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Bowdish et al.

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(54) **PHAGEMID VECTORS**

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(21) Appl. No.: **11/986,963**

(22) Filed: **Nov. 26, 2007**

Related U.S. Patent Documents

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 Filed: **Apr. 26, 2002**

U.S. Applications:

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(51) **Int. Cl.**
C12N 15/63 (2006.01)
C12N 15/70 (2006.01)
C12N 15/73 (2006.01)
C12N 15/64 (2006.01)
C12N 15/65 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **435/320.1**; 536/23.1; 536/23.4;
 536/23.7; 536/24.1; 536/23.2; 435/69.1;
 435/5

(58) **Field of Classification Search** None
 See application file for complete search history.

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Map of pComb3X vector. printed in 2006.

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Primary Examiner—Jennifer Dunston

(74) *Attorney, Agent, or Firm*—Ropes & Gray LLP

(57) **ABSTRACT**

Phagemid vectors containing a sequence of features between a Col E1 origin and an fl origin are useful for display of polypeptides or proteins, including antibody libraries.

3 Claims, 46 Drawing Sheets

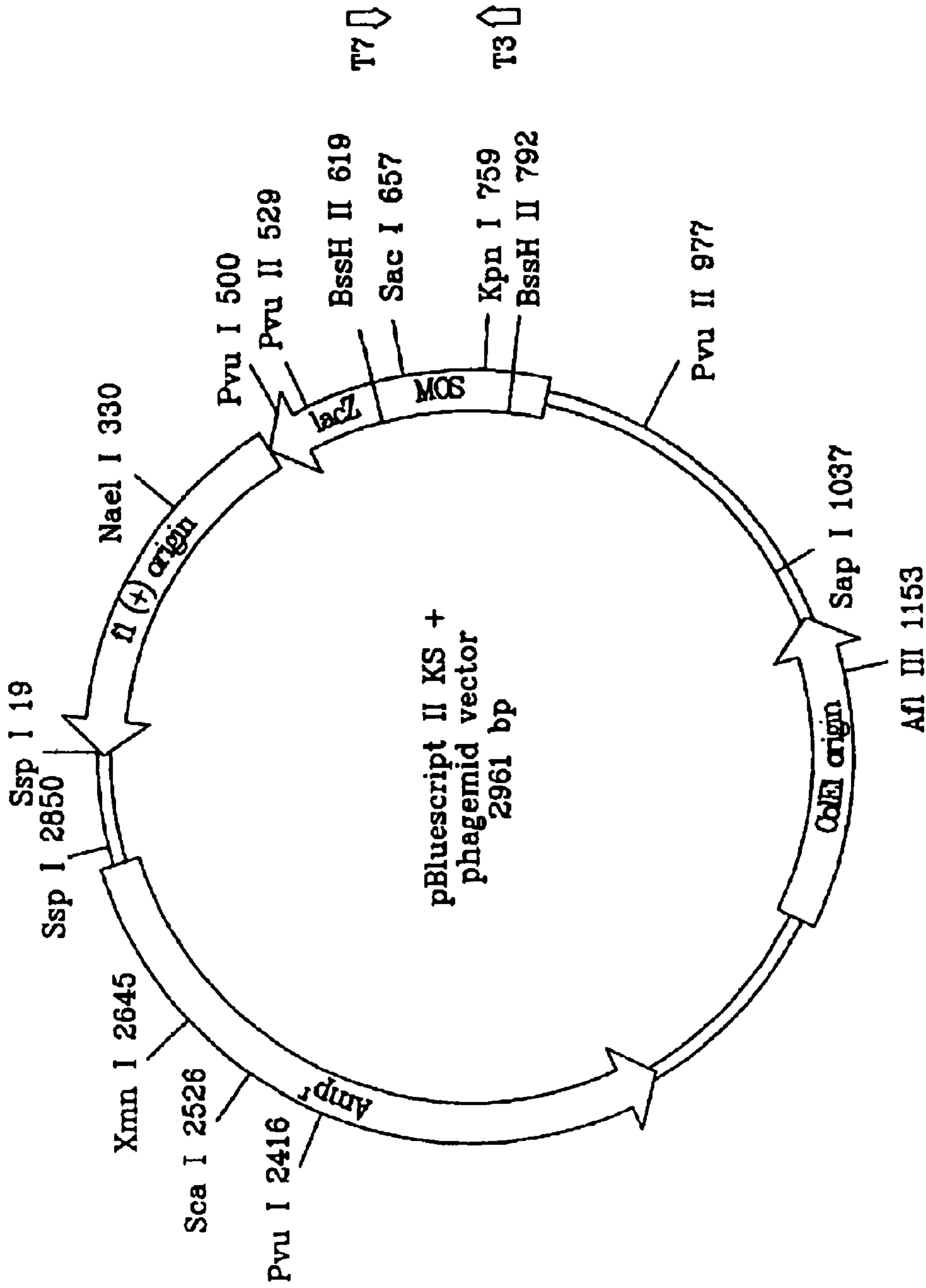
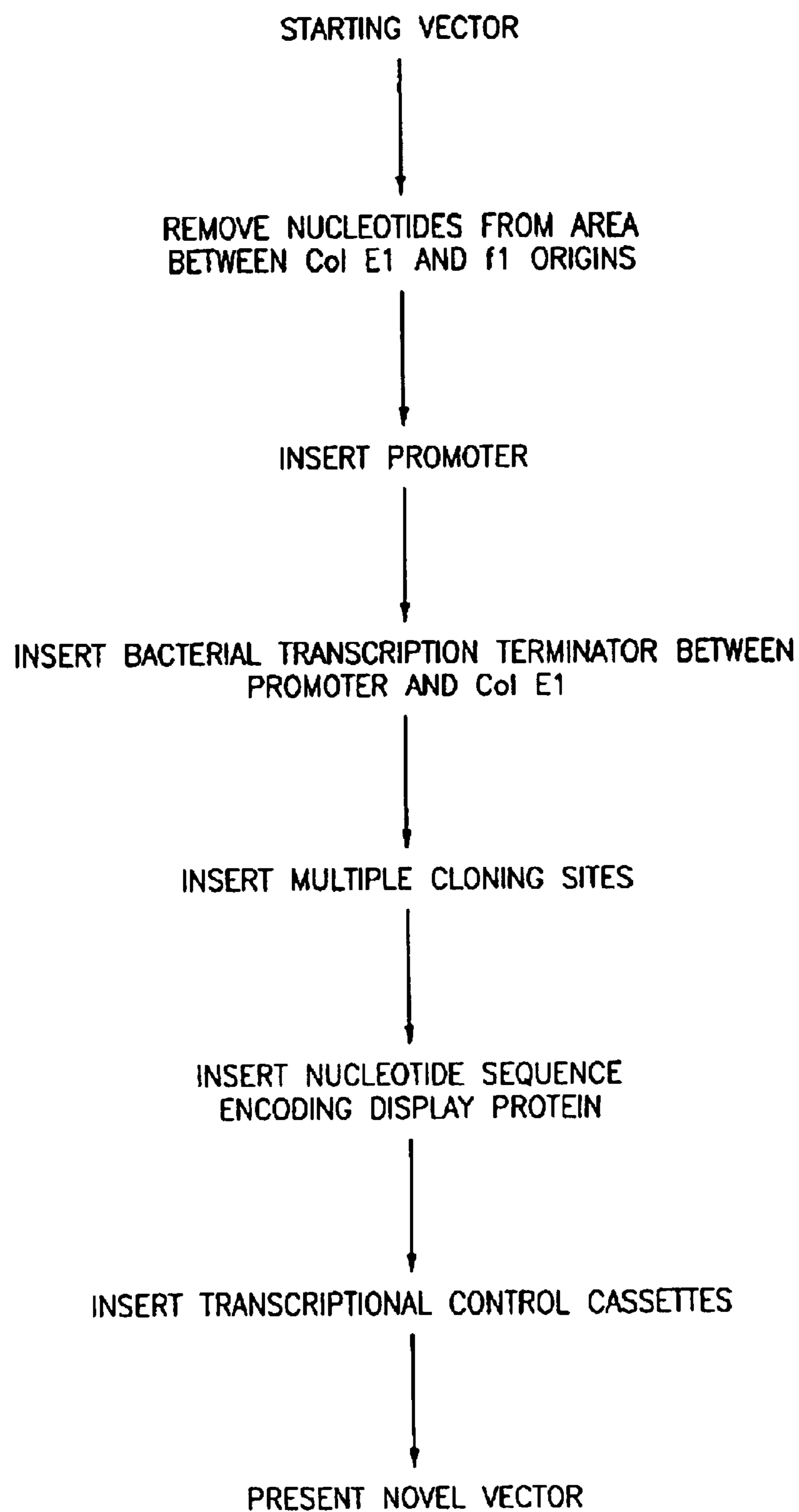


FIG. 1

**FIG. 2**

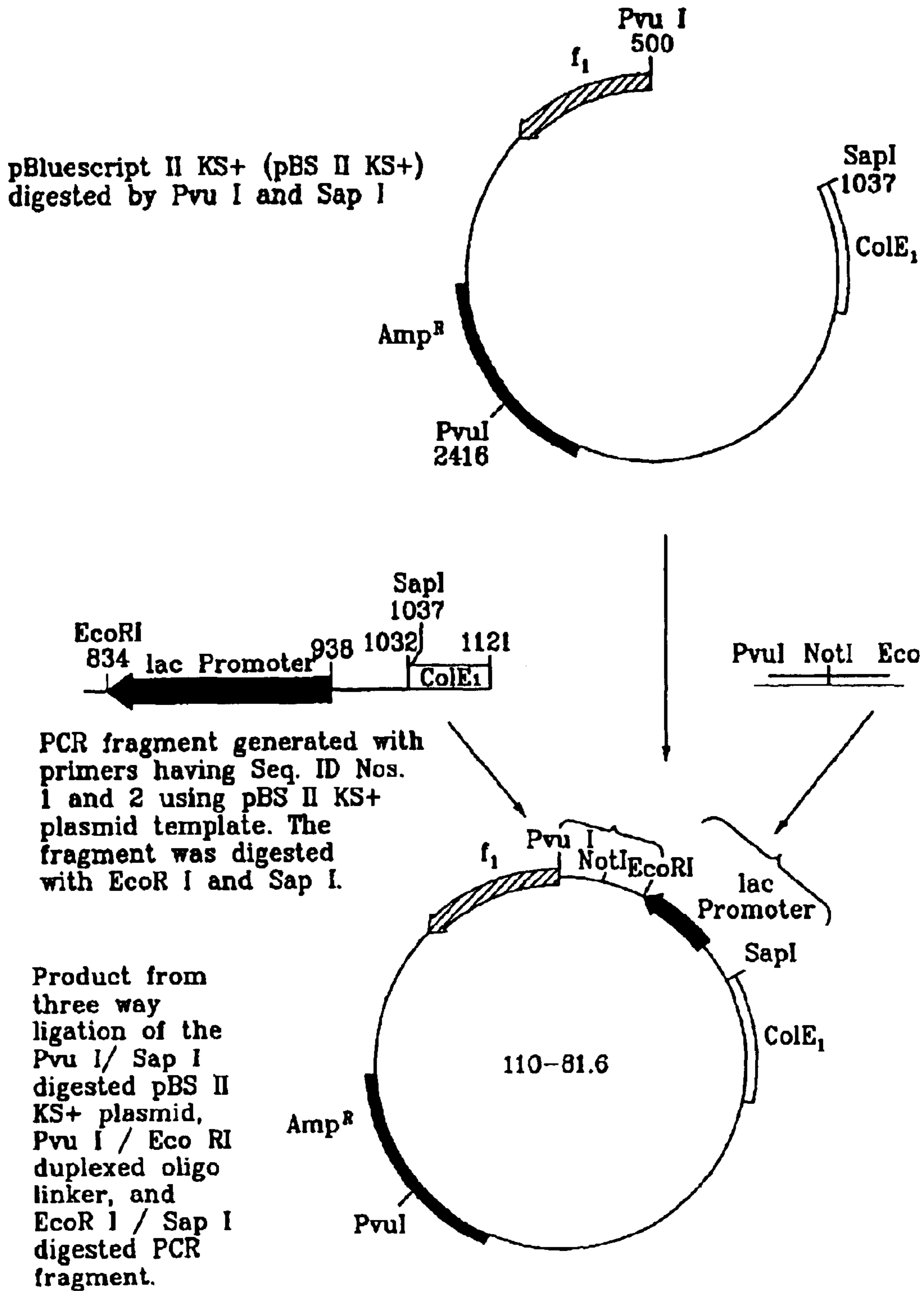


FIG. 3

(Seq. ID No. 19)

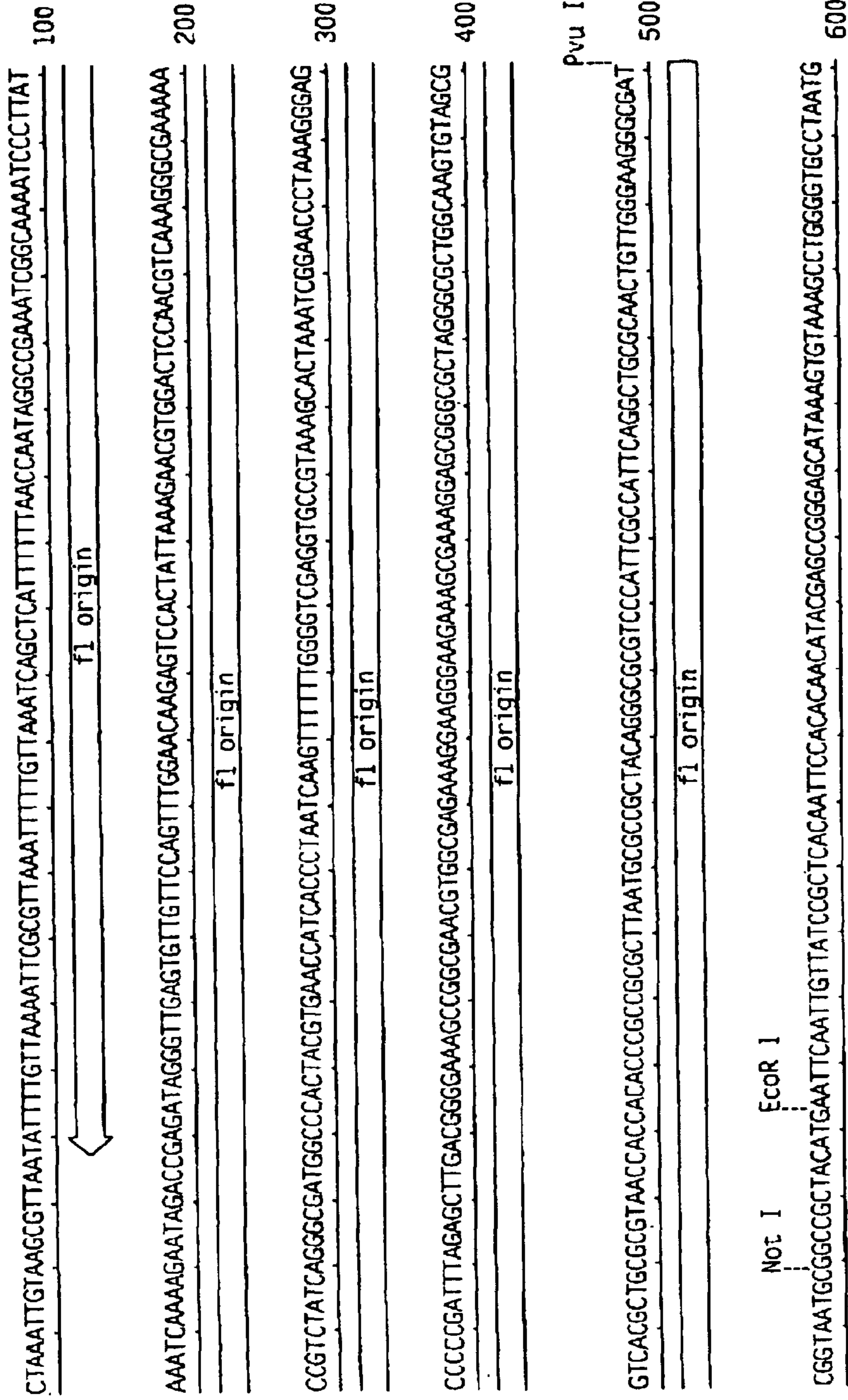


FIG. 4a

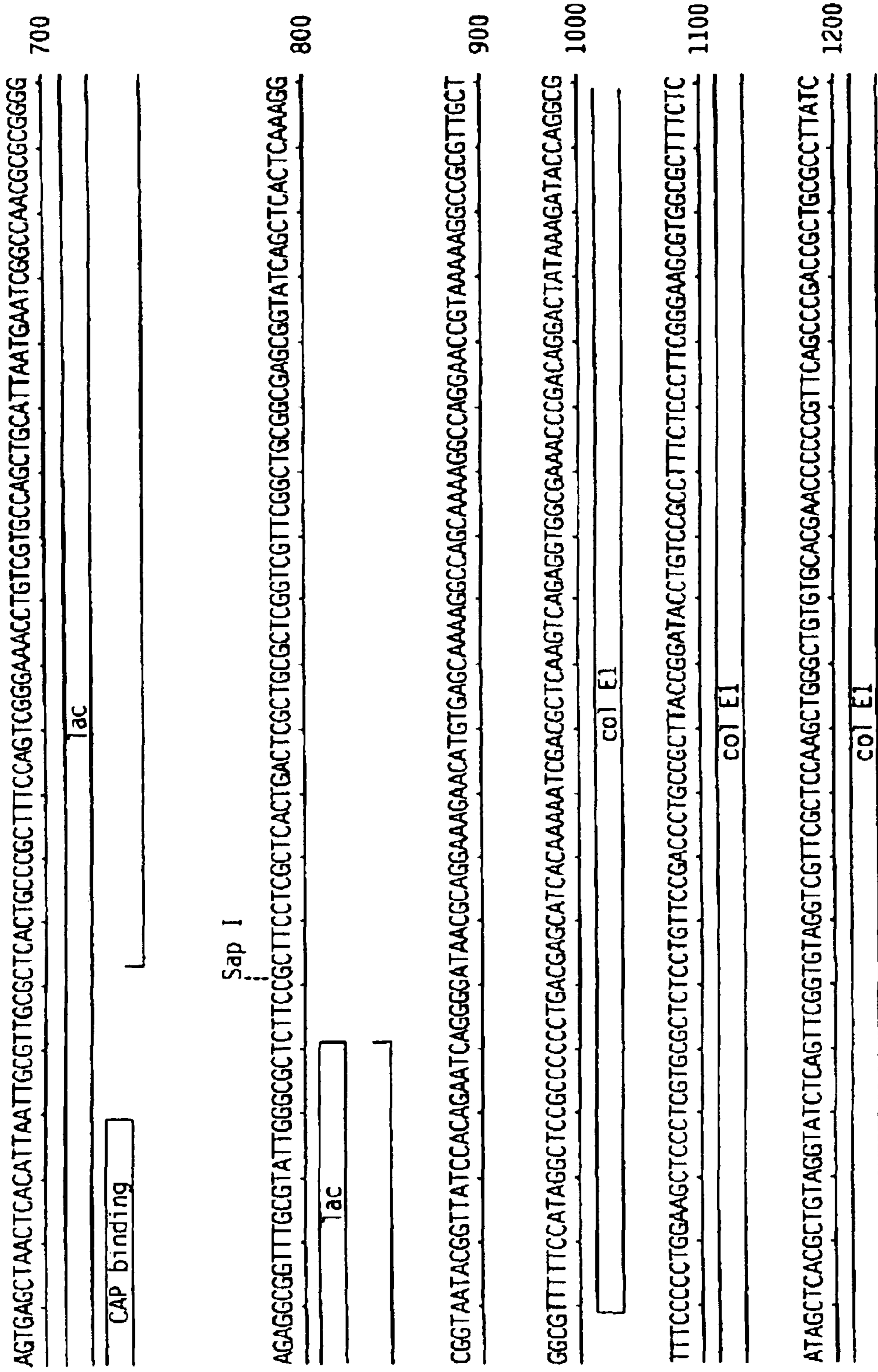


FIG. 4a (Cont.)

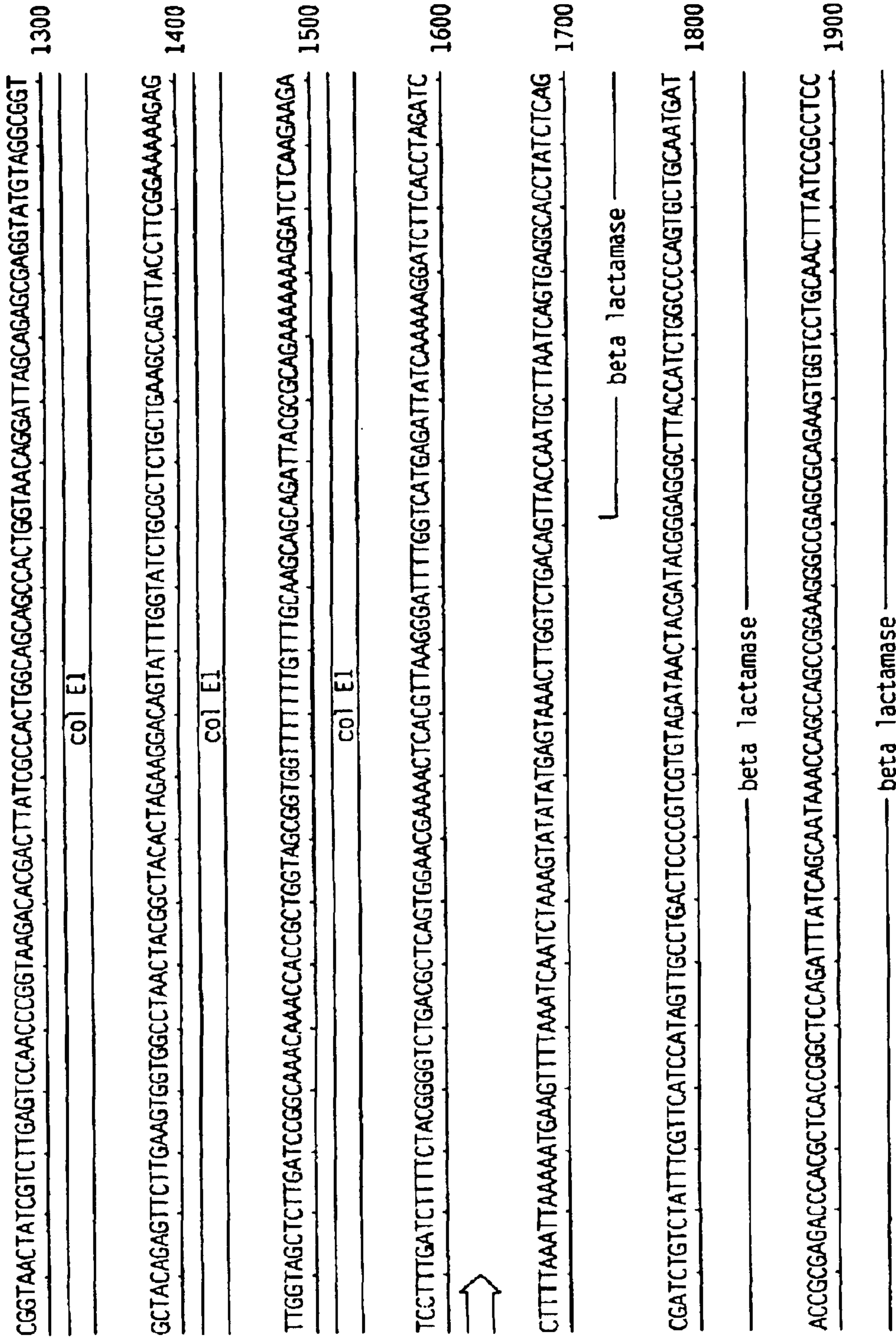


FIG. 4b

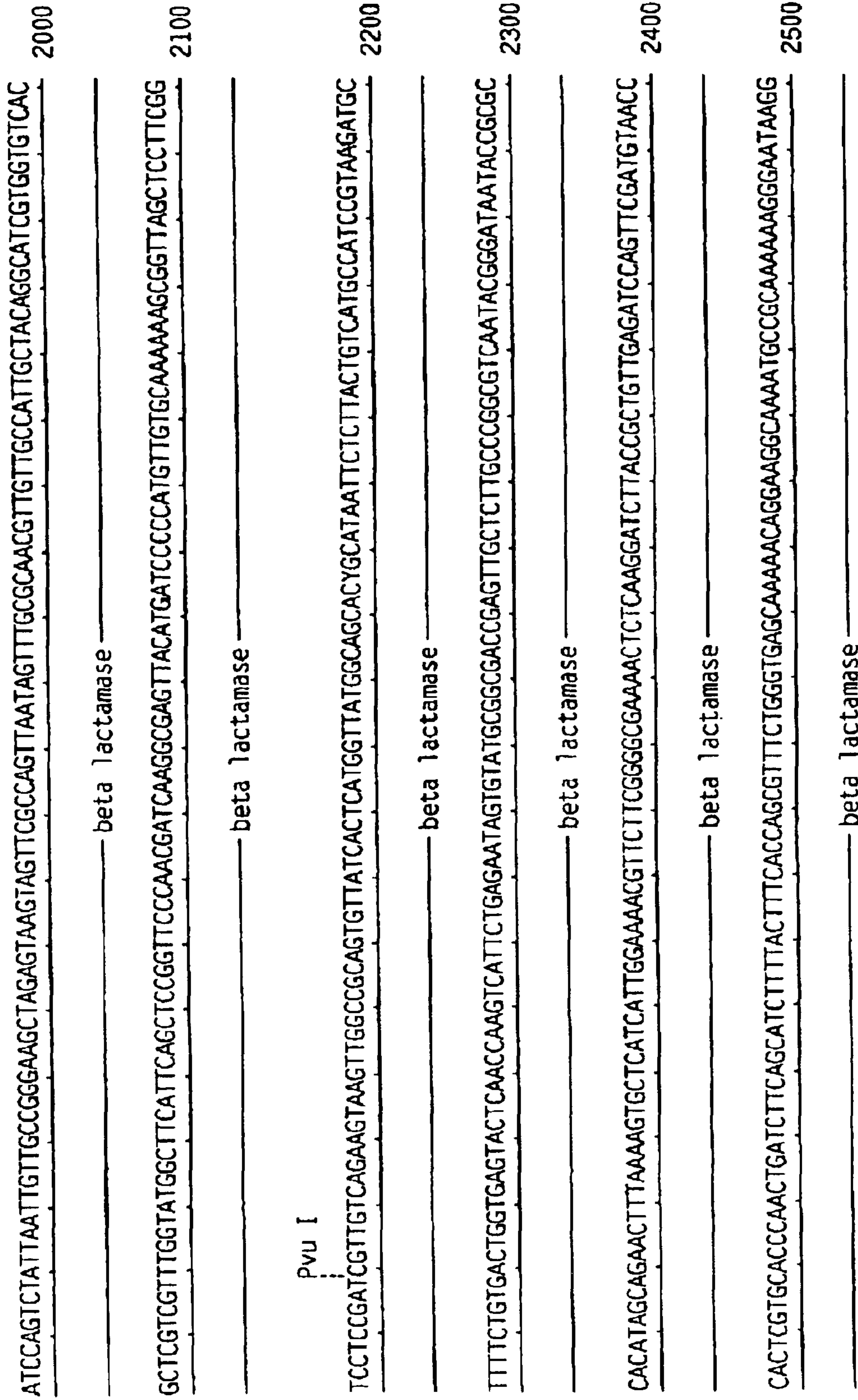


FIG. 4b (Cont.)

