 6. Considerable differences were found in the amounts of cro-β-galactosidase fusion protein directed by the different pCL plasmids. For the most proximal fusions, the amount of protein synthesized was approximately proportional to the length of the *cro* gene segment present. pCL70 produced the highest level of fusion protein amounting to ~30% of the total SDS-extractable protein. In contrast, the most proximal fusion, pCL72, produced 12 times less protein, while a 2-fold lower level was found with pCL47. Since all gene fusions have identical promoter and ribosome binding site sequences, these findings demonstrate that sequence rearrangements occurring downstream of the initiator AUG influence the efficiency of translation.

When supernatants of lysates were analyzed on SDS-polyacrylamide gels considerably less fusion protein was found than in the total cell extracts, in particular in strains synthesizing very high levels of fusion protein. The non-extractable protein was found to remain in the cell pellet, indicating that the cro-β-galactosidase fusion proteins precipitated intracellularly, as previously reported for other β-galactosidase fusion proteins (Goeddel *et al.*, 1979; Shine *et al.*, 1980; Davis *et al.*, 1981). The specific activity of the cro-β-galactosidase fusion proteins recovered in the lyates was, in all cases, very similar to that found for wild-type β-galactosidase. In contrast, a much lower specific activity was measured in cell cultures producing enhanced levels of cro-β-galactosidase fusion protein. We therefore conclude that a large part of the overproduced protein which is non-extractable, is also enzymatically inactive. The enzymatic assay may thus grossly underestimate the amount of β-galactosidase protein, in particular when very high levels are synthesized.

Discussion

We have described the construction of hybrid plasmids in which the P_R promoter and the beginning of the *cro* gene have been fused to a fragment of the *lacZ* gene. Two distinct types of plasmids expressing β-galactosidase activity have been isolated which differed primarily in the junction of the translational reading frames of the *cro* and *lacZ* genes. The type I plasmids carry *cro-lacZ* gene fusions in which the translational reading frames are in-register. These plasmids can direct the synthesis of very high levels of cro-β-galactosidase proteins under permissive conditions. The type II plasmids in which the genes are fused out-of-register, express low levels of β-galactosidase activity. In these P_R-*lacZ* promoter-gene fusions, translation is presumably initiated at a secondary site within the *lacZ* fragment.

The results presented here emphasize the importance of being able to control gene expression when constructing gene fusions. The isolation of the type I *cro-lacZ* gene fusions was possible only in strains producing λ repressor so that the expression of the hybrid *cro-lacZ* gene from the P_R promoter was repressed. In this case it was not the product of the fused gene itself which was deleterious to the cell, but rather a function encoded downstream of the *lacZ* gene. However, one cannot predict whether novel or hybrid proteins will adversely affect the growth of the cell, especially when expressed at high levels. Similar deleterious effects are frequently observed with strains over-producing proteins under the control of the P_L promoter of phage λ (Remaut *et al.*, 1981; M. Zabeau, unpublished results).

The observed correlation between inhibition of cell growth and elevated synthesis of cro-β-galactosidase fusion protein, indicates that the inhibitory effect results from the concomitant over-production of a function encoded downstream of the *lacZ* gene. A similar growth inhibition has been reported

