Polycistronic Viral Vectors

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Abstract: Traditionally, vectors for gene transfer/therapy experiments were mono- or bicistronic. In the latter case, vectors express the gene of interest coupled with a marker gene. An increasing demand for more complex polycistronic vectors has arisen in recent years to obtain complex gene transfer/therapy effects. In particular, this demand is stimulated by the hope of a more powerful effect from combined gene therapy than from single gene therapy in a process whose parallels lie in the multi-drug combined therapies for cancer or AIDS. In the 1980's we had only splicing signals and internal promoters to construct such vectors: now a new set of biotechnological tools enables us to design new and more reliable bicistronic and polycistronic vectors.

This article focuses on the description and comparison of the strategies for co-expression of two genes in bicistronic vectors, from the oldest to the more recently described: internal promoters, splicing, reinitiation, IRES, self-processing peptides (e. g. foot-and-mouth disease virus 2A), proteolytic cleavable sites (e.g. fusagen) and fusion of genes. I propose a classification of these strategies based upon either the use of multiple transcripts (with transcriptional mechanisms), or single transcripts (using translational/post-translational mechanisms).

I also examine the different attempts to utilise these strategies in the construction of polycistronic vectors and the main problems encountered. Several potential uses of these polycistronic vectors, both in basic research and in therapy-focused applications, are discussed.

The importance of the study of viral gene expression strategies and the need to transfer this knowledge to vector design is highlighted.

FROM VIRUSES TO TWO-GENE VIRAL VECTORS

One of the critical steps for gene transfer/therapy experiments is the availability of a suitable vector to carry the genetic information. Viruses represent a natural biological system of gene transfer to eukaryotic cells. Retroviruses were one of the initial systems to be developed for gene transfer/therapy and remain the most common vectors in gene therapy (Mountain, 2000). In nature, they mobilize cellular genes in the form of oncogenes, which has given rise to defective retroviruses (the oncogene replaces, partially or completely, the viral genes). An interesting exception is the Rous sarcoma virus, RSV, where the oncogene v-*src* is placed after the retroviral genes. The seminal development of designed, defective, retroviruses suitable to insert heterologous genes into cells, appeared in early 1980's. Other viral vectors are now available: adeno-viruses, adeno-associated virus, herpesviruses, chimeric viruses, etc. (for a recent review, see Kay *et al.*, 2001).

Although it is possible to construct vectors expressing only one gene, under certain circunstances it may be desirable to co-express a marker gene (drug resistance, colour, fluorescence, surface antigen, etc.) with the gene of interest. These markers make it easier to identify transduced cells and to detect the vector. A typical construct containing a gene of interest and a marker gene introduces a new problem: how to co-express two genes from a single (viral) vector? The more immediate answer is, again, to turn back to the viruses, not as sources of vectors, but as models for gene co-expression. The lessons gained are also useful for non-viral vectors, because the problem of gene co-expression is a universal one for all kinds of vectors. During the 1980's and 1990's, many different virus-based strategies were explored to co-express two genes from vectors (Table **1** and Fig. **1**). Taking into account the 5'-3' linearity of the genetic messages, there are several strategies used by viruses to co-express two genes that have been tested in viral and non-viral vectors.

Internal Promoters

Here there are two transcriptional units, each with its own open reading frame (ORF), producing two proteins. The advantage of this strategy is that there is a large set of promoters with particular characteristics described (specificity: -species, tissue, tumor; regulability: -drugs such as tetracycline, metals, radiation, etc.). For a recent general review, see Fussenegger (2001), for reviews more focused in the gene therapy field see: Walther and Stein (1996); Dachs *et al.* (1997); Harrington *et al.* (2000).

One major disadvantage is the uncoupled transcription of both genes. In the case of the retroviruses, this may lead to the transcription of only one gene due to transcriptional

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Table 1. Main Strategies Used to Co-Express Two Genes from a Single Vector

Expression strategy	Promoters	mRNAs	ORFs	Proteins
Internal promoters	2	2	2	2
Splicing	1	2	2	2
Internal initiation (IRES)	1	1	2	2
Reinitiation	1	1	2	2
Self-processing peptides (CHYSEL)	1	1	1	2
Proteolytic processing	1	1	1	2
Fusions	1	1	1	1

interference (initially described among the two long terminal repeats, LTRs; Cullen *et al.*, 1984; Boerkoel and Kung, 1992; Gama Sosa *et al.*, 1994; but also described with internal promoters, Bandyopadhyay and Temin, 1984; Nakajima *et al.*, 1993), or promoter suppression (Emerman and Temin, 1984a; 1984b; 1986a; 1986b; Palmer *et al.*, 1987; Hippenmeyer and Krivi, 1991; Zaboikin and Schuening, 1998). Furthermore, the behavior of each combination of cell type/internal promoter has been shown to be unpredictable (Li *et al.*, 1992; Mentz *et al.*, 1996). This has lead to loss of the desired properties of some promoters (Paulus *et al.*, 1996). Some researchers have also reported the influence on gene expression of the relative positions of the component genes in the vector (Xu *et al.*, 1989; McLachlin *et al.*, 1993). In addition, each inserted sequence performs in a site-specific manner (Xu *et al.*, 1989). In some cases rearrangements (including deletions) have been observed in the vectors (Emerman and Temin, 1984a; Bandyopadhyay and Temin, 1984; Olsen *et al.*, 1993; Breuer *et al.*, 1993).

Despite these problems, historically these vectors have been very popular. There are well known series of retroviral vectors of this type: e.g. Babe's (Morgenstern and Land, 1990) and LX's (Miller *et al.*, 1993). Internal promoters are also widely used in other viral vectors, where some of the disadvantages seen in retroviruses are alleviated.

Splicing

One transcriptional unit is used to produce two mature mRNAs. Typically, the unspliced mRNA is used to translate the first ORF. The flanking splicing signals lead to cleavage of the mRNA, removing the first ORF to obtain a spliced mRNA. This "mature" mRNA is used to translate the second ORF. The advantage here is the avoidance of the problems produced from the presence of two promoters, and a smaller size of the splicing signals compared with promoters. This strategy has been largely used in retroviral vectors, mainly because wild-type retroviruses employ it to co-express the *gag-pol* and *env* genes. However, presently, this is not a very common strategy, although interest has been stimulated recently by development of lentiviral vectors for the expression of two or even three genes (Reiser *et al.*, 2000; Zhu *et al.*, 2001).

A disadvantage of this approach is that in retroviruses, as part of the genomic mRNA population is spliced, the viral titer decreases (Gattas *et al.*, 1991; Hildinger *et al.*, 1998). Unfortunately, it is also difficult to achieve control of the splicing mechanism. The variability of the ratio among the retrovirus genomic and the subgenomic spliced mRNAs, has been described (Stoltzfus *et al.*, 1987; 1989; Arrigo *et al.*, 1988; Katz *et al.*, 1988; Berberich *et al.*, 1991). Design of these vectors must be very careful, as small variations can have a profound effect: inhibition of the splicing, rearrangements, activation of cryptic splicing signals, etc. (Joyner and Bernstein, 1983; Miller and Temin, 1986; Dougherty and Temin, 1986; Korman *et al.*, 1987; Bowtell *et al.*, 1988; Benchaibi *et al.*, 1989; Claudio *et al.*, 1989; Pulsinelli and Temin, 1991; Breuer *et al.*, 1993).

Internal Initiation

There are two sites of translation initiation from a single transcriptional unit. Translation of the first ORF is cap dependent, translation of the second ORF depends upon an internal sequence called an internal ribosomal entry site (IRES). IRES sequences, first described in picornaviruses in the late 1980's, are able to bind ribosomes internally and to initiate translation of the downstream ORF. During the last decade, several IRES elements have been found among the genomic mRNA population of eukaryotic cells (for a recent review see Hellen and Sarnow, 2001). Furthermore, other viruses apart from picornaviruses contain IRES elements. In retroviruses, both, unspliced and spliced mRNAs are translated from an IRES in their 5' untranslated regions (5'UTRs) (Vagner *et al.*, 1995; Berlioz and Darlix, 1995; Lopez-Lastra *et al.*, 1997; Deffaud and Darlix, 2000a; 2000b). A list of references about IRESes may be found in www.rangueil.inserm.fr/iresdatabase. Recently it has been shown, using a bicistronic vector, that the encephalomyocarditis virus (EMCV) IRES has low, but detectable, activity in plants -enabling its use in the vegetal context (Urwin *et al.*, 2000). One of the recently described IRESes in the picorna-like virus, *Rhopalosiphum padi* virus, has been shown to work in *in vitro* mammalian, *Drosophila* and also plant systems (Woolaway *et al.*, 2001). Furthermore, an IRES from the crucifer-infecting tobamovirus (crTMV) has shown a remarkable activity in plants, animal cells and yeast (Dorokhov *et al.*, 2002).