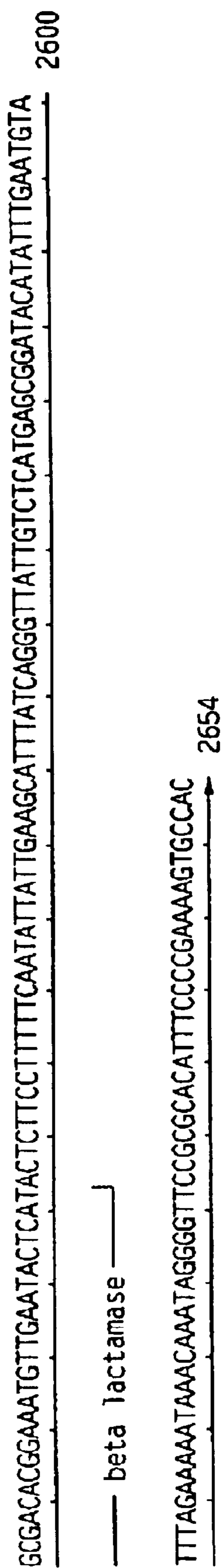


FIG. 4c

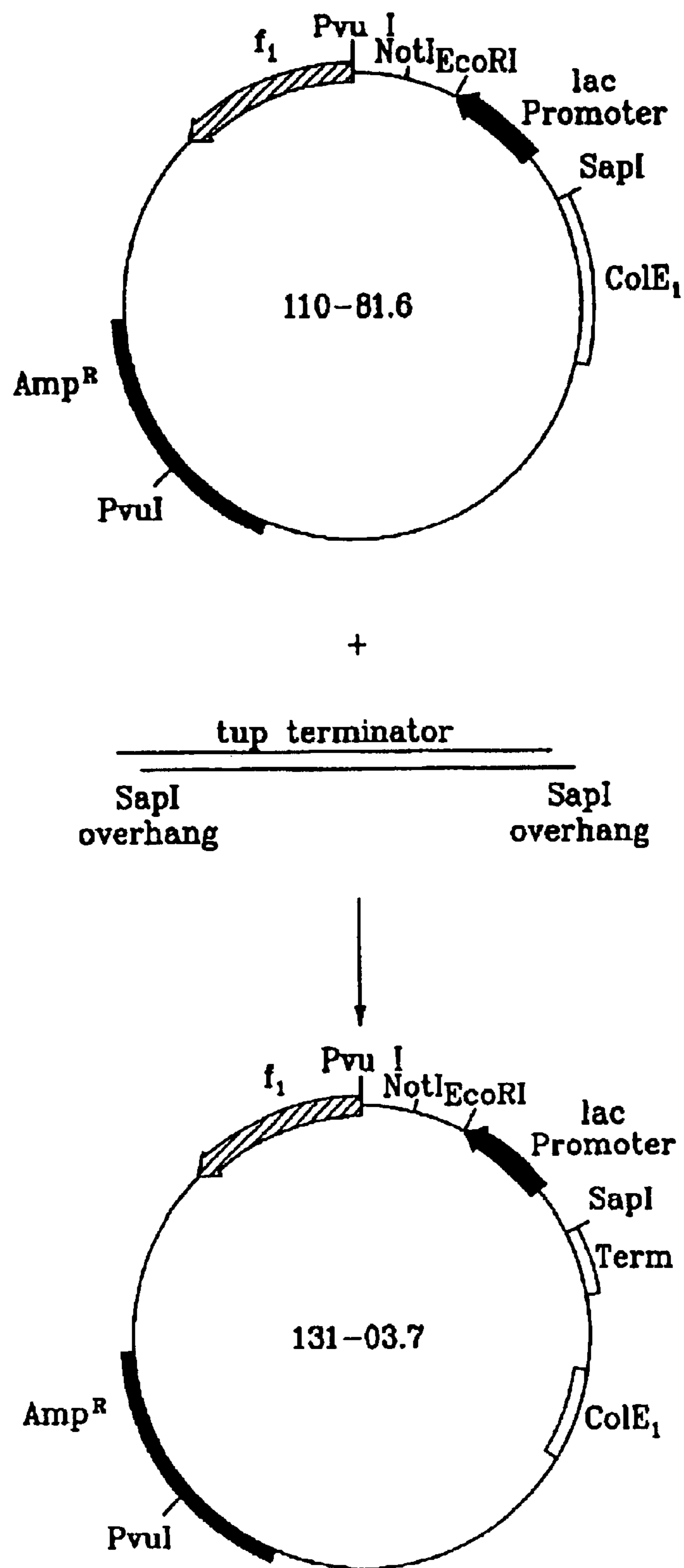


FIG. 5

(Seq. ID No. 20)

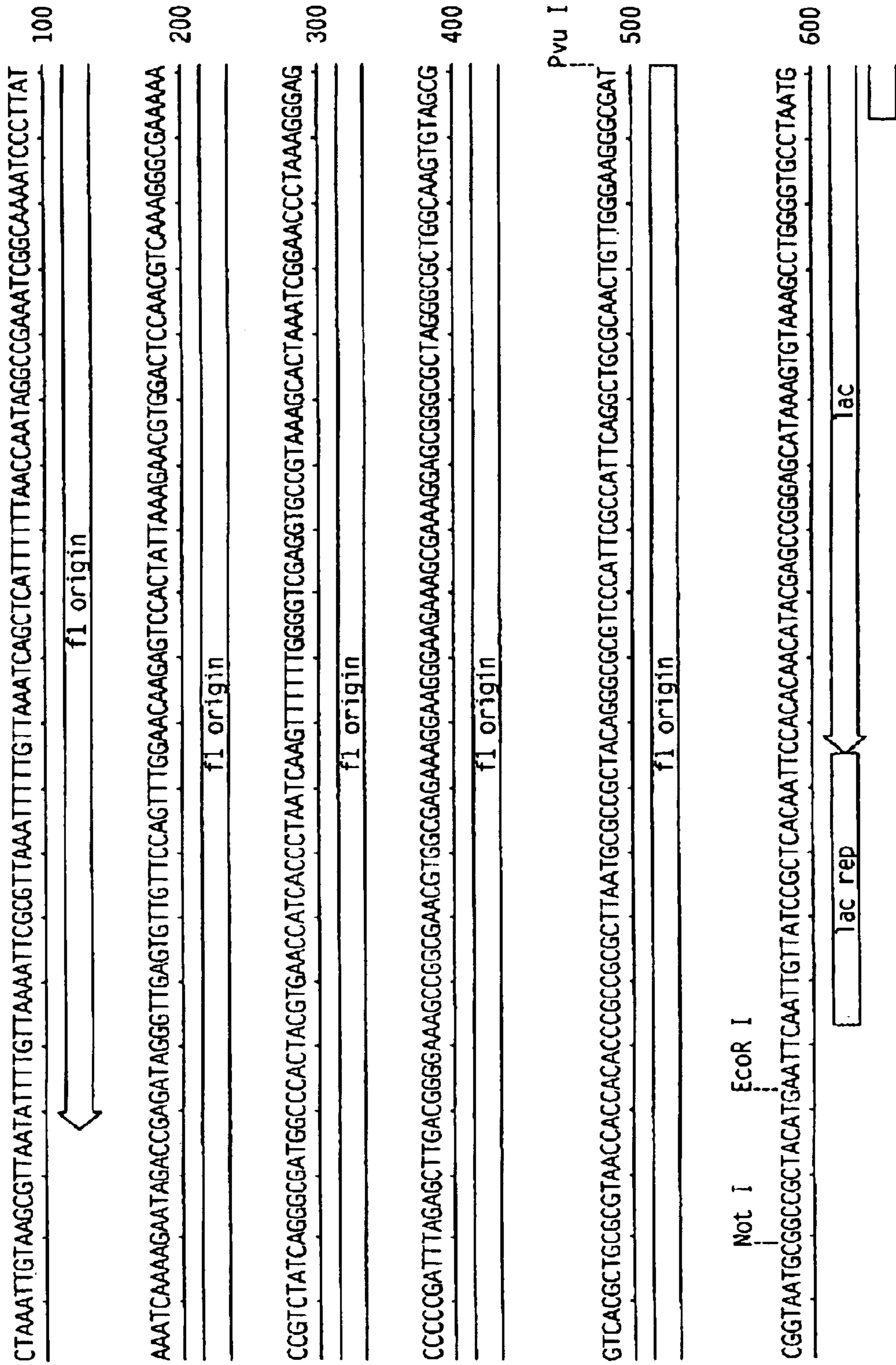


FIG. 6a

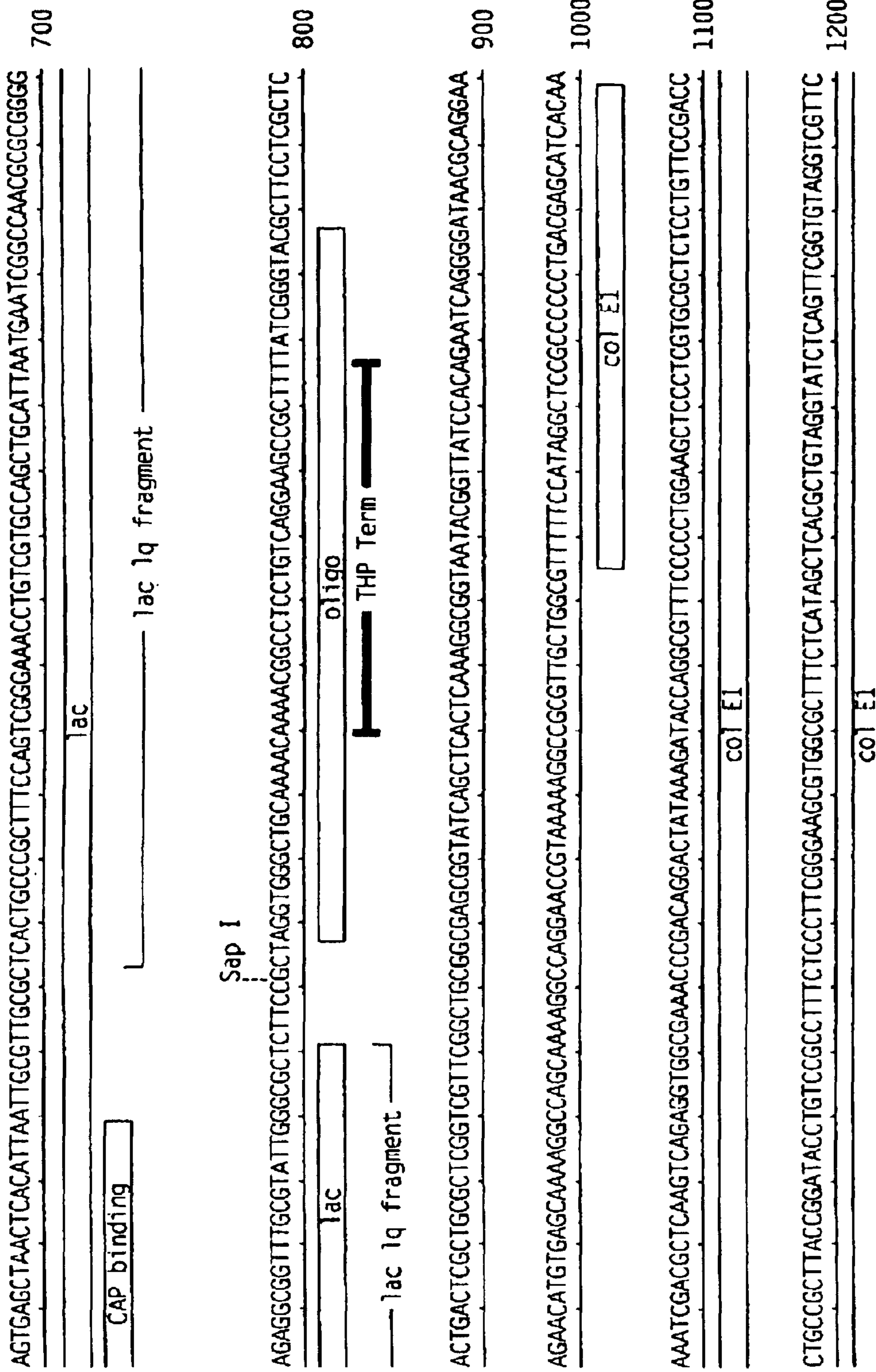


FIG. 6a (Cont.)

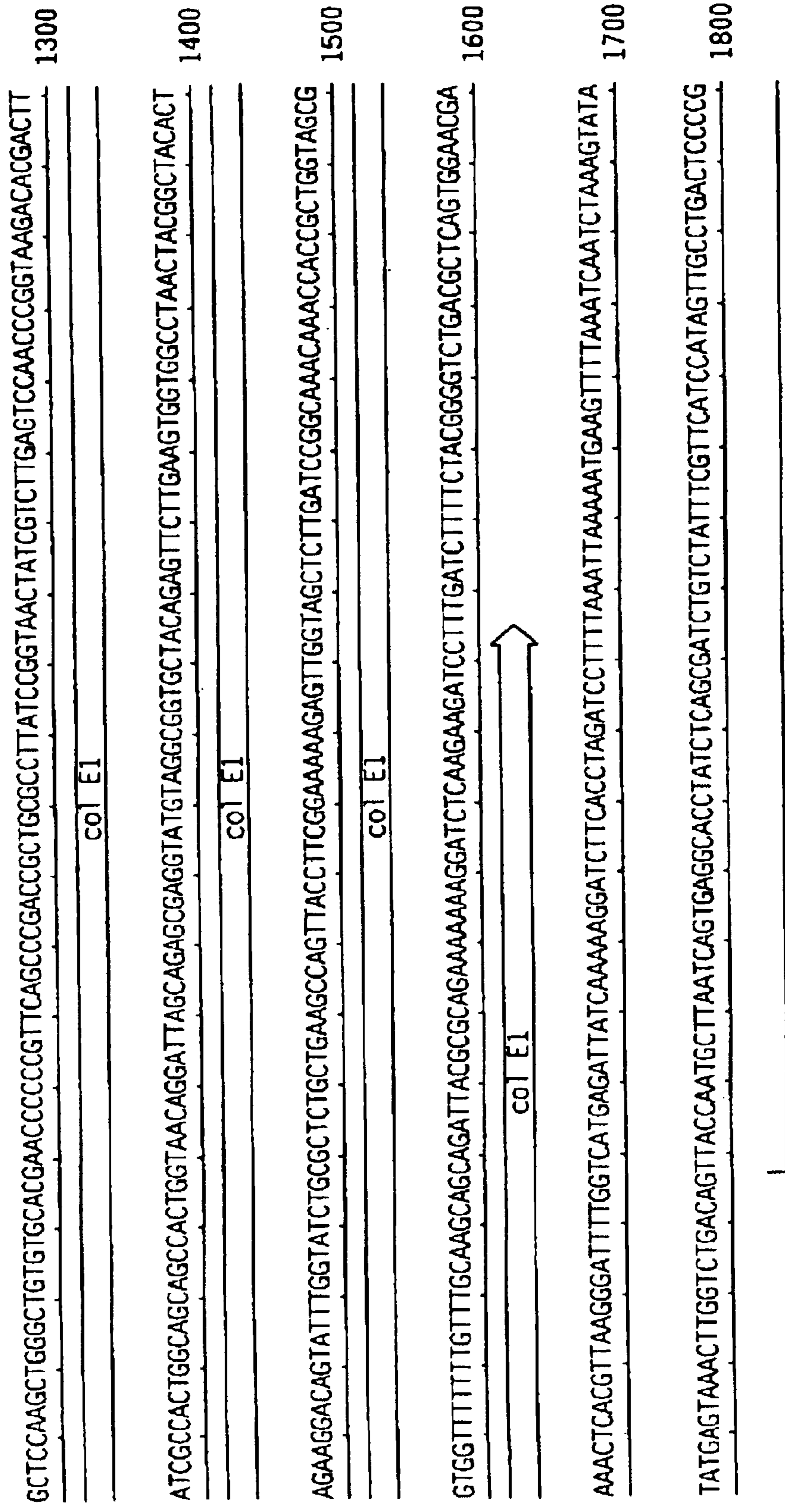


FIG. 6b

1900
TCGTAGATAACTACGGAGGGCTTACCATCTGGCCCGAGTGTGCAATGATACGGGAGACCAGCTCACGGCTCCAGATTTATCAGCAAT
2000
AAACCAGCCGGAAGGCCGAGCCGAGAAGTGGTCTGCAACTTTATCCGCTCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAGTAGT
2100
TCGCCAGTTAATAGTTGCGCAAGTTGTTGCCATTGCTACAGGCATGGTGGTGTACGGCTCGTGGTTGGTATGGCTTCATTACAGCTCCGGTCCCAAC
2200
GATCAAGCGGAGTTACATGATCCCCCATGTTGTGCAAAAAGCGGTTAGCTCCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTATC
2300
ACTCATGGTTATGGCAGCACIGCATAAATTCCTTACTGTCATGCCATCCGTAAGATGCTTTCGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAA
2400
TAGTGTATGGCGCAGCGAGTTGCTCTTGCCCGGGTCAATACGGGATAATACCGGCCACATAGCAGAACTTTAAAAGTGTCTCATCTGGAAAACGTT
2500
CTTCGGGGGAAACTCTCAAGGATCTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTGGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTCAC

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|

FIG. 6b (Cont.)

CAGCGTTTCTGGTGAGCAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTGAACTACTCTCTTCCCTTTTCAA 2600

TATTATTGAAGCATTATCAGGGTTATTGTCATGAGCGGATACATAATTTGAATGTAATAGAAAAATAAACAAATAGGGGTTCCGGGCACACATTTCCCC 2700

GAAAAGTGCCAC → 2712

FIG. 6c

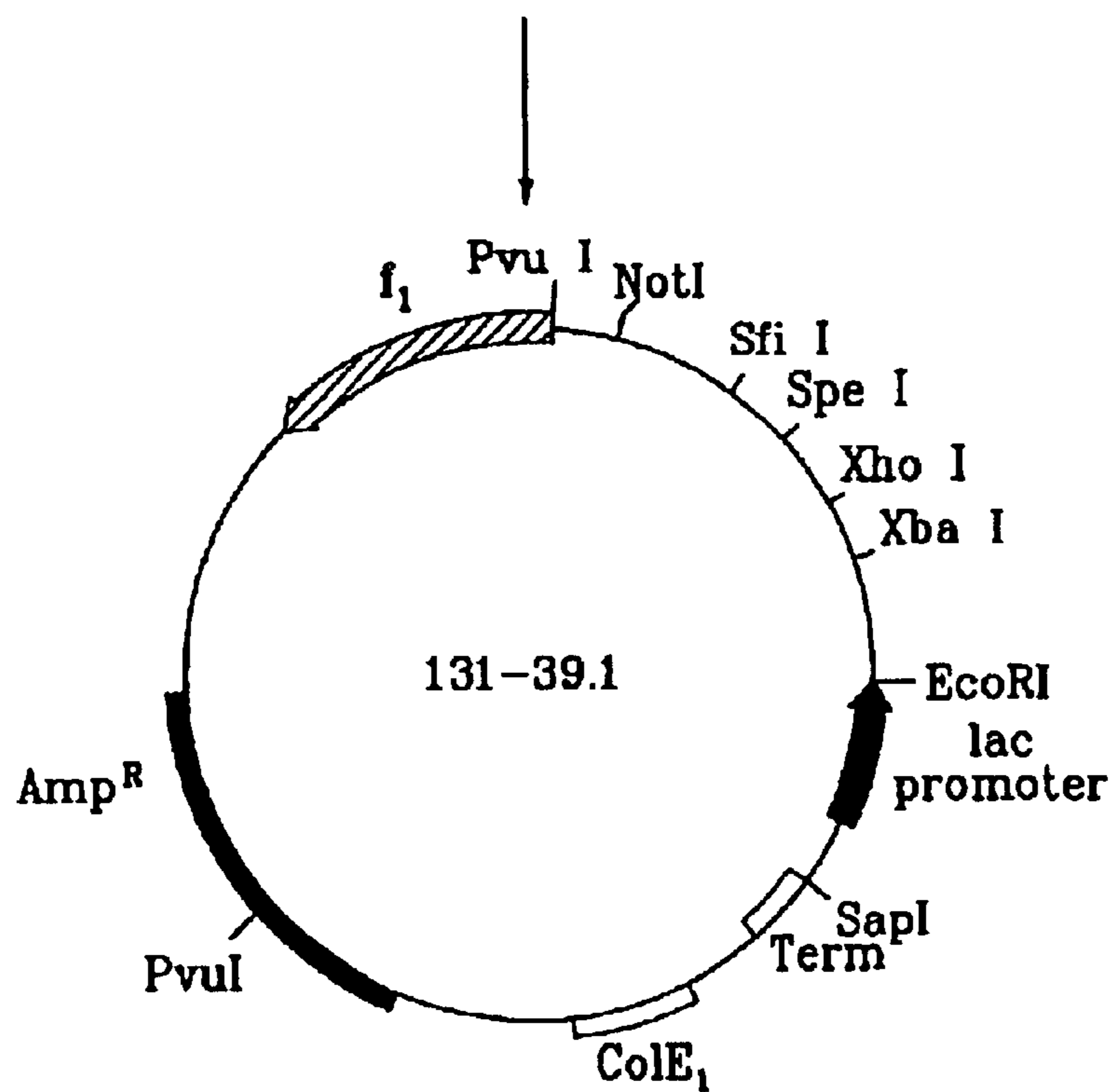
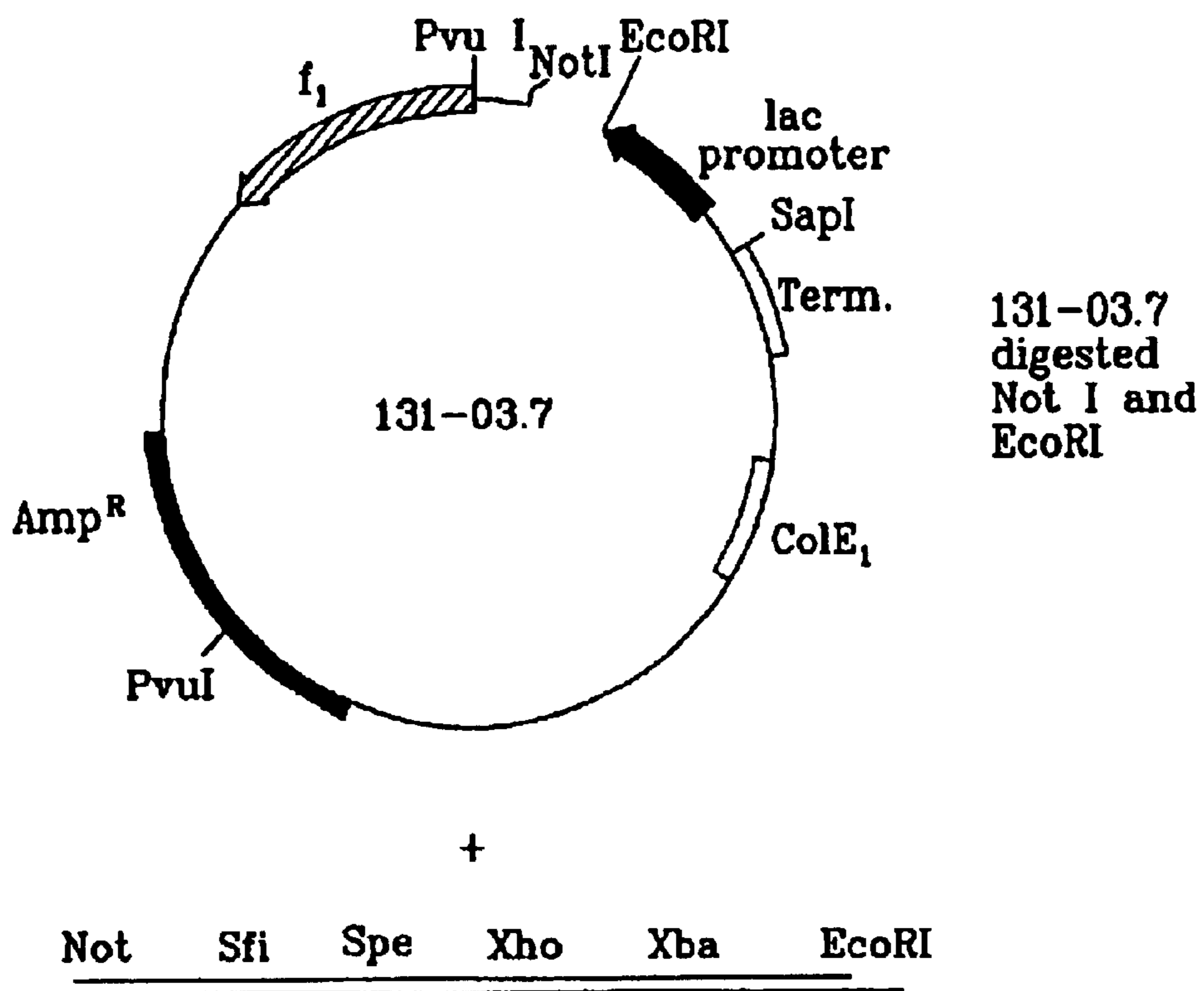


