UNIVERSIDAD DE CHILE
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CHARACTERIZATION AND USE OF RETROVIRAL INTERNAL RIBOSOME ENTRY SEGMENTS (IRES) FOR THE DEVELOPMENT OF BICISTRONIC RETROVIRAL VECTORS FOR AN EFFICIENT GENE TRANSFER

MARCELO ANDRES LOPEZ LASTRA

1999

Characterization and use of retroviral internal ribosome entry segments (IRES) for the development of bicistronic retroviral vectors for an efficient gene transfer.

Tesis
Entrega a la
Universidad de Chile
en cumplimiento parcial de los requisitos
para optar al grado de
Doctor en Ciencias con mención en Biología

Facultad de Ciencias

por

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Noviembre, 1999.

Director de Tesis: **Dr. Jean-Luc Darlix**
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FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

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TESIS DE DOCTORADO

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato.

MARCELO ANDRES LOPEZ LASTRA

Ha sido aprobada por la Comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Biología, en el Examen de la Defensa rendido el día 2 de noviembre de 1999.

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A mi gringuita, finalmente lo logramos,

Gracias.

AGRADECIMIENTOS / ACKNOWLEDGMENTS/ REMERCIEMENTS

Me gustaria por comenzar agradeciendo a todos los miembros de mi comision y a todos aquellas personas amigas que me apoyaron en la idea loca de hacer mi tesis en Francia.

Gracias a mis padres por haberme dado educacion. Gracias Anita y Eugenio por haberme dado las herramientas basicas durante mi tesis de pregrado y por haberme alentado a partir. Sabemos que no fue facil y que no resulto a la primera. En todo caso lo que mas cuesta, mas se disfruta. Gracias.

J-L thanks for giving me the chance, thanks for your trust and faith in my work and crazy ideas. I still do not know why you chose me, but I thank you for that, AMIGO.

I would like to thank all my old and current colaborators specially Dr. Derrington. Thanks my friend for showing me how poor my knowleds in the CNS is. Theo dont worry the gels will be done on time for summitting the article. Vive la vie de funtionaire. Thank you Mike, for all those night you spent reading my manuscripts. Thanks Louis for letting Mike work a bit at home.

Les travaux présentés dans cette thèse n'auraient pas pu être réalisés sans la présence et le soutien de Helga, la bonne humeur de Daniela, Valerie, Hirosan, Verinique, Lionnel , Pascal et Françoise; l'experénce de Caroline, ED et Christine; le long discusion cientifiques avec le gran chef J.L. et tous les petites chefs Didier, Theo, Val, Mike et ED. Merci a tous. Merci

Finalmente me gustaria agradecer a Gabi. Gracias negrita por salvar esta tesis en el último instante. Te pasaste Merci ....Merci ...Merci...

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ABBREVIATIONS

Act D: Actinomycin D

CA: Virus capsid
CTE: Nuclear/cytoplasmic RNA transport element

DLS: Dimer Linkage structure

E: packaging signal
E+: Extended packaging signal
eIF: Eukaryotic initiation factor
EMCV: Encephalomyocarditis virus
Env: Virus envelope

FMDV: Foot and mouth disease virus
FrMLV: Friend murine leukemia virus

GALV: Gibbon ape leukemia virus

HaMSV: Harvey murine sarcoma virus
HAV: Hepatitis A virus
HCV: Hepatitis C virus
HIV: Human immunodeficiency virus
HSV-tk: Herpes simplex virus thymidine kinase.
HTLV: Human T-cell leukemia virus

IN: Virus integrase
IRES: Internal ribosome entry segment

LTR: Long terminal repeat

MLV: Murine leukemia virus
MoMuLV: Moloney murine leukemia virus

NC: virus nucleocapsid protein
neo: Neomycin phosphotransferase activity
NBCS: Newborne calf serum

PBS: Primer binding site
plap: Human placental alkaline phosphatase
PPT: Polypurine tract
PR: Virus protease
PV: Poliovirus

Rap: Rapamycin
REV-A: Reticuloendotheliosis virus type A
RT: Virus reverse transcriptase
RSV: Rouse Sarcoma virus

SIV: Simian immunodeficiency virus
SNV: Spleen necrosis virus
SU: Surface protein

TM: Transmembrane protein
TU/ml: Transducing units per ml

UTR: Untranslated region
VL30: Virus like 30S

I. RESUMEN

Los vectores retrovirales se han perfilado como uno de las herramientas más importantes en la transferencia de genes. De hecho, han permitido la transducción de genes con potencialidad terapéutica a múltiples tipos celulares (Gunzburg & Salmons, 1995; Salmons *et al.*, 1995). Diversas estrategias de transferencia génica actualmente en desarrollo requieren de una expresión simultanea de más de un producto proteíco. El uso de elementos génicos tales como los segmentos de entrada interna de ribosomas, IRES (internal ribosomal entry segment) han demostrado ser una de las formas más eficientes para expresar dos genes exógeneos en una célula. Inicialmente descrito en picornavirus, los IRES son estructuras de ARN capaces de reclutar los factores necesarios para la iniciación de la traducción de manera independiente a la estructura cap, característica de los ARN mensajeros eucarióticos. La utilización de IRES de picornavirus en la construcción de vectores ha permitido la traducción de un mensajero policistrónico en un sistema eucariótico (Adam *et al.*, 1991). Diversos estudios han reportado la presencia de un IRES en la región 5' no traducida (UTR), "secuencia leader", de los retrovirus oncogénicos murinos (MLV) FrMLV, MoMLV y HaMSV (Berlioz & Darlix, 1995; Berlioz *et al.*, 1995; Vagner *et al.*, 1995b). Las regiones leader de los virus MLV no presentan homología de secuencia sin embargo, la estructura de esta región es conservada entre todos los retrovirus de tipo MLV y MLV-like (Koning *et al.*, 1992). Basados en estudios de homología estructural se estableció como hipótesis que los retrovirus MLV-like presentaba un IRES en su secuencia 5'leader. Como modelos de estudio se seleccionó el retrovirus de la reticuloendoteliosis aviar de subtipo A (REV-A) y el retrotransposon de ratón VL30 (VL30m). El presente trabajo describe la caracterización del IRES presente en la secuencia 5' leader del virus REV-A y aquel presente en la región 5' UTR VL30m. Ambos IRESes han sido utilizados en el desarrollo de nuevos vectores retrovirales bicistronicos de tipo MLV-IRES de alto título. La actividad de los IRES y utilidad de los vectores ha sido evaluada en diferentes tipos celulares. Este estudio muestra que en el sistema nervioso central (SNC) la actividad de los IRESes de origen viral no esta regulada

por el estado de diferenciación celular. Por tanto, los vectores desarrollados abren nuevas posibilidades en el diseño de estrategias de terapia génica para SNC. Finalmente, se evaluó la actividad del IRES de MoMuLV en el contexto viral. Los resultados indican que el virus puede utilizar el IRES para dirigir la síntesis de las proteínas virales y de esta manera escapar al control impuesto por la célula a nivel de la iniciación de la traducción.

SUMMARY

The murine leukemia virus (MLV)-type C viruses constitute a major class of retroviruses which includes a large number of exogenous and endogenous mammalian viruses and the related avian spleen necrosis viruses (SNV) (Coffin *et al.*, 1997). The genome of MLVs has a long and multifunctional 5' untranslated leader involved in key steps of the viral life cycle such as: genomic RNA splicing, translation, dimerization, encapsidation and reverse transcription. Moreover it has been shown that the 5' leader of Friend murine leukemia virus (FrMLV), Harvey murine sarcoma virus (HaMSV), and Moloney murine leukemia virus (MoMuLV) possesses an internal ribosome entry segment (IRES) which directs cap independent translation of gag precursor proteins (Berlioz & Darlix, 1995; Berlioz *et al.*, 1995; Vagner *et al.*, 1995b). These data, together with structural homology studies (Konings *et al.*, 1992; Yang & Temin, 1994; Mougel *et al.*, 1996; Mougel & Barklis, 1997; Fisher & Goff, 1998), prompted us to undertake a search for new IRESes of retroviral origin. My work describes the characterization of two IRESes of retroviral origin and their use for the development of novel bicistronic MLV-IRES retroviral vectors. The presence of an IRES in the 5' leader of avian reticuloendotheliosis virus type A (REV-A) genomic RNA, an exogenous retrovirus, and in the 5'region of mouse virus-like 30S RNA (VL30m), a mouse retrotransposon, are reported. These elements were characterized both in the rabbit reticulocyte lysate system using bicistronic RNAs and in cell culture using retroviral vectors. Data show that the REV-A 5' IRES maps downstream of the packaging/dimerization (E/DLS) signal (Watanabe & Temin, 1982) and that the minimal IRES sequence appears to be within a 129 nt fragment (nucleotides 452-580 of the 5'leader) immediately upstream of the gag AUG codon. In VL30m, we found that the putative packaging sequence is located within the 5' region (nt 362-1149) and that this region has an IRES activity. Both Rev-A and VL30m IRES have been utilized in the construction and production of novel high titer (10^7-10^9 TU/ml in NIH 3T3 cells) MLV-IRES based retroviral vectors.

In this study we also evaluated the ability of MLV-IRES bicistronic vectors to efficiently transduce and stably express more than one gene in different cell lines. Hence, the effect of cell differentiation on translation driven by viral IRESes (MoMuLV, REV-A, EMCV and VL30m) used in bicistronic vectors was examined. As cellular models, a human multipotent neural precursor cell line, Dev, derived from a pediatric medulloblastoma was used Results show that the initial co-expression of two genes, observed in transduced undifferentiated Dev cells, is maintained upon differentiation. These data emphasize the ability of the double IRES vectors to allow the co-expression of exogenous proteins in human neural precursor cells in a stable fashion and independent from the cell phenotype.

Finally, a series of experiments were designed to determine the ability of MoMuLV to utilize the 5' IRES in the synthesis of Gag polyprotein. The presented results confirm that there is an IRES in the MoMuLV genome capable of conferring internal initiation to the gag gene. Moreover, they suggest that Env synthesis can also occur though a cap-independent initiation mechanism.

Taken together, the data presented suggest that the mechanism of cap-independent translation initiation might be common to all MLV-like type C retroviruses. Moreover, we have shown that these genetic elements can be used in the construction of high titer bicistronic retroviral vectors with clear applications in gene transfer.

II. OVERVIEW

II. 1. Retroviruses as a model system.

Initially identified as disease causing agents with a pathogenic potential in human and animals, retroviruses have proved to be interesting models for the study of fundamental biological problems. An interesting association of concepts and characteristics of retroviruses can be proposed to reflect their relevance in different domains of biological sciences: (i) Ubiquity: retroviruses have been found in all vertebrate animals in which they have been sought, including fish, birds, rodents, cats, dogs, ungulants, nonhuman primates, and humans. (ii) Biochemistry: retroviruses are a family of animal viruses that contain a dimeric RNA genome that replicates through a DNA intermediate. Thus, the very name "retrovirus" embodies the biochemical property for which this class of viruses is most famous: the capacity to copy its RNA into DNA in a process termed reverse transcription. The retroviral life cycle depends upon unusual biochemical reactions, most obviously the reverse transcription and the orderly integration of viral DNA into host chromosomes to form proviruses. (iii) Molecular Biology: studies of retroviruses showed that transfer of information in biological systems was not limited to conventional transcription, translation and replication. The central dogma of unidirectional flow of genetic information from DNA to RNA to protein was first challenged when retrovirus replication was understood. From an applied point of view, retroviruses have also provided essential tools for molecular biology. Retroviral reverse transcriptase (RT) has the capacity to reverse transcribe any RNA template once provided with a suitable DNA or RNA primer. RT from avian and murine retroviruses are widely used to generate cDNA copies of RNA which then can be manipulated with relative ease for techniques such as molecular cloning and sequencing. (iv) Clinical diagnosis: The capacity of RT combined with the technique of PCR has allowed the development of new, accurate and relatively fast clinical diagnosis methods for RNA viruses (v) Genetics: retroviruses can perform genetic feats unique among animal viruses, including transduction and insertional mutations of host genes and entry of proviruses into the germ line through which they are vertically transmitted. (vi) Evolution: studies of the structure of proviruses and of reverse transcriptase have established evolutionary relationships among

retroviruses and a wide variety of transposable elements, viruses, and cellular genes, and have possible implications for early events in the origin of life. Some authors have argued that reverse transcription is logically a necessary event that must have occurred at a crucial stage in evolution to allow the transition from the earlier RNA-dominated world to our DNA-dominated one. (vii) Pathology: retroviruses cause a wide variety of diseases, most commonly cancers (leukemia's, sarcomas, and mammary carcinomas), but also immunodeficiencies, anemias, arthritis, and pneumonia; two such diseases, the acquired immune deficiency syndrome and adult T-cell leukemia-lymphoma, are important, newly recognized disorders of humans. (viii) Oncogenes: many retroviruses that cause cancer in experimental animals either carry oncogenes transduced from host genomes or modify the expression of the host proto-oncogenes by insertion mutation. The study of retroviruses has implicated nearly 50 cellular genes, such as *src* and *ras*, in oncogenesis. Since then, studies of retroviruses have brought to light a number of genes implied in oncogenesis, growth control and development. (ix) Gene transfer: the development of retroviral vectors takes advantage of the ability of retroviruses to integrate efficiently into genomic DNA of animal cells and be stably transmitted to their progeny. Such vectors have provided an important and versatile method of introducing and expressing genes in eukaryotic cells and organisms.

From all identified retroviruses the most extensively studied are those of murine origin. Even though historical survey of mouse leukemia, sarcoma, and carcinoma does not date as far back as that on the avian neoplasms described by Ellermann and Bang (1908) and Rous (1911), the availability of inbred strains of mice has greatly enhanced the identification and experimental manipulation of the mouse retroviruses. Recent studies on murine C-type retroviruses (MLV) have shown that like picornaviruses these simple retroviruses are able to initiate protein synthesis in a cap-independent fashion. These findings gave rise to several questions regarding basic and applied retrovirology. From these, my work addressed two: Are IRESes only present in MLVs or can they be also found other MLV-like retroviruses, and can these genetic elements be successfully used in the improvement of retroviral vectors.

III. INTRODUCTION

III. 1. Murine leukemia virus (MLV) and MLV-like type C retroviruses.

III. 1.1. Viral particle.

The murine leukemia virus-related type C viruses constitute one of the major classes of retroviruses. This class includes numerous exogenous and endogenous viruses of mammals and related spleen necrosis viruses (SNV) of birds (Coffin *et al.*, 1997). These retroviruses are assembled directly at the cell membrane with no visible cytoplasmic intermediate, and have an immature form with a large, open spherical core. This core matures into a central condensed structure with visible but not prominent surface projections. MLV are about 110 nm in diameter and are composed of an inner core surrounded by an outer envelope formed of a lipid bilayer of cellular origin in which viral glycoproteins are anchored. The inner core is formed by an outer shell of capsid protein molecules (CA) surrounding the nucleocapsid, where the dimeric, positive sense RNA genome is in tight association with nucleocapsid protein (NC) molecules, reverse transcriptase (RT), and integrase (IN) (Fig 1) (Coffin *et al.*, 1997). The viral genome resembles cellular mRNA in that they have a 5'cap structure and a 3'poly A tail (Rose *et al.*, 1976; Guntaka, 1993). The two RNA subunits are noncovalently linked near their 5' ends by a major structure called DLS (Paillart *et al.*, 1996). The retroviral genome is less than 10 Kb and of positive sense. Its prototypical organization is simple, comprising three genes encoding Gag (group-specific antigen in the virion core), Pol (RNA-dependent DNA polymerase) and Env (envelope glycoprotein) (Coffin *et al.*, 1997).

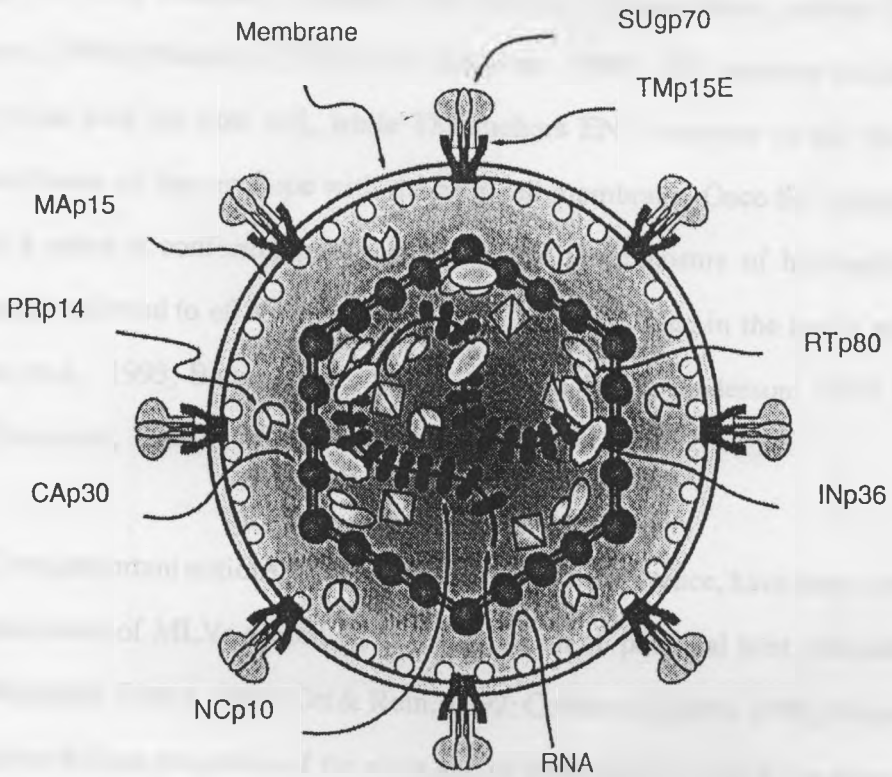


Figure 1. Schematic view of a MLV particle (Coffin et al, 1997). Two identical single strands of viral RNA and viral enzymes reverse transcriptase (RT, p80), integrase (IN, p36), and protease (PR, p14) together with the internal nonglicosylated structural proteins matrix (MA, p15), capsid (CA, p30), and the nucleocapsid (NC, p10) proteins are drawn within a viral core. The core is surrounded by an envelope derived from the host membranes enriched with the viral glycoproteins. These consist of the transmenbrane (TM, p15E) and the surface (SU, gp70) components linked together by disulfide bonds.

III. 1. 2. Viral life Cycle.

Infection process of MLV is initiated by an interaction between the viral envelope protein (Env) and a specific receptor molecule located on the host cell membrane (Fig 2). Retroviral ENVs are expressed as glycosylated polyproteins that are processed intracellulary by proteolytic cleavage into two subunits, a surface (SU) and a transmembrane protein (TM) (Ragheb & Anderson, 1994) (Pinter *et al.*, 1997) (Coffin *et al.*, 1997). SU contains the site for interaction of the virion with the host cell, while TM anchors ENV complex to the virus envelope and mediates fusion of the envelope with the host-cell membrane. Once SU interacts with the cell receptor a series of conformational changes lead to the exposure of hydrophobic domains on TM that are believed to effect membrane fusion by embedding in the host's plasma membrane (Morgan *et al.*, 1993; Battini *et al.*, 1994, 1995; Ragheb & Anderson, 1994; Lavillette *et al.*, 1998; Zhao *et al.*, 1998).

Two important notions, cell-tropism and viral interference, have been used as a basis for the classification of MLVs according to the spectrum of potential host species/cells (Gardner, 1978; Mitchell & Risser, 1992; Ott & Rein, 1992; Corbin & Sitbon, 1993; Nouvel *et al.*, 1994). Cell tropism defines properties of the virus and of those cells in which the virus will undergo a complete replication cycle, and is determined by the Env protein and its receptor on the target cell (Battini *et al.*, 1992; Miller, 1996). Infection of a cell by a replication-competent retrovirus results in the synthesis of a retroviral Env protein that binds to the receptor used for virus entry. This effectively blocks entry of any retrovirus that targets the same receptor, whereas entry of retroviruses that use different receptors is unaffected. This process in known as viral interference and in retroviruses has been shown to occur at the level of virus entry into cells and not at any other step in the virus life cycle (Gardner, 1978; Berkowitz & Goff, 1993; Corbin & Sitbon, 1993; Nouvel *et al.*, 1994).

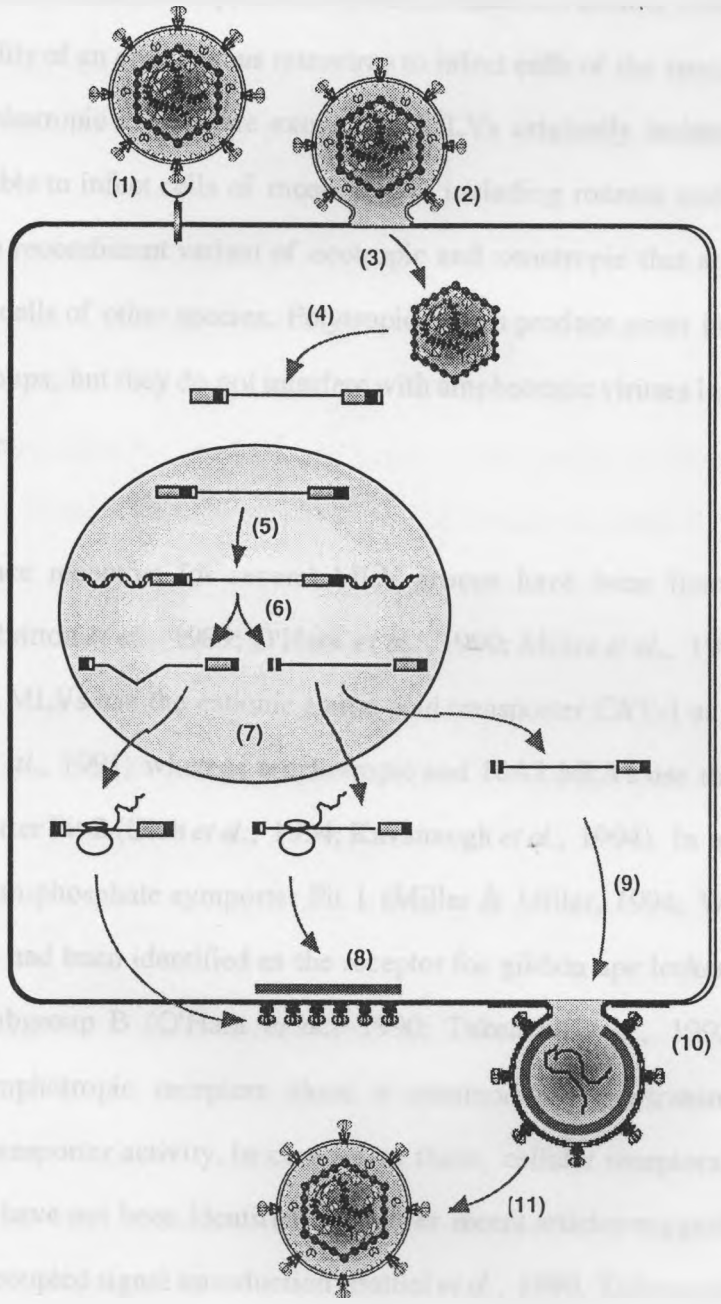


Figure 2. Retroviral life cycle (Coffin et al., 1997). The infection event is initiated by interaction bewteen the viral envelope proteins and the cell receptor **(1)** this leads to internalization of the virus **(2)**. The viral core is then released into the cell cytoplasm **(3)**. The viral RNA is reversed transcribed into a double stranded DNA, using the virion associated reverse transcriptase **(4)**. The DNA is integrates into the host cell chromosome, using a virally encoded integrase **(5)**. The integrated form, termed a provirus, is transcribed to give the viral RNAs **(6)** that code for viral proteins **(7)** and that provide the new viral genetic information. The viral proteins assemble at the cell periphery **(8)** and genomic RNA is then packaged **(9)**. The newly formed virus particles bud out of the cell, taking part of the envelope containing host cell membrane with it **(10)**. Once releaseed the process the virion proteolytic maturation is accomplished **(11)**.

MLV isolates that are able to infect only rodent cells are called ecotropic, whereas MLVs that infect exclusively cells from species other than rodents are termed xenotropic. Xenotropism defines the inability of an endogenous retrovirus to infect cells of the species. whose germ line it inhabits. Amphotropic viruses are exogenous MLVs originally isolated from wild mouse strains and are able to infect cells of most species including rodents and humans. Polytropic MLV represent a recombinant variant of ecotropic and xenotropic that are able to infect both rodent cells and cells of other species. Polytropic MLVs produce cross interference with both parental virus groups, but they do not interfere with amphotropic viruses indicating that they use different receptors.

Cell surface receptors for several MLV groups have been functionally cloned and characterized (Albritton *et al.*, 1989; O'Hara *et al.*, 1990; Miller *et al.*, 1994; van Zeijil *et al.*, 1994). Ecotropic MLVs use the cationic amino acid transporter CAT-1 as a receptor (Wang *et al.*, 1991; Kim *et al.*, 1991) whereas amphotropic and 10A1 MLVs use the sodium-dependent phosphate symporter Pit 2 (Olah *et al.*, 1994; Kavanaugh *et al.*, 1994). In addition, 10A1 MLV can use the sodium-phosphate symporter Pit 1 (Miller & Miller, 1994; Wilson *et al.*, 1994), which previously had been identified as the receptor for gibbon ape leukemia virus and feline leukemia virus subgroup B (O'Hara *et al.*, 1990; Takeuchi *et al.*, 1992). Interestigly, the ecotropic and amphotropic receptors share a common multiple-transmembrane spanning topology and a transporter activity. In contrast to these, cellular receptors for xenotropic and polytropic MLVs have not been identified. However recent articles suggest they might have a role in G protein-coupled signal transduction (Battini *et al.*, 1999, Tailor *et al.*, 1999).

Cells can be simultaneously infected with two enveloped viruses. In this case infected cells are able to produce phenotypically mixed viral populations (Lavignon & Evans, 1996). Such pseudotyped viruses carry the genetic information and core of one virus and an envelope containing Env proteins of either the second virus or both viruses. In consequence, pseudotyped viruses may have an extended infection spectrum with respect to both original viruses.

After internalization, conversion of genomic single stranded RNA into a double-strand proviral DNA by RT takes place (Fig 2) (Coffin *et al.*, 1997). The feature that distinguishes retrovirus from other viruses is the replication of a single-stranded RNA genome through a double-stranded DNA intermediate. Reverse transcription is believed to occur in the nucleocapsid substructure and involves two DNA strand transfers to generate the long terminal repeats (LTR) required for IN-mediated integration of the proviral DNA into the cellular genome and its subsequent expression (Coffin *et al.*, 1997). The linear DNA then enters the nucleus and is substrate for integration into the host cell genome. The integrated linear DNA form of the retrovirus genome is known as the provirus. As the double stranded viral DNA integrates at random into the host cell genome, the provirus can be located at a wide variety of sites, but it is uniform in structure, with the LTRs flanking an internal coding region. Integrated into the host chromosomes, the provirus is stably maintained and expressed like any other host gene and is transcribed by viral host RNA polymerase II (Coffin *et al.*, 1997).

A provirus serves both as a repository of viral genetic information in an infected cell and as a template for the synthesis of viral RNA (Figs 2 and 3). The main features of the provirus are; a) LTR, a tripartite structure containing sequences derived from the 5' (U5) and 3' ends of viral RNA (U3), and a short repeated sequence (R) found at the ends of the viral RNA. The LTRs are involved in two crucial aspects of the viral life cycle, the integration of proviral DNA into the host chromosome and the control of viral gene expression. The former is catalyzed by a virally encoded endonuclease, the viral IN, while the latter relies almost exclusively on the transcriptional machinery of the host cell. All retroviral LTRs contain recognizable variants of the DNA sequence motifs commonly associated with initiation of eukaryotic mRNA synthesis (5' LTR) and the signals for processing and polyadenylation (3' LTR). They also contain transcriptional enhancer elements

that can act as potent modulators of the activity of cis-linked promoters. (b) Short sequences; primer binding sites (PBS) and polypurine tract (PPT), required respectively for priming of the negative and positive DNA strand synthesis by RT. (c) The packaging signal (E) required for selection of viral RNA during assembly. (d) The structural genes gag, pol, env that are expressed from the retroviral promoter (retroviruses with simple genomes). (e) The viral splice donor and acceptor sequences (SD and SA) used to generate the sub-genomic RNA (Fig 3).

MLV produces two distinct RNAs: a full-length transcript and a smaller sub-genomic mRNA which has undergone splicing and serves as a template for translation of env gene products (Aloni, 1981). Splicing ensures that the subgenomic RNA does not become packaged into virion since the major determinant for packaging lies downstream of the splice donor site within the intron of the env mRNA. The full length transcript directs translation of the gag and pol genes, but also serves as the viral genome that is packed into the viral particle. The nascent viral particle now forms by budding of the plasma membrane and is released from the cell surface.

III. 1. 3. Viral Protein synthesis.

In infected cells, retroviral protein synthesis accounts for a small proportion of total cell protein synthesis. Translation of retroviral proteins takes place on cytoplasmic polysomes and is regulated by the host cell translational apparatus. The full-length viral transcript is translated on free ribosomes to give Gag and Gag-Pol polyprotein, while the spliced subgenomic mRNA is translated on membrane-bound ribosomes into Env polyprotein (Fig 3). Synthesis of MuLV Gag from the full-length viral transcript involves two pathways. In the major pathway, translation of gag initiates at the first AUG codon in a favorable context to produce a 65 kDa polyprotein that is subsequently assembled into virions and proteolytically processed during particle morphogenesis to produce mature core proteins. In a second pathway, a CUG codon upstream from the gag start codon in frame with the gag translation frame is recognized as an initiation codon for protein synthesis (Prats *et al.*, 1989). The CUG codon, which is also

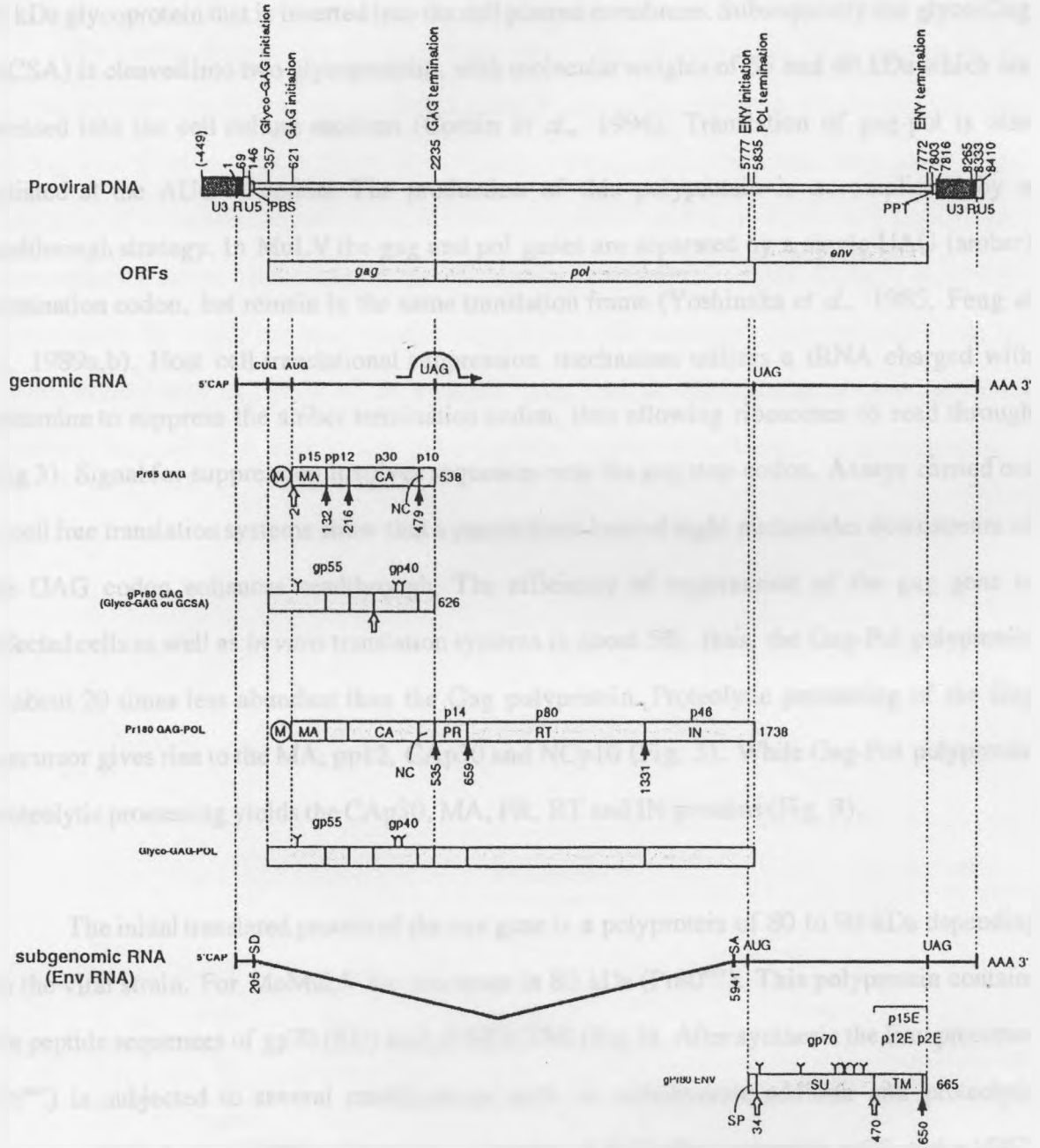


Figure 3. Structure and expression of MLV (Coffin et al., 1997).

present in a favorable context, directs the synthesis of a Gag polyprotein that has an N-terminal hydrophobic leader peptide. This form of MuLV Gag polyprotein is glycosylated to produce an 85 kDa glycoprotein that is inserted into the cell plasma membrane. Subsequently the glyco-Gag (GCSA) is cleaved into two glycoproteins, with molecular weights of 55 and 40 kDa which are released into the cell culture medium (Corbin *et al.*, 1994). Translation of gag-pol is also initiated at the AUG^{gag} codon. The production of this polyprotein is accomplished by a readthrough strategy. In MuLV the gag and pol genes are separated by a single UAG (amber) termination codon, but remain in the same translation frame (Yoshinaka *et al.*, 1985, Feng *et al.*, 1989a,b). Host cell translational suppression mechanism utilizes a tRNA charged with glutamine to suppress the amber termination codon, thus allowing ribosomes to read through (Fig 3). Signal for suppression involves sequences near the gag stop codon. Assays carried out in cell free translation systems show that a pseudoknot located eight nucleotides downstream of the UAG codon enhances readthrough. The efficiency of suppression of the gag gene in infected cells as well as *in vitro* translation systems is about 5%, thus, the Gag-Pol polyprotein is about 20 times less abundant than the Gag polyprotein. Proteolytic processing of the Gag precursor gives rise to the MA, pp12, CAp30 and NCp10 (Fig. 3). While Gag-Pol polyprotein proteolytic processing yields the CAp30, MA, PR, RT and IN proteins (Fig. 3).

The initial translated protein of the env gene is a polyprotein of 80 to 90 kDa depending on the viral strain. For MoMuLV the precursor is 80 kDa ($Pr80^{env}$). This polyprotein contains the peptide sequences of gp70 (SU) and p15(E) (TM) (Fig 3). After synthesis the Env precursor (Pr^{env}) is subjected to several modifications such as carbohydrate addition and proteolytic cleavage (Coffin *et al.*, 1997). Proteolytic cleavage of Pr^{env} first separates gp70 and p15(E). However, in virions a subsequent cleavage of p15(E) takes place giving rise to p12(E) (Fig 3).

III. 1. 4. Cis acting sequences in the 5' region of MLV-like C-type retroviruses.

The genetic organization of the 5' sequences of the MLV genomic RNA appears to be original and complex in that a long multifunctional 5' untranslated domain, the leader, precedes the gag gene (Coffin *et al.*, 1997). Even though little nucleotide sequence homology exists among the different members of the MLV-like C-type retroviruses they all present a high degree of secondary structure conservation in their 5' leader (Konings *et al.*, 1992, Cofin *et al.*,1997). Furthermore, the leader can be divided in independent well structured domains which can be directly related with key steps of the viral life cycle such as the initiation of proviral DNA synthesis, genomic RNA dimerization and encapsidation, and the initiation of Gag polyprotein.

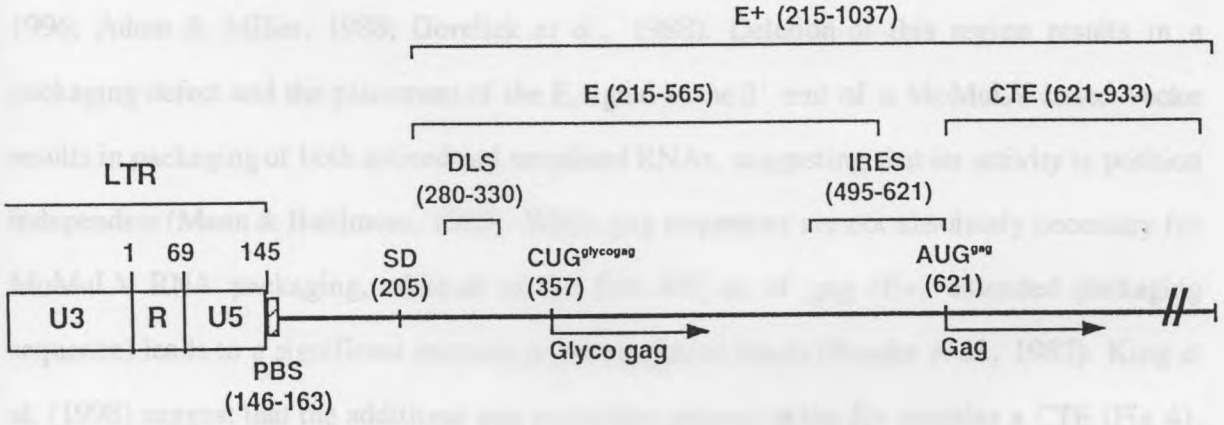


Figure 4. Features of the MoMuLV Leader

In MoMuLV the best studied cis-acting signals present in the leader are the PBS, the 5' packaging signal (E) (Adam & Miller, 1988), the extended packaging signal (E+) (Bender *et al.*, 1987), the dimerization (DLS) region (Prats *et al.*, 1990; Roy *et al.*, 1990; Girard *et al.*, 1996; De Tapia *et al.*, 1998), the splicing donor (SD), and the initiator codon $CUG^{glycogag}$ (Prats *et al.*, 1989). Other cis-acting signal present in the leader or in the E+ region are the internal ribosome entry signal (IRES) (Berlioz & Darlix, 1995; Berlioz *et al.*, 1995; Vagner *et al.*, 1995b) and the nuclear/cytoplasmic RNA transport element (CTE) (King *et al.*, 1998) (Fig 4).