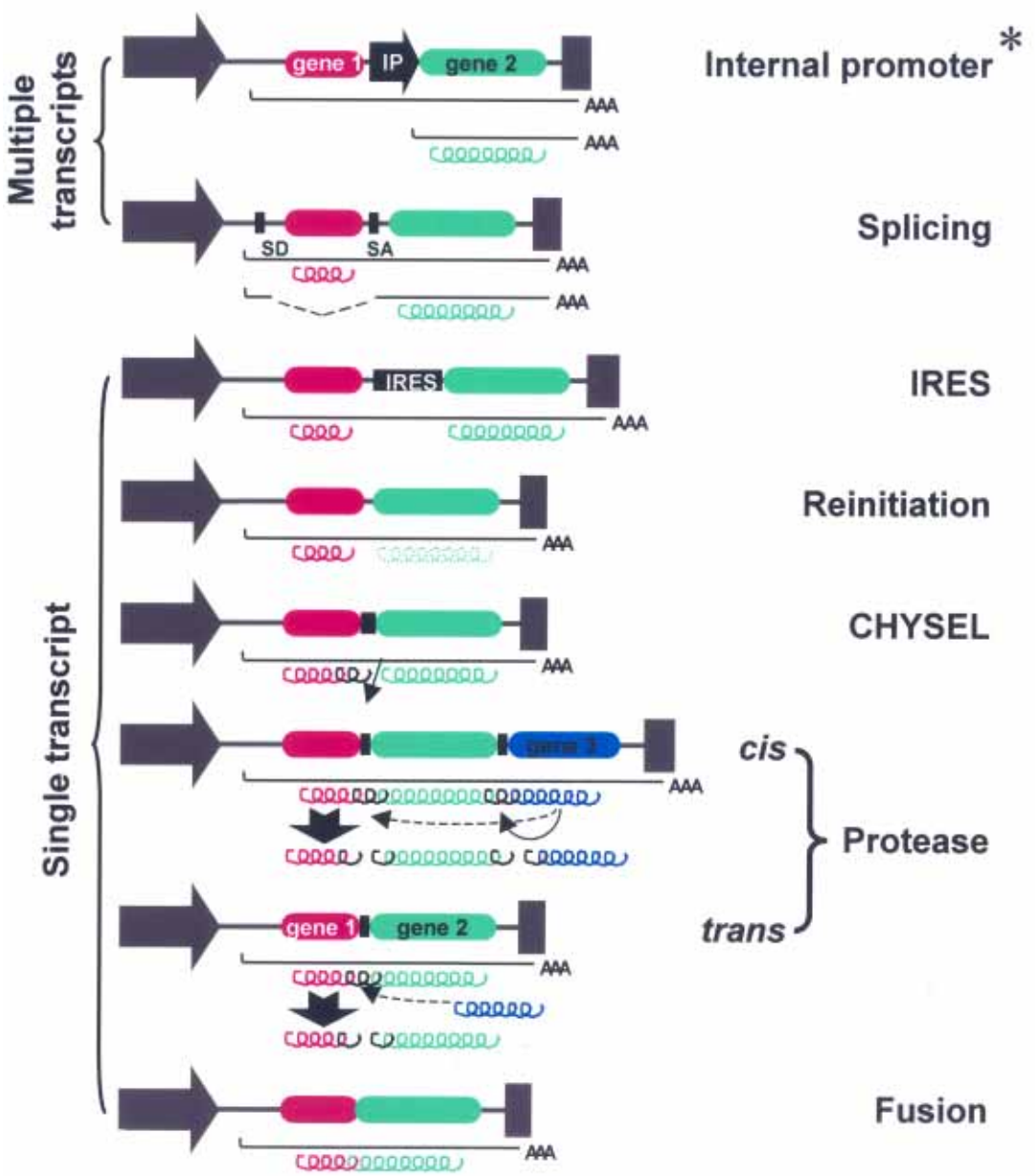

Fig. (1). Different strategies to co-express two genes. Thin arrows are for co-translational cleavage events; dotted arrows indicate post-translational proteolytic processing. * In this drawing there is only one poly(A) signal after the end of the second gene. However, it is possible to introduce another one between the end the first gene and the second promoter, so the first mRNA does not include the second cistron.

The majority of IRESes are found in the 5'UTR of an RNA from viral or cellular origin. Translation is driven by these complex RNA secondary structures in the 5' end of the RNA conferring a cap-independent mode of translation. IRES elements have been introduced between two cistrons to obtain bicistronic constructs for basic research (to explore IRES function) and in applied research (for co-expression in gene transfer/therapy). In nature a few cases have been described of organisms using IRESes to produce a polycistronic RNA, as in LINES-1 (McMillan and Singer, 1993) or the crTMV (Dorokhov *et al.*, 2002). An unusual IRES has been described in *Plautia stali* intestine virus and cricket paralysis virus (reviewed in RajBhandary, 2000; Hellen and Sarnow, 2001). This IRES lies in an intergenic region and directs the translation from a non-AUG codon without using a Met-tRNA$_i$. Another unusual example has been described from the PITSLRE protein kinase. This protein is translated in two alternative forms (58 and 110 kDa). Cornelis *et al.*, 2000, showed that the 58 kDa protein was translated from an IRES within the ORF of the 110 kDa protein. A tricistronic mRNA for the human cellular proto-oncogene c-*myc* has been reported from the first of four alternative promoters (P0), containing two different IRESes and three ORFs (Nanbru *et al.*, 2001).

The strategy of co-expression using IRESes has many advantages, probably the most significant being that they ensure the co-expression of the two genes: typically in more than 90% of cells (Ghattas *et al.*, 1991; Sugimoto *et al.*, 1995a; 1995b; Veelken *et al.*, 1996, Gallardo *et al.*, 1997; Saleh, 1997; Levenson *et al.*, 1998; Wagstaff *et al.*, 1998). Furthermore, a tight correlation between the level of expression of a marker protein downstream of the IRES and the expression level of the gene of interest cloned upstream of the IRES over an approximately 50-fold range has been shown in a bicistronic retroviral vector (Liu *et al.*, 2000). An additional advantage of IRESes is that they are active in situations where cap-dependent translation is inhibited, as in the reduction in overall protein synthesis in response to stress during certain steps of the cell cycle, apoptosis, etc. (reviewed in Hellen and Sarnow, 2001). In addition, IRESes have been used to enable translation from RNAs produced by RNA polymerases other than RNA polymerase II. T7 and T3 RNA polymerases, as well as RNA polymerase I produce RNAs without the 5'-terminal cap structure. An IRES in their 5' end enable ribosomes to use these RNAs as templates for translation (Elroy-Stein *et al.*, 1989; Zhou *et al.*, 1990; Palmer *et al.*, 1993).

Problems arise, however, when the expression levels of the genes upstream and downstream of the IRES are compared. In a retroviral vector, a gene was expressed at higher levels when cloned upstream of an EMCV IRES than when the same cDNA was translated from the IRES (Adam *et al.*, 1991; Sugimoto *et al.*, 1995b). The level of expression of the gene downstream of the IRES is typically 10% of that of the upstream gene (Kaufman *et al.*, 1991; Dirks *et al.*, 1993; Wakimoto *et al.*, 1997; Zhu *et al.*, 1999; Flasshove *et al.*, 2000; Paquin *et al.*, 2001). In experiments with bicistronic plasmid vectors comparing the expression of genes placed upstream and downstream of the EMCV IRES it was shown, *in vitro* and *in vivo*, that the gene downstream of the IRES was expressed at a lower level (generally 20-50%) in relation to the first gene (Mizuguchi *et al.*, 2000). These values varied depending on cell types and reporter genes used. Hennecke *et al.* (2001) have suggested that the coding sequence of the first cistron affects the strengh of downstream IRES-dependent translation. It means that protein expression levels directed by the IRES cannot be reliably predicted in base of the particular IRES element and cell type chosen. The choice of genes and their arrangement may have an important role in modulating IRES activity. None-the-less, IRESes have been the best available way to ensure the co-expression of two genes until now.

Another disadvantage of IRESes is their relatively large size (about 0.5 kb), similar or longer than commonly used promoters such as those derived from cytomegalovirus or simian virus 40. Furthermore, in the case of the Hepatitis C virus (HCV) IRES, part of the N-terminus of the original downstream viral protein (10 amino acids) is required for the full IRES activity, implying additional sequences need to be cloned and, necessarily, a final fusion protein product expressed (Reynolds *et al.*, 1995). A similar situation has been recently described affecting the encephalomyocarditis (EMCV) IRES (Qiao *et al.*, 2002). This is mainly a problem in small sized vectors such as adeno-associated virus (AAV), but not in other vectors or in plasmids, where the IRESes have been also used in the last years. However, since the description of the first IRES from picornaviruses, new IRESes have been described, with decreasing sizes, in genomic mRNAs or other viral genomes. Retroviruses or insect RNA viruses have IRESes of around 0.2 kb. In the polycistronic mRNA of the human c-*myc* proto-oncogene there is a 80 nt region with IRES activity (Nanbru *et al.*, 2001). But what has being really surprising is the recent isolation of "mini-IRES" sequences smaller than 0.1 kb. A 9 nt sequence from the 5' leader sequence of the mRNA encoding Gtx homeodomain protein has been identified with a significant internal initiation activity, that can be increased hundreds of times by linking 10 copies, performing even better than the classical EMCV IRES (Chappell *et al.*, 2000; Reiser *et al.*, 2000). This system has two important advantages over the traditional IRES: first, there is a substantial reduction in size and, in addition, it is possible to control the translational activity by varying the number of units. After that, new small sequences were identified from random pools (Owens *et al.*, 2001; Venkatesan and Dasgupta, 2001). However, in the case of using sequences that need to be repeated to obtain a useful activity, it is important to remember that in some vectors, such as retroviruses, direct repeats tend to be unstable (see below). A completely different approach from the IRES is to use the iron response element (IRE) derived from ferritin mRNA. Introduced between two genes, three copies of this sequence (less than 0.2 kb) can drive translation of the downstream cistron, when a fusion protein of the translation eukaryotic initiation factor G-iron regulatory protein (eIF4G-IRP) is provided in *trans.* The IRP binds the IRE, and the eIF4G mediates internal ribosomal entry (De Gregorio *et al.*, 1999). However, the IRE/IRP-4G-mediated translation is only about 5% as efficient as cap-dependent translation (less than the currently used IRESes).