FIG. 7

(Seq. ID No. 21)

GTGGCACTTTTCGGGGAAATGTGGCGGAACCCCTATTGGTTATTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCIGATAAAT 100
 GCTTCAATAATTGAAAAGGAGAGATGAGTATTCACATTTCCGTGTCGCCCTTATCCCTTTTGGGGCATTTTGCCTTCCIGTTTTGCTCAG 200
 CCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGTTACATCGAACTGGATCTCAACAGGGTAAGATCCTTGAGAGTT 300
 TTGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCGTATGTGGCGGGTATTATCCCGTATTGAGCCGGGCAAGAGCAACTCGGTCCG 400
 CCGCATACTATTTCAGAAATGACTTGGTTGAGTACTCACCAGTCACAGAAAGCATCTTACGGATGGCATGACAGTAAGAGAAATTATGCAGTGCCTGCC 500
 ATAACCATGAGTATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCAGGAGCTAACCGCTTTTTCACAACATGGGGGATCATGTAA 600

_____ beta lactamase
 _____ beta lactamase
 _____ beta lactamase
 _____ beta lactamase
 _____ beta lactamase
 _____ beta lactamase

Pvu I
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FIG. 8a

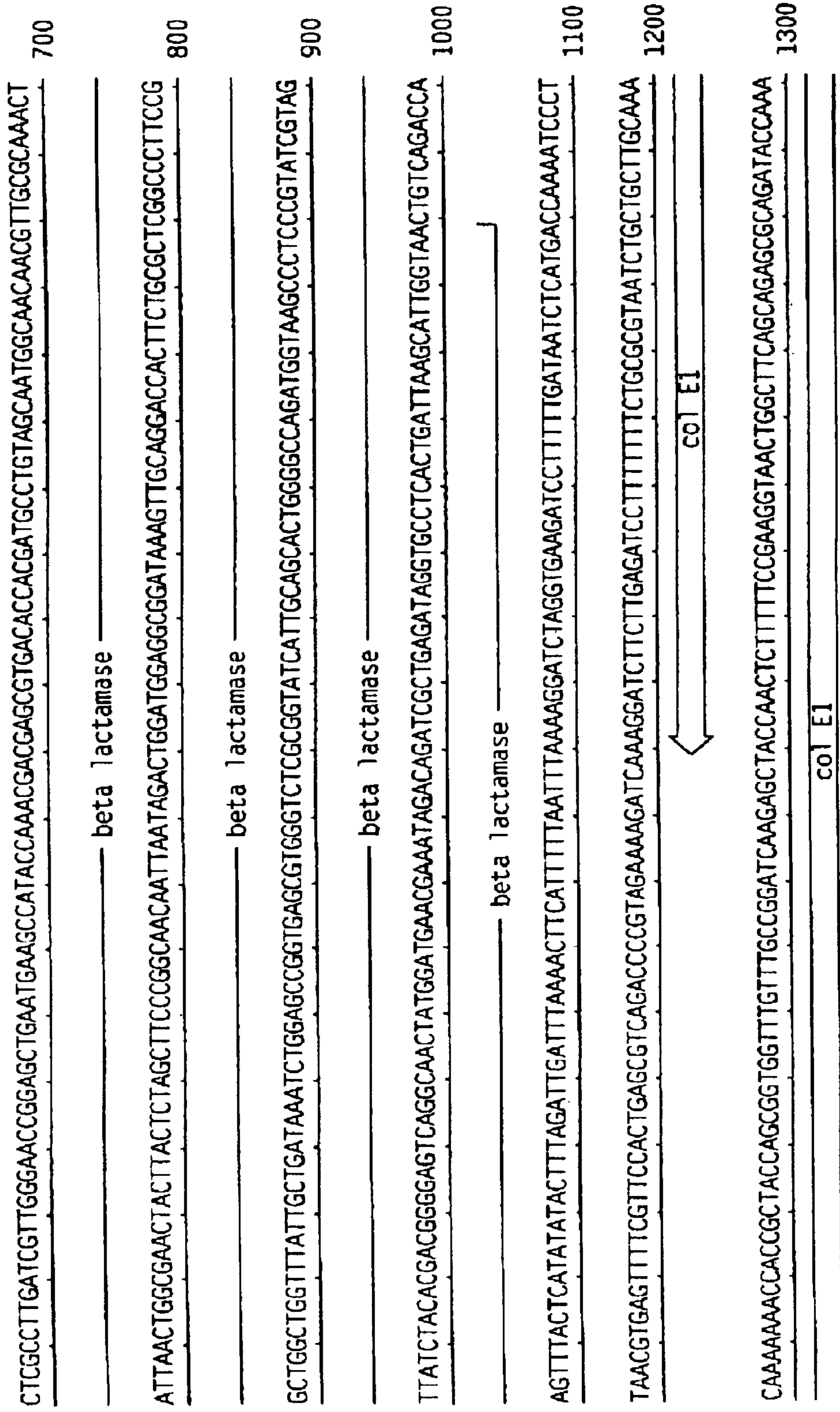


FIG. 8a (Cont.)

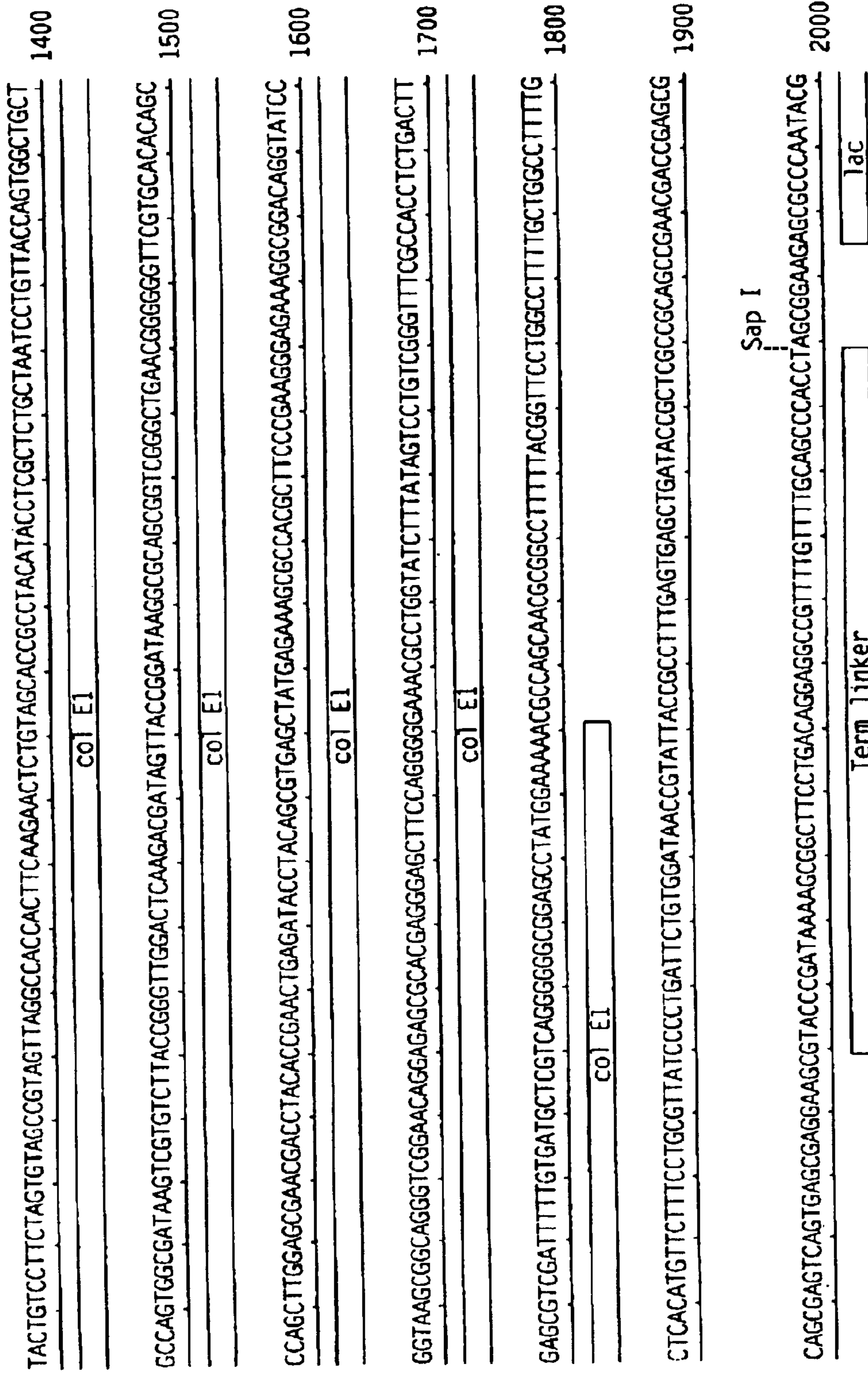


FIG. 8b

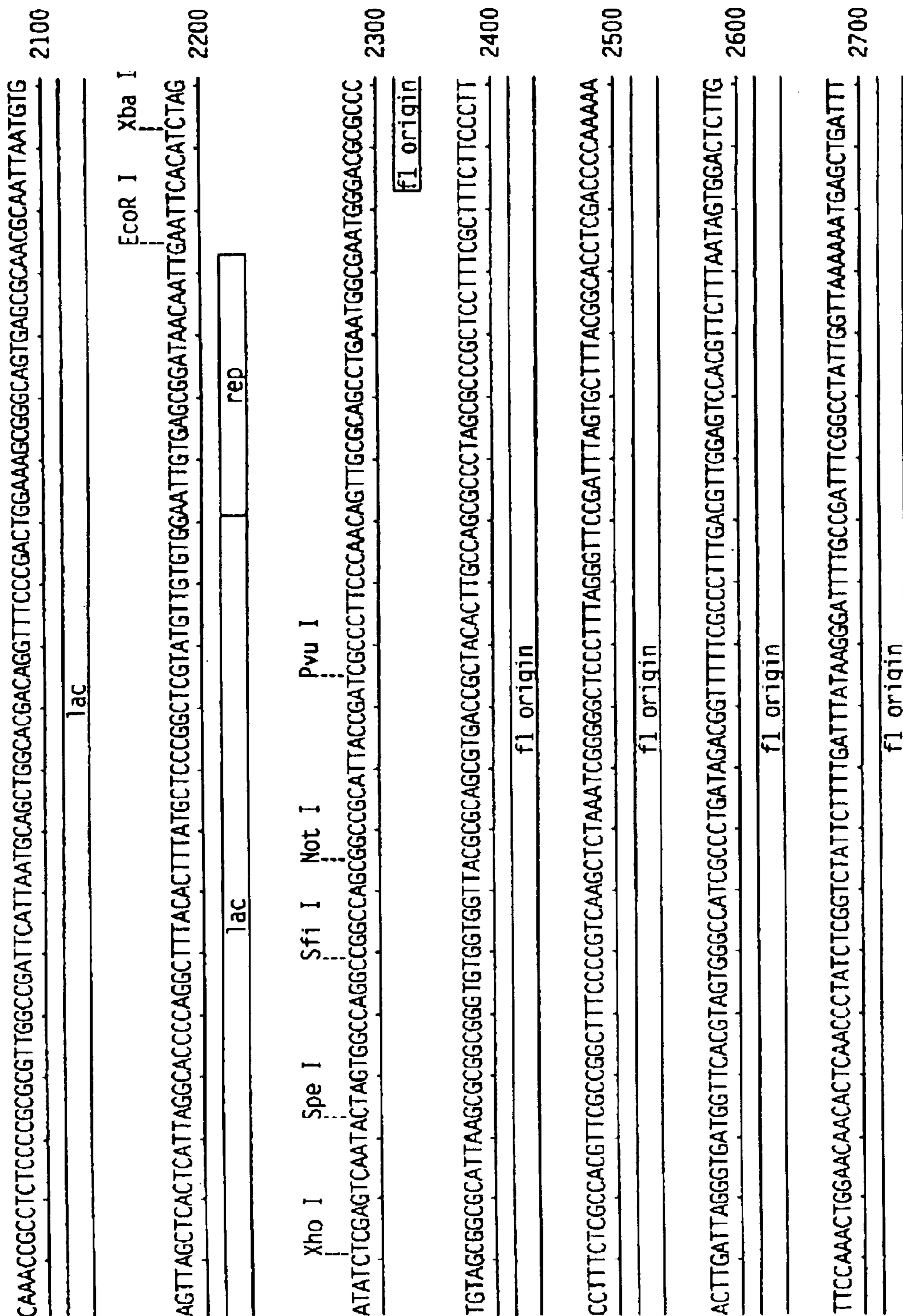


FIG. 8b (Cont.)

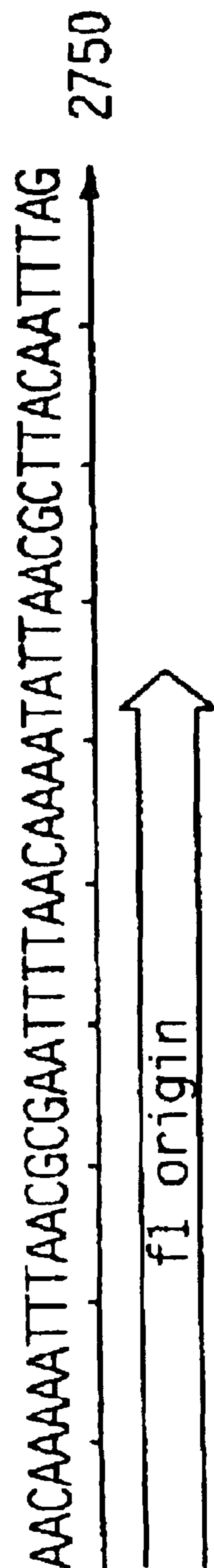


FIG. 8C

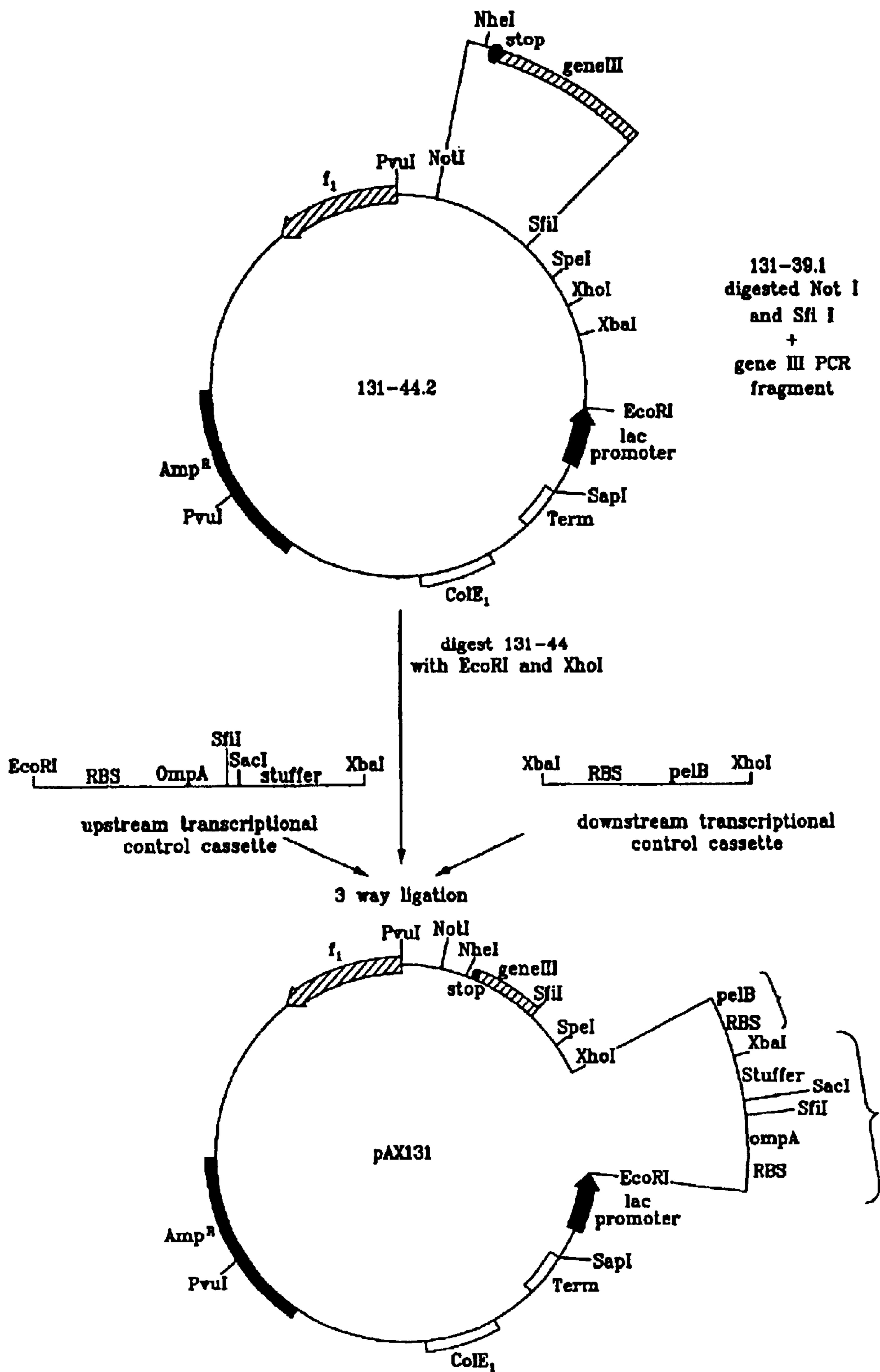


FIG. 9

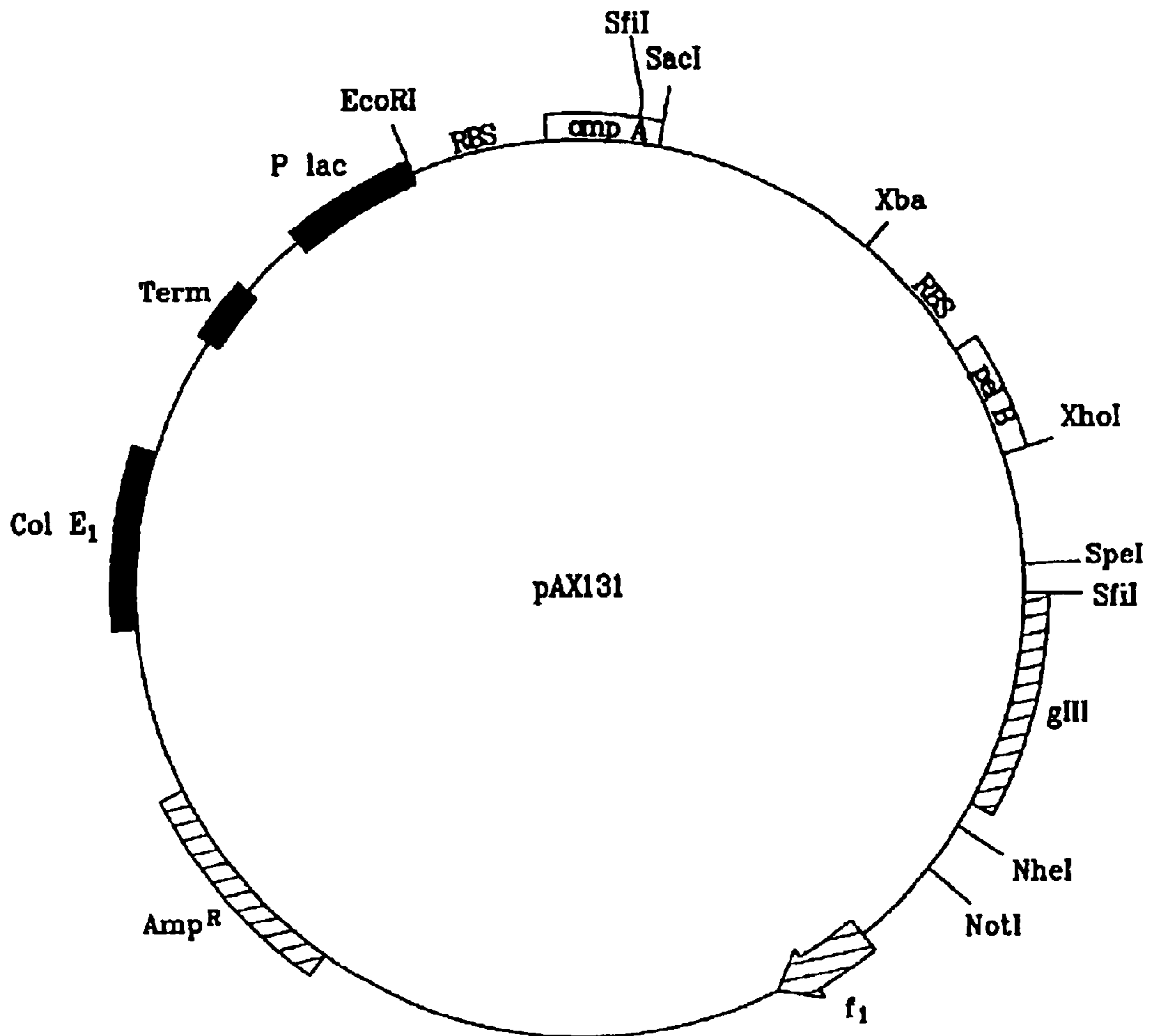


FIG. 10

Seq. ID No. 18

GTGGCCTTTTCGGGAAATGTGGCGGAACCCCTATTGTTTATTTCIAAATACATTCAAATATGATCCGCTCATGAGACAATAACCCCTGATAAAT 100

GCCTCAATAATTTGAA¹AAAGGAAGATGAGTATTCACATTCGGTGGCCCTTATCCCTTTTGGGGCATTTTGCCCTTCCTGTTTTGCTCAC 200

CCAGAAACCGCTGGTGAAGTAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTT 300

TTCGCCCCGAAGACGTTTTCCAATGATGAGCACTTTAAAGTCTGCTATGTGGCGGTATTATCCCGTATTGACGGCGGCAAGCAACTCGGTCCG 400

CCGCATACACTATTCTCAGAA TGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAAATTATGCAGTGTGCC 500

ATAACCATGAGTGATAACACTGGGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTCACACAATGGGGGATCATGTAA 600

beta lactamase

beta lactamase

beta lactamase

beta lactamase

beta lactamase

FIG. 11a

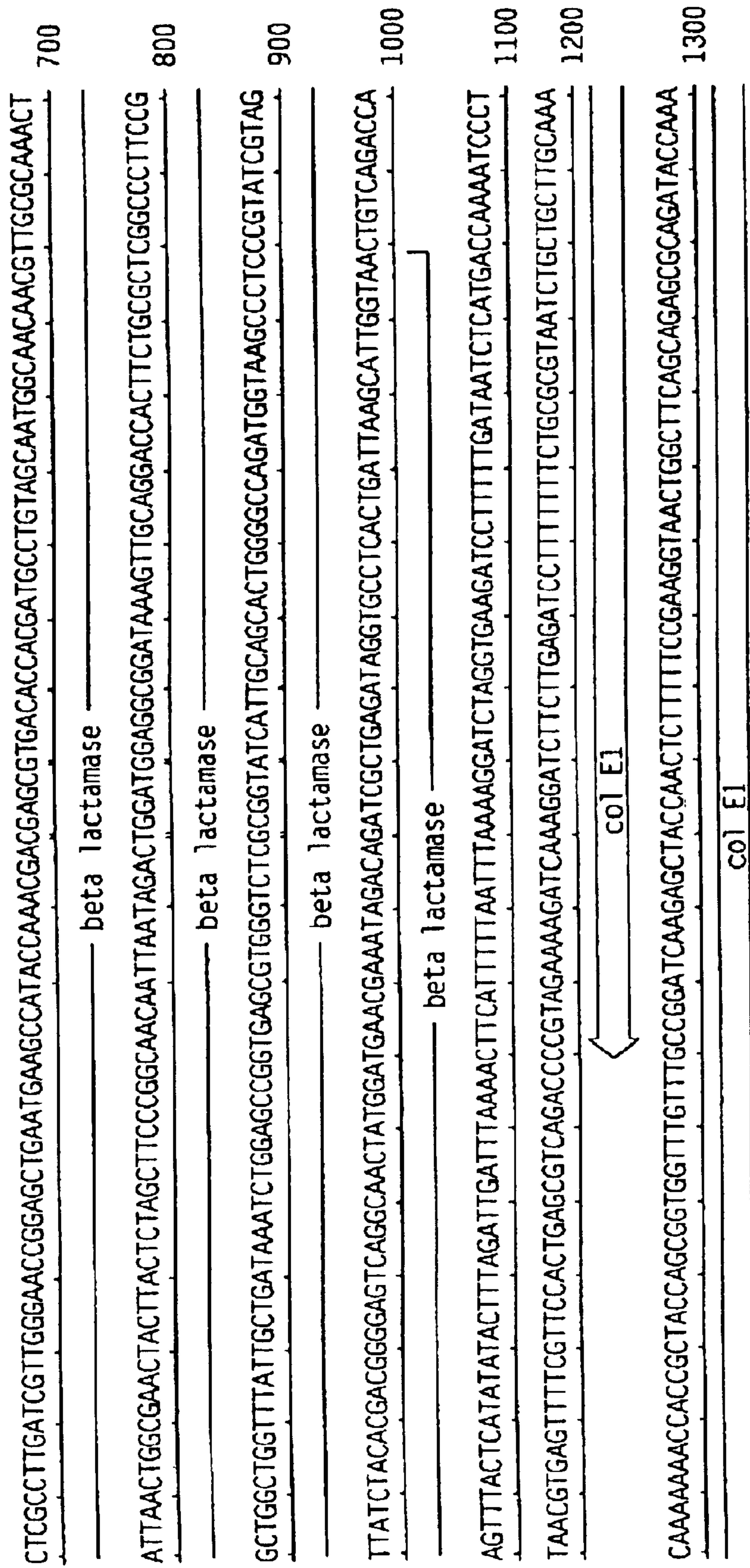


FIG. 11a (Cont.)

