The Plasmid, pBR322

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Genetic engineering can be defined as the transfer of DNA between hosts (or species) by in vitro enzymatic manipulations. This implies that the DNA to be transferred will be duplicated in the new host. Since most DNA fragments are incapable of self-replication in *E. coli* or any other host cell, an additional segment of DNA, capable of autonomous replication, must be linked to the fragment to be cloned. This autonomously replicating fragment is the molecular cloning vector and, by definition, plays a central role in recombinant DNA technology. Most cloning vectors were originally derived from naturally occurring extrachromosomal elements such as bacteriophage and plasmids. Bacteriophage vectors like M13 and lambda have proven to be very useful as cloning vectors and will be discussed in greater detail in later chapters (see Chapters 2–5).

Wild-type plasmids such as pSC101 (Cohen and Chang 1973, 1977; Cohen et al. 1973) and ColE1 (Clewell 1972; Helinski and Clewell 1972) served as two of the first cloning vectors. Although both plasmids possessed

certain features that made them useful as vectors (unique restriction sites, autonomous replication, and selectable markers), there were no vectors available at the time that possessed all of these features in one plasmid.

Although some of these features may not have been anticipated in the early days of plasmid construction, most researchers have adhered to the following criteria as a guide to the design of cloning vectors.

1.1   CRITERIA FOR PLASMID VECTOR DESIGN

1. A plasmid vector should be small, with little or no extraneous genetic information. Because the efficiency of transformation of many host cells decreases as the size of the plasmid approaches 15 kilobases (kb) and above, the vector should contribute as little as possible to the overall size of the recombinant molecule.
2. Vectors should be well characterized with respect to gene number and location, restriction enzyme cleavage sites, and nucleotide sequence.
3. The vector should be easily propagated in the desired host so that large quantities of vector and recombinant DNA molecules can be obtained.
4. The vector should have a selectable marker (gene) to allow cells harboring the vector to be distinguished from nontransformed cells.
5. An ideal vector should have an additional genetic marker that can be activated or inactivated by the insertion of foreign DNA fragments. The marker gene will allow cells harboring recombinant molecules to be distinguished from nonrecombinant molecules on the basis of altered phenotype.
6. Finally, the vector should possess the maximum number of unique restriction enzyme cleavage sites located in one or the other genetic marker. This provides maximum flexibility for cloning different kinds of restriction fragments.

1.2   CONSTRUCTION AND STRUCTURE

A good example of how plasmid vectors are constructed is shown in Figure 1–1, which illustrates the various steps involved in the construction of the plasmid pBR322 and its derivatives (Bolívar et al. 1977a,b; Bolívar 1978; Covarrubias et al. 1981; Covarrubias and Bolívar 1982). The salient points in the construction pathway involve the acquisition of a "relaxed mode" of plasmid DNA replication and the ampicillin and tetracycline resistance genes to serve interchangeably as genetic markers for selection and insertional inactivation.