A disadvantage of the IRES in comparison to promoters is that there is no known type of regulation or specificity of IRES elements. The studies with picornaviral IRESes showed only some degree of cell type specificity, the encephalomyocarditis (EMCV), foot-and-mouth disease virus (FMDV) and hepatitis C virus (HCV) IRESes commonly used in general vectors showing the least sensitivity to cell-type (Borman *et al.*, 1995; 1997; Roberts *et al.*, 1998; Shaw-Jackson and Michiels, 1999; Harries *et al.*, 2000). One way to modify the IRES activity is to mutate the IRES sequence to enhance or reduce its translational activity. Normally, mutations and/or deletions in the IRES lead to a reduced activity, but in some cases, mutations in the IRES can enhance its activity (reviewed in Fussenegger *et al.*, 2001, and references within). In addition, it is possible to clone the gene downstream of the IRES in a suboptimal position and/or in an AUG context far from the Kozak consensus sequence to produce lower translation. An increase in the IRES activity is helpful to obtain expression comparable to cap-dependent translation, whereas a partially disabled IRES has been also used to obtain a low production of the protein downstream. If a selection marker cloned in this way is the last cistron, a strong selection ensures survival of cells expressing the gene upstream of the IRES in a large excess in relation to the downstream marker. This arrangement of genes is called "autoselective configuration". The reason for this effect is that only cells harboring the

vector in chromosomal integration sites with overall high transcriptional activity, will express enough protein to resist the selection (for a review, see Fussenegger *et al.*, 2001).

Of great interest is the possibility to regulate IRES activity, as in the case of promoters. One step in this direction has been achieved by Poyry *et al.* (2001), introducing an iron responsive element (IRE) between an AUG at the 3' boundary of the FMDV or poliovirus (PV) IRES and the main functional internal translation initiation site downstream. In these IRESes, many ribosomes scan from the 3' end of the IRES to a downstream AUG, that is the real site of translation initiation. Insertion of the IRE element leads to a regulation of translation sensitive to the concentration of IRP. However, the range of regulation is limited (three to fourfold) and total inhibition of translation is not possible (as is the case when the IRE element is regulating translation by a ribosome scanning from the cap). Recently, a new range of specificities and potentials for regulation has emerged, in particular from the cellular IRESes. Remarkably, IRESes have been found in mRNAs that encode growth factors, oncogenes, proteins involved in apoptosis and cell proliferation, etc., and are able to sustain translation during situations of cellular stress: hypoxia, apoptosis, heat shock, oxidative stress, differentiation, γ-irradiation or mitosis (reviews: Sachs, 2000; Holcik *et al.*, 2000; Galy, 2000). IRES activity requires "IRES *trans*-acting factors" (ITAFs) provided by the cell. Different ITAFs are required by different IRESes, explaining specificity of the IRES and regulation at different stages of cell cycle, development, and stress (reviewed in Hellen and Sarnow, 2001; Martínez-Salas *et al.*, 2001; Vagner *et al.*, 2001).

The success of the IRES to co-express two genes and its wide acceptance among researchers has lead to the design of several IRES-containing families of vectors, such as retroviral vectors (Levenson *et al.*, 1998). IRESes have been introduced successfully in all types of viral vectors. Furthermore, some IRES-vectors are now commercially available. For a review on the biotechnological uses of IRES, see Martínez-Salas (1999).

Reinitiation

Two ORFs present in one mRNA are translated sequentially. Scanning ribosomes, after translation of the first ORF, reinitiate their activity and proceed to translate the second ORF. These vectors are able to co-express two proteins from the same mRNA, avoiding the necessity to generate two mRNAs, by introducing a "spacer" between the two ORFs. The spacer must be of about 100 nt and free of AUGs and secondary RNA structures impairing ribosomal scanning (Kozak, 1987; 1989; 1992). The main advantages here are a reduction in the size of the vector and the avoidance of all the inconveniences from introducing the signals discussed above.

Unfortunately, reinitiation is a very inefficient process. This means that the second protein is produced in a very low amount (about 1%) in relation to the first (Kaufman *et al.*, 1987; Dirks *et al.*, 1993; Mizuguchi *et al.*, 2000). This situation can be useful for expressing a selectable marker in the second position using the "autoselective configuration" (Gansbacher *et al.*, 1990; Levine *et al.*, 1991; Cosset *et al.*, 1995). What has not been described to date is a way to regulate the reinitiation mechanism. It has been shown that the efficiency of reinitiation decreases with the increase in time expended in translation of the upstream ORF (dependent upon the length of the cistron and the presence of RNA secondary structures) (Kozak, 2001). Changes in the length of the spacer (or the introduction of certain specific sequences) can make reinitiation even more inefficient, and further down-regulate the downstream gene. Interestingly, cauliflower mosaic virus contains a polycistronic RNA. Recently, it has been described that its translation by reinitiation is stimulated by a viral transactivator, overcoming the problems of this co-expression strategy (Park *et al.*, 2001 and references within).

Self-Processing Peptides

The proteins are fused with a self-processing peptide or CHYSEL (cis-acting hydrolase element) inserted in frame that undergoes a co-translational "self-cleavage". As in the previous strategy, there is only one mRNA but two proteins are produced upon translation. Here there is only one translational initiation event: the genes and the CHYSEL being fused in one ORF. There is not a termination of translation after the first gene and the ribosomes do not need to reinitiate their translating activity. This is due to the small CHYSEL sequence inserted in frame between the two genes (the minimal sequence comprises 19 amino acids, 57 nt). It produces a disruption of the translational process in the ribosome, and release of the first protein fused to the CHYSEL, while the ribosome continues translating the second gene. The proposed model is a "skipping" in the synthesis of one particular peptide bond (Gly-Pro) in the C-terminus of the CHYSEL sequence (Donnelly *et al.*, 2001a). This new co-translational activity was first discovered in the 2A peptide of a virus from the picornavirus group (foot-and-mouth disease virus, FMDV; Ryan *et al.*, 1991; Ryan and Drew, 1994). This peptide is active in heterologous contexts in all different eukaryotic cell-types tested to date (mammals, insects, plants, fungi, yeast), but not in prokaryotic cells (Donnelly *et al.*, 1997). Recently, this activity has been discovered in other viruses (Donnelly *et al.*, 2001b).

The main advantages of this strategy are the small size of the CHYSEL sequence compared with internal promoters or an IRES and, as happens when the two genes are fused (see below), that co-expression of both genes is ensured. However, an imbalance (due to an excess of the upstream protein) and a small amount of uncleaved fusion protein have been shown *in vitro* (Donnelly *et al.*, 1997; 2001a). This has not been a problem in achieving high levels of expression of the genes cloned upstream and downstream of the CHYSEL. Introduction of additional sequences from the FMDV upstream of the 2A (the last amino acids of the capsid protein 1D) has show that it is possible to achieve, even *in vitro*, a 100% cleavage and an equimolecular co-expression (Donnelly *et al.*, 2001b). A very recent report has shown that, not only the CHYSELs are able to produce reliable co-expression of two genes, but also that a gene downstream of a CHYSEL is expressed at a higher level than

if it was downstream of a PV or FMDV IRES (Klump *et al.*, 2001) or EMCV IRES (Furler *et al.*, 2001). This trend agrees with what we have observed using the EMCV IRES (Fig. 3A in de Felipe and Izquierdo, 2000). A potential problem is that the CHYSEL will be fused to the C-terminus of the first protein, and the second protein will have an extra Pro at the N-terminus. For the moment, all the different genes fused to the CHYSEL have shown activity. The same has been observed for the second proteins (with a N-terminal Pro), as expected (Varshavsky, 1992; 1996). Interestingly, as antibodies against FMDV 2A have been developed, this tag can be used to detect the gene cloned upstream (Ryan and Drew, 1994).

Until now, no system of regulating the activity of the FMDV 2A peptide has been described. Only the peptide itself and the ribosomal translational machinery seem implicated in this new activity, reducing the possibilities of regulation. From the recent report of Donnelly *et al.* (2001b), it seems possible to use different truncated and/or mutated 2A to obtain various degrees of "cleavage" of the fusion proteins. This has been confirmed with the study of several FMDV 2A-like sequences from different organisms, where a wide range of different balance ratios and amounts of fusion protein have been reported *in vitro*. These differences among FMDV 2A-mutants and deletions and FMDV 2A-like sequences are the only possibility, for the moment, to achieve regulation in this co-expression strategy. Deleted versions of FMDV 2A have been used to control the ratio of fused to cleaved proteins in the potato virus X (PVX) (Santa Cruz, *et al.*, 1997). As the gene downstream of the 2A is expressed at lower levels than the first, it can be also an advantage to introduce in the second position a marker gene to design plasmids over-expressing the first gene as in the two previous strategies (autoselective configuration). It seems feasible to have a set of 2A sequences to achieve different balances, as has been mentioned, between the three potential products: the fusion polyprotein and the two cleaved proteins, taking into account the differential rates of the three potential situations; readthrough, stop and reinitiation.