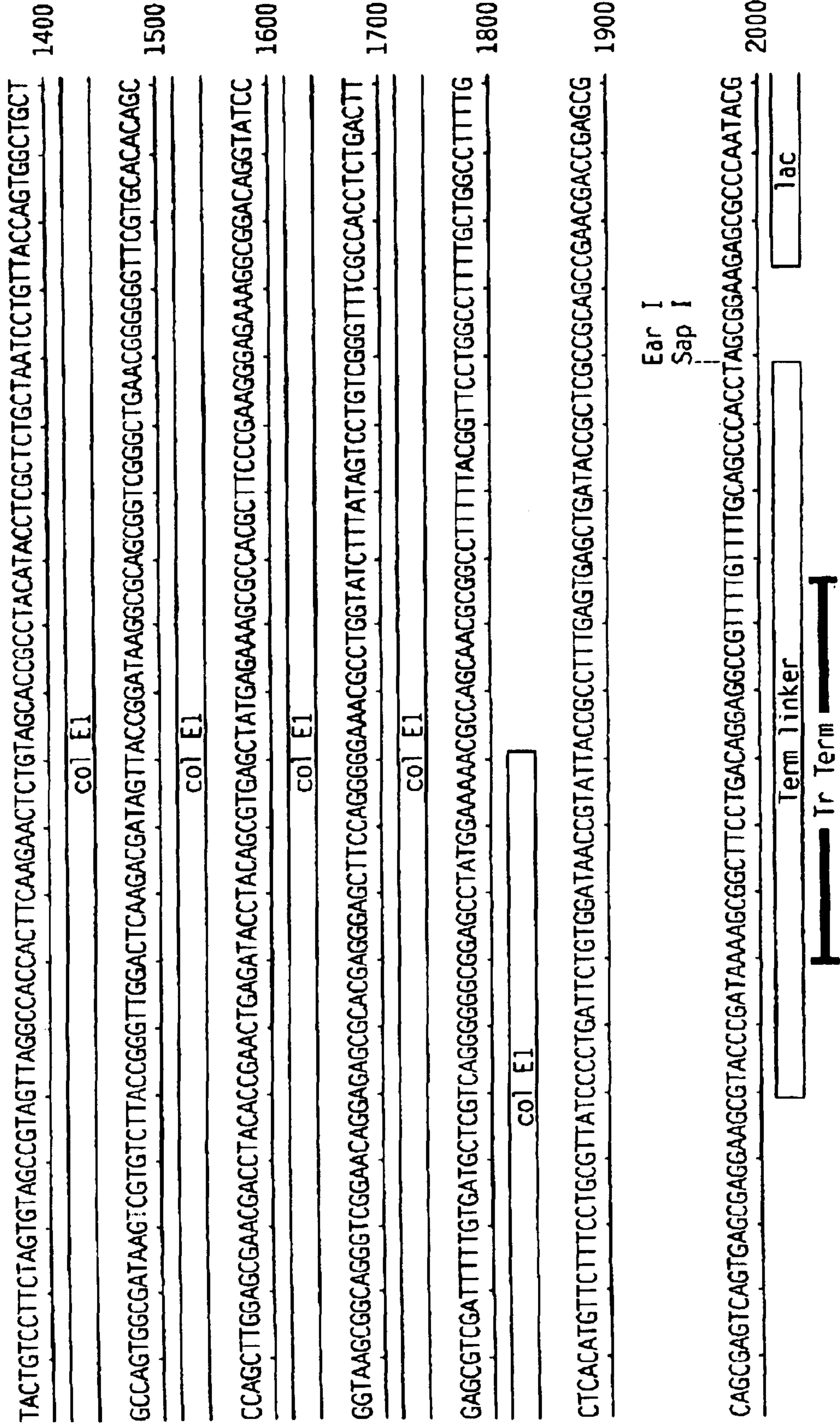


FIG. 11b

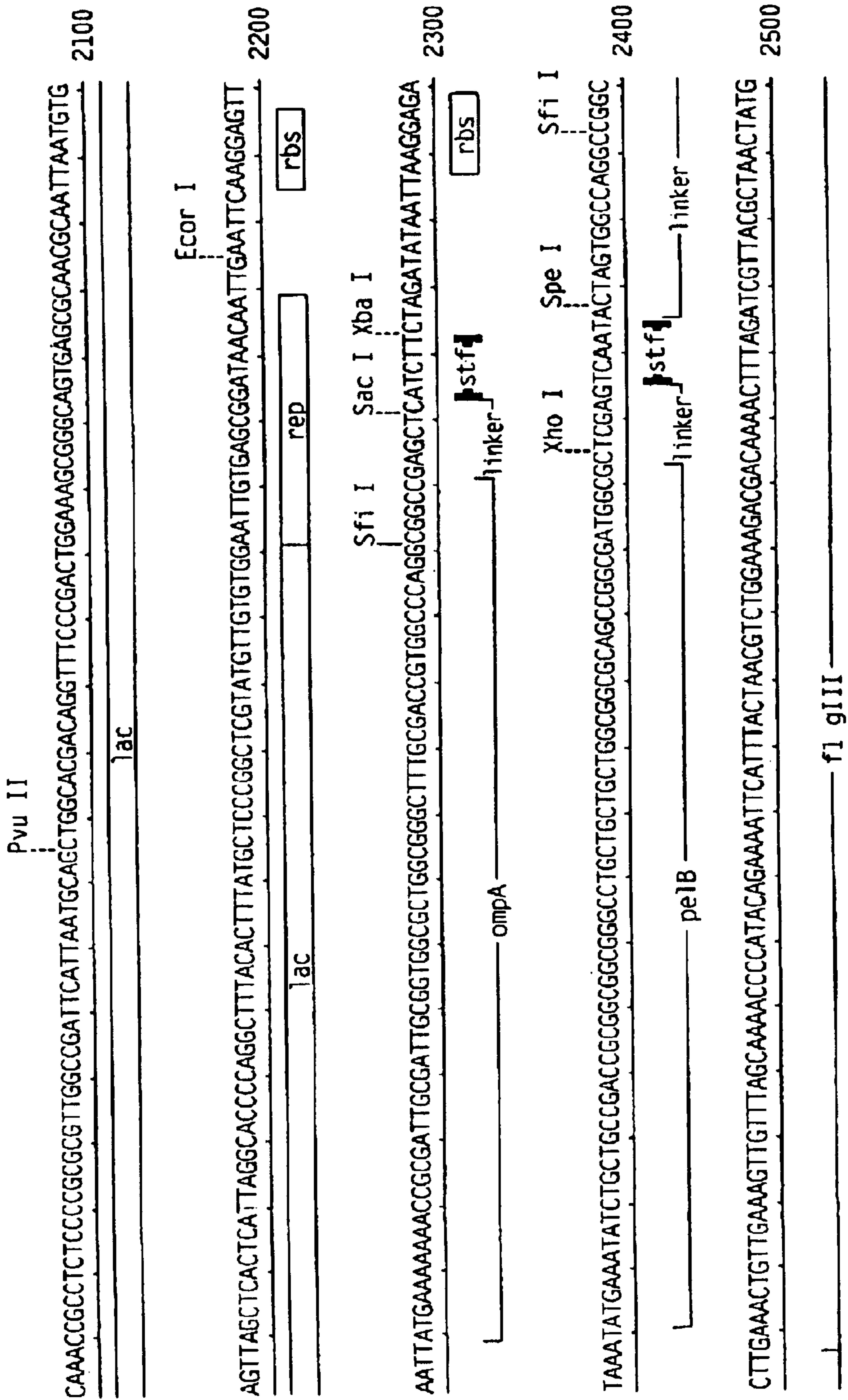


FIG. 11b (Cont.)

2600
AGGGCTGTCTGTGGAATGCTACAGGGTGTAGTTTGTACTGGTGACGAAACTCAGTGTACGGTACATGGGTTCTATTGGGCTTGCTATCCCTGAAAA
f1 gIII

2700
TGAGGGTGGTGGCTCTGAGGGTGGCGGCTCTGAGGGTGGCGGCTACTAAACCTCCTGAGTACGGTGATACACCTATTCCGGGCTAT
f1 gIII

2800
ACTTATCAACCCTCTCGACGGCATTATCCGCCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGCTCAGCCTCTTAATACTT
f1 gIII

2900
TCATGTTTCAGAAATAAGGTTCCGAAATAGGCAGGGGCATTAACTGTTTATACGGGCACCTGTTACTCAAGGCACCTGACCCCGTTAAAACCTTATACCA
f1 gIII

3000
GTACACTCCTGTATCATCAAAGCCATGATGACGCTTACTGGAACGGTAAATTCAGAGACTCGCTTCCATTCTGGCTTAAATGAGGATCCATTGCTT
f1 gIII

3100
TGTGAATATCAAGGCCAATCGTCTGACCCTGCCTCAACCTCCTGTTAATGCTGGCGGCGGCTCTGGTGGTGGTCTGAGGGTGGTGGCT
f1 gIII

3200
CTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCCGGTGGTGGCTCTGGTCCGGTGAATTTGATTAATAAAAGATGGCAAACGCTAA
f1 gIII

FIG. 11c

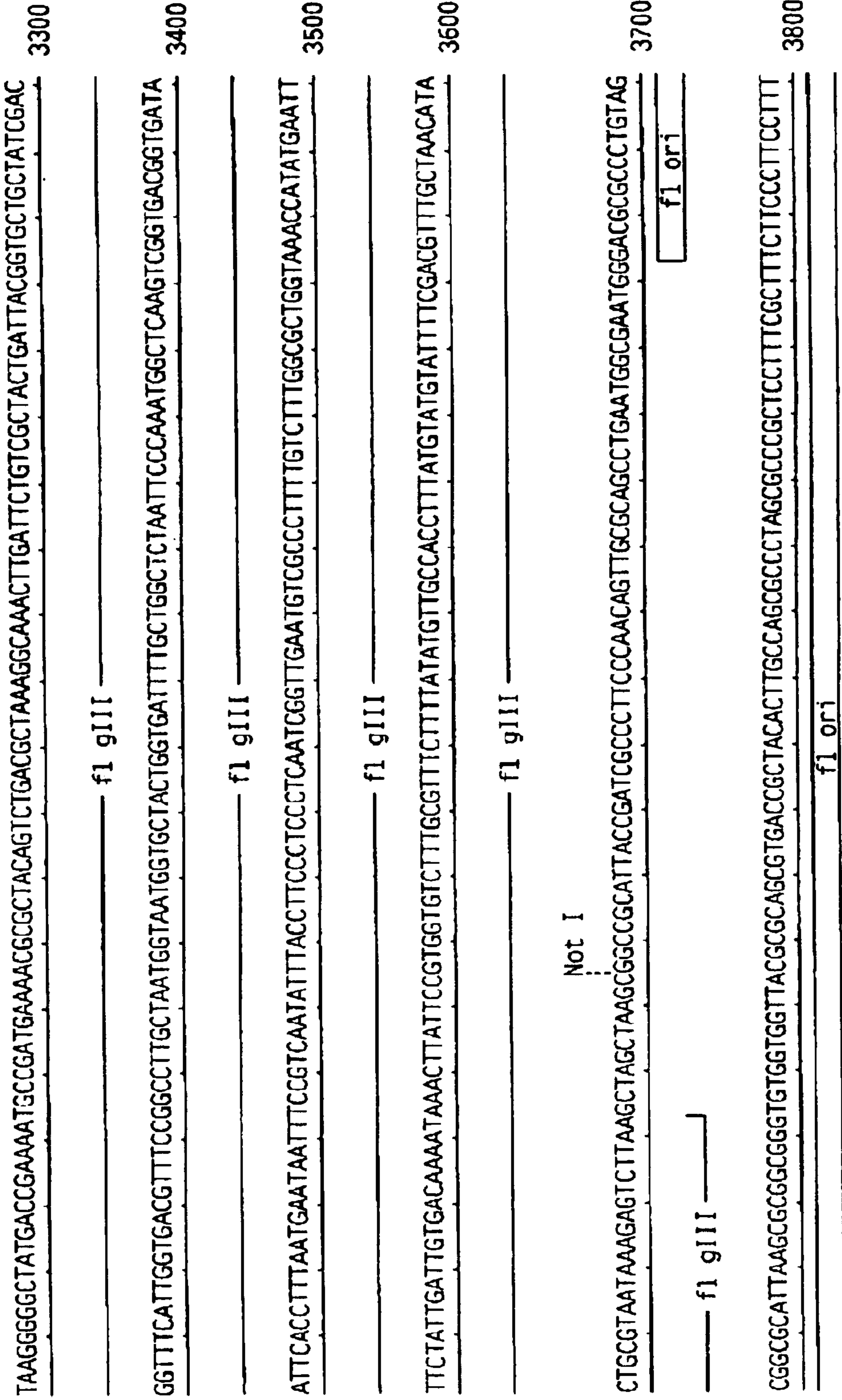


FIG. 11c (Cont.)

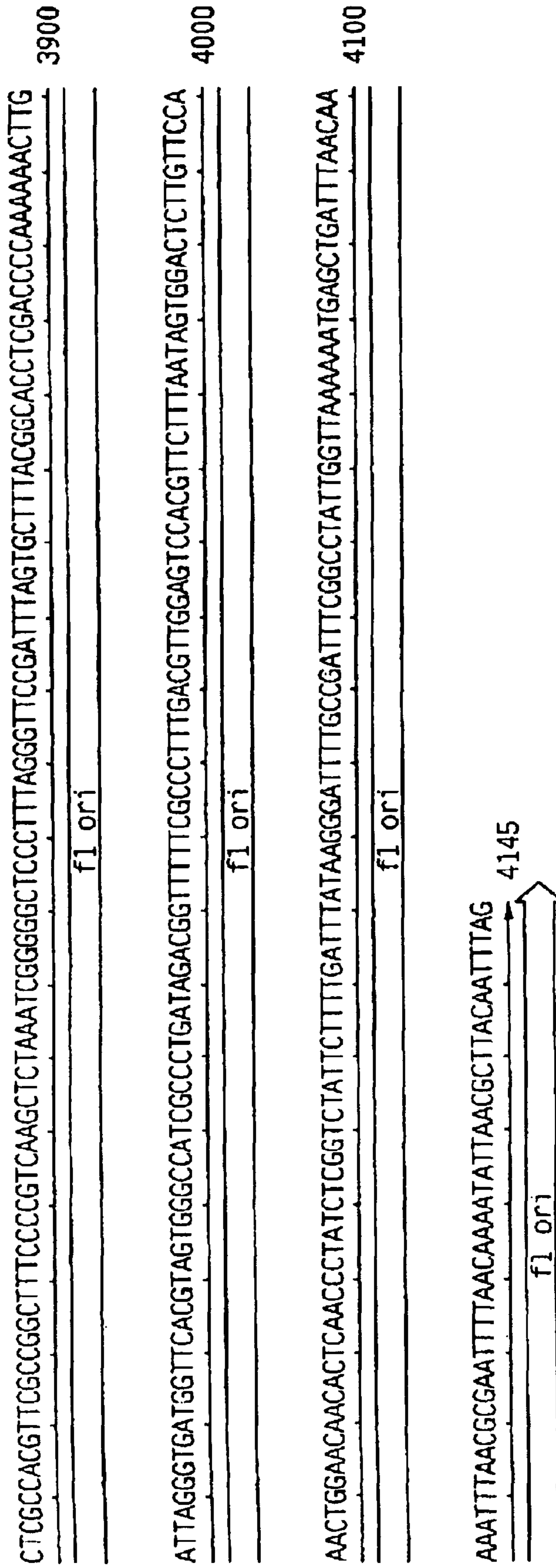


FIG. 11d

(SEQ. ID NO. 24)

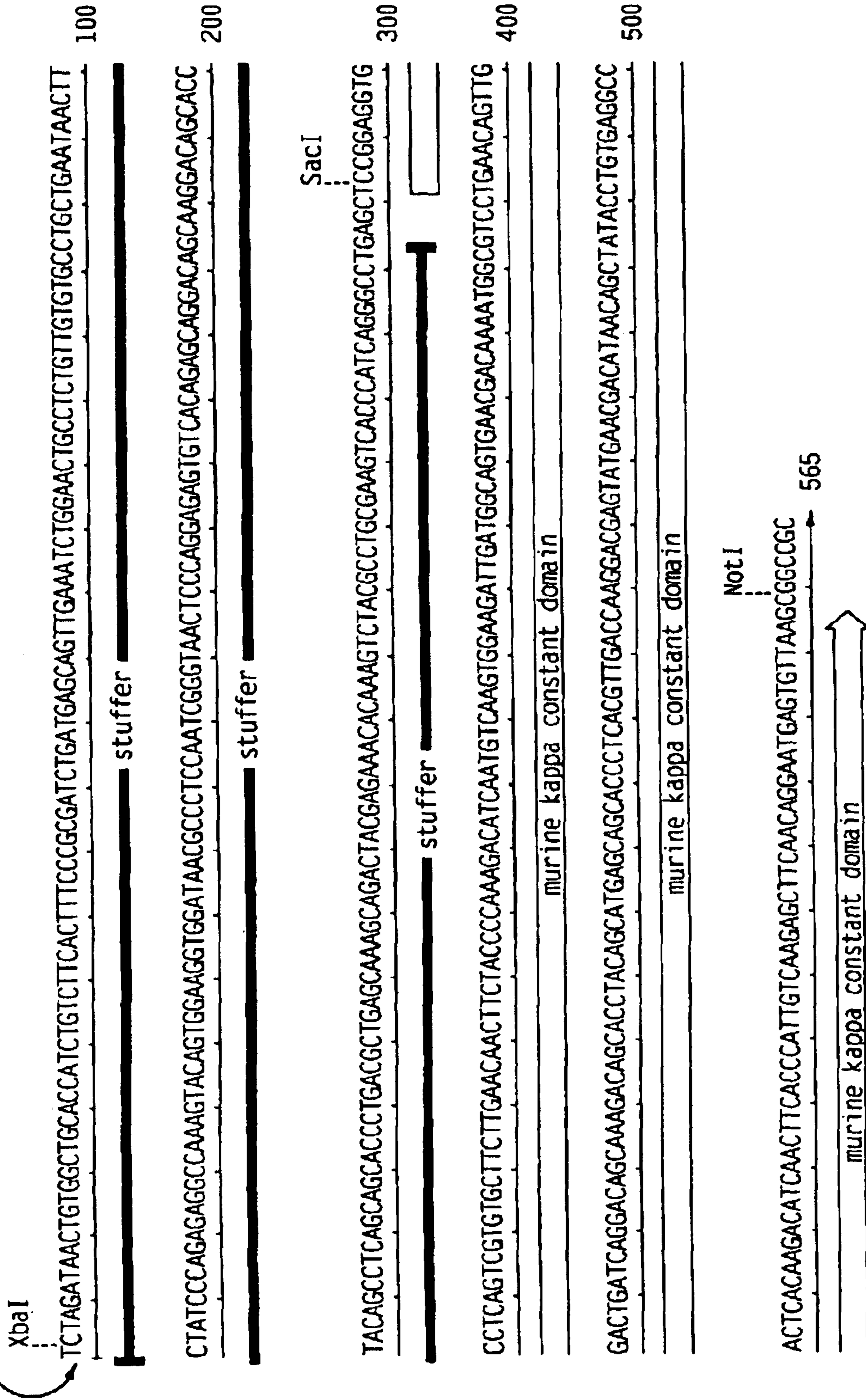


FIG. 12A

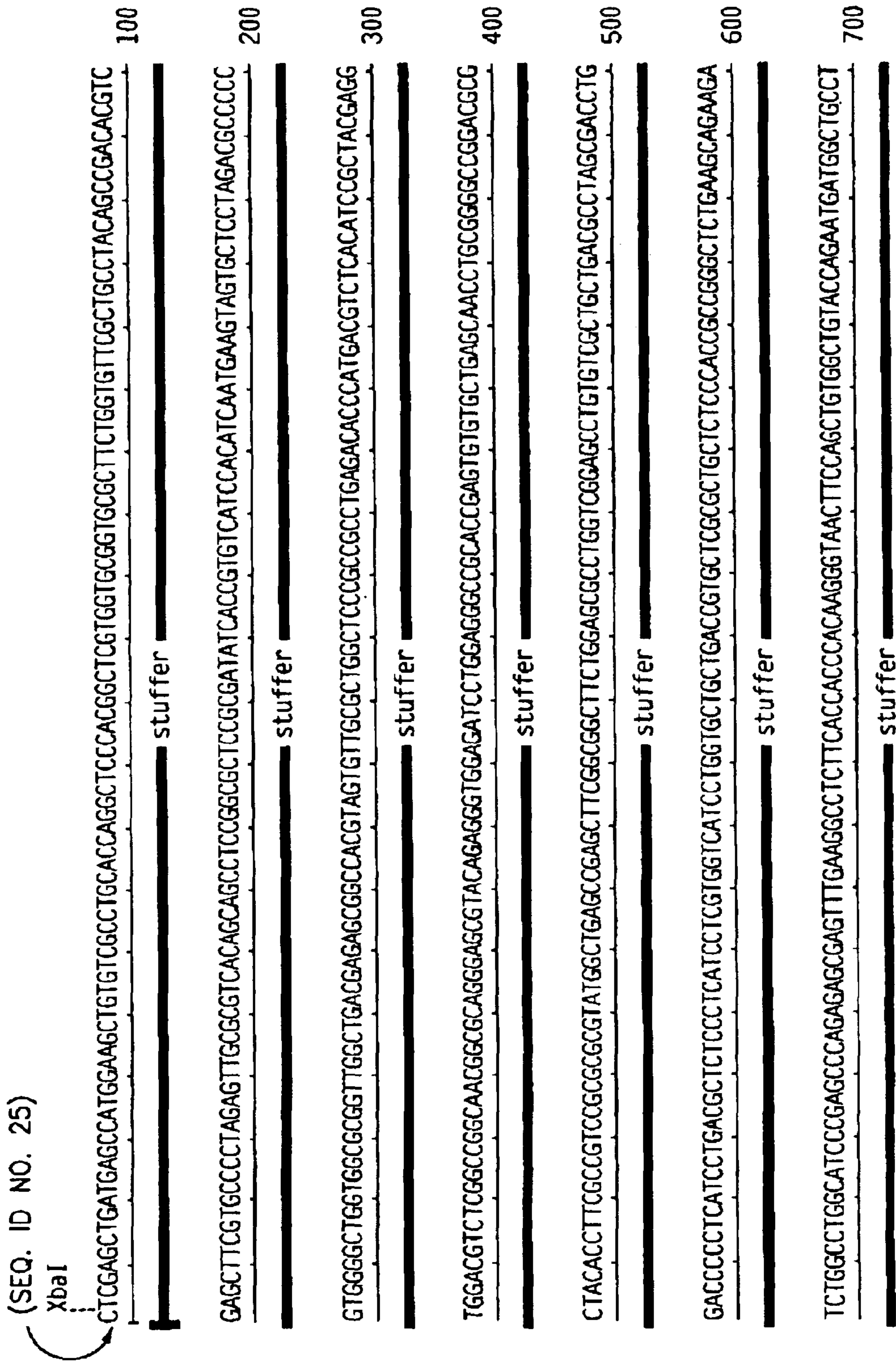


FIG. 12B

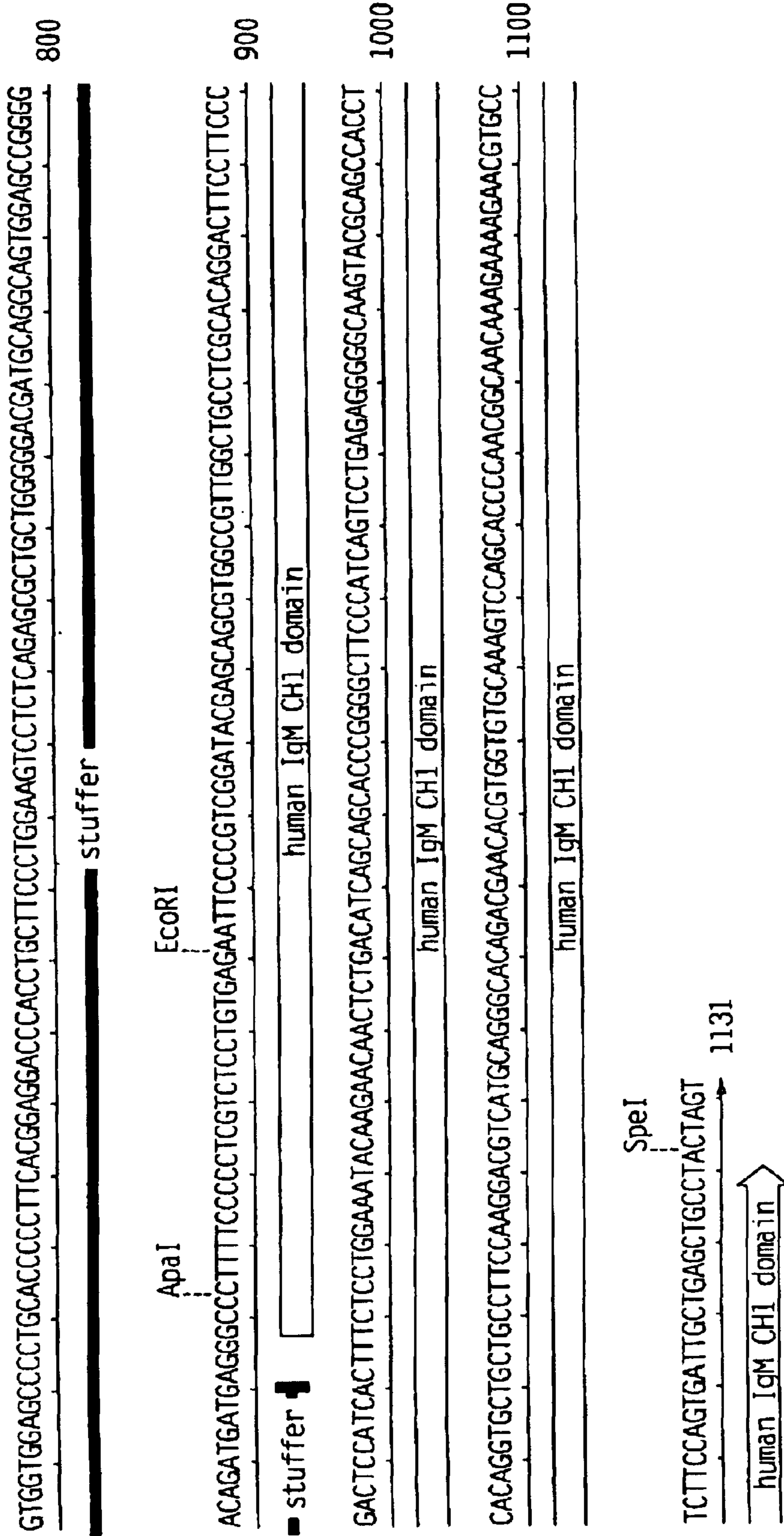


FIG. 12B (Cont.)

