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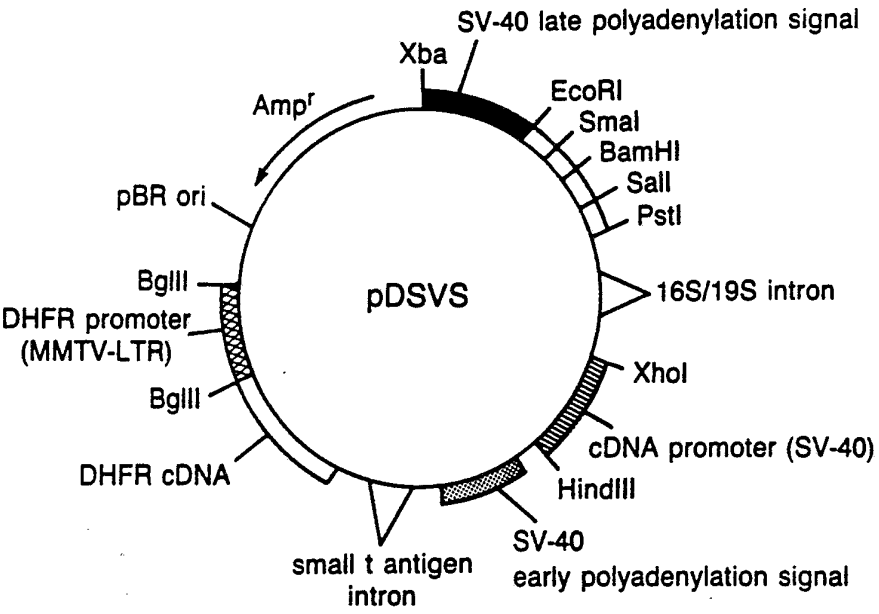

(57) Abstract

This invention provides novel recombinant plasmids which can be used to clone heterologous genes in *E. coli* and to clone and express such genes in mammalian cells. These plasmids contain a number of unique restriction sites that can be used to facilitate the deletion or replacement of genetic elements and the insertion of heterologous genes in the plasmids. The nature and placement of the unique sites is such that regeneration of the sites following endonuclease cleavage can readily be achieved with a minimum of manipulative effort. Host cells containing the recombinant plasmids are also provided by this invention.

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MAMMALIAN EXPRESSION VECTORS

BACKGROUND OF THE INVENTION

Much of the work in the recombinant DNA field to the present has focused on the use of bacterial host cells such as E. coli for the production of recombinant polypeptides and proteins. Yet, the use of bacterial cells has a number of undesirable aspects. For example, most proteins and polypeptides produced in E. coli accumulate in the cytosol or periplasmic space. Recovery of these gene products requires disruption of the cells and, often, extraction with a detergent or chaotropic agent such as urea or guanidine-hydrochloride. Proteins thus recovered are frequently in incorrectly folded forms and are difficult to purify because they must be isolated from the numerous other E. coli cellular constituents. Furthermore, bacteria cannot carry out glycosylation which is needed to complete the synthesis of many interesting gene products and are frequently incapable of forming the specific disulfide bonds which are essential for the proper conformation and biological activity of many eukaryotic proteins.

To overcome these deficiencies of bacterial expression systems, the attention of genetic engineers is increasingly turning to the use of eukaryotic host cells. Cells such as yeast and mammalian cells can secrete desired gene products into the culture medium and can carry out essential glycosylation processes as well. Yet, the use of

mammalian cells for recombinant DNA cloning and expression also presents a host of technical obstacles that must be overcome. For example, the endogenous plasmids that have proven to be so useful in bacteria are usually not replicated by higher eukaryotic cells. As a result, other approaches must be taken.

One approach has been to use the lower eukaryotic yeast, Saccharomyces cerevisiae, which can be grown and manipulated with the same ease as E. coli. Yeast cloning systems are available, and through the use of such systems the efficient expression in yeast of a human interferon gene has been achieved [Hitzeman et al., Nature (London) 293:717 (1981)]. It has been found, however, that yeast cells do not correctly transcribe at least one heterologous mammalian gene that contains introns, the rabbit β-globin gene [Beggs et al., Nature (London) 283:835 (1980)].

In another approach, heterologous genes have been inserted into mammalian cells by means of direct uptake. This has been accomplished, for example, by calcium phosphate co-precipitation of cloned genes, by which procedure about 1-2% of the cells can generally be induced to take up the DNA. Such a low level of uptake, however, produces only a very low level of expression of the desired gene product. Where mammalian cells can be found which lack the thymidine kinase gene (tk^- cells), better results can be obtained by co-transformation. Tk^- cells, which cannot grow in selective HAT (hypoxanthine-aminoterin-thymidine) medium, can regain this lost enzymatic activity by taking up exogeneous DNA (such as herpes simplex viral DNA) containing the tk gene through calcium phosphate co-precipitation. Other DNA covalently ligated to the tk DNA or merely mixed with it will also be taken up by the cells and will often be co-expressed [see Scangos et al., Gene 14:1 (1981)].

A number of investigators have developed expression vectors for use in eukaryotic cells. For example, Okayama et al. [Mol. Cell. Biol. 3:280 (1983)] have developed a "pcD" vector system which incorporates Simian virus 40 (SV40) control elements. Kaufman (U.S. Patent No. 4,740,461) has described eukaryotic cell vectors which incorporate SV40 elements and a selectable marker, for co-amplification. These vectors are said to be useful, e.g., for the production of tPA in CHO cells. Choo et al. [DNA 5:529 (1986)] have developed two recombinant vectors for the direct expression and amplification of cDNA in cultured mammalian cells. Pfarr et al. [DNA 4:461 (1985)] have developed a "modular" vector, pDSP1, which contains two independent mammalian transcription cassettes.

A variety of genetic engineering techniques are employed in the construction of such recombinant vectors. General methods have been described by Cohen et al. (U.S. Patent No. 4,237,224), Collins et al. (U.S. Patent No. 4,304,863) and Maniatis et al. (Molecular Cloning: A Laboratory Manual, 1982, Cold Spring Harbor Laboratory). Panayotatos [Gene 31:291 (1984)] has disclosed the complete end-filling of restriction site overhangs to generate new sites upon ligation, following the cleavage of palindromic restriction sites. Korch [Nuc. Acids Res. 15;3199 (1987)] and Hung et al. [Nuc. Acids Res. 12:1863 (1984)] have described ways to manipulate restriction endonuclease sites involving partial end-filling, to permit the joining of DNA fragments which normally cannot be ligated together.

SUMMARY OF THE INVENTION

This invention provides recombinant plasmids which can be used to clone heterologous genes in E. coli and to clone and express such genes in a variety of mammalian cells. The plasmids are characterized by strategically placed genetic elements and unique restriction sites, the nature of which facilitates the excision and/or

substitution of elements and the restoration of the original restriction sites.

The recombinant plasmids of the invention comprise DNA sequences which, in the direction of transcription, contain:

(a) a β-lactamase gene and a pBR322 origin of replication delimited by an XbaI and a BglII restriction site,

(b) an SV-40 or SRα promoter delimited by a HindIII and an XhoI restriction site,

(c) a polylinker containing one or more unique restriction sites delimited by a PstI and an EcoRI restriction site, and

(d) a polyadenylation signal sequence delimited by an EcoRI and an XbaI restriction site.

Preferably, the polyadenylation signal sequence is a human β-globin or an SV-40 early or late region polyadenylation signal sequence.

Some of the recombinant plasmids of the invention further comprise a dihydrofolate reductase (DHFR) transcription unit comprising, in the direction of transcription, a mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter, a DHFR cDNA, a splicing signal sequence and a polyadenylation signal sequence. Preferably, the splicing signal sequence is a small t antigen intron and the polyadenylation signal sequence is an SV-40 early region polyadenylation signal sequence.

When present in the plasmids, the DHFR transcription unit is positioned between the DNA sequences containing the β-lactamase gene and pBR322 origin of replication and the SV-40 or SRα promoter. The DHFR transcription unit is delimited, in the direction of transcription, by a BglII and a HindIII restriction site. The DNA sequence containing

the MMTV-LTR promoter within the transcription unit is delimited by two BglII restriction sites.

In other embodiments of the invention, the recombinant plasmids comprise DNA sequences containing a β-galactosidase (β-gal) or a chloramphenicol acetyltransferase (CAT) transcription unit in place of the DHFR transcription unit. When present, these sequences are also terminated by a BglII and a HindIII restriction site.

In still other embodiments, a splicing signal sequence is present between the DNA sequences containing the SV-40 or SRα promoter and the polylinker and is delimited, in the direction of transcription, by an XhoI and a PstI restriction site. This splicing signal sequence is preferably a 16S/19S intron.

BRIEF DESCRIPTION OF THE FIGURES

This invention can be more readily understood by reference to the accompanying Figures in which:

Fig. 1 is a schematic representation of recombinant plasmid pDSVS, showing unique restriction sites and elements.

Fig. 2, panels A-H, is a schematic representation showing the construction of plasmid pDSVS from various starting plasmids.

Fig. 3 is a schematic representation of plasmid pDSRS. This plasmid is similar to pDSVS except for the substitution of an SRα promoter for the SV-40 promoter of pDSVS.

Fig. 4 is a schematic representation of plasmid pSRS. This plasmid is similar to pDSRS except that the DHFR transcription unit has been deleted in pSRS.

Fig. 5, panels A and B, is a schematic representation of the construction of plasmids pDSRG and pSRG. These plasmids are similar to pDSRS and pSRS, respectively, except that plasmids pDSRG and pSRG contain a human β-globin polyadenylation signal sequence instead of an SV-40 late region polyadenylation signal sequence.

Fig. 6, panels A-C, is a schematic representation of the construction of plasmid pGSRG-hIL5, which is capable of directing the production of recombinant human interleukin-5 in mammalian cells.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, the term "transcription" means the synthesis of messenger RNA (mRNA) from a DNA template. A "promoter" is a DNA sequence that directs the binding of RNA polymerase and thus "promotes" transcription. "In the direction of transcription" means from upstream to downstream (i.e., from 5' to 3'), or in the direction of movement of RNA polymerase as it carries out the transcription of DNA. The SRα promoter used in this invention has been described by Takebe et al. [Mol. Cell. Biol. 8:466 (1988)]. The SV-40 promoter has been described by Okayama et al. [Mol. Cell. Biol. 3:280 (1983)].

An "enhancer" is a DNA sequence that can potentiate the transcription of a gene without regard to the nature of the gene. Although promoters must be positioned upstream of genes that are to be expressed, the positioning of enhancers is not critical. They can function whether upstream or downstream of a gene, and even when they are inverted with respect to the other DNA sequences (i.e., in the 3' to 5' orientation).

"Splicing signals" are DNA sequences which can also enhance the transcription of genes encoding some proteins. An "origin of replication" is a point in a plasmid at which replication of the plasmid by a host cell is initiated.

A "polyadenylation signal sequence" is a DNA sequence located downstream of the translated regions of a gene at which adenine ribonucleotides are added to form a polyadenylate tail at the 3' end of the mRNA.

A "polylinker" is a DNA sequence which contains one or more unique restriction sites. Polylinkers facilitate the insertion of heterologous genes (i.e., genes not normally present in a host cell) that are to be expressed in the plasmids of the invention. As used herein, the term heterologous "gene" includes both isolated genes and cDNAs prepared from mRNA.

"Restriction sites" are DNA sequences which define cleavage points for restriction endonucleases. Such sites are "unique" when only one of a given site is present in a plasmid. The plasmids of this invention may contain unique XbaI, EcoRI, SmaI, BamHI, SalI, PstI, XhoI and HindIII sites.

In the most basic embodiments, the plasmids of the invention comprise, in the direction of translation:

(a) a β-lactamase gene and a pBR322 origin of replication delimited by an XbaI and a BglII restriction site,

(b) an SV-40 or SRα promoter delimited by a HindIII and an XhoI restriction site,

(c) a polylinker containing one or more unique restriction sites delimited by a PstI and an EcoRI restriction site, and

(d) a polyadenylation signal sequence delimited by an EcoRI and an XbaI restriction site.

With a heterologous gene inserted into the polylinker, elements (b) - (d) together comprise a heterologous gene transcription unit.

The β-lactamase gene encodes an enzyme which degrades the antibiotic ampicillin. Because of this activity, the presence of the β-lactamase gene in a host cell confers on the cell resistance to ampicillin in the culture medium. As a result, host cells harboring the plasmids of the invention can be selected by growth in such medium, while other cells lacking such resistance will not survive. In the accompanying figures, the presence of the β-lactamase gene is indicated by "AMP" or "AMP^r".

A recombinant plasmid containing only the foregoing elements can be used for the transient expression of a heterologous gene inserted into the polylinker. Insertion of a gene into the polylinker is facilitated by the presence in the polylinker of unique restriction sites, including PstI, SalI, BamHI, SmaI and EcoRI sites. Gene insertion is easily achieved if the termini of the gene following isolation are compatible with the PstI and/or SalI, BamHI or EcoRI sites. If they are not, they can readily be modified using standard methods to be compatible. Alternatively, the gene termini can simply be blunt ended by standard methods and inserted into the SmaI-cleaved plasmid, because SmaI digestion produces blunt-ended cleavage.

The polylinker need not contain all of the restriction sites mentioned above; any undesired sites can be deleted by standard methods. Conversely, other sites can be added if need be, as long as they are unique sites so that cleavage with the corresponding restriction endonuclease does not also cut the plasmid elsewhere.

In other embodiments of the invention which can be used for transient expression, a β-gal or CAT transcription unit is inserted between the DNA sequences containing the β-lactamase gene and pBR322 origin of replication and the SV-40 or SRα promoter. Both the β-gal gene and the CAT gene are well known in the art, the former having been described by Hall et al. [J. Mol. Appl. Gen. 2:101 (1983)], the latter by Gorman et al. [Mol. Cell. Biol. 2:1044 (1982)]. When present in the plasmids, these genes provide useful marker activities for transformant/transfectant screening.

All of the foregoing plasmids are useful for transient expression in mammalian cells, but longer-term expression cannot be achieved with such plasmids. That is because there is no selective pressure to maintain the plasmids in host cells. Over time, they are lost. For more prolonged expression, the plasmids of the invention preferably contain a DHFR transcription unit in place of a β-gal or CAT transcription unit. The elements of the DHFR transcription unit have been described by Lee et al. [Nature 294:228 (1981)].