Starting with a clinical isolate harboring the colicin-producing plasmid pMB1, the relaxed origin of DNA replication was isolated in the form of plasmid pMB8 (Betlach et al. 1976). The tetracycline resistance (Tc^R) gene
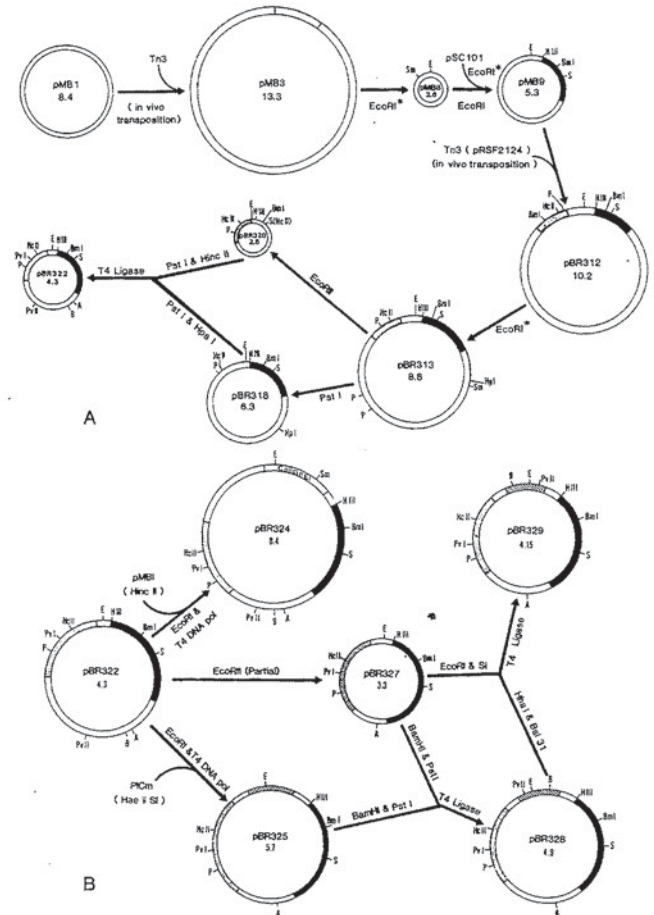
FIGURE 1–1   A. Schematic representation showing steps in construction of pBR322 from pMB1, pSC101, and pRSF2124. Restriction enzyme cleavage sites are represented as follows; A, *Ava*I; B, *Bal*I; BmI, *Bam*HI; E, *Eco*RI; HIII, *Hind*III; HcII, *Hinc*II; HpI, *Hpa*II; P, *Pst*I; PvI, *Pvu*I; PvII, *Pvu*II; S, *Sal*I; Sm, *Sma*I. **B.** Schematic representation showing steps in the construction of pBR329 from pBR322, pBR325, and pBR328. Solid bars = Tc^R; stippled bars = Ap^R; hatched bars = Cm^R. (Reproduced from Rodriguez, R. L., and Tait, R. C. [1983] *Recombinant DNA Techniques: An Introduction.* Benjamin Cummings, Menlo Park, Calif.)

from pSC101 and the ampicillin resistance (Ap^R) gene from the transposon Tn3 (carried on the plasmid pRSF2124) were incorporated into the plasmid pBR312 (Rodriguez et al. 1976; Bolivar et al. 1977a). This maneuver placed the Tc^R and Ap^R genes onto a relaxed replicon capable of amplification with inhibitors of protein synthesis (see section 1.4). All subsequent manipulations were designed to maximize the number of unique restriction enzyme cleavage sites and to minimize plasmid size. For example, the extra BamHI site near the Ap^R gene in pBR312 was removed by EcoRI* digestion (Gardner et al. 1982). Likewise, the two additional PstI cleavage sites in pBR313 were removed by a more complicated maneuver to give rise to pBR322. As can be seen in Figure 1–2, pBR322 contains twenty-one unique restriction enzyme cleavage sites, eleven of which reside in the Tc^R or Ap^R genes. Since the enzyme HincII also cleaves the SalI site, it is not included among the unique cleavage sites.

With the exception of pBR327 (Figure 1–1B), which lacks 1,089 bp of nonessential DNA present in pBR322, subsequent plasmid constructions were designed to introduce a unique EcoRI cleavage site within a plasmid gene. As shown in Figure 1–1B, both the colicin production gene in pBR324 (Bolivar 1978) and the chloramphenicol resistance gene in pBR329 (Covarrubias and Bolivar 1982) possess unique EcoRI cleavage sites. With these plasmids, EcoRI restriction fragments can be cloned and identified by the insertional inactivation of a plasmid encoded gene.

One feature of pBR322 that has contributed to its wide use as a cloning vector and research tool is the availability of a complete nucleotide sequence. The original sequence of pBR322 was reported by Sutcliffe (1979) to consist of 4,362 bp. This sequence was subsequently revised to 4,363 bp by incorporating the following two corrections (Figure 1–2). First, Heusterspreute and Davison (1983) raised the possibility of a mistake in the Tc^R gene sequence. This would explain the absence of an open reading frame consistent with earlier studies of the Tc^R protein. Minicell (Meagher et al. 1977) and maxicell (Sancar et al. 1979) studies revealed the Tc^R protein to be approximately 40 kDal, whereas the original nucleotide sequence predicted a polypeptide of about 20 kDal. This error was later confirmed by Peden (1983) and Backman and Boyer (1983), who showed that a GC base pair at position 526 in the coding region of the Tc^R gene had been omitted. The revised sequence now shows the Tc^R gene consisting of an open reading frame of 396 amino acids or 1,188 bp (position 86–1273) specifying a 41,500 kDal (M, 37,000) protein. This protein has been shown to actively exclude Tc from the cell (Tait and Boyer 1978) while simultaneously mediating the uptake of potassium (Dosch et al. 1984). There is no evidence to suggest, however, that these two functions for the Tc^R protein are related.

