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REVIEW

Components of Vectors for Gene Transfer and Expression in Mammalian Cells

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Progress in diverse scientific fields has been realized partly by the continued refinement of mammalian gene expression vectors. A growing understanding of biological processes now allows the design of vector components to meet specific objectives. Thus, gene expression in a tissue-selective or ubiquitous manner may be accomplished by selecting appropriate promoter/enhancer elements; stabilization of labile mRNAs may be effected through removal of 3′ untranslated regions or fusion to heterologous stabilizing sequences; protein targeting to selected tissues or different organelles is carried out using specific signal sequences; fusion moieties effect the detection, enhanced yield, surface expression, prolongation of half-life, and facile purification of recombinant proteins; and careful tailoring of the codon content of heterologous genes enhances protein production from poorly translated transcripts. The use of viral as well as nonviral genetic elements in vectors allows the stable replication of episomal elements without the need for chromosomal integration. The development of baculovirus vectors for both transient and stable gene expression in mammalian cells has expanded the utility of such vectors for a broad range of cell types. Internal ribosome entry sites are now widely used in many applications that require coexpression of different genes. Progress in gene targeting techniques is likely to transform gene expression and amplification in mammalian cells into a considerably less labor-intensive operation. Future progress in the elucidation of eukaryotic protein degradation pathways holds promise for developing methods to minimize proteolysis of specific recombinant proteins in mammalian cells and tissues.

In recent years progress in the design, sophistication, and availability of vectors for gene expression in mammalian cells has been phenomenal. Vectors have many applications, including the study of gene regulation, DNA sequencing, molecular cloning, protein production, antigen expression for vaccination, and gene therapy. There is a large number of vectors available; for example, Vector Database on the Web (Table 1) lists more than 2600 vectors. In spite of the plethora of available vectors, however, robust protein production in mammalian cells is not necessarily a routine matter. Efficient expression of genes in mammalian cells depends on many factors, including both transcriptional and translational control elements, RNA processing, gene copy number, mRNA stability, the chromosomal site of gene integration, potential toxicity of recombinant proteins to the host cell, as well as the genetic properties of the host. Gene transfer into mammalian cells may be effected either by infection with virus that carries the recombinant gene of interest or by direct transfer of plasmid DNA. Due to space limitations, the emphasis here is on nonviral vectors for high-level protein production. There is extensive literature on mammalian vectors of viral origin with applications in protein production, gene therapy, and vaccine development. The reader is referred to recent reviews (1–7) and to Table 2 for selected references on viral-based vectors. In addition, only brief coverage of inducible vector systems is provided here, as several excellent reviews have covered this topic in detail (8–13).

The choice of an expression system for production of recombinant proteins depends on many factors, including cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications and biological activity of the protein of interest, as well as regulatory issues and economic considerations in the production of therapeutic proteins (14–16). Key advantages of mammalian cells over other expression systems are the ability to carry out proper protein folding, complex *N*-linked glycosylation and authentic *O*-linked glycosylation, as well as a broad spectrum of posttranslational modifications (14). The essential elements of mammalian expression vectors (Table 3 and Fig. 1) include (1) a constitutive or inducible promoter capable of robust transcriptional activity; (2) optimized mRNA processing and transla-

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tional signals that include a Kozak sequence, translation termination codon, mRNA cleavage and polyadenylation signals, as well as mRNA splicing signals; (3) a transcription terminator; (4) selection markers (Table 4) for the preparation of stable cell lines and for gene amplification; and (5) prokaryotic origin of replication and selection markers for vector propagation in bacteria. The inclusion of the SV40 origin of replication facilitates transient gene expression in COS cells. Other genetic elements for specific applications include fusion moieties, protease cleavage sites, sequences for gene or protein targeting, and IRES elements for the construction of polycistronic vectors (Fig. 1D).

