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(54) Title: RECOMBINANT POXVIRUSES WITH FOREIGN POLYNUCLEOTIDES IN ESSENTIAL REGIONS

(57) Abstract

Defective poxviruses that lack a function imparted by an essential region of its parental poxvirus can be employed for protein production and vaccination, among other uses. A polynucleotide encoding the protein to be produced by the recombinant virus is inserted into the defective poxvirus and placed under transcriptional control of a promoter. The defective poxvirus is viable when the lost function of the essential region is complemented by a host cell, transgenic animal or helper virus.

RECOMBINANT POXVIRUSES WITH FOREIGN
POLYNUCLEOTIDES IN ESSENTIAL REGIONS

Recombinant poxviruses have been used as vectors for
expression of foreign genes from a variety of sources, such as
animals, plants, protozoa, fungi, bacteria, and viruses.
Additionally, recombinant poxviruses can be used to express
chimeric genes and genes of synthetic origins. Typically,
chimeric genes and synthetic genes are DNA sequences that are
derived from or based on naturally-occurring sequences. For
example, cDNA sequences generated from RNA transcripts,
including those from retroviruses, can be expressed with
recombinant poxvirus vectors.

Poxvirus vectors in present use contain foreign DNA
inserted into genomic regions that are not essential to the
viability of the virus. These poxviruses expressing foreign
genes are usually constructed by insertion of the foreign
sequences into genomic regions that are non-essential for growth
of the virus in cell culture by homologous recombination. See
Panicali and Paoletti, Proc. Nat'l Acad. Sci. USA 79: 4927-31
(1982); Mackett et al., Proc. Nat'l Acad. Sci. USA 79: 7415-19
(1982). Preferably, recombinant poxviruses can be constructed
via direct molecular cloning. See EP 0 561 034; Scheiflinger et
al., Proc. Nat'l Acad. Sci. USA, 89: 9977-81 (1992); Merchlinsky
and Moss, Virol. 190: 522-26 (1992). Viruses with foreign DNA
inserted into non-essential regions can grow autonomously in
their host organism and, thus, remain infectious to their
respective hosts.

The absence of the products of non-essential regions does
not unduly impair the viability of the recombinant poxvirus. In
the case of vaccinia, often considered the prototypic poxvirus,
one of the major non-essential regions for insertion is the
thymidine kinase gene. When a foreign gene is inserted into the
vaccinia thymidine kinase gene, the product of the gene is lost
to the virus. Nevertheless, the recombinant vaccinia is still
viable. Other poxviruses also are amenable to similar
manipulations in other non-essential regions. For example,

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recombinant avipox, such as fowlpox, have been developed where
foreign DNA has been inserted into non-essential regions.

Recombinant vaccinia can be used as live vaccines, in large-
scale expression systems, and a variety of other purposes. See
Miner & Hruby, TIBTECH 8: 20-25 (1990). The use of these
recombinant viruses for large-scale expression of proteins
presents significant risks, however, because the viruses retain
an infective capability. Accordingly, costly precautions and
cumbersome procedures are required when handling recombinant
vaccinia. For example, thermal inactivation of all media,
fluids and contaminated lab ware is required, as well as special
training for all personnel handling the virus. Several
approaches have been considered for minimizing the need for
these elaborate precautions. For example, highly attenuated
vaccinia strains have been developed. These highly attenuated
strains have limited infectivities. Accordingly, production of
proteins with these highly attenuated strains has been
attempted. Unfortunately, highly attenuated virus strains have
slow and limited growth, which often makes these strains poorly
suited for large-scale protein production.

In addition to the highly attenuated poxviruses, impaired
strains of other viruses are known. For example, impaired
retroviruses can be grown in helper cell lines that are capable
of complementing the impaired virus. See Kriegler, GENE
TRANSFER AND EXPRESSION (Stockton Press, 1990) at 33-39; Mann et
al., Cell 33: 153-59 (1983). Retroviruses, however, are small
RNA containing viruses that multiply by integration into the
genome of the host cell, which makes these viruses inappropriate
for use as expression vectors or vaccines.

Impaired adenoviruses grown in complementing cells have been
used as vaccines, as described by Eloit et al., J. Gen. Virol.
71: 2425-31 (1990). Adenoviruses are double-stranded DNA
viruses that, in contrast to vaccinia virus, replicate in the
nucleus of infected cells and have a narrow host range.
Additionally, the growth of impaired herpes virus in helper
cells has also been reported. See PCT publications WO 94/03595,
WO 94/21807 and WO 92/05263. Herpes virus also replicates in
the nucleus of infected cells. Naked herpes DNA, like

adenovirus DNA, is infectious. Accordingly, the promoters of
these viruses function normally in the host cell.

In contrast to poxviruses, herpes and adenoviruses are
nuclear viruses. Defective viruses, however, derived from
nuclear viruses have the potential of rescuing defective regions
from the host cell via homologous recombination. Such
recombinational events are undesirable and dangerous because the
impaired virus can potentially return to a wild-type state,
which results in increased infectivity and virulence, as well as
in the loss of the inserted foreign gene.

Because of the need for safer recombinant expression systems
and vaccines, inventive approaches are required for engineering
fundamentally different recombinant poxviruses. The approach
described here entails insertion of a foreign gene into an
essential region of the poxvirus. The loss in the poxvirus of
the product from the essential region is complemented by the
same or similar product from other sources, such as recombinant
host cells, helper virus or transgenic animals.

It thus is an object of the present invention to provide
recombinant poxviruses capable of expressing foreign
polynucleotides, such as genomic DNA and cDNA.

It is another object of the present invention to provide
recombinant poxviruses that are defective because they lack a
function imparted by an essential region of the poxvirus genome.

It is another object of the present invention to provide
defective poxviruses that possess foreign polynucleotides
inserted into essential regions of the poxvirus genome.

It is still another object of the present invention to
provide defective poxviruses that have all or part of an
essential region deleted.

It is still another object of the present invention to
provide defective poxviruses that can be used as vaccines.

It is yet another object of the present invention to provide
host cells, helper virus or transgenic animals that can
complement the lost function of a defective poxvirus.

In accomplishing these and other objects of the present
invention, there are provided, in accordance with one aspect of
the invention, defective poxviruses that lack a function

imparted by an essential region of its parental poxvirus, the
defective poxvirus comprising a foreign polynucleotide under
transcriptional control of a promoter. That is, the defective
poxvirus is derived from a parental poxvirus, but lacks a
function that is normally present in the parental poxvirus.

The promoter should be operable with the enzymes of the
defective poxvirus or the infected host. Such promoters include
poxvirus promoters and synthetic promoters based upon poxvirus
promoters. The foreign polynucleotide can be inserted into the
essential region or replace some or all of the essential region.

Additionally, a marker can substitute for the essential
region prior to insertion of the foreign polynucleotide into the
virus. The foreign polynucleotide can be then inserted into the
marker or replace the marker. Preferably, the parental poxvirus
is vaccinia and the essential functions to be lost are encoded
by open reading frames I7L, F18R, F13L, D13L, D6R, A8L, H4L,
J1R, J3R, A10L and A3L or open reading frames that encode a 14
kilodalton envelope protein or a 25 kilodalton structural
protein.

In accordance with another aspect of the present invention,
there are provided cell lines, transgenic animals and helper
viruses that have the ability to complement a lost function of a
defective poxvirus. The ability to complement is imparted by
expressibly incorporating a gene that encodes a product that
complements the lost function of the recombinant virus.

In accordance with still another aspect of the present
invention, there is provided a method of producing a protein,
comprising the step of providing a defective poxvirus that lacks
a function imparted by an essential region of its parental
poxvirus, wherein the defective poxvirus comprises a foreign
polynucleotide encoding the protein to be produced, and wherein
the polynucleotide is under transcriptional control of a
promoter. The promoter should be operable with the defective
poxvirus or infected host, such promoters include poxvirus
promoters. The polynucleotide can be inserted into the
essential region of the parental poxvirus. In another
embodiment, a marker, such as the gpt gene, can replace some or
all of the essential region and, in turn, the polynucleotide can

be inserted into the marker or replace some or all of the
marker. Loss of the marker function then will indicate that the
polynucleotide has been placed within the defective poxvirus.

In accordance with yet another aspect of the present
invention, there are provided vaccines comprising defective
poxviruses. These defective poxviruses comprise polynucleotides
that encode antigens against pathogens. The poxviruses for
vaccines of the present invention can be constructed in the same
or similar manner as the other defective poxviruses of the
present invention.

These and other aspects of the invention disclosed herein
will become apparent to the skilled artisan in view of the
disclosure contained herein.

FIGURE 1 schematically depicts the construction of plasmid
pRSET-I7L.

FIGURE 2 schematically depicts the construction of plasmid
pSV-I7L-EDH.

FIGURE 3 schematically depicts the construction of plasmid
pCR-I7Lf-ZG.

FIGURE 4 schematically depicts the construction of plasmid
pRSET-A8L-3'/2

FIGURE 5 schematically depicts the construction of plasmid
pSV-A8L-EDH.

FIGURE 6 schematically depicts the construction of plasmid
pCR-A8Lf-ZG.

FIGURE 7 schematically depicts the construction of plasmids
pRSET-D6R and pRSET-D6R-3'.

FIGURE 8 schematically depicts the construction of plasmid
pSV-D6R-EDH.

FIGURE 9 schematically depicts the construction of plasmid
pCR-D6Rf-ZG.

FIGURE 10 schematically depicts the construction of plasmid
pRSET-J1R.

FIGURE 11 schematically depicts the construction of plasmid
pSV-J1R-EDH.

FIGURE 12 schematically depicts the construction of plasmid
pCR-J1R-ZG.

FIGURE 13 schematically depicts the construction of plasmid pSV-H4L-EDH.

FIGURE 14 schematically depicts the construction of plasmid pCR-H4Lf-ZG.

FIGURE 15 schematically depicts the construction of plasmid pHS-I7L.

The present invention relates in one aspect to the use defective poxviruses for the production of proteins. A "defective poxvirus" according to the present invention is a poxvirus that lacks a function imparted by an essential region of a parental poxvirus upon which the defective poxvirus is based; as a consequence, the defective poxvirus is not viable or infective without complementation of the lost function by another source. An "essential region" is a region of the poxvirus genome that is needed for viability or infectivity of the parental poxvirus. The term "complementation" connotes a restoration of a lost function by another source, such as a host cell, transgenic animal or helper virus. Thus, a defective poxvirus is a non-viable or non-infective form of a parental poxvirus, and can become viable or infective in the presence of complementation.

A parental poxvirus can be a naturally-occurring poxvirus or poxvirus derived therefrom. Preferably, the parental poxvirus is a fast growing wild-type virus such as the Western Reserve ("WR") strain of vaccinia or related strains.

A defective virus is based upon (derived from) parental poxviruses such as naturally-occurring poxviruses or poxviruses derived from naturally-occurring poxviruses. These derived poxviruses for use as parental poxviruses can be obtained by techniques such as genetic engineering via region-specific and site-specific mutagenesis, in vivo recombination, passaging of virus through host cells in the presence or absence of mutagens, and any combination of the above. A defective poxvirus can be based upon any type of poxvirus in which a defect in an essential region can be introduced.

The defective poxvirus can be used as an expression system for proteins. The proteins produced by the defective poxviruses of the present invention are encoded by foreign polynucleotides,

such as genes or cDNA versions of RNA transcripts.   The term
"foreign polynucleotide" refers to a polynucleotide that is not
normally present in the parental poxvirus upon which the
defective poxvirus is based, or is a polynucleotide that is
present in a different location or form than would be found in
the parental poxvirus upon which the defective poxvirus is
based.   The foreign polynucleotides can be from a variety of
sources, such as animals, plants, protozoa, fungi, bacteria, and
viruses.

A defective poxviruses of the present invention can include
a promoter that is operable with the defective poxvirus or host
infected by the defective poxvirus in order to control the
transcription of the foreign polynucleotide within the defective
poxvirus. A "promoter" refers to a nucleotide sequence which
can initiate and/or direct transcription. Any promoter that is
operable with the defective poxvirus or within the infected host
can be used in the present invention. "Operability" denotes
recognizability or readability of the promoter by
transcriptional enzymes. Typically, the promoters are poxvirus
promoters or synthetic promoters based on promoters of poxvirus
origin.

A defective virus can have at least one foreign
polynucleotide inserted into an essential region of the
poxvirus. A promoter can be linked to the foreign
polynucleotide so that the promoter is inserted along with the
polynucleotide in order for the promoter to have transcriptional
control over the polynucleotide. In the alternative, the
promoter for controlling transcription of the foreign
polynucleotide can be already present in the defective poxvirus
in a location that will allow the promoter to control
transcription of the foreign polynucleotide.

The essential region may be fully deleted from the genome of
the defective virus so that no homologous recombination is
possible between the genome of the complementation source and
the defective virus. A defective virus with such a deletion can
only grow in the presence of the complementation source, such as
a helper cell line that provides the function of the essential
region in trans. Pure stocks of the defective virus can be

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grown in the helper cell line while growth and multiplication in
normal cells and organisms remain impossible. The host range of
this type of virus can be restricted to helper cell lines that
are specifically engineered to complement the defect of the
defective virus.   A marker gene can be used to replace an
essential region in the defective poxvirus. The foreign
polynucleotide can then be inserted into the marker or replace
all or part of the marker by homologous recombination. This
approach provides a selection system for poxviruses containing
inserts of interest.

One such marker is the gpt gene, which can be used for
reverse gpt-selection in a complementing cell line such as A9
cells (derivatives of mouse LM(TK-) cells) or STO cells (which
are resistent to 6-thioguanine, allowing reverse gpt-selection).
This system provides a fast and efficient way to obtain pure
stocks of the desired defective virus. Many rounds of plaque
purification usually needed to obtain pure stocks are avoided by
active killing of the wild-type virus (which is not achieved by
dominant selection procedures such as gpt-selection).

The present invention is useful for more than large scale
expression of proteins. The defective poxviruses of the
invention also can be used for vaccination purposes. For
example, if an appropriate essential gene is inactivated, the
defective viruses still retain their ability to penetrate into
cells and start an abortive life cycle. Such a gene would be
one that is required late in the life cycle and, therefore, the
virus still able to replicate its DNA, eventually form immature
particles and express its genomic information, which includes
the foreign polynucleotide. In this respect, these defective
viruses could have the effect of the "dead live vaccines."
Taylor and Paoletti, Vaccine 6: 466-68 (1988). A defective
poxvirus that is used as a vaccine would comprise a DNA
polynucleotide that encodes an antigen. Antigens are molecules
that an organism recognizes as being foreign. These antigens
can induce an immunological response from the organism exposed
to the antigen. Antigens include proteins of bacterial, viral,
fungal and protozoan origin. A preferred parental vaccinia
strain for the construction of defective viruses to be used as

vaccines would be vaccine strains such as the New York City
Department of Health Laboratories strain (ATCC VR-325).

The defective poxviruses of the present invention can be
grown in host cells that can complement the lost essential
function of the defective virus. Typically, these host cells
are from cell lines that are specifically engineered to
complement the lost function. Typically, the host cell will
have the same or similar poxvirus essential region inserted in
the host cell in such a manner that the essential region is
expressed by the host cell or the essential region in the cell
is inducible upon infection of the host cell.

The cell line chosen for complementation and subsequent
production of virus and protein should be easy to handle, allow
virus plaque formation, and be able to carry out all steps
required for functionality of the product. Vero cells (ATCC No.
CCL 81) and Mouse LM(TK-) cells fulfill these requirements and
are suitable hosts. Negative selection for virus lacking the tk
gene can be performed on TK-negative Vero cells. TK-negative
Vero cells are obtained by repeated subcloning of the cell line
in presence of bromo-deoxyuridine. Vero cells can be grown to
high cell densities and have successfully been used for the
production of recombinant proteins (Barrett et al., AIDS Res.
Hum. Retrovir. 5: 159-71 (1989).

The A9 cell line (ATCC CCL1.4), a derivative of the mouse
LM(TK-) cell line, also is resistant to 6-thioguanine, allowing
reverse gpt selection, a feature that is useful for applying
direct selective pressure for the isolation of defective virus.
Isaacs et al., Virol. 78: 626-30 (1990).

Dominant selection markers like the hygromycin
phosphotransferase or the neomycin phosphotransferase genes are
used to facilitate integration of the complementing gene. The
foreign genes may be linked on one plasmid, or, alternatively,
co-transformation procedures may be applied (Wigler et al., Cell
16: 777-785 (1979). Other selection markers such the herpes
simplex thymidine kinase gene may used with the mouse LM(TK-)
cells.

The promoter driving the poxvirus essential region for
complementation ("a complementation agent") should be active in

the respective cell line. A preferred promoter is from SV40. SV40 has a strong constitutive promoter that is active in mouse L cells and Vero cells. Other promoters that are usually used for expression in permanent cell lines include the actin promoter, Gunning, Proc. Nat'l Acad. Sci. USA 84: 4831-35 (1987), and the human cytomegalovirus promoter, Boshart et al., Cell 41: 521-30 (1985), both of which may also be used in the present invention.