The presence of a DHFR transcription unit in the plasmids provides two advantages. Firstly, when introduced into a host cell lacking dihydrofolate reductase (e.g., into CHO-dhfr$^-$ cells), the unit provides a means of selection. Cells harboring the plasmids can easily be selected from other cells in medium lacking hypoxanthine and thymidine. Secondly, cells containing high levels of the dhfr cDNA will survive culture in medium containing high levels of methotrexate, an inhibitor of de novo purine synthesis, while cells expressing low levels of the cDNA will not. Such conditions will thus enable selection for cells which have increased or amplified the number of copies of the dhfr gene.

When a heterologous gene that is to be expressed is also present in a plasmid with the dhfr gene, expression of the heterologous gene will be co-amplified as the host cell makes many copies of the dhfr

gene. In the examples below, culturing cells harboring a plasmid containing the DHFR transcription unit in the presence of methotrexate caused increased production of both dihydrofolate reductase and interleukin-5 (IL-5).

In the examples below, an SV-40 late region [Okayama et al., Mol. Cell. Biol. 3:280 (1983)] or human β-globin [Lawn et al., Cell 21:647 (1980)] polyadenylation signal sequence was used downstream of the polylinker. These sequences are interchangeable and can also be used in the DHFR transcription unit in place of the SV-40 early region sequence (Lee et al., supra). The SV-40 early region sequence could also be used downstream of the polylinker. The only requirement for such substitutions is that the sequences used in such positions in the plasmids must be terminated by the restriction sites indicated in Fig. 1, if it is desired to later remove the polyadenylation signal sequence. Since the nucleotide sequences of all three are known, they can be chemically synthesized and/or adapted for such use using conventional methods.

The plasmids of the invention may optionally contain enhancer regions. Useful enhancers are obtained from animal viruses such as SV-40, polyoma virus, bovine papilloma virus, cytomegalovirus, retroviruses or adenoviruses. Ideally, the enhancer used should be from a virus for which the host cells are permissive (i.e., from a virus which normally infects cells of the host type).

The enhancer regions of a number of viruses are known [see, e.g., Luciew et al., Cell 33:705 (1983)]. It would be a routine matter to excise these regions based upon published restriction maps for a virus selected and, if necessary, to modify the termini to enable splicing the enhancer into the recombinant plasmids. Alternatively, the enhancers can be synthesized from published sequence data.

The only requirement for a selected enhancer is that it preferably not contain a restriction site(s) that would destroy the uniqueness of the above-mentioned sites. One of skill in the art could readily determine this from the published sequence data and the known restriction site sequences. Although the enhancers from many viruses can be used, the enhancer from SV-40 (Takebe et al., supra) is preferred for use in this invention. Although the location of the enhancers within the plasmids is not critical, the HindIII restriction site is a convenient point for insertion.

A wide range of heterologous genes can be inserted into the plasmids, including but not limited to genes encoding blood clotting or fibrinolytic enzymes, regulatory proteins such as the various lymphokines or hormones, growth factors, oncogene products and soluble or cell-surface antigens and receptors. One of the plasmids of the invention has been used to express a gene encoding a soluble form of a gamma interferon receptor. In the examples below, the use of another plasmid to express a gene encoding IL-5 is illustrated.

Such genes will often contain wild-type translated sequences encoding prepro-polypeptides (e.g., secretory leaders), which may be desirable for the structure, stability and/or secretion of the mature proteins. Such polypeptides are removed during post-translational processing by the host cells. Alternatively, such prepro-sequences can be deleted by well known methods prior to insertion of the genes into the plasmids. The genes may also contain 5' and/or 3' noncoding sequences.

The genes or cDNAs to be expressed must contain translation initiation and stop codons, although the SV-40 or SRα promoter used provide the necessary transcription initiation signals.

Although the preferred order of elements in the plasmids is as described above, any element inserted upstream of the promoter for the heterologous gene, such as the β-gal or CAT transcription unit or the DHFR transcription unit, can be inserted downstream of the heterologous gene transcription unit instead. This can conveniently be done by the use of the XbaI restriction site.

The placement of the genetic elements and unique restriction sites within the plasmids facilitates the substitution of elements or the removal of elements for use in other plasmids or vectors. It also permits easy restoration of the original restriction sites after cleavage has been carried out.

For example, the DHFR transcription unit is easily removed. BglII/Klenow followed by HindIII/exoVII regenerates the BglII site. HindIII/Klenow followed by BglII/exoVII regenerates the HindIII site. Treatment of the polyadenylation signal sequence in the EcoRI/XbaI fragment by XbaI/EcoRI double digestion followed by Klenow polymerase and religation excises the sequence and regenerates both sites. XhoI/Klenow followed by PstI/exoVII and religation removes the XhoI/PstI fragment splicing signals and regenerates an XhoI site.

The plasmids of the invention can be used in many mammalian host cells, including primary explants from various tissues. The use of established and/or transformed cell lines, however, is preferred. Cell lines that can be used include but are not limited to the African green monkey kidney (COS), Chinese hamster ovary (CHO), NS-1, SP2/0, NIH 3T3, NIH 3T6, C127, CV-1, HeLa, mouse L and Bowes cell lines. Where co-amplification using the DHFR transcription unit is desired, dihydrofolate reductase-deficient cells such as CHO-dhfr$^-$ cells [Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)] are preferred.

Plasmid DNA can be introduced into mammalian cells in a number of ways. Transient expression studies in mammalian cells such as COS cells can be carried out using the DEAE-dextran method with chloroquine boost [Yokota et al.,Proc. Natl. Acad. Sci. USA 82:68 (1985)]. Transfection of suspension cells (e.g., NS-1) can be accomplished using the method of electroporation [Potter et al., Proc. Natl. Acad. Sci. USA 81:7161 (1984)]. Fibroblasts such as CHO cells can be transfected using the calcium phosphate precipitation method [Graham et al., Virology 52:456 (1973)]. All three of these methods are described below in detail.

Identification of cells harboring the plasmids can be made using standard methods. For example, host cell genetic material can be probed by Southern blot analysis. Expression of the desired heterologous gene can be detected by standard immunochemical or enzymatic analysis, or by bioassay.

EXAMPLES

In the examples that follow, percentages for solids in solid mixtures, liquids in liquids, and solids in liquids are given on a wt/wt, vol/vol and wt/vol basis, respectively, unless otherwise indicated. Sterile conditions were maintained during cell culture.

General Methods

Small scale isolation of plasmid DNA from saturated overnight cultures was carried out according to the procedure of Birnboim et al. [Nuc. Acids Res. 7:1513 (1979)]. This procedure allows the isolation of a small quantity of DNA from a bacterial culture for analytical purposes. Unless otherwise indicated, larger quantities of plasmid DNA were prepared as described by Clewell et al. [J. Bacteriol. 110:1135 (1972)].

Specific restriction enzyme fragments derived by the cleavage of plasmid DNA were isolated by preparative electrophoresis in agarose followed by electroelution (Maniatis et al., supra, p. 164). Gels measuring 9 x 5 1/2 cm were run at 50 mA for 1 hour in Tris-Acetate buffer (Maniatis et al., supra, p. 454) and then stained with 1 µg/ml ethidium bromide to visualize the DNA. Appropriate gel sections were excised and melted at 65°C for 10 minutes and then diluted with 5 ml of a low salt buffer containing 0.2 M NaCl, 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The DNA was then concentrated using a Elutip-D column (Schleicher and Schuell Inc., Keene, NH) following the manufacturer's instructions and precipitated at -20°C with ethanol in the presence of 10 µg of yeast tRNA carrier (Bethesda Research Laboratories, Bethesda, MD).

The restriction enzymes, DNA polymerase I (Klenow fragment) and T4 DNA ligase were products of New England Biolabs, Beverly, MA, and the methods and conditions for the use of these enzymes were essentially those of the manufacturer. T4 DNA ligation was carried out for 16 hours at 4°C in a buffer containing 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP and 50 mg/ml bovine serum albumin. Klenow blunt-ending of single-stranded DNA ends was carried out in restriction enzyme buffer which had been adjusted to contain 1 mM dGTP, dATP, dCTP and TTP.

Chemical Synthesis

Oligonucleotides used as linkers were either purchased from New England Biolabs, Beverly, MA, or prepared by standard methods using a Model 380A DNA synthesizer (Applied BioSystems, Foster City, CA).

Transformation and Transfection

E. coli cultures were transformed essentially as described by Maniatis et al., supra.

For transient expression in COS cells, the plasmid DNAs were introducted by DEAE-dextran treatment with a chloroquine boost [Yokota et al., Proc. Natl. Acad. Sci. USA 82:68 (1985)]. Briefly, 10 ml of a suspension of COS cells were plated onto 100 mm culture dishes at approximately 10^5 cells/ml in DME medium, penicillin/streptomycin (pen/strep) and 2 mM glutamine (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). After the cells reached approximately 50% confluence, the medium was removed and the cells were washed with 10 ml of serum-free DME.

A plasmid DNA-DME-DEAE-dextran mixture was prepared as follows: for each plate, DME medium (4.2 ml) supplemented with 50 mM Tris-HCl, pH 7.4, glutamine and antibiotics as described above was mixed with 80 µl of DEAE-dextran (20 mg/ml in sterile, distilled water; Pharmacia, Piscataway, NJ). Plasmid DNA (10 µg/dish) was added to 4.2 ml of the DME-DEAE-dextran mixture, and the mixture was added to the washed cells. Each 100 mm culture dish was incubated for 4 hours, the DNA solution was aspirated from the plate, and the plate was washed with 5 ml of serum-free DME. After the addition of 5 ml of chloroquine solution (150 µM chloroquine in DME, with 7% fetal bovine serum), the cells were incubated for 3 hours. After this incubation, the chloroquine was removed by aspiration and the plate washed with 5 ml of DME and aspirated. Each plate then received 10 ml of DME (10% fetal bovine serum, antibiotics and glutamine as above) and was incubated for approximately 72 hours. The cells were collected or the conditioned medium was harvested for bioassay.

For stable transfection of myeloma cells, the plasmid DNAs were introducted by electroporation [Potter et al., Proc. Natl. Acad. Sci. USA 81:7161 (1984)]. Briefly, approximately 10 ml of myeloma cells growing in suspension in complete DME medium supplemented as above (5×10^5 cells/ml) were centrifuged and resuspended in 1 ml of complete DME. Plasmid DNA (40 µg) was added to the 1 ml suspension and the cell/DNA mixture was divided into 0.25 ml aliquots. Each aliquot of cells was placed in an electroporation chamber and shocked with an electric pulse of 300 volts and 960 microFd capacitance using a Gene Pulser (Bio-Rad, Rockville Centre, NY). The cells were held at room temperature for 10 minutes and then the four aliquots were combined and placed in a plastic T75 tissue culture flask in a total volume of 10 ml of complete DME. After a 3-day incubation period, the cells were diluted with selection medium to a final concentration of 0.5 µg/ml mycophenolic acid (BRL/Life Technologies, Gaithersburg, MD), 100 µg/ml Xanthine (Sigma, St. Louis, MO), 15 µg/ml Hypoxanthine (Sigma), 10% fetal bovine serum and 880 µg/ml glutamine. The cells were diluted to give approximately 1,000 cells/0.2 ml/well of a 96-well tissue culture plate. Colonies of transfected cells could usually be seen forming in 7-14 days. Cells were collected or conditioned medium was harvested for assay as described above.

The calcium phosphate precipitation method was used for the stable transfection of fibroblasts with plasmid DNAs. The protocol included in the Mammalian Transfection Kit (Stratagene, LaJolla, CA) was followed. Briefly, fibroblasts were plated into a 100 mm tissue culture dish at 5×10^5 cells/dish in complete DME medium prepared as described above plus 1X nonessential amino acids (100X stock from Gibco), and 4 µg/ml thymidine and 15 µg/ml hypoxanthine (both contained in 100X stock from Gibco). After a 24 hour incubation, 10 µg of plasmid DNA (as a calcium phosphate precipitate) were added per plate. The cells were incubated for 24 hours, and the plates were washed with serum-free DME medium and aspirated.

The cells were incubated for 24 hours, split 1:10 and allowed to grow for 24 hours before the addition of selection medium. The selection medium contained DME, glutamine and nonessential amino acids as described above, plus 5% dialyzed fetal bovine serum (Gibco) without antibiotics. After colonies began to form, medium containing methotrexate was added stepwise until cells were obtained which could grow at a 1 μM level of methotrexate. This produced amplification of the integrated locus [Kaufman et al., J. Mol. Biol. 159:601 (1982)]. Cells were harvested or conditioned medium was collected for assay as described above.

Cell Cultures

E. coli strain C600 (ATCC 23724) was used for plasmid constructions. The cells were grown in 20:10:5 TYE medium (20 g tryptone, 10 g yeast extract, 5 g NaCl per liter).

COS cells (ATCC CRL 1650) were cultured in DME medium (Gibco, Grand Island, NY) plus antibiotics, 2 mM glutamine (Gibco) and 10% fetal bovine serum (Hyclone, Logan, UT) as described above. NS-1 cells (ATCC TIB 18) were cultured in DME medium like the COS cells. CHO dhfr$^-$ cells (clone DXB-11) were obtained from Dr. L. Chasin, Columbia University, NY. These cells were routinely carried in complete DME medium with added 1X nonessential amino acids, thymidine and hypoxanthine (from 100X stock, Gibco).

Construction of Plasmid pDSVS

Some of the starting plasmids and elements contained in them have been described by Lee et al. [Nature 219:228 (1981)], Zavodny et al. [J. Interferon Res. 8:483 (1988)] and Okayama et al. [Mol. Cell. Biol. 3:280 (1983)].

The unique BglII site of plasmid pMTV-dhfr was destroyed by digestion with BglII followed by fill-in of the protruding 5' overhangs with DNA polymerase Klenow fragment to form plasmid pMTV-DHFR. A DNA fragment containing the murine dihydrofolate reductase cDNA and SV-40 early region polyadenylation signal was obtained by elution of the large EcoRI/NdeI DNA restriction fragment of pL23 followed by restriction with HindIII.

The resulting HindIII/EcoRI fragment of pL23 (containing the SV-40 polyadenylation signal and dhfr cDNA) was inserted into EcoRI/HindIII-digested plasmid pMT11S to form plasmid pL12R-A. The unique HindIII site of plasmid pL12R-A was converted to a BglII site by Klenow polymerase fill-in of the HindIII-generated 5' overhangs followed by ligation of BglII linkers (CAGATCTG; No. 1036, New England Biolabs), BglII digestion, ligation and transformation of E. coli strain C600 to form plasmid pL13R-B. The unique BamHI site of plasmid pL13R-B was converted to a HindIII site by Klenow polymerase fill-in of the BamHI-generated 5' overhangs followed by ligation of HindIII linkers (CAAGCTTG; No. 1022, New England Biolabs), HindIII digestion, ligation and transformation of E. coli strain C600 to form pL14R.