The rare bibliography using this strategy in the middle 90's (for early reviews, see Ryan and Flint, 1997; Ryan *et al.*, 1998) has increased in the last years with applications to different plasmid and viral vectors in various living organisms (for a recent review, see Halpin *et al.*, 2001; an updated list of CHYSEL-related publications is available at cbms.st-and.ac.uk/academics/ryan/Index.htm).

Proteolytic Processing

Two proteins are fused with a sequence inserted in-frame that is the cleavage target of a protease. After translation, the polyprotein is cleaved by the protease to produce two proteins that remain fused with part of the protease target peptide. There are few examples of these precursor polyproteins. Some are from polypeptide prohormones in higher eukaryotes (reviewed in Douglass *et al.*, 1984; Smeekens, 1993) and also we can find examples in prokaryotes, such as the two different subunits of *Escherichia coli* penicillin acylase which are derived from a single precursor (Böck *et al.*, 1983; other examples are discussed in Luke and Ryan, 2001). Picornaviruses and flaviviruses, for example, have all the proteins linked in a single ORF. The precursor polyprotein is then cleaved by viral protease/s encoded in a portion(s) of this polyprotein. This strategy of co-expression is similar to the self-processing method in many ways, the main difference being that proteolytic cleavage is really produced in a polyprotein previously released from the ribosome, so the production of two proteins is an entirely post-translational process. The other main difference is that the process involves a third element apart from the polyprotein and the ribosome, the protease.

The localization of the protease enables us to divide this strategy in two subgroups, as the protease can be provided in *trans* or in *cis* with relation to the target polyprotein. Cellular polyprotein examples generally use an endogenous protease provided in *trans*. The protease in some groups of viruses, such as picornaviruses or retroviruses, is included in *cis*. There are theoretical advantages and disadvantages of each system. A viral protease encoded in the vector can be a very attractive possibility to make an autonomous vector useful in any target cell. However, the protease will use part of the precious coding capacity of the vector and it must retain some cleavage activity in the fusion polyprotein, at least until some free protease is produced. Unfortunately, the target sequences of viral proteases are not always very easily defined or cloned. Viral proteinases are often toxic, as they may cleave cellular proteins. Another possibility is to find a suitable endogenous protease, and clone it as part of the polycistronic vector. The other potential alternative is to use a well-defined target sequence of a cellular protease provided in *trans*. It is important to mention a common requirement of all proteolytic processing strategies, as with the CHYSEL, the three elements (two genes and the target sequence) must be cloned in frame.

For the moment, few laboratories have employed these strategies. As an example of the *cis* protease version of the strategy, we can examinate the use of the nuclear inclusion proteinase (NIa) from tobacco etch potyvirus (TEV) (Marcos and Beachy, 1994; 1997; Ceriani *et al.*, 1998). A similar system has been developed using the tobacco vein mottling potyvirus (TVMV) NIa (von Bodman *et al.*, 1995). These are viral proteinases responsible for processing the viral polyprotein. They have been used to co-express two different proteins flanking them at their N- and C-termini. The cleavage sites were cloned in-frame between the proteins and the NIa protease. However, there have been some problems with this strategy. First, the expression levels in plants were low. The expected equimolecular yield of the two proteins of interest was not achieved. It has been shown that the protein cloned at the C-terminus was produced in a lower amount than the N-terminal protein. Marcos and Beachy (1997), mentioned possible premature termination of translation as a mechanism for imbalance. The reason may be the nuclear localization signals (NLSs) present in the NIa, targeting the polyprotein to the nucleus. The removal of NLSs has improved results, but there is still a positional effect of the genes in the cassette (Ceriani *et al.*, 1998). In this last report, up to three genes were co-expressed *in vitro* in addition to the NIa protease. A different system has been recently

developed using the Human rhinovirus (HRV14) 2A protease (Cowton, 2000). This protease is cloned in the 3' end of a construction with the blue fluorescent gene (BFP) as an N-terminal fusion. The HRV $2A^{Pro}$ produces a co-translational cleavage in its own N-terminus. In order to expand this vector to a tricistronic, the glucoronidase (GUS) gene was cloned N-terminal to the BFP with a small 14 amino acid linker between them derived from eukaryotic initiation factor 4G (eIF4G, a cellular protein target of HRV14 2A). As a result, the polyprotein is cleaved and the three proteins are obtained in both bacteria and in plants. It is easily conceivable that this strategy can be expanded to obtain even more complex polycistronic vectors. Unfortunately, the toxicity of HRV14 $2A^{Pro}$ protease makes it impossible to be used in mammalian cells.

There is an example in plants of the *trans* protease proteolytic processing strategy. Two proteinase inhibitors have been linked as a polyprotein. The spacer was derived from a plant metallothionein-like protein, and the expected cleavage products were detected (Urwin *et al.*, 1998). In the mammalian context, Gäken *et al.* (2000), have reported the successful use of a furin target sequence to cleave different combinations of cytokines (IL-2, IL-4, IL-12) and the trasmembrane protein B7.1, as fusion proteins in the context of a retroviral vector. They have called this system "fusagen". Furin is a highly conserved ubiquitous eukaryotic endoprotease localized in the lumen of the *trans*-Golgi apparatus that processes precursor polyproteins secreted through the constitutive pathway. The target consensus sequence is Arg.X.Arg/Lys.Arg. Normally Gly residues are introduced flanking it to facilitate access of the furin to its target sequence. In addition, they have reported the use of a tricistronic plasmid containing the two subunits of IL-12 separated by IL-2 and two furin cleavage sites. The main advantage of this strategy is that the protease target sequence is very small (smaller than the CHYSEL), co-expression is ensured (as with CHYSEL) and the cleavage is in the center of the sequence, so it leaves smaller tags on the proteins expressed (but on both of them).

However, it is important to be aware of several limitations derived from the use of furins. The first is that as these proteins reside in the Golgi, this strategy is only useful for co-expressing proteins targeted to the cellular secretion pathway (transmembrane or extracellular proteins); this means that cytoplasmic proteins cannot be expressed in this way. Another important thing to take into account is that as the furin activity is situated in the lumen of the Golgi, the cleavage sequence must be exposed to the interior of this subcellular compartment. This means that two extracellular proteins or one extracellular and one transmembrane protein can be easily co-expressed and cleaved using furins. However to process one transmembrane and one extracellular proteins or two transmembrane proteins, the furin target sequence must be exposed to the lumen of the Golgi, so the number of transmembrane sequences and the positions of the N- and C-termini of the proteins are very important. To illustrate this point, Gäken and co-workers have shown the successful cleavage of the construction containing IL-2 (soluble extracellular protein) and B7.1 (transmembrane protein with an extracellular domain in the N-terminus and a short intracellular C-terminus). When the order of the genes was reversed, the furin cleavage sequence and the IL-2 were positioned in the cytosolic side of the Golgi and cleavage was not produced. Two extracellular proteins were also successfully co-expressed and cleaved (IL-4 and IL-2). It is also important to mention that, since this strategy relies on the enzymatic activity of the furins, the kinetic properties of these enzymes are important. Gäken *et al.* (2000), have described the saturation of furin-mediated cleavage, that can be circumvented by over-expressing the furins (from a furin expression vector). It is potentially possible to simplify this solution by including *in cis* the furin gene in the same polycistronic vector. This is a solution that resembles the role of proteases in the picornaviruses and other viruses that use polyproteins. This would also be a way to avoid the low endogenous expression levels in certain cells types (e.g. COS cells: discussed in Gäken *et al.*, 2000). Finally, the authors mention that the percentage of the fusion protein cleaved is approximately 50% and this can be affected by the amino acid sequence of the cleavage site. Although this strategy is mechanistically much simpler than the CHYSEL, the proportion of fusion protein is higher. More studies, including the over-expression of furins, will be necessary to fully characterize this system in order to determine if it is possible to improve this figure.

There are two theoretical possibilities of regulating this mechanism of co-expression. One relies on variations of the target cleavage sequence that may regulate the cleavage rate of the fusion protein; the other is dependent on potential regulation of the activity of the protease to cleave the target site in the fusion protein substrate. In the case of furins, potential regulation may arise from the normal cellular regulation of this particular family of proteases. Furins are ubiquitously expressed endoproteases, but it would be interesting to use, in a similar way, endoproteases with tissue (or other) specificities. Furthermore, the search for new endoproteases will lead to the use of not only Golgi-associated proteases, but also others in the cytosol or in other compartments. This may allow development of cleavage activity in specific subcellular locations that may be of interest for targeting of certain proteins. It would also be desirable to use well known regulatable and/or specifically expressed proteases.

Fusions

This is the most obvious way to connect two genes, an in-frame fusion. Many proteins have been fused in this way and there is a long tradition of fusions using popular markers such as βGAL or GFP. The new chimeric polyprotein will ideally display a dual activity. In the field of gene therapy, such fusions include MDR1+ADA (Germann *et al.*, 1989), HSV1TK+CD (Rogulski *et al.*, 1997; Blackburn *et al.*, 1998; 1999), HSV1TK+EGFP (Degreve *et al.*, 1998; Loimas *et al.*, 1998; Di Florio *et al.*, 2000; Paquin *et al.*, 2001), HSV1TK+BLE (Kuiper *et al.*, 2000), CD+UPRT (Erbs *et al.*, 2000; Chung-Faye *et al.*, 2001); four additional fusions are available containing the negative selectable CODA protein fused to a panel of positive selectable proteins: PAC, HPH, BSD and NEO (Karreman, 1998), and fusions of HSV1*tk* to the same selectable genes are reviewed by Karreman (2000). A large number of fusions has been

produced involving screenable/selectable markers that can be very useful in tracking the fate of the vectors in cells and animals or humans: GUS+NEO (Datla *et al.*, 1991), βGAL+NEO (Friedrich and Soriano, 1991), βGAL+BLE (Baron *et al.*, 1992), ADH+BLE (ADH, alcohol dehydrogenase, Gautier *et al.*, 1996), GPT+GUS (Cao and Upton, 1997), EGFP+BLE (Bennett *et al.*, 1998); aminoglycosidase 3'-adenyltransferase+GFP (Khan and Maliga, 1999), EGFP+PAC (Abbate *et al.*, 2001), (the gene order was also reversed in some cases).