A transcription system that may be especially suitable for vaccinia-infected cells includes the promoter from the human 70 kD heatshock protein ("Hsp70") along with untranslated upstream regions. See Hunt and Morimoto, Proc. Nat'l Acad. Sci. USA 82: 6455-59 (1985). Unlike the expression of many other genes, Hsp70 synthesis not deleteriously affected by vaccinia virus host protein shut-off in vaccinia infected cells. See Jindal and Young, J. Virol. 66: 5357-62 (1992).

Another host suitable for complementation may be mouse C127I cells (ATCC CRL 1616) or derivatives therefrom transfected with plasmids containing bovine papilloma virus (for example, ATCC 37110) vectors that carry the complementing poxvirus gene. To minimize the risk of homologous recombination in this system, the complementing poxvirus gene is preferably derived from a genus different from that of the defective virus. With this helper virus approach, the complementing poxvirus gene is only activated upon infection of the host cell with the defective poxvirus.

Other hosts may be used to propagate the defective poxviruses include animals transgenic for the vaccinia essential gene (hereafter, "complementing transgenic animals"). Such animals may provide the complementing function in every somatic cell or in a preferred tissue, which depends on the promoter chosen to express the poxvirus essential gene in the complementing transgenic animal. The animals, preferably mice, would constitute safe models for poxvirus infection studies and related studies. Selective expression of the transgene in a defined organ also can allow tissue specific gene expression studies and/or studies on the selective function or dysfunction of a particular organ.

Approaches for creating transgenic animals are known in the art. For example, transgenic animals can be created by microinjection, transformation of embryonic stem cells or use of retroviral vectors. Appropriate techniques are described in greater detail in GENETIC ENGINEERING OF ANIMALS, (Pühler ed., VCH, 1993).

Essential gene products suitable as complementing agents include, in general, gene products that are required late in the poxvirus life cycle. Illustrative of such gene products are those involved in morphogenesis or other post-replication events, as well as enzymes and regulatory proteins.

One essential gene product that is a suitable complementing agent is the 47-kDa protein (the "I7 protein") encoded by open reading frame ("orf") I7L, which is believed to be involved in viral genome organization. Kane and Shuman, J. Virol.67: 2689-98 (1993). The I7 protein is encapsulated with the virus core. A temperature sensitive (ts) mutation exists known as ts16. Condit et al., Virol. 128: 429-43 (1983). The I7 gene therefore fulfills a classic requirement of an essential gene, which is the existence of a conditional lethal mutant (temperature sensitivity).

Among the essential gene products that are suitable complementing agents in the present context is the 65kDa protein encoded by the D13L orf. This protein is expressed late in infection. If expression is prevented replication is unaffected while viral morphogenesis is blocked at an early stage. See Zhang and Moss, Virol. 187: 643-53 (1992). Other essential gene products that are suited as complementing agents are the vaccinia 14 kDa envelope protein, Lai et al., J. Biol. Chem. 265: 22174-80 (1990); the vaccinia 25kDa major structural protein, Weir and Moss, J. Virol. 56: 534-40 (1985); and other structural proteins such as P4a and P4b.

Another essential gene product that can be used as a complementing agent is the 11kDa phosphoprotein, Wittek et al., J. Virol. 49: 371-78 (1984), encoded by the F18R orf of WR wild type virus (known as "F17R" of the Copenhagen strain of vaccinia). See Goebel et al., Virol. 179: 247-66 (1990). The 11kDa phosphoprotein encoded by the F18R orf is an essential

gene that is required for correct virion assembly. See Zhang
and Moss, J. Virol. 65: 6101-10 (1991). Conditional lethal
vaccinia mutants in the F18R and F17R orfs form immature
particles with aberrant internal structures under certain
conditions. F18R overlaps with the orf A, however, and thus
generally would not be the essential region of first choice.
Goebel et al., supra.

Enzymes and regulatory proteins also can be employed as
complementing agents because these proteins need only be present
in catalytic amounts, which can be readily provided by the
complementing cell line. The subunits of the vaccinia virus
early transcription factor ("VETF") are suitable in this
respect. The VETF is essential for the initiation of
transcription of "early" vaccinia genes. Vaccinia RNA
polymerase lacking VETF is unable to transcribe double-stranded
DNA templates in vitro. See Broyles et al., J. Biol. Chem. 263:
10754-60 (1988). The factor is a heterodimer having a 77kDa
polypeptide and a 82kDa polypeptide, which are respectively
encoded by the D6R and A8L gene orf of the vaccinia WR genome.
See Gershon and Moss, Proc. Nat'l Acad. Sci. USA 87: 4401-05
(1990).

VETF proteins are expressed late in infection and packaged
into the nascent virus particles. A defective virus lacking the
D6R or the A8L gene isolated from VETF expressing cells should
therefore be able to perform an additional complete life cycle
on a non-complementing cell line without further limitations of
protein synthesis. Protein synthesis is an essential
requirement for the use of such defective viruses as "dead-live
vaccines."

Dead-live vaccines include avipoxviruses expressing foreign
genes that used to immunize mammalian hosts. In the mammalian
host, the viruses do not multiply, but rather undergo an
abortive life cycle. Nevertheless, this type of vaccine
stimulates immune response of particular T cells. See Taylor
and Paoletti, supra. A defective virus lacking VETF is
desirable because it should be able to perform an additional
life cycle in wild-type hosts as compared to viruses capable

only of performing abortive life cycles, which allows for
greater antigen production.

Another viral factor that is suitable for complementation is
the RNA polymerase associated protein RAP 94 or early
transcription specifity factor. RAP94 is encoded by the orf
H4L, Ahn and Moss, Proc. Nat'l Acad. Sci: USA 89: 3536-40
(1992), and it is a subunit of the viral DNA dependent RNA
polymerase that can be disassociated. The subunit confers
promotor specifity to the RNA polymerase complex for the
initiation of transcription of early stage genes. As
complementing agent it fulfills the same requirements as the
VETF subunits. RAP 94 is synthesized late in infection and
packaged into the nascent virus to mediate early transcription
in the following round of infection. Hence, phenotypically
intact virus particles lacking the H4L gene should be able to
perform one additional round of infection in a non-complementing
host without further limitations on protein synthesis. The
existence of conditionally lethal mutants, Condit and Motytzca,
Virol. 113: 224-41 (1981), confirms that H4L is an essential
gene. The protein is found to be present in the virion in
submolar amounts compared to other components of the RNA
polymerase complex. Ahn and Moss supra. Accordingly, low
expression levels of RAP94 in the complementing cell line should
not affect growth of the defective virus.

Another gene that may be used for complementation of
defective virus is the J1R gene, which is essential for virus
growth in cell culture. Although its gene product has not been
identified so far, this orf is of considerable interest due to
its location adjacent to the thymidine kinase gene on the
vaccinia virus genome. Deletion of a sequence that includes the
orf J1R and at least parts of the adjacent thymidine kinase gene
(J2R) results in a virus which is dependent on a complementing
cell line and which is at the same time selectable by negative
selection on tk-negative cells such as LM(TK-) cells or tk-
negative Vero cells. Pure stocks of defective virus are
rapidly obtained using this additional selection procedure.

The J3R gene is of interest in the same respect as the J1R
orf. Negative selection on tk-negative cells can be performed

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due to the simultaneous deletion of the J3R orf and the adjacent
J2R orf (thymidine kinase). The J3R gene encodes a
poly(A)polymerase subunit (VP39) that is required for the
formation of the 5' cap structure of mRNA and that stimulates
the formation of long poly(A) tails. Gershon et al., Cell 66:
1269-78 (1991); Schnierle et al., Proc. Nat'l Acad. Sci. USA 89:
2897-01 (1991).

Defective poxviruses of the present invention can be
produced with many of the same methods used to construct
recombinant viruses having inserts in non-essential regions.
For example, in vivo recombination via homologous flanking
region can be used. See Panicali and Paoletti, Proc. Nat'l
Acad. Sci. USA 79: 4927-31 (1982); Mackett et al., loc. cit. 79:
7415-19 (1982). An alternative method of producing defective
poxviruses is direct molecular cloning and heterologous
packaging. See EP 0 561 034; Scheiflinger et al., Proc. Nat'l
Acad. Sci. USA 89: 9977-81 (1992); Merchlinsky and Moss, Virol.
190: 522-26 (1992).

Pursuant to the present invention, a defective poxvirus with
unique restriction site(s), either in the essential gene or in
the former location of the essential gene (which is lost due to
deletion, including replacement by a marker) can be used as a
cloning vehicle. Alternatively, the essential gene of the virus,
including a selection marker if desired, can be flanked by
unique restriction sites, allowing co-excision of the essential
gene and the marker (if present). Following co-excision,
insertion of at least one foreign polynucleotide by forced
cloning, which favors ligation of the foreign polynucleotide to
the virus vector, is preferably undertaken.

The vector arms of the parental virus having its unique
site(s) in an essential region or adjacent to an essential
region may be ligated with the foreign polynucleotide and
packaged in the complementing cell line with fowlpox virus
("FPV") as helper virus. Because FPV cannot complete life
cycles and multiply in mammalian cells, this virus plays no
significant role as contaminant in the plaque assays that
follow. Re-ligated defective virus without an insert still may
form plaques, however. Careful preparation of vector arms and

forced cloning of the insert into the virus will solve this
problem, however.   The present invention is further illustrated
by the following examples.   These examples do not limit the
scope or spirit of the invention in any way, but rather are
provided to further describe the invention.

**Example 1:   Construction of a defective vaccinia virus lacking
            the I7L-protein ("d-I7L-ZG") and a complementing cell
            line based on Vero-cells ("V-I7L")**

Construction of Complementing Cell Line V-I7L

        The first step in the construction of the helper cell line
was the cloning of the I7L orf, by conventional, PCR-mediated
methodology, into an E. coli expression plasmid and into an
eukaryotic expression plasmid.   Expression of the I7L-orf in E.
coli allows rapid production of the protein for immunization of
rabbits to obtain anti-I7L-protein antibodies, which can be
later used to identify the I7L protein in permanent cell lines.

        For this purpose, the I7L orf was amplified by PCR and then
cloned into the plasmid pCRII (Invitrogen, Inc.).   The primers
oI7-1 (5'-AGT ACT CCA TGG AAA GAT ATA CAG ATT TAG-3') (SEQ ID
NO:1) and oI7-2 (5'-ATC CCG GGT TTT AGA GAC TTT GAA GCT ACT-3')
(SEQ ID NO:2) were used to amplify the I7L-gene as a 1.3kb
fragment.   This fragment was inserted into the plasmid pCRII,
which resulted in pCR-I7L.   Plasmid pCR-I7L was the source of
the NcoI-HindIII gene fragment that was later inserted by forced
cloning into the plasmid pRSET-B (InVitrogen, Inc.), yielding
pRSET-I7L (Figure 1).   Plasmid pRSET-I7L allowed over-expression
of the I7L orf, which was downstream of the T7 promoter.
Expression under the T7 promoter control requires T7 RNA
polymerase, which is provided by infection of the plasmid-
carrying E. coli by bacteriophage M13mp18/T7.

        The I7L protein was over-expressed in JM109 E. coli cells
infected with M13mp18/T7 and harboring plasmid pRSET-I7L.
Induction of the lac promoter drives the expression of the T7
polymerase in the bacteriophage M13mp18/T7 with the
isopropylthiogalactoside ("IPTG").   The E. coli expression
system used in this particular example was obtained from
InVitrogen, Inc. (USA), and includes the parental plasmids pRSET

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A, pRSET B and pRSET C and an M13-based helper phage M13mp18/T7.
Other appropriate expression systems also can be used with the
present invention.

Following expression, bacterial lysates were prepared and
subjected to polyacrylamide gel electrophoresis. The band
containing the I7L protein was detected and excised from the gel
and electroeluted. Rabbits were immunized with the electro-
eluted material and complete Freund's adjuvant (30 mg protein
per dose, i.m.). After one month, the animals are boosted with
the same dose in incomplete Freund's adjuvant and development of
antibodies is monitored by Western blots.

The Western blots may be performed essentially as described
by Towbin et al., Proc. Nat'l Acad. Sci. USA 76: 4350-54
(1979). The first antibody is a rabbit anti I7L antibody (see
above) used in a 1:100 dilution. The second antibody is a goat-
anti-rabbit IgG coupled with alkaline phosphatase (BioRad, Inc.)
used in a 1:1000 dilution. The reagents (BCIP and NBT) and
staining protocols are from Promega, Inc.

An NcoI-SmaI gene fragment from plasmid pCR-I7L is part of
the expression plasmid pSV-I7L-EDH. See Figure 2. The plasmid
pEDH1 (Figure 2) comprises the 'EDH gene cassette' including an
internal ribosome entry site, the dihydrofolate reductase
('dhfr') and hygromycin ('hph') genes as an EcoRV-SalI fragment.
This plasmid pEDH1 derives from the plasmid pB4/EDHPro
(Herlitschka, thesis), in which four additional restriction
sites (SalI, ClaI, SwaI and DraI) were inserted downstream of
the 'EDH gene cassette' between the sites SpeI and NotI. The
'EDH gene cassette' of pEDH1 was inserted as an EcoRV-SalI
fragment between the unique restriction sites SmaI and SalI of
the plasmid pSV-I7L. This construction yielded plasmid pSV-I7L-
EDH.

Plasmid pSV-I7L was obtained by inserting the 1.3kb NcoI-
SmaI fragment of pCR-I7L between the BbsI-SmaI sites of plasmid
pSV-Bbs. Plasmid pSV-Bbs is a derivative of the plasmid pSVß
(Clontech, Inc. USA), which lacks the ß-galactosidase gene but
contains a unique BbsI site. The BbsI site is introduced by a
linker including the oligonucleotides oBbs-1 (5'-CTA GGC TTT TGC
AAA AAG CTC CTC GAC CAT GGT GTC TTC A-3') (SEQ ID NO:3) and

oBbs-2 (5'-AGC TTG AAG ACA CCA TGG TCG AGG AGC TTT TTG CAA AAG
C-3') (SEQ ID NO:4). The linker was inserted between the AvrII
and the HindIII sites of the plasmid pSVß. See Figure 2.

In pSV-I7L-EDH, the I7L-orf is controlled by the SV40 early
promoter. The selection marker for obtaining permanent cell
lines is a fusion gene including the dhfr and hph genes, which
allows efficient screening and amplification of genes of
interest in permanent cell lines. Herlitschka, S. E. (1994),
Ph.D. thesis, Universität für Bodenkultur, Vienna, Austria.
Practice of the present invention is not limited to the use of
this marker, however.

This plasmid is transfected into monkey kidney Vero cells
(ATCC No. CCL 81). Vero cells were obtained from the American
Type Culture Collection (Rockville, MD). The cells were
transfected with the plasmid pSV-I7L EDH according to Graham and
van der Eb, Virol. 52: 456-67 (1973) and incubated in selective
medium based on hygromycin B (DMEM, 10% fetal bovine serum, 250
mg/ml hygromycin B). Colonies became visible after 10 to 14
days. These colonies were expanded, subcloned twice and then
characterized further. Permanent hph positive cell lines were
selected in the presence of the hygromycin B. The cell lines
were further characterized by PCR analysis for the presence of
the complementing gene construct (I7L). Western blots are used
to show that the gene of interest, the I7L-protein is expressed.
The final complementing cell line is named V-I7L.

Construction of Defective Virus d-I7L-ZG:

To construct the virus d-I7L-ZG, which lacks the I7L
product, the plasmid pCR-I7Lf was constructed as summarized in
Figure 3. This plasmid is based on the pCRII vector and
contains the I7L-orf, including about 0.5 kb of flanking region
on either side. The primers oI7-3 (5'-AGG AGT TAA TGA GGC CAA
TGG A-3') (SEQ ID NO:5) and oI7-4 (5'-GAC ATA GGT ATA GAA TCC
GGA-3') (SEQ ID NO:6) were used to obtain a PCR product of about
2.3 kb, which was subcloned into the pCRII plasmid to yield pCR-
I7Lf.

A double gene cassette including the E. coli genes lac Z and
gpt was excised as a SmaI fragment from the plasmid pLGb and
inserted into the unique HpaI site located within the I7L-orf,

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which inactivates the pCR-I7L gene in the resulting plasmid pCR-
I7Lf-ZG.