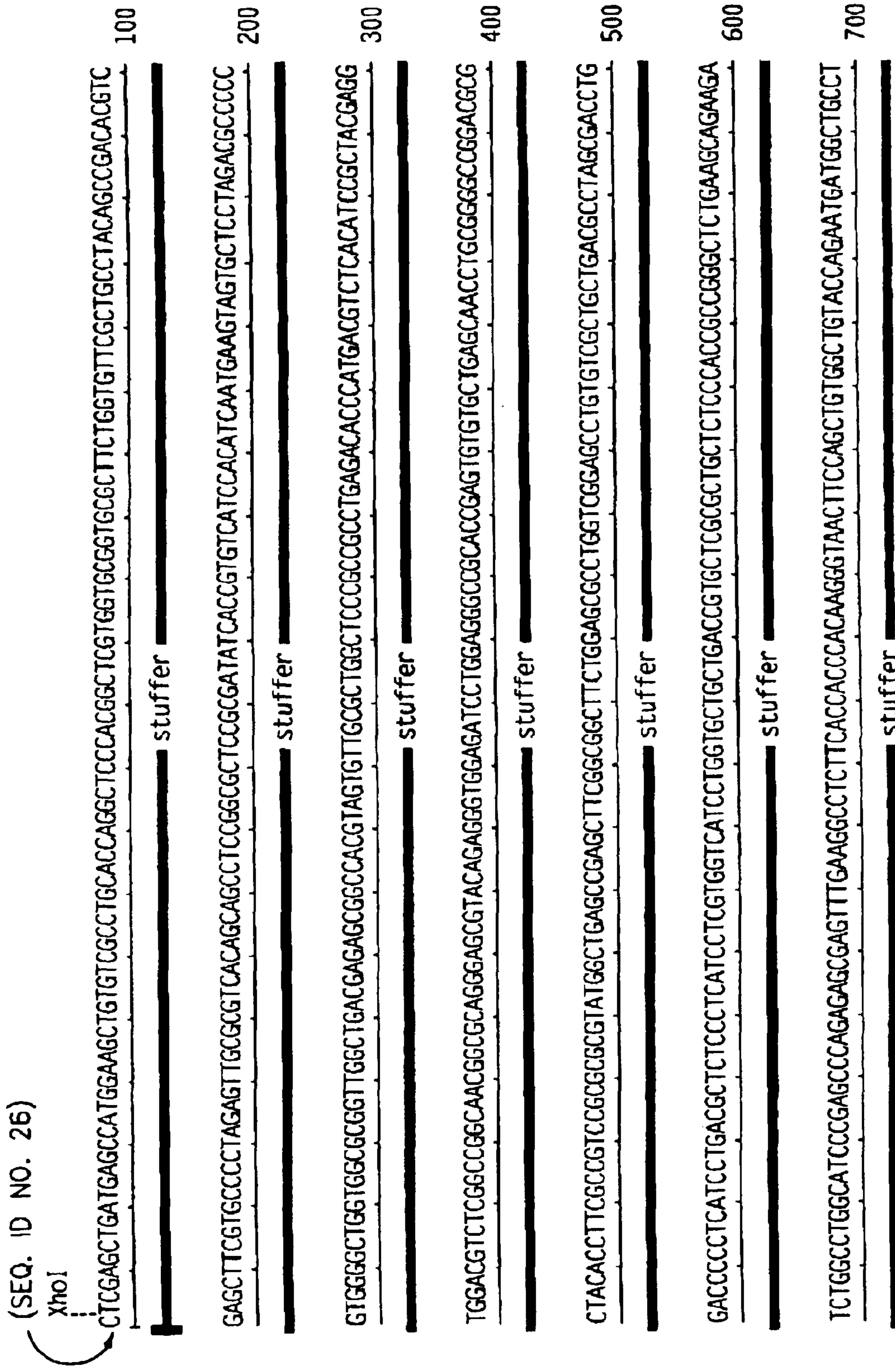


FIG. 12C

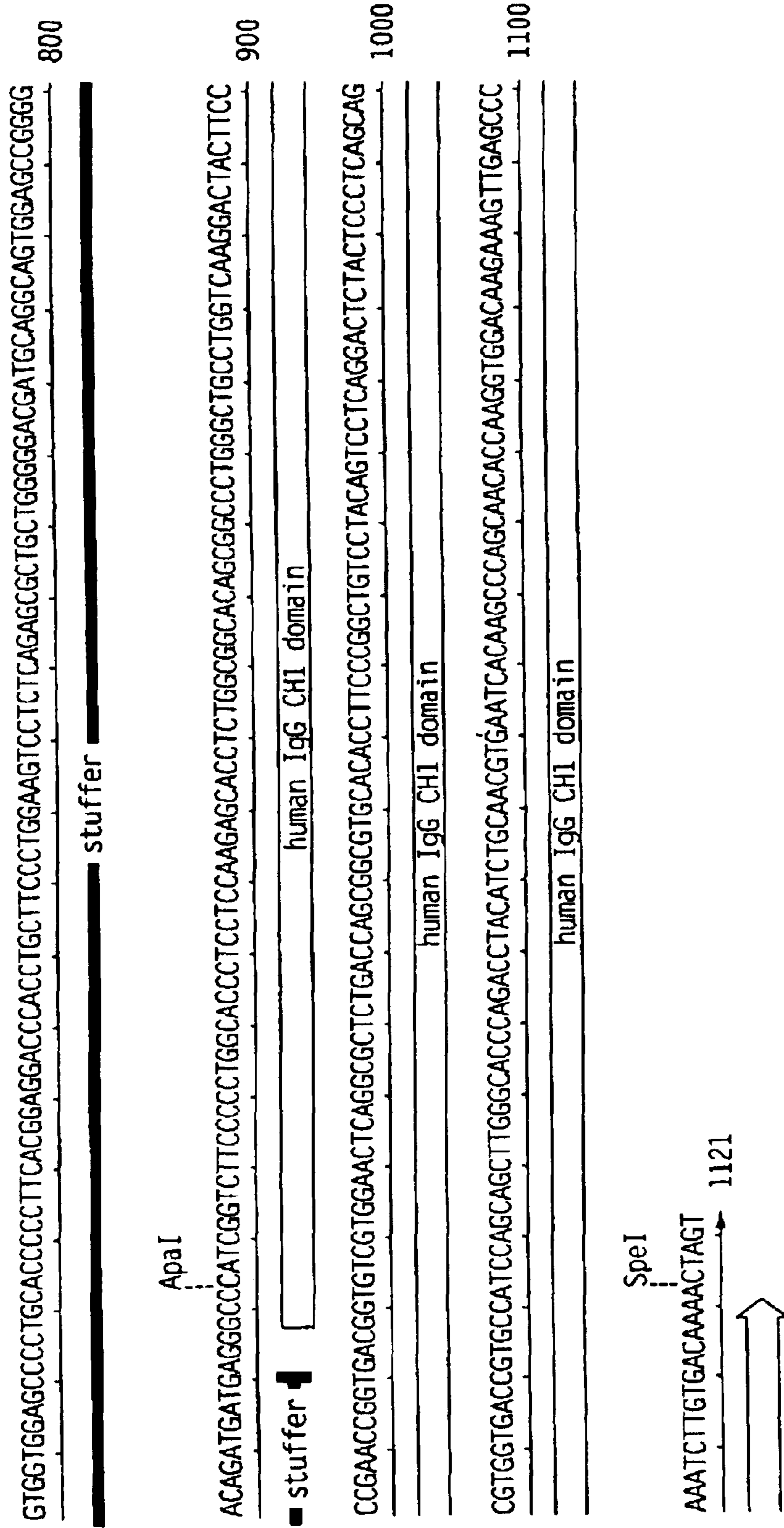


FIG. 12C (Cont.)

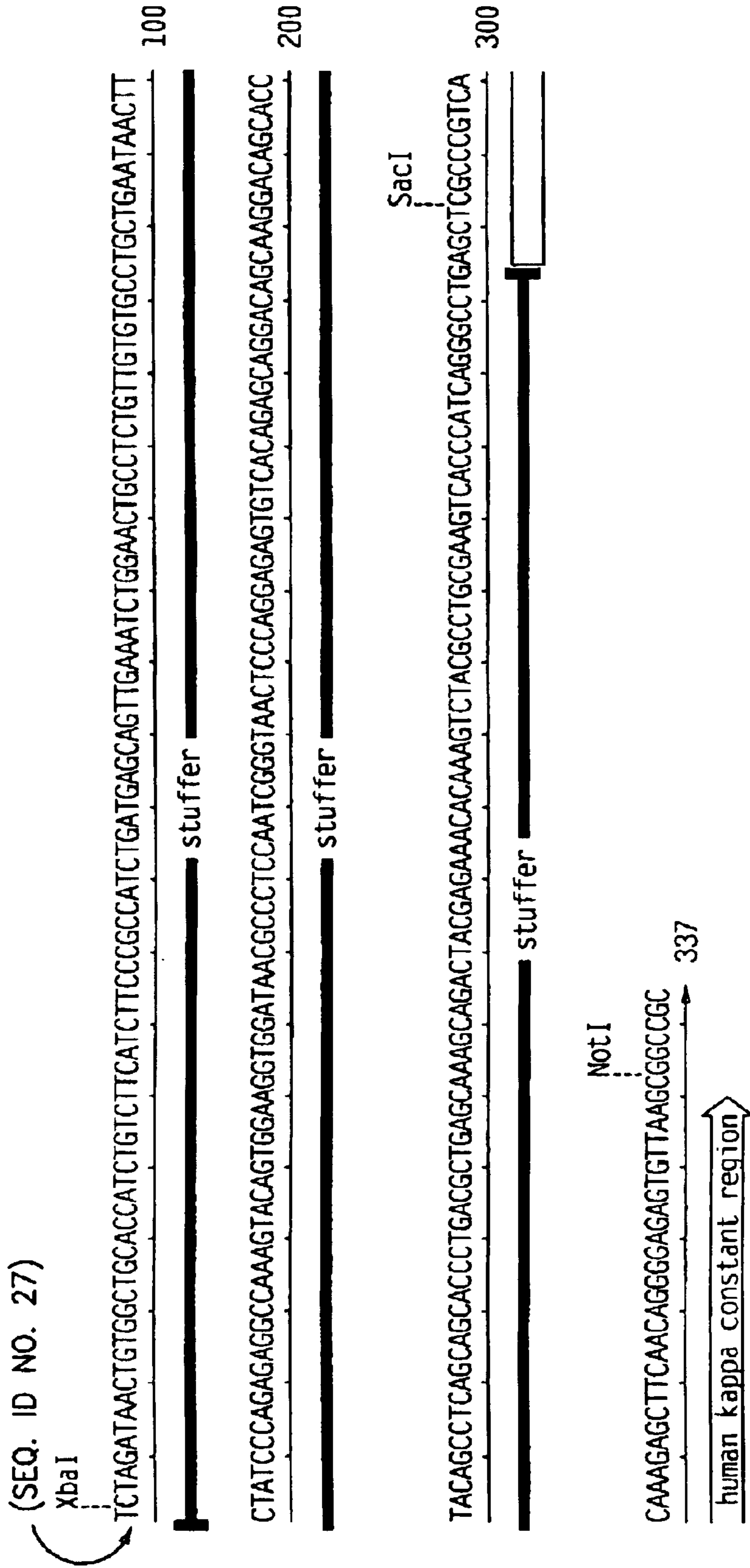


FIG. 12D

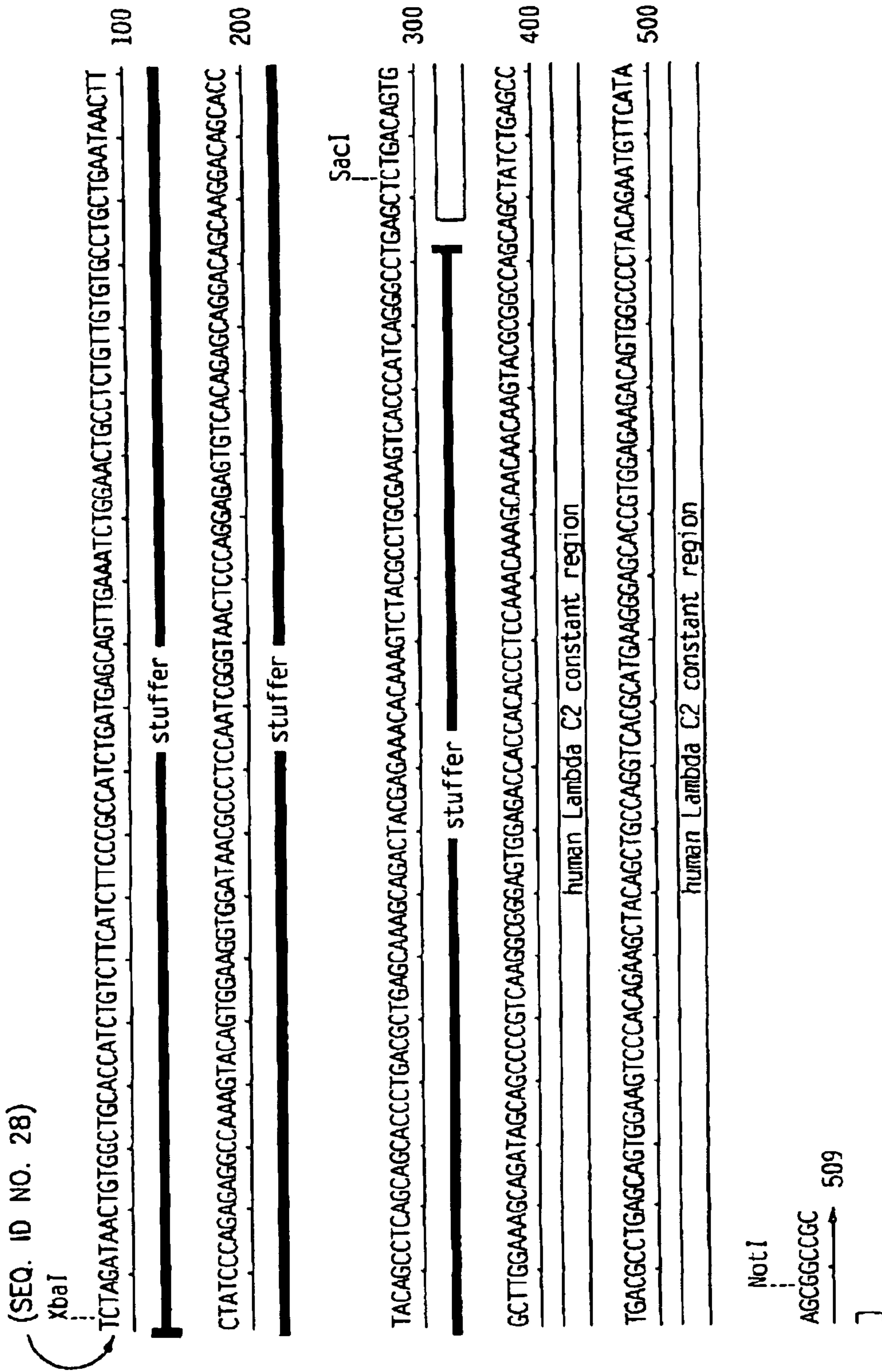


FIG. 12E

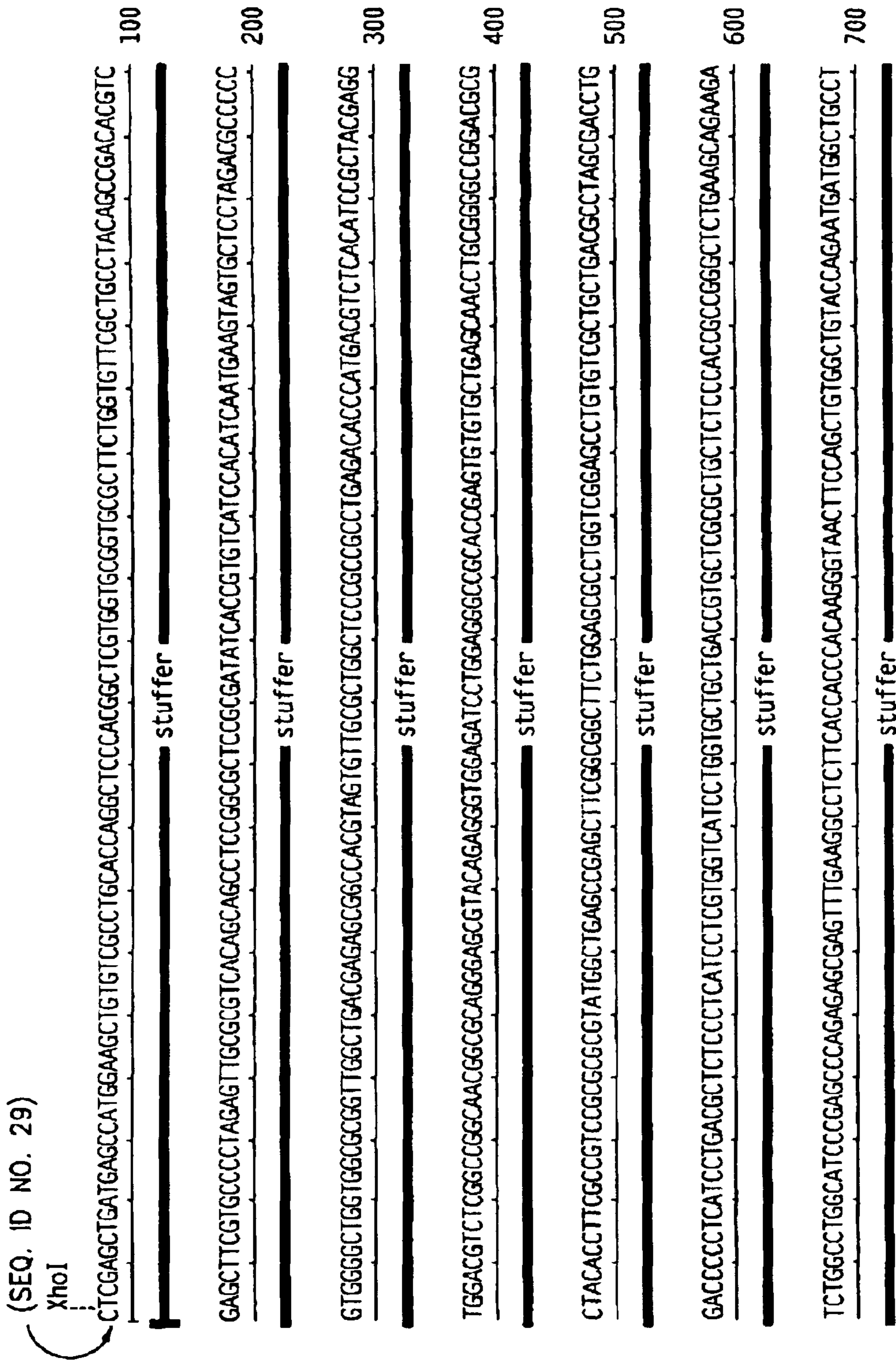


FIG. 12F

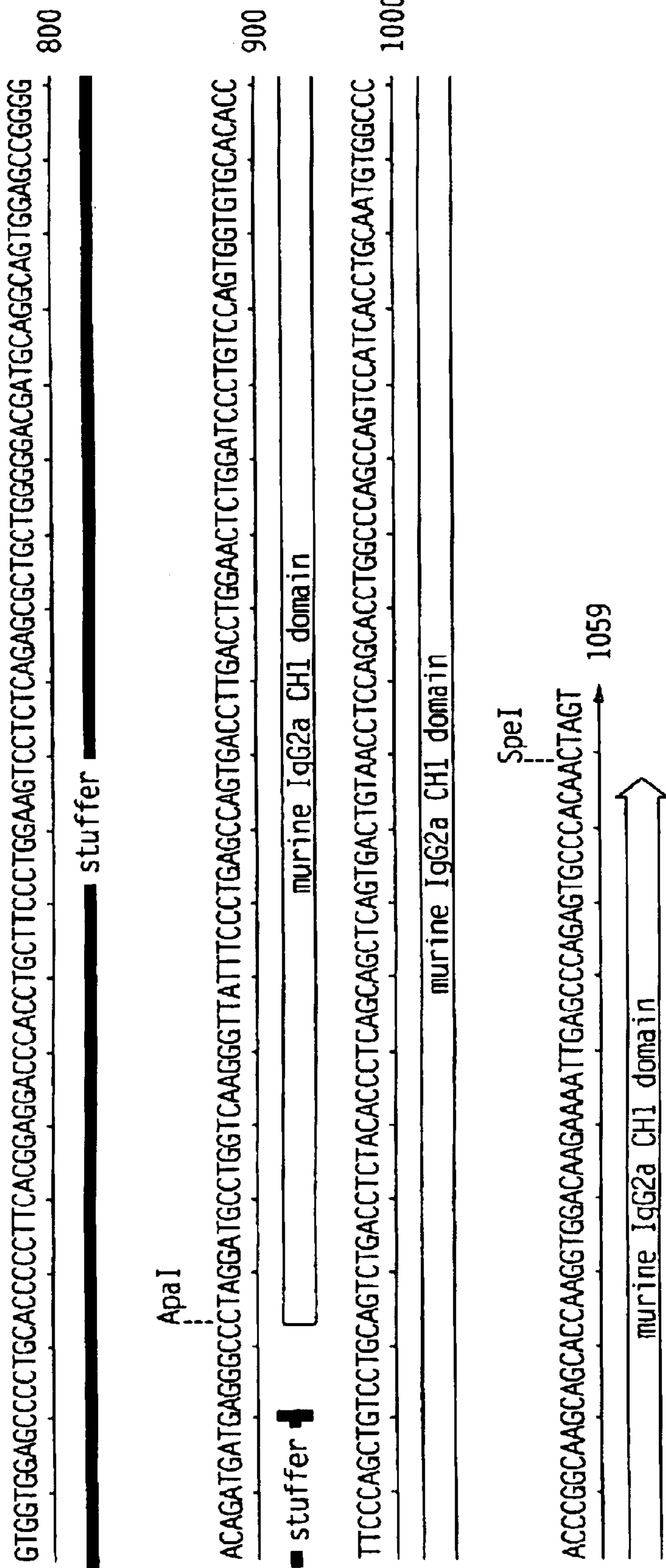


FIG. 12F (Cont.)