The fragment containing the pcD-derived SV-40 splicing signal sequence and murine gamma interferon cDNA was removed from plasmid pMgamma18 by XhoI digestion, and the plasmid backbone was religated to form plasmid pL27B. The DNA fragment containing the SV-40 early promoter and SV-40 late region polyadenylation signal of plasmid pL27B was removed by AccI restriction and Klenow polymerase fill-in of the 5' overhangs, ligation of PstI linkers (GCTGCAGC; No. 1024, New England Biolabs), and PstI/HindIII simultaneous digestion. The resulting HindIII/PstI fragment containing the SV-40 DNAs described above was ligated to HindIII/PstI-digested pL14R to generate plasmid pL15R-B. The BamHI restriction site immediately adjacent to the unique HindIII site of pL15R-B was removed by BamHI partial digestion to generate linearized DNA. This was followed by Klenow polymerase fill-

in of the 5' overhangs and religation of the plasmid to generate plasmid pL602.

The small EcoRI/PstI fragment of pL602 was removed by EcoRI/PstI digestion and replaced with a synthetic DNA containing compatible EcoRI/PstI overhangs but which resulted in the loss of both sites, due to lack of reconstitution of the complete recognition sequence. In addition, the synthetic DNA contained the recognition sequence for XbaI. The nucleotide sequences of the synthetic DNAs, AB176 and AB177, are shown in Fig. 2C. The resulting plasmid, pL603, lacked the EcoRI/PstI fragment of pL602 and contained a unique XbaI site in its place.

The SV-40 late region polyadenylation signal was removed from plasmid pCDV-1 by AccI/XhoI digestion, Klenow polymerase fill-in of the 5' overhangs, ligation to EcoRI linkers (GGAATTCC; No. 1020, New England Biolabs), EcoRI digestion and insertion into EcoRI-digested plasmid pMT11S to form plasmid pKMP-1. The unique XhoI site of plasmid pKMP-1 was removed by XhoI digestion, Klenow polymerase fill-in of the 5' overhangs and religation to form plasmid pKMP-2.

The DNA fragment containing the SV-40 early promoter and intron from plasmid pL1 was removed by PstI digestion, S1 Nuclease digestion of the 3' overhang, Klenow polymerase fill-in (of any 5' overhangs produced by S1 digestion), ligation of XbaI linkers (CTCTAGAG; No. 1032, New England Biolabs), and simultaneous restriction with XbaI and HindIII. The resulting HindIII-XbaI-linkered fragment was inserted into HindIII/XbaI-restricted plasmid pMT11S to form plasmid pKML-1. The DNA fragment containing the SV-40 early promoter and intron was removed from plasmid pKML-1 by HindIII/BglII digestion and inserted into HindIII/BamHI-digested pUC13 to create plasmid pUCL1-E. The overhangs created by BamHI and BglII are identical, so the DNA fragments annealed. Both sites, however, were

destroyed because the entire recognition sequences for both enzymes were lost.

The BamHI site of pUCL1-E was destroyed by BamHI digestion, Klenow polymerase fill-in of the 5' overhangs and religation to create pUCL2-A. The SV-40-containing HindIII/XbaI fragment of pUCL2-A was inserted into HindIII/XbaI-cut plasmid pKMP-2 to generate plasmid pcDL-4. The BglII and XbaI sites of plasmid pcDL-4 were destroyed by BglII/XbaI restriction followed by exonuclease VII digestion of the protruding 5' overhangs and religation to generate plasmid pL64G. The XhoI/BamHI fragment of plasmid pL64G containing the SV-40 intron was inserted into XhoI/BamHI-digested pL603 to generate plasmid pT443-2.

The unique NdeI site in plasmid pcDV-1 was converted to an XbaI site by NdeI digestion, Klenow polymerase fill-in of the 5' overhangs, ligation to XbaI linkers (CTCTAGAG; No. 1032, New England Biolabs), XbaI digestion and religation to form plasmid pT512-2. The SV-40 polyadenylation signal-containing DNA fragment of plasmid pT443-2 was replaced with the SV-40 polyadenylation signal-containing fragment of pT512-2 by BamHI/XbaI digestion and ligation to form plasmid pT514-2. A synthetic DNA fragment containing restriction sites for BamHI, SmaI and EcoRI (the original BamHI site of pT514-2 was lost on cloning) was inserted between the BamHI and SalI sites of plasmid pT514-2 to create plasmid pT519-1. The nucleotide sequences of the DNAs used, AB199 and AB200, are shown in Fig. 2G. The two HindIII sites of plasmid pL23 were converted to BglII sites by HindIII digestion, Klenow fill-in of the 5' overhangs on both DNA fragments, ligation of BglII linkers (CAGATCTG; No. 1036, New England Biolabs), digestion with BglII and ligation of the DNA mixture to create plasmid pLT7-A.

Finally, the DNA fragment containing the MMTV-LTR promoter was removed from plasmid pLT7-A by digestion with BglII and inserted into the unique BglII site of plasmid pT519-1, to create the general eukaryotic expression vector pDSVS.

Construction of Plasmid pDSRS

To replace the SV-40 promoter in pDSVS with an SRα promoter [Takebe et al., Mol. Cell. Biol. 8:466 (1988)], the SRα promoter was excised from plasmid pcDL-SRα296 by digestion with HindIII and and PstI. The DNA fragment containing the SRα promoter was electroeluted from an agarose gel and ligated to HindIII/PstI - digested pDSVS to generate pDSRS. This construction is shown schematically in Fig. 3.

Construction of Plasmid pSRS

Synthetic oligonucleotides AB361 and AB362 (Fig. 4) were synthesized as described above. When annealed, these oligonucleotides produced a double-stranded DNA with a BglII overhang at one end and a HindIII overhang at the other. The synthetic DNAs were annealed by heating to 95°C in a volume of 20 microliters and then allowed to reach room temperature.

The general expression plasmid pDSVS was digested with BglII and HindIII and electroeluted from an agarose gel. The annealed oligonucleotides were ligated to BglII/HindIII-digested pDSVS to produce the plasmid pSVS. The result was the removal of the dhfr transcription unit, with the regeneration of both restriction sites. The SRα promoter was then substituted for the SV40 promoter of pSVS. The SRα promoter was isolated from plasmid pcDL-SRα296 by digestion with HindIII and XhoI. The promoter element was ligated to HindIII/XhoI-digested pSVS to generate plasmid pSRS.

Construction of Plasmids pDSRG and pSRG

Synthetic oligonucleotides AB523 and AB524 (Fig. 5) were synthesized as described above. When annealed, these olibonucleotides introduced restriction sites for XhoI, BamHI, BglII, and SalI between the existing EcoRI and HindIII sites of pUC19. The AB523 and AB524 synthetic oligonucleotides were annealed as above and ligated to EcoRI/HindIII-digested pUC19 to produce plasmid p679-2.

The human beta globin 3' untranslated region was obtained by BamHI digestion of plasmid pBut-7 and ligated to BglII-digested p679, to produce plasmid p658J. This plasmid was used at the source for the human beta globin polyadenylation signal in the SRα promoter containing expression plasmids. Plasmid p658J was digested with HindIII, end-filled with DNA Klenow fragment, the Klenow was inactivated, and the plasmid was digested with EcoRI. This fragment was ligated to XbaI-digested, Klenow-treated and EcoRI-digested pDSRS and pSRS, to produce pDSRG and pSRG, respectively.

Construction of Plasmid pGSRG-hIL5

Human IL-5 cDNA was removed from plasmid pcD-SRα-hIL5 by the polymerase chain reaction (PCR) [Saiki et al., Science 239:487 (1988)]. The forward primer, AB790 (Fig. 6), was designed to introduce SalI restriction site upstream of a modified initiation region representing an optimum Kozak configuration [Kozak, J. Cell. Biol. 108:229 (1989)]. The reverse primer, AB791 (Fig. 6), was designed to introduce an EcoRI restriction site downstream of the termination codon for human IL-5. The directions of the primers are shown by the arrows in Fig. 6. After 30 cycles of PCR (95°C for 2 min., 40°C for 2 min. and 72°C for 2 min.) the amplified DNA fragment was digested with SalI/HindIII and ligated to SalI/HindIII-digested pSRS to produce the expression plasmid pSRS-hIL5. The expression cassette for human IL-5 containing the SRα promoter, splice sites, and hIL-5

cDNA up to the EcoRI site was removed from plasmid pSRS-hIL5 by HindIII/EcoRI digestion and ligated to HindIII/EcoRI-digested pDSRG and pSRG, to produce pDSRG-hIL5 and pSRG-hIL5, respectively. The dominant marker, E. coli xanthine-guanine phosphoribosyltransferase (gpt), was then introduced into the hIL-5 expression plasmid pSRG-hIL5. The region containing the E. coli. gpt transcription unit, a portion of the rearranged immunoglobulin in the heavy chain variable region (Vh1), amplicillin marker and E. coli plasmid origin of replication was removed from plasmid pD8Ch2UK by digestion with XhoI and SalI, end-fill with Klenow polymerase as above, inactivation of the Klenow enzyme and digestion with XbaI. The region from XbaI to SalI containing the elements described above is identical to the XbaI to SalI region in the plasmid pSVD8tbeta [Schnee et al., Proc. Natl. Acad. Sci. USA 84:6904 (1987)]. This XbaI/SalI fragment was inserted into HindIII-digested, Klenow treated and XbaI-digested pSRG-hIL5 to produce pGSRG-hIL5. This human IL-5 expression plasmid will express human IL-5 using the SRα promoter, SV40 splice signals and human beta globin 3' untranslated region.

Interleukin-5 Bioassay

The erythroleukemic human cell line TF-1 was obtained from Dr. T. Kitamura (Tokyo University), although the murine BCL_1 cell line (ATCC TIB 197) described by Dutton et al. [J. Immunol. 132:2451 (1984)] could have been used instead. TF-1 cells were routinely cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum, 1 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), and pen/strep. For bioassay, the cells were washed twice in phospate buffered saline (PBS, Gibco) and aliquoted into 96-well plates in RPMI 1640 at 4 x 10^4 cells/well. An equal volume containing test sample in RPMI 1640 and 10% fetal bovine serum was added to the cells. The cells were incubated for 48 hours at 37°C, 10 µg of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT,

Sigma) dye dissolved in PBS were added to each well, and the cells were incubated for 6 hours.

A 1:1 dilution in each well was made using acid-isopropanol (0.1M HCl:95% isopropanol), and the contents of the wells were thoroughly mixed. The colorimetric assay of Mosmann [J. Immunol. Methods 65:55 (1983)] was carried out using a V-max spectrophotometer (Molecular Devices, Palo Alto, CA) set at 570 nm. Blank absorbance at 630 nm was subtracted from the measurements at 570 nm. Activity (expressed in units/ml) was defined as the inverse of the dilution of the sample required to give half-maximal proliferative induction of TF-1 cells when cultured for 48 hours.

Use of pDSVS Derivatives to Produce Recombinant Human IL-5

The Human IL-5 expression plasmids described above were transfected into three different cell lines and the conditioned media from the cultures were assayed for human IL-5 biological activity, using the TF-1 cell bioassay. Conditioned medium from COS cells was prepared as described above. Recombinant CHO cells, either unamplified or selected to grow in 1 μM methotrexate, were seeded into 5 ml of DME medium (plus 10% fetal bovine serum, 1X nonessential amino acids and 880 μg/ml glutamine without antibiotics) a 5×10^5 cells/ml and cultured for 72 hours. Cell culture conditioned medium was harvested and passed through a 0.22 μ Nalgene low protein binding syringe filter in preparation for bioassay. Recombinant myeloma cells (NS-1, ATCC TIB 18) were seeded into 5 ml of HB101 medium (Hana Biological, Alameda, CA) supplemented with 800 μg/ml glutamine at 5×10^5 cells/ml and cultured for 72 hours as described above for CHO cells. After incubation, the conditioned medium was harvested for bioassay, with the results shown in Table I.

Table I
Expression Level (units/ml) of Human IL-5

Expression Plasmid	COS cells	CHO Cells[a]	CHO cells[b]	NS-1
pSRS-hIL5	2,000	n.d.	n.d.	n.d.
pDSRG-hIL5	n.d.	<2,000	20,000	n.d.
pSRG-hIL5	2,000	n.d.	n.d.	n.d.
pGSRG-hIL5	n.d.	n.d.	n.d.	12,000

[a] Plasmids were unamplified.
[b] Plasmids were amplified in 1 μM methotrexate.
n.d. = not determined.

The data of Table I show that human IL-5 was efficiently produced in all three cell types. The IL-5 biological activity in the conditioned media ranged from 2,000 units/ml in COS cells (transient expression) to 20,000 units/ml in amplified CHO cells. Production in the CHO cells increased more than ten fold upon selection with methotrexate. The unamplified production of IL-5 in the NS-1 cells, in view of the variability of the bioassay used, was roughly comparable to the amplified production in the CHO cells.

Plasmid Deposits

Plasmids pDSVS, pDSRS, pDSRG, pSRS and pSRG were deposited February 21, 1990 with the American Type Culture Collection, Rockville, MD, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the

Purposes of Patent Procedures. They have been assigned Accession Nos. ATCC 68231, 68232, 68233, 68234 and 68235, respectively.

Many modifications and variations of this invention may be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant plasmid comprising DNA sequences which, in the direction of transcription, contain:

(a) a β-lactamase gene and a pBR322 origin of replication delimited by an XbaI and a BglII restriction site,

(b) an SV-40 or SRα promoter delimited by a HindIII and an XhoI restriction site,

(c) a polylinker containing one or more unique restriction sites delimited by a PstI and an EcoRI restriction site, and

(d) a polyadenylation signal sequence delimited by an EcoRI and an XbaI restriction site.

2. The recombinant plasmid of claim 1 which further comprises a DHFR transcription unit comprising, in the direction of transcription, an MMTV-LTR promoter, a DHFR cDNA, a splicing signal sequence and a polyadenylation signal sequence, which transcription unit is between the β-lactamase gene and pBR322 origin of replication and the SV-40 or SRα promoter and is delimited by a BglII and a HindIII restriction site.

3. The recombinant plasmid of claim 1 which further comprises a β-gal transcription unit between the β-lactamase gene and pBR322 origin of replication and the SV-40 or SRα promoter, which β-gal transcription unit is delimited by a BglII and a HindIII restriction site.

