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(54) **VECTEURS RETROVIRAUX DERIVES DE VIRUS DE
SARCOMES-LEUCOSES AVIAIRES PERMETTANT LE
TRANSFERT DE GENES DANS DES CELLULES DE
MAMMIFERES ET LEURS EMPLOIS THERAPEUTIQUES**
(54) **RETROVIRUS VECTORS DERIVED FROM AVIAN SARCOMA
LEUKOSIS VIRUSES PERMITTING TRANSFER OF GENES
INTO MAMMALIAN CELLS AND THERAPEUTIC USES
THEREOF**

(57) L'invention concerne des vecteurs rétroviraux présentant un éventail d'hôtes étendu, dérivés de virus de sarcomes-leucoses aviaires (ASLV) de recombinaison. L'éventail d'hôtes est étendu par le remplacement du gène d'enveloppe de l'ASLV par un gène d'enveloppe provenant d'un virus capable d'infecter aussi bien les cellules mammaliennes que les cellules aviaires. Les vecteurs rétroviraux dérivés de l'ASLV de recombinaison ainsi obtenus peuvent se répliquer efficacement dans des cellules aviaires, infecter aussi bien les cellules de mammifères que celles d'oiseaux à titre élevé, et présentent un défaut de réplication dans les cellules de mammifères. Ils sont par conséquent assez inoffensifs et peuvent être utilisés avec profit en thérapie génique et dans des vaccins.

(57) Recombinant avian sarcoma leukosis virus (ASLV)-derived retrovirus vectors having an expanded host range are described. The host range is expanded by the replacement of the ASLV envelope gene by an envelope gene from a virus capable of infecting both mammalian and avian cells. The resulting recombinant ASLV-derived retroviral vectors can replicate efficiently in avian cells, infect both avian and mammalian cells in high titer, and are replication-defective in mammalian cells. Thus, they are quite safe and advantageous for use in gene therapy and vaccines.

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(57) Abstract

Recombinant avian sarcoma leukosis virus (ASLV)-derived retrovirus vectors having an expanded host range are described. The host range is expanded by the replacement of the ASLV envelope gene by an envelope gene from a virus capable of infecting both mammalian and avian cells. The resulting recombinant ASLV-derived retroviral vectors can replicate efficiently in avian cells, infect both avian and mammalian cells in high titer, and are replication-defective in mammalian cells. Thus, they are quite safe and advantageous for use in gene therapy and vaccines.

TITLE OF INVENTION

RETROVIRUS VECTORS DERIVED FROM AVIAN SARCOMA LEUKOSIS
VIRUSES PERMITTING TRANSFER OF GENES INTO
MAMMALIAN CELLS AND THERAPEUTIC USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of
U.S. Patent Application Serial No. 08/445,462, filed May
22, 1995.

FIELD OF INVENTION

This invention relates to the fields of genetic
engineering and gene transfer. More specifically, the
invention relates to recombinant retrovirus vectors
derived from avian sarcoma leukosis viruses (ASLVs) having
an expanded host range. In particular, this invention
relates to ASLV recombinant retrovirus vectors wherein a
viral *env* gene derived from a virus capable of infecting
both mammalian and avian cells is substituted for the ASLV
env gene, allowing the vectors to efficiently infect a
wide range of host cells, including mammalian and
particularly human cells, in high titers. Additionally,
this invention encompasses therapeutic applications
employing these vectors.

BACKGROUND OF INVENTION

Retroviral vectors carrying and expressing
nucleic acid sequences of interest are powerful tools for
the transfer of genes into a broad range of mammalian
cells and into animals, including humans. Indeed,
retroviruses offer substantial advantages for use as
vectors carrying and expressing desired nucleic acid
sequences in both cultured cells and intact animals.
(Weiss et al, RNA Tumor Viruses (1982)).

First, the retrovirus life cycle lends itself to
the efficient transfer of genes into host cells. The
infectious retroviral agent is called a viral particle or

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a virion. Virions consist of a capsid containing the
viral genome and any inserted nucleic acid sequences and
an envelope made up of glycoproteins. The envelope
glycoproteins on the surface of the virion recognize
receptors on the host cell that mediate entry of the RNA
retroviral genome into the host cell. Once inside the
host cell, a double stranded DNA copy of the virion RNA
genome and any inserted nucleic acid sequences of interest
is made by a viral enzyme, reverse transcriptase. This DNA
copy integrates into the host genome at a precise point on
the viral DNA molecule and at random, or nearly random
sites on host chromosonal DNA. The integrated viral DNA
copy is called a provirus. Since a DNA copy of the viral
genome integrates into the host genome, the progeny of a
single infected host cell are all infected, and the
provirus is located in the same place in the genome of
each of the progeny cells.

Second, in completing their replicative process,
retroviruses usually do not lyse the host cell. Thus, the
retroviruses constitute an efficient mechanism for the
introduction and high level expression of genes in living
host cells.

Third, retroviral genomes are small, making it
relatively easy to manipulate a cloned DNA copy of the
genome. Moreover, the viruses are efficient; in culture,
essentially all of the cells can be infected.

The ability of the retroviral replication
machinery to introduce genetic information into the genome
of the target cell provided the inspiration for the
development of recombinant retrovirus vectors containing a
nucleic acid sequence of interest as a vehicle for the
stable transfer of genes. Moreover, recombinant
retroviral vectors have been used in a number of
applications in addition to the expression of genes of
interest, including insertional mutagenesis, cell lineage
studies and the creation of transgenic animals.

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A desirable property useful for the retroviral vector is the ability to replicate in certain easily manipulated host cells, (e.g., avian cells) allowing rapid replication in these cells without aid of a helper or packaging cell line. This permits generation of high titer virus stocks by simply passaging transfected cells and allowing the virus to spread.

Another useful property for a retroviral vector is the ability to infect a wide range of host cells, including mammalian, and particularly human, cells in high titers. Preferably, the retroviral vector is unable to replicate in mammalian cells. Thus, once the vector enters the mammalian host cell, it becomes a stable provirus, integrated in the host cell genome and incapable of further rounds of infection in either the present or subsequent generations.

A number of retroviral vector systems have been described, including systems based on both mammalian (murine leukemia virus, Cepko, et al., (1984) _Cell_ 37:1053-1062, Cone and Mulligan, (1984) _PNAS_ (U.S.A.) 81:6349-6353; mouse mammary tumor virus, Salmons et al., (1984) _Biochem. Biophys. Res. Commun._ 159:1191-1198; gibbon ape leukemia virus, Miller et al. (1991) _J. Virology_, 65:2220-2224; human immunodeficiency virus, Buchschacher and Panganiban, (1992) _J. Virology_ 66:2731-2739, Page et al., (1990) _J. Virology_ 64:5270-5276) Shimada et al., (1991) _J. Clin. Invest._ 88:1043-1047); and avian retroviruses (Boerkoel et al., (1993) _Virology_ 195:669-679, Cosset et al., (1990) _J. Virology_ 64:1070-107ε Greenhouse et al., (1988) _J. Virology_ 62:4809-4812, Hughes et al., (1986) _Poult. Sci._ 65:1459-1467, Petropoulos and Hughes, (1991) _J. Virology_ 65:3728-3737, Valsessia et al., (1992) _J. Virology_ 66:5671-5676). However, none of these vector systems combines all of the above features. Indeed, each of the available retroviral vectors suffers from certain disadvantages.

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For example, one of the most widely used
retroviral vectors is a replication-defective derivative
of Moloney murine leukemia virus (MLV). The main
advantage of MLV is that it has a wide host range and can
infect mammalian host cells, including human cells.
However, the vectors derived from this virus are
replication-defective. MLV vectors contain all of the
cis-active elements necessary for viral replication, but
lack the genes for the viral structural proteins. These
proteins must be provided in trans by a helper or
packaging cell line.

MLV and other replication-defective vectors have
two major disadvantages. First, the titers of recombinant
retrovirus produced by a helper or packaging cell line are
not always sufficient for some applications, for example,
for in vivo gene transfer experiments or gene therapy.
(See, e.g. Hopkins, (1993) PNAS (U.S.A.) 90:8759-8760).
Second, recombination events between the helper or
packaging cell line genome and the replication-defective
vector can occur and can result in the generation of wild-
type virus. (Ott et al., (1994) Hum. Gene Ther. 5:567-
575). Contamination of the recombinant retroviral vector
stock with replication-competent MLV can interfere with
gene transfer and present potentially serious problems if
the vector is used for gene therapy. For example,
leukemias and lymphomas were induced in primates infected
by the wild-type MLV contaminating retroviral vector
stocks. (Donahue et al., (1992) J. Exp. Med. 176:1125-
1135; Vanin et al., (1994) J. Virology 68:4241-4250).
Finally, in order to use a helper or packaging cell line,
a selectable marker must be introduced into the retroviral
vector. However, with a helper-independent system there
is no need to introduce a selectable marker into the
vector, since any sequence present in the vector will be
carried along passively during replicants.

Other frequently used retroviral vectors are

derived from avian sarcoma leukosis viruses (ASLVs),
particularly the Rous sarcoma virus (RSV). (Hughes and
Kosik (1984) Virology 136:89-99; Hughes et al., (1987) J.
Virology 61:3004-3012). RSV is the only known
replication-competent retrovirus carrying an additional
gene, oncogene v-src, which is dispensable for viral
replication. This oncogene can be deleted from the RSV
derived vector and replaced with a gene or genes of
interest without affecting the ability of the virus to
replicate. For example, retroviral vectors derived from
RSV in which the v-src sequences present in the parental
RSV have been replaced with a unique restriction site, Cla
I, which can be used to insert the gene or genes of
interest have previously been described. These vectors
are designated the RCAS series. (Hughes et al., (1987) J.
Virology 61:3004-3102). The stability of these vectors
was improved by removal of the direct repeat upstream of
the src region. (Hughes et al., (1987) J. Virology
61:3004-3102). The construction and advantages of these
vectors are described in Petropoulos and Hughes (1991) J.
Virology 65:3728-3737. (See also Hughes and Kosik (1984)
Virology 136:89-99). Retrovirus vectors derived from
replication competent endogenous Rous associated virus
type-0 (RAV-0) are designated RCOS (Greenhouse, et al.,
(1988) J. Virology 62:4809-4812). Vectors without splice
acceptors are designated RCON and RCAN. (Hughes et al.,
U.S. Patent No. 4,997,763 (filed July 31, 1987, issued
March 5, 1991), Hughes et al., (1987) J. Virology 61:3004-
3012, Petropoulos and Hughes, (1991). J. Virology
65:3728-3737, Greenhouse, et al., (1988) J. Virology
62:4809-4812).

In contrast to the replication-defective
vectors, recombinant retrovirus vectors based on RSV or
other replication competent ASLVs do not require a
packaging or helper cell line. Thus, these vectors can
replicate in avian cells without the assistance of helper

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or packaging cell lines. Consequently, high-titer viral
stocks may be easily prepared by transfecting a plasmid
containing the vector into cultured chicken embryo
fibroblasts (CEFs) or other avian cells, and passaging the
transfected cells and allowing the virus to spread. The
simplicity of the virus stock preparation and the high
titers that are easily achievable with the replication-
competent retroviral vectors are significant advantages.
Additionally, these vectors have the desirable property of
being unable to replicate in mammalian cells. (Federspiel
et al., (1994) PNAS (U.S.A.) 91:11241-11245). RSV-derived
RCAS vectors have been used to express a number of genes
and to make transgenic chickens. (Hughes et al., (1990)
J. Reprod. Fertil. Suppl. 41:39-49; Petropoulos and
Hughes, (1991) J. Virology 65:3728-3737; Petropoulos et
al., (1992) J. Virology 66:3391-3397; Salter et al.,
(1986) Poult. Sci. 65:1445-1458; Salter et al., (1987)
Virology 157:236-240).

An important limitation of RSV and other ASLV-
based vectors is their host range. The ASLV-derived
vectors disclosed prior to the instant invention could not
efficiently infect mammalian cells. In order to infect a
host cell, the envelope (env) glycoprotein of a retrovirus
must specifically bind to a cognate receptor on the
surface of the host cell. Thus, host range is defined by
the binding capability of the env glycoprotein. In RSV
and other ASLVs, the env glycoprotein is restricted to
binding to avian cell receptors. Thus, these viruses
cannot infect mammalian cells efficiently.

One method that has been used to overcome this
limitation is to make transgenic mice that express the
cellular receptor of subgroup A avian leukosis sarcoma
viruses. (Federspiel et al., (1994) PNAS (U.S.A.)
91:11241-11245). RSV-derived vectors are able to transfer
and stably express alkaline phosphatase and
chloramphenicol-acetyltransferase (CAT) genes in the

muscle of subgroup A receptor transgenic mice.
(Federspiel et al., (1994) PNAS (U.S.A.) 91:11241-11245).
Although the transfer of genes is efficient, ASLVs do not
replicate in mammalian cells (there is a defect in virion
assembly) and the RSV-derived vectors are constitutively
replication-defective in mammalian cells. Moreover, use
of the RSV-derived vectors is limited to the small number
of mammalian host cells carrying the subgroup A avian
leukosis virus receptor.

        Efforts to expand the host range of retroviral
vectors to a number of different cell types in a variety
of mammalian species have utilized the ability of
retroviral capsids to assemble with or be "packaged" by
the envelope glycoproteins of other viral species.
Through a mechanism that is not well understood, a
pseudotyped virus bearing envelope glycoprotein that is a
mixture of the two viruses is generated. (Emi et al.
(1991) J. Virology 65:1201-1207). The pseudotyped virus
has the host range of the virus donating the envelope
protein. (Burns et al. (1993) PNAS (U.S.A.) 90:8033-
8037).

        For example, Emi et al. (1991) J. Virology
65:1201-1207 and Burns et al. (1993) PNAS (U.S.A.)
90:8033-8037 describe the generation of pseudotyped
viruses by co-infection of the same cells with MLV and the
vesicular stomatitis virus (VSV) helper/packaging virus.
The resulting pseudotyped viruses have the increased host
cell range of VSV (i.e. they can infect hamster cells,
which MLV generally cannot infect) but at a low titer).