The E element is necessary and sufficient for viral genomic RNA packaging (Watanabe & Temin, 1982; Mann *et al.*, 1983; Mann & Baltimore, 1985; Adam & Miller, 1988). In retroviruses this element defined as an RNA structure has been located at the 5' end of the viral genome (Konings *et al.*, 1992; Yang & Temin, 1994; Mougel *et al.*, 1996; Mougel and Barklis, 1997; Fisher & Goff, 1998). In simple retroviruses this signal is between the 5' major splice site and the AUG^{gag}. Therefore, E is absent in subgenomic mRNA (Fig. 3). Selection of viral RNA during assembly is governed by interactions between the NC domain of Gag and E in the viral RNA (Housset *et al.*, 1993; Rein *et al.*, 1994; Berkowitz *et al.*, 1995; Housset & Darlix, 1996; Adam & Miller, 1988; Gorelick *et al.*, 1988). Deletion of this region results in a packaging defect and the placement of the E signal at the 3' end of a MoMuLV based vector results in packaging of both spliced and unspliced RNAs, suggesting that its activity is position independent (Mann & Baltimore, 1985). While gag sequences are not absolutely necessary for MoMuLV RNA packaging, addition of the first 407 nt of gag (E+, extended packaging sequence) leads to a significant increase in encapsidation levels (Bender *et al.*, 1987). King et al. (1998) suggest that the additional gag sequences present in the E+ contains a CTE (Fig 4). The authors argue that the CTE would enhance genomic RNA nuclear export and it is this cytoplasmic increase that would favor encapsidation. The U5 region of the LTR has been also proposed to play a role as an enhancer but is not a requirement for packaging, since deletion of the 5' end of U5 from the full-length cloned provirus was shown to significantly reduce MoMuLV packaging (Murphy & Goff, 1989).

It has been proposed that the secondary structure preservation is important for retroviral RNA specific recognition and encapsidation (Konings *et al.*, 1992; Yang & Temin, 1994; Mougel, *et al.*, 1996; Mougel & Barklis, 1997; Fisher & Goff, 1998). The relationship between packaging function and structure of the E sequence can be illustrated by analyzing the spleen necrosis viruses (SNV) E element. As for MoMuLV the E SNV is located between the 5' SD and the AUG^{gag} and appears to work in a position-independent manner (Watanabe & Temin, 1982; Embretson & Temin, 1987). Using linker scanning and site directed mutagenesis, two potential stem-loop structures within the SNV E were defined (Yang &Temin, 1994). Destroying the secondary structure of any of the stem abolished packaging of an SNV vector by a helper virus (Yang & Temin, 1994). The function of the two stem-loops, does not appear redundant, as deletion of either structure reduces packaging by over 50 fold. Additionally, the replacement of the first stem-loop with another copy of the second stem-loop results in a 20 fold reduction in packaging as compared to the wt E sequence. As previously stated, the primary sequences of SNV an MLV packaging signal do not contain significant sequence homology, but they have similar double hairpin secondary structures (Konings *et al.*, 1992; Yang & Temin, 1994; Mougel and Barklis, 1997). Despite the lack of homology, MLV vector RNA can be packaged by a SNV helper virus (Embretson & Temin, 1987). SNV-MLV chimeric E elements constructed in a SNV backbone do not show a reduction in their packaging levels (Yang &Temin, 1994). Furthermore, Yang and Temin (1994) replaced the SNV E by that of MoMuLV in an SNV vector without affecting the efficiency of encapsidation by an SNV helper virus. The last set of results clearly illustrates the importance of RNA structures in retroviral life cycle.

In the genome of Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaSV) and Avian reticuloendotheliosis virus (REV) a region within the E signal known as the DLS, directs formation of the dimeric RNA genome (Darlix et al., 1992; Torrent et al., 1994a,b; Girard et al., 1995, De Tapia, et al., 1998). Dimerization of the genomic RNA seems to be an essential process since all known retroviruses genomes are packaged as dimers (Coffin

et al., 1997). On the other hand retroviral dimerization plays a crucial role in various phases of the viral life cycle, such as recombination, reverse transcription and regulation of translation.

III. 2. Cap-independent translation initiation of MLV mRNA.

III. 2. 1. Cap-dependent translation initiation; the scanning model.

Translation initiation is a sequential, multistep process aimed at positioning the ribosome at the initiator AUG codon of the messenger RNA (mRNA) (Fig 5) (Pain, 1996). The mRNA structure has been show to play an important role in the modulation of translation initiation (Kozak, 1991b). In this respect, mRNA elements such as (i) the 5' cap, (ii) the primary sequences or context surrounding the AUG codon (Kozak, 1989b, 1995, 1997), (iii) the position of the AUG codon (Kozak, 1984, 1989b), (iv) secondary structures upstream and downstream from the AUG codon (Kozak, 1986b, 1989a), and (v) the leaders length, can strongly modify initiation of protein synthesis (Kozak, 1991a).

Initiation of protein synthesis involves the sequential binding of first the 40S and then the 60S ribosomal subunit to a messenger RNA molecule (mRNA) (Fig 5) (Pain, 1996). In eukaryotes this process can be divided into three steps. The first implies the association of initiator tRNA (Met-tRNA) with eIF2-GTP, to form the ternary complex. This complex would later associate with several eukaryotic initiation factors (eIF) and the 40S ribosomal subunit to form the 43S preinitiation complex. Most probably the ternary complex binding to the 40S ribosomal subunit occurs in concert with the binding of eIF1A-eIF3-eIF5 complex (Asano *et al.*, 1999; Chaudhuri *et al.*, 1999). The second step involves the binding of the 43S preinitiation complex to the mRNA, followed by its positioning on to the correct AUG initiation codon (Kozak, 1981, 1989b, 1997, Pestova *et al.*, 1998). The binding of the 43S preinitiation complex to the mRNA is greatly facilitated by the eIF4F protein complex, formed of four components, eIF4A, eIF4B, eIF4E and eIF4G (Jackson & Wickens, 1997). Each component has a specific function in the initiation process (Thach, 1992). eIF4A exhibits RNA-dependent

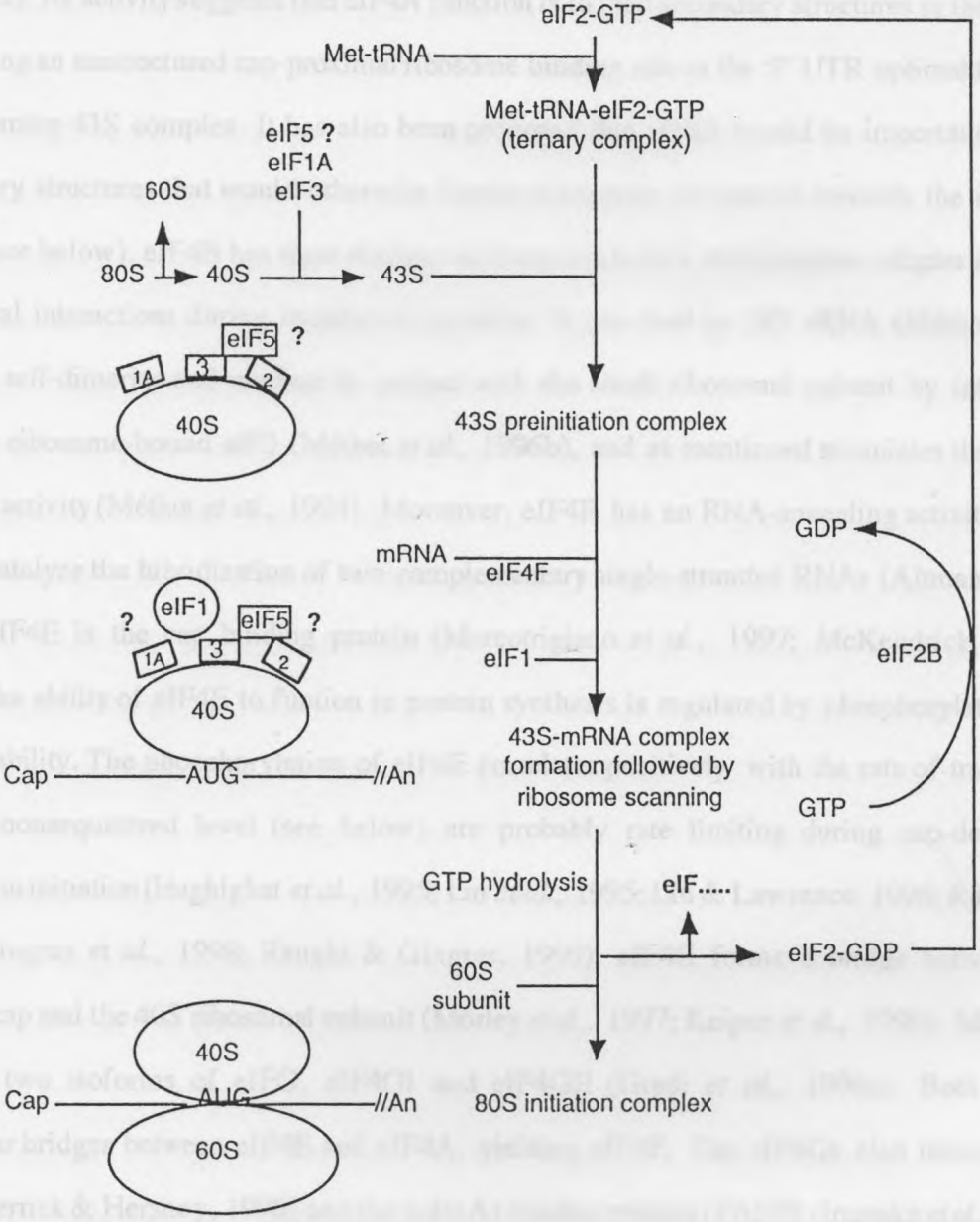


Figure 5. A simplified model of the mechanism of initiation of protein synthesis. The 40S ribosomal subunit is captured for initiation by binding eIF1A-eIF3-eIF5. Initiator tRNA binds in the form of a ternary complex with eIF2 and GTP to give rise to the 43S preinitiation complex. In the cap dependent mechanism the 43S preinitiation complex binds to mRNA at the 5' terminal cap structure with the help of the eIF4F protein complex, and then migrates along the mRNA towards the AUG initiation codon. In this process the funtions of eIF1 and eIF1A would be to monitor the accuracy of the start-site selection throughout scanning. When the 43S preinitiation complex stops at the initiation codon, the GTP molecule introduced as part of the eIF2 complex is hydrolysed to GDP, powering the ejection of the initiation factors bound to the 40S ribosomal subunit. The release of these factors allows the association of the 60S ribosomal subunit to reconstitute an 80S ribosome at the initiation codon. As shown upon release the eIF are recycled and the process is ready to restart. The ? sign indicated hypothetical interactions.

ATPase and ATP-dependent RNA duplex unwinding activities dependent on eIF4B (Rogers *et al.*, 1999). Its activity suggests that eIF4A function is to melt secondary structures in the mRNA generating an unstructured cap-proximal ribosome binding site in the 5' UTR optimal to recive the incoming 43S complex. It has also been proposed that eIF4A would be important to melt secondary structures that would otherwise impare ribosomes movement towards the initiation codon (see below). eIF4B has three domains and appears to be a multipurpose adapter involved in several interactions during translation initiation. It can bind to 18S rRNA (Méthot *et al.*, 1996a), self-dimerize and mediate its contact with tha small ribosomal subunit by interacting with the ribosome-bound eIF3 (Méthot *et al.*, 1996b), and as mentioned stimulates the eIF4A helicase activity (Méthot *et al.*, 1994). Moreover, eIF4B has an RNA-annealing activity that is able to catalyze the hibridization of two complementary single-stranded RNAs (Altmann *et al.*, 1995) eIF4E is the cap binding protein (Marcotrigiano *et al.*, 1997; McKendrick, et al., 1999).The ability of eIF4E to funtion in protein synthesis is regulated by phosphorylation and its availability. The phosphorylation of eIF4E correlates positively with the rate of translation and its nonsequestred level (see below) are probably rate limiting during cap-dependent translation initiation (Haghighat *et al.*, 1995; Lin *et al.*, 1995; Lin & Lawrence, 1996; Rau *et al.*, 1996; Gingras *et al.*, 1998; Raught & Gingras, 1999). eIF4G forms a bridge between the mRNA cap and the 40S ribosomal subunit (Morley *et al.*, 1997; Keiper *et al.*, 1999). Mammals possess two isoforms of eIFG, eIF4GI and eIF4GII (Gradi *et al.*, 1998a). Both act as molecular bridges between eIF4E and eIF4A, yielding eIF4F. The eIF4Gs also interact with eIF3 (Merrick & Hershey, 1996) and the poly(A)-binding protein (PABP) (Imataka *et al.*, 1998; Fraser *et al.*, 1999). The first is a multisubunit translation initiation factor associated with the 40S ribosomal subunit, which enables eIF4F to recruit the 40S ribosomal subunit to the 5'end of the mRNA (Merrick & Hershey, 1996).The interaction between eIF4G and PABP implies the circularization of the mRNA, event that has been observed by atomic force microscopy using recombinant yeast eIF4G, eIF4E and PABP (Wells *et al.*, 1998). The circularization of the mRNA has been suggested to enhance translation by shunting terminating ribosomes directly to the 5' end of the mRNA. Another mechanism of translational enhancement by

poly(A)tail proposed by Preiss and Hentze (1998) is that the poly(A) tail acts as a translational promoter by increaseing the concentration of eIF4G on the mRNA.

The eIF4E-eIF4G interaction is of central importance for cap-dependent initiation, and can be blocked by small regulatory proteins that bind to eIF4E, known as 4E binding proteins (4E-BPs, also known as PHASs, for phosphorylated heat and acid-stable) (Mader *et al.*, 1995; Feigenblum & Shneider, 1996). Mammalian 4E-BP1, 4E-BP2 and 4E-BP3, a family of small (10-15 kD) acidic proteins, inhibit cap-dependent protein synthesis by binding to eIF4E (Pause *et al.*, 1994; Poulin *et al.*, 1998). 4E-BPs act as molecular mimics of eIF4Gs, and biochemical studies have shown that eIF4Gs and the 4E-BPs occupy mutually exclusive binding sites on the surface of eIF4E (Haghighat *et al.*, 1995; Mader *et al.*, 1995; Rau *et al.*, 1996; Marcotrigiano *et at.*, 1999). The interaction of mammalian 4E-BPs with eIF4E is modulated by the extent of 4E-BPS phosphorylation (Gingras *et al.*, 1999). The 4E-BPs strongly interact with eIF4E when in their hypophosphorylated state and dissociate from eIF4E upon hyperphosphorylation (Lin *et al.*, 1994; Pause *et al.*, 1994; Gingras *et al.*, 1999). As for eIF4G, 4E-BP binding to eIF4E enhances cap affinity (Haghight & Sonenberg, 1997; Ptushkina *et al.*, 1999). Thus, the binding of 4E-BPs is expected to trap eIF4E in an active complex with high affinity for capped mRNA that cannot interact with eIF4G (Ptushkina *et al.*, 1999).

In general and depending on how the 43S preinitiation complex is recruited translation initiation can take place by a cap-dependent or a cap-independent fashion (Iizuka *et al.*, 1995; Jackson *et al.*, 1995; Sachs *et al.*, 1997). Cap-dependent translation initiation involves binding of this 43S initiation complex at the 5' 7mGpppG-cap structure of the mRNA (Kozak, 1981, 1991b). In the cap-independent translation initiation or internal initiation the recruitment of the translation initiation factors is mediated by a RNA secondary (and presumably also tertiary) structure, the IRES, located within the 5'region of certain mRNA (Le *et al.*, 1993, 1996; Jackson & Kaminski, 1995; Le & Maizel, 1997). Therefore, the IRES element promotes direct internal ribosome entry to the initiation codon: This genetic element can be considered as functionally equivalent to the Shine and Dalgarno motif in prokaryotic mRNAs, which is

usually present upstream of the initiation site of each cistron of a polycistronic mRNA and acts as an "identifier" for independent initiation at each cistron: It remains an open question whether this functional equivalence implies a similar mechanism, and that IRESes therefore promote internal initiation by base pairing with rRNA (Jackson *et al.*, 1995; Sachs *et al.*, 1997).

Upon binding the 43S preinitiation complex scans downstream until it locates the initiation codon. The successful scanning and recognition of the initiator AUG in the right context is assured by eIF1 and eIF1A (Pestova *et al.*, 1998). These two factors also cooperatively destabilize aberrantly assembled complexes, enabling them to dissociate and enter another round of initiation. The optimal context consists of the sequence GCC(A/G)CCAUGG. The purine (A or G) -3 bases and the G (+4) have been shown to be important. In fact, experiments showed that mutation of purine -3 or +4 greatly decreased the chance of an AUG being recognized as the initiator codon (Kozak, 1984, 1989b, 1995, 1997). Even though the AUG codon is the main recognized initiation codon CUG, ACG or GUG are occasionally used.

The third and last step of the initiation process involves the hydrolysis of the GTP molecule introduced as part of the eIF2 complex, the release of all initiator factors associated with the 40S ribosomal subunit, and the joining of the large 60S ribosomal subunit to reconstitute an 80S ribosome at the initiation codon poised to commence the elongation stage of translation (Fig 5).

The canonical scanning mechanism rules initiation of most mRNAs, but at least three alternative non-classical cap-dependent initiation mechanisms have been described, leaky scanning, ribosomal shunting and termination-reinitiation mechanisms. It is believed that these alternatives allow the scanning complex to overcome limitations imposed by the 5' untranslated regions (UTR) (Fig 6). The scanning model predicts that ribosomes should initiate at the first AUG codon encountered by a scanning 40S subunit. However, the first encountered AUG codon can be by-passed if it is present in a poor Kozak context. In this case the 40S subunit will initiate at an AUG in a better context further downstream, in a process known as leaky scanning

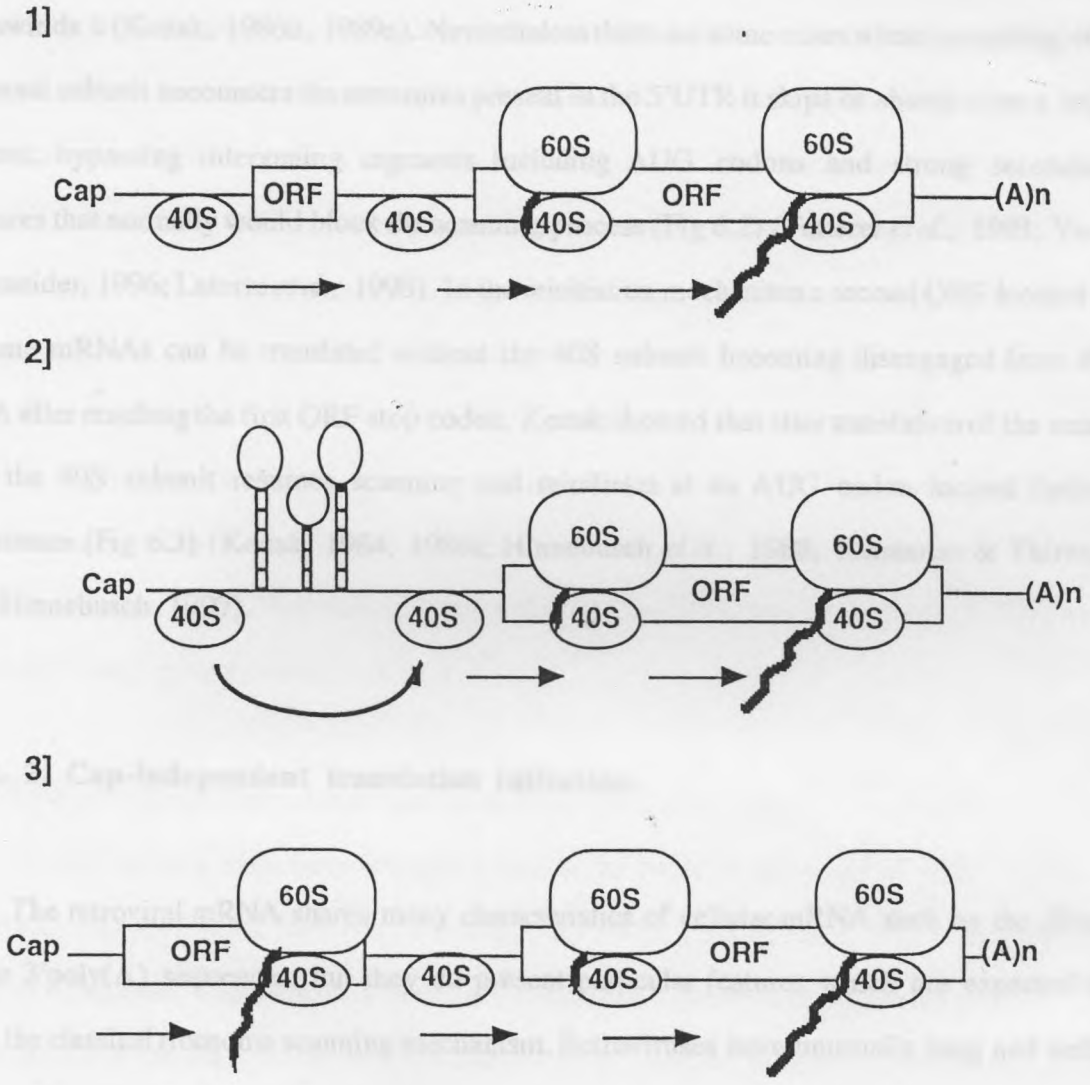


Figure 6. Scheme of alternative cap-dependent translational mechanisms. Alternative machanisms can be use to avoid inhibory effects of multiple open reading frames (ORF) or secondary structures. Ribosomes initially recruited by the 5' cap structure may (1) scan an upstream AUG without initiating until reaching the downstream AUG codon (leaky scaning), (2) Jump over the secondary structure or upstream initiation codons in a process termed ribosomal shunt, (3) initiate at an upstream AUG codon and translate the upstream ORF, and reinitiate at a downstream AUG codon.

(Fig 6.1) (Lin & Lo, 1992; Kozak, 1995). The scanning model postulates that when a scanning 40S ribosomal subunit encounters a hairpin loop in the 5'UTR, it does not skip over the loop but unwinds it (Kozak, 1986b, 1989a). Nevertheless there are some cases when a scanning 40S ribosomal subunit encounters the structures present in the 5'UTR it skips or shunts over a large segment, bypassing intervening segments including AUG codons and strong secondary structures that normally would block the scanning process (Fig 6.2) (Futterer *et al.*, 1993; Yueh & Schneider, 1996; Latorre *et al.*, 1998). In the reinitiation mechanism a second ORF located in the same mRNAs can be translated without the 40S subunit becoming disengaged from the mRNA after reaching the first ORF stop codon. Kozak showed that after translation of the small ORF, the 40S subunit resumes scanning and reinitiates at an AUG codon located further downstream (Fig 6.3) (Kozak, 1984, 1986a; Hinnebusch *et al.*, 1988; Tzamarias & Thireos, 1988; Hinnebusch, 1997).

III. 2. 2. Cap-independent translation initiation.

The retroviral mRNA shares many characteristics of cellular mRNA such as the 5'cap and the 3'poly(A) sequences, but they do present particular features which are expected to inhibit the classical ribosome scanning mechanism. Retroviruses have unusually long and well-structured leader (Corbin & Darlix, 1996). As other viral families like picornavirus and flavivirus, the untranslated leader of MLV may use an IRES to overcome the translation impediment imposed by its secondary structure (Konings *et al.*, 1992; Mougel *et al.*, 1996; Mougel & Barklis, 1997).

A number of experimental criteria are widely used to functionally discriminate an IRES from other 5' mRNA secondary structures (Jackson *et al.*, 1995). The most common involves the comparative test of the efficiency of translation of capped versus uncapped forms of the same mRNA species. The measurement of the effect of cap analogues on the efficiency of translation. The evaluation of the effect of eIF4F activity on the efficiency of translation of a given mRNA, either as a result of cleavage of eIF4G or reduction of eIF4E concentration. Most

of the studies have employed bicistronic mRNAs to identify IRES elements that can mediate cap-independent translation. In these constructs the first cistron in a capped mRNA can be translated by a cap dependent scanning mechanism (Pelletier & Sonenberg, 1988). The second cistron should not be translated unless preceded by an IRES. In this system it is important to test whether the second cistron is indeed translated from an intact bicistronic mRNA. As an alternative to bicistronic mRNAs some groups have developed assays to identify IRESes based on circular mRNA (Chen & Sarnow, 1998). The principle behind this strategy relies on the observation that in cell free systems eukaryotic ribosomes are unable to bind to small circular RNAs, 25-110 nucleotides in length. This finding suggested that eukaryotic ribosomes could only bind RNAs via free 5'end. However, Chen and Sarnow (1995) showed that spatial constraints imposed by circularization of IRES-containing RNA molecules does not interfere with IRES function. Thus, their data confirmed that IRESes allow recruitment of the ribosomal 40S subunit independently from the 5' end.

Several strategies have been used to test the translation initiation mechanism used by MLVs. Stable hairpins have been inserted between the cap and the putative IRES element. Capped and uncapped RNA have been translated in vivo to measure the effect of the 5'cap on the rate of translation. Capped RNAs have been translated in presence of poliovirus protease 2A and the ability of the MLV leader to drive translation of a second cistron in a bicistronic RNA construct has been determined. Together these assays allowed workers to discard any cap-dependent initiation mechanism and strongly favor the presence of an IRES in the 5'leader of MLV retroviruses (Berlioz & Darlix, 1995; Berlioz *et al.*, 1995; Vagner *et al.*, 1995b). Up to date very little is known about the MLV IRES and its presence raises a number of question regarding its function and its implication on viral replication. Some of these questions will be addressed in the results and discussion sections.

III. 2. 3. Picornavirus model.

Internal translation initiation was first discovered in picornaviruses and has since been found in other families of RNA viruses and several cellular mRNAs (for examples see references: Pelletier & Sonenberg, 1988; Pelletier *et al.*, 1988a,b; Tsukiyama-Kohara *et al.*, 1992; Jackson & Kaminski, 1995; Vagner *et al.*, 1995a; Attal *et al.*, 1996; Bernstein *et al.*, 1997; Ivanov *et al.*, 1997; Nanbru *et al.*, 1997; Ye *et al.*, 1997; Akiri *et al.*, 1998; Gan *et al.*, 1998; Huez *et al.*, 1998; Kim *et al.*, 1998; Stein *et al.*, 1998; Stoneley *et al.*, 1998). Some examples of cellular mRNAs reported to possess IRESes able to confer cap-independent translation of a downstream cistron in a bicistronic chimeric mRNA, include those encoding the immunoglobulin heavy chain binding protein (BiP) (Macejak & Sarnow, 1991), the fibroblast growth factor 2 and insulin-like growth factor (Vagner et al., 1995a; Teerink et al., 1995), the translational initiation factor eIF4G (Gan and Rhoads, 1996), the product of the *Drosophila* homeotic gene *Antennapedia* (OH et al., 1992), and two yeast transcription factors TFIID and HAP4 (Iizuka et al., 1994).

The picornaviridae comprise one of the largest and most important families of human and agricultural pathogenic viruses. The family is currently divided into five genera: the enteroviruses, the cardioviruses, the rhinoviruses, the aphthoviruses and the hepatoviruses (Fields *et al.*, 1996). However, picornavirus can be classified into three different groups based on similarities in predicted RNA secondary structures and location of the initiator AUG of their IRES (entero and rhinoviruses, cardio and aphtoviruses, and hepatitis) (Fig 7) (Jackson *et al.*, 1994; Belsham & Sonenberg, 1996). The genome consists of a single plus strand RNA which is polyadenylated at the 3' terminus and which carries a small protein, Vpg, covalently attached to its 5'end. VPg, is rapidly cleaved off following uncoating of the virus and plays no important function in viral RNA translation (Belsham & Sonenberg, 1996; Fields *et al.*, 1996). The genomic RNA encodes a single large polyprotein containing at least one, and in many

species two, protease domains that cleave the polyprotein into four mature viral capsid proteins and several non-structural proteins (Fields *et al.*, 1996).

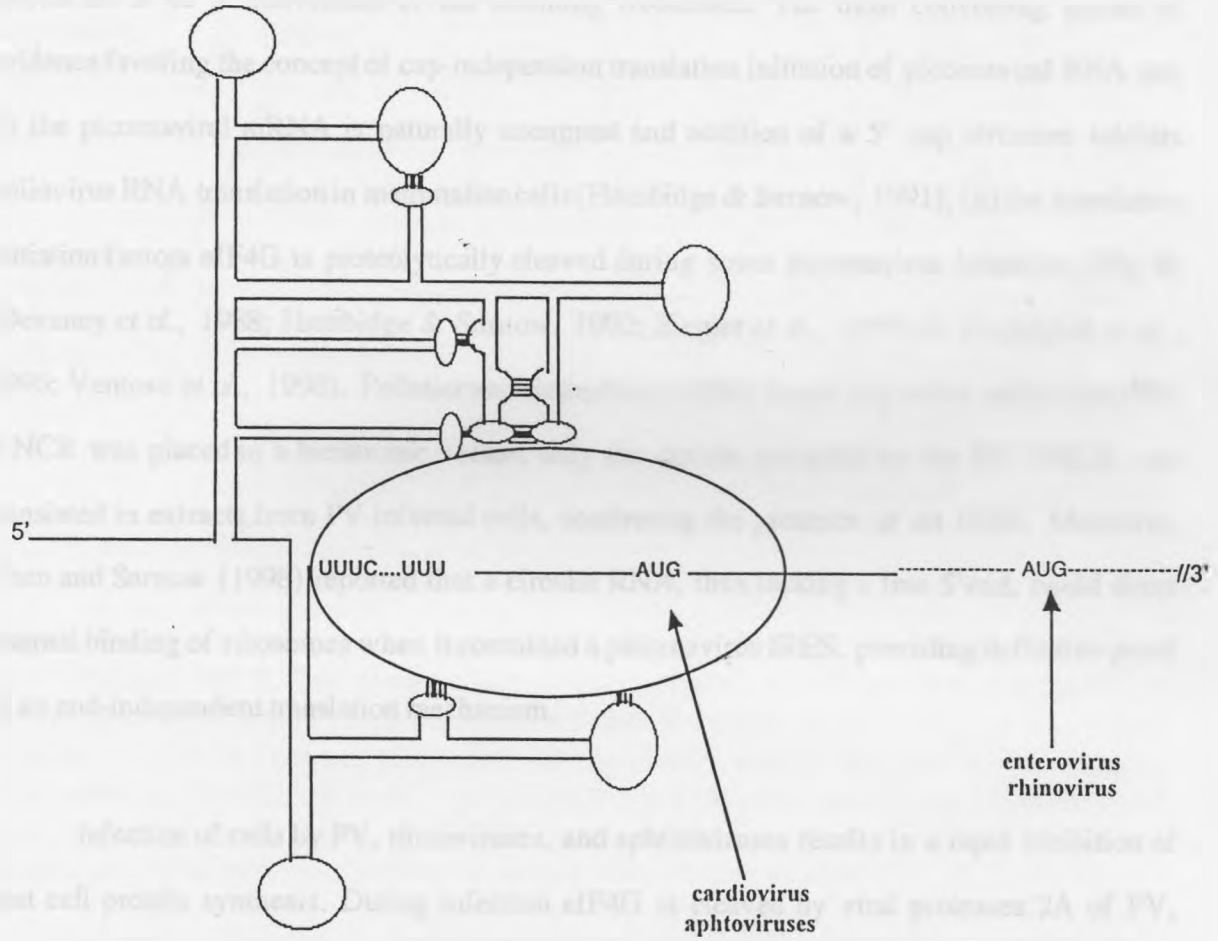


Figure 7. A model for internal initiation of translation of picornavirus RNA (Jackson and Kaminski, 1995). The IRES consists of a number of stem-loops, with possible tertiary structure interactions between the loops and bulges. The AUG at the 3'end of the IRES is the authentic initiation site for viral polyprotein synthesis in the cardio- and aphto-viruses. In the case of the entero- and rhino-viruses, this AUG is likewise the ribosome entry site but is not used as an initiation site, the ribosomes most probably are able to scann until the authentic initiation site, further downstream is found.

The 5' nontranslated region of picornaviral genomic RNA is unusually long compared to homologous regions of cellular mRNA, it ranges in size from 625 bases in human rhinovirus

(HRV) to nearly 1200 in aphthovirus (Fields *et al.*, 1996). The RNA is uncapped and possesses several AUG upstream from the true initiation codon (Jackson *et al.*, 1994; Jackson & Kaminski, 1995; Belsham & Sonenberg, 1996). In addition the 5'untranslated region of picornavirus RNA is composed of extensive secondary structures, which would be expected to inhibit the 5' to 3' movement of the scanning ribosomes. The most convincing pieces of evidence favoring the concept of cap-independent translation initiation of picornaviral RNA are; (i) the picornaviral mRNA is naturally uncapped and addition of a 5' cap structure inhibits poliovirus RNA translation in mammalian cells (Hambidge & Sarnow, 1991); (ii) the translation initiation factors eIF4G is proteolytically cleaved during some picornavirus infections (Fig 8) (Devaney *et al.*, 1988; Hambidge & Sarnow, 1992; Ziegler *et al.*, 1995a,b; Haghighat *et al.*, 1996; Ventoso *et al.*, 1998). Pelletier and Sonenberg (1988) found that when poliovirus (PV) 5'NCR was placed in a bicistronic vector, only the cistron preceded by the PV 5'NCR was translated in extracts from PV-infected cells, confirming the presence of an IRES. Moreover, Chen and Sarnow (1998) reported that a circular RNA, thus lacking a free 5'end, could direct internal binding of ribosomes when it contained a picornavirus IRES, providing definitive proof of an end-independent translation mechanism.

Infection of cells by PV, rhinoviruses, and aphthoviruses results in a rapid inhibition of host cell protein synthesis. During infection eIF4G is cleaved by viral proteases 2A of PV, coxsackie virus and human rhinovirus or the leader (L) protease of FMDV (Devaney, et al., 1988; Belsham & Brangwyn, 1990; Hambidge & Sarnow, 1992; Ziegler, et al., 1995a,b; Haghighat, et al., 1996). In PV, rhinovirus, and aphthovirus infections eIF4G is cleaved into two fragments an amino terminal, which has the eIF4E-binding site, and a carboxy-terminal which has the binding site for eIF3 and eIF4A (Fig 8). Although eIF4G proteolysis provides an explanation for the shutoff phenomenon, several earlier findings obtained with PV are inconsistent with such a model. For example , there is no good temporal correlation between the proteolysis of eIF4G and the shutoff of host translation in poliovirus-infected HeLa cells (Etchison *et al.*, 1982; Perez & Carrasco, 1992). While the cleavage of eIF4G occurs 2h postinfection the shutoff is observed at2.5 h or even later. These data suggest that the complete

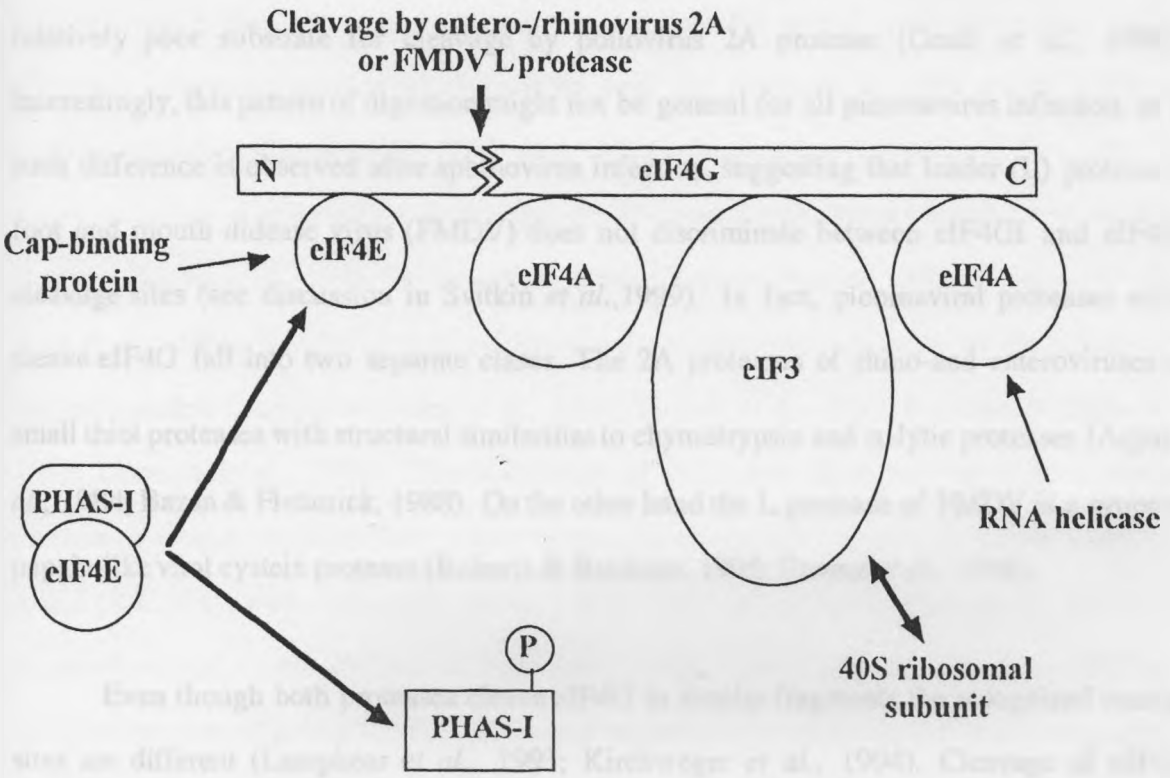


Figure 8. Scheme of the cleavage of eIF4G by the picornavirus protease (Jackson and Wickens, 1997). The picornavirus protease cleaves eIF4G into two fragments: an amino-terminal one-third fragment (N), which has the eIF4E-binding site, and a carboxy-terminal two-thirds fragment (C), which has the binding site for eIF3 and eIF4A. PHAS-I also known as 4E-BP1 binds eIF3 and sequesters it from entry into the eIF4F holoenzyme complex but phosphorylation of 4E-BP1 by the rapamycin-sensitive pathway results in a much-reduced affinity for eIF4E and releases it for binding to eIF4G.

inhibition of host cell protein synthesis following poliovirus infection requires a second event in addition to the cleavage of eIF4G (Bonneau & Sonenberg, 1987). As mentioned in a previous section recently a functional homolog of eIF4G, termed eIF4GII has been identified (Gradi *et al.*, 1998a). eIF4GII is 46% identical to the original eIF4G, which has been renamed eIF4GI. eIF4GII is cleaved both in vivo and *in vitro* by poliovirus 2A protease (Gradi *et al.*, 1998b; Svitkin *et al.*, 1999). However, after PV or human rhinovirus infection, the cleavage of eIF4GII is slower than that of eIF4GI and, in contrast to the cleavage of eIF4GI, it coincides with the shutoff of host protein synthesis (Gradi *et al.*, 1998b; Svitkin *et al.*, 1999). Moreover,

in contrast to the efficient cleavage of eIF4GI in HeLa cytoplasmic extract, eIF4GII is a relatively poor substrate for cleavage by poliovirus 2A protease (Gradi *et al.*, 1998b). Interestingly, this pattern of digestion might not be general for all picornavirus infection, as no such difference is observed after aphthovirus infection, suggesting that leader (L) protease of foot and mouth didease virus (FMDV) does not discriminate between eIF4GI and eIF4GII cleavage sites (see discussion in Svitkin *et al.*,1999). In fact, picornaviral proteases which cleave eIF4G fall into two separate clases. The 2A proteases of rhino-and enteroviruses are small thiol proteases with structural similarities to chymotrypsin and α-lytic proteases (Argos *et al.*, 1984; Bazan & Fletterick, 1988). On the other hand the L protease of FMDV is a proposed papain-like viral cystein protease (Roberts & Belsham, 1995; Guarné *et al.*, 1998).

