
Overproduction of the *EcoR* V endonuclease and methylase

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ABSTRACT

Strains overproducing the *EcoR* V endonuclease and methylase have been obtained by inserting each of the two genes in expression vectors containing the lambda P_L promoter. The methylase is overproduced to a level reaching 5-10% of the total cellular proteins, which represents a 50-100 fold increase. A 30 fold overproduction of endonuclease was achieved by randomly positioning the *EndRV* gene downstream of the lambda P_L promoter. The situation in the endonuclease overproducing clone resembles that encountered in maxi-cells. The strains described here allowed a quick purification of both enzymes in sufficient amounts for crystallisation attempts.

INTRODUCTION

Although restriction enzymes are widely used in genetic engineering very little is known about their structure and genetics. In a previous paper (1) we characterised the *EcoR* V restriction and modification system, which like all the other type II systems, is composed of two enzymes of low molecular weight: a restriction endonuclease (29 Kd) and a modification methylase (32 Kd). Both enzymes recognize the same short specific DNA sequence, namely the hexanucleotide GATATC. The *EcoR* V endonuclease cleaves this sequence after the first T, generating blunt ends (2). The *EcoR* V methylase modifies one of the two adenines (R. Brown; Personal communication). Because both the endonuclease and the methylase are able to interact with the same specific DNA segment, they constitute an ideal system to study specific protein-DNA interactions at the structural level. The fact that these enzymes are generally produced in very small amounts by their natural hosts, has hampered their purification in the relatively large quantities required for physicochemical studies. This problem can be solved by engineering strains overproducing specific enzymes (3). A first step in overproducing such enzymes can be achieved by cloning the system in a multicopy plasmid, as has been done with *Pst* I (4). In the *EcoR* V case, the system is already plasmid borne, and its cloning in pBR322 did not lead to a substantial

increase of production (1). This problem can be circumvented by using expression vectors based on regulatable promoters.

One of the main difficulties in overproducing restriction enzymes, resides in their potential lethality. The role of the methylase is to protect the DNA from the action of the endonuclease, it is thus easily conceivable that a substantial increase in the endonuclease synthesis could be suicidal. Since the two genes are transcribed divergently (1) we chose to engineer each gene separately, so as to construct separate strains overproducing the EcoR V endonuclease and methylase. In this paper we present the different strategies that enabled us to construct strains overproducing these enzymes.

MATERIAL AND METHODS

Strains:

K-12ΔH1Δtrp (lacZ_{am}, strA, r_K⁺m_K⁺, λ bio252, cI857, ΔH1)(6) was used as a recipient for transformation. Strain K514 (C600 r_K⁻m_K⁺) and strain K-12 H1 trp were both used as indicator strains for restriction and modification tests. For the determination of the 'de novo' synthesis of proteins in induced cells, the strain 1100(pRK248) was used as a recipient. Plasmid pRK248 (7) codes for the thermosensitive lambda repressor and the resistance to tetracycline. This plasmid is compatible with the expression vectors used in this work. The cells were grown in L-Broth or on L-Agar. When necessary, ampicillin or chloramphenicol were added to the medium to a final concentration of 100 μg/ml and 25 μg/ml respectively. Phage tests were done on BBL-Agar plates supplemented with 0.1M MgSO₄.

Plasmids:

Plasmid pLB6 (which carries the complete EcoR V system) and pLB7 (which carries the EcoR V methylase alone) have been described in a previous paper (1) and were used as a source of DNA for cloning experiments. The construction of the pLK50 and pLK30 plasmid series is described elsewhere (8). Plasmid pLK331 contains the EndRV and MetRV fragment from pLB6 inserted in pLK33, in the opposite direction. The principal characteristics of these plasmids are shown on Figure 1.

Restriction and modification tests:

Phage lambda vir was used for all tests. Restriction spot test: dilutions of a 10¹⁰ pfu/ml non-methylated lambda vir.0 stock were spotted onto a lawn of the cells to be tested, as well as onto a non-restricting indicator strain (K514 or K-12ΔH1Δtrp). The efficiencies of plating were compared and the ratio eop(K514)/eop(cell) gives the efficiency of restriction. Methylation test: 0.1 ml of an appropriate dilution of lambda vir (10⁵ to 10⁹ pfu/ml respectively for non-restricting and restricting clones) was mixed with 0.1 ml of freshly grown cells. The mixture was incubated 15 minutes at 37°C without agitation to allow adsorption of phage lambda. 3 ml of BBL top layer agar containing MgSO₄ was then added to the mixture and poured onto a BBL-Agar plate. Because of the extremely high restriction efficiency of the EcoR V system, the methylation could be tested by simply toothpicking single plaques on a nonrestricting strain and on the appropriate restricting strain. The methylated phages give plaques on both plates whereas the non-methylated phages are unable to grow on the restricting strain. This allows the discrimination between methylating and non-methylating clones.

Viability tests:

Recombinant clones were inoculated in 3 ml L-Broth and allowed to grow for 3 hours. Dilutions of these cultures (10^{-2} , 10^{-4} , 10^{-6}) were then spotted (10 μ l/spot) onto two L-Agar plates which were incubated at 28°C and 42°C. The viability was checked after 18 hours incubation by comparing the growth on the two plates. Clones which had an eoc 42°C/eoc 28°C of 10^{-4} were considered as non-viable.