4. The recombinant plasmid of claim 1 which further comprises a CAT transcription unit between the β-lactamase gene and pBR322 origin of replication and the SV-40 or SRα promoter, which CAT transcription unit is delimited by a BglII and a HindIII restriction site.

5. The recombinant plasmid of any one of claims 1 to 4 which further comprises a splicing signal sequence between the SV-40 or SRα promoter and the polylinker, which splicing signal sequence is delimited by an XhoI and a PstI restriction site.

6. The recombinant plasmid of any one of claims 1 to 5 which further comprises a viral enhancer.

7. The recombinant plasmid of claim 1 which is pDSVS.

8. The recombinant plasmid of claim 1 which is pDSRS.

9. The recombinant plasmid of claim 1 which is pSRS.

10. The recombinant plasmid of claim 1 which is pDSRG.

11. The recombinant plasmid of claim 1 which is pSRG.

12. The recombinant plasmid of claim 1 which is pGSRG-hIL5.

13. The recombinant plasmid of claim 1 which is pDSRG-hIL5.

14. An E. coli bacterium harboring the recombinant plasmid of any one of claims 1 to 13.

15. A mammalian cell harboring the recombinant plasmid of any one of claims 1 to 14.

Figure 1

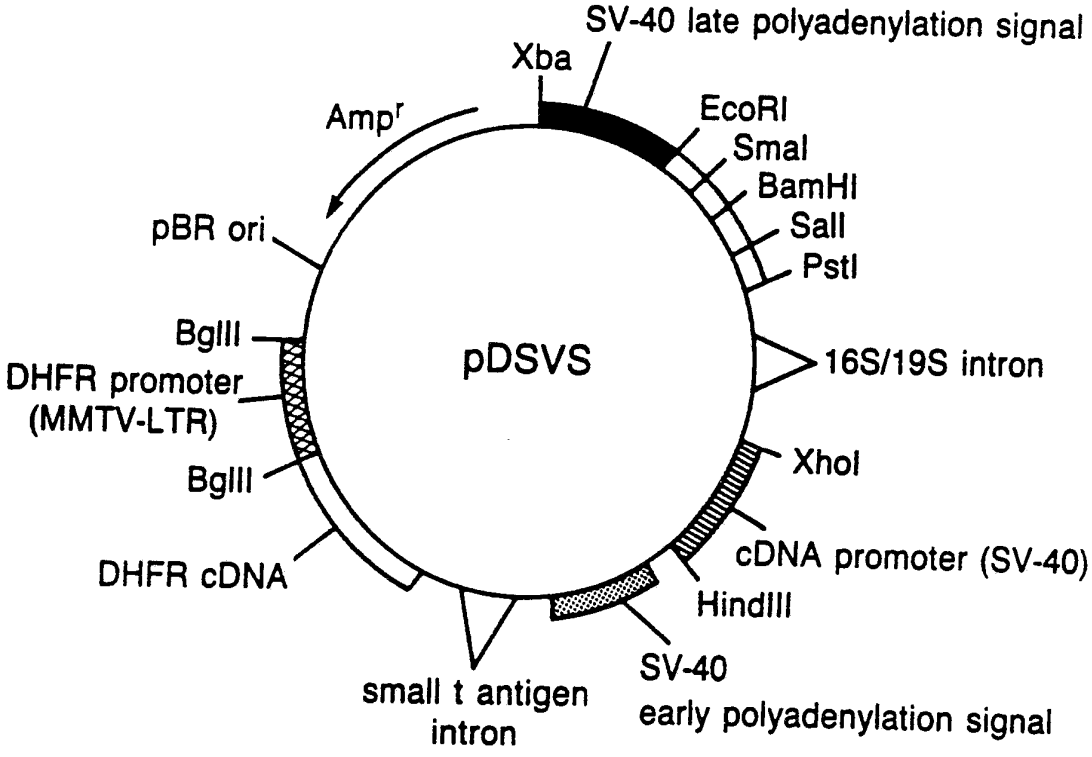
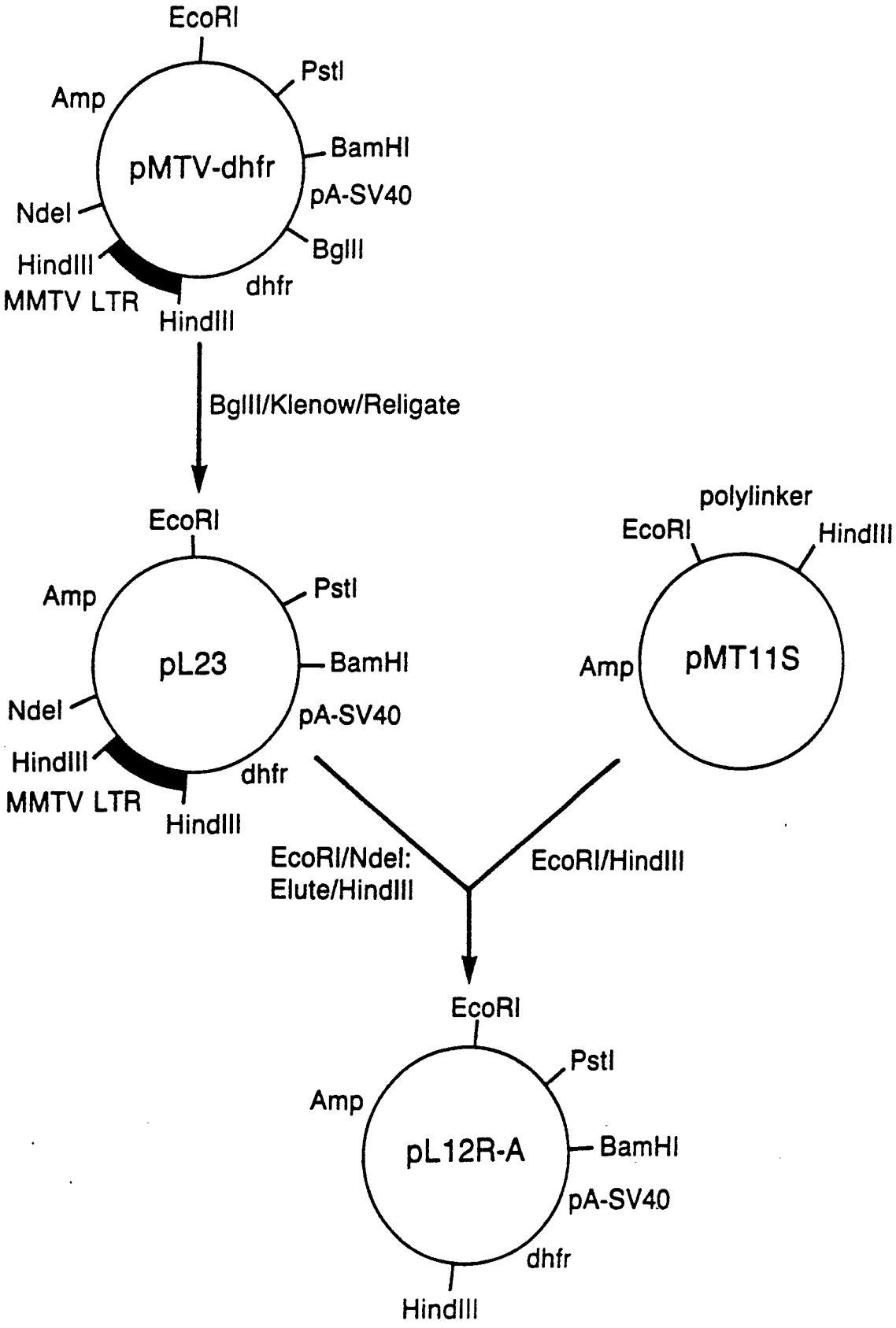

SV-40 late polyadenylation signal
Xba
Amp^r
EcoRI
SmaI
BamHI
SalI
PstI
pBR ori
BglII
DHFR promoter
(MMTV-LTR)
pDSVS
16S/19S intron
BglII
XhoI
DHFR cDNA
cDNA promoter (SV-40)
HindIII
SV-40
early polyadenylation signal
small t antigen
intron

Figure 2A


EcoRI
PstI
Amp
pMTV-dhfr
BamHI
pA-SV40
NdeI
BglII
HindIII
MMTV LTR
dhfr
HindIII
BglII/Klenow/Religate
EcoRI
Amp
PstI
pL23
BamHI
NdeI
pA-SV40
HindIII
MMTV LTR
dhfr
HindIII
polylinker
EcoRI
HindIII
Amp
pMT11S
EcoRI/NdeI:
Elute/HindIII
EcoRI/HindIII
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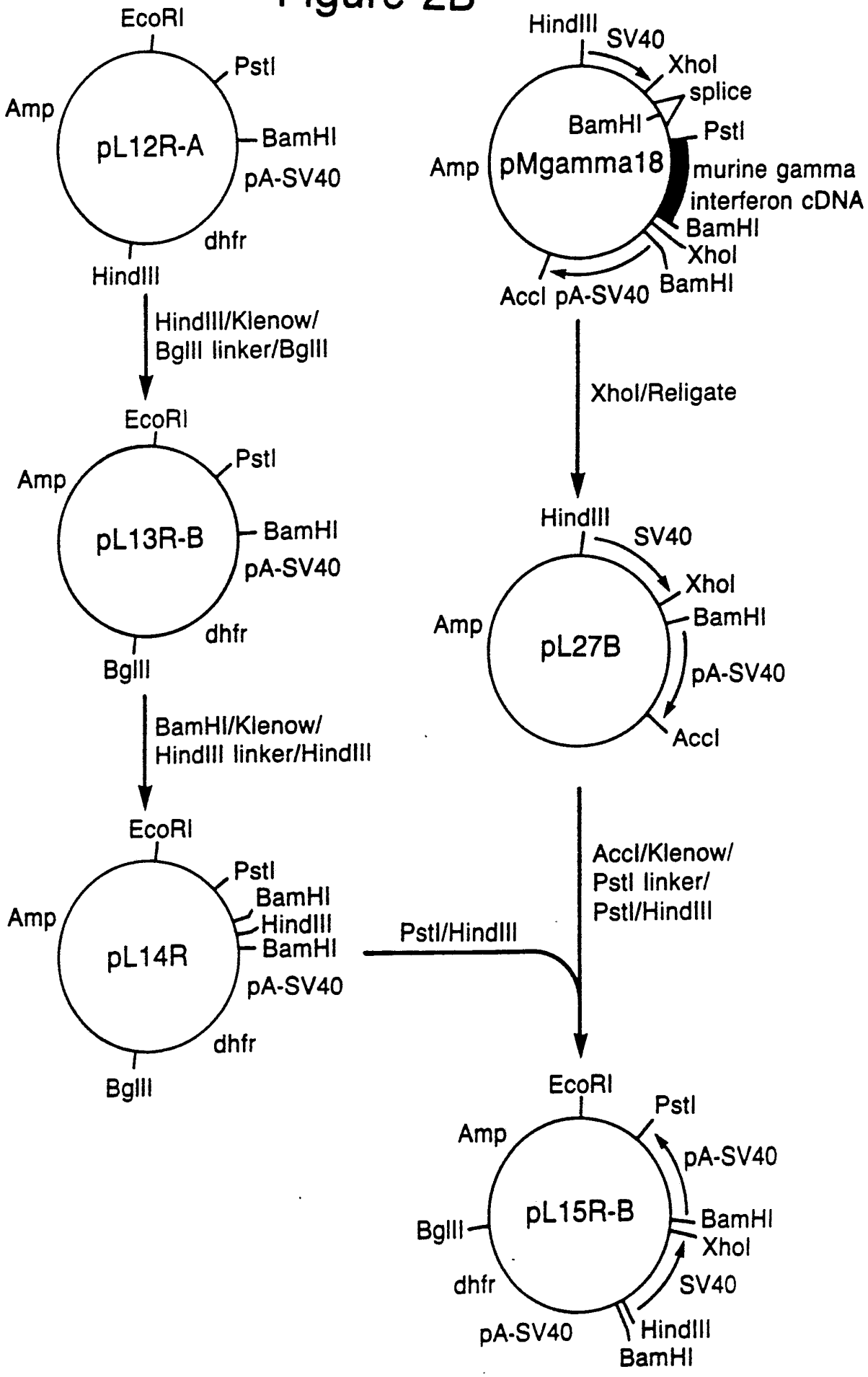
PstI
Amp
pL12R-A
BamHI
pA-SV40
dhfr
HindIII