As shown in Figure 1–1A, the Tc^R gene of pBR322 was derived as an EcoRI* fragment from the naturally occurring R-factor, pSC101 (Cohen and Chang 1977). The Tc^R mechanism of pSC101 belongs to the class C resistance group and is closely related to the class A (RP1/Tn1721) and class B (Tn10)
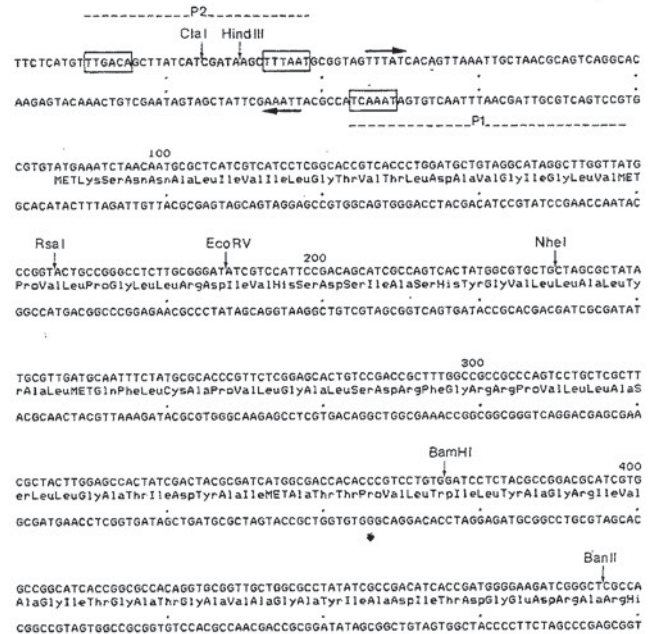
FIGURE 1–2 Nucleotide sequence of pBR322. The symbols and letters appearing throughout sequence are used according to the following convention: (a) Restriction enzyme cleavage sites that are present no more than three times on pBR322 are indicated by vertical arrows at the site of cleavage. (b) Structural genes are indicated by corresponding amino acid sequence written above or below the coding strand. (c) Promoters are indicated by the designation P$_N$, where N refers to the identity of the promoter according to numbering system of Stüber and Bujard 1981 and Brosius et al. 1982. Boxed sequences indicate the location of the -10 and -35 conserved sequences commonly found in most prokaryotic promoters (Rosenberg and Court 1979). Horizontal arrows indicate start point and direction of transcription for each promoter. Location of transcription terminators can be found in Figure 1–3. (d) Origin of replication is indicated by end-on-end arrows, starting at the first base of nacent DNA attached to the RNA primer. Horizontal lines are used to highlight regions of dyad symmetry thought to be biologically relevant. In the region around position 3000, the symmetrical sequences involved in the regulation of DNA replication are identified with Roman numerals according to standard nomenclature (Tomizawa and Itoch 1981). (From Balbas et al. 1987.)

Ban II
500
CTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCT
sPheGlyLeuMETSerAlaCysPheGlyValGlyMETValAlaGlyProValAlaGlyGlyLeuLeuGlyAlaIleSerL

GAAGCCCGAGTACTCGCGAACAAAGCCGCACCCATACCACCGTCCGGGGCACCGGCCCCCTGACAACCCGCGGTAGAGGA

Sph I
600
TGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCAT
euHisAlaProPheLeuAlaAlaAlaValLeuAsnGlyLeuAsnLeuLeuLeuGlyCysPheLeuMETGlnGluSerHis

ACGTACGTGGTAAGGAACGCCGCCGCCACGAGTTGCCGGAGTTGGATGATGACCCGACGAAGGATTACGTCCTCAGCGTA

Acc I / Hinc II
Sal I
700
AAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGT
LysGlyGluArgArgProMETProLeuArgAlaPheAsnProValSerSerPheArgTrpAlaArgGlyMETThrIleVa