        Landau and Littman, (1992), J. Virology,
66:5110-5113, describe the production of replication-
defective pseudotyped viruses wherein the MLV genome bears
either the MLV or ecotropic or the RSV envelope
glycoprotein. The packaging system is produced by
transient expression of the env genes in cells infected
with replication defective MLV. The resulting MLV

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pseudotyped viruses have expanded host ranges.

        Miller et al., U.S. Patent No. 4,861,719 (filed
April 25, 1986; issued August 29, 1992) and Temin et al.,
U.S. Patent No. 5,124,263 (filed January 12, 1989; issued
June 23, 1992) describe packaging/helper cell lines used
to alter the host range of replication defective
retroviral vectors they are co-cultivated with. Both
references describe, <u>inter</u> <u>alia</u>, helper/packaging cell
lines derived from amphotropic MLV.

        Researchers have attempted to "package" the ASLV
genome in the envelope glycoprotein of a virus with a
broader host range. For example, Weiss et al., (1977)
<u>Virology</u> 77:808-825, describe superinfection of cells
producing RSV with temperature sensitive mutants of VSV in
an effort to expand the host range of the RSV-based
vectors. Two types of pseudotyped viruses resulted: VSV
genomes bearing RSV envelope antigens and RSV genomes
bearing VSV envelope antigens. The RSV genomes bearing
the VSV envelope antigens possessed the host range of the
VSV virus and were capable of infecting mammalian cells,
but at a lower titer than chicken cells.

        Weiss and Wong, (1977) <u>Virology</u> 76:826-834
describe mixed infection of cultured avian cells by RSV
and MLV. The appearance of RSV particles infectious for
mammalian cells was observed. However, the MLV *env*
protein does not appear to compete efficiently with RSV
env to form pseudotyped virus. In addition, the resulting
RSV pseudotyped viruses with the xenotropic and ecotropic
MLV *env* antigens were shown to infect mammalian cells only
at a very low titer (on the order of 10^2/ml).

        In each of these references the expanded host
range avian pseudotyped viruses depend on the production
of an envelope protein by a helper virus or packaging
cell. Thus, these vector systems are susceptible to
recombination between the two viral genomes and the
instability and potential contamination with wild-type

virus recombination engenders. Consequently, they are not suitable for gene therapy.

Some researchers have attempted to expand the host cell range of avian leukosis virus-based vectors by creating recombinant vectors which express chimeric proteins with expanded host cell binding capacity. For example, Dong et al., (1992) J. Virology 66:7374-7382, describe a recombinant RSV-based vector expressing a chimeric influenza virus hemagglutinin (HA). Plasmids containing chimeric HA genes comprised of the coding sequence for the RSV env signal peptide fused to the hemagglutinin (HA) structural genes or a combination of HA and RSV structural genes were used to co-infect cells with plasmids carrying RSV gag-pol-sequences. Viral particles that contained HA were formed and could be used to infect mammalian cells. However, the replication-competence of the vector in avian cells was not demonstrated and the efficiency of infection of mammalian cells was low, on the order of 10^2/ml.

Valsessia-Wittman et al., (1994) J. Virology 68:4609-4619, describe the replacement of the putative receptor-binding domain of the subgroup (A) RSV envelope protein gp85 (SU) with the peptide known to be the target for cellular integrin receptor. Viral particles coated with the modified envelope were shown to infect mammalian cells which are resistant to infection by subgroup (A) ASLVs. However, infectivity of this vector in mammalian cells also appeared to be quite low, on the order of 10-10^2/ml.

In spite of numerous attempts in the prior art to develop vectors that were simultaneously able to: 1) efficiently infect a broad range of cell types in a variety of avian and mammalian species, independent of helper or packaging virus cell lines; and 2) unable to replicate once inside the mammalian host cell, until the present invention, such a vector remained elusive.

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SUMMARY OF INVENTION

The present invention, in general, provides recombinant avian sarcoma leukosis virus (ASLV) -derived retroviral vectors having an expanded host range, thereby allowing the vector to be used for gene transfer in a variety of species. In particular, the ASLV envelope gene is replaced by the *env* region derived from a virus capable of infecting both mammalian and avian cells. The recombinant retroviral vectors of this invention can replicate efficiently in avian cells, allowing for production of high titer stock and they are able to infect both mammalian and avian cells at a high titer. However, the ASLV-derived vectors of this invention are replication defective in mammalian cells, thereby providing retroviral vectors that are quite safe for use in gene therapy.

It is a general object of the present invention to provide recombinant retroviral vectors that are capable of carrying one or more nucleic acid sequences of interest and expressing them in a broad range of mammalian cells.

It is a further object of the present invention to provide host cells, particularly avian or mammalian cells, carrying the recombinant retroviral vectors and capable of expressing one or more nucleic acid sequences of interest.

It is also an object of the present invention to provide methods for using the recombinant retroviral vectors to transfer nucleic acid sequences of interest into a broad range of host cell types,, both _in_ _vitro_ and _in_ _vivo_, in a variety of species, including avian and particularly mammalian species.

It is yet another object of this invention to provide a method for using the recombinant retroviral vectors to produce transgenic animals.

It is a further object of the invention to provide transgenic animals carrying the recombinant retroviral vectors in at least one cell.

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It is a further object of the invention to provide methods for treating or preventing diseases involving transfer by the recombinant retroviral vectors of all or part of a nucleic acid sequence of interest to a mammal, preferably a human.

DESCRIPTION OF THE FIGURES

Figure 1A: A schematic depiction of the construction of plasmid RSVgagpol.

Figure 1B: A schematic depiction of the construction of recombinant retroviral vector RCAS-M(4070A).

Figure 2A: The structure of plasmids RSVgagpol and MLVenv shown schematically. RSVgagpol contains the gag-pol open reading frame derived from the retroviral vector RCASBP(A) Petropoulos and Hughes, (1991) J. Virology 65:3728-3737. MLVenv carries a region encoding the signal peptide, surface glycoprotein gp70 and transmembrane protein p15E of an amphotropic MLV, clone 4070A (Ott et al., (1990) J. Virology 64:757-766, Ott et al., (1992) J. Virology 66:4632-4638).

Figure 2B: Immunoblotting analysis of the viral particles harvested from the complementation assay with RSVgagpol and MLVenv. Antibodies against RSV p19 (MA) and MLV gp70 (SV) were used. Lanes 1 and 2 show MLVenv recovered from supernatants of cells transfected with MLVenv plasmid alone. Lanes 3 and 4 show RSVgagpol recovered from supernatants of cells transfected with RSVgagpol plasmid alone and Lanes 5 and 6 show virus particles recovered from supernatants of cells co-transfected with both plasmids.

Figure 3A: Schematic depictions of the structures of retroviral vectors RCAS-M(4070A) and RCASBP(A). RCAS-M(4070A) contains a chimeric env gene derived from an amphotropic MLV in place of the env gene of RCASBP(A). Retroviral vector RCAS BP(A) was modified to create RCAS-M(4070A) by replacing the env gene stop

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codon with a unique NotI cleavage site. Additionally, RSV
env coding sequences were removed and the chimeric
envelope coding region, including a new stop codon, was
inserted generating the recombinant retroviral vector
RCAS-M(4070A).

Figure 3B: SDS-PAGE gel. Lanes 1 and 2 contain
viral particles recovered from the transfection of CEFs
with RCAS-M(4070A). Lanes 3 and 4 are controls and
contain the viral particles recovered from CEFs infected
with pR4070A, the molecular clone of the amphotropic MLV
from which the MLV *env* gene was derived.

Figure 4A: Stained cells expressing gp70 from
RCAS-M(4070A). To measure the ability of RCAS-M(4070A) to
replicate in CEFs, cells were transfected with RCAS-
M(4070A) DNA and passaged six times to allow the virus to
spread. At passages 1, 4 and 6 a small number of cells
were plated separately. Those containing virus were
identified by staining with antibodies that react with
gp70. Only a small number of positively stained cells are
seen in passage 1. This number increases significantly by
passage 4. At passage 6 virtually all cells are infected
and expressing gp70.

Figure 4B: Immunoblot analysis of the viral
particles recovered from the harvested CEFs cell medium at
each passage with antibodies against p19 and gp70. The
amount of viral proteins in the supernatant increases by
passage 4 and reaches a high level by passage 6.

Figure 5: Puromycin-resistant colonies of D17
(dog) cells produced by infecting D17 cells with serial
dilutions of RCAS-M(4070A)Puro.

Figure 6A: Schematic Structure of RCAS-
M(4070A)NEO. Restriction endonuclease sites are
indicated. "E" refers to EcoRI, "B" refers to BamHI.

Figure 6B: Detection of RCAS-M(4070A)NEO
provirus in the genomic DNA of infected NIH 3T3 cells by
Southern blot hybridization. Lanes 1-19 show fragments

derived by cleavage of the DNA of G418^r clones by EcoRI and BamHI. Lane C shows fragments derived by cleavage of plasmid RCAS-M(4070A) DNA by EcoRI and BamHI.

Figure 6C: Detection of RCAS-M(0470A)NEO provirus in the genomic DNA of infected HeLa cells by Southern blot hybridization. Lanes 1-15 show fragments derived by cleavage of the DNA of G418^r clones by EcoRI and BamHI. Lane C shows fragments derived by cleavage of plasmid RCAS-M(4070A) DNA by EcoRI and BamHI.

Figure 7: Immunoblot analysis of viral particles recovered from the culture of cells infected with RCAS-M(4070A). Antibodies against p15E were used. Lane 1 shows wild type MLV p15E proteins Lane 2 shows p15E proteins from a strain of MLV with a defective protease gene. Lane 3 shows RCAS-M(4070A) p15E proteins.

Figure 8: A schematic depiction of mutations in the amphotropic *env* gene of RCAS-M(4070A)NEO. CEFs were infected with RCAS-M(4070A)NEO after 3 passages of the virus. Full-length clones of the viral DNA were derived from the library of a low-molecular- weight DNA which was extracted from infected CEFs. *Env* genes of clones *env*1, 3, 6, 8 and 9 were sequenced and are depicted.

Figure 9A: Replication of RCAS-M2(4070A)Puro and RCAS-M2(4070A)Puro 1, 6, 8, and 9 in CEFs as measured by a RT assay. CEFs were transfected with plasmid DNA and passaged. 24 h after transfection and at each passage, virus particles were recovered from the culture fluid by centrifugation and quantified by determination of the RT activity. The control, uninfected cells, is designated a "mock." RCAS-M2(4070A)Puro is designated "env (wt)" and RCAS-M2(4070A)Puro 1, 6, 8 and 9 are designated "env 1", "env 6", "env 8", and "env 9", respectively.

Figure 9B: Titers of RCAS-M2(4070A)Puro1, 6, 8 and 9 on D17 cells. Cells were infected with the serial 10-fold dilutions of virus-containing culture fluid harvested at each passage. Resistant clones were selected

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in the medium containing puromycin. pac^r colonies were
stained with Giemsa stain and counted. RCAS-M2(4070A)Puro
1, 6, 8 and 9 are designated "env 1", "env 6", "env 8",
and "env 9", respectively. "Cfu" means colony-forming
units.

Figure 9C: Titers of RCAS-M2(4070A)Puro8
(designated "RCAS-M2(4070A)Puro") and RCAS-M2C(4070A)Puro
on D17 cells. Values shown are the average of two
independent determinations.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to recombinant retroviral
vectors capable of infecting a broad range of host cells,
in particular both mammalian and avian cells, in high
titer. In general, the recombinant retroviral vectors of
this invention comprise at least one long terminal repeat
(LTR), a *gag* region, a *pol* region, and an *env* region, or
functional equivalents thereof. More specifically, the
recombinant retroviral vectors comprise LTR, *pol* and *gag*
regions derived from avian sarcoma leukosis viruses (ASLV)
and an *env* region derived from a virus capable of
infecting both avian and mammalian cells, such as
amphotropic MLV. The recombinant vectors are capable of
carrying and expressing one or more nucleic acid sequences
of interest, replicate efficiently in avian cells and are
able to efficiently infect and transfer genes into a broad
range of mammalian cells. The recombinant retroviral
vectors of the invention are replication-defective in
mammalian cells, greatly enhancing their safety.

Gag is the retroviral gene which encodes the
structural proteins that form the virion core. The *gag*
region of the vector may be derived from the *gag* region of
any member of the ASLV family. Examples of the genomes
from which the *gag* sequence may be derived include, but
are not limited to, Rous sarcoma virus (RSV), MC29-
associated virus (MAV), Rous associated virus (RAV), RAV-

O, avian erythroblastosis virus (AEV), avian myoblastosis
virus (AMV), other members of this virus family and their
associated helper viruses. The *gag* region may comprise
all or parts of the *gag* gene. For a replication competent
vector, it is preferable that a sequence sufficient to
encode a functional *gag* protein is used. In a preferred
embodiment the *gag* region is derived from RSV.

   Pol is the retroviral gene which encodes the
reverse transcriptase and integrase. The *pol* region may
be derived from the *pol* region of any member of the ASLV
family. Examples of the genomes from which the *pol*
sequences may be derived, include, but are not limited to,
RSV, MAV, RAV, RAV-O, AEV, AMV, other members of this
virus family and their associated helper viruses. The *pol*
region may comprise all or parts of the *pol* gene. For a
replication competent vector it is preferable that a
sequence sufficient to encode a functional *pol* protein is
used. By way of example, a *pol* region derived from the
Bryan high titer RSV strain may be used. (Petropoulos and
Hughes (1991) <u>J. Virology</u>, 65:3728-3737).

   Env is the retroviral gene which encodes the
envelope antigens that determine the antigenic and
subgroup specificity of the virus. The *env* sequence is
preferably derived from the envelope region of a virus
capable of infecting both mammalian and avian cells.
Examples of the genomes from which the *env* sequences may
be derived include, but are not limited to, mammalian
viruses capable of infecting avian species, such as the
amphotropic Moloney murine leukemia virus (MLV) *env*
sequence. Weiss et al., (1982) <u>RNA Tumor Viruses</u>; Weiss
(1985) Supplement to <u>RNA Tumor Viruses</u>. The *env* sequence
may comprise all or parts of the *env* gene. For a
replication competent virus, it is preferable that a
sequence sufficient to encode a functional envelope
protein is used. By way of example, a sequence encoding
the complete amphotropic MLV *env* region may be used in the

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recombinant retroviral vector of this invention.