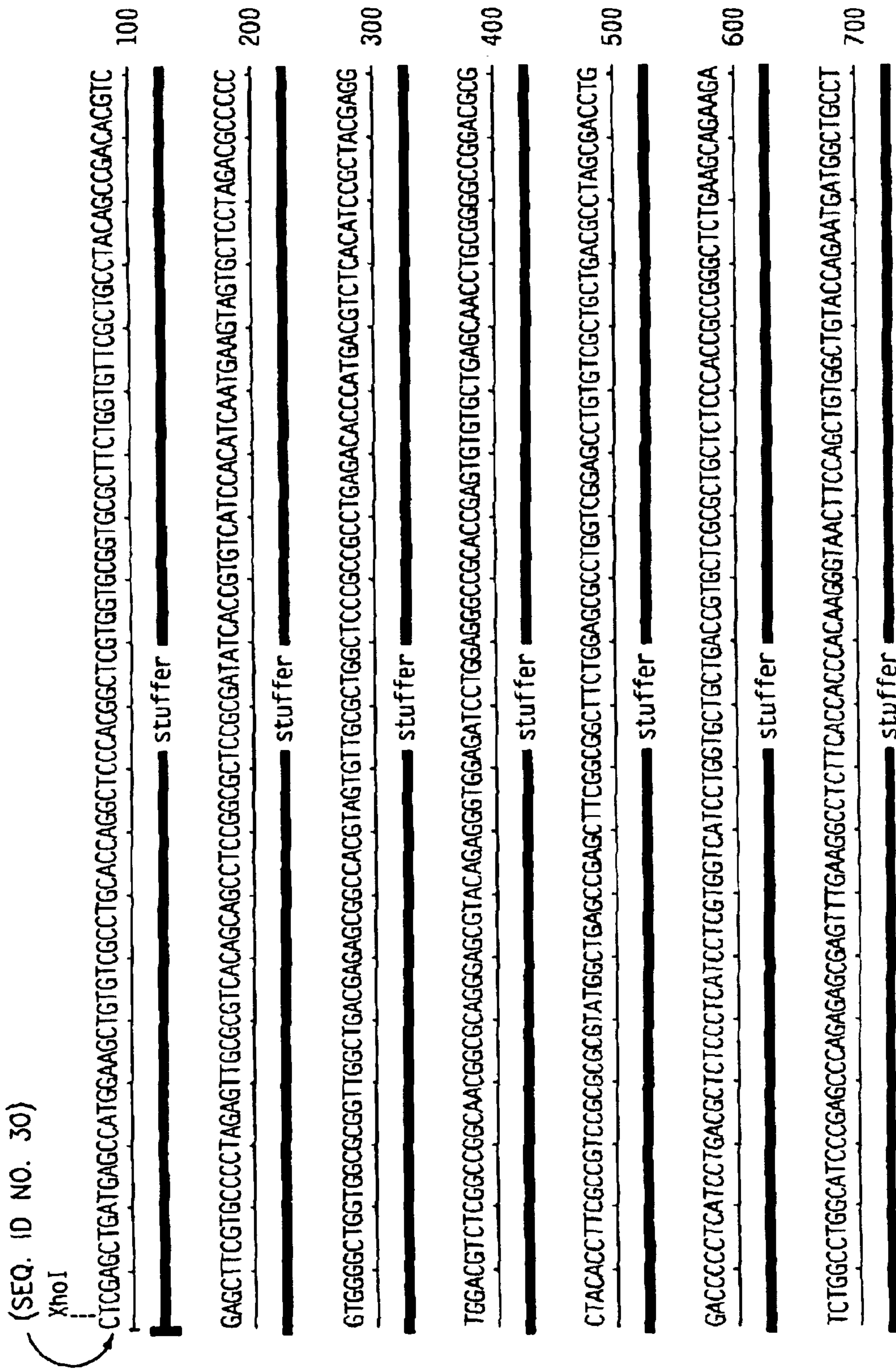


FIG. 12G

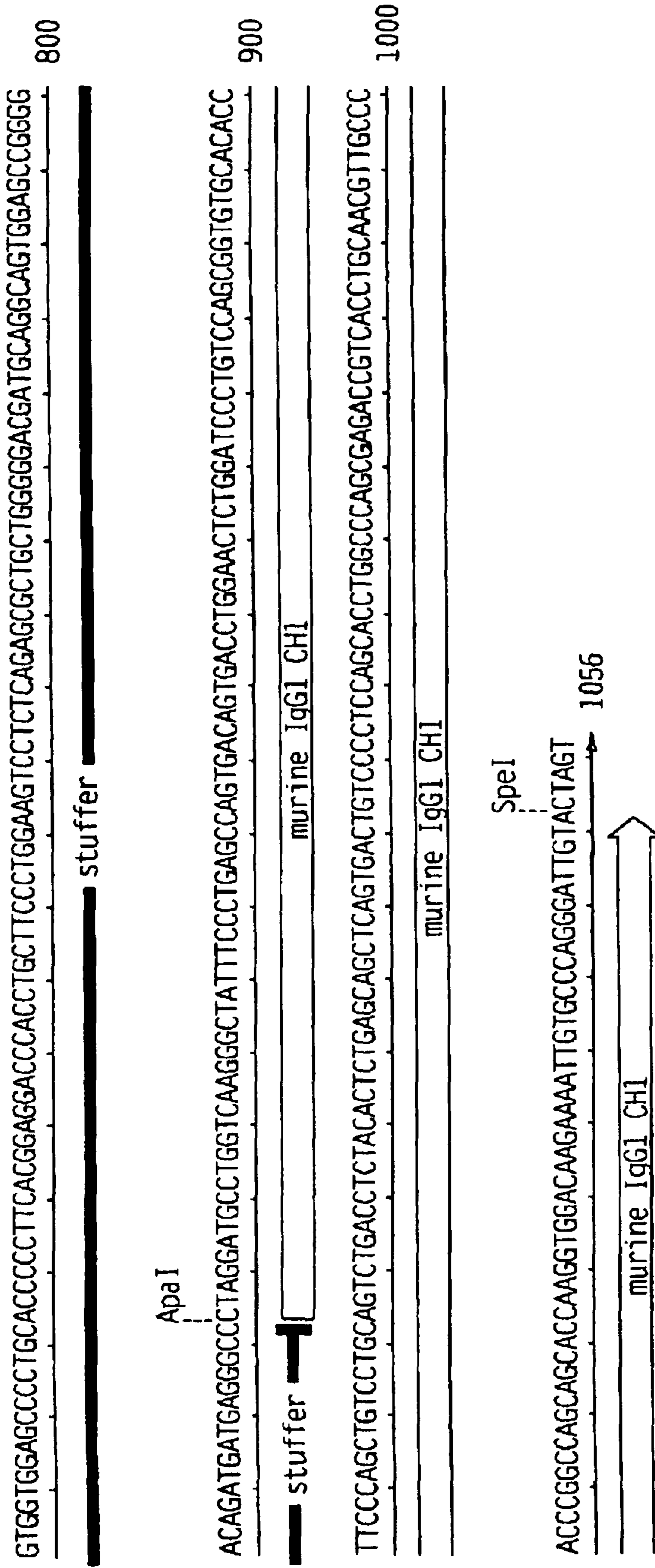


FIG. 12G (Cont.)

(SEQ. ID NO. 31)

GTGGCAGCTTTTCGGGAAATGTGGCGGAACCCCTATTGTTATTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAAT 100

GCTTCAATAATATTGAAAAGGAGAGATGAGIATTCACATTCGCGTGGCCCTTATCCCTTTTGGGGCATTTGGCTTCCCTGTTTTGGCTCAC 200

CCAGAAACGCTGGTGAAGTAAAGATGCTGAAGATCAGTTGGTGCACGAGTGGTTACATCGAATCGGATCTCAACAGCGGTAAGATCCTTGAGAGTT 300

TTGCCCGAAGACGTTTCCAATGATGAGCAGTTTAAAGTTCTGCTATGTGGCGGTATTATCCGTTATGACCGCGGGCAAGAGCAACTCGGTGG 400

CCGCATACACTATTCTCAGATGACTGGTTGAGTACTCACCAGTACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAAATTAATGCAGTGGCTGCC 500

ATAACCATGAGTATAACACTGGGCGCAACTTACTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTCACACAACATGGGGGATCATGTAA 600

CTGGCCTTGATCGTTGGGACCGGAGCTGAATGAAGCCATACCAACGACGAGGTGACACCAGTGGCTGTAGCAATGGCAACACAGTTGGGCAAACT 700

beta lactamase

beta lactamase

beta lactamase

beta lactamase

beta lactamase

beta lactamase

FIG. 13A

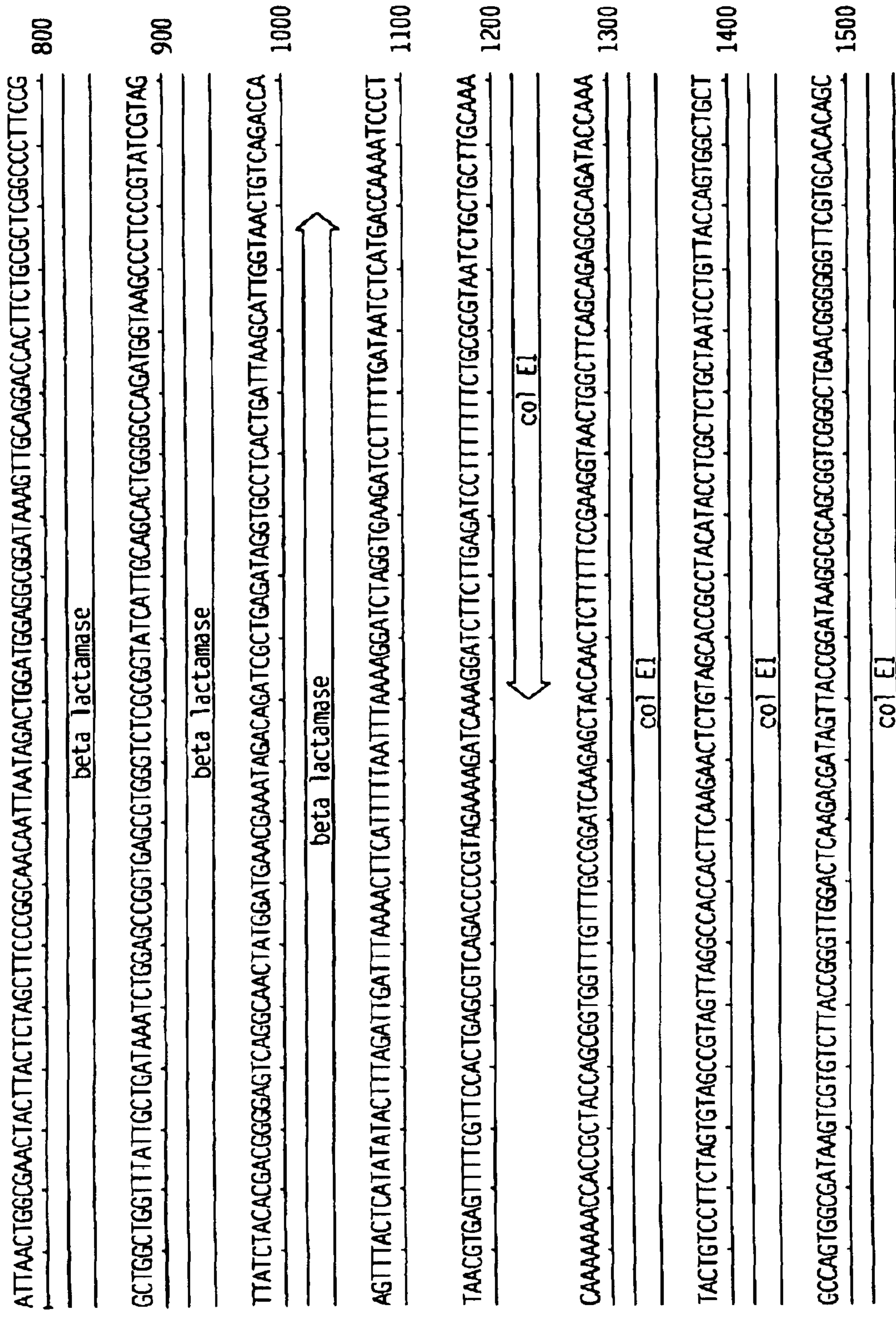


FIG. 13A (Cont.)

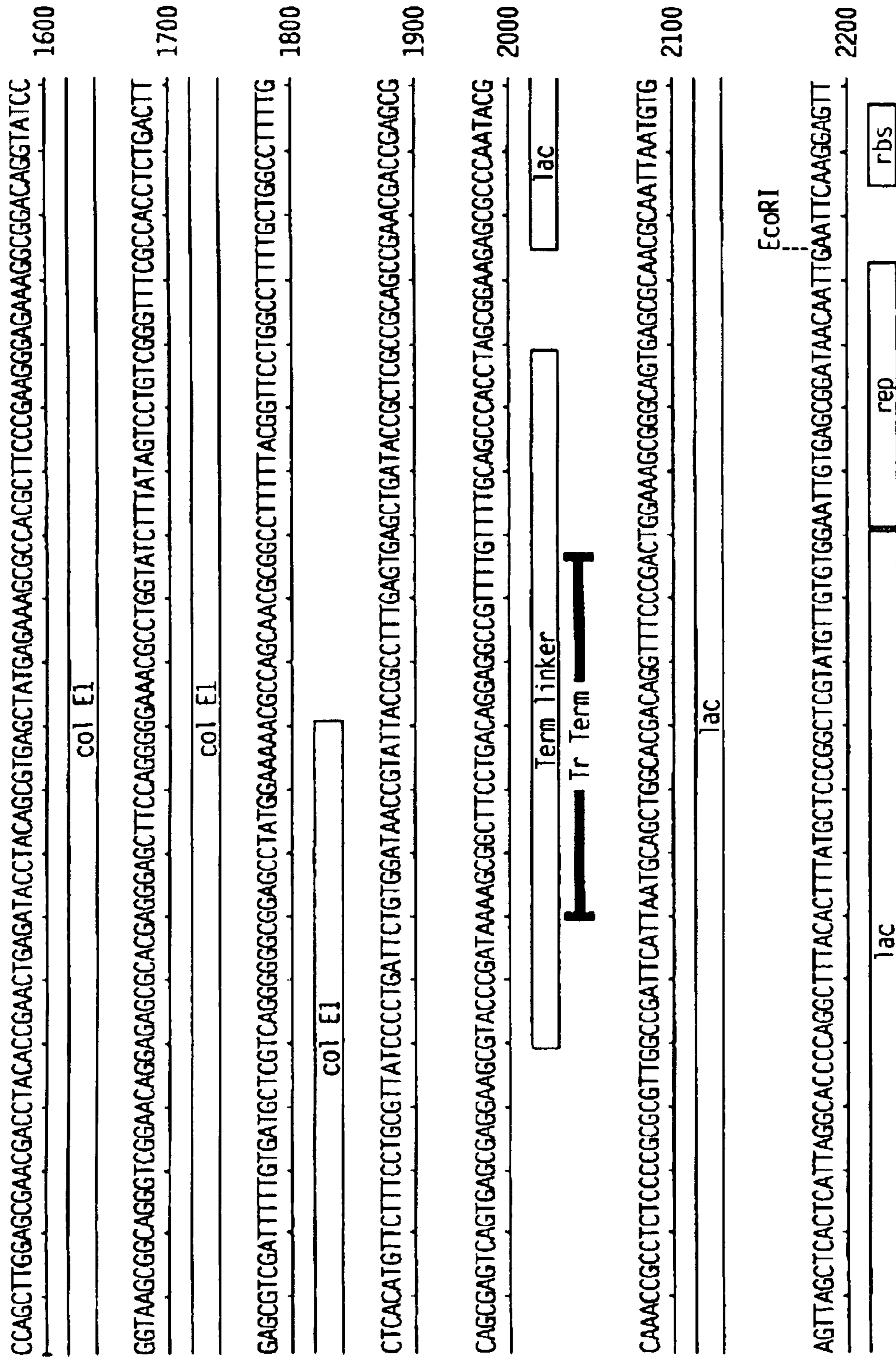


FIG. 13B

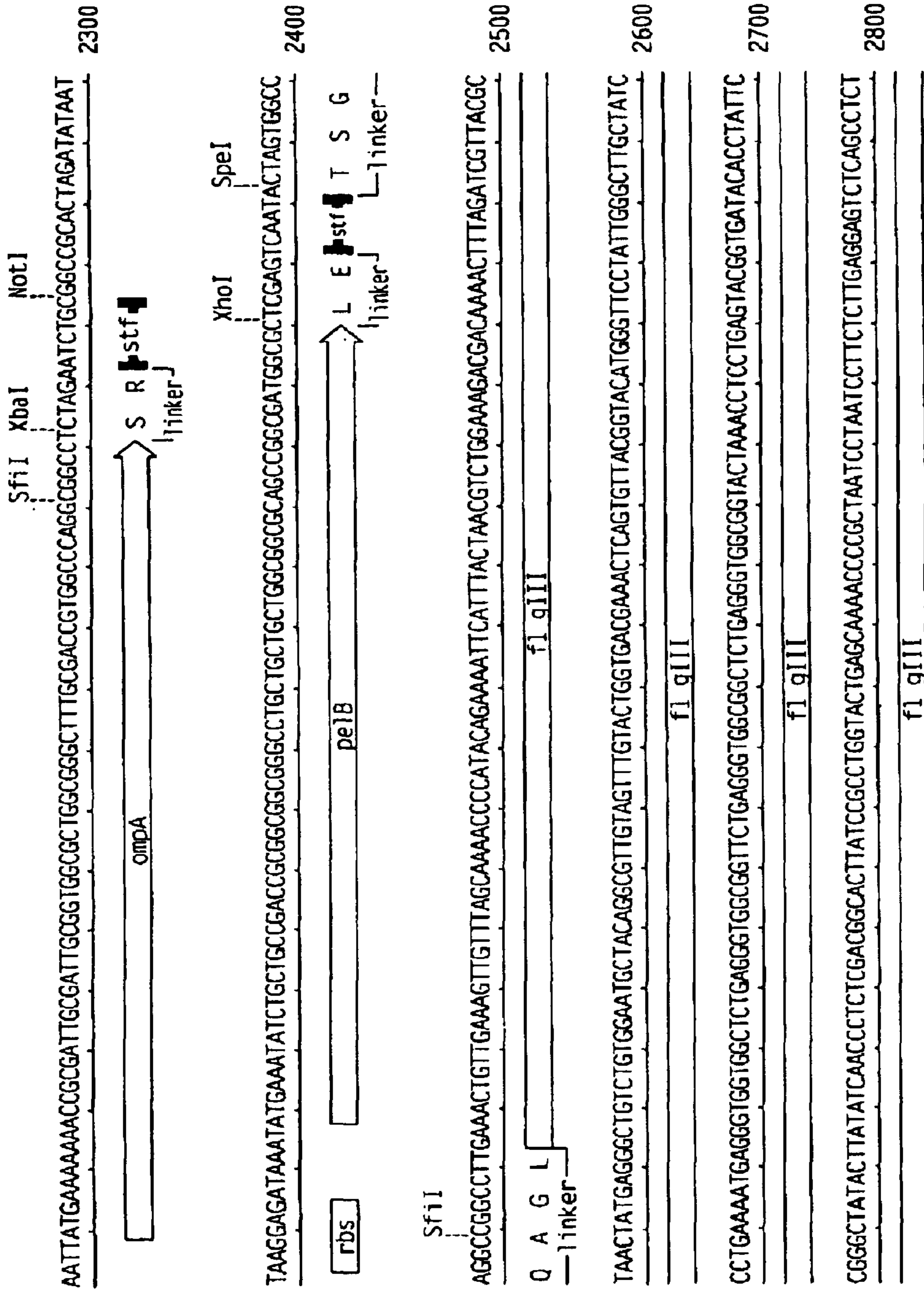


FIG. 13B (Cont.)

2900
TAATACTTTCATGTTTCAGATAATAGGTCGAAATAGGCABGGGGCATTAACTGTTTATACGGGCACGTGTTACTCAAGGCACCTGACCCCGTTAAAACT
f1 qIII

3000
TATTACCAGTACACTCCTGTATCATCAAAGCCATGATGACGCTTACTGGAACGGTAATAATCAGAGACTGGCGCTTCCATTTCGGCTTTAATGAGGATC
f1 qIII

3100
CATTCCGTTTGIGAATATCAAGGCCAATCGTCTGACCTGCCCTCAACCTCCTGTTAAATGCTGGCGGGCGCTCTGGTGGTGGTTCCTGGGCTCTGCGGG
f1 qIII

3200
TGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGGGCTCTGAGGGTGGCGGTTCCGGTGGTGGCTCTGGTCCGGTGATTTTGATTATGAAAAGATGGCA
f1 qIII

3300
AACGCTAATAGGGGGCTATGACCGAAATGCCGATGAAACGGCTACAGTCTGACGCTAAAGGCAACTTGATTCCTGTCGCTACTGATTACGGTGGCTG
f1 qIII

3400
CTATCGACGGTTTCATTGGTGACGTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGGCTGCTAATCCCAAAATGGCTCAAGTCGGTGA
f1 qIII

3500
CGGTGATAATTCACCTTTAATGAATAATTCGGTCAATAATTCACCTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCCTTGGCGCTGGTAAACCA
f1 qIII

3600
TAATGAATTTTCIATGATTGACAAAATAAACTTATTCGGTGGTCTTTGGGTTCTTTTATATGTTGCCACCCTTATGTAIGTATTTTCGACGTTTG
f1 qIII

FIG. 13C

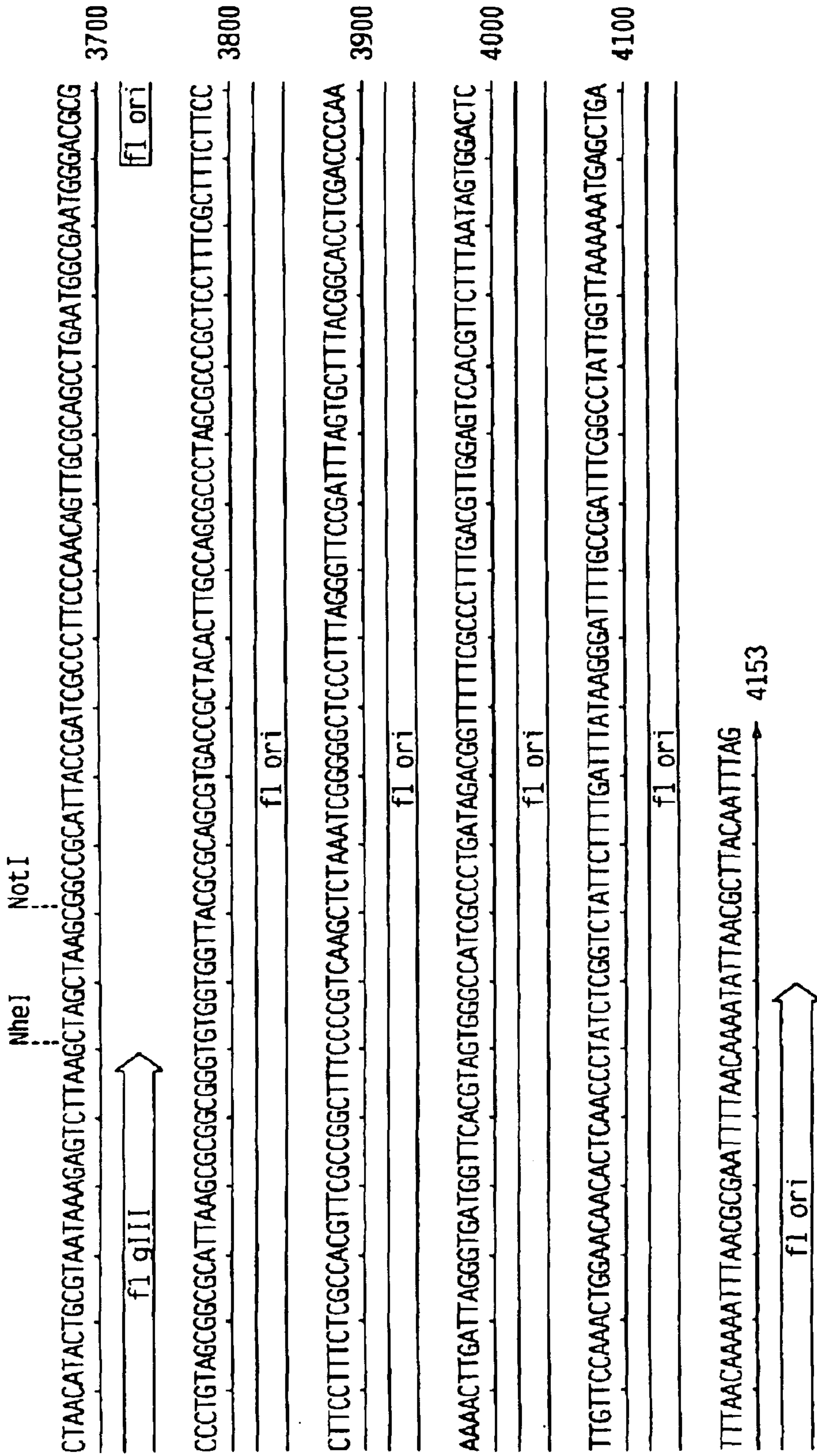


FIG. 13C (Cont.)

PHAGEMID VECTORS

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

This application claims priority under 35 USC 119(e) to provisional application 60/287,355, filed Apr. 27, 2001.

BACKGROUND

1. Technical Field

This disclosure relates to cloning vectors. More specifically, phagemid vectors useful in the cloning and expression of foreign genetic information are disclosed.

2. Background of Related Art

Plasmids are extrachromosomal genetic elements capable of autonomous replication within their hosts. Bacterial plasmids range in size from 1 Kb to 200 Kb or more and encode a variety of useful properties. Plasmid encoded traits include resistance to antibiotics, production of antibiotics, degradation of complex organic molecules, production of bacteriocins, such as colicins, production of enterotoxins, and production of DNA restriction and modification enzymes.