TABLE 1

Web Databases for Gene Expression Vectors

Vector Database
http://vectordb.atcg.com
VectorDB contains information on more than 2600 vectors, including phage, plasmid, phagemid, phasmid, cosmid, viral, and YAC vectors. The database has a search engine and contains annotation and sequence information for many of the vectors. In addition, vectors which are also in GenBank have direct links to that database.

Gene Transfer Vector Core
http://www.uiowa.edu/~gene
This site is from the University of Iowa College of Medicine. The core produces viral and nonviral vectors in quantities necessary for gene transfer in research experiments or preclinical studies. Core staff work closely with investigators to plan and develop gene transfer vectors to fit individual project requirements. Customized virus constructions are available on a fee for service basis.

Gene Therapy Vectors
http://www.wiley.co.uk/genetherapy/vectors.html
This site deals with all aspects of gene therapy, including an overview of gene delivery systems and illustrations of the most widely used gene transfer vectors. The site also provides comprehensive summaries of clinical trials in gene therapy worldwide and has links to information from meetings, published material, regulatory agencies, and related databases.

Course BS335: Virology
http://www-micro.msb.le.ac.uk/335/BS335.html
This course on the main principles of virology is organized by Alan Cann from the University of Leicester. The site includes a detailed section on viral vectors for gene transfer and therapy organized by David Peel.

National Gene Vector Laboratories (NGVL)
http://www.iupui.edu/~iucc/ngvl
The NGVL are funded by the National Institutes of Health and are composed of a group of academic laboratories whose goal is to provide eligible investigators with clinical grade vectors for gene therapy. The home page includes the following links: *NGVL at Indiana University* produces retroviral and AAV vectors. *NGVL at the University of Michigan* produces nonviral vectors. *NGVL at the University of Pennsylvania* produces adenoviral vectors.

Sindbis Virus Gene Expression Vectors
http://www.microbiology.wustl.edu/Sindbis/sinVectors
This site from Washington University in St. Louis provides a detailed description of the molecular biology of Sindbis virus, including a bibliography and types of Sindbis virus gene expression vectors.

TABLE 2

Viral-Based Vectors for Gene Transfer and Expression in Mammalian Cells

Viral-based vector	Reference No.
DNA viruses	
Cytomegalovirus	148
Herpes simplex virus	149
Epstein-Barr virus	150, 151
Simian virus 40	20, 21
Bovine papillomavirus	49
Adeno-associated virus	152
Adenovirus	153, 154
Vaccinia virus	155, 156
Baculovirus	157
RNA viruses	
Semliki Forest virus[a]	158, 159
Sindbis virus	160, 161
Poliovirus	162, 163
Rabies virus	164
Influenza virus	165, 166
SV5	167
Respiratory syncytial virus	168
Venezuelan equine encephalitis virus	169, 170
Kunjin virus	171
Sendai virus	172
Vesicular stomatitis virus	173
Retroviruses	2, 174, 175
Chimeric viral vectors	
Adenovirus-Sindbis virus	176
Adenovirus-adeno-associated virus	177

[a] A DNA-based self-amplifying SFV vector has been developed (178).

1. TRANSIENT GENE EXPRESSION

Transient gene expression is a convenient method for the rapid production of small quantities of protein for initial characterization. In addition, the method lends itself to the rapid testing of vector functionality as well as optimization of different combinations of promoters and other elements in expression vectors. Thus, once the appropriate vector has been constructed, results from transient expression assays can be obtained in 2 to 3 days and allow one to proceed with confidence to the more time-consuming task of preparing permanent stable cell lines for protein production on a larger scale. There are several cell types used for transient gene expression, including COS, baby hamster kidney (BHK)[1], and human embryonic kidney (HEK)-293 cells, as well as genetically modified HEK-293 cells (reviewed in 17). The most widely used transient expression system utilizes COS cells (reviewed in

[1] Abbreviations used: BHK, baby hamster kidney; HEK, human embryonic kidney; S/MAR, scaffold/matrix attached region; CHO, Chinese hamster ovary; Cre, cyclization recombination; MDCK, Madin–Darby canine kidney cells; MMP13, metalloproteinase 13; IPTG, isopropyl β-D-thiogalactopyranoside; UTR, untranslated region; OTC, ornithine transcarbamylase; GFP, green fluorescent protein; IRES, internal ribosome entry site.