Plasmid pLGb was obtained from p2T, which is based on
plasmid p1Ta and pTZ19R (Pharmacia). First, the large PvuII
vector fragment of pTZ19R was ligated with annealed
oligonucleotides P-1T(1): 5'- AGT TTA AAC GGC GCG CCC GGG CTC
GAG AGG CCT CTG CAG ATG CAT CCA TGG GGA TCC GAA TTC 3' (SEQ ID
NO:7) and P-1T(2): 5' - GAA TTC GGA TCC CCA TGG ATG CAT CTG CAG
AGG CCT CTC GAG CCC GGG CGC GCC GTT TAA ACT (SEQ ID NO:8). This
yielded p1Ta, which was then cleaved with EcoRI and BamHI. The
cleaved p1Ta was then ligated to annealed oligonucleotides P-
2T(1): 5'-GAT CCT ACG TAT CTA GAA TTA ATT AAT GGC CAA TTT AAA
TGC CCG GGA-3' (SEQ ID NO:9) and P-2T(2): 5'-AAT TTC CGG GCA TTT
TAA ATT GGC CAT TAA TTA ATT CTA GAT ACG TAG-3' (SEQ ID NO:10).
This yielded plasmid p2T, which was then cleaved with SmaI. A
double gene cassette including the E. coli genes lacZ and gpt
was inserted as a HindIII, Sal I and Klenow polymerase treated
fragment. The double gene cassette is obtained from the plasmid
pZgpt-a, which is based on plasmid pTNa and related plasmids.

For the construction of the d-I7L-ZG virus, plasmid pCR-
I7Lf-ZG was inserted into the WR strain vaccinia virus by in
vivo recombination in CV-1 cells. First, 5 x 106 CV-1 cells
were infected with 0.1 pfu/cell of vaccinia WR, grown for 1
hour, transfected with a calcium phosphate precipitate that
includes 20 µg of the plasmid pCR-I7Lf-ZG, and then grown for 3
days. See Graham and van der Eb, supra. A viral crude stock is
prepared and used for plaque cells in the presence of gpt
selection, Falkner and Moss, J. Virol. 62: 1849-54 (1988), and
blue plaque screening, Chakrabarti et al., Mol. Cell. Biol. S.
5: 3403-09 (1985).

The defective virus grows in V-I7L cells only and is gpt and
lacZ positive, while the wild-type virus grows in both cell
lines, but is gpt and lacZ negative. The purification is
performed ten times. The purified defective viruses are then
grown to larger scale and examined by Southern blotting. The
absence of wild-type virus and presence of the predicted bands
confirms that the correct defective genomes have formed. Plaque
assays of the defective viruses on the complementing cells and

on wild-type cells confirm that the host range of the defective
viruses are limited to the complementing cell line. Animal
studies confirm that the defective viruses are non-pathogenic.

Example 2: Construction of a defective vaccinia virus expressing
          the HIV env gp160 gene (d-I7L MN) in the
          complementing cell line V-I7L

To demonstrate foreign gene expression in the novel system,
the HIV gp160 gene of the HIV MN strain is inserted into the
essential region I7L. For this purpose, a gene cassette from a
SmaI fragment of plasmid pSep-ST2 is obtained. Plasmid pSep-ST2
contains the HIV-1 gp160MN sequences controlled by the strong
semi-synthetic poxvirus promoter Sep and a selection cassette
that includes the P7.5 gpt gene. See Falkner and Moss, J.
Virol. 62: 1849-54 (1988).

To construct the plasmid pSep-ST2, first the plasmid pSep(1)
was constructed. To obtain pSep(1), the semi-synthetic poxvirus
promoter Sep was constructed by combination of the late
fowlpoxvirus promoter P2 (EP 0 538 496 A1, Dorner et al) with a
synthetic early promoter sequence. Briefly, the HpaI/NcoI
digested vector pS2gpt-P2 (European Patent Application
Publication Number 0 561 034 A2) was ligated with the annealed
oligonucleotides P-Sep(3) & P-Sep(4). The sequence of the
oligonucleotides was, P-Sep(3), 5'-CTCGTAAAAA TTGAAAAACT
ATTCTAATTT ATTGCACGGT CGCGA-3' (SEQ ID NO:11), and P-Sep(4), 5'-
CATGGTACGT ACCGTGCAAT AAATTAGAAT AGTTTTTCAA TTTTTACGAG-3' (SEQ
ID NO:12). The resulting plasmid was designated pSep(1). To
obtain pSep-ST2, first a 2.65kb StuI/PvuII fragment derived from
the plasmid pMNenv2 (described in EP 0 561 034 A2), containing
the HIV1-MN env gene, was ligated with the SnaBI linearised
plasmid pSep(1). The resulting plasmid, containing the insert
in the proper orientation with respect to the "Sep-promoter" was
designated pSep-gp160mn. In order to repair a point mutation
located within the gp160-orf, a 1.9kb NsiI/SalI fragment of
pSep-gp160mn was replaced by an equivalent fragment derived from
the plasmid pMN-ST2. The resulting plasmid was designated pSep-
ST2. The plasmids pMNenv1 and pMN-ST2 were provided by Marvin
Reitz (N.C.I. Bethesda, Maryland, USA).

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The cassette from pSep-ST2 contains the HIV gp160 gene and
E. coli gpt gene, and is inserted into the unique HpaI site of
pCR-I7Lf resulting in the plasmid pCR-I7Lf-MN. This plasmid is
used in an in vivo recombination experiment in CV-1 cells
involving WR wild type virus to obtain a viral crude stock that
is a mixture of: (1) defective viruses, which have undergone two
cross-over events to become a recombinant virus; (2) viruses
that have undergone only one cross-over event and (3) wild-type
helper viruses. An overall explanation of cross-over events is
contained in Falkner and Moss, J. Virol. 64: 3108-11 (1990).

This mixture of viruses is plaque purified in the
complementing cell checked for purity as described in Example 1,
except that the plaque assays are based solely on gpt-selection.
The resulting virus is designated d-I7L-MN and used for
expression of recombinant gp160 in V-I7L cells. The virus d-
I7L-MN is used in combination with its complementing cell line
to express gp160. V-I7L-cells are infected with 0.1 pfu of d-
I7L-MN and grown for three days. The recombinant protein is
detected by Western blots using an anti-gp41 monoclonal antibody
in a method according to Towbin et al., supra. Animal
experiments in mice are carried out to demonstrate that d-I7L-MN
is non-pathogenic but still able to prime immune response.

Example 3: Construction of a defective vaccinia virus lacking
            the large subunit of the early transcription factor
            (d-A8L-ZG) and a complementing cell line for
            propagating the virus
Construction of a helper cell line based on Vero cells (V-A8L):
    The VETF factor is a heterodimer comprised of a 77kD
polypeptide and a 82kD polypeptide, which are encoded
respectively by the D6R and the A8L orfs of the vaccinia-WR
genome. Gershon and Moss, Proc. Nat'l Acad. Sci. USA 87: 4401-
05 (1990). VETF proteins are expressed late in infection and
packaged into the nascent virus particles. A defective virus
lacking VETF is desirable because it should be able to perform
an additional life cycle in wild-type hosts as compared to
viruses capable only of performing abortive life cycles.

The D6R subunit contains a possible ATP binding site and has been found to bind to the early promotor DNA sequence. See Broyles and Li, J. Virol 67: 5677-80 (1993). Temperature sensitive mutations have been mapped to the D6R gene. The A8L orf was chosen in order to avoid undesirable protein-DNA interactions in the complementing cell line.

The first step in the construction of the helper cell line was cloning of the A8L orf by PCR mediated techniques into an E. coli expression plasmid and into an eukaryotic expression plasmid. Expression of the orf in E. coli allowed rapid production of the protein for immunization of rabbits to obtain anti-VETF large subunit antibodies that are subsequently used to identify the protein in the permanent cell lines.

For this purpose, the A8L-orf was amplified by PCR and the cloned into the plasmid pCRII. The primers oA8-9t (5'-AAA CTG CAG GAT GCG ATA TAT AGT AAG TCC GCA AAT GGT ATT A-3') (SEQ ID NO:13) and oA8-2t (5'-AGG AAT TCA GGC CTG TTT AAT TAA TTT GTG CTC TTC-3') (SEQ ID NO:14) were used to amplify the A8L-gene as a 2.1 kb fragment. This fragment was inserted into the plasmid pCRII (InVitrogen Inc.), resulting in pCR-A8Lt.

Plasmid pCR-A8Lt is the source of a 0.9 kb BamHI-EcoRI gene fragment. The BamHI site (present in the A8L gene) and the EcoRI site (introduced by the multiple cloning site of pCRII) were cleaved to obtain the fragment which was inserted by forced cloning into the plasmid pRSET-B (InVitrogen, Inc.) resulting in the plasmid pRSET-A8L-3'/2. See Figure 4. The plasmid contains a fusion gene encoding the C'-terminal portion of the VETF large subunit with a N'-terminal 6-histidine-tag. The truncated A8L-protein was over expressed in E. coli cells (strain JM109) harboring the plasmid pRSET-A8L-3'/2.

The bacteria were thereafter infected by the helper phage M13mp18/T7 in presence of IPTG, which induces the lac promoter driving the expression of T7 polymerase in the bacteriophage M13mp18/T7. The recombinant truncated A8L-protein including the his-tag was purified from bacterial lysates by affinity chromatography on nickel-NTA columns, according to the manufacturer's protocol (Diagen, Inc.). The purified material emulsified in complete Freund's adjuvant was used to immunize

rabbits (80 mg protein per dose, s.c. and i.m.). After one
month, the animals were boosted with the same dose and
development of antibodies was monitored by Western blots.

To characterize the rabbit anitsera the A8L-proteins in
bacterial lysates were detected by Western blots. The Western
blots were done essentially as described by Towbin et al.,
supra. The first antibody was a rabbit anti-A8L protein
antibody used in a 1:100 dilution. The second antibody was a
goat-anti-rabbit IgG coupled with alkaline phosphatase (BioRad,
Inc.) used in a 1:1000 dilution. The reagents (BCIP and NBT)
and staining protocols were from Promega, Inc.

To obtain the expression plasmid pSV-A8L-EDH the A8L gene
was isolated from the plasmid pCR-A8Lt as a 2.1kb PstI-StuI gene
fragment. The sites PstI and StuI were introduced by the PCR
primers oA8-9t and oA8-2t, respectively. This fragment and a
EcoRV-ClaI gene fragment from the plasmid pEDH1 comprising the
"EDH gene cassette" were ligated into the PstI-ClaI cut
expression vector pSVMCS#51, which is derived from pSVß
(Clontech, Inc.) by substitution of the ß-galactosidase gene by
a multiple cloning site ("msc") including the restriction sites
ApaI, AvrIII, SpeI, HindIII, PstI, SalI, XbaI, SmaI, EcoRI and
ClaI. For this purpose the vector pCMVβ was cut with NotI and
religated resulting in the plasmid pCMV. This plasmid was cut
with SalI and HindIII, treated with Klenow polymerase and
religated resulting in an intermediate plasmid. This plasmid
was cut with XhoI and a double stranded linker, coding for the
restriction sites ApaI, AvrIII, SpeI, HindIII, PstI, SalI, XbaI,
SmaI, EcoRI and ClaI was inserted resulting in the plasmid
pSVMCS#51. See Figure 5.

In plasmid pSV-A8L-EDH, the A8L-orf is controlled by the
SV40 early promoter. The selection marker for obtaining
permanent cell lines is a fusion gene including the dhfr gene
and the hph gene as described in Example 1.

The plasmid pSV-A8L-EDH was transfected into monkey kidney
Vero cells (ATCC No. CCL 81) in a method according to Graham &
van der Eb, supra, and was incubated in selective medium (DMEM,
10% fetal bovine serum, 250 µg/ml hygromycin) and further
treated as described in Example 1. Permanent hygromycin

phosphotransferase positive cell lines were selected in the
presence of the antibiotic hygromycin B.   After five passages
integration of the foreign DNA was demonstrated by PCR using
primers specific for a 0.5kb segment of the A8L gene.

Western blots are used to determine whether the gene of
interest, the VETF large subunit, is expressed.   The cell lines
expressing the highest levels of VETF large subunit is further
characterized by PCR analysis and used as the complementing cell
line and named V-A8L.

Construction of the defective virus d-A8L-ZG:

To construct the virus d-A8L-ZG the plasmid pCR-A8Lf-ZG was
cloned as schematically shown in Figure 6.   Regions (sized
between 0. 5kb and 0. 7kb) flanking the A8L orf at either sides
were amplified by PCR and subcloned into the plasmid pCRII to
obtain pCR-A8L-fl and pCR-A8L-fr respectively.

The PCR-primers for the amplification of the A8L 5'-flanking
region were oA8-8 (5'-AGC GCC GCT ACT AGC ACT CC-3') (SEQ ID
NO:15) and oA8-5 (5'-GAA CGT TAA CAT TTA TAT CGT GGG GTA AAG TGA
AAA TC-3')(SEQ ID NO:16).   The primers for the A8L 3'-flanking
region were oA8-4 (5'- TTG GAG AAC TTG ATA CGC CG-3') (SEQ ID
NO:17) and oA8-6 (5'-CAT ATG CAA TTG TAA ATT AAA CAA CTA AAT CTG
TAA ATA AAT A-3') (SEQ ID NO:18).   The A8L 5'-flanking region as
0. 7kb NotI-SpeI fragment from pCR-A8L-fl was ligated between
the sites NotI and XbaI right upstream of the A8L 3'-flanking
region in pCR-A8L-fr yielding the plasmid pCR-A8L-flanks.

In order to facilitate further cloning procedures, a
synthetic linker containing the rare restriction sites SmaI,
NotI, SfiI and RsrII was ligated between the A8L-flanking
regions in pCR-A8L-flanks.   The oligonucleotides used for the
construction of the linker were oA8-11 (5'-AAC GGT CCG GCC CGG
GCG GCC GCC-3') (SEQ ID NO:19) and oA8-12 (5'-AAT TGG CGG CCG
CCC GGG CCG GAC CGT T-3')(SEQ ID NO:20).   The plasmid pCR-A8L-
flanks was thereafter linearized with MunI and HpaI (both sites
are introduced by the PCR primers oA8-5 and oA8-6,
respectively).   The resulting plasmid, designated pdA8L, was
ligated with a 3.9kb SmaI double gene cassette from the plasmid
pLGb described in Example 1 to yield pCR-A8Lf-ZG.   In this
construct, the A8L orf is deleted completely in order to prevent

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rescue of the defective virus by the complementing cell line via homologous recombination.

For the construction of the d-A8L-ZG virus the plasmid pCR-A8L-ZG was inserted into the vaccinia virus WR. The plasmid pCR-A8Lf-ZG was used for in vivo recombination in CV-1 cells. First, 5 x 106 CV-1 cells were infected with 0. 1 pfu/cell of vaccinia WR, incubated for 1 hour, transfected with a calcium phosphate precipitate, according to Graham & van der Eb, supra, which contained 20 µg of the plasmid pCR-A8L-ZG and grown for 3 days.

This resulted in a crude stock containing wild-type viruses (helper) and defective viruses. To obtain pure stocks of the defective virus, plaque assays were carried out in V-A8L-cells that are able to complement the defective virus. The viral crude stock was prepared and used for plaque assays on V-A8L cells in the presence of gpt selection (Falkner and Moss (1988), supra) and blue plaque screening (Chakrabarti et al., supra). The plaque purification is repeated ten times. The purified defective poxviruses are grown to larger scale and examined by Southern blotting. The absence of wild-type virus and presence of the predicted bands confirms that the correct defective genomes have formed. Plaque assays of the defective viruses on the complementing cells and on wild-type cells confirm that the host range of the defective viruses are limited to the complementing cell line. Animal studies confirm that the defective viruses are non-pathogenic.

Example 4: Construction of a defective vaccinia virus lacking the small subunit of the early transcription factor (d-D6R-ZG) and of a complementing cell line

Construction of a helper cell line based on Vero cells (V-D6R):

The small subunit of the early transcription factor VETF is a polypeptide of 77kD in size. As described in Example 3, VETF contains a possible ATP binding site and has been found to bind to the early promotor DNA sequence. Broyles and Li, supra. The D6R orf is chosen for complementation because temperature sensitive mutations have been mapped to this gene, which is an

element of the definition of an essential gene. Seto et al.,
Virol. 160: 110-19 (1987).

The steps in the construction of the helper cell line V-D6R
are in general identical to the construction of the cell line V-
A8L in Example 3. First the D6R orf was cloned into the plasmid
pCRII (InVitrogen, Inc.) by PCR mediated techniques. The
resulting plasmid is the source of the D6R gene fragment for the
construction of an eukaryotic expression vector and an E. coli
expression plasmid. Expression of the orf in E. coli allows
rapid production of the protein for immunization of rabbits to
obtain anti-VETF small subunit antibodies that are subsequently
used to identify the protein in the permanent cell lines.

For this purpose, the D6R-orf was amplified as a 1.9 kb
fragment by PCR using the primers oD6-1 (5'-CTG CAG AAT GAA TAC
CGG AAT TAT AGA TTT-3') (SEQ ID NO:21) and oD6-2 (5'-CCC GGG TTA
TGG AGA AGA TAC CAC GTT-3') (SEQ ID NO:22), and cloned into
pCRII resulting in the plasmid pCR-D6R.