Inductions of enzyme synthesis:

The potential overproducers were grown at 28°C in L-Broth until they reached an OD_{560nm} = 0.2 and transferred at 42°C. Aliquots of these cultures were taken at various times to prepare crude extracts (10 ml for in vitro activity and 1.5 ml for loading on SDS-PAGE). For large scale inductions the cultures are diluted 1:1 in LB prewarmed at 56°C prior to transfer at 42°C so as to immediately reach the desired temperature.

Preparation of crude extracts:

10 ml of cells were spun for 10 minutes at 5Krpm, the pellet was resuspended in 1 ml TES + 100 μ l of lysozyme 10 mg/ml (TES :50 mM Tris-HCl, 5mM EDTA, 15% glucose). They were cooled for 20 minutes on ice and 1 ml of a 1.6 M NaCl, 4 mM DTT solution was added. The solution was sonicated for 15 seconds and spun for 30 minutes at 20 Krpm. Aliquots of those extracts were used for the titration of the endonuclease activity on lambda DNA.

Endonuclease titration in vitro:

The crude extracts were serially diluted (1:5 in restriction enzyme buffer) and 10 μ l of the dilutions was incubated with 10 μ l of a mixture containing 0.5 μ g of lambda DNA in restriction enzyme buffer. The mixtures were incubated for 15 minutes at 37°C and loaded onto a 1.4% agarose gel.

Ligations and transformations:

Ligation reactions were performed at 15°C in 50 mM Tris pH=7.9, 50 mM NaCl, 10 mM MgCl₂, 5 mM DTT, 0.5 mM ATP, using a concentration of 5 pm/ml for each DNA fragment. T4 DNA ligase was a gift from E. Remaut. Prior to transformation the DNAs were phenol and chloroform extracted, and isopropanol precipitated. Transformations were done as described by Dagert and Ehrlich (9).

Determination of the 'de novo' protein synthesis:

The strain 1100(pRK248)(pLBM)(pTZ115) was grown in M9 medium supplemented with 1 mM MgSO₄, 0.2% glucose, 0.05% casamino acids and 50 μ g/ml L-tryptophan. When the OD_{560 nm} reached 0.2 to 0.3, the cultures were shifted to 42°C. At various times after induction, aliquots from the 42°C cultures were labeled with 2.5 μ Ci/ml ¹⁴C protein hydrolysate (Amersham). An aliquot taken prior to temperature shift was treated the same way. These samples were analysed on a 12.5% SDS gel. Gels were treated with Amplify (Amersham) and after drying were exposed onto a X-ray Fuji film at -70°C. The protein pattern obtained that way could then be compared to the Coomassie protein pattern.

Plasmid DNA preparation and gel electrophoresis:

Large scale plasmid DNA preparation was according to Clewell (10) and for small scale preparations a modification of the procedure described by Gough (11) was used. Gel electrophoresis was done on vertical agarose gels ranging from 1.2 to 2 % agarose according to the molecular weights to be detected. The gels were run in Tris-Acetate buffer, stained in a 0.5 mg/ml EtBr solution and photographed under short wave U.V. light.

Enzymes:

All enzymes were purchased from Boehringer Mannheim, Biolabs or B.R.L. and used according to the suppliers recommendations.

RESULTS

In order to overproduce the EcoR V endonuclease and methylase the

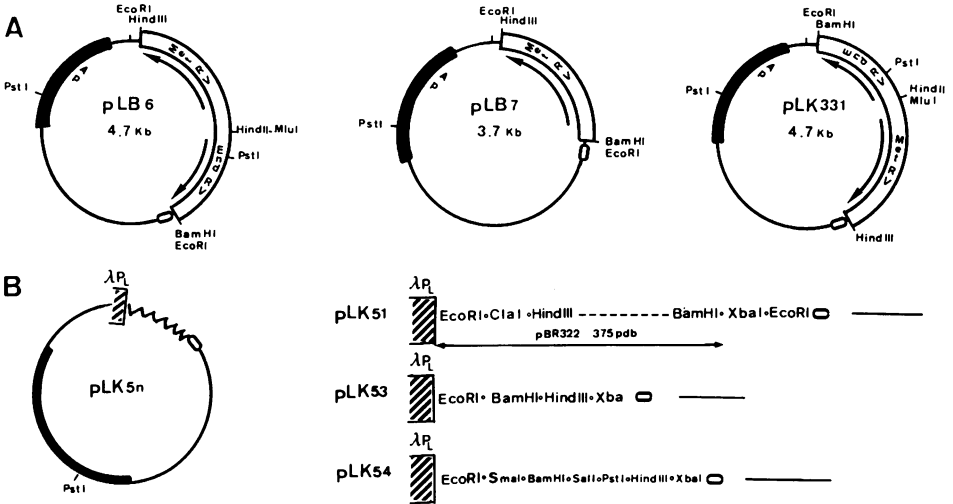


Figure 1 : Structure of the plasmids used. The hatched box represents the Lambda P_L promoter and the black boxes the gene coding for the resistance to ampicillin (Ap). The oval shape indicates the position of the fd terminators. A) Structure of plasmids pLB6 (EndRV, MetRV, Ap), pLB7 (MetRV, Ap), pLK331 (EndRV, MetRV, Ap). The direction of transcription of the MetRV and EndRV gene is indicated. B) Structure of the expression vector family pLK5n. The diagram on the right shows an enlargement of the adaptor region for each of the pLK5n plasmids used.