In a preferred embodiment, the coding sequence
of a virus capable of infecting both mammalian and avian
cells such as amphotropic MLV, is "adapted" to permit
initial replication rates similar to those observed in
wild type RSV via one or more mutations of the DNA of the
env coding sequence, including, but not limited to, the
substitution of the proline at position 242 with
isoleucine. As described more fully in the examples, the
"adapted" env sequence may be produced by passaging the
virus and selecting for fast replicating clones.
Additionally, one skilled in the art will recognize that
the env sequence may be "adapted" by a number of other
methods including, but not limited to, use of site
directed mutagenesis to produce the desired mutation(s).

Alternatively, a chimeric envelope sequence may
be used in constructing the recombinant retroviral vector
of this invention, comprising the coding region of a wild
type or "adapted" env gene sequence of a virus capable of
infecting mammalian and avian cells and the N terminal
signal peptide sequence of an ASLV.

Long terminal repeats (LTRs) facilitate
integration of the viral genome into the host genome and
contain promoters for transcription of the viral genome.
The retroviral vectors of this invention have at least
one, preferably two LTR's. A LTR derived from an ASLV
family member may be used. These include, but are not
limited to, RSV, MAV, RAV, RAV-O, AEV, AMV and other
members of this family, and their associated helper virus.
(Hughes and Kosik, (1984) Virology 136:89-99, Hughes et
al., (1987) J. Virology 61:3005-3102). For a replication
competent virus, preferably the entire LTR is used.
Alternatively, in another embodiment, a retroviral vector
with a single LTR may be used. If a single LTR circular
plasmid is used, a two LTR proviral form can be simply
derived by cleavage at an appropriate restriction site and

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ligation.   (Hughes and Kosik, (1984) <u>Virology</u> 136:89-99).

          The RSV-derived vector designated RCAS may be
used to generate the recombinant retroviral vector of this
invention by providing the LTR, *pol* and gag sequences.
(Hughes et al., <u>J. Virology</u> (1987) 61:3004-3012; Hughes
and Kosik (1984) <u>Virology</u> 136:99-99, herein incorporated
by reference).   Additionally, in another embodiment the
RCASBP vectors are used.   (Petropoulos and Hughes, (1991)
<u>J. Virology</u> 65:3728-3737).   In the construction of RCAS,
the genome of a circular DNA form of the Schmidt Rappin A
(SR-A) strain of RSV, was recloned in proviral DNA form
with the *E coli* replicon between the LTRs of the provirus.
The v-src coding region was removed and was replaced with
the cleavage site for the restriction endonuclease Cla I.
One copy of the direct repeats flanking v-src in SR-A was
removed.   To construct RCASBP, the RCAS vector was used
and the *pol* gene of the SR-A strain was replaced by the
pol gene of the Bryan high titer strain of RSV.

          Conventional methodology may be used to replace
the RSV-derived *env* sequence present in the RCAS or RCASBP
vector with the *env* sequence of the amphotropic MLV
retrovirus (see Example 2).   The complete MLV *env* coding
sequence may be used or a chimeric *env* sequence comprising
the MLV *env* coding sequence having the RSV signal peptide
in place of the MLV signal peptide may be constructed and
cloned into the vector.   The resulting retrovirus,
designated RCAS-M (4070A), is shown in Figure 3.   The
functional equivalents of the recombinant retrovirus shown
in Figure 3 are also intended to be encompassed by this
invention.

          In a preferred embodiment, the of RCAS-M (4070A)
is "adapted" for fast initial replication by introduction
of one or more mutations into the env sequence, including
substitution of the proline at position 242 with an
isoleucine.   The resulting retrovirus is designated RCAS-
M2(4070A).   The functional equivalents of the RCAS-M2

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(4070A) recombinant retrovirus are also intended to be
encompassed by this invention.

In yet another embodiment of this invention, the
envelope region of the recombinant retroviral vectors of
the invention is derived from a virus that recognizes
mammalian, but not avian cell receptors. In order for a
vector containing such an envelope sequence to infect
avian host cells, the avian cells may be engineered to
express a receptor recognized by the chosen envelope
region.

The recombinant retroviral vectors of this
invention may be used for expression of nucleic acid
sequences of interest. In this embodiment, the vector
further comprises one or more nucleic acid sequences
encoding a protein of interest. One skilled in the art
will recognize that virtually any nucleotide sequence from
virtually any genome may be used. In particular, nucleic
acid sequences encoding a therapeutic or immunogenic
protein may be used. Alternatively, nucleic acid
sequences encoding the antisense strand for one or more
proteins may be used to inhibit the expression of the
proteins. The nucleic acid sequences may be inserted into
the retroviral vector of this invention using conventional
methodology known to those skilled in the art. Examples
include, but are not limited to, cleavage by a specific
restriction endonuclease and ligation or homologous
recombination.

Conventional methodology including, but not
limited to site directed mutagenesis, may be used to
introduce one or more restriction endonuclease sites into
the vectors of this invention. (Sambrook et al (eds)
(1989) "Molecular Cloning, A Laboratory Manual, Cold
Spring Harbor Press, Plainview, New York and Ausubel et
al. (eds) (1987) in "Current Protocols in Molecular
Biology" John Wiley and Sons, New York, New York). To
retain the ability of the vectors to replicate in avian

cells it is important not to delete any of the *gag*, *pol*, or *env* sequences necessary for virus replication. Any restriction endonuclease site may be generated, preferably an endonuclease unique to the retroviral expression vector or an endonuclease site that does not appear frequently in the vector. One of skill in the art will know how to select and create the appropriate restriction endonuclease sites.

In one embodiment, RSV is used to generate the recombinant retroviral vectors of the invention and the nucleic acid sequences of interest may be inserted in place of the RSV v-src oncogene using restriction endonucleases and ligation. In a preferred embodiment, the nucleic acid sequences of interest are inserted into the Cla I site of the RSV-derived RCAS or RCASBP vectors and at least one copy of the direct repeats flanking the v-src site are removed to increase the stability of the vector (see Example 2).

The desired nucleic acid sequences inserted into the recombinant expression vector of this invention may be transcribed under the direction of the LTR promoter. By way of example, the RSV-derived RCAS or RCASBP vectors may be used to generate the recombinant retroviral vectors of the invention when the LTR will direct the expression of the nucleic acid sequence of interest. (Hughes and Kosik, (1984) <u>Virology</u> 136:89-99, Hughes et al., (1987) <u>J. Virology</u> 61:3004-3012).

Alternatively, the expression of the desired nucleic acid sequences may be under the direction of an internal promoter within the recombinant retroviral vector of this invention. By way of example, tissue specific promoters such as α sk actin (Petropoulos and Hughes (1991) <u>J. Virology</u> 65:3728-3737), inducible promoters such as metallothionien (Petropoulos and Hughes (1991) <u>J. Virology</u> 65:3728-3737), a cell cycle or stage specific promoters may be used to direct expression of the desired

nucleic acid sequences. One of skill in the art will know
what promoter should be used based on the intended
application of the gene product. By way of example, the
RSV-derived RCAN vectors, (Hughes and Kosik, (1984)
Virology 136:89-99; Hughes et al. (1987) _J. Virology_
6:3004-3012) may be used to generate the recombinant
retroviral vectors of the invention when an internal
promoter will be used. Conventional methodology may be
used to incorporate these promoters into the recombinant
retroviral vectors.

        Additionally, one skilled in the art will
recognize that a splice acceptor or an IRES (internal
ribosome entry site) may be introduced in the retroviral
vector to permit expression of an inserted gene.

        The recombinant vectors of this invention may
further include additional expression control elements,
including, but not limited to enhancer sequences.
Conventional methodology can be used to incorporate these
additional expression control elements into the vector.
(Sambrook et al (eds (1989) "Molecular Cloning, A
Laboratory Manual, Cold Spring Harbor Press, Plainview,
New York and in Ausubel et al. (eds) (1987) in "Current
Protocols in Molecular Biology" John Wiley and Sons, New
York, New York).

        Another aspect of the present invention is to
provide a method for using the recombinant retroviral
vectors to transfer one or more nucleic acid sequences of
interest to a broad range of host cells from a variety of
species, particularly mammalian species. The recombinant
retroviral vectors of the present invention may carry one
or more nucleic acid sequences of interest. The virions
encapsulating the nucleic acid sequences of the invention
are capable of infecting a broad range of mammalian cell
types. Once inside the cell, the recombinant retroviral
vectors make a DNA copy of the retroviral genome and the
nucleic acid sequences of interest, which is stably

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inserted into the host genome and can be expressed by the
host cell.

The recombinant retroviral vectors of the
invention may be directly introduced into a host cell or
may be inserted into a plasmid or other construct which is
then inserted into a host cell. For example, to generate
large quantities of the retroviral vector DNA of this
invention, the recombinant retroviral vectors of the
invention may be cloned into a plasmid for propagation of
the DNA in appropriate cells. Examples of the cells that
may be used include but are not limited to *E. coli*.
Construction of the plasmid containing the retroviral
vector DNA can be performed by conventional methods.

By way of example the pBR322 plasmid or a
derivative thereof may be used to propagate the vector
DNA. In a preferred embodiment the retroviral vector of
the invention is inserted into the *E. coli* replicon pPH,
derived from pBR322. (Hughes et al. (1987) <u>J. Virology</u>
61:3004-3012).

It will be understood by one skilled in the art
that the plasmid carrying the recombinant retroviral DNA
should contain additional elements necessary for transfer
and subsequent replication of the construct in the host
system being used to propagate the DNA. Examples of such
elements include but are not limited to origins of
replication and selectively. It will be understood by one
skilled in the art that the correct combination required
or preferred elements to be used.

The means by which the recombinant retroviral
vectors may be introduced into the host cell include, but
are not limited to, microinjection, electroporation,
transduction or transfection using DEAE-dextran,
lipofection, calcium phosphate or other procedures known
to one skilled in the art (Sambrook et al. (1989) in
"Molecular Cloning. A Laboratory Manual," Cold Spring
Harbor Press, Plainview, New York).

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Another aspect of this invention is to provide host cells into which the recombinant retroviral vector containing all or part of nucleic acid sequences of interest has been inserted. The host cells containing the recombinant retroviral vector of this invention include eukaryotes, such as avian species including ducks, chickens, turkeys, and quail, and mammalian species including mice, dogs, humans. Preferred host cells include, but are not limited to, CEF cells, NIH 3T3 cells, D17 cells and HeLa cells.

The retroviral vectors of this invention are replication competent in avian cells. Avian cells including but not limited to, chicken, turkeys, quail, and duck may be used to generate a viral stock. The viral stock may be generated by methods known to those skilled in the art. The recombinant retroviral vector cloned into a plasmid may be introduced into the avian cells by conventional methodology and the cells may be passaged to allow the virus to spread throughout the culture. Subsequent generations of the virus may be used to infect other cells. Alternatively, a virus carrying the recombinant retroviral vector may be used to infect the avian cells. The viral stock produced may be used to infect other host cells. Examples of cells that may be infected include but are not limited to, duck, chicken, turkeys cells or mammalian cells. Examples of mammalian cells include but is not limited to hematopoietic stem cells, islet cells, or T-cells.

Host cells containing a recombinant retroviral vector of the invention can be used _in vitro_ to produce recombinant proteins. The recombinant proteins can be isolated and purified by conventional methods. (Sambrook et al (eds) (1989) "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, New York and in Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New

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York).   Additionally, host cells may be used in vivo to
supply recombinant proteins.

In another embodiment, the recombinant
retroviral vectors of the invention may be used to
transfer nucleic acid sequences encoding one or more
therapeutic proteins to a mammal, preferably a human, in
need of gene therapy. The retroviral vector carrying the
nucleic acid sequence of interest may be administered to
an individual in need of such therapy in a variety of
ways. Retroviral supernatants from avian host cells
transfected with and producing the virus may be
administered to the individual in need of gene therapy.
Additionally, a substantially purified form of the virus
may be administered to the mammal in need of such
treatment alone or in the form of a pharmaceutical
composition.

Alternatively, the gene therapy may be
accomplished by inserting the nucleic acid sequences
encoding the therapeutic protein(s) into the recombinant
retrovirus vector of the invention and introducing it into
a host cell. The host cell which contains the recombinant
retroviral vector and expresses the desired therapeutic
protein is then administered to or implanted in the
individual in need of gene therapy. The cells then
express the therapeutic protein recombinantly in the
mammal.

The host cells may be from virtually any
species. In one embodiment, the host cells are taken from
the individual in need of the gene therapy. Examples of
such host cells include, but are not limited to,
hematopoietic stem cells or T cells. In another
embodiment the host cells are not from the individual
receiving the therapy, but are from different species.
For example, in one embodiment, which takes advantage of
the ability of the retroviral vectors of the invention to
replicate and produce virus in avian but not mammalian

cells, avian host cells containing the retroviral vector
are administered to a mammal in need of therapy.

Means of administering the host cell containing
the recombinant retroviral vectors of the invention which
recombinantly express the proteins of interest include,
but are not limited to, intravenous, intramuscular,
intralesional, subcutaneous or intraperitoneal injection
or implantation. Alternatively, the cells containing the
recombinant retroviral vectors may be administered locally
by topical application, direct injection into an affected
area or implantation of a porous device containing cells
from the host or another species in which the recombinant
retroviral vectors are inserted and which express the
proteins of interest.

Examples of diseases that may be suitable for
gene therapy include, but are not limited to,
neurodegenerative diseases or disorders, Alzheimer's,
schizophrenia, epilepsy, neoplasms, cancer and AIDS.

In yet another aspect of the invention, the
recombinant retroviral vectors can be used to generate
transgenic animals carrying the recombinant vector in at
least one cell. In a preferred embodiment, the
recombinant retroviral vector carrying one or more nucleic
acid sequences of interest is introduced into an animal or
an ancestor of the animal at an embryonic stage. The
transgenic animal can be made by several methods,
including, but not limited to, introducing the recombinant
retroviral vector carrying the nucleic acid sequences of
interest into the embryonic animal by infection or
injection. Additionally, the recombinant retroviral
vector may be inserted into host cells which are then
introduced into the developing embryo. By way of example,
chicken cells containing the recombinant retroviral vector
and carrying the gene of interest_can be implanted into
the blastocyst stage of a mammalian embryo.