Even though both proteases cleave eIF4G in similar fragments the recognized cleavage sites are different (Lamphear *et al.*, 1993; Kirchweger *et al.*, 1994). Cleavage of eIF4Gs following picornavirus infection results in the inactivation of the eIF4F complex with respect to its ability to recognize capped mRNAs and hence in a severe inhibition of cap-dependent translation initiation (Ohlmann *et al.*, 1995; Ziegler, et al., 1995a,b). Interestingly, eIF4Gs cleavage enhances cap-independent translation initiation (Ohlmann *et al.*, 1995). Thus, through this mechanism picornaviruses are able to modify the specificity of the host translational machinery, favoring translation of viral mRNA.

In contrast to the enteroviruses, rhinoviruses, and aphthoviruses, the cardioviruses do not induce cleavage of eIF4G (Mosenkis *et al.*, 1985). It has been proposed that inhibition of host cell protein synthesis following infection by cardioviruses correlates with the dephosphorylation of 4E-BPI (Gingras *et al.*, 1996, 1999). As an exception to what is observed in other picornaviruses Hepatitis A does not inhibits host cell protein synthesis and its IRES requires an intact eIF4G protein to functional (Brown *et al.*, 1994; Borman & Kean, 1997).

Picornaviruses use the translational apparatus of the host cell to assure their own protein synthesis. In terms of IRES driven translation initiation eIF4E is the only factor which has been

shown not to be requierd. All the other components of the eIF4F complex have been suggested to play a role in the binding of the ribosomes to the mRNA in a 5' end-independent manner. Therefore, ribosome attachment depends on both the structural integrity of the IRES and specific interactions between the IRES with different translation components. Favouring this notion a number of reports have shown that eIF4G I and eIF4B directly interact with the IRES structure (Meyer *et al.*, 1995; Pestova *et al.*, 1996b; Kolupaeva *et al.*, 1998; Ochs *et al.*, 1999; Rust *et al.*, 1999). Several lines of evidence indicate that cellular proteins are also involved in cap-independent translation (Chang *et al.*, 1993; Toyoda *et al.*, 1994; Belsham & Sonenberg, 1996; Borman *et al.*, 1997). The translation of poliovirus does not occur in a cell-free translation system prepared from wheat germ and occurs inefficiently in rabbit reticulocyte lysate (RRL). However, the poor translation in RRL, is markedly improved by addition of factors from HeLa cells (Pelletier & Sonenberg, 1989). Other IRESes, such as the IRESes of encephalomyocarditis virus (EMCV) RNA and hepatitis C virus RNA, are highly expressed in the RRL system, indicating that quantitative and/or qualitative differences in host factors might be needed for the expression of specific IRESes. Among cellular proteins which are not related to the translational apparatus, so far identified to interact with viral IRESes are the La autoantigen (Rojas-Eisenring *et al.*, 1995; Belsham and Sonenberg, 1996), the polypyrimidine tract-binding protein (PTB) (Borovjagin *et al.*, 1994; Kolupaeva *et al.*, 1996; Niepmann, et al., 1997), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Schultz *et al.*, 1996), unr a cellular cytoplasmic RNA-binding protein (Hunt *et al.*, 1999), and the poly(rC) binding protein 2 (PCBP2) (Blyn *et al.*, 1996, 1997; Graff *et al.*, 1998). Up to date the real function of these cellular proteins is not clear, but they are thought to stabilize secondary structures needed for the correct IRES folding (Belsham & Sonenberg, 1996; Kaminski & Jackson, 1998, Hunt & Jackson, 1999).

A parallel can be drawn between picornaviral and retroviral IRESes. Even though no structural homology has yet been found between picornaviral and retroviral IRESes data from Vagner et al. (1995b) suggest that the same transacting factors may interact with both genetic elements. On the other hand, several authors have used MoMuLV IRES to drive translation of

the first cistron in a bicistronic retroviral vector construct (Adam *et al.*, 1991; Ghattas *et al.*, 1991; Torrent *et al.*, 1996). In these constructs the E+ sequence was used, but in all AUG^{gag} was mutated to avoid production of a Gag fusion protein. Together these data suggest that upon recruitment by MoMuLV IRES the 43S preinitiation complex is able to scan until the fist AUG initiation codon is found. Therefore, MoMuLV IRES would have a global initiation mechanism similar to that of entero and rhinoviruses. Another characteristic of MLV IRES that links it to IRESes of picornaviruses (with the exception of Hepatitis A, see above) is that clevage of eIF4G enhances its activity (López-Lastra *et al.*, 1997, 1999).

III. 3. IRESes in the development of polycistronic retroviral vectors.

11I. 3. 1. Retroviruses, transduction and cell transformation.

Many features of retroviruses favor their choice for the purpose of gene transfer as an alternative to nude-DNA or other potential physical, chemical or viral vector systems (Gunzburg & Salmons, 1995; Treco & Selden, 1995; Hope *et al.*, 1998; Prince, 1998; Robbins *et al.*, 1998; Neumann *et al.*, 1999). The most important advantages of retroviruses are their natural capacity to carrying foreign genes and their ability to integrate into the genomic DNA of animal cells and be stably replicated and transmitted to all of the progeny of these cells. On the other hand, retroviral integration is site-specific with respect to the viral genome at the extreme ends of the proviral LTRs. Thus, post integration, the structure of the viral genes that lie within the LTRs are intact. Finally, retroviruses appear as versatile tools since, they tolerate a tremendous amount of manipulation, their genomes are highly plastic, and their host range can be modulated (for reviews, see Salmons *et al.*, 1995; Gunzburg *et al.*, 1996; Miller, 1997).

Retroviruses can cause cancer in their natural hosts with an efficiency and rapidity that ranks them among the most potent carcinogens known (for reviews, see Weiss *et al.*, 1982; Fan, 1994; Patarca, 1998). However, the oncogenic properties of most retroviruses are

mediated by genetic information that is of cellular rather than viral origin. This observation first pointed to the existence of proto-oncogenes in the DNA of somatic cells. Studies of these viruses laid much of the foundation for our current understanding of the molecular mechanisms involved in tumor induction (for review, see Bishop, 1991). Transforming retroviruses can be divided into two classes: acute transforming and non-acute transforming retroviruses. Non-acute transforming retroviruses are standard replication-competent retroviruses (van Lohuizen & Berns, 1990; Kung *et al.*, 1991). They induce neoplasms with relative long latencies, and they do not morphologically transform cells in culture. Infection with non-acute transforming retroviruses is thought to be only the first step in a series of events that are required for the generation of a transformed cell (Fan, 1997). Acute transforming retroviruses are derived from non-acute retroviruses, but they carry onocogenes which endow them with the capacity to rapidly induce tumors and morphologically transform cells in culture. Thus, they are directly responsible for causing diseases in infected animals (for reviews, see Gallo & Wong-Staal; 1982; Weiss *et al.*, 1982; Fan, 1994).

Viral oncogenes result from retroviral capture of normal cell proto-oncogenes, that encode proteins involved in growth control (Chiu, 1989). During the capture process, known as transduction, alterations such as deletions, fusions and/or point mutations occur in the proto-oncogene that convert it into an oncogene (Swain & Coffin, 1992). With the exception of RSV, the acquisition of a proto-oncogene occurs at the expense of part of the viral genome. Therefore, most acute-transforming retroviruses are replication-defective and are unable to replicate autonomously (for review, see Weiss *et al.*, 1982; Fan, 1994; Coffin *et al.*, 1997). Their continued propagation is dependent on viral structural proteins and enzymes supplied in *trans* by an associated, non-defective helper virus. Tumorigenesis by acute transforming retrovirus can be viewed as viral introduction of a potent transforming gene into cells, which induces them to develop into tumors. Due to retrovirus random integration and to the fact that acute transforming retroviruses are defective in replication and can only infect individual cells, tumors arising from their infection are polyclonal.

It was the study of oncogenic retroviruses that led to the development of retroviral vectors (Rein *et al.*, 1979; Tabin *et al.*, 1982). These studies showed that after the formation of a viral particle, no further viral protein synthesis is required before retrovirus integration. In fact all of the viral proteins coding regions can be removed from a replication competent virus without affecting the ability of the viral RNA to be encapsidated, reverse-transcribed and integrated. The phenomenon of transduction emphasized that retroviruses can naturally carry foreign genes and that propagation of replication-defective retroviruses involves *trans* complementation by a helper virus. However, certain *cis* acting sequences must be present in order to allow replication-defective virus propagation. Proof of principle was accomplished in 1982 when Tabin et al. inserted an intact herpes simplex virus thymidine kinase gene (HSVtk) into the backbone of a MoMuLV proviral clone and rescued an infectious retrovirus that conferred hypoxanthine, aminopterin, and thymidine (HAT) resistance to tk^- cells (Tabin *et al.*, 1982).

III. 3. 2. IRES based retroviral vectors in gene transfer.

Gene transfer involves the delivery to target cells of an expression cassette made up of one or more genes and the sequences controlling their expression. In the case of human gene transfer this process can be carried out *ex vivo* in a procedure in which the cassette is transferred to cells in laboratory conditions and modified cells are then administrated to the recipient (Vile *et al.*, 1996; Miller, 1997). Alternatively, this process can be done *in vivo*, in a procedure in which the expression cassette is transferred directly to cells within the individual. In both cases the transfer process is usually aided by a vector that delivers the cassette to the intracellular site where it can function appropriately (Vile *et al.*, 1996; Miller, 1997).

The retroviral life cycle requires the presence of virally encoded transacting factors. Gag molecules, which form the structural framework of the viral core and interacts with the E sequence playing a key role in the incorporation of viral RNA into the virion core during virion formation (Rein, 1994). Reverse transcriptase and integrase responsible for the conversion of the genomic RNA into the double stranded proviral DNA and its integration in the hosts

chromosome. The viral envelope glycoprotein required for virus attachment to the uninfected cell and for viral spread (Miller, 1996). Therefore, in order to be infectious retroviral vectors consist of two components, the recombinant retroviral vector which carries the gene(s) of interest and the construct(s) providing retroviral structural proteins in *trans*. The recombinant vectors contain viral sequences required in *cis* for recombinant RNA packaging, reverse-transcription, and integration, thus ensuring a successful gene transfer. These sequences are the 5' and 3' LTR, PBS, PPT and E signal, in addition to the genes of interest (Fig. 9) (for reviews, see Salmons *et al.*, 1995; Gunzburg & Salmons, 1996; Miller, 1997).

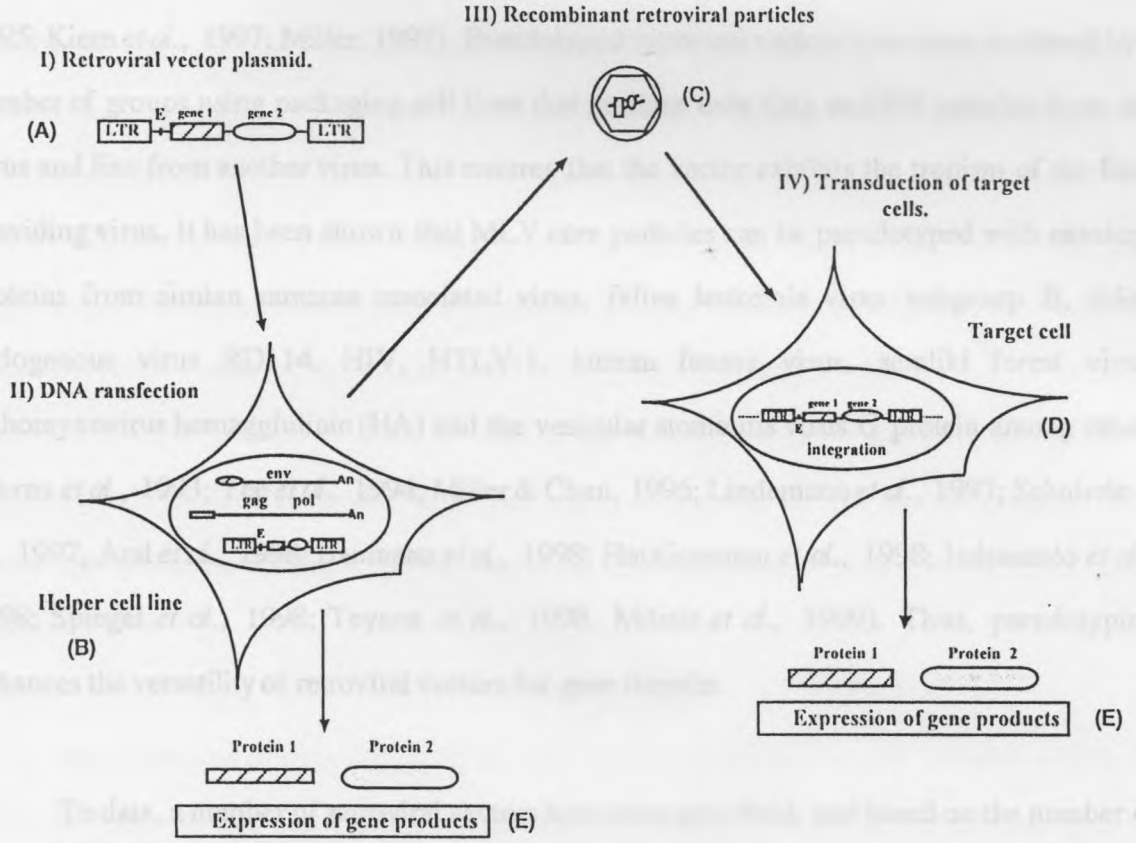


Figure 9. Retroviral vector system. Packaging of retroviral RNA is limited by the size of transcripts. Thus, the introduction of genes of interest into retroviral vectors (I) between the LTRs requires the removal of gag, pol, and env (A). Therefore, retroviral vectors are replication defective because they are unable to synthesize the viral structural proteins and enzymes. These proteins can be provided in trans by a packaging cell line (B), which carry constructs expressing Gag, Pol and Env proteins, but, due to the lack of E, they recombinant viruses cannot package the RNAs encoding the viral proteins. The retroviral vector is transfected (II) into the packaging cell where it is expressed (B). The E (+) vector RNA is recognized by the viral proteins to generate recombinant virus (III, C). This virus is used to transduce (III) target cells (D). Reverse transcription then proceeds and the integrated recombinant provirus can stably express the transduced genes (E). As the vector lacks the gag, pol, and env genes no replication competent virus can be produced by the transduced cells.

As production of retroviral virions has been accomplished only in intact cells, the most currently used helper systems correspond to engineered packaging cell lines which are designed to produce all of the *trans*-acting factors required by a replication-defective retrovirus (Fig 9) (Miller, 1990). Packaging cells synthesize all retroviral proteins, Gag precursor polyprotein and Env required for assembly of high-titer infectious virus. As viral proteins are translated from E minus RNAs, helper cell lines should not produce replication competent virus.

A primary consideration in the selection of the appropriate packaging cell line is the host range of the vectors produced by the cells. Host range has been constantly expanding with the development of new packaging lines. Pseudotyping has allowed the development of retroviral vectors with an extended infection spectrum with respect to wild type MLV (Friedmann & Yee, 1995; Kiem *et al.*, 1997; Miller, 1997). Pseudotyped retroviral vectors have been produced by a number of groups using packaging cell lines that produce only Gag and Pol proteins from one virus and Env from another virus. This ensures that the vector exhibits the tropism of the Env-providing virus. It has been shown that MLV core particles can be pseudotyped with envelope proteins from simian sarcoma associated virus, feline leukemia virus subgroup B, feline endogenous virus RD114, HIV, HTLV-1, human foamy virus, semliki forest virus, orthomyxovirus hemagglutinin (HA) and the vesicular stomatitis virus G protein among others (Burns *et al.*, 1993; Yee *et al.*, 1994; Miller & Chen, 1996; Lindemann *et al.*, 1997; Schnierle *et al.*, 1997, Arai *et al.*, 1998; Baumann *et al.*, 1998; Hatziioannou *et al.*, 1998; Indraccolo *et al.*, 1998; Spiegel *et al.*, 1998; Teysset *et al.*, 1998, Miletic *et al.*, 1999). Thus, pseudotyping enhances the versatility of retroviral vectors for gene transfer.

To date, a number of retroviral vectors have been described, and based on the number of genes they carry they fall into one of the following groups: a) Monocistrinic vectors. These are the simplest retroviral vectors which carry only one gene. The major disadvantage of a single gene vector is the lack of a suitable selection for the presence and expression of the recombinant virus. Therefore, these vectors are best employed to transmit genes which elicit a clearly distinguishable phenotype. b) Polycistronic vectors. Genes with no convenient selection, pose

the problem of how to identify transfected or infected cells harboring the recombinant provirus. This has been solved by inserting a second gene in the same construct encoding a selectable phenotype.

Three different strategies have been used in the development of polycistronic mRNAs (for review, see Miller, 1997). In most examples, the gene proximal to the 5' LTR is expressed from the genome length RNA. Three different strategies have been used to express the more distal gene: (i) it can be expressed from a separate subgenomic mRNA upon splicing of the viral RNA, (ii) from an internal promoter inserted within the vector, or (iii) from an internal ribosome entry segment (IRES). The first strategy represents the most natural approach to the construction of bicistronic vectors. Splicing vectors mimic the mechanism employed by the parental retrovirus and uses the viral splice donor and acceptor sites to form a subgenomic RNA analogous to env RNA. The spliced vectors are designed to express both inserted genes from the viral promoter. In these vectors the first protein gene is translated from the full-length viral mRNA whereas the second is translated from the spliced mRNA (Cepko *et al.*, 1984). The most common problem encountered with the bicistronic splicing vectors is that foreign sequences are inserted into the intron of the spliced gene. Thus, the efficiency of splicing can be severely altered, leading to poor expression of one of the two genes, since the ratio of spliced to unspliced mRNA has been shown to be highly dependent on the vector context. This arrangement results in the higher production of the upstream protein relative to the downstream protein.

An alternative strategy has been the use of an additional promoter inserted within the vector in such a position as to drive expression of the distal gene, while the proximal gene is expressed from the viral LTR. Thus, each gene has its own independent promoter. An important consideration for applications of these two promoter vectors requiring long-term expression, is promoter competitive shut down. In some cases, integrated viral DNA persists but transcription of one of the genes is reduced to undetectable levels, by an epigenetic suppression (Emerman & Temin, 1984, 1986a,b). As an alternative, the second transcription

unit may be inserted within the 3' U3 region. In this arrangement, the second transcription unit is duplicated in the 5' U3 after reverse transcription. This strategy can provide more balanced protein production; however, a second promoter in the LTR can lead to unstable transcription. On the other hand viral regulatory sequences may also have a negative effect on the internal promoter.

The third strategy consists in using IRES to drive cap-independent translation of one or more genes encoded by a single transcript (Adam *et al.*, 1991; Ghattas *et al.*, 1991; Koo *et al.*, 1992; Morgan *et al.*, 1992; Torrent *et al.*, 1996). The initial aim was to use the viral LTR to express a single bicistronic transcript. The upstream cistron is expected to be expressed by a classical cap-dependent translation mechanism, whereas the downstream cistron is translated from the IRES. Insertion of an IRES element into retroviral vectors is compatible with the retroviral replication cycle and has been shown to avoid transcriptional and RNA splicing interferences.

The first bicistronic IRES based vector was constructed by Adam et al. (1991) and took advantage of the polio-IRES. Adam et al. (1991) constructed MoMuLV-based retroviral vectors that positioned a selectable marker or a reporter gene either downstream of the 5' LTR, downstream of an IRES sequence, or downstream of an internal simian virus 40 (SV40) early promoter. Virus titers were similar among the vectors, suggesting that the IRES did not alter virus replication. The level of reporter protein produced was similar when the gene was directly downstream of the LTR or an IRES. Expression of the selectable marker, neomycin phosphotransferase (neo), was similar when neo was positioned downstream from an IRES or the SV40 early promoter. Ghattas et al. (1991) in another survey of IRES function used Rous sarcoma virus (RSV)-based vectors and also found virus titers was not affected by the EMCV IRES. Reporter gene activity from EMCV IRES was twofold higher than from an internally promoted mRNA.

Gene expression from splicing vectors or internal promoters vectors in long-term culture can be at a low level or absent. With polycistronic IRES vectors, selection for one gene should ensure expression of the second gene. Koo et al (1992) constructed a bicistronic spleen necrosis virus (SNV) vector, which contained two selectable markers between the SNV LTRs, the genes conferring resistance to hygromycin B (hyg), and G418 (neo). The EMCV IRES was inserted upstream of neo. Virus titers on target cells after selection with hygromycin, G418, or hygromycin and G418 were similar, suggesting that both hyg and neo were expressed in the same cells. Two months after the initial drug selection, populations of cells selected with hygromycin, G418, or doubly selected were analyzed. In each population the provirus of the recombinant virus remained intact, the predicted single vector transcript was detected using a neo-specific probe, and the Neo protein was stably expressed. The level of Neo protein produced from the IRES vector was similar to the level of the control vector that expressed neo directly from the SNV LTR. These results indicate that selection of either markers on a bicistronic RNA allows expression of both markers. Since the reports of Berlioz and Darlix (1995), Vagner et al. (1995b) and Berlioz et al. (1995) showing the presence of an IRES in the 5' leader of MLV a new generation of double IRES vectors was constructed. MLV IRES conserves all the properties described for picornavirus IRESes when used to develop bicistronic retroviral vectors. Moreover, Torrent et al. (1996) have proposed an additional advantage to these IRESes. As this element is contained within the packaging sequence, the same sequence can be used to guide both mRNA translation and RNA packaging, representing a space economy in the design of retroviral vectors.

III. 4. Objectives:

III. 4. 1. Characterization of an IRES in the 5' leader of Avian reticuloendotheliosis virus type A and mouse VL30 retrotransposon, and their use in the development of bicistronic retroviral vectors.

Studies on retroviruses provide growing evidence that higher-order RNA structural features have regulatory properties. These structural features occur in both protein-coding and non-coding regions. Comparisons of available data shows that in spite of the little nucleotide sequence homology a common regulatory function in different retroviruses can be implemented by unique RNA structures. In this respect Koning et al. (1992) described three highly conserved regions that presented, what they define as unusual folding properties regions (UFR) in terms of free energy. One region present in all MLV-related viruses reflects the location of the hairpin-like structure involved in the gag/pol reading frame transition. A second common UFR, present in most viruses includes the tRNA primer binding site. The third UFR varies in length for the different type C retroviruses but is present in all of them. This UFR is located in the 5' leader downstream of the env splice donor. This last structure is also present in endogenous murine virus and the mouse VL30 retro-element. The mouse VL30 elements constitute a family of retrotransposons present at 100 to 200 copies dispersed in the mouse genome. A typical element is 5 to 6 kilobases (kb) in length with long terminal repeats (LTRs) of about 500 nucleotides (nt) with an organization resembling that of retroviral LTRs, namely U3-R-U5. Mouse VL30 elements do not encode viral-like proteins, have little sequence homology to Moloney murine leukemia virus (MoMuLV) and have not been implicated in retroviral carcinogenesis (Fan et al., 1976 ; Keshet & Shaul, 1981; Keshet & Itin, 1982; French & Norton, 1997). However, VL30 RNA possesses the unusual property of being packaged into MLV particles that are propagated in cell culture (Howk et al., 1978; Scolnick et al., 1979; Carter et al., 1986; Hatzoglou et al., 1990). As a consequence, VL30 elements are able to retrotranspose at high frequency from cell to cell via MLV particles culture (Howk et al., 1978; Scolnick et al., 1979; Carter et al., 1986; French & Norton, 1997).

Since a functional IRES was found in the leader of FrMLV, HaMLV and MoMLV, and considering that IRES can be defined as RNA structures, and that structures are highly conserved in type-C retro-elements, we have hypothesized that most if not all MLV-like retro-elements possess an IRES. Thus the main objective of my work was to characterize new IRESes from retroviral origin and determine their potential to develop novel bicistronic retroviral vectors.

III. 4. 2. Translation initiation driven by IRESes from retroviral origin is not inhibited by neural differentiation.

Retroviruses provide an effective way of transferring genes into many cell types. Several approaches have been used to construct retroviral vectors able to express more than one exogenous product within individual cells. In this respect, internal ribosome entry segments, IRESes, were found to be an efficient means of expressing two exogenous genes in cells without the need for two promoters or a regulated splicing mechanism transcript (Adam *et al.*, 1991; Ghattas *et al.*, 1991; Koo *et al.*, 1992; Torrent *et al.*, 1996). However, recent studies have shown that in certain systems transition from a cap-dependent to a cap-independent mode of translation is a regulated process (van der Velden & Thomas, 1999). For example, utilization of the *anntenapedia* and *ultrabithorax* IRES is spatially and temporally regulated during Drosophila development and the PDGF-B IRES is specifically used upon megakaryocytic differentiation (Bernstein *et al.*, 1997; Ye *et al.*, 1997). Based on these observations the objective of this second part was to determine the effect of cell differentiation on the activity of IRES of viral origin and to determine the capacity of (MLV)-based bicistronic vectors to efficiently transduce and stably express more than one gene product in a human cell line.

III. 4. 3. Determination of IRES activity in the MLV virus context.

The IRES present within the 5' leader of FrMuLV, HaMSV and MoMuLV has been identified by its ability to mediate the translation of a second cistron in a heterologous bicistronic mRNA (Pelletier & Sonenberg, 1988; Berlioz & Darlix, 1995; Berlioz, et al., 1995; Jackson *et*

al., 1995; Vagner *et al.*, 1995b). However, it remains unknown whether the MLV IRES is functional when located in the natural capped mRNA. The objective of this third part was to determine the ability of MoMuLV to utilize the 5' IRES in the synthesis of Gag polyprotein.

IV MATERIALS AND METHODS

IV. 1 Plasmid construction. Standard procedures were used for restriction nuclease digestion and plasmid DNA construction (Sambrook, 1989). *Escherichia coli* HB101 strain 1035 (*recA*$^-$) was used for plasmid DNA amplification. Details of the constructions are given below. Numbering is with respect to the genomic RNA cap site (position +1).

IV. 1. 1. Constructs for *in vitro* translation assays. Segments of the REV-A leader from positions 1 to 580, 265-580 or 452-580 were generated by PCR using plasmid pREVSC-1 as template (Darlix, et al., 1992) followed by digestion with *Nhe*I (PCR added restriction site). To construct the bicistronic plasmids **pREV-CB54**, **pREV-CB55** and **pREV-CG58** each respective fragment was inserted between neo and lacZ of pEMCV-D260-837 previously digested with *Nhe*I (Berlioz *et al.*, 1995). In these constructs, initiation of lacZ translation requires the AUG of REV-A gag at position 580. To generate **pREV-CG52**, the REV-A leader of plasmid pREVSC-1 was digested with *Kpn*I and *Sac*I (Darlix *et al.*, 1992). After Klenow treatment, the plasmid was religated and used as template for the PCR amplification of fragment 1 to 580 containing a deletion from 268 to 452. The PCR product was digested with *Nhe*I and inserted between neo and lacZ of pEMCV-D260-837 previously digested with *Nhe*I (Berlioz, et al., 1995).The inverted REV-A leader, fragment 580 to 1, was generated by PCR using plasmid pREVSC-1 as template (Darlix *et al.*, 1992), followed by digestion with *Nhe*I. The fragment was inserted between Neo and lacZ of pEMCV-D260-837 previously digested with *Nhe*I to give **pREV-CG50**. In these constructs lacZ translation was promoted by an AUG in the optimal Kozak context. **pKT403:** This plasmid contains the mouse VL30 1,9 Kb Hind III fragment in pSP64 (NLV-3, kindly provided by S. Adams (Adams *et al.*, 1988). **pVL30m bicistronic RNAs:** The different VL30 DNA fragments [362-1144, 362-461, 362-575, 576-1144, and 462-1144] were generated by PCR, digested with NheI and inserted between the neo and lacZ genes of pMLV-CB28 (Berlioz & Darlix, 1995) digested with NheI. The neo-VL30-

lacZ sequences are under the control of the T7 RNA polymerase promoter for *in vitro* expression and the cytomegalovirus early promoter for expression in eukaryotic cells. In these constructs, the initiation of ß-galactosidase (ß-gal) translation was from an AUG in a favorable context (GCCAUGG) which was generated by PCR. pEMCV-CBD260-837 was used as a positive control while pEMCV-D837-260 containing the same EMCV IRES fragment but in reverse orientation was the negative control (Berlioz *et al.*, 1995).

IV. 1. 2 Recombinant retroviral constructs. All retroviral constructs were made by standard procedures using pBR322 as a backbone bacterial vector. **pEMCV-CBT4** used as an EMCV positive control was described in Torrent et al. (1996). **pREV-HW 1;** The REV-A leader (positions 265 to 580) was generated by PCR, digested by *Nhe*I and cloned between plap and neo of pMLV-CB71 (Berlioz & Darlix, 1995). **pREV-HW 2;** The *Eco*RI fragment of pVL CBT5 (Torrent *et al.*, 1996), containing the MLV 5'LTR and VL30 E+ sequence was cloned into pREV-HW1/*Eco*RI. **pREV-HW 3;** The *Eco*RI fragment of pCB-100 [pEMCV-CBT4] (Torrent, et al., 1996), containing the MLV 5'LTR and MLV E+ sequence was cloned into pREV-HW1/EcoRI. **pREV-HW 4;** The REV-A 5' leader (positions 452 to 580) was generated by PCR, digested with *Nhe*I and cloned between plap and neo of pMLV-CB71. **pREV-HW 5;** The *Eco*RI fragment of pVL CBT5, containing the MLV 5'LTR and VL30 E+ sequence was cloned into pREV-HW4/*Eco*RI. **pREV-HW 6;** The *Eco*RI fragment of pEMCV-CBT4, containing the MLV 5'LTR and MLV E+ sequence was cloned into pREV-HW4/*Eco*RI. **pVL30m-SJE1:** The VL30 DNA (position 362-575) generated by PCR (pKT403 template) was digested with BalI and NcoI and cloned into pLNPOZ (Adam *et al.*, 1991) digested with BalI (position 800) and NcoI (position 3449) containing the lacZ gene and the two MLV LTRs. ß-gal expression was promoted by an AUG in a favorable context as for pVLD362-575. **pVL30m-SJE2:** The VL30 DNA (position 362-1149) generated by PCR was digested with BalI and NcoI and cloned into pLNPOZ as described for pVLEL362-575. **pVL-SJE3:** The oligonucleotide CCAGCTGAAGCTTGCC was cloned into pLNPOZ digested with BalI (position 800) and NcoI (position 3449). In this construct which served as a negative control for RNA packaging, ß-gal expression was promoted by an AUG in a favorable context

(GCCAUGG), generated by PCR. pMLV-LacZ+: The two LTRs of pMLVK and the MLV Psi$^+$ packaging sequence to position + 1035 (Bender et al., 1987) were inserted into Bluescript pKS. The fragment of pCH110 (Pharmacia) containing the lacZ gene was also inserted into this construct. ß-gal expression was promoted by an AUG in a favorable context. This plasmid served as a positive control for RNA packaging into MLV virions. **pVL30m-SU8:** VL30 DNA (position 362 to 575) generated by PCR and digested with NheI and cloned between plap and neo of pMLV-CB71 (Berlioz & Darlix, 1995). **pVL30m-SU9:** VL30 DNA (position 362 to 1149) generated by PCR and digested with NheI and cloned between plap and neo of pMLV-CB71 (Berlioz & Darlix, 1995). **pVL30m-SU11:** The EcoRI fragment of pEMCV-CBT4 (Torrent *et al.*, 1996), containing the MLV 5'LTR and MLV E+ sequence was cloned into pVL30m-SU9/EcoRI. **pVL30m-SU12:** VL30 DNA (position 362 to 1149) generated by PCR was digested with EcoRI and cloned 5'of plap in pVL-CBT2 which contains rat VL30 between plap and neo (Berlioz *et al.*, 1995).

IV. 2. *In vitro* RNA synthesis. Capped and uncapped RNAs were synthesized using a DNA template and T7 RNA polymerase (mMessage mMachine^{TM} or MEGAscript^{TM}, Ambion) according to the manufacturer's protocol. 1-2 μg of plasmid DNA digested with SspI (position 1240 in the lacZ gene) was used for RNA synthesis in a 20 μl final volume reaction. Transcription was terminated by digestion of the template DNA with DNaseI, and RNA precipitated by lithium chloride. RNA was resuspended in 50 μl of TE buffer and further purified and desalted through MicroSpin^{TM} S-300 columns (Pharmacia BioTech) according to the manufacturer's protocol. Integrity of RNAs was monitored by 0.7% agarose gel electrophoresis and RNA concentration determined spectrophotometrically.

IV. 3. Translation in nuclease-treated rabbit reticulocyte lysate system (RRL). Rev-A containing capped and uncapped RNAs were translated in nuclease-treated rabbit reticulocyte lysate (RRL, Promega) at 50% concentration with 8 μg/ml of RNA and 0,6 mCi/ml of [^{35}S] methionine (Amersham) at 31°C for 1 hr. The assay was supplemented

with potassium acetate to a final concentration of 60 mM, 0,5 mM magnesium acetate and 40 mM KCl. Capped and uncapped VL30m containing RNAs were translated in Flexi-RRL (Promega) at 50% concentration with 25 μg/ml of RNA and 0,6 mCi/ml of [^{35}S] methionine (Amersham) at 31°C for 1 hr. The assay was supplemented with potassium choride to a final concentration of 80 mM. For EMCV RNA 0,5 mM magnesium acetate was added. The effect of foot and mouth disease virus (FMDV) L protease on translation of the capped bicistronic RNAs was assayed in the Flexi RRL (Promega) as previously described, but in the presence of 80 mM KCl. Flexi RRL was pretreated with 1,2 μg/ml of purified recombinant L protease [kindly provided by Dr. S.J. Morley, Department of Biochemistry, The University of Sussex, UK] (Ohlmann *et al.*, 1995, 1996). After translation samples were heated at 96 °C for 3 min in 62.5 mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, 0,02% bromophenol blue and labelled proteins analyzed by 12 -15% SDS-PAGE. Bands were quantified using a Phosphorimager (Molecular Dynamics).

IV. 4. Cell culture. Murine NIH-3T3 cells, and the retroviral packaging cell lines GP+E-86 (Markozwitz et al., 1988) and PG-13 (ATCC,CRL-10686) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) with 10% newborn calf serum (NBCS) at 37°C in presence of 5% CO_2. TE-671 cells (ATCC, CRL-8805) were maintained in DMEM with 10% fetal calf serum (FCS). The packaging cell lines FlyA and FLYRD (Cosset *et al.*, 1995), and TE-FLY-GALV, TE-FLY-A and TE-FLY-10A1 (S. Chapel-Fernanadez & F-L. Cosset; unpublished) were maintained in DMEM with 10% fetal calf serum (FCS), at 37°C in a 5% CO2 atmosphere. The Dev cell line (used between passages 30 - 60 in these experiments), which was originally generated from an undifferentiated human medulloblastoma, was obtained from the Neurobiotec, Lyon Neurological Hospital, and maintained in RPMI 1640 glutamax 1, suplemented with 20% FCS in a 5% CO2 atmosphere.

IV. 5. Transfection, infection and titration. Packaging cells were seeded at 5x10^5 cells per 100 mm plate 24 hr prior to transfections using calcium phosphate with 20 μg of

DNA (Chen & Okayama, 1988). For infections, freshly harvested viruses from stably expressing helper cells were filtered (0.45 μm pore sizes filter) and polybrene was added at a concentration of 8 μg/ml. Vector containing supernatants were overlaid onto cells which were then incubated at 37°C and 5% CO_2 for 24 hr, after which the medium was replaced. Infected cells were grown for a total of 48 hr and subsequently passaged and placed under G418 selection at 0.5 to 0.8 mg/ml (Dev cells) or 1 mg/ml (helper cell lines) or stained for expression of ß-galactosidase or plap. For helper cells after two months of selection the G418 concentration was increased to 1,5 mg/ml. Titrations were performed by adding diluted virus containing medium to target cells seeded at $2x10^4$ in a 24 well plate. The recombinant viral titer was determined by counting the number of lac Z or plap positive cells 48 hr post-infection in limiting-dilution infections. Titer, as transducing units/ml (TU/ml), was calculated by: (number of colonies) X (dilution of infecting retrovirus) / (total volume in ml of diluted vector overlaid onto cells).

