

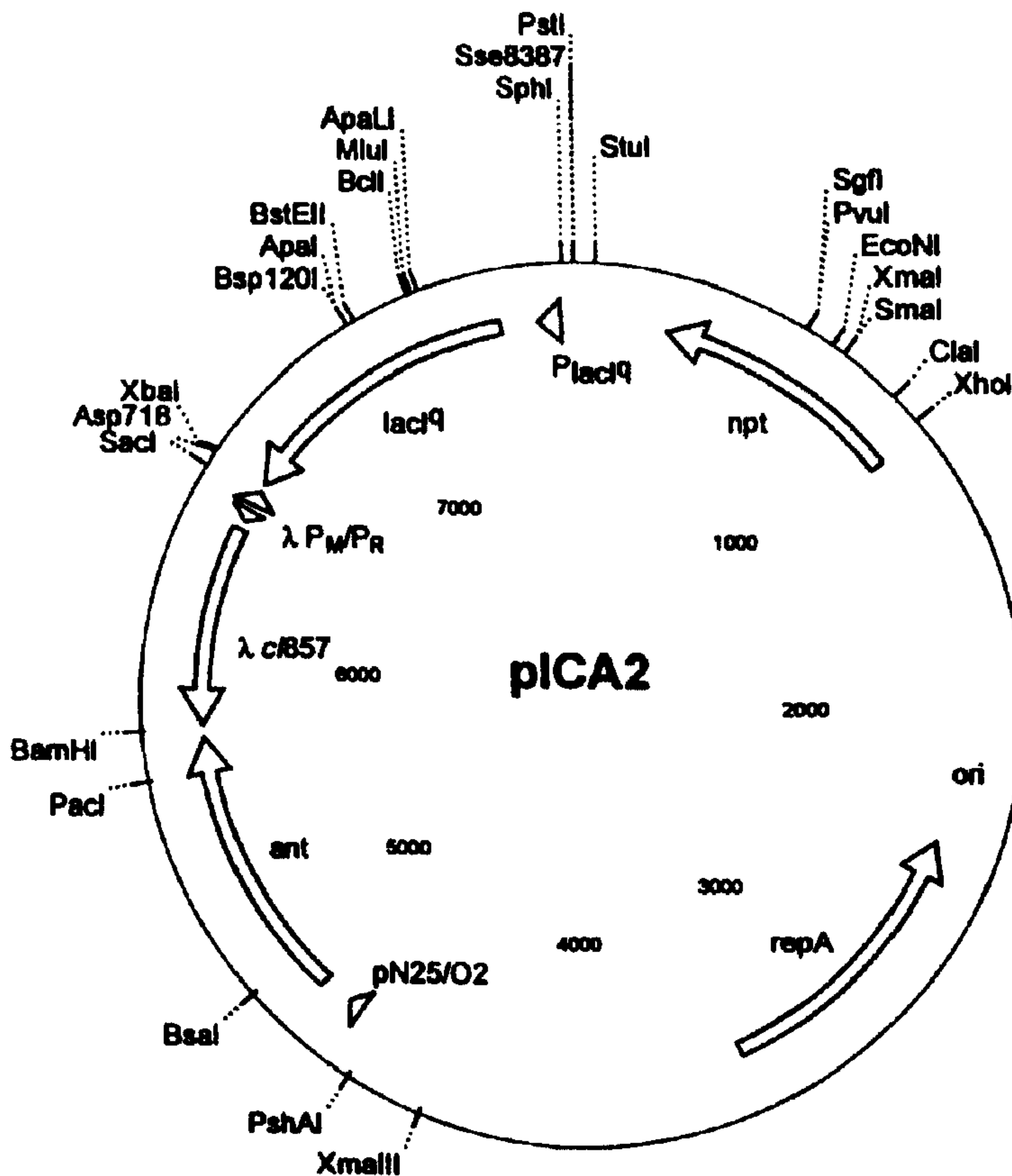


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(54) Titre : SYSTEME REGULATEUR DESTINE A L'EXPRESSION INDUITE DE GENES AU MOYEN DE PROMOTEURS LAMBDOIDES

(54) Title: REGULATORY SYSTEM FOR INDUCIBLE EXPRESSION OF GENES WITH LAMBDOID PROMOTERS



(57) Abrégé/Abstract:

The invention relates to a regulation system for inducible expression of genes, comprising a lambdoid promoter, a gene coding for a repressor for the lambdoid promoter and a gene coding for an antirepressor of the repressor, which antirepressor is under the

(57) Abrégé(suite)/Abstract(continued):

influence of an inducible promotor. The invention further relates to a regulatory replicon, comprising said gene coding for an antirepressor, an expression system, comprising said regulatory replicon, and an expression vector based on a lambdoid promotor, and also to a method for producing a gene product in a heterologous host, by providing a culture of a host comprising a heterologous sequence which codes for the gene product, wherein the expression of the heterologous sequence is under the control of a regulation system, a gene coding for a repressor for the lambdoid promotor and a gene coding for an antirepressor, and by inducing the promotor of the antirepressor gene.

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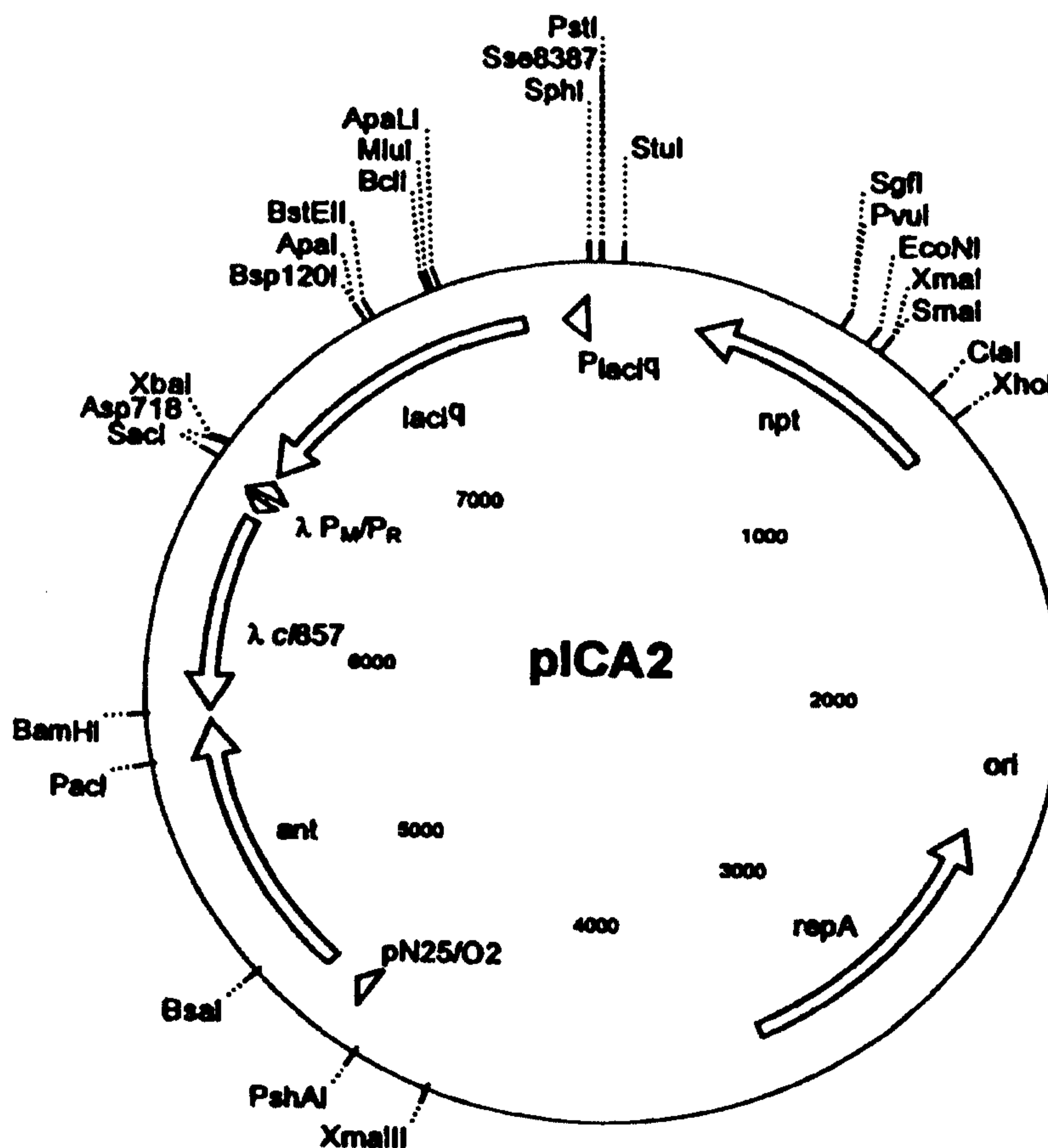
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(54) Title: REGULATORY SYSTEM FOR INDUCIBLE EXPRESSION OF GENES WITH LAMBDOID PROMOTERS

(57) Abstract

The invention relates to a regulation system for inducible expression of genes, comprising a lambdoid promoter, a gene coding for a repressor for the lambdoid promoter and a gene coding for an antirepressor of the repressor, which antirepressor is under the influence of an inducible promoter. The invention further relates to a regulatory replicon, comprising said gene coding for an antirepressor, an expression system, comprising said regulatory replicon, and an expression vector based on a lambdoid promoter, and also to a method for producing a gene product in a heterologous host, by providing a culture of a host comprising a heterologous sequence which codes for the gene product, wherein the expression of the heterologous sequence is under the control of a regulation system, a gene coding for a repressor for the lambdoid promoter and a gene coding for an antirepressor, and by inducing the promoter of the antirepressor gene.



**REGULATORY SYSTEM FOR INDUCIBLE EXPRESSION OF GENES
WITH LAMBDOID PROMOTERS**

The present invention relates to a new regulatory system for inducible expression of genes based on lambdoid promoters. The invention further relates to a regulatory replicon and a method for producing
5 heterologous proteins.

In order to enable production of human or animal proteins in sufficient quantities, the gene which codes for the protein is usually cloned in the bacteria Escherichia coli. This bacteria has a high synthesis
10 capacity and is well characterized at molecular level. Bacterial regulation signals are also required for expression of the cloned gene in the bacterial host.

It has been found that the strongest regulation signals for E.coli do not originate from the bacteria
15 itself but from the bacteria-challenging bacteriophages. There exist so-called non-temperate and temperate phages.

The first type are the phages with unregulated promoters. Genes under the control of such promoters are continuously expressed. This results in a high protein
20 production, which can be detrimental or even lethal to the host bacteria.

The other type, the so-called temperate phages, can insert their DNA in a non-active form in the host genome and therefore co-replicate passively with this
25 host genome. By induction of particular promoters the host is stimulated to produce phage protein or, in the case of expression vectors based on phage promoters, to produce the heterologous protein. As long as there is no induction, expression from the promoter is shut off by
30 means of repressor molecules binding cooperatively to the promoter. The promoters of temperate phages are among the strongest, but also the best expressed and controllable promoters from E. coli known (Lanzer & Bujard (1988); Knaus & Bujard (1988)).

35 The combination of intrinsic strength and superior regulation make these promoters preferable to

other regulated or non-regulated E.coli promoters for obtaining heterologous expression on industrial scale.

The best known and prototype phage from the group of temperate phages is the E.coli phage λ . There are many λ -related or lambdoid phages such as 21, ϕ 80, ϕ 81, 82, 424, 434, P22, etc. These phages usually have a different immunity, inter alia through the use of different promoter sequences, repressor molecules and operator sequences.

Many expression plasmids for heterologous protein production which are used in E.coli are based on the λP_L promoter. The λP_L promoter is very strong and can be well regulated. The best known and most controllable regulation mechanism makes use of a thermosensitive mutant of the original repressor molecule. Induction of protein synthesis from the promoter can in this case be started by increasing the temperature from 28°C to 42°C. The repressor molecule is deactivated by this temperature increase. However, this higher temperature can also be unfavorable for production of many proteins because the protein, instead of remaining soluble, then precipitates for the greater part in the form of so-called inclusion bodies, wherein it loses its activity.

Inclusion bodies are in fact an aggregate of incorrectly folded polypeptide chains. It is a phenomenon which is observed on both laboratory scale and industrial scale when an attempt is made to produce large quantities of a specific protein in E.coli. Inclusion bodies can per se be separated quite easily from the other cellular proteins in one step. However, after isolation of the inclusion bodies the protein must first be denatured by means of for instance urea or guanidine hydrochloride and then slowly refolded into the natural spatial structure. This refolding of the protein from inclusion bodies is not always successful and generally results in a considerable loss of material and entails extra costs in the scale-up process due to an increase in the number of steps in the final processing. The frequent occurrence of

inclusion bodies has resulted in it not always being possible to fully utilize the potential of an economically advantageous expression host for heterologous protein production.

5 It has been found that the formation of inclusion bodies can sometimes be prevented by reducing the fermentation temperature. The reason herefor may be either that the lower temperature has a different effect on the folding of the overproduced protein or that there
10 are fewer newly synthesized protein molecules per unit of time and volume.

When protein synthesis at a lower temperature is desired, it is no longer possible to use the currently existing and much used temperature induction in
15 combination with the strong and well regulated promoters derived from phage lambda and related promoters.

It is therefore the object of the present invention to provide a simple, well controllable regulation system for strong and highly repressable
20 promoters derived from lambdoid phages, with which induction at a lower temperature becomes possible.

This is achieved by the invention with a regulation system for expression vectors, comprising a
25 lambdoid promoter, a gene coding for a repressor for the lambdoid promoter and a gene coding for an antirepressor of the repressor, which antirepressor gene is under the control of an inducible promoter. This promoter can originate from a gene other than the antirepressor gene
itself and is preferably inducible at lower temperatures.