Examples of animals into which the recombinant

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retroviral vector can be introduced include, but are not limited to, non-human primates, cows, sheep, dogs, mice, rats, or other rodents. Such transgenic animals may be useful as biological models for the study of disease and to evaluate diagnostic or therapeutic methods for disease.

It will be appreciated by those skilled in the art that the recombinant retroviral vectors of this invention may be used to generate transgenic animals without additional nucleic acid sequences (e.g. insertional mutagenesis). Alternatively, nucleic acid sequences of interest may be inserted into the recombinant retroviral vectors of the invention such that the transgene animal expresses the desired gene(s).

It is a further aspect of the invention to use the recombinant retroviral vectors to deliver a prophylactic or therapeutic vaccine for a wide variety of mammalian, and particularly human, diseases.

A prophylactic vaccine is provided in advance of any evidence of the disease of interest and serves to prevent or attenuate the disease in a mammal. In a preferred embodiment, mammals, preferably humans, at high risk for the disease of interest, are treated prophylactically with the vaccines of this invention.

When provided therapeutically, the vaccine is provided at or shortly after the onset of the disease of interest (or symptoms of the disease of interest) to enhance the immune response of a patient (a mammal, preferably a human) to the disease of interest and to attenuate the disease.

The vaccine, which acts as an immunogen, may be in the form of a cell transfected with the recombinant retroviral vector of the invention carrying nucleic acid sequences encoding all or part of one or more immunogenic peptides associated with the disease of interest. The vaccine may also be in the form of a culture supernatant from cells producing viral particles or a preparation of

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viral particles containing the retroviral vectors of
interest with the inserted nucleic acid sequences of
interest.

When the vaccine is used in the form of a host
cell, the recombinant retroviral vector of the invention
can be introduced into virtually any mammalian cell. The
means by which the vector carrying the gene may be
introduced into a cell include, but are not limited to,
infection or other procedures known to one skilled in the
art (Sambrook et al. (eds) (1989) Molecular Cloning. A
Laboratory Manual, Cold Spring Harbor Press, Plainview,
New York). The mammalian cells infected with the
recombinant retrovirus vectors of the invention and
expressing the immunogenic protein can be administered to
mammals and serve as a vaccine. Cells expressing the
immunogenic protein of interest can be administered
intravenously, intramuscularly, intraperitoneally,
intralesionally or subcutaneously.

Additionally, the vaccine may be administered as
a preparation of viral particles. Viral particles
containing the recombinant retroviral vectors may be
directly administered to the mammal several ways,
including, but not limited to exposure of cells to virus
ex vivo or injection of the retrovirus into the affected
tissue or intravenously. Alternatively, viral particles
carrying all or part of the nucleic acid sequences of
interest may be administered locally by direct injection
into an affected area or by topical application in a
pharmaceutically acceptable carrier.

Examples of mammals to which the vaccine may be
administered include, but are not limited to, mice, rats,
dogs, non-human primates, and humans with a family history
of the disease of interest. Veterinary uses are intended
to be encompassed by this invention.

Vaccination can be conducted by conventional
methods. The recombinant viral vectors containing the

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nucleic acid sequences encoding the immunogenic protein
may be administered once or at periodic intervals until a
significant titer of antibody or immune cells against the
immunogen protein of interest is produced. One skilled in
the art will know the proper immunoassays and other
methods for detecting and measuring the presence of immune
cells against the protein of interest.

The recombinant retroviral vector expressing the
immunogen may be administered in a pure or substantially
pure form. Additionally, it is possible to present it as
a pharmaceutical composition, formulation or preparation.

The pharmaceutical compositions, formulations or
preparations of the present invention, both for veterinary
and for human use, may comprise an immunogen as described
above, together with one or more pharmaceutically
acceptable carriers and, optionally, other therapeutic
ingredients. The carrier(s) must be "acceptable" in the
sense of being compatible with the other ingredients of
the formulation and not deleterious to the recipient
thereof. The formulations may conveniently be presented
in unit dosage form and may be prepared by any method
well-known in the pharmaceutical art.

All methods include the step of bringing into
association the active ingredient with the carrier which
constitutes one or more accessory ingredients. In
general, the formulations are prepared by uniformly and
intimately bringing into association the active ingredient
with liquid carriers or finely divided solid carriers or
both, and then, if necessary, shaping the product into the
desired formulation.

Formulations suitable for intravenous,
intramuscular, subcutaneous, or intraperitoneal
administration conveniently comprise sterile aqueous
solutions of the active ingredient with solutions which
are preferably isotonic with the blood of the recipient.
Such formulations may be conveniently prepared by

dissolving solid active ingredient in water containing physiologically compatible substances such as sodium chloride (e.g. 0.1-2.0M), glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering said solution sterile. These may be present in unit or multi-dose containers, for example, sealed ampoules or vials.

The formulations of the present invention may incorporate a stabilizer. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids which may be used either on their own or as admixtures. These stabilizers are preferably incorporated in an amount of 0.11-10,000 parts by weight per part by weight of immunogen. If two or more stabilizers are to be used, their total amount is preferably within the range specified above. These stabilizers are used in aqueous solutions at the appropriate concentration and pH. The specific osmotic pressure of such aqueous solutions is generally in the range of 0.1-3.0 osmoles, preferably in the range of 0.8-1.2. The pH of the aqueous solution is adjusted to be within the range of 5.0-9.0, preferably within the range of 6-8. In formulating the immunogen of the present invention, anti-adsorption agent may be used.

After immunization, the efficacy of the vaccine can be assessed by production of antibodies or immune cells that recognize the antigen, as assessed by techniques known to those skilled in the art. If the mammal to be immunized is already afflicted with the disease of interest, the vaccine can be administered in conjunction with other therapeutic treatments.

One of skill in the art will know the parameters to determine the correct titer of particles to be administered. The quantity of recombinant retroviral vector or virus carrying all or part of the nucleic acid

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sequence encoding the immunogenic protein of interest to be administered may be based on the titer of virus particles. The amount of virus to be administered is in no way limited to a particular concentration and may vary depending upon the individual being healed. Based on clinical parameters the treating physician will determine the therapeutically effective amount of the virus containing the gene of interest to be administered to a given individual. Such therapy may be administered as often as necessary and for the period of time judged necessary by the treating physician. The therapeutic methods described herein may be used alone or in conjunction with additional therapy known to those skilled in the art for the treatment of a given disease or condition in a mammal.

All books, articles, or patents referenced herein are incorporated by reference. The following examples illustrate various aspects of the invention, but in no way are intended to limit the scope thereof.

Example 1

The amphotropic MLV env protein
efficiently assembles with RSV gag proteins

In order to investigate directly the ability of RSV *gag* and MLV *env* to assemble, a complementation assay in which *gag* proteins of RSV and the envelope glycoproteins of MLV were transiently expressed in chicken embryo fibroblasts (CEFs) derived from EV0 chicken embryos was developed. (Astrin et al., (1979) Nature 282:339-341. See also Hughes and Kosik (1984) Virology 136:89-99, Hughes et al., (1987) J. Virology 61:3004-3012). The CEFs used in this experiment and the other examples were maintained in Dulbecco modified Eagle medium (DMEM, GIBCO BRL, MD) supplemented with 5% fetal bovine serum, 5% newborn calf serum, 10% tryptose-phosphate broth (GIBCO BRL), 100 u/ml penicillin and 100 μg/ml streptomycin.

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Construction of the Plasmids

Two plasmids, RSV*gagpol* (which contains the *gag-pol* open reading frame derived from the retroviral vector RCASBP(A) (Petropoulos and Hughes, (1991) <u>J. Virology</u> 69:3728-3737)) and MLV*env* (which carries a region encoding the signal peptide, surface glycoprotein gp70 and transmembrane protein p15E of an amphotropic MLV (clone 4070A))(Fig. 2A) were constructed by standard methods. <u>See</u> Sambrook et al., (eds) (1989) <u>Molecular Cloning</u>. <u>A Laboratory Manual</u>, Cold Spring Harbor Press, Plainview, New York. Recombinant clones were isolated in <u>E. coli</u> DH5a.

Plasmid RSV*gagpol* was constructed as follows: plasmid DNA RCASBP(A) (Petropoulos and Hughes, (1991) <u>J. Virology</u> 69:3728-3737 was cleaved by restriction endonucleases Sac I and Xba I (Fig. 1A) and the fragment containing the complete *gag* region and the 3'-end of *pol* was isolated. In a separate reaction, RCASBP(A) was digested by Xho I. The Xho I ends were filled in using T4 DNA polymerase and the DNA was subsequently digested by Xba I. The fragment Xba I-Xho I(blunt) containing the 3'-end of the *pol* open reading frame was isolated. The Sac I-Xba I and Xba I-Xho I (blunt) fragments were ligated in the presence of pBluescript KS II DNA that had been cleaved by Sac I and Eco RV. The resulting plasmid pT7*gagpol* contained the whole *gag-pol* region flanked by unique Sac I and Cla I sites. RCASBP(A) was digested by Sac I and Cla I to remove *gag-pol-env* region, and Sac I-ClaI fragment from pT7*gagpol* was inserted.

To construct plasmid MLV*env*, the *env* coding region of the amphotropic MLV was PCR-amplified from a plasmid pR4070A (Ott et al., (1990) <u>J. Virology</u> 64:757-766, Ott et al., (1992) <u>J. Virology</u> 66:4632-4638) (kindly provided by Alan Rein, ABL-Basic Research Program) with the forward primer AMPH-F (AAAAGAGCTCGGCCGACACCCAGAGTGGAC) located just upstream *env* initiation codon and the reverse

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primer AMPH-R (AAAAGAGCTCTCATGGCTCGTACTCTATGGGTT) spanning
the *env* termination codon. The resulting Sac I
recognition sites were included in the 5'-ends of both
primers. PCR product was cleaved by Sac I and inserted
into the vector TFA-NEO (Federspiel et al., (1989) <u>J.
Virology</u> 173:167-177), generating MLV*env*. This construct
expresses amphotropic *env* gene under the transcriptional
control of the RSV LTR.

*Transfection of cells and
preparation of virus particles*

        Plasmids RSV*gagpol* and MLV*env* were introduced
into CEFs by transfection both separately and in the
mixture. Calcium phosphate-mediated transfection of
plasmid DNAs into CEFs was performed according to standard
procedures Graham and Van der Eb, (1973) <u>J. Virology</u>
52:456-467. Precipitates containing 10 μg of DNA per 10
mm-plate were incubated with subconfluent CEF monolayers
for 6 hr at 37°C followed by the incubation with the
medium containing 15% glycerol for 5 min at 37°C. Cells
were washed twice with the phosphate-buffered saline (PBS)
and incubated in a growth medium for 24 hr. When
necessary, transfected cells were passaged to allow the
virus to spread through the culture.

        Virus-containing culture fluid was then
harvested. 24 hr after transfection, the culture medium
was harvested and viral particles were recovered by
ultracentrifugation. To prepare virus particles, culture
fluid was clarified by low-speed centrifugation and the
virus was pelleted through 15% sucrose cushion in SW41
rotor (Beckman) at 35,000 rpm for 1 hr at +4°C. The
resulting pellet was resuspended in protein sample buffer
and heated at 100°C for 4 min before loading on a gel.

Immunoblot Analysis

        Viral proteins were resolved by electrophoresis
in a 4-20% gradient sodium dodecyl sulfate-polyacrylamide
gel (SDS-PAGE). After electrophoresis proteins were

electroblotted onto a nitrocellulose membrane (BA85, Schleicher & Schuell, NH). RSV matrix protein was detected by incubation with rabbit antiserum against p19 (MA) (generously provided by Volker Vogt, Cornell University). MLV envelope glycoproteins were detected with goat antiserum against gp70 (SU). Protein bands were visualized using enhanced chemiluminescence (ECL) detection system (Amersham).

The immunoblotting analysis (Fig.2B) showed that MLV*env* plasmid, when introduced into CEFs, expresses surface glycoproteins that are anchored on a cell membrane and can be detected by immunoblotting in a membrane protein fraction (data not shown) but are not secreted into the medium (Fig. 2B, lanes 1 and 2). Transfection of RSV*gagpol* results in a synthesis of capsid proteins that assemble into virus-like particles that can be recovered by ultracentrifugation (Fig. 2B, lanes 3 and 4). More than one band was detected by the anti-p19 antibodies. Since RSV MA is phosphorylated in several positions, additional bands usually seen on immunoblots apparently represent proteins with different degree of phosphorylation. Burstein et al., (1992) J. Virology 66:1781-1785.

Co-transfection of RSV*gagpol* and MLV*env* causes the co-expression of both capsid and envelope proteins in the same cell. Immunoblotting analysis shows that both the RSV *gag* and MLV *env* are present in the particles produced upon co-transfection (Fig. 2B, lanes 5 and 6) suggesting that pseudotyped virions are formed.

This data shows that the amphotropic MLV envelope can associate efficiently with an RSV virion if competing RSV envelope is not present.

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Example 2

The recombinant retroviral vector RCAS-M(4070A)

Construction of RCAS-M(4070A)

          Given the apparent efficiency with which the MLV
envelope protein assembles with RSV _gag_, recombinant RSV
genome was constructed in which the envelope gene in the
parental virus was replaced with the _env_ gene of an
amphotropic MLV. To ensure the efficient intracellular
transport of the envelope precursor and the signal peptide
cleavage in avian cells, a chimeric _env_ gene in which a
sequence encoding a mature MLV gp70 was fused to the RSV
envelope signal peptide. RSV _env_ coding sequences were
removed and the chimeric envelope coding region, including
a new stop codon was inserted, generating the recombinant
retroviral vector RCAS-M(4070A).

          The recombinant retroviral vector RCAS-M(4070A)
was prepared as follows. First, an Eco RI fragment of
RCASBP(A) spanning the 3'-end of the RSV _env_ gene and U3R
segment of the 3'-LTR was subcloned in pUC19, giving rise
to pUCenvRI (Fig. 1B). The termination codon for _env_ was
replaced by a Not I site using site-directed mutagenesis.
Clones containing mutant plasmids with the Not I site were
propagated in _Escherichia coli_ BMH 71-18 mutS (Clontech)
and selected by Not I cleavage. The resulting construct
was called pUCenvRINOT (Fig. 1B).