genes were inserted downstream of a strong promoter. The lambda P_L promoter was chosen as it is well established that it is one of the most efficient promoters, and because its expression can be strictly controlled in a host strain providing a thermosensitive cI repressor. Two main reasons directed our choice: i) Overexpression of a protein may by itself be deleterious to the host cell. ii) The endonuclease is a potentially lethal enzyme and its overproduction could be suicidal for the host cell. It was thus an absolute prerequisite to be able to strictly control the transcription. We thus chose the strain K-12ΔH1Δtrp (which harbors a cryptic lambda cI857 prophage) as a recipient for our constructions. This strain produces a temperature sensitive lambda repressor: at 28°C, the repressor is active and blocks the lambda P_L promoter, while at 42°C the repressor is inactivated and transcription may start from the lambda P_L promoter. Recombinant clones were always selected at 28°C so that potential overproduction was prevented. Furthermore the genes were cloned in improved expression vectors containing phage fd transcription terminators and were inserted in between the lambda P_L promoter and the fd terminators, so as to limit the extension of overex-

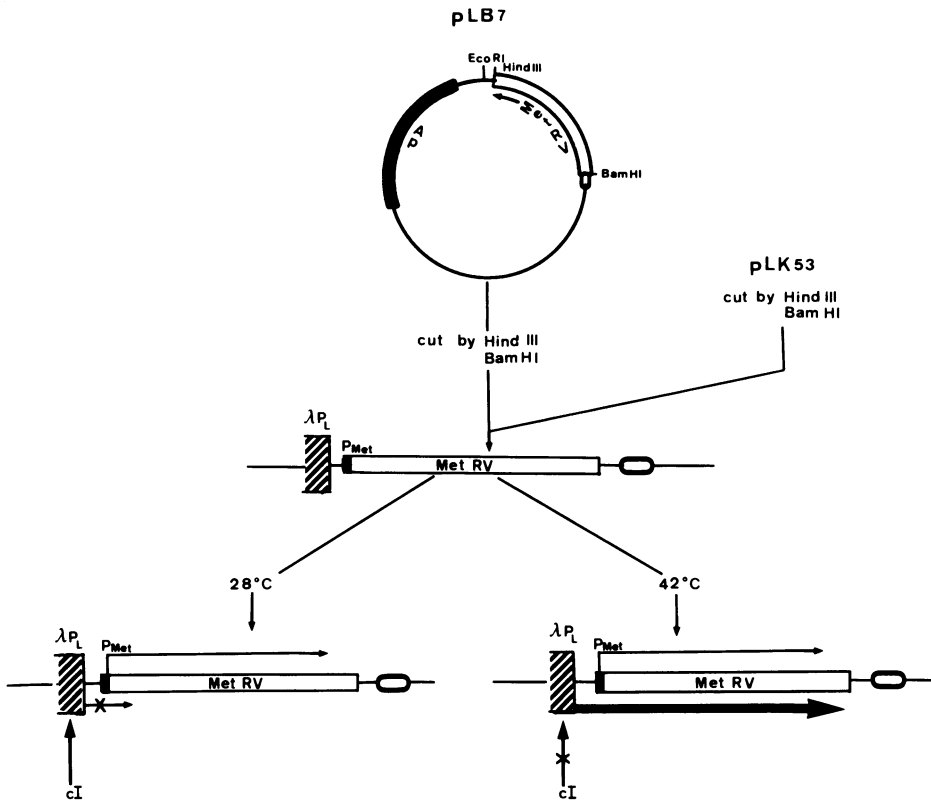


Figure 2 : Construction of a methylase overproducing strain. The oval shape represents the **fd** terminators, the hatched box the **lambda P_L** promoter, the **Ap** black box the gene coding for resistance to ampicillin and the small black region noted **P_{Met}**, the methylase promoter.

pression to the cloned gene (12).

In a first attempt we tried to obtain overproducing strains by inserting the whole **EndRV MetRV** fragment in the expression vectors **pLK53** and **pLK51** (Figure 1). These two vectors differ only by the respective order of **BamH I** and **Hind III** sites in their polylinkers, thus allowing the positioning of the genes downstream of **lambda P_L** promoter in both orientations. The two genes **EndRV** and **MetRV** being transcribed divergently from a 310 bp intergenic region (1), each of these two orientations should give rise to an overproducing clone: the overexpression of the methylase gene should be obtained by insertion in **pLK53** and the overexpression of the endonuclease gene in **pLK51**, although

in both cases transcription would have to proceed through 1 Kb of non-coding DNA.

The plasmid pLB6, containing the entire EndRV-MetRV segment, flanked by a BamH I and a Hind III site, was used as a source of DNA for this experiment. Recombinant clones which contained plasmids with the expected structure were obtained after random screening. These clones exhibited the same phenotype as K-12 Δ H1 Δ trp (pLB6)- e.g. normal restriction properties and normal viability at 42°C. This result indicated that this type of gene fusion did not result in an enhanced synthesis of either gene product and that the unexpressed DNA present between the promoter and the gene prevented efficient expression of the genes. Therefore we chose to further engineer the system and to separately clone each of the genes downstream of the lambda P_L promoter.

Construction of a methylase overproducing strain.

