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**CELL-SPECIFIC TARGETING OF RETROVIRAL VECTORS VIA
LIGAND-RECEPTOR INTERACTION**

by

Noriyuki Kasahara

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHYSIOLOGY/ENDOCRINOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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Noriyuki Kasahara

Dedication

I would like to dedicate this thesis to my parents, who have believed in, supported, and encouraged me throughout, and to Michelle, who stood by me when times were darkest.

Preface

This work is the culmination of a long, often agonizing, frustrating, and Sisyphean process which started with a relatively simple and seemingly straightforward idea--targeting viruses to specific cells by tagging them with a ligand, directing them to preferentially infect cells with the appropriate receptor, a classic "lock-and-key" interaction. Maybe it could be done as a nifty little rotation project. Yeah, right. "Hindsight is 20-20," as the saying goes, and indeed, if I had known then the sheer depth of my naiveté in the realm of retroviruses, I might never have undertaken this project. However, six years, two ligands, and literally hundreds of packaging cell subclones later, I would like to acknowledge the many people who have helped me reach this point. First, of course, my parents, for their persistent belief that I would someday finish my thesis, and Michelle, for sharing and propagating this concept...Drs. Reg Kelly and Keith Yamamoto, for teaching at the 1985 Cold Spring Harbor summer course that nearly decapitated Jim Watson and firmly entrenched my desire to come to U.C.S.F., and Keith especially for sage advice and great BioReg lectures...Drs. Dick Weiner and Mary Dallman, for taking me in to the Endo program, and for constant encouragement, guidance, and support, a memorable bottle of Wild Turkey and great Thanksgiving parties...Drs. Tim Reudelhuber, Keith Duncan, and Fred Schaufele, for teaching me practically everything I know about molecular biology and many things I probably shouldn't know...like how to make a killer Hurricane...also everyone in the Metabolic Research Unit, especially Drs. Peter Kushner and Margot LaPointe, for encouragement and advice, and of course Dr. Dave Gardner for adopting me after Tim left, for tremendous help with

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CELL-SPECIFIC TARGETING OF RETROVIRAL VECTORS VIA LIGAND-RECEPTOR INTERACTION

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Abstract

The host range of retroviral vectors has been altered so that they are now capable of tissue-specific infection via ligand-receptor interaction. Sequences encoding the peptide hormones atrial natriuretic factor (ANF) and erythropoietin (EPO) were engineered into the Moloney murine leukemia virus (MoMLV) *env* gene and expressed in the ecotropic packaging cell line Ψ2 and amphotropic packaging cell line PA317. Western blot and flow cytometric analyses showed that some of the chimeric ligand-envelope constructs could be expressed as protein and could reach the cell surface. The packaging cell lines expressing EPO-*env* chimeric envelope were used to package viral vectors carrying the neomycin resistance gene and infect target cells with or without a transfected EPO receptor. Viruses bearing the recombinant EPO-*env* protein showed two- to six-fold increased infectivity on cells with the EPO receptor. More strikingly, the chimeric EPO-*env* protein enabled the ecotropic virus to infect human erythroleukemic cells such as HEL and K562, which express the EPO receptor. This demonstrates that the host range of the ecotropic virus can be modified so that it will now cross species and specifically infect human cells bearing the appropriate receptor.

Furthermore, by changing the targeting ligand, this method can be used to create a wide variety of vectors which would target to specific cell types. By achieving efficient tissue-specific gene transfer, such vectors will simplify preliminary preparation steps in *ex vivo* infection procedures, increase the likelihood of properly regulated expression of the therapeutic gene, and may represent a delivery system which is potentially applicable *in vivo*.

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CHAPTER 1.

The retroviral life cycle, mechanisms of host cell entry, and determinants of specificity

The focus of this thesis is on the mechanisms by which retroviruses attach to and enter host cells, and how these mechanisms can be manipulated to alter the host range and tissue specificity of the virus. I will first present a general overview of the retroviral life cycle, followed by a review of the more pertinent aspects of virus-host cell interaction, and finally a discussion of the experimental approach I have used in these experiments.

The retroviral life cycle

Retroviral virions contain two copies of a (+) strand RNA genome encased within an icosahedral nucleocapsid, which is itself surrounded by an envelope consisting of viral proteins embedded in a lipid bilayer [1]. Generally, the retroviral envelope proteins consist of two subunits: a larger glycoprotein which is situated exterior to the lipid bilayer, and a smaller protein which is embedded in the bilayer. These two envelope proteins are both derived from a common precursor polypeptide, and are linked by disulfide bonds [1]. The outer viral envelope glycoprotein mediates attachment of the virion to the host cell surface by specifically recognizing and binding cell surface proteins referred to as "viral receptors". After adsorption, the retroviral contents gain access to the cytoplasm by a sequence of events which is still relatively unclear; the traditional view has been that internalization of the virus is effected via receptor-mediated endocytosis and subsequent fusion between the viral envelope and acidified endosomal vesicle membranes [2] though recent evidence suggests that fusion may take place at the cell surface [3] in the case of some retroviruses.

The actual membrane fusion event seems to be mediated by the inner transmembrane subunit, which contains a fusogenic peptide sequence. This sequence is normally concealed by the outer glycoprotein subunit, but binding of the outer subunit to its host cell receptor triggers a conformational change in the outer subunit which thus exposes the inner subunit fusogenic sequence.

Once inside the cytoplasm, a double-stranded DNA copy of the RNA genome is synthesized by viral reverse transcriptase. This proviral DNA is then transported to the nucleus and integrated into the host cell DNA, a process which is mediated by viral integrase and by long terminal repeat (LTR) sequences which flank the genome; once stably integrated, the proviral DNA serves as a template to direct viral RNA synthesis, and is replicated along with the host DNA (Fig.1-1).

Typical retroviral genes include *gag*, which encodes capsid proteins; *pol*, which encodes reverse transcriptase and integrase; and *env*, which encodes the envelope glycoproteins. In addition, a "packaging signal", *psi*, helps viral genomic RNA and core proteins in the cytoplasm associate with capsid proteins and envelope glycoproteins embedded in the host cell plasma membrane; as this viral assembly occurs, mature virion particles bud off from the surface of the infected cell.

It has been possible to construct replication-defective retroviral vectors from which *gag*, *pol*, and *env* have been deleted, but which still retain the *psi* signal [4]. Such vectors may thus be packaged into intact virions by introduction into a "packaging cell line" which provides the *gag*, *pol*, and *env* functions in trans [5] (Fig. 1-2).

Figure 1-1.

The retrovirus life cycle. Viruses bind to host cell surface proteins which serve as receptors, and are internalized and uncoated either at the cell surface (in the case of amphotropic virus) or in an endosomal compartment (in the case of ecotropic virus). Reverse transcription of the viral RNA to double stranded complementary DNA also results in duplication of the U5 and U3 regions at each end of the virus genome, to create the long terminal repeat (LTR) sequences. After translocation to the nucleus and integration into the host genome, the provirus directs the transcription of full length genomic viral RNAs as well as spliced envelope (*env*) RNAs, both of which are translated into viral structural proteins. These proteins assemble around genomic viral RNA, which contains a packaging signal *psi* (Ψ), and bud off from the cell surface. See text for further details.

Retrovirus life-cycle:

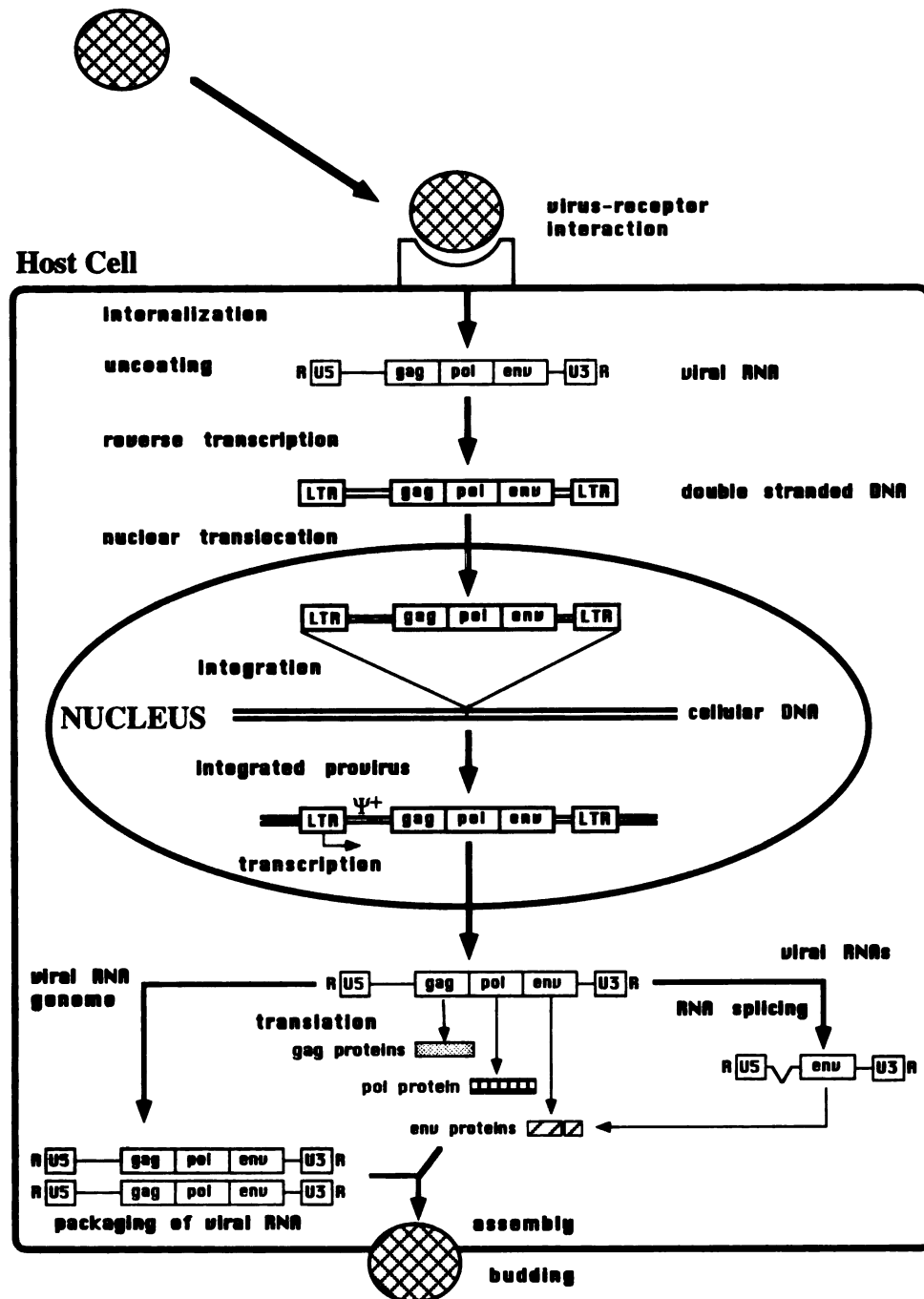
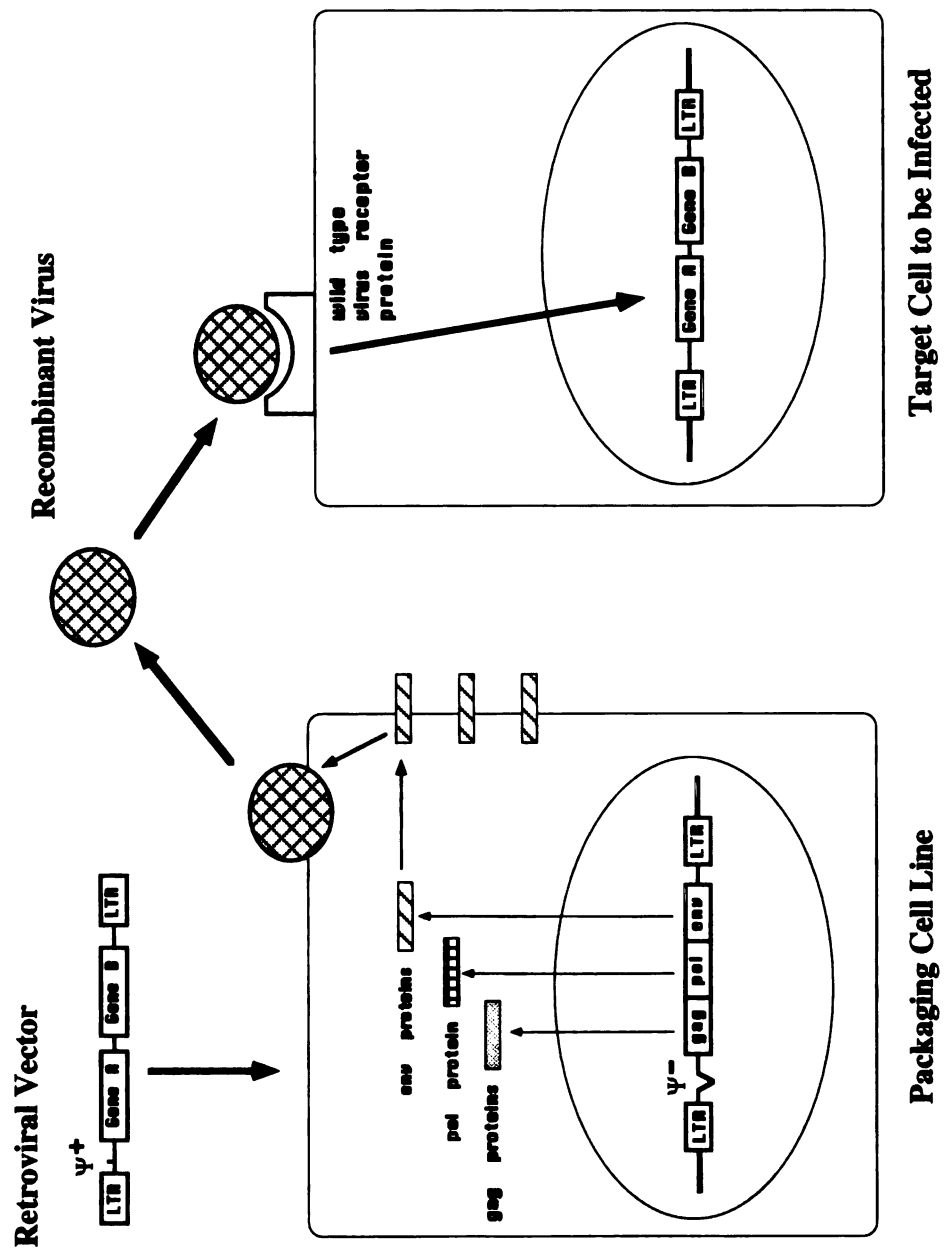


Figure 1-2.

The use of packaging cells to create replication-deficient retroviral vectors. Retroviral packaging cells contain integrated viral genes lacking the *psi* packaging signal (Ψ^-); the RNAs transcribed from these viral genes thus cannot be packaged. However, the viral protein products of these genes can be used to supply retroviral functions *in trans*, to package vectors which do contain the packaging signal (Ψ^+) and are flanked by long terminal repeat (LTR) sequences. As these vectors usually carry non-viral genes (genes A and B) and lack viral structural genes, they are dependent on the viral genes contained within the packaging cell for assembly into viral form, and thus are replication-defective after infection of target cells. See text for details.



The virions produced in this manner will successfully infect and integrate in the genome of target cells, but will not be capable of reproducing themselves in the target cells; this is an important safety feature which prevents undesirable consequences of unchecked replication and spread of the vector virus.

The mammalian retroviral packaging cell lines most commonly used at present utilize the *gag*, *pol*, and *env* functions of the Moloney murine leukemia virus (MoMLV). MoMLV is a prototypical mammalian retrovirus first isolated in 1960 from a sarcoma passaged in BALB/c mice; injection of MoMLV into newborn mice causes the rapid development of a lymphocytic leukemia [1]. The wild-type virus is capable of autonomous replication and possesses a full complement of genes, which are arranged linearly in the order 5'-LTR-*gag-pol-env* -LTR-3' and which have been entirely sequenced [6].

Based on their ability to infect cells of various species, murine leukemia viruses are classified as ecotropic (mouse-infecting), xenotropic (nonmouse-infecting), and amphotropic (both mouse- and nonmouse-infecting). Wild type MoMLV is an ecotropic retrovirus, that is, it will only infect murine cells. There is a packaging cell line, called Ψ 2, derived from stable integration of *psi* (packaging signal)-deleted wild type MoMLV into NIH 3T3 cells [5]. Replication-defective vectors packaged by this cell line will, accordingly, have an ecotropic host specificity, and will not infect cells from other mammalian species. However, there are variants of MoMLV which are known to infect non-murine cells, or both murine and non-murine cells, i.e., xenotropic and amphotropic variants [1]. One particular amphotropic strain, known as 4070A, is thought to have arisen from a

recombination event in the *env* gene, and 4070A sequences have been used to create packaging cell lines such as PA12, PA317, ΨCRIP, GPEam12, etc., which produce retroviral vectors with a broad mammalian host range [7, 8].

Determinants of host specificity

What are the viral factors which determine species- or tissue-specificity? In principle, tissue-specificity of viral infection can be mediated either by specific delivery to the target tissue or by specific expression in the target tissue, or by a combination of both mechanisms. Thus there seem to be two factors involved: one is the ability of the virus to actually attach and enter its target cell, and the other is its ability, once inside the cell, to integrate and express its genome efficiently.

As mentioned above, adsorption and entry to the host cell are mediated by viral envelope (*env*) proteins; in retroviruses, these two steps in the process of infection seem to be mediated by, respectively, the outer (extramembranous) envelope subunit and the inner (anchoring) subunit. The fact that the binding of viral envelope proteins to the host cell occurs via a specific interaction with cell surface proteins is well documented. The best characterized example is the lymphotropism and neurotropism of HIV mediated by binding of the HIV envelope glycoprotein gp120 to the CD4 antigen [9]. In the case of wild type ecotropic Moloney murine leukemia virus (MoMLV), the outer envelope subunit gp70 has been shown to bind avidly to a variety of mouse cells but poorly, if at all, to cells of other mammalian species. However, as mentioned above, variants of MoMLV

exist which exhibit differences in their species specificity, and this seems to correlate with differences in their respective *env* genes. The *env* gene of ecotropic MoMLV contains one large open reading frame extending for 2 kilobases [6], and encodes a polyprotein precursor which is post-translationally cleaved to produce the two envelope subunit species: one cleavage product is gp70, a glycoprotein of approximately 70,000 daltons, and the other is a non-glycosylated protein of 15,000 daltons (p15E) [10]. Topographically, gp70 is situated mainly outside the envelope lipid membrane and is linked to the highly hydrophobic p15E protein, which serves as its membrane anchor and which contains a membrane fusogenic sequence, by disulfide linkages [11].

Even prior to sequencing of the MoMLV genome in 1981 by Shinnick et al. [6], various attempts were made to correlate host range with specific domains of the virus by, for example tryptic peptide mapping [12] or by heteroduplex analysis [13]. Once the genome was sequenced, comparison of the ecotropic MoMLV *env* gene with that of xenotropic or amphotropic variants identified non-conserved regions of the gp70 coding sequence which were theorized to be involved in determining host range [13-17]. Interestingly, one of the most variable sites is located in the central portion of the molecule, in a highly proline-rich region which may form complex alpha-helical structures and was originally thought to be involved in receptor recognition [14]. Other hypotheses focused on naturally-occurring envelope mutants which altered the tropism of the virus while maintaining its viability, which are unfortunately exceedingly rare [1]. One such mutant, known as *ts1*, is a neurovirulent temperature-sensitive variant of MoMLV which causes progressive hindlimb paralysis in mice; however, it was subsequently found that the *env* mutations in *ts1* did not mediate

neurotropism per se, but rather resulted in inefficient processing of gp70 precursors which then accumulated in the infected neurons and resulted in neurodegeneration [17]. However, more recent studies involving saturation mutagenesis of the gp70 sequence, as well as mix-and match experiments creating chimeric fusion proteins containing various portions of the ecotropic and amphotropic envelopes, have indicated that it is in fact the amino terminal portion of the gp70 glycoprotein which mediates binding to its receptor [18-21]. This region contains two variable regions, designated VR-A and VR-B. In 1992 Battini et al. [18] studied chimeras in which the VR-A, VR-B, and proline-rich regions of amphotropic, xenotropic, and polytropic envelopes were exchanged, and suggested that the VR-A region recognizes the receptor, while downstream regions such as VR-B and the proline-rich domain are important for structural stability. These results were subsequently confirmed last year by detailed mutational analysis of gp70 by Gray and Roth [19], and extended by Morgan et al. [20], who further localized the host range-determining regions to the first 88 amino acids of ecotropic MoMLV envelope and the first 157 amino acids of amphotropic MLV variant 4070A. At the time my project was first conceived, such detailed structural analyses of the gp70 molecule had not yet been performed and even the full sequence of the amphotropic 4070A MLV virus envelope had not been published; thus it was not until quite recently, in fact, that the amino-terminal portion of gp70 was established as the receptor-binding moiety.

Studies by Cunningham and coworkers [22] have shown that murine cells contain a transmembrane receptor that specifically recognizes the envelope protein of ecotropic Moloney retrovirus. This receptor, apart from

its role in mediating MoMLV infection, normally functions as a transporter for basic amino acids such as arginine and lysine [23, 24]. The human homologue of this protein has also been cloned and has been shown to contain small differences in its amino acid sequence which render it incapable of binding ecotropic MoMLV envelope [25]; amino acid substitutions which change portions of the human ecotropic receptor homologue sequence to that of the mouse have been shown to confer the capability to bind ecotropic MoMLV envelope and thus mediate ecotropic MoMLV infection in human cells [26]. More recently, the receptor for the amphotropic Moloney retrovirus has also been cloned, and proves to be an entirely separate protein. The amphotropic has been putatively identified as a phosphate transporter, and appears to be related to the receptor for Gibbon ape leukemia virus (GALV) [27, 28]. Thus it seems that the gp70 envelope glycoprotein, or variants thereof, are responsible for species-specificity of adsorption of MoMLV particles to target cells, by virtue of their interaction with cell surface receptors.

The tissue distribution of both ecotropic and amphotropic Moloney virus receptors is largely ubiquitous, which is to be expected considering that they are normally employed by the cell as, respectively, amino acid and phosphate receptors. On the other hand, although most tissues can readily be infected, they are not necessarily transformed, and the expression of wild type MoMLV is thymotropic [1]. Therefore, although the binding of viral ecotropic or amphotropic envelope proteins to their respective host cell receptors appear to constitute the basis for **species** specificity among MLV variants, since the tissue distribution of the receptors is largely ubiquitous within a given species, cell-specific delivery is probably **not** the determinant of **tissue** specificity in this case. It has been suggested that the U3 region of

the long terminal repeat (LTR) sequence, is responsible for thymus-specific expression, on the basis of sequence comparisons with variant viruses exhibiting differing tissue-specificity [29]. This was recently confirmed by Couture et al. [30], who exchanged the U3 promoter/enhancer region of MoMLV with the corresponding region from five related murine retroviruses, and found differences in expression levels with respect to specific cell types.

Experimental strategy

Therefore, since it is known that 1) the envelope proteins are involved in viral attachment and entry into host cells, and that 2) the attachment process seems to involve a specific recognition and binding interaction between the envelope glycoprotein and host cell surface receptors, one can envision various approaches to dissecting the details of this envelope-receptor interaction. One approach, of course, is to saturate the virus envelope and/or the host cell receptor with mutations to disrupt the binding interaction and thus determine which regions are important. As an alternative approach, it seems logical to seek to alter retroviral tissue-specificity by introducing modifications into the *env* sequence.

At present, retrovirus vectors are packaged in the appropriate envelope protein by transfecting the virus into a packaging cell line. As mentioned above, two classes of packaging cell lines are available: one class consists of ecotropic packaging cell lines (such as Ψ 2), which contain the *gag*, *pol*, and *env* genes of the wild type MoMLV, but lack the packaging signal (*psi*) sequence. Viruses thus packaged are ecotropic, that is, they will only infect

cells from mice and rats. The second class is composed of amphotropic packaging cell lines (e.g., PA12, PA317, ΨAM), which were constructed similarly but derived from amphotropic viruses. Retroviruses packaged by these amphotropic cell lines will infect most mammalian cells.

Although viral vectors packaged in this way show species-tropism, they can potentially invade all the tissues of that species, like wild-type retroviruses. Hence, to deliver genes to a specific target tissue, that tissue must be infected *in vitro*. Therefore in this project I have attempted to target retrovirus to specific tissues or cell types by making packaging cell lines that produce genetically altered viral envelope proteins.

Tissue tropism of retroviral attachment and entry is determined by specific interactions between the viral envelope glycoproteins and host cell surface receptors. Thus, modification of the envelope glycoprotein should result in alteration of the tissue-specificity of retroviral infection. In particular, introduction of a sequence encoding a particular ligand into an appropriate region of the *env* gene should produce a mutant glycoprotein, which can target the virion to cells bearing the specific receptor for that ligand (Fig. 1-3).

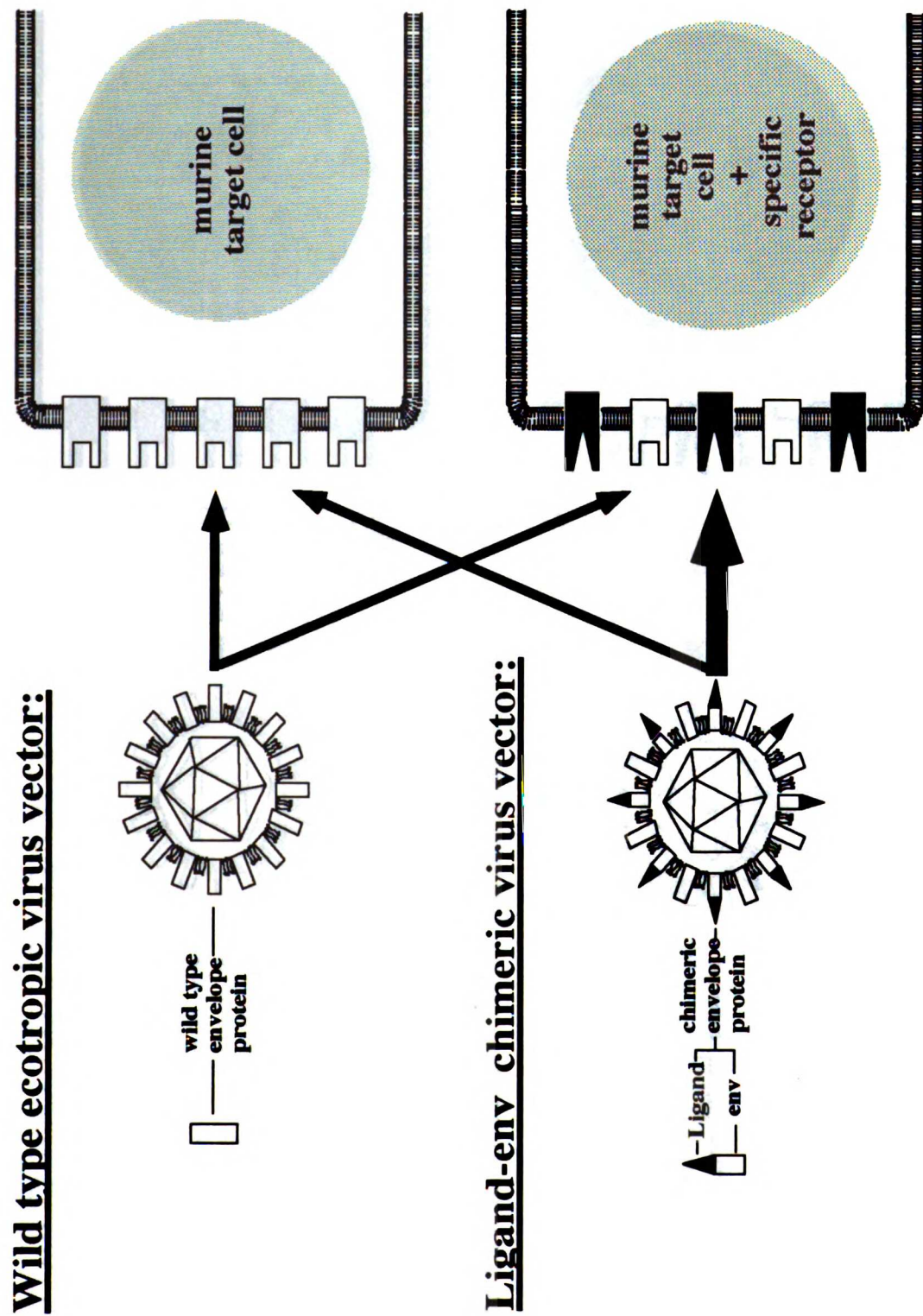
However, manipulation of retroviral envelope genes has historically been a risky endeavor. Most mutations introduced into the envelope gene result in non-expression of the gene and/or loss of viability of the virus [1, 19, 31]. Thus, I designed two main types of envelope construct. In the first type, I directly inserted a small sequence encoding part of a peptide hormone, atrial natriuretic factor (ANF), into various locations in the *env* gene. In the second type of construct, I made larger deletions of the gene and replaced these stretches with the entire coding sequence for another peptide hormone, erythropoietin (EPO).

Figure 1-3

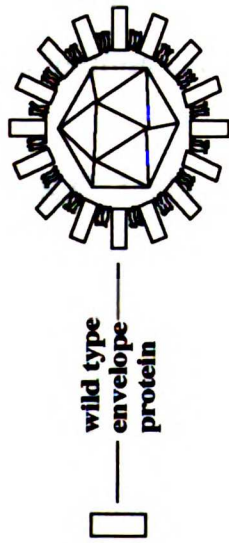
Strategies for tissue-specific targeting via ligand-receptor interaction.

Panel A. Strategy for enhancing tissue-specificity by ligand-receptor interaction. If the targeting ligand is placed on an ecotropic virus, this will enhance the affinity of that virus for infection of murine cells with the specific receptor.

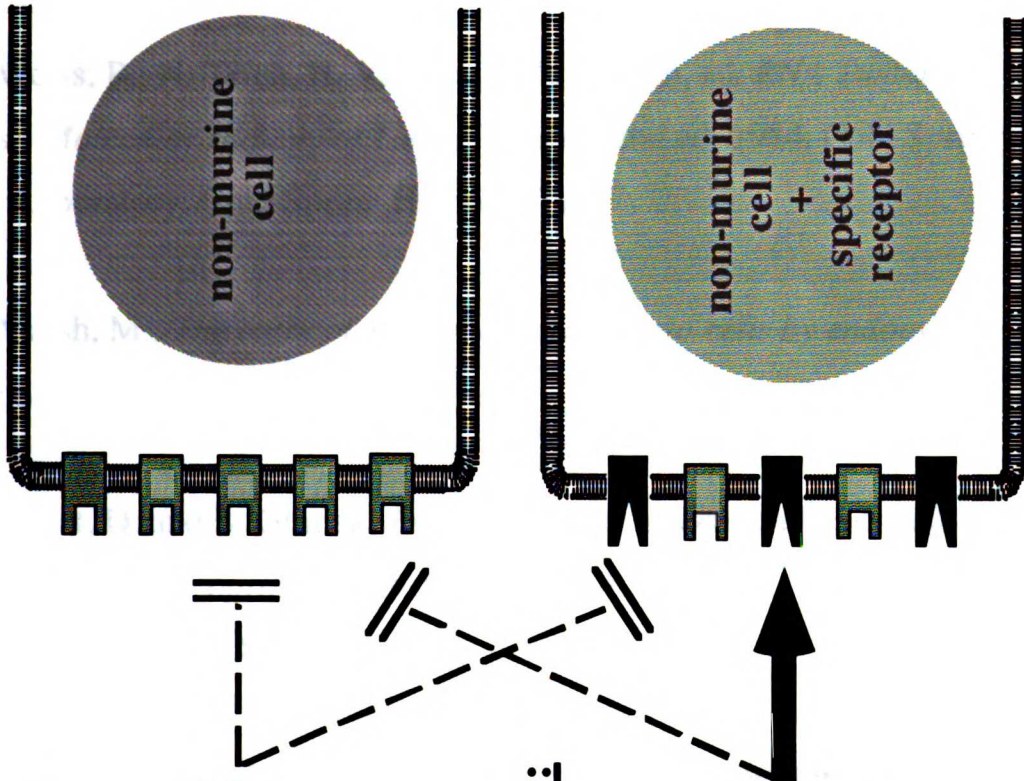
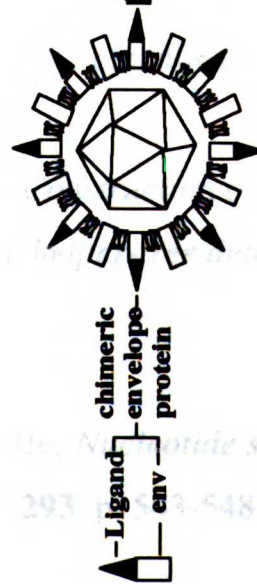
Panel B. Strategy for cross-species, tissue-specific infection via ligand-receptor interaction. Ecotropic virus does not normally infect non-murine cells. The targeting ligand may, however, now enable the virus to specifically infect only those non-murine cells expressing the specific receptor for that ligand.



Wild type ecotropic virus vector:



Ligand-env chimeric virus vector:



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CHAPTER 2.

Some recombinant envelope constructs containing ANF as the targeting ligand can be expressed in packaging cells, but the ANF epitope does not appear to be recognized.

Introduction

The outer subunit of the Moloney murine leukemia virus (MoMLV) glycoprotein gp70, mediates adsorption of the retrovirus to the host cell by binding to specific cell surface proteins which act as viral receptors. Therefore, attempts to alter the host range of the virus by changing its binding specificity would logically focus on modification of the gp70 molecule. However, it has been shown previously that expression levels of the retroviral envelope appear to be highly dependent on its structural integrity, and mutagenesis of the envelope gene often results in lack of expression. This may be a consequence of low transcription from envelope constructs, low stability and short half-life of the altered envelope mRNA, or rapid degradation of the translated protein product. Thus it was important to first construct a MoMLV *env* gene expression vector which would express at high levels in packaging cells. Three different classes of expression vector were tested. The first expression vector consisted of the ecotropic MoMLV *env* gene simply linked to a strong heterologous promoter, in this case the Rous sarcoma virus (RSV) promoter. The *env* gene carried its own polyadenylation signals, but no other processing signals, such as splice donor or acceptor sequences, were included. The second class of *env* expression vector again used a heterologous promoter, the mouse mammary tumor virus (MMTV) promoter, and in addition, contained splice and polyadenylation signals from simian virus 40 (SV40) at the 3' end of the *env* gene. The third class of expression vector consisted of the MoMLV *env* gene left relatively intact, being driven by its own (MLV) promoter and retaining splice donor and acceptor sequences from the MLV genome upstream of the *env* coding sequence, as well as part of the 3' LTR

containing its own polyadenylation signal. Each of these expression vectors was used to construct and test recombinant *env* genes containing a targeting ligand sequence. My first targeting constructs consisted of a small ligand sequence simply inserted into various locations in the *env* gene without any deletions of the envelope sequence itself.

As a targeting ligand, I first used the human peptide hormone atrial natriuretic factor (ANF), also called atrial natriuretic peptide (ANP). ANF is a recently discovered peptide hormone that regulates salt and water balance, and blood pressure [1]. It is derived by cleavage from a precursor protein produced mainly by the cardiac atrium, and is usually released in response to increases in circulating blood volume and arterial blood pressure. Though ANFs of various sizes have been described, the predominant circulating form of the hormone is a 26-amino acid peptide which exhibits potent natriuretic, diuretic, and vasodilatory activities [2]. Disulfide linkage of two cysteine residues in this peptide cause it to form a ring structure [3].

Through pharmacological and biochemical studies, there are now known to exist at least three distinct ANF receptor populations. Two of these receptors are coupled to intracellular cyclic GMP (cGMP) production and they probably mediate many of the biological effects of ANF; these receptors are referred to as the ANF A- and B-receptor, respectively, and both have stringent structural requirements for recognition of the ANF ligand [1, 4]. Conversely, a third type of ANF receptor, referred to as the "ANF C-receptor" is not linked to cGMP production and probably functions in the sequestration and metabolic clearance of circulating ANF [5, 6]. This receptor is capable of binding ring-deleted and shortened linear analogues of ANF containing as few as 9 amino acids with high affinity [5]. The C-receptor has been purified, sequenced, and cloned from aortic smooth

muscle cells [6]. Initially, a sequence encoding the 15 amino acids which lie between the two cysteine residues in the human ANF peptide was inserted into various positions in the *env* gene. This 15 amino acid form of ANF is still capable of binding to the C-receptor [5]. This cysteine-deleted linear sequence was used instead of the full sequence because it was thought that the presence of cysteines in the insert might cause adverse conformational changes in the envelope glycoprotein by interaction with other cysteine residues in the envelope. The recombinant *env* gene containing this ANF sequence was to be transfected into packaging cells, so that the transfected cells would then produce virions encoated by the ANF ligand, and these virions would be tested for enhanced ability to infect cells displaying ANF receptors. In fact, the infectivity of such virions could be tested on cells from non-murine species, as long as those cells possessed ANF receptors.

ANF was chosen as an initial targeting ligand for the following reasons: (1) its relatively small size made it easy to engineer into the envelope glycoprotein, (2) it was hoped that it was not so large as to cause drastic conformational changes in the envelope glycoprotein which might preclude normal assembly or function, yet not so small as to be buried in the host protein without exerting any discernable effect, and (3) because the C-receptor has non-stringent requirements for recognition of linear fragments of the ANF ligand and clears them from the circulation, a cysteine-deleted linear sequence could be used as an insert, thus avoiding possible conformationally adverse interactions with other cysteine residues in the envelope, and this linear ANF insert would still be recognized by the C-receptor, resulting in receptor-mediated endocytosis of the viral particle into the target cell.

Two packaging cell lines were used for transfection experiments. Initially, envANF constructs were transfected into Ψ 2 cells, which contain *gag*, *pol*, and the ecotropic wild type *env* [7]. Subsequently, another cell line, GP101, was also used in some experiments. GP101 is a packaging cell line which contains *gag* and *pol* genes, but does not contain the *env* gene [8]. Since no endogenous envelope proteins are produced by GP101 cells, if the recombinant envANF proteins were successfully expressed in these cells and assembled correctly into virion particles, the resultant virions would be encoated completely by ANF ligand-containing envelope. Obviously this would be the most desirable result. However, the possibility that such virions might not be produced also had to be taken into consideration, for the following reasons: mutagenesis of the *env* gene with the ANF insert would undoubtedly cause some conformational changes to occur in the tertiary structure of the envelope glycoprotein; as mentioned above, hopefully these changes would be relatively minor and non-disruptive, but there still remained the possibility that even minor structural changes might be magnified when similarly altered glycoproteins attempted to assemble, thus inhibiting efficient production of intact virions. It seemed at least equally likely that viable virions would form, if there were instead a mixture of normal and recombinant envelope proteins, which would hopefully dilute out the conformational changes wrought by the insertional mutations, thus producing a chimeric virion. This was the rationale for using Ψ 2, an established packaging cell line which already has an endogenous wild type (ecotropic) *env* sequence, for my initial transfection experiments. Furthermore, replication-defective vectors normally produced by the packaging cell line Ψ 2 have an ecotropic host specificity, and do not infect

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cells from other mammalian species. Thus the presence of wild type envelope proteins in the chimeric virion (or of any purely wild type viruses which might also be produced by Ψ 2 cells transfected with the envANF constructs) should not cause binding to occur between the viruses and non-murine, ANF receptor-positive cells which would be used in infectivity assays; that is, any binding which does occur to such cells should solely be due to the presence of the ANF insert in the recombinant envelope proteins.

Materials and Methods

Construction of recombinant envelope expression plasmids

pRSVenvANF constructs:

The first construct consisted of a sequence encoding the 15 amino acids that lie between the two cysteine residues in the human ANF peptide into a unique BamH1 site in the central portion of the wild type ecotropic MoMLV *env* gene, in an expression vector driven by the Rous sarcoma virus (RSV) promoter.

The plasmid pZAP, which contains the entire MoMLV proviral genome (total length: 8332 nucleotides), was digested with restriction enzymes in order to release the *env* gene sequence (nucleotide positions 5777-7774). The 3' end was cut first with the enzyme Afl II and filled in with Klenow enzyme to produce a blunt end, after which the 5' end was cut with Xba I. The enzymes Xba I and Afl II cut at positions 5766 and 7851 respectively, and so a fragment of approximately 2.1 kb which contains the entire *env* gene and the polyA signal in the 3' LTR was released. This

fragment was isolated from an agarose gel using DEAE membrane paper, eluted, and ligated between the Xba I and Sma I sites in the polylinker of the cloning vector pUC12, with the Klenowed Afl II end being compatible with the Sma I site, also a blunt end. The gene was driven by an RSV promoter sequence (a Nde I - Hind III fragment cleaved from the plasmid pRSVCAT) which was cloned into the Nde I and Hind III sites just upstream of the *env* gene insert (Fig 2-1). Transformed *E. coli* subclones with plasmids containing the proper inserts were identified by restriction digest of plasmid mini-preps purified by the alkaline lysis method, and plasmids were purified from large scale cultures of the positive subclones by cesium chloride gradient centrifugation.

A unique Bam H1 recognition site exists in the gp70 envelope glycoprotein coding sequence at position 6537, just upstream of the region encoding the proline-rich helical domain. As the literature at the time suggested that this domain of the envelope might be involved in binding to its host receptor, this site was first chosen for insertion of the ANF ligand (Fig 2-2). The pRSV*env* plasmid was therefore cut at this position and phosphatased. In the meantime, oligonucleotides encoding the 15 amino acid sequence situated between the two cysteine residues in the ANF peptide, as well as the complementary minus strand sequence, were synthesized. These oligonucleotides were designed with flanking sequences which, when the plus and minus strand oligonucleotides are annealed together, created staggered ends which were cohesive and compatible with the Bam H1 restriction site (Fig. 2-3). This oligonucleotide duplex with the corresponding cohesive ends was kinased and ligated into the pRSV*env* plasmid. *E. coli* transformants were first screened by restriction digest for the presence of the insert. Though the oligonucleotide sequence had been

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Figure 2-1 Cloning of first MoMLV envelope vector
pRSVenv. See text for details.

The plasmid pZAP contains the complete genome of Moloney murine leukemia virus (MoMLV). The envelope (*env*) gene was isolated from this plasmid and cloned into the vector pUC, to create the vector pUCenv. The Rous sarcoma virus (RSV) long terminal repeat, which contains a promoter/enhancer, was then isolated from pRSVCAT and cloned into pUCenv upstream of the *env* gene to drive transcription.

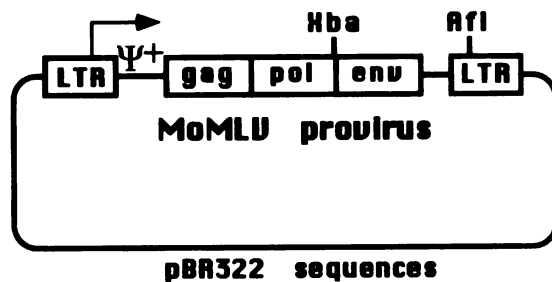
LTR: long terminal repeat.

Ψ^+ : *psi* packaging signal.

polyA: *env* gene polyadenylation signal

CAT: chloramphenicol acetyltransferase gene.