          The _env_ gene of RCASBP(A) was replaced with the
one carrying Not I site. RCASBP(A) was digested by Kpn I
and Cla I and the fragment containing _env_ region was
removed. The _env_ region containing the unique Not I site
was introduced by ligating a Kpn I-Eco RI fragment of
RCASBP(A) and the Eco RI-Cla I fragment from pUCenvRINOT
with RCASBP(A) that had been cleaved by Kpn I and Cla I,
generating plasmid RCASBP(A)NOT (Fig. 1B).

          Overlap extension, Horton et al., (1990)
Biotechniques 8:528-535, was used to construct a chimeric
amphotropic _env_ gene in which the sequence coding for the

N-terminal signal peptide of MLV was replaced with the equivalent sequence from the RSV *env* gene. A fragment spanning the unique Kpn I site, the *env* splice acceptor site and the signal peptide was amplified from RCASBP(A) by PCR using primers RSV-FOR (GGACGAGGTTATGCCGCTGTG) and RSV-BACK (ACATTAAAGACCTGATGGGGGCTAACATCAGCTCTTACCCCCGTAA) (Fig. 1B). The fragment beginning with the codon for the first serine residue of the mature gp70 and spanning the whole *env* open reading frame followed by the unique Not I site was amplified from pR4070A with the primers MLV-FOR (AGCCCCCATCAGGTCTTTAATGT) and MLV-BACK (AGCGGCCGCTCATGGCTCGTACTCTATGGGTT) (primer MLV-FOR overlapped RSV-BACK). These two fragments were fused and a product amplified by PCR with the primers RSV-FOR and MLV-BACK. The resulting PCR product which contained a chimeric *env* gene was cleaved by Kpn I and Not I. The *env* gene was removed from RCASBP(A)NOT by digesting with Kpn I and Not I, and a chimeric Kpn I-Not I fragment was inserted, generating a final plasmid RCAS-M(4070A). The correct sequence of the region containing the junction between RSV and MLV *env* sequences was confirmed by sequencing using Sequenase Sequencing Kit (USB, OH). The structure of RCAS-M(4070A) is shown schematically on Fig. 3A.

Production of Recombinant Viral Particles by RCAS-M(4070A)

To test the expression of a chimeric *env* gene in a context of an RSV genome and the ability of the vector to produce particles that had incorporated the MLV surface glycoproteins, CEFs were transfected with the RCAS-M(4070A) DNA, as described in Example 1. CEFs transfected with the pR4070A, the molecular clone of the amphotropic MLV from which the MLV *env* gene was derived served as a positive control. 24 hr after transfection, cell culture medium was harvested and viral particles recovered by ultracentrifugation as described in Example 1. As can be

seen in Fig. 3B, CEFs transfected with RCAS-M(4070A)
generate particles that contain the MLV surface
glycoprotein (lanes 1 and 2). Lanes 3 and 4 of Fig. 3B
contain the control viral particles from the cells
transfected with the amphoteric MLV. The incorporation of
gp70 into a particles produced by RCAS-M(4070A) appeared
to be approximately as efficient as the incorporation into
MLV virions (compare lanes 1 and 2 with lanes 3 and 4).

Replication of RCAS-M(4070A) in CEFs

Having demonstrated that the recombinant
retroviral vector RCAS-M(4070A) produced chimeric viral
particles, its ability to replicate in CEFs was measured.
CEF cells were transfected with RCAS-M(4070A) DNA as
described in Example 1 and passaged to allow the virus to
spread. A total of six passages were done. At passages
1, 4 and 6, a small number of cells was plated separately,
and the ability of the virus to spread was measured by the
staining of CEFs by the antibodies that react with
expressed gp70.

The indirect immunofluorescence microscopy assay
was performed as follows. CEFs infected with RCAS-
M(4070A) were grown on glass slides. Cells were fixed
with methanol, reacted with anti-gp70 goat serum (diluted
1:200 in PBS-1% bovine serum albumin), washed 3 times for
5 min with PBS containing 0.1% Triton X-100 and incubated
with fluorescein isothiocyanate (FITC)-conjugated rabbit
anti-goat secondary antibody. Photomicroscopy was
performed by using a Nikon Microphot-FXA microscope (Nicon
Inc., Melville, NY). As shown in Fig. 4A, only a small
number of positively stained cells are seen initially
(passage 1). This number increases significantly by
passage 4 and at passage 6 virtually all cells are
infected and expressing gp70.

To monitor the production of virus particles,
cell culture medium at each passage was harvested and
particles were recovered by ultracentrifugation as

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described in Example 1. The viral particles were analyzed
by immunoblotting with the antibodies against p19 and gp70
as described in Example 1. The amount of viral proteins
in the supernatant increases by passage 4 and reaches a
high level by passage 6 (see Fig. 4B). The ability of
RCAS-M(4070A) to spread in the CEF culture is comparable
to the rates of spreading seen with the parental vector
RCASBP(A).

The data show that the chimeric retrovirus
replicates in the avian cells at rates comparable to the
original RSV-based retroviral vector. The particles of
RCAS-M(4070A) generated after transfection into CEFs
contain approximately the same amount of the gp70 as do
particles of the amphotropic MLV, and can infect mammalian
cells. Taken together, these data suggest that the
initial association of the RSV *gag* and the amphotropic MLV
env proteins is efficient enough to allow the assembly of
the infectious virions.

Table 1. Titer of RCAS-M(4070A)Puro on mammalian cells.

Species	Cell line	Titer
Mouse	NIH 3T3	7.1×10^4
Dog	D17	$5-7 \times 10^5$
Human	HeLa	3.3×10^4

Gene transfer to
mammalian cells by RCAS-M(4070A).

To demonstrate the ability of RCAS-M (4070A)
viral particles to infect and transfer genes to mammalian
cells, the marker gene pac, that confers resistance to the
antibiotic puromycin (de la Luna, et al. (1988) Gene
62:121-126, Vara et al., (1986) Nucleic Acids Res.
14:4617-4624), was introduced into the Cla I cleavage site
downstream of the *env* gene and splice acceptor to generate

the vector RCAS-MC (4070A) Puro.

        To facilitate cloning into RCAS-M(4070A), a
silent point mutation was used to eliminate the Cla I site
in the amphotropic *env* region. It was introduced by site-
directed mutagenesis. The Kpn I-Not I-fragment of RCAS-
M(4070A) was first subcloned into pBluescript KS II for
mutagenesis. Mutant clones selected by Cla I cleavage
were propagated in *Escherichia coli* BMH 71-18 mutS. The
env region in RCAS-M(4070A) was replaced with the mutant
Kpn I-Not I fragment, giving rise to a vector RCAS-
MC(4070A).

        To construct RCAS-MC(4070A)Puro, the puromycin
resistance gene (pac) was amplified by PCR from the
plasmid pSVpac (de la Luna et al., (1988) Gene 62:121-126;
Vara et al., (1986) Nucleic Acids Res. 14:4617-4624; with
the primers that appended Nco I and Hind III sites to the
5'- and 3'-ends of pac respectively. The product was then
cloned into the adaptor plasmid Cla12Nco. Hughes et al.,
(1987) J. Virology 61:3004-3012. The pac gene was
isolated from the adaptor construct as a Cla I-fragment
and introduced into RCAS-MC(4070A).

        CEFs were transfected with RCAS-MC(4070A)Puro as
described in Example 1 and passaged 6 times. Virus-
containing culture fluid was used to infect fresh cells
that were passaged two more times. The resulting virus
was titered on murine cells (NIH 3T3), human (HeLa) cells
and D17, dog kidney cells. Titration on mammalian cells
was performed as follows. Virus-containing culture fluid
was harvested and filtered through a 0.45 μm membrane.
Host cells were plated in 60-mm plates (5 x 10^5 cells per
plate) and grown overnight. Cells were infected with
serial dilutions of the retroviral vector stocks in the
presence of polybrene (10 μg/ml) for 24 h, trypsinized and
plated in a selective medium containing G418 (400 μg/ml,
GIBCO BRL) or puromycin (2.5 μg/ml, Sigma). Ten days
later, colonies that developed from resistant cells were

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fixed with methanol, stained with Giemsa stain and
counted. In some experiments, individual colonies were
isolated and expanded into cell lines for further
analysis.

Puromycin-resistant colonies of D17 cells
produced by infecting the cells with the serial dilutions
of RCAS-MC(4070A)Puro are shown in Fig.5.

Table 1 shows that RCAS-MC (4070A) Puro was able
to infect each of the mammalian cells tested in high
titers. The titer of RCAS-MC(4070A)Puro on D17 (dog)
cells was approximately a one order of magnitude higher
than the titer on NIH 3T3 (mouse) or HeLa (human) cells.
The variable efficiency with which RCAS-MC(4070A)Puro
infects these cells could be attributed to the differences
in the amphotropic receptor density on the surface of
mammalian cells of different tissues and species.

Replication and Infectivity of RCAS-M(4070A)NEO

Additionally, the marker gene neo was introduced
into RCAS-M(4070A). The resulting vector, RCAS-
M(4070A)NEO (Fig. 6A), was used to infect and transfer
genes to mammalian cells. RCAS-M(4070A)NEO was constructed
as follows: The ClaI-fragment containing the neo gene was
isolated from RCASBP(A)NEO and cloned into the ClaI site
of RCASBP(A)NOT. The chimeric *env* gene was transferred
into this plasmid as a KpnI-NotI fragment.

CEFs were transfected with RCAS-M(4070A)NEO as
described in Example 1, and viral stocks that were
generated 24 hours after transfection were titered on
murine NIH 3T3 and human HeLa cells as described above.
The virus obtained by the transient expression of the
RCAS-M(4070A)NEO in CEFs had the titer 2-3 X10^3 colony
forming units (cfu/ml) on both cell lines. Genomic DNA
isolated from the G418^r clones was analyzed by southern
blot hybridization. Cells were infected by RCAS-
M(4070A)NEO and G418^r clones were selected. Their genomic
DNA was digested with *Eco*RI and *Bam*HI. Fragments were

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resolved in agarose gel, transferred onto charged nylon
membrane, and hybridized with digoxygenin-labeled EcoRI-
digested RCAS-M(4070A)NEO DNA. Membranes were incubated
with alkaline phosphatase-conjugated anti-digoxygenin
antibody, and bands were detected using Lumi-Phos 530
reagent. As shown in Figs. 6B and C, the majority of the
G418[r] clones derived from NIH 3T3 and HeLa cells contained
provirus that was structurally indistinguishable from the
RCAS-M(4070A)NEO.

The transfected CEFs were passaged and the
produced virus was used to infect both fresh CEFs and
mammalian cells. By immunoblotting analysis of the viral
particles collected at each passage, it was determined
that the virus replicated reasonably efficiently in CEFs;
however, the ability to transfer neo[r] into NIH 3T3 cells
decreased relatively rapidly (Table 2) and was several
orders of magnitude lower than the titer of
RCAS-M(4070A)Puro at a similar passage (compare Table 1
and Table 2). Sequencing of the proviral DNA as described
in Example 5 showed that the neo gene was somewhat
unstable in the context of the amphotropic env gene and
specifically that a number of proviruses suffered
deletions involving the splice acceptor site, initiation
codon and 5' end of the neo gene.

It appears that the difference in stability
between the neo and pac selectable markers is not a
function of the vector, but rather a measure of the
relative cytotoxicity of these markers on the host cell.
It seems unlikely that there is a much greater tendency
for the neo insert to rearrange because this would imply
that there are certain preferred sites for rearrangement
in the neo insert. However, the neo deletions that were
analyzed were all different.

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TABLE 2.   Titer of RCAS-M(4070A)NEO on NIH 3T3 cells

Virus	Titer, cfu/ml
24 h posttransfection	2.6×10^3
Virus passage 1	10^1
Virus passage 2	10^1
Virus passage 3	5×10^2

Example 3

Transmembrane protein p15E is correctly processed in RCAS-M(4070A) particles

In the murine leukemia viruses, the *env* precursor is initially cleaved by a cellular protease into gp70 (SU) and pre-p15E (TM). After the virus particle is released from the cell, the viral protease removes the C-terminal 16 residues from the cytoplasmic domain of pre-p15E, yielding the mature p15E and p2E. Rein et al., (1994) J. Virology 68:1773-1781. This cleavage activates the membrane fusion capability of the *env* protein and is essential for viral infectivity.

Virions produced by cells infected with RCAS-M(4070A) do not contain MLV protease. However, the chimeric virus is infectious, suggesting that pre-p15E is cleaved. Viral particles were prepared as described in Example 1 and the proteins were fractionated on 16% SDS-PAGE and analyzed by immunoblotting with rabbit antiserum against p15E (kind gift of Alan Rein) (Fig. 7). The data shows that the chimeric virus particles formed by RCAS-M(4070A) contain the processed p15E, although the MLV protease is not present. The transmembrane protein processing in the RCAS-M(4070A) particles appears to be as efficient as in the wild type MLV virions (compare lanes 1 "MLV" and 3 "RCAS-M (4070A)" of Figure 7), suggesting that

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either RSV protease or the protease of the avian cells can
effectively process pre-p15E.

<center>Example 4</center>

*Virus particles are not produced by
mammalian cells infected with RCAS-M(4070A)*

Recent studies demonstrated the development of
the lymphomas in the non-human primates after gene
transfer as a result of infection and spreading of the
replication-competent helper MLV which was present in the
retroviral vector stocks. (Donahue et al., (1992) J. Exp.
Med. 176:1125-1135; Vanin et al., (1994) J. Virology
68:4241-4250). This illustrates the considerable
importance of the issue of a retroviral vector safety.
One of the advantages of using avian retrovirus-based
vectors in a mammalian cell system is that the mammalian
cells transfected or infected with RSV do not produce
infectious viral particles (Federspiel et al., (1994) PNAS
(U.S.A.) 91:1124-11245) and therefore the vector cannot
spread in the mammalian host. Inability of RSV to
synthesize or to export into the cytoplasm a full-length
genomic RNA, (Berberich et al., (1990) J. Virology
64:4313-4320; Knight et al., (1993) J. Gen Virology
74(P112):2629-2636; Quintrell et al., (1980) J. Mol. Biol.
143:363-393), as well as its inability to process *gag*
precursor protein in mammalian cells (Vogt et al., (1982)
J. Virology 44:725-730) were implied as the possible
defects that prevent the synthesis of the virus structural
proteins and production of the virus particles.