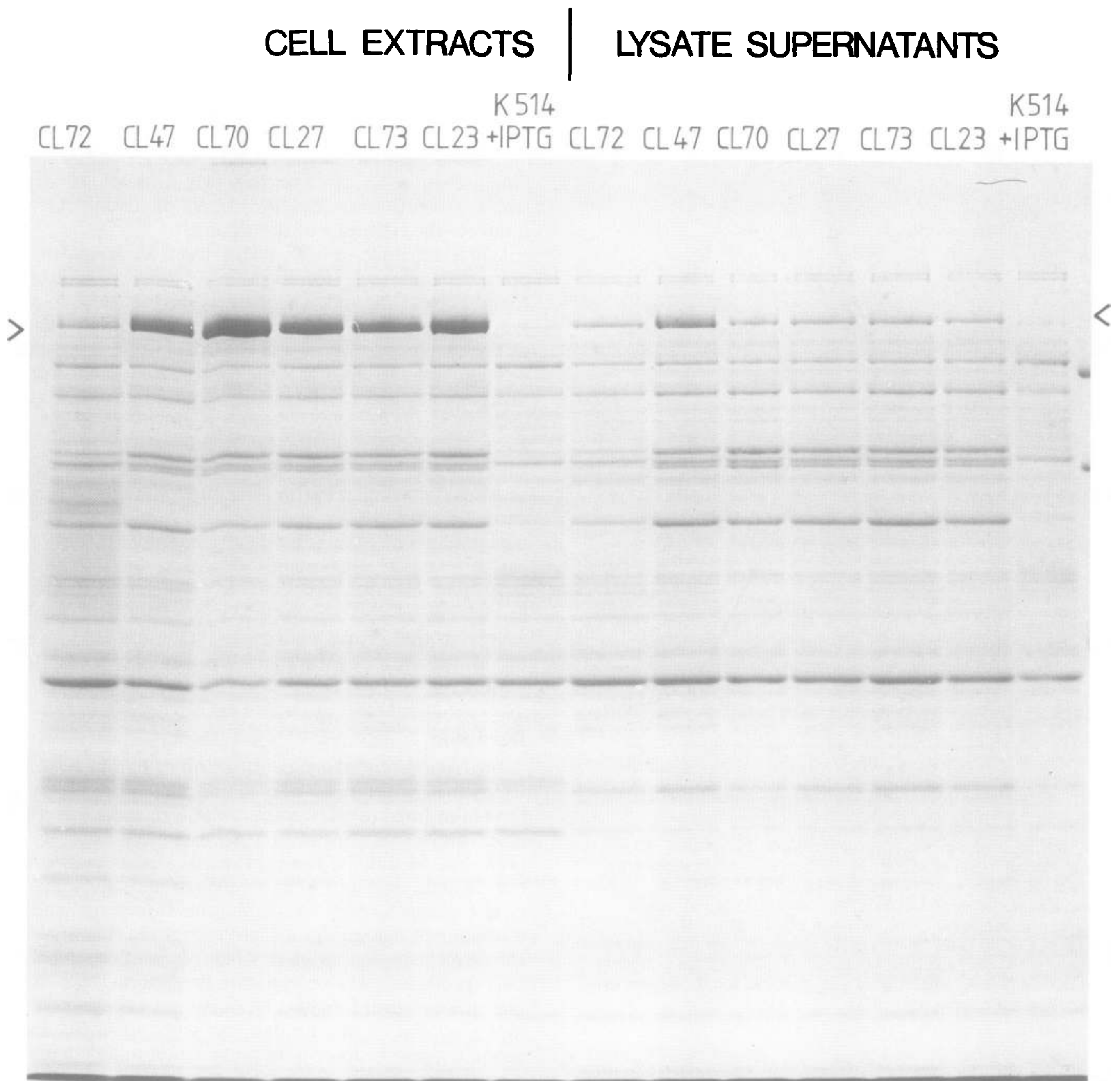

Fig. 6. SDS-polyacrylamide gel analysis of the cro-β-galactosidase fusion proteins. Samples of the cultures and the lysate supernatants from Table IV were run on a 10% SDS-polyacrylamide gel as described. For the total cell extracts ~8 x 10^7 cells were loaded per track, and for the lysate supernatants ~25 μg of protein was loaded.

for plasmids carrying the entire *lac* operon (Teather *et al.*, 1978). In the pCL plasmids the segment between the *lacZ* gene and the region bearing the origin of replication was derived from pLG400 and carries the beginning of the *lacY* gene joined to sequences of bacteriophage λ (Guarente *et al.*, 1980). Thus, it is plausible that the inhibition of growth might be caused by over-expression of a protein fragment specified by the *lacY* gene segment. This finding is particularly relevant in view of the fact that the same sequences are present in the plasmids pLG200 and pLG400 which were designed to maximize the expression of cloned genes (Guarente *et al.*, 1980). In this procedure, a gene fragment coding for the amino-terminal portion of the protein is first fused to the *lacZ* fragment, whereafter a promoter is positioned randomly in front of the fused gene. The most efficient promoter-gene fusions will, however, also efficiently express the inhibitory *lacY* gene product. Consequently, there will be selection against these recombinant plasmids. This might explain why in previous studies the strain that produced the highest amount of a β-globin-β-galactosidase fusion protein made only 10 000 – 30 000 monomers per cell (Guarente *et al.*, 1980), while our type I pCL plasmids direct the synthesis of ~10 times more fusion protein under appropriate conditions. No such counter-selection should occur with the modified pCL △Y plasmids which lack the *lacY* gene segment. It should also be noted that the β-galactosidase enzyme assay cannot always be used with confidence to monitor levels of protein synthesized. In particular, when high levels were produced, only a small fraction of the cro-β-galactosidase protein was enzymatically active. The quantitation of the amount of fu-