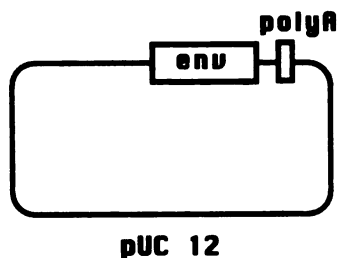
pZAP



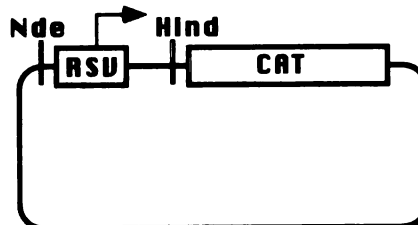
- Afl II digest
- Klenow
- Hba I digest
- isolate 2.1 kb env fragment

-clone into Hba I/Sma I sites in pUC 12 polylinker

pUCenv



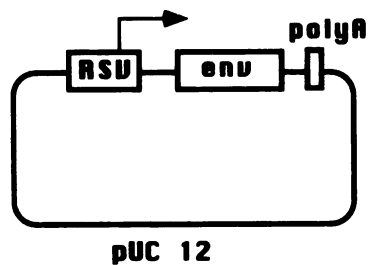
pRSUCAT



- Nde I & Hind III digest
- isolate 600 bp RSU promoter

-clone into Nde I/Hind III sites in pUC 12 polylinker

pRSUenv

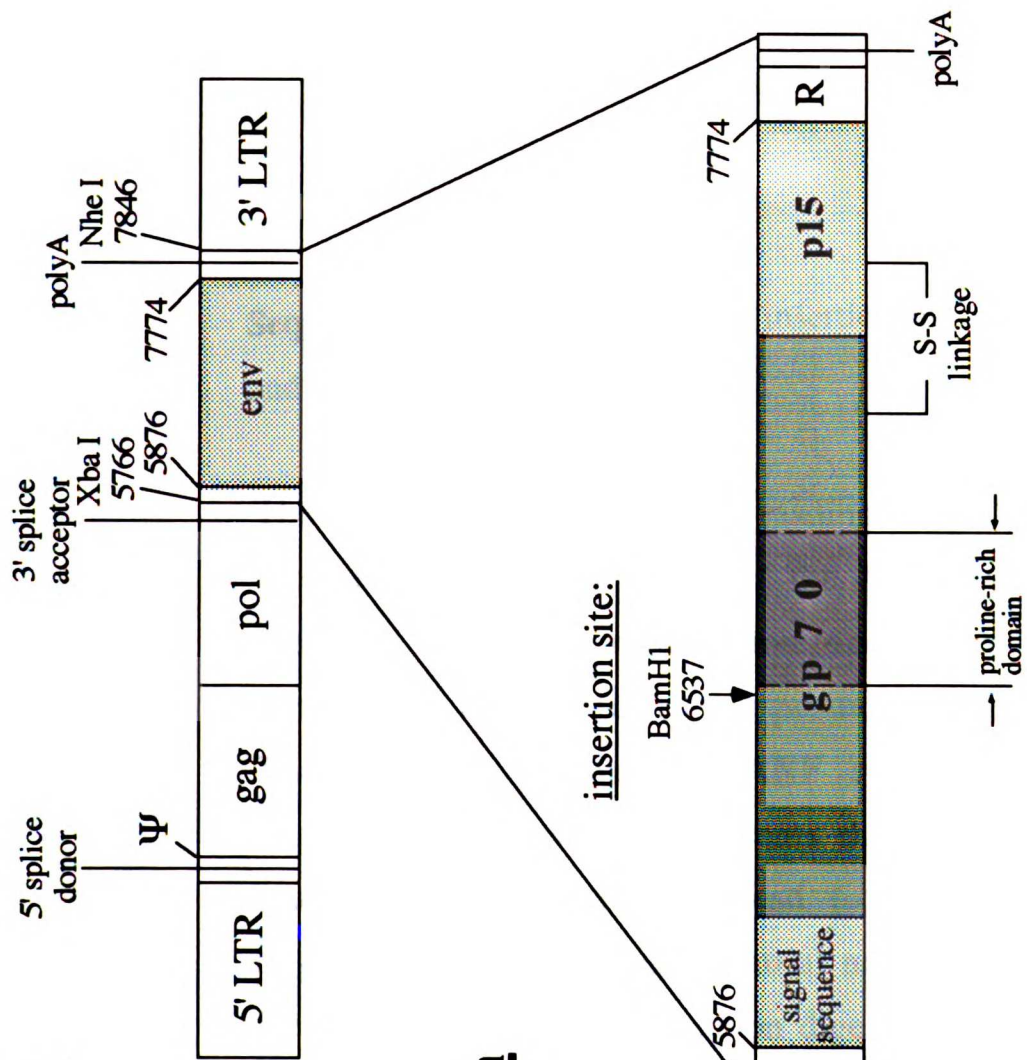


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Figure 2-2 **Location of BamH1 insertion site for ANF ligand.**

The BamH1 site used for insertion of the atrial natriuretic factor (ANF) ligand sequence is shown in relation to the proline-rich hypervariable domain of the Moloney murine leukemia virus (MoMLV) envelope outer glycoprotein (gp70). The BamH1 site is located just upstream of the 5' end of the proline-rich domain, which extends from position 6559 to 6720 of the envelope (*env*) gene.

MoMLV genome:



RSVenvANF-Bam construct:

Figure 2-3 **Sequences of pRSVenvANF-Bam insertion site and ANF ligand insert.**

The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus envelope gene surrounding the BamH1 site at position 6537. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (–) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the BamH1 site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

RSV: Rous sarcoma virus promoter/enhancer.

env: MoMLV envelope gene.

polyA: polyadenylation signal.

pRSUenv:



env reading frame:

BamH1 site

5'... CCA GGG CTT ACA TTT G **G G G A T C** C G A C T C A G A T A C ...3'
 3'... GGT CCC GAA TGT AAA C **C C C T A G** G C T G A G T C T A T G ...5'

Pro Gly Leu Thr Phe Gly Ile Arg Leu Arg Tyr

5'... G A T C T T C G G G G G C A G G A T G G A C A G G A T T G G A G C C C C A G A G C G G A C T G G G - - - ...3'
 3'... - - - A A G C C C C C G T C T T A C C T G C C T A A C C T C G G G T C C G C C T G A C C C C T A G ...5'

Ile Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Ile

ANF sequence oligonucleotide insert

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designed so that the insert would still maintain the proper *env* reading frame, this was checked by dideoxynucleotide sequencing of the region around the insert site in the positive subclones. Constructs which contained the ANF sequence in frame, in the correct orientation, were designated pRSVenvANF-Bam, and large scale plasmid preparations purified by cesium gradient centrifugation were used for transfection experiments.

pMTVenvANF constructs:

The pMAM vector contains multiple cloning sites within a polylinker, which is flanked by the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) at the 5' end, and simian virus 40 (SV40) splice and polyadenylation signals at the 3' end (Fig. 2-4). The MMTV-LTR, which is known to express at high levels in mouse fibroblasts, additionally contains multiple glucocorticoid response elements (GREs), which makes it possible to greatly increase levels of transcription by the addition of dexamethasone [9, 10]. The pMAM vector also contains the *E. coli gpt* gene, which encodes a purine salvage pathway enzyme, xanthine-guanine phosphoribosyl transferase (XGPRT; EC 2. 4. 2. 22). This vector therefore makes it possible to isolate stable transformants by selection with medium containing mycophenolic acid, aminopterin, xanthine and hypoxanthine [10, 11].

Plasmids pRSVenv and pRSVenvANF-Bam were therefore digested with restriction enzymes in order to release their respective *env* gene sequences. The enzymes Xba I and Nhe I cut at positions 5766 and 7846 respectively, and so a fragment of approximately 2.1 kb which contains the entire wild type *env* gene (nucleotide positions 5777-7774) was released in the case of pRSVenv, and the entire *env* gene with the 48-bp ANF sequence already inserted into the unique BamHI site at position 6537 was released in

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the case of pRSVenvANF-Bam. These fragments were isolated from an agarose gel using DEAE membrane paper, eluted, and ligated into the Nhe I site in the polylinker of the eukaryotic expression vector pMAM, taking advantage of the fact that the enzymes Xba I and Nhe I produce compatible cohesive ends (Fig. 2-4). After screening *E. coli* transformants by restriction digest of plasmid mini-preps, positive subclones with constructs (which will be referred to as pMTVenv and pMTVenvANF) containing the inserts in the proper orientation were identified, and cesium gradient-purified large scale preps were used for transfection experiments.

pMLVenvANF constructs:

Plasmids containing Moloney murine leukemia virus (MoMLV) genes [8], were generously provided by Drs. A. Bank and D. Markowitz (Columbia University).

The plasmid p3PO contains an ecotropic MoMLV provirus, along with some flanking rat genomic sequences, cloned into the Hind III and EcoRI sites of pBR322. The provirus contains a 134 base pair deletion of the *psi* packaging signal, from Bal I at position 212 to Xma III at position 346 of the retroviral genome [12], which was replaced by another EcoRI restriction site.

The plasmid pMLVenv was derived from p3PO by Bgl II and Nhe I digestion to release the entire wild type ecotropic MoMLV *env* gene (from position 5407 to position 7846 of the retroviral genome). This fragment includes sequences at the 3' end of the *pol* gene, between positions 5440 and 5501, which contain four heptanucleotide stretches which can serve as lariat structures and three AG dinucleotides that can serve as splice acceptor sites [13] for the *env* message, including the dominant splice acceptor site at

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Figure 2-4 **Cloning of second MoMLV envelope vector pMTVenvANF. See text for details. The construction of the pRSVenv and pRSVenvANF vectors, from which pMTVenv and pMTVenvANF, respectively, are derived, are also detailed in the text. (See also Fig. 2-1 and 2-3.)**

**RSV: Rous sarcoma virus promoter/enhancer
sequence (long terminal repeat).**

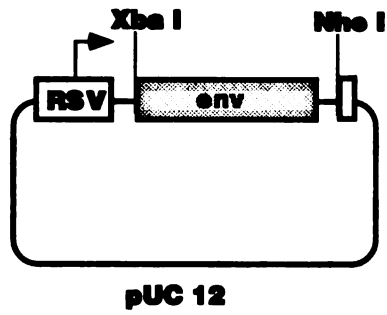
env: Moloney murine leukemia virus *env* gene.

ANF: atrial natriuretic factor sequence.

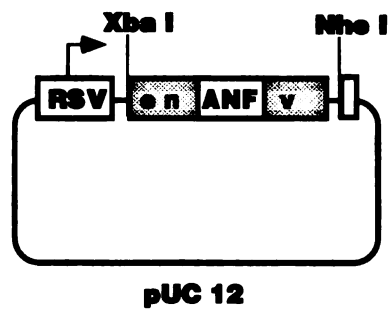
**MMTV: mouse mammary tumor virus
promoter/enhancer (long terminal repeat).**

**SV40: splice and polyadenylation signals from
simian virus 40.**

pRSVenv1



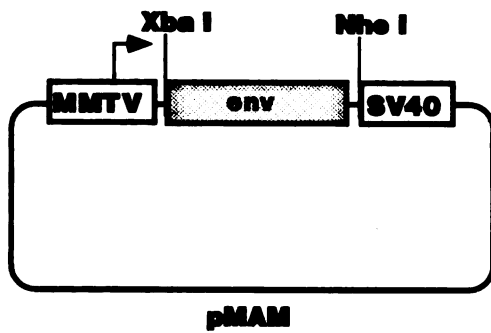
pRSVenvANF-Bam1



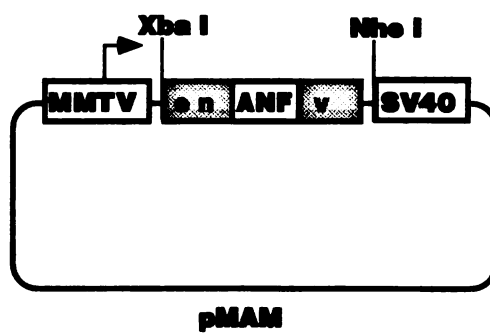
-Xba I and Nhe I digest
-isolate 2.1 kb env fragment

-clone into Nhe I site in pMAM polylinker:

pMTVenv1



pMTVenvANF-Bam1



position 5490, as determined by sequencing of *env* message cDNAs [13, 14]. The overhangs on this 2.4 kb *Bal* I–*Nhe* I *env* gene fragment were filled in with Klenow enzyme and dNTPs, and *Eco*RI linkers were ligated to both ends. This was then cloned into a 6.2 kb vector derived from *Eco*RI digestion of p3PO. This vector consists of pBR322 with the 5'LTR of MoMLV and the 5' splice donor sequences for the *env* message, located between position 204 and 210, just upstream of the *Eco*RI site replacing the deleted *psi* packaging signal. The correctly ligated product was identified by restriction enzyme digest of transformed *E. coli* strain HB101 subclones, and designated pMLVenv (Fig. 2-5). This plasmid thus consists of the wild type ecotropic *env* gene whose transcription is driven by the 5'-MoMLV-LTR and which retains the 5' and 3' splicing signals for the *env* mRNA; the intervening *gag* and *pol* genes, which are normally spliced out of the *env* message, were deleted, leaving only a small sequence in between the splicing signals remaining to be spliced.

Recombinant envelope genes containing the ANF ligand sequence were then constructed using pMLVenv, according to the same strategy described above for pRSVenvANF-Bam. That is, 48-mer oligonucleotides encoding the (+) and (–) strands of the 15 amino acid ANF ligand sequence with compatible *Bam*H1 ends were annealed and ligated in frame into the unique *Bam*H1 site at position 6537 (Fig. 2-6). Mini prep DNAs from *E. coli* HB101 transformants were screened for presence of the insert and correct orientation by restriction digest and positive subclones were confirmed by dideoxynucleotide sequencing. This construct was designated pMLVenvANF-Bam. In addition, four other constructs were similarly designed, with ANF inserts at positions 5923, 6657, 6728, and 7054 of the *env* gene, corresponding to sites for the restriction enzymes *Bst*EII, *Dra* III,

Acc I, and Nsi I, respectively (Fig. 2-7). In order to eliminate additional sites for these enzymes which were found to exist within the pBR322 vector, the plasmid pMLVenv was truncated by digestion with HindIII and Nde I and removal of the intervening vector sequences, after which the overhangs were filled in using Klenow enzyme and the blunt ends re-ligated, thus yielding the plasmid pMLVenv2 (Fig. 2-8). This not only eliminates unwanted restriction sites, but also results in the deletion of the tetracycline resistance gene as well as the "poison" sequences which act as a repressor of replication. Plasmid pMLVenv2 was digested to completion with BstEII, PflMI (compatible with the Dra III site at position 6657), or Nsi I, or was partially digested with AccI and the linearized band isolated by agarose gel electrophoresis. ANF oligonucleotide duplexes designed for each insert site were ligated to the cut vectors, and positive subclones identified and confirmed as above. The resultant constructs were designated pMLVenvANF-Bst (Fig. 2-9), pMLVenvANF-Dra (Fig. 2-10), pMLVenvANF-Acc (Fig. 2-11), and pMLVenvANF-Nsi (Fig. 2-12), respectively. The ANF insert in pMLVenvANF-Bst is at the 5' end of the *env* gene, just downstream of the signal peptide cleavage site. As mentioned above, pMLVenvANF-Bam contains the ANF insert just upstream of the proline-rich variable domain in the central portion of the *env* gene, while pMLVenvANF-Dra and pMLVenvANF-Acc contain the ANF insert within and just downstream of the proline-rich domain, respectively (Fig. 2-7).

Figure 2-5.

Cloning of the third MoMLV envelope vector pMLVenv. The plasmid p3PO contains a Moloney murine leukemia virus genome lacking the psi packaging signal (Ψ^-). The viral structural genes were first entirely deleted from this vector, and a fragment containing the envelope (*env*) gene subsequently re-introduced, thus resulting in deletion of *gag* and *pol* genes. The splice donor sequence located just downstream of the 5' long terminal repeat (LTR) and the splice acceptor sequences located just upstream of the *env* gene are retained, however, as well as the polyadenylation signal (polyA) in the 3' LTR. See text for details.

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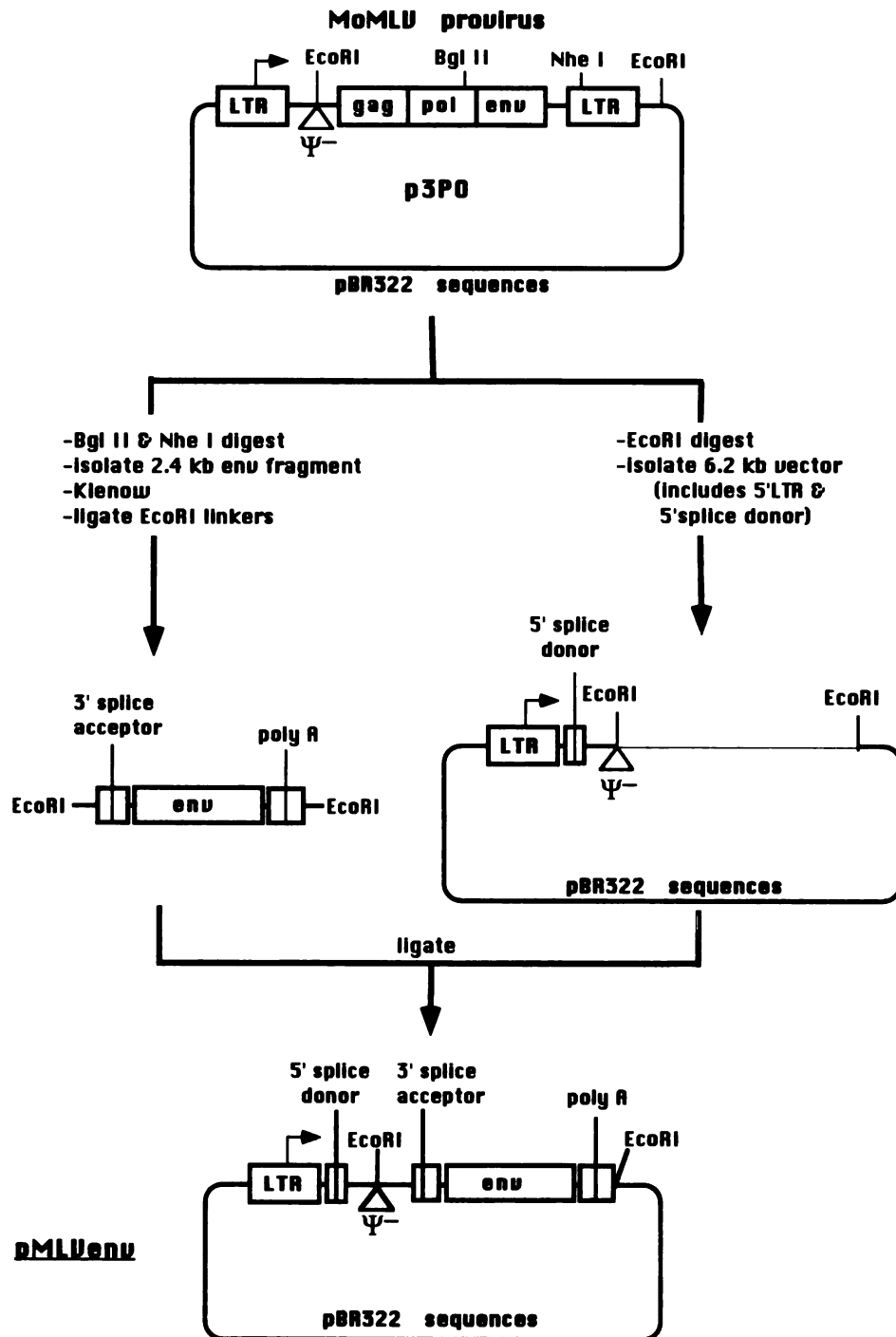


Figure 2-6

Sequences of pMLVenvANF-Bam insertion site and ANF ligand insert. In the schematic diagram of pMLVenv, the BamH1 site is shown in relation to the proline-rich domain and the other insert sites used. The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus (MoMLV) envelope gene surrounding the BamH1 site at position 6537. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (–) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the BamH1 site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

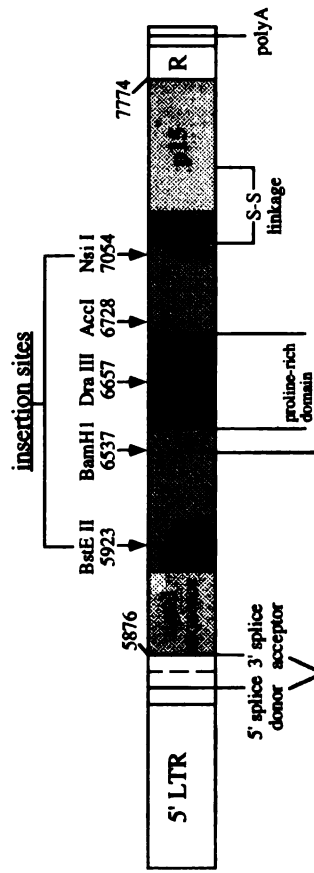
LTR: long terminal repeat.

gp70: MoMLV envelope gene glycoprotein gp70.

p15: MoMLV envelope gene subunit p15.

polyA: polyadenylation signal.

pMLUenv:



env reading frame:

BamH1 site

5'... CCA GGG CTT ACA TTT GGG ATC GGA CTC AGA TAC ...3'
 3'... GGT CCC GAA TGT AAA CCG TAG CTT GAG TCT ATG ...5'

Pro Gly Leu Thr Phe Gly Ile Arg Leu Arg Tyr

5'... G ATC TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GG - - - ...3'
 3'... - - - AAG CCC CCG TCT TAC CTG TCC TAA CCT CGG GTC TCG CCT GAC CCC TAG ...5'

Ile Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Ile

ANF sequence oligonucleotide insert

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Figure 2-7. **Location of ANF ligand insertion sites in pMLVenv vector.** The restriction sites used for insertion of the atrial natriuretic factor (ANF) ligand sequence are shown in relation to the proline-rich hypervariable domain of the Moloney murine leukemia virus (MoMLV) envelope outer glycoprotein (gp70), which extends from position 6559 to 6720 of the envelope (*env*) gene. The BamH1 site is located just upstream, the Dra III site is located within, and the Acc I site is located just downstream of the proline-rich domain. The BstE II site is located at the amino-terminal end of the gene, just downstream of the signal sequence. The Nsi I site is located at the carboxy-terminus of the gene, just upstream of the p15 subunit.

MoMLV genome:

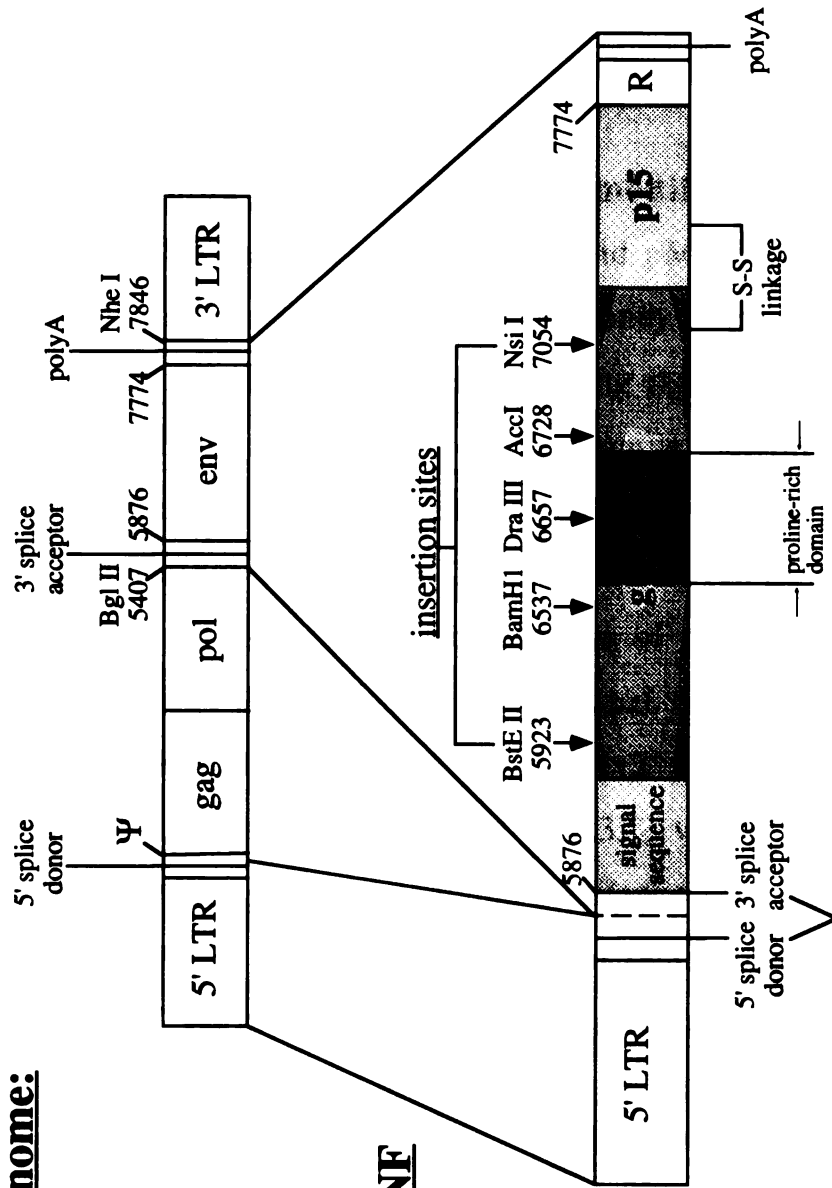
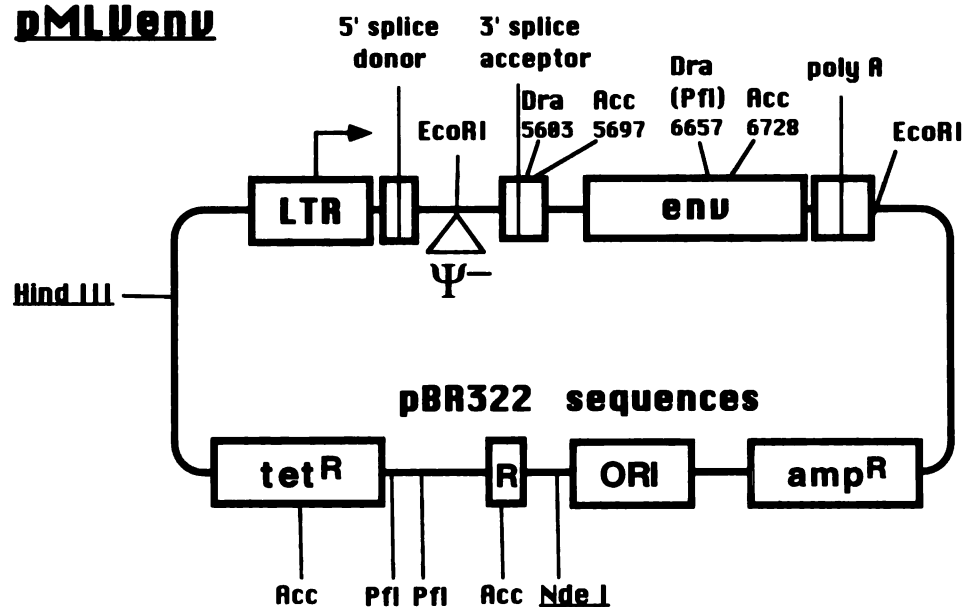


Figure 2-8. **Cloning of pMLVenv2, a modification of the pMLVenv vector.** The plasmid pMLVenv contains the envelope (*env*) gene of Moloney murine leukemia virus, and lacks the psi packaging signal (Ψ^-) as well as the *gag* and *pol* genes, but retains the splice donor sequence located just downstream of the 5' long terminal repeat (LTR) and the splice acceptor sequences located just upstream of the *env* gene, as well as the polyadenylation signal (polyA) in the 3' LTR. The plasmid pMLVenv2 is derived from pMLVenv by deletion of pBR322 vector sequences containing the tetracycline resistance gene (tet^R) and repressor of replication (R), but retains the origin of replication (ORI) and ampicillin resistance gene (amp^R). See text for details.

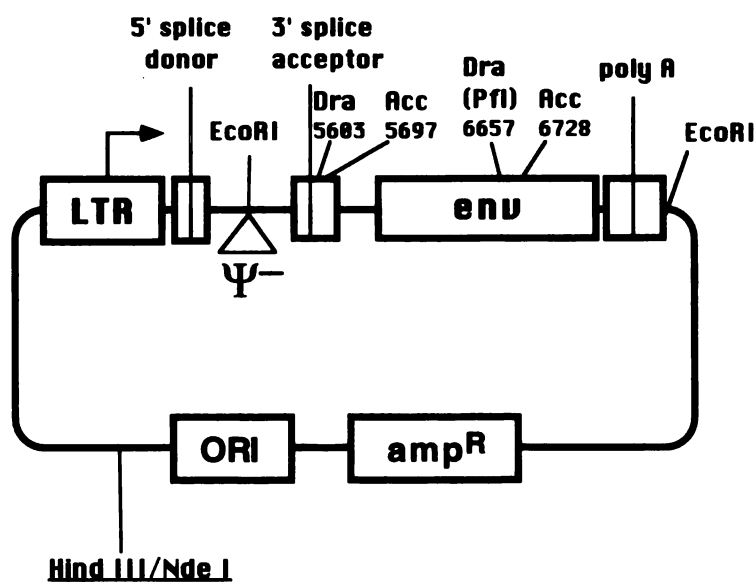
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pMLUenv



-Hind III & Nde I digest
-isolate 6.4 kb env fragment
-Klenow
-re-ligate

pMLUenv2



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Figure 2-9. **Sequences of pMLVenvANF-Bst insertion site and ANF ligand insert.** In the schematic diagram of pMLVenv, the BstE II site is shown in relation to the proline-rich domain and the other insert sites used. The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus (MoMLV) envelope gene surrounding the BstE II site at position 5923. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (-) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the BstE II site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

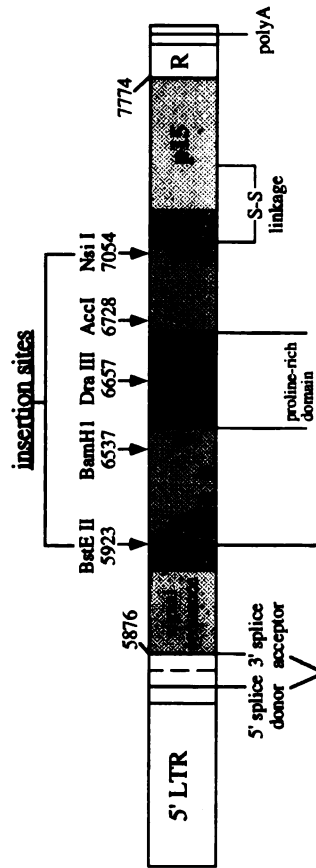
LTR: long terminal repeat.

gp70: MoMLV envelope gene glycoprotein gp70.

p15: MoMLV envelope gene subunit p15.

polyA: polyadenylation signal.

pMLUenv:



env reading frame:

BstE II site

5'... ACCTGG GA **ATG** ACC **AA**T GGA GAT ...3'
 3'... TGG ACC **CTC** **TAT** TGA **TTA** CCT CTA ...5'

Thr Trp Glu Val Thr Asn Gly Asp

5'... GTA ACT TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GGG ... 3'
 3'... - - - - A AAG CCC CCG TCT TAC CTG TCC TAA CCT CGG GTC TCG CCT GAC CCC CAT TG ...5'

Val Thr Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly (Val) (Thr)

ANF sequence oligonucleotide Insert

Figure 2-10. **Sequences of pMLVenvANF-Dra insertion site and ANF ligand insert.** In the schematic diagram of pMLVenv, the Dra III site is shown in relation to the proline-rich domain and the other insert sites used. The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus (MoMLV) envelope gene surrounding the Dra III site at position 6657. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (-) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the Dra III site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

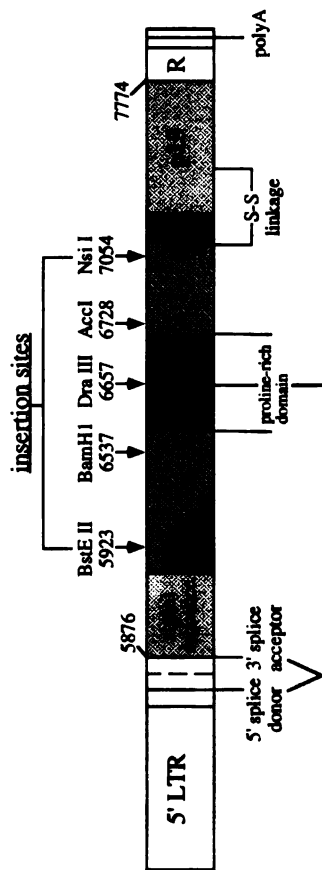
LTR: long terminal repeat.

gp70: MoMLV envelope gene glycoprotein gp70.

p15: MoMLV envelope gene subunit p15.

polyA: polyadenylation signal.

pMLUenv:



env reading frame:

p11M 1 site
(Dra III site)



5'... - - - - GTTTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC CCC A - - 3'
3'... - GG TCAAAG CCC CCG TCT TAC CTG TCC TAA CCT CGG GTC TCG CCT GAC CCG G - - 5'
(Pro) Ser Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Pro (Ser)

ANF sequence oligonucleotide insert

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Figure 2-11. Sequences of pMLVenvANF-Acc insertion site and ANF ligand insert. In the schematic diagram of pMLVenv, the Acc I site is shown in relation to the proline-rich domain and the other insert sites used. The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus (MoMLV) envelope gene surrounding the Acc I site at position 6728. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (-) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the Acc I site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

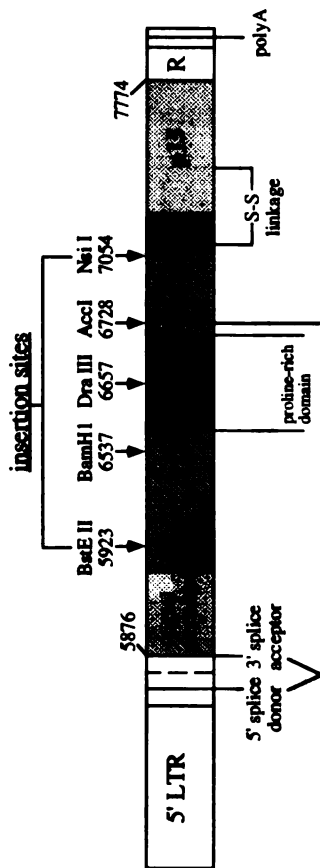
LTR: long terminal repeat.

gp70: MoMLV envelope gene glycoprotein gp70.

p15: MoMLV envelope gene subunit p15.

polyA: polyadenylation signal.

pMLUenv:



env reading frame:

Acc I site

5'... CTA AAC TTA GGA GCC TAC ...3'
 3'... GAT TTG AAT CCT CGG ATG ...5'

Leu Asn Leu Val Asp Gly Ala Tyr

5'... -- A GAT TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC GT- ---...3'
 3'... -- -TA AAG CCC CCG TCT TAC CTG TCC TAA CCT CGG GTC TCG CCT GAC CCG CAT C- -...5'
 (Val) Asp Phe Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly (Val) (Asp)

ANF sequence oligonucleotide insert

Figure 2-12. Sequences of pMLVenvANF-Nsi insertion site and ANF ligand insert. In the schematic diagram of pMLVenv, the Nsi I site is shown in relation to the proline-rich domain and the other insert sites used. The sequence shown as "env reading frame" represents the codon usage of the Moloney murine leukemia virus (MoMLV) envelope gene surrounding the Nsi I site at position 7054. The sequence shown as "ANF sequence oligonucleotide insert" represents the codon usage of the annealed (+) and (–) strand oligonucleotides encoding the atrial natriuretic factor (ANF) ligand. Restriction digest of the Nsi I site and insertion of the annealed ANF oligonucleotides thus maintains the reading frame of the envelope gene.

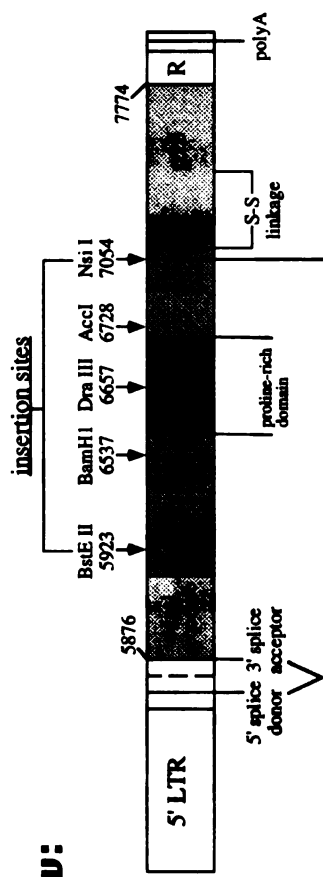
LTR: long terminal repeat.

gp70: MoMLV envelope gene glycoprotein gp70.

p15: MoMLV envelope gene subunit p15.

polyA: polyadenylation signal.

pMLUenv:



env reading frame:

Nsi I site

5'... CTT ACT CC TCC ACC ACC...3'
 3'... GAA TGA GGG ACC TGG TGG...5'

Leu Thr Pro Cys Ile Ser Thr Thr

5'... -TC TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC TGC A ---...3'
 3'... ACG TAG AAG CCC CCG TCT TAC CTG TCC TAA CCT CGG GTC TCG CCT GAC CCG ---...5'
 (Cys) (Ile) Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys (Ile)

ANF sequence oligonucleotide insert

pN2

The plasmid pN2 encodes a replication-defective retroviral vector which contains the *psi* packaging signal sequence and the neomycin resistance (*neo*^R) gene flanked by MoMLV long terminal repeat (LTR) sequences [15]. This plasmid was grown in *E. coli* strain TG1, purified by cesium chloride gradient ultracentrifugation, and used for co-transfection into the packaging cells along with the different recombinant envelope constructs.

pSV-ANPCR

A pGEM (Promega) -based plasmid containing cDNA encoding the ANF C-receptor was generously provided by Drs. D. Gardner and M. LaPointe (University of California San Francisco). As pGEM is a prokaryotic expression vector, the cDNA had to be re-cloned in order to express in eukaryotic cells. The plasmid was therefore digested with Xba I and Pvu II to release the 2.2 kb ANF receptor insert. This fragment was isolated by agarose gel electrophoresis and transferred to DEAE membrane paper, eluted, and purified by ethanol precipitation. Pvu II creates a blunt end, and this enabled me to ligate the fragment into the Xba I and Sma I sites in the polylinker of the mammalian expression vector pSVL (Clontech), as Sma I also creates a blunt end. Genes inserted into the multiple cloning site of pSVL are transcribed by the SV40 late promoter, and the vector also contains the SV40 VP1 intron and late polyadenylation signals for processing of the transcripts. Positive *E. coli* transformants were identified by restriction enzyme digest of mini-prep DNA, and large scale cultures were purified by cesium chloride gradient, as above.

pFR400

The plasmid pFR400 contains the methotrexate-resistant dihydrofolate reductase (dhfr) gene [16]. Dihydrofolate reductase (EC 1.5.1.3; tetrahydrofolate dehydrogenase) is an important enzyme which catalyzes the NADPH-dependent reduction of folate to produce N⁵, N¹⁰-methylene tetrahydrofolate, which serves as an essential cofactor in the synthesis of deoxythymidine monophosphate. Methotrexate (MTX), a folate analogue, normally binds dihydrofolate reductase and inactivates it, disrupting the *de novo* pathway of pyrimidine nucleotide synthesis, ultimately resulting in death of the cell. The dhfr gene contained in pFR400, however, was isolated from a MTX-resistant strain of murine 3T6-R400 cells, and has been shown to contain a point mutation at the binding site for methotrexate which greatly decreases the affinity of the enzyme for the toxic substrate, and hence can be used for positive selection [16]. An additional advantage of using dhfr as a selectable marker is the fact that it can under some circumstances undergo gene amplification with increasing concentrations of methotrexate [17]. The construct is driven by the SV40 early promoter, and contains polyadenylation signals from the hepatitis B virus surface antigen gene [16]. This plasmid was grown and purified as above, and used for co-transfection with pSV-ANPCR to create target cell lines expressing the ANF C receptor.

Packaging cell lines and transfections

The ecotropic packaging cell lines Ψ2 [7] and GP101 [8] were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% newborn calf serum (CS) and 100 µg/ml

penicillin/streptomycin (PS), with the addition of the following reagents at regular intervals to maintain the phenotype of the cell lines, both of which were originally selected for guanine phosphoribosyltransferase (gpt) resistance: hypoxanthine (15 µg/ml), xanthine (250 µg/ml), mycophenolic acid (25 µg/ml), thymidine (10 µg/ml) and aminopterin (2 µg/ml) (Sigma).

All transfections were performed using the calcium phosphate precipitation method [18, 19]. Twenty-four hours after seeding approximately 10^5 cells on 60-mm plates, the cultures were co-transfected with 10-20 µg of recombinant envelope construct and retroviral vector pN2 plasmid DNA, in a 10:1 molar ratio. The following day the medium was changed, and 48 hours after transfection the cells were either lysed for harvesting of cytoplasmic RNA, or trypsinized and re-plated at 1:5 or 1:10 dilution on 100-mm plates in selective medium containing 400 µg/ml of the neomycin analogue G418 (Gibco). Colonies surviving G418 selection were isolated and transferred to 24-well plates and subsequently expanded for extraction of genomic DNA and cytoplasmic RNA. In some experiments, the G418-resistant colonies were trypsinized and pooled for characterization of recombinant *env* gene expression. All stable transfectants were maintained in selective medium except for 24-48 hours prior to harvesting of the supernatant culture medium for use in infection experiments.

DNA and RNA analyses

Genomic DNA from these clones was isolated by lysis of 10^6 cells with 0.65% NP-40/0.01M Tris (pH 7.8)/0.15M NaCl/1.5mM MgCl₂, centrifugation of the lysate at 2000 rpm for 10 min at 4°C to pellet the

nuclei, followed by overnight digestion of the nuclear pellets with 0.5 mg/ml proteinase K, phenol/chloroform extraction and ethanol precipitation.

For Southern blot analysis, approximately 10 µg of DNA per sample was digested with Hinc II, ethanol precipitated, and separated by electrophoresis on a 0.8% agarose gel in 1x TEA buffer. The gel was denatured in 0.4N NaOH/1M NaCl and transferred to Hybond nylon membrane filters (Amersham) in 20x SSC overnight, which were then probed with the same ANF oligonucleotides as those used for construction of the RSVenvANF-Bam insert. The ANF oligonucleotide probe was end-labelled with γ -³²P-ATP and T4 polynucleotide kinase and fractionated through a Sephadex G25 column. After pre-hybridization of the filters, 2 x 10⁶ cpm/ml of the purified probe was hybridized in 5x SSC/20 mM NaHPO₄ (pH 7.2)/7% SDS/10% dextran sulfate/10x Denhardt's solution with 100 µg/ml of salmon sperm DNA at 55°C overnight. The filters were also probed with a 1.3 kb *env* gene fragment which was isolated after digestion of pZAP with Hpa I and nick translated with α -³²P-dCTP. After pre-hybridization, the filters were hybridized with 2 x 10⁶ cpm/ml of Sephadex column-purified *env* probe in 6x SSC/0.5% SDS/10% dextran sulfate at 65°C overnight. Hybridized filters were washed in 2x – 0.5x SSC/1% SDS at 55° – 65°C for 30 min and exposed to film overnight.

For polymerase chain reaction (PCR) analysis, approximately 0.1 µg of genomic DNA per sample was incubated with 50 pmoles of (+) and (–) strand primers and 25 mM of dNTPs per 100 µl reaction, in PCR buffer containing 50mM KCl/10mM Tris (pH 8.3)/0.1% (w/v) gelatin and 1.5 to 5 mM MgCl₂ (final concentration) depending on the primers used. The sequence of the (+) strand primer was 5'–CTGGGGCTTACGTTTGTATG–3', corresponding to the MoMLV *env* gene sequence between positions 6487

and 6506. Two different (–) strand primers were used. One (–) strand primer, 5'–TTGGGTTTTGTCAGGACTGG–3', was complementary to the MoMLV *env* gene sequence between positions 6759 and 6778, approximately 220 to 240 base pairs downstream from the ANF insert site. The other (–) primer, 5'–CCCAGTCCGCTCTGGGCTCCAATCCTGTCC–3', corresponds to sequences within the (–) strand of the ANF insert itself. The PCR reactions were begun with denaturation at 95°C for 2–3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 20 sec, and elongation at 72°C for 30 sec. The PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Cytoplasmic RNA from the cells was isolated by lysis of the cells in the NP40-containing buffer and centrifugation as described above, after which urea buffer (7M urea/10mM Tris (pH 7.4)/10mM EDTA/0.35M NaCl/1% SDS) was added to the supernatant, followed by phenol extraction and ethanol precipitation of the RNA.

For Northern blots of pRSVenvANF-Bam transfectants, 20 µg of RNA per sample was incubated with 1 M glyoxal, 10 mM NaPO₄, and 50% DMSO at 50°C for 1 hour, and separated by electrophoresis on a 1% agarose gel in 10 mM NaPO₄ buffer (pH 7.0). The RNA samples were transferred to Hybond nylon membrane filters (Amersham) in 20x SSC, and the filters were pre-hybridized, hybridized with either end-labelled (–) strand ANF oligonucleotide probe or nick-translated *env* gene probe, and washed, as described above.

For PCR analysis of cDNA reverse-transcribed from cytoplasmic RNA (RT-PCR), approximately 5 µg of cytoplasmic RNA per sample was first denatured at 95°C for 2-3 min, followed by annealing of 20 pmoles of a

(-) strand primer per 20 µl reaction, at 65°C for 5 min, in the presence of 40U of RNasin (NEBL). The primers consisted of (-) strand sequences corresponding to either the *env* gene downstream of the insert site or to the ANF insert itself. After annealing, an additional 40U of RNasin was added, along with 10mM of dNTPs and 20U of AMV reverse transcriptase (BRL) per 20 µl reaction in RT buffer containing 40 mM KCl/50mM Tris (pH 8.3)/8 mM MgCl₂/0.4mM DTT (final concentration), and the reaction allowed to proceed at 42°C for 1 hour. One half of this reaction (10µl) was then used for PCR, according to the same protocol as described above for genomic DNA samples.