An advantage of this strategy is that it is a simple way to co-express two genes, as it is not necessary to include intervening sequences. Sometimes a short linker is inserted between both proteins comprising only a few amino acids, ideally several Gly residues to allow more free spatial freedom to both proteins, as in some fusions of the two subunits of IL-12 that enabled researchers to produce a single-chain IL-12 protein (Anderson *et al.*, 1997; Lee *et al.*, 1998; Harries *et al.*, 2000). Another advantage of using fusion of genes is that this strategy ensures co-expression of both genes, as for the transcriptional/translational machinery, there is only one ORF.

There are, however, important disadvantages. One is the possibility of losing or lowering the activity of one or both fused proteins (as in Karreman, 1998; Thomas and Maule, 2000). A more difficult problem to solve is presented when each protein has to be targeted to a different subcellular compartment. Paquin *et al.* (2001), have compared retroviral vectors containing GFP gene alone, the GFP-HSV1TK fusion and the TK-IRES-GFP cassette. The fluorescence from the chimeric protein was 30 times more intense than the GFP expressed downstream of the IRES, but 20 times lower than GFP alone. Furthermore, as the HSV1TK has nuclear localization signals, the fluorescence of the chimera was mainly localized in the nucleus, instead of the normal cytoplasmic fluorescence from GFP. It is not a problem in this case, but it can be an inconvenience with other proteins, where different and incompatible subcellular localizations are required (for an example, see Kotlizky *et al.*, 2000; Klump *et al.*, 2001).

Many different strategies have been developed to co-express two genes from vectors in the last twenty years. May this experience help us to develop efficient polycistronic vectors?

HOW TO MOVE FROM BICISTRONIC TO POLYCISTRONIC VECTORS?

The diverse strategies of co-expression described above (summarized in Table **1**) may be arranged in different groups based upon the mechanism used to produce the two proteins involved (Table **2**):

Multiple Transcripts

Transcriptional mechanisms such as splicing signals and internal promoters generate initially one or two mRNAs, but finally, both produce two mRNAs (Table **2**, rows A and B).

Single Transcript

In this case there is only one mRNA to translate. As it is necessary, in the retroviral vectors, to produce a genomic mRNA from the 5'LTR to the 3'LTR, it is also desirable to use the same molecules to translate the genes cloned in it, without producing additional mRNAs. The differences among the several translational/post-translational strategies available lie in the moment of the translational process when the two proteins are produced:

- Translation initiation: reinitiation and IRES have two AUGs to initiate translation. That means that in this strategy two ORFs are necessary (Table **2**, row C).
- Co-translational: during the translation of a unique chimeric ORF, the CHYSEL sequence produces the release of the first protein, without affecting the ribosome procesivity into the downstream cistron(s) (Table **2**, row D).
- Post-translational: after the translation of a unique chimeric ORF, a protease cleaves the polyprotein at a target amino acid sequence introduced between the two proteins (Table **2**, row E).

Table 2. Classification of the Expression Strategies Based on the Mechanism Used to Co-Express N Number of Proteins

	Expression strategy	Promoters	mRNAs	ORFs	Proteins	Co-expression mechanism		
A	Internal promoters	N	N	N	N	Transcription		Multiple transcripts
B	Splicing	1	N	N	N			
C	Internal initiation (IRES)	1	1	N	N	Translation initiation	Translation	Single transcript
	Reinitiation	1	1	N	N			
D	Self-processing peptides (CHYSEL)	1	1	1	N	Co-translation		
E	Proteolytic processing	1	1	1	N	Post-translation		
F	Fusions	1	1	1	1	Chimeric protein translation		

N=2, 3, 4, etc.

The best way to achieve the maximum co-ordination among two or more genes, is to have a single ORF. This means that we need only one promoter, one mRNA, one site of translation initiation (ORF) and one chimeric protein (Table **2**; row F). In fact, this is a monocistronic vector expressing a single chimeric protein. This is the most simple and compact way of expressing several proteins, but it has a very limited utility, even in the case of fusing only two proteins. As we have seen it is a possible, but not a good solution in all cases, because normally it is necessary to obtain discrete proteins. For polycistronic vectors it will produce a large polyprotein, potentially non-functional in all its domains.

To obtain each protein separate from the others using a polycistronic vector, the ideal would be to obtain an scheme as close as possible to row F in Table **2**. The smallest modification is to change the number of proteins from 1 to N (Table **2**; rows D and E). Until now, other strategies have been tested. Those in rows A and B in Table **2** have produced a large number of problems even when N=2 and have been used only in few occasions with N>2. On the other hand, our proposal of a system as in rows D and E in Table **2**, has been tested successfully with N=2. There is nothing that implies that it can not be extended to obtain polycistronic vectors using several CHYSEL and/or Fusagen strategies (a plasmid with three genes and two fusagen has been reported by Gäken *et al.*, 2000; we have successfully constructed a tricistronic plasmid vector using two different CHYSELs, unpublished, similar to the one mentioned in Halpin *et al.*, 2001).

However, the most widely used strategy in the polycistronic field is with the mechanism in Table **2**, row C. The use of the reinitiation strategy has been very limited, but the use of two IRESes to obtain tricistronic vectors has become routine in recent years in spite of the problems discussed. What has been the experience from these real polycistronic vectors constructed to date?

WORKING WITH POLYCISTRONIC VECTORS: BASIC AND APPLIED SCIENCE

With the tools available in the 1980's, a limited number of polycistronic vectors was constructed. Two internal promoters, in addition to the promoter in the 5'LTR, were used to construct a triple gene retroviral vector that suffered severe rearrangement (Emerman and Temin, 1984a). Overell and co-workers (1988), had a low expression of the second and third genes (relative expression values from the promoters were LTR (42): SV40 (6): HSV1*tk* (1)). In spite of these results, Paulus and co-workers (1996) were successful with a new retroviral vector using two internal promoters. Recently, Reiser and co-workers (2000), constructed a successful triple gene lentiviral vector using internal promoters and splicing signals. Another strategy tested was that of reinitiation, but the second gene was expressed in the proportion of 1/100 in relation to the first, expression of the third gene was even lower, 1/300 (Kaufman *et al.*, 1987).

By the middle of the 1990's, the IRES strategy dominated the field as the paradigm for co-expression of two genes. One of the initial articles reporting the use of the IRES to co-express two genes (Jang *et al.*, 1989) also reported the construction of two complex tetracistronic plasmids (one with one IRES and two identical internal promoters and the other with a reinitiation, an IRES and an internal promoter). The discovery of the IRES elements lead soon to construction of polycistronic retroviral vectors, comparing related bi- and tricistronic vectors (Morgan *et al.*, 1992). The first IRESes used in vector design were derived from poliovirus (PV) and encephalomyocarditis (EMCV), so the idea was to combine both in a tricistronic vector. The results were encouraging: co-expression of all the genes, good viral titers and stability of the integrated proviruses.

Other groups developed polycistronic vectors using different designs. It is possible to design a polycistronic vector using a single co-expression strategy (e.g.: IRESes) or a mixed strategy. Mixed strategies can combine either different co-expression mechanisms (as one transcriptional and one translational: internal promoter and IRES) or strategies belonging to the same co-expression mechanism (e.g., internal promoter and splicing signals). Mixed strategies have been common in the 1990's, in particular using an internal promoter and an IRES for a tricistronic vector, probably because these sequences were well described and freely available (Morgan *et al.*, 1992). This design has been also used in the transcriptionally regulated systems that rely on the regulation of an internal promoter (see below). However in this design there are at least two promoters, so all the problems related with this situation in the two-gene vectors could be potentially relevant for these three-gene ones, particularly in a retroviral context.

To avoid these potential problems, many groups have continued using the IRES for bicistronic vectors. Some groups have, however, made a variation in the model of Morgan and co-workers using two copies of the same IRES (EMCV) (Zitvogel *et al.*, 1994; Tahara and Lotze, 1995; Tahara *et al.*, 1995; Metz *et al.*, 1998; Okada *et al.*, 1999; 2000; Gautam *et al.*, 2000; Laufs *et al.*, 2000). Some of these groups have experienced problems in the co-expression of all the inserted genes. It may be advisable to avoid direct repetition of sequences of some 0.5 kb, in particular in retroviral vectors, where there are many instances of deletions by homologous recombination with repetitions of sequences comprising just a few nucleotides (Omer *et al.*, 1983; Rhode *et al.*, 1987; Pathak and Temin, 1990; Junker *et al.*, 1995; Zhang et Sapp; 1999; Delviks and Pathak, 1999; Li and Zhang, 2000; Logg *et al.*, 2001b). Recombination between identical sequences can also be observed at the proviral DNA level, although at much lower frequency (Li and Zhang, 2000). Recombination at the DNA level may also affect repetitions in integrating non-retroviral vectors, such as adeno-associated vectors, as well as cellular genomic repeats.