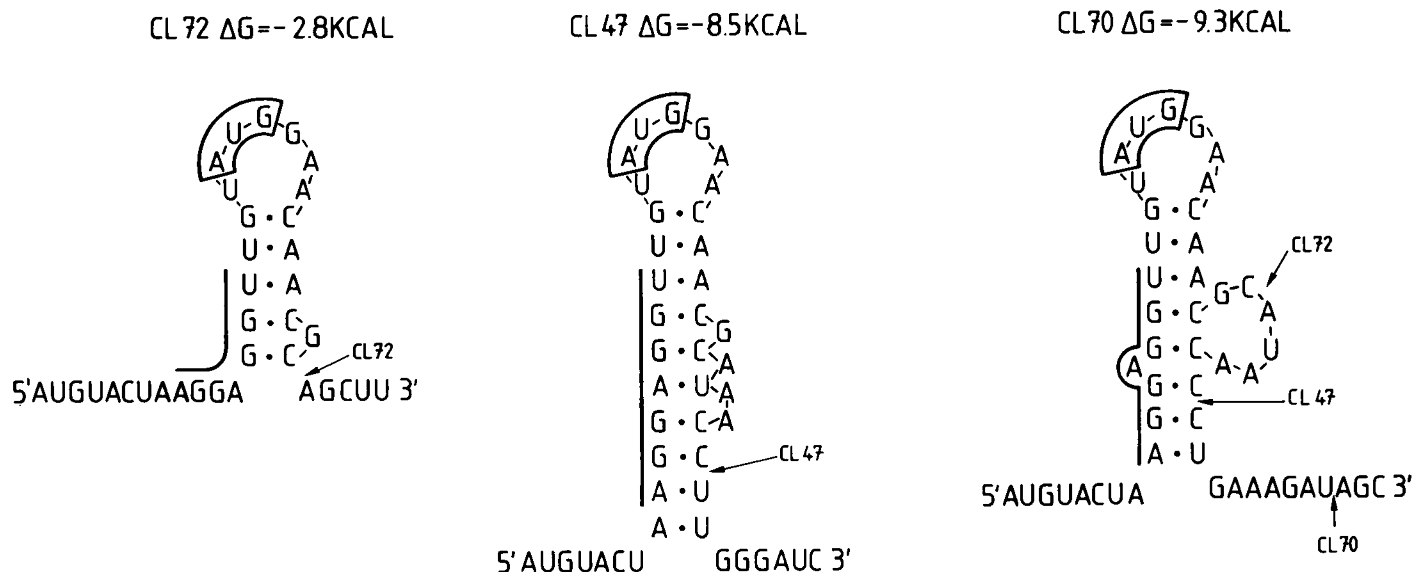

Fig. 7. Possible structures of the translation initiation regions of the *cro-lacZ* mRNAs. The thermodynamic stabilities were calculated according to Salser (1977) and are expressed as the free energy ($\triangle G$) of the structure. Arrows indicate the positions of the fusion points between the *cro* and *lacZ* genes. The Shine and Delgarno sequence is indicated by a solid line and the initiator AUG of *cro* is boxed. The structure of the *cro-lacZ* mRNAs of the fusions distal to CL70 were identical to the one shown for CL70.

sion protein in total SDS extracts on SDS-polyacrylamide gels proved the only reliable method for assaying levels of protein directed by the plasmids.