30 The regulation of the heterologous protein expression can now be controlled by the expression of the antirepressor. The absence or presence of the antirepressor determines the suppression respectively
activation of the promoter of the protein to be produced.
35 The presence or absence of the antirepressor is in turn regulated by whether or not the promoter of the antirepressor is induced.

Regulation of the antirepressor gene can occur in different ways. Use can thus be made for instance of a promoter regulatable by lactose, arabinose or the absence of amino acids or of any other regulatable promoter.

5 The different components of the regulation system according to the invention can be located on the chromosome of the host as well as on one or more individual replicons, such as plasmids. In a particular embodiment the antirepressor gene can lie on a regulatory
10 plasmid together with the gene coding for the repressor of the promoter of the antirepressor gene. Such a regulatory plasmid can then be combined with any random expression vehicle containing the heterologous gene and its promoter and repression system. Optionally the
15 repression system of the promoter of the heterologous gene can also be situated on the regulatory vehicle. All components can also lie on different replicons.

In a preferred embodiment of the invention the antirepressor is the ant of the lambdoid phage P22. Ant
20 is coded in the immI region of phage P22 from Salmonella typhimurium and engages into a non-covalent interaction with the C-terminal part of the P22c2 repressor and thereby prevents the dimerization of the c2 repressor required for repressor activity and thereby binding to
25 the operator.

In a preferred embodiment of the regulation system according to the invention the expression of this anti-repressor is under the control of an inducible promoter such as $P_{N25/02}$. Repression of the $P_{N25/02}$ promoter
30 takes place for instance by means of the lacI repressor of E.coli. The induction of this promoter is based on derepression and preferably takes place by means of administering IPTG.

The regulation system according to the
35 invention is a flexible system wherein according to a preferred embodiment the induction of the desired heterologous protein synthesis can take place in two ways. On the one hand the production of the antirepressor

can already be initiated at low temperature by adding IPTG, which leads to derepression of the lambdaoid promoter. It is also possible to use the same bacterial culture, wherein the conventional temperature-dependent induction can still be applied.

The regulation system can be applied with any expression vector derived from lambda. As regulatable promoter of the heterologous gene the λP_L promoter is particularly recommended, but the invention is certainly not limited thereto. The λP_R promoter or any lambdaoid promoter which can be repressed by a repressor with sufficient homology in the C-terminal region to P22c2 to be recognized by ant can also be used.

The principle of the invention, i.e. regulating a promoter inducible by a repression system by means of an antirepressor to be expressed in regulated manner, can of course also be applied within the scope of the present invention in configurations other than those specifically described herein.

The invention further relates to a regulatory replicon, comprising a gene coding for an antirepressor, which antirepressor gene is under the control of an inducible promoter. The replicon can further comprise a gene coding for a repressor of the inducible promoter of the antirepressor gene. A gene which codes for a repressor for a lambdaoid promoter can moreover also be present in the replicon.

In a preferred embodiment a replicon according to the invention comprises the gene coding for the P22ant protein of S.typhimurium, under the control of the $P_{N25/02}$ promoter, the $lacI^q$ gene under the control of the $pLacI^q$ promoter and the gene coding for the cI857 repressor.

A preferred embodiment of a regulatory replicon according to the invention is shown in Figures 1 and 3. Both figures show the plasmid, designated herein pICA2. Fig. 3 shows the general structure and Fig. 1 the restriction map. The construction of this plasmid is described in the examples.

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In an alternative embodiment of the replicon according to the invention the replicon can further comprise the regulation signals, including the lambdoid promoter, required for expression of a heterologous gene.

5 In such an embodiment there are not therefore two separate vectors for expression and regulation, but only one.

The invention moreover relates to an expression system, comprising a regulatory replicon and an expression
10 vector derived from phage lambda. Examples of expression vectors derived from phage lambda are pLT10T or pLR10T.

The invention further relates to a regulation system for inducible expression of a gene or genes of interest (GOI), comprising a lambdoid promoter operably
15 linked to the GOI, the lambdoid promoter being under control of a repressor, a gene coding for the repressor, a gene coding for an antirepressor of the repressor and an inducible promoter regulating the expression of the antirepressor.

20 The invention still further relates to a method for producing a gene product in a heterologous host cell, comprising: culturing a host cell comprising a heterologous sequence which codes for said gene product, wherein said heterologous sequence is under control of a regulation
25 system as described above; and inducing expression of said heterologous sequence by inducing the inducible promoter of said regulation system.

Finally, the invention relates to a method for producing a gene product in a heterologous host, comprising
30 the steps of: providing a culture of a host comprising a heterologous sequence which codes for the gene product,

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wherein the expression of the heterologous sequence is under the control of a regulation system comprising a lambdoid promoter operably linked to the heterologous sequence, a gene coding for a repressor for the lambdoid promoter and a
5 gene coding for an antirepressor, which antirepressor gene is operably linked to an inducible promoter; and inducing the promoter of the antirepressor gene. If the inducible promoter of the antirepressor gene is the $P_{N25/02}$ promoter, it can be induced by adding IPTG to the culture.

10 Following below is a summary of the definitions used in this application.

Antirepressor: protein which can neutralize a repressor and thus activates the repressed promoter.

Phage: (bacteriophage) a virus whose host is a
15 bacteria.

Phage λ : (bacteriophage lambda) temperate bacteriophage which infects Escherichia coli, belongs to the Styloviridae family.

Phage P22: (bacteriophage P22) temperate phage
20 which infects Salmonella typhimurium, belongs to the Podoviridae family.

Gene expression: expression of a gene by transcription and translation to a polypeptide or a protein (functional protein).

Temperate phage: phage which can pass on its genetic information through infection as well as via the cell division of the host after insertion in the genome.

Immunity: (phage immunity) superinfection resistance for phages with the same or homologous regulatory elements.

Inclusion bodies: discrete structures consisting of non-native folded, coagulated protein.

LMBP: Laboratory of Molecular Biology Plasmid collection, recognized deposit body for plasmids, part of the Belgian Coordinated Collection of Micro-organisms (BCCM).

Plasmid: extra-genomic replication unit.

Promoter: DNA sequence which allows transcription to initiate.

Replicon: a unit of DNA replication.

Repressor: protein which prevents transcription initiation on one or more determined promoters.

Vector: a biological entity which can ensure the multiplication of genetic information.

In the examples below the invention is illustrated on the basis of the prokaryote lacZ gene and the eukaryote genes coding for human interferon- γ (hIFN γ), murine Interleukin 2 (mIL2) and human Interleukin 2 (hIL2) as model system for protein synthesis. It will be apparent to a person skilled in the art that in an analogous manner other genes can be expressed in a regulated manner with the system described here without any inventive work having to be performed for this purpose.

EXAMPLES

The materials and methods used are first elucidated hereinbelow. Thereafter the invention is illustrated in the examples. In support of most of the methods reference is further made to Sambrook et al. (1989) Molecular cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA, and Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, NY.

10

MATERIALS AND METHODS1. Bacteria strains, phages and plasmids

All cloning experiments were carried out in E.coli MC1061 (hsdR mcrB araD139 Δ (araABC-leu)7697 Δ lacX74 galU galK rpsL thi) (Casadaban and Cohen, 1980) lysogenized with λ when multiplying λP_L -containing plasmids. Expression experiments were carried out in MC1061 transformed with a regulatory plasmid (for instance pcI857 or pICA2).

20

The repressor came from pcI857 (LMBP537), a vector with a high copy number (P15A replicon), which carries an autogenously regulated cI857 gene coding for a thermosensitive cI mutant (Remaut et al., 1983).

S. typhimurium LT2 (ATCC 19 585) was grown in Nutrient Broth (Difco 0001) supplemented with 0.5% NaCl. Phage P22 (ATCC 19 585-B1) stocks were obtained from confluent lysis plates prepared by using an excess of plaque-forming units with freshly prepared nutrient agar plates. The macerated soft agar was cleared by centrifugation to isolate the phage particles. P22 DNA was prepared by phenol extraction of purified phage particles.

30

pLR10T is a vector derived from pLT10T (Mertens et al., 1995 B) in which the actual translation initiation site is preceded by a small, well-translated cistron which resulted from a fusion of the N-terminal region of T7g10 and the C-terminal piece of the E.coli trpB gene (fig. 1).

35

pDMI,1 (LMBP1594) was obtained from Dr. Dietrich Stüber (Hoffman-La Roche, Basel, Switzerland). (See fig. 2)

5 2. Plasmid construction (fig. 3)

Plasmid DNA purification was carried out as described in Sambrook et al., 1989. All enzymes used for plasmid cloning were obtained from New England Biolabs or Boehringer Mannheim and used according to the
10 recommendations of the supplier.

The P22 ant region was amplified by means of PCR with Vent DNA polymerase (New England Biolabs) using 5'-ATCAGAATTCGCGGTAACAGTCAGGGCTTCGG-3' as forward and 5'-TTAAGGATCCGAAGCTGGGTCGTTGCGTTGG-3' as backward primer.
15 This amplifies a 1054 bp DNA region spanning the coordinates 498-1531 of the POP22IMM Genbank sequence (Sauer et al., 1983). This includes the ant coding region with its own ribosomal binding site and adds a BamHI restriction site to the 3' end.

20 The amplified fragment was trimmed with BamHI and ligated in a pDS12 vector opened with SmaI and BamHI (Stüber et al., 1984). An XhoI-BamHI fragment from pDS12ant (with the $P_{N_{25/02}}$ -ant combination) was combined with the cI857 gene on an EcoRI-BamHI fragment derived
25 from pAT153cI857 (Mertens et al., 1995 A), (LMBP1065), a lacI^q-containing fragment from pUC18lacI^q (LMBP3259) trimmed with AatII and EcoRI, and an AatII-SalI pBR322 (LMBP140) vector part. The resulting pICA1 is a multi-copy number plasmid which contains a combination of lacI^q,
30 cI857 and $P_{N_{25/02}}$ ant which is useful according to the invention. From this plasmid an EagI-PstI fragment was combined with a pUC18Kan (Pharmacia, Sweden) trimmed with PstI and XhoI and containing an npt gene (Km^R (kanamycin resistance)) and an XhoI-EagI vector fragment from pLG339
35 (Stoker et al., 1982) in order to obtain the plasmid pICA2 according to the invention. pICA2 (fig. 1) is a low copy number plasmid compatible with all current

expression vectors in respect of replication origin (pSC101) and antibiotic selection.

3. Gene induction and protein analysis

5 Expression strains containing pCI857, pICA1 or pICA2 were kept at a non-permissive temperature (28°C) during manipulations preceding induction. λP_L -dependent temperature induction was carried out in MC1061 with either pICA2 according to the invention or the known
10 pCI857 by raising the culture temperature from 28°C to 42°C. MC1061 (pICA1) and MC1061 (pICA2) were induced at temperatures of 28°C or lower by adding IPTG to a final concentration of 1 mM. The cells were harvested, resuspended in sonication buffer (SB, 10 mM Tris-Cl pH
15 7.5; 0.1 M NaCl; 5 mM DTT; 10% glycerol) and frozen at -20°C. Aliquots (usually 200 μ l) were thawed at 37°C and cooled on ice. The cells were subsequently opened by sonication on ice using a Sonics & Materials (Danbury ct, USA) sonicator with a microtip. Lysates were then cleared
20 by centrifugation at 15 000 G for 15 minutes.

Prior to cytokine assay the lysates were diluted in SB and filtered over a cellulose-acetate 0.22 μ m pore-size filter.

25 β -Galactosidase was assayed using ONPG as substrate (Miller, 1972).

mIL2 titers were determined by a proliferation assay using the IL2-dependent cytotoxic T-cell line CTLL-2 (Guisez et al., 1993).

30 Human interferon- γ activity was determined on human FS4 cells by a cytopathic effect reduction assay using encephalomyocarditis virus as challenge virus (Devos et al., 1982).

EXAMPLE 1

35 Construction of a regulatory plasmid for IPTG induction of λP_L

The ant gene of phage P22 of S. typhimurium was amplified from purified P22 DNA and cloned in the pDS12

expression vector, as described in Materials and Methods. In this manner the gene was under the control of the $P_{N25/O2}$ promoter (Strueber et al., 1984) inducible by means of IPTG. Induction of the resulting expression plasmid pDS12 ant resulted in a high production of the repressor P22 ant (figure 4B).

The ant gene was subsequently combined with $lacI^q$ and $\lambda cI857$ in a manner such that the different promoters did not interfere with the expression of each individual gene. The resulting combination (pICAL) was transferred to a low copy number replicon which is ColE1-compatible and derived from pSC101 and also carried a kanamycin resistance selection marker (Stoker et al., 1982). The resulting plasmid pICA2 contains all necessary information for repression of λP_L or λP_R (by means of $cI857$) and repression (by means of $lacI^q$) and induction (from the $P_{N25/O2}$ -ant promoter) of the antirepressor, and can therefore be used as a suitable expression regulatory plasmid for IPTG-induced λP_L or λP_R expression when it is combined with an expression plasmid containing a gene under the transcriptional control of the λP_L or λP_R promoter.

EXAMPLE 2

Tight regulation and expression at low temperatures with the P22 ant-based expression system.

In order to quantify the characteristics of the new expression system an expression vector was used containing as model gene a λP_L -driven $lacZ$ gene for protein synthesis. This vector is capable of inducing high levels of functional B-galactosidase by means of translationally-coupled translation initiation (Mertens et al., 1995 A; Mertens et al., 1997).

The non-induced levels of B-galactosidase, which could be influenced by a possible continuous presence of low non-induced levels of antirepressor protein, were comparably low when pICA2 or $pcI857$ (without antirepressor) plasmid were used. This means

therefore that low, non-induced levels of antirepressor protein are probably not present, since if this were indeed the case a higher expression of lacZ would be expected.