Plasmid pLB7 (containing the MetRV gene flanked by BamH I and Hind III sites) was used as the source of the DNA fragment containing only the methylase gene to construct methylase overproducing strains. The MetRV gene segment was excised and inserted in the pLK53 expression vector (Figure 2). Recombinant clones selected at 28°C were tested for viability at 42°C and for methylating activity. The recombinants obtained by insertion of the methylase gene in pLK53 (pLB73) were properly methylating at 28°C but poorly growing at 42°C ($eoc=10^{-2}$). Crude extracts of these recombinant clones were loaded onto SDS-PAGE before and after temperature induction. Already 30 minutes after induction a new protein band, with an apparent molecular weight of 32 Kd (Figure 3), appeared. This protein was comigrating with the purified EcoR V methylase (Data not shown). Two hours after induction we estimated the methylase to represent 5-10% of the total cell proteins. This constitute a 50-100 fold increase in methylase synthesis, as compared to the parental pLB7 strain (R. Brown, personal communication).

Construction of endonuclease overproducing clones.

To overproduce the endonuclease we had to remove the methylase gene preceeding the endonuclease gene on the DNA segment derived from pLB6. On the otherhand, the presence of the methylase is necessary to ensure the viability of the strain. We, therefore, constructed a plasmid compatible with the pLK vectors, harboring the MetRV gene (Figure 4). The MetRV gene was transferred from pLB7 to pACYC184 (13), using the BamH I and Hind III sites present in the tetracycline resistance gene. This plasmid pLBM directs the production of enough methylase to fully methylate lambda DNA, although

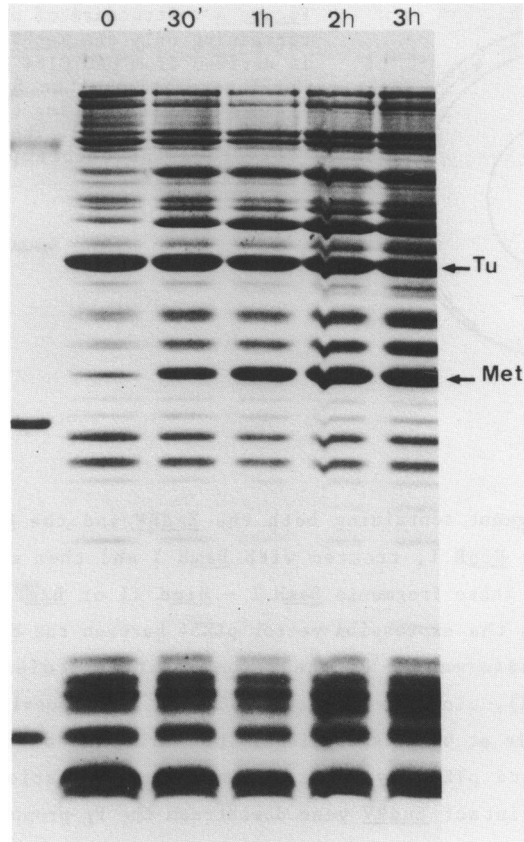
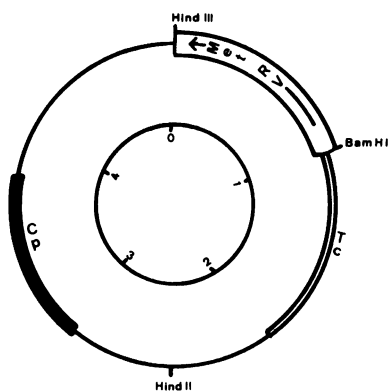


Figure 3 : Overproduction of the methylase. Crude extracts of an induced culture of the overproducing strain K12 H1 trp(pLB73) were loaded on a 12.5% SDS-polyacrylamide gel. Channel 0 correspond to an aliquot taken just prior to temperature shift, the others to different induction times as indicated on top of the gel. The protein band corresponding to the elongation factor tu (corresponding to 5 -10% of the total protein content) is indicated as well as the EcoR V methylase band.

its copy number is 2 or 3 fold lower than that of pBR322 (Data not shown). The strain K-12 Δ H1 Δ trp (pLBM) could be used as a receptor to isolate new plasmid molecules containing only the EndRV gene.

The first approach to overproduce the endonuclease is described in Figure 5. The plasmid pLK331 (identical to pLB6, but carrying the EcoR V system in the inverted orientation) was used as a source of DNA for the constructions and the overlapping restriction sites for Hind II and Mlu I, which are present in the intergenic region, were used to separate the two



pLBM 5 Kb

Figure 4 : Structure of pLBM, a plasmid containing only the MetRV gene. This plasmid is derived from pACYC184 by insertion of the MetRV gene between the Hind III and BamH I site, thus inactivating the tetracycline resistance gene (Tc). The region noted Cp represents the chloramphenicol resistance gene.

genes. The DNA segment containing both the EndRV and the MetRV genes was first excised by EcoR I, treated with BamH I and then cleaved by either Hind II or Mlu I. These fragments BamH I - Hind II or BamH I - Mlu I were then inserted in the expression vector pLK54 between the EcoR I and BamH I sites. After transformation of the recombinant molecules in the strain K-12ΔH1Δtrp(pLBM), clones were isolated that were poorly restricting at 28°C and not viable at 42°C. Analysis of the structure of the recombinant plasmids (pTZ1 and pTZ2) by restriction enzyme digestion confirmed the presence of the intact EndRV gene downstream the P_L promoter. A 8-15 fold increase in endonucleolytic activity 'in vitro' was found -as compared to the parental strain pLK331- after induction (Table 1), but only a weak new protein band was observed in the expected molecular weight range.