TTCCCTCTCGCAGCTGGCTACGGGAACTCTCGGAAGTTGGGTCAGTCGAGGAAGGCCACCCGCGCCCCGTACTGATAGCA

800
CGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGG
IAlaAlaLeuMETThrValPhePheIleMETGlnLeuValGlyGlnValProAlaAlaLeuTrpValIlePheGlyGluA

GCGGCGTGAATACTGACAGAAGAAATAGTACGTTGAGCATCCTGTCCACGGCCGTCGCGAGACCCAGTAAAAGCCGCTCC

ACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGCTCAAGCCTTC
spArgPheArgTrpSerAlaThrMETIleGlyLeuSerLeuAlaValPheGlyIleLeuHisAlaLeuAlaGlnAlaPhe

TGGCGAAAGCGACCTCGCGCTGCTACTAGCCGGACAGCGAACGCCATAAGCCTTAGAACGTGCGGGAGCGAGTTCGGAAG

Bgl I Xma III
900
GTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGT
ValThrGlyProAlaThrLysArgPheGlyGluLysGlnIleIleAlaGlyMETAlaAlaAspAlaLeuGlyTyrVa

CAGTGACCAGGGCGGTGGTTTCGCAAAGCCGCTCTTCGTCCGGTAATAGCGGCCGTACCGCCGGCTGCGCGACCCGATGCA

Nru I
1000
CTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCG
lLeuLeuAlaPheAlaThrArgGlyTrpMETAlaPheProIleMETIleLeuLeuAlaSerGlyGlyIleGlyMETProA

GAACGACCGCAAGCGCTGCGCTCCGACCTACCGGAAGGGGTAATACTAAGAAGAGCGAAGGCCGCCGTAGCCCTACGGGC

1100
CGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTCTTACCAGC
laLeuGlnAlaMETLeuSerArgGlnValAspAspHisGlnGlyGlnLeuGlnSerLeuAlaAlaLeuThrSer

GCAACGTCCGGTACGACAGGTCCGTCCATCTACTGCTGGTAGTCCCTGTCGAAGTTCCTAGCGAGCGCCGAGAATGGTCG

Bgl I
1200
CTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGAT
LeuThrSerIleThrGlyProLeuIleValThrAlaIleTyrAlaAlaSerAlaSerThrTrpAsnGlyLeuAlaTrpIl

GATTGAAGCTAGTGACCTGGCGACTAGCAGTGCCGCTAAATACGGCGGAGCCGCTCGTGTACCTTGCCCAACCGTACCTA

TGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGG
eValGlyAlaAlaLeuTyrLeuValCysLeuProAlaLeuArgArgGlyAlaTrpSerArgAlaThrSerThr

ACATCCGCGGCGGGATATGGAACAGACGGAGGGGCGCAACGCAGCGCCACGTACCTCGGCCCGGTGGAGCTGGACTTACC

Bsm
1300
AAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGC

TTCGGCCGCCGTGGAGCGATTGCCTAAGTGGTGAGGTTCTTAACCTCGGTTAGTTAAGAACGCCTCTTGACACTTACGCG

Sti I
1400
Ava I
AAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGG

CTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGGAGGTCGTCGGCGTGCGCCGCGTAGAGCCCGTCGCAACCC

Bal I
1500
TCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCA

AGGACCGGTGCCCACGCGTACTAGCACGAGGACAGCAACTCCTGGGCCGATCCGACCGCCCCAACGGAATGACCAATCGT

1600
GAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGG

CTTACTTAGTGGCTATGCGCTCGCTTGCACTTCGCTGACGACGACGTTTTGCAGACGCTGGACTCGTTGTTGTACTTACC

TCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCA

AGAAGCCAAAGGCACAAAGCATTTCAGACCTTTGCGCCTTCAGTCGCGGGACGTGGTAATACAAGGCCTAGACGTAGCGT

1700
GGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTG

CCTACGACGACCGATGGGACACCTTGTGGATGTAGACATAATTGCTTCGCGACCGTAACTGGGACTCACTAAAAAGAGAC

1800
GTCCCGCCGCATCCATACCGCCAGTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTA

CAGGGCGGCGTAGGTATGGCGGTCAACAAATGGGAGTGTTGCAAGGTCATTGGCCCGTACAAGTAGTAGTCATTGGGCAT

1900
TCGTGAGCATCCTCCTCTCGTTTCATCGGTATCATTACCCCCATGAACAGAAATTCCCCCTTACACGGAGGCATCAAGTGA
METT
AGCACTCGTAGGAGAGAGCAAAGTAGCCATAGTAATGGGGGTACTTGTCTTTAAGGGGGAATGTGCCTCCGTAGTTCACT