5 Investigation of the induction kinetics of the lacZ gene was carried out in MC1061[pICA2] at 18°C, 24°C, 28°C, 37°C or 42°C after adding 1 mM IPTG (figure 4A). Temperatures above 28°C likewise denature the temperature-sensitive cI857 λ -repressor and are an
10 indication of the level of expression obtainable with this vector by thermo-induction. From figure 4A can be concluded that maximum B-galactosidase levels can be obtained by adding IPTG at lower temperatures. As expected, the induction kinetics were slower at
15 temperatures under 28°C, because under these sub-optimal growth conditions a lower growth rate and a lower metabolism are obtained.

Figure 4B compares the level of the induced proteins in MC1061[pICA2][pLR10 β gal] and
20 MC1061[pDMI,1][pDS12 ant], which shows that the high expression level of P22 ant obtained using the high copy number expression vector pDS12 ant disappears by transferring the P_{N25/02}-ant combination to a low copy number plasmid (6-10 copies/cell), while still retaining
25 the ability to induce the lambdoid promoter by induction of ant.

Decreasing the synthesis of the repressor antagonist to <1% of the total protein synthesis is advantageous because the ultimate concern is to obtain a
30 large quantity of a particular protein - the gene of which has been inserted behind the strong lambdoid promoter - and the ultimate yield of this target protein can be adversely affected if the repressor antagonist must be produced in large quantities.

EXAMPLE 3Induction at low temperature can improve the production of soluble, heterologous proteins

Induction resulting in a high expression level, particularly at increased temperatures where the *E. coli* metabolism is high, often results, as already indicated above, in the production of inclusion bodies, while at the same time further cell growth is often inhibited. For reasons which are still not entirely clear, induction at low temperatures is more favourable when production of correctly folded, soluble protein is required. (Lin et al., 1990; Shirano & Shibata, 1990; Schein and Noteborn, 1988; Bishia et al., 1987; Mizukami et al., 1986).

This example therefore investigates whether the different methods of induction (temperature increase or IPTG) result in different quantities of soluble protein.

Used for this purpose were expression vectors derived from pLT10T (Mertens et al., 1995) containing one of the following genes: prokaryotic T7g10 or thioredoxin, two proteins which can be readily expressed in *E. coli*); human interferon- γ (hIFN γ) and murine interleukin-2 (mIL2).

Fig. 5 compares production levels obtained either after thermo-induction or after low temperature IPTG induction. It can be clearly inferred from the prokaryotic examples, T7g10 and thioredoxin, that in the same time-span the same quantity of heterologous protein can be induced. The strains induced by means of IPTG continued to grow and eventually synthesized a larger quantity of host proteins (fig. 5B, lane B). This resulted in a lower yield in comparison with the total protein content (% of the total protein), but gave practically the same absolute yield (mg protein per litre culture). In the figure equivalent quantities of bacterial cultures are compared to each other.

Figure 6 shows the activities of mIL2 and hIFN γ obtained after induction by temperature increase or by IPTG induction.

In these experiments with the eukaryotic proteins human interferon- γ and murine interleukin-2, two proteins which do aggregate readily in E.coli, less heterologous protein is formed after the IPTG induction at low temperature (figure 5A). However, the high expression at 42°C results exclusively in the forming of inclusion bodies, while after IPTG induction at 28°C a significant quantity of soluble protein is produced (fig. 6A and 6B). The soluble protein fraction visible on gel corresponds with the obtained amount of activity after a biological titration.

EXAMPLE 4

Use of the ant-based induction system from different replicons

In a preferred embodiment of the invention the synthesis of the repressor, the antirepressor and the repressor of the promoter controlling the gene of the antirepressor takes place from a low copy number plasmid. This example illustrates the use of the ant system from other replicons.

As the case arises, the regulatory genes (repressor cI857 controlled by the auto-regulatory promoter P_M , antirepressor ant controlled by the $P_{N25/02}$ promoter and the lacI gene controlled by the constitutive P_{lacI}^q -promoter) were induced from a high copy number plasmid and a ColE1/pMB1 replication origin (figure 7A). The gene for expressing (human interleukin 2, hIL2) was linked on a plasmid with a high copy number and a broad host range to the λP_L promoter and a prokaryote ribosome binding site (originating from the ner gene of phage Mu). This plasmid also contains an extra copy of the λP_L repressor gene cI857. The pPLGNIhIL2 plasmid also contains the required functions also enabling replication in other bacteria (figure 7B).

Induction was investigated after adding 0, 0.1 or 1 mM of the inducer IPTG, which provides induction of ant from the $P_{N25/02}$ promoter, which in turn brings about

inhibition of the λP_L repressor cI857 and thus activates the λP_L , which results in overexpression of, in this case, hIL2 (figure 7C). These inductions were carried out at 28°C and compared with the classic temperature
 5 deactivation of cI857 by growing the bacteria further at 42°C.

In this example the relative production of hIL2 using the ant system and by temperature induction are roughly equal. The temperature induction at 42°C was
 10 however found to cause a greater growth-inhibiting effect (the gel shows equivalent samples of culture medium).

EXAMPLE 5

An ant-based expression system for the lambdoid promoters
 15 P22P_L and P22P_R

Induction of a repressor antagonist in order to induce a promoter can in principle also be applied to promoters other than the λP_L . In this example a vector system is described which makes use of the P22 ant gene
 20 to deactivate the homologous P22c2 repressor and thus obtain induction of the P22P_L or the P22P_R.

Constructed first for this purpose was the pICA3 plasmid which is a derivative of pICA2 but which contains the P22c2 repressor gene instead of the λ cI857
 25 repressor gene. The λP_L promoter in pLT1OT3 was further replaced by both the P22P_L (pLT22T3) and the P22P_R (pRT22T3). Both pICA3 and pLT22T3 and pRT22T3 are shown in figure 8.

30 EXAMPLE 6

An ant-based regulatory system for λP_L and λP_R that is inducible with L-arabinose

In the pICA2 regulatory plasmid, the induction of ant is controlled by the IPTG-inducible P_{TS N25/02}.
 35 Control of ant-gene expression can in principle come from any inducible promoter. It is preferred that the promoter of choice is inducible also at lower temperatures (28°C or lower). It is also preferred that the promoter of

choice be well regulated. If this would not be the case, the level of expression of the uninduced culture can be sufficient to initiate continues expression. Uncontrolled expression is likely to result in eventual loss of the functional expression strain.

In this example a regulatory plasmid was constructed containing the promoter region of the E.coli arabinose operon (P_{araBAD}) and the gene encoding the araC repressor of this promoter. The ant-gene is in this way controlled by a promoter which is inducible by the addition of L-arabinose.

The ParaBAD promoter and the araC repressor gene encoding the repressor for this promoter were amplified from a wild type E.coli K12 bacterial strain, using the PCR primers NM73 (ATATATCCAAGGTTATGCAATCGCCATCGTTTCACTCC) and NM72 ATATCGGCCGTTATGACAACTTGACGGCTACATC. PCR amplification was performed with Vent DNA polymerase (New England Biolabs) and the resulting fragment was cloned between the XmaIII and the Styl restriction sites present in pICA2. The resulting pICA5 plasmid was characterized by restriction site mapping, PCR analysis and the PCR amplified insert was sequenced. Figure 9 shows the pICA5 plasmid.

EXAMPLE 7

Comparison of the λP_L -ant induction system with other IPTG inducible expression systems

Although the λP_L promoter is amongst the strongest promoters recognized by the E.coli transcriptional machinery, the T7 promoter, which is recognized by the extremely active T7 RNA-polymerase (T7RNAP), allows for a far greater amount of mRNA to be formed (Studier and Moffatt, 1986). A well-controlled, IPTG-based induction system for the T7RNAP that also resides on a pSC101-derived low-copy number plasmid system was previously described (Mertens et al., 1995b). The pLT10mIL2T and the pLT10hIFN γ T expression plasmids were used, which contain both the λPL and the P_{T7}

promoters (Mertens et al., 1995a), to compare the expression obtained from either the P_{T7} and the λP_L induction system upon addition of IPTG (Fig. 10A).

Strikingly, cells containing the T7-based induction system stopped growing almost immediately after induction, while those induced by the λP_L -ant system continued to proliferate. This resulted in an almost 10-fold higher biomass after 4 h of induction at 28°C. Remarkably, after induction of the P_{T7} T7RNAP system all of the mIL2 and almost all of the hIFN γ were in the insoluble phase, while when using the λP_L -ant system about 50% of the heterologues protein was found in the soluble phase.

The coding sequence of hIFN γ combined with the strong RBST7g10 resulted in a favorable translation initiation region (Mertens et al., 1995a). When combined with a strong promoter on a high-copy number plasmid, abundant expression was obtained after induction. To emphasize the difference in promoter strength between various IPTG-inducible promoters such as P_{trc} (Amann et al., 1988), $P_{T5 N25/02}$ (Stüber et al., 1984) and P_{T7} (Mertens et al., 1995b; Studier et al., 1990) and the λP_L -ant system, the RBS-gene-terminator combination combined with the aforementioned promoters was transferred to an RK2 replicon (Blatny et al., 1997). This resulted in expression plasmids with a much lower copy-number than the normally used ColE1-derived vectors. Subsequently the induction of hIFN γ was then compared using these vectors. Using the stronger P_{T7} resulted in a higher level of production, but all of the hIFN γ produced was insoluble. Employing the P_{trc} and $P_{T5 N25/02}$ promoters did not result in visually detectable levels of induced protein from this low-copy number vector after SDS-PAGE staining with Coomassie Brilliant Blue. However, when the λP_L -ant system was used a clearly detectable level of huIFN γ was synthesized, while the protein remained completely soluble (Fig. 10B).

Fig. 10 shows that induction at 28°C using the $\lambda P_L/ant$ system is more efficient in producing functional protein than other IPTG-inducible expression systems. Fig. 10A demonstrates the SDS-PAGE analysis of soluble (S) and pelleted (P) proteins after induction of pLT10mIL2T or pLT10hIFN γ T in either MC1061 [pT7POL26] (inducing the T7 promoter) or MC1061 [pICA2] (inducing the λP_L -promoter). Induction was obtained by growing for 5 h at 28°C in the presence of IPTG. Clearly, more soluble mIL2 was obtained by using the $\lambda P_L/ant$ induction system. Unlike the T7-system, the latter system also allowed the cultures to continue growing, and thus resulted in a higher biomass accumulation.

Fig 10B is a comparison of expression of the RBS_{T7g10}-hIFN γ -T7T ϕ module combined with some different IPTG-inducible promoters on an RK2-derived low-copy number plasmid. (S=soluble, P=pelleted fraction). Arrow points indicate the position of the induced proteins. Protein markers (M) are 94; 67; 43; 30; 21 and 14kDa.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE

(ii) TITLE OF INVENTION: REGULATORY SYSTEM FOR INDUCIBLE EXPRESSION OF
GENES WITH LAMBDOID PROMOTERS

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: ONT

(E) COUNTRY: CANADA

(F) ZIP: K1P 5Y6

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII (text)

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: CA 2,287,541

(B) FILING DATE: 23-APR-1998

(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: NL 1005884

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: FETHERSTONHAUGH & CO.

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30 (C) REFERENCE/DOCKET NUMBER: 29723-5

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23

(A) TELEPHONE: (613)-235-4373

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(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 32

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: synthetic construct

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 1:

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(2) INFORMATION FOR SEQ ID NO.: 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 31

(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: synthetic construct

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 2:

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20 (2) INFORMATION FOR SEQ ID NO.: 3:

(i) SEQUENCE CHARACTERISTICS

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(ii) MOLECULAR TYPE: DNA

(A) ORGANISM: synthetic construct

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 3:

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(ii) MOLECULAR TYPE: DNA

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(A) ORGANISM: synthetic construct

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 4:

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34

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CLAIMS:

1. A regulation system for inducible expression of a gene or genes of interest (GOI), comprising a lambdoid promoter operably linked to the gene or GOI, the lambdoid promoter being under control of a repressor, a gene coding for the repressor, a gene coding for an antirepressor of the repressor and an inducible promoter regulating the expression of the antirepressor.
2. The regulation system as claimed in claim 1, wherein the inducible promoter originates from a gene which is not the antirepressor gene.
3. The regulation system as claimed in claim 1 or 2, wherein the antirepressor is the *ant* protein of the lambdoid phage P22 from *Salmonella typhimurium* and is coded in the *immI* region thereof.
4. The regulation system as claimed in any one of claims 1 to 3, wherein the inducible promoter is P_{N25/02}.
5. The regulation system as claimed in claim 4, wherein repression of the P_{N25/02} promoter takes place by means of the *lacI* repressor of *E. coli*.
6. The regulation system as claimed in any one of claims 1 to 5, wherein the system comprises a plurality of replicons.
7. The regulation system as claimed in claim 6, wherein a regulatory replicon of the plurality of replicons comprises the gene coding for the antirepressor and the inducible promoter regulating the expression of the antirepressor.

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8. The regulation system as claimed in claim 7, wherein the inducible promoter originates from a gene other than the antirepressor gene.

9. The regulation system as claimed in claim 7 or 8, wherein the replicon further comprises a gene coding for a repressor of the inducible promoter of the antirepressor gene.

10. The regulation system as claimed in any one of claims 7 to 9, wherein the replicon further comprises the gene which codes for the repressor for the lambdoid promoter.

11. The regulation system as claimed in claim 10, wherein the replicon comprises the gene coding for the P22 ant protein of *S typhimurium* under the control of a P_{N25/02} promoter, a lacI^q gene under the control of a pLacI^q promoter and the gene coding for a c1857 repressor.

12. The regulation system as claimed in claim 11, wherein the replicon is the plasmid pICA2 as shown in Fig. 1.

13. The regulation system as claimed in any one of claims 7 to 12, wherein the replicon further comprises the regulation signals, including the lambdoid promoter, required for expression of the GOI.

14. The regulation system as claimed in claim 13, wherein the replicon further comprises the GOI operably linked to the lambdoid promoter.

15. The regulation system according to any one of claims 7 to 12, comprising an expression vector comprising the lambdoid promoter operably linked to the GOI.

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16. The replicon as defined in claim 11.