In the light of these problems it is advisable to maintain the approach of Morgan *et al.* (1992), if two or more IRESes are used, particularly in the retroviral context. As the picornavirus IRES sequences are very different, it is possible to clone two different IRESes in the same vector, without the risk of recombination, to obtain a tricistronic retroviral vector. Fan *et al.* (1999), and Qian *et al.* (2001), have

combined the PV and EMCV IRESes while Laufs *et al.* (2000), and Wen *et al.* (2001), have combined the EMCV and FMDV IRESes successfully. The work of Borman *et al.* (1997), has showed that picornaviral IRESes from FMDV, HCV and EMCV are less sensitive to the cell type used than the IRESes from picornaviruses such as PV. For wide use of the polycistronic vectors, IRESes from cardio and aphthovirus may be recommended. A mixed translational strategy was used to construct tricistronic retroviral vectors combining one CHYSEL and one IRES in de Felipe and Izquierdo (2000). A pure translational strategy for a tricistronic retroviral vector using only two protease cleavable sequences is described in Gäken and co-workers (2000), and we have successfully obtained a tricistronic plasmid using two CHYSELs (unpublished).

When the level of complexity increases from tricistronic to tetracistronic retroviral vectors, few reports are found in the literature. Two copies of PV IRES combined with one EMCV IRES have been used in the plasmid vector family pQuattro, derived from the tricistronic vector family pTRIDENT (for reviews: Moser *et al.*, 2000; Fussenegger, 2001) and three identical IRESes have been combined in another plasmid vector to obtain a tetracistronic mRNA (Dr. S. Hobbs, personal communication). In the retroviral context, two identical IRESes and an internal promoter were used by Wang and co-workers (1996) and Sun and co-workers (2000). The first report described recombinations in the vector that impaired its use. This problem did not appear in the second report, but was described by Le Guern and co-workers (1994), in a vector with three copies of the cellular human immunoglobulin heavy chain-binding protein (BiP) IRES. The tetracistronic retroviral vectors described in de Felipe and Izquierdo (2000), used a mixed strategy relying on translational mechanisms (a CHYSEL and one or two different IRESes). All vectors showed good titers and were able to co-express the genes cloned *without* rearrangements (individual cell clones showed the feasibility of obtaining cells co-expressing up to four genes).

Finally, vectors co-expressing five genes have been derived from HSV and vaccinia using several promoters (Krisky et al, 1998; Carroll *et al.*, 1998). Our last experiments combining a CHYSEL and three different IRESes showed positive results for a pentacistronic retroviral vector (De Felipe and Izquierdo, submitted for publication).

As the bibliography about polycistronic vectors is growing, it is becoming clear that they are not merely an academic exercise of searching for complexity. Positive results with test vectors using marker genes have lead researchers to design polycistronic vectors for practical purposes. Some reports are more focused in taking advantage of these vectors to co-express several genes. In other cases, the aim is gene therapy. Polycistronic vectors have been used in several fields:

Co-Expression of Several Genes to Obtain a Combined and/or Synergistic Effect

Researches in cancer gene therapy have used several strategies to destroy malignant cells by the introduction of different genes (suicide, immunoactive, anti-angiogenic, etc.). Initial results are encouraging but not yet good enough for a human therapy, and interest in combined therapies has been rising in recent years. This interest may be considered as a parallel process to the increasing attention paid to combined therapies using conventional pharmacological compounds (Lowenstein *et al.*, 1999). Many different combinations of viral antigens and immune modulators have been widely used, in particular with vaccinia-derived vectors, for immunization (see Johnson, *et al.*, 2001 and references within). Some combinations of anti-tumor genes have been tested in bicistronic vectors (without marker genes) or in polycistronic vectors (two or more genes plus a marker gene), as shown in Table **3**. Of particular interest are the vaccinia vector containing five immunoactive and marker transgenes described by Carroll *et al.* (1998; B7.1 or MHA, the two subunits of IL-12; *lacZ* and the selection gene, *gpt*) and the HSV vector described by Krisky et al, 1998, expressing a combination of five suicide and immunoactive genes (IL-2, GM-CSF, B7.1, HSV1TK and IFNγ).

Another approach is to develop resistance in some cell types, particularly hematopoietic cells, to the toxicity of anticancer drugs. Bone marrow suppression is the main toxicity problem in chemotherapeutic treatments of cancer. Increasing the chemoresistance of bone marrow cells may protect them against chemotherapy and allow a more aggressive treatment without toxicity. As combined cancer treatments use multiple drugs, a combined gene therapy approach co-expressing several chemoresistance genes is necessary (Table **3**). In addition, a chemoresistance gene can be combined with HSV1TK as a safety tool for selective killing of unintentionally transduced tumour cells, and even as a way of enhancing chemoresistance (Table **3**).

In general, an enhanced therapeutic effect has been observed with these various polycistronic vectors. In spite of this, each particular combination of genes has to be carefully chosen and well tested, as not all researchers have described an enhanced therapeutic effect (e.g.: B7.1 and IL-12; Sun *et al.*, 2000).

The opposite effect is sought in the transformation/ immortalisation of cell lines using oncogenes. Two oncogenes have been combined in a single vector to ensure transformation (Table **3**). In a different approach, Hoshimaru *et al.* (1996), constructed a retroviral vector expressing v-*myc* downstream of a promoter regulated by the tetracycline-controlled transactivator, also cloned in the same tricistronic vector, with the resistance marker *neo*. This vector enabled control of the oncogenic activity of *myc*.

Other combinations have been produced in different contexts, such as in transgenic plants, to confer resistance against plant viruses (Table **3**).

Co-Expression of Genes Coding for Subunits of Hetero-Multimeric Proteins

The initial approaches for gene therapy were based on the introduction of genes coding for monomeric or homo-multimeric proteins. However, many proteins of interest are

Table 3. Co-Expression of Several Genes Using bi- or Polycistronic Vectors to Obtain Combined and/or Synergistic Effects

Combinations of genes	Examples	References
Suicide genes	HSV1TK and CD	Rogulski *et al*., 1997 Blackburn *et al*., 1998 Blackburn *et al*., 1999
Immunoactive genes	GMC-SF and IL-4 IL-12 and B7.1 B7.1 or MHA and IL-12 IL-2 and B7.1 LYM and IL-2 or IL-12 MHC I K^b and IL-2 IL-2 and B7.1	Wakimoto *et al*., 1997 Pützer *et al*., 1997 Sun *et al*., 2000 Carrol *et al*., 1998 Emtage *et al*., 1998 Emtage *et al*., 1999 Qian *et al*., 2001 Mazzocchi *et al*., 2001
Suicide and immunoactive genes	HSV1TK and IL-2 HSV1TK and GM-CSF HSV1TK and IL-7 IL-2, GM-CSF, B7.1, HSV1TK and IFNγ HSV1TK and IL-4	Ram *et al*., 1994 Castleden *et al*., 1997 Pizzato *et al*., 1998 Palù *et al*., 1999 Kuiper *et al*., 2000 Castleden *et al*., 1997 Miller *et al*., 1998 Sharma *et al*., 1997 Krisky *et al*., 1998 Okada *et al*., 1999 Okada *et al*., 2000
Suicide and bystander effect enhancing genes	HSV1TK and Cx43	Marconi *et al*., 2000
Chemoresistance genes	MDR1 and GST pi MDR1 and a mutant DHFR MDR1 and MGMT MGMT and MDR1 MDR1 and ATase MDR1 and a mutant ATase ALDH-1 and MDR1 MDR1 and gamma-GCS MGMT and MDR1	Doroshow *et al*., 1995 Galipeau *et al*, 1997 Suzuki *et al*., 1997 Suzuki *et al*., 1998 Jelinek *et al*., 1999 Baum *et al*., 2000 Wang *et al*., 2001 Rappa *et al*., 2001 Wang *et al*., 2002
Chemoresistance and suicide genes	MDR1 and HSV1TK HSV1TK and DHFR	Sugimoto *et al*., 1997 Mineishi *et al*., 1997
Oncogenes	V-MYC and V-HA-RAS V-MYC and V-HA-RAS SV40 large T Ag and H-RASval 12	Schwartz *et al*., 1986 Overell *et al*., 1988 Wang *et al*., 1996
Viral coat genes	TMV CP and SMV CP	Marcos and Beachy, 1997

hetero-multimeric: immunoglobulins, receptors, membrane channels, interleukins, enzymes, transcription factors, etc. The demand for vectors to express these complex proteins is increasing. It is possible to use several bicistronic vectors (with different markers) as for the two chains of the human major histocompatibility complex class II antigen HLA-DR (Korman *et al.*, 1987; Yang *et al.*, 1987) and for the four chains of the *Torpedo californica* acetylcholine receptor (AchR; Claudio *et al.*, 1989). Four polypeptides have been co-expressed in plants to produce functional antibodies (Ma *et al.*, 1995).

From the difficulties highlighted in some of those reports, it is advisable to use just one polycistronic vector, if the size limit permits. One of the most studied examples is the co-expression of the two subunits of IL-12 using IRES, CHYSEL or fusagen. Some researchers have even co-expressed it with other genes (see Table **3**). Two further examples have been recently described. Fan and co-workers (1998), using a bicistronic AAV vector containing an IRES, have co-expressed the genes of Apolipoprotein A-I (apo A-I) and lecithin-cholesterol acyltransferase (LCAT), constituents of circulating high-density lipoprotein (HDL) particles which play an important role in 'reverse cholesterol transport'. Later, they reported the successful construction of a tricistronic retroviral vector co-expressing these two genes plus *neo* (Fan *et al.*, 1999). Another report from Kawashima and co-workers (1998) describes a tricistronic plasmid co-

expressing *neo* and two subunits (P2X2 and P2X3) of an ATP-gated cation channel.