Fluorescence-activated cell sorting (FACS) analysis

A 15-amino acid ANF peptide, corresponding to the human ANF sequence used for cloning, was kindly synthesized by Dr C. Turck (Howard Hughes Medical Institute (HHMI), University of California San Francisco). Three mg of this peptide was conjugated to thyroglobulin as a carrier protein and used as an immunogen to raise anti-ANF antibodies in rabbits (CalTag). The crude anti-ANF antiserum was incubated with an acetone powder made from GP101 cell proteins at 4°C for 30 min to absorb the non-specific binding constituents, centrifuged at 10,000 g for 10 min, and the supernatant used for flow cytometric analysis of the transfected packaging cells, to examine cell surface expression of the ANF ligand-containing recombinant envelope constructs. Cells were grown to confluence on 100-mm plates, detached and dispersed with 1x phosphate-buffered saline (PBS) containing 2 mM EDTA, and pre-incubated with normal goat serum (CalTag) in PBS

containing 2% bovine serum albumin (BSA) for 30-60 min on ice. After washing twice in PBS with 2% BSA, the cells were incubated with the ANF-specific rabbit antiserum diluted 1:100 in PBS with 2% BSA for 30-60 min, washed again repeatedly, incubated with fluorescein-conjugated goat anti-rabbit antibody (CalTag) similarly diluted 1:200, and washed again. After addition of 1 μ g/ml propidium iodide to stain and exclude the non-viable cells, the samples were analyzed using a Becton-Dickinson FACScan and FACS IV cell sorter, the latter operated by P. Dazin (HHMI, University of California San Francisco). In some experiments, an affinity-purified goat anti-*env* gp70 antiserum (#79S-771; NCI Repository & Microbiological Associates, Inc.) was used as the primary antibody, and fluorescein-conjugated swine anti-goat immunoglobulin (CalTag) was used as the secondary antibody instead. In addition, cells were also incubated with preimmune rabbit or goat serum and the respective fluorescein-conjugated secondary antibody, or with the secondary antibody alone, to control for non-specific binding.

Electron microscopy

For electron microscopy, overnight culture medium from virus-producing cells was filtered through a 0.2 μ m filter (Millipore) and centrifuged at 150,000 g in an SW40 rotor for 1 hour at 4 °C to pellet the virus particles. The pellet was re-suspended in 100 μ l of 1x PBS, after which the samples were fixed in glutaraldehyde, placed on copper mesh grids, stained with phosphotungstic acid, and examined under an electron

microscope, operated by M.-L. Wong (HHMI, University of California San Francisco).

Target cells and viral infections

For determination of virus titer, NIH3T3 mouse fibroblasts were used as target cells. Wild type NIH3T3 cells were maintained in DMEM supplemented with 10% CS and 100 µg/ml PS. For tissue-specific infection experiments, bovine arterial smooth muscle (BASM) cells (provided by Dr. D. Leitman, University of California San Francisco) and human lung fibroblast (HLF) cells (ATCC), which are both known to express high levels of the ANF C-receptor [5, 6], were used as target cells. BASM and HLF cells were maintained in DMEM with 10% fetal bovine serum (FBS) and 100 µg/ml PS. Both cell lines were characterized for expression of endogenous ANF receptors by ¹²⁵I-labelled ANF radioligand binding assay. Cells grown to confluence in 24-well plates (approximately 10⁵ cells) were incubated with 1 x 10⁶ cpm of ¹²⁵I-ANF (Amersham) in 1 ml of serum-free DMEM with 2% bovine serum albumin (BSA) for 1 hour at 37°C, washed 3 times with 1x PBS containing 2% BSA, lysed with 1 ml of 1 N NaOH, and the radioactivity retained in the cell lysates counted by gamma counter to determine the amount of total binding. Some incubations were performed in the presence of 10⁻⁶ M of cold ANF peptide (Sigma) to determine the amount of non-specific binding, and the difference between total and non-specific binding was considered the amount of specific binding to ANF receptors. The total number of receptors per cell was then estimated based

on the total number of cells in each well and the specific activity of the radioligand. In addition, the ANF C-receptor was expressed in HeLa cells by stable transfection of plasmid pSV-ANPCR. Briefly, 20 μ g of pSV-ANPCR and 2 μ g of pFR400 were co-transfected into HeLa cells by calcium phosphate precipitation, and 48 hours later the cells were placed under selection with increasing levels of methotrexate ranging from 0.2 to 10 μ M. Colonies surviving methotrexate selection were also characterized for expression of the ANF C-receptor by 125 I-ANF binding assay and used as target cells for infection experiments. Target cell response to different doses of G418 was determined, and all target cell lines were found to be completely killed by 400 μ g/ml of G418 within 12 - 14 days.

For transient transfection experiments, the culture medium from the packaging cells was replaced 24 hours after calcium phosphate transfection with fresh DMEM/10%CS/PS without any gpt selection reagents, and was harvested and filtered through a 0.2 μ m filter (Millipore) to remove cells and debris 48 hours after transfection. For stably transfected packaging cell clones or pools which were constitutively producing virus, the culture medium was simply changed to non-selective DMEM 24 hours prior to harvesting. One ml of filtered virus-containing medium was used for infection of approximately 10^5 target cells in a 60-mm dish, in the presence of 7.5 μ g/ml polybrene (final concentration). In some experiments, the packaging cells were incubated in serum-free DMEM overnight prior to viral harvesting, and the infections were also carried out in serum-free medium. The target cells were trypsinized and re-plated at 1:10 dilution into selective medium (DMEM/10%CS/PS with 400 μ g/ml G418) on 100-mm plates at 48 hours post-infection. The medium was replenished every 3 days, and target

cell colonies surviving G418 selection were fixed with methanol/acetone (1:1) and counted 12 - 14 days later.

Results

The pRSVenvANF-Bam construct was successfully transfected into Ψ 2 packaging cells, but shows no expression.

The recombinant *env* construct pRSVenvANF-Bam was first co-transfected into Ψ 2 cells along with the viral vector construct pN2, using the calcium phosphate precipitation method, at a molar ratio of pRSVenvANF-Bam : pN2 = 10:1. The cytoplasmic RNA from the transiently transfected cell pools, as well as wild type Ψ 2 control cells transfected with pN2 alone, was analyzed by Northern blot using both a 50 bp minus-strand oligonucleotide complementary to the ANF sequence end-labelled with ^{32}P , and a nick-translated probe against the *env* sequence. Although the *env* probe showed hybridization to a specific band in both control and pRSVenvANF-transfected Ψ 2 cells, the ANF oligo probe showed no specific hybridization to the pRSVenvANF-transfected cell RNA (data not shown). One possible explanation for these negative results was low transfection efficiency; the viral titer from the supernatant medium 48 hours post-transfection was only on the order of 10^2 per ml, as assayed by the number of colonies of infected NIH3T3 target cells surviving selection in 400 $\mu\text{g/ml}$ of the neomycin analog G418.

Therefore, co-transfection of pRSVenvAVF-Bam and pN2 into Ψ 2 cells was repeated, and this time stably transfected packaging cell clones were isolated by selection in 400 μ g/ml G418 (Fig. 2-13). The genomic DNA samples were digested with HincII and analyzed by Southern blot, again using a nick-translated *env* probe as well as an end-labelled ANF oligonucleotide probe. Although there was extensive cross-reactivity with high molecular weight bands, a specific 1 kb band corresponding to the transfected MoMLV *env* sequence was also seen after hybridization with the *env* probe. Furthermore, the ANF oligo probe also showed hybridization to the same band in two of the clones (Fig. 2-14). Since these two packaging cell clones, clone 12 and 17 respectively, appeared to have successfully integrated the pRSVenvANF construct, the relative copy number and transcription levels were then characterized by the polymerase chain reaction (PCR) technique. This technique, rather than standard Northern blot analysis, was chosen as an assay this time because of difficulties previously encountered with non-specific hybridization using the ANF oligo probe on Northern blots. Furthermore, PCR amplification is potentially a more sensitive technique, capable of detecting even single copies [20]. Genomic DNA and cytoplasmic RNA were prepared from each clone as described previously for Southern and Northern blots, and cDNA specific for the MoMLV envelope sequence was prepared from the RNA samples by reverse transcription using a minus strand oligonucleotide primer annealing to the *env* sequence at position 6778, approximately 50 base pairs downstream from the ANF insert site. These cDNA samples, as well as the genomic DNA samples, were then used as templates for PCR amplification using the downstream primer in combination with a plus strand oligonucleotide primer annealing approximately 50 base pairs upstream of the ANF insert site at

position 6487 of the *env* sequence. Thus, the amplified sequence bounded by the two primers would be longer or shorter, depending on the presence or absence of the ANF insert, and thus the PCR product derived from the recombinant envelope could be distinguished from that of the wild type. From the genomic DNA samples, two bands could indeed be amplified and separated by polyacrylamide gel electrophoresis, the larger corresponding to the recombinant sequence and the smaller corresponding to the wild type sequence (Fig. 2-15). Wild type pRSVenv and recombinant pRSVenvANF-Bam plasmids mixed together in varying molar ratios (1:0, 0:1, 1:1, 10:1, 1:10) were used as templates for control reactions to ensure that relative copy number could be determined by this assay. The relative intensity of the two bands thus gave a rough estimate of copy number in clone 17 as approximately 10 copies per cell. Conversely, the recombinant envANF band amplified from clone 12 genomic DNA was approximately 10-fold less intense than the endogenous wild type *env* band, suggesting that the gene was not present in all cells in this population.

In contrast with the results obtained from the genomic DNA templates, when PCR reactions were performed using cDNA prepared by reverse transcription of cytoplasmic RNA as the template (RT-PCR), no recombinant envANF band was seen with either clone (Fig. 2-15). The RT-PCR assay was consistently negative for the recombinant envANF band throughout repeated assays performed under various reaction conditions, while the endogenous wild type *env* band was consistently positive, serving as an internal control for the PCR reaction. Thus it was concluded that either this construct was transcriptionally silent, or the RNA message was extremely unstable.

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Figure 2-13. **Protocol for transfection of pRSVenvANF constructs into Ψ 2 cells.** The plasmid pRSVenvANF was co-transfected with the plasmid pN2 into the Ψ 2 packaging cell line. G418-resistant subclones were isolated and characterized for expression of the ANF-containing envelope by Southern, Northern, and RT-PCR analyses. See text for details.

RSV: Rous sarcoma virus promoter/enhancer
sequence (long terminal repeat).

env: Moloney murine leukemia virus *env* gene.

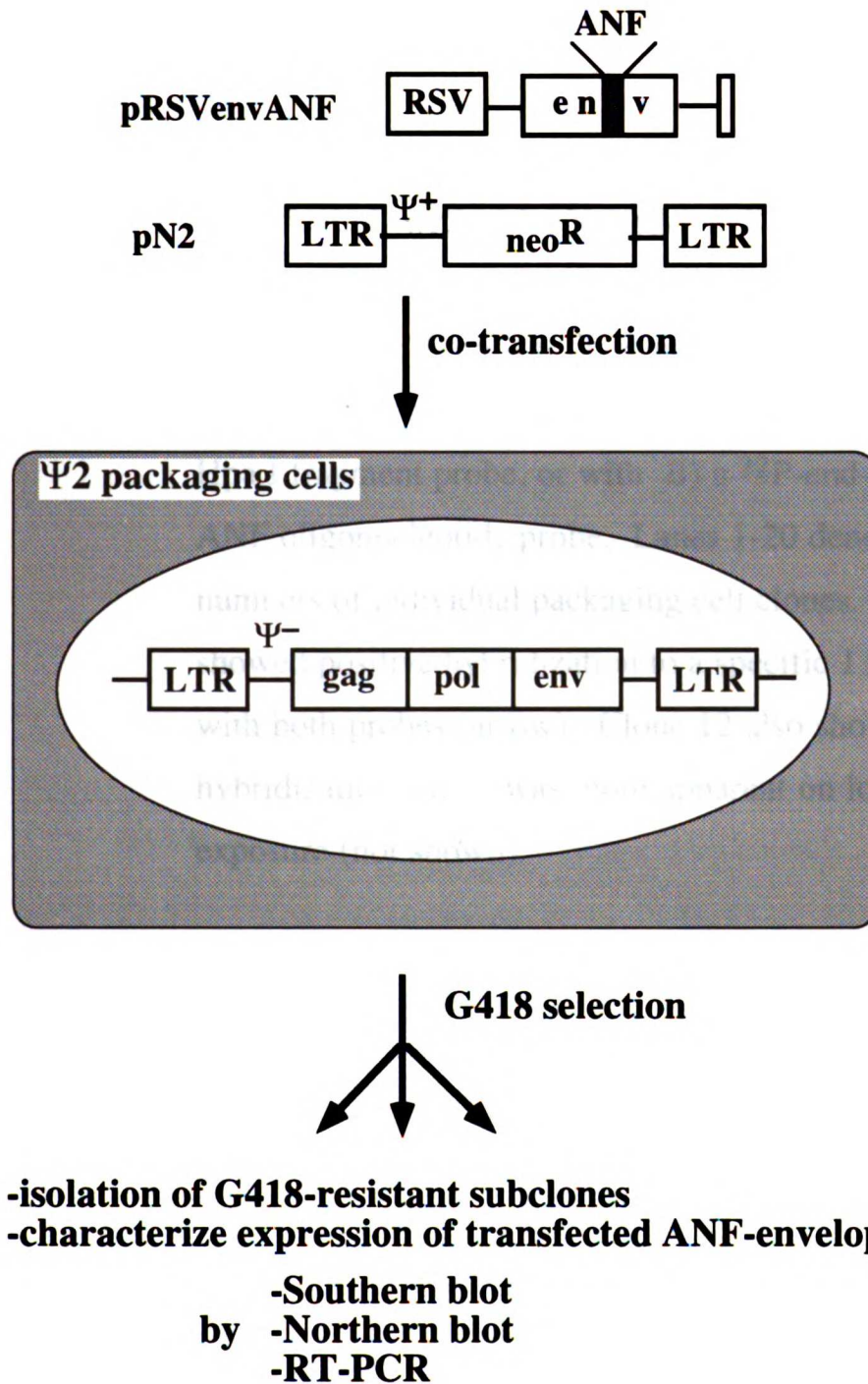
ANF: atrial natriuretic factor sequence.

LTR: long terminal repeat.

neo^R: neomycin (G418) resistance gene.

Ψ^+ : psi packaging signal present.

Ψ^- : psi packaging signal deleted.



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Figure 2-14. **Southern blot analysis of Ψ 2 cell subclones transfected with pRSVenvANF-Bam.**

Genomic DNA from Ψ 2 cells stably transfected with pRSVenvANF-Bam was digested with Hinc II and hybridized with A) a ^{32}P -nick-translated Moloney *env* Hpa I fragment probe, or with B) a ^{32}P -end-labelled ANF oligonucleotide probe. Lanes 1-20 denote the numbers of individual packaging cell clones. Clone 17 showed positive hybridization to a specific 1 kb band with both probes (arrow). Clone 12 also showed faint hybridization which was more apparent on longer exposure (not shown).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

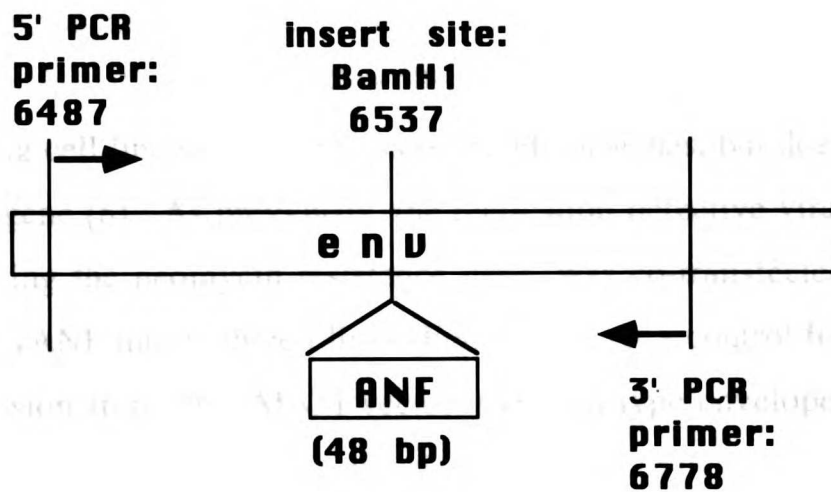


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Figure 2-15. PCR analysis of Ψ 2 cell subclones transfected with pRSVenvANF-Bam.

- A. Strategy of PCR assay for recombinant ANF-env detection in genomic DNA and reverse-transcribed RNA (cDNA) samples from stably transfected packaging cell clones.
- B. Results of PCR assay. Lane M: Φ X174 marker.
- Lane 1: cDNA reverse-transcribed from pRSVenvANF-Bam transfected clone 12 RNA.
- Lane 2: cDNA reverse-transcribed from pRSVenvANF-Bam transfected clone 17 RNA.
- Lane 3: genomic DNA from clone 12.
- Lane 4: genomic DNA from clone 17.
- Lane 5: 10 fM pRSVenv plasmid DNA control.
- Lane 6: 10 fM pRSVenvANF-Bam plasmid prep DNA (positive control).
- Lane 7: 5 fM pRSVenv + 5 fM pRSVenvANF.
- Lane 8: 10 fM pRSVenv + 1 fM pRSVenvANF.
- Lane 9: 1 fM pRSVenv + 10 fM pRSVenvANF.

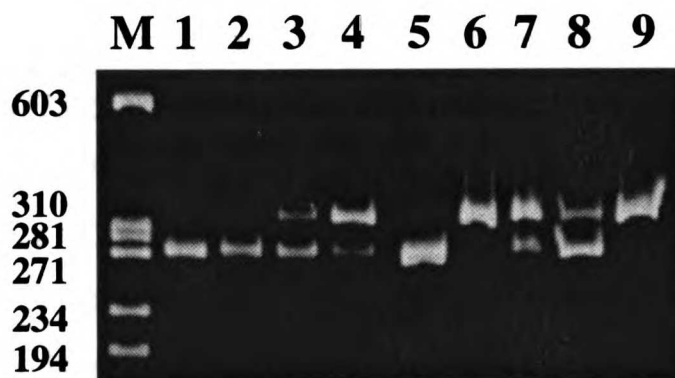
A.



-amplified wild type env
band size should be:
 $6778 - 6487 = \underline{291}$ bp

-amplified recombinant env
band size should be:
 $291 + 48 = \underline{339}$ bp

B.



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dexamethasone treatment (Fig. 2-17 B). However, Ψ 2 cells similarly co-transfected with pMTVenvANF-Bam and pN2 showed good amplification of the wild type *env* sequence but no amplification of the recombinant sequence (Fig. 2-17 A & B). No pMTVenvANF expression could be detected in the Ψ 2 cells by RT-PCR even after overnight treatment with 10^{-6} M dexamethasone (Fig. 2-17 B). Controls using GP101 cells transfected with pN2 alone showed no amplification, and GP101 cells transfected with wild type pMTVenv and pN2, Ψ 2 cells transfected with pMTVenv and pN2, or pN2 alone, showed amplification of the wild type *env* sequence only, as expected.

The MLVenvANF constructs also show expression of recombinant envelope mRNA as well as proteins.

As previously, both Ψ 2 and GP101 packaging cell lines were transfected with the replication-defective viral vector pN2 by calcium phosphate precipitation, and this time co-transfections were performed with or without pMLVenvANF in 10-fold molar excess (Fig. 2-18). After G418 selection, the surviving colonies were pooled and PCR analysis of cytoplasmic RNA isolated from these cells was initially performed as previously by RT-PCR, with oligonucleotide primers corresponding to plus and minus strand *env* sequences approximately 50 base pairs up- and downstream, respectively, of the ANF insert site (Fig. 2-19 A). Again, as with the pMTVenvANF transfectants, GP101 cells stably co-transfected with pMLVenvANF-Bam and pN2 showed good amplification of the ANF insert (+) *env* sequence, demonstrating successful transcription of the transfected recombinant *env* gene. Control GP101 cells transfected with pN2 alone

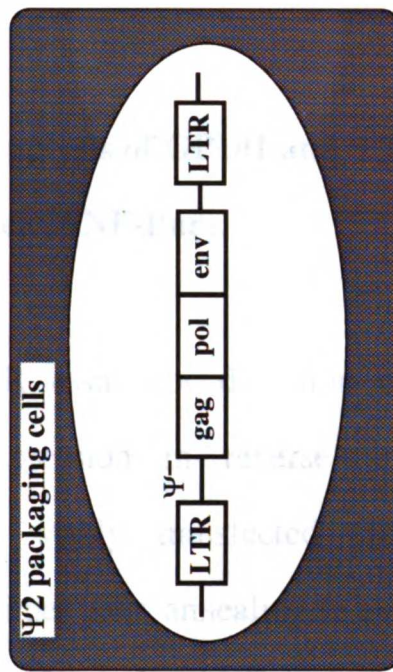
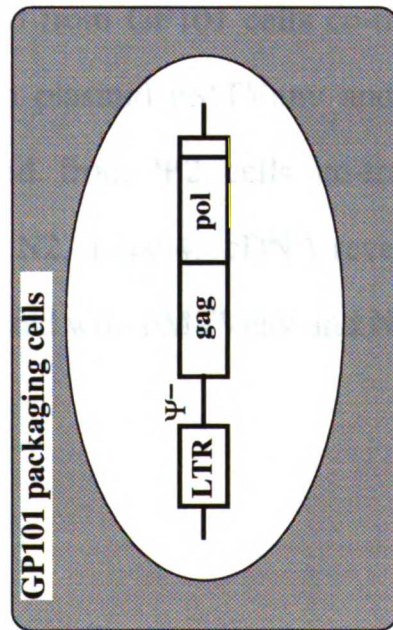
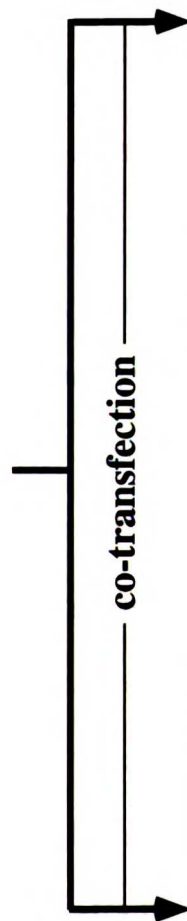
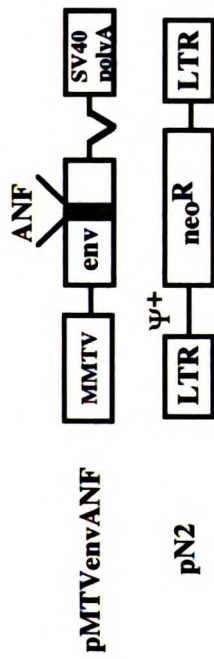
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showed no amplification of any band, as expected, and control Ψ 2 cells transfected with pN2 alone showed amplification of the wild type *env* sequence, also as expected. However, Ψ 2 cells co-transfected with pMLVenvANF-Bam and pN2 again showed amplification of the wild type but no amplification of the recombinant *env* sequence (Fig. 2-19B).

In order to increase the likelihood that the recombinant envANF sequence would be amplified if it was present, RT-PCR assays were subsequently repeated using the 5'-oligonucleotide primer corresponding to the plus strand of the *env* sequence upstream from the ANF insert site in conjunction with two different 3'-primers, one of which corresponded to the (–) strand of the *env* sequence downstream from the insert site, as previously, and the other which consisted of sequences within the (–) strand of the ANF insert (Fig. 2-20 A). The GP101 cells stably transfected with pMLVenvANF-Bam showed the larger band containing the ANF insert amplified by the 5'-*env* primer and 3'-*env* primer, as well as a specific ANF band amplified by the 5'-*env* primer and the 3'-ANF primer (Fig. 2-20 B). Interestingly, the Ψ 2 cells transfected with pMLVenvANF showed this specific ANF band also, even though the 5'-*env*/3'-*env* primer pair again resulted in amplification of only the smaller band corresponding to the wild type envelope sequence. When blotted onto nitrocellulose and hybridized with a 32 P-labelled ANF oligo probe, the presence of the ANF sequence in the band amplified from these Ψ 2 cells using the 5'-*env*/3'-ANF primer pair was confirmed (Fig. 2-20 C). Thus it was concluded that the recombinant pMLVenvANF-Bam construct was indeed expressing at detectable levels in both GP101 and Ψ 2 cells, but in the Ψ 2 cells the wild type sequence was being preferentially amplified by RT-PCR when the downstream *env* (–) strand primer was used.

Figure 2-16. **Protocol for transfection of pMTVenvANF constructs into GP101 and Ψ 2 cells.** The plasmid pMTVenvANF was co-transfected with the plasmid pN2 into the GP101 and Ψ 2 packaging cell lines. G418-resistant subclones were isolated and characterized for expression of the ANF-containing envelope by RT-PCR analyses. See text for details.

MMTV: mouse mammary tumor virus
promoter/enhancer (long terminal repeat)
env: Moloney murine leukemia virus *env* gene.
ANF: atrial natriuretic factor sequence.
LTR: long terminal repeat.
neo^R: neomycin (G418) resistance gene.
 Ψ^+ : psi packaging signal present.
 Ψ^- : psi packaging signal deleted.



-characterize expression of ANF-envelope in G418-resistant cell pools by RT-PCR

Figure 2-17. RT-PCR analysis of GP101 and Ψ 2 subclones transfected with pMTVenvANF-Bam.

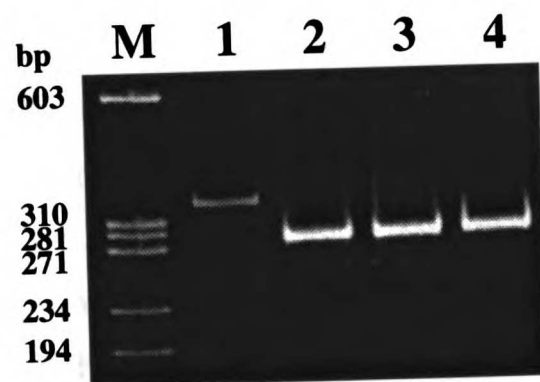
A. Results of RT-PCR assay for detection of recombinant pMTVenvANF-Bam expression in reverse-transcribed RNA (cDNA) samples from stably transfected GP101 and Ψ 2 packaging cells, using primer pairs annealing to *env* sequences 50 bp up- and down-stream from the ANF insert site. Lane M: Φ X174 marker. Lane 1: cDNA reverse-transcribed from GP101 cells co-transfected with pMTVenvANF-Bam and N2. Lane 2: cDNA reverse-transcribed from GP101 cells co-transfected with wild type *env* expression plasmid pMTVenv and N2. Lane 3: cDNA reverse-transcribed from Ψ 2 cells co-transfected with pMTVenvANF-Bam and N2. Lane 4: cDNA reverse-transcribed from Ψ 2 cells co-transfected with pMTVenv and N2.

Figure 2-17 (continued). RT-PCR analysis of GP101 and Ψ 2 subclones transfected with pMTVenvANF-Bam.

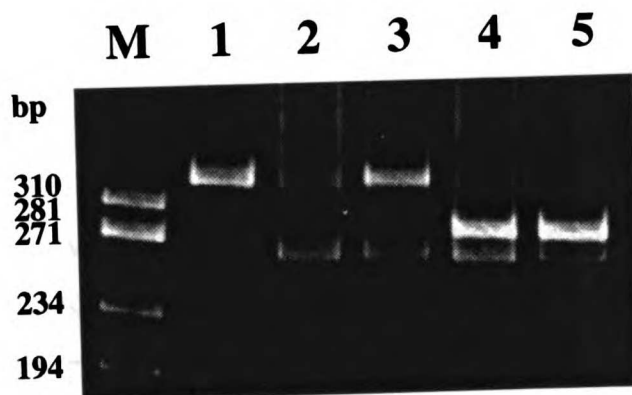
B. Results of RT-PCR assay before and after treatment of cells with 10^{-6} M dexamethasone. Lane M: Φ X174 marker. Lane 1: positive control genomic DNA from GP101 cells co-transfected with pMTVenvANF-Bam and N2. Lane 2: cDNA reverse-transcribed from GP101 cells co-transfected with pMTVenvANF-Bam and N2, before dexamethasone treatment. Lane 3: cDNA reverse-transcribed from GP101 cells co-transfected with pMTVenvANF-Bam and N2, after dexamethasone treatment. Lane 4: cDNA reverse-transcribed from Ψ 2 cells co-transfected with pMTVenvANF-Bam and N2, before dexamethasone treatment. Lane 5: cDNA reverse-transcribed from Ψ 2 cells co-transfected with pMTVenvANF-Bam and N2, after dexamethasone treatment.

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A.



B.



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Figure 2-18. Protocol for transfection of pMLVenvANF constructs into GP101 and Ψ 2 cells.

The pMLVenvANF constructs (pMLVenvANF-Bst, -Bam, -Dra, -Acc, and -Nsi, respectively) were each co-transfected with the plasmid pN2 into the GP101 and Ψ 2 packaging cell lines. G418-resistant subclones were isolated and characterized for expression of the ANF-containing envelope by RT-PCR and FACS analyses. Virus production was examined by electron microscopy and titration on NIH3T3 cells. Specific infectivity was tested on bovine aortic smooth muscle (BASM) cells and human lung fibroblast (HLF) cells. See text for details.

env: Moloney murine leukemia virus *env* gene.

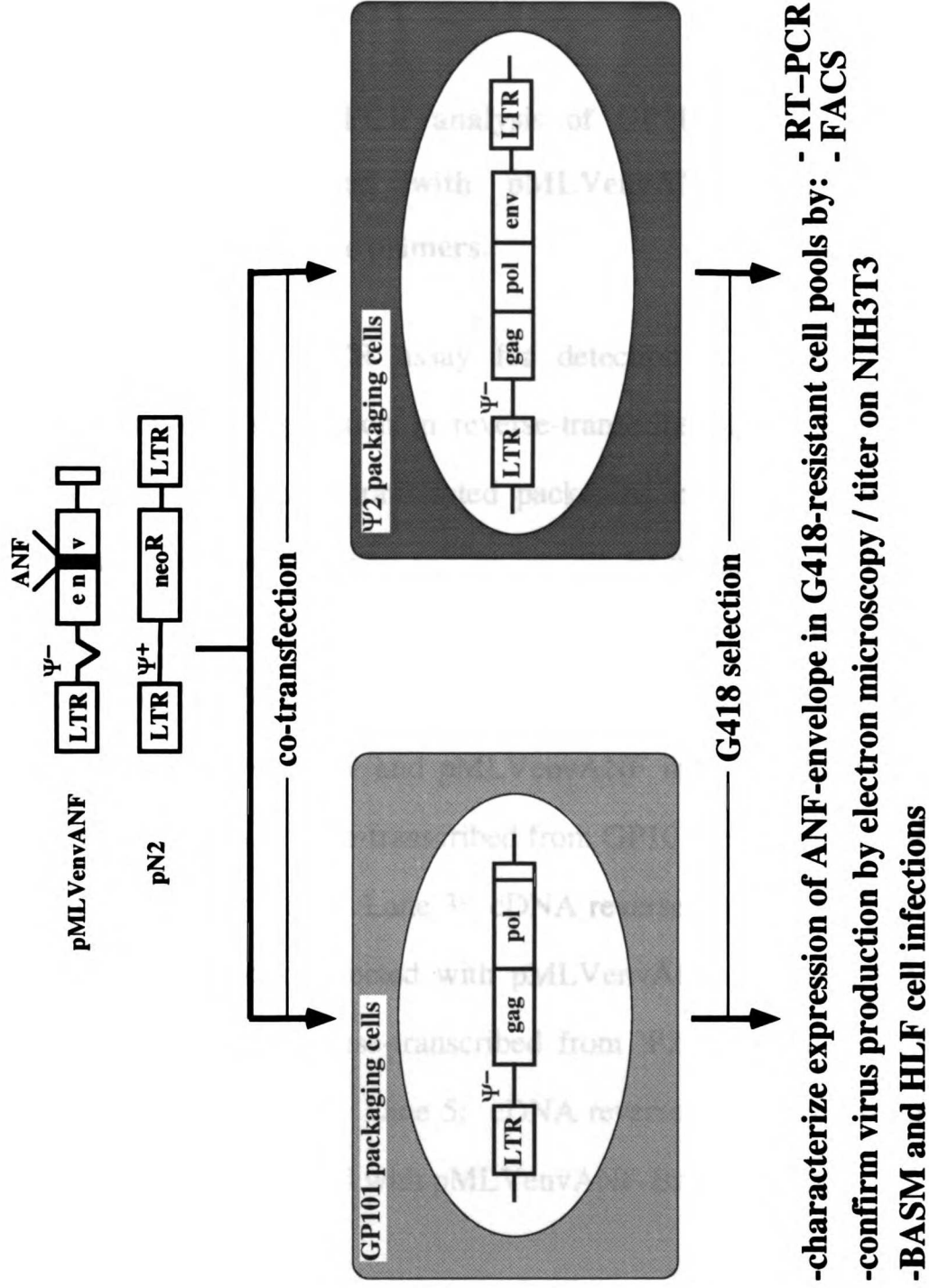
ANF: atrial natriuretic factor sequence.

LTR: long terminal repeat.

neo^R: neomycin (G418) resistance gene.

Ψ^+ : psi packaging signal present.

Ψ^- : psi packaging signal deleted.



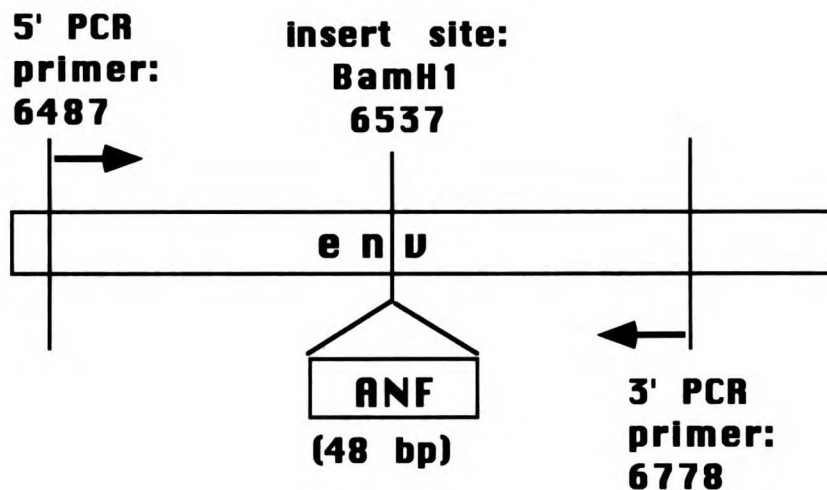
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Figure 2-19. RT-PCR analysis of GP101 and Ψ 2 cell subclones transfected with pMLVenvANF-Bam, using flanking *env* sequence primers.

A. Strategy of PCR assay for detection of recombinant MLVenvANF expression in reverse-transcribed RNA (cDNA) samples from stably transfected packaging cell clones, using primer pairs annealing to *env* sequences 50 bp up- and downstream from the ANF insert site. B. Results of RT-PCR assay. Lane M: Φ X174 marker. Lane 1: positive control DNA mixture of plasmids pMLVenv and pMLVenvANF in 1:1 molar ratio. Lane 2: cDNA reverse-transcribed from GP101 cells transfected with N2 vector alone. Lane 3: cDNA reverse-transcribed from GP101 cells co-transfected with pMLVenvANF-Bam and N2. Lane 4: cDNA reverse-transcribed from Ψ 2 cells transfected with N2 vector alone. Lane 5: cDNA reverse-transcribed from Ψ 2 cells co-transfected with pMLVenvANF-Bam and N2.

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A.



-amplified wild type env
band size should be:
 $6778 - 6487 = \underline{291}$ bp

-amplified recombinant env
band size should be:
 $291 + 48 = \underline{339}$ bp

B.

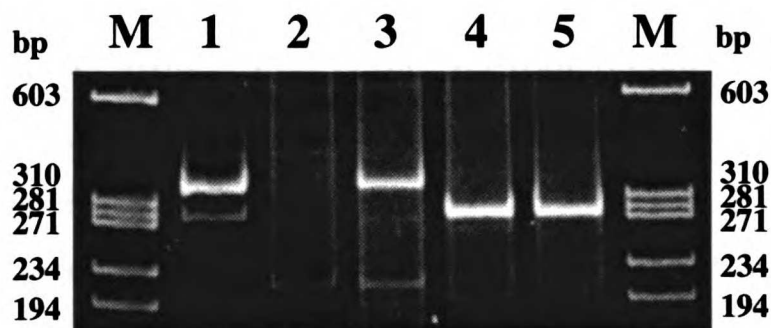


Figure 2-20. RT-PCR analysis of GP101 and Ψ2 cell subclones transfected with pMLVenvANF-Bam, using an ANF sequence-specific primer.

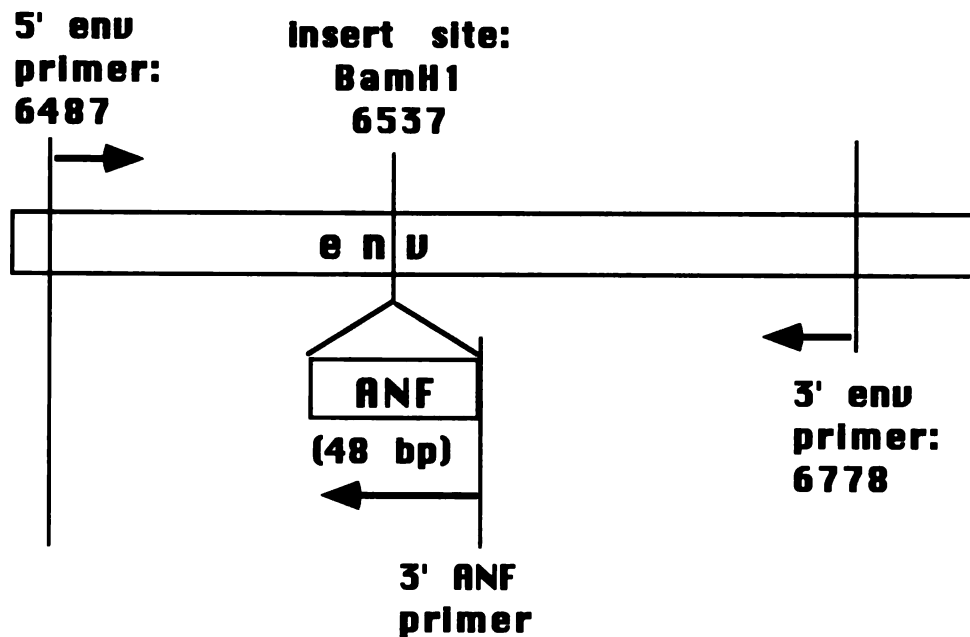
A. Strategy of PCR assay for recombinant MLVenvANF detection in reverse-transcribed RNA (cDNA) samples from stably transfected packaging cell clones, using *env*-specific or ANF-specific 3'-primers.

B. Results of RT-PCR assay. Lane M: ΦX174 marker. Lane 1: positive control pMLVenvANF-Bam plasmid DNA, using 5'-*env* /3'-ANF primer pair only. Lane 2: cDNA reverse-transcribed from GP101 cells transfected with N2 vector alone, using 5'-*env* primer with both 3'-*env* and 3'-ANF primers. Lane 3: cDNA reverse-transcribed from GP101 cells co-transfected with pMLVenvANF-Bam and N2, using 5'-*env* primer with both 3'-*env* and 3'-ANF primers. Lane 4: cDNA reverse-transcribed from Ψ2 cells transfected with N2 vector alone, using 5'-*env* primer with both 3'-*env* and 3'-ANF primers. Lane 5: cDNA reverse-transcribed from Ψ2 cells co-transfected with pMLVenvANF-Bam and N2, using 5'-*env* primer with both 3'-*env* and 3'-ANF primers.

C. Autoradiogram of gel in Fig. 4-2 B, blotted onto nitrocellulose and probed with ³²P-labelled ANF-specific probe.

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A.



-amplified wild type env
band size, using 5' env
and 3' env primers,
should be:

$$6778 - 6487 = \underline{291} \text{ bp}$$

-amplified recombinant env
band size, using 5' env
and 3' env primers,
should be:

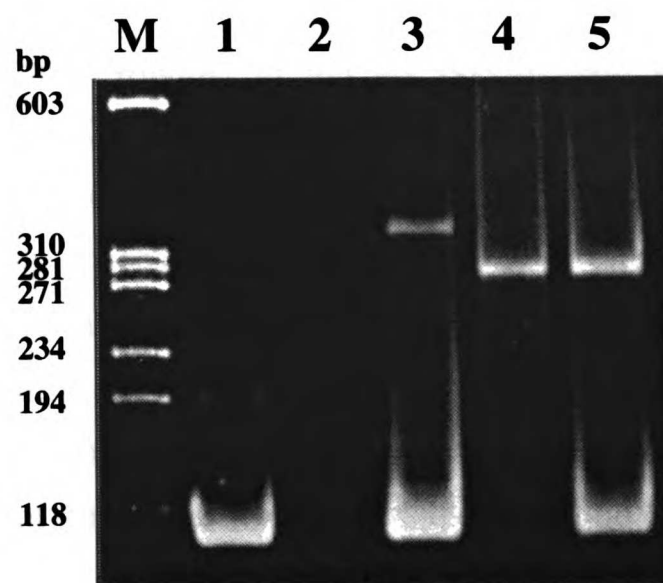
$$291 + 48 = \underline{339} \text{ bp}$$

-amplified recombinant env
band size, using 5' env
and 3' ANF primers,
should be:

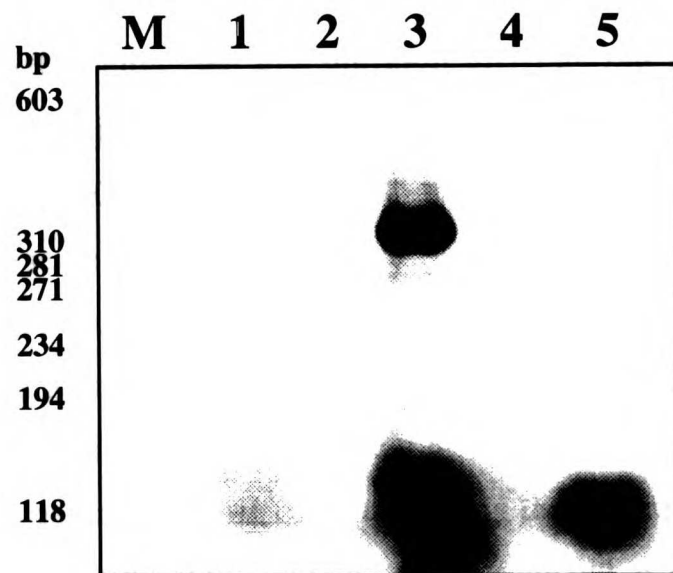
$$6537 - 6487 + 48 = \underline{98} \text{ bp}$$

11227 11228 11229

B.



C.



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Cell surface expression of *env* epitopes but not of ANF epitopes is detectable by FACS analysis of packaging cell lines expressing MLVenvANF constructs.

In order to determine whether or not the recombinant envelope transcripts detected by RT-PCR were actually being translated into protein and expressed on the surface of the packaging cells, to be subsequently incorporated into budding virions, FACS analysis of the transfected cells was performed. This was most readily accomplished in the GP101 cells, which do not originally have the MoMLV *env* gene; thus, any cell surface expression of MLV envelope proteins must necessarily derive from the transfected recombinant envelope constructs. Accordingly, GP101 packaging cells stably co-transfected with pN2 and the ANF ligand-containing envelope construct pMLVenvANF-Bst, pMLVenvANF-Bam, pMLVenvANF-Dra, pMLVenvANF-Acc, or pMLVenvANF-Nsi, respectively, were incubated with an affinity-purified polyclonal goat antiserum specific for MLV envelope glycoprotein gp70 as the primary antibody, and fluorescein-conjugated swine-anti-goat IgG as the secondary antibody, and analyzed by FACS (Fig. 2-21). Untransfected parental GP101 cells incubated with the same primary and secondary antibodies under the same conditions served as a baseline control, and untransfected wild type Ψ 2 packaging cells served as a positive control for expression of the ecotropic MoMLV envelope. In each histogram, the stable transfectants are shifted to the right compared to the parental GP101 cells, indicating that the respective MLV envelope proteins are being expressed and are recognized as epitopes on the cell surface.

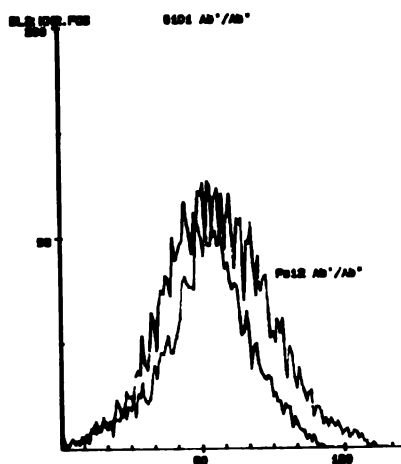
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Figure 2-21. FACS analysis of MLVenvANF-transfected GP101 packaging cell lines using MLV env-specific antiserum.

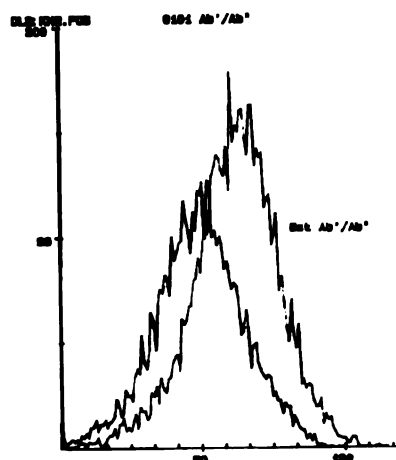
Untransfected Ψ 2 packaging cells as positive controls (A) and GP101 packaging cells stably co-transfected with pN2 and pMLVenvANF-Bst (B), pMLVenvANF-Bam (C), pMLVenvANF-Dra (D), pMLVenvANF-Acc (E), or pMLVenvANF-Nsi (F), respectively, were incubated with an affinity-purified polyclonal goat antiserum specific for MLV envelope glycoprotein gp70 as the primary antibody, and fluorescein-conjugated swine-anti-goat IgG as the secondary antibody, and analyzed by fluorescence-activated cell sorting (FACS). In each histogram, the baseline peak on the left represents untransfected parental GP101 cells incubated with the same primary and secondary antibodies under the same conditions as a control. The shifts to the right seen in each histogram indicate that the respective MLV envelope proteins are being expressed and are recognized as epitopes on the cell surface.