17. The replicon as defined in claim 12.

18. The replicon as defined in claim 14.

19. A method for producing a gene product in a
5 heterologous host cell, comprising:

culturing a host cell comprising a heterologous
sequence which codes for said gene product, wherein said
heterologous sequence is under control of a regulation
system according to any one of claims 1 to 15; and

10 inducing expression of said heterologous sequence
by inducing the inducible promoter of said regulation
system.

20. The method as claimed in claim 19, wherein the
inducible promoter is the P_{N25/02} promoter and is induced by
15 adding IPTG to the culture.

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PATENT AGENTS

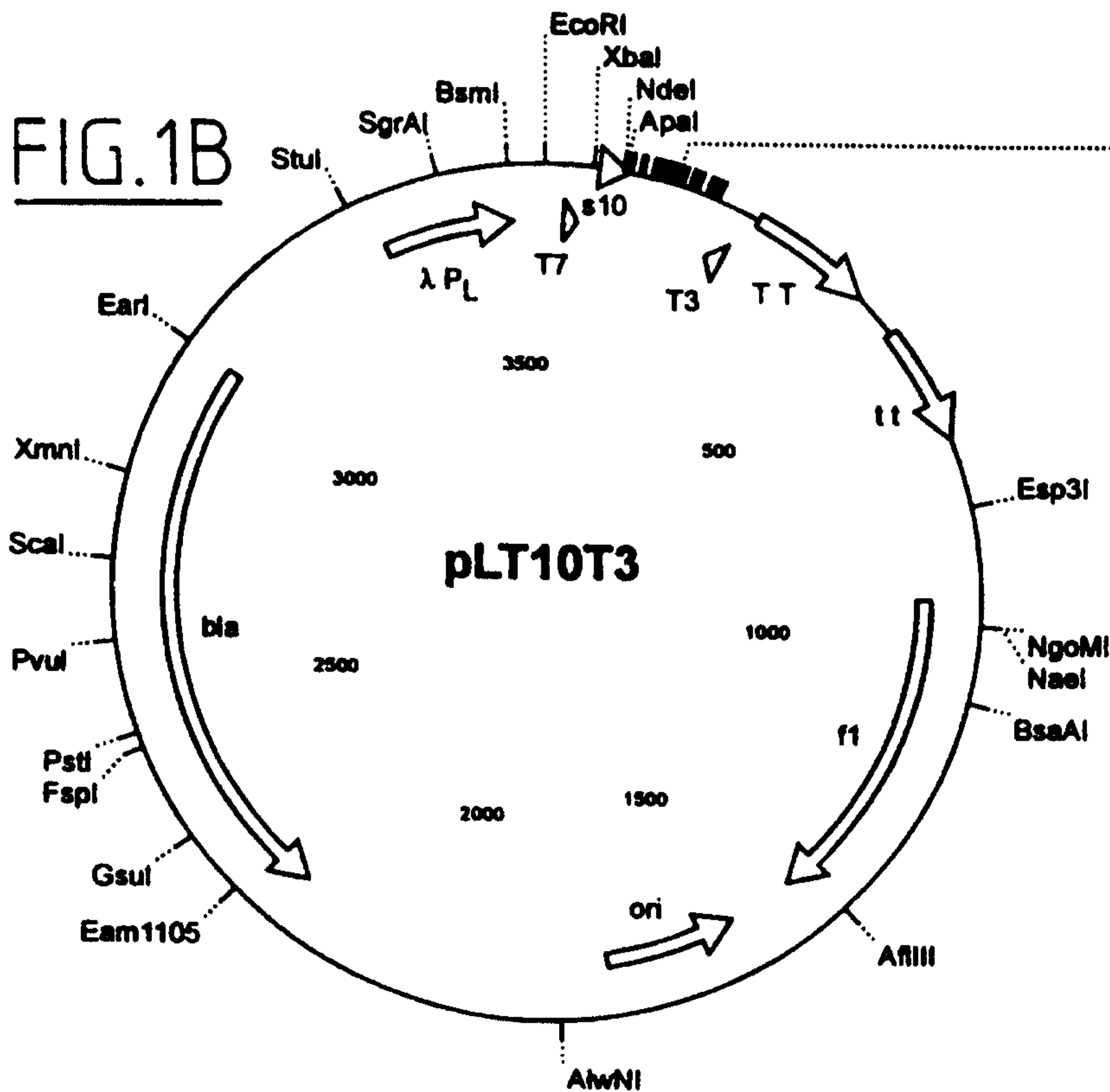
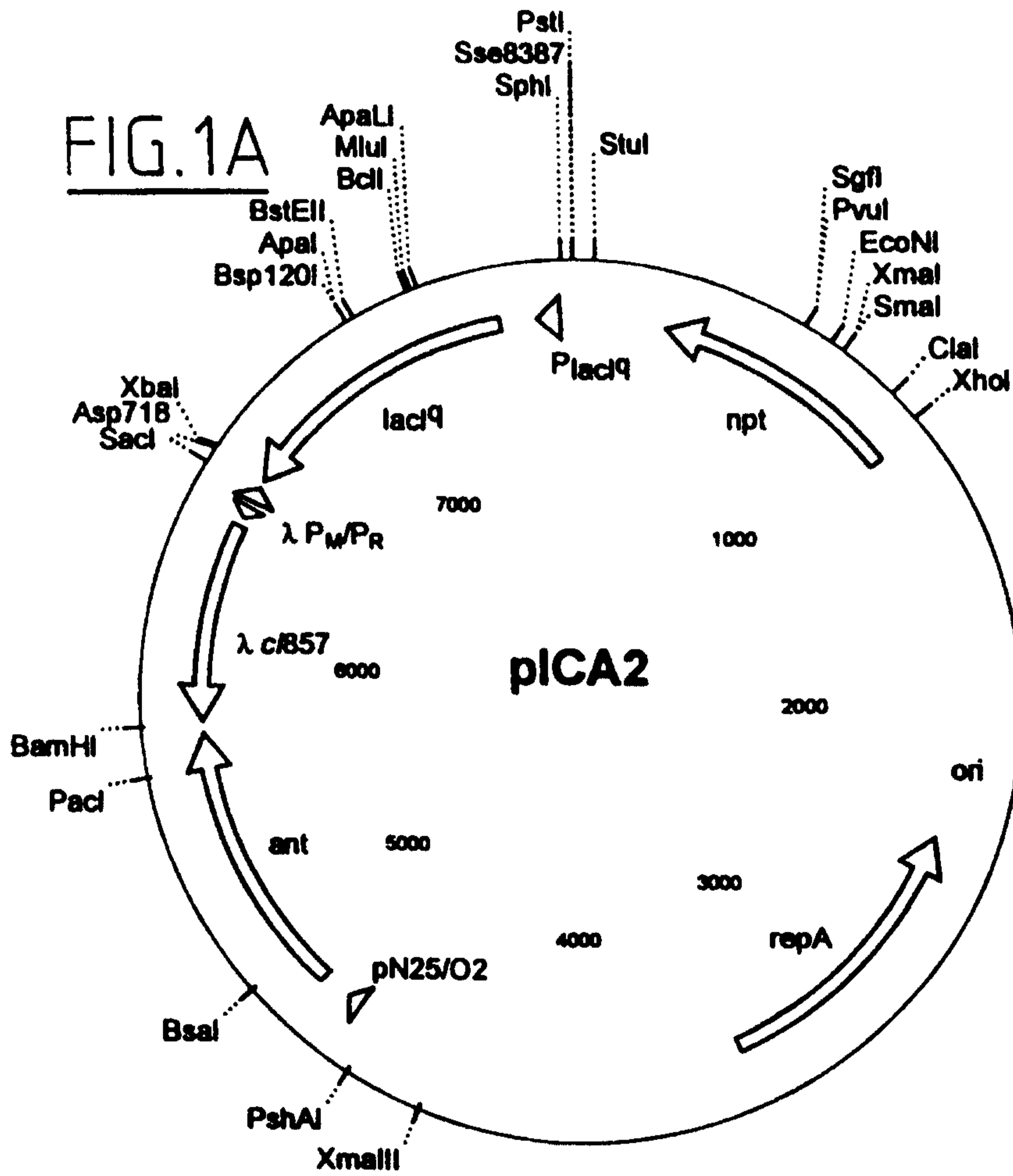
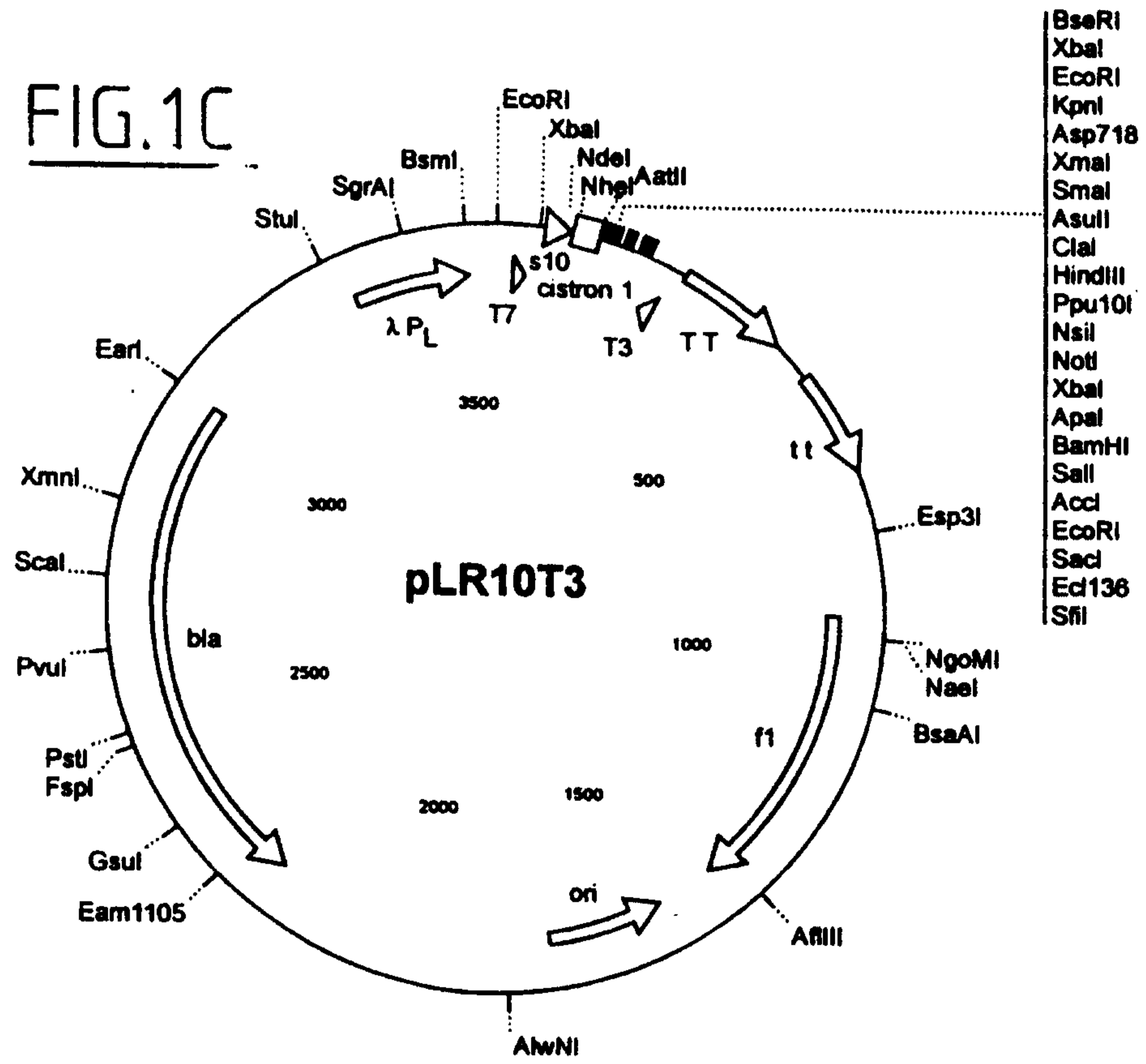
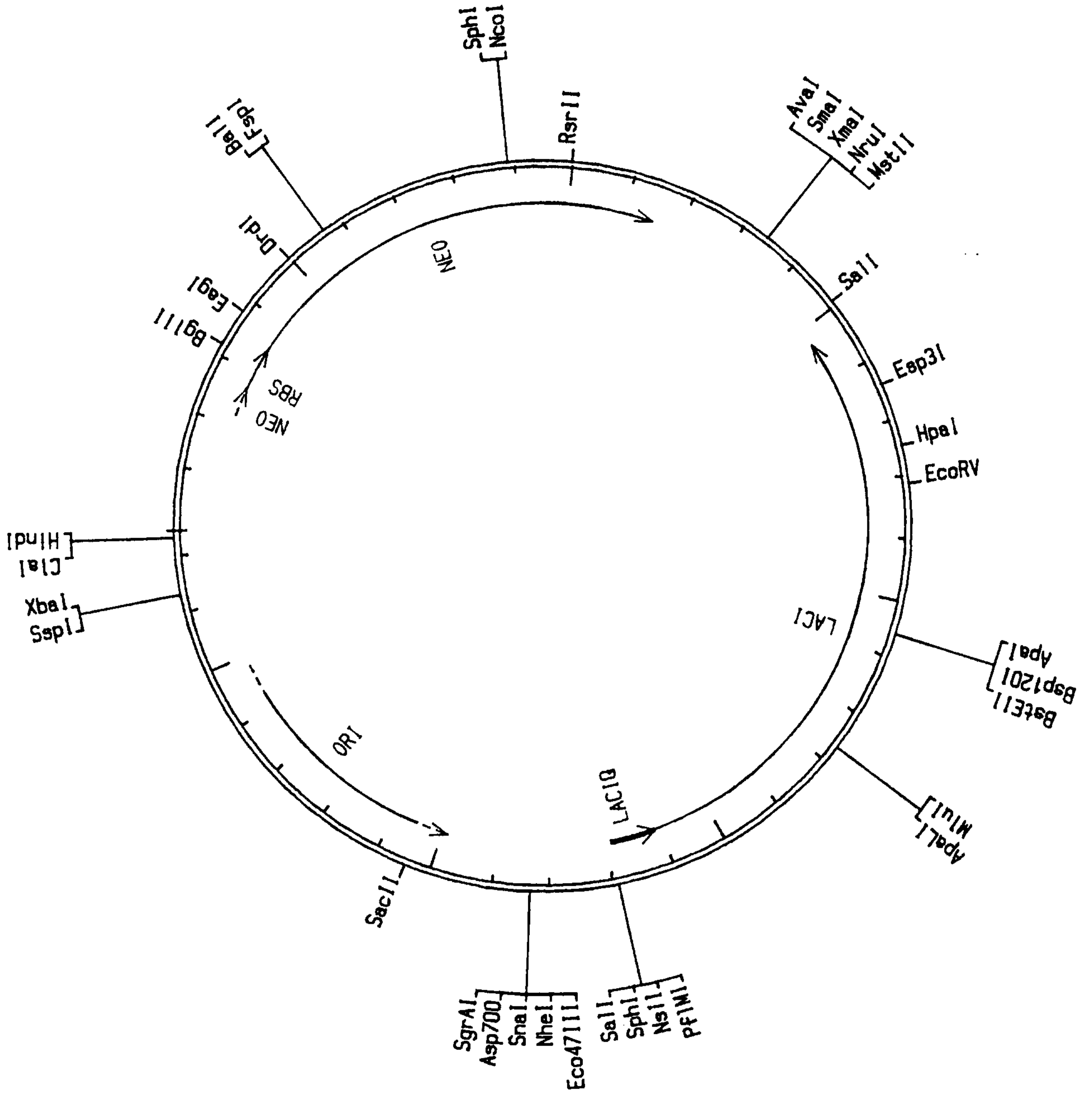