Most of the type I *cro-lacZ* gene fusion plasmids directed the synthesis of similar amounts of fusion protein, a finding entirely consistent with the fact that these gene fusions have identical promoter and ribosome binding site sequences. However, the two gene fusions in which the *lacZ* segment was fused most closely to the initiator AUG of the *cro* gene expressed considerably lower levels of protein. Previous studies on fusions between the *lac* promoter and the *cro* gene showed that sequence alterations within the 5′ untranslated mRNA strongly influenced translation efficiency (Roberts *et al.*, 1979). These effects have been correlated with substantial alterations in the RNA secondary structure, suggesting that folding of the mRNA plays an important role in translation initiation (Iserentant and Fiers, 1980). Figure 7 shows a possible structure of the translation initiation region of the proximal *cro-lacZ* mRNAs derived from the structure of the *cro* mRNA segment proposed by Inserentant and Fiers (1980). No structures with lower free energy were found using the computer folding programme of Zucker and Stiegler (1981). In these structures, the initiator AUG was situated in a hairpin loop whereas the Shine and Delgarno sequence (Shine and Delgarno, 1974) was located in a hydrogen-bonded structure. Two correlations between efficiency of translation and RNA structure are found: first, all distal fusions, starting from CL70, which direct the synthesis of large amounts of protein also exhibit identical RNA structures, while a different RNA structure is found in the proximal fusions CL72 and CL47 which produce lower levels of protein. Secondly, the differences in amounts of protein synthesized (Table IV) closely parallel the thermodynamic stabilities of the RNA secondary structure as indicated by the $\triangle G$s shown in Figure 7. This interpretation, that the efficiency of translation depends on the secondary structure recognized by the ribosomes, is corroborated by the fact that the points of fusion in pCL72 and pCL47 are located within the segment of the *cro*-mRNA protected by the ribosome (Steege, 1977), while the junction in pCL70 occurs downstream of this region. In contrast to other studies (Iserentant and Fiers, 1980; Schwartz *et al.*, 1981), our results indicate that the less efficiently translated CL72 has a more accessible Shine and Delgarno sequence (Figure 7). This finding suggests that other aspects of the secondary structure are important.

The pCL plasmids described here constitute a novel type of plasmid vector in which cloned DNA segments can be expressed at very high levels. Two types of fusion proteins can be obtained. First, polypeptides can be fused to the amino terminus of *cro* by inserting DNA fragments in the unique *Bam*HI site. It should be noted that the plasmid series pCL48, pCL19, and pCL70 can be used to fuse DNA segments to the *cro* gene in all three translational reading frames. Moreover, the *Bam*HI site in these plasmids is located downstream of the region that influences the efficiency of translation. Consequently, fusion proteins should be expressed at very high levels, comparable to that of the cro-β-galactosidase fusion protein produced by pCL70. Secondly, DNA fragments inserted in the *Bam*HI site between the *cro* and *lacZ* genes can be expressed as part of a fusion protein in which the novel polypeptide is sandwiched between a cro 'head piece' and an enzymatically active fragment of β-galactosidase. The ability to express any piece of DNA in the form of a hybrid fusion protein can be used to identify cloned DNA fragments by immunological screening of expressed antigenic determinants (Broome and Gilbert, 1978). In this respect, the type II pCL plasmids are particularly useful since a proportion of inserted DNA fragments bring the translational reading frames of the *cro* and *lacZ* genes back into register, thus allowing the expression of high levels of β-galactosidase. The β-galactosidase activity thus acts as a marker for expression of the DNA fragment. While this manuscript was in preparation, a report describing a similar cloning vector has appeared (Koenen *et al.*, 1982).

Materials and methods

Bacterial strains and plasmids

E. coli strain K514, a $r_k^- m_k^+$ derivative of strain C600 (Wood, 1966) was used as host strain for plasmid constructions. Strain LK111 is a *lacI*$^-$, *lacZ*△M15, *lacY*$^+$ derivative of K514 isolated by F′ homogenotisation using the F′*lac-pro* (*lacZ*△M15) from strain 71-18 (Mesing *et al.*, 1977). K-12△H1△*trp* is the designation for the *lacZ*$^-$ am strain carrying the defective λ prophage λ*N*am7, *N*am53, *c*I857△H1 (Bernard *et al.*, 1979). This strain produces a temperature-sensitive *c*I857 repressor. Plasmid pLG400 (Guarente *et al.*, 1980) was obtained from G. Lauer. Plasmid pcI857 is a multicopy plasmid derived from pACYC187 containing a *c*I857 temperature-sensitive λ repressor gene and was obtained from E. Remaut.

Plasmid pLK5 was derived from plasmid pBλH3 which contains a 900-bp fragment of the phage λ chromosome inserted in the *Hind*III site of pBR322 (Pirrotta *et al.*, 1980). This fragment bears an almost complete *c*I gene, the P_R promoter, and the first 96 bp of the *cro* gene. The P_R promoter controls the expression of the *tet*^{R} gene. Plasmid pLK5 was obtained by deleting the *c*I gene from pBλH3: pBλH3 was opened with *Eco*RI, resected with *Bal*31, ligated with *Eco*RI linkers, cut with *Bam*HI, and then ligated with pBR322 cut with *Eco*RI and *Bam*HI. Recombinant plasmids carrying a functional P_R promoter were isolated in the *c*I^{ts} strain K-12△H1△*trp* and were identified by scoring transformants which are resistant to tetracycline at high temperature (42°C) but sensitive at low temperature (30°C). One of these plasmids, pLK5 (Figure 1) carried a deletion ending in the O_R3 operator, as confirmed by DNA sequence analysis.