Construction of improved endonuclease overproducers

We tried to optimize the constructions by shortening the distance between the lambda P_L promoter and the beginning of the gene (Figure 6). To this end, the plasmid pLK331 was cut with Mlu I and then treated very shortly with the exonuclease Bal31, to remove all or part of the 90 bp region upstream of the AUG codon. The resected EndRV DNA fragments were then isolated using the enzyme BamH I, and inserted in the expression vector pLK54.

A series of recombinant clones was obtained with a phenotype similar to that of pTZ1 and pTZ2 (non-viable at 42°C and poorly restricting at 28°C). A 30-35 fold increase in the 'in vitro' endonucleolytic activity was found in the two recombinants mapping closest to the AUG start codon. The other recombinant clones exhibited activities similar to those found with

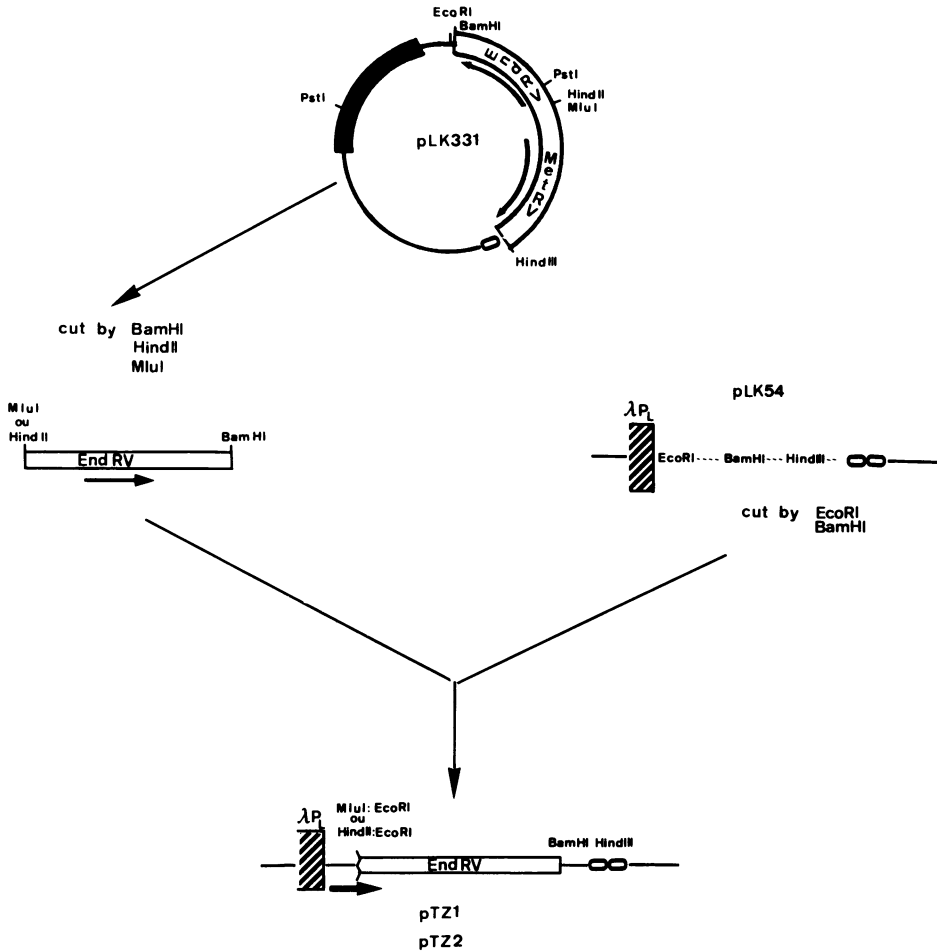


Figure 5 : Construction of strains overproducing the EcoRV endonuclease. Direct insertion in pLK54 of the BamH I-Hind II DNA segment containing the EndRV gene.

pTZ1 and pTZ2 (Table 1). The analysis, on SDS-PAGE, of the protein content of the recombinant clones exhibiting the highest endonucleolytic activity (namely K-12 Δ H1 Δ trp(pTZ115) and K-12 Δ H1 Δ trp(pTZ225)), revealed the appearance of a new protein band in the expected range of molecular weight (29 Kd)(1). That band amounted to roughly 2% of the total cellular proteins and could be detected after prolonged incubation - this band appears after 2 hours induction and continues to increase to reach the highest level after 6 to 8 hours (Figure 7).