Co-Expression of Genes Coding for Metabolic Pathways

There have been reports describing the co-expression of small metabolic pathways using polycistronic vectors. With regard to metabolic therapies, Laufs and co-workers (1998), using two different retroviral vectors, expressed the enzymes GTP cyclohydrolase I (GTPCH) and 6-pyruvoyltetra-hydropterin synthase (PTPS). These enzymes are the first and second of a three step metabolic pathway in the biosynthesis of tetrahydrobiopterin (BH_4), an essential cofactor for the aromatic amino acid hydroxylases. Mutations in these enzymes produce several neurological diseases. In a more recent article, Laufs and co-workers (2000), were able to construct a tricistronic vector combining these two genes plus *neo*. The enzymes from this vector reconstructed the metabolic pathway of BH_4 in cells expressing endogenously only the third enzyme, sepiapterin reductase (SR). Another field that can benefit from these advances is Parkinson's disease. Shen and co-workers (2000), have reported a behavioral recovery in Parkinsonian rats after a triple transduction with three AAV vectors expressing tyrosine hydroxylase (TH), aromatic-L-amino-acid decarboxylase (AADC), and GTPCH. A polycistronic approach seems, therefore, feasible. Wang *et al.* (2001), have constructed a HSV-1 amplicon co-expressing TH and AADC.

The reconstitution of a metabolic pathway may, however, have other utilities apart from metabolic therapies. Adachi and co-workers (2000) and Chung-Faye and co-workers (2001), have introduced in an adenoviral vector the genes for *Escherichia coli* cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT). Traditionally, CD was used to convert the prodrug 5-fluorocytosine (5-FC) into the toxic compound 5-fluorouracil (5-FU). The combination of CD and UPRT increased the killing of rat tumour cells in culture and *in vivo* (similar results were obtained with the *S. cerevisiae* versions of those genes; Erbs *et al.*, 2000). In a different context, a small metabolic pathway has been reconstructed in plants co-expressing enzymes from the mannityl opine biosynthetic pathway: mannityl opine conjugase gene (mas2) and mannityl opine reductase (mas1), using a protease-mediated polycistronic vector (von Bodman *et al.*, 1995).

Regulated Systems

The availability of tricistronic vectors has enabled some groups to develop regulated systems encoded by only one vector. The basic scheme is one gene as a marker, a second gene for the regulating protein and a third gene for the regulated protein. As the best studied regulated systems operate at the level of transcription (regulatable promoters), these were the first systems to be introduced in tricistronic vectors. A good example is the tetracycline system that was introduced in retroviral vectors few years ago (Paulus *et al.*, 1996; Iida *et al.*, 1996; Hwang *et al.*, 1996; Hoshimaru *et al.*, 1996; Watsuji *et al.*, 1997). Some promoters, however, lose their properties in the context of retroviral vectors (Paulus *et al.*, 1996). Translational systems can be used to link the marker and the regulating protein, but as expression of the regulated protein depends upon an internal promoter, it is not possible to construct such a vector with only one RNA. Presently it is at the transcriptional level, mainly using appropriate enhancer/promoters, where it is possible to find regulated and specific systems (development, stress response, differentiation, cell cycle, cell-type, species-specificity, etc.). To eliminate internal promoters it will be necessary to develop regulated and specific systems at the translational level. As discussed above, it may be possible to regulate the activity of IRESes, but further development is required. The possibilities of regulation with other translational/post-translational co-expression strategies have not yet been explored.

However, as the vectors grow in size and complexity, a question arises: what are the limits for polycistronic vectors?

THE PACKAGING SIZE LIMIT

One of the mayor difficulties in modifying viruses for biotechnological purposes is the limited coding capacity of these organisms. The most common viruses used in biotechnology can only accommodate small inserts. The only way to overcome this limitation is to use defective recombinant viruses as vectors, with deletions in sequences that can be provided in *trans* (such as genes coding for capsids and envelop proteins). However, the overall packaging size limit remains a major limitation (reviewed by Walther and Stein, 2000; Kay *et al.*, 2001).

Retroviruses have genomic sizes from 7.5 to 9.5 kb, varying from one group to other: 7.7 kb for spleen necrosis virus (SNV), 8.3 kb for Moloney murine leukemia virus (MoMLV), 9.3 kb for Rous sarcoma viruses (RSV) (reviewed by Weiss *et al.*, 1985). In the widely used murine retroviruses, mutations in the polyadenylation signal that produced readthrough of the 3'LTR and long RNAs, leads to a severe reduction (80-95%) of the viral titer (Zhang *et al.*, 1998). This is a good safety factor for gene therapy, but it confers a lower flexibility for the inclusion of additional 3' sequences. Transforming retroviruses include oncogenes in their genomes replacing viral genes, instead of adding them. Only RSV is able to accommodate, naturally, an oncogene, *src* (aprox.1.6 kb), and has been modified to replace *src* with heterologous sequences of up to 2 kb (Foster and Hanafusa, 1983; Hughes and Kosik, 1984; Kornbluth *et al.*, 1986; Hughes *et al.*, 1987; Greenhouse *et al.*, 1988; Petropoulus and Hughes, 1991; Barsov and Hughes, 1996; Murakami *et al.*, 1997). Functional murine leukemia retroviruses, used as vectors, are not always very stable and frequently revert to the natural genome length. These viruses have been engineered to include an extra insert of a maximum size of about 1.5 kb (Goff *et al.*, 1981; Lobel *et al.*, 1985; Reik *et al.*, 1985; Jørgensen *et al.*, 1988; Stuhlmann *et al.*, 1989a; 1989b; Dillon *et al.*, 1991; Coulombe *et al.*, 1996; Jespersen *et al.*, 1999; Smith *et al.*, 2000; Logg *et al.*, 2001a; 2001b). A study testing the size of inserts introduced into SNV, showed that insertions of 0.4 to 1.6 kb in the 3' untranslated region (between the *env* gene and the 3'LTR) disrupt its

ability to replicate (Yin and Hu, 1999) in spite of previous observations (Gelinas and Temin, 1986). An unusualy long defective bicistronic vector of 10.5 kb has been described using the Friend-mink cell focus forming/murine embryonic stem cell virus hybrid (FMEV; Hildinger *et al.*, 1998). In single replication cycle experiments, it has been possible to package up to 19.2 kb MoMLV-derived genomes (Shin *et al.*, 2000). However, the rate of errors is high, so for practical biotechnological purposes, it seems that the wildtype limits have to be roughly maintained. We have recently obtained some results in agreement with that conclusion. Pentacistronic MoMLV-derived retroviral vectors, differing only in the length of one of the five cistrons, were constructed. A vector with a genome of 7.9 kb was functional, but another of 8.5 kb suffered severe rearrangements (De Felipe and Izquierdo, submitted for publication). With regard to the packaging size constraints, lentivirus vectors cannot accommodate much larger inserts than the traditional oncoretroviral vectors (Akkina *et al.*, 1996; Lee *et al.*, 1997; Page *et al.*, 1997; Jamieson and Zack, 1998). Human foamy viruses, another retroviral family of 11.7 kb, have been modified to obtain viruses up to 12.7 kb (Schmidt and Rethwilm, 1995).

Adeno-associated viruses (AAV) have been intensively studied for gene transfer and therapy, mainly due to their ability to integrate chromosomally in a non-pathogenic manner. An important limitation of AAV is their small packaging size of 4.7 kb of exogenous DNA (including the enhancer/promoter and the polyadenylation signal). It has been shown that they can be extended only to a 119% of wildtype (an extra insert of 0.9 kb; Hermonat *et al.*, 1997). Recently, two new systems using a two-vector approach have allowed researchers to nearly double the packaging capacity of AAV. The *trans*-splicing system uses two vectors that are able to form an intermolecular head-to-tail concatemer and reconstruct a long heterologous cassette. The interruption introduced in the junction is removed by splicing signals. The overlapping approach relies upon the homologous recombination between overlapping regions in two different AAV vectors, one containing an enhancer and other the expression cassette driven by a minimal promoter (for a review, see Flotte, 2000; for a comparison of the two systems see Duan *et al.*, 2001).

Adenoviruses have also a very limited flexibility in their size. It has been shown that their genome can be enlarged by only some 105% of the wildtype genome length (less than 1.2 kb) (Bett *et al.*, 1993). The initial vectors had a capacity to encode about 8 kb of exogenous DNA. However, as adenoviruses have a genome of about 36 kb, there is more potential to introduce deletions to accommodate larger sequences. The development of new "high-capacity, HC", "helper-dependent, HD" or "gutless" adenoviral vectors with major deletions in the adenoviral genome, have "freed" more space (about 30 kb) to clone long genes and regulative sequences or several genes (for reviews, Kochanek, 1999; Russell, 2000; Parks, 2000; Kochanek *et al.*, 2001).