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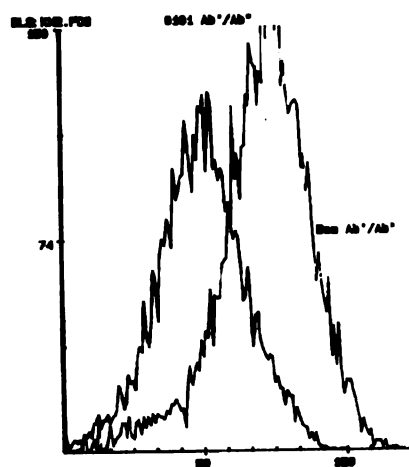
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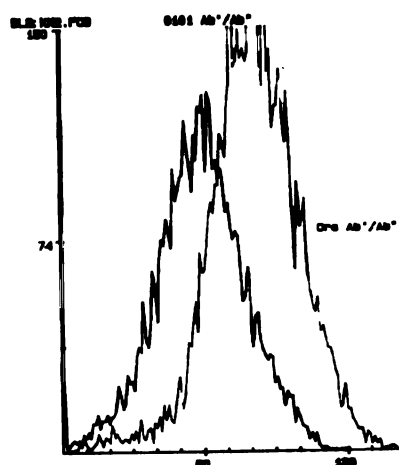
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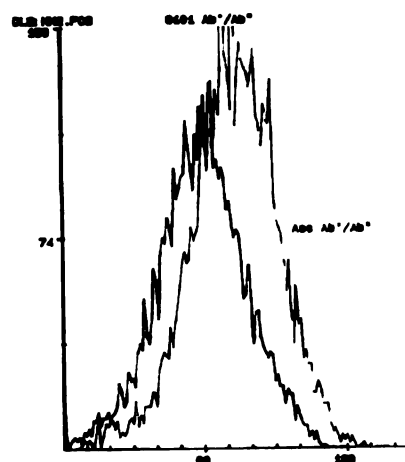
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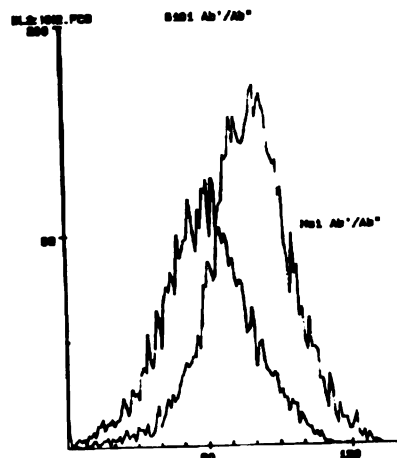
D.



E.



F.



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If ANF ligand epitopes on the surface of the transfected packaging cells could be recognized by ANF-specific antibodies, this would further suggest that the ligand, although expressed in the context of a larger protein, could still mediate binding to its specific receptor. Therefore, anti-ANF antibodies were raised in rabbits by immunization with a synthetic 15-amino acid ANF peptide, representing the same sequence as that used in designing the envelope constructs, and this antiserum was used as the primary antibody for FACS analysis of the transfected GP101 cells. However, in this case, no shifts were seen in any of the transfected packaging cell lines compared to the parental GP101 cells, regardless of which recombinant envelope construct was used (Fig. 2-22). In control experiments, the packaging cells were also incubated with the fluorescein-conjugated goat anti-rabbit secondary antibody alone, without the primary antibody, and showed much lower levels of fluorescence, indicating that non-specific binding by the secondary antibody was not a confounding factor (Fig. 2-22 A & C).

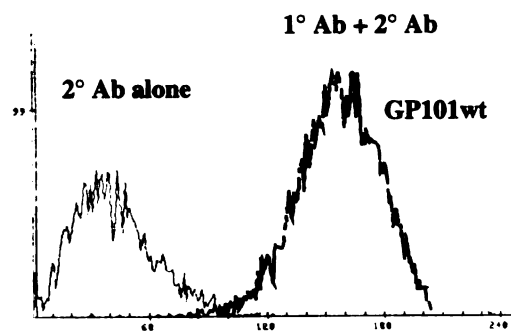
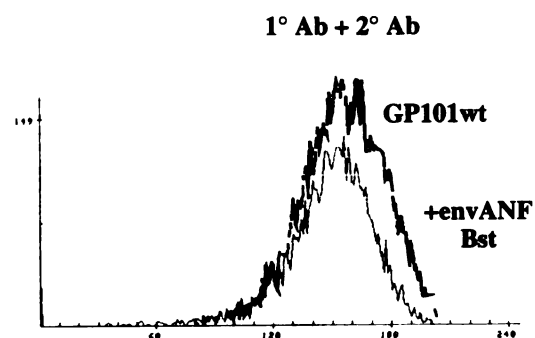
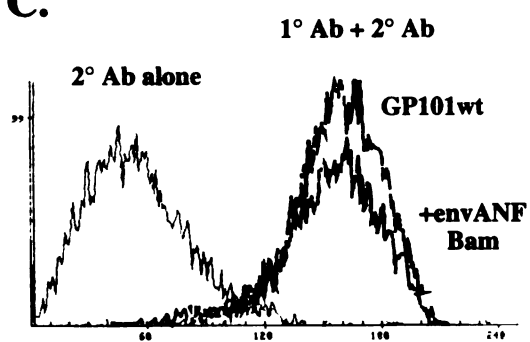
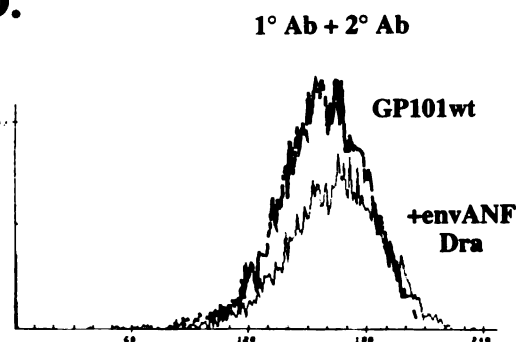
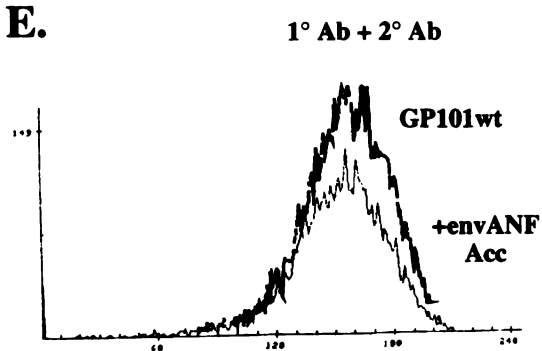
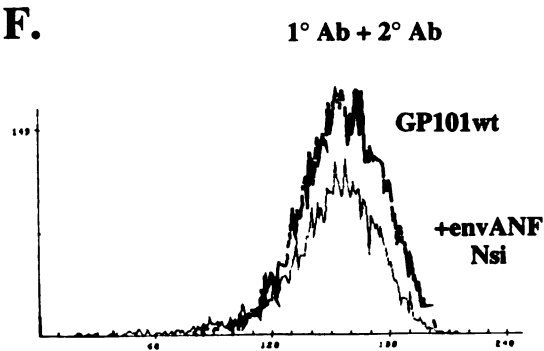
The incubations with the primary antibodies were performed on parental GP101 cell controls, which produce no endogenous *env* proteins, under exactly the same conditions as the transfectants. Positive shifts were seen compared to the parental cells with the *env*-specific antibody, yet no shifts were observed with the ANF-specific antibody. Thus, these results suggested that although the recombinant envelope constructs were successfully being expressed as proteins on the surface of the packaging cells, the ANF ligand sequence contained within them was not being recognized as an epitope. However, as the affinity and specificity of the antiserum for ANF was not established at the time, the possibility that the antiserum was simply incapable of detecting ANF epitopes efficiently remains to be formally ruled out.

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Figure 2-22. FACS analysis of MLVenvANF-transfected GP101 packaging cell lines using ANF-specific antiserum.

Untransfected GP101 packaging cells as baseline controls (A) and GP101 packaging cells stably co-transfected with pN2 and pMLVenvANF-Bst (B), pMLVenvANF-Bam (C), pMLVenvANF-Dra (D), pMLVenvANF-Acc (E), or pMLVenvANF-Nsi (F), respectively, were incubated with rabbit antiserum raised against the 15 amino acid ANF peptide sequence as the primary antibody, and fluorescein-conjugated goat anti-rabbit IgG as the secondary antibody, followed by FACS analysis. In (A) and (C), the peak on the left represents the respective packaging cells incubated with secondary antibody alone as a control for non-specific binding. In each histogram, the higher peak on the right represents untransfected parental GP101 cells incubated with the same primary and secondary antibodies under the same conditions, as a baseline control. When compared to this baseline control, no shifts in the peaks representing the transfected cell populations are seen in any of the histograms. One possible interpretation of these results is that, although the respective ANF sequence-containing MLV envelope proteins are being expressed, none of them are recognized by ANF-specific antibodies on the cell surface, perhaps because the ANF epitopes are inaccessible within the conformational context of the envelope protein.

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A.**B.****C.****D.****E.****F.**

Packaging cell lines expressing MLVenvANF produce virions but do not infect BASM or HLF cells.

Viral particles were visualized by electron microscopy of the culture medium taken from packaging cells stably co-transfected with pMLVenvANF-Bam and pN2. Pelleted viruses isolated from the medium were fixed in glutaraldehyde, stained with phosphotungstic acid, and examined at 10,000x to 25,000x magnification. Virions were present in relatively low abundance (1-3 per high power field), but were clearly visible as particles surrounded by an electron-dense outer wall and a clear inner wall, some of which showed a trailing "tail" of plasma membrane remaining as vestigial evidence of the process of budding off from the packaging cell [21] (Fig. 2-23). Viral particles of identical appearance were also observed in samples of medium from Ψ 2 cells transfected with pN2 alone, as well as GP101 cells co-transfected with pMLVenvANF and pN2 (data not shown).

The viral medium was harvested and used to infect BASM and HLF cells, which are known to express high levels of ANF C-receptor, on the order of 100,000 to 300,000 receptors per cell [5, 6]. The human and bovine ANF sequences show a high degree of homology, and human ANF is known to cross-react quite well with the bovine receptor in BASM cells [22]. By radioligand binding assay using ^{125}I -conjugated human ANF, the BASM cells used for these infection experiments were found to express approximately 200,000 receptors per cell (mean $\pm$ S.D.: $196,534 \pm 17,134$ ($n = 6$)), and the HLF cells expressed approximately 150,000 receptors per cell (mean $\pm$ S.D.: $145,821 \pm 14,112$ ($n = 4$)). However, when exposed to culture medium from the transfected packaging cell lines, neither BASM nor HLF cells showed any evidence of infection regardless of the packaging cell line

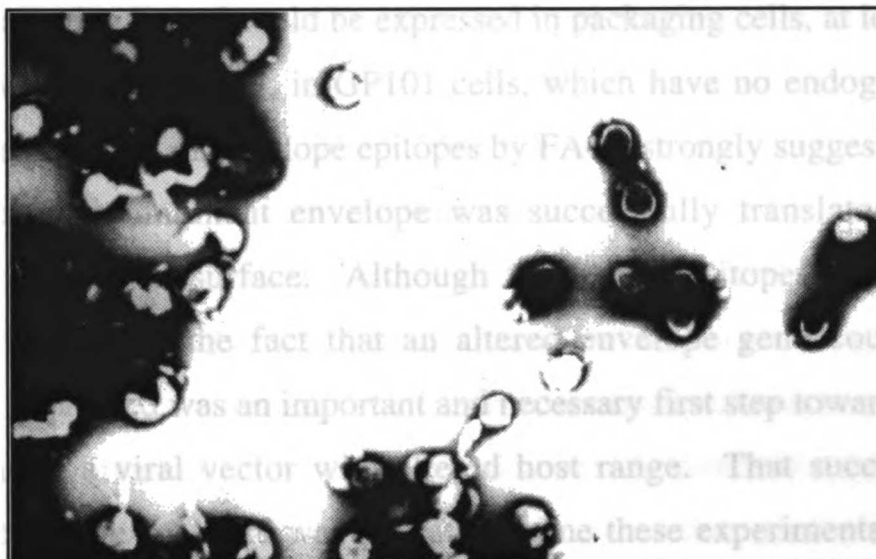
used, that is, no G418-resistant colonies were formed. These experiments were repeated three times using regular DMEM with 10% serum, as well as two times using serum-free medium for viral harvest and target cell incubation, with the same negative result. At this point, these primary cells began to show a progressive decrease in their capability to grow in culture, and ultimately proved to have a finite lifetime of 40 to 50 population doublings before they stopped dividing entirely. Therefore, HeLa target cells expressing the ANF C-receptor were created by co-transfection of plasmids pSV-ANPCR and pFR400, followed by selection in increasing concentrations of MTX. Subclones isolated at a selection level of 10 μ M MTX were screened for expression of the receptor by 125 I-ANF binding assay, and those expressing the highest levels of receptor (mean $\pm$ S.D.: $52,554 \pm 17,183$ (n = 6)) were also used as target cells. However, once again, no evidence of infection was observed after incubation of the HeLa + ANF receptor cells with culture supernatant from GP101 cells transfected with pN2 alone, GP101 cells transfected with pMLVenvANF-Bam and pN2, Ψ 2 cells transfected with pN2 alone, or Ψ 2 cells transfected with both pMLVenvANF-Bam and pN2. Both of the transfected Ψ 2 cell lines did, however, produce virus which conferred neomycin resistance to NIH3T3 cells at a titer of approximately 2 to 5 x 10³/ml. Culture supernatant from the transfected GP101 cells showed no titer on NIH3T3 cells.

Figure 2-23. Electron microscopy of virions obtained from medium of Ψ 2 cells stably co-transfected with pMLVenvANF and pN2.

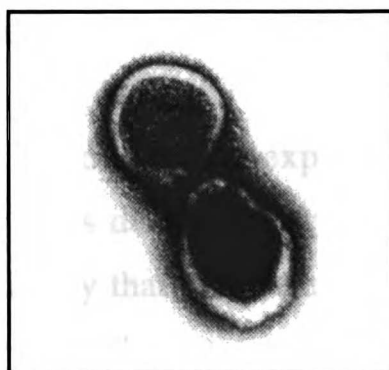
Viruses were pelleted by ultracentrifugation of the medium from Ψ 2 packaging cells which had been stably co-transfected with pMLVenvANF and pN2. The medium was spun in an SW40 tube at 150,000g for 1 hour, the supernatant was discarded, and the pellet was re-suspended in 100 μ l Tris/EDTA. The pelleted virus sample was fixed in glutaraldehyde, stained with phosphotungstic acid, and examined under an electron microscope.

A: 10,000x magnification. B: 24,000x magnification.

A.



B.



Discussion

In these experiments, it was possible to demonstrate that, with the appropriate construct, a recombinant envelope gene containing sequences encoding a targeting ligand could be expressed in packaging cells, at least at the RNA level. Furthermore, in GP101 cells, which have no endogenous *env* gene, the detection of envelope epitopes by FACS strongly suggests that the transfected recombinant envelope was successfully translated and transported to the cell surface. Although the ligand epitopes were not recognized by FACS, the fact that an altered envelope gene could be successfully expressed was an important and necessary first step towards the development of a viral vector with altered host range. That successful expression could be achieved was not, at the time these experiments were initiated, something which could be taken for granted, and in fact, mutagenesis of the *env* gene had hitherto been considered to be more likely than not to lead to loss of expression. Indeed, naturally occurring viral envelope mutants which continue to be viable or even alter the cell specificity of the virus are known to be exceedingly rare [23].

Thus, the lack of detectable expression encountered with the RSVenvANF construct was disappointing but not surprising, although it remains a formal possibility that the construct was in fact expressing, but that the RT-PCR assay using the *env* sequence 3'-primer downstream of the ANF insert site was incapable of detecting it, as was the case for the pMTVenvANF and pMLVenvANF constructs when transfected into Ψ 2 cells. Unfortunately, as the GP101 cell line was not available to us at the time this construct was being used, this possibility could not be directly

tested by introducing the RSVenvANF construct into GP101 cells. However, when the Ψ 2 cells which had been transfected with the RSVenvANF construct were later re-assayed by RT-PCR using the (-) strand ANF-specific primer, there was still no evidence of expression (data not shown), and so it was concluded that the construct was most probably not capable of expressing in these cells.

If this is indeed the case, it still remains to be formally demonstrated whether this construct is transcriptionally inactive, or whether the transcripts are made but are highly unstable. If transcriptional silencing were the cause, this was presumed to be a consequence of the construct itself and not due to an effect of the integration site, since the same results were obtained with two independent clones. It was noted at this point that promoter and polyadenylation sequences required for transcription in eukaryotic cells were provided in the construct, and so failure of transcription was considered to be a less likely cause, unless the RSV promoter functioned very poorly in this cell type (mouse fibroblasts). On the other hand, the splice donor and acceptor sequences normally present in the MoMLV genome had been deleted in this construct, and this might account for the lack of detectable RNA message even when assayed by RT-PCR. The splice donor site is located 5' of the *gag* gene in the MoMLV genomic sequence, while several possible splice acceptor sites exist 5' of the *env* gene. These splice sites are known to be utilized by the wild type virus to generate a specific mRNA consisting of the *env* sequence alone, splicing out *gag* and *pol*, and may be important in stabilizing the *env* mRNA. Thus, a second construct was made to address these potential pitfalls and to ensure adequate levels of expression in the transfected packaging cells, using the eukaryotic expression vector

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pMAM which not only contains a promoter known to function at high levels in mouse fibroblasts and polyadenylation signals, but also splicing signals which would contribute to the stability of the mRNA transcripts. The MMTV-LTR is a strong promoter in NIH3T3 cells, and in addition contains multiple glucocorticoid response elements (GREs) which act essentially as conditional enhancers, resulting in greatly increased levels of transcription in the presence of dexamethasone [9, 10].

In stably transfected GP101 cells, the pMTVenvANF-Bam construct was indeed shown to express at levels detectable by RT-PCR using the downstream *env* 3'-primer. However, the recombinant envelope band was still not observed in the Ψ 2 cells transfected with pMTVenvANF-Bam. My first interpretation of this result was that the Ψ 2 cells co-transfected with pMTVenvANF-Bam and pN2 simply did not express the recombinant *env* at high enough levels, and I attempted to increase expression levels by treating these cells with dexamethasone. After overnight treatment with 10^{-6} M dexamethasone, an increase in expression was seen in the transfected GP101 cells, but no apparent effect was seen on pMTVenvANF expression in the Ψ 2 cells by RT-PCR. This suggested some defect in the RT-PCR assay, at least in the Ψ 2 cells, as alluded to above. Furthermore, given the high sensitivity of the PCR assay and the fact that detectable expression levels of the recombinant *env* were readily achieved in the GP101 cells transfected with either pMTVenvANF-Bam or pMLVenvANF-Bam, this possibility seemed increasingly likely, i.e., that the recombinant *env* was actually being expressed but the RT-PCR assay was not amplifying the recombinant sequence in the Ψ 2 cells, perhaps due to preferential amplification of the shorter wild type sequence. In fact, when the RT-PCR assays were

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subsequently repeated with addition of another 3'-primer, specific for the ANF insert sequence, both GP101 and Ψ 2 cells transfected with pMLVenvANF showed a specific ANF band, suggesting that the recombinant *env* transcript was indeed being made in both cells, even though in Ψ 2 cells the 5'-*env*/3'-*env* primer pair again resulted in amplification of only the smaller band corresponding to the wild type envelope sequence.

In order for the recombinant envelope construct to be incorporated into the viral coat, it is further necessary for the transcript to be translated and the protein transported to the surface of the packaging cell. It is entirely conceivable that altered envelope proteins might be retained in the endoplasmic reticulum and degraded without reaching the cell surface. FACS analysis of the transfected GP101 cells using *env*-specific antibodies, however, showed detectable MLV envelope epitopes on all transfectants. As the untransfected parental GP101 cells have no endogenous *env* gene, this provides direct evidence that the transfected recombinant envelope was being correctly expressed on the cell surface. However, FACS analysis using an ANF-specific antiserum was unable to detect the ANF ligand epitope in any of the GP101 transfectants. Thus it appeared that, despite the presence of the recombinant envelope proteins on the cell surface of transfected GP101 cells as demonstrated by *env*-specific antibody, the ANF ligand was perhaps conformationally inaccessible or otherwise unable to function as a ligand in the context of the larger *env* protein. If true, this result obviously has adverse implications for infection experiments using viruses encoated with these envelope constructs.

The results of the infection experiments indeed proved negative for all attempts at cross species infection of target cells via the ANF receptor. As the packaging cell lines used were ecotropic, this result could be a consequence of (1) complete lack of virus assembly despite the presence of detectable envelope on the packaging cell surface, (2) inadequate levels of overall virus titer, or (3) production of virus which nevertheless does not bind ANF receptor, either because the recombinant envelope protein is not being incorporated into the virus coat despite being present on the packaging cell surface or because the ligand is not accessible or otherwise non-functional despite being present in the virus coat.

The first possibility appeared to be less likely, since viral particles could actually be visualized by electron microscopy, albeit at low abundance. However, the transfected GP101 cells still showed no titer on NIH3T3 cells, suggesting that virus production was extremely low, or that the ability of the viruses produced to infect ecotropically was impaired. The latter could conceivably be the result of introducing the ANF sequence into a structurally important region of the envelope. This could result in low overall expression levels of the envelope in the transfected GP101 cells due to instability of the protein, leading to the production of uncoated virions largely devoid of envelope proteins. Alternatively, introduction of ANF into the envelope could have resulted in adequate expression levels of the protein and encoating of the virions, but impairment of their ability to bind the ecotropic receptor. On the other hand, the transfected Ψ 2 cell lines did show detectable titer on NIH3T3 cells, confirming that viruses were being produced, but this would be expected in any case as these cells already produce the wild type ecotropic envelope. It could not be determined

whether viruses produced by the transfected Ψ 2 cell lines were encoated purely by wild type envelope, or whether they had chimeric envelopes consisting of both the wild type and recombinant ANF-containing envelope proteins. The possibility remains that perhaps the overall expression levels of the recombinant envelope were low, or that the recombinant envelope proteins were not being efficiently incorporated into the virus coat, thus resulting in the production of viruses which are essentially no different from wild type ecotropic viruses. However, if incorporation into the virus coat was not a limiting factor, and the recombinant envelope proteins were present on the virus surface in abundance, the fact that virus produced by the transfected Ψ 2 cells nevertheless could not infect human or bovine target cells bearing the ANF receptor would indicate that the recombinant ecotropic envelope was still incapable of mediating virus entry into non-murine cells despite the presence of the ANF sequence. If this were the case, it could be due to lack of accessibility of the ligand sequence within the conformational context of the envelope protein, as suggested by the FACS analysis results.

As it has been shown that even non-viral proteins on the cell surface can be carried along during the budding process and incorporated into the virus coat [24], I believe that the most likely explanation for these results is that the pMLVenvANF-Bam construct produces an envelope protein which is impaired in its ability to bind the ecotropic receptor, yet still unable to bind the ANF receptor, possibly because the ligand sequence is buried in the protein and inaccessible. Although it has recently been shown that the binding domain for the ecotropic receptor is in the amino-terminal 100 or so amino acids of the envelope protein [25], global conformational effects

resulting from insertions into other parts of the envelope which are important for the overall structural integrity of the protein could also impair receptor binding. In fact, Gray and Roth [26], in a saturation mutagenesis study of the *env* gene published just last year, showed that mutants with linker insertions in the same region as that of the BamH1 site were generally non-viable. This would explain why the transfected GP101 cells produced virus which could not infect NIH3T3 cells, whereas virus from the transfected Ψ 2 cells could still infect NIH3T3 cells via their endogenously produced wild type ecotropic envelope. However, the possibility that the recombinant envelope protein was defective for incorporation into budding virus coats still remains to be formally ruled out.

Although five different constructs were transfected, only pMLVenvANF-Bam was actually tested in infection experiments using ANF receptor bearing target cells. This was because the literature at the time these experiments were initiated suggested that the proline-rich central domain was more important in determining receptor binding, and because the discouraging results obtained from FACS analysis led me to abandon insertional mutant constructs in favor of constructs which swapped large regions of the envelope, described in the following chapters. However, these initial experiments did allow me to demonstrate that it is possible to express recombinant envelope proteins correctly on the surface of packaging cells, given the appropriate construct. Furthermore, and perhaps more importantly, in attempting to interpret the results obtained from these experiments, I realized how important it was to have some means of confirming the actual incorporation of recombinant envelope proteins from the packaging cell surface into the virion coat. In addition, these

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experiments clearly demonstrated the fact that negative results obtained with cross-species infections using ecotropic-based virus can be quite uninformative, as one cannot distinguish between failure of the recombinant envelope protein to encoat the virus, and failure of the virus to bind and enter the non-murine target cell via ligand-receptor interaction despite adequate encoating by the recombinant envelope. Thus I realized that in subsequent experiments, it would be advantageous to create a murine target cell line which has stably integrated the specific target receptor and expresses it at high levels, enabling a comparison of specific ligand-receptor mediated infectivity with the baseline infectivity mediated by wild type ecotropic envelope.

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CHAPTER 3.

Expression of EPO receptor-targeting ligands in amphotropic packaging cells results in a virus showing enhanced infectivity for EPO receptor -bearing target cells.

Introduction

Ligands for targeting retroviruses to the erythropoietin receptor

1. Erythropoietin

As the next targeting ligand for the ecotropic *env* gene, I used the peptide hormone erythropoietin. Erythropoietin (EPO) is a sialoglycoprotein hormone responsible for maintenance of physiological levels of circulating erythrocyte mass by regulating the rate of erythrocyte formation [1]. EPO is produced primarily by the kidney in adult life and by the liver during fetal life, and stimulates erythroid progenitors in the adult bone marrow, fetal liver, and adult spleen, to proliferate and differentiate into mature red blood cells [1].

The human EPO gene has been cloned and codes for a 193-amino acid primary translation product, which includes a 27-amino acid signal peptide at the amino terminus and a 166-amino acid mature protein [2, 3]. The mature hormone contains two disulfide linkages, between residues 7-161 and 29-33 [4], and three N-linked glycosylation sites [2, 3]; though glycosylation seems to be important for the biologic activity of EPO *in vivo* and the glycosylation sites are highly conserved across species, the carbohydrate moiety seems to play no role in the specific interaction between EPO and its receptor *in vitro* [1, 4, 5]. Immunochemical studies of human EPO using site-specific antibodies have localized a putative receptor-binding domain to residues 99-129 [5].

The EPO receptor is expressed exclusively in hematopoietic cells of the erythroid and megakaryocytic lineages, which are dependent on EPO for

their growth and differentiation [1, 6]. A cDNA sequence encoding a high affinity EPO receptor has recently been cloned from murine erythroleukemia (MEL) cells [7]. The receptor is a 507 amino acid polypeptide which binds EPO with a Kd of 30 pM, and can be expressed in COS cells at high levels. However, recent evidence also suggests that there may be an accessory component of the EPO receptor, required for high affinity binding of the ligand, which is present in some cell types but not in others.

The value of using targeting ligands which recognize the EPO receptor lies in its high degree of tissue specificity. The ability to target retroviral vectors specifically to erythroid cells would be useful in future studies aimed at the in vivo treatment of red blood cell disorders at the genetic level. Furthermore, targeting of retroviral infection to erythroid cells would provide the optimal cellular milieu for expression of genes from erythroid-specific promoters or enhancers.

The previous series of pMLVenv-based constructs, in which a sequence encoding 15 amino acids of ANF was directly inserted into various locations in the *env* gene, were proven to be capable of expression in packaging cells. Thus the EPO ligand-containing envelope constructs were created using the pMLVenv vector as well. However, in the previous constructs the ANF ligand did not appear to be recognized on the cell surface as an epitope, and was perhaps buried within the envelope protein or otherwise inaccessible or non-functional as a ligand. In the case of EPO, the mature peptide hormone consists of 166 amino acids, and so it was anticipated that incorporation of a sequence of this size would allow the ligand epitope to be more accessible. On the other hand, the direct insertion of 166 amino acids into the envelope glycoprotein might have deleterious

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effects on its overall conformation. Therefore, rather than direct insertion, I decided to first delete and then replace large stretches of the wild type ecotropic MoMLV envelope protein with the mature EPO peptide sequence. Further support for this sequence replacement strategy comes from the success of "mix-and-match" studies, which have swapped subunits of various receptor proteins or transcription factors with consequent alteration of functions [8, 9]. In fact, the various types of murine leukemia viruses appear to have arisen from recombination events which resulted in swapping of portions of the envelope gene, with concomitant alterations in host range [10]. Conversely, it has been previously shown that insertion of as few as four or five amino acids can be sufficient to completely disrupt the viability of the virus [11]. Thus it was thought to be feasible that deletion of large domains of the envelope with subsequent replacement by the EPO sequence might still result in a functional molecule with the capability to bind the inner envelope protein p15 and to package virus, and additionally confer an altered binding specificity.

Portions of the envelope to be removed and replaced with the EPO coding sequence were therefore selected. In the center of the envelope gp70 protein lies a hypervariable proline-rich region which, at the time these experiments were initiated, was considered to be one of the most likely candidate domains for cell receptor recognition [12, 13]. Other sites of interest included two variable regions which flank a region of homology in the amino-terminal portion of the molecule [14], and sites at which point mutations give rise to a neurotropic, paralytogenic mutant of MoMLV [15]. At the same time, portions of the gp70 sequence which encode the *env* signal peptide at the amino terminus, as well as the cysteine residues in the

boxyl-terminal region which participate in sulfhydryl bonding with the other envelope subunit p15, had to be left intact [10, 12]. Owing to these considerations, two constructs were prepared (Fig. 3-1). In one construct, designated pEPOenv $\Delta 6257$, the EPO ligand replaced the central portion of the gp70 envelope, directly overlapping the proline-rich domain. In the other construct, designated pEPOenv $\Delta 5923$, sequences in the amino terminal region of gp70 including both variable sites A and B were replaced by EPO.

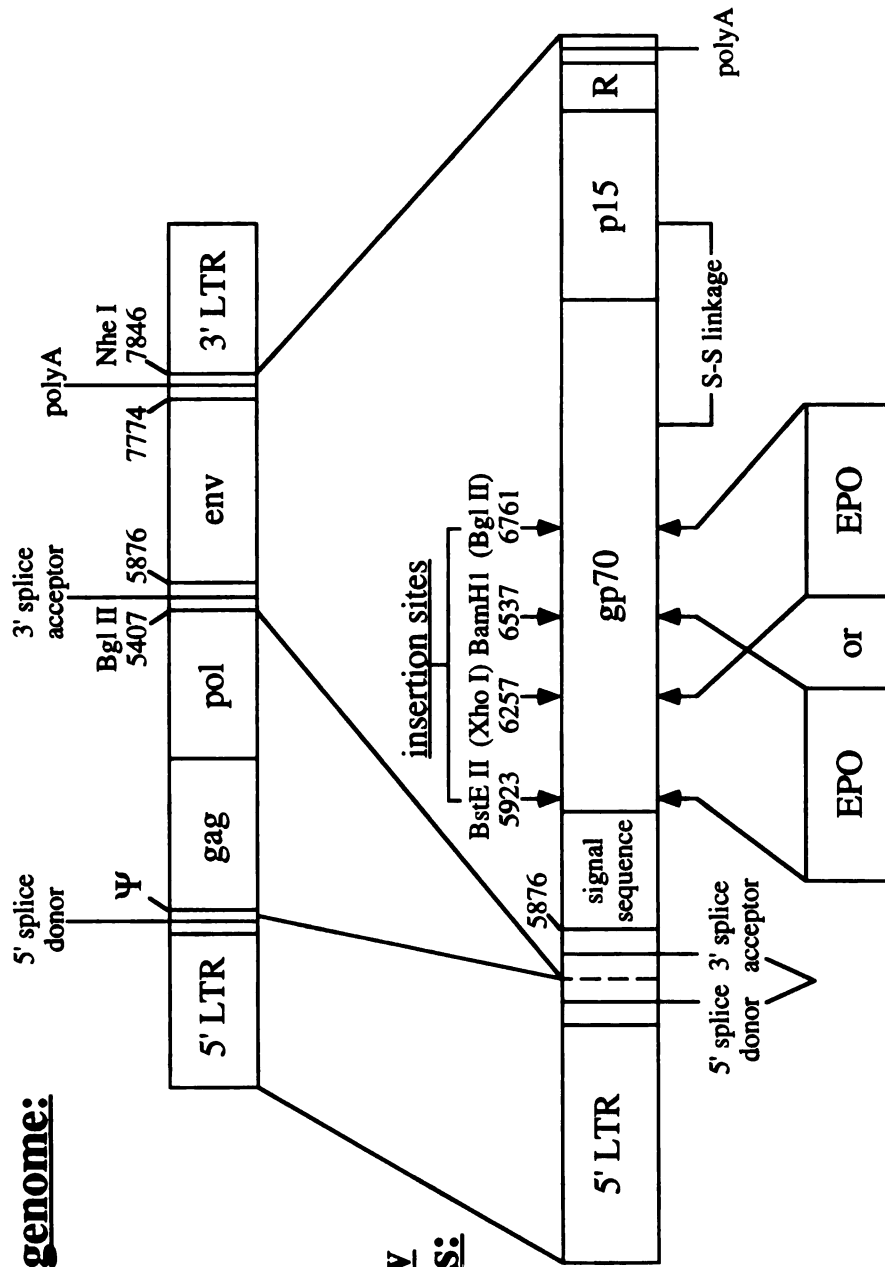
Friend virus glycoprotein gp55

In some cases, naturally-occurring viral envelope proteins are known to bind surface receptors which are specific to certain tissues. One of the best well characterized examples is the HIV envelope glycoprotein gp120, which specifically binds CD4 [16, 17]. Recently, Shimada and Nienhuis [18] developed a retroviral packaging system based on HIV, and demonstrated targeted infection of cells expressing CD4. In the case of myeloid cells, it has been shown that the envelope glycoprotein of the replication-defective Friend spleen focus-forming virus, known as gp55 or gp52, binds directly to the EPO receptor, and that this interaction may be responsible for the MEL cell phenotype [19]; that is, gp55 binding may stimulate the receptor in MEL cells and thus bypass the normal requirement for EPO, resulting in prolonged proliferation and leukemogenesis. The mechanism is thought to involve a continuous intracellular autocrine stimulation process; i.e., the Friend virus gp55 *env* gene integrated into the host genome results in the continuous production of gp55 protein, which binds and activates the EPO receptor intracellularly (probably while

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Figure 3-1. **Location of EPO ligand insertion sites in pMLVenv vector.** The restriction sites used for insertion of the erythropoietin (EPO) ligand sequence are shown in this schematic diagram of the Moloney murine leukemia virus (MoMLV) envelope outer glycoprotein (gp70), which extends from position 5876 to 7183 of the envelope (*env*) gene. The BamH1 site at position 5923 and the BstE II site at position 6537 were used to delete the intervening gp70 sequences at the amino-terminal end of the gene, and replace them with sequences encoding EPO. Xho I and Bgl II sites were created at positions 6257 and 6761, respectively, to replace the intervening gp70 sequences in the proline-rich hypervariable domain, located in the central portion of the gene, with EPO. See text for details.

MoMLV genome:



EPO - env constructs:

still in the endoplasmic reticulum), resulting in an autocrine stimulation of cell division. The gp55 protein appears to have arisen from a recombination event between the parental ecotropic Friend murine leukemia virus and possibly a xenotropic mink cell focus-forming virus in the region of the *env* gene [20]. In gp55 there is also a 585 base pair deletion spanning what would normally be the gp70 C-terminal and p15 N-terminal regions of the *env* gene, resulting in a single transmembrane fusion protein [21] (Fig. 3-2). Since gp55 is known to bind EPO receptor so avidly, it might also serve as a targeting ligand which would direct viral particles to EPO receptor-bearing cells. I therefore initially also attempted to express Friend gp55 in packaging cell lines as a targeting ligand, again to test whether viruses thus packaged would preferentially infect cells expressing EPO receptor.

Creation of retroviral vectors which target the EPO receptor

Development of a system for production of viruses which target the EPO receptor was divided into 4 phases:

- 1) construction of chimeric EPO-*env* genes and Friend gp55 expression vectors,
- 2) preparation and characterization of packaging cell lines that contain these constructs,
- 3) using these cell lines to package replication-defective retroviral vectors containing the neomycin-resistance (neo^R) gene,
- 4) testing the tissue specificity of viruses thus packaged by infection of murine and non-murine cells bearing the EPO-receptor.

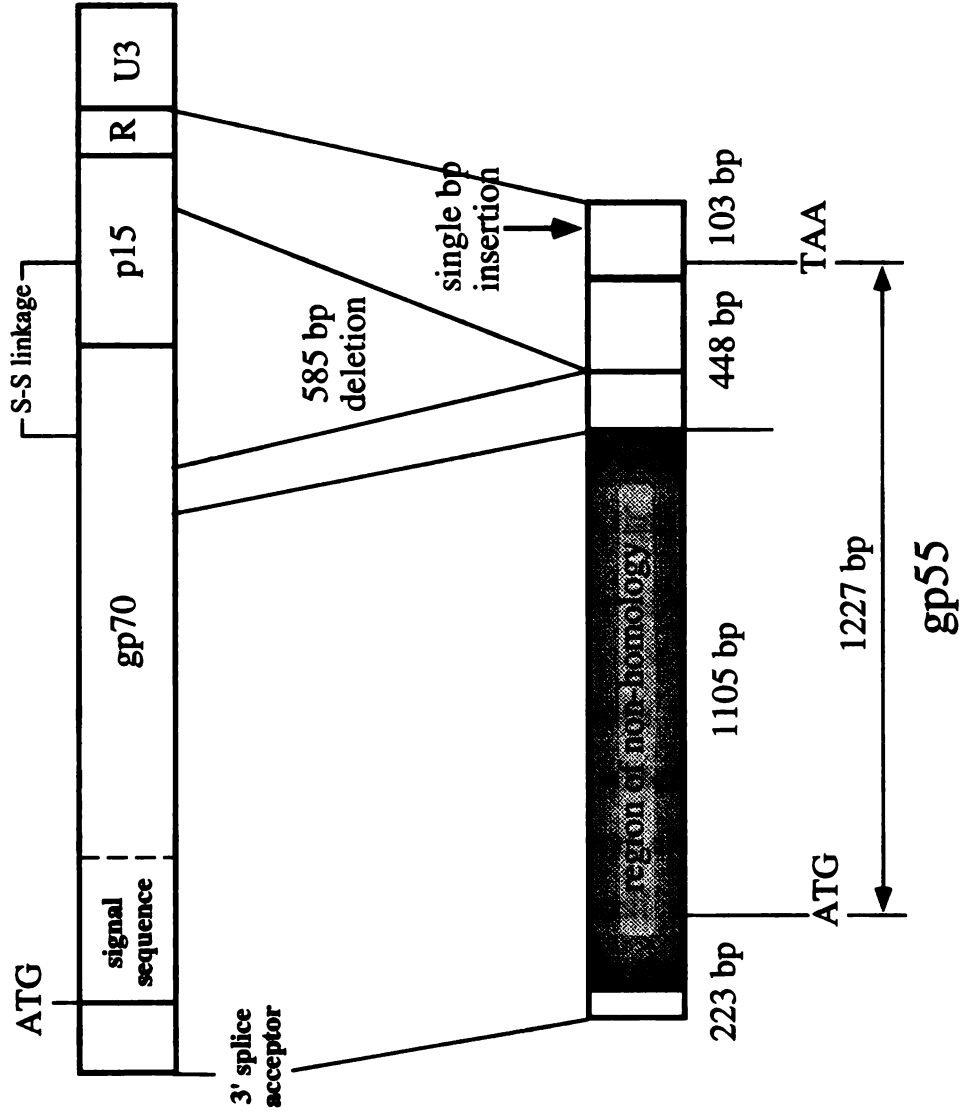
Figure 3-2

Structure of Friend spleen focus-forming virus envelope glycoprotein gp55.

The Friend spleen focus-forming virus (SFFV) envelope glycoprotein (gp55) appears to have arisen through a recombination event between the envelope genes of mink cell focus-forming (MCF) virus and Moloney murine leukemia virus (MoMLV). This has resulted in a chimeric envelope gene which encodes a single chain polypeptide derived from deletion of the carboxy-terminal end of MoMLV gp70 and the amino terminal end of p15, and fusion of the remaining sequences. The amino terminal and central domains of gp70 have been replaced by a 1.3 kb stretch of non-homologous sequence, presumably derived from the MCF virus. This protein, known as SFFV glycoprotein gp55, is known to bind the EPO receptor.

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MoMLV env:



SFFV gp55:

UUCU LIDNHN!

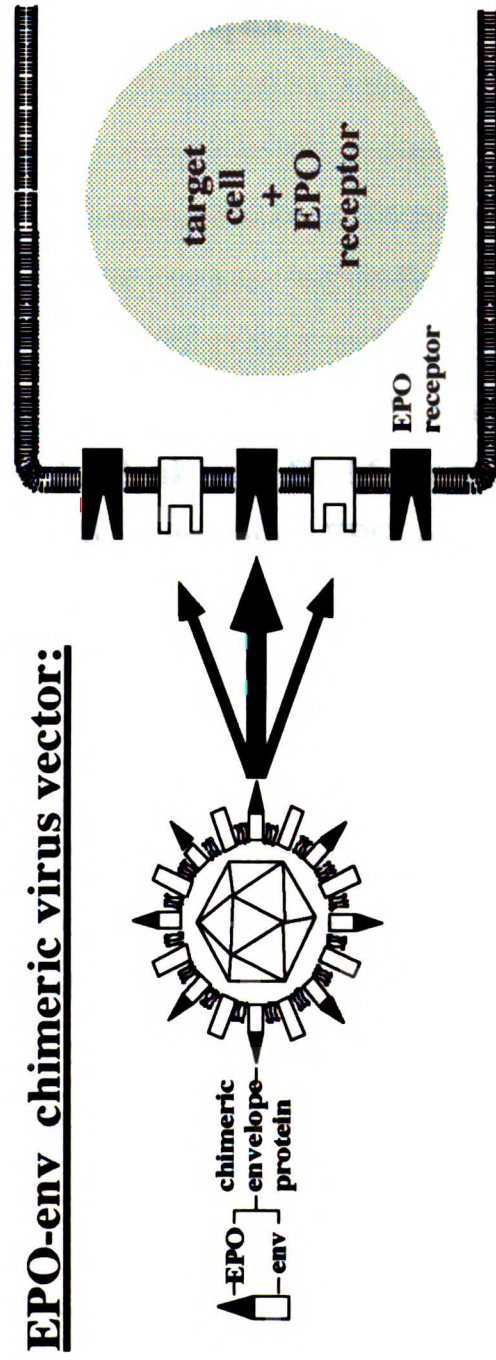
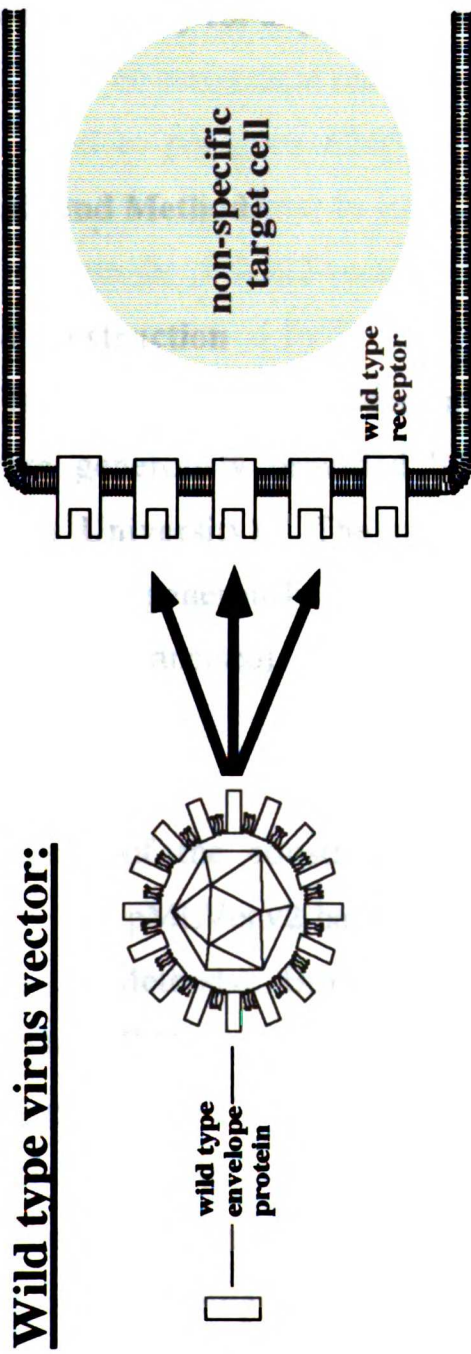
Retrovirus vectors bearing the recombinant envelope with the EPO sequence were thus generated and used to infect murine and non-murine cells which had been stably transduced with the EPO receptor (Fig. 3-3). Recalling the difficulties which were encountered with the use of primary cells such as BASM and HLF as targets, these target cell lines were created from NIH3T3 and HeLa cells, both transformed permanent cell lines. In addition, the use of the untransfected wild type NIH3T3 and HeLa cells as negative control target cells provided a cellular background which was identical to that of the positive target cells except for the additional presence of the transfected EPO receptor, enabling a more reliable comparison of the relative infection efficiency, with fewer variables than if two totally different cell types had been used.