FIG. 1C





pDMI, 1

SN : 1594

max. cutting freq. = 2
32 unique sites

FIG. 2

length : 3740
divisions : 500
subdiv. : 100

FIG.3A

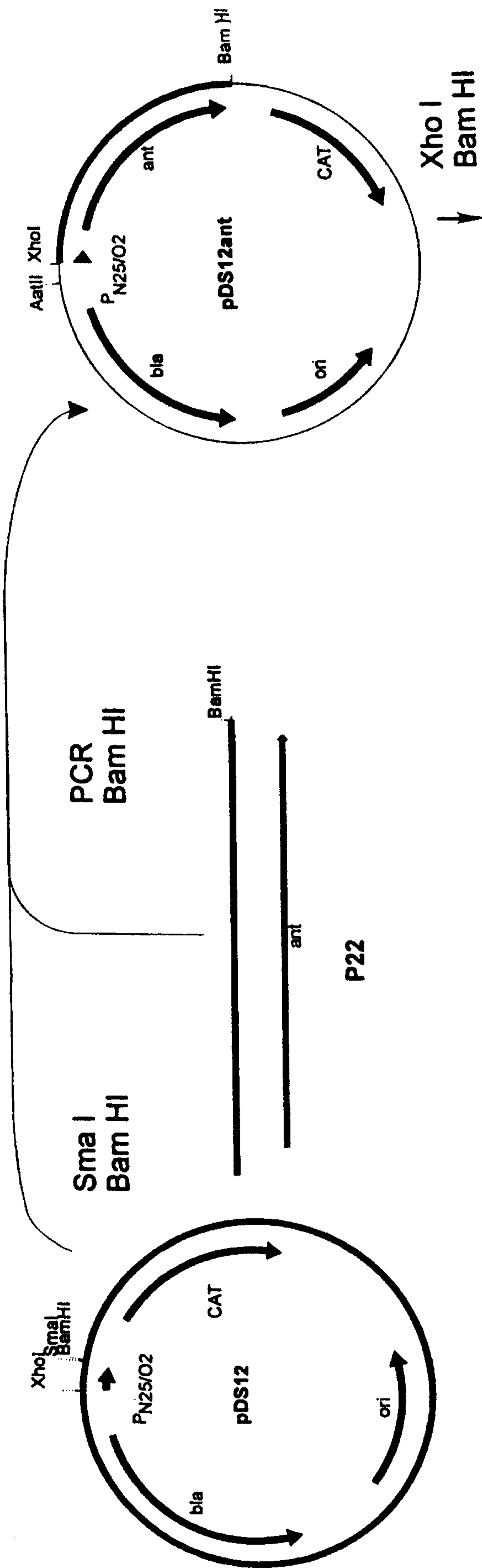


FIG. 3B

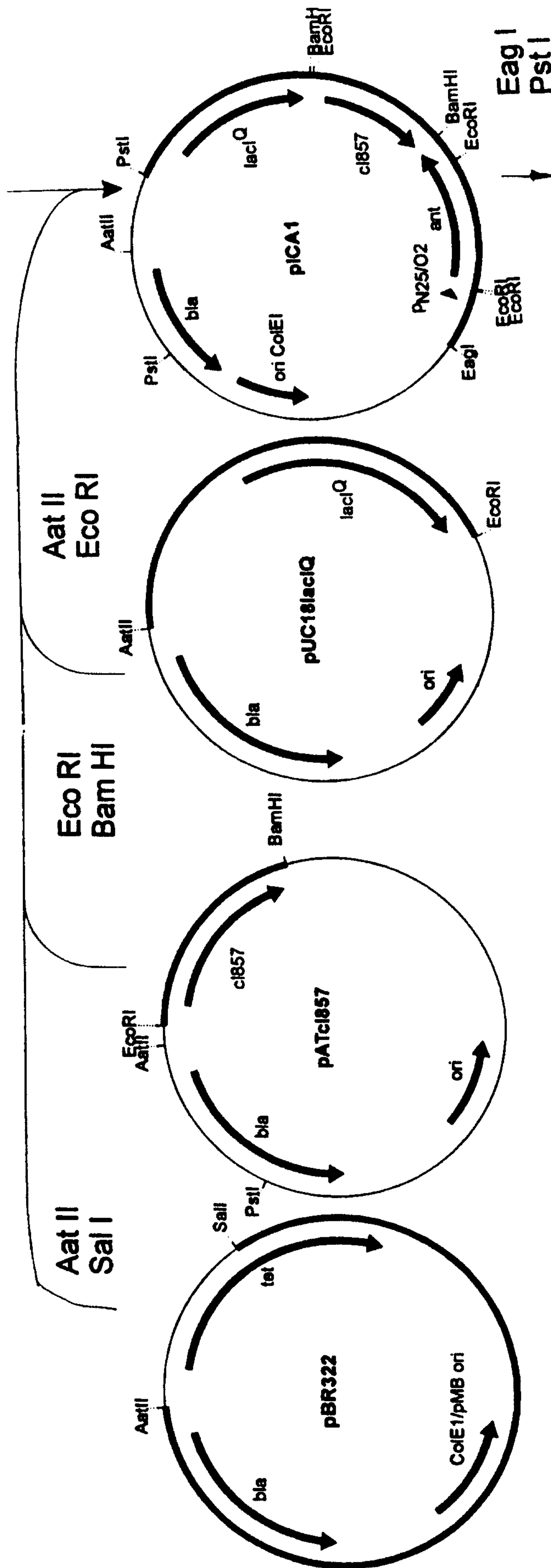
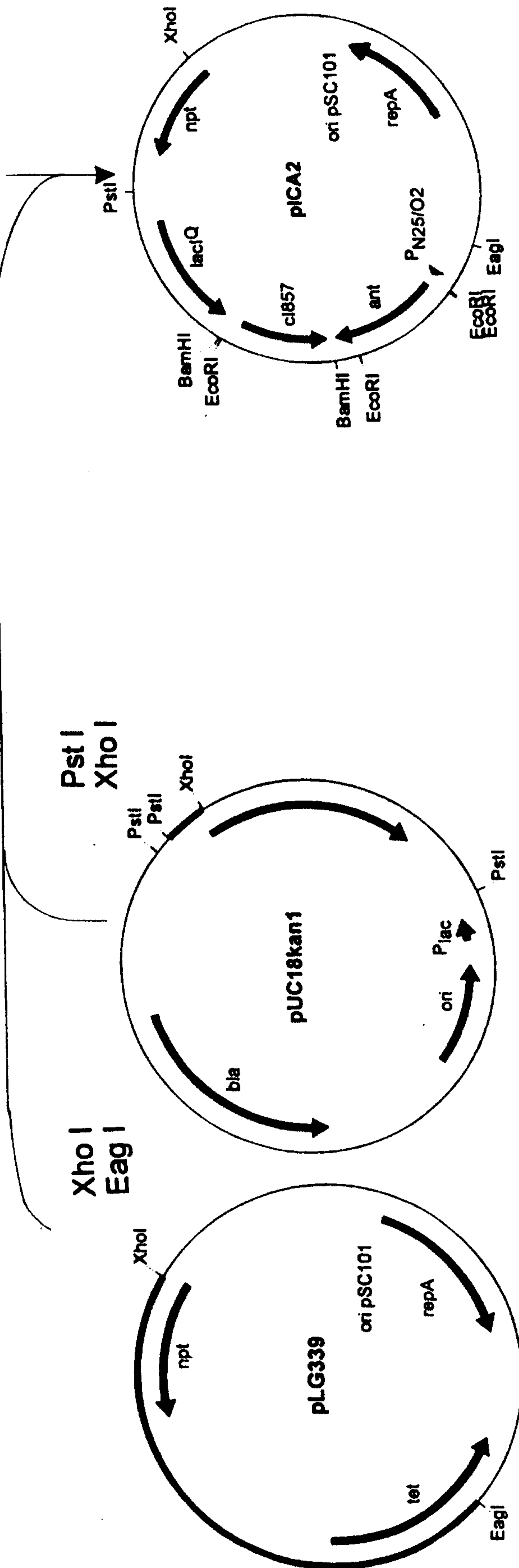


FIG.3C



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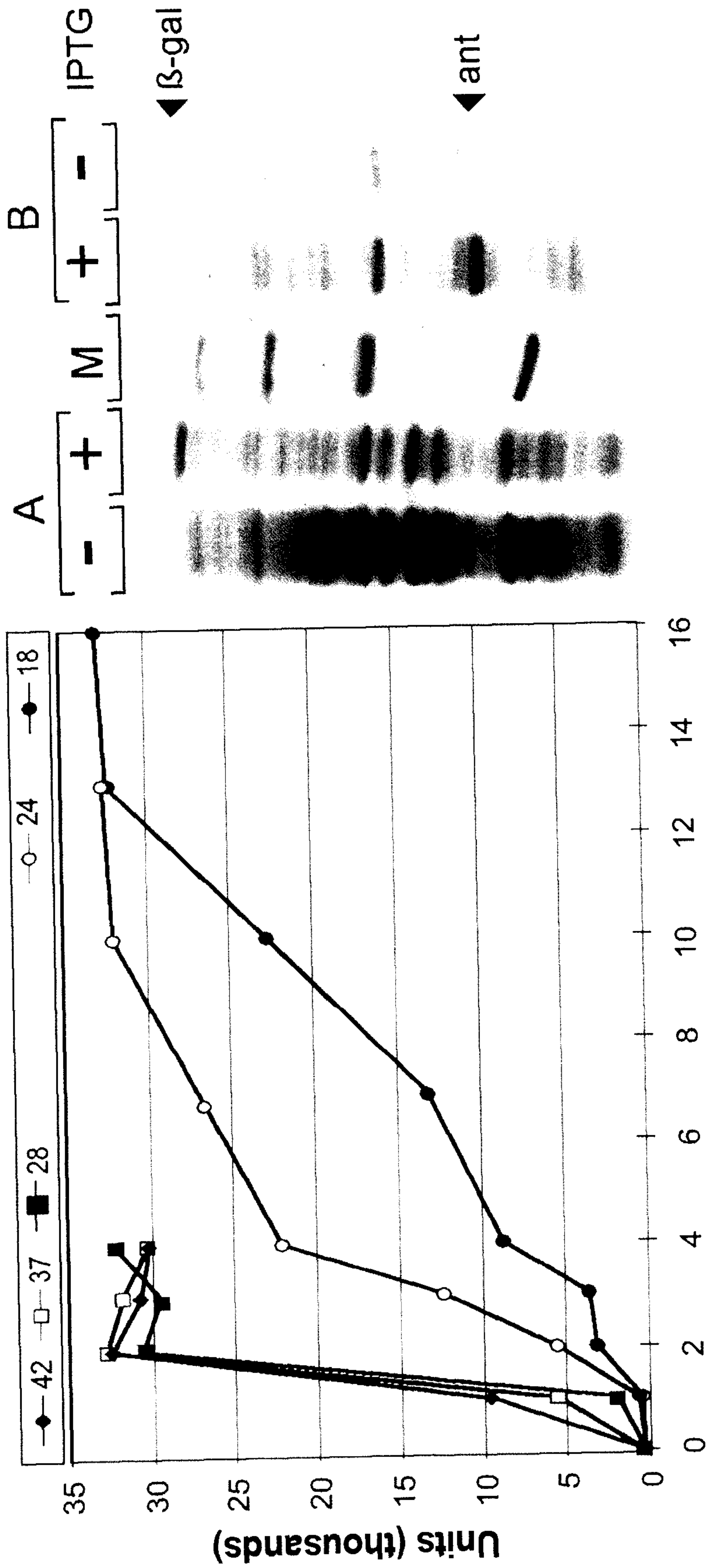