To analyze long term virus production of vector which did not express a selection gene, 2 μg of pSV2neo (Southern & Berg, 1982) coding for the neomycin phosphotransferase gene under the control of the SV40 promoter were cotransfected with 18 μg of the pVL plasmids into GP+E-86 cells. 72 hours after transfection, GP+E-86 cells were diluted and placed under selection in a medium supplemented with G418 at 0.8 mg/ml. After 3 weeks of selection, harvested virus was used to infect NIH 3T3 cells as described above.

IV. 6. Metabolic labeling. Cells were serum-starved for 48 h and pre-incubated 1 h in methionine-free medium (Life technologies-Gibco BRL) before [^{35}S]Methionine (100 μCi/plate) (Amersham pharmacia biotech) and 10 % NBCS (Boehringer Mannheim) was added (Beretta *et al.*, 1996) (Morley & McKendrick, 1997). At each sampling time supernatant was collected and cells were lysed in NP40 buffer as previously described. Virus containing supernatant was filtered (0.45 μm pore size filter, Millipore) and virus was partially purified by ultracentrifigation at 25,000 rpm for 150 min (rotor Beckman SW41) through a 20% sucrose cushion in 1x TNE (100 mM NaCl, 50 mM Tris-HCL pH 7,5, 1 mM EDTA). Virus pellets

were resuspended in 300 μl of NP40 buffer and radioactivity incorporated into TCA precipitable material was measured (Ready filter™ Beckman) (Beretta *et al.*, 1996; Morley and McKendrick, 1997).

IV. 7. Histochemical staining. Cells were fixed in phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.2% glutaraldehyde. For LacZ, after two washes in PBS, cells were stained with 5-bromo-4chloro-indolyl ß-D galactosidase (X-gal). For placental alkaline phosphatase (plap) histochemical staining, cells were fixed in phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.2% glutaraldehyde. After two washes in 1x PBS, they were incubated at 65°C for 30 min in 1X PBS. Cells were washed twice with AP buffer (100 mM Tris-HCl pH 9.5, 100mM NaCl, and 5 mM $MgCl_2$ in ddH_2O) and incubated for 5 hr in staining solution (0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 1 mg/ml nitroblue tetrazolium salt (NBT), and 1 mM levamisole in 1xAP buffer).

IV. 8. Enzymatic Activities. Cell extracts were used as substrate for subsequent enzymatic assays: cells were washed twice with cold 1x PBS, scrapped using a rubber policeman, collected by centrifugation at 600 x g, and resuspended in NP40 buffer (0.5% NP-40, 140 mM NaCl, 30 mM Tris-HCl, pH 7,5). Nuclei were removed by a 10 min centrifugation at 14000 x g. Protein concentration was determined using the Micro BCA* protein assay reagent (Pierce). Plap activity in cell extract was determined spectrophotometrically (Alkaline Phosphatase substrate kit, BIO-RAD) using commercial Calf intestine alkaline phosphatase (Boehringer Mannheim) as an activity standard. Neomycin phosphotransferase activity (neo) was measured by [γ-^{32}P]ATP phosphate transfer to neomycin as previously described (Ramesh & Osborne, 1991).

IV. 9. Immunocytochemistry. For immunocytochemistry cells were transferred to 8 well labtek chamber slides (Nunc, Naperville, IL) and differentiation was induced by supplementing medium with 10% of FCS. Cells were maintained for up to 10 days with or

without G418 (0.5 mg/ml). Cells were fixed with 4% paraformaldehyde in 100 mM sodium phosphate buffer pH 7.4 at 4°C for 30 min. After 3 washes in PBS, cells were incubated for 30 min in a blocking solution of PBS containing 5% BSA and 1% normal goat serum prior to staining with anti-galactocerebroside, or the same solution including 0.2% Tween 20 for all other antibodies. Monoclonal anti-neurofilament (clone 2F11) and polyclonal rabbit anti-plap were from Dako, (Copenhagen, Denmark); monoclonal anti-GFAP, (clone GA5) and monoclonal anti-galactocerebroside were from Boehringer Mannheim. Double-labelling was performed by simultaneous staining with a monoclonal anti-neural cell type-specific antigen and the polyclonal rabbit anti-plap. Antibodies were diluted at 1/200, in blocking solution (except anti-galactocerebroside which was diluted to 1/50) and incubated for 2 hours. Bound mouse antibodies were revealed with FITC-conjugated goat anti-mouse IgG1 antibodies except anti-galactocerebroside which was revealed with FITC-conjugated goat anti-mouse IgG3. Bound rabbit antibodies were revealed by a Cy3-conjugated goat anti-rabbit IgG antibody. Controls included no primary antibodies and uninfected (plap-negative) Dev cells. Slides were washed 3 times in PBS, mounted with moviol and analyzed with an Zeiss Axioplan fluorescence microscope.

IV. 10. Flow cytometry. Infected and uninfected cells were suspended in PBS, 1 mM with respect to EDTA, fixed in 1% parformaldehyde in 100 mM phosphate buffer pH 7.4 for 1 hour at 4°C, and then washed in PBS 3 X 5 min. Cells were preincubated in 5% BSA in PBS for 30 min then stained with a 1/100 dilution of mouse anti-plap antibody (DAKO). Bound antibodies were labeled with an FITC-conjugated goat anti-mouse antibody (DAKO) and cell staining was analyzed with a Becton-Dickinson FACSTAR flow cytometer. Data were analyzed with Cell Quest software on a Macintosh 650 Quadra..

IV. 11. MoMuLV chronically infected NIH 3T3 cells. NIH 3T3 cells were transfected by the calcium phosphate method (Chen and Okayama, 1988) with pMLVH2-neo which contains the complete MoMuLV provirus followed by the selection marker neomycin phosphotransferase (neo) under the control of SV40 promoter, in a pBR322 backbone (Housset

et al., 1993; Housset & Darlix, 1996). Upon selection, high virus producing clones were identified by RT activity assay and used as virus producer cells. The virus-containing medium was used to infect fresh NIH 3T3 cells in the presence of 8 μg/ml of polybrene (Sigma).

IV. 12. Rapamycin (Rap) and Actinomycin D (Act D) assays. Stably transduced or MoMuLV infected NIH 3T3 cell were grown to 70-80% confluency in 100 mm. Cells were serum-starved for 48 h prior to the addition of DMEM containing serum (10% NBCS) and either 50 ng/ml Rap (Sigma) (time 0) or vehicle alone (Beretta *et al.*, 1996; Morley and McKendrick, 1997). In all assays in which Act D (Sigma) was used, the drug was added to a final concentration of 1 μg/ml 30 min before time 0 (Levin & Rosenak, 1976). At sampling times cells were scrapped using a rubber policeman, collected by centrifugation at 600 x g, directly resuspended in NP40 buffer (0.5% NP-40, 140 mM NaCl, 30 mM Tris-HCl, pH 7,5), and nuclei were removed by a 10 min centrifugation at 14000 x g. The supernatant was transferred to a new tube and protein concentration was determined using the Micro BCA* protein assay reagent (Pierce). For transduced cells the level of reporter gene expression, measured by enzymatic activity in presence or absence of rapamycin was used to calculate the effect of the drug as a percentage increase or decrease relative to untreated cells.

IV. 13. Western Blot. Cells were washed twice with PBS, trypsinized and collected by centrifugation at 600xg. Cells were directly resuspended in NP40 buffer (0.5% NP-40, 140 mM NaCl, 30 mM Tris-HCl, pH 7,5), followed by 10 min centrifugation at 14000 xg. The supernatant was transferred to a new tube and the protein concentration was determined using the Micro BCA* protein assay reagent (Pierce). Once quantified, 10 µg of total protein were subjected to electrophoresis on 15% ployacrylamide/SDS. Proteins were transferred to PVDF membrane (Boehringer Mannheim) by semi-dry transfer in a 30%methanol Tris-glicine buffer. The filter was blocked with 5% fat-free dried milk in TBST (10mM Tris-HCL, pH 7.4, 150mM NaCl and 0.05% Tween 20). The membrane was incubated for 1 h at room temperature in a 1:800 dilution of rabbit-anti neomycin phosphotransferase II antibody (5 Prime→3Prime, Inc®,

USA), a 1:800 dilution of HR187 anti-p30 antibody (Chesebro *et al.*, 1983) or a 1/2,000 dilution of anti-gp70 antibody (Lavillette *et al.*, 1998) (Quality Biotech Inc., Camdem, N.J.) in blocking buffer. After two 15 min wash in TBST, the membrane was incubated as before in a 1:800 dilution of biotinilated anti-rabbit IgG-antibody, anti-rat or anti-goat antibody (Bio Sys, France). After two washes with TBST, the membrane was incubated for 30 min in VECTRASTAIN® Elite® ABC avidin-peroxidase solution (Vector laboratories, USA) and developped by ECL (Amersham, UK) according to the manufacturer's protocol.

IV. 14. Electron Microscopy. The cells were covered for 30 min at 4 °C with 5 ml of 2% glutaraldehyde in 0.1 M Sörensen phosphate buffer, pH7.4. They were then washed 3x 10 min with phosphate buffer containing 0.2M sucrose and post-fixed in 1% O_sO_4 plus 1.5% ferrocyanure potassium for 1 h at room temperature. Cells were dehydrated through graded ethanol and embedded in Epon 812. Thin sections were cut and picked up on 200 mesh copper grids, stained with uranyl acetate and counter-stained with lead citrate. Specimens were analyzed with a Philips CM120 electron microscope (CMEABG-Villeurbanne-France).

V. RESULTS

V. 1- Characterization of an IRES in the 5' leader Avian reticuloendotheliosis virus type A and of mouse VL30 retrotransposon.

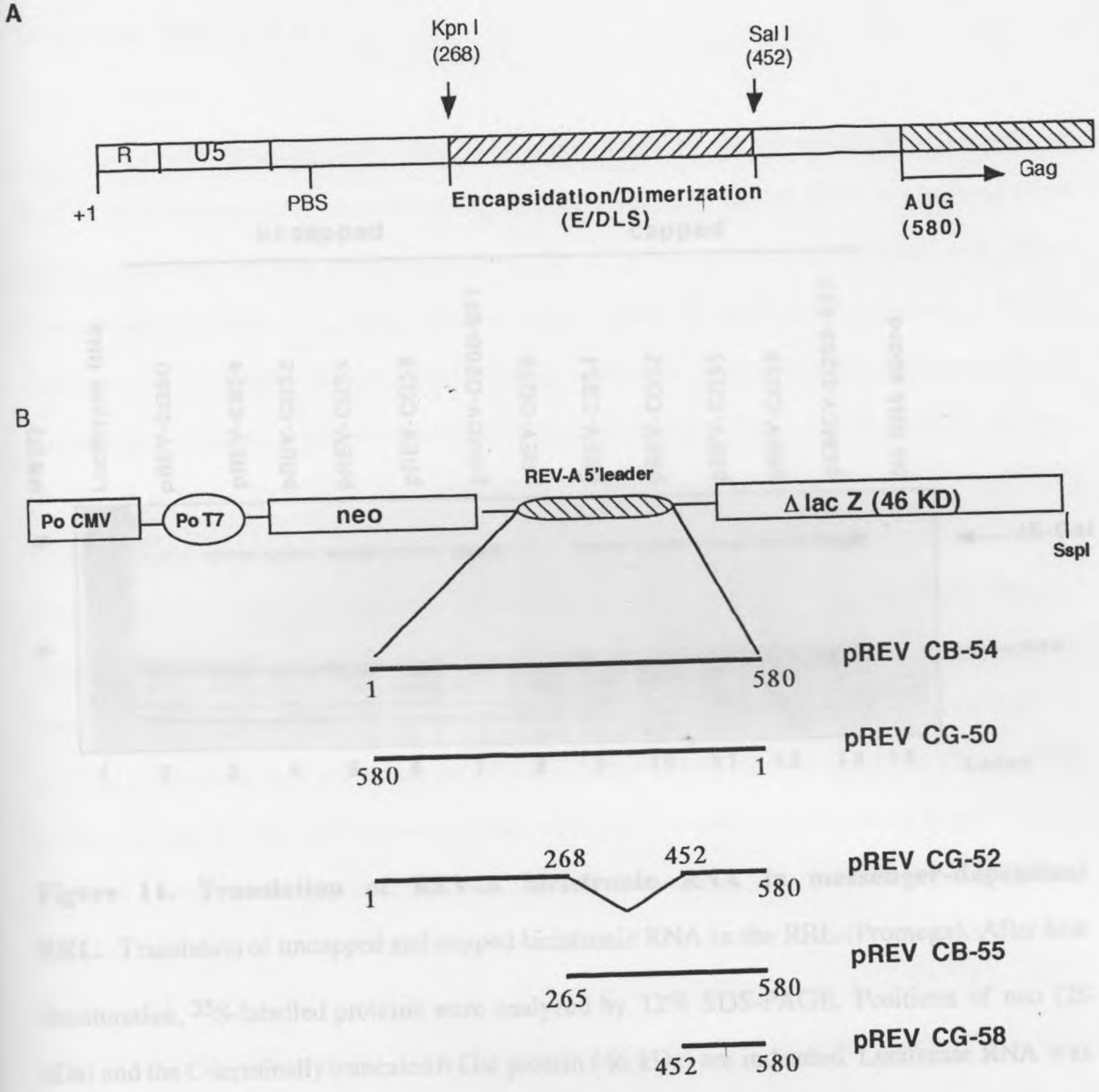
V. 1. 1- The 5' leader of REV-A RNA directs *in vitro* translation of the second cistron in a bicistronic RNA.

The REV-A 5' leader having a structural organization similar to that of the 5' leader of both MoMLV and FrMLV prompted us to examine the possibility that REV-A RNA also shares the ability to direct cap independent translation initiation (Konings *et al.*, 1992; Coffin *et al.*, 1997). Capped and uncapped monocistronic RNAs, containing different segments of the 5'leader of REV-A were generated and used in *in vitro* translation assays. For all REV-A segments used in this assay, translation initiation of monocistronic RNAs was cap-independent. Thus, to further examine the translational properties of the REV-A 5'leader, we generated capped bicistronic mRNAs where different REV-A 5' leader sequences were inserted between the neomycin (*neo*) and a C-terminal truncated (46 kD) *lacZ* reporter genes (fig.10). The first cistron lies downstream of a short 5' capped noncoding region (54 nt) and was expected to be translated by conventional cap-dependent scanning (Kozak, 1991b; Pain, 1996). However, the second cistron, encoding ß-Gal, can be translated only if the REV-A 5' leader contains an IRES or through a termination reinitiation mechanism (Pelletier & Sonenberg, 1988; Jackson *et al.*, 1995).

As shown (fig. 11), RNA pREV-CB54, containing the complete REV-A 5' leader (lanes 3 and 9), as well as, RNAs pREV-CG52 (lane 4 and 10), pREV-CB55 (lanes 5 and 11), and pREV-CG58 (lanes 6 and 12), with deletions in the 5'leader, were capable of promoting expression of ß-Gal in a cap-independent fashion, expression independent of the first cistron. These data suggest the presence of an IRES within the 5'leader of REV-A RNA and that, at

least *in vitro* , the IRES function is independent of the packaging sequence since its absence (pREV-CB52 and pREV-CG58) did not reduce the IRES activity. The fact that pREV-CG52 and pREV-CG58 possess IRES activity suggests that the minimal REV-A IRES structure is contained within a 129 nt fragment. In these experiments RNAs pREV-CG50, with the full REV-A leader in reverse orientation, was used as a negative control (lanes 2 and 8) and pEMCV-CBD260-837 (lanes 7 and 13) containing the EMCV IRES was used as control for cap-independent translation initiation. The relative intensities of the bands on the gel (fig 11) were quantified and the cap/uncapped ratio was calculated. The mean ratio for neo translation was 6,4, indicating a high dependence on the 5'cap structure, while for lacZ the mean ratio was 1,1, suggesting that translation of the second cistron was cap-independent.

Figure 10. Scheme of the REV-A leader and the bicistronic constructs. A) Representation of the 5' leader of REV-A RNA, the restriction sites (DNA) of interest are shown above the scheme. The important genetic elements such as PBS, the tRNA-binding site corresponding to the initiation of minus-strand cDNA synthesis, and the E/DLS signal necessary for viral genomic RNA dimerization and encapsidation are shown below. Numbering is with respect to the genomic RNA cap site (position +1). **B)** Schematic representation of the bicistronic plasmid constructs containing different portions of the REV-A 5' leader located between the neomycin and LacZ genes under the control of the T7 promotor (PoT7) for *in vitro* experiments. Numbering is with respect to the genomic RNA cap site (position +1).


A
Kpn I
(268)
Sal I
(452)
R
U5
PBS
+1
Encapsidation/Dimerization
(E/DLS)
AUG
(580)
Gag
B
Po CMV
Po T7
neo
REV-A 5'leader
Δ lac Z (46 KD)
SspI
pREV CB-54
1
580
pREV CG-50
580
1
pREV CG-52
268
452
1
580
pREV CB-55
265
580
pREV CG-58
452
580

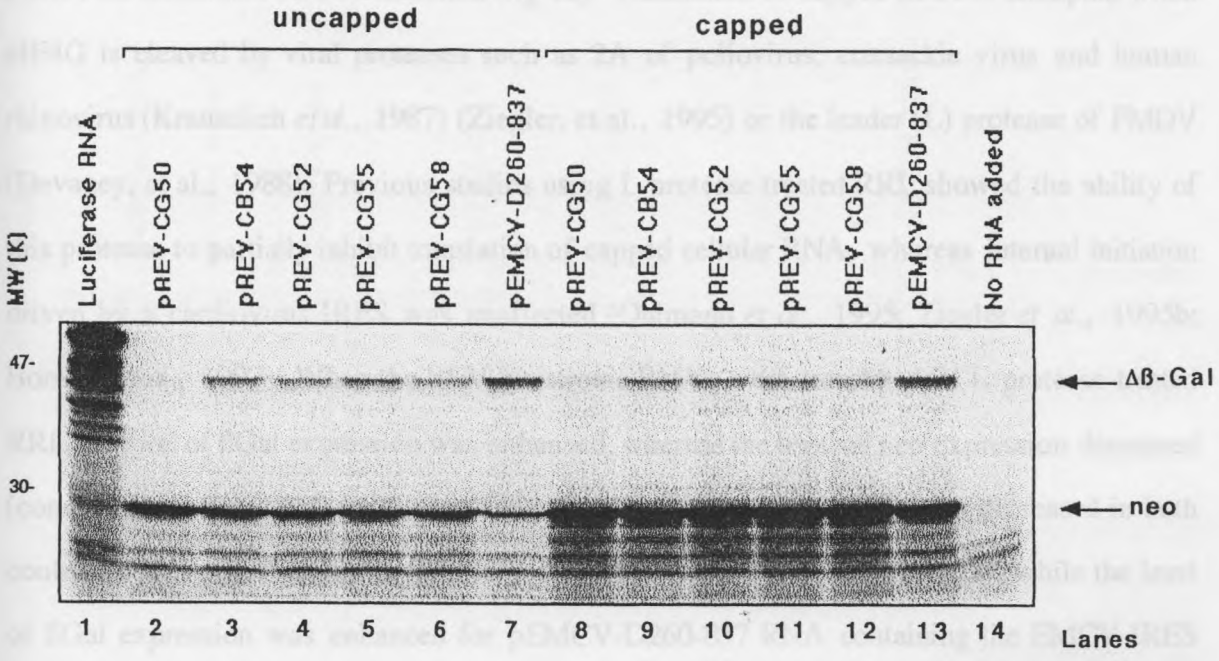


Figure 11. Translation of REV-A bicistronic RNA in messenger-dependent RRL. Translation of uncapped and capped bicistronic RNA in the RRL (Promega). After heat denaturation, ^{35}S-labelled proteins were analyzed by 12% SDS-PAGE. Positions of neo (28 kDa) and the C-terminally truncated ß-Gal protein (46 kDa) are indicated. Luciferase RNA was used as a translation (+)Control (lane 1), while for the translation (-) Control no RNA was added (lane 14).

V. 1. 2- Influence of the L protease of FMDV on *in vitro* translation of bicistronic REV-A RNAs.

To substantiate the above data further, we examined the effect of the L protease of FMDV on bicistronic RNA translation (fig 12). Translation of capped RNA is disrupted when eIF4G is cleaved by viral proteases such as 2A of poliovirus, coxsackie virus and human rhinovirus (Krausslich *et al.*, 1987) (Ziegler, et al., 1995) or the leader (L) protease of FMDV (Devaney, et al., 1988). Previous studies using L protease treated RRL showed the ability of this protease to partially inhibit translation of capped cellular RNA, whereas internal initiation driven by a cardiovirus IRES was unaffected (Ohlmann *et al.*, 1995; Ziegler *et al.*, 1995b; Borman *et al.*, 1997). When the REV-bicistronic RNAs were translated in L protease treated RRL the level of ßGal expression was enhanced, whereas the level of neo expression decreased (compare lanes 5 and 6, 7 and 8, 9 and 10). The level of neo expression also decreased in both control vectors pREV-CG50 and pEMCV-D260-837 (lanes 3 and 4, 11 and 12), while the level of ßGal expression was enhanced for pEMCV-D260-837 RNA containing the EMCV IRES (lanes 11 and 12). No *lacZ* translation was observed for pREV-CG50 (lanes 3 and 4). The effect of L protease on expression of neo and ß-Gal was measured by scanning densitometry and data summarized in Table 1. Quantification data strongly suggest the presence of an IRES within the 5'leader of REV-A RNA. In addition, these data indicate that the minimal REV-A IRES is probably contained within a 129 nt fragment (nucleotides 452-580) of the REV-A RNA 5'leader, immediately upstream of the gag AUG codon (see pREV-CG58 in fig 12; lanes 9 and 10).

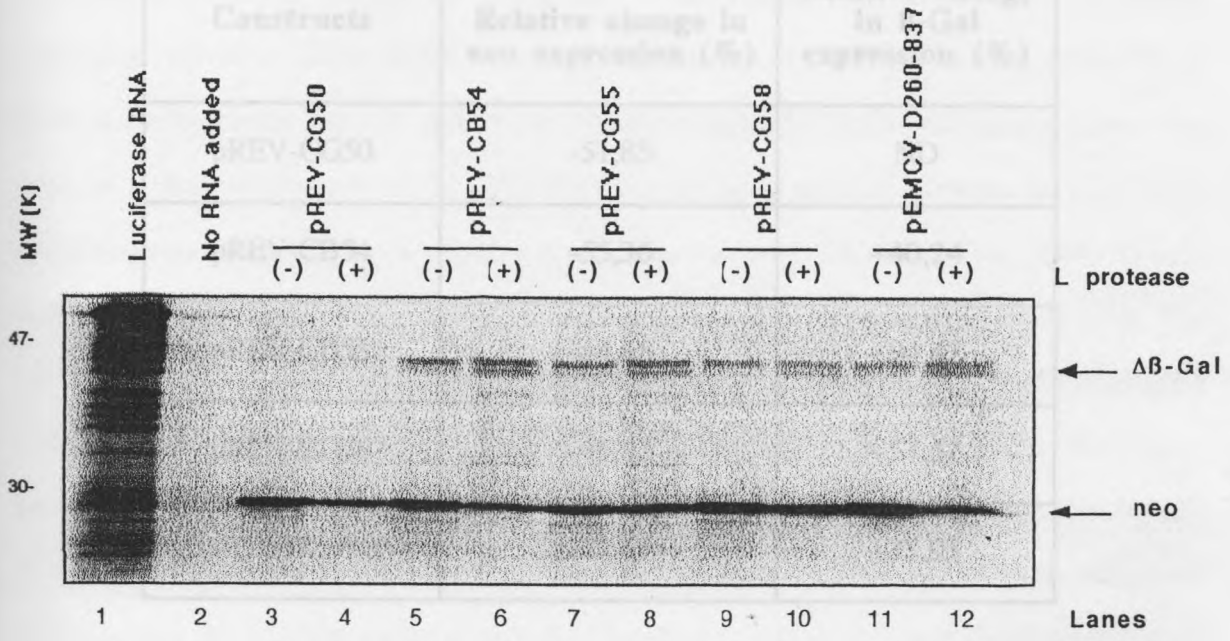


Figure 12. Effect of FMDV L Protease on Rev-A bicistronic RNA translation. Translation of bicistronic capped RNA in the Flexi-RRL system (Promega) with or without L protease. After heat denaturation, ^{35}S-labelled proteins were analyzed by 12% SDS-PAGE. Positions of neo (28 kDa) and the C-terminally truncated ß-Gal protein (46 kDa) are indicated. Luciferase RNA was used as a translation (+)Control (lane 1), while for the translation (-) Control no RNA was added (lane 2).

Constructs	Relative change in neo expression (%)	Relative change in ß-Gal expression (%)
pREV-CG50	-51,85	ND
pREV-CB54	-55,36	+40,24
pREV-CB55	-34,65	+84,42
pREV-CG58	-45,07	+57,51
pEMCV-D260-837	-79,51	+33,93

Table 1. Relative change in reporter gene expression caused by FMDV L protease (%). The gel shown in fig. 12 was scanned and the data used to calculate the effect of L protease as a percentage (%) increase or decrease relative to translation in untreated Flexi-RRL [100% - (level of reporter gene in the presence of L protease) x 100%/ (level of reporter gene in the absence of L protease)]. ND stands for not determined.

V. 1. 3- Recombinant MLV/VL30m vectors allow expression of LacZ in transduced NIH-3T3 cells.

To evaluate if the 5' region of VL30m can replace the 5' leader of MoMLV in a recombinant construct, two regions downstream of the VL30m PBS were chosen as putative packaging sequences. These regions were placed upstream of LacZ in a monocistronic MLV-based retroviral vector (Fig.13). pMLV-LacZ+ containing the MLV E+ packaging sequence was used as a positive control, while pVL-SJE3 containing a random sequence in place of a packaging signal was used as a negative control. Vectors were transfected into GP+E-86 cells and virus containing medium was later recovered and used to transduce NIH 3T3 cells. The number of NIH 3T3 LacZ positive cells obtained after transduction with vectors pVL30m-SJE1 and pVL30m-SJE2 was within the same order of magnitude as MLV-LacZ E+, the control vector (data not shown). This preliminary observation prompted us to determine recombinant viral titer of stably producing helper cell line, set up by cotransfecting GP+E-86 cells with pSV2neo and the different vector constructs. As shown in table 2, upon G418 selection the titers obtained with the MLV-VL30m vectors are within the same order of magnitude as those of the control MLV-vector. These data not only confirm the work of Chakraborty et al. (1995), which suggested the ability of the 5' region of VL30m to drive packaging of a recombinant RNA, but as LacZ is expressed they also show that VL30m can direct translation of a 3' cistron in the context of a monocistronic retroviral vector.

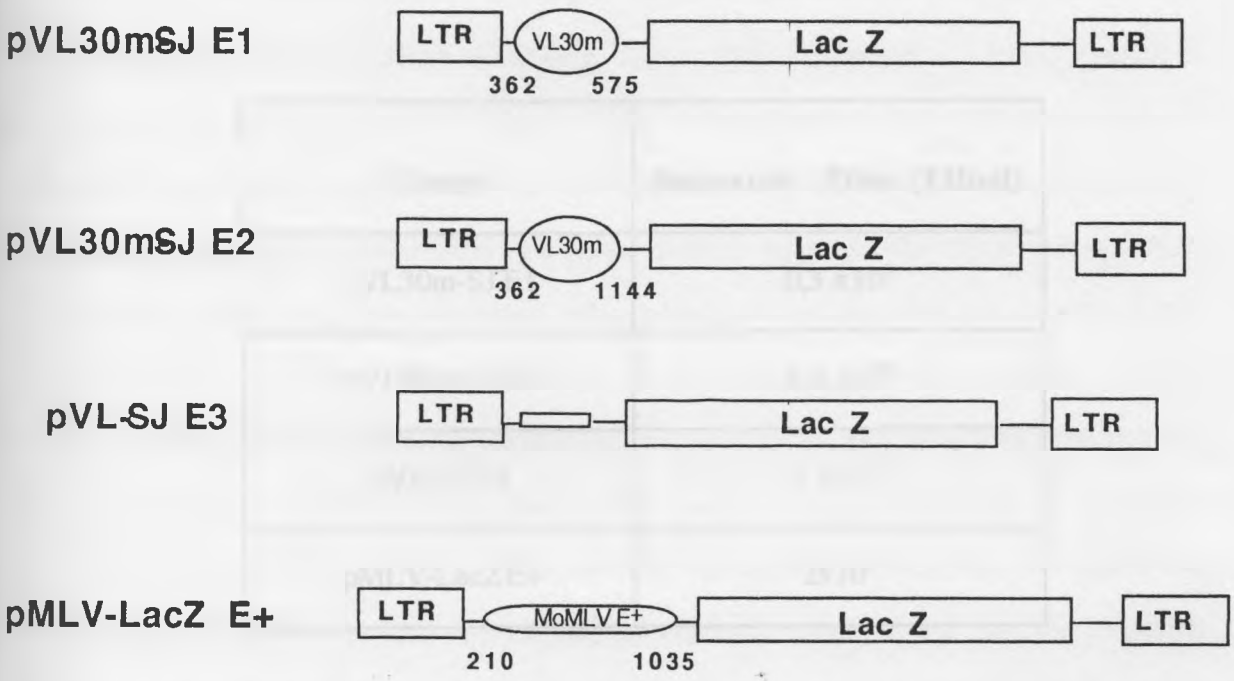


Figure 13. Schematic representation of MLV/VL30m-Lac Z monocistronic retroviral vectors. All MLV/VL30m vectors contain the 5' LTR and the primer binding site (PBS) of MLV. In vectors pVL30m-SJ E1 and pVL30m-SJ E2 different segments of the 5' region of NLV-3 VL30 sequences were inserted upstream of the lacZ reporter cistron. The pMLV-LacZ+ vector used as a positive control contains the MLV Psi+ encapsidation sequence. The pVL-SJE3 used as a negative control contains no encapsidation sequence. Numbering is with respect to VL30m RNA cap site (position +1).

Vector	Retroviral Titer (TU/ml)
pVL30m-SJ E1	$0,3 \times 10^5$
pVL30m-SJ E2	$1,3 \times 10^5$
pVL-SJ E3	$< 1 \times 10^2$
pMLV-LacZ E+	2×10^5

Table2 : Recombinant monocistronic MLV/VL30m-Lac Z retroviral titer. Viral titer was determined by the method of serial dilutions followed by lacZ histochemical staining. NIH 3T3 cells were seeded 24 hr prior to infection with virus containing medium harvested from stably transformed ecotropic GP+E-86 cells. The viral titer is reported as $lacZ^+$ transducing units per milliliter (TU/ml) of collected medium and corresponds to the mean of triplicate experiments. S.D. did not exceed 10% of the mean values.

V. 1. 4- The 5' region of VL30m directs expression of a 3' cistron of a bicistronic RNA in rabbit reticulocyte lysate (RRL).

Based on data published for MoMuLV, HaMSV, and FrMLV we hypothesized that VL30m contains a functional IRES (Berlioz & Darlix, 1995; Berlioz, et al., 1995; Vagner, et al., 1995b). As a first approach in the characterization of the putative VL30m IRES, capped and uncapped monocistronic RNAs, with different segments of VL30m 5'RNA upstream from LacZ, were translated in rabbit reticulocyte lysate. Results suggested that translation of RNAs containing VL30m 5' sequence proceeded independently of the cap (data not shown). These data promted us to test the translational ability of these sequences when contained within a bicistronic mRNA (fig. 14). In pVL30m bicistronic RNAs the VL30m sequences from position 362-1144, 362-461, 362-575, 576-1144, or 462-1144 were inserted between the neomycin (neo) and the lacZ gene, as previously described (fig 14). In all constructs, the first cistron lies downstream of a short 5' capped or uncapped untranslated region (54 nt), and the 3' cistron (lacZ) would be expressed only if the VL30m sequence has IRES activity or through a termination reinitiation mechanism. As a positive control for cap-independent translation we used pEMCV-CBD260-837 RNA (fig 14) containing the EMCV IRES, while pEMCV-D837-260, with the complete EMCV leader in reverse orientation, was used as a negative control.

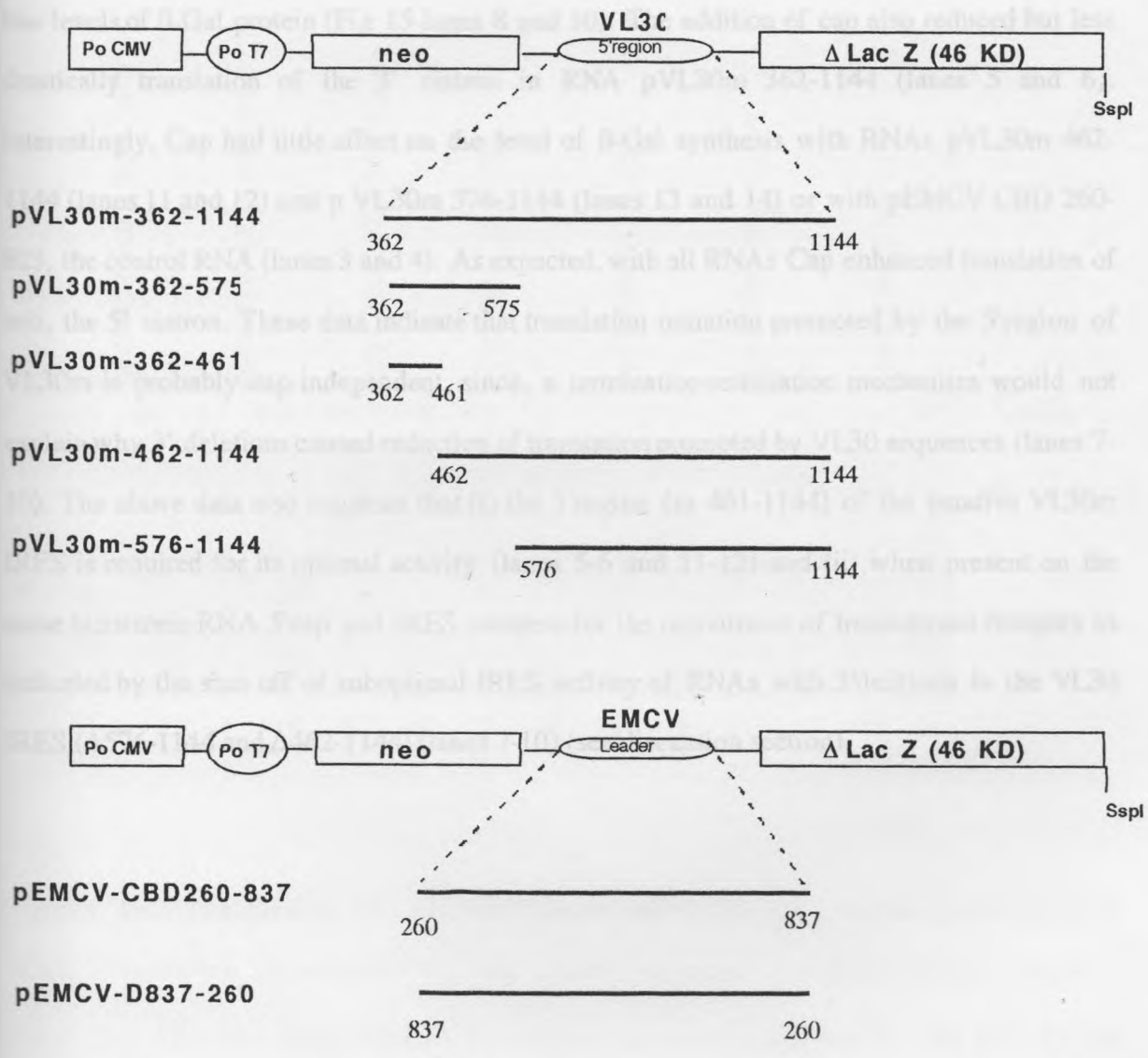


Figure 14. Schematic representation of the VL30m bicistronic plasmids. Constructs containing different portions of the VL30 5'RNA located between the neomycin and LacZ genes under the control of the T7 promoter (PoT7) for *in vitro* expression. Numbering is with respect to the genomic RNA cap site (position +1).

Results show that in uncapped pVL30m RNAs (Fig 15, lanes 5, 7 9, 11, and 12) the putative VL30m IRES was capable of promoting synthesis of ß-Gal. With capped RNAs the putative VL30m IRES activity of pVL30m 362-575 and pVL30m 362-461 was reduced to very low levels of ß-Gal protein (Fig 15 lanes 8 and 10). The addition of cap also reduced but less drastically translation of the 3' cistron in RNA pVL30m 362-1144 (lanes 5 and 6). Interestingly, Cap had little effect on the level of ß-Gal synthesis with RNAs pVL30m 462-1144 (lanes 11 and 12) and p VL30m 576-1144 (lanes 13 and 14) or with pEMCV CBD 260-823, the control RNA (lanes 3 and 4). As expected, with all RNAs Cap enhanced translation of neo, the 5' cistron. These data indicate that translation initiation promoted by the 5'region of VL30m is probably cap-independent, since, a termination-reinitiation mechanism would not explain why 3' deletions caused reduction of translation promoted by VL30 sequences (lanes 7-10). The above data also suggests that (i) the 3'region (nt 461-1144) of the putative VL30m IRES is required for its optimal activity (lanes 5-6 and 11-12) and (ii) when present on the same bicistronic RNA 5'cap and IRES compete for the recruitment of translational complex as indicated by the shut off of suboptimal IRES activity of RNAs with 3'delitions in the VL30 IRES (Δ576-1144 and Δ462-1144) (lanes 7-10) (see discussion section).