As shown in Figure 7, the E. coli expression plasmid pRSET-
D6R-3' was constructed by ligating a 1.6 kb EcoRI fragment from
pCR-D6R into the vector pRSET-B (InVitrogen, Inc.). Plasmid
pRSET-D6R-3' contains a fusion gene encoding a N'-terminal 6-
histidine-tag fused to the VETF small subunit lacking its first
100 amino acids. Bacterial expression, the tag-mediated
purification of the recombinant protein and the production of
anti-VETF small subunit antibodies were performed as outlined in
Example 3.   The expression plasmid pSV-D6R-EDH was constructed
as follows: The 1.9kb gene fragment D6R orf was isolated from
pCR-D6R by cutting the plasmid with PstI and SmaI and ligated
into the vector pSVMCS#51 (Herlitschka, supra) by forced cloning
to obtain the plasmid pSV-D6R. The sites PstI and SmaI were
introduced by the PCR primers oD6-1 (5'-CTGCAGAATG AATACCGGAA
TTATAGATTT -3') (SEQ ID NO:21) and oD6-2 (5'-CCCGGGTTAT
GGAGAAGATA CCACGTT-3') (SEQ ID NO:22), respectively. Ligation
of an EcoRV-ClaI gene fragment from the plasmid pEDH1 comprising
the "EDH gene cassette" into SmaI-ClaI cut pSV-D6R yielded the
expression plasmid pSV-D6R-EDH. See Figure 8.

In plasmid pSV-D6R-EDH, the D6R-orf is controlled by the
SV40 early promoter. The selection marker for obtaining

permanent cell lines is a fusion gene having the dhfr gene and the hph gene as described in Example 1. Transfection and screening procedures for the construction of the complementing cell line are identical to those in Example 3.

Construction of the defective virus d-D6R-ZG:

To construct the virus d-D6R-ZG the plasmid pCR-D6Rf-ZG was cloned as schematically shown in Figure 9. Regions (sized between 0.5 kb and 0.6 kb) flanking the D6R orf at either sides were amplified by PCR mediated techniques and subcloned into the plasmid pCRII to obtain pCR-D6R-fl and pCR-D6R-fr, respectively. The PCR-primers for the amplification of the D6R 5'-flanking region are oD6-3 (5'-TGA GAA GAA TTG CCG TCG-3') (SEQ ID NO:23) and oD6-5 (5'-GTC GAC GAT ATC TTC TAT AAA TAT ATG AGC-3') (SEQ ID NO:24). The primers for the D6R 3'-flanking region are oD6-4 (5'-TAC AGT ACC CGA ATC TCT AC-3') (SEQ ID NO:25) and oD6-6 (5'-ACA ATT GGT ATT AAG TAT AAC GAC TCC-3') (SEQ ID NO:26). The D6R 5'-flanking region as 0.6kb SacI-SpeI fragment from pCR-D6R-fl was ligated between the sites SacI and XbaI right downstream to the D6R 5'-flanking region in pCR-D6R-fl yielding the plasmid pCR-D6R-flanks. In order to facilitate further cloning procedures a synthetic linker containing the rare restriction sites SmaI, NotI, SfiI and RsrII was ligated between the D6R-flanking regions in pCR-D6R-flanks. The oligonucleotides used for the construction of the linker are oA8-11 and oA8-12. See Example 3. The plasmid pCR-D6R-flanks is therefore linearized with SalI, treated with Klenow Polymerase and cut with MunI (both restriction sites are introduced by the PCR primers oD6-5 and oD6-6, respectively). The resulting plasmid designated pdD6R is ligated with a 3.9kb SmaI double gene cassette from the plasmid pLGb described in Example 1 to yield pCR-D6Rf-ZG. In this construct, the D6R orf is deleted completely in order to prevent rescue of the defect virus by the complementing cell line due to homologous recombination.

For the construction of the d-D6R-ZG virus the plasmid pCR-D6RF-ZG is inserted into the vaccinia virus WR. The plasmid pCR-D6Rf-ZG is used for in vivo recombination in CV-1 cells. First, 5 x 106 CV-1 cells are infected with 0.1 pfu/cell of vaccinia WR, incubated for 1 hour, transfected with a calcium

phosphate precipitate (according to Graham & van der Eb, supra)
containing 20 µg of the plasmid pCR-D6R-ZG and grown for 3 days.
This results in a crude stock containing wild-type viruses
(helper) and defective viruses. To obtain pure stocks of the
defective virus, plaque assays are carried out in V-D6R-cells
that are able to complement the defective virus. The viral
crude stock is prepared and used for plaque assays on V-D6R
cells in the presence of gpt selection (Falkner and Moss, supra
(1988) and blue plaque screening (Chakrabarti et al., supra).
The plaque purification is repeated ten times. The purified
defective poxviruses are grown to larger scale and examined by
Southern blotting. The absence of wild-type virus and presence
of the predicted bands confirms that the correct defective
genomes have formed. Plaque assays of the defective viruses on
the complementing cells and on wild-type cells confirm that the
host range of the defective viruses are limited to the
complementing cell line. Animal studies confirm that the
defective viruses are non-pathogenic.

Example 5: Construction of a defective vaccinia virus lacking
            the J1R gene product (d-J1R-ZG) and of a
            complementing cell line

Construction of a helper cell line based on Vero cells (V-J1R):
        The first step was expression of the J1R orf in E. coli
(using the InVitrogen pRSET-vector system). First, the plasmid
pCR-J1R was constructed by cloning the J1R PCR product obtained
with the primers oJ1R-1 (5'-GCCATGGATC ACAACCAGTA TCTCTT-3')
(SEQ ID NO:27) and oJ1R-2 (5'-ACCCGGGTTA ATTAATTATT GTTCACTTTA-
3') (SEQ ID NO:28) into the pCRII vector. An NcoI-EcoRI
fragment was then inserted into pRSET-B yielding the E. coli
expression vector pRSET-J1R (Figure 10). With this plasmid,
high amounts of J1R protein were obtained which, notably, were
not immunogenic in rabbits. Antibodies for the further
identification of the protein in the permanent cell lines were
thus raised against a conjugate of a synthetic J1R peptide and
the carrier protein KLH. The synthetic peptide with the amino
acid sequence SLATTAIDPIRYIDP, corresponding to amino acid
positions 118 to 132 of the J1R protein, was coupled to KHL

(preactivated KLH from Pierce, USA). The purified conjugate in
complete Freund's adjuvant was used to immunize rabbits (100 mg
of protein per dose, s.c. and i.m.). After one month, the
animals were boosted with the same dose and development of
antibodies are monitored by Western blots.

Proteins are detected by Western blots. The Western blots
are done essentially as described by Towbin et al., supra. The
first antibody is a rabbit anti-J1R protein antibody used in a
1:100 dilution. The second antibody is a goat-anti-rabbit IgG
coupled with alkaline phosphatase (BioRad, Inc.) used in a
1:1000 dilution. The reagents (BCIP and NBT) and staining
protocols are from Promega, Inc (USA).

To obtain the expression plasmid pSV-J1R-EDH (Fig. 11) for
transformation into the Vero cells a 0.5kb NcoI- SmaI gene
fragment encoding the complete J1R gene was isolated from the
plasmid pCR-J1R. The sites NcoI and SmaI were introduced by
the PCR primers oJ1R-1 and oJ1R-2 (see above), respectively.
This fragment was ligated into the BbsI cut expression vector
pSV-Bbs (see Example 1). Insertion of a EcoRV-ClaI gene
fragment from plasmid pEDH1 comprising the "EDH gene cassette"
into SmaI-ClaI cut pSV-J1R yielded pSV-J1R-EDH. See Figure 11.
In plasmid pSV-J1R-EDH the J1R-orf is controlled by the SV40
early promoter. The selection marker for obtaining permanent
cell lines is a fusion gene including the dhfr gene and the hph
gene as described in Example 1.

The plasmid pSV-J1R-EDH was transfected into monkey kidney
Vero cells (ATCC No. CCL 81) in a method according to Graham &
van der Eb, supra, and incubated in selective medium (DMEM, 10%
fetal bovine serum, 250 µg/ml hygromycin) and further treated as
described in Example 1. Permanent hygromycin phosphotransferase
positive cell lines were selected in the presence of the
antibiotic hygromycin B. After five passages integration of the
foreign DNA was demonstrated by PCR using primers specific for a
0.5kb segment of the J1R gene. Western blots are used to
determine whether the gene of interest, the J1R protein, are
expressed. The cell lines expressing the highest levels of J1R
protein is further characterized by Southern blotting and used
as the complementing cell line and named V-J1R.

Construction of the defective virus d-J1R-ZG:

    To construct the virus d-J1R-ZG the plasmid pCR-J1Rf-ZG was
cloned as schematically shown in Figure 12. A 1.5 kb gene
fragment comprising the J1R orf and about 0.5kb of flanking
sequences at either sides was amplified from vaccinia virus
(strain WR) by PCR and subcloned into the plasmid pCRII to
obtain pCR-J1Rf.

    The PCR-primers were oJ1-3 (5'-TCC ACA TCC ATG AGA TTG AAT-
3') (SEQ ID NO:29) and oJ1-4 (5'-CGA TTG ATA CAT ATC ATT ACC-3')
(SEQ ID NO:30). Deletion of the complete J1R gene and the
first 40 nucleotides of the adjacent thymidine kinase gene was
also performed by PCR mediated techniques. The complete
plasmid pCR-J1Rf, excluding the sequences to be deleted, was
amplified via PCR resulting in a 6kb product. Polynucleotide
kinase treatment and subsequent re-ligation of the PCR product
yielded the plasmid pCR-J1R-flanks. The primers oJ1-5 (5'-
AACAATTGCA TCATCTGCGA AGAACATCG-3') (SEQ ID NO:31) and oJ1-6
(5'-CAGTTAACTT TTCAGGTAAA AGTACAGAAT-3') (SEQ ID NO:32) that
were employed for this reaction introduce the restriction sites
MunI and HpaI. The plasmid was cleaved at these sites and the
linker (oA8-11 and OA8-12 as described in Example 3) was
inserted to obtain pdJ1R. Analogous to Example 3 a lacZ-gpt
double marker cassette was ligated into the SmaI site of pdJ1R
resulting in the recombination vector pCR-J1Rf-ZG. (This
plasmid only contains 40 base pairs of the J1R gene, at its 5'
terminus, which are necessary for the integrity of the adjacent
L5R orf in the defective virus.)

    For the construction of the d-J1R-ZG virus the plasmid pCR-
J1R-ZG was inserted into the vaccinia virus WR. The plasmid
pCR-J1Rf-ZG was used for in vivo recombination in CV-1 cells.
First, 5 x 106 CV-1 cells were infected with 0.1 pfu/cell of
vaccinia WR, incubated for 1 hour, transfected with a calcium
phosphate precipitate (according to Graham & van der Eb, supra)
containing 20 micrograms of the plasmid pCR-J1R-ZG and grown for
3 days.

    This procedure resulted in a crude stock containing wild-
type (helper viruses) and defective viruses. To obtain pure
stocks of the defective virus, plaque assays were carried out in

30

V-J1R-cells that are able to complement the defective virus.
The viral crude stock was prepared and used for plaque assays on
V-J1R cells in the presence of gpt selection (Falkner and Moss
(1988), supra) and blue plaque screening (Chakrabarti et al.,
supra), alternating with plaque assays on tk-negative V-J1R
cells in the presence of BUdR (Mackett et al, supra). The
plaque purification is repeated ten times. The purified
defective poxviruses are grown to larger scale and examined by
Southern blotting. The absence of wild-type virus and presence
of the predicted bands confirms that the correct defective
genomes have formed. Plaque assays of the defective viruses on
the complementing cells and on wild-type cells confirm that the
host range of the defective viruses are limited to the
complementing cell line. Animal studies confirm that the
defective viruses are non-pathogenic.

Example 6: Construction of a defective vaccinia virus lacking
            the H4L gene product (d-H4L-ZG) and of a
            complementing cell line

        The H4L orf is chosen for complementation as conditional
lethal temperature sensitive mutations have been mapped to this
gene. Seto et al., supra.

        The steps in the construction of the helper cell line V-H4L
are in general identical to the construction of the cell line V-
A8L in Example 3. First, the H4L orf was cloned into the
plasmid pCRII (InVitrogen, Inc.) by PCR mediated techniques.
The resulting plasmid is the source of the H4L gene fragment for
the construction of the Vero cell expression vector pSV-H4L-EDH
as shown in Figure 13. Antibodies to identify the H4L gene
product (RAP94) in the permanent cell lines are raised against a
KLH conjugate of a synthetic peptide. The conjugate was
prepared by linkage of the synthetic peptide KIYKNSFSEDHNNSLD
corresponding to amino acid positions 242 to 258 in the H4L orf,
Kane and Shuman, J. Virol. 66: 5752-62 (1992), to activated KLH
(KLH coupling Kit, Pierce Inc., USA). This conjugate was used
for the production of anti-H4L antibodies analogous to Example
5.

For the construction of the Vero cell expression vector, the
H4L-orf was amplified as a 2.4kb fragment by PCR using the
primers oH4-1 (5'-ACC ATG GAC TCT AAA GAG ACT AT-3') (SEQ ID
NO:33) and oH4-2 (5'-CAA TTG CCC GGG TTA ATT AAT GAA ATT AAT CAT
ATA CAA CTC-3') (SEQ ID NO:34) and cloned into pCRII resulting
in the plasmid pCR-H4L. The expression plasmid pSV-H4L-EDH was
constructed as follows: The H4L orf was isolated as a 2.4kb gene
fragment from pCR-H4L by cutting the plasmid with NcoI and SmaI
and ligated into the vector pSV-Bbs (see Example 1) by forced
cloning to obtain the plasmid pSV-H4L. The sites NcoI and SmaI
were introduced by the PCR primers oH4-1 and oH4-2,
respectively. Ligation of an EcoRV-SalI gene fragment from the
plasmid pEDH1 comprising the "EDH gene cassette" into SmaI-SalI
cut pSV-H4L yielded the expression plasmid pSV-H4L-EDH. See
Figure 13.

In plasmid pSV-H4L-EDH, the H4L-orf is controlled by the
SV40 early promoter. The selection marker for obtaining
permanent cell lines is a fusion gene including the dhfr gene
and the hph gene as described in Example 1. Transfection and
screening procedures for the construction of the complementing
cell line were done as described in Example 3, except that H4L-
specific reagents were used.
Construction of the defective virus d-H4L-ZG:

To construct the virus d-H4L-ZG, the plasmid pCR-H4Lf-ZG was
cloned as schematically shown in Figure 14. Regions (sized
between 0.5kb and 0.6kb) flanking the H4L orf at either sides
were amplified by PCR mediated techniques and subcloned into the
plasmid pCRII to obtain pCR-H4L-fl and pCR-H4L-fr, respectively.

The PCR-primers for the amplification of the H4L 5'-
flanking region are oH4-3 (5'-CCT TTA GGT CAG AA GAT CGC-3')
(SEQ ID NO:35) and oH4-5 (5'-GTC GAC AAT TGT TAA AG TAC AAA CAA
CTA GGA-3') (SEQ ID NO:36). The primers for the H4L 3'-flanking
region are oH4-4 (5'-GCT AAC ATC ATA CCC TCC TG-3') (SEQ ID
NO:37) and oH4-6 (5'-GTC GAC GTT AAC AGA AGC ATT GGA ATA CGC AT-
3') (SEQ ID NO:38). The H4L 3'-flanking region as 0.5 kb SalI-
SpeI fragment from pCR-H4L-fr was ligated between the sites SalI
and XbaI right downstream to the H4L 5'-flanking region in pCR-
D6R-fl, yielding the plasmid pCR-H4L-flanks.

In order to facilitate further cloning procedures, a
synthetic linker containing the rare restriction sites SmaI,
NotI, SfiI and RsrII was ligated between the H4L-flanking
regions in pCR-H4L-flanks. The oligonucleotides used for the
construction of the linker are oA8-11 and oA8-12. The plasmid
pCR-H4L-flanks was thereafter linearized with MunI and HpaI
(both sites are introduced by the PCR primers oH4-5 and oH4-6,
respectively). The resulting plasmid designated pdH4L was
ligated with a 3.9 kb SmaI double gene cassette from the plasmid
pLGb described in Example 1 to yield pCR-H4Lf-ZG. In this
construct the H4L orf is deleted completely in order to prevent
rescue of the defect virus by the complementing cell line due to
homologous recombination.

The construction of the d-H4L-ZG virus by in vivo
recombination and plaque purification is done according to the
scheme described in Example 1.

Example 7: Construction of a complementing cell line with
            heatshock-inducible I7L function

The effect of the vaccinia host protein shut-off varies from
cell type to cell type, which influences the effectiveness of
complementation and thus determines the amount of plaque
purifications necessary to obtain pure stocks of defective virus
and the titers obtainable with the host cell lines. In order to
make complementation more efficient, the essential open reading
frame coding for the complementing function is cloned downstream
of the promoter of the 70 kd human heatshock protein (Hsp70)
gene (Hunt & Morimoto, supra). Transcription of the Hsp70-gene
is induced by vaccinia infection and Hsp70 has been proposed to
be involved in the assembly of the vaccinia virion (Jindal and
Young, supra).