FIG. 4B

FIG. 4A

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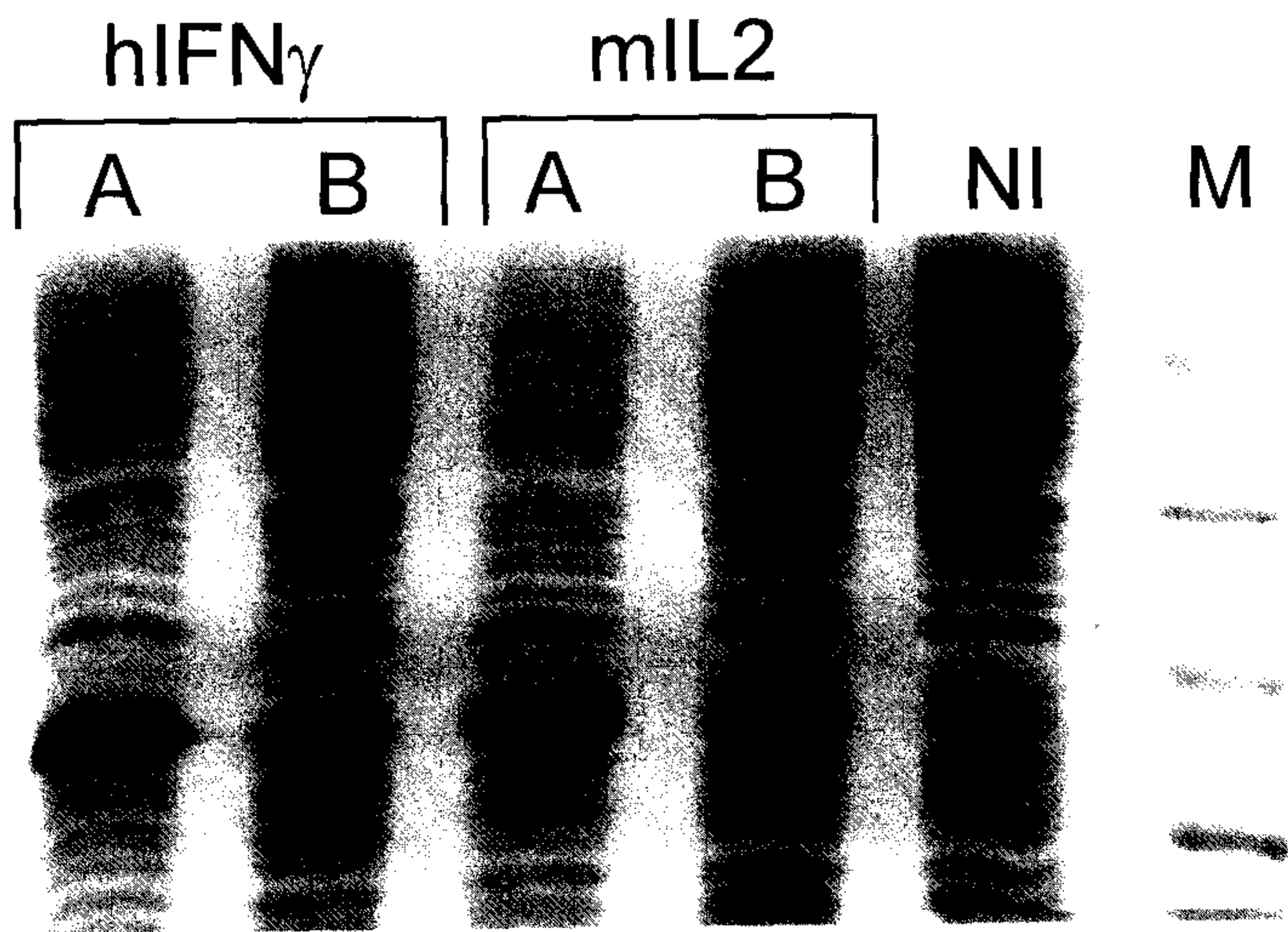