18,19). COS cell lines were generated by the transfection of African green monkey kidney CV1 cells with an origin-defective SV40 (20,21). COS cells express the SV40 T antigen, which allows replication of plasmids containing the SV40 origin of replication. This host/vector system facilitates high-level plasmid amplification and protein production, followed by lysis of the cells 3 to 4 days from the time of transfection. COS cells have been used for the transient expression of numerous genes, including the production of a wide range of monoclonal antibodies (19). It is also possible to use COS cells on a large scale for the production of milligram quantities of protein, thus obviating the need for multiple transfections (17).

2. STABLE GENE EXPRESSION

In contrast to transient gene expression, preparation of stable cell lines that "permanently" express the gene of interest depends on the stable integration of plasmid into the host chromosome. It is also possible, however, to generate stable cell lines that harbor vectors extrachromosomally. For example, vectors that carry the Epstein-Barr virus nuclear antigen (EBNA-1) and the origin of replication (oriP) (Table 3) can be maintained episomally in primate and canine cell lines but not in rodent cell lines (22). Recently, an episomal replicating vector has been described that does not express any viral proteins, thus avoiding cell transformation (23). The vector contains the SV40 origin of replication and the scaffold/matrix attached region (S/MAR) from the human interferon-β gene. S/MARs are DNA sequences associated with chromosomal origins of bidirectional replication. The vector was shown to replicate at very low copy numbers (below 20) in Chinese hamster ovary (CHO) cells and was stably maintained without selection for more than 100 generations (23).

The choice of host cell may have a significant impact on gene expression levels. For example, myeloma cells have been mainly used for high-level production of monoclonal antibodies (19). However, amplifiable expression systems using CHO cells have been widely used for the successful production of proteins of therapeutic interest. There are many genes that confer drug resistance upon amplification (24); however, the two most widely used amplification systems rely on the dihydrofolate reductase and glutamine synthetase genes. Thus, by growing cells in increasing concentrations of selection drugs it is possible to amplify significantly the copy number of the cotransfected gene of interest and concomitantly elevate the amount of protein produced (24).

The generation of stable cell lines, particularly the selection of amplified and high-expressing clonal cells, necessitates the screening of large numbers of transfected cells, both during the initial transfection and at each subsequent amplification step. This is mainly due to the wide variation in the level of expression and amplification of the transfected gene, depending on the chromosomal site of plasmid integration (25–27). For example, in CHO cells, gene amplification frequency in one transformant was 100-fold that of the others, and in another, amplification of transfected genes inserted near a centromere resulted in chromosome instability and rearrangements (25). These observations have theoretical implications for the method of gene introduction into mammalian cells. Often the gene of interest and the selectable gene marker (or, in the case of antibodies, the heavy and light chains) are located on two separate plasmids. These are cotransfected into the host cell where they recombine and integrate as a unit into the host chromosome (28). It is possible, therefore, that the two genes integrate in separate chromosomal loci of different transcriptional activity, necessitating the screening of large numbers of transfected cells. This potential problem may be resolved by placing both genes of interest on a single vector. To date, however, there is no firm evidence that a single expression vector is advantageous over two vectors. In the case of antibody expression, it has been concluded that equivalent levels of production and stability of the resulting cell lines have been obtained using single- and double-vector systems (19).