The first step is cloning of the Hsp70 promoter by PCR
techniques. The oligonucleotides oHS-1, 5'- TACGTATGGA
GACCAACACC CTTCC-3' (SEQ ID NO:39) and oHS-2, 5'- CCATGGATAT
CGGGTTCCCT GCTCTCTGTC GG-3' (SEQ ID NO:40) were used to together
with human DNA (Clontech, Inc.) as a template to obtain the
human Hsp70 promoter sequences. The PCR product is cloned into
the pCRII vector (InVitrogen, Inc.) yielding the plasmid pCR-

Hsp. The SnaBI-NcoI promoter fragment is used to substitute the
SV40 promoter in the plasmid pSV-Bbs (see Example 1) resulting,
after removal of the SV40 intron present in this plasmid, in the
plasmid pHS. This vector is used to insert open reading frames
such as I7L, by ligating the NcoI-SmaI fragment of pSV-I7L
(Example 1) with pHS yielding the plasmid pHS-I7L. See Figure
15.

    To construct the complementing cell line, the co-
transformation procedure (Wigler, supra) is performed. A
suitable selection marker, such as the neomycin resistance gene
present on the plasmid pSV2-neo (Southern, P.J. and Berg, P., J.
Mol. Appl. Genet. 1: 327 (1982)) is used together with the
plasmid pHS-I7L in a transfection experiment into Vero cells.
The cells are transfected according to Graham and van der Eb,
supra, and incubated in selective medium including the
antibiotic G418 (DMEM, 10% fetal bovine serum, 500 µg/ml G-418).
After 10-14 days, colonies become visible. These colonies are
expanded, subcloned twice and then characterized further. The
cell lines are further characterized by PCR analysis for the
presence of the complementing gene construct. Western blots are
used to show that the gene of interest, the I7L-protein is
expressed upon induction. The final complementing cell line is
named VHsp-I7L. The cell line is characterized as described in
Example 1.   Construction of defective virus d-I7L-ZG/Hsp is
done as described in Example 1 for the virus d-I7L-ZG except
that the cell line VHsp-I7L is used for complementation and that
only five rounds of plaque purification are done to obtain the
defective virus.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: IMMUNO AG
        (B) STREET: Industriestr. 67
        (C) CITY: Wien
        (E) COUNTRY: AUSTRIA
        (F) POSTAL CODE (ZIP): A-1221

    (ii) TITLE OF INVENTION: Recombinant poxviruses with foreign
        polynucleotides in essential regions

    (iii) NUMBER OF SEQUENCES: 40

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/235,392
        (B) FILING DATE: 29-APR-1994

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

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

AGTACTCCAT GGAAAGATAT ACAGATTTAG   30

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

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

ATCCCGGGTT TTAGAGACTT TGAAGCTACT   30

(2) INFORMATION FOR SEQ ID NO: 3:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 40 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

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

CTAGGCTTTT GCAAAAAGCT CCTCGACCAT GGTGTCTTCA   40

(2) INFORMATION FOR SEQ ID NO: 4:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 40 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

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

AGCTTGAAGA CACCATGGTC GAGGAGCTTT TTGCAAAAGC   40

(2) INFORMATION FOR SEQ ID NO: 5:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 22 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGAGTTAAT GAGGCCAATG GA   22

(2) INFORMATION FOR SEQ ID NO: 6:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 21 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACATAGGTA TAGAATCCGG A   21

(2) INFORMATION FOR SEQ ID NO: 7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 63 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGTTTAAACG GCGCGCCCGG GCTCGAGAGG CCTCTGCAGA TGCATCCATG GGGATCCGAA   60

TTC   63

(2) INFORMATION FOR SEQ ID NO: 8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 63 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAATTCGGAT CCCCATGGAT GCATCTGCAG AGGCCTCTCG AGCCCGGGCG CGCCGTTTAA   60

ACT   63

(2) INFORMATION FOR SEQ ID NO: 9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 48 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCCTACGT ATCTAGAATT AATTAATGGC CAATTTAAAT GCCCGGGA   48

(2) INFORMATION FOR SEQ ID NO: 10:

        (i) SEQUENCE CHARACTERISTICS:

           (A) LENGTH: 48 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AATTTCCCGG GCATTTAAAT TGGCCATTAA TTAATTCTAG ATACGTAG   48

(2) INFORMATION FOR SEQ ID NO: 11:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 45 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCGTAAAAA TTGAAAAACT ATTCTAATTT ATTGCACGGT CGCGA   45

(2) INFORMATION FOR SEQ ID NO: 12:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 50 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CATGGTACGT ACCGTGCAAT AAATTAGAAT AGTTTTTCAA TTTTTACGAG   50

(2) INFORMATION FOR SEQ ID NO: 13:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 43 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

38

AAACTGCAGG ATGCGATATA TAGTAAGTCC GCAAATGGTA TTA   43

(2) INFORMATION FOR SEQ ID NO: 14:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AGGAATTCAG GCCTGTTTAA TTAATTTGTG CTCTTC   36

(2) INFORMATION FOR SEQ ID NO: 15:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGCGCCGCTA CTAGCACTCC   20

(2) INFORMATION FOR SEQ ID NO: 16:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 38 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAACGTTAAC ATTTATATCG TGGGGTAAAG TGAAAATC   38

(2) INFORMATION FOR SEQ ID NO: 17:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTGGAGAACT TGATACGCCG   20

(2) INFORMATION FOR SEQ ID NO: 18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 43 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CATATGCAAT TGTAAATTAA ACAACTAAAT CTGTAAATAA ATA   43

(2) INFORMATION FOR SEQ ID NO: 19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AACGGTCCGG CCCGGGCGGC CGCC   24

(2) INFORMATION FOR SEQ ID NO: 20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AATTGGCGGC CGCCCGGGCC GGACCGTT   28

(2) INFORMATION FOR SEQ ID NO: 21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid

```
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTGCAGAATG AATACCGGAA TTATAGATTT                               30

(2) INFORMATION FOR SEQ ID NO: 22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CCCGGGTTAT GGAGAAGATA CCACGTT                                  27

(2) INFORMATION FOR SEQ ID NO: 23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TGAGAAGAAT TGCCGTCG                                            18

(2) INFORMATION FOR SEQ ID NO: 24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)




    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GTCGACGATA TCTTCTATAA ATATATGAGC                               30
```

(2) INFORMATION FOR SEQ ID NO: 25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TACAGTACCC GAATCTCTAC   20

(2) INFORMATION FOR SEQ ID NO: 26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ACAATTGGTA TTAAGTATAA CGACTCC   27

(2) INFORMATION FOR SEQ ID NO: 27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GCCATGGATC ACAACCAGTA TCTCTT   26

(2) INFORMATION FOR SEQ ID NO: 28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

42

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

ACCCGGGTTA ATTAATTATT GTTCACTTTA   30

(2) INFORMATION FOR SEQ ID NO: 29:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCACATCCA TGAGATTGAA T   21

(2) INFORMATION FOR SEQ ID NO: 30:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CGATTGATAC ATATCATTAC C   21

(2) INFORMATION FOR SEQ ID NO: 31:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 29 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

AACAATTGCA TCATCTGCGA AGAACATCG   29

(2) INFORMATION FOR SEQ ID NO: 32:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 30 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

43

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CAGTTAACTT TTCAGGTAAA AGTACAGAAT   30

(2) INFORMATION FOR SEQ ID NO: 33:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 23 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

ACCATGGACT CTAAAGAGAC TAT   23

(2) INFORMATION FOR SEQ ID NO: 34:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 42 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CAATTGCCCG GGTTAATTAA TGAAATTAAT CATATACAAC TC   42

(2) INFORMATION FOR SEQ ID NO: 35:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 20 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCTTTAGGTC AGAAGATCGC   20

(2) INFORMATION FOR SEQ ID NO: 36:

44

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 32 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GTCGACAATT GTTAAAGTAC AAACAACTAG GA   32

(2) INFORMATION FOR SEQ ID NO: 37:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GCTAACATCA TACCCTCCTG   20

(2) INFORMATION FOR SEQ ID NO: 38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GTCGACGTTA ACAGAAGCAT TGGAATACGC AT   32

(2) INFORMATION FOR SEQ ID NO: 39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

TACGTATGGA GACCAACACC CTTCC   25

(2) INFORMATION FOR SEQ ID NO: 40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

CCATGGATAT CGGGTTCCCT GCTCTCTGTC GG   32

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Claims:

1.   A defective poxvirus that lacks a function imparted by
an essential region of its parental poxvirus, said defective
poxvirus comprising a foreign polynucleotide under
transcriptional control of a promoter.

2.   A defective poxvirus according to claim 1, wherein said
promoter is operable with enzymes of said defective poxvirus.

3.   A defective poxvirus according to claim 1, wherein said
promoter is a poxvirus promoter.

4.   A defective poxvirus according to claim 1, wherein said
foreign polynucleotide is inserted into said essential region.

5.   A defective poxvirus according to claim 1, wherein said
essential region has been deleted from said defective poxvirus.

6.   A defective poxvirus according to claim 5, wherein a
marker replaces said essential region.

7.   A defective poxvirus according to claim 6, wherein said
foreign polynucleotide is inserted into said marker.

8.   A defective poxvirus according to claim 1, wherein said
parental poxvirus is vaccinia.

9.   A defective poxvirus according to claim 8, wherein said
essential region is an open reading frame selected from the
group consisting of I7L, F18R, D13L, D6R, A8L, J1R, J3R, H4L,
A10L and A3L.

10.   A defective poxvirus according to claim 8, wherein said
essential region is an open reading frame encoding the 14
kilodalton envelope protein or the 25 kilodalton structural
protein.

11.   A cell line having the ability to complement a lost
function of a defective poxvirus according to claims 1 to 10.

12.   A cell line according to claim 11, wherein said ability
is imparted by an essential region of a parental poxvirus of
said defective poxvirus.

13.   A cell line according to claim 12, wherein said ability
is imparted by an essential region of a parental poxvirus of
said defective poxvirus said essential region being controlled
by a promoter induced by poxvirus infection.

14.   A cell line according to claim 13, wherein said
promoter is an Hsp70 promoter.

15.   A transgenic animal having the ability to complement a
lost function of a defective poxvirus according to claims 1 to
10.

16.   A transgenic animal according to claim 15, wherein said
ability is imparted by an essential region of a parental
poxvirus of said defective poxvirus.

17.   A helper virus having the ability to complement a lost
function of a defective poxvirus according to claims 1 to 10.

18.   A helper virus according to claim 17, wherein said
ability is imparted by an essential region of a parental
poxvirus of said defective poxvirus.

19.   A method of producing a protein, comprising the step of
providing a defective poxvirus that lacks a function imparted by
an essential region of its parental poxvirus, wherein said
defective poxvirus comprises a foreign polynucleotide encoding
said protein, and said polynucleotide is under transcriptional
control of a promoter.

20. A method according to claim 19, wherein said promoter is operable with enzymes of said defective poxvirus.

21. A method according to claim 19, wherein said promoter is a poxvirus promoter.

22. A method according to claim 19, wherein said providing step is accomplished by inserting said polynucleotide into said essential region of said parental poxvirus.

23. A method according to claim 19, wherein said providing step is accomplished by replacing said essential region of said parental poxvirus with said polynucleotide.

24. A method according to claim 19, wherein said providing step is accomplished by replacing said essential region of said parental poxvirus with a marker.

25. A method according to claim 24, wherein said polynucleotide is inserted into said marker.

26. A method according to claim 24, wherein said polynucleotide replaces said marker.

27. A method according to claim 24, wherein said marker is a gpt gene.

28. A vaccine comprising a defective poxvirus that lacks a function imparted by an essential region of its parental poxvirus, wherein said defective poxvirus comprises a polynucleotide encoding an antigen and said polynucleotide is under transcriptional control of a promoter.

29. A vaccine according to claim 28, wherein said promoter is operable with enzymes of said defective poxvirus.

30. A vaccine according to claim 28, wherein said promoter is a poxvirus promoter.

31. A vaccine according to claim 28, wherein said parental poxvirus is vaccinia.

32. A method of producing a vaccine, comprising the step of providing a defective poxvirus that lacks a function imparted by an essential region of its parental poxvirus, wherein said defective poxvirus comprises a polynucleotide encoding an antigen and said polynucleotide is under transcriptional control of a promoter.

33. A method of producing a vaccine according to claim 32, wherein said promoter is operable with enzymes of said defective poxvirus.

34. A method of producing a vaccine according to claim 32, wherein said promoter is a poxvirus promoter.

35. A method of producing a vaccine according to claim 32, wherein said parental poxvirus is vaccinia.

36. A defective poxvirus comprising a foreign polynucleotide under transcriptional control of a promoter, wherein said defective poxvirus lacks a function imparted by an essential region of a parental poxvirus from which said defective poxvirus is derived.

37. A method of producing a vaccine, comprising the step of providing a defective poxvirus comprising a foreign polynucleotide under transcriptional control of a promoter, wherein said defective poxvirus lacks a function imparted by an essential region of a parental poxvirus from which said defective poxvirus is derived.

38. A vaccine comprising a defective poxvirus comprising a foreign polynucleotide under transcriptional control of a promoter, wherein said defective poxvirus lacks a function

imparted by an essential region of a parental poxvirus from which said defective poxvirus is derived.

39. The use of a defective poxvirus according to any of claims 1 to 10 for the preparation of a vaccine.

Construction of the plasmid pRSET-I7L
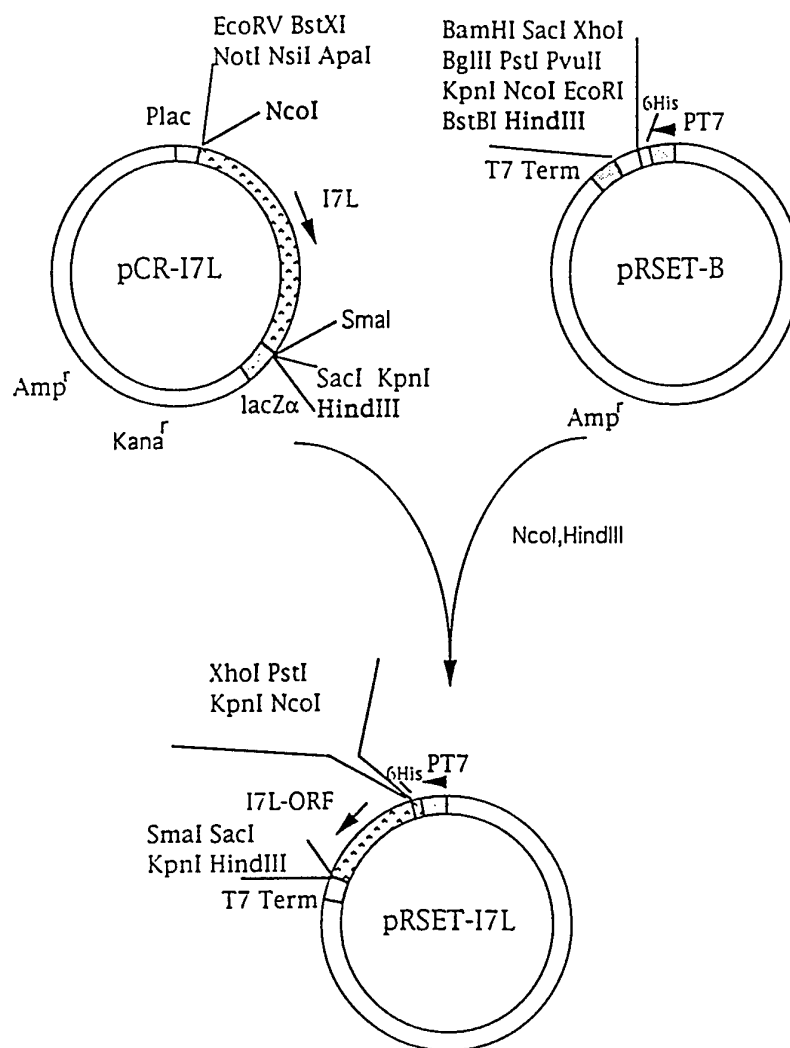
Figure /1