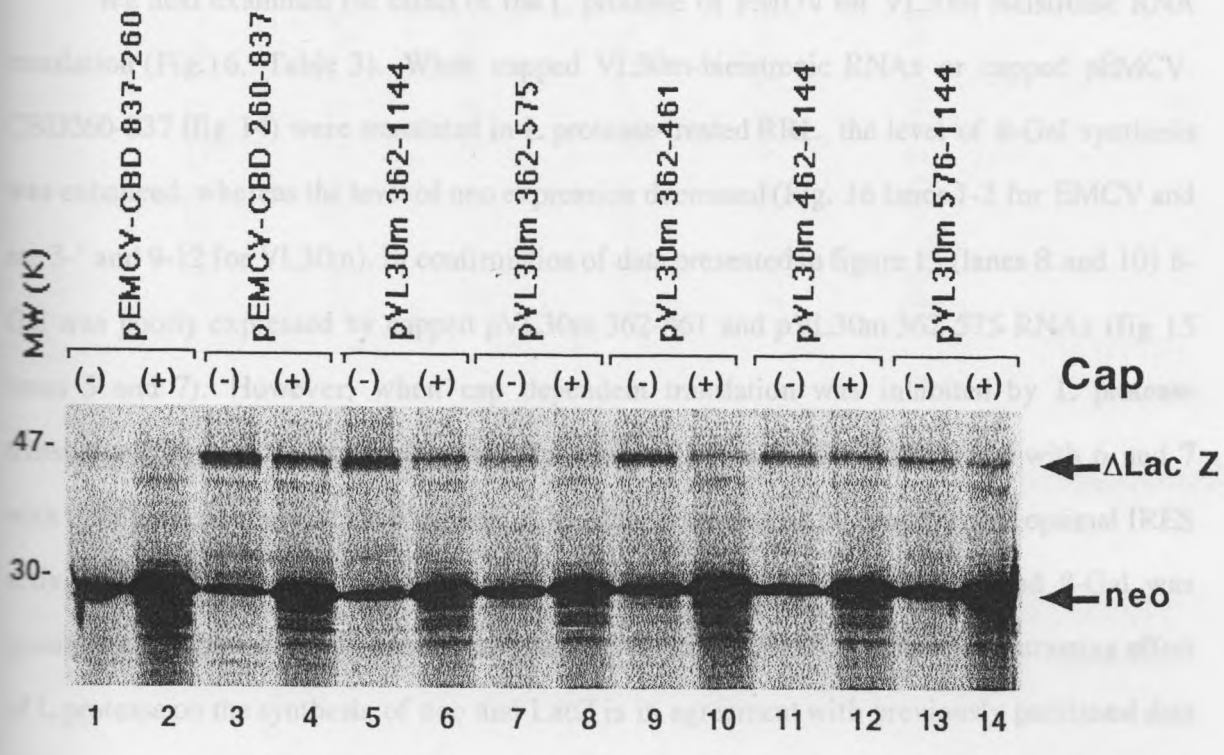


Figure 15. Translation of VL30m bicistronic RNA in messenger-dependent RRL. Translation of uncapped (-) and capped bicistronic (+) RNA in the flexi-RRL (Promega). After heat denaturation, ^{35}S-labelled proteins were analyzed by 15% SDS-PAGE. Positions of neo (28 kDa) and the C-terminally truncated ß-Gal protein (46 kDa) are indicated. Lanes 1-4 correspond to the control RNAs containing the EMCV IRES (see materials and methods). Lanes 7-10 correspond to RNAs containing different 3' deletions in the putative VL30m IRES. Lanes 5-6 and 11-14 correspond to RNAs containing the 5'region VL30m RNA or 5' deletions of this sequence.

V. 1. 5- Influence of FMDV L protease on *in vitro* translation of bicistronic VL30m RNAs.

We next examined the effect of the L protease of FMDV on VL30m bicistronic RNA translation (Fig.16, Table 3). When capped VL30m-bicistronic RNAs or capped pEMCV-CBD260-837 (fig 14) were translated in L protease-treated RRL, the level of ß-Gal synthesis was enhanced, whereas the level of neo expression decreased (Fig. 16 lanes 1-2 for EMCV and see 3-' and 9-12 for VL30m). In confirmation of data presented in figure 15 (lanes 8 and 10) ß-Gal was poorly expressed by capped pVL30m 362-461 and pVL30m 362-575 RNAs (fig 15 lanes 5 and 7). However, when cap dependent translation was inhibited by L protease translation from the 3' cistron was partially restored (figure 16 compare lanes 5 with 6 and 7 with 8). This confirms that the 3' region of VL30m 5' sequences is important for optimal IRES activity (fig 16, lanes 5-8). The effect of L protease on expression of neo and ß-Gal was quantitated by scanning densitometry and data are summarized in Table 3. The contrasting effect of L protease on the synthesis of neo and LacZ is in agreement with previously published data (Ohlmann, et al., 1995, 1996; Ziegler, et al., 1995b) and confirm the presence of a functional IRES within the 5' region of VL30m RNA. Our *in vitro* assays show that the presence of cap is able to shut down IRES activity from RNA pVL30m 362-575 effect that can be abolished by treatment with FMDV L protease (Fig 15, lanes 7 and 8; Fig 16 lanes 5 and 6). It should also be pointed out that in the *ex vivo* assays (Fig 13, Table 2) monocistronic retroviral vector, pVL30m-SJ E1 containing the 5' VL30m region from nt 362-575 was able to promote synthesis of ß-Gal in murine cells.

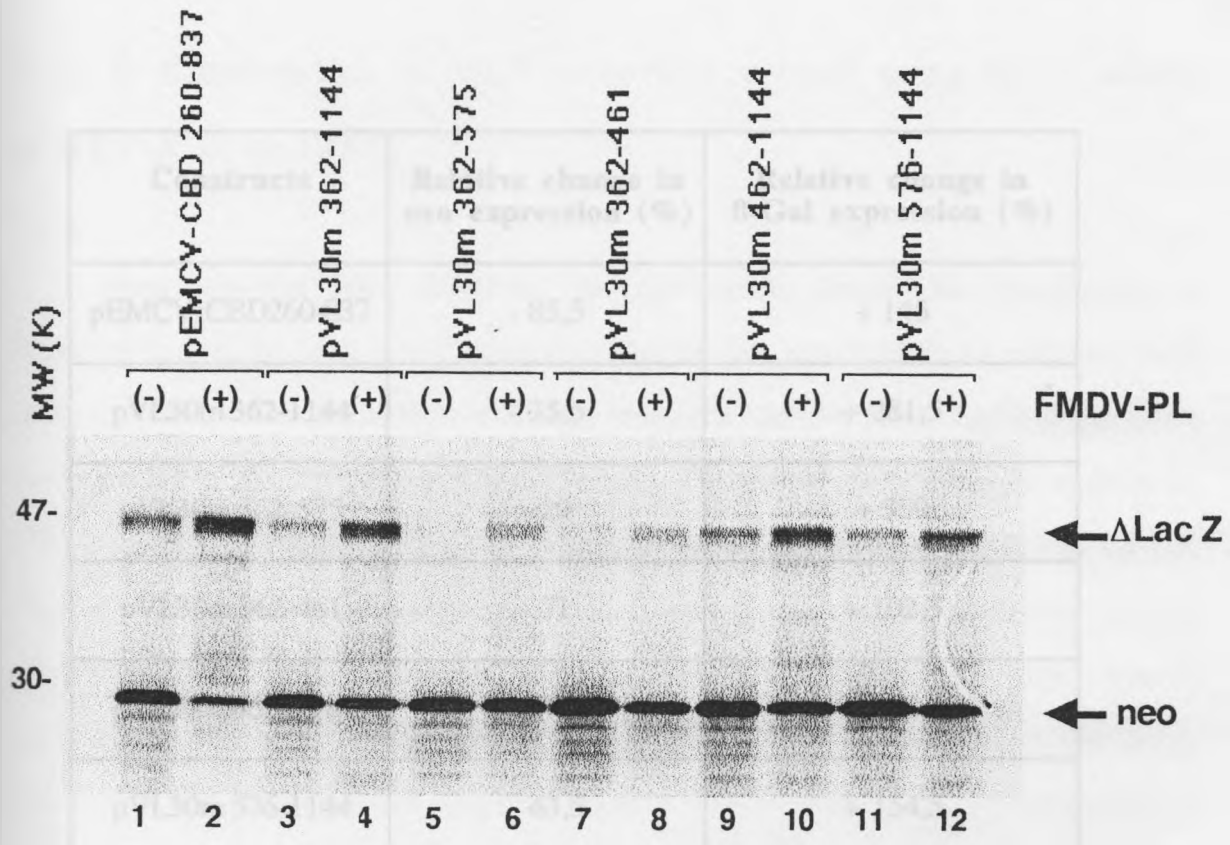


Figure 16. Effect of FMDV L Protease on bicistronic VL30m RNA translation. Translation of bicistronic capped RNA in the Flexi-RRL system (Promega) with (+) or without L (-) protease. After heat denaturation, ^{35}S-labelled proteins were analyzed by 15% SDS-PAGE. Positions of neo (28 kDa) and the C-terminally truncated ß-Gal protein (46 kDa) are indicated. Lanes 1-2 correspond to the control RNAs containing the EMCV IRES (see materials and methods). Lanes 5-8 correspond to RNAs containing different 3' deletions in the putative VL30m IRES. Lanes 3-4 and 9-12 correspond to RNAs containing the 5'region VL30m RNA or 5' deletions of this sequence.

Constructs	Relative change in neo expression (%)	Relative change in ß-Gal expression (%)
pEMCV-CBD260-837	- 85,5	+ 145
pVL30m 362-1144	- 35,5	+ 281,5
pVL30m 362-575	- 29	+ 359
pVL30m 362-461	- 71	+ 102,5
pVL30m 462-1144	- 59,5	+ 302,5
pVL30m 576-1144	- 63,5	+ 154,5

Table 3. Relative change in reporter gene expression in VL30m RNAs caused by FMDV L protease. The gel shown in fig. 16 was scanned and data were used to calculate the effect of L protease in percentage (%) increase or decrease relative to translation in untreated Flexi-RRL [100% - (level of reporter gene in the presence of L protease) x 100%/ (level of reporter gene in the absence of L protease)].

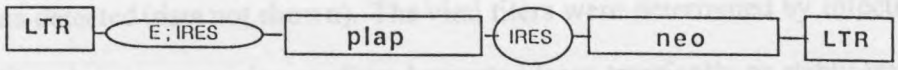
V. 2- Use of the VL30m and Rev-A IRESes in the development of bicistronic retroviral vectors.

V. 2. 1- Construction of MLV retroviral vectors using the 5' leader of REV-A as an IRES.

Many strategies being developed for gene transfer demand the coexpression of heterologous gene products. This fact together with the data presented above prompted us to construct a series of retroviral vectors in order to test the ability of the 5' leader of REV-A to enable cap independent translation initiation in cells. The general vector design is show in fig 17A, the pREV-HW series in fig 17B, and the control vector pEMCV-CBT4 in fig 17C (Torrent *et al.*, 1996). In all vectors, the first cistron encodes the human placental alkaline phosphatase (plap), while the second codes for neomycin phosphotransferase (neo). Vectors pREV-HW1, pREV-HW2, and pREV-HW3 contain the REV-A 5' leader from position 265 to 580 inserted between plap and neo (fig 8B). This 315 nt segment has been previously shown to contain the REV-A RNA packaging signal (E) (Watanabe &Temin, 1982). In vectors pREV-HW4, pREV-HW5 and pREV-HW6 the second cistron is preceded by the 129 nt fragment (position 452 to 580) of the REV-A 5' leader, suggested to contain the minimal IRES sequence (see figs 11 and 12). Vectors pREV-HW2 and pREV-HW5 contain the rat retrotransposon virus-like 30S (VL30) packaging element (E+) and IRES function (position 205-794) (Torrent *et al.*, 1994a, b; Berlioz et al., 1995). In vectors pREV-HW3 and pREV-HW6 the first cistron is preceded by the MoMLV E+ packaging sequence (position 210-1035) (Bender *et al.*, 1987), which also possesses IRES activity (Berlioz and Darlix, 1995; Vagner *et al.*, 1995b; Corbin & Darlix, 1996). In all constructs the LTRs are those of MoMLV.

Figure 17. pREV-HW vector series. **A**) Schematic representation of the bicistronic retroviral vector design. Constructs possess two IRESes of different retroviral origin, and one or two packaging sequences. Placental alkaline phosphatase (plap) and neomycin phosphotransferase (neo) were used as marker genes. **B**) The retroviral vector series pHW, MLV-based vectors with a pBR322 backbone. VL30 E+ corresponds to the 5' untranslated region of HaMSV while MLV E+ corresponds to the enhanced packaging region of MLV. **C**) Control vector pEMCV-CBT4 possesses two IRESes, the first from MLV, which also directs packaging (E+), and the second from EMCV. In all cases numbering is with respect to the genomic RNA cap site (position +1).

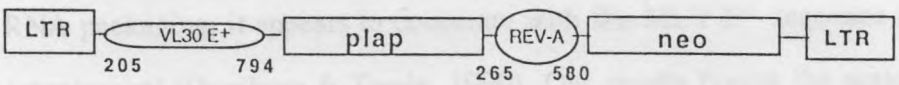
A



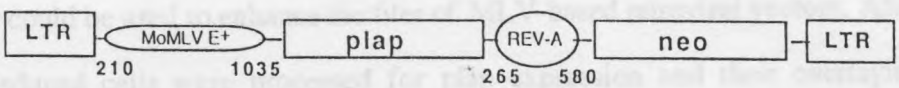
B

pREV-HW1

pREV-HW2

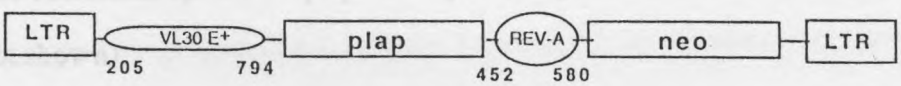


pREV-HW3

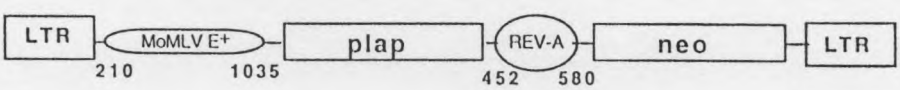


pREV-HW4

pREV-HW5

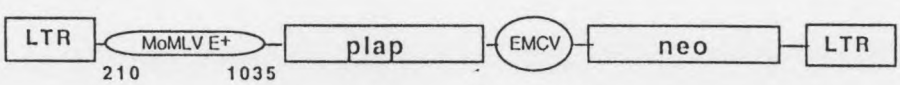


pREV-HW6



C

pEMCV-CBT4



The pREV-HW vectors were used to transfect ecotropic NIH 3T3 derived GP+E-86 cells (Markowitz et al., 1988). To confirm that *in vivo* the second cistron was indeed translated from a bicistronic messenger RNA, and to rule out the possibility of a cryptic promoter and/or cryptic splice sites, cellular RNAs were extracted from GP+E-86 cells 3 days after transfection and subjected to Northern analysis using a ^{32}P-labeled neo probe. Only one major RNA of the expected size was detected (data not shown). The viral titers were determined by infecting NIH 3T3 cells with diluted virus containing medium harvested from transiently or stably-transfected helper cells (after G418 selection) (Table 4). The highest viral titer obtained with the pREV-HW series was in the range of 10^9 TU/ml for pREV-HW3. For both pREV-HW1 and pREV-HW4, no infectious virus was obtained either from transciently or stably transfected helper cells. These results suggest that even though the REV-A E element alone, in a MLV-LTR context, cannot promote recombinant RNA packaging, it appears to cooperate with the MLV E^+ sequence to enhance the recombinant retroviral (Dornburg & Temin, 1990). Our results favour the notion that the REV-A leader could be used to enhance the titer of MLV-based retroviral vectors. After G418 selection, transduced cells were processed for plap expression and their overlaying medium was used to infect new NIH 3T3 cells and assayed for RT activity (Goff *et al.*, 1981). Results showed that more than 90% of the initially transduced cells scored positive for plap staining, yet neither an horizontal spread of plap activity or drug resistance, nor RT activity were detected (data not shown).

Vector	Transient expression (TU/ml)	Stable transformant expression (TU/ml)
pREV-HW2	$0,2 \times 10^4$	$3,2 \times 10^6$
pREV-HW3	$1,6 \times 10^4$	$1,4 \times 10^9$
pREV-HW5	$1,3 \times 10^4$	$6,5 \times 10^5$
pREV-HW6	$2,0 \times 10^4$	$4,5 \times 10^8$
pEMCV-CBT4	$1,1 \times 10^4$	$2,1 \times 10^8$

Table 4: Recombinant MLV/REV-A retroviral titer. Viral titer was determined by the method of serial dilutions followed by plap histochemical staining. NIH 3T3 cells were seeded 24 hr prior to infection with virus containing medium harvested from transiently or stably transformed ecotropic GP+E-86 cells.

V. 2. 2- Effect of Rapamycin on alkaline phosphatase and neomycin phosphotransferase enzymatic activity expressed by the recombinant vectors.

To confirm the above data, the effect of rapamycin on plap and neo expression was examined. Rapamycin has been shown to block phosphorylation of the negative regulator of cap binding protein 4E-BP1, known as PHAS-1. In its dephosphorylated form PHAS-1 acts as a natural repressor of the cap binding protein, eIF4E (Gingras, et al., 1998; Sonenberg & Gingras, 1998; Gingras, et al., 1999; Raught and Gingras, 1999), whose non sequestred levels are probably rate limiting during cap-dependent translation initiation (Rau, et al., 1996). Phosphorylation of PHAS-I, results in the release of eIF4E and increased translational activity (Pause *et al.*, 1994). Beretta et al. (1996) have shown that in NIH 3T3 cells rapamycin blocks PHAS-I phosphorylation, inhibiting cap-dependent, but not cap-independent translation. We used the protocol of Beretta et al. (1996), to determine the effect of rapamycin on the expression of plap and neo in stably transfected GP+E-86 cells (following three months of G418 selection). On the basis of the above data, translation of plap was expected to be cap-dependent in vectors pREV-HW1 and pREV-HW4, while neo translation cap-independent. pREV-HW2, pREV-HW3, pREV-HW5, pREV-HW6 and the control vector pEMCV-CBT4 are double IRES vectors, therefore we predicted translation of both cistrons to be cap-independent. As shown in table 5 the presence of rapamycin reduced cap dependent translation (see pREV-HW1 and pREV-HW4), in agreement whith the data of Beretta et al. (1996). Furthermore, in all cases where translation was expected to be cap-independent rapamycin actually enhanced enzymatic activity (table 5). These results are in agrement witn the *in vitro* data obtained using L protease (fig. 12). To further assess these data the pREV-stable cell line (following three months of G418 selection) were transfected with vector pMLP-P2A which codes for poliovirus p2A protease (Berlioz and Darlix, 1995; Berlioz et al., 1995). It could be seen that the presence of P2A only reduced cap-dependent translation while IRES driven protein expression was not affected or enhanced (data not shown) (Ziegler, et al., 1995a). These *in vivo* results validate those of Beretta et al. (1996) and illustrate a simple method of assaying cap-independent

translation *in vivo*.

These data further confirm the presence of an IRES within the 5'leader of REV-A and emphasize its potential in the construction of polycistronic retroviral vectors for efficient coexpression of genes of interest. In addition these *in vivo* data also discount a translation reinitiation within vectors pREV-HW4, pREV-HW5 and pREV-HW6 containing the minimal REV-A IRES since a decrease in the expression of both plap and neo, should have been observed in the presence of rapamycin (table 3 and 4). Finally, and in agreement with the *in vitro* data (fig. 11 and 12, table 1), these data strongly suggest that the 5' and 3' borders of the REV-A IRES are around positions 452 and 580 and that the IRES activity is independent from the REV-A packaging signal (E).

Table 5. Effect of rapamycin on translation. The ecotropic retroviral packaging cell line GP+E-86 were transfected with recombinant vector DNA. After three months of G418 selection rapamycin was added to a final concentration of 20 ng/ml. Total proteins were extracted 20 h later from both treated and non-treated cells and enzymatic activities were determined as described (see material and methods). The presented data correspond to the average of three independent experiments. The mean values of alkaline phosphatase and neomycin phosphotransferase specific activities as well as the standard deviation for each set of experiments are shown. ND stands for non-detectable. The level of reporter gene expression, measured as enzymatic activity, in the presence and absence of rapamycin were used to calculate the effect of the drug as a percentage increase or decrease relative to untreated cells [100% - (level of reporter gene in the presence of drug) x 100%/ (level of reporter gene in the absence of drug)].

Cell Line	Alkaline phosphatase			Neomycin phosphotransferase		
	Activity (U/μg of total protein)		relative variation	Activity (cpm/min/μg of total protein) ($x10^4$)		relative variation
	Rapamycin			Rapamycin		
	(-)	(+)	(%)	(-)	(+)	(%)
GP+E-86	ND	ND	____	$1,2 \times 10^{-6}$	$5,5 \times 10^{-6}$	____
pREV-HW1	$9,7 \pm 0,7 \times 10^{-5}$	$3,5 \pm 0,7 \times 10^{-5}$	-63,9	$2,5 \pm 0,7$	$3,4 \pm 0,7$	+36,0
pREV-HW2	$1,8 \pm 0,8 \times 10^{-4}$	$2,2 \pm 0,2 \times 10^{-4}$	+22,2	$1,6 \pm 0,7$	$2,2 \pm 0,9$	+37,5
pREV-HW3	$1,6 \pm 0,7 \times 10^{-4}$	$2,8 \pm 0,3 \times 10^{-4}$	+75,0	$3,2 \pm 0,7$	$5,5 \pm 0,5$	+71,8
pREV-HW4	$2,3 \pm 0,8 \times 10^{-6}$	$7,0 \pm 0,6 \times 10^{-7}$	-69,6	$1,6 \pm 0,5$	$1,7 \pm 0,2$	+6,25
pREV-HW5	$6,7 \pm 0,7 \times 10^{-6}$	$1,0 \pm 0,4 \times 10^{-5}$	+49,2	$1,0 \pm 0,4$	$1,5 \pm 0,9$	+50,0
pREV-HW6	$2,5 \pm 0,5 \times 10^{-5}$	$3,8 \pm 0,5 \times 10^{-5}$	+52,0	$1,4 \pm 0,5$	$1,8 \pm 0,3$	+28,0
pEMCV-CBT4	$2,7 \pm 1,0 \times 10^{-4}$	$3,3 \pm 0,5 \times 10^{-4}$	+22,2	$1,7 \pm 0,6$	$2,2 \pm 0,5$	+29,0

V. 2. 3 - Construction of MLV-based bicistronic retroviral vectors using the 5' region of VL30m.

Retroviral vectors incorporating mouse VL30 sequences have been proposed to have potential use in gene therapy. In order to evaluate the use these VL30m 5' region (E/IRES) in gene transfer and to test thir function in cells we constructed bicistronic retroviral vectors.

The pVL30m-SU vectors as well as control vectors are shown in Figure 18. In all constructs, the first cistron encodes human placental alkaline phosphatase (plap) while the second codes for neomycin phosphotransferase (neo). In vectors pVL30m-SU8 and pVL30m-SU9 the 5' MoMLV E sequence has been deleted and the putative VL30m E/IRES alone (position 362-575 and 362-1149 respectively) was expected to promote both packaging of the recombinant RNA and cap-independent translation of the second cistron in a position-independent manner. In vector pVL30m-SU11 the first cistron is preceded by the MoMLV E+ packaging sequence (position 210-1035), previously shown to contain an IRES, while the second cistron is preceded by the putative VL30m IRES (position 362-1149). In the construct pVL30m-SU 12 the first cistron is preceded by the putative VL30m E/IRES (position 362-1149) and the second cistron by the previously described rat VL30 E/IRES (position 205-794) (Torrent *et al.*, 1994 a, b; Berlioz *et al.*, 1995).

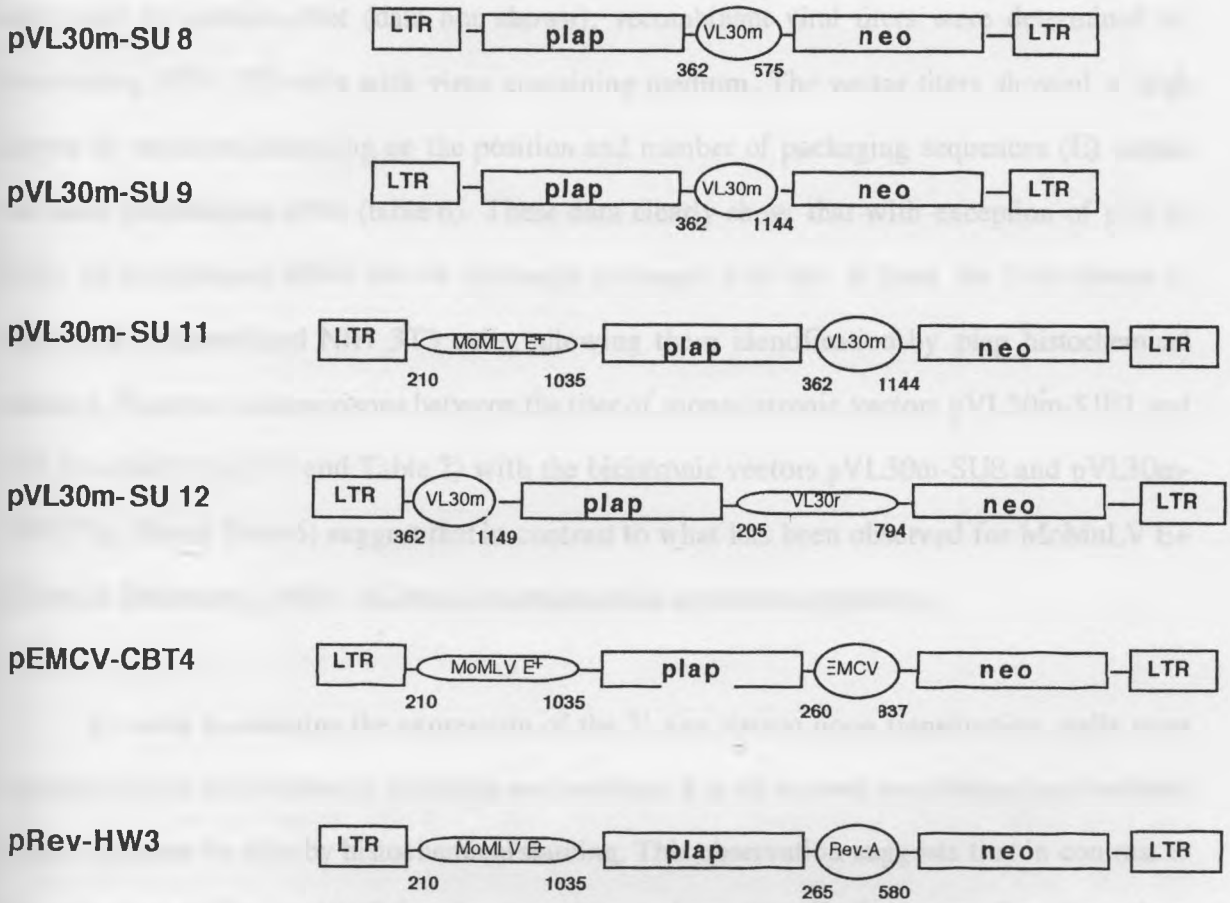


Figure 18. Schematic representation of the VL30m/MLV bicistronic retroviral vectors. The VL30m/MLV-based retroviral vectors are built on a pBR322 backbone. VL30m corresponds to the 5' RNA region of mouse VL30 retrotransposon, VL30r corresponds to the 5' untranslated region of HaMSV, while MLV E+ corresponds to the extended packaging region of MLV. Placental alkaline phosphatase (plap), and neomycin phosphotransferase (neo) were used as marker genes. The control vectors pEMCV-CBT4 and pRev-HW3 possess two IRESes, the first from MLV, which also directs packaging (E+), and the second from EMCV or Rev-A, respectively. In all cases numbering is with respect to the genomic RNA cap site (position +1).

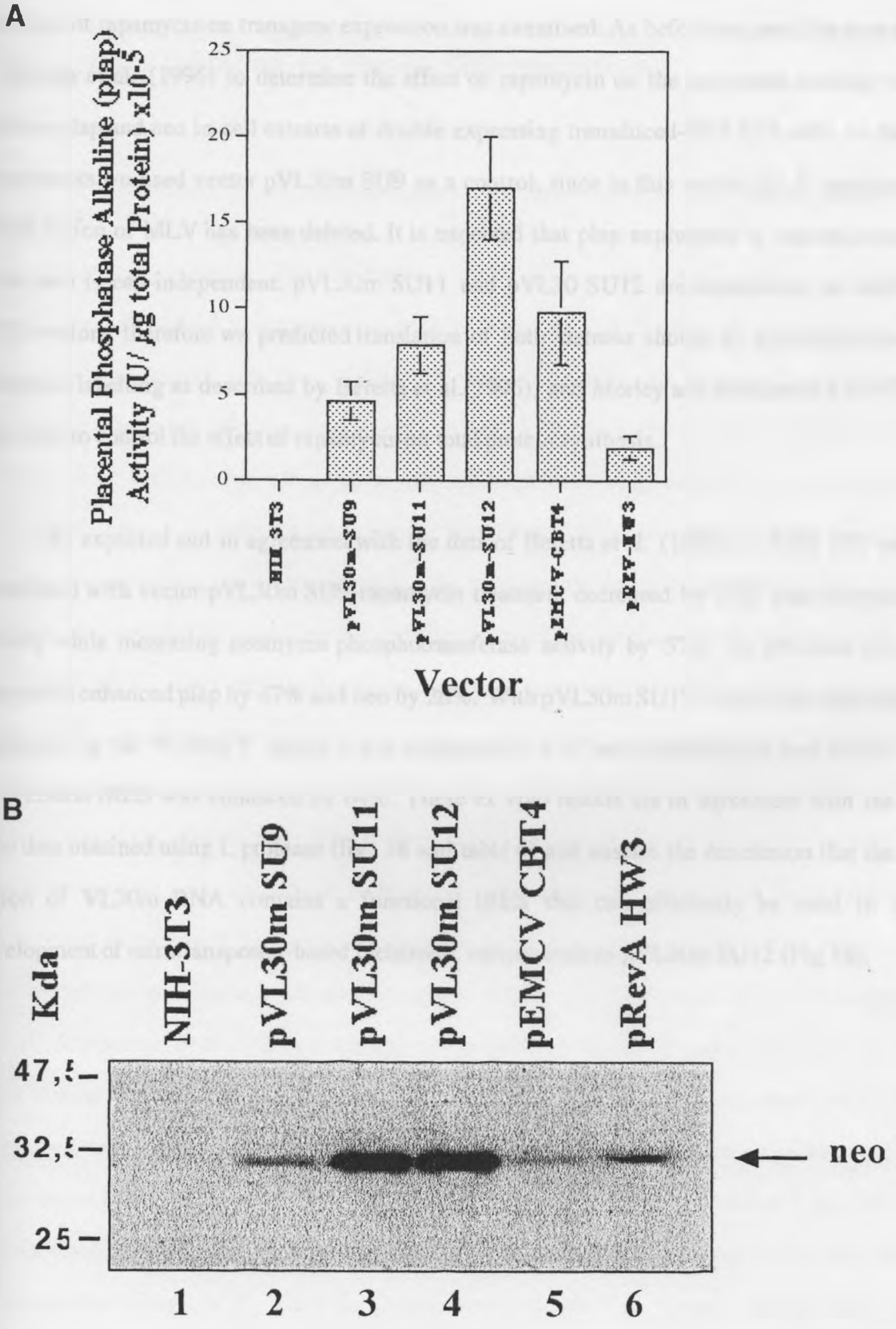
Vectors were used to transfect ecotropic helper cells, GP+E-86, and G418 resistant clones were selected. All transfected GP+E-86 cells were found to stably express both genes (plap and neo), for at least two months. Once the integrity of the polycistronic RNA was confirmed by northern blot (data not shown), recombinant viral titers were determined by transducing NIH 3T3 cells with virus containing medium. The vector titers showed a high degree of variation depending on the position and number of packaging sequences (E) within the same recombinant RNA (table 6). These data clearly show that with exception of pVL30 SU8, all recombinant RNA can be eficiently packaged and that at least the first cistron is expressed in transduced NIH 3T3 cells, allowing there identification by plap histochemical staining. However, comparisons between the titer of monocistronic vectors pVL30m-SJE1 and pVL30m-SJE2 (Fig. 13 and Table 2) with the bicistronic vectors pVL30m-SU8 and pVL30m-SU9 (Fig 18 and Table 6) suggest that in contrast to what has been observed for MoMuLV E+ (Mann & Baltimore, 1985), VL30m E seems to act in a position dependent.

In order to examine the expression of the 3' neo cistron upon transduction, cells were selected (G418) as indicated in materials and methods. For all vectors, we obtained neo resistant clones positive for plap by histochemical staining. This observation suggests that in contrast to the packaging ability, the IRES function is position independent. To further confirm these data, expression of both proteins was examined in cellular extracts from transduced cells. The level of plap expression was determined by an biochemical assay while the level of neo expression was determined by western blotting (Fig 19). In agreement with histochemical staining and drug resistance, both proteins were detected. Interestigly and despite the fact that 100% of the cells were plap positive and G418 resistant, expression of each cistron varied depending on the vector. These type of variation in gene translation may be due to the general vector context and/or competition for the recruitment of factors necessary for translation. These data suggest that both packaging and IRES functions of 5'VL30 region can be efficiently used in the development of retroviral vectors. However, the efficiency of packaging and protein expression depends on the position of the E sequence and on the combination of IRESes used in the vector construct.

Vector	Retroviral Titer (TU/ml)
pVL30m-SU 8	2 (*)
pVL30m-SU 9	$1,4x10^3$
pVL30m-SU 11	$2,0x10^7$
pVL30m-SU 12	$1,0x10^5$
pEMCV-CBT4	$3,3x10^8$

Table 6: Recombinant MLV/VL30m bicistronic retroviral titer. Viral titer was determined by the method of serial dilutions followed by plap histochemical staining. NIH 3T3 cells were seeded 24 hr prior to transduction with virus containing medium harvested from stably transformed ecotropic GP+E-86 cells. (*) 1 ml of viral containing medium was directly used to transduce $8x10^5$ NIH 3T3 cells, plap (+) colonies were counted after 15 days of G418 selection. The viral titer is reported in plap positive (+) transducing units per milliliter (TU/ml) of collected medium and corresponds to the mean of triplicate experiments. S.D. did not exceed 15% of mean values.

Figure 19. Monitoring double transgene expression. Proteins extracted from transduced plap positive neo resistant NIH-3T3 cells were used to determine the level of expression of each transgene by vector constructs. **A**) Enzymatic activities of plap was determined as described in materials and methods. The mean values of alkaline phosphatase specific activities as well as the standard deviation for each set of experiments are shown. Data correspond to the average of three independent experiments. **B**) 10μg of total proteins were loaded per lane and subjected to 15% ploy acrylamide/SDS gel electrophoresis. Proteins were transferred to PVDF membrane and probed with a rabbit anti-neomycin phosphotransferase II antibody. The membrane was then incubated with a biotinilated anti-rabbit IgG-antibody and an avidin-peroxidase solution, and finally developed by ECL. Lane 1 the negative control, corresponds to protein extract from non-transduced NIH-3T3 cells. Lanes 2 through 6 represent protein extracts from cells transduced with the different retroviral vectors (rv).


A
Placental Phosphatase Alkaline (plap) Activity (U/ µg total Protein) x10-5
25
20
15
10
5
0
NIH 3T3
pVL30m-SU9
pVL30m-SU11
pVL30m-SU12
pEMCV-CBT4
pREV-HW3
Vector
B
Kda
NIH-3T3
pVL30m SU9
pVL30m SU11
pVL30m SU12
pEMCV CBT4
pRevA HW3
47,5
32,5
25
neo
1
2
3
4
5
6

In order to further confirm that the 5'region of VL30m RNA containes a funtional IRES the effect of rapamycin on transgene expression was examined. As before we used the protocol of Beretta et al. (1996) to determine the effect of rapamycin on the enzymatic activity both proteins plap and neo in cell extracts of double expressing transduced-NIH 3T3 cells. In these experiments we used vector pVL30m SU9 as a control, since in this vector the 5' packaging /IRES region of MLV has been deleted. It is expected that plap expression is cap-dependent, while neo is cap-independent. pVL30m SU11 and pVL30 SU12 are expected to be double IRES vectors, therefore we predicted translation of both cistrons should be cap-independent. Metabolic labelling as described by Beretta et al.(1996), and Morley and McKendrick (1997), was used to control the effect of rapamycin on total protein synthesis.

As expected and in agreement with the data of Beretta et al. (1996), in NIH 3T3 cells transduced with vector pVL30m SU9 rapamycin treatment decreased by 13% plap enzymatic activity while increasing neomycin phosphotransferase activity by 57%. In pVL30m SU11 rapamycin enhanced plap by 47% and neo by 28%. With pVL30m SU12 where plap expression is directed by the VL30m 5' region it was enhanced by 16% and expression of neo driven by the VL30rat IRES was enhanced by 64%. These *ex vivo* results are in agreement with the *in vitro* data obtained using L protease (fig. 16 and table 4) and sustain the conclusion that the 5' region of VL30m RNA contains a functional IRES that can efficiently be used in the development of retrotransposon-based bicistronic vectors such as pVL30m SU12 (Fig 18).

V. 3- Translation initiation driven by IRESes from retroviral origin is not inhibited by neural differentiation.

V. 3. 1- Production of recombinant retroviral vectors for transduction of human cell lines.

Retroviral vectors pEMCV-CBT4, pREV-HW1 and pREV-HW3, containing combinations of MoMuLV and EMCV or MoMuLV and REV-A IRESes, were transfected into the different packaging cell lines (Table7). Transfected helper cell lines were submitted to selection pressure for 15 days using G418. Gene double-expression was verified by plap histochemical staining for all vectors used. Clones surviving selection were mixed in subsequent cultures to generate homogenous populations. Vectors were harvested at various passages immediately prior to transduction. We used several different helper cell lines, which produce recombinant viruses posessing different envelope proteins, including amphotropic MLV (AM-MLV), 10A1 MLV, gibbon ape leukemia virus (GALV) and the feline endogenous virus RD114, to allow us to compare and hence optimize the capacity of vectors to transduce human multipotent neural precursors. PG-13 derived from NIH-3T3 cell line, produces viral particles containing gibbon ape leukemia virus (GALV) envelope. Due to its origin this cell line was expected to be comparable to GPE-86 in terms of the long term stability of the recombinant MLV-based vectors used. However, because the packaging cell line is of murine origin, the viral vectors it generates are inactivated by human complement (Takeuchi *et al.*, 1994; Cosset *et al.*, 1995; Pensiero *et al.*, 1996; Takeuchi *et al.*, 1996). This renders them inappropriate for most clinical in vivo gene transfer applications. Therefore, we also tested and compared several packaging cell lines designed to produce human complement-resistant recombinant vectors. These novel helper cell lines were derived from the human rhabdomyosarcoma cell line TE-671 or from the human fibrosarcoma cell line HT-1080 and produce recombinant vectors that resist human serum (Cosset *et al.*, 1995; S. Chapel-Fernanadez & F-L. Cosset; unpublished). The recombinant viruses produced by these helper cells have been shown to resist inactivation by human serum. In all assays the vector pREV-HW1, which has been shown not to be packaged

in MLV-based packaging systems, was used as a negative control. As an additional negative control pEMCV-CBT4 and pREV-HW3 vectors, that were produced by the ecotropic GPE-86 packaging cell line were used.