Plasmid pBRT12 carries the 99-bp *Eco*RI* fragment bearing the terminator of bacteriophage fd (Sugimoto *et al.*, 1977), inserted in two tandem copies between the *Eco*RI and *Pvu*II sites of pBR322 and was constructed as follows. Plasmid pBRT7 (Reiss, 1982; H. Schaller, unpublished results) carries the 99-bp *Eco*RI* fd terminator fragment inserted between the *Eco*RI and *Hin-d*III sites of pBR322. Plasmid pBRT11 was derived from pBRT7 by deleting the *Hind*III-*Pvu*II fragment carrying the *tet*^{R} gene. The second copy of the terminator in pBRT12 was inserted by substituting the *Pst*I-*Eco*RI fragment of pBRT11 with a *Pst*I-*Hind*III fragment of pBRT7.

DNA constructions

Restriction enzymes were prepared by H. Cambier and digestions were performed using the conditions recommended by New England Biolabs. Digested DNA samples were analyzed by electrophoresis on 1.2% agarose gels (Sugden *et al.*, 1975). Digestions were terminated by adding 0.1 volume of phenol, followed by chloroform extraction and precipitation with 0.5 volume of isopropanol in the presence of 0.5 M $NaClO_4$. 5′ Protruding ends were filled with DNA polymerase I 'Klenow' fragment purchased from Boehringer/Mannheim A.G. by adding 0.02 units of enzyme/pmol of ends to the digested DNA in the presence of 100 μM dXTPs, and incubating for 10 min at 20°C. The reactions were terminated as above. Resections with exonuclease *Bal*31, obtained from Bethesda Research Laboratories, were performed using conditions recommended by the supplier. The extent of resection from each end was monitored by agarose gel electrophoresis, after recleaving the resected DNAs with an appropriate restriction enzyme. *Bal*31 digests were terminated as above. DNA fragments were isolated following the method described by Maxam and Gilbert (1980). Ligation reactions were performed at 15°C in 50 mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.5 mM ATP using a concentration of 5 pmol/ml for each DNA fragment. T4 DNA ligase was the gift of E. Remaut. Prior to transformation, the ligated DNAs were extracted and precipitated as described in order to ensure a reproducible recovery of transformants. Transformations were performed as described by Dagert and Ehrlich (1979). Large scale preparation of plasmid DNA was according to Clewell (1972) and for small scale preparation a modification of the procedure described by Gough *et al.* (1980) was used.

β-Galactosidase assays

Since the type I *cro-lacZ* gene fusion plasmids could not be propagated in the absence of *c*I repressor, β-galactosidase synthesis was measured after temperature induction in strains producing a *c*I^{ts} repressor. Fresh overnight cultures were diluted 1:200 and grown at 30°C to OD_{600} = 0.6. β-Galactosidase synthesis was induced by adding an equal volume of broth preheated to 54°C and the cultures were incubated further at 42°C. For induction times exceeding 90 min, the cells were maintained at a concentration of $1-4 \times 10^8$ cells/ml by successive dilution in prewarmed medium. β-Galactosidase activity was measured according to Miller (1972) by lysing aliquots of the cultures with chloroform and SDS. Lysate supernatants were prepared by lysing cells treated with lysozyme in the presence of 1% Triton X-100 and removing the cell debris by centrifugation.

SDS-gel electrophoresis

Bacterial cultures (OD_{600} = 0.6 − 1.0) were cooled, spun down, and resuspended in 1/20th of their original volume. 10 − 20 μl samples were taken up in sample buffer (containing 100 mM Tris HCl (pH 8.8), 4% (w/v) SDS, 1 mM EDTA, 15% (w/v) sucrose, 10 mM DTT and bromophenol blue), and run on 10% acrylamide gels according to Laemmli (1970). Gels were stained with Serva blue and quantitated by scanning 35 mm negatives of the gels on a rotating drum microdensitometer at 50 μm resolution and analysing the area under peaks on a Nord 10S mini computer.

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