Construction of the plasmid pSV-I7L-EDH
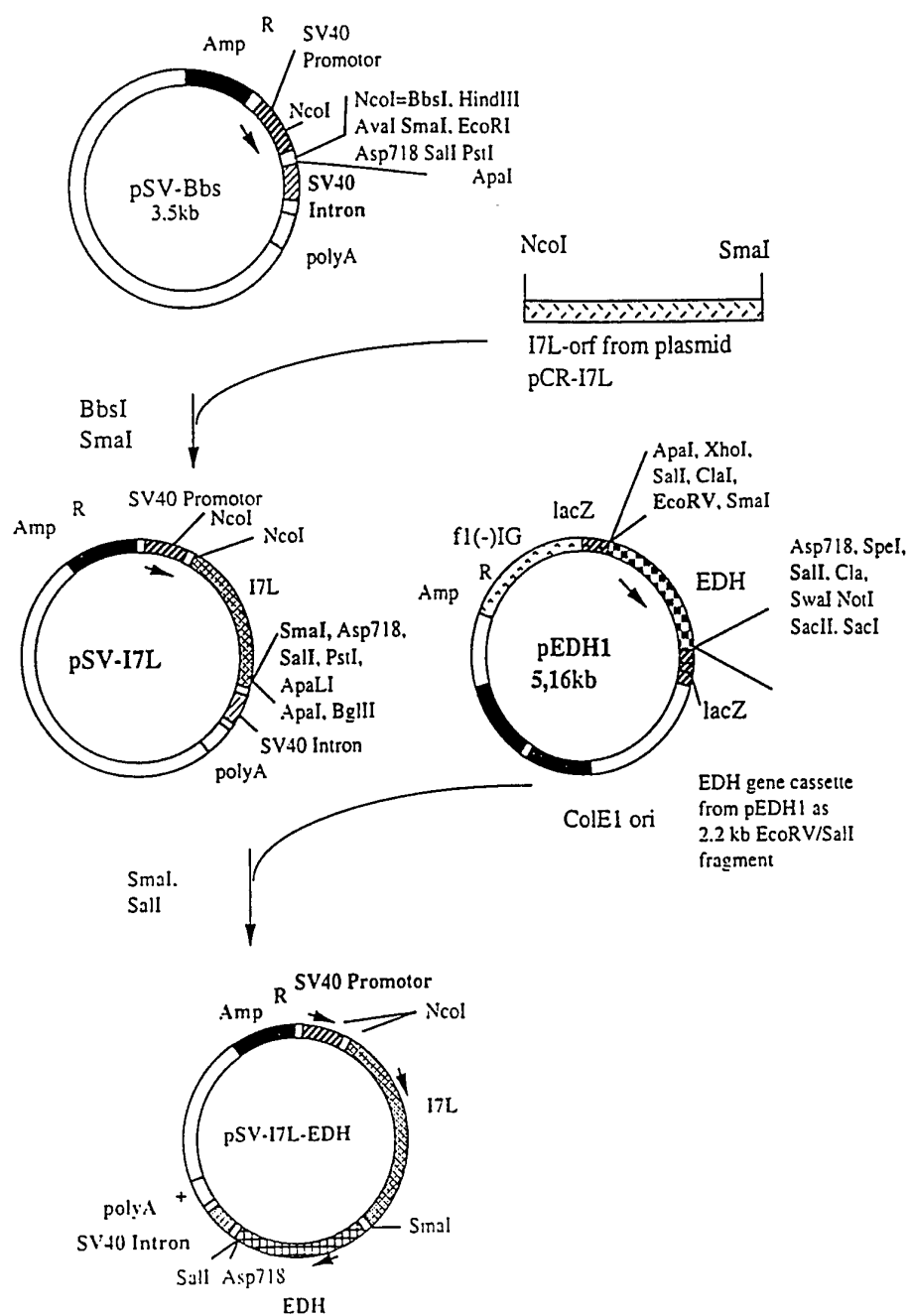
Figure 2

0 3 / 1 5
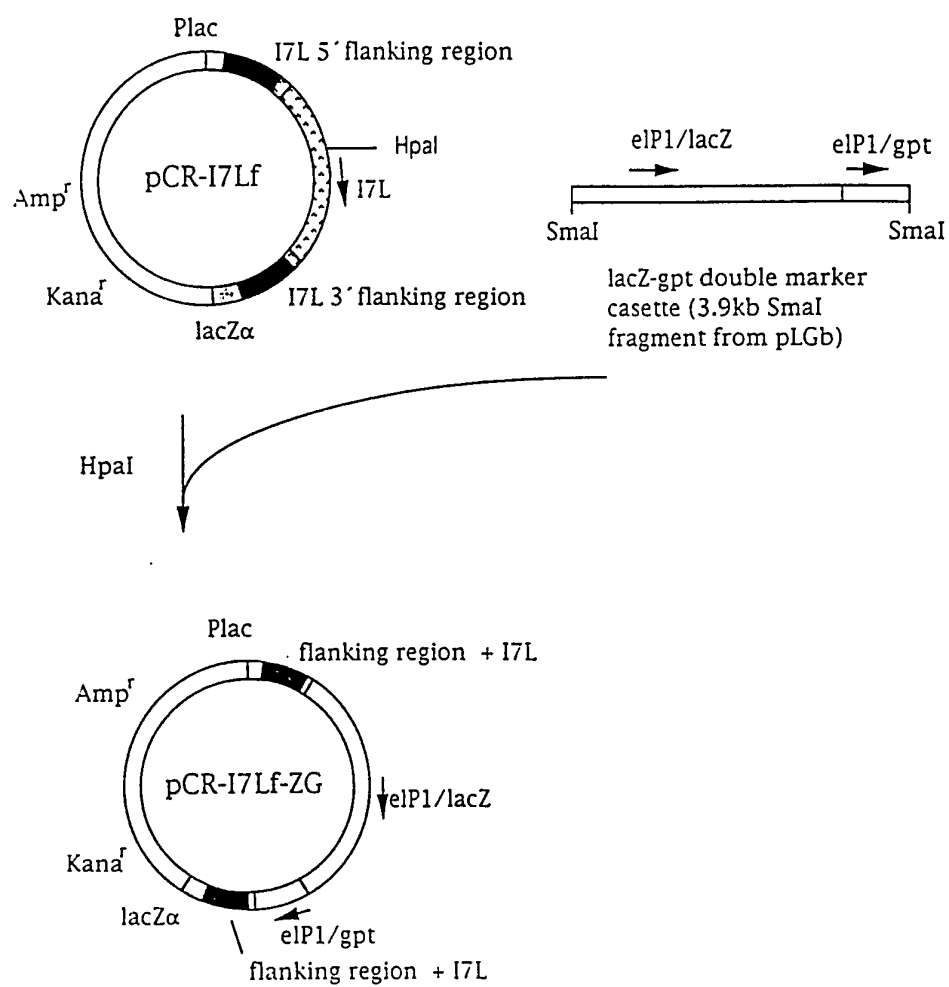
Construction of the plasmid pCR-I7Lf-ZG
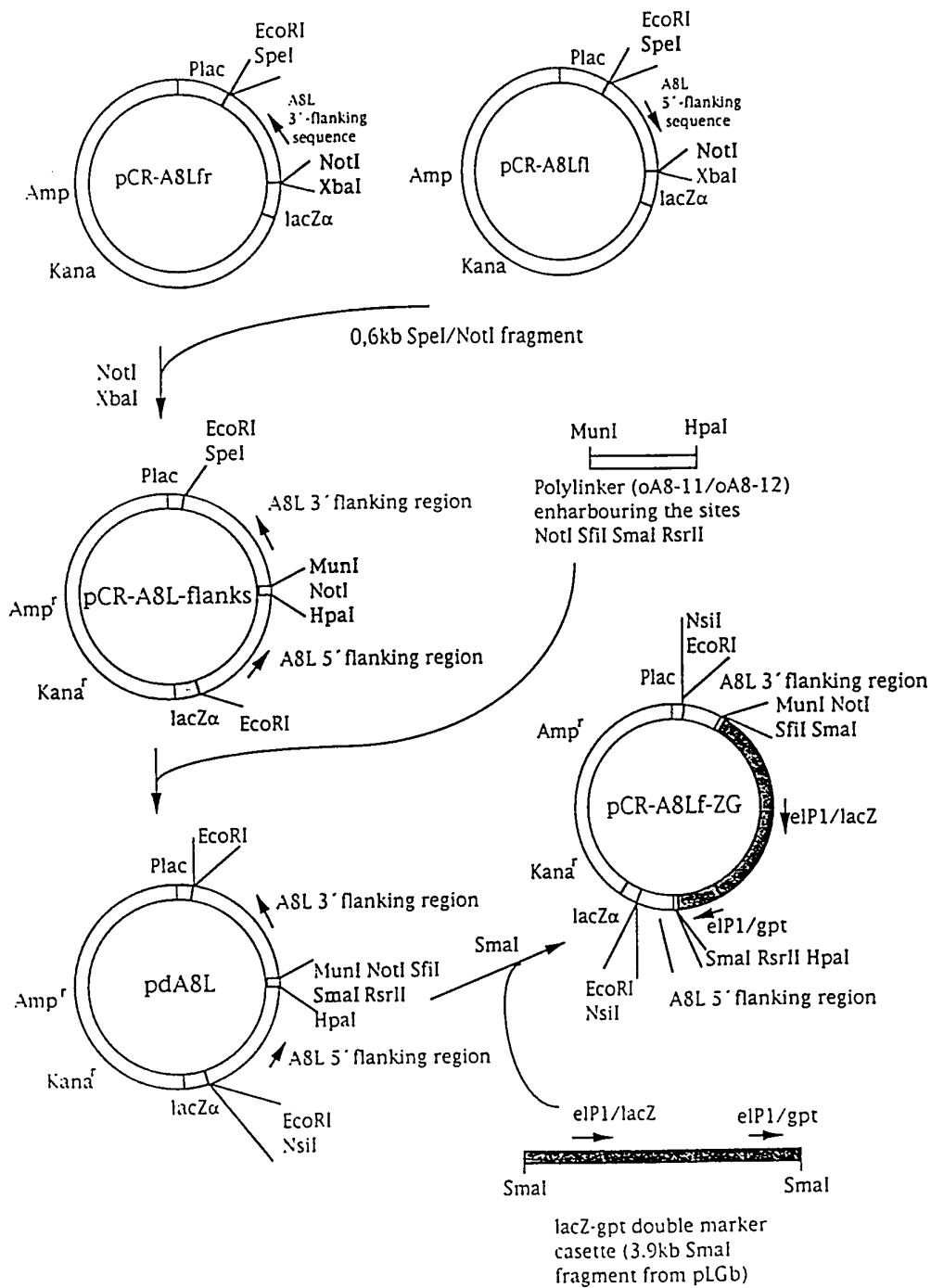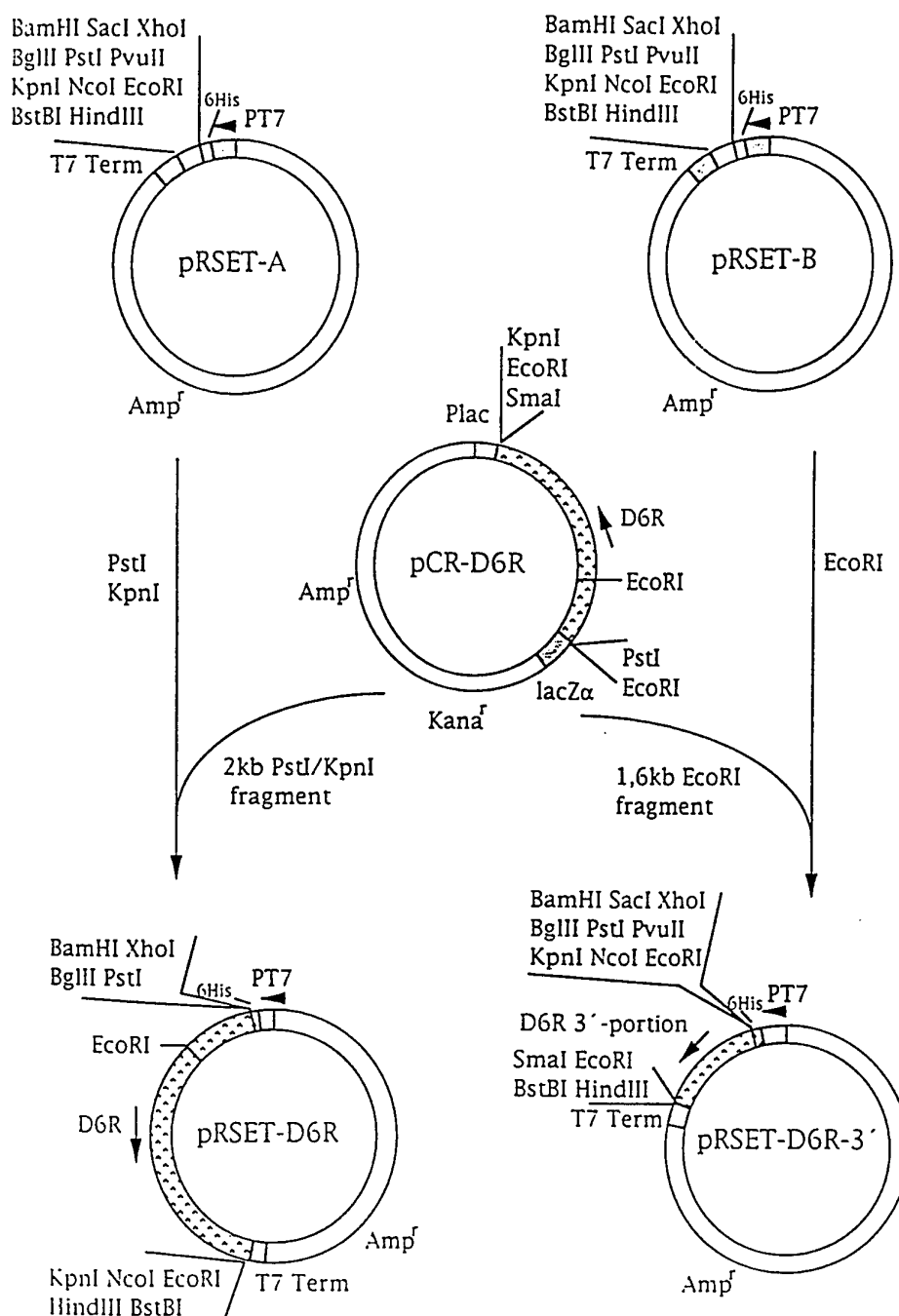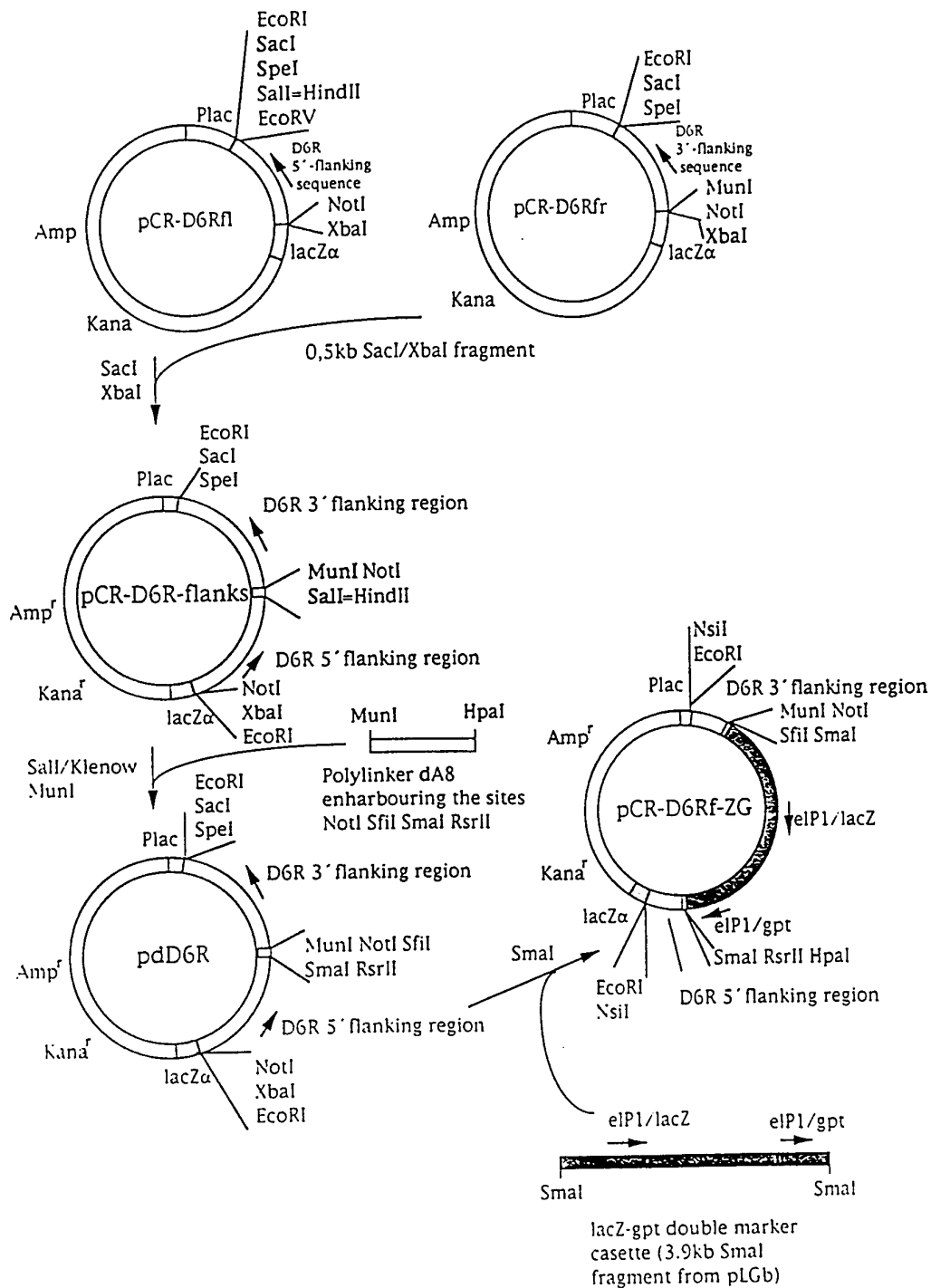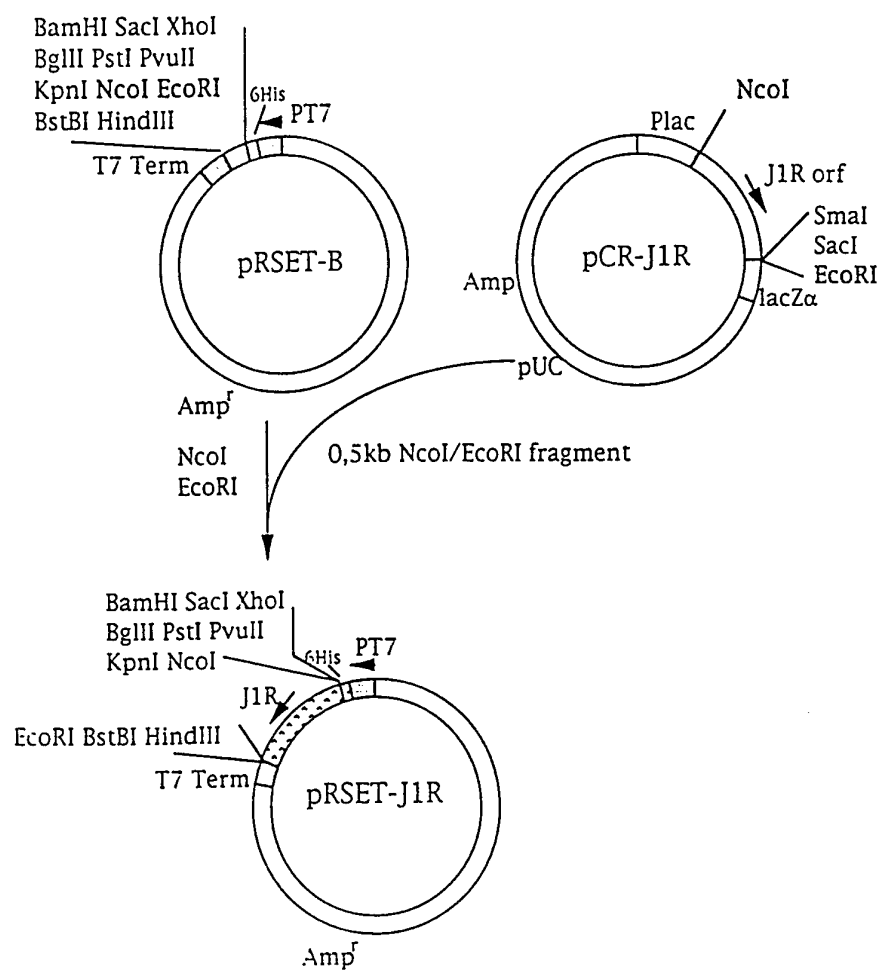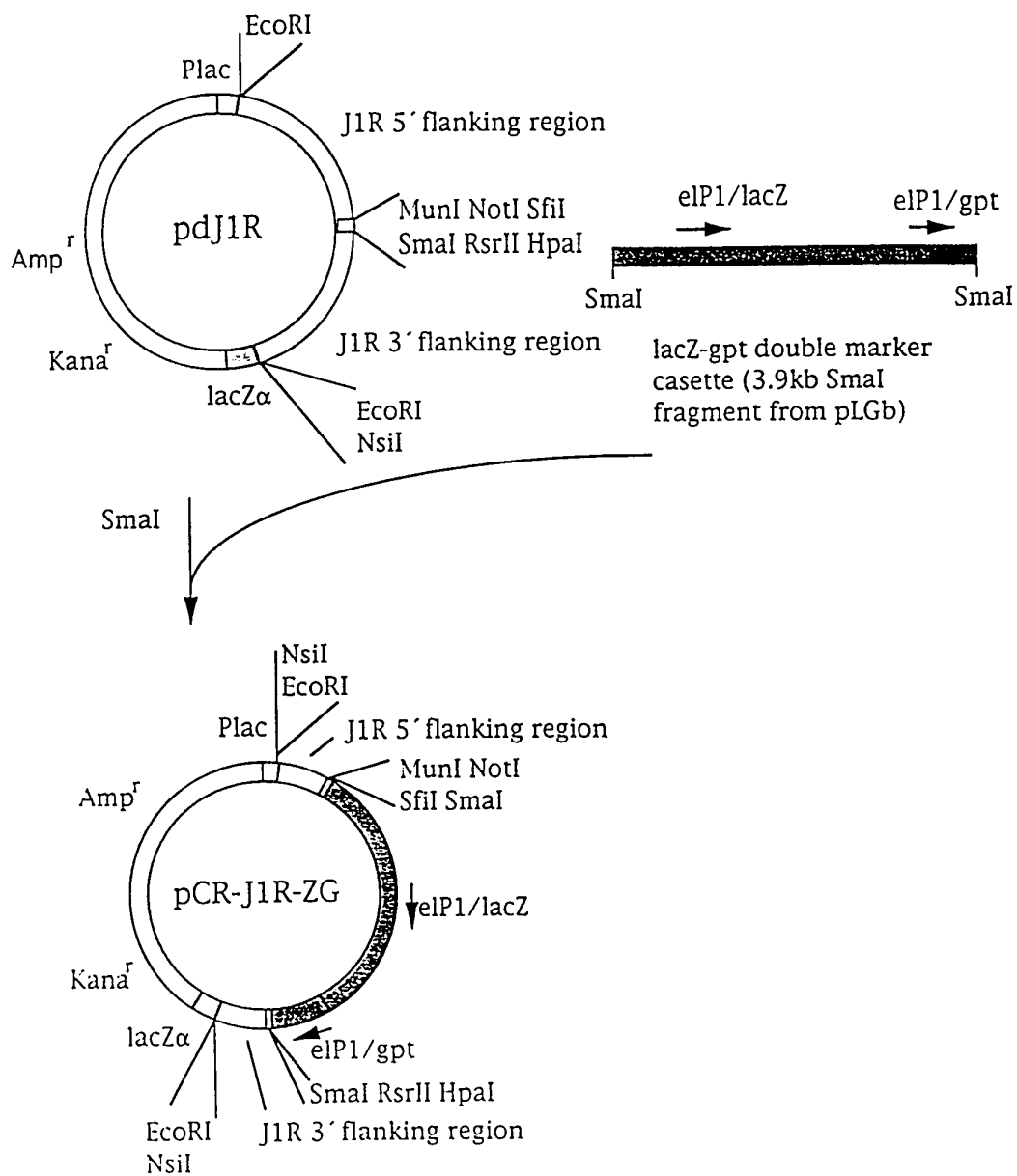