TABLE 1: Endonucleolytic activity of the endonuclease overproducing strains.

| Plasmid | Activity at 28°C | Activity at 42°C | Distance to AUG | Amplification |
|---------------|------------------|------------------|-----------------|---------------|
| pLK331 | 0.5 | 0.5 | | 1 |
| pTZ1,pTZ2 | <4 | 4-7 | 90 | 8-14 |
| pTZ159 | " | 3.6-7 | 80 | 7-14 |
| pTZ236,pTZ242 | " | 4.4-7.4 | 65 | 9-15 |
| pTZ100 | " | 4.8-8.4 | 50 | 9-17 |
| pTZ272 | " | 3.8-7.8 | 40 | 8-16 |
| pTZ98,pTZ90 | " | 3.4-7 | 25 | 7-14 |
| pTZ225 | " | 12.2-17 | 12 | 24-34 |
| pTZ115 | " | 15-18 | 5 | 30-36 |

The activity is expressed in 10^4 u/OD (Unit per OD). One unit corresponds to the amount of enzyme required to fully degrade 1 ug of lambda DNA in one hour at 37°C. The distance to the AUG has been calculated by restriction enzyme digestions and is therefore given with a 10 bp precision, except for plasmid pTZ115 and pTZ225 where the nucleic sequence of the fusion is available.

The exact fusion point between P_L and the EndRV gene was sequenced in these two cases (pTZ115 and pTZ225)(Figure 8). In pTZ225 the fusion point is located just upstream of the putative Shine and Dalgarno sequence (GGA), while in pTZ115 the original Shine and Dalgarno sequence is removed and the fusion occurs 5 bp in front of the AUG. Fortunately the same sequence element (GGA) is brought by the P_L DNA fragment and this clone directs a slightly higher synthesis of endonuclease.

The viability of the cells rapidly decreases after induction (data not shown) while the protein synthesis continues for at least 6 hours. To check whether there is still a 'de novo' protein synthesis after prolonged incubation, bacterial cultures were pulse labeled with a ^{14}C protein hydrolysate at regular intervals as described in Material and Methods. The synthesis of the EcoR V endonuclease continues through the whole period of induction, while the synthesis of cellular proteins is shut off after 2 hours (Figure 9). The accumulation of the EcoR V endonuclease is paralleled by that of another protein which we believe is the chloramphenicol acetyl transferase

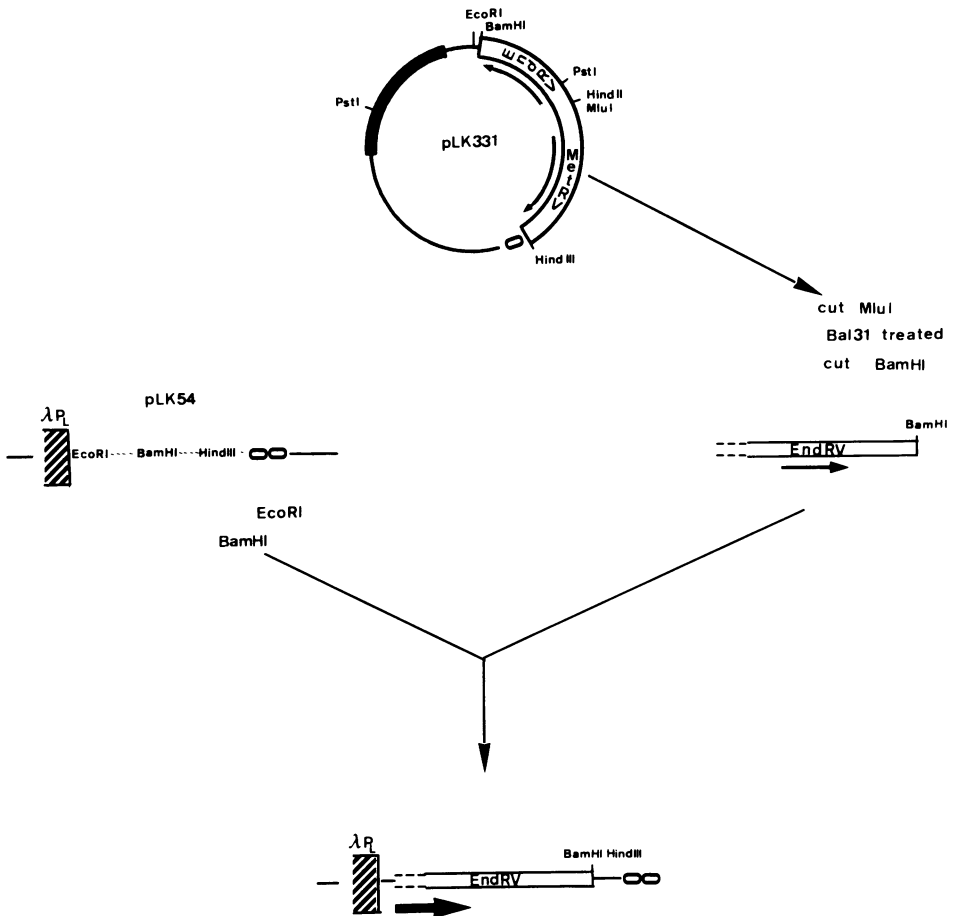


Figure 6 : Optimization of the construction by shortening the length of DNA between the Lambda P_L promoter and the beginning of the *EcoRV* endonuclease gene.

on the basis of its apparent molecular weight. Moreover, the corresponding promoter (P_{cat}) is known to be very efficient and is supposed to direct the synthesis of high levels of proteins such as the one observed in this experiment (14).

The clone (K-12 Δ H1 Δ trp (pTZ115)) was successfully used to purify the *EcoRV* endonuclease in large quantities (0.9 mg of purified *EcoRV* endonuclease were obtained per gramm of bacteria - S. Halford, personal communication). All of the overproduced enzyme can be recovered and is fully active.