Figure 2B


EcoRI
PstI
Amp
pL12R-A
BamHI
pA-SV40
dhfr
HindIII
HindIII/Klenow/
BglII linker/BglII
EcoRI
PstI
Amp
pL13R-B
BamHI
pA-SV40
dhfr
BglII
BamHI/Klenow/
HindIII linker/HindIII
EcoRI
PstI
BamHI
Amp
HindIII
pL14R
BamHI
pA-SV40
dhfr
BglII
PstI/HindIII
HindIII
SV40
XhoI
splice
BamHI
PstI
Amp
pMgamma18
murine gamma
interferon cDNA
BamHI
XhoI
AccI
pA-SV40
BamHI
XhoI/Religate
HindIII
SV40
XhoI
BamHI
Amp
pL27B
pA-SV40
AccI
AccI/Klenow/
PstI linker/
PstI/HindIII
EcoRI
PstI
Amp
pA-SV40
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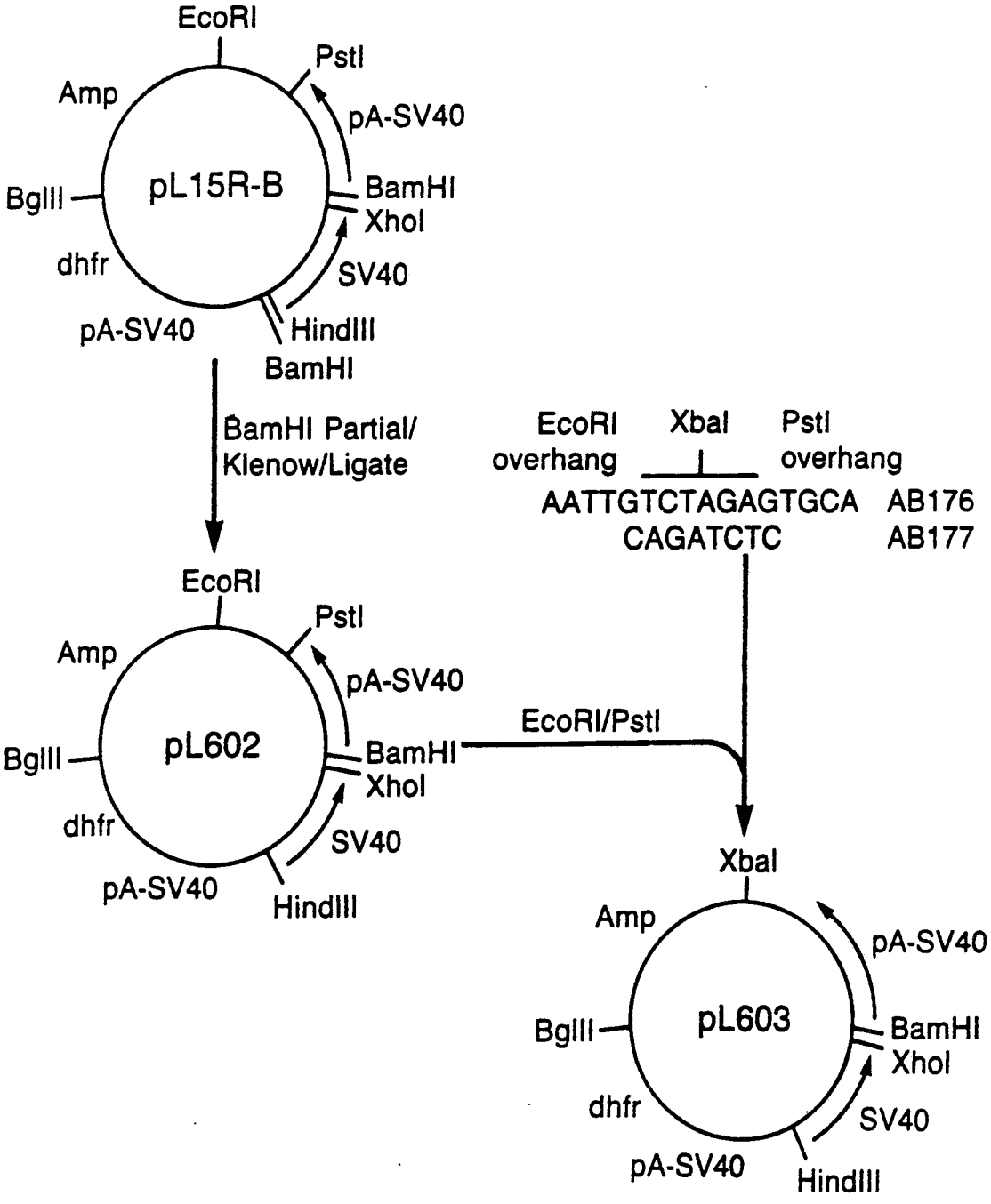
BamHI
BglII
XhoI
dhfr
SV40
pA-SV40
HindIII
BamHI

Figure 2C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PstI
Amp
pA-SV40
pL15R-B
BglII
BamHI
XhoI
dhfr
SV40
pA-SV40
HindIII
BamHI
BamHI Partial/
Klenow/Ligate
EcoRI
Xbal
PstI
overhang
overhang
AATTGTCTAGAGTGCA AB176
CAGATCTC AB177
EcoRI
PstI
Amp
pA-SV40
pL602
EcoRI/PstI
BglII
BamHI
XhoI
dhfr
SV40
pA-SV40
HindIII
Xbal
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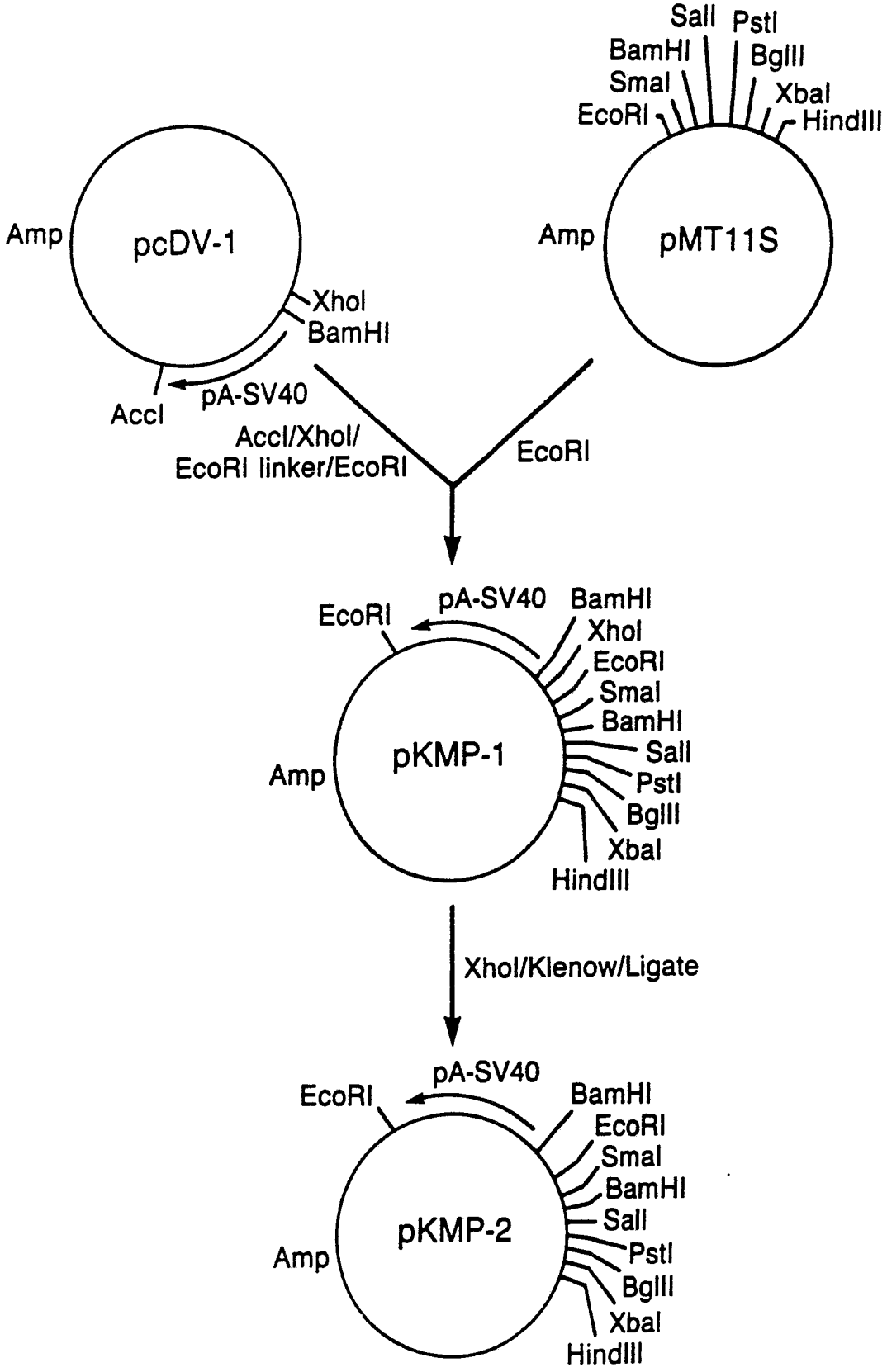
XhoI
dhfr
SV40
pA-SV40
HindIII

Figure 2D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PstI
BamHI
BglII
SmaI
XbaI
EcoRI
HindIII
Amp
pcDV-1
XhoI
BamHI
AccI
pA-SV40
AccI/XhoI/
EcoRI linker/EcoRI
Amp
pMT11S
EcoRI
EcoRI
pA-SV40
BamHI
XhoI
EcoRI
SmaI
BamHI
SalI
PstI
BglII
XbaI
HindIII
Amp
pKMP-1
XhoI/Klenow/Ligate
EcoRI
pA-SV40
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PstI
BglII
XbaI
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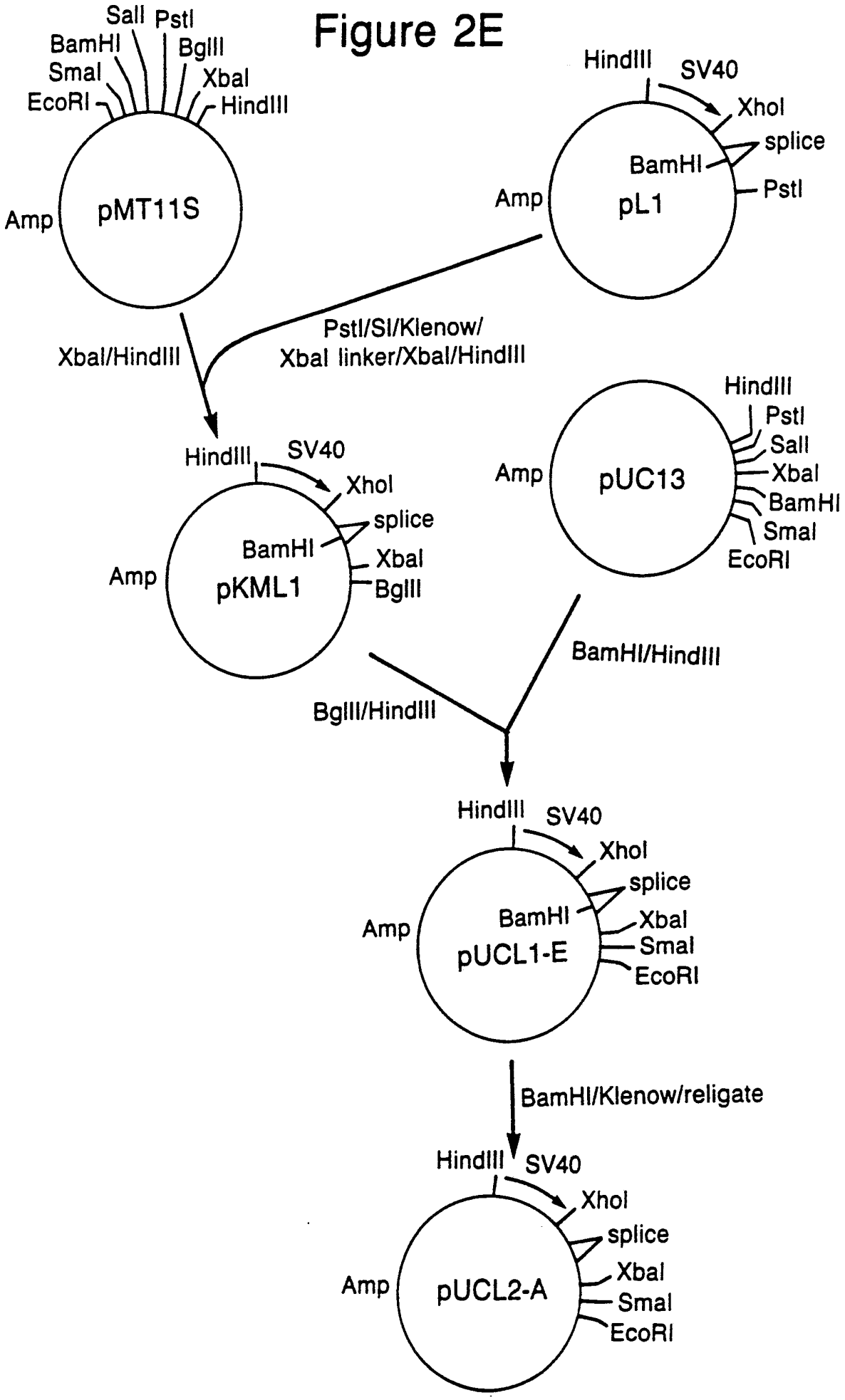
Amp
pKMP-2

Figure 2E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PstI
BamHI
BglII
SmaI
XbaI
EcoRI
HindIII
Amp
pMT11S
HindIII
SV40
XhoI
splice
BamHI
PstI
Amp
pL1
XbaI/HindIII
PstI/SI/Klenow/
XbaI linker/XbaI/HindIII
HindIII
SV40
XhoI
splice
BamHI
XbaI
Amp
pKML1
BglII
HindIII
PstI
SalI
XbaI
BamHI
SmaI
EcoRI
Amp
pUC13
BamHI/HindIII
BglII/HindIII
HindIII
SV40
XhoI
splice
BamHI
XbaI
SmaI
EcoRI
Amp
pUCL1-E
BamHI/Klenow/religate
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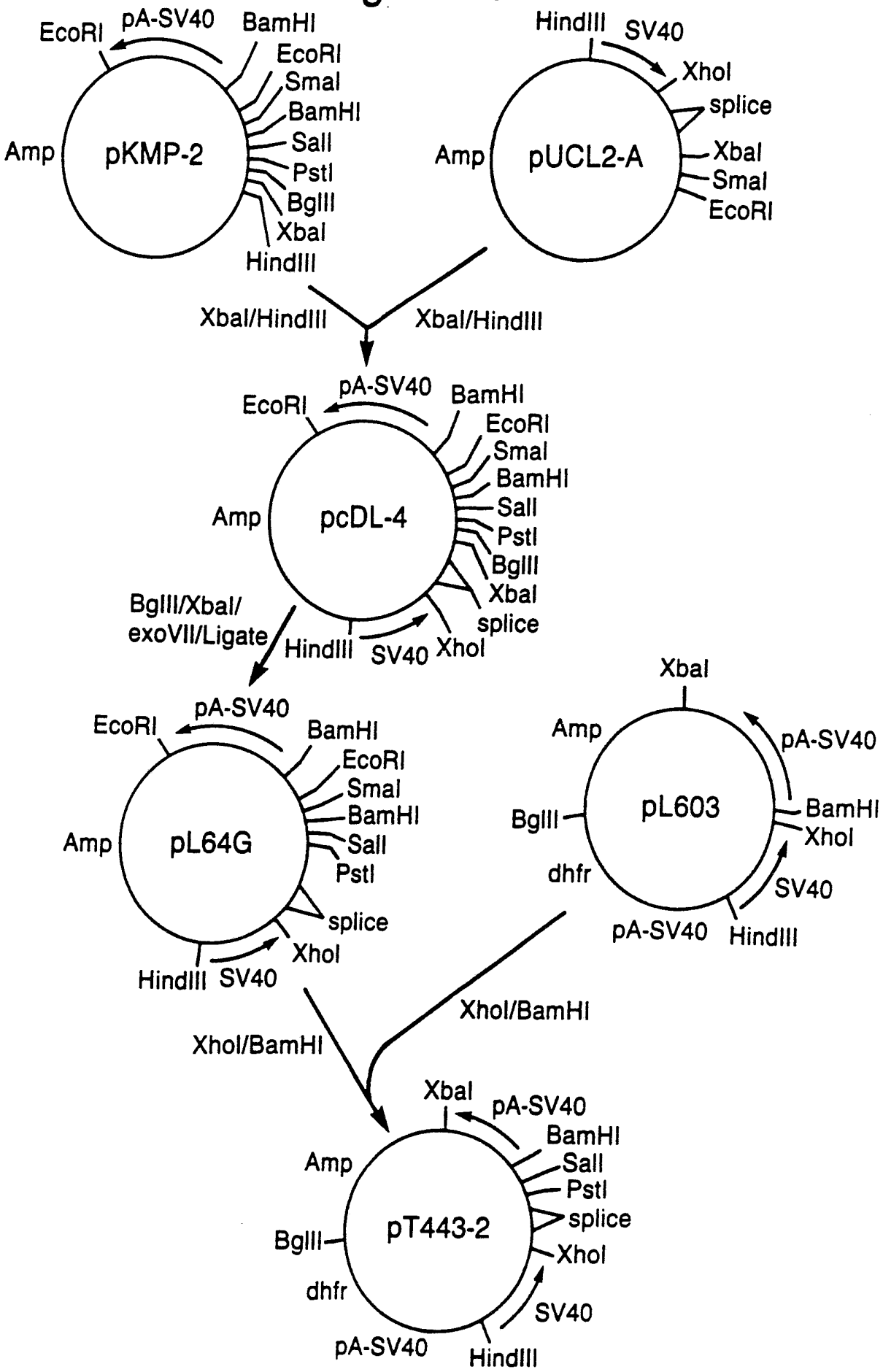
SV40
XhoI
splice
XbaI
SmaI
EcoRI
Amp
pUCL2-A