2000
CCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACGAG
hrLysGlnGluLysThrAlaLeuAsnMETAlaArgPheIleArgSerGlnThrLeuThrLeuLeuGluLysLeuAsnGlu

GGTTTGTCCTTTTTTGGCGGGAATTGTACCGGGCGAAATAGTCTTCGGTCTGTAATTGCGAAGACCTCTTTGAGTTGCTC

FIGURE 1–2 Continued

Xmn I

Pvu II

CTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCG
LeuAspAlaAspGluGlnAlaAspIleCysGluSerLeuHisAspHisAlaAspGluLeuTyrArgSerCysLeuAlaAr

GACCTGCGCCTACTTGTCCGTCTGTAGACACTTAGCGAAGTGCTGGTGCGACTACTCGAAATGGCGTCGACGGAGCGCGC

2100
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gPheGlyAspAspGlyGluAsnLeuEND

AAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTCGAACAGACATTCGCCTACGGCCCT

2200
GCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGC

CGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCCACAGCCCCGCGTCGGTACTGGGTCAGTGCATCGCTATCG

AccI/SnaI   RELAXATION NICK   RsaI   NdeI
                                                   2300
GGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCAC

CCTCACATATGACCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTG
--------P_CRP-----

2400
AGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCT

TCTACGCATTCCTCTTTTATGGCGTAGTCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGA

                                                              AflIII
GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT

CGCCGCTCGCCATAGTCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCCTATTGCGTCCTTTCTTGTACA

2500
GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGAC

CTCGTTTTCCGGTCGTTTTCCGGTCCTTGGCATTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTG
                                            REPLICATION ORIGIN

2600
GAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGG

CTCGTAGTGTTTTTAGCTGCGAGTTCAGTCTCCACCGCTTTGGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACC

2700
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TTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGGAAGCCCTTCGCACC

2800
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GCGAAAGAGTATCGAGTGCGACATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTTGGG

CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACT

GGGCAAGTCGGGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGA

2900
GGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT
-------P4-------
CCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGA
                      IV

                                          3000
ACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCT

TGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTCTCAACCATCGAGA
        III   III'   III'   II

                                                                3100
TGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATC
ACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAAT GCGCGTCTTTTTTTCCTAG
II'   I   1'   ---------Pp------

                                                                          3200
TCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGA
AGTTCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAATTCCCTAAAACCAGTACT

                    DraI   DraI
GATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAA
CTAATAGTTTTTCCTAGAAGTGGATCTAGGAAAATTTAATTTTTACTTCAAAATTTAGTTAGATTTCATATATACTCATT

                    3300
ACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGC
                  ENDTrpHisLysIleLeuSerAlaGlyIleGluAlaIleGlnArgAsnArgGluAspMETThrAla
TGAACCAGACTGTCAATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGTATCAACG

                    3400
CTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACC
GlnSerGlyThrThrTyrIleValValIleArgSerProGlyLeuAlaAlaAlaIleIleGlyArgSerGl
GACTGAGGGGCAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGGGGTCACGACGTTACTATGGCGCTCTGG

                            BglI
                                    3500
CACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTA
yArgGluGlyAlaGlySerLysAspAlaIlePheTrpGlyAlaProLeuAlaSerArgLeuLeuProGlyAlaValLysA
GTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAGGACGTTGAAAT

                                                    3600
TCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGT
spAlaGluMETTrpAspIleLeuLeuGlnGlnArgSerAlaLeuThrLeuLeuGluGlyThrLeuLeuLysArgLeuThrThr
AGGCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATTATCAAACGCGTTGCAACA

FIGURE 1–2 *Continued*

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