Although plasmids have been studied for a number of years in their own right, particularly in terms of their replication, transmissibility, structure and evolution, with the advent of genetic engineering technology the focus of plasmid research has turned to the use of plasmids as vectors for the cloning and expression of foreign genetic information. In its application as a vector, the plasmid should possess one or more of the following properties. The plasmid DNA should be relatively small but capable of having relatively large amounts of foreign DNA incorporated into it. The size of the DNA insert is of concern in vectors based on bacteriophages where packing the nucleic acid into the phage particles can determine an upper limit. The plasmid should be under relaxed replication control. That is, where the replication of the plasmid molecule is not strictly coupled to the replication of the host DNA (stringent control), thereby resulting in multiple copies of plasmid DNA per host cell. The plasmid should express one or more selectable markers, such as the drug resistance markers, mentioned above, to permit the identification of host cells which contain the plasmid and also to provide a positive selection pressure for the maintenance of the plasmid in the host cell. Finally the plasmid should contain a single restriction site for one or more endonucleases in a region of plasmid which is not essential for plasmid replication. A vector as described above is useful, for example, for cloning genetic information, by which is meant integrating a segment of foreign DNA into the vector and reproducing identical copies of that information by virtue of the replication of the plasmid DNA.

The next step in the evolution of vector technology was the construction of so-called expression vectors. These vectors are characterized by their ability not only to replicate the inserted foreign genetic information but also to promote the transcription of the genetic information into mRNA and its subsequent translation into protein. This expression requires a variety of regulatory genetic sequences including but not necessarily limited to promoters, operators, transcription terminators, ribosomal binding sites and protein synthesis initiation and termination codons. These expression elements can be provided with the foreign DNA segment as parts thereof or can be integrated within the vector in a

region adjacent to a restriction site so that when a foreign DNA segment is introduced into the vector it falls under the control of those elements to which it is now chemically joined.

Filamentous bacteriophage consist of a circular, single-stranded DNA molecule surrounded by a cylinder of coat proteins. There are about 2,700 molecules of the major coat proteins pVIII that envelope the phage. At one end of the phage particle, there are approximately five copies of each of gene III and VI proteins (pIII and pVI) that are involved in host cell binding and in the termination of the assembly process. The other end contains five copies of each of pVII and pIX that are required for the initiation of assembly and for maintenance of virion stability. In recent years, vectors have been developed and utilized for the display of foreign peptides and proteins on the surface of filamentous phage or phagemid particles.

The display of peptides and proteins on the surface of phage or phagemid particles represents a powerful methodology for selection of rare members in a complex library and for carrying out molecular evolution in the laboratory. The ability to construct libraries of enormous molecular diversity and to select for molecules with predetermined properties has made this technology applicable to a wide range of problems. A few of the many applications of such technology are: i) phage display of natural peptides including, mapping epitopes of monoclonal and polyclonal antibodies and generating immunogens; ii) phage display of random peptides, including mapping epitopes of monoclonal and polyclonal antibodies, identifying peptide ligands, and mapping substrate sites for proteases and kinases; and iii) phage display of protein and protein domains, including directed evolution of proteins, isolation of antibodies and cDNA expression screening.

Vectors have been developed which incorporate DNA from plasmids and bacteriophage. These phagemid vectors are derived by modifications of a plasmid genome containing an origin of replication from a bacteriophage, (e.g. f1, M13, fd) as well as the plasmid origin of replication. Phagemids are useful for the expression of foreign genetic information.

One known phagemid vector is [pBluescript] *PBLUE-SCRIPT*TM II KS+ (pBS II KS+) (Stratagene, La Jolla, Calif.), which is a useful starting point for the construction of the present vector because of its small size and the fact that it contains the colE1 plasmid origin of replication and the phage f1 origin of replication in the desired orientation. The plasmid also carries an ampicillin resistance gene.

Vectors which due to their structures provide enhanced functionality would be desirable.

SUMMARY

Novel plasmid vectors capable of replication and expression of foreign genetic information in bacteria, such as, for example, cyanobacterium and *E. coli* are described herein. These new vectors contain a specific sequence of features after the ColE1 origin but before the f1 origin. Specifically, the present phagemid vector contains, after the ColE1 origin but before the f1 origin, a bacterial transcription terminator, a promoter, a first ribosomal binding site, a first leader sequence and a first cloning region, a second ribosomal binding site, a second leader sequence and a second cloning region. The second cloning region is adapted to receive a gene encoding a polypeptide to be displayed and a nucleotide sequence encoding at least a functional domain of a display protein.

The vectors described herein are constructed through a series of steps which convert a starting vector through a series of intermediate plasmids to the present novel vector which can be used for display of antibody libraries.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically illustrates the structure of pBS II KS+, a useful starting vector for making the novel vectors described herein;

FIG. 2 is a flow chart illustrating the method of making the novel vectors described herein;

FIG. 3 schematically illustrates the digestion of the starting vector and insertion of the promoter;

FIGS. 4A–C show the sequence (Seq. ID No. 19) of intermediate vector p110-81.6;

FIG. 5 schematically illustrates the insertion of the terminator;

FIGS. 6A–C show the sequence (Seq. ID No. 20) of intermediate vector p131-03.7;

FIG. 7 schematically illustrates the insertion of multiple cloning sites;

FIGS. 8A–C show the sequence (Seq. ID No. 21) of intermediate vector p131-39.1;

FIG. 9 schematically illustrates the insertion of the nucleotide sequence encoding the display protein and the two transcriptional control cassettes;

FIG. 10 is a map of plasmid pAX131; and

FIGS. 11A–D show the nucleic acid sequence (Seq. ID No. 18) of plasmid pAX131, including the domains corresponding to particular genes.

FIGS. 12A–G show the nucleic acid sequences of illustrative stuffer sequences.

FIGS. 13A–C show the nucleic acid sequence of plasmid pAX131 Xba/Not.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present novel phagemid vectors are useful for display of polypeptides such as, for example, antibody libraries. The vectors described herein can be prepared using any commercially available vector containing a ColE1 and an fl origin of replication as the starting material. Such starting materials are known and are commercially available. One suitable starting material is the vector pBS II KS+ which is commercially available from Stratagene Corp., La Jolla, Calif. (See FIG. 1).

FIG. 2 is a flow-chart showing one embodiment of the steps involved in converting a starting vector into one of the present novel vectors. Those skilled in the art will readily envision other schemes for preparing the present vectors. Accordingly, the present disclosure is not limited to the sequence of steps shown in FIG. 2.

In the first step, the starting vector is digested with restriction enzymes to remove a substantial portion of the vector between the ColE1 origin and the fl origin of replication. Typically, the portion to be removed from the starting vector includes multiple cloning sites. Depending on the particular restriction sites present in the starting vector, suitable methods for digesting the starting vector are known to and readily selected by those skilled in the art.

Next, a promoter is inserted downstream of the ColE1 origin of the digested starting vector. Any promoter recognized by a host cell can be employed. Suitable promoters

include, but are not limited to, ara, lac and trc promoters. The promoter drives expression of other sequences inserted into the vector, such as, for example expression of polypeptides. In particularly useful embodiments, a promoter sequence generated from the starting vector is employed as the promoter inserted downstream of the ColE1 origin as described in more detail below.

In the next step, a bacterial transcription terminator is inserted downstream of the ColE1 origin, and upstream of the promoter. Any terminator recognized by a host cell can be employed. Suitable terminators include, but are not limited to, the t_{HP} terminator, the bgIG terminator, and the crp terminator. It should be noted that bioinformatics analysis has allowed the identification of over 100 rho-independent transcription terminators in the E. coli genome, all of which should be suitable for this purpose (Ermolaeva, et al, J. Mol Biol 301:27–33 (2000)).

In the next step, multiple restriction sites are inserted downstream of the promoter. The restriction site can be any known restriction site. Suitable restriction sites for insertion include, but are not limited to Nhe I, Hind III, Nco I, Xma I, Bgl II, Bst I, Pvu I, etc. The number of restriction sites inserted is not critical, provided a sufficient number of restriction sites are inserted to allow completion of the balance of the steps needed to create the present novel vectors. Thus as few as 2 to as many as 10 or more restriction sites can be inserted in this step. It should be understood that if one or more of the restriction sites selected for insertion is present in the starting vector, it may be desirable to remove or disable the native restriction site to avoid unwanted digestion during further processing. The restriction site can be inserted using any technique known to those skilled in the art. A particularly preferred combination of restriction sites inserted in this step is Not I, Sfi I, Spe I, Xho I, Xba I and EcoR I.

The next step involves inserting a nucleotide sequence encoding a product that enables display of a polypeptide on the surface of a phagemid particle. The product encoded can thus be considered at least a functional domain of a display protein. The display protein can be any natural or synthetic polypeptide to which a polypeptide to be displayed can be fused and which can present the polypeptide to be displayed for screening processes. Suitable display polypeptides include proteins that can be incorporated into the coat of a phage particle. As those skilled in the art will appreciate, filamentous bacteriophage consist of a circular, single-stranded DNA molecule surrounded by a cylinder of coat proteins. There are about 2,700 molecules of the major coat protein pVIII that encapsidate the phage. At one end of the phage particle, there are approximately five copies each of gene III and VI proteins (pIII and pVI) that are involved in host-cell binding and in the termination of the assembly process. The other end contains five copies each of pVII and pIX that are required for the initiation of assembly and for maintenance of virion stability. A nucleotide sequence encoding any of these coat proteins can be employed in making the novel vectors herein. Particularly preferred are nucleotide sequences encoding at least a functional domain of pIII. The nucleotide sequence encoding at least a functional domain of pIII can be natural or synthetic. The nucleotide sequence inserted can encode a truncated pIII provided the display function of the protein is maintained. An example of a synthetic or artificial coat protein useful herein is that disclosed in Weiss et al., Mol Biol., 300(1), 213–219 (2000), the disclosure of which is incorporated herein by reference.

In the next step, two transcriptional control cassettes are inserted, an upstream transcriptional control cassette and a

downstream transcriptional control cassette. Each of the transcriptional control cassettes include a ribosomal binding site, a leader sequence and a cloning site for receiving a gene encoding a polypeptide to be expressed. Any known ribosomal binding site (RBS) and leader sequence recognized by the host cell can be employed. Preferably, the RBS and leader sequence employed is optimized for expression in *E. coli*. The cloning site is a region of the nucleic acid between two restriction sites, typically with a nonessential region of nucleotide sequence (commonly referred to as a "stuffer" sequence) positioned therebetween. Alternatively, the stuffer sequence may contain a non-essential region and a portion of an antibody constant domain. Suitable stuffer sequences include, for example, those shown in FIGS. 12A–G.

The downstream transcriptional control cassette is inserted adjacent to the nucleotide sequence encoding at least the functional domain of the display protein. In this manner, a fusion protein will be expressed when a gene encoding a polypeptide to be displayed is inserted at the cloning site of the downstream transcriptional control cassette. As those skilled in the art will appreciate, a suppressible stop codon could be positioned between the gene encoding the polypeptide to be displayed and the nucleotide sequence encoding at least a functional domain of the display protein such that fusion display is obtained in a suppressing host (as long as the gene is inserted in-frame) and a secreted protein without the display protein is obtained in a non-suppressing host.

The upstream transcriptional control cassette is inserted upstream of the downstream transcriptional control cassette. The upstream transcriptional control cassette provides a second cloning region for receiving a second gene encoding a polypeptide that can dimerize with the polypeptide to be displayed. For example, where the vector expresses a heavy chain Fd fused to a display protein, the second gene preferably encodes an antibody light chain. As with the cloning site of the downstream transcriptional control cassette, the cloning site of the upstream transcriptional control cassette is a region of the vector between two restriction sites, typically with a stuffer positioned therebetween. It should of course be understood that where a polypeptide other than an antibody is to be displayed (such as, for example, where monomeric display of a single polypeptide or protein is intended) a second gene need not be cloned into the vector at the cloning site of the upstream transcriptional control cassette. In such cases the second cloning site can simply remain unused. As those skilled in the art will also appreciate, where a single chain antibody is encoded by the gene inserted at the cloning site of the downstream transcriptional control cassette, there is no need to insert a second gene into the vector at the cloning site of the upstream transcriptional control cassette.

Thus, the phagemid vector produced by the process illustrated in FIG. 2 will contain, after the ColE1 origin but before the fl origin, a terminator, a promoter, a first ribosomal binding site, a first leader sequence and a first cloning region, a second ribosomal binding site, a second leader sequence and, a second cloning region for receiving a gene encoding a polypeptide to be displayed and a nucleotide sequence encoding at least a functional domain of a display protein.

The present vectors also include a selectable marker. Either an ampicillin resistant or a CAT resistant vector can be produced in accordance with the present disclosure. The ampicillin or CAT resistance can be provided by simply choosing a starting vector having the desired resistance. Alternatively, if the starting vector is ampicillin resistant to produce a CAT resistant vector, the ampicillin resistant gene is removed and replaced with the chloramphenicol trans-

ferase gene. Techniques for providing either ampicillin or CAT resistance in the present vectors will be readily apparent to those skilled in the art. Other suitable selectable markers include, but are not limited to, tetracycline or kanamycin resistance.

The vectors described herein can be transformed into a host cell using known techniques (e.g., electroporation) and amplified. The vectors described herein can also be digested and have a first gene and optionally a second gene ligated therein in accordance with this disclosure. The vector so engineered can be transformed into a host cell using known techniques and amplified or to effect expression of polypeptides and/or proteins encoded thereby to produce phage particles displaying single polypeptides or dimeric species. Those skilled in the art will readily envision other uses for the novel vectors described herein.

The following examples illustrate the present invention without limiting its scope. The steps involved in constructing the vectors described herein are discussed in detail in the Examples. Those skilled in the art possess knowledge of suitable techniques to accomplish the steps described below without the need for undue experimentation, such techniques being well known to those skilled in the art.

EXAMPLE 1

This example illustrates methods and compositions for the construction of one embodiment of a phagemid vector according to the present disclosure. The starting phagemid selected for construction was pBS II KS+ which contains an ampicillin resistant gene which results in a final vector, pAX131, which is ampicillin resistant.

Digestion of Starting Vector and Insertion of Promoter

The commercially available vector pBS II KS+ (Stratagene, LaJolla, Calif.) was digested with Pvu I and Sap I to generate a 2424 bp pBS II KS+ fragment which lacks the bases at positions 500 to 1037 corresponding to the multiple cloning region. The resulting fragment contains the Ampicillin resistant gene (AmpR), phage fl origin, and the Col E1 origin. (See FIG. 3.) Next, two mutagenic primers were used with the pBS II KS+ fragment in a PCR reaction followed by digestion with EcoR I and Sap I to generate a 209 bp fragment containing the lac promoter. The primers used were as follows:

5' AAC CGT ATT ACC GCC TTT GAG TG 3' (SEQ. ID. NO. 1);

and

5' CCT GAA TTC AAT TGT TAT CCG CTC ACA ATT CCA C 3'
(SEQ. ID. NO. 2).

The 2424 bp fragment and the 209 bp fragment were combined in a three-way ligation reaction with two overlapping oligonucleotides which contain a Not I, EcoR I and Pvu I sites to form a first intermediate plasmid (designated p110-81.6). (See FIG. 3.) The oligonucleotides used for this reaction were:

5' CGG TAA TGC GGC CGC TAC ATG 3' (SEQ. ID. NO. 3);

and

5' AAT TCA TGT AGC GGC CGC ATT ACC GAT 3' (SEQ. ID. NO. 4).

The resulting plasmid p110-81.6 was digested and sequenced in the altered region to identify a clone with the correct incorporation of the lac promoter, Pvu I, Sap I, EcoR,

and Not I sites. The sequencing of p110-81.6 revealed a nucleic acid change at position 875 within the lac promoter. The published sequence of pBS II KS+ had an adenine at position 875. However, sequencing of p110-81.6 and the original pBS II KS+ revealed a guanine at position 875. The sequence (Seq. ID No. 19) of intermediate plasmid p110-81.6 is shown in FIGS. 4A-C.

Insertion of Terminator

A transcription termination sequence was inserted into the first intermediate plasmid (p110-81.6) upstream of the lac promoter at the Sap I site. (See FIG. 5.)

Plasmid 110-81.6 was digested with Sap I to create an insertion point for the oligonucleotides which contained a t_{HP} terminator (Nohno et al., Molecular and General Genetics, Vol. 205, pages 260-269 (1986). The oligonucleotides used in this ligation were:

5' AGC GTA CCC GAT AAA AGC GGC TTC CTG ACA GGA
GGC CGT TTT GTT TTG CAG CCC ACC T 3'; (SEQ. ID. No.
5);

and

5' GCT AGG TGG GCT GCA AAA CAA AAC GGC CTC CTG
TCA GGA AGC CGC TTT TAT CGG GTA C 3' (SEQ. ID. NO.
6).

The resulting intermediate vector (designated p131-03.7) was digested and sequenced in the altered region to determine its identity. The sequence (Seq. ID No. 20) of intermediate vector p131-03.7 is shown in FIGS. 6A-C.

Insertion of Multiple Restriction Sites

Oligonucleotides containing the Xba I, XhoI, SpeI and Sfi sites were then inserted into intermediate plasmid p131-03.7. (See FIG. 7.)

Intermediate vector p131-03.7 was digested with EcoR I and Not I and then gel purified. Then overlapping oligonucleotides containing the Xba I, Xho I, Spe I and Sfi I sites were ligated into the p131-03.7 backbone. The oligonucleotides inserted were:

5' AAT TCA CAT CTA GAT ATC TCG AGT CAA TAC TAG TGG
CCA GGC CGG CCA GC 3' (SEQ. ID. NO. 7);

and

5' GGC CGC TGG CCG GCC TGG CCA CTA GTA TTG ACT
CGA GAT ATC TAG ATG TG 3' (SEQ. ID. NO. 8).

The resulting intermediate plasmid (designated p131-39.1) was sequenced and analyzed to determine its identity. The sequence (Seq. ID No. 21) of intermediate plasmid p131-39.1 is shown in FIGS. 8A-C.

Construction of Nucleotide Sequence Encoding Display Protein

Single stranded DNA from phage f1 (ATCC #15766-B2) was used as a template for the cloning of gene III. (See FIG. 9.)

The primers used were:

5' AGT GGC CAG GCC GGC CTT GAA ACT GTT GAA AGT
TGT TTA GCA AA 3' (SEQ. ID. NO. 9)

which contains the Sfi I site, bases to maintain the coding frame and a portion of gene III; and

5 TCT GCG GCC GCT TAG CTA GCT TAA GAC TCT TTA TTA
CGC AGT ATG TTA GCA 3' (SEQ. ID. NO. 10);

which contains the end of gene III in which an internal ribosome binding site ordinarily used for the next downstream

gene has been removed by changing a silent third base position in the corresponding codon. This oligonucleotide also contains a stop codon, Nhe I site for potential use in removal of the fusion, a second stop codon for use with the fusion, and the Not I site for cloning. The PCR fragment was digested with Sfi I and Not I and inserted into p131-39.1 digested with Sfi I and Not I to create intermediate vector p131-44.2. The integrity of the gene III region and flanking sequences was confirmed by sequence analysis.

Creation of the Upstream Transcriptional Control Cassette

Plasmid 131-39.1 was utilized as a shuttle vector for cloning the oligonucleotides containing the ompA signal peptide coding sequence. The upstream transcriptional control cassette was generated within intermediate plasmid 131-39.1 by inserting a pair of oligonucleotides which contain EcoR I, the ompA signal peptide leader, followed by a Sac I site, a small stuffer region, and a ribosome binding site. (See FIG. 9.) The oligonucleotides used were:

Eco Xba:

5' AAT TCA AGG AGT TAA TTA TGA AAA AAA CCG CGA
TTG CGA TTG CGG TGG CGC TGG CGG GCT TTG CGA
CCG TGG CCC AGG CGG CCG AGC TCA TCT T 3' (SEQ.
ID. NO. 11);

and

Xba Eco:

5' CTA GAA GAT GAG CTC GGC CGC CTG GGC CAC GGT
CGC AAA GCC CGC CAG CGC CAC CGC AAT CGC AAT
CGC GGT TTT TTT CAT AAT TAA CTC CTT G 3' (SEQ. ID.
NO. 12).

The RBS and leader sequences included in the upstream transcriptional control cassette are optimized for use in E. coli. These novel sequences are:

5' AAG GAG 3' (Seq. ID No.13)

for the RBS; and

5' ATG AAA AAA ACC GCG ATT GCG ATT GCG GTG GCG
CTG GCG GCC TTT GCG ACC GTG GCC CAG GCG GCC 3'
(Seq. ID No. 14)

for the ompA leader. The resulting plasmid was sequenced to confirm the identity of the insert and digested at the EcoRI and XbaI sites to generate a 94 bp fragment which is the upstream transcriptional control cassette.

Creation of the Downstream Transcriptional Control Cassette

Intermediate plasmid 131-39.1 was utilized as a shuttle vector for cloning the oligonucleotides containing the pelB signal peptide coding sequence. The downstream transcriptional control cassette was generated within intermediate plasmid 131-39.1 by inserting a pair of oligonucleotides containing the pelB signal peptide, Xba I, site, and a ribosome binding site. The oligonucleotides used were:

XbaXho:

5' CTA GAT ATA ATT AAG GAG ATA AAT ATG AAA TAT CTG
CTG CCG ACC GCG GCG GCG GGC CTG CTG CTG CTG
GCG GCG CAG CCG GCG ATG GCG 3' (SEQ. ID. NO. 15);

and

XhoXba:

5' TCG AGC GCC ATC GCC GGC TGC GCC GCC AGC AGC
AGC AGG CCC GCC GCC GCG GTC GGC AGC AGA TAT

TTC ATA TTT ATC TCC TTA ATT ATA T 3' (SEQ. ID. NO. 16).

The novel pelB leader sequence was optimized for use in *E. coli* and had the sequence

5' TAT GAA ATA TCT GCT GCC GAC CGC GGC GGC GGG
CCT GCT GCT GCT GGC GGC GCA GCC GGC GAT GGC G
3' (Seq. ID No. 17).

The resulting plasmid was sequenced to confirm the identity of the insert and digested at the XbaI and XhoI sites to generate a 91 bp fragment which is the downstream transcriptional control cassette.

Construction of pAx131 Vector

The upstream transcriptional control cassette and the downstream transcriptional control cassette were combined with intermediate plasmid p131-44.2 digested with EcoRI and XhoI in a 3-way ligation reaction to produce pAX131 (See FIG. 9). FIG. 10 is a map of the resulting pAX131 vector. The pAX131 was analyzed to determine its nucleic acid sequence (SEQ. ID. NO. 18) which is shown in FIGS. 11A-D.

EXAMPLE 2

Insertion of an alternate upstream transcriptional control cassette

PAX131 vector was digested with Not I restriction enzyme. The resulting DNA overhangs were then filled in with Klenow fragment Polymerase to blunt end the DNA followed by ligation. This was performed to remove the existing Not I site. The Not I deleted PAX131 vector was digested with EcoR I/Xba I, and ligated with a duplexed oligo containing EcoR I and Spe I overhangs (Xba I, and Spe I have compatible ends).

Eco/Spe oligo:

5' AAT TCA AGG AGT TAA TTA TGA AAA AAA CCG CGA
TTG CGA TTG CGG TGG CGC TGG CGG GCT TTG CGA
CCG TGG CCC AGG CGG CCT CTA GAA TCT GCG GCC
GCA 3' (SEQ. ID NO. 22)

Spe/Eco oligo:

5' CTA GTG CGG CCG CAG ATT CTA GAG GCC GCC TGG
GCC ACG GTC GCA AAG CCC GCC AGC GCC ACC GCA
ATC GCA ATC GCG GTT TTT TTC ATA ATT AAC TCC TTG
3' (SEQ. ID NO. 23)

The resulting vector (pAX131 Xba/Not) had Xba I, and Not I sites for cloning of a gene, such as light chains, rather than Sac I and Xba I. FIGS. 13A-C show the nucleic acid sequence for vector (pAX131 Xba/Not).

It is contemplated that the present novel vectors can be used in connection with the production and screening of

libraries made in accordance with conventional phage display technologies. Both natural and synthetic antibody repertoires have been generated as phage displayed libraries. Natural antibodies can be cloned from B-cell mRNA isolated from peripheral blood lymphocytes, bone marrow, spleen, or other lymphatic tissue of a human or non-human donor. Donors with an immune response to the antigen(s) of interest can be used to create immune antibody libraries. Alternatively, non-immune libraries may be generated from donors by isolating naive antibody B cell genes. PCR using antibody specific primers on the 18st strand cDNA allows the isolation of light chain and heavy chain antibody fragments which can then be cloned into the display vector.

Synthetic antibodies or antibody libraries can be made up in part or entirely with regions of synthetically derived sequence. Library diversity can be engineered within variable regions, particularly within CDRs, through the use of degenerate oligonucleotides. For example, a single Fab gene may be modified at the heavy chain CDR3 position to contain random nucleotide sequences. The random sequence can be introduced into the heavy chain gene using an oligonucleotide which contains the degenerate coding region in an overlap PCR approach. Alternatively, degenerate oligo cassettes can be cloned into restriction sites that flank the CDR(s) to create diversity. The resulting library generated by this or other approaches can then be cloned into a display vector in accordance with this disclosure.

Upon introduction of the display library into bacteria, phage particles will be generated that have antibody displayed on the surface. The resulting collection of phage-displayed antibodies can be selected for those with the ability to bind to the antigen of interest using techniques known to those skilled in the art. Antibodies identified by this system can be used therapeutically, as diagnostic reagents, or as research tools.