Initially PA317-based packaging cell lines transfected with EPO receptor-targeting constructs were tested extensively because the amphotropic-based viruses produced would always show a baseline level of infection, no matter what species of target cell was used. This was considered to be important because, in the event that incorporation of the EPO sequence rendered viruses thus encoated non-viable or the packaging function of the envelope were somehow compromised and only very low titers were obtained, a negative result would be uninformative unless compared to a baseline level of infection. For example, if an ecotropic-based virus containing the EPO ligand failed to infect HeLa cells transfected with the EPO receptor, it could be due to lack of EPO receptor binding by the chimeric envelope, or it could simply be due to lack of overall titer despite sufficient intrinsic binding capability.

Figure 3-3

Strategy for targeting of amphotropic retroviral vectors to EPO receptor-bearing cells.

Schematic diagram of the strategy for enhancing tissue-specificity by ligand-receptor interaction. If the EPO targeting ligand is placed on an amphotropic virus, this will enhance the affinity of that virus for infection of target cells with the EPO receptor.



In this chapter, I will therefore describe primarily the results obtained from experiments using the PA317 packaging cell line to express EPO receptor targeting envelope constructs and produce viruses which show enhanced infectivity for target cells bearing the EPO receptor.

Materials and Methods

Plasmid construction

Plasmids containing Moloney murine leukemia virus (MoMLV) genes [22] were generously provided by Drs. A. Bank and D. Markowitz (Columbia University). The plasmid pE49f, containing EPO cDNA sequences was generously provided by Dr. K. Shannon (University of California San Francisco).

pEPOenv Δ 6257

Details of the construction of the MoMLV env gene vectors pMLVenv and pMLVenv2 have been described previously (see Chapter 2, Materials and Methods). Briefly, both plasmids contain the entire wild type ecotropic MoMLV *env* gene (from position 5407 to position 7846 of the retroviral genome [12]), cloned directly downstream from the 5'-MoMLV-LTR, which drives transcription of the gene. The *psi* sequence has been deleted, but the vector retains the 5' and 3' splicing signals for the *env* mRNA; the intervening *gag* and *pol* genes, which are normally spliced out of the *env* message [23], were deleted, thus leaving only a small sequence in between the splicing signals remaining to be spliced (see also Fig. 2-5). The

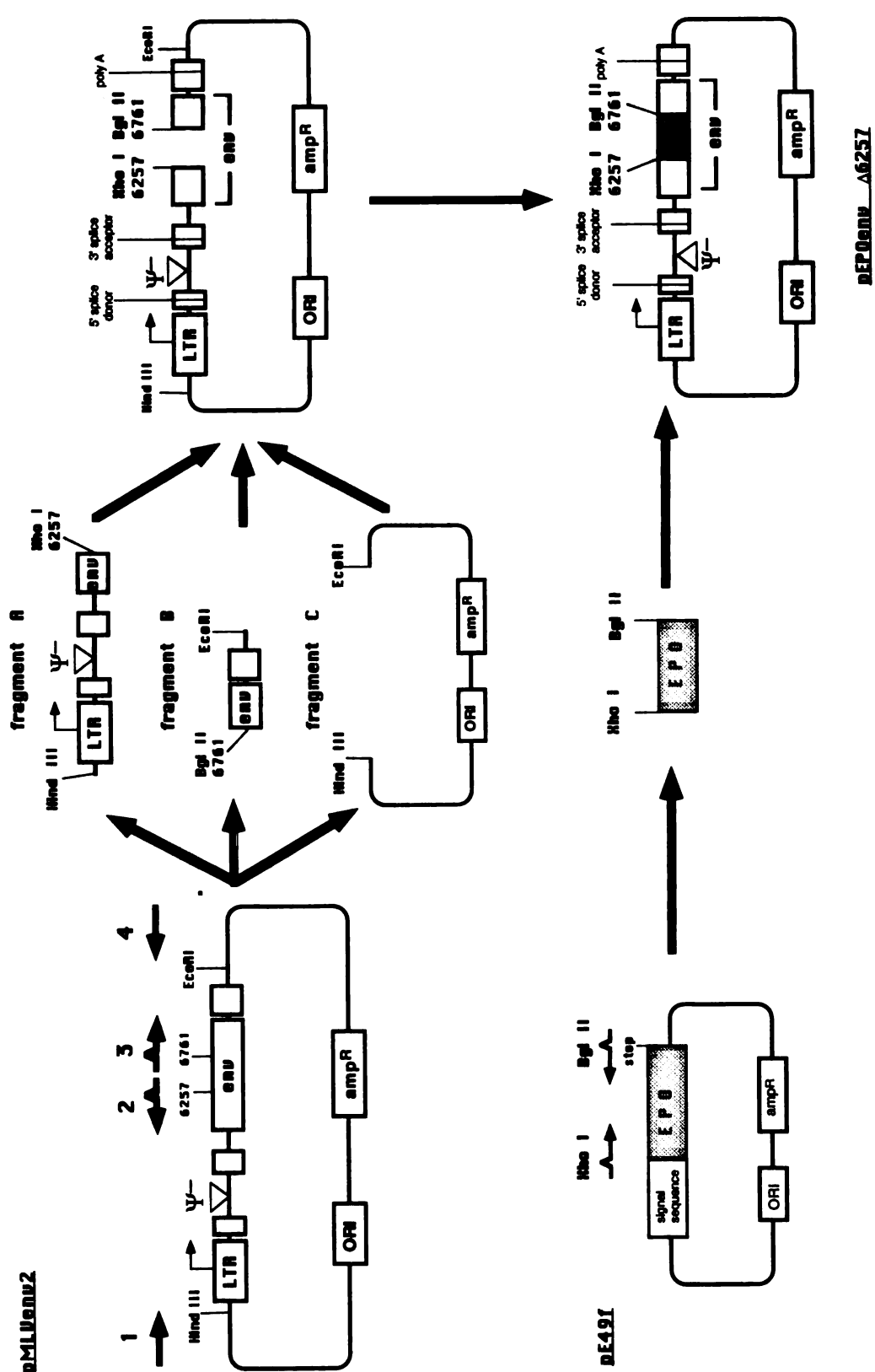
UNIVERSITY OF CALIFORNIA

two plasmids differ only in the amount of pBR322 vector sequence retained (Fig. 2-8).

In this construct the EPO sequence was used to replace *env* sequences in the central region of the molecule, directly overlapping the proline-rich region. Since no convenient restriction sites exist in this region, unique sites were created at positions 6250 and 6750 by means of PCR-mediated mutagenesis [24]. In order to introduce these restriction sites, fragments extending from the pBR322 vector sequence to the *env* gene region flanking the sites were amplified by PCR, using one pBR322 primer and one *env* primer containing mismatches which would create the restriction site, and a 3-piece ligation was done to re-create the entire vector. The primers 5'-GTAAATTGACACTATTTGA-3' and 5'-CATGACTCGAGTTCTCGG-3' were used to amplify a 1.9 kb fragment extending from the pBR322 vector just upstream of the 5' LTR to the *env* gene at position 6269. The underlined nucleotides identify those which were mismatched compared to the wild type *env* sequence in order to introduce an Xho I site at position 6257. This fragment thus contains 5' flanking pBR322 vector sequences which contain a unique Hind III site, the entire 5' LTR, the 5'- and 3'-splicing signals, and one-third of the *env* gene at the amino-terminal end, including the signal sequence (fragment A in Fig. 3-4). The amplified fragment was then cut with Hind II and Xho I, isolated from an agarose gel with DEAE membrane paper, eluted, and purified by ethanol precipitation. Similarly, oligonucleotide primers 5'-AACCTCACCAGATCTGACAAAACC-3' and 5'-GTATCACGAGGCCCTTTCGT-3' were used to amplify a 1.1 fragment extending from the *env* gene at position 6752 to the pBR322 vector just downstream of the gene. Again, the underlined nucleotides identify those which were mismatched compared to the wild type *env* sequence in order to

introduce a Bgl II site at position 6761. This fragment thus contains one-third of the *env* gene at the carboxy-terminal end, including the entire p15 coding sequence, the polyadenylation signal, and 3' flanking pBR322 vector sequences which contain a unique EcoR I site (fragment B in Fig. 3-4). This fragment was cut with Bgl II and EcoR I, and isolated as above. Finally, pBR322 vector itself was cut with Hind III and EcoR I (fragment C in Fig. 3-4), isolated, and ligated with the two amplified fragments to re-create the *env* gene expression vector with deletion of the sequences between the newly created Xho and Bgl sites at 6257 and 6761 respectively.

The EPO cDNA sequence coding for the mature 166-amino acid peptide hormone without the 27 amino acid signal peptide [2-4], was then used to replace the MoMLV *env* sequences which had been deleted in the central region of the envelope protein. Again, PCR-mediated mutagenesis was used to create compatible ends on the EPO sequence, so that it could be inserted via the appropriate restriction sites while maintaining the proper reading frame. The 5'-primer 5'-CCCAGTCCTCGAGGCCCCACCACGC-3' and the 3'-primer 5'-GGACACACCTGGAGATCTGTCCCCTGT-3' were used to introduce Xho I and Bgl II sites, respectively, via mismatches at the underlined nucleotides. The Bgl II site mutagenizes the stop codon while maintaining the reading frame with respect to *env*. These primers were used to amplify EPO sequence from the plasmid pE49f, and the amplified product was cut, purified by ethanol precipitation, and ligated to the vector prepared above (Fig. 3-4). *E. coli* strain TG1 transformants were screened by restriction digest of mini-preps, positive clones were confirmed by sequencing, and large scale cultures were purified by cesium chloride gradient ultracentrifugation. This construct was designated pEPOenv Δ6257.



pEPOenv Δ5923 construct

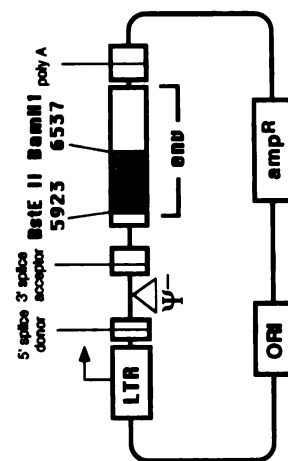
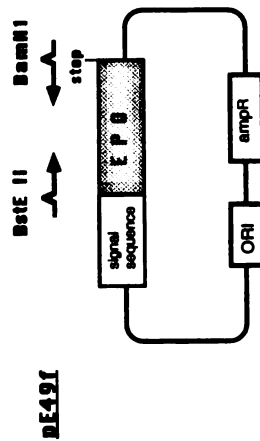
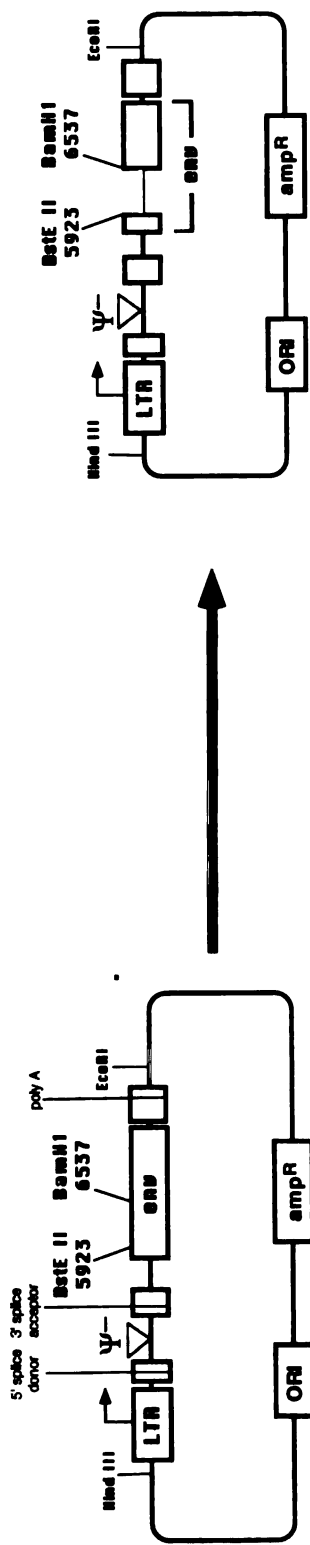
Unique restriction sites exist at position 5923 (BstEII) and position 6537 (BamHI) of the *env* gene; deletion of the envelope sequence between these two sites preserved the *env* signal sequence but allowed the EPO sequence (approximately 500 base pairs in length) to be inserted, in frame, at the amino-terminal end of gp70.

The EPO cDNA sequence encoding the mature 166-amino acid peptide hormone without the 27 amino acid signal peptide, was used to replace the MoMLV *env* sequences which had been deleted. An EPO sequence with compatible ends was created by PCR-mediated mutagenesis, with 5'-GGCCTCCCAGTGGTAACCGCCCCACCACGC-3' as the 5'-primer and 5'-GGACACTCCTGGGATCCTGTCCCCTGTCCT-3' as the 3'-primer, so that it could be inserted via the appropriate restriction sites while maintaining the proper reading frame, followed by restriction digest of the PCR product with BstE II and Bam H1. The ligated product was used to transform *E. coli* strain TG1, positive clones were identified and confirmed as above, and large scale cultures were purified by cesium chloride gradient centrifugation. This construct was designated pEPOenv Δ5923 (Fig. 3-5).

pFR400

The plasmid pFR400, which contains the methotrexate (MTX)-resistant dihydrofolate reductase (*dhfr*) gene [25], has been described previously (see Chapter 2, Materials and Methods). This plasmid was co-transfected into packaging cells with the targeting envelope constructs, as a selectable marker.

pMLUanuv2



pE49I Δ5923

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pXMgp55

This plasmid, provided by Dr. Alan D'Andrea (Dana Farber Cancer Institute), contains the gene encoding Friend spleen focus-forming virus envelope glycoprotein gp55 [26-28], cloned into the eukaryotic expression vector pXM. Transcription of the gp55 gene is driven by the adenovirus major late promoter, and splice and polyadenylation signals are provided by dihydrofolate reductase gene sequences downstream [7]. This plasmid was also grown in *E. coli* strain TG1, and large scale cultures were purified as above.

pCRIP-SVlac:

The plasmid pCRIP-SVlac is a modification of the replication-defective retroviral vector pCRIPdhfr provided by Dr. R. Scharfmann (Salk Institute). The original vector pCRIPdhfr contains a *psi* packaging signal, a neomycin resistance gene driven by the 5'-LTR, and the β -galactosidase gene driven by the dihydrofolate reductase promoter [29]. However, since the 5'-LTR appeared to cause silencing of the internal promoter, as evidenced by a large percentage of G418-resistant but β -galactosidase-negative colonies after selection of stably transfected packaging cells (data not shown), I sought to replace the original promoter with a stronger one. Therefore, I deleted the dihydrofolate reductase promoter by restriction digest of a unique *Stu* I site at the 5' end of the promoter, which results in a blunt end, and a unique *Hind* III site at the 3' end. To replace the deleted internal promoter, I inserted a fragment containing the SV40 origin of replication and promoter sequences, derived from the plasmid pCH110 (Clontech) by digestion with *Pvu* II (which also results in a blunt end) and *Hind* III. *E. coli* strain TG1 transformants were screened by restriction

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digest, and large scale cultures of positive clones were purified by cesium chloride gradient ultracentrifugation. This plasmid was designated pCRIP-Vlac, and used for transfection of packaging cells to produce replication-effective virus vectors.

XM EPO-R

The plasmid pXM EPO-R (generously provided by Dr. A. D'Andrea Dana Farber Cancer Institute) contains the murine EPO receptor cDNA [7] cloned into the mammalian expression vector pXM, described above. This plasmid was grown and purified as above, and used for transfection of NIH3T3 and HeLa cells to produce target cell lines stably expressing the EPO receptor.

packaging cell lines

The amphotropic packaging cell line PA317 [30] was maintained in improved modified Eagle's (IME) medium (Biofluids) containing 10% fetal bovine serum (FBS) and 100 μ g/ml penicillin/streptomycin (PS). To create amphotropic packaging cell lines expressing the targeting envelope constructs, 1×10^5 PA317 cells were co-transfected with 20 μ g of pEPOenv 6257, pEPOenv Δ 5923, or pXMgp55 respectively, and 1 μ g of pFR400. All transfections were performed in 60-mm dishes using the calcium phosphate precipitation method [31, 32]. The medium was changed 24 hours later, and 48 hours post-transfection the cells were trypsinized, replated at 1:10 dilution in 100-mm plates, and subsequently selected with gradually increasing concentrations of MTX (Sigma) ranging from 0.2 μ M to 10 μ M, at which point subclones were isolated from the surviving colonies and characterized (Fig. 3-6).

Western blot analysis

Subclones were grown to confluence in 100-mm plates, washed with phosphate-buffered saline (PBS), and lysed in 4% SDS/10% glycerol/10% β -mercaptoethanol/50 mM Tris (pH 6.7), boiled for 10 minutes, and stored frozen at -20°C until analyzed. After thawing, the samples were boiled again briefly, separated by electrophoresis on an 8% SDS-polyacrylamide gel, and electro-transferred to nitrocellulose (Hybond ECL; Amersham). The filters were then blocked in TBST buffer (0.02% Tween-20/150 mM NaCl/50 mM Tris (pH 7.5)) with 5% dried milk (BioRad) at room temperature for 1 hour, or in some cases, at 4°C overnight. Filters from packaging cells transfected with pEPOenv Δ 6257 or pEPOenv Δ 5923 were incubated with a monoclonal anti-EPO antibody (Genzyme) as the primary antibody, followed by washing in TBST buffer with 0.5% dried milk and incubation with a horseradish peroxidase-conjugated goat anti-mouse antibody (CalTag) as the secondary antibody. Filters from packaging cells transfected with pXMgp55 were incubated with a primary polyclonal goat anti-*env* antiserum (Microbiological Associates), followed by washing and incubation with a secondary horseradish peroxidase-conjugated swine anti-goat antibody (CalTag). After washing again, the filters were incubated in ECL chemiluminescent immunodetection reagents (Amersham) and exposed to film. The anti-*env* antiserum also cross-reacts with MoMLV gp70, and so filters from the pEPOenv Δ 6257- or pEPOenv Δ 5923-transfected cell lines were also re-blocked and re-probed using the anti-*env* antiserum as the primary antibody, followed by horseradish peroxidase-conjugated swine anti-goat as the secondary antibody.

DNA and RNA analyses

Subclones were grown to confluence in 100-mm plates; genomic DNA from these clones was isolated by lysis of 10^6 cells with 0.65% NP-40/0.01M Tris (pH 7.8)/0.15M NaCl/1.5mM $MgCl_2$, centrifugation of the lysate at 2000 rpm for 10 min at 4°C to pellet the nuclei, followed by overnight digestion of the nuclear pellets with 0.5 mg/ml proteinase K, phenol/chloroform extraction and ethanol precipitation.

For Southern blot analysis, approximately 10 μ g of DNA per sample was digested with EcoR I, ethanol precipitated, size-fractionated on a 1% agarose gel, and transferred through 0.4N NaOH/1M NaCl to a nylon membrane (Hybond N; Amersham) in 20x SSC. The membrane was hybridized to a 500 bp EPO probe generated by PCR and labelled with digoxigenin (Boehringer Mannheim) at 42°C overnight, and washed two times at room temperature in 2 $\times$ saline sodium citrate (SSC), 0.1% SDS, and two times at 65°C in 0.5 $\times$ SSC, 0.1% SDS. Detection was performed by incubation of the membrane with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and AMPPD (Tropix), and exposure to film at room temperature.

Cytoplasmic RNA from the cells was isolated by lysis of the cells in the NP40-containing buffer and centrifugation as described above, after which urea buffer (7M urea/10mM Tris (pH 7.4)/10mM EDTA/0.35M NaCl/1% SDS) was added to the supernatant, followed by phenol extraction and ethanol precipitation of the RNA.

For PCR analysis of cDNA reverse-transcribed from cytoplasmic RNA (RT-PCR), approximately 5 μ g of cytoplasmic RNA per sample was first denatured at 95°C for 2-3 min, followed by annealing of 20 pmoles of a (–) strand primer per 20 μ l reaction, at 65°C for 5 min, in the presence of 40U of RNasin (NEBL). The primer consisted of (–) strand sequences corresponding to the downstream EPO-*env* recombinant joint. After annealing, an additional 40U of RNasin was added, along with 10mM of dNTPs and 20U of AMV reverse transcriptase (BRL) per 20 μ l reaction in RT buffer containing 40 mM KCl/50mM Tris (pH 8.3)/8 mM MgCl₂/0.4mM DTT (final concentration), and the reaction allowed to proceed at 42°C for 1 hour.

One half of this reaction (10 μ l) was then used for PCR, using the same (–) strand primer used for reverse transcription as the 3' primer, and a 5' primer corresponding to sequences spanning the upstream EPO-*env* recombinant joint. The PCR reactions were begun with denaturation at 95°C for 2–3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 20 sec, and elongation at 72°C for 30 sec. PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. In some experiments, the PCR products were transferred to a nylon membrane (Hybond, Amersham) after electrophoresis, and hybridized to a 500 bp EPO probe generated by PCR and labelled with digoxigenin (Boehringer Mannheim). Detection with alkaline phosphatase-conjugated antibody (Boehringer Mannheim) and AMPPD (Tropix) was performed as described above.

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Fluorescence-activated cell sorting (FACS) analysis.

Polyclonal anti-EPO antiserum #8C295 generously provided by Dr. J. Egrie (AmGen) and fluorescein-conjugated secondary antibodies (CalTag) were used for flow cytometry, using a Becton-Dickinson FACScan and FACS IV cell sorter. Cells were grown to confluence on 100-mm plates, detached and dispersed with phosphate-buffered saline (PBS) containing 2 mM EDTA, and pre-incubated with normal goat serum (CalTag) in PBS containing 2% bovine serum albumin (BSA) for 30-60 min on ice. After washing twice in PBS with 2% BSA, the cells were incubated with pre-immune rabbit serum (CalTag) or the polyclonal EPO-specific rabbit antiserum (#8C295; AmGen) diluted in PBS with 2% BSA for 30-60 min, washed again repeatedly, incubated with fluorescein-conjugated goat anti-rabbit antiserum (CalTag), washed again, and subjected to flow cytometry after addition of propidium iodide. In some experiments, a molar excess of soluble EPO peptide (Amgen) was added at the time of primary anti-EPO antiserum incubation, to compete for specific binding.

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Target cells and infection experiments

Target cells were created by co-transfection of HeLa and NIH3T3 cells with the plasmids pXM EPO-R and pFR400 by means of the calcium phosphate precipitation method, followed by selection in increasing concentrations of MTX. HeLa and NIH3T3 cells were maintained in

1125 1200

DMEM supplemented with 10% FBS and 100 $\mu\text{g/ml}$ PS until transfection. Stably transfected subclones were isolated at a selection stage of 10 mM MTX, and screened for expression of the EPO receptor by assaying binding of ^{125}I -labelled EPO peptide (Amersham); target cells were grown to confluence in 24-well plates, and subsequently incubated in DMEM with 2% bovine serum albumin (BSA) and approximately 10^6 cpm of radiolabelled EPO, with or without excess cold EPO (AmGen), for 90 min. at 37°C in a humidified incubator, washed with PBS, lysed in 1N NaOH, and counted in a gamma counter. The approximate EPO receptor number per cell was estimated based on the specific activity of the radiolabelled EPO.

Infectious virus was generated from pEPOenv-transfected PA317 packaging cells by transient transfection with the replication-defective retroviral vector pCRIP-SVlac. The packaging cells were incubated in non-selective culture medium (DMEM/10% CS/100 $\mu\text{g/ml}$ PS) prior to transfection with pCRIP-SVlac, using the calcium phosphate precipitation method. The medium was changed after 24 hours, and the virus-containing medium was filtered through a 0.2 μm filter into medium containing 8 $\mu\text{g/ml}$ of polybrene and used to infect the target cells 48 hours after transfection. The target cells were subsequently trypsinized and re-plated at low density, selected in 400 $\mu\text{g/ml}$ G418 (Sigma), and the number of surviving G418-resistant colonies was counted after fixation with methanol/acetone (1:1) and Giemsa staining.

Results

Recombinant envelope constructs pEPOenv $\Delta 6257$ and pEPOenv $\Delta 5923$ can be successfully expressed in PA317 cells.

To introduce the peptide hormone erythropoietin (EPO) into the ecotropic MoMLV envelope, a portion of the MoMLV envelope (*env*) gene [12] in the central portion or at the amino-terminal end of gp70 was removed and replaced, in frame, with sequences coding for EPO [2, 3]; these constructs were designated pEPOenv $\Delta 6257$ and $\Delta 5923$, respectively. Portions of the gp70 sequence which encode the *env* signal peptide at the amino terminus, as well as the cysteine residues in the carboxyl-terminal region which participate in sulfhydryl bonding with the inner envelope subunit p15, were left intact (Fig. 3-1). Each of these EPO-*env* hybrid constructs was co-transfected with the plasmid pFR400, containing the methotrexate (MTX)-resistant dihydrofolate reductase (*dhfr*) gene [25], into PA317 packaging cells [30] by calcium phosphate precipitation. The transfected cells were selected with increasing concentrations of MTX ranging from 0.2 μM to 10 μM , and subclones were isolated and screened at each selection stage (Fig. 3-6).

These subclones were first screened for production of the EPO ligand-containing envelope glycoprotein recombinant by Western blot, using a monoclonal EPO-specific antibody. Several of the subclones transfected with pEpoenv $\Delta 6257$ (Fig. 3-7) or pEpoenv $\Delta 5923$ (Fig. 3-8) showed a specific 70 kD band. The same band was visualized after re-probing the Western blot with polyclonal anti-*env* antiserum, demonstrating co-expression of both epitopes in the same 70 kD protein (Fig. 3-8 B). Specific subclones showing positive bands were chosen for further characterization.

Figure 3-6

Protocol for transfection of EPOenv constructs into packaging cells.

The targeting constructs were each co-transfected into both Ψ 2 cells and PA317 cells along with the plasmid pFR400, which contains the methotrexate (MTX) -resistant dihydrofolate reductase (dhfr) gene as a selectable marker. Independent subclones were isolated at MTX selection levels of 0.2, 2, and 10 μ M, and the presence of the transgene was determined by Southern blot, and expression levels were characterized by Western blot, RT-PCR, and FACS analysis. See text for details.

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Creation of packaging cell lines expressing targeting ligands:

Ψ2 cells (Ψ^+ , gag $^+$, pol $^+$, ecotropic env $^+$)

or

PA317 cells (Ψ^+ , gag $^+$, pol $^+$, amphotropic env $^+$)

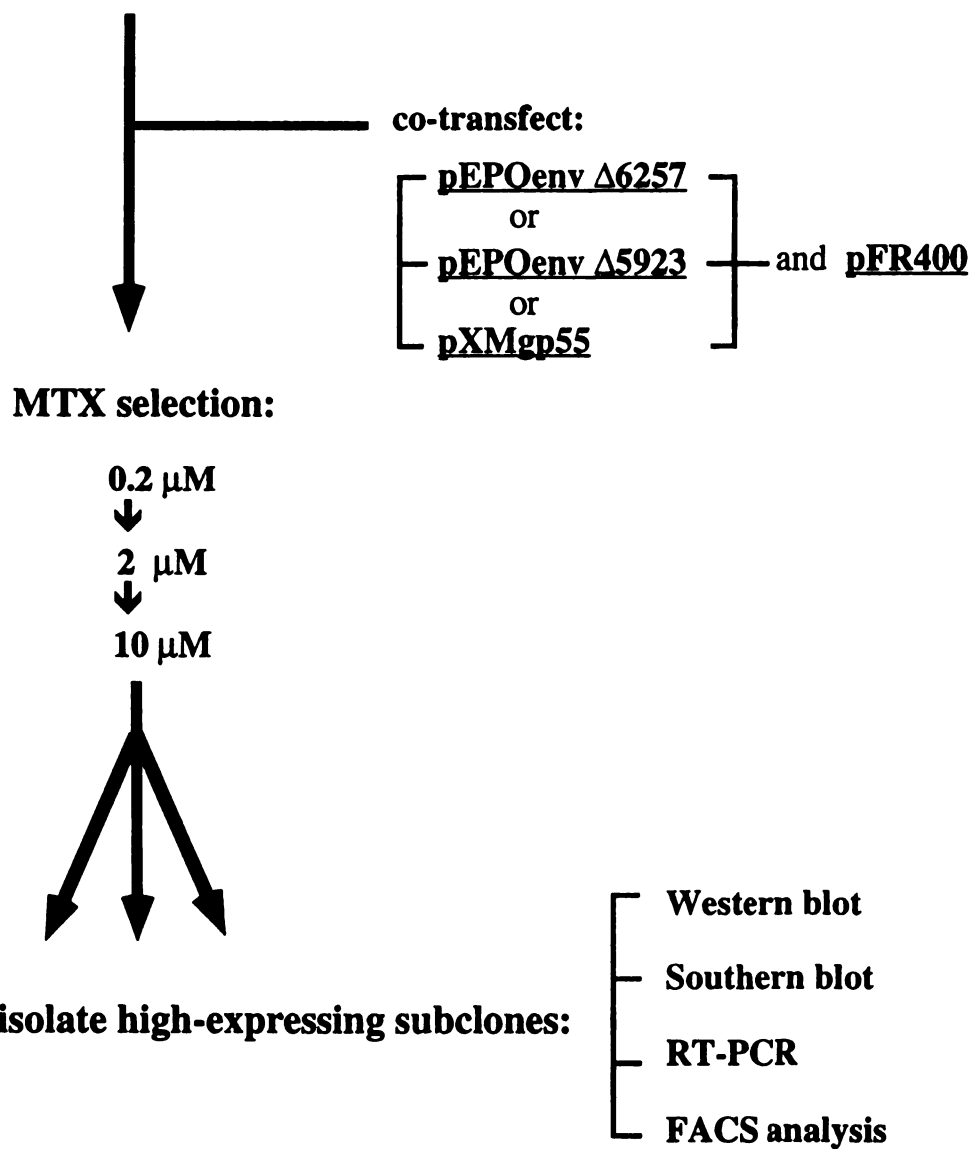


Figure 3-7. Western blot analysis of PA317 cell subclones stably transfected with pEPOenv Δ 6257.

Panel A: PA317 cells transfected with pEPOenv Δ 6250 and selected in 0.2 μ M MTX. Protein extracts from individual subclones were analyzed by Western blot using a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase-conjugated goat-anti-mouse antibody as the secondary antibody.

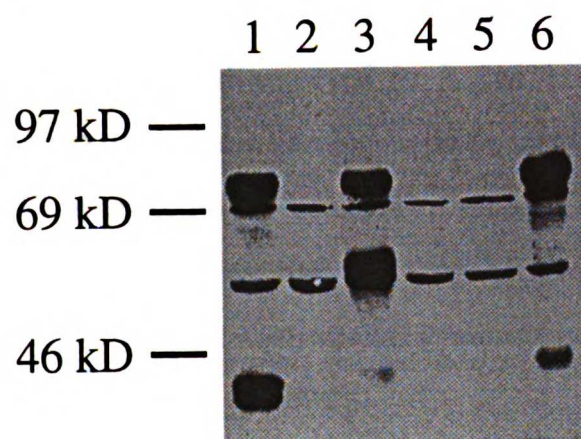
1: subclone 1C. 2: subclone 2B. 3: subclone 2C. 4: subclone 3A. 5: subclone 3B. 6: subclone 3C.

Panel B: PA317 cells transfected with pEPOenv Δ 6250 and selected in 2 and 10 μ M MTX. Protein extracts from pooled cells or individual subclones were analyzed by Western blot using a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase-conjugated goat-anti-mouse antibody as the secondary antibody.

1: 2 μ M MTX pool. 2: 10 μ M MTX subclone 1-1. 3: 10 μ M MTX subclone 1-2. 4: 10 μ M MTX subclone 1-4. 5: 10 μ M MTX subclone 3-1. 6: 10 μ M MTX subclone 3-2. 7: 10 μ M MTX subclone 3-3.

140

A.



B.

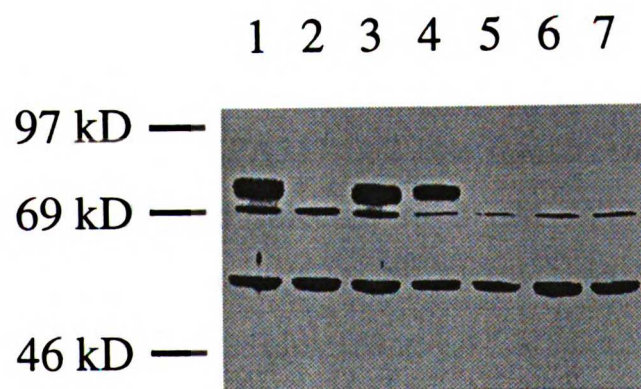


Figure 3-8. Western blot analysis of PA317 cell subclones stably transfected with pEPOenv Δ 5923.

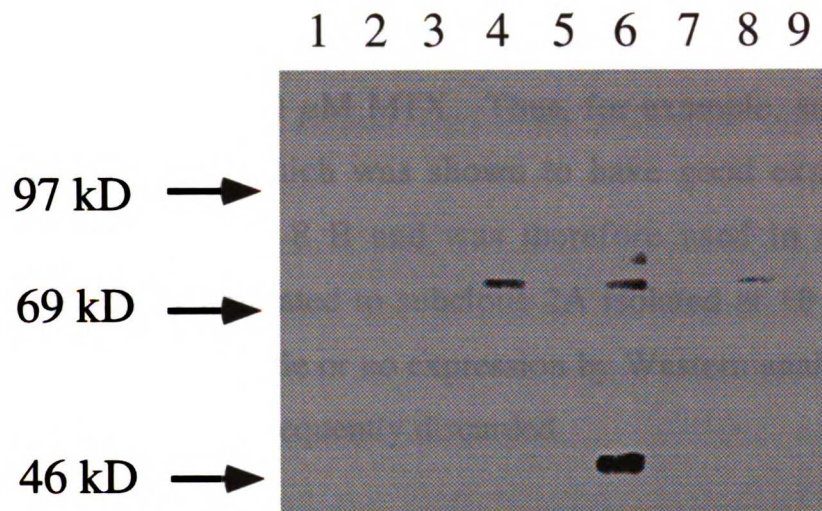
Panel A: PA317 cells transfected with pEPOenv Δ 5923 and selected in 2 and 10 μ M MTX. Protein extracts from pooled cells or individual subclones were analyzed by Western blot using a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase-conjugated goat-anti-rabbit antibody as the secondary antibody.

Lane 1: 10 μ M MTX subclone 2C. Lane 2: 10 μ M MTX subclone 2B. Lane 3: 10 μ M MTX subclone 2A. Lane 4: 2 μ M MTX subclone 4C. Lane 5: 2 μ M MTX subclone 4B. Lane 6: 2 μ M MTX subclone 4A. Lane 7: 2 μ M MTX pool 4. Lane 8: 10 μ M MTX pool 4. Lane 9: 10 μ M MTX subclone 3C.

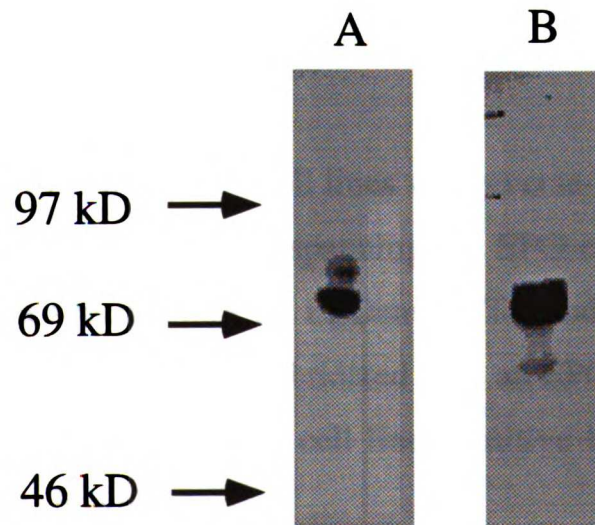
Panel B: PA317 cell line transfected with pEPOenv Δ 5923 and selected in 2 μ M MTX, subclone 2A. Protein extract from 2 μ M subclone 2A was analyzed by Western blot using (A) a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase (HRP)-conjugated goat-anti-mouse antibody as the secondary antibody, or (B) a polyclonal anti-env antibody as the primary antibody and HRP-conjugated swine anti-goat antibody as the secondary antibody.

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A.



B.



It should be noted that independent subclones were isolated at each selection stage; thus, selection plates were prepared in triplicate, and once colonies were formed at 0.2 μ M MTX, at least 5 colonies were isolated from each plate, the remaining colonies were trypsinized and pooled, and again plated in triplicate plates and placed under 2 μ M MTX selection, and so on, up to a selection level of 10 μ M MTX. Thus, for example, subclone 2A isolated at 2 μ M MTX, which was shown to have good expression by Western analysis in Fig. 3-8 B and was therefore used in subsequent experiments, is totally unrelated to subclone 2A isolated at 10 μ M MTX, which was shown to have little or no expression by Western analysis in Fig. 3-8 A and was therefore subsequently discarded.

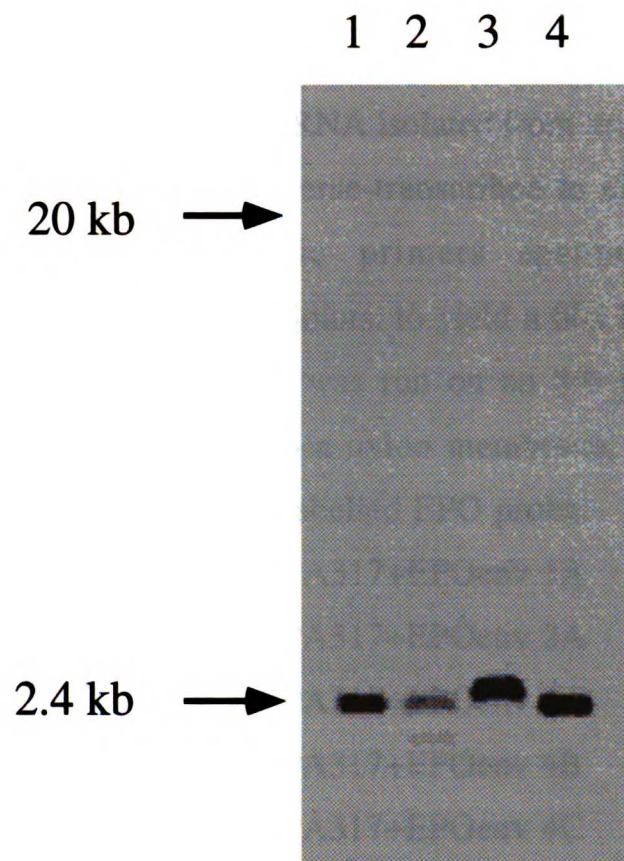
Genomic DNA from selected packaging cell lines was digested with EcoR1 and the Southern blot was hybridized with a digoxigenin-labelled EPO probe, which revealed the expected 2.4 kb restriction fragment containing the recombinant EPOenv sequence. This band was observed in all cell lines which had been positive by Western blot (Fig. 3-9 and data not shown).

Messenger RNA from selected cell lines was reverse-transcribed, and then amplified by PCR, using primers spanning the EPO-env recombinant joints. This yielded a specific 603 bp EPOenv PCR product, which was transferred to a nylon membrane and hybridized with an EPO probe. Again, the expected band was observed in all cell lines positive by Western blot (Fig. 3-10 and data not shown).

Figure 3-9. Southern blot analysis of PA317 cell subclones stably transfected with pEPOenv Δ 5923.

Genomic DNA, from subcloned PA317 cell lines transfected with pEPOenv Δ 5923 and selected at 2 μ M MTX, was digested with EcoR I and hybridized with a digoxigenin-labelled 504 bp EPO probe, which revealed the expected 2.4 kb restriction fragment containing the recombinant EPOenv sequence.

Lane 1: 2 μ M subclone 2A. Lane 2: 2 μ M subclone 4A. Lane 3: 2 μ M subclone 4B. Lane 4: 2 μ M subclone 4C.



1000 100 10 1

Figure 3-10. **RT-PCR analysis of PA317 cell subclones stably transfected with pEPOenv Δ 5923.**

Cytoplasmic RNA isolated from selected packaging cell lines was reverse-transcribed to cDNA and amplified with specific primers spanning the EPO-env recombinant joints, to yield a 603 bp EPOenv product. The reaction was run on an 8% polyacrylamide gel, transferred to a nylon membrane, then probed with a digoxigenin-labelled EPO probe.

Lane 1: PA317+EPOenv 1A
2: PA317+EPOenv 2A
3: PA317+EPOenv 4A
4: PA317+EPOenv 4B
5: PA317+EPOenv 4C

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דוד אבינו

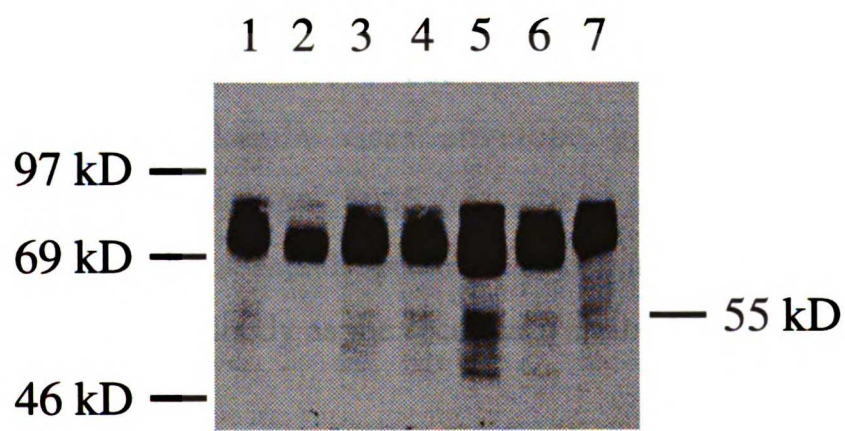


The Friend virus mutant envelope gp55 can be expressed in PA317 cells, but is not transported to the cell surface efficiently.

PA317 cells transfected with pXMgp55 were selected with increasing concentrations of MTX ranging from 0.2 mM to 10 mM, and subclones were isolated and screened for production of the gp55 envelope glycoprotein by Western blot, using a polyclonal anti-*env* antiserum, which cross reacts with both MoMLV gp70 and Friend gp55 [19]. Some of the transfected subclones showed a specific 55 kD band (Fig. 3-11).

It was interesting to note that when the gp55 construct was transfected into GP101 cells, however, which do not express endogenous envelope, no expression of gp55 could be detected in any of the subclones tested (Fig. 3-12 A). On the other hand, when the construct was transfected into Ψ 2 cells, gp55 protein was again detectable in some subclones (Fig. 3-12 B). This suggested that gp55, a mutated form of envelope glycoprotein, might require wild type envelope proteins to be expressed concurrently in order to be expressed itself. It is possible that the presence of "normal" wild type envelope proteins somehow rescues the mutated gp55 protein from degradation. However, it was subsequently found that even if gp55 proteins are synthesized, they are not efficiently transported to the cell surface, and for the most part retained in the endoplasmic reticulum (D. Kabat, personal communication; see also [21]). Thus this construct was abandoned as a targeting ligand in subsequent experiments.

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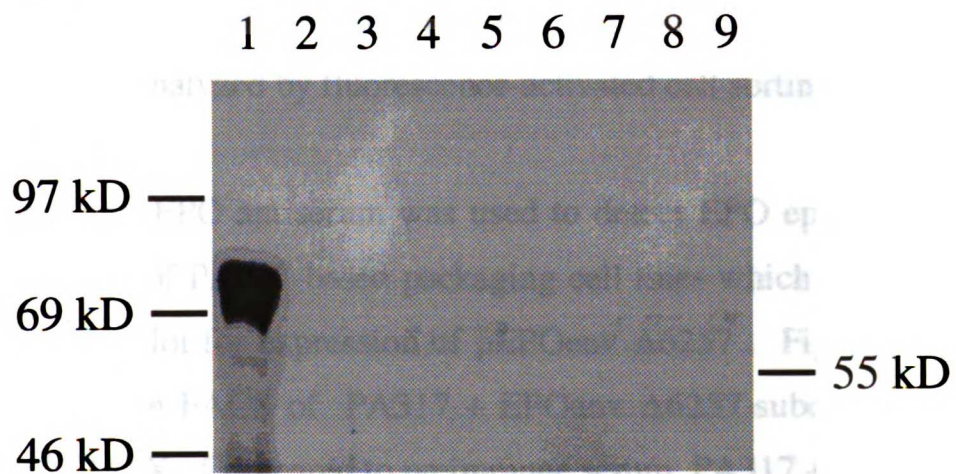
Figure 3-12. Western blot analysis of GP101 and Ψ2 cell subclones stably transfected with Friend gp55.