An alternative strategy for the efficient preparation of stable cell lines is site-specific gene integration using recombination systems such as Cre/*loxP* (29) and FLP/*FRT* from yeast (30). Cre (cyclization recombination) recombinase of bacteriophage P1 recombines DNA at 34-bp sites called *loxP* (locus of crossover of P1). The FLP recombinase from the 2-μm circle of *Saccharomyces cerevisiae* recognizes *FRT* (the FLP recombination target). It should be possible to engineer a cell line using a reporter gene to select a transcriptionally active chromosomal locus. Such a cell line could then be used for the routine excision and replacement of the reporter construct with the gene of interest. Progress in this area is ongoing (31,32), and a commercially available vector/host system makes use of the FLP/*FRT* elements (pOG vector, Stratagene, La Jolla, CA).

Recently, a well-differentiated epithelial cell line, Madin–Darby canine kidney (MDCK), was shown to be capable of producing large amounts of protein (33). The cells were transfected with a plasmid containing the cytomegalovirus promoter controlling the expression of matrix metalloproteinase 13 (MMP13). The yield of MMP13 was 10 mg/liter of conditioned medium, an amount that rivals yields obtained from CHO amplification systems. The authors point out that the unusually high yield could be attributed partially to the properties of MDCK cells, since CHO cells transfected with the same vector yielded much less protein (33).

TABLE 3

Vectors for Gene Expression in Mammalian Cells

Vector	Promoter	Induction	Selection marker		f1/M13 origin	Reporter/ Purification tag/epitope	MCS	Vector size (kbp)	Comments	Commercial source or reference[a]
			Mammal	Bacteria						
pSVK3	SV40 Early			Amp	f1		8	3.9	Transient	Amersham Pharmacia (www.apbiotech.com)
pSVL	SV40 Late			Amp			5	4.9	Transient	Amersham Pharmacia
pMSG	MMTV-LTR (mouse mammary tumor virus)	Dexamethasone	gpt	Amp			4	7.6	Transient/stable	Amersham Pharmacia
pCH110	SV40 Early			Amp			1	7.1	Promoter screen	Amersham Pharmacia
pTargeT	hCMV-IE[b] (cytomegalovirus immediate early)		Neo	Amp	f1		10	5.7	Transient/stable	Promega (www.promega.com)
pSI	SV40 Early			Amp	f1		10	3.6	Transient	Promega
pCI	hCMV-IE			Amp	f1		11	4.0	Transient	Promega
pCI-neo	hCMV-IE		Neo	Amp	f1		9	5.5	Transient/stable	Promega
pOPRSVI/MCS	RSV-LTR (Rous Sarcoma virus)	IPTG[c]	Neo	Amp	f1		8	5.6	Requires cotransfection with pCMVLacI	Stratagene (www.stratagene.com)
pBK-CMV	hCMV		Neo	Kana	f1		17		Phagemid	Stratagene
pBK-RSV	RSV-LTR		Neo	Kana	f1		17		Phagemid	Stratagene
pCMV-Script	hCMV		Neo	Kana	f1		17	4.3		Stratagene
pDual	hCMV (mutated)		Neo	Kana	f1	CBP[d]		5.5	Mammalian and bacterial expression	Stratagene
pCMV-Tag series	hCMV		Neo	Kana	f1	FLAG[e], *c-myc*	1–13	~4.3		Stratagene
pFLAG series	hCMV			Amp	f1	FLAG			Transient	Sigma (www.sigma-aldrich.com)
pSFV1								11.0	Vector serves as template for *in vitro* synthesis of RNA	Life Technologies (www.lifetech.com)
pTet-Splice	Tet	Tetracycline		Amp	f1		4	5.2	Requires cotransfection with pTet-tTAk	Life Technologies
pTRE	hCMV*-1[f]	Tetracycline or doxycycline[g]		Amp			4	3.1	Requires cotransfection with pTet-On or pTet-Off	Clontech (www.clontech.com)
pRev-TRE	hCMV*-1	Tetracycline or doxycycline[g]	Hyg	Amp			6	6.5	Requires cotransfection with pRevTet-On or pRevTet-Off	Clontech
pRetro-On pRetro-Off	hCMV*-1	Tetracycline or doxycycline[g]	Puro	Amp			3	6.8 7.0	Do not require cotransfection with other vectors	Clontech
pLNCX	hCMV-IE		Neo	Amp			3	6.6	Retroviral vector (Transient/stable)	Clontech
pLXSN	5′ LTR		Neo	Amp			4	5.9	Retroviral vector (Transient/stable)	Clontech
pLXIN	5′ LTR		Neo	Amp			3	6.1	Retroviral vector (Bicistronic)	Clontech
pSIR	5′ LTR		Neo	Amp			4	8.1	Retroviral vector (Self-inactivating)	Clontech
pLAPSN	5′ LTR		Neo	Amp		AP[h] (fusion)		7.9	Retroviral vector (Transient/stable)	Clontech
pIRES-bleo/hyg/neo/puro	hCMV-IE		Bleo, hyg, neo, puro	Amp			5–9	5.3–5.7	Bicistronic vector	Clontech
pIRES-EGFP pIRES-EYFP	hCMV-IE			Amp		EGFP[i] EYFP[i]	5	5.2	Bicistronic vector	Clontech