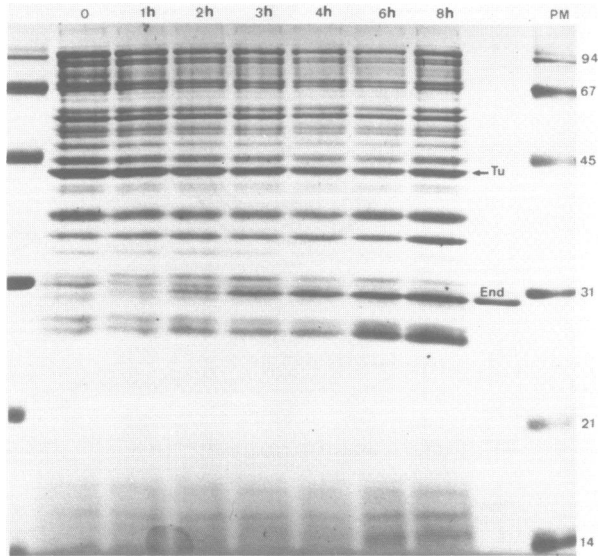
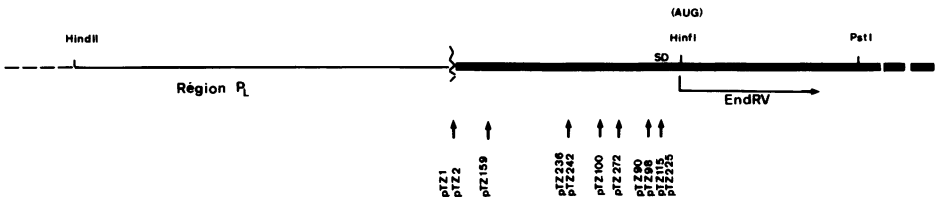


Figure 7 : Overproduction of the endonuclease by the strain K12 H1 trp(pTZ115). Crude extracts of the overproducing strain were loaded on a 12.5% SDS-polyacrylamide gel. The time of induction corresponding to each sample are indicated at the top of the gel. Channel 0 corresponds to the aliquot taken just prior to induction. The bands corresponding to the elongation factor tu and to the EcoR V endonuclease are indicated.



pTZ225 AAGCATTGGA TGGAAAAAAAACATGAGTC
 pTZ115 AAGCATTGGA AAAACATGAGTC

Figure 8 : Position of the fusion points between the lambda P_L promoter and the endonuclease gene in the recombinant plasmid obtained by random fusion. The second half of the figure shows the sequence of the fusion in the cases of plasmids pTZ115 and pTZ225.

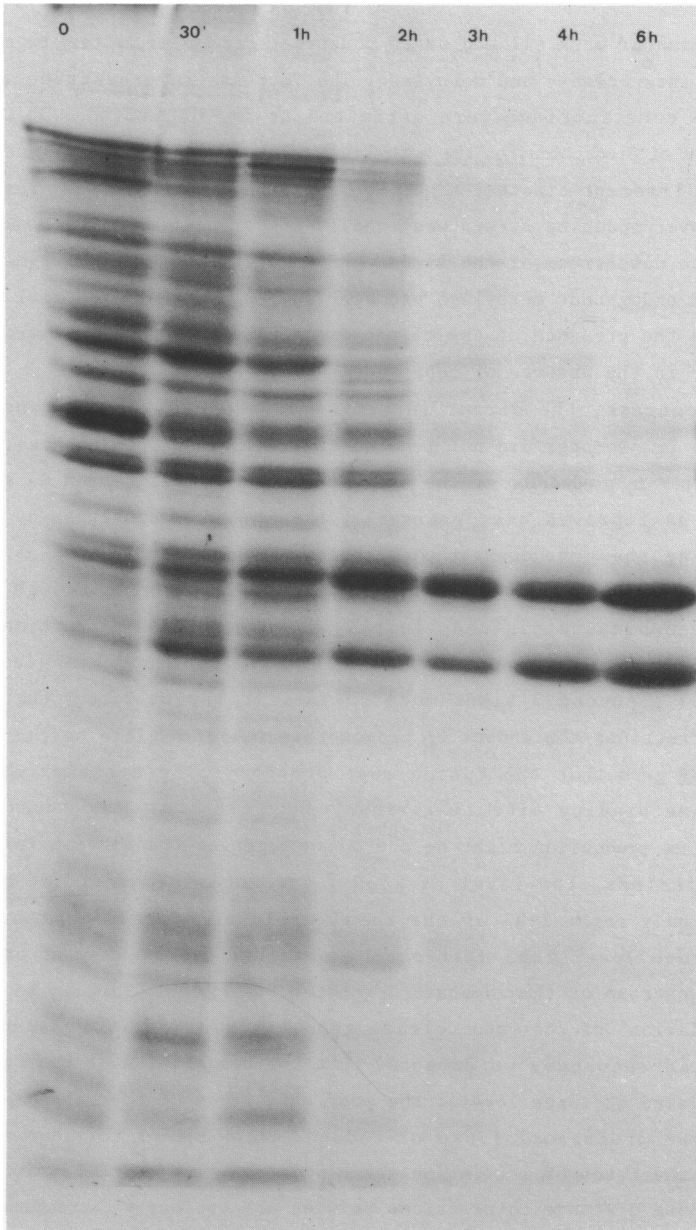


Figure 9 : Pulse labelling experiment. The clone used is 1100(pRK248)(pLBM)-(pTZ115). Aliquots of the culture were taken after different induction times, (indicated at the top of the gel) and pulse labelled for 20 minutes with ^{14}C protein hydrolysate. Two bands appear: the upper one is the *EcoR* V endonuclease and the lower one the chloramphenicol acetyl transferase.