Helper cell line	Viral Envelope	Recognized receptor	References
PG-13	GALV	Glvr-1 (Pit-1)	ATCC, CRL-10686 Miller, 1996.
TE-FLY-Galv	GALV	Glvr-1 (Pit-1)	S. Chapel-Fernandes & F-L. Cosset (Unpublished). (Miller, 1996).
TE-FLY-A	AM-MLV	Ram-1 (Pit-2)	S. Chapel-Fernandes & F-L. Cosset (Unpublished). Miller, 1996.
FLY-A	AM-MLV	Ram-1 (Pit-2)	Cosset et al., 1995. Miller, 1996.
FLY-RD	RD-114	Not Identified	Cosset et al., 1995. (Sommerfelt & Weiss, 1990). (Sommerfelt *et al.*, 1990).
TE-FLY-10A1	10AI-MLV	Glvr-1 and Ram-1	S. Chapel-Fernandes & F-L. Cosset (Unpublished). (Miller and Chen, 1996)

Table 7. Envelope characteristics of helper cell lines. The PG-13 (ATCC) was derived from NIH-3T3 cells. Helper cell lines from the TE-FLY series were derived from the human rhabdomyosarcoma cell line TE-671 (ATCC), whereas those from the FLY series were derived from the human fibrosarcoma cell line HT-1080 (ATCC).

V. 3. 2- Transduction of Human Neuroectodermal Precursor-like Cells

Recombinant AM-MLV, 10A1 MLV, RD114 and GALV pseudotyped virus produced in the presence of serum and titered on TE-671 cells gave results in the same order of magnitude as previously described (Cosset, et al., 1995). However in the present series of experiments infections were carried out in the absence of serum and titers were lower (10^4 - 10^5 transducing units/ml). As expected pseudotyped pREV-HW1 vectors and ecotropic pEMCV-CBT4 and pREV-HW3 were not able to transduce TE-671 cells (no plap signal could be detected).

Once titered on TE-671 cells, and normalized, we evaluated the potential of these pseudotyped viruses to achieve significant levels of gene transfer to human multipotent neural precursor-like cells. Previous studies suggest that the unique features of the medulloblastoma cell line Dev make it a particularly interesting model system in which to evaluate strategies for gene transfer therapy in human brain (Giraudon *et al.*, 1993; Dufay *et al.*, 1994; Derrington *et al.*, 1998). Medulloblastoma occurs when pluripotent neuroectodermal precursor cells or their immature progeny escape constraints on their proliferation, or programmed cell death, and proliferate anarchicly to generate primitive neuroectodermal tumours (Rorke *et al.*, 1997). Dev cells demonstrate a phenotype that is remarkably similar to that of normal multipotent precursors. Thus, like the normal cells from which they are thought to have derived, Dev cells are undifferentiated in defined medium and express the markers nestin and MAP5, specific to undifferentiated neuroectodermal precursors in the brain (Lendahl *et al.*, 1990; Derrington et al., 1998). Similarly, in a manner highly reminiscent of normal multipotent neural precursors, Dev cells respond to FGF2 by proliferation and will subsequently differentiate by expressing neuronal and glial phenotypes on adherent substrates or in response to FCS (Derrington *et al.*, 1998).

Multipotent precursors from the CNS require trophic support from FGF2 to induce their proliferation and to allow them to express the full complement of neural phenotypes (Kilpatrick

& Bartlett, 1993; Vescovi *et al.*, 1993; Gritti *et al.*, 1996; Johe *et al.*, 1996). We therefore evaluated and compared the transduction efficiency of the different pseudotyped viruses in Dev cells grown in the minimum medium required for survival and in the same medium supplemented with FGF2. Our results show large (fig 20) variations in the ability of recombinant viruses to transduce the cells of this human undifferentiated multipotent neural precursor cell line that presumably reflect specific differences in the ability of the different envelopes tested to bind to membrane receptors. Vectors produced by TE-FLY-10A1 helper cells proved to produce the highest transduction efficiency as measured by the percentage of Dev cells transduced (40-50 % of plap positive cells) in presence of FGF2 (fig. 20). Relatively high levels of transduction were also obtained when vectors were produced by FLY-RD114 (25-30 %) (fig. 20).

The absence of helper virus was verified for all helper cell lines tested. Neither horizontal spread of plap activity and G418 resistance, nor RT activity were detected in supernatants of transduced Dev cells. Verification was carried out monthly after cell transduction. As expected, the pREV-HW1 vector showed no capacity to transduce plap activity or G418 resistance to Dev cells. The same observations were made when the ecotropic version of the vectors were used to transduce Dev cells.

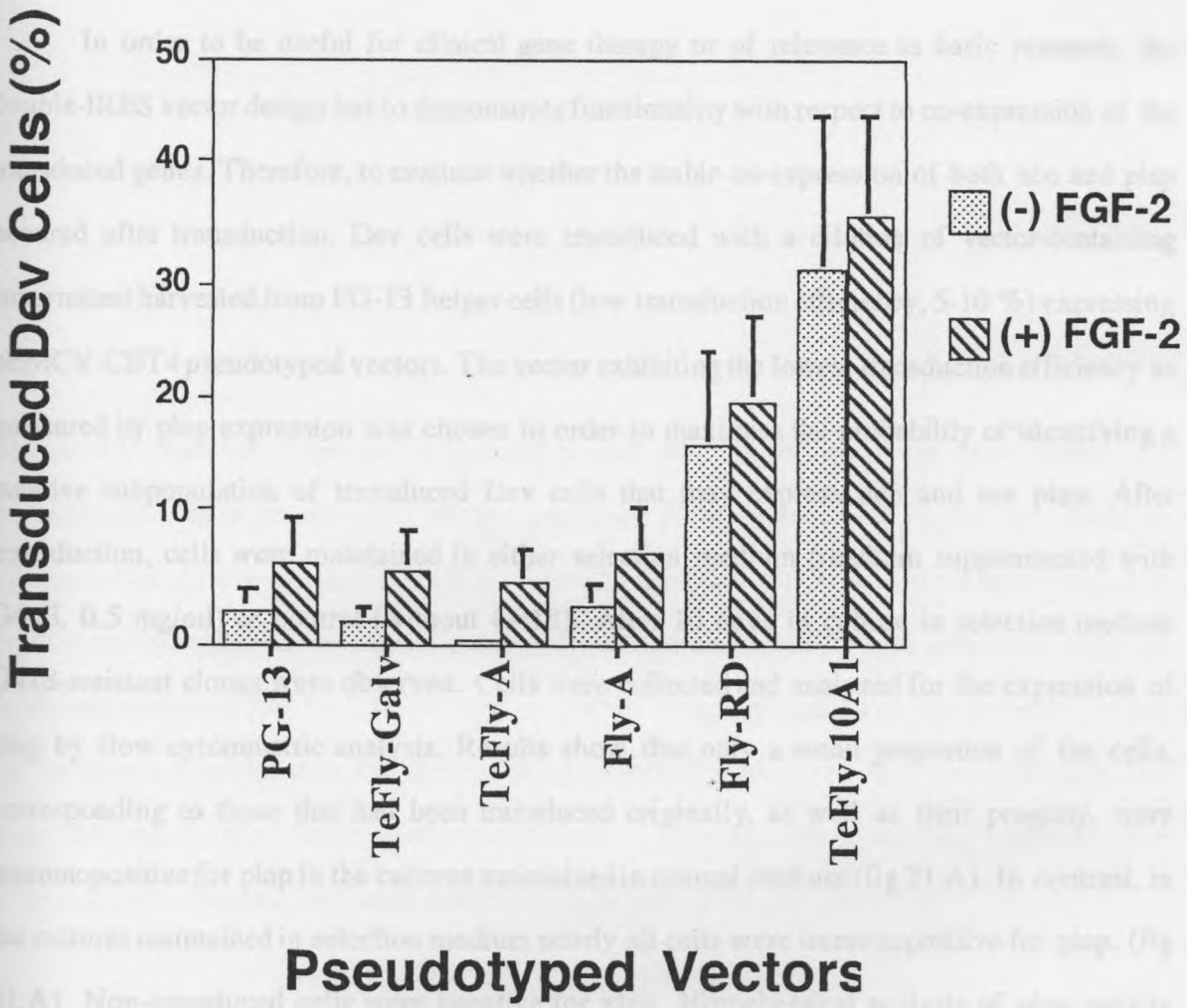


Figure 20. Transduction of Dev Cells. Vector pEMCV-CBT4 was transfected into a series of helper cell lines. Recombinant AM-MLV, 10A1 MLV, RD114 and GALV pseudotypes virus, produced in the absence of serum, were used to transduce Dev cells in presence or absence of FGF-2. The percentage of transduction was determined by FACS analysis and expressed in respect to total number of cells analyzed. The presented data correspond to mean values of four independent assays. Error bars are standard deviations.

V. 3. 3- Co-expression of two gene products

In order to be useful for clinical gene therapy or of relevance in basic research, the double-IRES vector design has to demonstrate functionality with respect to co-expression of the transduced genes. Therefore, to evaluate whether the stable co-expression of both neo and plap occured after transduction, Dev cells were transduced with a dilution of vector-containing supernatant harvested from PG-13 helper cells (low transduction efficiency, 5-10 %) expressing pEMCV-CBT4 pseudotyped vectors. The vector exhibiting the lowest transduction efficiency as measured by plap expression was chosen in order to maximise the probability of identifying a putative subpopulation of transduced Dev cells that may express neo and not plap. After transduction, cells were maintained in either selection medium (medium supplemented with G418, 0.5 mg/ml) or control (without G418). After 10 days in culture in selection medium G418-resistant clones were observed. Cells were collected and analyzed for the expression of plap by flow cytommetric analysis. Results show that only a small proportion of the cells, corresponding to those that had been transduced originally, as well as their progeny, were immunopositive for plap in the cultures maintained in normal medium (fig 21.A). In contrast, in the cultures maintained in selection medium nearly all cells were immunopositive for plap. (fig 21.A). Non-transduced cells were negative for plap. Histochemical analysis of plap activity staining confirmed the flow cytommetric data by showing that all cells that had survived selection, (i.e. neo-positive), also expressed plap activity (fig 21.B). Similar results were obtained with cells transduced by each of the vectors (not shown). These results confirm the coexpression of both gene products in transduced cells. Co-expression over periods as long as six months could be demonstrated in cultures maintained in the presence of serum and G418.

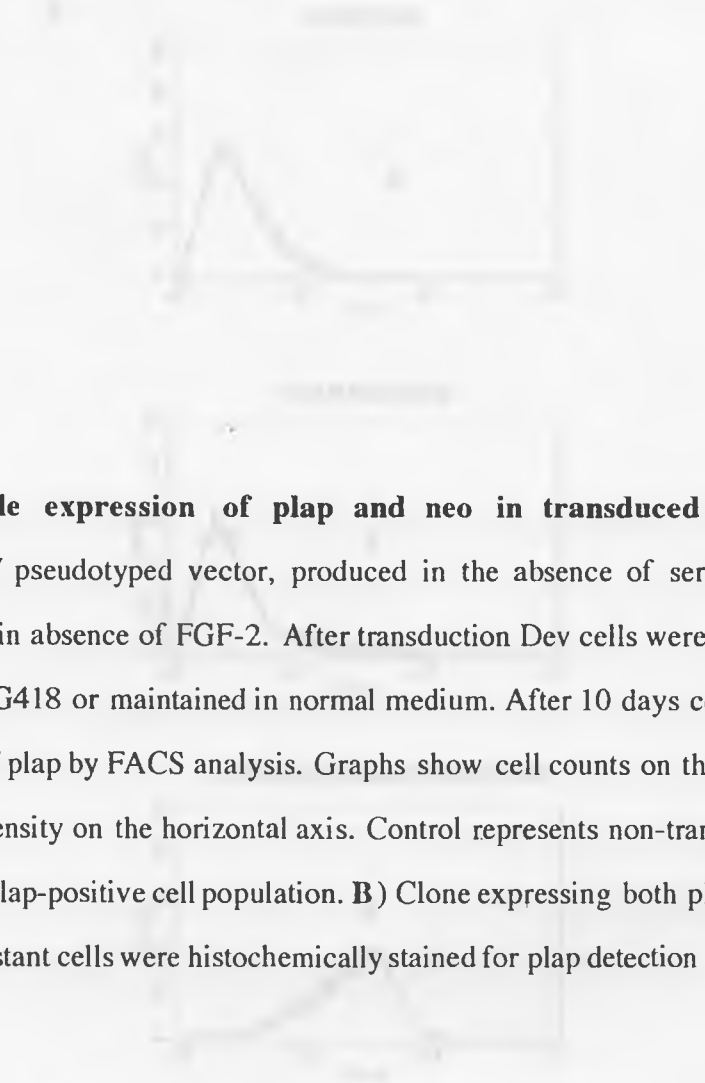
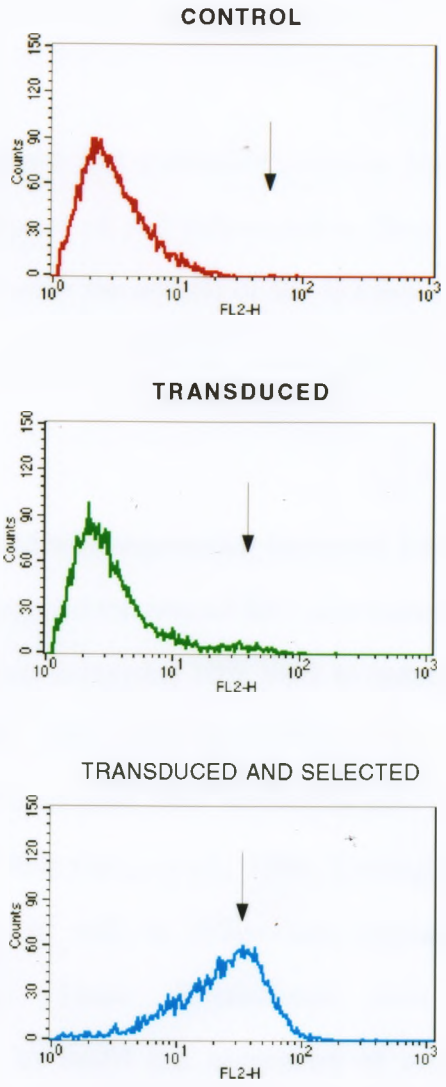
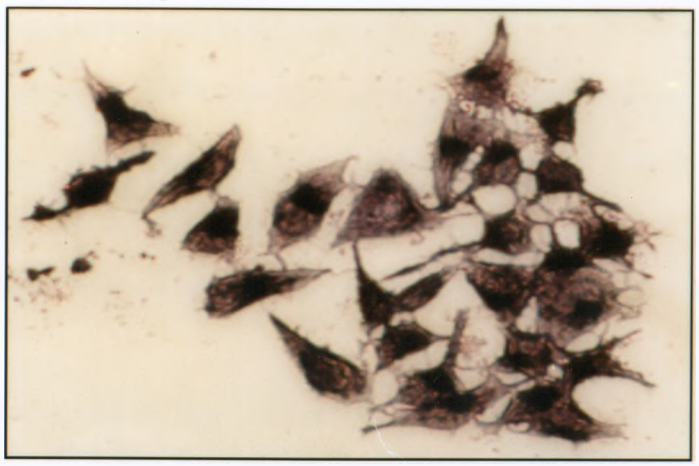


Figure 21. Double expression of plap and neo in transduced Dev cells. A) Recombinant GALV pseudotyped vector, produced in the absence of serum, was used to transduce Dev cells in absence of FGF-2. After transduction Dev cells were either selected in medium containing G418 or maintained in normal medium. After 10 days cells were analyzed for the expression of plap by FACS analysis. Graphs show cell counts on the vertical axis and log fluorescence intensity on the horizontal axis. Control represents non-transduced cells. The arrow indicates the plap-positive cell population. **B)** Clone expressing both plap and neo. After G418 selection, resistant cells were histochemically stained for plap detection

A


CONTROL
Counts
FL2-H
TRANSDUCED
Counts
FL2-H
TRANSDUCED AND SELECTED
Counts
FL2-H

B



V. 3. 4- Neural differentiation does not modulate translation of transduced proteins

Recent studies have shown that translation initiation driven from certain IRESes is a regulated process that may depend on cell differentiation (Bernstein *et al.*, 1997; Ye *et al.*, 1997). Therefore we asked whether the activity of the IRESes of viral origin used to initiate translation in the bicistronic vectors might be regulated by the state of differentiation of transduced Dev cells.

Dev cells were transduced with supernatant harvested from TE-FLY-10A1 helper cells. Following transduction, non-selected transduced Dev cells were transfered to labtek wells and grown in differentiation medium containing 10% FCS to induce differentiation in either the presence or absence of G418. Dev cells are endogenously undifferentiated and require exogenous stimulation for differentiation. This stimulation can be provided by specific stimuli or by FCS (Giraudon *et al.*, 1993; Dufay *et al.*, 1994; Derrington *et al.*, 1998). Addition of FCS induces distinct clusters of cells to differentiate expressing neuronal, astrocytic or oligodendrocytic phenotypes. These differentiated cells can be evidenced by immunocytochemical analysis to reveal the expression of markers specific to terminally-differentiated neurons (e.g. neurofilament proteins; NF), astrocytes (glial fibrillary acidic protein; GFAP) and oligodendrocytes (galactocerebroside; Gal-C). Double-labelling enabled us to test whether the expression of the transgene plap was co-expressed by these differentiated cells (Fig 22 and 23A). Clusters of plap-positive cells were scattered among unstained cells and it was evident that neural differentiation did not switch off MoMuLV IRES because co-expression of plap and the various neuronal and glial anigens was observed (fig 23A).

By growing the cells in differentiation medium supplemented with G418 we were able to simultaneously evaluate the effect of differentiation on translation of proteins driven by the IRESes of REV-A and EMCV. Results show that after selection in G418, all cells were

apparently plap-positive, confirming that the subpopulation of transduced cells and their progeny had been selected, and that co-expression of plap and neo occured in transduced cells as suggested by the results shown above. Furthermore after selection in G418, clusters of differentiated cells expressing neural cell type-specific markers were still observed, demonstrating that neural differentiation did not switch off the IRES driving neo selection. Results obtained with the vector pREV-HW3 are shown (Fig 23B), similar results were obtained with pEMCV-CBT4. Together, these results indicate co-expression of both transgenes in the differentiated subpopulations of cells, and therefore that the MoMuLV, EMCV and REV-A IRESes, utilized in the construction of the bicistronic vectors, are not switched off by neural differentiation in human cells.

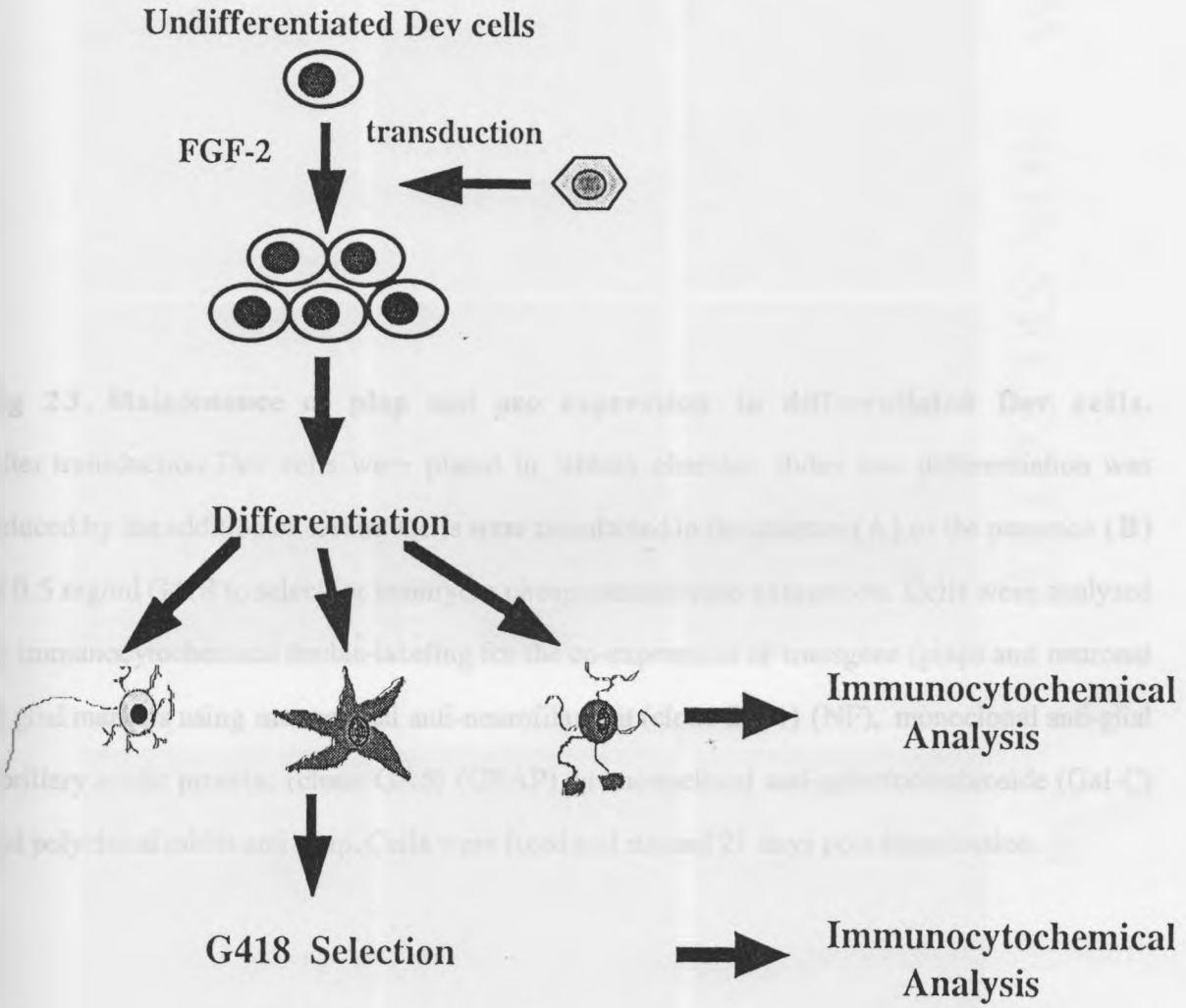
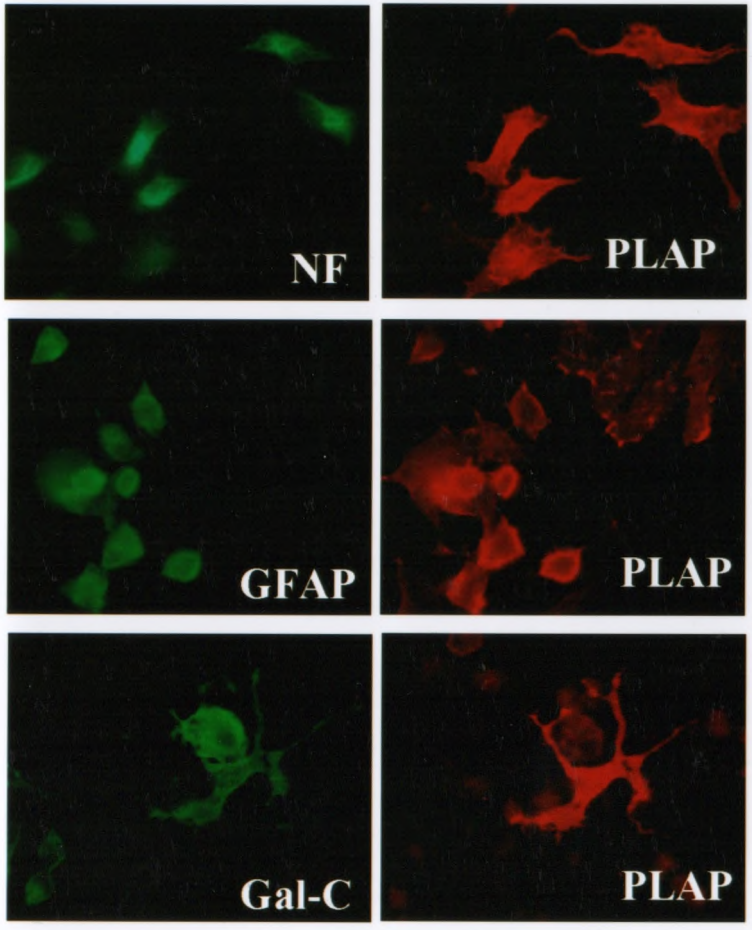


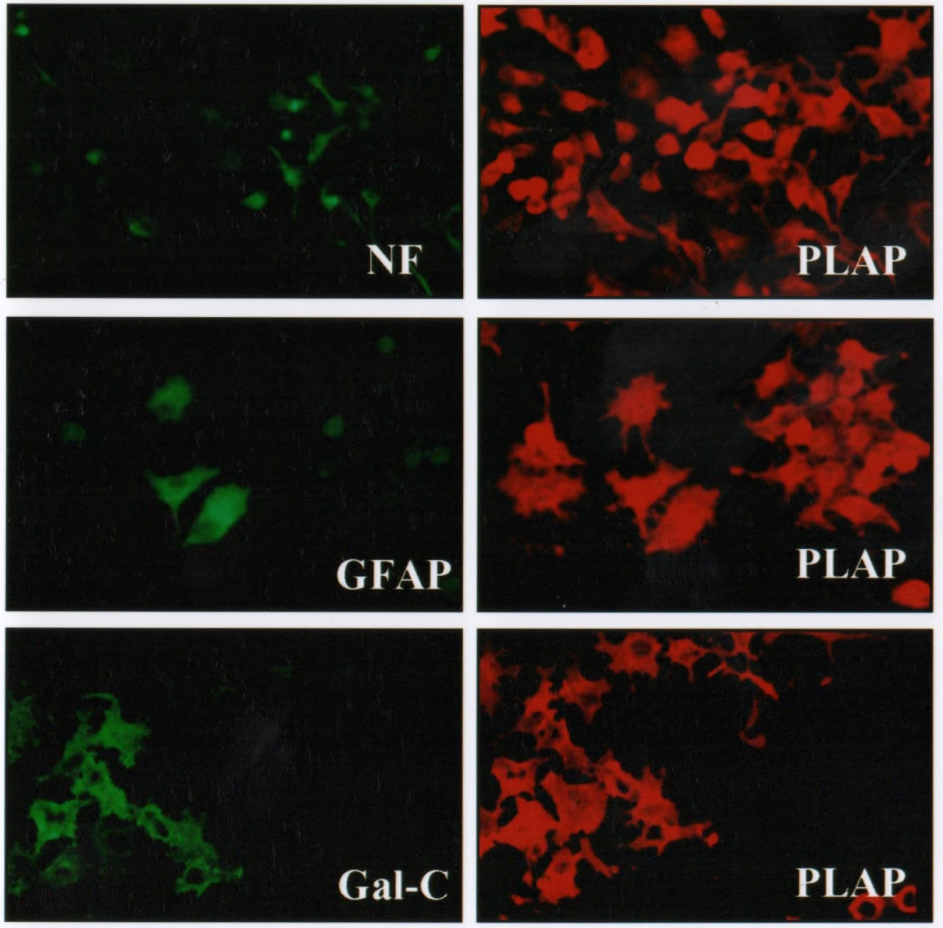
Fig 22. Maintainance of plap expression in differenciated Dev cells a schematic representation of the experimental design. Recombinant 10A1 MLV pseudotyped vector, produced in the absence of serum, was used to transduce Dev cells in the presence of FGF-2. After transduction differentiation was induced by the addition of serum and cells were analized by immunocytochemical double-labeling for the co-expression of the transgene and neuronal or glial markers

Fig 23. Maintenance of plap and neo expression in differentiated Dev cells. After transduction Dev cells were plated in labteck chamber slides and differentiation was induced by the addition of serum. Cells were maintained in the absence (**A**) or the presence (**B**) of 0.5 mg/ml G418 to select for neomycin phosphotransferase expression. Cells were analyzed by immunocytochemical double-labeling for the co-expression of transgene (plap) and neuronal or glial markers using monoclonal anti-neurofilament (clone 2F11) (NF), monoclonal anti-glial fibrillary acidic protein, (clone GA5) (GFAP) or monoclonal anti-galactocerebroside (Gal-C) and polyclonal rabbit anti-plap. Cells were fixed and stained 21 days post transduction.

23A NON-SELECTED



23B SELECTED



V. 4- Evidences indicating that MoMuLV IRES is used for the synthesis of GAG polyprotein during virus replication.

V. 4. 1- Effect of rapamycin on the cell cycle and protein synthesis in MLV-infected cells.

The effect of rapamycin (Rap) on cell cycle is well documented (Heitman *et al.*, 1991; Hultsch *et al.*, 1992; Albers *et al.*, 1993; Morice *et al.*, 1993; Terada *et al.*, 1993; Javier *et al.*, 1997; Metcalfe *et al.*, 1997; Hashemolhosseini *et al.*, 1998), addition of Rap along with serum, to G0 arrested 3T3 cells results in a significant delay in the entry of stimulated cells into S phase and reduced the rate of cell proliferation (Chung *et al.*, 1992). As an experimental control, it we determined the effect of Rap on the cell cycle and total protein synthesis of MoMuLV infected NIH 3T3 cells upon release from G0. In these experiments, the degree of synchrony of cells and the effect of the drug upon release from G0 was estimated by measuring the distribution of DNA content per cell in cell populations by propidium iodide staining followed by flow cytometric analysis (FACS). Results obtained from a series of cultures released from G0 arrest are shown in Fig 24A. In agreement with the report of Chung et al. (1992), addition of Rap and serum to G0 arrested MoMLV chronically infected NIH 3T3 cells resulted in a delay but not blockage in the entry of stimulated cells into S phase (Fig 24A). As expected under these experimental conditions Rap also impaired the enhancement of protein synthesis in response to serum stimulation (fig 24B) (Beretta *et al.*, 1996; Morley &McKendrick, 1997).

Figure 24: Effect of rapamycin on cell cycle and protein synthesis of MLV infected NIH-3T3 cells. Panel A MoMuLV-infected NIH 3T3 cells were serum starved for 48 h prior to the addition of DMEM containing serum (10% final dilution) and either 50 ng/ml rapamycin (+Rap) or vehicle alone (-Rap). Cells were harvested, fixed, permeabilized with saponin and stained with propidium iodide for flow cytometry analysis. The modal distribution of DNA content per cell in cultures are shown at the times after the addition of serum, indicated to the right of each histogram. Panel B, MoMuLV-infected NIH 3T3 cells were serum starved for 48 h prior to the addition of DMEM containing serum (10% final dilution), 100 μCi [35S] methionine and either 50 ng/ml rapamycin (+Rap) or vehicle (-Rap). Cells were harvested at times indicated, and the incorporation of radioactive methionine into trichloroacetic acid-precipitable material was determined.

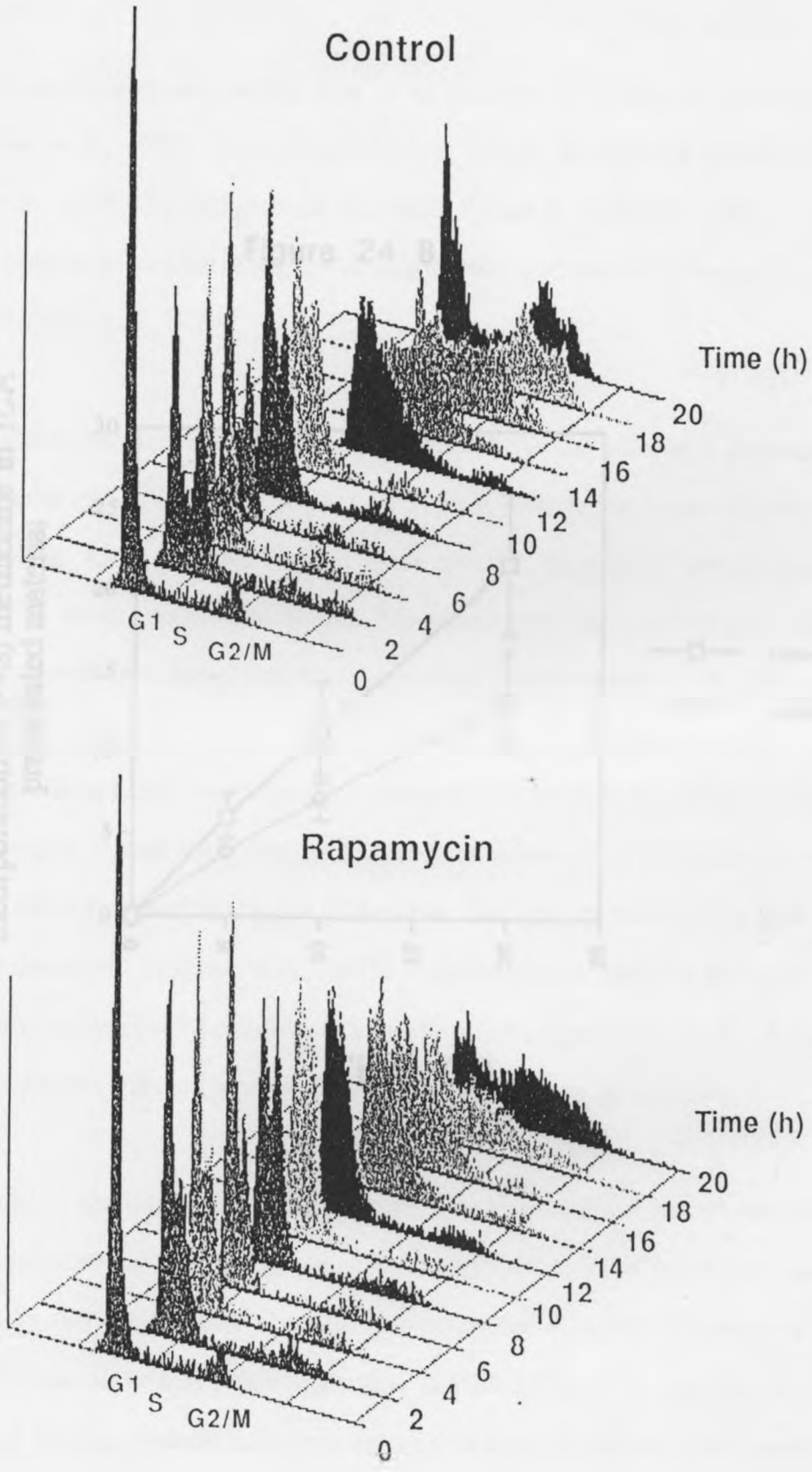
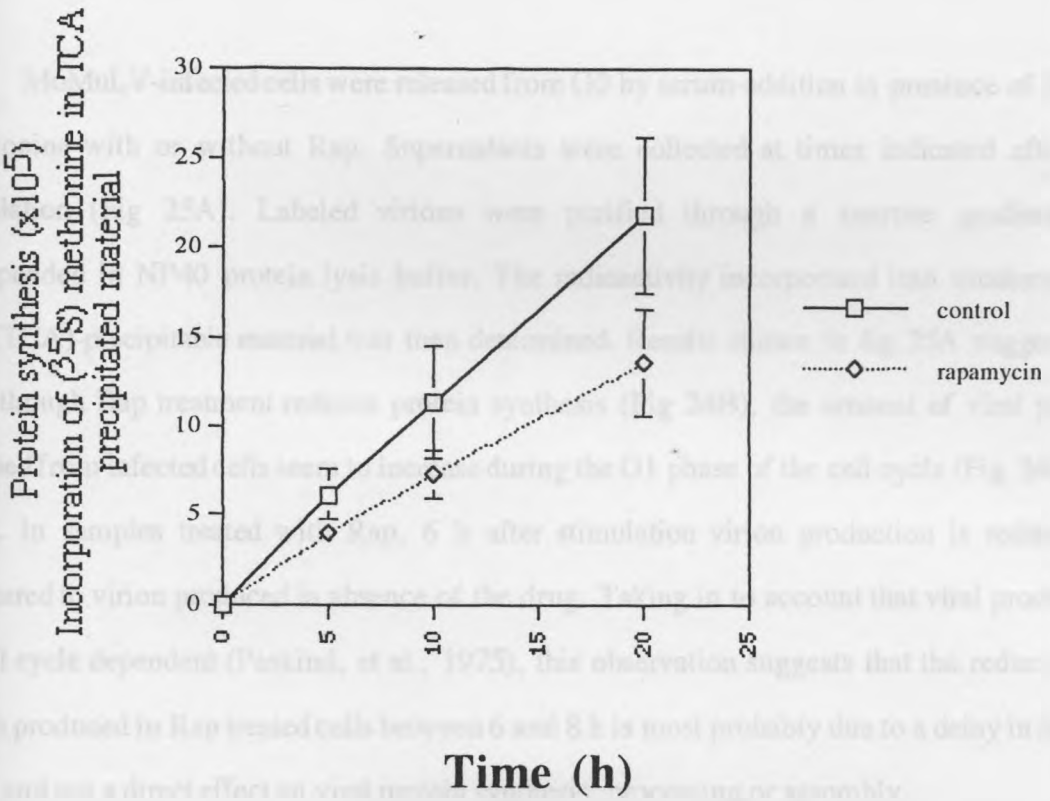

Control
Time (h)
20
18
16
14
12
10
8
6
4
2
0
G1
S
G2/M
Rapamycin
Time (h)
20
18
16
14
12
10
8
6
4
2
0
G1
S
G2/M

Figure 24 B


Protein synthesis (x10⁵)
Incorporation of (35S) methionine in TCA precipitated material
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (h)
control
rapamycin

V. 4. 2- Effect of Rapamycin on virion production.

The immunosuppressive drug Rap, is an inhibitor of G1 cell cycle progression (Fig 24A) (Chung, et al., 1992). Thus, as cell cycle is known to influence MoMuLV production (Sherton *et al.*, 1976; Guttman-Bass *et al.*, 1980; Balazs & Caldarella, 1981, Gloger *et al.*, 1985), we examined the effect of Rap on viral production and infectivity during G1 phase of the cell cycle (Paskind *et al.*, 1975).