pCDM8	hCMV			*sup*F	M13		6	4.4	Transient	Invitrogen (www.invitrogen.com)
pcDNA1.1	hCMV			*sup*F	M13		12	4.0	Transient	Invitrogen
pcDNA1.1/Amp				Amp			11	4.8		
pcDNA3.1-neo/zeo/hyg	hCMV		Neo, Zeocin, Hyg	Amp	f1		15	5.0–5.6	Stable	Invitrogen
pcDNA3.1/His/Myc-His/V5-His	hCMV		Neo	Amp	f1	$(His)_6$, *c-myc*, V5	11–13	5.5	Stable	Invitrogen
pRc/CMV2	hCMV		Neo	Amp	f1		6	5.5	Stable	Invitrogen
pRc/RSV	RSV-LTR		Neo	Amp	f1		6	5.2	Stable	Invitrogen
pSecTag2 pSecTag2/hyg	hCMV		Zeocin, Hyg	Amp	f1	$(His)_6$, *c-myc*	12, 15		Igκ leader for protein secretion	Invitrogen
pDisplay	hCMV		Neo	Amp Kana	f1	*c-myc*, HA	8	5.3	Igκ leader, PDGFR TM domain for surface display	Invitrogen
pZeoSV2	SV40		Zeocin (P_{CMV})	Zeocin (P_{EM-7})[j]	f1		17	3.5	Transient/stable	Invitrogen
pREP series	RSV-LTR		Hyg, HisD, Neo	Amp			7–9	9.5–11.8	EBV *ori*P and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pCEP4	hCMV-IE		Hyg	Amp			9	10.4	EBV *ori*P and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pEBVhis	RSV-LTR		Hyg	Amp		$(His)_6$, Ab	8	10.3	EBV *ori*P and nuclear antigen (EBNA-1) for episomal replication	Invitrogen
pcDNA4/HisMax	hCMV		Zeocin (P_{SV40})	Zeocin (P_{EM-7})	f1	$(His)_6$, Ab	12	5.3	Contains translational enhancer QBI SP163	Invitrogen
pVP22/myc-His	hCMV		Neo	Amp	f1	*c-myc*, $(His)_6$	8	6.4	VP22 fusions for nuclear targeting	Invitrogen
pIND series	ΔHSP (Heat shock protein)	Ecdysone	Neo, Hyg	Amp	f1	V5, $(His)_6$	16	5.0–5.7	Requires cotransfection with pVgRXR	Invitrogen
pSin series	SG (Sindbis subgenomic promoter)			Amp		$(His)_6$, Ab	6		Viral vector	Invitrogen
pEF series	hEF-1α (Elongation factor 1α)		Neo	Amp	f1	*c-myc*	4, 5		Targeting to cytoplasm, nucleus, mitochondria, ER	Invitrogen
pCMV series	hCMV		Neo	Amp	f1	*c-myc*	4, 5		Targeting to cytoplasm, nucleus, mitochondria, ER	Invitrogen
pTracer series	hCMV-IE, SV40, hEF-1α		Zeocin (P_{CMV})	Zeocin (P_{EM-7})	f1	GFP	9–13	4.2–6.2	Monitor transfection status by fluorescent microscopy	Invitrogen
pGT series									Modular bicistronic plasmids for gene therapy	InvivoGen (www.invivogen.com)
pCMV-LIC	hCMV-IE			Amp	f1			4.6	Transient, stable by cotransfection	PharMingen (www.pharmingen.com)
pBacMam-1	Hybrid: hCMV–IE/			Amp			6	11.6	Baculovirus-based	Novagen
pBacMam-2	avian actin			Amp	f1		8	7.6	vector for mammalian cells	(www.novagen.com)
pPOP	mPGK/lacO (phosphoglycerate kinase)	IPTG	Neo				8			(179)