It is contemplated that single and double stranded versions of the vectors described herein are within the scope of the present invention. It is well within the purview of those skilled in the art to prepare either single or double stranded vectors having the features described herein.

It will be understood that various modifications may be made to the embodiments described herein. For example, as those skilled in the art will appreciate, a first gene encoding a fusion protein having an antibody light chain to be fused to and displayed by pVIII and a second gene encoding a heavy chain Fd can be inserted into the vector at the newly created restriction site to provide effective antibody display. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

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<213> ORGANISM: artificial sequence

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<223> OTHER INFORMATION: pelB leader

<400> SEQUENCE: 17

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gatggcg 67

<210> SEQ ID NO 18

<211> LENGTH: 4154

<212> TYPE: DNA

<213> ORGANISM: artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: plasmid vector

<400> SEQUENCE: 18

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ggaagagtat gagtattcaa cttttccgtg tcgcccttat tccctttttt gcggcatttt 180

gccttctgt ttttgctcac ccagaaacgc tggtgaaagt aaaagatgct gaagatcagt 240

tgggtgcacg agtgggttac atcgaactgg atctcaacag cggtaagatc cttgagagtt 300

ttcggcccga agaacgtttt ccaatgatga gcacttttaa agttctgcta tgtggcgcg 360

tattatcccg tattgacgcc gggcaagagc aactcggctg ccgcatacac tattctcaga 420

atgacttggg tgagtactca ccagtcacag aaaagcatct tacggatggc atgacagtaa 480

gagaattatg cagtgtgcc ataaccatga gtgataaac tgccggccaac ttacttctga 540

caacgatcgg aggaccgaag gagctaaccg cttttttgca caacatgggg gatcatgtaa 600

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ctcgccttga	tcgttgggaa	cgggagctga	atgaagccat	accaaacgac	gagcgtgaca	660
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ctctagcttc	ccggcaacaa	ttaatagact	ggatggaggc	ggataaagtt	gcaggaccac	780
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cccgttaaaa	cttattacca	gtacactcct	gtatcatcaa	aagccatgta	tgacgcttac	2940
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cactcaacc tatctcggtc tattcttttg attataagg gattttgccg atttcggcct 4080
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cgcttacaat ttag 4154

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<210> SEQ ID NO 19

<211> LENGTH: 2654

<212> TYPE: DNA

<213> ORGANISM: artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: plasmid vector

<400> SEQUENCE: 19

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gataggggtg agtgttgttc cagtttgtaa caagagtcca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
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cacaccgcc gcgcttaatg ccgcgctaca gggcgctcc cattcgccat tcaggctgcg 480
caactgttgg gaagggcgat cggtaatgcg gccctacat gaattcaatt gttatccgct 540
cacaattcca cacaacatac gagccggaag cataaagtgt aaagcctggg gtgcctaatg 600
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gtcgtgccag ctgcattaa gaatcgcca acgcgcgggg agaggcggtt tgcgtattgg 720
gcgctcttcc gcttctcgc tcaactgactc gctgcgctcg gtcgctcggc tgcggcgagc 780
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cactcgtgca cccaactgat cttcagcatc tttactttc accagcgttt ctgggtgagc 2460
aaaaacagga aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga aatggtgaat 2520
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cggatacata tttgaatgta tttagaaaaa taaacaaata ggggttccgc gcacatttcc 2640
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<210> SEQ ID NO 20

<211> LENGTH: 2712

<212> TYPE: DNA

<213> ORGANISM: artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: plasmid vector

<400> SEQUENCE: 20

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gatagggttg agtggtgttc cagtttgtaa caagagtcca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240

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ctcgtgcacc	caactgatct	tcagcatctt	ttactttcac	cagcgtttct	gggtgagcaa	2520
aaacaggaag	gcaaaatgcc	gcaaaaaagg	gaataagggc	gacacggaaa	tgttgaatac	2580

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<210> SEQ ID NO 21

<211> LENGTH: 2750

<212> TYPE: DNA

<213> ORGANISM: artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: plasmid vector

<400> SEQUENCE: 21

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ggaagagtat gagtattcaa catttccgtg tgcgccctat tccctttttt gcggcatttt	180
gccttctctgt ttttgctcac ccagaaacgc tggtgaaagt aaaagatgct gaagatcagt	240
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caacgatcgg aggaccgaag gagctaaccg cttttttgca caacatgggg gatcatgtaa	600
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ctcacatggt ctttctgctg ttatccctg attctgtgga taaccgtatt accgcctttg	1860
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<210> SEQ ID NO 22
<211> LENGTH: 102
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide

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<400> SEQUENCE: 22

```

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aattcaagga gttaattatg aaaaaaacg cgattgcgat tgcgggtggcg ctggcgggct 60
ttgcgaccgt ggcccaggcg gcctctagaa tctgcggccg ca 102

```

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<210> SEQ ID NO 23
<211> LENGTH: 102
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: oligonucleotide

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<400> SEQUENCE: 23

```

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ctagtgcggc cgcagattct agaggccgcc tgggccacgg tcgcaaagcc cgccagcgc 60
accgcaatcg caatcgcggt ttttttcata attaactcct tg 102

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<210> SEQ ID NO 24
<211> LENGTH: 565
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: stuffer sequence

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<400> SEQUENCE: 24

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tctagataac tgtggtgca ccactgtct tcacttccc gccatctgat gagcagttga 60
aatctggaac tgctctgtt gtgtgcctgc tgaataactt ctatcccaga gaggccaaag 120
tacagtggaa ggtggataac gccctccaat cgggtaactc ccaggagagt gtcacagagc 180
aggacagcaa ggacagcacc tacagcctca gcagaccct gacgctgagc aaagcagact 240
acgagaaaca caaagtctac gcctgcgaag tcacccatca gggcctgagc tccggaggtg 300
cctcagtcgt gtgcttctt aacaacttct accccaaaga catcaatgct aagtggaaga 360

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ttgatggcag tgaacgacaa aatggcgtcc tgaacagttg gactgatcag gacagcaaag	420
acagcaccta cagcatgagc agcaccctca cgttgaccaa ggacgagtat gaacgacata	480
acagctatac ctgtgaggcc actcacaaga catcaacttc acccattgtc aagagcttca	540
acaggaatga gtgttaagcg gccgc	565

<210> SEQ ID NO 25
 <211> LENGTH: 1131
 <212> TYPE: DNA
 <213> ORGANISM: artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: stuffer sequence

<400> SEQUENCE: 25

ctcgagctga tgagccatgg aagctgtgtc gctgcacca ggctcccacg gctcgtggtg	60
cggtgcgctt ctggtgttcg ctgcctacag ccgacacgtc gagcttcgtg ccctagagt	120
tgcgcgtcac agcagcctcc ggcgctccgc gatatcaccg tgtcatccac atcaatgaag	180
tagtgctcct agacgcccc gtggggctgg tggcgcggtt ggctgacgag agcggccacg	240
tagtgttgcy ctggctcccg ccgcctgaga cacccatgac gtctcacatc cgctacgagg	300
tggacgtctc ggccggcaac ggcgagggga gcgtacagag ggtggagatc ctggagggcc	360
gcaccgagtg tgtgctgagc aacctgcggg gccggacgcy ctacaccttc gccgtccgcy	420
cgcgtatggc tgagccgagc ttggcggtt tctggagcgc ctggteggag cctgtgtcgc	480
tgtgacgcc tagcgacctg gacccctca tctgacgct ctccctcatc ctcgtggtca	540
tctgggtgct gctgaccgtg ctgcgctgc tctcccaccg ccgggctctg aagcagaaga	600
tctggcctgg catcccagc ccagagagcy agtttgaagg cctcttcacc acccacaagg	660
gtaacttcca gctgtggctg taccagaatg atggctgect gtggaggagc ccctgcaccc	720
ccttcacgga ggaccacct gcttccctgg aagtcctctc agagcgtgc tgggggacga	780
tgaggcagt ggagccggg acagatgatg agggccctt tccccctcgt ctctgtgag	840
aattccccgt cggatacagc cagcgtggcc gttggctgcc tcgcacagga ctctctccc	900
gactccatca ctttctctg gaaatacaag aacaactctg acatcagcag caccggggc	960
ttcccatcag tctgagagg gggcaagtac gcagccacct cacagggtgct gctgccttcc	1020
aaggacgtca tgcagggcac agacgaacac gtggtgtgca aagtccagca cccaacggc	1080
aacaaagaaa agaactgtcc tcttcagtg attgctgagc tgctactag t	1131

<210> SEQ ID NO 26
 <211> LENGTH: 1121
 <212> TYPE: DNA
 <213> ORGANISM: artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: stuffer sequence

<400> SEQUENCE: 26

ctcgagctga tgagccatgg aagctgtgtc gctgcacca ggctcccacg gctcgtggtg	60
cggtgcgctt ctggtgttcg ctgcctacag ccgacacgtc gagcttcgtg ccctagagt	120
tgcgcgtcac agcagcctcc ggcgctccgc gatatcaccg tgtcatccac atcaatgaag	180
tagtgctcct agacgcccc gtggggctgg tggcgcggtt ggctgacgag agcggccacg	240
tagtgttgcy ctggctcccg ccgcctgaga cacccatgac gtctcacatc cgctacgagg	300
tggacgtctc ggccggcaac ggcgagggga gcgtacagag ggtggagatc ctggagggcc	360
gcaccgagtg tgtgctgagc aacctgcggg gccggacgcy ctacaccttc gccgtccgcy	420

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cgcgtatggc tgagccgagc ttggcgggct tetggagcgc ctggteggag cctgtgtcgc 480
tgctgacgcc tagcgacctg gacccccctca tcctgacgct ctccctcatc ctctgtgtca 540
tcctgggtgct gctgaccgtg ctgcgctgc tctcccaccg ccgggctctg aagcagaaga 600
tctggcctgg catcccgagc ccagagagcg agtttgaagg cctcttcacc acccacaagg 660
gtaacttcca gctgtggctg taccagaatg atggctgcct gtgggtggagc ccctgcaccc 720
ccttcacgga ggaccacct gcttccctgg aagtcccttc agagcgctgc tgggggacga 780
tgcaggcagt ggagccgggg acagatgatg agggcccatc ggtcttcccc ctggcacct 840
cctccaagag cacctctggc ggcacagcgg ccctgggctg cctgggtcaag gactacttcc 900
ccgaaccggg gacggtgtcg tggaactcag gcgctctgac cagcggcgtg cacaccttcc 960
cggtgtctct acagtctca ggactctact ccctcagcag cgtgggtgacc gtgccatcca 1020
gcagcttggg caccagacc tacatctgca acgtgaatca caagcccagc aacaccaagg 1080
tggacaagaa agttgagccc aaatcttgtg aaaaaactag t 1121

```

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<210> SEQ ID NO 27
<211> LENGTH: 337
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: stuffer sequence

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```

<400> SEQUENCE: 27

```

```

tctagataac tgtggctgca ccactctgtct tcacttccc gccatctgat gacgagttga 60
aatctggaac tgcctctggt gtgtgcctgc tgaataactt ctatcccaga gaggccaaag 120
tacagtggaa ggtggataac gccctccaat cgggtaactc ccaggagagt gtcacagagc 180
aggacagcaa ggacagcacc tacagcctca gcagcacct gacgctgagc aaagcagact 240
acgagaaaca caaagtctac gcctgcgaag tcacccatca gggcctgagc tcgcccgtca 300
caaagagctt caacagggga gagtgtaag cggccgc 337

```

```

<210> SEQ ID NO 28
<211> LENGTH: 509
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: stuffer sequence

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```

<400> SEQUENCE: 28

```

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tctagataac tgtggctgca ccactctgtct tcacttccc gccatctgat gacgagttga 60
aatctggaac tgcctctggt gtgtgcctgc tgaataactt ctatcccaga gaggccaaag 120
tacagtggaa ggtggataac gccctccaat cgggtaactc ccaggagagt gtcacagagc 180
aggacagcaa ggacagcacc tacagcctca gcagcacct gacgctgagc aaagcagact 240
acgagaaaca caaagtctac gcctgcgaag tcacccatca gggcctgagc tctgacagtg 300
gcttgaaag cagatagcag ccccgtaag gcgggagtgg agaccaccac accctccaaa 360
caaagcaaca acaagtacgc ggccagcagc tatctgagcc tgacgctga gcagtggaag 420
tcccacagaa gctacagctg ccaggtcacg catgaaggga gcaccgtgga gaagacagtg 480
gccctacag aatgttcata agcggccgc 509

```

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<210> SEQ ID NO 29
<211> LENGTH: 1059
<212> TYPE: DNA
<213> ORGANISM: artificial sequence

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<220> FEATURE:

<223> OTHER INFORMATION: stuffer sequence

<400> SEQUENCE: 29

```

ctcgagctga tgagccatgg aagctgtgtc gcctgcacca ggctcccacg gctcgtggtg    60
cggtgcgctt ctggtgttcg ctgcctacag ccgacacgtc gagcttcgtg cccctagagt    120
tgcgcgtcac agcagcctcc ggcgctccgc gatatcaccg tgtcatccac atcaatgaag    180
tagtgctcct agacgcccc gtggggctgg tggcgcggtt ggctgacgag agcggccacg    240
tagtgttgcg ctggctcccg ccgcctgaga caccatgac gtctcacatc cgctacgagg    300
tggacgtctc ggccggcaac ggcgcagggg gcgtacagag ggtggagatc ctggagggcc    360
gcaccgagtg tgtgctgagc aacctgcggg gccggacgcg ctacacctc gccgtccgcg    420
cgcgtatggc tgagccgagc ttcggcggct tctggagcgc ctggtcggag cctgtgtcgc    480
tgctgacgcc tagcgacctg gacccccctca tctgacgct ctccctcatc ctcgtggtca    540
tcttggtgct gctgaccgtg ctgcgctgc tctcccaccg ccgggctctg aagcagaaga    600
tctggcctgg catcccgagc ccagagagcg agtttgaagg cctcttcacc acccacaagg    660
gtaacttcca gctgtggctg taccagaatg atggctgcct gtggtggagc ccctgcaccc    720
ccttcacgga ggaccacct gcttcctgg aagtcctctc agagcgctgc tgggggacga    780
tgcaggcagt ggagccgggg acagatgatg agggccctag gatgcctggt caagggttat    840
ttccctgagc cagtgacctt gacctggaac tctggatccc tgtccagtgg tgtgcacacc    900
tcccagctg tctgacgac tgacctctac accctcagca gctcagtgac tgtaacctcc    960
agcacctggc ccagccagtc catcacctgc aatgtggccc acccggcaag cagcaccaag   1020
gtggacaaga aaattgagcc cagagtgccc acaactagt                               1059

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<210> SEQ ID NO 30

<211> LENGTH: 1056

<212> TYPE: DNA

<213> ORGANISM: artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: stuffer sequence

<400> SEQUENCE: 30

```

ctcgagctga tgagccatgg aagctgtgtc gcctgcacca ggctcccacg gctcgtggtg    60
cggtgcgctt ctggtgttcg ctgcctacag ccgacacgtc gagcttcgtg cccctagagt    120
tgcgcgtcac agcagcctcc ggcgctccgc gatatcaccg tgtcatccac atcaatgaag    180
tagtgctcct agacgcccc gtggggctgg tggcgcggtt ggctgacgag agcggccacg    240
tagtgttgcg ctggctcccg ccgcctgaga caccatgac gtctcacatc cgctacgagg    300
tggacgtctc ggccggcaac ggcgcagggg gcgtacagag ggtggagatc ctggagggcc    360
gcaccgagtg tgtgctgagc aacctgcggg gccggacgcg ctacacctc gccgtccgcg    420
cgcgtatggc tgagccgagc ttcggcggct tctggagcgc ctggtcggag cctgtgtcgc    480
tgctgacgcc tagcgacctg gacccccctca tctgacgct ctccctcatc ctcgtggtca    540
tcttggtgct gctgaccgtg ctgcgctgc tctcccaccg ccgggctctg aagcagaaga    600
tctggcctgg catcccgagc ccagagagcg agtttgaagg cctcttcacc acccacaagg    660
gtaacttcca gctgtggctg taccagaatg atggctgcct gtggtggagc ccctgcaccc    720
ccttcacgga ggaccacct gcttcctgg aagtcctctc agagcgctgc tgggggacga    780
tgcaggcagt ggagccgggg acagatgatg agggccctag gatgcctggt caagggctat    840

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ttccctgagc cagtgacagt gacctggaac tctggatccc tgtccagcgg tgtgcacacc	900
ttcccagctg tcctgcagtc tgacctctac actctgagca gctcagtgac tgtcccctcc	960
agcacctggc ccagcgagac cgtcacctgc aacgttgccc acccggccag cagcaccaag	1020
gtggacaaga aaattgtgcc cagggattgt actagt	1056

<210> SEQ ID NO 31
 <211> LENGTH: 4153
 <212> TYPE: DNA
 <213> ORGANISM: artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: plasmid vector

<400> SEQUENCE: 31

gtggcacttt tgggggaaat gtgcgcggaa ccctatttg tttatTTTTc taaatacatt	60
caaatatgta tccgctcatg agacaataac cctgataaat gcttcaataa tattgaaaaa	120
ggaagagtat gagtattcaa catttccgtg tgcaccttat tccctTTTTt gcggcatttt	180
gccttccctgt ttttgetcac ccagaaacgc tggtgaaagt aaaagatgct gaagatcagt	240
tgggtgcacg agtgggttac atcgaactgg atctcaacag cggtaagatc cttgagagtt	300
ttcgccccga agaacgtttt ccaatgatga gcactTTTTa agttctgcta tgtggcgcg	360
tattatcccg tattgacgcc gggcaagagc aactcggctc cgcatacac tattctcaga	420
atgacttggg tgagtactca ccagtcacag aaaagcatct tacggatggc atgacagtaa	480
gagaattatg cagtgtgcc ataacctga gtgataacac tgcggccaac ttacttctga	540
caacgatcgg aggaccgaag gagctaaccg cttttttgca caacatgggg gatcatgtaa	600
ctcgccttga tcggtgggaa cgggagctga atgaagccat accaaacgac gagcgtgaca	660
ccacgatgcc tgtagcaatg gcaacaacgt tgcgcaaaact attaactggc gaactactta	720
ctctagcttc ccggcaacaa ttaatagact ggatggaggc ggataaagtt gcaggaccac	780
ttctgcgctc ggcccttccg gctggctggg ttattgctga taaatctgga gccggtgagc	840
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taggtgcctc actgattaag cattggtaac tgtcagacca agtttactca tatatacttt	1020
agattgattt aaaacttcat ttttaattta aaaggatcta ggtgaagatc ctttttgata	1080
atctcatgac caaaatccct taacgtgagt tttcgttcca ctgagcgtca gaccccgtag	1140
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caaaaaaac accgctacca gcggtggttt gtttgccgga tcaagagcta ccaactcttt	1260
ttccgaaggt aactggcttc agcagagcgc agataccaaa tactgtcctt ctagtgtagc	1320
cgtagttagg ccaccacttc aagaactctg tagcaccgcc tacatacctc gctctgctaa	1380
tctgttacc agtggctgct gccagtgccg ataagtctg tcttaccggg ttggactcaa	1440
gacgatagtt accggataag gcgcagcggg cgggctgaac ggggggttcg tgcacacagc	1500
ccagcttggg gcgaacgacc tacaccgaac tgagatacct acagcgtgag ctatgagaaa	1560
gcgccacgct tcccgaaggg agaaaggcgg acaggtatcc ggtaagcggc agggtcggaa	1620
caggagagcg cacgagggag cttccagggg gaaacgcctg gtatctttat agtccctgctg	1680
ggtttcgcca cctctgactt gagcgtcgat ttttgtgatg ctcgtcaggg gggcggagcc	1740
tatggaaaaa cgccagcaac gcggcctttt tacggttcct ggcccttttg tggccttttg	1800
ctcacatggt ctttctgctg ttatccctg attctgtgga taaccgtatt accgcctttg	1860

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agtgagctga	taccgctcgc	cgcagccgaa	cgaccgagcg	cagcgagtca	gtgagcgagg	1920
aagcgtaccc	gataaaagcg	gcttcctgac	aggaggccgt	tttgttttgc	agcccaccta	1980
gcggaagagc	gcccataacg	caaaccgect	ctccccgcgc	gttggccgat	tcattaatgc	2040
agctggcacg	acaggtttcc	cgactggaaa	gcgggcagtg	agcgcaacgc	aattaatgtg	2100
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tgtggaattg	tgagcggata	acaattgaat	tcaaggagtt	aattatgaaa	aaaaccgcca	2220
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gcggccgcac	tagatataat	taaggagata	aatatgaaat	atctgctgcc	gaccgcggcg	2340
gcgggcctgc	tgctgctggc	ggcgcagccg	gcgatggcgc	tcgagtcaat	actagtggcc	2400
aggccggcct	tgaactgtt	gaaagttggt	tagcaaaacc	ccatacagaa	aattcattta	2460
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agggtggcgg	ctctgagggt	ggcggtaacta	aacctcctga	gtacgggtgat	acacctatc	2700
cgggctatac	ttatatcaac	cctctcgacg	gcacttatcc	gcctggact	gagcaaaacc	2760
ccgctaatac	taactcctct	cttgaggagt	ctcagcctct	taatactttc	atgtttcaga	2820
ataatagggt	ccgaaatagg	cagggggcat	taactgttta	tacgggcact	gttactcaag	2880
gcactgacct	cgtaaaact	tattaccagt	acactcctgt	atcatcaaaa	gccatgtatg	2940
acgcttactg	gaacggtaaa	ttcagagact	gcgctttcca	ttctggcttt	aatgaggatc	3000
cattcgtttg	tgaatatcaa	ggccaatcgt	ctgacctgcc	tcaacctcct	gttaatgctg	3060
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gttctgaggg	tgccggctct	gagggtggcg	gttccggctg	tggtctggt	tccggtgatt	3180
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ctatcgacgg	ttcattggt	gacgtttccg	gccttgctaa	tggtaatggt	gctactggtg	3360
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tgaataattt	ccgtcaatat	ttaccttccc	tcctcaatc	ggttgaatgt	cgcccttttg	3480
tctttggcgc	tggtaaacca	tatgaatttt	ctattgattg	tgacaaaata	aacttattcc	3540
gtggtgtctt	tgcgtttctt	ttatatggtg	ccacctttat	gtatgtattt	tcgacgtttg	3600
ctaacatact	gcgtaataaa	gagtcttaag	ctagctaagc	ggccgcatta	ccgatcgccc	3660
ttcccaacag	ttgcgcagcc	tgaatggcga	atgggacgcg	ccctgtagcg	gcgcattaag	3720
cgccggcgggt	gtggtgggtta	cgcgcagcgt	gaccgctaca	cttgccagcg	ccctagcgcc	3780
cgctcctttc	gctttcttcc	cttcctttct	cgccacgttc	gccggctttc	cccgtaagc	3840
tctaaatcgg	gggctccctt	tagggttccg	athtagtct	ttacggcacc	tcgaccccaa	3900
aaaacttgat	tagggtgatg	gttcacgtag	tgggcatcg	ccctgataga	cggtttttcg	3960
ccctttgacg	ttggagtcca	cgttctttaa	tagtggtctc	ttgttccaaa	ctggaacaac	4020
actcaacctt	atctcggtct	attcttttga	ttataaggg	atthtgccga	tttcggccta	4080
ttggttaaaa	aatgagctga	tttaacaaaa	atthaacgcg	aatthtaaca	aaatattaac	4140
gcttacaatt	tag					4153

We claim:

- [1.]** A phagemid vector comprising:
 a selectable marker;
 a ColE1 origin;
 an fl origin; and
 after the ColE1 origin but before the fl origin, further
 comprising the following features:
 a bacterial transcription terminator;
 a promoter,
 a first ribosomal binding site;
 a first leader sequence;
 a first cloning region;
 a second ribosomal binding site;
 a second leader sequence;
 a second cloning region for receiving a gene encoding a
 polypeptide to be displayed; and
 a nucleotide sequence encoding a product that enables
 display of a polypeptide on the surface of a phagemid
 particle.]
- [2.]** A phagemid vector as in claim 1 wherein at least one of
 the first or second ribosomal binding sites comprises Seq. ID
 No. 13.]
- 3.** [A phagemid vector as in claim 1] *A phagemid vector
 comprising:*
a selectable marker;
a ColE1 origin;
an fl origin; and
after the ColE1 origin but before the fl origin, further
comprising the following features: a bacterial tran-
scription terminator and a promoter wherein the bacte-
rial transcription terminator is upstream of the pro-
moter;

a first ribosomal binding site;
a first leader sequence;
a first cloning region;
a second ribosomal binding site;
a second leader sequence;
a second cloning region for receiving a gene encoding
a polypeptide to be displayed; and
a nucleotide sequence encoding a product that enables
display of a polypeptide on the surface of a phagemid
particle,

wherein at least one of the first or second leader sequences
 comprises a sequence selected from the group consist-
 ing of Seq. ID No. 14 and Seq. ID No. 17.

[4.] A phagemid vector as in claim 1 wherein the nucle-
 otide sequence encoding a product encodes a protein
 selected from the group consisting of pIII and pVIII.]

[5.] A phagemid vector as in claim 1 wherein the nucle-
 otide sequence encoding a product encodes a truncated pIII.]

[6.] A phagemid vector as in claim 1 wherein the nucle-
 otide sequence encoding a product encodes a synthetic pIII.]

[7.] A phagemid vector as in claim 1 wherein the selectable
 marker is selected from the group consisting of ampicillin
 resistance, chloramphenicol transferase resistance, tetracy-
 cline resistance and kanamycin resistance.]

8. A phagemid vector comprising Seq. ID No. 18.

9. A vector comprising a sequence selected from the
 group consisting of Seq. ID Nos. 19, 20 and 21.

* * * * *