To determine whether the mammalian cells
infected by RCAS-M(4070A) generate virus particles, we
infected fresh NIH 3T3 cells with the supernatants of
several NIH 3T3/RCAS-M(4070A)NEO clones carrying un-
rearranged proviruses. Cells were then subjected to G418
selection. No G418-resistant colonies were detected.
This sensitive assay showed that infectious viral
particles were not produced by the murine NIH 3T3 cells

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containing non-rearranged RCAS-M(4070A)NEO provirus.
Additionally, this experiment was repeated using RCAS-M2
(4070A)Puro to infect NIH 3T3 cells. Again, no viral
particles were detected. Thus, the RSV-based vector RCAS-
M(4070A), being inherently replication defective in the
mammalian cells, has substantially improved safety
features required for sensitive applications such as human
gene therapy.

<u>Example 5</u>

Construction of RCAS-M2(4070A),
A Fast Replicating and Stable Vector

Initially the chimeric virus RCAS-M(4070A)
replicates at a considerably lower rate than the parental
vector RCASBP(A). Indeed, after transfection of
RCAS-M(4070A) into CEFs, 3-4 cell passages were required
before detectable amounts of the virus were produced.
However, after 5-6 passages on CEFs, the chimeric virus
infects these cells efficiently and spreads quickly
throughout the entire culture.

To investigate the possibility that genetic
changes in the viral genome during this period of initial
slow replication permitted the chimeric virus to adapt and
grow more efficiently, the *env* regions of molecular clones
of RCAS-M(4070A) provirus containing the neo marker gene
(RCAS-M(4070A)NEO) were sequenced.

Cloning of RCAS-M(4070A)NEO Provirus

RCAS-M(4070A)NEO provirus was cloned as follows.
Low-molecular-weight DNA was isolated from CEFs infected
with RCAS-M(4070A)NEO at passage 3 by the Hirt extraction
procedure, Hirt (1967) <u>J. Mol. Bio.</u> 26:365-369. The low-
molecular-weight DNA was cleaved with the restriction
endonuclease *Sac*I, ligated with *Sac*I-cleaved vector λZAP
Express (Stratagene, CA) and packaged using GigaPack II
Gold packaging extract (Stratagene, CA). A library of 4.7
x 10^6 independent clones was screened by hybridization
with a ^{32}P-labeled amphotropic *env*-specific probe

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(*SalI/ClaI* fragment derived from pR4070A). Positive phage
clones were converted into plasmids by co-infection with
ExAssist Helper phage (Stratagene, CA). These clones were
designated env1, env3, env6, env8, and env9. RCAS-
M(4070A)NEO proviral DNA in the resulting plasmid clones
was analyzed by cleavage with restriction endonucleases.

*Sequencing of the env regions of the
RCAS-M(4070A)NEO Proviruses*

The *env* gene sequences of the RCAS-M(4070A)NEO
provirus clones and the parental plasmid RCAS-M(4070A)NEO
were determined by cycle sequencing of both strands using
PRISM™ Ready Reaction DyeDeoxy Terminator Cycle
Sequencing Kit (Applied Biosystems, CA). Sequencing
reactions were analyzed using the Automated 373A DNA
Sequencer (Applied Biosystems, CA). The complete
sequences of the *env* genes were assembled using Sequencher
software (Gene Codes Corporation, MI).

A limited spectrum of mutations in the *env*
region of the individual proviral clones was discovered.
The mutations were located primarily in the central and C-
terminal regions of the gp70 coding sequence (Fig. 8). In
all of the clones, two adjacent cytosines in the region
encoding gp70 were mutated to A and T, resulting in the
substitution of Ile for Pro at position 242. The same
region of the *env* gene in the parental plasmid RCAS-
M(4070A)NEO, had a proline codon at position 242, thus
confirming that the Pro242Ile mutation had been selected
during the passage of the vector on CEFs, and had not
occurred in the course of the vector construction. In
addition to this mutation, other substitutions were found,
including Lys308Gln (clones env1 and env9), Ala310Gly
(clones env3 and env6), and Met446Leu (clones env6 and
env9). Some clones had additional amino acid changes,
which were seen only in that clone. In addition, in the
clone env3, the sequence encoding the C-terminal end of
the gp70 and almost all of the p15E was deleted.

The Pro242Ile mutation appears to be critically important for efficient replication, since it is present in all of the clones we derived from passaged RCAS-M(4070A)NEO virus. Furthermore, this mutation alone is sufficient to dramatically increase the efficiency of the virus replication, since it is the only mutation found in the *env* gene of clone env8, the virus that replicates most efficiently in CEFs and produces the highest titers on mammalian cells.

The replacement of Pro242 with the uncharged isoleucine residue could indicate an important structural change in the gp70, since proline residues are often located at the points of β-turns. This change could reflect the adjustment necessary for the gp70 to recognize both avian and mammalian types of the amphotropic receptor or, alternatively, could enhance the interaction between RSV capsid and the *env* protein from the amphotropic MLV. It is also possible that the mutation affects the structure and/or function of viral RNA.

The mammalian cellular receptor for amphotropic MLV has recently been cloned and characterized. Miller, et al., (1994) PNAS(USA) 91:78-82; van Zeijl et al., (1994) PNAS(USA) 91:1168-1172. However, the gene for the avian amphotropic receptor has not been unambiguously identified. van Zeijl et al., (1994) PNAS(USA) 91:1168-1172. Passaging of RCAS-M(4070A) through CEFs could select for the viral variants that have increased affinity for the avian amphotropic receptor. No mutations were seen either in the N-terminal half of gp70, the region that appears to determine host range (Ott and Rein (1990) J. Virol 64:757-766; Ott and Rein (1992) J. Virol 66:4632-4638) or in the hypervariable proline-rich region. If the alteration in gp70 increases the affinity of the protein for the avian receptor, it does not compromise the ability of the virus to infect mammalian cells.

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Construction of RCAS-M2(4070A)

To construct an "adapted" version of RCAS-M(4070A) which takes advantage of the genetic changes which occurred in the viral genome during the period of initial slow replication, vectors designated RCAS-M2(4070A)Puro1, 6, 8 and 9 were constructed. These vectors contain the (gag-pol)^{RSV}-env^{MLV} regions derived from clones *env* 1, *env* 6, *env* 8 and *env* 9 of RCAS-M(4070A)NEO in place of the (gag-pol)^{RSV}-env^{MLV} region of RCAS-M(4070A)Puro.

To construct RCAS-M2(4070A)Puro, DNA fragments containing the (gag-pol)^{RSV}-env^{MLV} region were derived from the plasmid clones by digestion with *Sac*I and *Not*I and ligated with the LTR-Puro-LTR cassette that was obtained by *Sac*I/*Not*I cleavage of RCAS-MC(4070A)Puro.

The resulting RCAS-M2(4070A)Puro1, 6, 8, and 9 vectors were transfected into CEFs, and the cells were passaged to generate virus. At each passage, the production of viral particles was quantified by determining the virion-associated RT activity, and the number of viruses able to infect mammalian cells was determined by titration on D17 cells as described in Example 2.

Determination of the virion-associated RT activity was performed as described in Whitcomb et al., (1995) *J. Virol* 69:6228-6238. Briefly, virus particles were recovered from the culture fluid by centrifugation at 14,000 rpm for 30 min at 4°0C. Pellets were resuspended in RT buffer (50 mM Tris, pH 8.3, 60 mM NaCl, 12 mM MgCl$_2$, 20 mM dithiothreitol, 0.1% NP-40) containing oligo(dG) (5 μg/ml), poly(rC) (10 μg/ml), dGTP (10 μM) and 10 μCi of [α-^{32}P]dGTP (800 Ci/mmol, Amersham, IL). Reaction mixtures were incubated at 37°C for 1 h. Nucleic acids were precipitated by adding 1 ml of 10% trichloracetic acid (TCA), collected on the GF/C glass microfiber filters (Whatman, OR) and washed with 10% TCA and 95% ethanol.

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Filters were dried and counted in a TriCarb 1500 Liquid Scintillation analyzer (Packard).

As shown in Fig. 9A, the initial rates of replication of the "adapted" vectors are much faster than the parental vector, RCAS-M(4070A)Puro. Virus production is detectable at passage 1 and reaches a maximum at passages 3-4, which is similar to the initial rates of replication of RCASBP(A). In this experiment, the production of the parental vector, RCAS-M(4070A)Puro, designated "env (wt)," could not be detected until passage 6. The "adapted" RCAS-M2(4070A)Puro vectors are infectious for mammalian cells, exhibiting significant titers on D17 cells (Fig. 9B). The fastest replicating virus (clone env8) reaches the maximum titer on D17 cells by passage 2 (compare Figs. 9A and B).

Construction of RCAS-M2C(4070A)

To facilitate cloning into RCAS-M2(4070A), the *ClaI* site in the *env* gene of RCAS-M2(4070A)Puro (derived from the clone env8) was eliminated by introducing a silent point mutation by site-directed Mutagenesis as described in the construction of RCAS-MC(4070A) in Example 2. The DNA of the resulting vector RCAS-M2C(4070A)Puro was transfected into CEFs as described in Example 1 and the cells were passaged. At each passage, the number of virus particles able to infect mammalian cells was quantified by titration on D17 cells as described in Example 2. As can be seen in Fig. 9C, the RCAS-M2C(4070A)Puro replicates efficiently in CEFs, and the titer of a virus stock produced at each passage is the same as the titer of the RCAS-M2(4070A)Puro virus from which it was derived.

In clinical applications of retroviral gene transfer, both the titer and the safety of the vector are of critical importance. The titer of the RCAS-M2C(4070A)Puro vector exceeds 10^6 cfu/ml. This titer can be obtained by 2-3 cell passages after transfection of

CEFs. Not only is the vector simple to use and the titer
high, but the vector should also be quite safe. RSV-
infected mammalian cells do not produce infectious viral
particles and the vector cannot spread in the mammalian
host. The inability of RSV to efficiently export a full-
length genomic RNA into the cytoplasm (Berberich et al.,
(1990) _J. Virol_ 64:4313-4320; Knight et al., (1993) _J. Gen
Virol_ 74(Pt 12):2629-2636; Nasioulas et al., (1995)
PNAS(USA) in press; Quintrell et al., (1980) _J. Mol. Biol._
143:363-393) as well as its inability to assemble, export,
and process the Gag precursor protein in mammalian cells
(Nasioulas et al., (1995) _PNAS(USA)_ in press, Vogt et al.,
(1988) _J. Virol_ 44:725-730) appear to prevent the
production of infectious virus particles. To determine
whether the mammalian cells infected by RCAS-M(4070A)NEO
generate infectious virus particles, we infected fresh NIH
3T3 cells with the supernatants of several NIH 3T3/RCAS-
M(4070A)NEO clones carrying unrearranged proviruses as
described in Example 4. Following G418 selection, no
G418-resistant colonies were detected, indicating that the
mammalian cells infected with RCAS-M(4070A) do not produce
infectious particles. No infectious viral particles were
detected in the murine NIH 3T3 cells containing non-
rearranged RCAS-M(4070A)NEO provirus were detected using
G418 selection as described in Example 4. Similar
experiments were performed using RCAS-M2(4070A)Puro to
infect the mammalian cells and no virus particles were
detected in the infected murine NIH 3T3 cells. Since it
is inherently defective in the mammalian cells, the RSV-
based vector RCAS-M2C(4070A) appears to have the safety
features required for such sensitive applications as human
gene therapy.

        For purposes of clarity of understanding, the
present invention has been described in some detail by the
use of illustration and examples. However, it will be
obvious to those skilled in the art that certain

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modifications may be practiced within the scope of the
appended claims.

We Claim:

1.   An isolated nucleic acid comprising:

    a.   at least one long terminal repeat (LTR) derived from an avian sarcoma leukosis virus(ASLV);

    b.   a first nucleic acid sequence selected from a *gag* region derived from ASLV;

    c.   a second nucleic acid sequence selected from a *pol* region derived from an ASLV; and

    d.   a third nucleic acid sequence selected from a viral envelope region, said third sequence encoding a protein capable of binding to both avian and mammalian cells whereby a viral particle harboring said nucleic acid can infect both avian and mammalian cells in titers of at least 10^4/ml, which is replication competent in avian cells but is replication defective in mammalian cells.

2.   The nucleic acid of claim 1 further comprising at least a fourth nucleic acid sequence.

3.   The nucleic acid of claim 1 further comprising a restriction endonuclease site allowing for the insertion of a further nucleic acid sequence.

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4. The nucleic acid of claim 1 wherein the LTR, the gag and the pol sequences are derived from Rous sarcoma virus (RSV).

5. The nucleic acid of claim 1 wherein the envelope sequence is derived from the amphotropic murine leukemia virus (MLV).

6. The nucleic acid of claim 1 wherein the envelope sequence is a chimeric envelope sequence comprising a sequence encoding the N terminal signal peptide of an ASLV *env* region and a sequence selected from the coding region of an amphotropic MLV *env* sequence.

7. The nucleic acid of claim 1, 5 or 6 wherein the envelope sequence is adapted for increased initial replication due to the presence of one or more mutations in the envelope sequence.

8. The nucleic acid of claim 1, 5 or 6 wherein the envelope sequence contains an isoleucine at position 242.

9. The nucleic acid of claim 1 wherein said nucleic acid comprises RCAS-M (4070A) shown in Figure 3 or an equivalent nucleic acid having the functional properties and features of said nucleic acid.

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10.   The nucleic acid of claim 1 wherein said nucleic acid comprises RCAS-M2(4070A)Puro 8 or an equivalent nucleic acid having the functional properties and features of said nucleic acid.