TABLE 3—*Continued*

Vector	Promoter	Induction	Selection marker		f1/M13 origin	Reporter/ Purification tag/epitope	MCS	Vector size (kbp)	Comments	Commercial source or reference[a]
			Mammal	Bacteria						
pEF-LAC	hEF-1α/lacO	IPTG	Neo							(180)
pEF-BOS	hEF-1α									(181)
pBPVMT1	mMT-I (metallothionein I)	Cd^{2+}, Zn^{2+}, PMA[k]								(49)
pMT	hMT-II (metallothionein II)	Cd^{2+}, Zn^{2+}, PMA	Neo							(182)
pMT302	hMT-IIA (mutant)	Cd^{2+}, Zn^{2+}, PMA							High inducibility, low basal activity	(51)
pIFP	hIFN-α, (interferon-α)	Viruses	Neo, dhfr	Amp			2	8.9		(183)
pGRE5	5XGRE/Ad2MLP (Glucocorticoid response element/ adenovirus major late promoter)	Dexamethasone	Neo	Amp			7	4.0		(184)
pRDB	DRE/MMTV (dioxin response element)	TCDD[l]	Neo	Amp					5- to 10-fold inducibility	(185)
pLTRpoly	MoMLV-LTR (Moloney murine leukemia virus)		Neo	Amp			10	4.3		(186)
pKX	hLS (Leukosialin (CD43))			Amp					Selective for T cells	(187)
pMU1	mU1a, U1b snRNA (small nuclear RNA)									(188)
pSRα	Hybrid: SV40/HTLVI-LTR									(189)
pRD133	CMV/Ad2TPL (adenovirus tripartite leader)			Amp			18			(190)
pHEKneo	κ Light chain		Neo	Amp					Selective for myeloma cells	(191)
pTIF-1	Variable heavy chain		Neo						Ig expression in lymphoid cells	(192)
pIE	HSV (Herpes simplex virus)								Requires cell line expressing HSV VP16 transactivator	(193)
p753	hUbiquitin C									(43, 44)
pEPI-1	CMV		Neo	Kana				6.7	Non-viral-based vector for stable episomal replication	(23)

[a] References for commercially available vectors may be found in the respective company catalogs.
[b] h, human; m, murine.
[c] Isopropyl-β-D-thiogalactopyranoside.
[d] Calmodulin-binding peptide.
[e] Synthetic peptide sequence DYKDDDDK.
[f] Compound promoter consisting of the tetracycline-responsive element (TRE), which contains seven direct repeats of the 42-bp *tetO* sequence and the minimal immediate early promoter of CMV, which lacks the CMV enhancer. This promoter is silent unless activated by (r)tTA.
[g] Induction or repression, depending on the cotransfected vector.
[h] Alkaline phosphatase.
[i] Enhanced green (yellow) fluorescent protein.
[j] Synthetic promoter.
[k] 4-β-Phorbol 12-myristate 13-acetate.
[l] 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