DISCUSSION

The present work allowed us to construct strains separately overproducing the EcoR V endonuclease and methylase. The increase in production obtained with these constructions were estimated at 50 -100 and 30 - 35 times the natural level of production for the methylase and the endonuclease respectively.

Two different strategies had to be designed to reach this result. The methylase overproducing strain was readily constructed by direct insertion of the gene downstream of the lambda P_L promoter. The cloned fragment still contains the endogenous methylase promoter and, consequently, normal activity is found in the presence of the cI repressor. The 50 - 100 fold overexpression is obtained in the absence of repressor.

In contrast, the direct insertion of the EndRV gene downstream of the lambda P_L promoter did not lead to a very substantial increase, neither in activity nor in production of the endonuclease. For that reason we attempted to obtain an improved overproducer of the EcoR V endonuclease by randomly positioning the gene downstream of the lambda P_L promoter, so as to bring the gene and the promoter in an optimal configuration. Although most of the new recombinants yielded levels of endonuclease production similar to those obtained with plasmids pTZ1 and pTZ2, two recombinants (pTZ115 and pTZ225) produced 3 times more enzyme. It is noteworthy that in both these constructions the lambda P_L promoter is fused so close to the beginning of the EndRV gene that the fusion most probably alters the structure of the ribosome binding site. This result suggest that this enhanced level of endonuclease production might be due to an improved translation initiation.

Nonetheless, the level of EcoR V endonuclease specified by pTZ115 and pTZ225 only reach 2-5% of the total cellular protein content, which is still much lower than what can be obtained in the case of other gene fusions downstream of the lambda P_L promoter (8, 15, 16). This may be explained by a 'modulation' of the endonuclease gene translation (1). Based on the nucleic acid sequence, we proposed that the translation of the EndRV gene may be modulated at three levels: the poor efficiency of the ribosome binding site and the preferential use of codons corresponding to minor tRNAs may reduce both the rates of initiation and elongation. The third level, involves potential long distance interactions between mRNA secondary structure elements and will be discussed below. Each of these separate features could contribute to the low level of endonuclease production.

Several lines of evidence support that the EndRV ribosome binding site is very inefficient. First, the improved overproducers (pTZ115 and

pTZ225) probably result from a fortuitous rearrangement of the ribosome binding site configuration, thereby increasing the efficiency of translation initiation. However, even in this case, the level of overproduction is far from being optimal. The poor efficiency of translation initiation in pTZ115 has been confirmed by constructing EndRV - lacZ gene fusions. First, the lambda P_L promoter together with the beginning of the EndRV gene (in the pTZ115 configuration) have been fused to the lacZ gene in the correct reading frame, then the cro ribosome binding site has been substituted to that of the EndRV gene. The first construction (EndRV ribosome binding site) directs the production of a low level (2-5% of the total cellular proteins) of β -galactosidase, while the β -galactosidase production amounts to 30% of the total proteins in the substitution constructions (cro ribosome binding site)(J. Botterman, unpublished data).

Although in the case of the EcoR I endonuclease, where roughly the same codons are used (17, 18), very high levels of production were obtained (8), the codon usage might also contribute to the low level of the EcoR V endonuclease overproduction as was shown in other instances (19, 20).

The third level of translation modulation is suggested by the potential secondary structure of the EndRV gene mRNA: the terminator stem-loop structure could interact with the sequence surrounding the initiation codon, and thus block the translation initiation (1). This interaction would only allow the translation of nascent mRNAs, but not that of mature fully transcribed mRNAs. This could provide a rigorous control of the level of endonuclease in the cell. Preliminary results show that removing part of the terminator structure in pTZ115 results in an enhancement of endonuclease production (J. Botterman, unpublished data). This would indicate that the structure of the mRNA contributes to the low level of endonuclease overproduction although site directed mutagenesis experiments would be required to fully confirm this point. The plasmids pTZ115 and pTZ225 do offer a good way to study in more detail the expression and regulation of the EcoR V system.

Another interesting feature emerges from the pulse labeling experiment. It appears that the cellular protein synthesis stops after two hours induction, while, the plasmid encoded proteins are still synthesised and accumulate. That situation closely resembles the pattern of protein synthesis in the maxicell system (21). In our case, the chromosomal DNA is probably degraded when the level of endonuclease present in the cell becomes critical, thus causing the chromosome directed protein synthesis to stop. In contrast, the two plasmids pLBM and pTZ115, being devoid of EcoR V sites, are not subject

to degradation by the endonuclease and are still able to direct protein synthesis.

The overproducing strains described in this paper have been successfully used to purify large amounts of both enzymes, thus allowing crystallisation attempts. Crystals of the native endonuclease, as well as crystal of the complex formed between this enzyme and its substrate (a decanucleotide containing an EcoR V site) were readily obtained (5). That should permit the resolution of the tridimensionnal structure of this enzyme in the near future.

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