GP101 and Ψ2 cells were transfected with Friend virus envelope gp55 and selected in 10 μM MTX. Protein extracts from individual subclones were analyzed by Western blot using a polyclonal goat anti-*env* antibody, which cross-reacts with both Moloney murine leukemia virus envelope gp70 and Friend virus envelope gp55, as the primary antibody, and horseradish peroxidase-conjugated swine-anti-goat antibody as the secondary antibody.

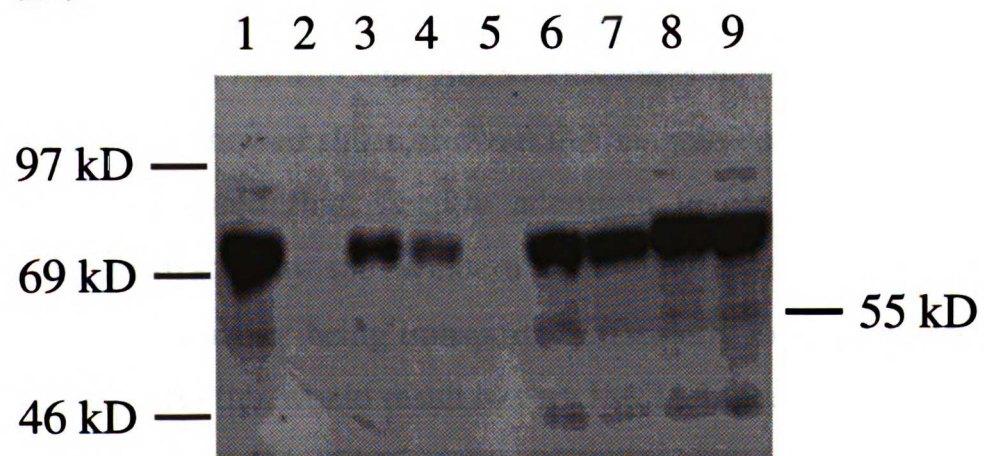
Panel A: GP101 cell subclones transfected with gp55. Lane 1: wild type Ψ2 cell control. Lane 2: GP101 + gp55 subclone 1. Lane 3: subclone 2. Lane 4: subclone 3. Lane 5: subclone 4. Lane 6: subclone 5. Lane 7: subclone 6. Lane 8: subclone 7. Lane 9: subclone 8.

Panel B: Ψ2 cell subclones transfected with gp55. Lane 1: Ψ2 + gp55 subclone 1. Lane 2: subclone 2. Lane 3: subclone 3. Lane 4: subclone 4. Lane 5: subclone 5. Lane 6: subclone 6. Lane 7: subclone . Lane 8: subclone 8. Lane 9: subclone 9.

A.



B.



Packaging cells expressing pEPOenv Δ 6257 by Western blot show little expression on the cell surface.

To ascertain whether the chimeric pEPOenv Δ 6257 envelope protein is transported to and oriented correctly in the cell membrane, the transfected packaging cells were analyzed by fluorescence-activated cell sorting (FACS) analysis.

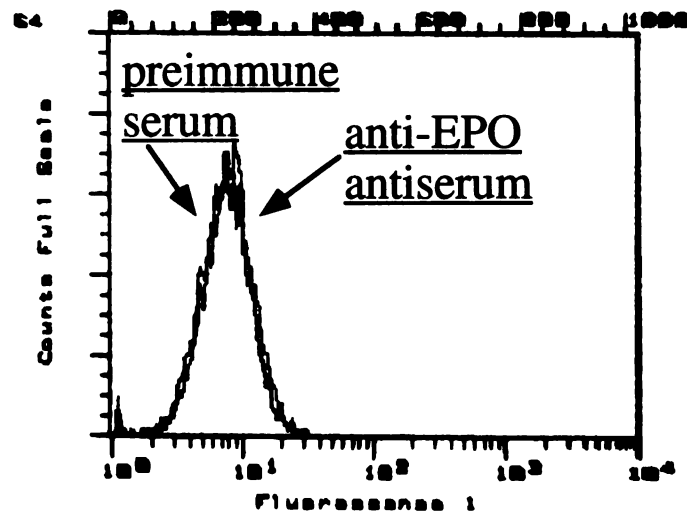
Polyclonal anti-EPO antiserum was used to detect EPO epitopes by FACS on the surface of PA317-based packaging cell lines which had been positive by Western blot for expression of pEPOenv Δ 6257. Figure 3-13 shows a representative FACS of PA317 + EPOenv Δ 6257 subclone 1-4, selected at 10 μ M MTX. Compared to preimmune serum, PA317 + EPOenv Δ 6257 subclone 1-4 cells showed a very small but consistent shift in fluorescence when incubated with anti-EPO antiserum (panel B). This shift was not seen in untransfected wild type PA317 control cells (panel A). When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, the shift in fluorescence was abolished (data not shown). Overall, most of the PA317 + EPOenv Δ 6257 subclones which had been positive by Western blot showed shifts of about this magnitude, i.e., barely detectable. This suggested that, despite adequate levels of protein expression as demonstrated by the Western blot results, the chimeric EPOenv Δ 6257 protein was not being transported efficiently to the surface of the packaging cells. As this would result in very low incorporation of the ligand-bearing chimeric envelope protein into virions budding from the cell surface, packaging cells expressing this construct were not subsequently utilized in infection experiments.

UCSF | **IRB** | **IRB**

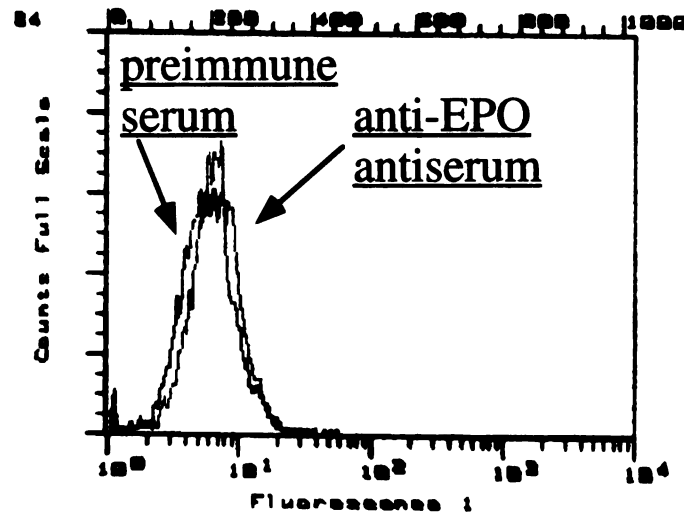
Figure 3-13. FACS analysis of pEPOenv Δ 6257-transfected PA317 packaging cell line using EPO-specific antiserum.

Polyclonal anti-EPO antiserum was used to detect EPO epitopes on the surface of packaging cell line PA317 + EPOenv Δ 6257, subclone 1-4, selected at 10 μ M MTX. Compared to preimmune serum, PA317 + EPOenv Δ 6257 subclone 1-4 cells showed a small but consistent shift in fluorescence when incubated with anti-EPO antiserum (panel B). This shift was not seen in untransfected wild type PA317 control cells (panel A). When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, the shift in fluorescence was abolished (data not shown).

A. wild type PA317



B. PA317+EPOenv $\Delta 6257$



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Packaging cells expressing pEPOenv Δ 5923 by Western blot show good cell surface expression of the EPO epitope.

Similarly, PA317 packaging cell subclones which had been positive by Western blot for expression of pEPOenv Δ 5923 were then analyzed by FACS, using polyclonal anti-EPO antiserum, to test for cell surface expression of the EPO epitope.

Figure 3-14 shows the FACS results of a representative packaging cell line, PA317 + EPOenv Δ 5923 subclone 2A, selected at 2 μ M MTX. Compared to preimmune serum, PA317 + EPOenv Δ 5923 subclone 2A cells showed a significant shift in fluorescence when incubated with anti-EPO antiserum (panel A). This shift was not seen in wild type PA317 control cells (data not shown, but see also Fig. 3-13 A). When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, the shift in fluorescence was abolished (panel B). Significant shifts, of varying magnitudes, were observed in several pEPOenv Δ 5923-transfected PA317 subclones. These results suggested that the chimeric EPOenv Δ 5923 protein was being transported efficiently to the surface of the packaging cells, and therefore were available on the surface of the packaging cells for incorporation into budding virions. As such virions would now contain the EPO ligand-bearing chimeric envelope protein as well as the endogenously expressed wild type amphotropic envelope protein, it was feasible to further examine their infectivity on target cells with and without the EPO receptor. The 2 μ M MTX-selected subclone 2A proved to have one of the largest shifts in fluorescence, and was therefore chosen for use in subsequent infection experiments.

Figure 3-14. FACS analysis of pEPOenv Δ5923-transfected PA317 packaging cell line using EPO-specific antiserum.

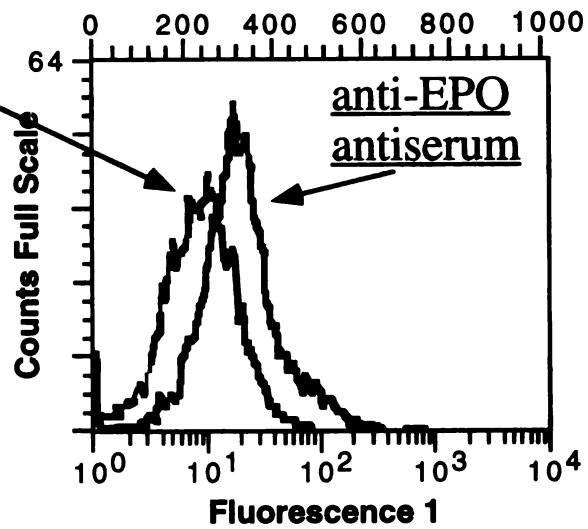
Polyclonal anti-EPO antiserum was used to detect EPO epitopes on the surface of packaging cell line PA317+EPOenv Δ5923, subclone 2A selected at 2 μM MTX.

Panel A: Compared to preimmune serum, PA317+EPOenv Δ5923 subclone 2A cells showed a clear shift in fluorescence when incubated with anti-EPO antiserum. This shift was not seen in wild type PA317 control cells (data not shown).

Panel B: When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, the shift in fluorescence was abolished.

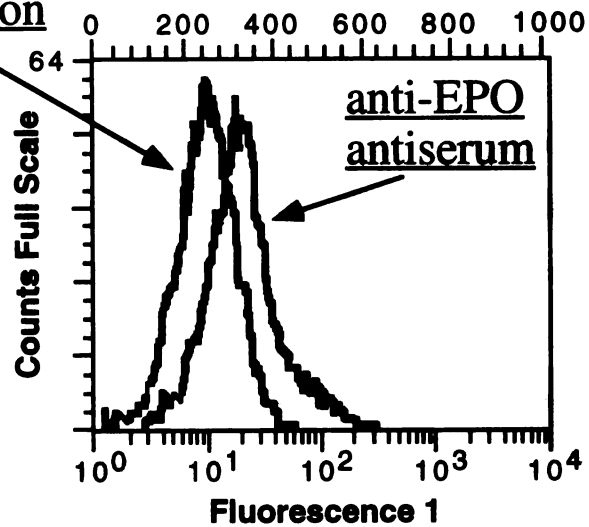
A.

preimmune
serum



B.

anti-EPO antiserum
+ EPO competition



PA317-based packaging cell line pEPOenv Δ 5923 2 μ M subclone 2A shows 2-fold higher infectivity on target cells with EPO-receptor.

HeLa and NIH3T3 target cells stably expressing EPO receptor were generated by co-transfection of pXM EPO-R and pFR400, followed by MTX selection to 10 μ M, and screened by 125 I-EPO radioligand binding assay. Subclones showing the highest levels of 125 I-EPO binding, corresponding to approximately 1,000 receptors per cell in the case of HeLa + EPO receptor cells (mean $\pm$ S.D.: $1,069 \pm 374$ (n=5)), and approximately 10,000 receptors per cell in the case of NIH3T3 + EPO receptor cells (mean $\pm$ S.D.: $11,557 \pm 1998$ (n=9), see also Chapter 4: Results), were chosen for use in infection experiments. Infectious retroviral particles packaging the CRIP SVlac vector were generated by transient transfection of packaging cell line PA317+EPOenv Δ 5923 2 μ M subclone 2A. After infection with the PA317 + EPOenv virus, or untransfected wild type PA317-derived virus as a control, the EPO receptor-bearing target cells were selected with 400 mg/ml G418 and the surviving G418-resistant colonies were fixed and stained (Fig. 3-15).

Two variables then had to be taken into account in interpreting the results and determining the efficiency of infection. First, differences in initial plating density and growth rates of the two target cell lines were corrected for by comparing the number of G418-resistant EPO receptor-positive colonies with the number of G418-resistant wild type (EPO receptor-negative) colonies after infection by wild type PA317 virus. Thus, as the same virus is infecting both types of target cell, the titer should theoretically be the same on both, and any significant differences actually

seen in the number of resistant colonies must be the result of differences in initial plating density or cellular growth rates.

Second, differences in the overall titers of the wild type and EPOenv-containing viruses were corrected for by comparing the number of G418-resistant wild type colonies after infection with wild type PA317 virus with that of wild type colonies after infection with PA317+EPOenv virus. That is, as the two different virus preparations are infecting the same (wild type) target cell type, which when initially plated was distributed equally among all plates used in the experiment, and should divide in a uniform manner, any significant differences actually seen in the number of G418-resistant wild type colonies must reflect the difference in overall titer between the two viruses.

The relative infection efficiency was thus calculated by normalizing the ratio of infection events in NIH3T3+EPO receptor vs. NIH3T3 wild type (wt) target cells after PA317+EPOenv virus infection, to the respective ratio of infection events after wild type PA317 virus infection (Fig. 3-16 A):

Relative infection efficiency

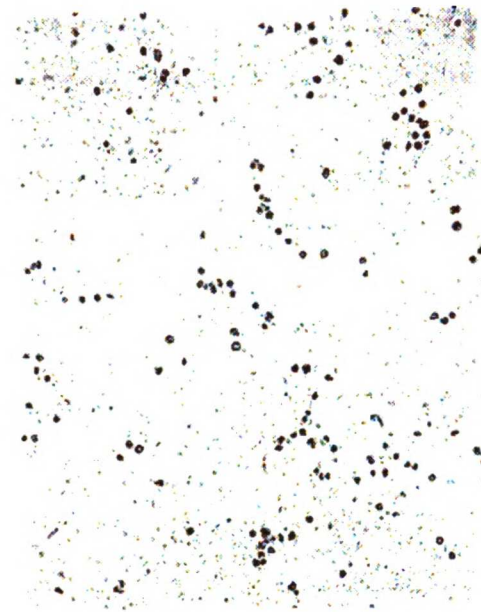
$$= \frac{\frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies}}{\text{no. of G418-resistant NIH3T3 wt colonies}} \text{ for EPOenv virus}}{\frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies}}{\text{no. of G418-resistant NIH3T3 wt colonies}} \text{ for wild type virus}}$$

The relative infection efficiency thus derived, expressed as the mean $\pm$ the standard deviation, was 2.39 ± 0.38 (n=5) (Fig. 3-16 B). The EPOenv 2A virus thus showed a doubling of infection efficiency on target cells bearing the EPO receptor, as compared to wild type PA317 virus (p<0.05).

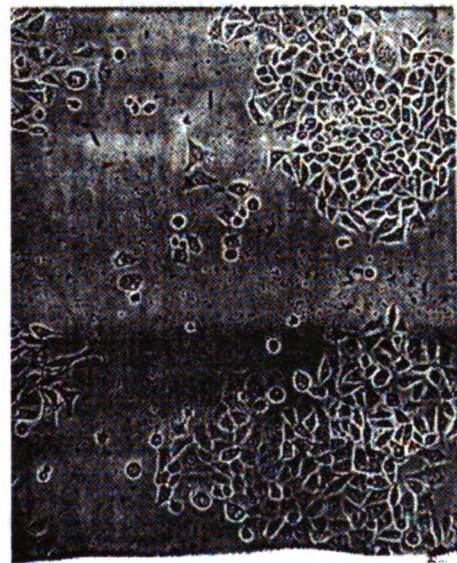
Figure 3-15. **Infection of HeLa cells with and without the EPO receptor, by viruses from EPOenv Δ 5923-transfected PA317 packaging cell line 2A.** Representative fields are shown from wild type (wt) HeLa target cells and HeLa target cells stably expressing the EPO receptor (+ EPO-R), after G418 selection. The upper panels show negative controls which had not been exposed to virus, and the lower panels show target cells which had been infected with virus produced by the packaging cell line PA317 + EPOenv Δ 5923, 2 μ M subclone 2A (EPOenv virus).



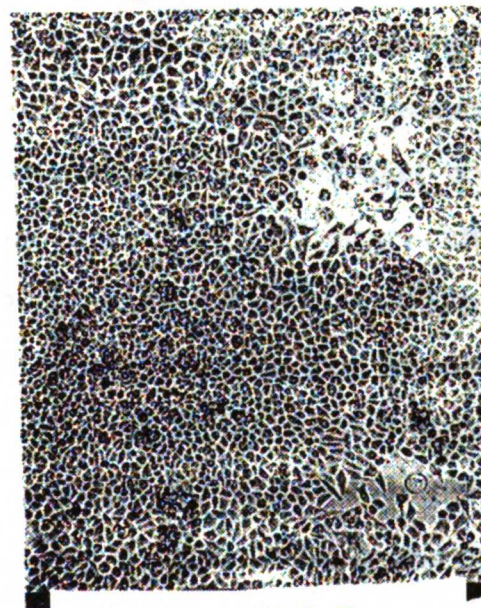
wt HeLa
(negative control)



HeLa + EPO-R
(negative control)



wt HeLa
(EPOenv virus)



HeLa + EPO-R
(EPOenv virus)

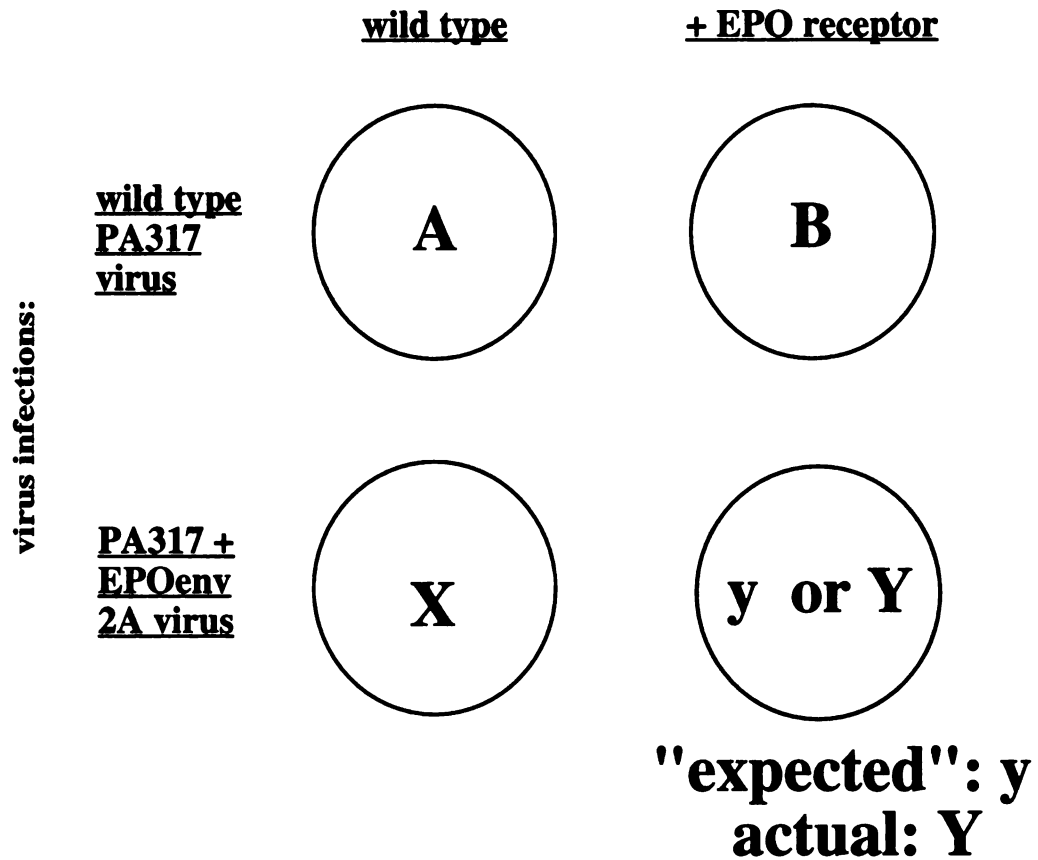
Figure 3-16. Infection of NIH3T3 cells with and without the EPO receptor, by viruses from EPOenv Δ 5923-transfected PA317 packaging cell line 2A.

Panel A: The variables "A" and "B" represent the numbers of G418-resistant colonies after using the same amount of wild type virus to infect, respectively, wild type and EPO receptor-bearing NIH3T3 target cells. The ratio A:B reflects the differences in initial plating density and growth rate between the two target cell lines, as the titer of wild type virus should theoretically be the same on both. The variable "X" represents the number of G418-resistant colonies after infection of wild type NIH3T3 target cells with EPOenv virus. The variable "y" represents the "expected" number of G418-resistant colonies after infection of EPO receptor-bearing NIH3T3 target cells with EPOenv virus if the null hypothesis, i.e., that there is no difference in infectivity between wild type and EPOenv virus, is true. The ratio X:y therefore equals the ratio A:B, with differences in the actual numbers merely reflecting the relative overall titer of the two viruses. The variable "Y" represents the actual number of G418-resistant colonies counted after infection of EPO receptor-bearing NIH3T3 target cells with EPOenv virus. Thus, if the null hypothesis is true and no difference in infectivity exists between wild type and EPOenv viruses, then the ratio X:Y should equal X:y. However, if there does exist a difference in infectivity, this can be quantitated as the relative infection efficiency by the ratio Y/y. Since $A:B = X:y$, and therefore $y = B/A \cdot X$, this results in the formula $[Y/X] \div [B/A]$ for the relative infection efficiency.

Panel B: Bar graph showing the relative infection efficiency of PA317 + EPOenv Δ 5923-derived virus on NIH3T3 target cells with and without EPO receptor, as compared with wild type PA317-derived virus.

A.

NIH3T3 target cells

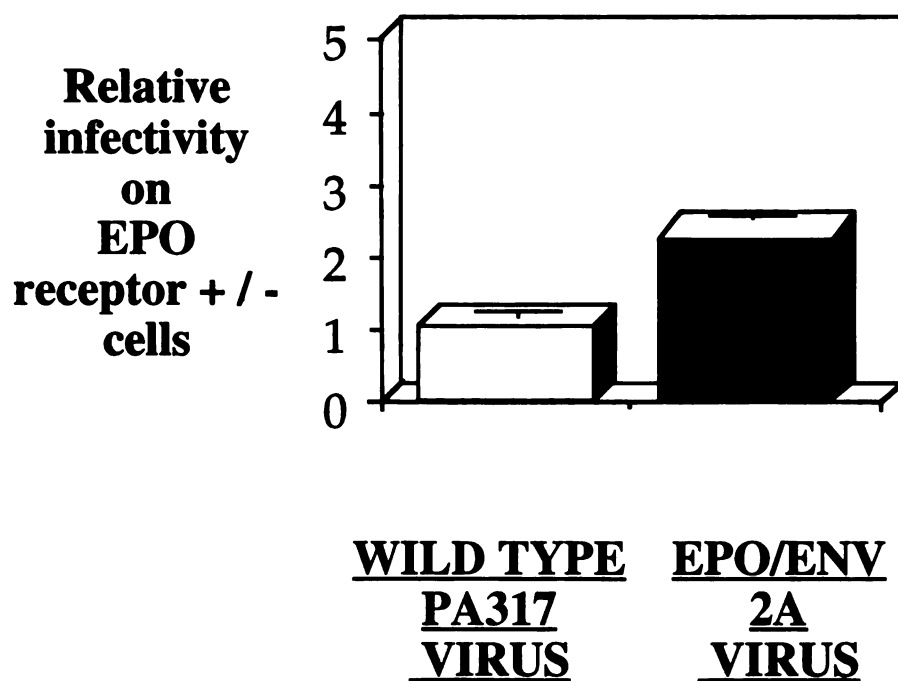


Relative infection efficiency:

$$\begin{aligned}
 & \frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies (Y)}}{\text{no. of G418-resistant NIH3T3 wt colonies (X)}} \text{ for EPOenv virus} \\
 = & \frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies (B)}}{\text{no. of G418-resistant NIH3T3 wt colonies (A)}} \text{ for wild type virus}
 \end{aligned}$$

B.

**Relative infectivity of
wt vs. EPOenv viruses on
EPO receptor + vs. – cells**



Discussion

In these experiments, I was able to show that, in one case, deletion of large portions of the ecotropic gp70 envelope glycoprotein and replacement with a peptide hormone targeting ligand resulted in a functional chimeric ligand-envelope protein which could be successfully expressed in packaging cell lines. The data suggest that replacement of the amino terminal domain of gp70 is more likely to produce a protein which is expressed at detectable levels and correctly transported to the cell surface. Moreover, the chimeric protein thus expressed can be recognized by EPO-specific antibodies, and appears to be able to encoat viruses and confer enhanced infectivity on target cells bearing the EPO receptor.

Expression of chimeric EPO-*env* genes and Friend gp55 in the PA317 packaging cell line

Following the lessons learned from the previous set of experiments involving expression of the ANF-containing constructs, the transfected packaging cell line subclones were analyzed at each stage of virus production for successful expression of the transfected EPO receptor-targeting constructs. That is, Southern blot analyses were performed to confirm integration of the transfected genes at the DNA level, polymerase chain reaction amplification of reverse-transcribed cDNA (RT-PCR) was done to confirm transcription at the mRNA level, Western blot analyses were performed to ensure that the transcripts were being translated into protein, and fluorescence-activated cell sorting (FACS) analyses were done

to determine if the proteins were then being transported to the packaging cell surface and oriented correctly in the plasma membrane.

It is of interest that the pEPOenv $\Delta 6250$ construct in PA317-derived subclones was not detected on the cell surface at significant levels, even though Western blots of total cell lysate were positive. Not only does it underscore the importance of checking each step as the protein is synthesized and subsequently progresses through the cell to the surface from which it buds off with the virion, but it also implies that replacement of the central domain of gp70 is deleterious to its ability to be correctly transported to the cell surface, or results in a molecule which is otherwise defective. In fact, when the $\Delta 6250$ construct was co-transfected into $\Psi 2$ cells with pFR400 as the selectable marker, subclones which were MTX-resistant nevertheless did not appear to express the recombinant envelope at significant levels even by Western blot (data not shown). Perhaps the disruption of the central, proline-rich, helical region of the envelope compromises the structural integrity of the molecule to such an extent that it is sequestered and rapidly degraded in the endoplasmic reticulum; this finding is in agreement with recent reports, published long after I had first designed and tested these constructs, that mutations in the central region of the MoMLV envelope are generally non-viable [11]. Conversely, it appears that the EPOenv $\Delta 5923$ construct, with replacement of the amino terminal domain, results in a functionally intact chimeric protein which is correctly transported to the cell surface, and which displays the EPO epitope in an antigenically recognizable conformation. Again, this is consistent with recent studies which localize the ecotropic receptor binding domain to the amino terminal region of the envelope [33, 34].

While the Friend virus gp55 construct provided yet another object lesson in how alteration of the structure of the envelope gene can lead to defects in processing and transport of the synthesized protein, it was additionally interesting to note that although the protein could be expressed at levels detectable by Western blot in PA317 and Ψ 2 cells, none of the GP101 transfectants tested showed any evidence of gp55 expression. It is tempting to speculate that this is an example of an "abnormal" envelope protein, which would ordinarily be trapped and degraded in the endoplasmic reticulum as was perhaps the case in the GP101 cells, instead being "rescued" by the presence of the normal envelope protein in PA317 and Ψ 2 cells, and ultimately transported to the cell surface. This would therefore extend the notion that the wild type envelope protein needs to be present along with the recombinant envelope, not only to help assembly of the chimeric coat, but even for the recombinant envelope to be successfully expressed and correctly processed in the first place, and could be another reason why numerous attempts to pseudotype retroviruses using purely artificial coats without any wild type envelope have usually been unsuccessful in the past. This idea is further supported by the fact that all attempts to express either pEPOenv Δ 6257 or pEPOenv Δ 5923 in GP101 cells, which have no endogenous wild type envelope to rescue the recombinant, ended in failure, with all subclones surviving selection showing rearrangement or truncation of the recombinant envelope when tested by Western blot (data not shown).

Packaging of replication-defective retroviral vectors containing the neomycin-resistance (neo^R) gene

PA317-based packaging cell lines which showed good expression of pEPOenv Δ5923 were transfected with a replication-defective retrovirus vector containing the neomycin-resistance gene. This vector contains the *psi* packaging signal, and therefore is packaged by the retroviral proteins produced in the cell. Envelope proteins, including the transfected envelope products, encoat the virus as it buds from the cell surface. Initially experiments were done by transient transfection of the vector construct and harvesting of the viruses produced 48 hours later. Although this procedure results in relatively low titer, usually about one order of magnitude lower than that obtainable from a subcloned cell line stably producing virus, this was sufficient to show a difference in the relative infectivity of EPO ligand-bearing viruses compared to wild type viruses.

As an interesting sidelight, attempts were subsequently made to create stable virus-producing cell lines in order to increase the amount of virus available for further characterization of the envelope proteins present on the coat. However, although stable virus producer cell lines from Ψ2-derived packaging cell lines transfected with pEPOenv Δ5923 could readily be created by subsequent infection with a replication-defective amphotropic virus containing CRIP-SVlac packaged by PA317 (see Chapter 4), all attempts to create the equivalent virus producer cell line from the PA317-derived packaging cell line 2A have repeatedly been unsuccessful. That is, all attempts at infecting the 2A cell line with a replication-defective ecotropic virus containing CRIP-SVlac packaged by Ψ2, have not to date resulted in any surviving G418-resistant colonies, despite the fact that

control cells infected by the same virus showed numerous colonies (data not shown); thus, I would postulate that infection was inhibited, perhaps due to restriction of ecotropic viral entry by the prior presence of the recombinant EPO-*env* protein, which is itself derived from the ecotropic MoMLV envelope. If so, this implies that, despite replacement of the amino-terminal region of the ecotropic envelope with EPO, the recombinant EPO-*env* protein was still able to bind or otherwise sequester the ecotropic viral receptor protein in the 2A cells, thus making it unavailable at the cell surface and rendering the cell resistant to subsequent infection by ecotropic virus, analogous to the mechanism by which infection by one ecotropic virus renders the host cell resistant to superinfection by other ecotropic viruses. This suggests that regions other than the amino-terminal domain might also be important in binding of the ecotropic envelope to its host receptor.

Testing the tissue specificity of viruses thus packaged by infection of murine and non-murine cells bearing the EPO-receptor

These experiments showed a doubling of the infection efficiency of EPO ligand-bearing viruses on EPO receptor-expressing target cells. Although the increase in infection efficiency is relatively small, I believe that it provides sufficient demonstration of the principle that the infectivity of retroviral vectors can be manipulated by engineering a ligand into the coat.

The relatively small increase in infection efficiency shown by viruses packaged by the PA317-derived EPOenv 2A cell line might be due simply to the overall level of expression of the recombinant EPOenv protein, or

perhaps it could be related to the fact that the viruses are chimeric and require assembly of two different envelope proteins; it is possible that the amphotropic wild type envelope may not pack together with the recombinant EPO-containing envelope, which is derived from ecotropic MoMLV, as well as does the ecotropic wild type envelope. Creation of more PA317-derived cell lines with different levels of EPOenv expression or the use of the amphotropic *env* gene to create recombinant ligand-containing envelopes, may provide further insight.

The amphotropic PA317-based transfectants were first pursued because the positive baseline level of infection even in non-murine cells ensured that any results obtained would usually be interpretable. However, the fact that this strategy for the development of viral vectors targeting the EPO receptor was thus proven to be feasible in an amphotropic system, thus implied that the same strategy would be equally successful in ecotropic packaging cells.

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CHAPTER 4.

EPO-containing envelope proteins can alter the host range of ecotropic viruses and mediate cell-specific infection via ligand-receptor interaction.

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Introduction

The aim of this project has been to develop a model system for the targeted delivery of genes by creating tissue-specific retroviral vectors bearing ligands which recognize and bind specific cell surface receptors. The experimental strategy has been to incorporate a sequence encoding the targeting ligand into the viral envelope, thus targeting the virus to cells which express the receptor for that ligand. Thus far, I have been able to demonstrate that amphotropic viruses bearing a targeting ligand show an increase in infectivity for target cells expressing the specific receptor, probably due to the enhanced affinity for binding to the target cell contributed by the ligand-receptor interaction. As the modified virus was amphotropic-based, a background level of "non-specific" infection of both murine and non-murine cells, mediated via the ubiquitous amphotropic receptor, was unavoidable. In fact, this non-specific background infection was utilized as a baseline to quantitate the magnitude of the increase in infection efficiency.

However, for viral infection (or retrovirus-mediated gene delivery) to be truly tissue-specific, there should ideally be no background infection at all. Thus for the next series of experiments, the chimeric envelope construct containing the targeting ligand was expressed in an ecotropic packaging cell line, and the resultant virus tested for its ability to infect non-murine target cells with and without the specific receptor. Since the virus is now ecotropic-based, it should be normally be incapable of entering non-murine cells, thus background infection is eliminated. If, however, the targeting ligand can confer the ability to cross species and exclusively infect non-

murine cells which display the receptor for that ligand, then this can be considered to be a truly tissue-specific vector system. In this chapter I will present the results of such cross species infection experiments using virus derived from Ψ 2 cells transfected with pEPOenv Δ 5923.

Materials and Methods

Plasmid construction

pEPOenv Δ 5923

Details of the construction of plasmid pEPOenv Δ 5923, which contains the targeting ligand EPO in the ecotropic MoMLV *env* gene, have been described previously (see Chapter 3, Materials and Methods). The plasmid pEPOenv Δ 5923 contains the EPO sequence in the amino terminal portion of the *env* gene (Fig. 3-1).

pFR400

The plasmid pFR400, which contains the methotrexate (MTX)-resistant dihydrofolate reductase (*dhfr*) gene [1], has been described previously (see Chapter 2, Materials and Methods). This plasmid was co-transfected into NIH3T3 cells with pXM EPO-R, as a selectable marker.

pCRIP-SVlac:

Details regarding the construction of plasmid pCRIP-SVlac, a modification of the replication-defective retroviral vector pCRIP*dhfr* provided by Dr. R. Scharfmann (Salk Institute), have been described

previously (see Chapter 3, Materials and Methods). This plasmid contains the psi packaging signal, the neomycin resistance gene driven by the 5'-LTR, and the β -galactosidase gene driven by an internal SV40 promoter.

pXM EPO-R

The plasmid pXM EPO-R (generously provided by Dr. A. D'Andrea (Dana Farber Cancer Institute) contains the murine EPO receptor cDNA [2] cloned into the mammalian expression vector pXM, as described previously (see Chapter 3, Materials and Methods). Transcription of the EPO receptor cDNA is driven by the adenovirus major late promoter, with splicing and polyadenylation signals provided by the dhfr gene.

Packaging cell transfections

The ecotropic packaging cell line Ψ 2 [3] was maintained in Dulbecco's modified Eagle's (DME) medium (Cellgro) containing 10% calf serum (CS) and 100 μ g/ml penicillin/streptomycin (PS). To create ecotropic packaging cell lines expressing the targeting envelope constructs, 1×10^5 Ψ 2 cells were co-transfected with 20 μ g of pEPOenv Δ 6257, pEPOenv Δ 5923, or pXMgp55 respectively, and 1 μ g of pFR400. All transfections were performed in 60-mm dishes using the calcium phosphate precipitation method [4, 5]. The medium was changed 24 hours later, and 48 hours post-transfection the cells were trypsinized, re-plated at 1:10 dilution in 100-mm plates, and subsequently selected with gradually increasing concentrations of MTX (Sigma) ranging from 0.2 μ M to 10 μ M, at which point subclones were isolated from the surviving colonies and characterized.

Creation of virus producer cell lines

Virus-producing cell lines were created by first transiently transfecting amphotropic PA317 packaging cells with pCRIP-SVlac. PA317 cells [6] were grown in improved modified Eagle's (IME) medium (Biofluids) containing 10% fetal bovine serum (FBS) and 100 units per ml penicillin/streptomycin. After plating approximately 1×10^5 PA317 cells on 60-mm dishes, 20 μ g of pCRIP-SVlac was transfected using the calcium phosphate precipitation method [4, 5], the medium was changed after 24 hours, and at 48 hours post-transfection the virus-containing medium was harvested. Cellular debris was removed by passage through a 0.2 μ m filter. The filtrate was used to infect wild type Ψ 2 ecotropic packaging cells, as well as Ψ 2-derived packaging cells expressing recombinant envelope, in medium containing 8 μ g/ml of polybrene (final concentration). The infected packaging cells were subsequently trypsinized and re-plated at low density, selected in 400 μ g/ml G418 (Gibco), and individual G418-resistant colonies were isolated. Subcloned colonies were re-tested for expression of the EPO epitope by Western blot and FACS analysis. Wild type amphotropic virus-producing cells were created by a similar strategy, in this case using transient transfection of wild type Ψ 2 cells with pCRIP-SVlac to generate virus for infection of PA317 packaging cells, followed by G418 selection.

Western blot analyses

Subclones were grown to confluence in 100-mm plates, washed with phosphate-buffered saline (PBS), and lysed in 4% SDS/10% glycerol/10% β -mercaptoethanol/50 mM Tris (pH 6.7), boiled for 10 minutes, and stored

frozen at -20°C until analyzed. After thawing, the samples were boiled again briefly, separated by electrophoresis on an 8% SDS-polyacrylamide gel, and electro-transferred to nitrocellulose (Hybond ECL; Amersham). The filters were then blocked in TBST buffer (0.02% Tween-20/150 mM NaCl/50 mM Tris (pH 7.5)) with 5% dried milk (BioRad), and subsequently incubated with a primary monoclonal anti-EPO antibody (Genzyme) followed by washing in TBST buffer with 0.5% dried milk and incubation with a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (CalTag). After washing again, the filters were incubated in ECL chemiluminescent immunodetection reagents (Amersham) and exposed to film. The filters were also re-blocked and re-probed using a polyclonal goat anti-*env* antiserum (Microbiological Associates) as the primary antibody, followed by horseradish peroxidase-conjugated swine anti-goat (CalTag) as the secondary antibody.

DNA and RNA analyses

Subclones were grown to confluence in 100-mm plates; genomic DNA was extracted and digested to completion with EcoRI, size-fractionated on a 1% agarose gel, and transferred to a nylon membrane (Hybond N; Amersham). The membrane was hybridized to a 500 bp EPO probe labelled with digoxigenin (Boehringer Mannheim) at 42°C overnight, and washed two times at room temperature in 2 × saline sodium citrate (SSC), 0.1% SDS, and two times at 65°C in 0.5 × SSC, 0.1% SDS. Detection was performed by incubation of the membrane with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and AMPPD (Tropix), and exposure to film at room temperature.

FACS analyses

Polyclonal anti-EPO antiserum #8C295 generously provided by Dr. J. Egrie (AmGen) and fluorescein-conjugated secondary antibodies (CalTag) were used for flow cytometry, using a Becton-Dickinson FACScan and FACS IV cell sorter. Cells were grown to confluence on 100-mm plates, detached and dispersed with phosphate-buffered saline (PBS) containing 2 mM EDTA, and pre-incubated with normal goat serum (CalTag) in PBS containing 2% bovine serum albumin (BSA) for 30-60 min on ice. After washing twice in PBS with 2% BSA, the cells were incubated with pre-immune rabbit serum (CalTag) or the polyclonal EPO-specific rabbit antiserum (#8C295; AmGen) diluted in PBS with 2% BSA for 30-60 min, washed again repeatedly, incubated with fluorescein-conjugated goat anti-rabbit antiserum (CalTag), washed again, and subjected to flow cytometry after addition of propidium iodide.

Sucrose density gradient fractionation

Viral particles in overnight cell culture medium from virus producer cells expressing the chimeric envelope were purified by centrifugation at 150,000 g through 2 ml 20% sucrose onto a 1 ml 60% sucrose cushion in an SW40 rotor for 1 hour at 4 °C. Virus particles removed at the interface were diluted to 3 ml, layered on top of a 9 ml 20% to 45% sucrose gradient and centrifuged at 150,000 g overnight in an SW40 rotor at 4°C. Fractions of 1 ml were collected from the bottom of this gradient using a peristaltic pump, and each fraction was diluted 1:3 before pelleting at 190,000 g in an SW50

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rotor for 1 hour at 4°C. Pelleted samples were lysed in 4% SDS/10% glycerol/10% β -mercaptoethanol/50 mM Tris (pH 6.7), boiled for 10 min, and stored frozen at -20°C until analyzed by Western blot as above, using either the monoclonal EPO-specific antibody (Genzyme), the polyclonal *env*-specific antiserum (Microbiological Associates), or a total MLV-specific antiserum (Microbiological Associates).

Electron microscopy

For electron microscopy, overnight culture medium from producer cells was centrifuged at 150,000 g through 2 ml 20% sucrose onto a 1 ml 60% sucrose cushion in an SW40 rotor for 1 hour at 4 °C. Virus particles removed at the interface were incubated with rabbit polyclonal anti-EPO antiserum (AmGen) and protein A-gold (Sigma), or goat polyclonal *env*-specific antiserum (Microbiological Associates) and protein G-gold (Sigma), for 1 hour at room temperature, after which the samples were fixed in glutaraldehyde, stained with phosphotungstic acid, and examined by electron microscopy.

Target cell lines and radioligand binding assays.

Wild type mouse fibroblast (NIH3T3) cells, grown in DME medium with 10% CS, and NIH3T3 cells stably expressing the EPO receptor, were used as target cells. To generate the latter, NIH3T3 cells were co-transfected with pXM EPO-R and pFR400, and selected in increasing concentrations of MTX ranging from 0.2 to 10 μ M. Expression of the EPO receptor was assayed by binding of 125 I-labelled EPO peptide (Amersham). Target cells were grown to confluence in 24-well plates, and subsequently incubated in

serum-free DME medium with 2% BSA and approximately 10^6 cpm of ^{125}I -EPO, with or without excess cold EPO peptide (Sigma). After incubation at 37°C for 90 min, the cells were washed 2-3 times in PBS with 2% BSA to eliminate unbound radioligand, lysed in 1N NaOH, and the bound radioactivity was counted in a gamma counter. The approximate EPO receptor number per cell was estimated based on the specific activity of the ^{125}I -EPO and the approximate number of cells contained within the wells. The human erythroleukemia cell lines HEL and K562 were also used as target cells, and were similarly assayed for ^{125}I -EPO binding, except that cells were incubated with ^{125}I -EPO in suspension, and were spun for 5 min at 1000 rpm in a Beckman desktop centrifuge to pellet the cells after each wash. Non-erythroid human cell lines used as target cells include HeLa (human cervical carcinoma) and Raji (human lymphoma). All human cell lines were cultured in IME medium with 10% FBS.

Target cell infections

For production of infectious virus, producer cells were incubated in non-selective culture medium (MTX- and G418-free DME medium without serum) for 8 to 24 hours prior to harvesting. The virus-containing medium was filtered through a $0.2\ \mu\text{m}$ filter into serum-free medium containing $8\ \mu\text{g/ml}$ of polybrene and used to infect the target cells. Some infections were done in the presence of 5-50 μg of competing soluble EPO peptide (Amgen). The target cells were trypsinized 24 hours later and re-plated at low density in DME medium containing 10% CS or FBS, selected for 12 to 14 days in 400-1000 $\mu\text{g/ml}$ G418 (Gibco) depending on the target cell type, and the number of surviving G418-resistant colonies was counted after fixation in methanol/acetone (1:1 vol/vol) and Giemsa staining.

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Results

Recombinant envelope constructs pEPOenv Δ 6257 and pEPOenv Δ 5923 can be successfully expressed in Ψ 2 cells.

To introduce the peptide hormone erythropoietin (EPO) into the ecotropic MoMLV envelope, a portion of the MoMLV envelope (*env*) gene [7] at the amino-terminal end of gp70 was removed and replaced, in frame, with sequences coding for EPO [8, 9]; this construct was designated pEPOenv Δ 5923 (see Fig. 3-1). This EPO-*env* hybrid construct was co-transfected with the plasmid pFR400, containing the methotrexate (MTX)-resistant dihydrofolate reductase (*dhfr*) gene [1], into Ψ 2 packaging cells [3] by calcium phosphate precipitation. The transfected cells were selected with increasing concentrations of MTX ranging from 0.2 μ M to 10 μ M, and subclones were isolated and screened at each selection stage (see Fig. 3-6).