Figure 3

Construction of the plamid pRSET-A8L-3´/2
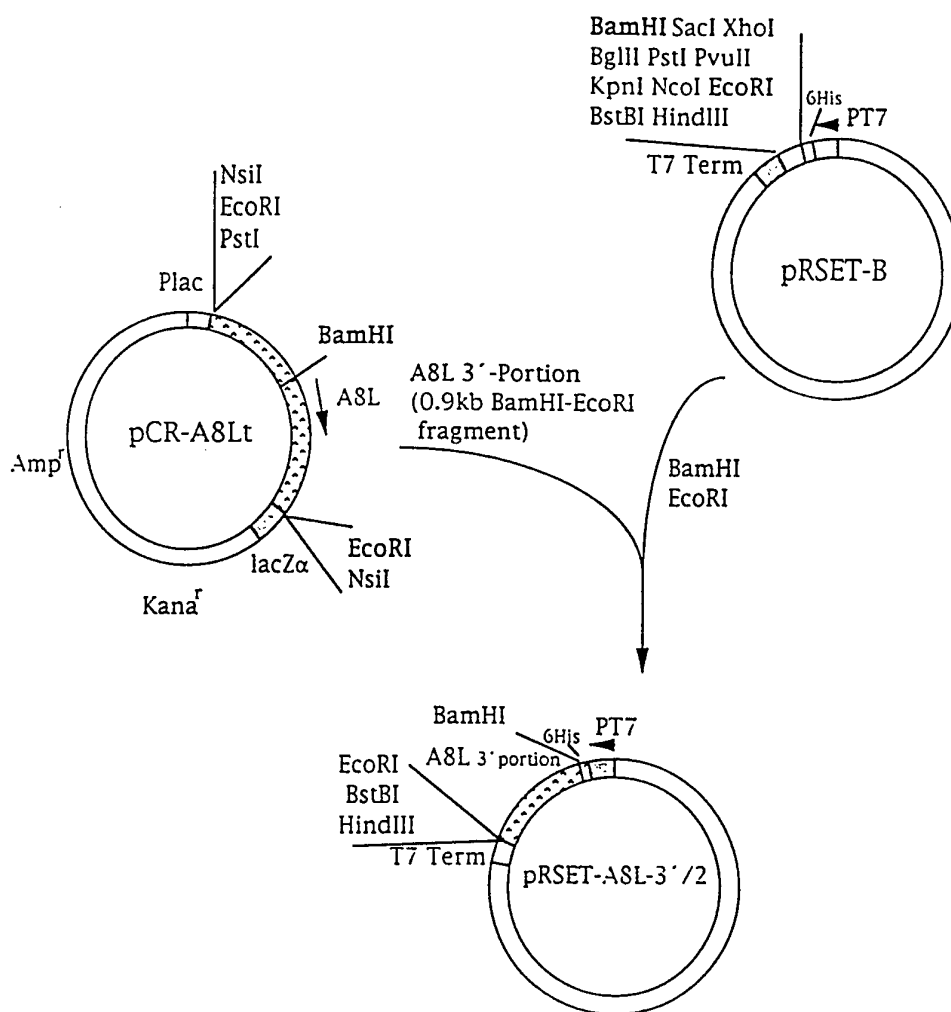
Figure 4

Construction of the plasmid pSV-A8L-EDH
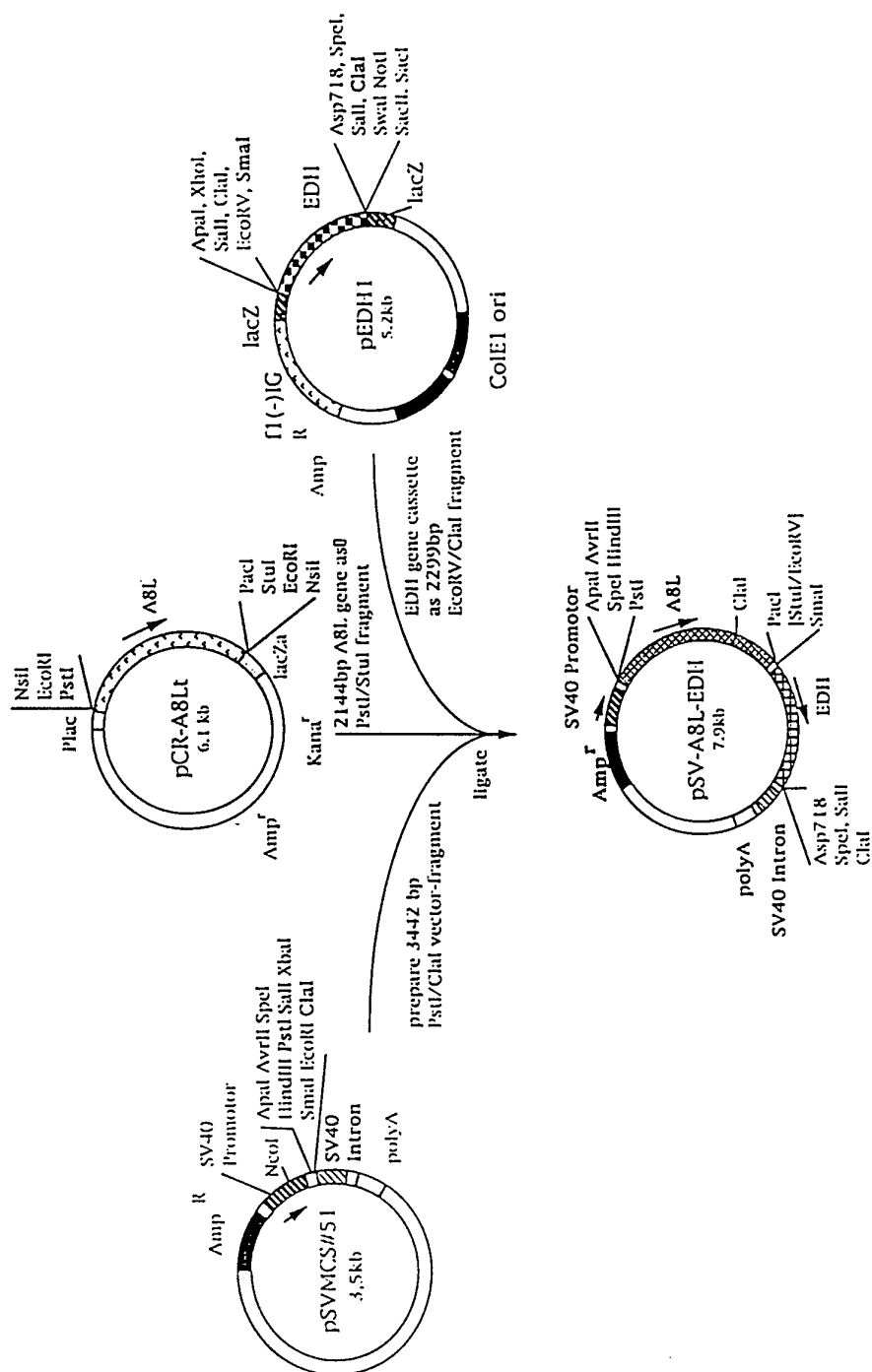
Figure 5

Construction of the plasmid pCR-A8Lf-ZG

Figure 6

Construction of the plasmid pRSET-D6R and pRSET-D6R-3´

Figure 7

Construction of the plasmid pSV-D6R-EDH
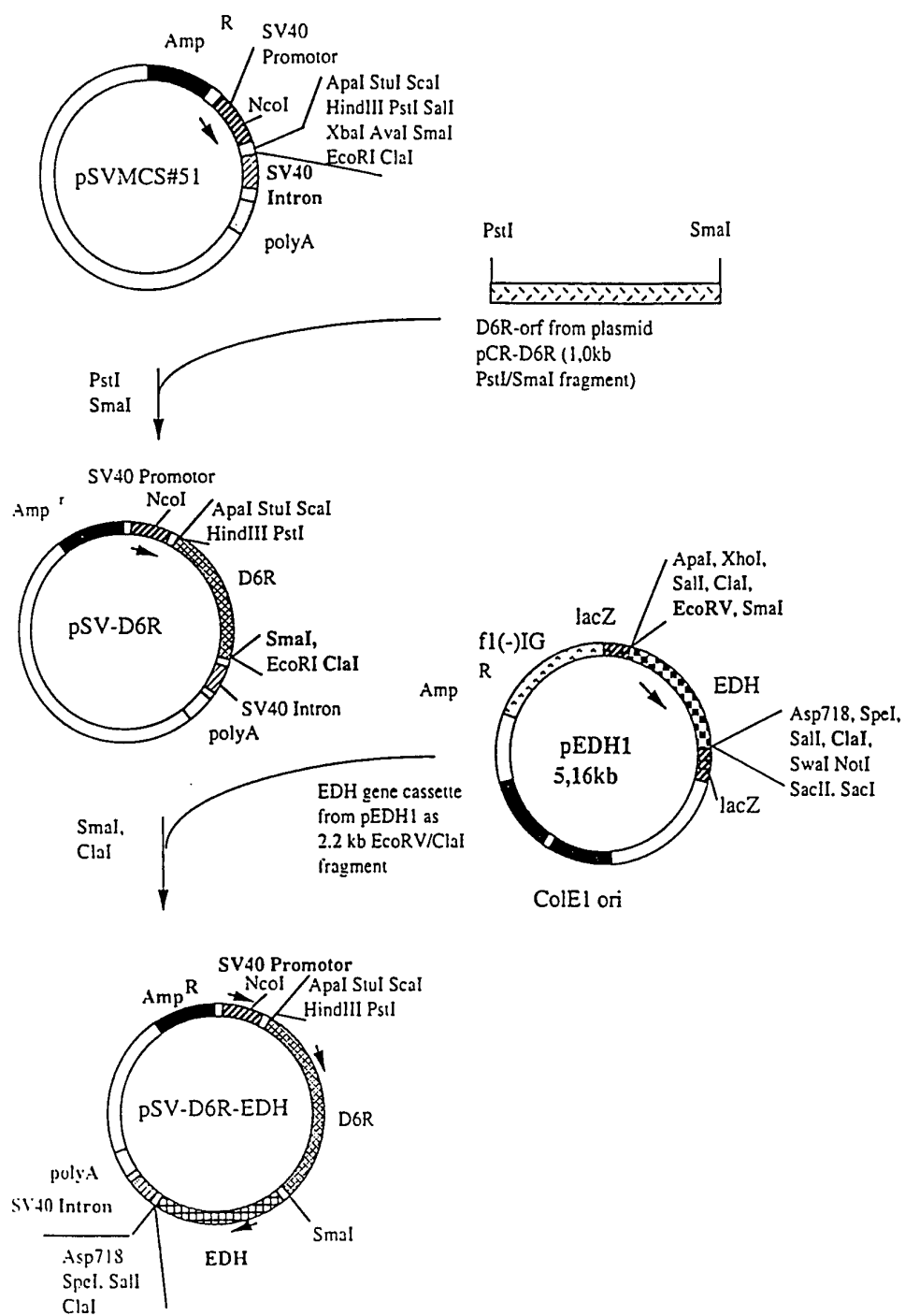
Figure. 8