Figure 2F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pA-SV40
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SmaI
BamHI
SalI
PstI
BglII
XbaI
HindIII
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pKMP-2
HindIII
SV40
XhoI
splice
XbaI
SmaI
EcoRI
Amp
pUCL2-A
XbaI/HindIII
XbaI/HindIII
EcoRI
pA-SV40
BamHI
EcoRI
SmaI
BamHI
SalI
PstI
BglII
XbaI
splice
Amp
pcDL-4
BglII/XbaI/
exoVII/Ligate
HindIII
SV40
XhoI
EcoRI
pA-SV40
BamHI
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
Amp
pL64G
HindIII
SV40
XbaI
Amp
pA-SV40
BamHI
XhoI
SV40
HindIII
pA-SV40
BglII
dhfr
pL603
XhoI/BamHI
XhoI/BamHI
XbaI
pA-SV40
BamHI
SalI
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XhoI
SV40
HindIII
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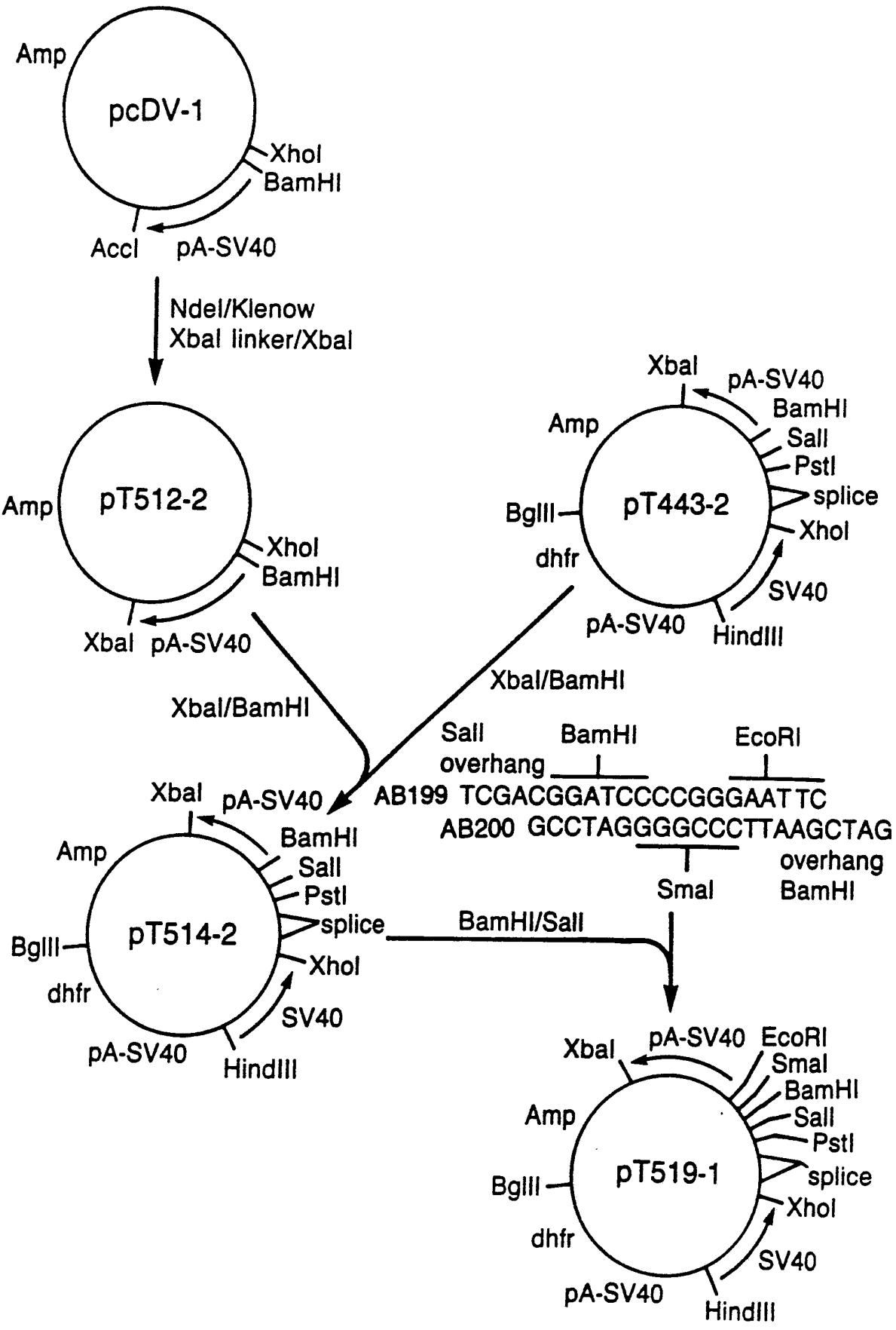
BglII
dhfr
pA-SV40
pT443-2

Figure 2G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XhoI
BamHI
AccI
pA-SV40
NdeI/Klenow
XbaI linker/XbaI
Amp
pT512-2
XhoI
BamHI
XbaI
pA-SV40
XbaI
pA-SV40
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
BglII
dhfr
Amp
pT443-2
pA-SV40
XbaI/BamHI
XbaI/BamHI
SalI
overhang
BamHI
EcoRI
AB199 TCGACGGATCCCCGGGAATTC
AB200 GCCTAGGGGCCCTTAAGCTAG
SmaI
overhang
BamHI
XbaI
pA-SV40
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
BglII
dhfr
Amp
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pA-SV40
BamHI/SalI
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
BglII
dhfr
Amp
pT519-1
pA-SV40

Figure 2H

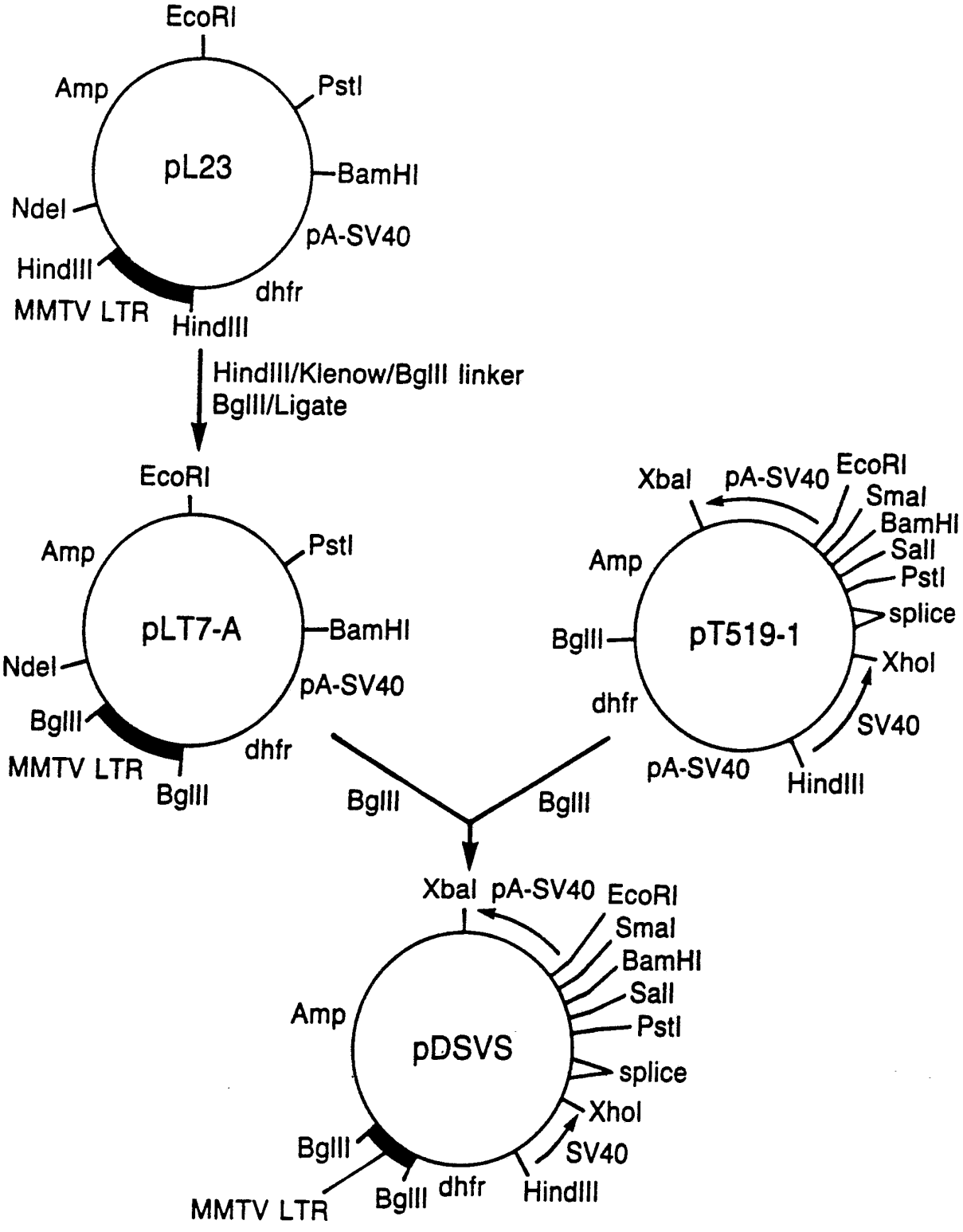

EcoRI
Amp
PstI
pL23
BamHI
NdeI
pA-SV40
HindIII
dhfr
MMTV LTR
HindIII
HindIII/Klenow/BglII linker
BglII/Ligate
EcoRI
Amp
PstI
pLT7-A
BamHI
NdeI
pA-SV40
BglII
dhfr
MMTV LTR
BglII
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
Amp
BglII
pT519-1
dhfr
pA-SV40
BglII
BglII
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
Amp
pDSVS
BglII
MMTV LTR
BglII
dhfr

Figure 3

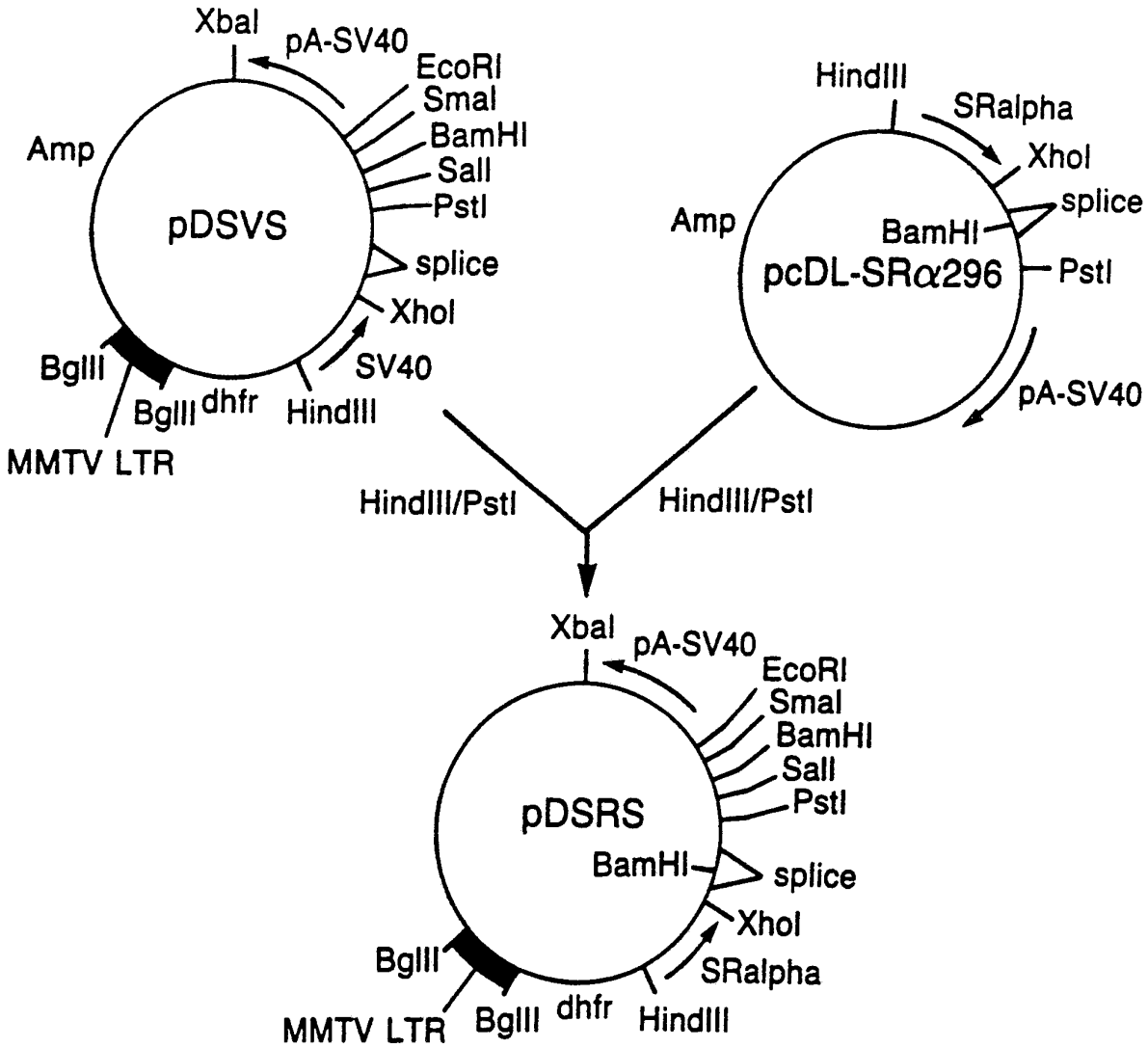

XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
dhfr
BglII
BglII
MMTV LTR
Amp
pDSVS
HindIII
SRalpha
XhoI
splice
PstI
pA-SV40
BamHI
pcDL-SRα296
Amp
HindIII/PstI
HindIII/PstI
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
BamHI
splice
XhoI
SRalpha
HindIII
dhfr
BglII
BglII
MMTV LTR
pDSRS