Subclones were isolated from pools of Ψ 2 cells stably transfected with pEpoenv Δ 5923 at selection levels of 0.2, 2, and 10 μ M MTX, respectively. Several of the subclones transfected with pEpoenv Δ 5923 showed a specific 70 kD band when cell lysates were subjected to Western analysis using a monoclonal anti-EPO antibody. The same band was visualized after re-probing the Western blot with polyclonal anti-*env* antiserum, demonstrating co-expression of both epitopes in the same 70 kD protein (Fig. 4-1). In particular, a Ψ 2-derived subclone isolated at a selection level of 10 μ M MTX, designated Ψ EPOenv 8 was chosen for further characterization.

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Figure 4-1. Western blot analysis of Ψ 2 cell subclones transfected with pEPOenv Δ 5923.

Ψ 2 cells were transfected with pEPOenv Δ 5923 and selected in MTX up to 10 μ M. Protein extracts from individual subclones were analyzed by Western blot using a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase-conjugated goat-anti-mouse antibody as the secondary antibody. In some subclones, a specific 70 kD band was recognized by the EPO-specific antibody, suggesting that the EPO sequence-containing envelope gp70 construct was being expressed as protein. Lane numbers denote the designated numbers of individual subclones.

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Genomic DNA from selected packaging cell lines was examined by Southern blot, using a digoxigenin-labelled EPO probe, which revealed the expected 2.4 kb restriction fragment containing the recombinant EPO env sequence, and the presence of pEPO env mRNA transcripts was confirmed by RT-PCR, as previously (data not shown).

Packaging cells expressing pEPO env Δ 5923 by Western blot can be sorted for high expressing subclones.

Fluorescence-activated cell sorting (FACS) analysis, using a polyclonal anti-EPO antiserum, was performed to demonstrate that the chimeric envelope protein transfected into the Ψ 2-based packaging cells is transported to and oriented correctly in the cell membrane. Figure 4-2 confirms the detection of EPO epitopes on the surface of packaging cell line Ψ EPO env 8. Compared to preimmune serum, Ψ EPO env 8 cells showed a biphasic shift in fluorescence when incubated with anti-EPO antiserum (top panel). This shift was not seen in wild type Ψ 2 control cells (data not shown). When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, the shift in fluorescence was abolished (middle panel). Because the biphasic pattern of fluorescence suggested the existence of a mixed population of cells, the cells with high fluorescent activity were sorted by FACS and re-subcloned, resulting in a monophasic population of cells expressing the EPO epitope at high levels (bottom panel). The monophasic shift in fluorescence could also be abolished by competition with soluble EPO peptide (data not shown). Two subcloned packaging cell lines showing the largest shifts in fluorescence, designated Ψ EPO env 8.1 and 8.19, were selected for further studies.

Figure 4-2. **FACS analysis of pEPOenv Δ 5923-transfected Ψ 2-based packaging cell line using EPO-specific antiserum.**

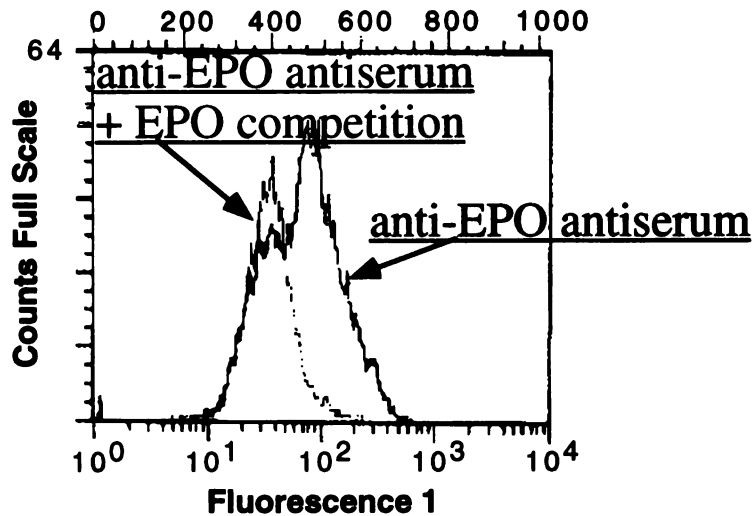
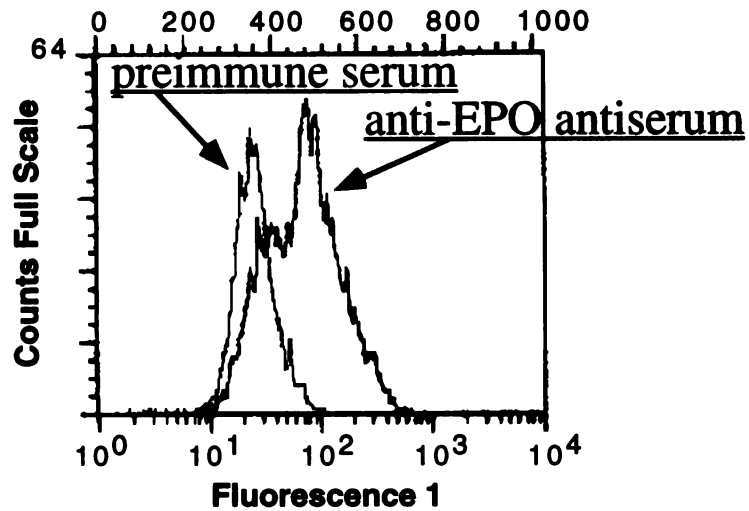
Polyclonal anti-EPO antiserum was used to detect EPO epitopes on the surface of packaging cell line Ψ EPOenv Δ 5923, subclone 8, selected at 10 μ M MTX.

Prior to sorting: Compared to preimmune serum, Ψ EPOenv Δ 5923 subclone 8 cells showed a biphasic shift in fluorescence when incubated with anti-EPO antiserum (upper panel). This shift was not seen in wild type Ψ 2 control cells (data not shown). The shifted peak could be abolished by competition with soluble EPO peptide (lower panel). This suggested that a mixed population of cells was present with respect to expression of the EPOenv construct.

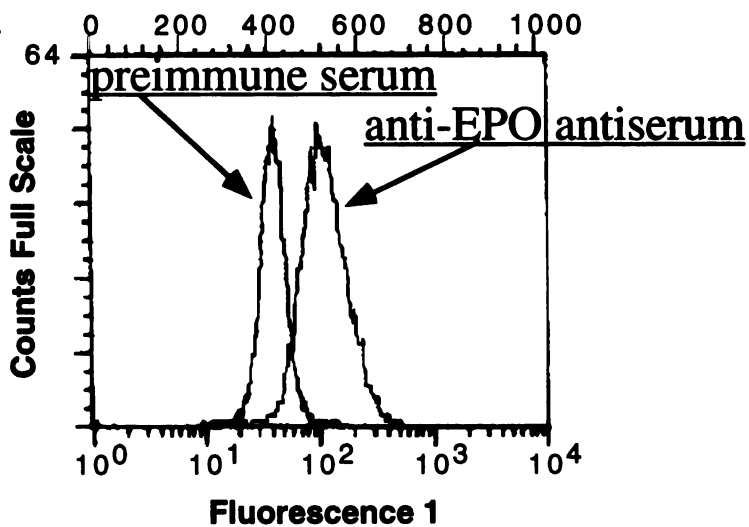
After sorting: The shifted peak was sorted to isolate the high expressing cells, and the sorted population now showed a monophasic peak which was shifted with EPO-specific antiserum. When an excess of EPO peptide was added to compete for specific anti-EPO antibody binding, this shift in fluorescence was abolished (data not shown).

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Prior to sorting:



After sorting:



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After sorting, Southern blot analysis was re-done and showed that these cell lines, as expected, also carried the 2.4 kb Eco R1 restriction fragment containing the chimeric EPO-*env* sequence (Fig.4-3). Furthermore, Western blots of the sorted packaging cell lines re-confirmed expression of the protein (Fig. 4-4). Thus, not only was the integrated gene expressed in these packaging cells, but the chimeric envelope protein was correctly transported through the endoplasmic reticulum to the cell surface, where it becomes available for incorporation into budding virus particles.

Producer cells can be generated from Ψ 2-based packaging cell lines Ψ EPOenv 8.1 and 8.19, and continue to show expression of the recombinant envelope.

In order to maximize titer for use in infection experiments, as well as to create a source of constitutively produced virus to further characterize the expression of the targeting ligand on the virion surface, stable virus-producing cell lines were then generated. The Ψ EPOenv 8.1 and 8.19 packaging cells were infected with virion-containing cell culture medium harvested after transient transfection of wild type PA317 packaging cells [6] with pCRIP-SVlac, a Ψ (+) viral vector which contains both the neomycin resistance gene and the β -galactosidase gene (Fig. 4-5). The reason for introducing these vectors into the Ψ 2-derived cell lines via viral infection rather than direct transfection using calcium phosphate is that, viral infection ensures that only one copy of the retroviral vector will be introduced into the cell [10], whereas transfection often results in multiple copies which are prone to genetic recombination, rearrangement, and deletion, thus leading to low viral titers.

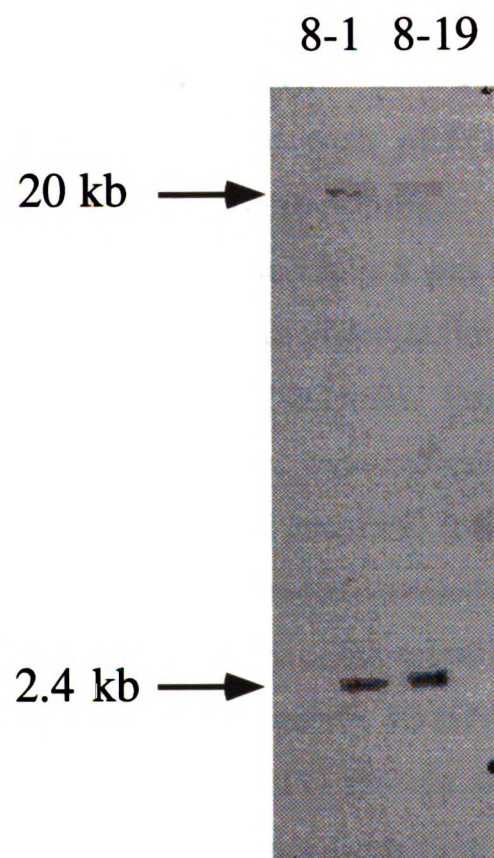
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Figure 4-3. Southern blot analysis of Ψ 2 cell subclones stably transfected with pEPOenv Δ 5923, after sorting and re-subcloning.

Genomic DNA, from sorted and re-subcloned Ψ 2 cell lines transfected with pEPOenv Δ 5923, was digested with EcoR I and hybridized with a digoxigenin-labelled 504 bp EPO probe, which revealed the expected 2.4 kb restriction fragment containing the recombinant EPOenv sequence.

Lane 8-1: Ψ EPOenv Δ 5923 subclone 8.1. Lane 8-19: Ψ EPOenv Δ 5923 subclone 8.19.

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Figure 4-4. Western blot analysis of Ψ 2 cell subclones stably transfected with pEPOenv Δ 5923, after sorting and re-subcloning.

Ψ EPOenv Δ 5923 subclone 8 was sorted by FACS, and the sorted cells were plated at limiting dilution to re-subclone individual colonies. Protein extracts from individual subclones were analyzed by Western blot using a monoclonal anti-EPO antibody as the primary antibody and horseradish peroxidase-conjugated goat-anti-mouse antibody as the secondary antibody. In some subclones, a specific 70 kD band was again recognized by the EPO-specific antibody, suggesting that the EPO sequence-containing envelope gp70 construct was being expressed as protein. Lane numbers denote the designated numbers of individual subclones.

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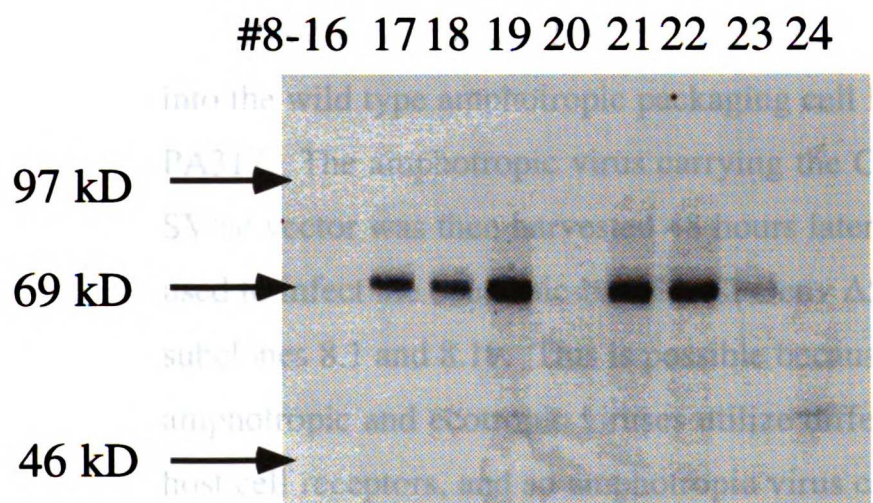
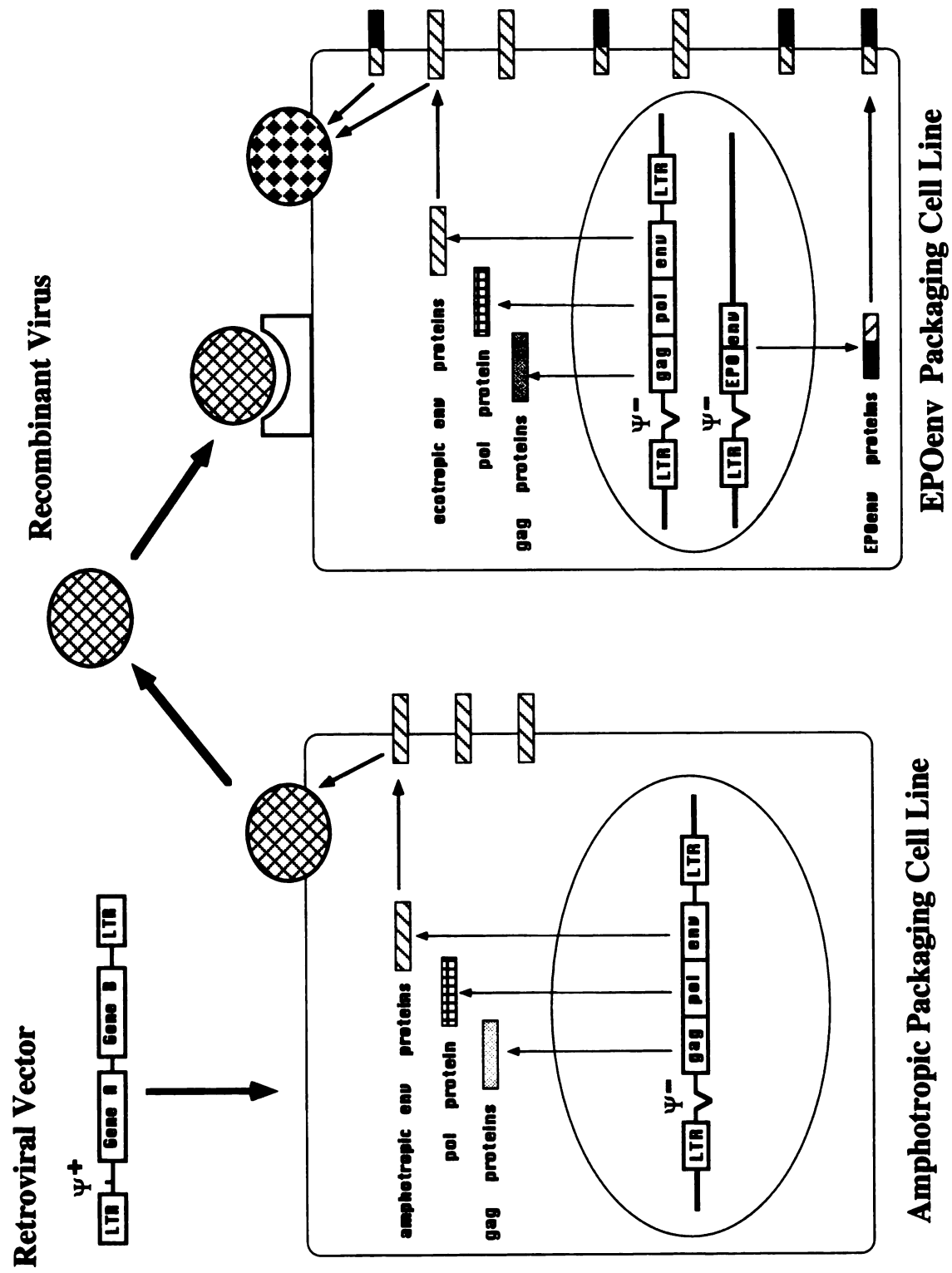


Figure 4-5. Strategy for creation of stable virus-producing cell lines. In order to make a permanent virus producer cell line from the sorted Ψ EPOenv Δ 5923 subclones 8.1 and 8.19, the replication-defective pCRIP-SVlac vector was first transiently transfected into the wild type amphotropic packaging cell line PA317. The amphotropic virus carrying the CRIP-SVlac vector was then harvested 48 hours later, and used to infect the ecotropic-based Ψ EPOenv Δ 5923 subclones 8.1 and 8.19. This is possible because the amphotropic and ecotropic viruses utilize different host cell receptors, and so amphotropic virus can still super-infect ecotropic packaging cells without interference. The advantage of this strategy is that, by infection, usually only one copy of the vector is transmitted to the recipient packaging line; in contrast, if the CRIP-SVlac vector were directly transfected into the Ψ EPOenv Δ 5923 packaging line, multiple copies might integrate but this would lead to a high frequency of recombination and rearrangement due to the presence of multiple long terminal repeats, ultimately resulting in loss of titer.

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G418-resistant subclones of Ψ EPOenv 8.1 and 8.19 were re-tested for expression of the EPO-*env* proteins by Western blot and FACS analysis (data not shown), and positive cell lines, designated Ψ EPOenv 8.1.8 and Ψ EPOenv 8.19.9, were obtained.

Virions produced from Ψ EPOenv 8.1.8 and 8.19.9 cells show incorporation of the EPO targeting ligand in the viral envelope.

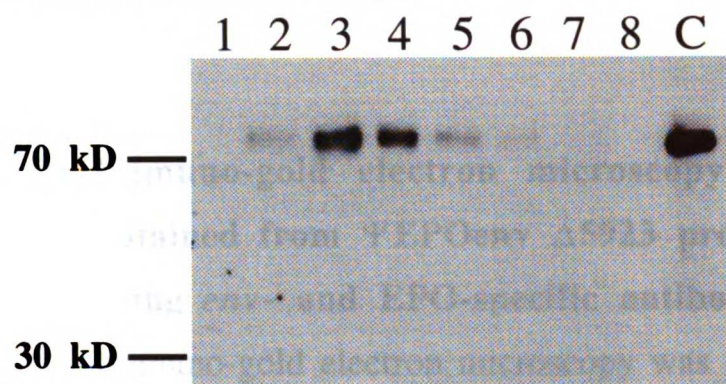
To show that the virions produced by this cell line contain the chimeric envelope protein, culture medium from these cell lines was fractionated by sucrose density gradient centrifugation, and the fractions were subjected to Western analysis using three different antisera specific for EPO, *env*, and total MoMLV. Before fractionation, the samples were first pre-spun through 20% sucrose onto a 60% sucrose cushion, which excludes free proteins and concentrates intact virions at the 20%/60% interface. This interface was then tapped and fractionated through a three-step sucrose density gradient. The same 70 kD band was detected by the anti-EPO antibody as well as the anti-*env* antisera predominantly in two fractions which also contain other components of the MoMLV virus such as the p15 transmembrane subunit of the envelope and the *gag* proteins (Fig. 4-6 A-C). This demonstrates that the chimeric EPO-*env* proteins were indeed incorporated into intact virions released by the producer cells into the medium. This was further confirmed morphologically by immuno-gold electron microscopy of purified virions using *env*- and EPO-specific antibodies (Fig. 4-7 A and B, respectively). While the conditions were not optimal for quantitation of the relative numbers of wild type versus EPO ligand-containing envelope proteins on the virion surface, the ratio appeared to be on the order of 10 to 1.

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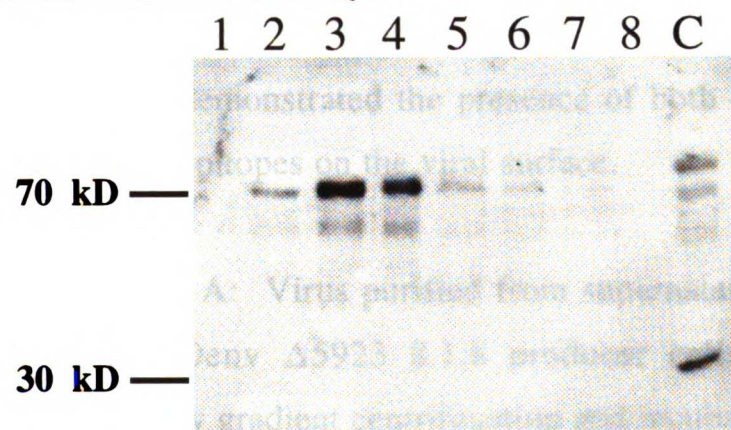
Figure 4-6. Sucrose density gradient fractionation of culture medium from Ψ EPOenv Δ 5923 producer cells and analysis by Western blot, using EPO-, *env*-, and total MLV-specific antibodies.

Culture medium from Ψ EPOenv Δ 5923 producer cell line 8.1.8 was cleared of free proteins by pre-spinning the sample through a 20% sucrose layer onto a 60% sucrose cushion. Intact virions at the 20%/60% interface were tapped and spun through a sucrose density gradient, and 1 ml fractions were collected starting from the bottom of the gradient. These fractions were analyzed by Western blot using EPO-specific (upper panel), murine leukemia virus (MLV) envelope-specific (middle panel), and total MLV-specific (lower panel) antisera. The 70 kD EPO epitopes co-fractionate (and in fact co-migrates) with the envelope epitopes, as well as the p30 gag and p15 transmembrane subunit epitopes detected by the antiserum raised against total MLV, confirming that the EPO ligand is present in intact virions produced by the Ψ EPOenv Δ 5923 producer cells. Lane numbers denote serial fractions collected from the bottom of the gradient. Lane C is control whole cell lysate from Ψ EPOenv Δ 5923 producer cells.

anti-EPO antibody:



anti-env antibody:



anti-MLV antibody:

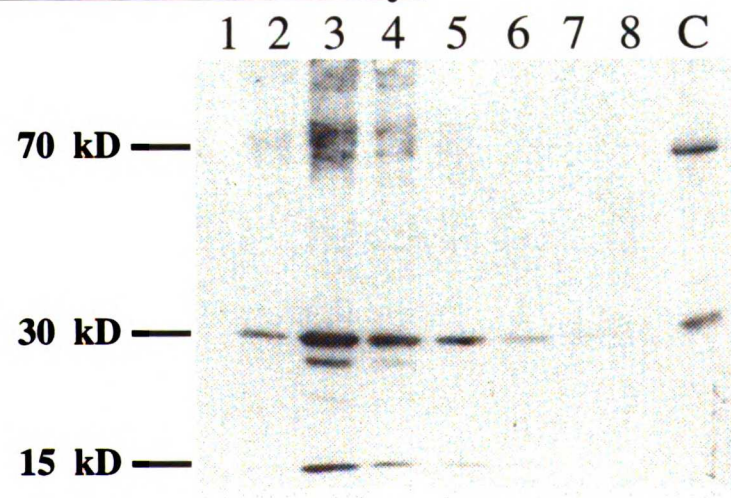


Figure 4-7. Immuno-gold electron microscopy of virions obtained from Ψ EPOenv Δ 5923 producer cells, using *env*- and EPO-specific antibodies.

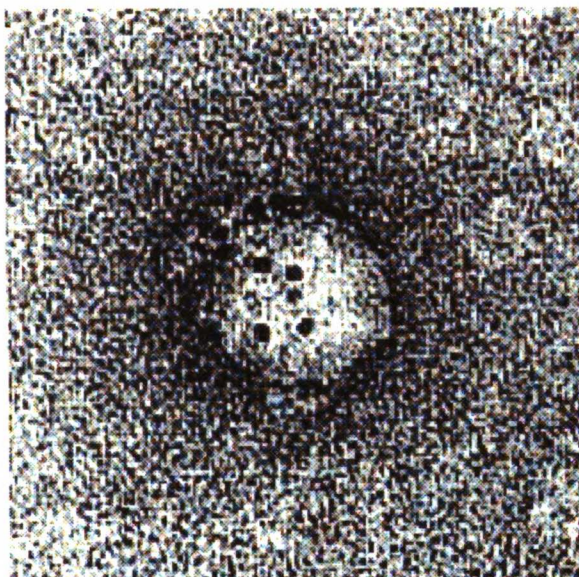
Immuno-gold electron microscopy was performed on virions obtained from the cell culture medium of Ψ EPOenv Δ 5923 producer cells, using both MLV envelope-specific antibody and EPO-specific antibody. This demonstrated the presence of both envelope and EPO epitopes on the viral surface.

Panel A: Virus purified from supernatant medium of Ψ EPOenv Δ 5923 8.1.8 producer cells by sucrose density gradient centrifugation and incubated with goat anti-envelope gp70 antibody/protein G-gold.

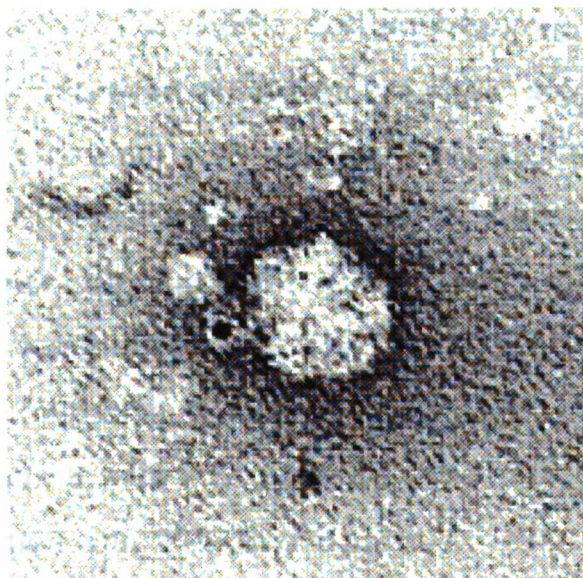
Panel B: The same virus preparation incubated with rabbit anti-EPO antibody/protein A-gold.

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A.



B.



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Viruses produced from Ψ EPOenv 8.1.8 and 8.19.9 cells show up to 6-fold higher infectivity on murine target cells with EPO-receptor.

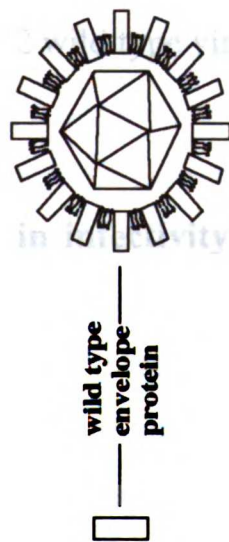
To test if the EPO ligand-bearing ecotropic viruses show an increased affinity for murine cells with the EPO receptor (Fig. 4-8), I had created an EPO receptor-containing target cell line by stably transfecting wild type NIH3T3 cells with a cDNA encoding the EPO receptor [2]. Subclones were screened by ^{125}I -EPO radioligand binding assay, and those showing the highest levels of ^{125}I -EPO binding, corresponding to approximately 10,000 receptors per cell (mean $\pm$ S.D.: $11,557 \pm 1998$ (n=9)), were chosen for use in infection experiments (see also Chapter 3). The wild type (NIH3T3 wt) and EPO receptor-containing (NIH3T3 + EPO receptor) target cells were infected with the CRIP-SVlac vector either packaged by wild type Ψ 2 cells or produced by the Ψ EPOenv 8.1.8 producer cell line. The infections were scored by counting the number of surviving target cell colonies after selection in G418. As previously, two variables then had to be taken into account in interpreting the results and determining the efficiency of infection. First, differences in initial plating density and growth rates of the two target cell lines were corrected for by comparing the number of G418-resistant NIH3T3 +EPO receptor colonies with the number of G418-resistant NIH3T3 wt colonies after infection by wild type Ψ 2 virus. Second, differences in the overall titers of the wild type and Ψ EPOenv viruses were corrected for by comparing the number of G418-resistant NIH3T3 wt colonies after infection with wild type Ψ 2 virus with that of NIH3T3 wt colonies after infection with Ψ EPOenv virus.

Figure 4-8.

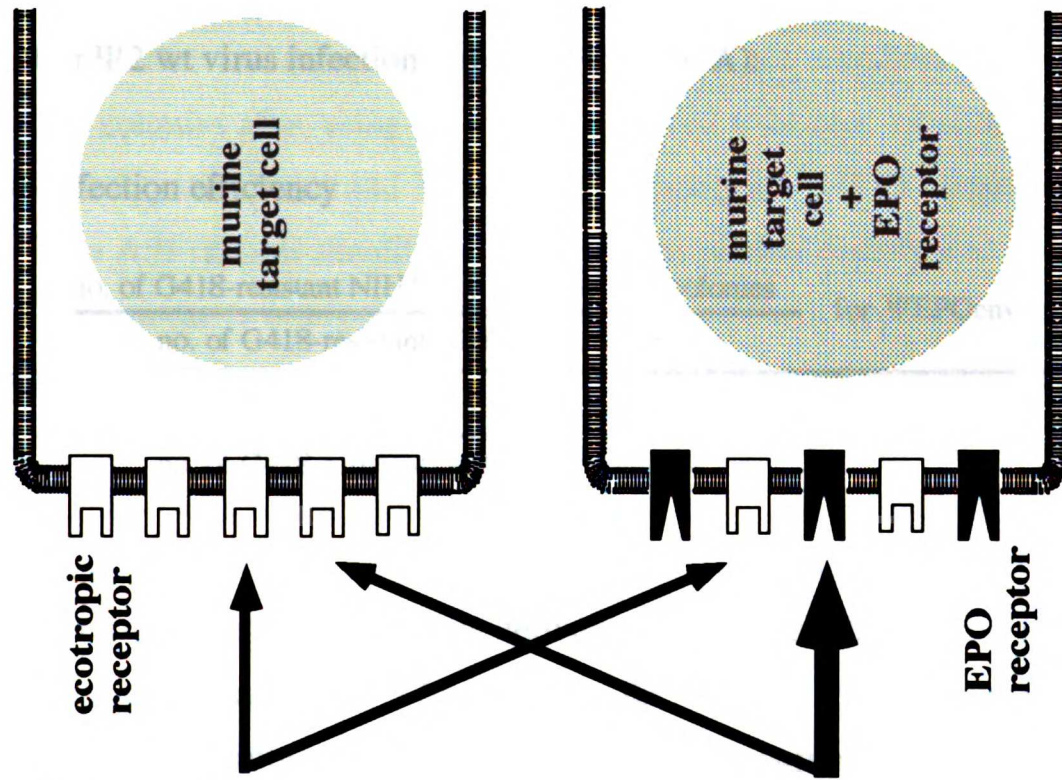
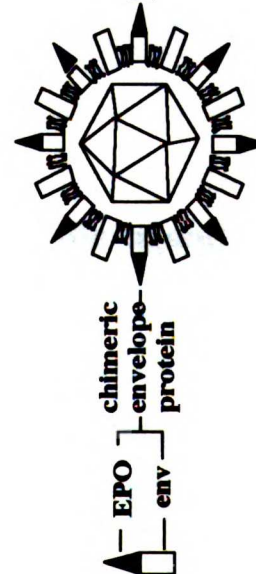
**Strategy for targeting of murine cells
expressing the EPO receptor.**

Schematic diagram of the strategy for enhancing tissue-specificity by ligand-receptor interaction. If the EPO targeting ligand is placed on an ecotropic virus, this will enhance the affinity of that virus for infection of target cells with the EPO receptor. The background level of infection due to the wild type envelope-ecotropic receptor interaction can serve as a baseline for quantitation of the increase in infectivity resulting from the EPO ligand-EPO receptor interaction.

Wild type ecotropic virus vector:



EPO-env chimeric virus vector:



The relative infection efficiency was thus calculated by normalizing the ratio of infection events in NIH3T3+EPO receptor vs. NIH3T3 wt target cells after Ψ EPOenv virus infection, to the respective ratio of infection events after Ψ 2 wt virus infection (see also Fig. 3-16 A):

Relative infection efficiency

$$= \frac{\frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies}}{\text{no. of G418-resistant NIH3T3 wt colonies}} \text{ for } \Psi\text{EPOenv virus}}{\frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies}}{\text{no. of G418-resistant NIH3T3 wt colonies}} \text{ for } \Psi 2 \text{ wt virus}}$$

The relative infection efficiency thus derived, expressed as the mean $\pm$ the standard deviation, was 6.1 ± 1.2 (n=6). Thus, the Ψ EPOenv virus showed a 6-fold increase in infectivity on NIH3T3+EPO receptor target cells compared to Ψ 2 wild type virus (Fig. 4-9).

The increase in infectivity is mediated via a specific ligand-receptor interaction.

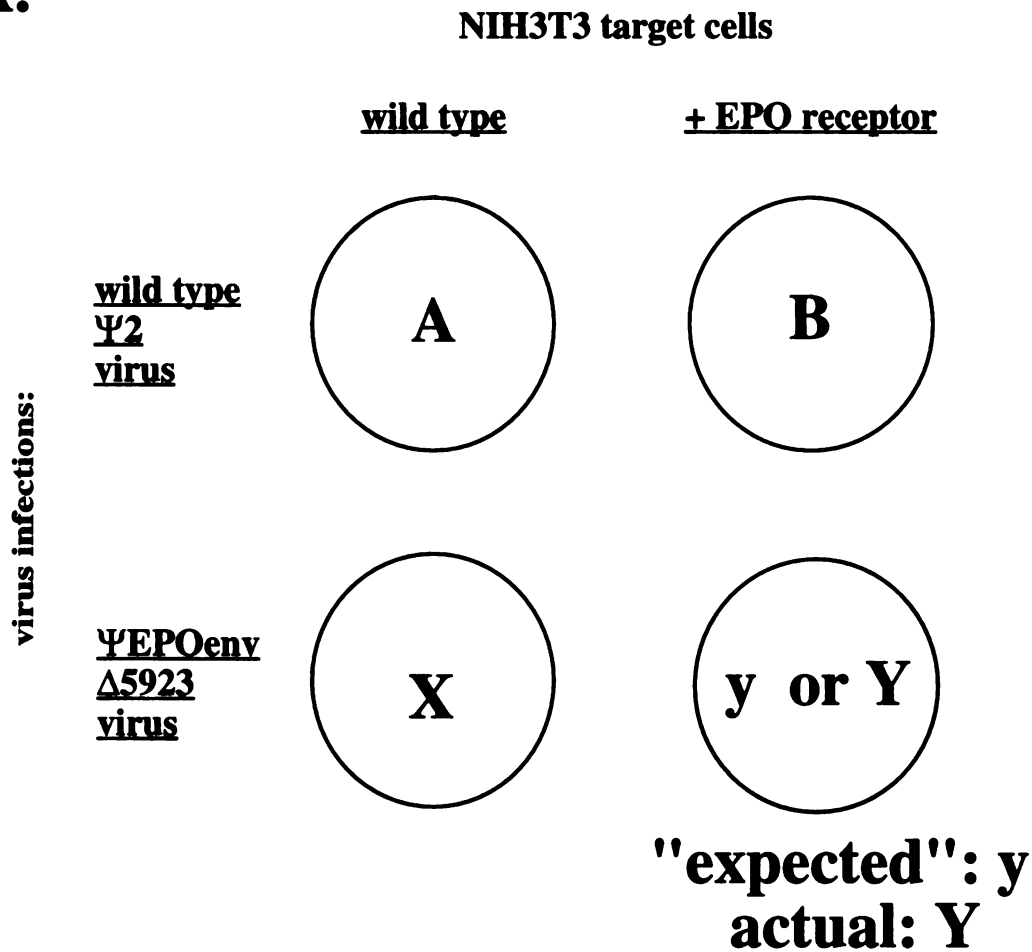
This increased infection efficiency is specifically mediated through the EPO ligand-receptor interaction, as the increase in infection events is abolished in a dose-dependent manner by addition of soluble EPO peptide at the time of infection (Fig. 4-10).

Figure 4-9. Results from infection of NIH3T3 cells with and without EPO receptor, by viruses derived from Ψ EPOenv Δ 5923 producer cells.

Panel A: The variables "A" and "B" represent the numbers of G418-resistant colonies after using the same amount of wild type virus to infect, respectively, wild type and EPO receptor-bearing NIH3T3 target cells. The ratio A:B reflects the differences in initial plating density and growth rate between the two target cell lines, as the titer of wild type virus should theoretically be the same on both. The variable "X" represents the number of G418-resistant colonies after infection of wild type NIH3T3 target cells with EPOenv virus. The variable "y" represents the "expected" number of G418-resistant colonies after infection of EPO receptor-bearing NIH3T3 target cells with EPOenv virus if the null hypothesis, i.e., that there is no difference in infectivity between wild type and EPOenv virus, is true. The ratio X:y therefore equals the ratio A:B, with differences in the actual numbers merely reflecting the relative overall titer of the two viruses. The variable "Y" represents the actual number of G418-resistant colonies counted after infection of EPO receptor-bearing NIH3T3 target cells with EPOenv virus. Thus, if the null hypothesis is true and no difference in infectivity exists between wild type and EPOenv viruses, then the ratio X:Y should equal X:y. However, if there does exist a difference in infectivity, this can be quantitated as the relative infection efficiency by the ratio Y/y. Since $A:B = X:y$, and therefore $y = B/A \cdot X$, this results in the formula $[Y/X] \div [B/A]$ for the relative infection efficiency.

Panel B: Bar graph showing the relative infection efficiency of Ψ EPOenv Δ 5923-derived virus on NIH3T3 target cells with and without EPO receptor, as compared with wild type Ψ 2-derived virus.

A.



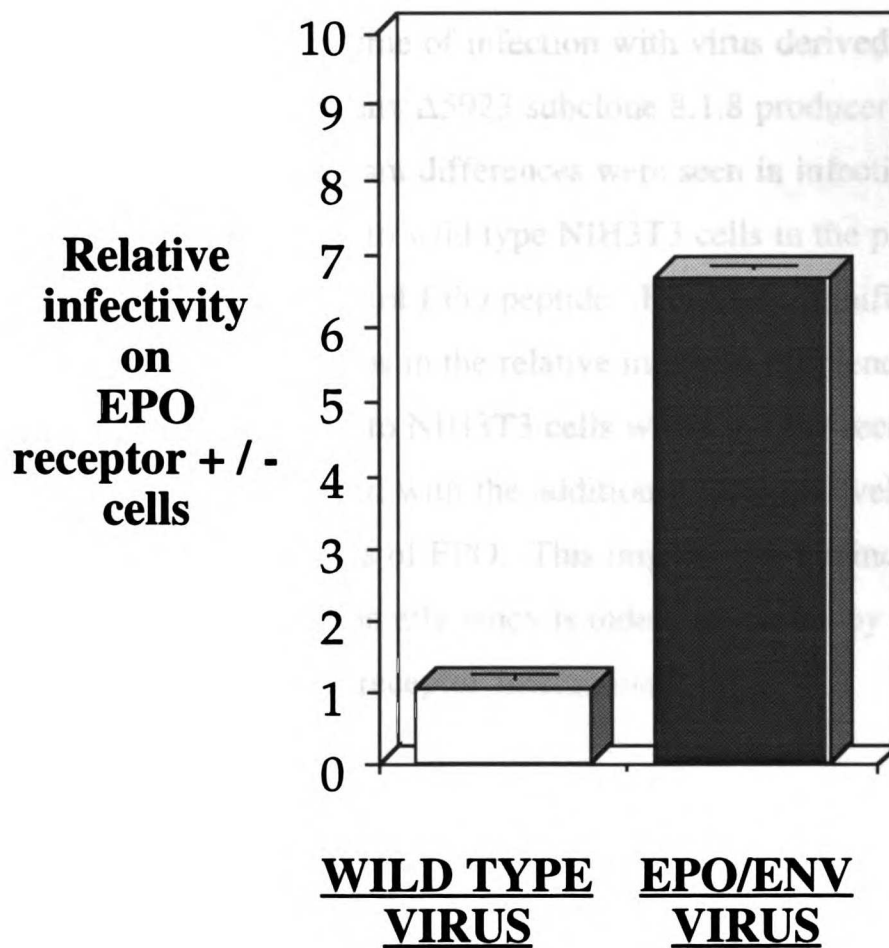
Relative infection efficiency:

$$\begin{aligned}
 & \frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies (Y)}}{\text{no. of G418-resistant NIH3T3 wt colonies (X)}} \text{ for EPOenv virus} \\
 = & \frac{\text{no. of G418-resistant NIH3T3 + EPO receptor colonies (B)}}{\text{no. of G418-resistant NIH3T3 wt colonies (A)}} \text{ for wild type virus}
 \end{aligned}$$

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B.

**Relative infectivity of
wt vs. EPOenv viruses on
EPO receptor + vs. – cells**



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Figure 4-10. Dose-dependent competition of enhanced ΨEPOenv Δ5923 virus infectivity on NIH3T3 +EPO receptor target cells by addition of excess soluble EPO.

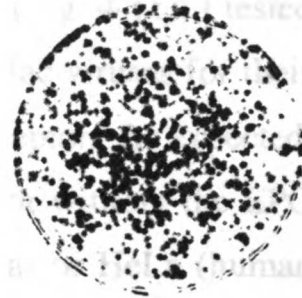
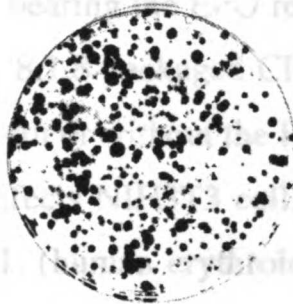
Soluble EPO peptide was added to the NIH3T3 target cell culture medium at concentrations of 5 to 50 µg/ml at the time of infection with virus derived from ΨEPOenv Δ5923 subclone 8.1.8 producer cells. No significant differences were seen in infectivity with respect to wild type NIH3T3 cells in the presence or absence of EPO peptide. However, significant decreases in the relative infection efficiency with respect to NIH3T3 cells with the EPO receptor were observed with the addition of progressively larger amounts of EPO. This implies that the increased infection efficiency is indeed mediated by the EPO ligand-receptor interaction.

NIH3T3 target cells:

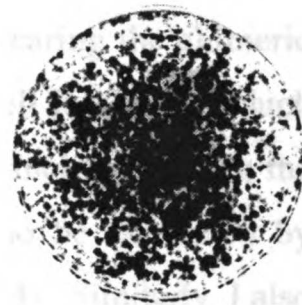
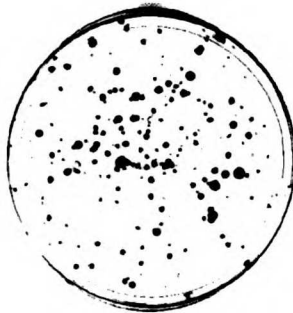
wild type

+ EPO receptor

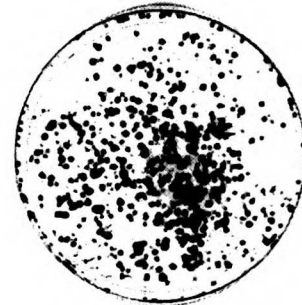
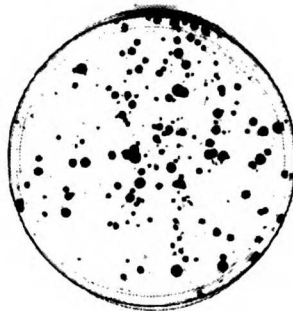
Ψ2
wild type
virus



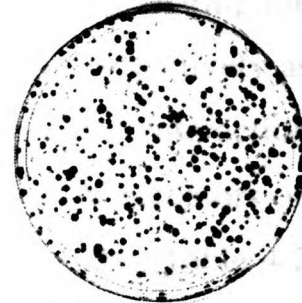
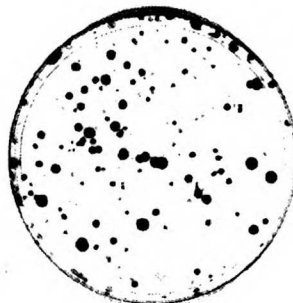
EPOenv
8.1.8
virus



EPOenv
8.1.8
+ EPO
5 μg/ml



EPOenv
8.1.8
+ EPO
50 μg/ml



virus infections:

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Viruses produced from Ψ EPOenv 8.1.8 and 8.19.9 cells can cross-species and specifically infect non-murine cells with the EPO-receptor.