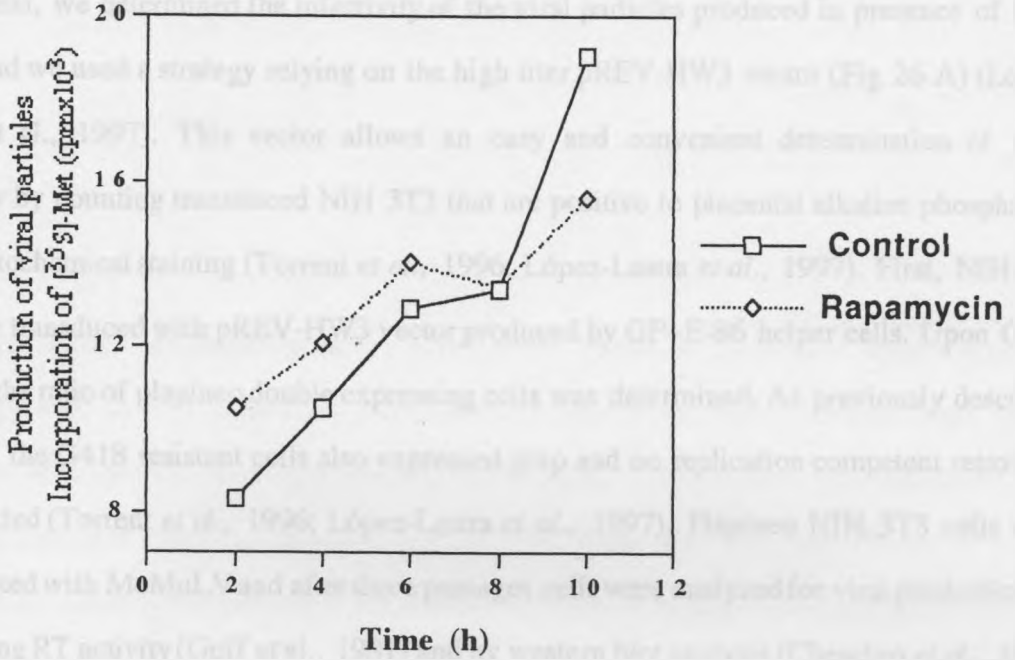
MoMuLV-infected cells were released from G0 by serum-addition in presence of 35[S]-methionine with or without Rap. Supernatants were collected at times indicated after cell stimulation (Fig 25A). Labeled virions were purified through a sucrose gradient and resuspended in NP40 protein lysis buffer. The radioactivity incorporated into trichloroacetic acid (TCA)-precipitable material was then determined. Results shown in fig 25A suggest that even though Rap treatment reduces protein synthesis (Fig 24B), the amount of viral particle released from infected cells seem to increase during the G1 phase of the cell cycle (Fig 24A and 25A). In samples treated with Rap, 6 h after stimulation virion production is reduced as compared to virion produced in absence of the drug. Taking in to account that viral production is cell cycle dependent (Paskind, et al., 1975), this observation suggests that the reduction of virion produced in Rap treated cells between 6 and 8 h is most probably due to a delay in the cell cycle and not a direct effect on viral protein synthesis, processing or assembly.

In order to confirm the above data, experiments were repeated, but western blotting was used as an alternative method of determining virion production. Virus production was followed during 12 h by analyzing MoMuLV capsid p30 production (CAp30) (Chesebro *et al.*, 1983) (Fig 25B). Results show that virion production increased (Fig 25B, lane 10, 12 and 13 vs. Lane 4, 6 and 7) when producer cells were treated with Rap. As before, virion production does not seem to increase between 6 and 8 h after Rap treatment (lanes 10 and 11). However, 10-12 h after Rap treatment production of viral particles is again enhanced (Fig 16B, compare lanes 12

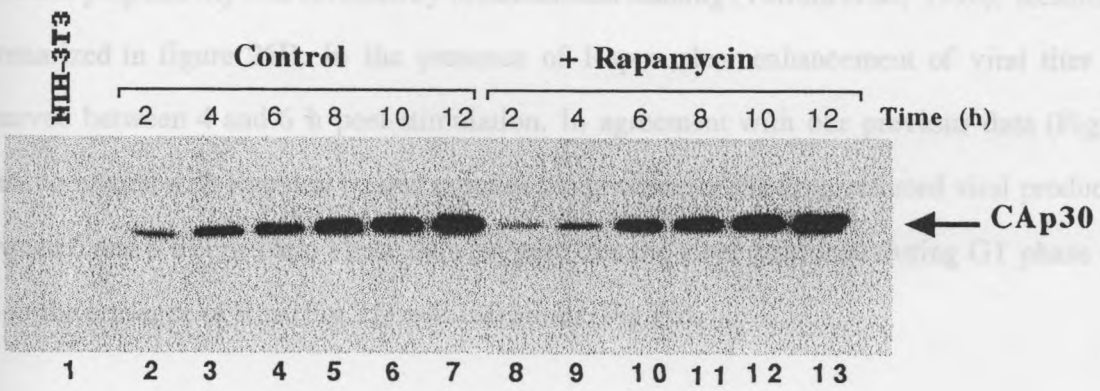
and 13 with lanes 6 and 7). As for other IRES containing viruses Rap seems to have no effect on virus protein synthesis, processing or particle assembly (Beretta *et al.*, 1996; Svitkin *et al.*, 1998). According to the data (Fig 24 and 25) reduction of virus production between 6 and 8 h is most probably due to an effect of Rap on the cell cycle and not on viral protein synthesis.

Figure 25. Effect of rapamycin on virion production. MoMuLV-infected NIH 3T3 cells were serum starved for 48 h prior to the addition of 6 ml of DMEM containing: **Panel A**, either 50 ng/ml rapamycin (+Rap) or vehicle alone (-Rap), 100 μCi [35S] methionie, and serum (10% NBCS). Supernatant was harvested at times indicated, virions were partially purified though a 20% sucrose cushion and the incorporation of radioactive methionine into trichloroacetic acid-precipitable material was determined. **Panel B**, either 50 ng/ml rapamycin (+Rap) or vehicle (-Rap), and serum (10% NBCS). Supernatant was harvested at times indicated and produced virions were partially purified as indicated above. An aliquot of the resuspended viral particles was analyzed by western blot using an anti CA-p30 antibody as described in material and methods.

A


Production of viral particles
Incorporation of [35S]-Met (cpmx10³)
20
16
12
8
0
2
4
6
8
10
12
Time (h)
Control
Rapamycin

B


NIH-3T3
Control
+ Rapamycin
2 4 6 8 10 12 2 4 6 8 10 12
Time (h)
CAp30
1 2 3 4 5 6 7 8 9 10 11 12 13

V. 4. 3- Effect of Rapamycin on virus infectivity.

Next, we determined the infectivity of the viral particles produced in presence of Rap. To this end we used a strategy relying on the high titer pREV-HW3 vector (Fig 26 A) (López-Lastra, et al., 1997). This vector allows an easy and convenient determination of virus infectivity by counting transduced NIH 3T3 that are positive to placental alkaline phosphatase (plap) histochemical staining (Torrent *et al.*, 1996; López-Lastra *et al.*, 1997). First, NIH-3T3 cells were transduced with pREV-HW3 vector produced by GP+E-86 helper cells. Upon G418 selection the ratio of plap/neo double expressing cells was determined. As previously described 100 % of the G418 resistant cells also expressed plap and no replication competent retrovirus was detected (Torrent *et al.*, 1996; López-Lastra *et al.*, 1997). Plap/neo NIH 3T3 cells were then infected with MoMuLV and after three passages cells were analyzed for viral production by determining RT activity (Goff et al., 1981) and by western blot analysis (Chesebro *et al.*, 1983) (data not shown). Plap/neo NIH 3T3 cells infected with MoMuLV were plated and depleted of serum for 48 h, after which they were stimulated in presence or absence of Rap by addition of serum (Beretta *et al.*, 1996, Morley &McKendrick, 1997). Cells supernatant were collected at indicated times post-stimulation and used to infect fresh NIH-3T3 cells. Infected cells were fixed and plap activity was revealed by histochemical staining (Torrent *et al.*, 1996). Results are summarized in figure 26B. In the presence of Rap a clear enhancement of viral titer was observed between 4 and 6 h post-stimulation. In agreement with our previous data (Fig 25) when compared with Rap non treated samples the presence of the drug reduced viral production between 6 and 8 h (Fig 26B) These data suggest that the virus produced during G1 phase (Fig 24) in the presence of Rap (Fig 25) was infectious (Fig 26).

Taken together (Figs, 24, 25 and 25) these data indicate that specific inhibition of cap-dependent translation in MoMuLV infected cells does not interfere with viral protein synthesis, genomic RNA encapsidation, virus assembly and infectivity.

Figure 26. Effect of rapamycin on viral titer. Panel A. Schematic representation of the strategy used to titer MoMuLV. pREV-A-HW3 transduced NIH 3T3 cells were serum starved for 48 h infected upon infection with wt MoMuLV. Supernatant was harvested upon serum stimulation at times indicated and used to infect fresh NIH 3T3 cells. **Panel B.** Viral titer was determined by the method of serial dilution followed by plap histochemical staining.

Figure 26 A

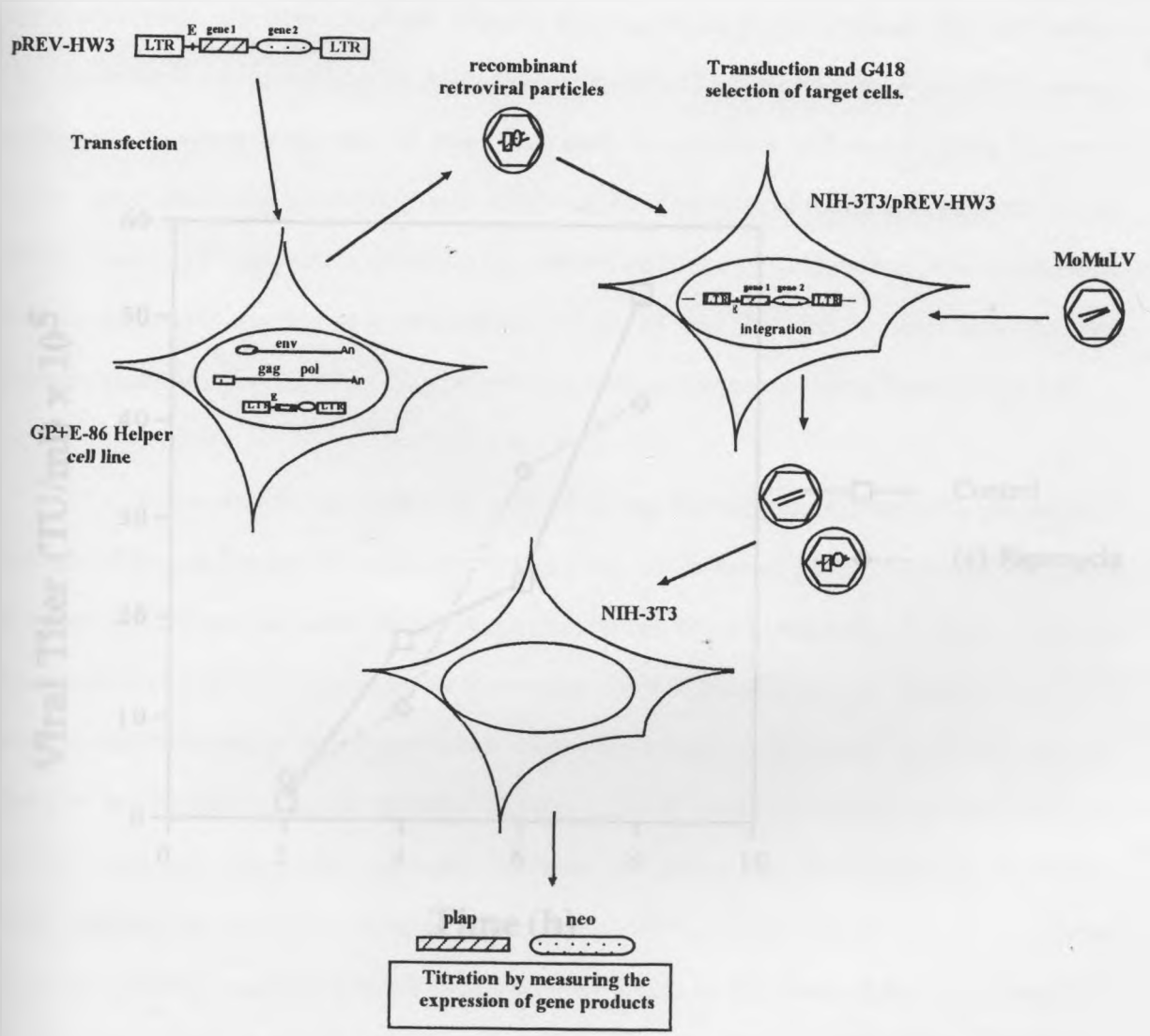
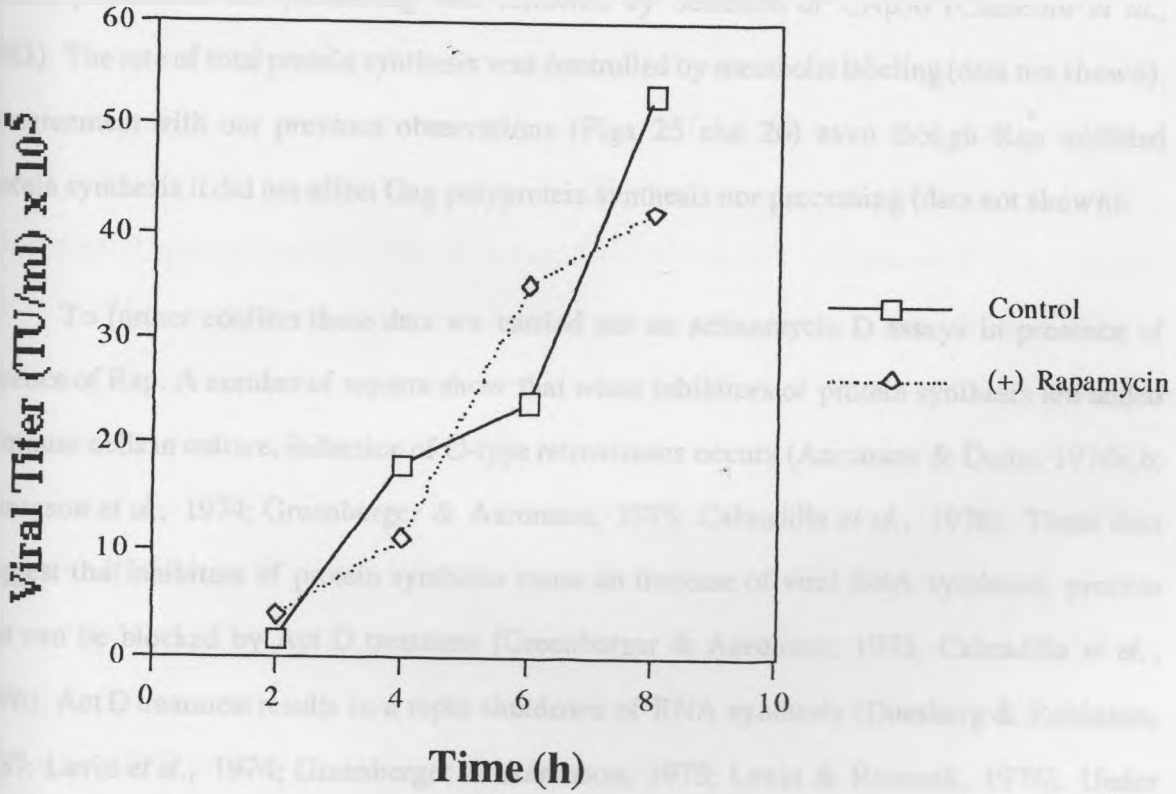

pREV-HW3
LTR
E
gene1
gene2
LTR
Transfection
recombinant
retroviral particles
Transduction and G418
selection of target cells.
NIH-3T3/pREV-HW3
MoMuLV
integration
env
An
gag
pol
An
GP+E-86 Helper
cell line
NIH-3T3
plap
neo
Titration by measuring the
expression of gene products

Figure 26B


Viral Titer (TU/ml) x 10^5
60
50
40
30
20
10
0
0
2
4
6
8
10
Time (h)
Control
(+) Rapamycin

V. 4. 4- Effect of rapamycin on viral protein synthesis.

Our present results indicate that the MLV IRES is most probably used by the virus to drive Gag synthesis when cap-dependent translation is repressed (Figs 25 and 26). To confirm our observations, we determined the effect of Rap on virus protein synthesis and processing. Cellular extracts recovered from MoMuLV-infected NIH-3T3 cells released from G0 by serum addition in presence or absence of Rap were used in a western blot assays. Gag precursor protein production and processing was followed by detection of CAp30 (Chesebro *et al.*, 1983). The rate of total protein synthesis was controlled by metabolic labeling (data not shown). In agreement with our previous observations (Figs 25 and 26) even though Rap inhibited protein synthesis it did not affect Gag polyprotein synthesis nor processing (data not shown).

To further confirm these data we carried out an actinomycin D assays in presence of absence of Rap. A number of reports show that when inhibitors of protein synthesis are added to mouse cells in culture, induction of C-type retroviruses occurs (Aaronson & Dunn, 1974a,b; Aaronson *et al.*, 1974; Greenberger & Aaronson, 1975; Cabradilla *et al.*, 1976). These data suggest that inhibitors of protein synthesis cause an increase of viral RNA synthesis, process that can be blocked by Act D treatment (Greenberger & Aaronson, 1975, Cabradilla *et al.*, 1976). Act D treatment results in a rapid shutdown of RNA synthesis (Duesberg & Robinson, 1967; Levin *et al.*, 1974; Greenberger & Aaronson, 1975; Levin & Rosenak, 1976). Under these experimental conditions RNA synthesis was found to be inhibited by more than 95% (Levin *et al.*, 1974; Levin & Rosenak, 1976). A number of reports show that despite the absence of ongoing RNA synthesis, MLV particles containing the appropriate amount of all viral proteins continue to be produced for at least 8 h after the addition of the Act D (Levin *et al.*, 1974; Paskind *et al.*, 1975; Levin & Rosenak, 1976). These results indicates that viral mRNA is stable and can be translated after the cessation of viral RNA synthesis.

As viral RNA synthesis has been shown not to be affected by serum depletion (Guttman-Bass *et al.*, 1980), ActD treatment of G0-arrested cells is expected to produce an

initial pool of viral mRNA stable for about 6 h (Levin, et al., 1974) (Levin & Rosenak, 1976). Serum stimulation is not only expected to enhance translation but also to affect the rate of transcription of a number of mRNAs (Lau & Nathans, 1985; for review see Treisman, 1992; Kleijn *et al.*, 1998). Therefore, as RNA synthesis will not be enhanced a reduced amount of viral proteins should be expected in the Act D treated cells. On the other hand, Rap favors cap-independent translation initiation thus, under these experimental condition, if MoMuLV RNA contains an IRES viral protein synthesis should be favored in the presence of both drugs.

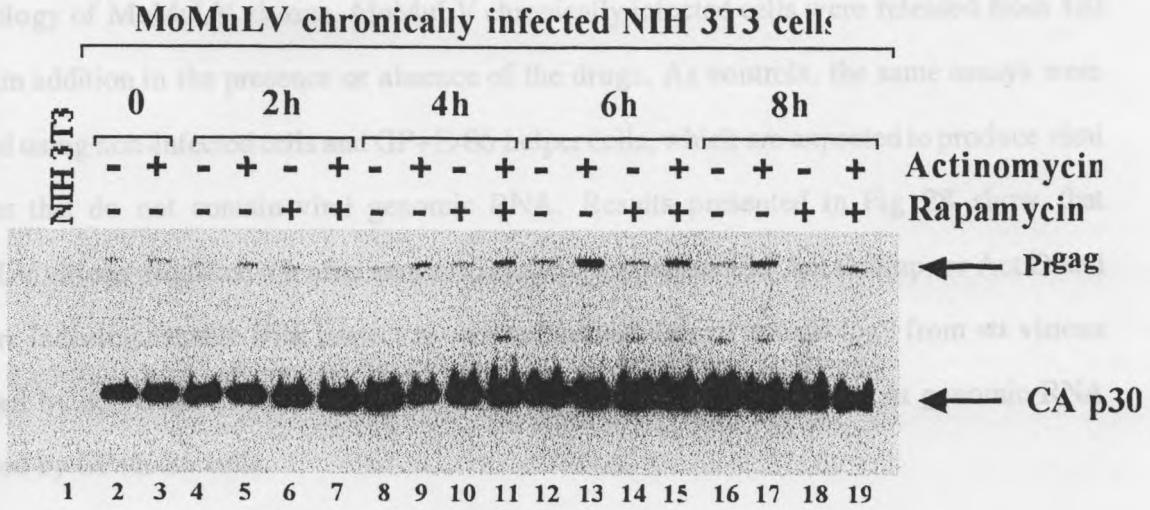
As expected results show that Rap enhances full length viral mRNA translation (Fig 27A lanes 6, 10 and 14). A comparatively lower amount of CAp30 protein was detected in ActD treated cells (Fig 27A lanes 5, 9, 13 and 17). This reduction is not only associated with a reduced RNA synthesis but also with an apparent accumulation of non-processed Gag precursor (Pr) (Fig 27A lanes 5, 9, 13 and 17). A reduced protein synthesis was observed in ActD (Fig 18A lane 17) and ActD-Rap (Fig 18A lane 19) treated cells 8 h after serum addition. This observation correlates with the expected 6h stability of the viral mRNA (Levin *et al.*, 1974; Levin & Rosenak, 1976). These results confirms that the effect of Rap is at the level of mRNA translation (Beretta *et al.*, 1996). As expected Act D does not reduce the enhancement of viral protein synthesis in Rap treated cells (Fig 27A lanes 7, 11 and 15), suggesting that both drugs act at different levels (mRNA synthesis or translation). These data further sustain the presence of an active IRES in MLV capable of driving Gag synthesis.

Data show that CAp30 synthesis (Fig 27A) as well as virus production and infectivity (Figs 16 and 17), are not affected by Rap. However, viral infectivity is known to be dependent on the presence of the viral envelope protein (Env). Therefore, our results would support those of Deffaud and Darlix (1999) which suggest the presence of an IRES in the 5' leader of the subgenomic viral mRNA. To further confirm this observation, it was interesting to analyses the effect of Rap on the synthesis of the Env precursor (Pr^{env}) (Fig 27B). In agreement with observations of Deffaud and Darlix (1999), the rate of Pr^{env} synthesis is not reduced by Rap treatment (Fig 27B lanes 6, 11, 14, 18) and is even enhanced in the presence of both ActD and

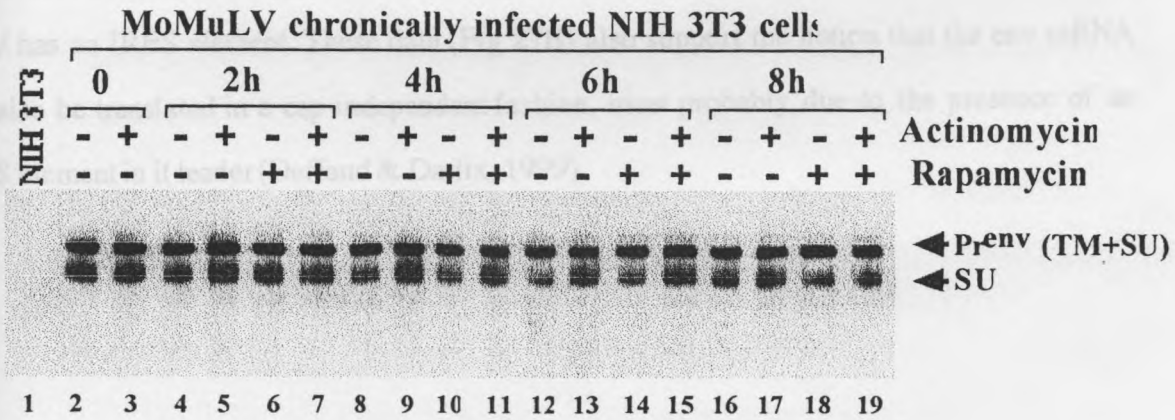
Rap (Fig 27B lanes 7, 11, 15, 19) suggesting that env mRNA can also be translated in a cap-independent fashion. These data agree with our previous observations (Figs 25 and 26) and can explain why viral infectivity is not affected by Rap.

Figure 27: Effect of drug treatment on viral protein synthesis. MoMuLV-infected NIH 3T3 cells were serum starved for 48 h prior to the addition of 6 ml of DMEM containing either 1μg/ml actinomycin D, 50 ng/ml rapamycin, both drugs or vehicle alone and serum (10% NBCS). Cells were harvested at times indicated, and protein extracts were prepared (see materials and methods). Equal amounts of total protein (10 μg) were loaded and submitted to gel electrophoresis and latter transferred to a PVDF membrane. Membranes were analyzed using an anti-CAp30 antibody (**panel A**) or an anti-gp70 (Env) antibody (**panel B**). After an incubation in the appropriated biotinilated secondary antibody blots were developed by ECL. Proteins extracted from non-infected NIH 3T3 cells were used as negative control (lane 1). Time 0 corresponds to protein extracted from non stimulated infected cell with (lane 3) or without ActD treatment (lane 2).

A


MoMuLV chronically infected NIH 3T3 cells
NIH 3T3
0
2h
4h
6h
8h
Actinomycin
Rapamycin
PrgagPr
CA p30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

B

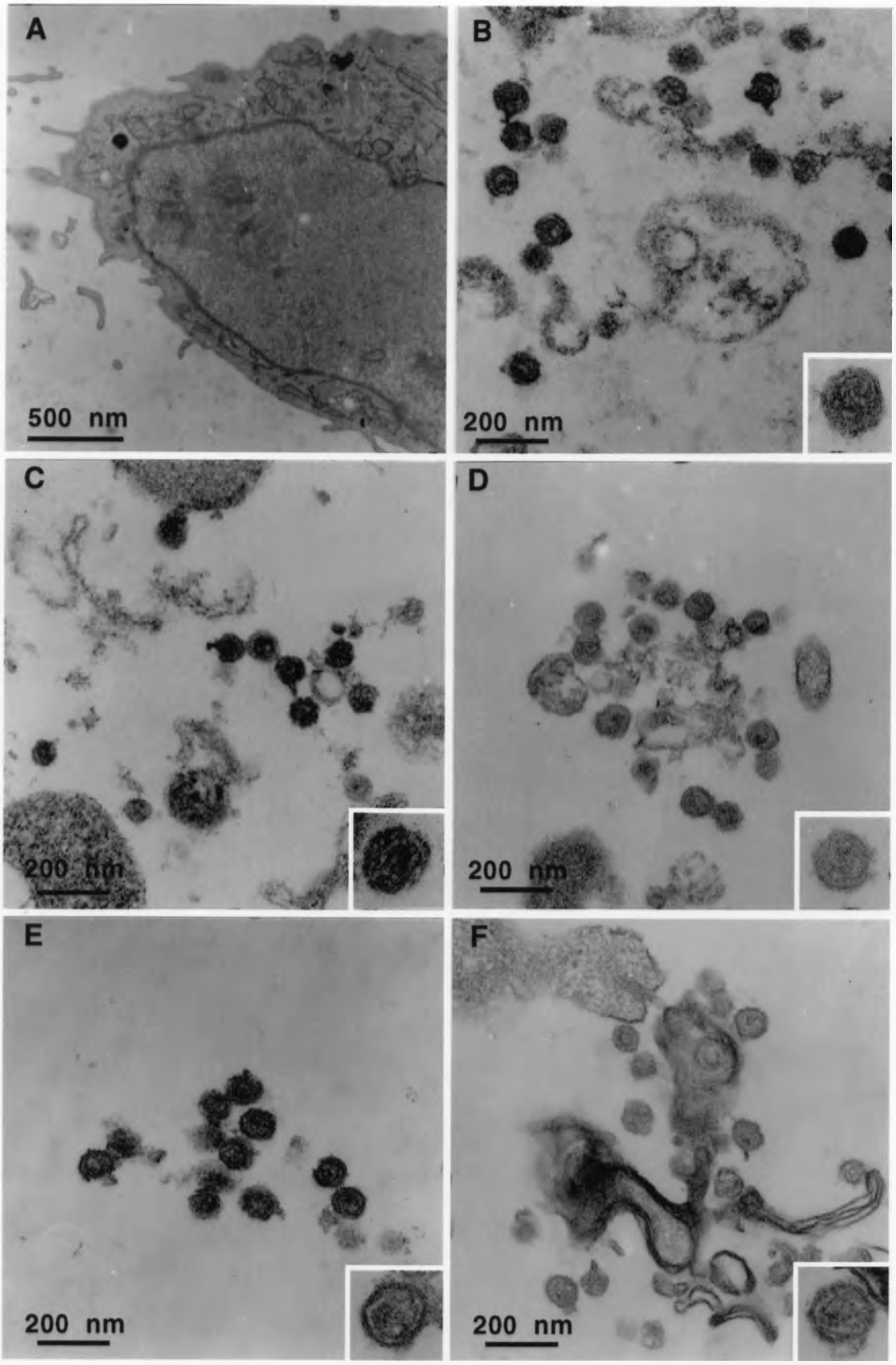

MoMuLV chronically infected NIH 3T3 cells
NIH 3T3
0
2h
4h
6h
8h
Actinomycin
Rapamycin
PrenvPr (TM+SU)
SU
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

V. 4. 5- Rapamycin does not modify MoMuLV morphology.

To extend this study we examined the effect of Rap and ActD-Rap treatments on the morphology of MoMuLV virions. MoMuLV chronically infected cells were released from G0 by serum addition in the presence or absence of the drugs. As controls, the same assays were repeated using non-infected cells and GP+E-86 helper cells, which are expected to produce viral particles that do not contain viral genomic RNA. Results presented in Fig 28 show that MoMuLV virions produced 4 h after serum-stimulation in presence of Act D, Rap, or Act D and Rap, are indistinguishable with respect to size and ultrastructural morphology from wt virions produced by non-treated MoMuLV-infected NIH 3T3 or from virions without genomic RNA produced by GP+E-86 cells.

Together, these data confirm that there is an IRES in the MoMuLV genome capable of conferring internal initiation of translation of Gag and support the conclusions of Berlioz and Darlix (1995), Berlioz et al. (1995) and Vagner et al. (1995b) which suggest the 5' leader of MLV has an IRES element. These data (Fig 27B) also support the notion that the env mRNA can also be translated in a cap-independent fashion, most probably due to the presence of an IRES element in it leader (Deffaud & Darlix, 1999).

Figure 28: Electron microscopy analysis. NIH 3T3 cells (**panel A**) or MoMuLV-infected NIH 3T3 cells were serum starved for 48 h prior to the addition of 6 ml of DMEM containing either serum (10% NBCS) (**panel B**), serum and 1μg/ml actinomycin D (**panel C**), serum and 50 ng/ml rapamycin (**panel D**), or serum and both drugs (**panel E**). As a control for particles that do not contain genomic RNA we used GP+E-86 helper cells (**panel F**). Cells were fixed 4 h after serum stimulation and processed for thin-section electron microscopy, no morphological particle changes were observed among the different treatments. Inset Mx 90 000. (Dr C. Péchoux)


A
500 nm
B
200 nm
C
200 nm
D
200 nm
E
200 nm
F
200 nm

VI. DISCUSSION

The two main aims of this work were to determine if IRESes could be found in retroelements other than FrMLV, MoMuLV and HaMSV and if IRESes from retroviral origin could be efficiently used in bicistronic retroviral vectors. The existence of an IRES in the 5' leader of REV-A and mouse VL30 retrotransposon was predicted by the long 5' untranslated region and the structural resemblance of this region with that of MoMuLV, FrMLV and HaMSV. This section will not only focus on the presented data but will intend to establish the importance and possible advantage of an IRES during virus replication. A possible role of the IRES in determining the fate of the full length RNA will be proposed. However, in order to sustain the different proposition that will be presented a parallel with other viral families mainly picornaviruses will be drawn.

VI. 1. Identification of Rev-A and VL30m IRESes.

Murine leukemia virus (MuLV) and reticuloendotheliosis virus (REV-A) are type C retroviruses with a long, highly structured and multifunctional 5' leader RNA (Coffin *et al.*, 1997). Interestingly, despite the almost non existing sequence homology, retroviruses from the REV group and VL30 retrotransposons present structural homologies with FrMuLV, HaMSV, and MoMuLV (Koning *et al.*, 1992; Yang & Temin, 1994), found to possess an IRES within the 5' leader (Berlioz & Darlix, 1995; Vagner et al; 1995b). These observations prompted us to examine the functional features of the 5'leader of REV-A RNA and the 5' UTR of mouse VL30 retrotransposon (VL30m) with respect to translation initiation.

In order to characterize the REV-A and VL30m IRES we used the canonical strategy of bicistronic constructs, first described by Pelletier and Sonenberg (1988) (figs 11 and 15). Results, presented in figs 11 and 15, show that both REV-A and VL30m 5' region allow

translation of the downstream cistron in a bicistronic construct independently from the first cistron. Thus, these data suggest that both REV-A and 5' VL30m sequence are capable of recruiting ribosomes by an internal mechanism. Moreover, FMDV L protease known to specifically shut off cap dependent translation enhanced translation driven by the REV-A and VL30m 5' region, while reducing expression of neo, the cap dependent gene (figs 12 and 16; tables 1 and 3). These data suggest that as for other IRESes the cap binding protein of the translation pre-initiation complex, eIF4E, may not be required for retroviral IRES driven translation initiation (Ohlmann *et al.*, 1995, 1996; Krausslich *et al.*, 1987; Ventoso *et al.*, 1998). The observed increase of cap-independent protein expression in presence of L protease (fig. 12, 16 and tables 1, 3) or rapamycin (table 5) is probably be due to competition between 5' cap structures and IRESes for the recruitment of canonical translation initiation factors, in agreement with the data of Rosen et al. (1982), Evstafieva et al. (1990) and Duke et al. (1991), Ohlmann et al. (1995) and Ziegler et al. (1995a and b), Pestova et al. (1996 a and b). The observation that IRESes might compete with the cap structure should be taken into account when designing retroviral vectors. If competition is established between the 5' cap structure and an IRES within the same transcriptional unit (see pREV-HW1 and pREV-HW4, table 5), significant variations in the relative expression of both genes should be expected. These variations are likely to depend on the relative strength of the IRES (Borman et al., 1995), the cell type (Borman et al., 1997), and cell physiology (Ye et al., 1997). Similarly, competition for the translational machinery is likely between two or more IRESes present within the same transcriptional unit. However, data presented herein suggest that the use of double IRES constructs containing both the MLV and REV-A IRESes, such as pREV-HW3 (tables 4 and 5), allow stable and high level expression of both genes in a bicistronic vector in a cap-independent fashion.

For REV-A IRES its 5' and 3' borders appears to be between positions 452 and 580, respectively, just upstream of the gag AUG start codon. In REV-A, the IRES function is independent from the E/DLS domain (see pREV-CG58 in fig 10 and 11 and table 1; see vectors

pREV-HW4, pREV-HW5 and pREV-HW6 in fig 17 and tables 4 and 5) and therefore, the IRES structure is not expected, by itself, to promote viral packaging (see vectors pREV-HW1 and pREV-HW4 in fig 17 and table 4). The size and location within the 5' leader of the 129 nt fragment REV-A IRES is in agreement with the data reported for MoMuLV (Vagner *et al.*, 1995b)

With respect to VL30m, several lines of evidence such as sequence analysis which revealed that the VL30m 5' region contains stop codons in all reading frames (Adams *et al.*, 1988), suggest that VL30m has no translational activity. This is supported by the fact that to date no VL30m encoded polypeptides have been identified (French & Norton, 1997). Nevertheless, numerous aminoacid homology blocks exist between VL30 and retroviral genes, suggesting that VL30m RNA contains the remnants of ancestral gag and pol genes (Adams, et al., 1988). This, together with the presence of an IRES may reflect an ancient translational activity which can explain why VL30m RNA has been found associated with polyribosomes (Fan & Muller-Lantzsch, 1976) (Johannes & Sarnow, 1998). Moreover, it should be pointed out that in Mus dunni endogenous virus (MDEV), which appears to be a chimera between VL30m and a virus similar to GALV, genomic RNA packaging and gag polyprotein expression are most probably driven by the VL30m 5' region herein reported to contain a functional IRES (Wolgamot *et al.*, 1998)

Taken together these results further validate the structural homology studies made by Koning et al. (1992) on MLV-type C retroviruses and allow us to predict the presence of an IRES within the 5' leader of the other MLV-related type C retroviruses, such as feline leukemia virus (FeLV) and gibbon ape leukemia virus (GaLV), included in their study.

VI. 2. Development of bicistronic retroviral vectors.

VI. 2. 1. High titer MLV/REV-A vectors.

The presented data not only reports the presence of an IRES within the leader of REV-A and mouse VL30 retrotransposon, but they show that these IRESes can be used to develop bicistronic retroviral vectors. The promise of gene transfer and its potential to generate effective treatments for human diseases has been a subject of much debate. It is now well recognized that gene delivery technology presents a major obstacle to the success of this field, and a consensus has emerged that the development of vectors that can deliver and appropriately express relevant gene products in specific tissues in vivo is much needed. For this reason, significant effort has been placed on expanded studies in molecular virology and gene expression relevant to gene-transfer technology. To data studies in vectorology are aimed to develop gene transfer vectors based on the following standards: vectors should be injectable, targetable to specific sites *in vivo*, regulatable, able to maintain long-term gene expression, and they should be nonimmunogenic. Even though retroviral vectors developed in this work do not fulfill the requirements to be considered as ideal vectors, they strongly contribute to the field of retroviral vector development. As pointed out in the introduction there are several reasons why retroviruses are useful vectors (Gunzburg & Salmons, 1996; Gunzburg *et al.*, 1996; Federspiel & Hughes, 1997). However, retroviral vectors also have limitations: (i) Infection with MLV based retroviral vectors is limited to dividing cells and (ii) a relative low titer (10^5-10^7) when compared to other viral vectors systems such as adenovirus ($10^9 - 10^{11}$) (Gunzburg & Salmons, 1995; Robbins *et al.*, 1998). Thus, understanding why we obtained high titers with vector pREV-HW3 might allow the development of a new series of high titer retroviral vectors.