Figure 4

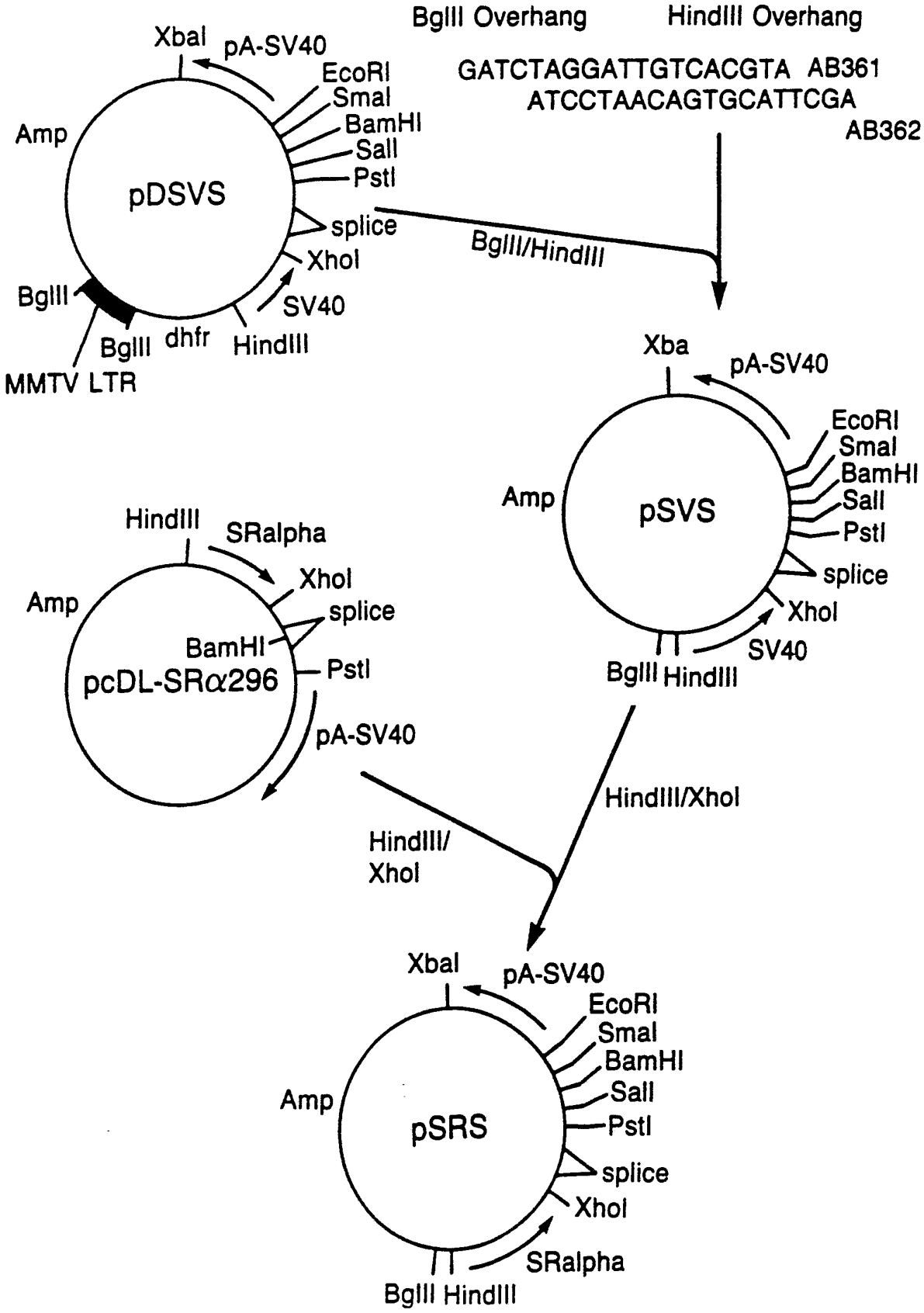

BglII Overhang
HindIII Overhang
GATCTAGGATTGTCACGTA AB361
ATCCTAACAGTGCATTCGA
AB362
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
HindIII
dhfr
BglII
MMTV LTR
Amp
pDSVS
BglII/HindIII
Xba
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SV40
BglII HindIII
pSVS
HindIII
SRalpha
XhoI
splice
BamHI
PstI
pA-SV40
pcDL-SRα296
HindIII/XhoI
HindIII/
XhoI
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SRalpha
BglII HindIII
pSRS

Figure 5A

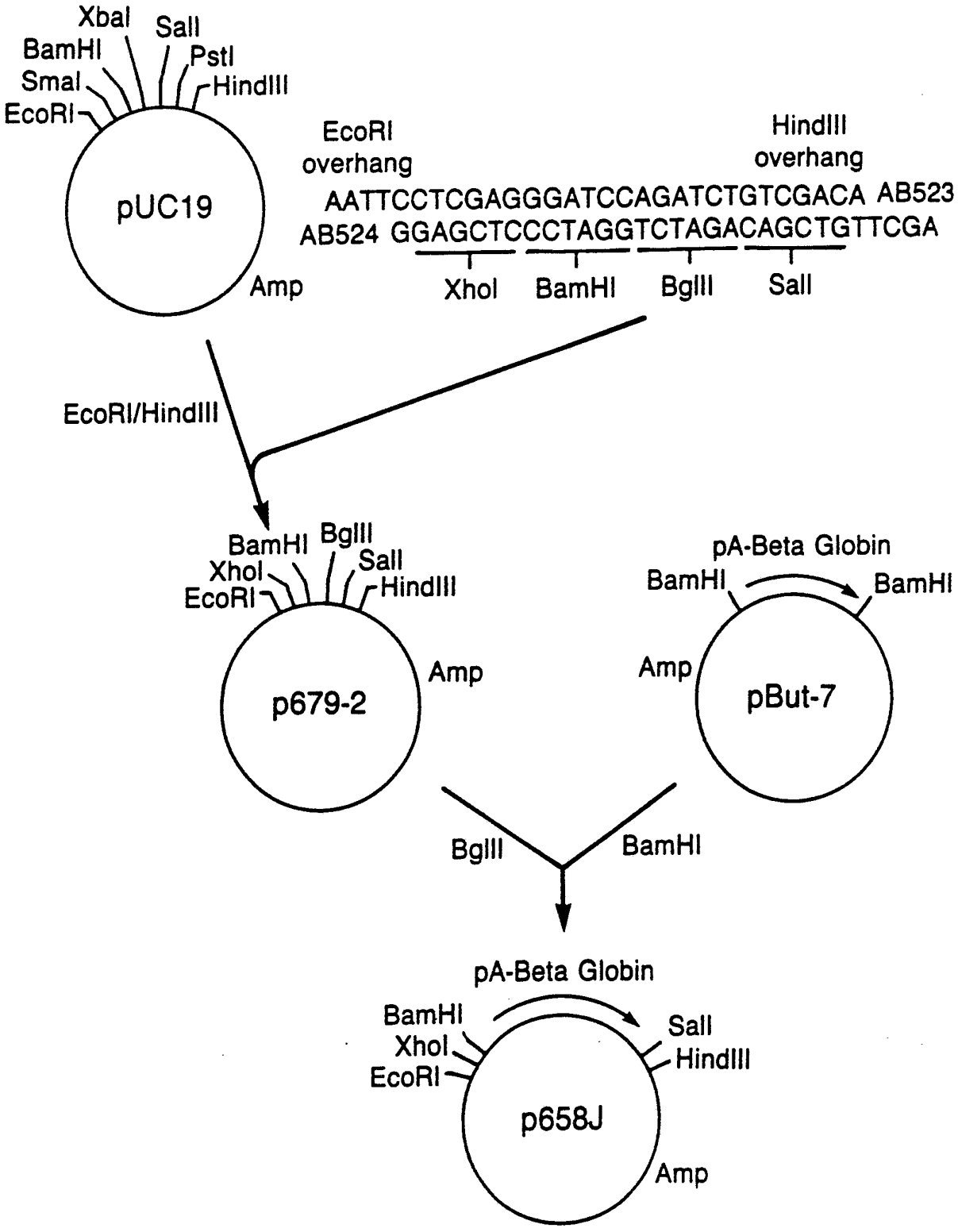

XbaI
SalI
BamHI
PstI
SmaI
HindIII
EcoRI
pUC19
Amp
EcoRI overhang
HindIII overhang
AATTCCTCGAGGGATCCAGATCTGTCGACA AB523
AB524 GGAGCTCCCTAGGTCTAGACAGCTGTTCGA
XhoI
BamHI
BglII
SalI
EcoRI/HindIII
BamHI
BglII
XhoI
SalI
EcoRI
HindIII
p679-2
Amp
pA-Beta Globin
BamHI
BamHI
Amp
pBut-7
BglII
BamHI
pA-Beta Globin
BamHI
SalI
XhoI
HindIII
EcoRI
p658J
Amp

Figure 5B

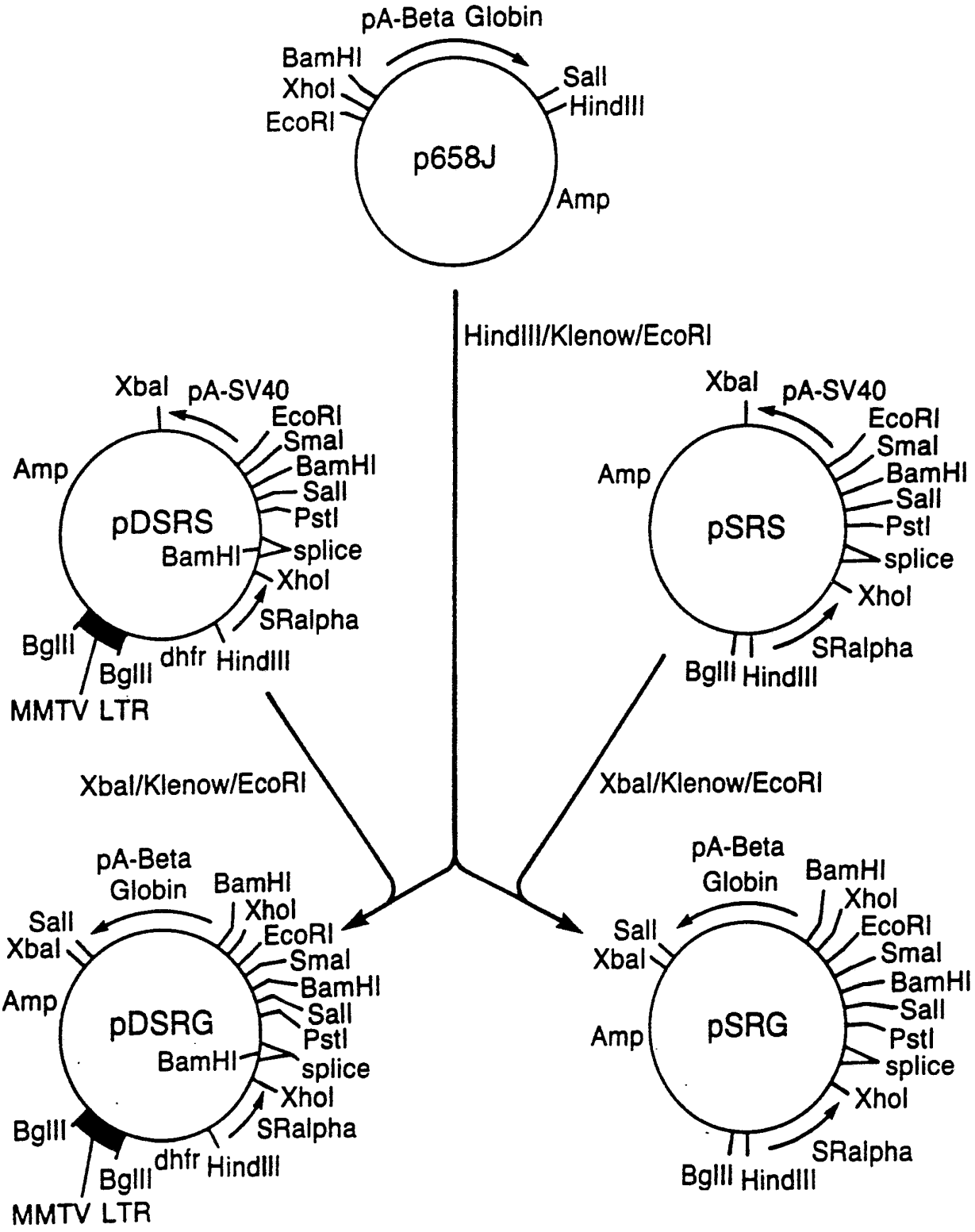

pA-Beta Globin
BamHI
XhoI
EcoRI
SalI
HindIII
p658J
Amp
HindIII/Klenow/EcoRI
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SRalpha
Amp
pDSRS
BamHI
BglII
BglII
dhfr
HindIII
MMTV LTR
pSRS
XbaI/Klenow/EcoRI
pA-Beta Globin
BamHI
XhoI
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SRalpha
SalI
XbaI
Amp
pDSRG
pSRG
BglII
HindIII
MMTV LTR