Normally ecotropic viruses do not infect human cells. To test if the ecotropic virus which bears the chimeric EPO-*env* envelope will now cross species to infect human cells bearing the EPO receptor (Fig. 4-11), I tested Ψ 2-, PA317-, and Ψ EPOenv 8.1.8-packaged CRIP-SVlac viruses for their ability to infect human cells with or without the EPO receptor. As expected, Ψ 2-packaged virus, which infects NIH3T3 cells with or without the EPO receptor, does not infect HEL (human erythroleukemia) or HeLa (human cervical carcinoma) cells, whereas PA317-packaged virus infects all of the cell lines. Ψ EPOenv 8.1.8-packaged ecotropic virus bearing the chimeric EPO-*env* envelope specifically infects the erythroid cell line HEL, which bears the EPO receptor, but does not infect HeLa cells, which do not bear the EPO receptor (Fig. 4-12). This infectivity could also be competed by addition of soluble EPO at the time of infection (Fig. 4-13). Similarly, I also found that this virus infects the human erythroid cell line K562, but not the human lymphocytic cell line, Raji (Fig. 4-14). The EPO receptor number was measured by ^{125}I -EPO binding, and it was determined that the HEL and K562 cells contained approximately 1000 receptors (mean $\pm$ S.D.: 1018 ± 144 (n=4)) and 800 receptors (mean $\pm$ S.D.: 867 ± 196 (n=4)) per cell, respectively. Table 4-1 shows a representative assay, using NIH3T3 + EPO receptor cells as a positive control. Since the ecotropic virus bearing the chimeric EPO-*env* envelope infects HEL and K562 cells as effectively as the PA317-packaged amphotropic virus, the ligand-receptor interaction appears to be efficient and does not require large numbers of receptors on the target cell surface in crossing species to infect human cells. Table 4-2 summarizes the results of cross species infections using the Ψ EPOenv virus.

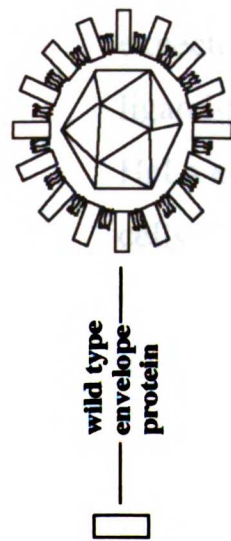
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Figure 4-11.

Strategy for tissue-specific targeting of non-murine cells expressing the EPO receptor.

Ecotropic virus does not normally infect non-murine cells, because the critical residues for virus envelope binding to the ecotropic receptor are conserved only in murine species. In other species, the homologue of the ecotropic receptor differs in those residues and therefore cannot mediate binding. The EPO targeting ligand may, however, now enable the virus to cross species and specifically infect only those non-murine cells which express the EPO receptor, via the EPO ligand-receptor interaction.

Wild type ecotropic virus vector:



EPO-env chimeric virus vector:

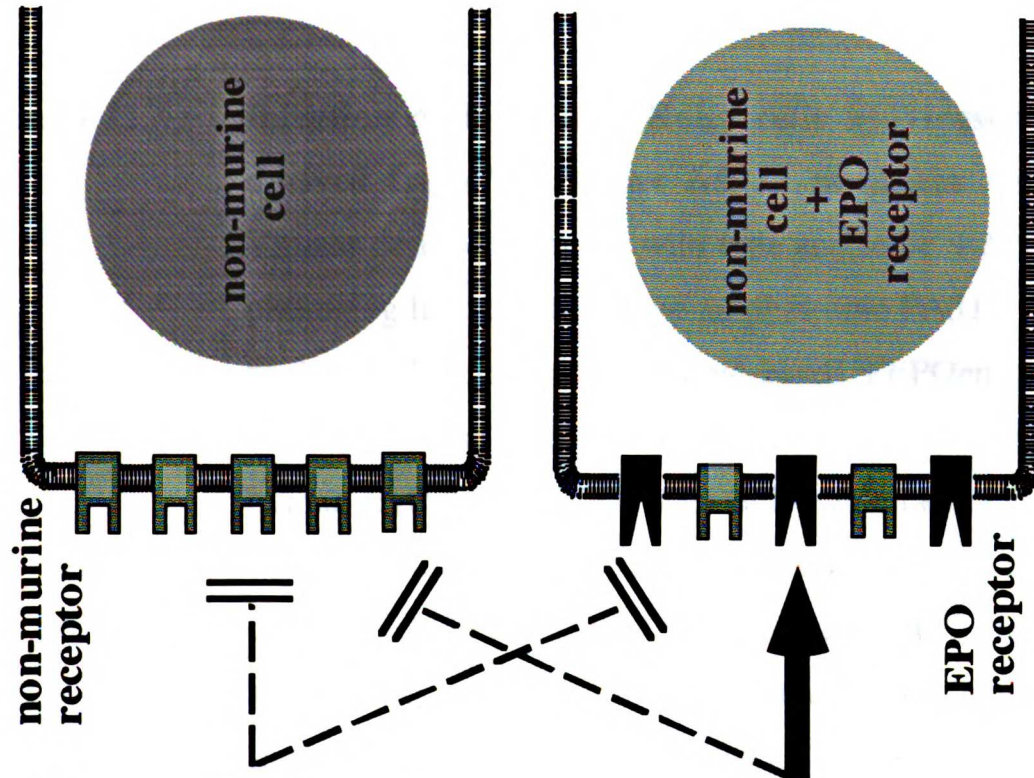
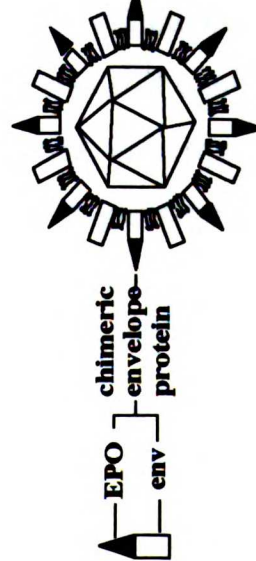


Figure 4-12.

Infection of HEL and HeLa cells by viruses derived from Ψ EPOenv Δ 5923 producer cells.

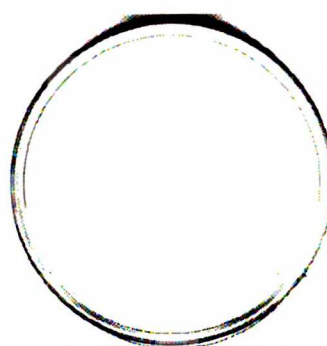
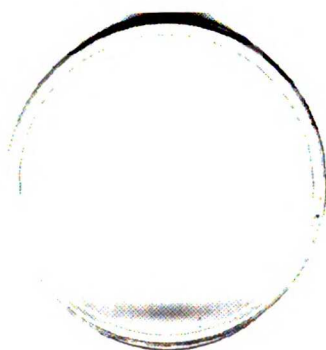
Viruses derived from the wild type ecotropic Ψ 2 packaging line, the wild type amphotropic PA317 packaging line, and the ecotropic-based Ψ EPOenv Δ 5923 subclone 8.1.8 producer line were used to infect human erythroleukemia (HEL) cells, which express the EPO receptor, and HeLa (human cervical carcinoma) cells, which do not.. Wild type ecotropic (Ψ 2) virus, as expected, showed no infectivity on either human cell line. Wild type amphotropic (PA317) virus, as expected, infected both cell lines efficiently. The EPO ligand-bearing (Ψ EPOenv) virus still could not infect HeLa cells, but did show efficient infection of HEL cells. This implies that the ecotropic-based Ψ EPOenv virus can now cross species to infect specific non-murine target cells expressing the EPO receptor.

target cells

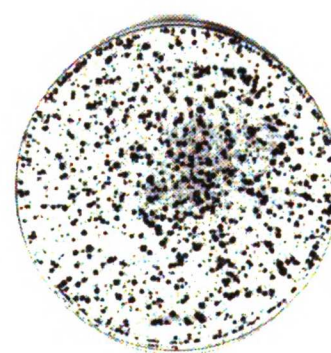
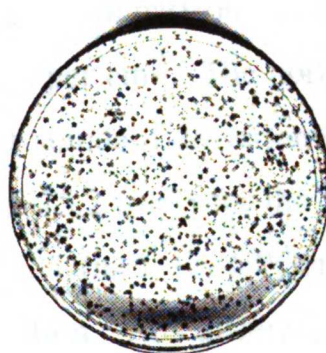
HeLa

HEL

wild type
 Ψ 2 virus



wild type
PA317 virus



Ψ EPOenv
8.1.8 virus

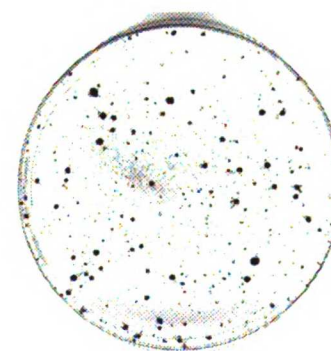
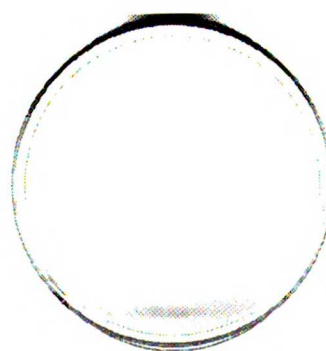


Figure 4-13. **Competition of cross-species Ψ EPOenv Δ 5923 virus infectivity on HEL cells by addition of excess soluble EPO.**

Panel A: G418-resistant colonies on HEL cell plates after infection with EPOenv 8.1.8 virus or EPOenv 8.1.8 virus competed by addition of 5 μ g/ml soluble EPO peptide at the time of infection.

Panel B: A 10-fold magnified view of representative 1 cm² fields.

Panel C: Comparison of the number of G418 resistant colonies per cm². The horizontal bars denote the mean $\pm$ standard deviation for each group, which were 122.7 ± 15.2 (n=3) for the EPOenv virus alone, and 66.3 ± 2.1 (n=3) for EPOenv virus with EPO competition. This difference was statistically significant by Student's *t* test, with $p < 0.005$

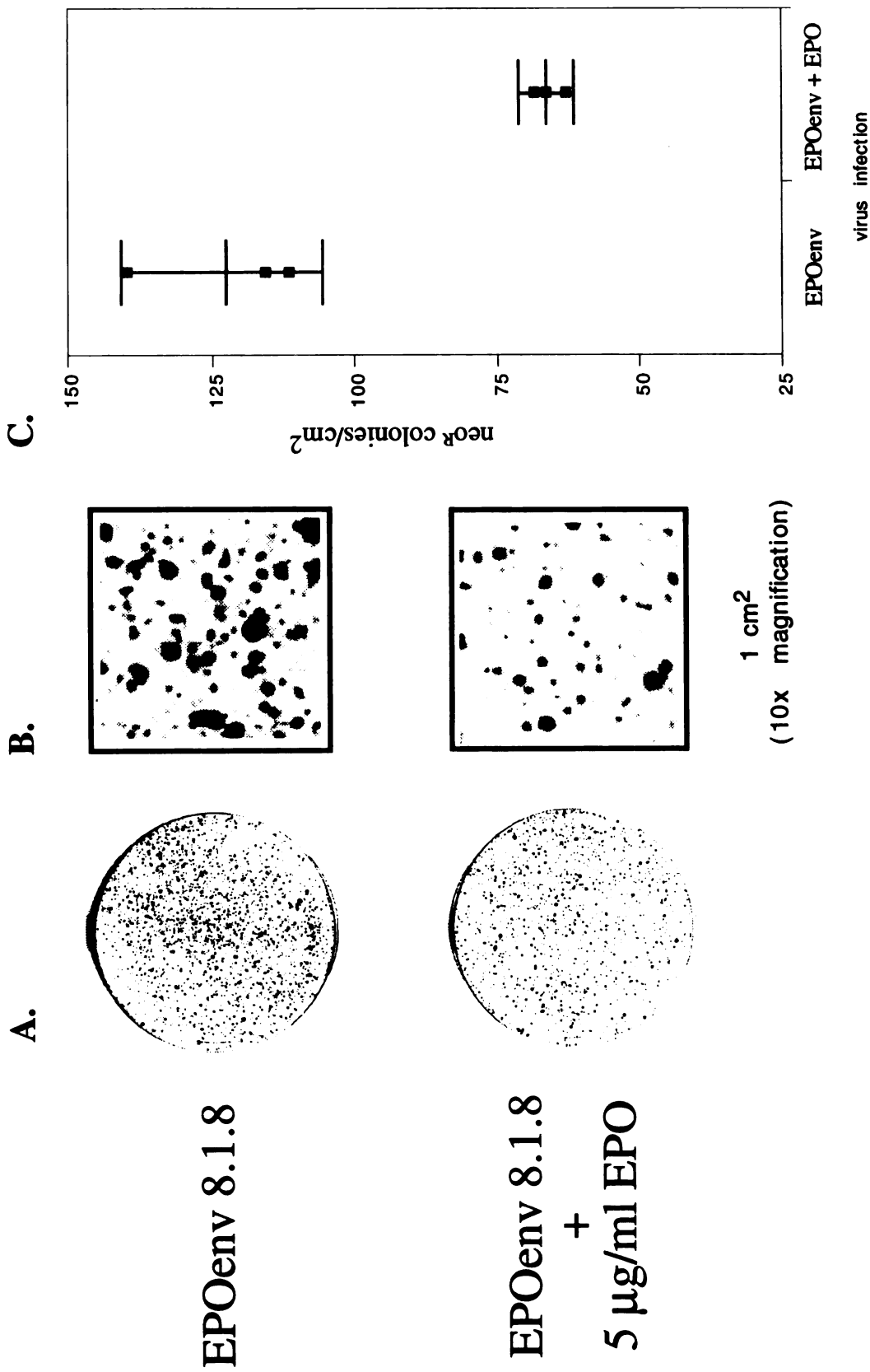


Figure 4-14.

Infection of K562 and Raji cells by viruses derived from Ψ EPOenv Δ 5923 producer cells.

Viruses derived from the wild type ecotropic Ψ 2 packaging line, the wild type amphotropic PA317 packaging line, and the ecotropic-based Ψ EPOenv Δ 5923 subclone 8.1.8 producer line were used to infect K562 (human erythroleukemia) cells, which express the EPO receptor, and Raji (human lymphoma) cells, which do not. As these are non-adherent cells, the infections were scored by selecting the cells in G418, periodically passing the cells through a Ficoll gradient to pellet the dead cells, counting the viable cells at the interface, and re-plating the viable cells. The surviving cell count is expressed as $\times 10^4$ per ml.

□: uninfected, $\diamond$: Ψ 2 virus infected, X: Ψ EPOenv virus infected, +: PA317 virus infected.

Wild type ecotropic (Ψ 2) virus, as expected, showed no infectivity on either human cell line. Wild type amphotropic (PA317) virus, as expected, infected both cell lines efficiently. The EPO ligand-bearing (Ψ EPOenv) virus still could not infect HeLa cells, but did show efficient infection of HEL cells. This implies that the ecotropic-based Ψ EPOenv virus can now cross species to infect specific non-murine target cells expressing the EPO receptor.

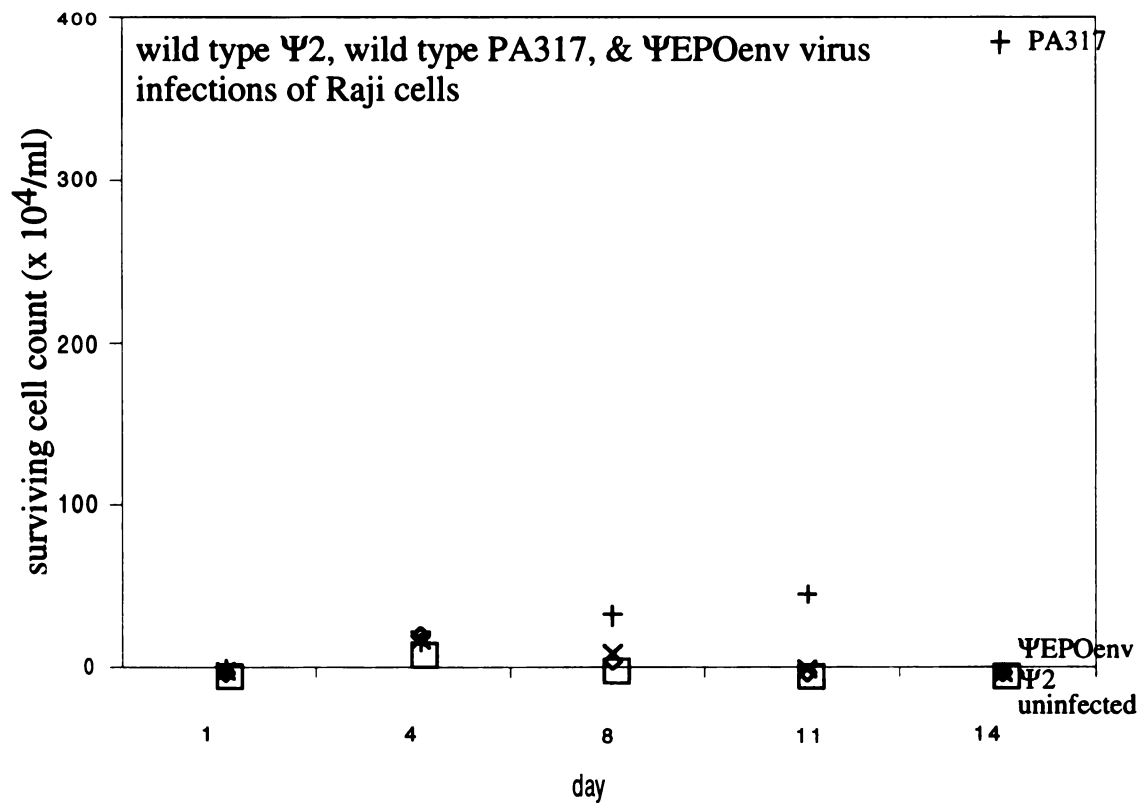
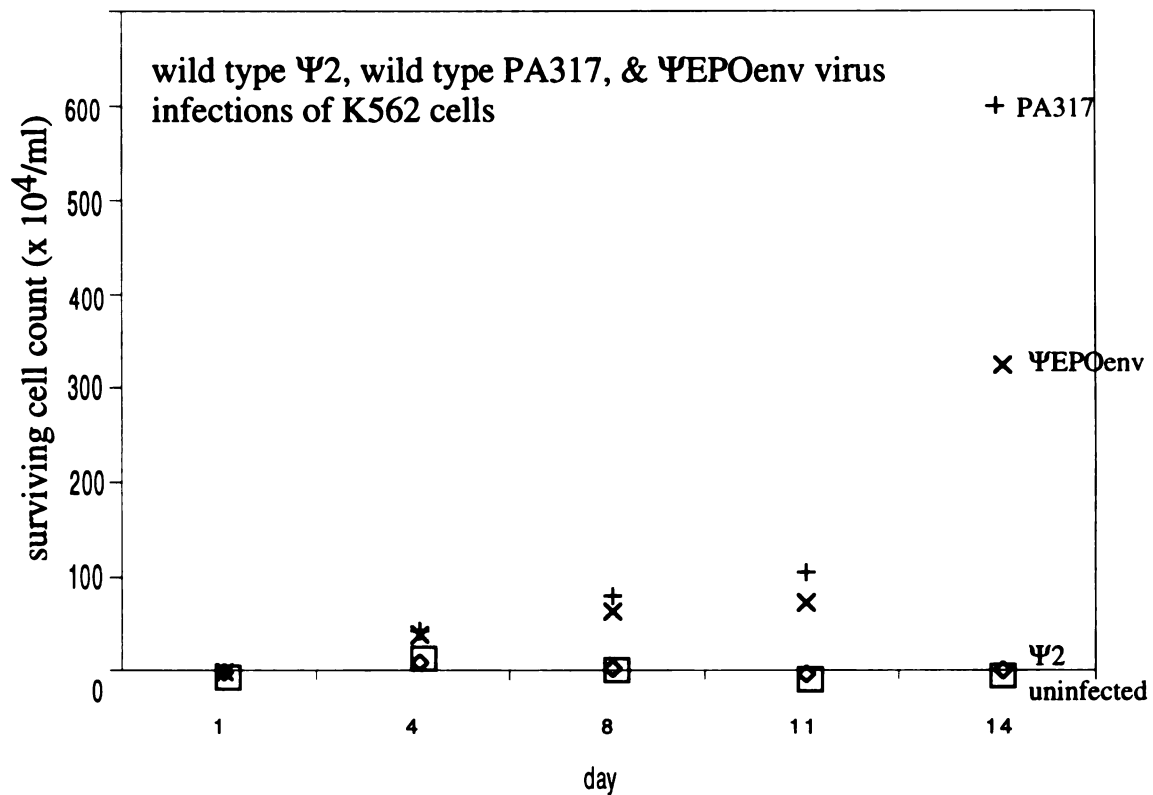


Table 4-1. Representative ^{125}I -EPO binding assay of target cells.

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^{125}I -EPO binding assay of target cells:

<u>target cell</u>	<u>cpm bound</u>	<u>^{125}I-EPO bound*</u>	<u>no. of EPO-R/cell**</u>
NIH3T3+EPO-R			
with ^{125}I -EPO	11718	1.7595	10,962
with cold EPO	4	0.0006	<10
HEL			
with ^{125}I -EPO	846	0.1270	791
with cold EPO	6	0.0009	<10
K562			
with ^{125}I -EPO	1098	0.1649	1027
with cold EPO	6	0.0009	<10

* ^{125}I -EPO bound = cpm bound per well divided by the specific activity at the time of assay (6660 cpm/ 10^{-15} M)

**no. of EPO-R/cell = moles of ^{125}I -EPO bound multiplied by 6.23×10^{23} molecules/M and divided by 1×10^5 cells per well

Table 4-2. Summary of results from cross-species infections.

The Ψ EPOenv virus can mediate cross-species infections which are specific for target cells bearing the EPO receptor. This table shows a summary of the results of cross-species infection experiments using the ecotropic-based EPOenv virus, as well as wild type ecotropic and amphotropic control viruses, to infect human cells with and without the EPO receptor.

human target cell	wild type Ψ 2 (ecotropic) virus	wild type PA317 (amphotropic) virus	EPOenv (tissue-specific) virus
HeLa (cervical carcinoma)	—	++	—
Raji (lymphocytic)	—	++	—
HEL (erythroleukemia)	—	++	+
K562 (erythroleukemia)	—	++	+

Discussion

These experiments have demonstrated that chimeric envelope proteins which contain sequences not present in naturally occurring retroviral envelopes can be expressed in virus-producing cells and incorporated into intact virions. Chimeric envelope proteins bearing targeting ligands can enhance the cell-specificity of the resultant virus, and enable ecotropic-based viruses to cross species and infect human cells with the appropriate receptor. These findings have obvious implications for targeted delivery of therapeutic genes via viral vectors.

Previously it has been shown that the host range of viruses can be altered by pseudotyping [11-13], however the alternative envelope proteins used in such experiments were derived from naturally-occurring viral sequences such as GALV, ALV, HIV, etc., and hence the resultant pseudotyped virions were still limited by the host range of the naturally-occurring virus. In some cases, it has been shown that viral targeting can be achieved by ligand-receptor interactions, mediated by bivalent antibodies using biotin-streptavidin as linkers [14], or by chemical modification with lactose, to produce an asialoglycoprotein [15]. These manipulations, which involve modifications to the virus after its production, usually result in low infection efficiency. A recombinant virus containing in its envelope a sequence encoding a single-chain antibody variable region was shown to bind to a solid matrix containing the appropriate polypeptide antigen, and the bound viruses, as expected, are infectious for NIH3T3 cells [16]. However, direct infection of target cells

by the virus through antigen-antibody interaction was not demonstrated. In these experiments, an ecotropic virus was engineered to bear a chimeric envelope containing a targeting ligand on its surface. This virus not only showed enhanced infectivity for murine cells which bear the appropriate receptor, but could also cross species and specifically infect the appropriate receptor-bearing human cells. These effects could be abolished in a dose-dependent manner by competition with excess ligand, thus demonstrating that they were mediated by specific ligand-receptor interactions.

Even more recently, as this manuscript was being prepared, Cosset and co-workers reported the use of the same strategy to re-direct the host range of avian leukosis virus (ALV)-based vectors, using an integrin ligand inserted into the envelope glycoprotein [17]. The ALV envelope contains four variable regions (vrI, II, III, and IV) which are postulated to be involved in binding of the envelope to its natural receptor. Cosset's group constructed a set of envelope mutants in which these regions were replaced by a sequence encoding a 16 amino acid RGD-containing peptide, designated FLA16, which is known to be a ligand for certain cell surface receptors of the integrin superfamily [18]. These constructs were expressed in the HNLB20 avian packaging cell line to generate viruses encoated by FLA16 ligand-containing envelope proteins. The HNLB20 cell line, derived from quail QT6 cells, produces ALV *gag* and *pol* proteins as well as the replication-defective avian retrovirus vector NLB, which carries the neomycin resistance and β -galactosidase genes, but does not produce any wild type envelope [19]. Avian virus vectors thus encoated by envelope proteins containing the FLA16 ligand in region II were able to infect HT1080 human fibrosarcoma and Rugli rat glioblastoma target cells,

which express integrin receptors that bind FLA16 [20, 21] and are normally resistant to infection by avian retroviruses. This cross species infection was specifically mediated by the integrin ligand-receptor interaction, as it could be competed by pre-incubation of the target cells with soluble FLA16. Thus the same targeting strategy which I employed for an ecotropic murine virus system also works in an avian retrovirus system.

It is as yet unclear whether the presence of the wild type envelope is necessary for these chimeric viruses to gain entry to the target cell after binding via the ligand/receptor interaction. In this case there appeared to be roughly a 10 to 1 ratio of wild type to EPO-containing envelope on the virion surface. This is a very soft number derived from the electron microscopy results, which were not optimal (note that the anti-env antibody should result in immunogold particles covering the entire surface of the virion, but this is not seen in Fig. 4-7 A), either because the antibody concentrations were not saturating, or because passing the sample through a sucrose gradient in order to concentrate the viruses resulted in shearing off of the outer envelope proteins, a phenomenon which is known to commonly occur due to the loose, non-covalent nature of the bonds which tether the gp70 proteins to the inner transmembrane subunit [22]. Possibly the efficiency of EPO receptor-specific infection may be further improved by increasing the number of EPO ligand-containing envelope proteins on the virus surface. However, it remains to be seen whether a viral coat composed exclusively of ligand-containing recombinant envelope proteins will still function to allow entry of the virus via membrane fusion after binding to the host cell surface. Normally the outer (gp70) envelope subunit mediates

attachment to the host cell, and this event triggers a conformational change that exposes the inner (p15) envelope subunit, which is linked to the carboxy-terminal region of gp70 by disulfide bonds and which, once exposed, catalyzes fusion of the lipid membranes of the virus and the cell. Although the carboxy-terminal region of the outer gp70 envelope subunit was not altered and the inner p15 subunit left intact in order to preserve this function as much as possible, it is still possible that the recombinant EPOenv protein might not be capable of achieving the conformational change necessary to expose the inner subunit upon binding to the EPO receptor. In that case, it may be important for the wild type envelope protein to be present, to allow subsequent fusion to occur. This implies that there is an inherent "on-off" rate of conformational change in the envelope protein, and that tethering the virus to the target cell surface long enough until the wild type gp70 envelope protein flips open spontaneously and exposes the inner subunit is sufficient to mediate entry.

Although the most straightforward way of testing this hypothesis is by creating packaging cell lines containing only the recombinant EPOenv gene, without the wild type envelope, in view of the difficulties encountered in trying to express the EPOenv constructs in GP101 cells, this may not be possible. Even if, however, the recombinant envelope needs to be rescued by the wild type envelope in order to be expressed in packaging cells and assembled into a viral coat, and successful infection of EPO receptor-bearing cells cannot be mediated in the absence of the the wild type envelope, it still appears to be possible to create ecotropic-based retroviral vectors containing wild type and ligand-bearing envelopes which are completely tissue-specific in a non-murine setting, because the wild type envelope cannot itself bind to

the cationic amino acid transporter of non-murine species. Thus the question of whether purely recombinant viral coats without wild type envelope could be made would be a peripheral issue from the standpoint of creating viral vectors, but is nevertheless interesting in terms of the mechanisms involved in virus entry into host cells.

Alternatively, if tethering the virus to the host cell surface long enough for the wild type envelope to initiate fusion is sufficient for entry, it could be imagined that the targeting ligand does not necessarily need to be part of the *env* protein itself; perhaps co-expressing the targeting ligand on the virion surface would be sufficient to mediate tissue-specific infection. It has been shown by Young et al. [23] that when CD4 or fusion proteins containing the CD4 transmembrane domain are expressed in avian retrovirus-producing cells, and virions are isolated from the cell culture supernatant, these proteins copurify with the avian retroviral envelope protein on a sucrose density gradient. Thus, it seems that exogenous cell surface proteins will mingle with the viral envelope and bud off along with the virus. It is therefore conceivable that if one can anchor a ligand in the plasma membrane of a packaging cell line, for example by fusing the ligand to the transmembrane domain of a cell surface protein, then this fusion protein might then be incorporated nonspecifically into the ecotropic viral envelope structure and displayed on the virion surface. Again, if tethering the virus to the target cell surface long enough for the wild type envelope fusion protein to spontaneously become exposed is sufficient, then this strategy should be equally effective in mediating cell-specific infection. The advantage of such a strategy is that one would be free from the conformational constraints of having to insert the ligand into the context of

the envelope protein, thus allowing a much larger variety of ligands to be used and greatly simplifying the technical aspects. If, however, this strategy does not work, it would suggest that it is indeed the recombinant envelope which undergoes a specific conformational change induced by ligand binding to the receptor, thus exposing the inner subunit.

Furthermore, it remains to be seen whether other processes, such as receptor-mediated endocytosis, might play a role in retroviral entry. While amphotropic MoMLV is known to enter at the host cell surface via a pH-independent process [24], ecotropic MoMLV appears to go through an endosomal compartment and enters via a pH-dependent process. In fact, very early studies on MoMLV reported that viral infection and replication could be inhibited by chloroquine, which disrupts the endosomal pH [25]. Thus it is possible that activation of the fusion protein requires an acidic pH environment; in that case, tethering the ecotropic wild type envelope to the host cell surface will probably not be sufficient to mediate entry. This could be tested in an interesting manner by changing the ligand on the chimeric envelope to one which utilizes a receptor that does not undergo endocytosis upon binding. Or, for example, ligands such as apolipoprotein B100 or E, which bind the low density lipoprotein (LDL) receptor, could be used to one's advantage since mutations in the LDL receptor which abolish receptor-mediated endocytosis are well documented.

The EPO receptor itself appears to be internalized after binding the ligand, as evidenced by surface labelling of EPO receptors with radioiodinated EPO ligand at 4°C, followed by internalization of the radioligand when the cells were warmed to 37°C [2]. However, it is not known what

cellular compartment the internalized receptors are then directed to; obviously, this may affect the efficiency of infection of any viruses which attach to the receptors, and perhaps internalize with them. Another interesting, though speculative, point is that there now seems to be evidence for a second cellular component of the EPO receptor, which when combined with the cloned receptor protein results in a binding site with higher affinity than that of the cloned protein alone. Goldwasser and colleagues have shown that some cells express this second component, and PEG-mediated fusion of these cells to CHO cells expressing exclusively the cloned protein results in an increase in high affinity binding sites. NIH3T3 cells may not express this second component, and this could be a reason for the increase in relative infection efficiency being limited to approximately 6-fold. On the other hand, HEL cells are erythroid in origin and produce endogenous high affinity EPO receptor, presumably with both components expressed, and this may result in more efficient infection via ligand-receptor interaction. Theoretically, as the ratio of the number of virions physically present to the effective titer is on the order of 1000 to 1 (at least for HIV [26]), that is, only one out of every 1000 virions present actually infects a target cells under normal circumstances, it is probably possible to increase the ligand-receptor mediated infectivity even more by designing interactions of higher affinity. That an increase in the binding affinity, or K_B , will result in an increased ratio of bound versus free ligand is readily demonstrable by the principles of mass action and application of the Scatchard plot. In the case of ligand-bearing virions, the particle is presumably multivalent, that is, there are many ligand moieties on the surface of a single virion, and this may also increase the efficiency of receptor-specific infection.

In summary, I have demonstrated that it is possible to purposefully alter the host range of ecotropic retrovirus vectors and achieve tissue-specific gene delivery by designing chimeric envelope proteins which contain targeting ligands. The approach demonstrated here can be applied more broadly to generate a wide variety of viruses which bear different ligands for specific receptors, and thus deliver genes to specific tissues or organs for the treatment of genetic diseases and cancer. Finally, in a general sense it should be further noted that, as in the examples given above, the process of engineering the viral coat to achieve a specific aim, i.e., tissue-specific gene delivery, can also serve to elucidate the basic mechanisms by which viruses enter cells.

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CHAPTER 5.

Discussion and perspectives: the use of tissue-specific vectors for gene therapy

Issues in gene therapy

Gene therapy, or the treatment of diseases at the genetic level, has in recent years finally but tentatively become a clinical reality. This entails the stable introduction of therapeutic genes into somatic cells. For this to be achieved, 1) the exogenous gene must be delivered to the correct target cells and stably remain there long enough to be effective, 2) the exogenous gene must be expressed in the target cell at an appropriate level, and 3) the foreign gene must not harm the host cells, or, by extension, the animal [1]. These three requirements may be summarized as delivery, efficacy, and safety. While the theme of this project directly addresses the first requirement, I believe that achieving tissue-specific gene delivery has implications for the issues of efficacy and safety as well.

Various approaches to gene transfer have been investigated in order to devise methods for highly efficient delivery of genetic information into target cells and which would serve as the optimal delivery system for attempts at gene therapy [1-3]. These include: 1) chemical techniques (such as calcium phosphate-mediated DNA uptake) [4], 2) physical techniques (e.g., electroporation [5] or microinjection [6]), 3) liposome fusion [7], and 4) viral vectors (both RNA [2, 8] and DNA [9] viruses). At present, viral vectors (retroviruses in particular) are the technique of choice for most gene therapy protocols.

The use of retroviral vectors in gene therapy

Retroviruses are a particularly advantageous vehicle for the delivery of genes into eukaryotic cells because of the following attributes: 1) high efficiency of transfer; potentially, up to 100% of cells can be infected by retroviruses (in contrast with the low efficiency of chemical techniques, in which typically less than one cell in 1000 is transduced), 2) many cells (typically 10^6 to 10^7) can be infected simultaneously (in contrast with physical techniques such as microinjection, in which each cell must be injected individually), 3) stable integration into the host cell DNA, being a natural part of the retroviral life cycle, is an efficient event; the integrated provirus is passed on to all daughter cells, and continues to direct the nonlytic production of its encoded products [8], thus readily allowing establishment of permanent cell lines which continuously make virus, and 4) replication-defective virus vectors can be created by the deletion of essential viral functions, which renders the vectors incapable of secondary horizontal infections of adjacent cells.

However, despite such progress in developing retroviral vectors which deliver genes to cells with high efficiency, the issues of efficacy and safety still remain to be addressed before gene therapy becomes a practical and feasible enterprise.

Ideally, for human gene therapy, it would be desirable to replace a defective gene and introduce its functional equivalent gene to substitute for it *in situ* . Since such *in situ* replacement would mean that the newly inserted gene is located in the correct chromosomal location within the

correct cell type (i.e., the cell type in which it is normally expressed), all the regulatory elements necessary for its proper functioning would be present, and thus it should express at sufficient levels under appropriate regulatory control, thereby enhancing both the efficacy as well as the safety of the procedure [1, 10].

Different approaches may be envisioned to achieve such tissue- and site- specific expression of exogenous genes. These include: 1) site-specific recombination or integration, 2) cis-acting signals which specify tissue-specific expression, and 3) vectors which mediate tissue-specific delivery. Of course, the best approach would combine all three, as a series of multiple fail-safes, so that the exogenous genes would only enter the correct cell type, only express in that cell type, and would be located in the proper chromosomal location.

Unfortunately, at present, severe difficulties have been encountered in the development of these approaches, with corresponding deleterious consequences ensuing. First, site-specific homologous recombination has been achieved using plasmid vectors transfected by electroporation; however, the relatively low efficiency of this procedure ($1:10^3$) seems to preclude its usefulness as a therapeutic tool [11, 12]. Second, the retroviral vectors used at present are non-specific in their host range, and the viral sequences which drive their expression (long terminal repeats, or LTR sequences) do not always seem to work efficiently in mammalian cells *in vivo*; efforts have therefore focused on specifying tissue-specific expression through specific cis-acting regulatory sequences in spite of non-specific delivery of foreign genes [1, 13]. Third, though encouraging results have

been reported in retroviral gene transfer experiments using cultured cells[14, 15], difficulties have been encountered when attempting to apply these procedures to whole animals [16-18]. These difficulties consist primarily of unstable or low expression of the inserted genes [16, 17], and lack of appropriate regulation of expression levels [18], in whole animals.

Such difficulties may stem, at least in part, from the fact that, the inserted genes may contain promoter or enhancer elements which are inappropriate for the tissue they are to be expressed in. If this is the case, then matters would be improved if the genes could be delivered specifically to the appropriate tissues in which expression would be properly regulated. While the retroviruses currently in general use exhibit species-tropism (i.e., they are either ecotropic, xenotropic or amphotropic [19]), unfortunately they are not tissue-tropic in each species. Thus, the virus will potentially infect cells of all tissues. Consequently, the standard procedure for retroviral gene transfer necessitates removing the target cells from the body, infecting these cells with the retrovirus vector in primary culture, and subsequently returning the treated cells to the body [3, 13]. This particular approach has obvious drawbacks. Target tissues are at present limited to those cell types which can be removed from the body, then maintained and manipulated in culture, such as bone marrow cells [16, 17], skin fibroblasts [18], and hepatocytes [14]. The procedure is tedious, and the amount of tissue (such as hematopoietic stem cells) which can be removed at one time is often insufficient. The second drawback is that the expression of the inserted gene may remain low [16, 17, 20]. There has been initial enthusiasm about using enzymes such as adenosine deaminase as a model system because the gene is small and high levels of expression are not required. However it was

subsequently found that these genes are expressed at low levels when inserted into hematopoietic tissue, presumably because the LTR (long terminal repeat) used in the retrovirus vector is not an appropriate enhancer in certain cells [21-23]. Furthermore, even the use of the original promoter for that gene may not always guarantee adequate or appropriate expression, because of the currently limited repertoire of target cells that can be removed and manipulated in culture. In fact, this limited repertoire of target tissues makes it inevitable that most exogenous genes will be introduced into cell types in which they are not normally expressed, thus leading to improper expression, which may be an important factor contributing to inefficacious or dangerous results. In other words, since the only tissues available for retroviral gene transfer *in vivo* are bone marrow, skin fibroblasts, hepatocytes, etc., it is not possible to introduce, for example, the insulin gene and its promoter into the appropriate cellular milieu; conversely, that introduction of an insulin promoter/gene construct into an inappropriate milieu can lead to inappropriate or unregulated gene expression with life-threatening consequences was dramatically illustrated by reports of diabetic mice which paradoxically died from hypoglycemia due to hyperinsulinemia caused by "therapeutic" insertion of the insulin promoter/gene into skin fibroblasts [18]. This is due to the logical inconsistency of attempting to put an exogenous gene under the control of a "tissue-specific" cis-acting regulatory element, such as an enhancer, into the "wrong" cell type. In fact, "tissue-specific" enhancers may require tissue-specific trans-acting factors to bind to their DNA sequences, for appropriate expression to be achieved [24]. When delivered into the wrong tissue, the "tissue-specific" enhancer would not encounter the correct trans-acting factor, leading to improper expression. Thus, in general, most exogenous promoters will most likely be introduced

into cell types in which they are not normally expressed, leading to low or deregulated expression. The third drawback occurs when gene replacement therapy is directed to tissues and cells that undergo terminal differentiation, such as hematopoietic cells. When retrovirally altered cells are returned to the animal, hematopoietic colonies containing inserted genes are established initially, but gradually disappear from the animal, probably because only few pluripotent stem cells are infected in the bone marrow specimens used for retroviral infection, and most infected cells are already terminally differentiated. The problem of loss is more severe in larger animals such as dogs [16] or primates [25].

The advantages of developing a tissue-specific vector

In view of the above considerations, it seems desirable to construct, if possible, a vector which would deliver genes into target tissues or organs not only with high efficiency, but also with high specificity; such a system would enable tissue-specific promoters and enhancers to work properly, by targeting delivery to the appropriate cell type. As mentioned above, at present, most retroviral vectors currently in use are not tissue-specific: these vectors are based on variants of Moloney murine leukemia virus, normally an "ecotropic" virus (which only infects mouse cells) [19, 26], that have been isolated as naturally occurring mutants which were found to be "amphotropic" (infecting both mouse and non-mouse cells) [19, 27-29], and they are known to infect a wide variety of tissues. Therefore in this project, I have pursued the development of a model system for retroviral vectors which exhibit tropism for specific cell types; such vectors would only attach

and enter one particular cell type, and would represent a delivery system which is potentially applicable *in vivo*. Furthermore, since such a vector would only infect the desired cell type specifically and efficiently, the therapeutic gene with its promoter could be delivered to the cells or tissues in which it is normally expressed; this would reduce the likelihood of subsequent silencing or deregulated expression of the delivered gene, thus increasing the efficacy and safety of the procedure.

In practical terms, this project has dealt primarily with the problem of demonstrating erythroid-specific retroviral gene delivery in an *in vitro* cell culture system. The specific targeting ligand used in these experiments was EPO, a sialoglycoprotein hormone which regulates the rate of erythrocyte formation [30], and is recognized by EPO receptors normally expressed only in hematopoietic cells of the erythroid and megakaryocytic lineage [31]. By using EPO as a targeting ligand, I have been able to confer to an ecotropic virus the ability to specifically infect non-murine cells expressing the EPO receptor in culture, mediated by ligand-receptor interaction. The *in vivo* equivalent of this target cell population would be the erythroid cells at the BFU-E (blood forming unit-erythroid) and CFU-E (colony forming unit-erythroid) stages. These cells are already terminally differentiated, and would not serve as suitable targets for most applications. However, infection of earlier hematopoietic progenitors could conceivably be achieved by changing the ligand to target c-kit [32, 33] or CD34 [34, 35], which are cell surface molecules present as early as the stem cell stage. A retrovirus vector with tissue tropism for this specific subpopulation of target cells could potentially be of use in gene therapy of red cell disorders such as

sickle cell anemia and thalassemias, as well as in developmental studies of erythroid cell differentiation.

For human gene therapy, an additional advantage of using ecotropic virus-based vectors bearing tissue-specific targeting ligands is that infection of human cells without the specific receptor will not occur, thus resulting in a safer vector. Tissue-specific vectors are also potentially more efficacious, as the input viruses are not wasted on irrelevant tissues, effectively increasing the titer with respect to cells expressing the appropriate receptor. This would be especially advantageous when attempting to transduce rare cell types such as the early hematopoietic stem cells, which occur at a frequency of 1 in 10,000 to 100,000 in the bone marrow cell population.

Once targeted delivery of genes into erythroid cells is accomplished, high levels of expression of the inserted gene can be achieved by utilizing erythroid-specific promoters and enhancers. This is an example of how the issue of gene delivery ties into the issue of efficacy. Recent work has shown that four major DNase I hypersensitive sites (HS) far upstream from the β -globin gene cluster are important for tissue-specific expression of the globin gene in erythropoietic cells. Several lines of evidence demonstrate the important role which the DNA sequences of these HS sites play in enhancing the expression of erythroid cell-specific genes: (1) these sites are only hypersensitive in erythroid cells [36, 37]; (2) patients in whom these sites are deleted exhibit impaired β -globin gene expression [38-40]; (3) the addition of these sites to β -globin gene constructs significantly enhances expression of these constructs up to levels comparable to endogenous globin expression in transgenic mice [41-43]; (4) the addition of these sites to

heterologous genes enhances their expression in an erythroid tissue-specific manner [44, 45]. Thus, incorporation of these HS site sequences as erythroid-specific enhancers would increase expression of genes targeted to erythroid cells.

One final issue deals with the possible *in vivo* application of this tissue-specific retroviral delivery system. If this system can be shown to work *in vitro*, the next logical step would be to attempt to replicate the results in whole animals; The recent work of Wu and Wu [46] shows that DNA may be targeted to a specific organ. They chemically coupled asialoglycoprotein to a DNA molecule and showed that this complex could target the linked DNA to liver cells. Thus Wu et al. have already demonstrated that, in principle, DNA can be targeted to specific tissues *in vivo*, after introduction into the general circulation. In their experiments, however, the DNA was chemically coupled to the targeting ligand, therefore resulting in only transient expression. The advantage of a tissue-specific retroviral delivery system is that stable integration into the host genome would be achieved only in the targeted tissues, and the inserted gene would then be passed on to the daughter cells, resulting in long-term expression. Though the feasibility of direct *in vivo* administration is uncertain, and a number of potential problems can be envisioned (e.g., host immune response resulting in the development of neutralizing antibodies), possible solutions may also be entertained (e.g., immunosuppressive therapy for the duration of the treatment); since at least one recent study has shown that direct retroviral infection *in vivo* can be efficiently achieved in a localized area of endothelium by means of catheterization [47], it seems that this would be an avenue of research which is certainly worth exploring.

Other applications

Finally, this mode of viral delivery may have even broader application beyond therapy for genetic diseases. If successful, this type of targeting could be applied to any system that has a specific receptor on a tissue cell. The repertoire of targeting ligands might be increased immensely if the antigen recognition sites of antibody molecules could be engineered into the viral envelope, to target the virus to cells which express that specific surface antigen. Furthermore, one can envision retroviral delivery of genes encoding cytotoxins to tumor cells via receptor- or antigen-specific targeting as a form of cancer chemotherapy, for example, or the utility of cell type-specific retroviruses as functional tools for the study and manipulation of developmental processes at various stages.

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