Both the high titer of bicistronic MLV-REV vectors such as pREV-HW3 (table 5) and high level of protein expression in individual cells (table 5) suggest that the presence of MLV-E and REV-E packaging sequences possessing IRES function within the same MLV based vector are able to enhance both vector expression and recombinant RNA packaging. This agrees with

the data of Dornburg and Temin (1990) indicating that addition of the MLV E sequence in an SNV-based vector with the SNV E sequence resulted in a viral titer enhanced by 2 orders of magnitude, favoring a functional interaction between both E sequences. Data of Soneoka et al. (1995) show that transcription of the recombinant RNA driven by the CMV promoter, instead of the LTR, enhances recombinant RNA synthesis and infectious titers, suggesting that the level of genomic RNA limits the viral titer. An alternative way of increasing viral titer may well be to stabilize viral genomic RNA. The presence of MLV E+ and REV-A E sequences within the same transcriptional unit, may stabilize the viral genomic RNA probably due to an enhanced dimerization of recombinant viral RNA. Dimeric REV-A RNA was found to by highly stable, and the minimal domain required for REV-A RNA dimerization *in vitro* was mapped between positions 268 and 452 (Kpn I and Sal I sites, see figure 1.A), corresponding to the E sequence (Darlix *et al.*, 1992). However, a higher stability of RNA dimers would be expected to reduce translation, phenomenon that we do not observe (Bieth *et al.*, 1990). When comparing vectors pREV-HW3 and pREV-HW6 it can be seen that only pREV-HW3, the high titer vector, contains the complete E/DLS region. Interestingly enough, the REV-A E sequence probably interacts in a selective manner, because recombinant viral titer was not enhanced when located downstream of VL30rat or VL30m (see titer for pREV-HW2 in fig17 and table 4 and pVL30m-SU11 in fig 18 and table 6). This result agrees with the data of Torrent et al. (1996), which show that the recombinant viral titer was not enhanced when MLV E+ and VL30 rat sequences are located within the same transcriptional unit. The possible interactions taking place between REV-A E and MLVE+ both in MLV and SNV based retroviral vectors are currently under study. However, new evidence that has been recently published suggesting a different interpretation of our data. Butsch et al. (1999) have shown that SNV possess a constitutive/cytoplasmic transporter element or CTE in a region between the LTR and the AUG^{gag}. Interestingly, the presented data indicate that an additional characteristic of the CTE containing region is that it enhances RNA-polysome association. Thus, most probably it overlaps with the REV-A IRES. In light of these new finding it is tempting to speculate that the high titer presented by vector pREV-HW3 is related to the presence of two CTEs (MoMuLV and Rev-A) within the same transcriptional unit (King *et al.*, 1998) (Butsch *et al.*, 1999).

Preliminary experiments show that in fact a relationship can be established between the reported vector titer and the amount of vector RNA present in the cytoplasm (data not shown). Therefore, the presence of two CTEs might simply provide a more efficient nuclear export as opposed to more efficient packaging. In other words, the recombinant RNA would no longer be the limiting factor for packaging (Soneoka *et al.*, 1995). However, the current available data do not let us exclude that both E complementation and CTEs activity are responsible for the high titer observed in our vectors. If our preliminary data are confirmed it can be proposed that retroviral titers could be enhanced by simply adding CTEs to the retroviral construct.

VI. 2. 2. VL30m for the development of safer vectors.

Retroviral vectors incorporating mouse VL30 sequences have been proposed to have potential use in gene therapy (Chakraborty *et al.*, 1995; French & Norton, 1995). In MLV-based vectors the packaging signal comprising the extended (E+) region of MLV encompasses gag and glyco-gag coding sequences (Bender *et al.*, 1987). These sequences which might be the cause of homologous recombinations possibly generating replication competent retroviruses (RCR) cannot be deleted without destroying the ability of the recombinant RNA from being encapsidated in to virions. Our results show that the VL30m 5' region is capable of directing both packaging (fig 13 and Table 2) and transgene expression (Figs 13 and 15; Table 2). We therefore constructed novel MLV/VL30 vectors (fig 18) to determine if the VL30m E/IRES characterized *in vitro* could be used in the development of bicistronic vectors.

Upon transduction of murine cells, the VL30m IRES was found to be functional, since it allowed expression of a 3' cistron of a bicistronic retroviral vector (figs 18 and 19; table 6). Moreover, by combining VL30m and rat VL30, we were able to develop an efficient IRES-based vector, pVL30 SU12, that contains only the LTRs of MLV, thus, reducing to a minimum sequences that can potentially recombine with gag and glyco-gag (fig 18). This improvement together with those of others (Chakraborty *et al.*, 1995; French & Norton, 1995) will allow the development of safer retrotransposon-based vectors, that are less likely to undergo homologous

recombination. Therefore, it can be concluded that the knowledge generated in this work shall be the starting point for the development of a novel generation of high titer and biologically safer retroviral vectors.

VI. 3. Uses of MLV/REV-A vectors in the central nervous system.

Neuropathologies that might theoretically be amenable to gene therapy are not lacking. However the vast number of different neural phenotypes as well as the highly complex macroscopic functional organization of neural cells presents an exciting challenge for gene therapy, if not an insurmountable barrier. Mature neural cells do not proliferate, and it was a long held dogma that neurons were not generated post-natally. This raises the question of how one might ensure the appropriate functional insertion of a gene or indeed a cellular vector in the brain ? Recently it was discovered that both neurons and glia derive from multipotent precursors (Snyder *et al.*, 1992; Johe *et al.*, 1996; Lundberg *et al.*, 1997). The finding that these cells can be cultivated *in vitro* and reinserted into a host brain where they will integrate functionally with a phenotype appropriate to their site of integration, in response to environmental cues, has opened a new avenue to prospective gene therapy in the brain (Snyder *et al.*, 1992; Gage *et al.*, 1995; Snyder & Wolfe, 1996; Lundberg *et al.*, 1997; Whittemore *et al.*, 1997).

It is easy to envisage situations in which it would be highly advantageous, if not an essential prerequisite, for cells to be transduced to co-express more than one gene product. This might be because it allows transduced cells to be selected prior to use, or because several enzymes might be implicated in a particular biosynthetic, or catabolic pathway. The double-IRES approach provides a more efficient means of inducing co-expression than a regulated-splicing mechanism or the use of two promoters (see introduction section). Single-IRES vectors can also be used to co-express two genes from a monocistronic mRNA, but the presence of 5' cap structures and IRESes within the same transcriptional unit can lead to competition for the recruitment of canonical translation initiation factors (Figs 12, 16, 19; Tables 1, 3, 5). This can lead to inefficient expression of one of the transgenes.

On the other hand, there is evidence that the capacity of IRESes to initiate translation may be cell-type or differentiation dependent (Bernstein *et al.*, 1997, Ye *et al.*, 1997). Differentiated neural cells do not proliferate and are therefore not amenable to transduction with MLV-based retroviral vectors. Therefore, if the double-IRES strategy is to be applied to transduce the co-expression of two proteins in neural precursor cells, it will be necessary to identify IRESes that are not subsequently switched off by neural differentiation.

In this study we also have sought to optimize MLV-based bicistronic vectors for transduction of human multipotent neural precursors. Furthermore, we have investigated whether neural differentiation alters translation driven from MoMLV, EMCV or REV-A IRESes in bicistronic vectors (fig 17). As vectors, we selected pEMCV-CBT4 and pREV-HW3 which have been shown to be efficient both with respect to high titer and expression stability in ecotropic packaging cell lines (fig 17; tables 4 and 5). As a negative control of these vectors we used the vector pREV-HW1 which is not packed in MLV-helper lines (fig 17 and table 4). As a cellular model we used a human multipotent neural precursor cell line, Dev, derived from a pediatric medulloblastoma. Dev cells have been established to express an antigenic profile similar to that of normal multipotent precursors and to respond to growth factors by proliferation and differentiation in a manner typical of such cells (Derrington et al., 1998). Previous demonstrations that Dev cells can be induced to express mature neuronal (Dufay *et al.*, 1994) and glial (Giraudon *et al.*, 1993) phenotypes suggest that this cell line provides a homogenous source of human multipotent neural precursor-like cells. For these reasons the Dev cell line provides an interesting and accessible model system in which to study and perfect gene therapy strategies to target this cell type in man.

A comparison of the efficiency of transduction obtained with the different recombinant vectors produced by several packaging cell lines under normalized standard conditions was performed. Our data show that the 10A1 MLV pseudotyped vectors are extremely efficient transducers of Dev cells (40 - 50 % of transduced cells in a population of 10^6 cells) (fig 20). In

human cells GALV uses exclusively the Glvr-1 receptor for entry whereas amphotropic (AM-MLV) retrovirus exclusively uses the Ram-1 receptor (Miller, 1996, Miller & Chen, 1996). The 10A1 MLV envelope can use either the Glvr-1 (Pit-1) or the Ram-1 (Pit-2) for infection of human cells, although in most cell lines examined to date Pit-2 is used (Miller & Chen 1996; F-L. Cosset, unpublished). Our results suggest that Pit-1 and Pit-2 may act synergistically to allow a highly efficient transduction of Dev cells. Alternatively distinct uncharacterized receptors may be utilized by the 10A1 MLV-pseudotyped vectors to transduce Dev cells. The receptor of the feline endogenous retrovirus envelope, RD114, which is a type C retrovirus, has yet to be identified. However, RD114 envelope, which also allowed a very efficient transduction, would be expected to use type D retroviral receptors on human and other susceptible mammalian cells (Sommerfelt & Weiss, 1990, Sommerfelt *et al.*, 1990).

FGF2, which it a potent enhancer of the proliferation of multipotent neural precursors (Kilpatrick & Bartlett, 1993; Vescovi *et al.*, 1993; Johe *et al.*, 1996), and Dev cells (Derrington *et al.*, 1998) induced a small but highly reproducible increase in the proportion of cells transduced. This increase was similar for all pseudotyped vectors tested (fig 20). This suggests that FGF2 is probably not acting via a capacity to stimulate the expression of receptors for the different viral envelopes. It is far more likely that a more general principal is responsible. One hypothesis is that enhanced cell proliferation, in response to FGF2, may favor the incorporation of virus into the host cell genome. This would be in agreement with data suggesting that the MLV integration complex gains entry to the cell nucleus only during mitosis by taking advantage of the nuclear envelope breakdown (Roe *et al.*, 1993).

Transduced Dev cells could be selected in G418 and expressed plap as shown by either cytochemistry or immunocytochemistry (fig 21, 22, 23). Various IRESes have been described to be switched on or off in a cell type- and developmental stage-specific manner. Our results, using the MLV-based vectors pEMCV-CBT4 and pREV-HW3, show that MoMuLV, EMCV and REV-A IRESes maintain co-expression of plap and neo-resistance after the induction of neural differentiation (fig 23). These data suggest that the double-IRES vector strategy using

two of the above viral IRESes can provide an acceptable means of ensuring the stable translation of two gene products in human undifferentiated neural precursor cells and in their differentiated neuronal and glial progeny.

VI. 4. MoMuLV IRES is functional in its natural context.

The data presented herein confirm the presence of IRES in the 5'leader of MoMuLV genomic mRNA. Interestingly, and in agreement with data of Deffaud and Darlix (1999), results presented in fig 27B suggest that there might also be an IRES in the 5' leader of the spliced env mRNA. This env IRES would explain why Rap has no effect on virus infectivity. Take together, these results allow us to speculate that the presence of IRESes in the 5' leader of both spliced and unspliced MLV mRNA might be a strategy to over come host control of viral protein expression. Cellular cap-dependent translation is strictly dependent upon the availability of the cap-binding initiator factor eIF4E, which is regulated to large extent by PHAS-I sequestration (Pause *et al.*, 1994; Haghighat *et al.*, 1995; Rau *et al.*, 1996). However, the IRES-mediated mechanism of translation does not require eIF4E (Ohlmann *et al.*, 1995, 1996; Pestova *et al.*, 1996a, b). Thus, translation driven by an IRES should allow retroviruses such as MoMuLV, FrMLV, HaMSV and REV-A to overcome cellular modulation of translation initiation that acts at the level of PHAS-I/eIF4E (for reviews see Hershey, 1991 and (Kleijn *et al.*, 1998).

Translation efficiency is defined as the rate of polypeptide synthesis per mRNA per unit time. In can be inferred that levels of protein synthesis would be determined not only by message abundance but also by the ability of the transcript to be successfully translated. Our data show that Rap enhances translation efficiency of MLV mRNAs (Fig 27). This is reflected by an increase of viral protein synthesis (Fig 27) and a concomitant increase of virion production (Fig 25 and 26). Due to the known effect of Rap on the cell cycle, we can not fully discard that the increase in virion production is a consequence of a prolongation of G1 phase (Paskind *et al.*, 1975). However, since our data are in agreement with those reported for other

viral IRESes (Beretta *et al.*, 1996; Svitkin *et al.*, 1998) and with previous observations showing that translation efficiency of retroviral IRES-based bicistronic vector is enhanced in the presence of Rap (table 5). We favor the idea that the increase of virus protein synthesis and virion production observed in presence of Rap is due to the ability of IRES containing transcript to be preferentially translated. Therefore, the presence of an IRES in the viral genome confers a competitive advantage to viral RNA over cellular mRNA, thereby promoting virus production under conditions where cellular translation is temporarily repressed.

In conclusion, the MLV 5' IRES is functional both in the context of the virus and in retroviral vectors (figs. 17, 18, 1921, 23, 25, 26, 27, 28 and tables 4, 5, 6) (Torrent *et al.*, 1996). However, there are no evidences that would indicate that cap-independent translation is the only initiation mechanism used by MLVs to accomplish gag mRNA translation. In fact, preliminary electron microscopic observation and ribosome mapping assays (Prats, 1988) show that *in vitro*, ribosomes can directly bind to internal sequences as well as to the 5' end of MoMuLV RNA. Based on these findings it can be suggested that MLVs have the ability to used both cap dependent and independent mechanisms for translation initiation. Recent studies have shown that in certain IRES containing euckaryotic mRNAs, transition from a cap-dependent to a cap-independent mode of translation is a regulated process that would depend on cellular factors (Toyoda *et al.*, 1994; Bernstein *et al.*, 1997; Nanbru *et al.*, 1997 Ye *et al.*, 1997; van der Velden and Thomas, 1999).

VI. 5. Why does VL30m possess an IRES?

It is tempting to speculate on the conservation of these retroviral IRESes in VL30 retrotranspososns. Due to the compact size of retrotransposon genomes, it is not surprising that the long 5' region participates in multiple transposon life-cycle functions. It is clear that higher ordered RNA structures can persist through evolution despite substantial changes in primary nucleotide sequence. Thus, it is a distinct possibility that, as for MoMLV, FrMLV and HaMSV, the VL30m IRES is contained within the region which determines dimerization and subsequent packaging of its RNA. Hence its maintenance would be a requirement for the retrotransposon to be preserved (Courtney *et al.*, 1982). To date no specific biological role has been described for the mouse VL30 retrotransposon. VL30s, despite their close association with oncogenic MLV viruses, have not been directly linked to oncogene activation (French & Norton, 1997). Since mouse VL30 sequences are actively transcribed under a variety of stimuli, they might act as insertional mutagens by gene activation, as with murine retroviruses (French & Norton, 1997). On the other hand, the variety of VL30 LTR promoters found in the mouse, together with their mobility, may also provide possible mechanisms for evolutionary changes in gene regulation as well as for the spread of genes across species (Bultman *et al.*, 1994; Norton & Hogan, 1988). Although, the data presented herein do not allow us to directly address the question of the biological role of this family of retroelements, they do support the hypothesis that VL30m may not only activate genes at the level of transcription but may also have an effect on translation due to its IRES activity (Carter *et al.*, 1986; Norton & Hogan, 1988).

VI. 6. Interplay between translation and genomic RNA packaging.

In retrovirus the full length RNA acts both as mRNA and as genomic RNA (see introduction). Based on the current models of genomic RNA packaging both functions do not seem to be compatible. Several authors favor the idea of two pools of RNA, one for translation with a high stability and a second one for packaging (Levin & Rosenak, 1976; Messer *et al.*, 1981). However, this model does not explain results reported by Paskind et al. (1975) showing that MoMuLV infected NIH 3T3 cells released from G0 in the presence of actinomycin D are

able to produce RNA containing particles. Therefore, an interesting hypothesis would be that the retroviral IRES is implicated in determining the fate of the full length viral RNA.

As a parallel, picornavirus genome has at least three major fates: it act as mRNA to direct the synthesis of viral proteins, it serves as the template for genome replication and it is packaged during viral assembly (Fields *et al.*, 1996). These processes must be properly balanced to allow efficient viral replication. Thus, once the viral RNA-dependent RNA polymerase and other essential proteins are synthesized, the genomic RNA must be used as a template for negative-strand RNA replication (Andino *et al.*, 1999). This creates a conflict between the translatation and replication machineries. While the ribosomes are moving along the viral RNA in a 5' to 3' direction, the polymerase initiates replication at the 3' end of the same RNA and moves in the opposite way, 3' to 5', as it synthesizes the complementary negative strand. The 5' untranslated region (UTR) of picornaviruses plays an important role in the interplay between translation and RNA replication (Pelletier & Sonenberg, 1988; Pelletier *et al.*, 1988; Trono *et al.*, 1988; Andino *et al.*, 1990; Andino *et al.*, 1993; Borman *et al.*, 1994; Rohll *et al.*, 1994). PV IRES overlaps with another genetic element involved in RNA replication. In fact the cloverleaf RNA structure at the 5' end of the PV genome has recently been defined as a bifunctional element involved in the regulation of both viral translation and RNA replication. This genetic element has also been proposed to coordinate the use of the genomic RNA for translation or replication. The regulation model proposed by Gamarnik and Andino (1998) suggests that after poliovirus entry, the genomic RNA interacts with translation initiation factors to begin protein synthesis. Once a critical amount of viral protein is reached the uncleaved precursor of the viral protease and polymerase binds to the 5' cloverleaf RNA structure. This binding changes the conformation of the 5' secondary structures, resulting in a shut down of viral translation and promotion of negative-strand synthesis (Gamarnik & Andino, 1998). This regulation mechanism, that involves the switch from translation to RNA replication, seems to be conserved among different viral families. In agreement with this notion the entire PV IRES can be replaced with the corresponding sequence of different members of the picornaviridae family and even by the HCV IRES, without major consequences for viral replication (Alexander *et al.*, 1994; Rohll *et al.*,

1994; Jia *et al.*, 1996; Lu & Wimmer, 1996; Zhao *et al.*, 1999). In this same line, Nugent et al. (1999) have proposed a direct coupling between PV genome replication and packaging. Thus, in this model RNA translation, replication and packaging would be sequentially regulated processes. Authors suggest that a requirement for successful translation of the PV genome before it is used as a template for RNA synthesis should prevent the replication of RNA genomes that contain frameshift and nonsense mutations. Then the coupling between RNA synthesis and RNA packaging imposes a requirement that RNAs to be packed must emerge from active replication complexes, ensuring that the templates for those RNAs were competent to form such complexes. This mechanism would impose a form of late proofreading to the error-prone RNA genome of PV (Nugent *et al.*, 1999).

In retroviruses a similar scenario probably occurs, since the E/DLS element overlaps with the IRES and both have been characterized as RNA structural elements (Konings *et al.*, 1992; Yang & Temin, 1994; Le *et al.*, 1996; Mougel & Barklis, 1997; Le & Maizel, 1997, 1998; Fisher & Goff, 1998). Dimerization of genomic retroviral RNA represents an essential feature since all known genomic retroviral RNAs are packaged as dimers in virions (Rein, 1994; Berkowitz *et al.*, 1996). Dimerization and encapsidation of the genomic RNA are regulated by the same transacting factor, the nucleocapsid protein (Gorelick *et al.*, 1988; Meric & Goff, 1989; Prats *et al.*, 1990; Rein *et al.*, 1994; Berkowitz *et al.*, 1995; Zhang & Barklis, 1995; Bonnet-Mathoniere *et al.*, 1996; Girard *et al.*, 1996). Mutation or deletions of the DLS motif have also been shown to reduce the RNA encapsidation level (Darlix *et al.*, 1992; Yang & Temin, 1994). These observations reinforce the idea that RNA dimerization and packaging are closely related processes. A number of reports show that *in vitro* dimerization of MoMuLV genome implies RNA structural rearrangements (Prats *et al.*, 1990; Tounekti *et al.*, 1992; Mougel *et al.*, 1993; Girard *et al.*, 1996; Mougel *et al.*, 1996; De Tapia *et al.*, 1998). Interestingly, important structural changes occur near the SD site and in the *gag* initiation subdomain, mostly affecting a U-rich region upstream of the AUG initiation codon (Mougel *et al.*, 1993).

Based on *in vitro* data Girard et al. (1996) proposed an interesting model to explain the overall dimerization process. Their model is divided in three steps (1) a temperature-dependent intramolecular rearrangement of the monomeric RNA (M → M*), leading to a "dimerizable" conformation of the RNA monomer, (2) a loop-loop interaction leading to an unstable dimer (M* + M* ↔ D1), and (3) reassociation of each stem to generate a stable dimer (D1 → D2). Results presented in the same study suggest that NCp10 lowers the energy barrier required for generation of a dimerizable conformation of the monomer RNA. At this point it is interesting to ask : How does this model apply to the in vivo data available for retroviral RNA packaging?

Genomic RNA is present in virions made with Gag as the only protein (Shields *et al.*, 1978; Sakalian *et al.*, 1996; Sakalian & Hunter, 1998). This observation implies that Gag is the only protein required for directing the selective packaging of viral RNA into virions. Several studies using MLV mutants have shown the implication of the NC domain of Gag protein in the specific encapsidation of the genomic RNA. Mutations in the NC result in virus that are defective for replication. Several phenotypes have been reported for these mutants. In most cases, there is a decrease in virion-associated genomic RNA (Gorelick *et al.*, 1988; Meric & Goff, 1989; Rein *et al.*, 1994; Housset & Darlix, 1996). Furthermore, evidence for specific RNA packaging into virions directed by the NC domain of Gag was obtained with an ASLV mutant in which its own NC was replaced with the MLV NC, resulting in partially altered packaging specificity (Dupraz & Spahr, 1992).

Newly released MLV particles contain a dimeric form of viral RNA that dissociates to monomers at a lower temperature relative to dimeric RNA in mature virions (Fu & Rein, 1993). This observation indicate that in agreement with the model proposed by Girard et al (1996) the viral RNA can exist in at least two distinct dimer states and that there is a shift to a more stable dimer in virions. Transition from unstable to stable viral RNA dimer is dependent upon the activity of the viral protease that cleaves the Gag precursors resulting in the accumulation of NCp10 in the virion core.

A theoretical model can be proposed to explain the switch between retroviral genomic RNA translation and encapsidation. The monomeric form of the genomic RNA would be used as template for protein synthesis. When a critical concentration of Gag polyprotein is reached, it would bind to the monomeric RNA favoring conformational RNA rearrangements. These RNA rearrangements would modify the IRES structure resulting in an inhibition of translation, thus favoring dimerization and encapsidation of the genomic viral RNA. This hypothesis is favored by several additional observations. Studies of dimer formation in Rous sarcoma virus and its effect on viral protein synthesis showed that monomeric RNA translated is more efficiently translated than the dimer (Bieth *et al.*, 1990). Moreover, nucleocapsid protein (NC)-induced RNA dimerization totally inhibited *in vitro* viral RNA translation (Bieth *et al.*, 1990). As for PV this model would assure that the retroviral genomic RNA packaged into particles can be efficiently used as a template, mainly because its genes can be translated (Nugent *et al.*, 1999). Interestingly, Kaye and Lever (1999) propose a similar model for the recognition of HIV-2 genomic RNA. The authors find that efficient encapsidation of HIV-2 RNA requires cotranslation of Gag protein containing a functional NC domain (Kaye & Lever, 1999). In the case of HIV-2 it is argued that selection of RNA for packaging occurs preferentially in *cis*. This would provide the required selectivity for unspliced RNA in a system in which the packaging signal is present on all of the HIV-2 messages (Kaye & Lever, 1999).

VI. 7. IRES and viral infection: the relationship between virus-cell tropism and viral protein synthesis.

In about 1% of humans infected with PV, the neurovirulent phenotype of PV is expressed, resulting in paralytic poliomyelitis (Fields *et al.*, 1996). Repeated passages of the three PV strains in animals and cultured cells generated the corresponding attenuated vaccine strains (Sabin types 1-3) (Fields *et al.*, 1996; Ogra, 1999). The improved ability of these PV variants to grow in non-nervous tissue compromised their ability to grow in the nervous system, as demonstrated by the decreased neurovirulence in monkeys (Fields *et al.*, 1996). Studies showed that several point mutations in the viral IRES contribute to a replication defect in the nervous system. These mutations, which affect the RNA secondary structure, not only

decrease neuroptropism of the Sabin strain, but also reduce translation levels of the viral RNA *in vitro*, suggesting that PV neurotropism and translation are interrelated. Attenuated strains of Sabin oral poliovirus vaccine replicate in guts and in rate cases cause vaccine-associated paralytic poliomyelitis (VAPP) (Georgescu *et al.*, 1997; Zimmerman & Spann, 1999). Reversion of vaccine strains towards a pathogenic phenotype has been proposed as the amin cause of VAPP. Studies show that reversions can be associated to the viral IRES (Evans *et al.*, 1985; Georgescu *et al.*, 1997).

Most PV strains only infect primates. The host range of PV was thought to be primarily determined by a cell surface molecule that functions as PV receptor, since transgenic mice are made PV sensitive by introducing the human PV receptor into their genome. The relative levels of neurovirulence of PV tested in these transgenic mice were shown to correlate well with the levels tested in monkeys suggesting that these mice are good second animal models for poliomyelitis. Using this transgenic (Tg) mouse model Shiroki et al (1997) characterized mutations in the PV IRES which allowed the mutant to replicate well in primate cells and in the CNS of monkeys but failed to replicate in mouse cells and in the CNS of Tg mice. They also confirmed that replication of the mutant strains in mouse cells was blocked at the level of translation initiation. These data suggest that interactions between the IRES and host factors are important determinants of host specificity of PV and stress the implication of the IRES in viral tropism (Shiroki *et al.*, 1997).

Hepatitis A virus (HAV) is the sole member of the hepatovirus genus of the picornaviridae (Fields, et al., 1996). It is characterized by its lack of sequence relatedness with other picornaviruses and by several unique biological characteristics, including slow noncytopathic growth in cell culture and an inability to shut down host cell protein synthesis (Lemon *et al.*, 1992; Whetter *et al.*, 1994). As is the case for other picornaviruses, HAV possesses an IRES in its 5' NTR (Glass *et al.*, 1993; Brown *et al.*, 1994). Interestingly, virus translation is the rate-limiting step for replication of HAV in human fetal lung fibroblasts, MRC-5 cells (Funkhouser *et al.*, 1994, 1997, 1999; Whetter *et al.*, 1994). Funkhouser et al (1994)

showed that certain mutations (MR) which naturally occur by serial passage in MRC-5 allow an efficient HAV replication in these cells. Upon analysis the MR mutations were localized within the HAV IRES (Funkhouser, et al., 1994). These MR mutations allowed a cell type specific adaptation and had no discernible impact on virus replication in fetal rhesus kidney-4 (FRhK-4) cells or in other tested cell types (Funkhouser *et al.*, 1996). A second group of mutations (AG) acquired during passage in African green monkey kidney (AGMK) which allowed enhanced translation initiation specifically in these cells, were also located within the IRES (Funkhouser, et al., 1994). In agreement with these observations Martinez-Salas et al. (1993) described a single point mutation in the IRES of FMDV which accounts for the hypervirulence of the virus for BHK-21 cells. Authors suggest that the mutation may facilitate the interaction of the IRES with cellular proteins needed for initiation of translation.

The relation of IRES and viral infectivity is not limited to picornaviruses. The majority of Hepatitis C virus (HCV), member of the flaviviridae, can be classified into six main genotypes 1 to 6. Studies have shown that the HCV IRES activity is different depending on the cell type (Tsukiyama-Kohara, et al., 1992) (Wang *et al.*, 1993; Buratti *et al.*, 1997). These data suggest that the interaction of the HCV IRES with host factors that modulate their activity differs between different cell types (Collier *et al.*, 1998). Furthermore, studies comparing the efficiency of the IRES element from different HCV genotypes have established that HCV 5' UTR belonging to different genotypes have different translational initiation capabilities (Buratti *et al.*, 1997; Kamoshita *et al.*, 1997; Collier *et al.*, 1998; Honda *et al.*, 1999; Jang *et al.*, 1999; Tang *et al.*, 1999). Buratti et al. (1997) have suggested that the biological differences between HCV genotypes in terms of quantity of virus in serum or sensitivity to interferon correlates with the replication efficiencies of the various genotypes. Again, initiation of protein synthesis seems to be an important factor affecting HCV replication. As many other RNA viruses HCV has a very high mutation rate and circulates as a population of closely related genomes, referred to as quasispecies (Jang *et al.*, 1999). This dynamic and the highly plastic nature of HCV has been postulated to play an important role in evading eradication by the host immune system. Jang et al (1999) analyzed the difference between circulating and replicating virus, by amplifying and

comparing serum and liver derived HCV sequences in patients with chronic hepatitis C. In their study they chose to amplify and analyze the 5' UTR (region that includes the IRES) and were able to establish quasispecies between the virus present in the serum and that in the liver. The authors propose that 5' UTR variations could represent viral adaptive mutations for different liver cell populations.

Taken together these data not only point out the importance of host factors in the regulation of cap-independent translation initiation but also suggest the importance of the IRES in viral replication and how this element is a target for adaptive mutations. Accumulating data that starts to establish a direct relationship between IRES activity and viral pathogenecity (Gromeier *et al.*, 1996, 1999; Carthy *et al.*, 1997; Shiroki *et al.*, 1997; Shaw-Jackson & Michiels, 1999).

It is interesting to speculate on the possible roles of retroviral IRESes in viral-host relationships. Up to date IRES elements have been identified in the 5' leader MoMuLV, HaMSV, FrMuLV, REV-A, mouse VL30 retrotransposon and HTLV-I (Beretta *et al.*, 1996a, b; Vagner *et al.*, 1995b; Attal *et al.*, 1996). This, strongly suggest that IRES elements may be present in most if not all retroviruses. In fact unpublished data strongly suggests that an IRES can also be found in the 5' leader of lentiviruses. Interestingly, a recent report shows that HIV poor replication in astrocytes is due to regulation at the level of translation. Gorry et al. (1999) reported that astrocytes acutely infected by HIV-1 produce high levels of viral mRNA, which is correctly spliced and efficiently translocated to the cytoplasm. However, despite high levels of available mRNA the major HIV-1 and Nef proteins are expressed at low levels during the acute infection phase. Data show that diminished expression of these proteins results from an HIV-1-specific restriction in the translation of gag, env, and nef mRNAs (Gorry *et al.*, 1999). If these data are related to the putative HIV IRES activity, internal initiation may not only represent an important and conserved retroviral mechanism to overcome cellular control at the level of translational initiation but also may play an important role in viral tropism.

VI. 8. Conclusions and perspectives. The final comments.

The use of IRESes of retroviral origin has shown to be important for the development of retroviral vectors. In this respect, major properties of retroviral IRESes have been identified that should be used in the development of vectors. It can be concluded that the retroviral IRES acts in a position independent fashion, they are able to compete with the 5' cap structure or with other IRESes for the recruitment of ribosomes and, at least in the CNS their activity is not dependent on the state of cell differentiation. Further experiments should aim to determine the best combination of IRESes to ensure a stable expression of and the IRES activity in many different cell types.

In theory human gene transfer should involve a wide range of strategies for the alleviation of any of a variety of diseases. The majority of these strategies have not progressed beyond the stage of laboratory studies, many of which have drawn attention to fundamental problems that must be overcome prior to clinical application. For example, suicide gene therapy for cancer requires development of delivery systems that allow gene therapies to be used in patients. This requirement demands that the vector itself has capacity to discriminate between target and nontarget cells. If the vector transduced all cells with which it came into contact, healthy cells would also sensitive to prodrug treatment, resulting in perhaps dangerous nonspecific toxicity. One might avoid this problem by taking advantage of the natural limitations of MLV-based retroviral vectors, that it the inability to transduce nondividing cells. Even though it might seem a simple strategy, it becomes of importance when considering the use of MLV-based retroviral vectors in for example the context of brain, muscle or liver tumors. In these tissues the only dividing cells would correspond to tumor cells. Therefore, the vector would be naturally targeted to cancer cells.

Up to date the MLV-IRES vectors carry a marker gene, plap and a selectable gene, neo. To render these vector of use in gene therapy, our work will focus on the identification of appropriate genes that can be transferred into target tissues. In cancer several prodrug-activating

genes are interesting candidates. A series of prodrug-activating strategies such as the herpes simplex virus thymidine kinase gene (HSV-tk) which was shown to induce chemosensitivity to ganciclovir or other drug/gene partners like cyclophosphamide/rat cytochrome P450 2B1 (CYP2B1) (Connors, 1995; Tabin *et al.*, 1982; Chen *et al.*, 1996; Rainov *et al.*, 1996; Chen *et al.*, 1997; Jounaidi *et al.*, 1998; Waxman *et al.*, 1999), 5-flurocytosine/*Escherichia coli* cytosine deaminase (Aghi *et al.*, 1998), 6-methylpurine deoxyribonucleoside/*E.coli* purine nucleoside phophorylase (Sorscher *et al.*, 1994; Parker *et al.*, 1997), and 6-thioxanthine (6-TX) or 6-thioguanine/E.coli guanine phosphoribosyl transferase (gpt) (Tamiya *et al.*, 1996; Ono *et al.*, 1997), should be tested. One important problem faced by vector gene therapy treatment of solid tumors is the limited action radius of the vectors. A strategy to achieve efficient gene delivery into brain tumors employs the steriotactic implantation of helper cells that produce a retroviral vector bearing the gene of interest. The rational behind this approach is that the helper cells will continue to produce retroviral vectors *is situ*, resulting in higher tumor transduction levels than those obtained by a simple injection of a vector. This method relies on both the bystander effect of the therapeutic protein and on the ability of the vector to transduce tumor cells (Wei *et al.*, 1995; Pope *et al.*, 1997). In the case of solid tumors a potent bystander effect is expected to abrogate the requirement of gene transfer to all tumor cells to elicit an antitumor response. An alternative would be to transduce genes such as those coding for connexins. Malignant cells usually show altered gap junctions leading to intercellular communication, and are often associated with aberrant expression or localization of connexins. Transfection of connexin genes into tumor cells restores normal cell growth, suggesting that connexins form a family of tumor suppressor genes (Huang *et al.*, 1998; Zhang *et al.*, 1998; Mehta *et al.*, 1999). Some studies also show that specific connexins may be necessary to control growth of specific cell types (Asamoto *et al.*, 1994). Studies have suggested that the bystander effect of HSV-tk is due to gap junction communication (Yang *et al.*, 1998). On the other hand, several authors have proposed a method to enhance killing of cancer cells by diffusion of therapeutic agents through gap junctions (Duflot-Dancer *et al.*, 1998; Ghoumari *et al.*, 1998; Touraine *et al.*, 1998; Estin *et al.*, 1999). Thus, the advantage of connexin genes is that they can exert dual effects in tumor control, that is, tumor suppression and a bystander effect in cancer therapy

Current treatments of the most common forms of adult primary tumors have failed to improve the survival rate of patients. Strategies have failed because of physical restrictions of the transfer system: (1) the producer cells are non-motile, so continuous transduction of the tumor cells by the vectors is not adequate; (2) normally the vectors are replication defective thus transduction occurs within only a few cell-distances from the producing cells; (3) The vector titer is not high enough, so relatively large number of helper cells would have to be inoculated into the tumor; and (4) human tumor proliferation rate has been described to be slower than that of the murine tumors or of the cell lines used as an experimental model. These factors explain why these procedures have shown to be of limited use and only in restricted treatment of small tumors when many inoculation tracts were used. In an attempt to face these problems our strategy will focus on the expression of a fusion protein herpes VP22/prodrug-activating gene. In order to test this approach we shall use the fusion gene VP22-tk (Phelan *et al.*, 1998; Dilber *et al.*, 1999). Though this procedure we intend to enhance the bystander effect of HSV-tk by taking advantage of the reported ability of VP22 to exit and re-enter cells through a cytoskeleton-mediated pathway (Elliott & O'Hare, 1997). Given the very high titer and level of protein expression of the MLV-IRES vectors, it can be anticipated that combined with VP22-tk or conexins-tk genes they will represent a major contribution to the development of novel gene therapy strategies.

From the fundamental point of view, several new questions have arisen from my work. For example: (i) why do viruses with a capped mRNA require an IRES? (ii) is cap-independent translation the only mechanism used by retrovirus to translate their mRNAs?. It can be proposed that retroviruses are able to initiate translation by both a cap dependent or a cap independent mechanism. It can even be hypothesized that upon cap recognition ribosomes would be able to shunt over existent secondary structures. This dual initiation mechanism would allow retroviruses to over come different restrictions imposed at the level of translation initiation as a by the cell as a defensive response to infection. (iii) Do IRES represent a key point in the modulation of the fate of viral RNA? If so, several concepts in retrovirology would have to be reanalyzed. For example the notion of two pools of RNA could be challenged and a relationship

between translation and packaging could be established. In this case it could be argued that the IRES/E-DLS would contribute to the balance between translation and packaging. (iv) Does the IRES participate in determining viral tropism, what cellular proteins would be able to modulate the retroviral IRES activity? And (v) do lentiviruses have an IRES and, if so, can the IRES be a target for the development of new anti-retroviral therapies? It should be pointed out that preliminary data would suggest the presence of an IRES in the 5' leader of simian immunodeficiency virus (SIV). IRES elements have so far been described in MLV, MLV-like C type retroviruses and oncoretroviruses such as HTLV-1. Therefore, it would not be striking that IRESes would represent a conserved element thus, also present in lentiviruses. It is interesting to note that a naturally occurring small yeast RNA that specifically inhibits IRES dependent translation initiation in picornaviruses and HCV has been identified (Das *et al.*, 1996, 1998a,b). These finding are encouraging and if the presence of an IRES is confirmed in HIV, they may represent a possible new strategy to face HTLV-1 and HIV infections.

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