11.   A viral particle comprising the nucleic acid of claim 1.

12.   A viral particle comprising the nucleic acid of claim 10.

13.   A method for inserting at least one nucleic acid sequence of interest into a host cell comprising introducing the nucleic acid of claim 2 or 10 into the host cell.

14.   A method for inserting at least one nucleic acid sequence of interest into a host cell comprising infecting the host cell with the viral particle of claim 11.

15.   A method for inserting at least one nucleic acid sequence of interest into a host cell comprising infecting the host cell with the viral particle of claim 12.

16.   A use for the nucleic acid of claim 1 or 10

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for the treatment or prevention of disease in a mammal
comprising administering an effective amount of said
nucleic acid.

17.   A use for the viral particle of claim 11 or
12 for the treatment or prevention of disease in a mammal
comprising administering an effective amount of said viral
particle.

18.   A host cell containing the nucleic acid of
claim 1.

19.   A host cell containing the nucleic acid of
claim 10.

20.   A transgenic animal, wherein at least one
cell contains the nucleic acid of claim 1 or 10.

21.   A use for the nucleic acid of claim 1 or 10
for providing gene therapy to an individual comprising
introducing the nucleic acid into a host cell and
administering the host cell to the individual.

22.   A use for the nucleic acid of claim 1 or 10
for providing gene therapy to an individual comprising
administering the nucleic acid to the individual.

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23.　A use for the viral particle of claim 11 or 12 for providing gene therapy to an individual comprising infecting a host cell with the viral particle and administering the infected host cell to the individual.

24.　A use for the viral particle of claim 11 or 12 for providing gene therapy to an individual comprising administering the viral particle to the individual.

25.　A composition comprising the host cell of claim 18 or 19 and a carrier.

26.　A composition comprising the viral particle of claim 11 or 12 and a carrier.

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FIG. IB

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FIG. 2A

FIG. 2B

RCASBP(A)

RCAS-M (4070A)

FIG. 3A

1   2   3   4

← gp 70
  (MLV)