Figure 6A

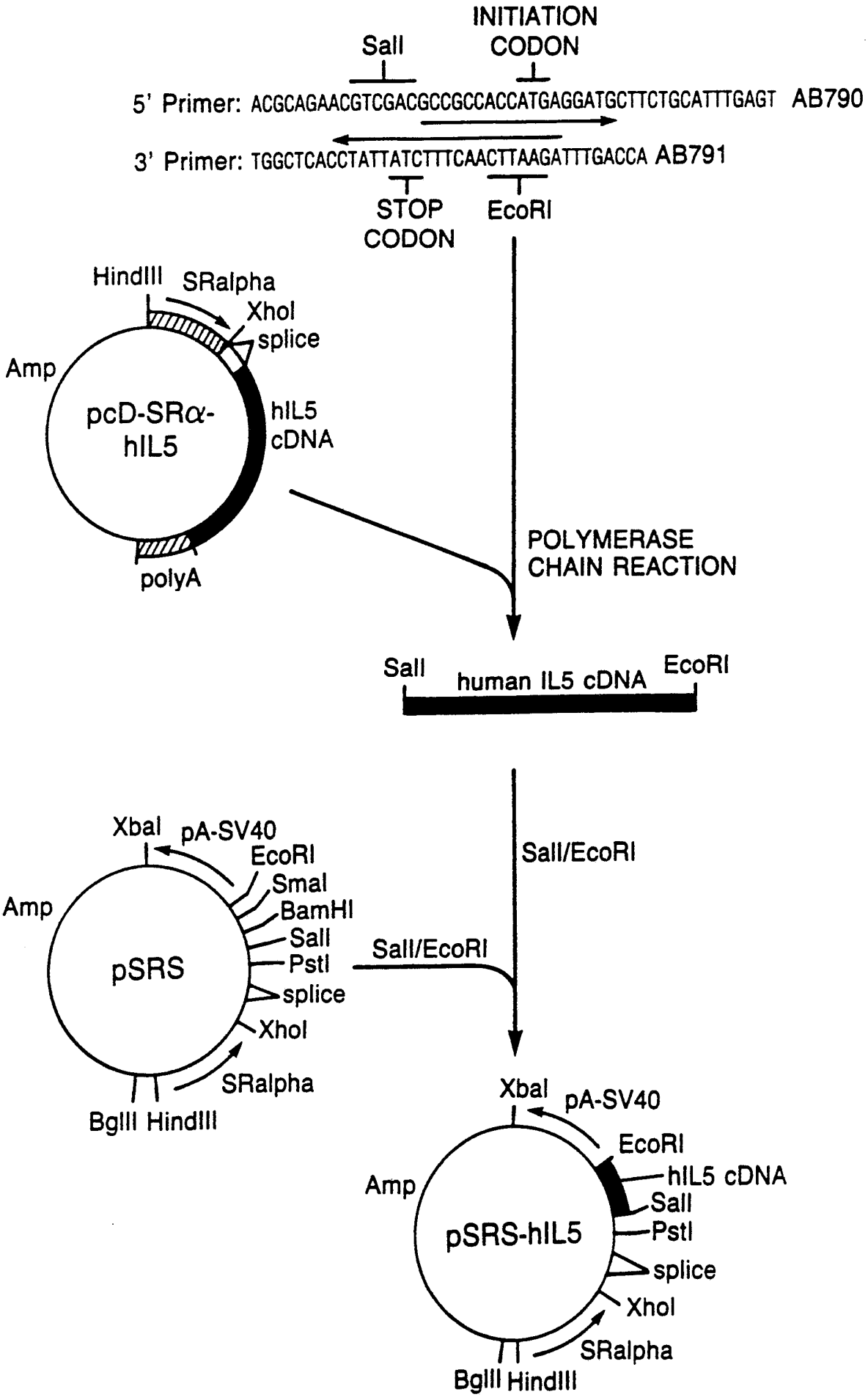

INITIATION CODON
SalI
5' Primer: ACGCAGAACGTCGACGCCGCCACCATGAGGATGCTTCTGCATTTGAGT AB790
3' Primer: TGGCTCACCTATTATCTTTCAACTTAAGATTTGACCA AB791
STOP CODON
EcoRI
HindIII
SRalpha
XhoI
splice
Amp
pcD-SRα-hIL5
hIL5 cDNA
polyA
POLYMERASE CHAIN REACTION
SalI
human IL5 cDNA
EcoRI
SalI/EcoRI
XbaI
pA-SV40
EcoRI
SmaI
BamHI
SalI
PstI
splice
XhoI
SRalpha
Amp
pSRS
BglII HindIII
SalI/EcoRI
XbaI
pA-SV40
EcoRI
hIL5 cDNA
SalI
PstI
splice
XhoI
SRalpha
Amp
pSRS-hIL5
BglII HindIII

Figure 6B

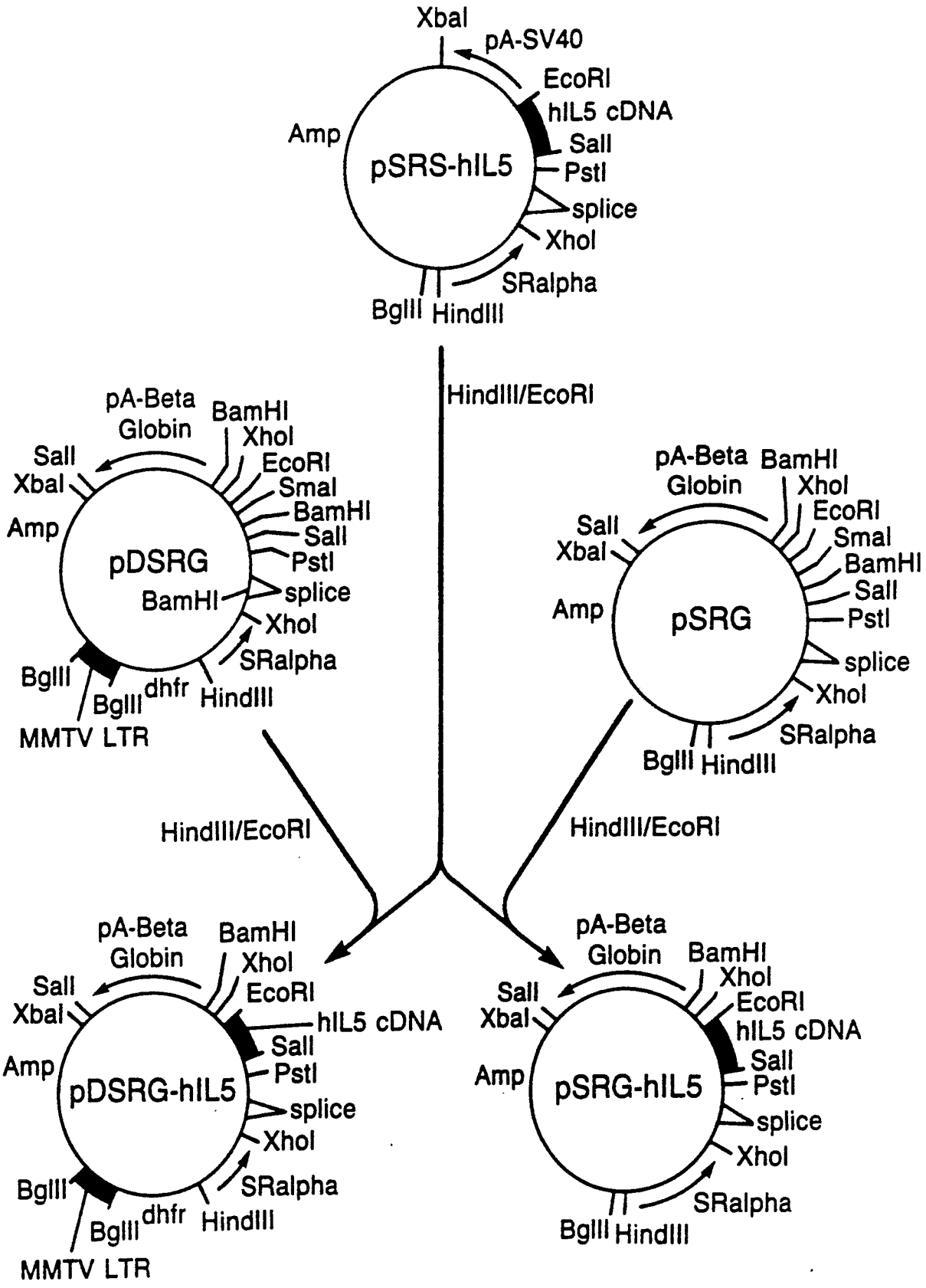
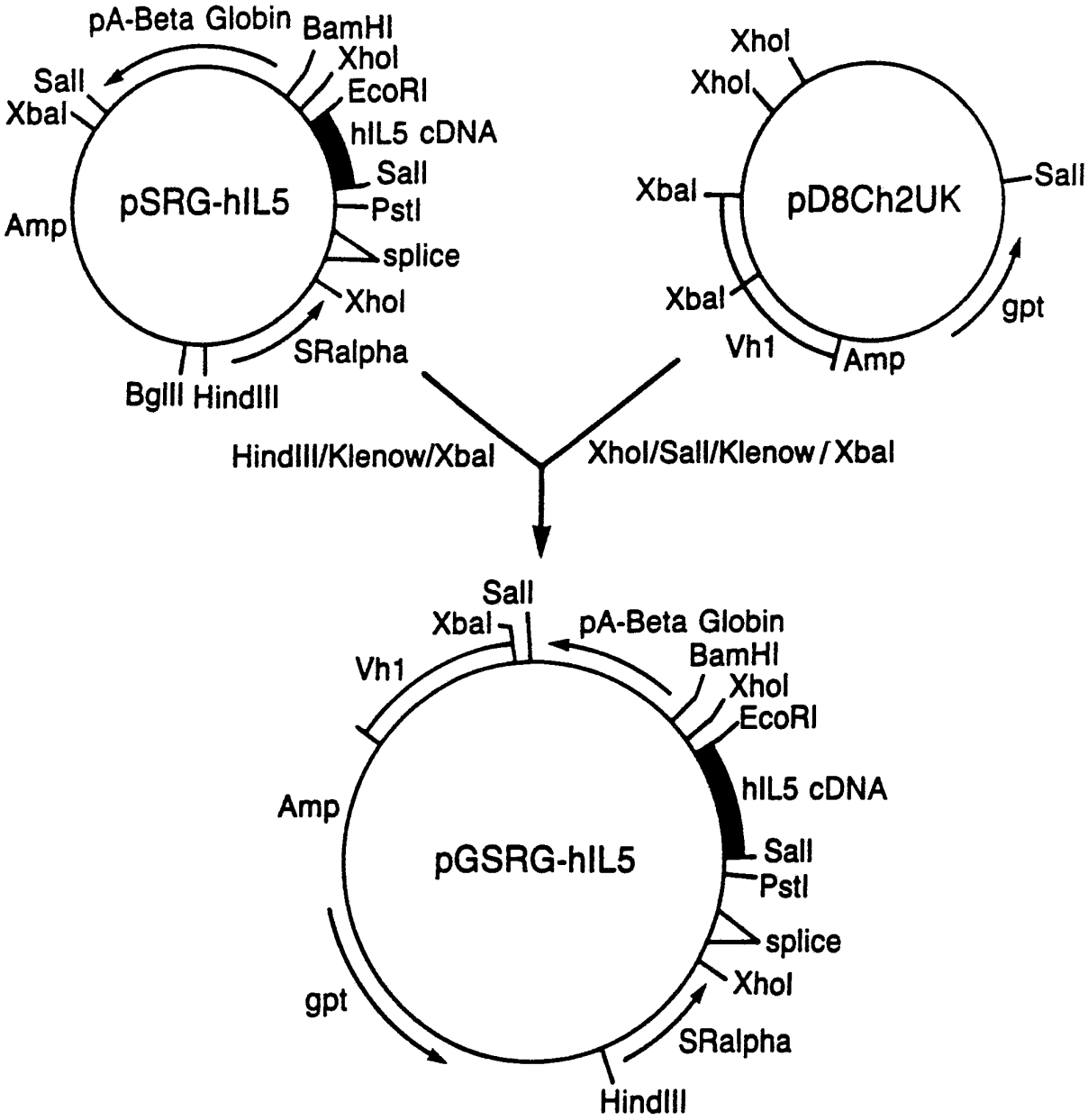

Xbal
pA-SV40
EcoRI
hIL5 cDNA
Sall
PstI
splice
Xhol
SRalpha
BglII
HindIII
Amp
pSRS-hIL5
HindIII/EcoRI
pA-Beta Globin
BamHI
Xhol
EcoRI
Smal
BamHI
Sall
PstI
splice
Xhol
SRalpha
HindIII
dhfr
BglII
MMTV LTR
Sall
Xbal
Amp
pDSRG
pSRG
pDSRG-hIL5
pSRG-hIL5

Figure 6C


pA-Beta Globin
BamHI
XhoI
EcoRI
hIL5 cDNA
SalI
PstI
splice
XhoI
SRalpha
HindIII
BglII
SalI
XbaI
Amp
pSRG-hIL5
XhoI
XhoI
XbaI
SalI
pD8Ch2UK
XbaI
gpt
Vh1
Amp
HindIII/Klenow/XbaI
XhoI/SalI/Klenow/XbaI
SalI
XbaI
pA-Beta Globin
BamHI
XhoI
EcoRI
Vh1
hIL5 cDNA
Amp
pGSRG-hIL5
SalI
PstI
splice
XhoI
gpt
SRalpha
HindIII

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/01078

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/85, 1/21, 5/10, (C 12 N 1/21; C 12 N 1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 4656134 (G.M. RINGOLD) 7 April 1987 see column 1, lines 49-59; column 2, lines 36-45; column 6, line 35 - column 7, line 9	1,2,14,15

X	Gene, volume 71, no. 1, 15 November 1988, Elsevier Science Publishers B.V., (Amsterdam, NL), H. Ikeda et al.: "Mulcos: a vector for amplification and simultaneous expression of two foreign genes in mammalian cells", pages 19-27 see page 22; figures 1	1,5,6,14,15
Y		3,4

	./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th May 1991	25. 06. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Journal of Molecular and Applied Genetics, volume 2, no. 1, 1983, Raven, (New York, US), C.V. Hall et al.: "Expression and regulation of escherichia coli lac Z gene fusions in mammalian cells", pages 101-109 see summary cited in the application	3
Y	Molecular and Cellular Biology, volume 2, no. 9, 1982, American Society for Microbiology, (US), C.M. Corman et al.: "Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells", pages 1044-1051 see summary; figure 1B cited in the application	4
X	EP, A, 0276730 (BEHRINGWERKE AG) 3 August 1988 see figure 1; page 2, lines 8-12, 28-34	1,5
A	Molecular and Cellular Biology, volume 8, no. 1, 1988, (US), Y. Takebe et al.: "SRα promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat", pages 466-472 cited in the application	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9101078 SA 45091

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 21/06/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4656134	07-04-87	None	
EP-A- 0276730	03-08-88	DE-A- 3702116	04-08-88
		AU-A- 1071188	28-07-88
		JP-A- 63196294	15-08-88