Herpesviruses have been also used for gene transfer/therapy purposes, in particular *Herpes simplex* virus 1 (HSV-1). Its genome is larger and more complex than the other viruses described above (152 kb); the expectation being an increased probability of being able of creating deletions. Recombinant HSV-1 vectors have been engineered to accommodate up to 30 kb, and the introduction of at least five different genes into the same construct has been reported (Krisky *et al.*, 1998). However, as in the previous cases, it is also possible to construct minimal vectors containing only a DNA cleavage/packaging signal and the origin of DNA replication of the virus. These vectors are called HSV-1 amplicons. In theory, they can accommodate up to 150 kb (for a review, see Fraefel *et al.*, 2000). A recent report has shown that 51 kb can be packaged in this vector system (Wang *et al.*, 2000). These vectors are the largest available and provide enough space to clone several genes and/or long regulatory sequences (enhancers, promoters, locus control regions, matrix attachment regions, etc.). Three genes in two transcription units (vector size of 31.3 kb) have been co-expressed from such an amplicon (Wang *et al.*, 2001).

In recent years, another strategy to extend the capacity of the traditional vectors has been developed: new chimeric or hybrid vectors. The large transgene capacity available in adenenovirus vectors and HSV-1 amplicons has provided the ideal platform to introduce other viral vectors (AAV, retrovirus, retrotransposons) to develop chimeric vectors (reviewed in Reynolds *et al.*, 1999; Fraefel *et al.*, 2000; Lam and Breakefield, 2000). Of particular interest are the new chimeras using AAV. An AAV/Ad hybrid vector has been developed by introducing adenoviral packaging sequences in AAV genomes. The chimeric genomes are encapsidated in adenoviral capsids and, for this reason, they can accommodate nearly 30 kb, dramatically increasing the packaging capacity of AAV vectors (Goncalves, 2001).

If the ability to accommodate all the genes and regulatory sequences that we need in our chosen vector is a major problem, how to ensure then the correct expression of all those genes?

THE CO-EXPRESSION AND BALANCE PROBLEMS

There are two important concerns regarding expression strategies in obtaining polycistronic vectors. The first is to ensure co-expression of all the genes cloned in the polycistronic vector. Strategies relying on the production of a single polyprotein (Table **2**, rows D and E) are able to produce the most tight "linkage" possible: one single ORF. A weakness of this strategy is that a mutation may change the frame or introduce a premature stop codon, affecting all the downstream proteins. It is advisable, therefore, to place the selection marker as the last polyprotein domain: selection pressure will ensure that only vectors maintaining the frame will survive. Only mutations or rearrangements maintaining expression of the selective marker (e.g.: a mutation or deletion upstream of the marker maintaining the frame) may lead to failure of expression of upstream domains. The second concern is how to achieve an appropriate balance among all the proteins produced from the vector. In contrast to other strategies, proteolytic processing seems an obvious way to obtain a 1:1:etc stoichiometry. The situation is more complex with the CHYSEL. The *in vitro* studies show that each particular CHYSEL has different properties. The

CHYSEL sequence from the porcine teschovirus-1, PTV1 (and the FMDV 2A with an N-terminal extension of 5, or even better 14, extra amino acids from 1D) produces a stoichiometry more close to 1:1 (Donnelly *et al.*, 2001b). However, the different properties of FMDV 2A and 2A-like sequences may enable us, as has been discussed above, to determine the stoichiometry of the products.

Another strategy that has been successful is the scheme in Table **2**, row C. Experiments carried out several years ago showed that this strategy cannot be done realistically by relying on the reinitiation mechanism (Kaufman *et al.*, 1987). However, IRESes sequences have been successful in obtaining functional polycistronic vectors with this scheme. As there are several independent ORFs and the production of the different proteins is not directly linked, it is possible to lose one of the proteins (by a mutation or rearrangement) while maintaining the others. In this case, introduction of a selective marker ensures that the construct is in the target cells, but it may not guarantee its structural integrity. The balance among the different proteins produced from these polycistronic vectors relies on the IRES activity. One of the disadvantages of the reinitiation strategy is a "positional" effect, meaning that translational efficiency is progressively decreased as we move from the first translational initiation in the 5' end of the mRNA to the last one in the 3' end. The only way to overcome this situation is to introduce a selective marker in the last position, to ensure a minimal expression of this protein. This does not change the imbalance, but simply selects those cells with an overall higher transcriptional activity (autoselective configuration). Apart from the particular situations where this strategy can have advantages (as an overexpression of the upstream genes), it will be more convenient to have an IRES to achieve a more balanced co-expression. It has been shown that the "positional" effect does not affect a tricistronic plasmid containing two copies of EMCV IRES (Zhu *et al.*, 1999). This report showed that the second protein was produced at a reduced level compared with the first one driven from the cap, but no further loss of expression was detected from the third gene. However, other authors have described a lower expression from the last gene that can also be used for the autoselective configuration, as in reinitiation or that can be corrected using improved versions of IRES (Fussenegger *et al.*, 2001). Some reports have described a decrease in the production of the second gene in other tricistronic constructions, explained as an interference due to the proximity of the two IRES sequences (Morgan *et al.*, 1992; Zitvogel *et al.*, 1994; Laufs *et al.*, 2000).

CONCLUSIONS

In 1991, Adam and co-workers suggested that several IRESes may one day enable us to obtain "retroviral vectors containing a string of coding regions". They also advanced the main potential problems for this particular type of viral polycistronic vector: size constraints on retroviruses and recombination between identical sequences. The experience in the 1990's and in the last few years with IRES sequences shows that it is possible to design and work with polycistronic vectors.

Furthermore, alternatives to IRESes in bi- and polycistronic vectors have become available. Translational strategies of co-expressing genes using self-processing peptides (CHYSEL) and proteolytic processing targets (fusagen) are now proven technologies. Further characterization of these new systems is required to use them in a more precise way. CHYSEL and fusagen employ small sequences producing a good stoichiometry among the proteins co-expressed, but they produce proteins fused with extra peptide extensions. These sequences employ a novel approach for the expression of several proteins from a single cistron: encoding multiple products as a single fusion protein that is processed co- or post-translationally into its active components. On the other hand, IRESes are long sequences leading to high imbalance in stoichiometry, but some of them may be used to produce proteins without any additional sequence extensions. In addition, IRES sequences enable an independent translation of the downstream cistron in relation to translation of the upstream cistron/s. A good knowledge of these three strategies may let us to progress towards a more rational design of polycistronic vectors, helping us to combine them in the most convenient way.

There are many potential applications of these new tools. Polycistronic vectors may be very useful either to co-express several proteins for a combined/synergistic effect or a heteromultimeric protein, and to reconstruct a metabolic pathway. Very complex and time-consuming co-introductions of several genes can be now shortened using polycistronic vectors.

Looking to the short history of vector technology, genetic engineering is imitating the natural viruses where the gene expression processes are highly optimized: small RNA viruses with IRES and protein fusions that are cleaved in different ways to obtain the final functional proteins. As our knowledge of (virus) protein biogenesis increases, we can look forward to using an expanded biomolecular "tool box".

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ABBREVIATIONS

AAV = Adeno-associated virus

ADA = Adenosine deaminase

ADH = Alcohol dehydrogenase

ALDH-1 = Aldehyde dehydrogenase class-1

ATase = O6-alkylguanine-DNA-alkyltransferase

BiP = Human immunoglobulin heavy chain-binding protein

BSD = Blasticidin S deaminase

BLE = Bleomycin resistance protein

CD = Cytosine deaminase

CODA = Cytidine deaminase

Cx43 = Connexin 43

DHFR = Dihydrofolate reductase

EGFP = Enhanced green fluorescent protein

eIF4G = Eukaryotic initiation factor 4G

EMCV = Encephalomyocarditis virus

FMDV = Foot-and-mouth disease virus

βGAL = β-galactosidase

γ-GCS = γ-glutamylcysteine synthetase

GFP = Green fluorescent protein

GM-CSF = Granulocyte, macrophage colony stimulating factor

GPT = Guanine phosphoribosyltransferase

GST pi = Glutathione S-transferase pi

GUS = Glucoronidase

HCV = Hepatitis C virus

HPH = Hygromycin B phosphotransferase

HRV = Human rhinovirus

HSV1TK = *Herpes simplex* virus-1 thymidine kinase

IFNγ = Interferon γ

IL = Interleukin

IRE = Iron response element

IRES = Internal ribosomal entry site

IRP = Iron regulatory protein

ITAFs = IRES *trans*-acting factors

LTRs = Long terminal repeats

LUC = Luciferase

LYM = Lymphotactin

MDR1 = Multidrug resistance 1 gene

MGMT = O6-methylguanine-DNA methyltransferase

MHA = Measles hemagglutinin

MHC = Major histocompatibility complex

NIa = Nuclear inclusion proteinase

NEO = Neomycin phosphotransferase

NLSs = Nuclear localization signals

ORF = Open reading frame

PAC = Puromycin N-acetyl transferase

PTV1 = Porcine teschovirus-1

PV = Poliovirus

PVX = Potato virus X

RSV = Rous sarcoma virus

SMV CP = Soybean mosaic potyvirus capsid protein

SV40 = Simian virus 40

T Ag = T antigen

TEV = Tobacco etch potyvirus

TVMV = Tobacco vein mottling potyvirus

TMV CP = Tobacco mosaic tobamovirus coat protein

UPRT = Uracil phosphoribosyltransferase

5'UTR = 5' untranslated region

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NOTE

In a recently published article Wu *et al*. (2002) have described the first example of an organism using two CHYSEL sequences (the *Perina nuda* picorna-like insect virus, PnPV). In another recent report, polycistronic vectors containing two or three copies of the FMDV 2A CHYSEL and three or four cistrons have been constructed (Ma and Mitra, 2002).

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