FIG. 5A

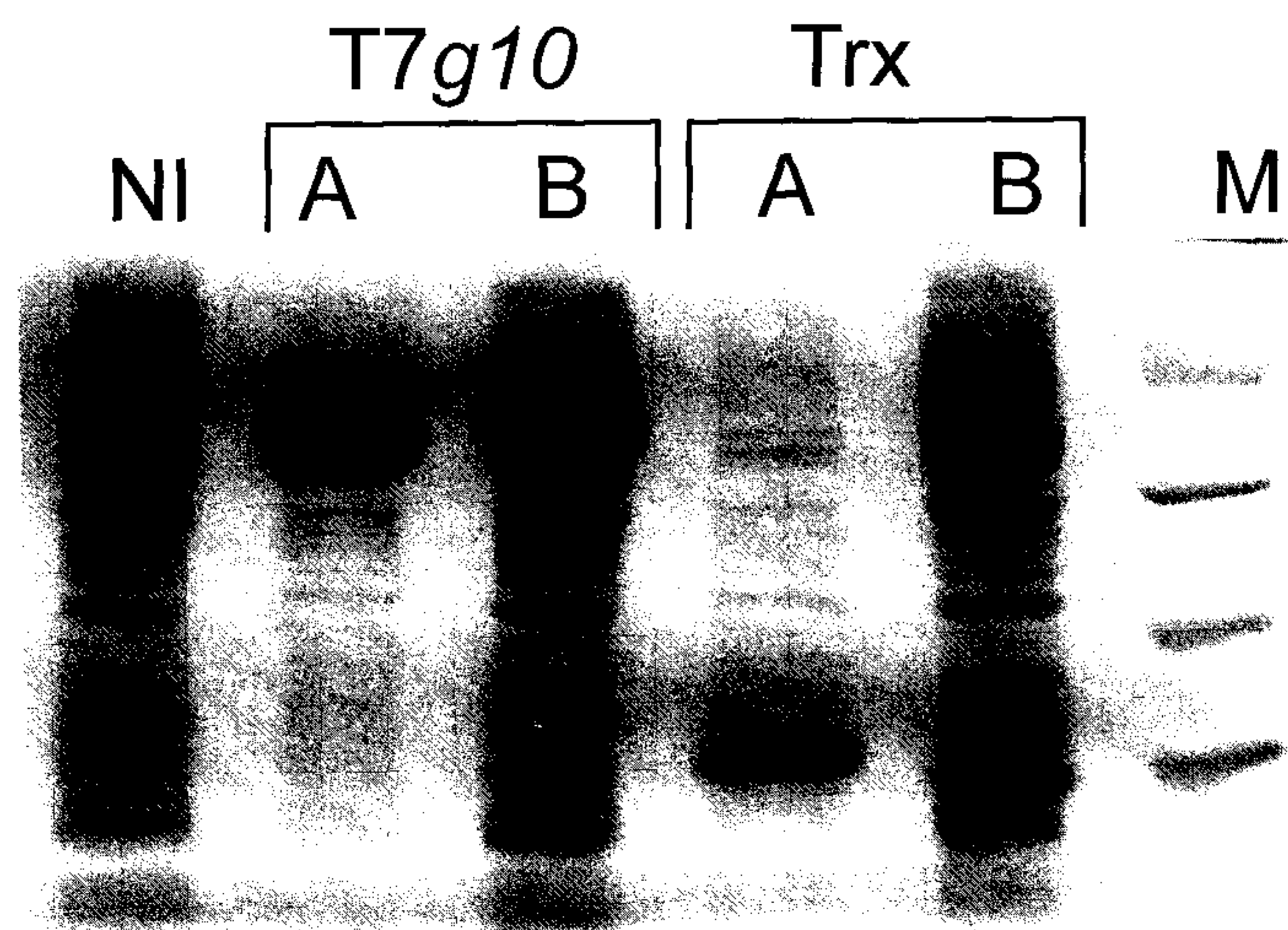


FIG. 5B

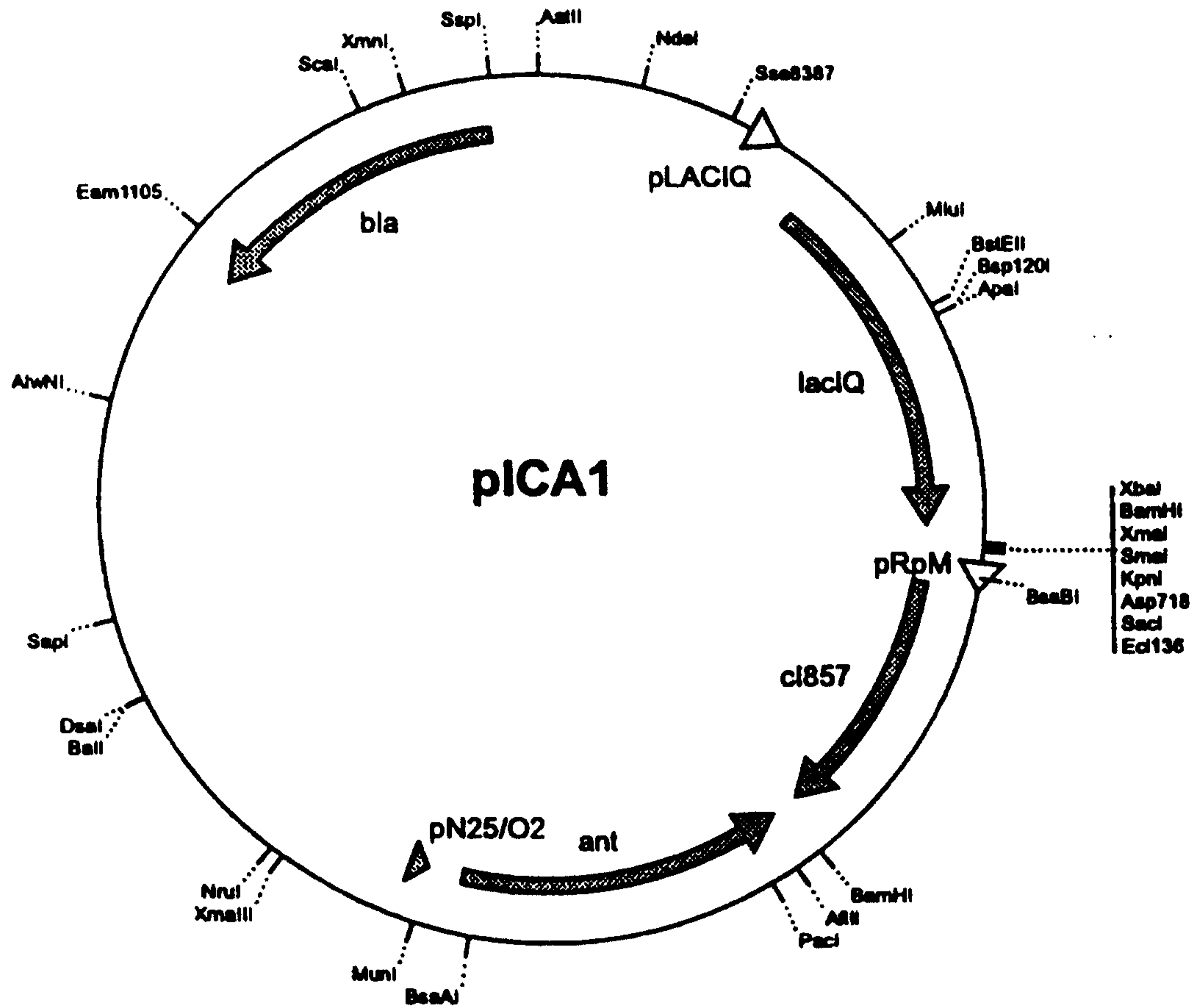


FIG. 7A

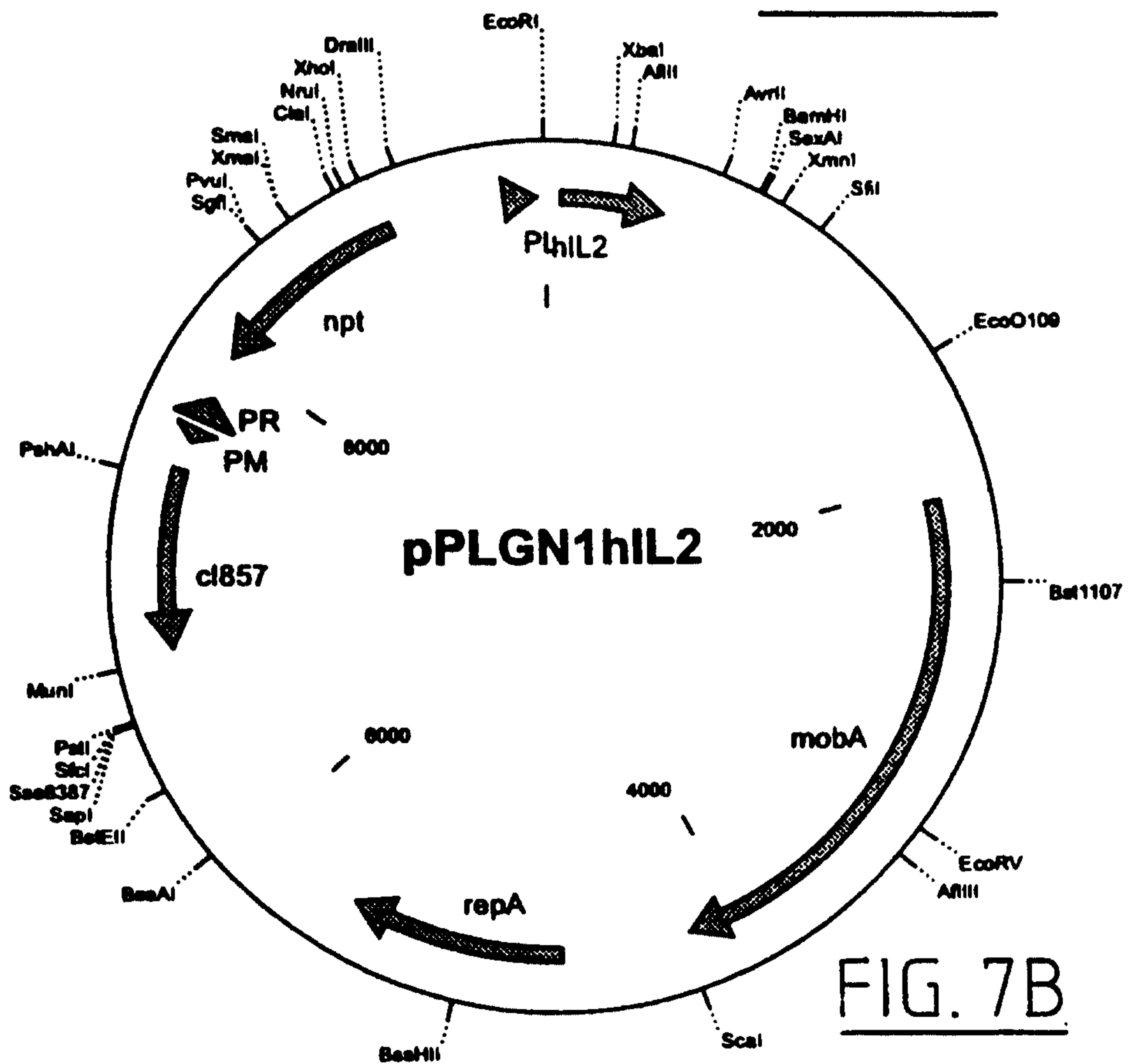


FIG. 7B

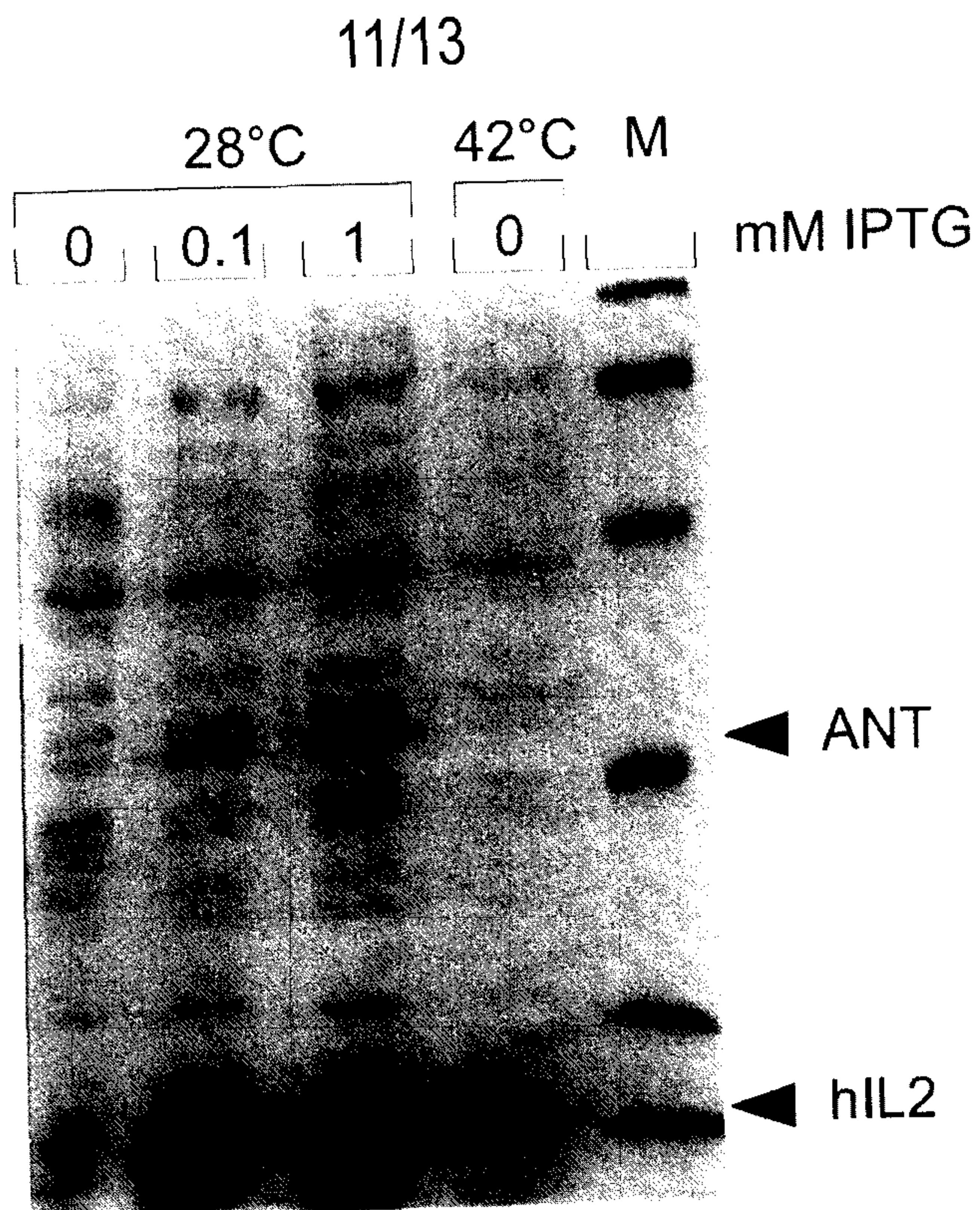


FIG. 7C

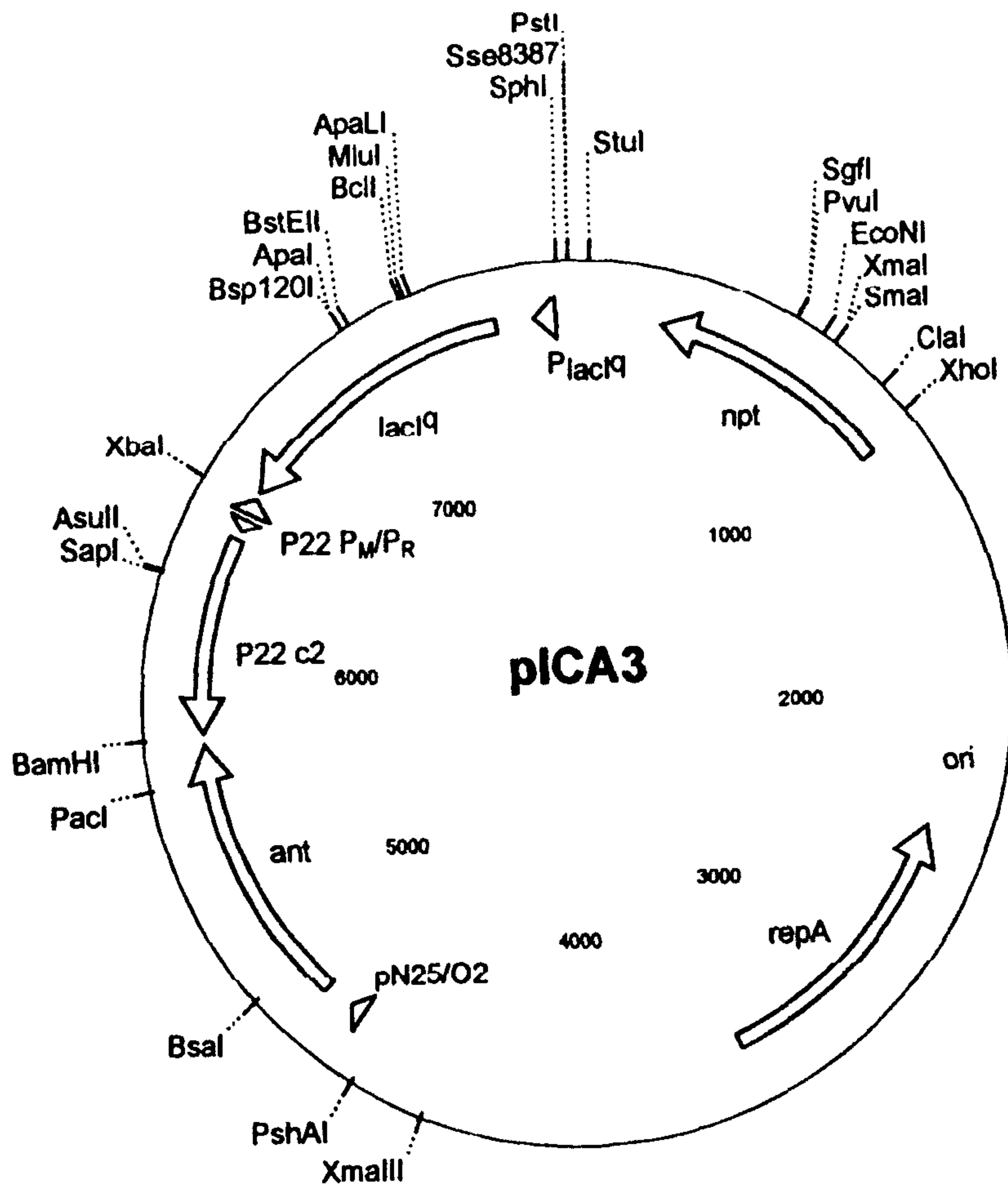


FIG. 8A

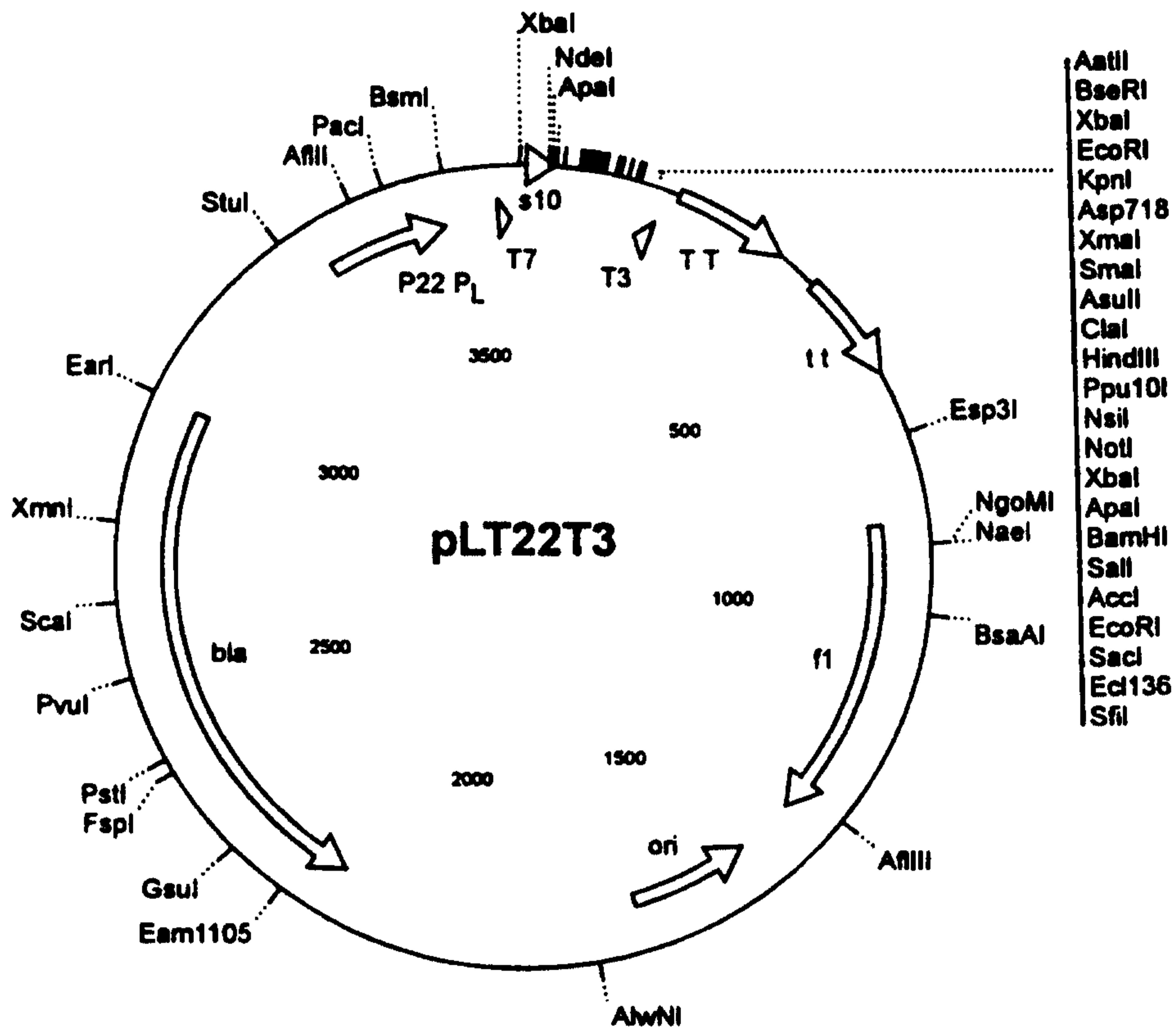