Construction of the plasmid pCR-D6Rf-ZG

Figure 9

Construction of the plasmid pRSET-J1R

Figure 10

Construction of the plasmid pSV-J1R-EDH
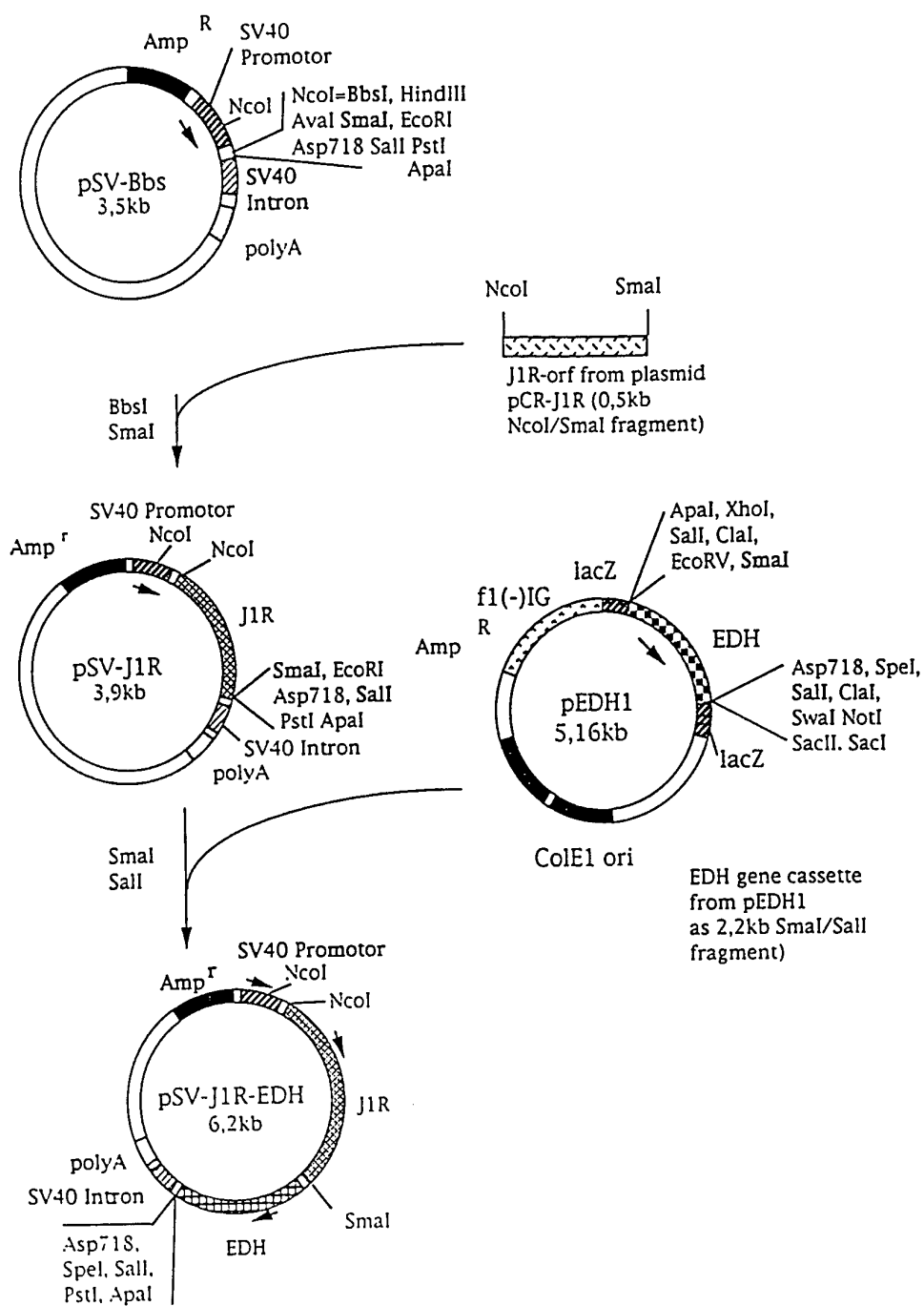
Figure 11

Construction of the plasmid pCR-J1R-ZG
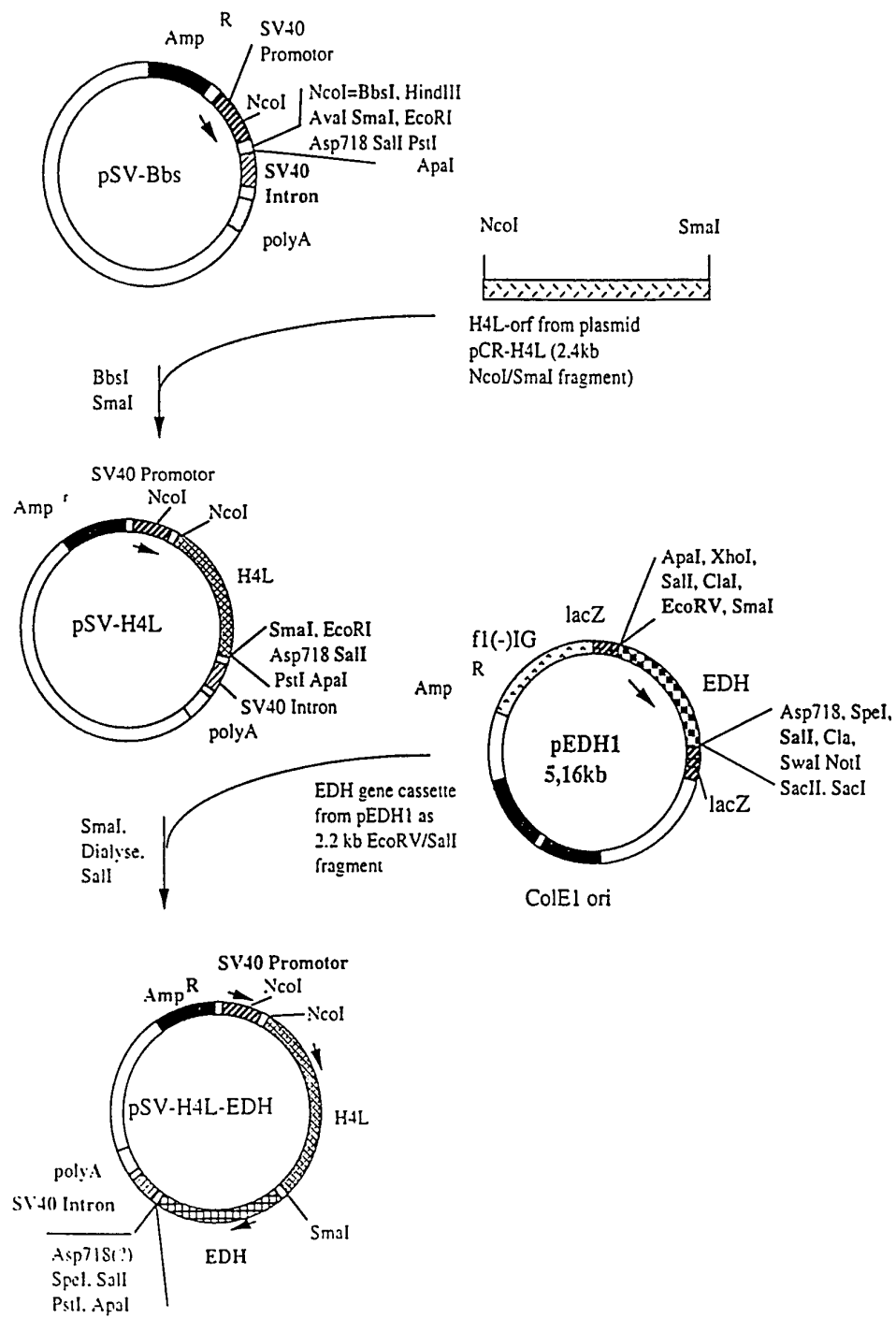

Figure /2

Construction of the plasmid pSV-H4L-EDH

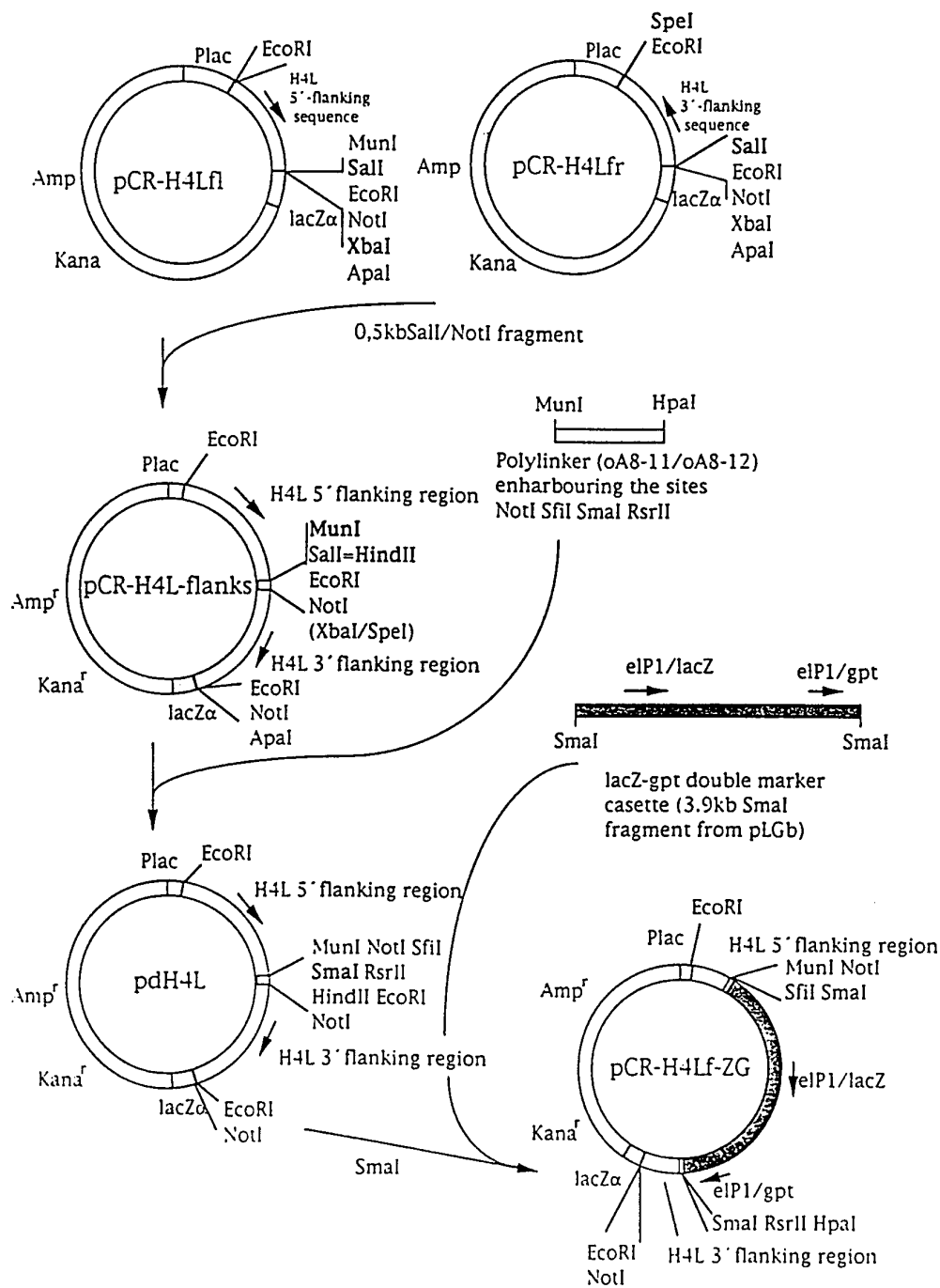Figure 13

Construction of the plasmid pCR-H4Lf-ZG

Figure 14

Construction of the plasmid pHS-I7L
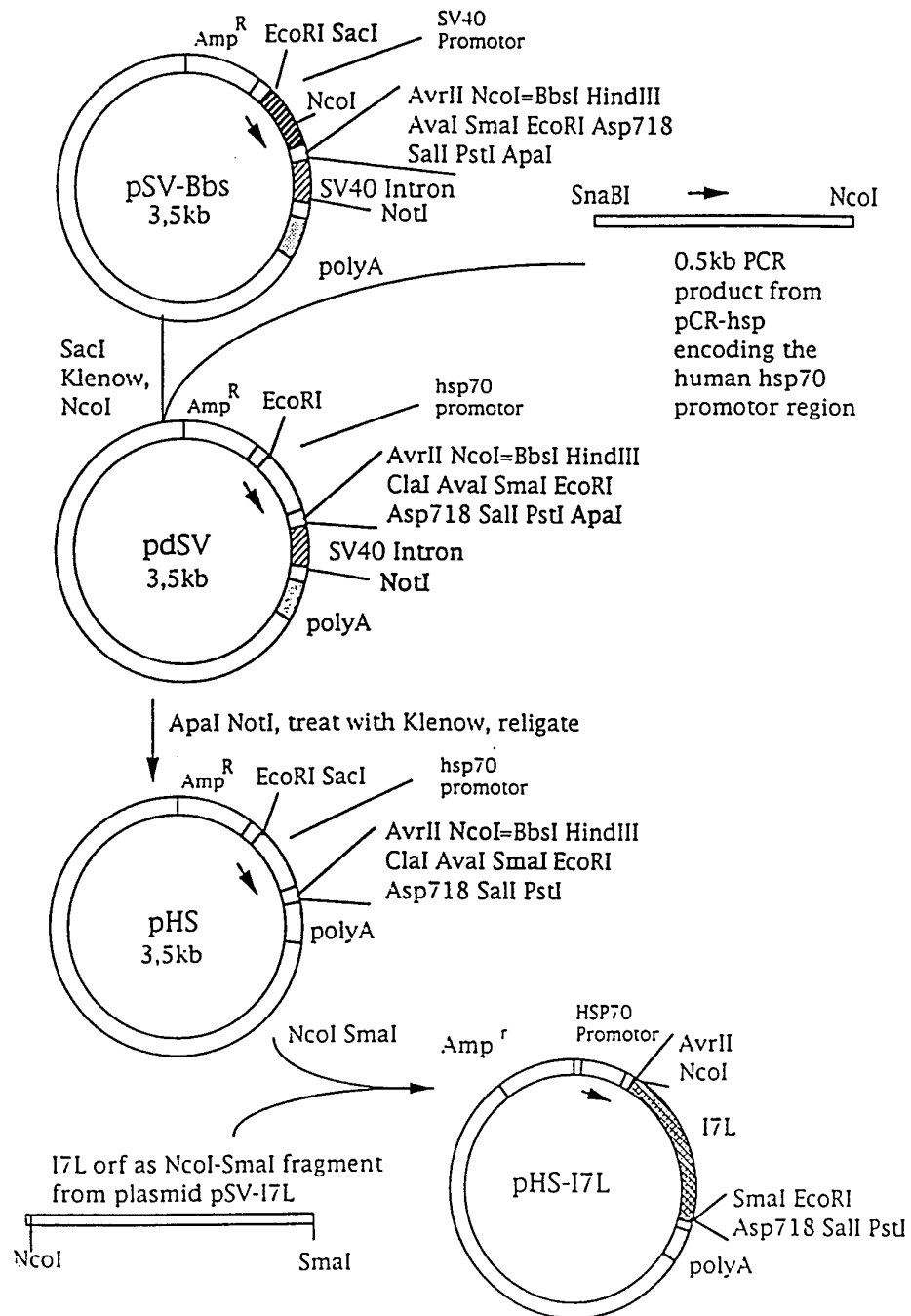

Figure 15