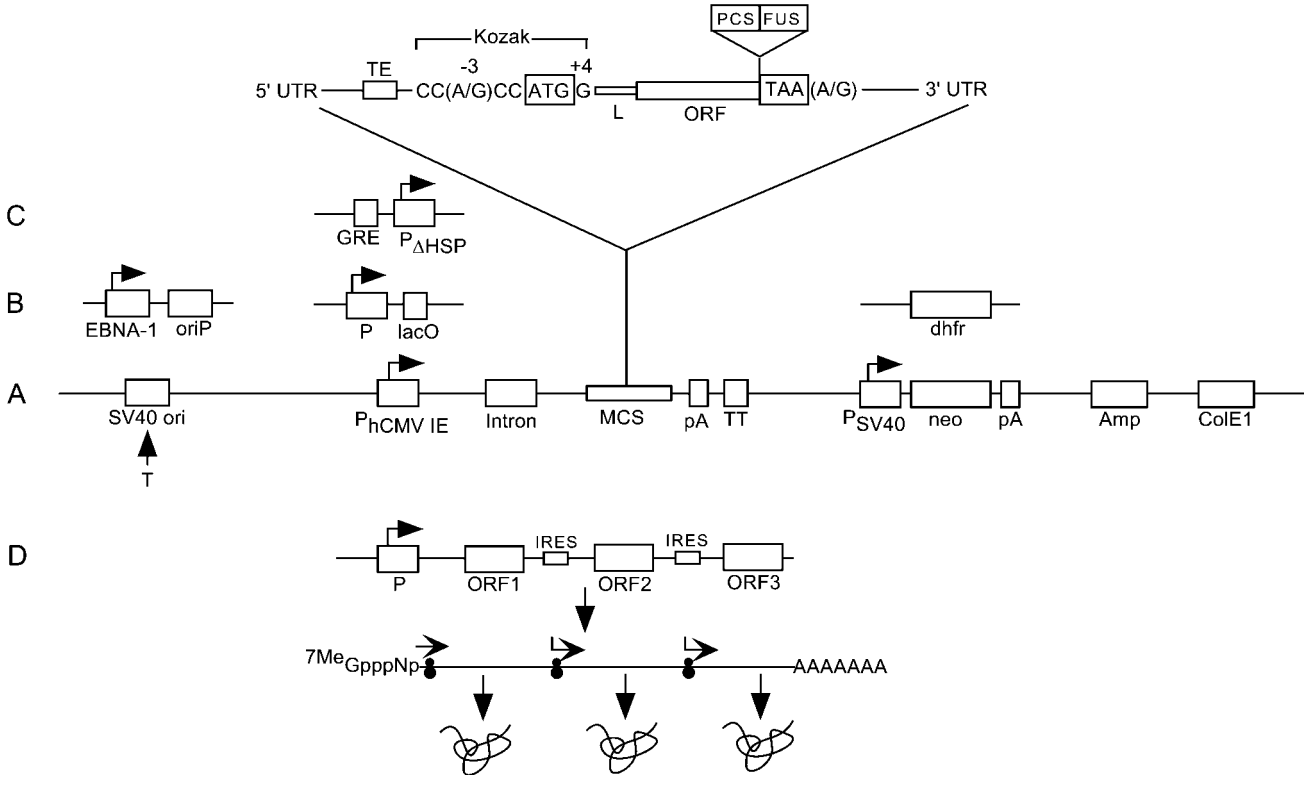

FIG. 1. Configuration of genetic elements in monocistronic (A) and polycistronic (D) expression vectors. Specific elements are shown for illustrative purposes and are not drawn to scale. The source, position, and combination of different components in the vector may vary in order to meet specific experimental requirements. SV40 ori is required for transient gene expression in COS cells. EBNA-1 and oriP facilitate high-copy episomal replication in primate and canine cell lines. The various promoter (P) elements allow constitutive (A) or inducible (B, C) expression. The optimal translational initiation sequence (Kozak) and termination codon followed by purines are shown. The ColE1 origin of replication and the ampicillin-resistance gene allow vector propagation in bacteria. The neomycin-resistance gene facilitates selection in mammalian cells, and the *dhfr* gene allows both selection and gene amplification. In a polycistronic vector (D) IRES elements allow multiple ORFs to be efficiently translated from a single transcript. See text for details. Amp, ampicillin resistance gene; ColE1, prokaryotic origin of replication; dhfr, dihydrofolate reductase; EBNA, Epstein-Barr virus nuclear antigen; FUS, fusion moiety; GRE, glucocorticoid response element; hCMV IE, human cytomegalovirus immediate early enhancer/promoter; HSP, heat shock protein; IRES, internal ribosome entry site; lacO, *lac* operator; L, leader (targeting sequence); MCS, multiple cloning site; neo, neomycin resistance gene; ORF, open reading frame; ori, origin of replication; oriP, Epstein-Barr virus origin of replication; P, promoter; pA, polyadenylation signal; PCS, protease cleavage site; T, SV40 large tumor (T) antigen; TE, translational enhancer; TT, transcription terminator; UTR, untranslated region.

3. TRANSCRIPTIONAL CONTROL ELEMENTS

Promoters and Enhancers

Although the physical boundaries between these two control elements are not always clear, promoters are operationaly defined as the site of transcription initiation, an event mediated through interactions of transcription factors with their cognate promoter and enhancer elements (34–36). Enhancers potentiate promoter activity, temporally as well as spatially (34,37). In general, promoters contain the TATA box, located upstream of the transcription initiation site, and the CAAT box, located upstream of the TATA box. Both regions bind transcription factors that facilitate transcription initiation; however, there are promoters that do not contain a TATA box (38,39).