FIG. 8B

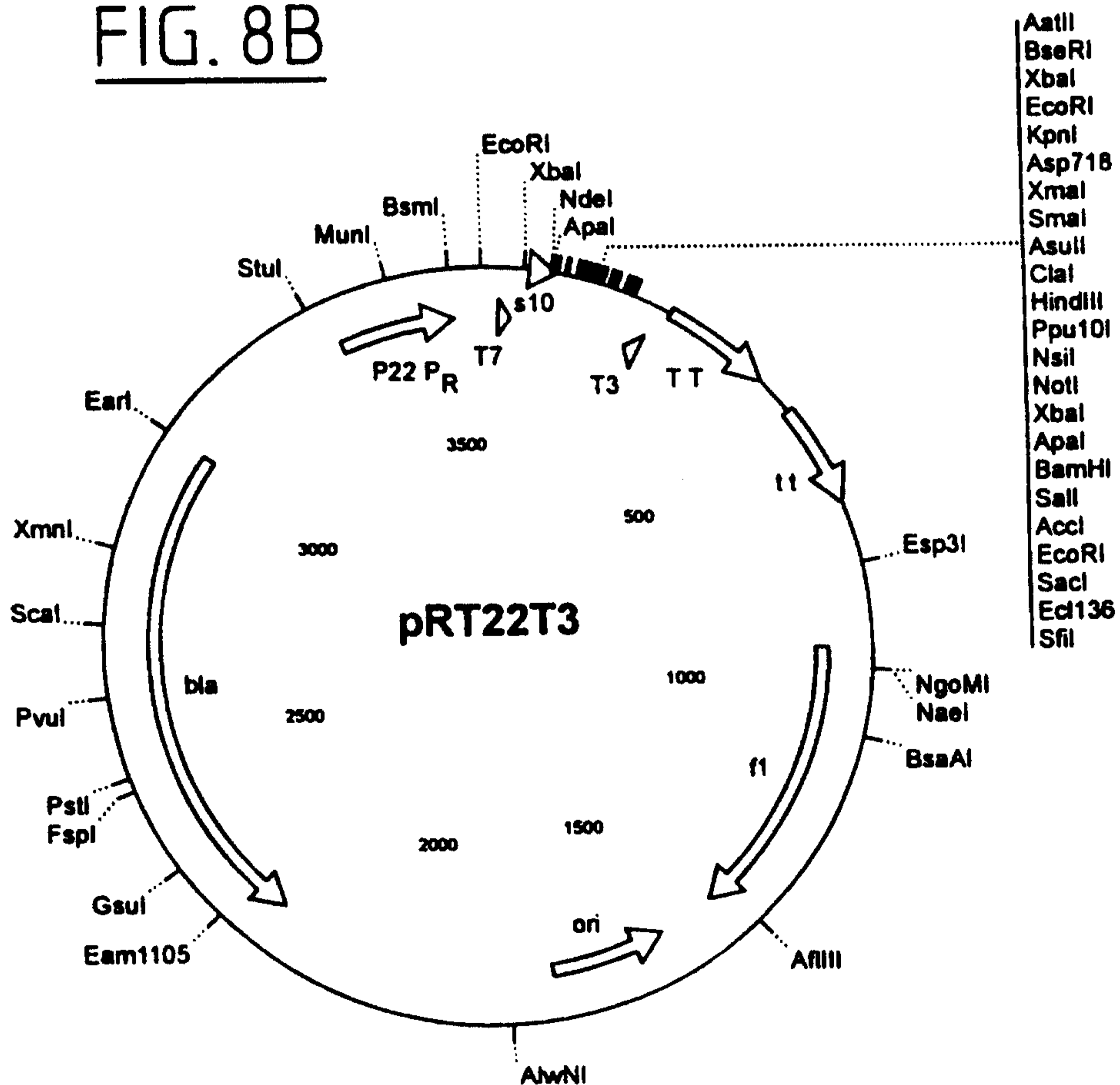


FIG. 8C

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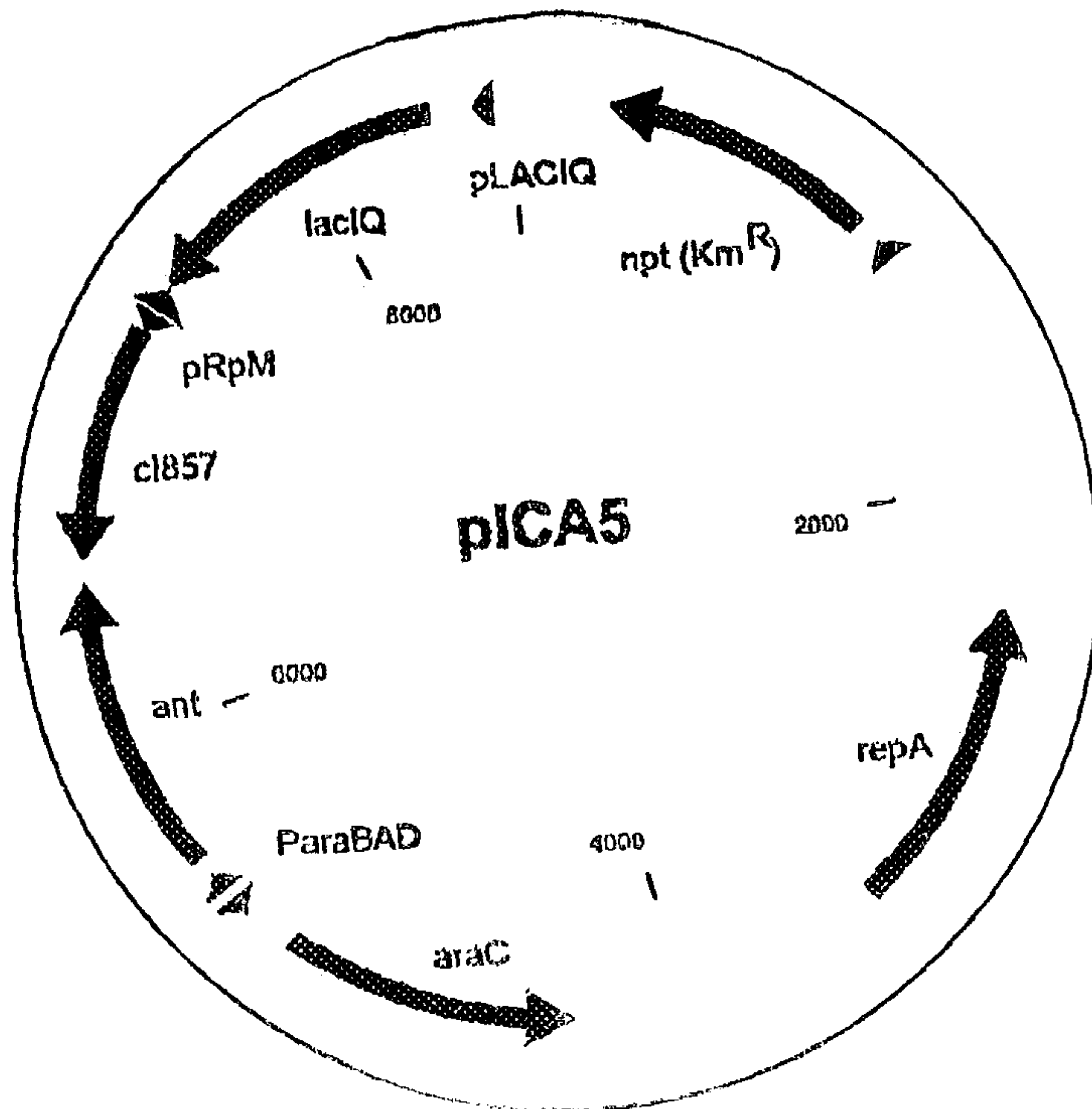


FIG. 9

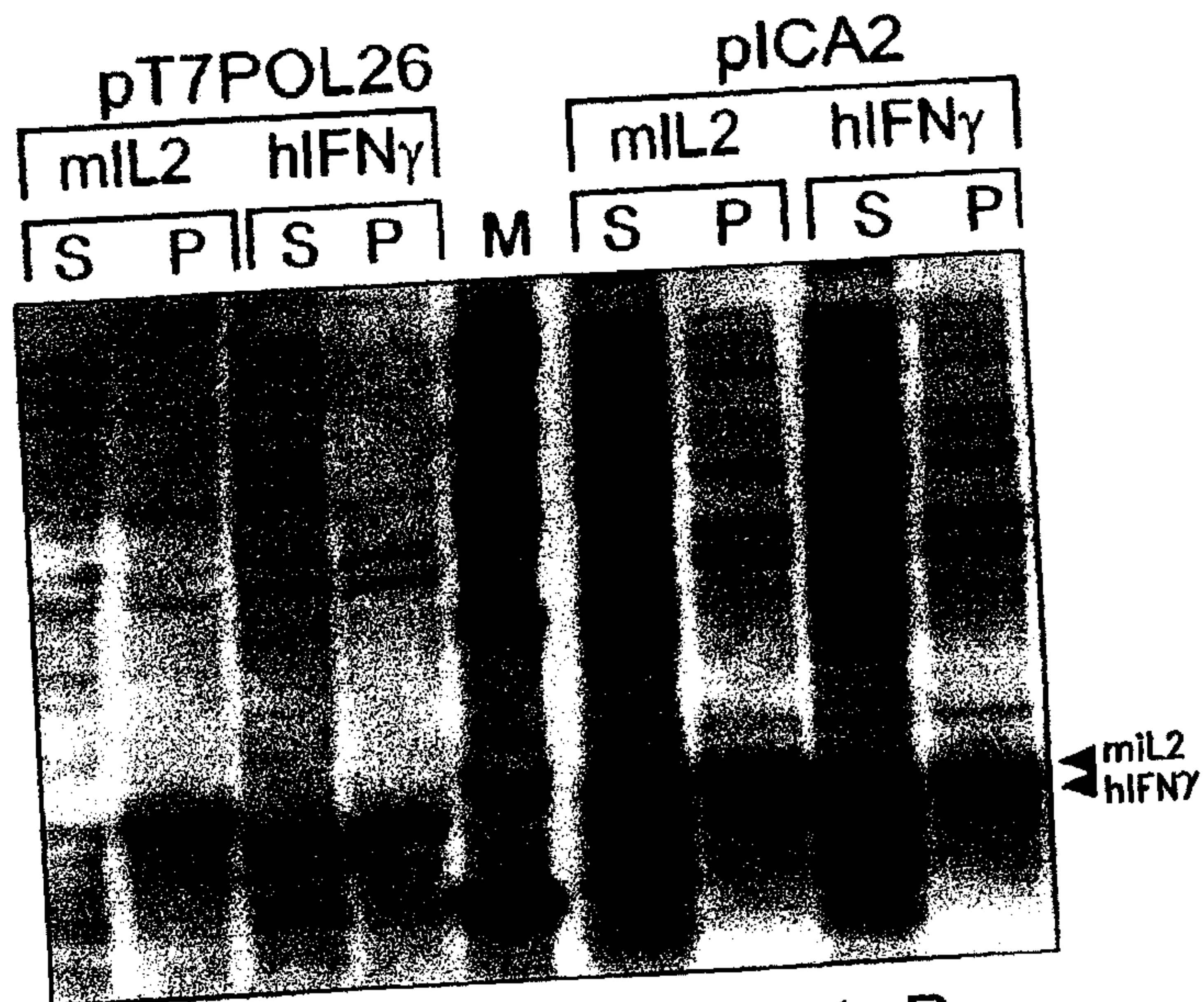


FIG. 10A

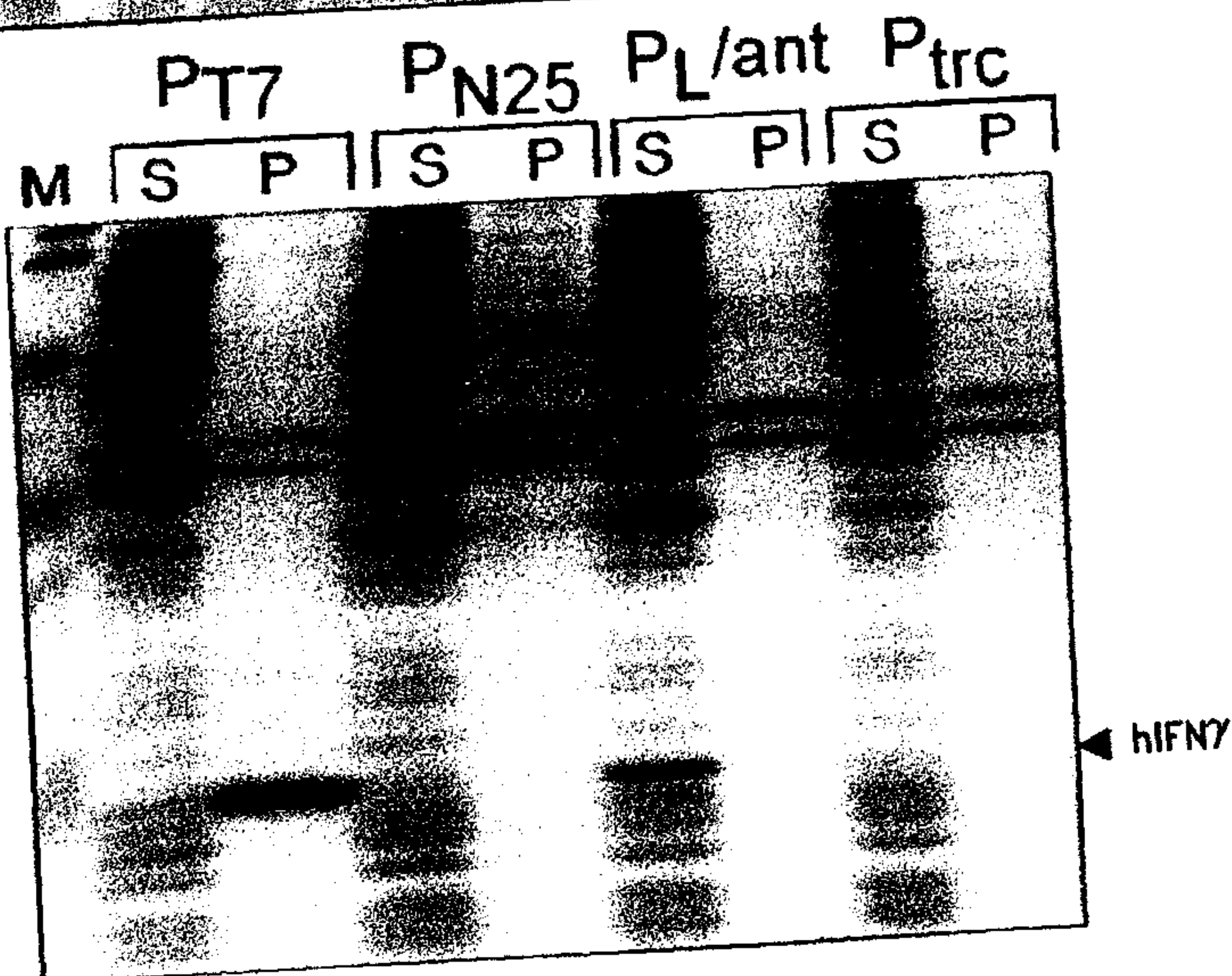


FIG. 10B