FIG.3B

Passage 1 **Passage 4** **Passage 6**

FIG.4A
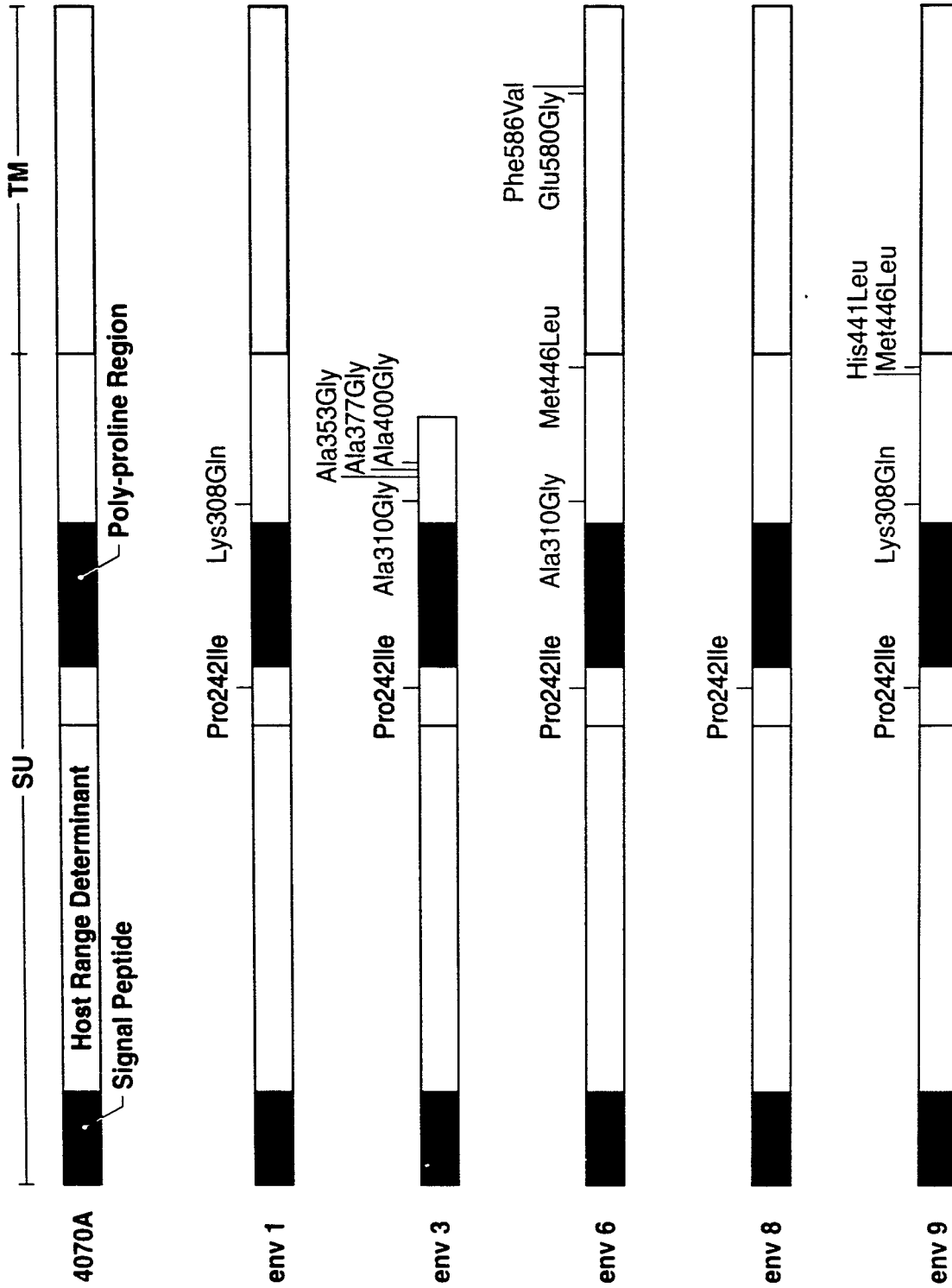
FIG.4B

FIG.5

FIG.6A

FIG.6B

FIG.6C

1   2   3

p30^{gag} →

pre-p15E →
p15E →
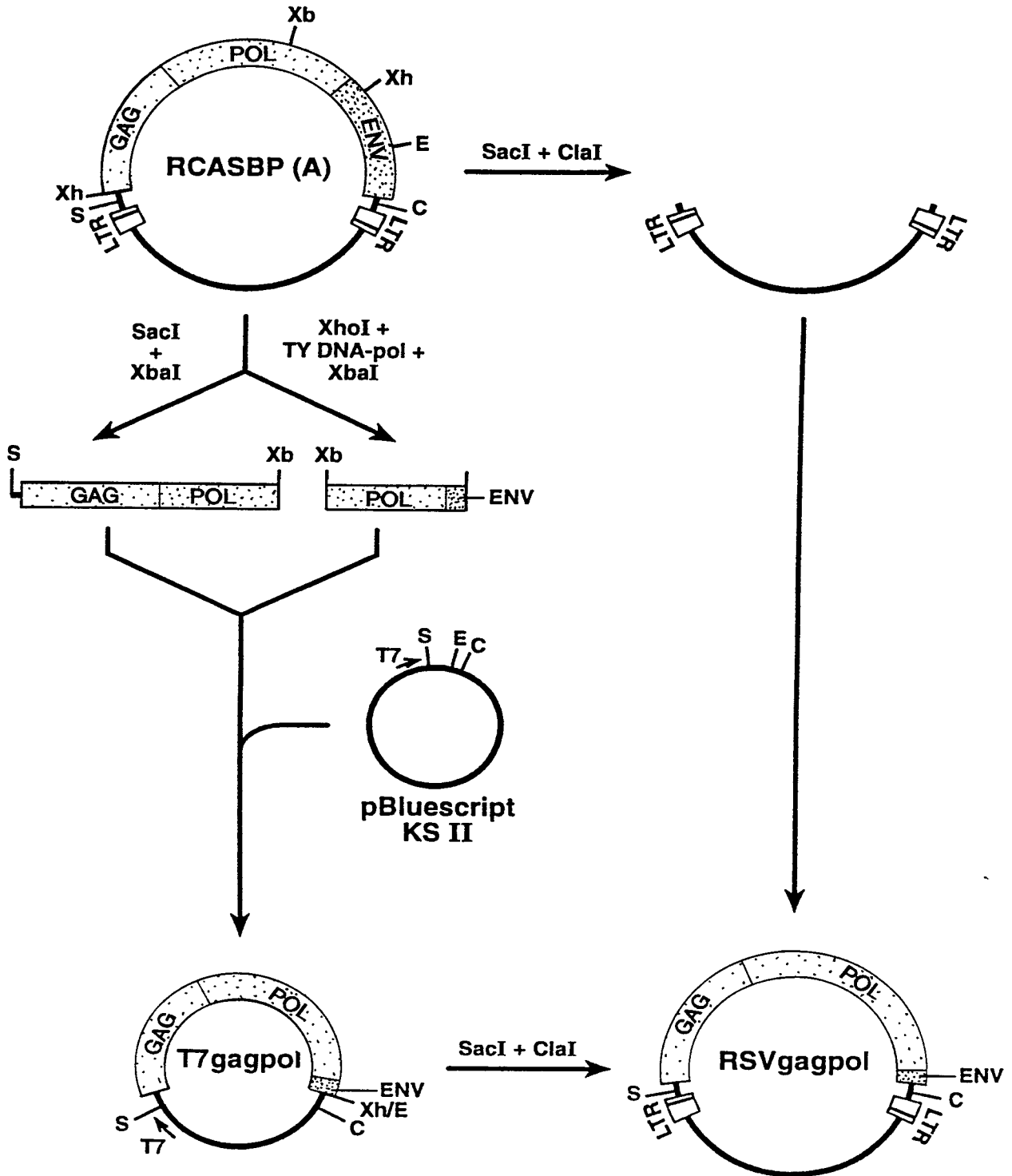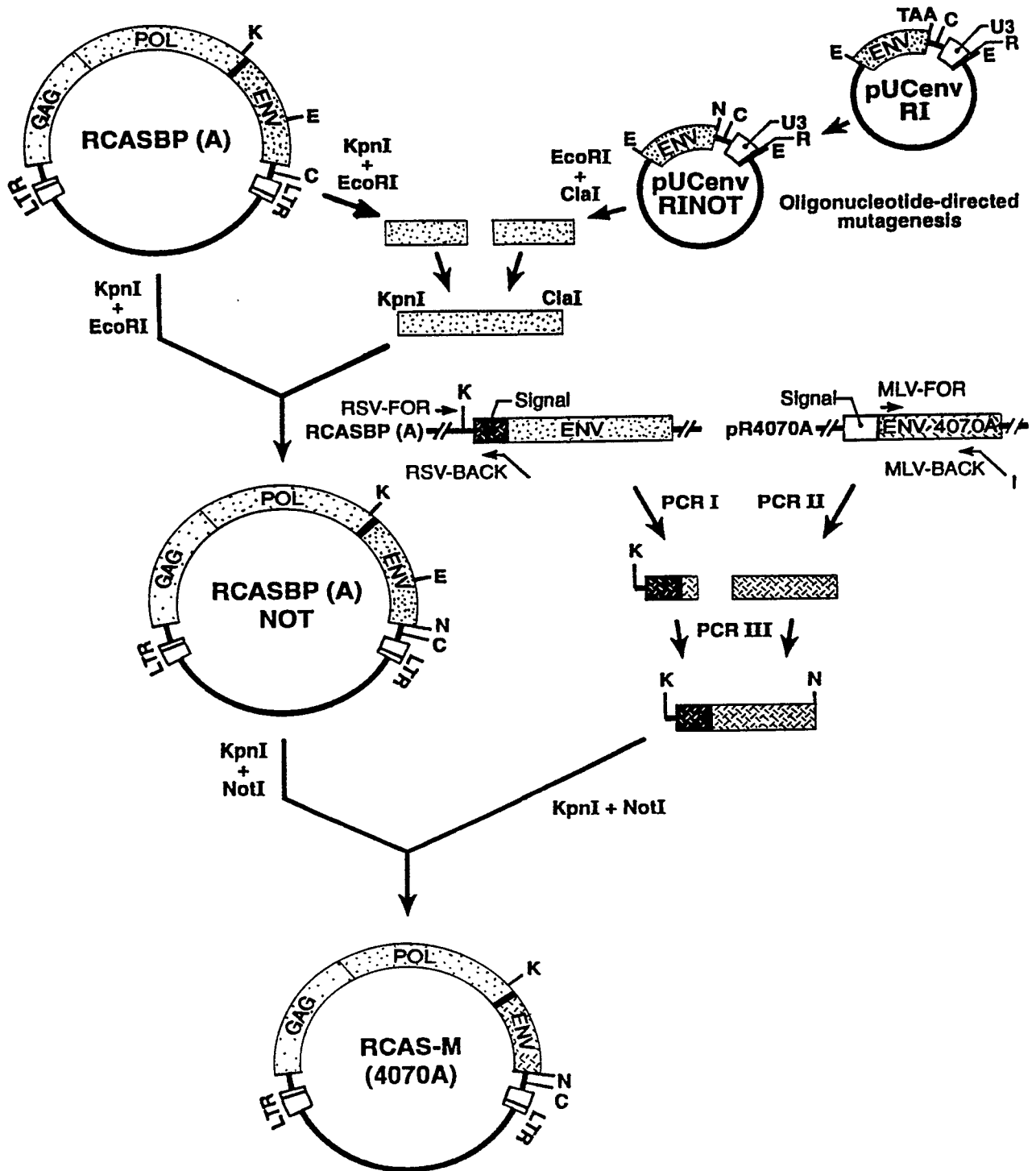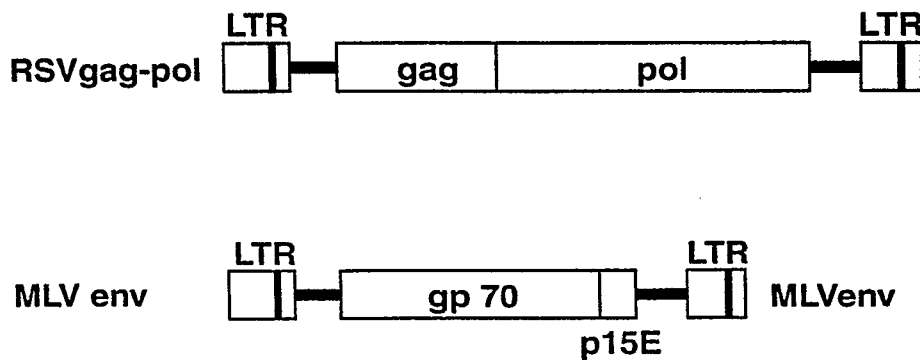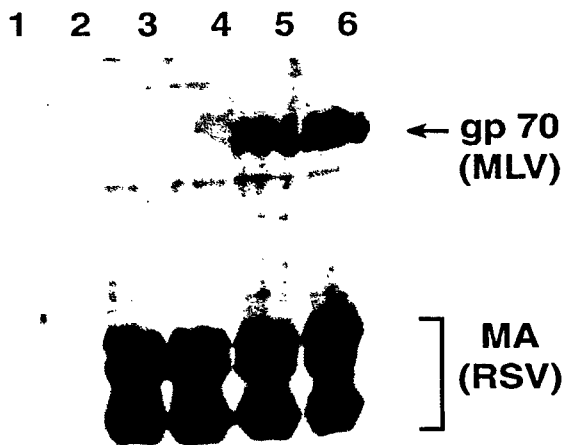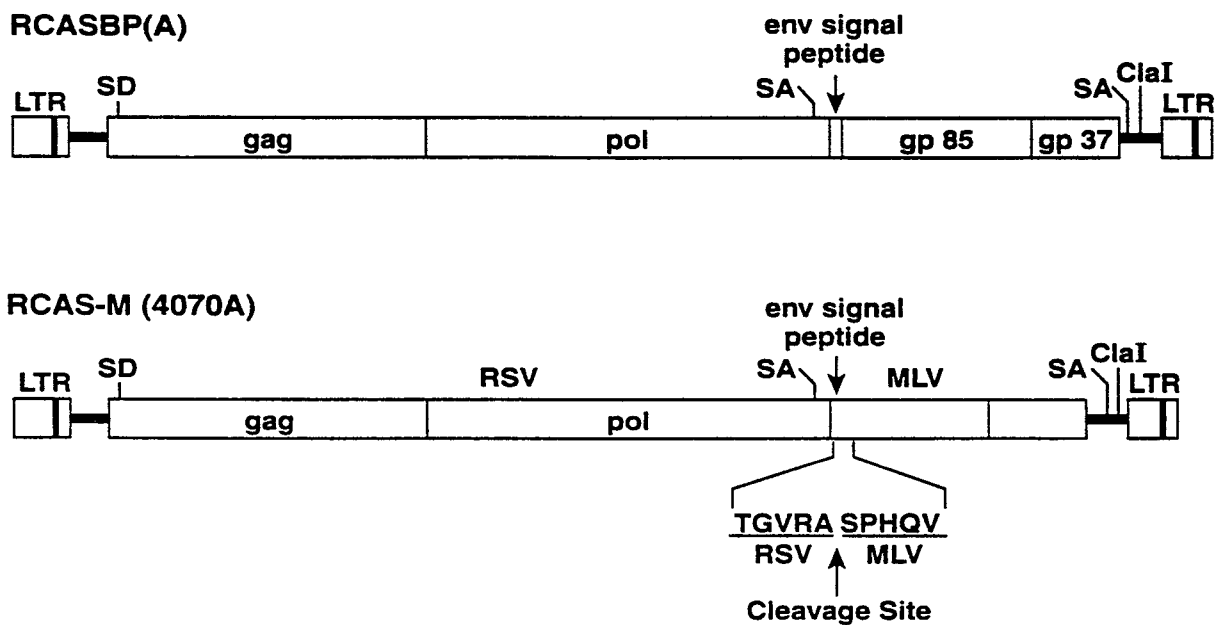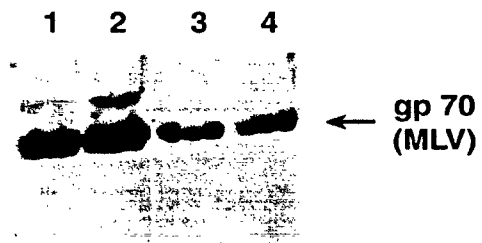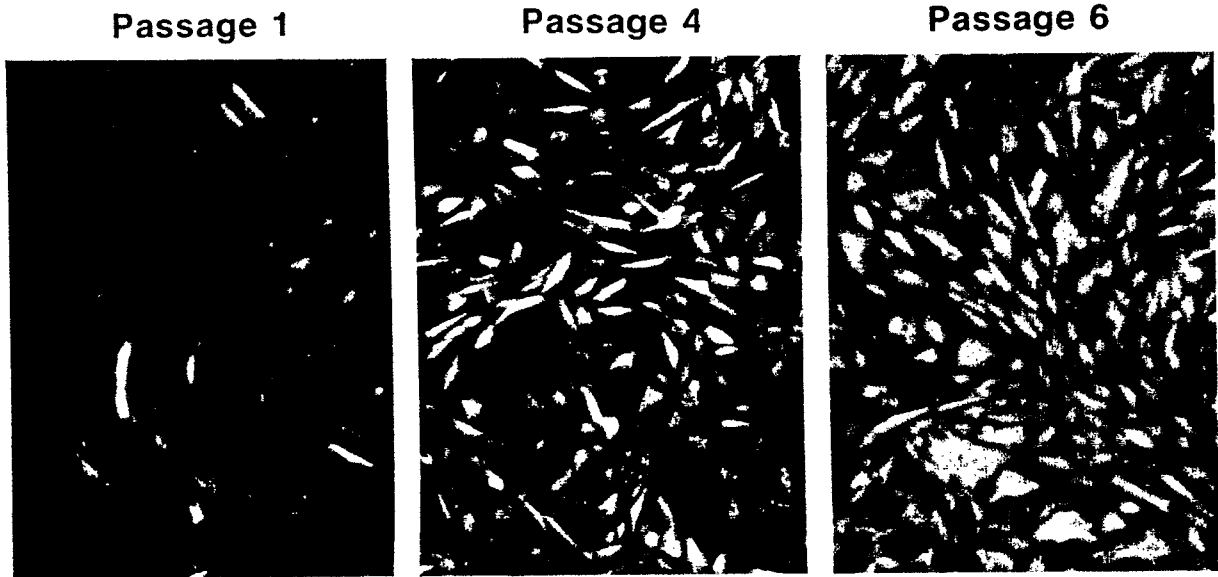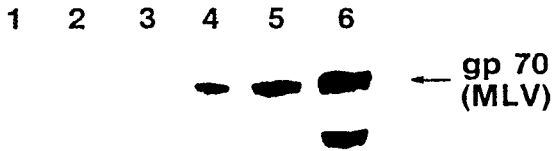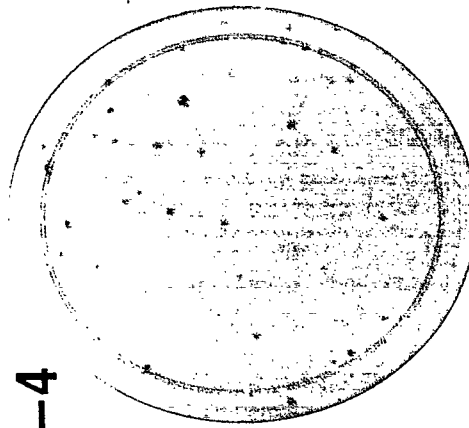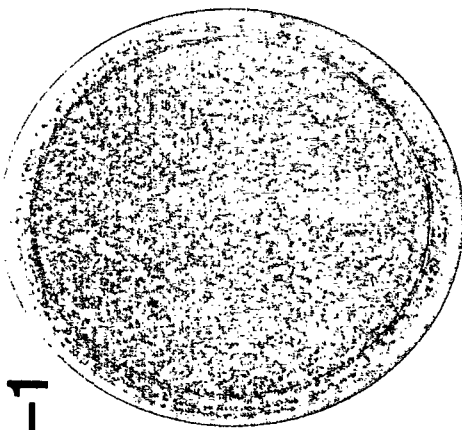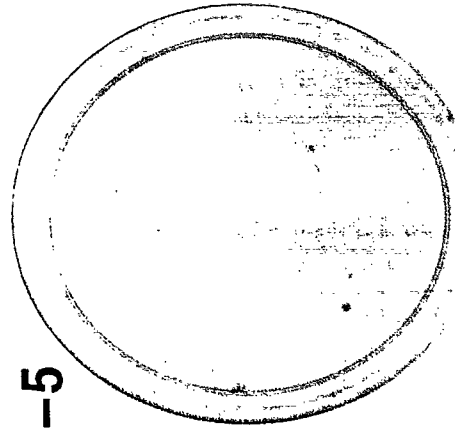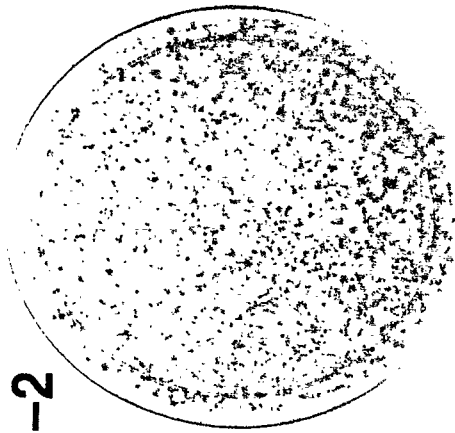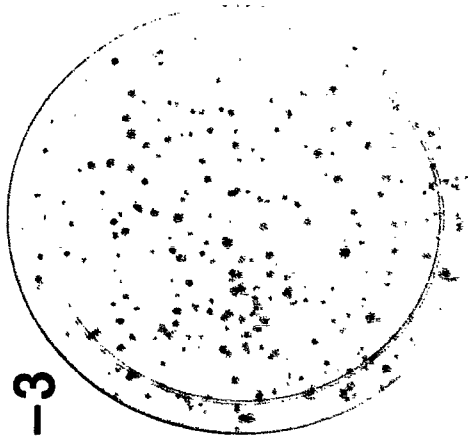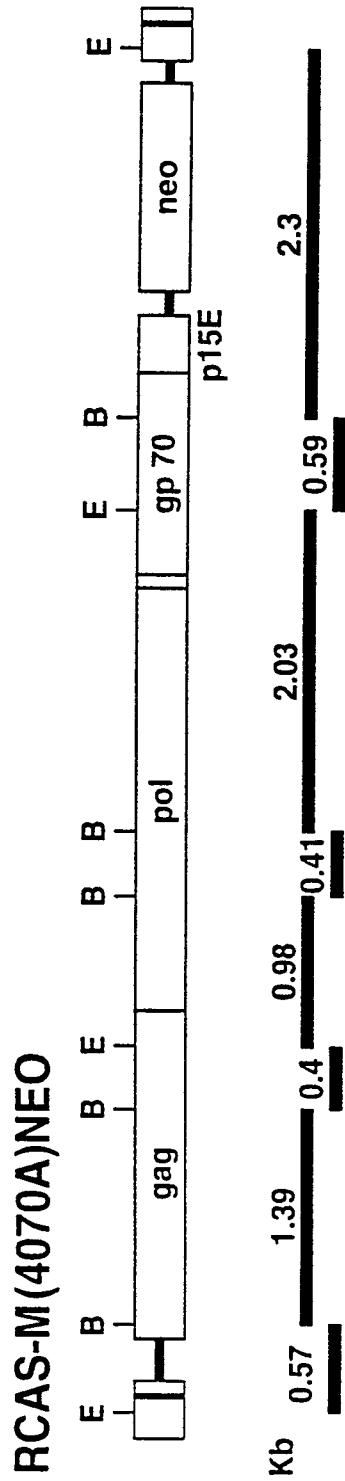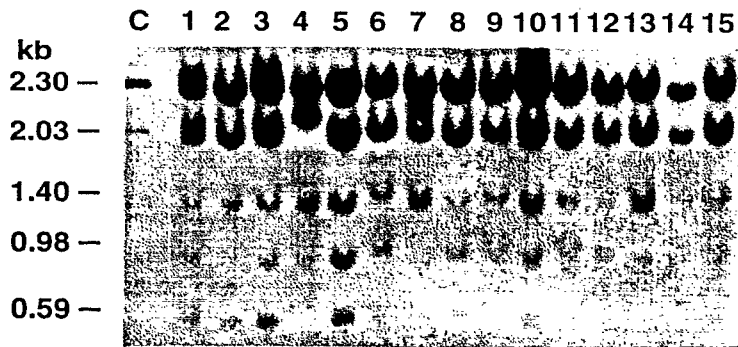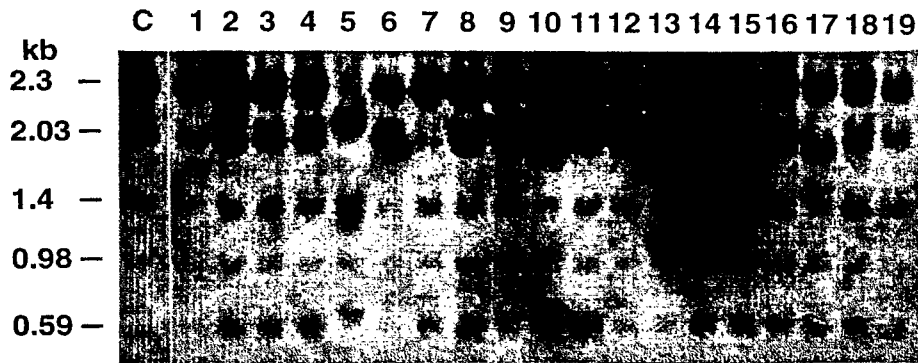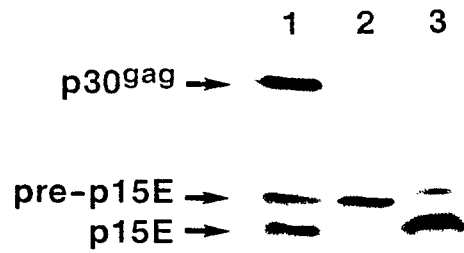
FIG. 7

FIG.8

FIG.9A

FIG.9B

FIG.9C