Many promoters are transcriptionally active in a wide range of cell types and tissues. However, most exhibit tissue specificity, a property that must be carefully considered prior to the construction and use of expression vectors (40,41). For example, the widely used cytomegalovirus promoter exhibits low transcriptional activity in hepatocytes (42). Strong constitutive promoters which drive expression in many cell types include the adenovirus major late promoter, the human cytomegalovirus immediate early promoter, the SV40 and Rous Sarcoma virus promoters, and the murine 3-phosphoglycerate kinase promoter. The human ubiquitin C promoter is active in tissue culture (43), and it is capable of high-level gene expression in a very broad range of tissues (44).

Tissue specificity of promoters is of particular interest in gene therapy applications. An interesting strategy was recently devised to enhance the transcriptional activity of weak promoters without loss of tissue specificity (45). The principle behind this strategy was to use a cell type-specific promoter to drive the simul-

TABLE 4

Selection Markers for Gene Expression in Mammalian Cells

Gene	Phenotype	Action of selective agent	Reference
	Positive selection		
dhfr (dihydrofolate reductase)	Resistance to MTX[a]	MTX is a competitive inhibitor of DHFR	194, 195, 196
xgprt (gpt) (xanthine–guanine phosphoribosyl transferase)	Xanthine as the source for guanine synthesis	Aminopterin and mycophenolic acid in dialyzed medium block *de novo* synthesis of GMP	197
aph (neo) (aminoglycoside phosphotransferase)	Resistance to G418[b]	G418 blocks mammalian protein synthesis	198, 199
hph (hyg) (hygromycin-B-phosphotransferase)	Resistance to hygromycin B	Hygromycin B blocks protein synthesis	200, 201
pac (puro) (puromycin-N-acetyl transferase)	Resistance to puromycin	Puromycin blocks protein synthesis	202, 203
ble (bleomycin)	Resistance to bleomycin, phleomycin, or zeocin	Bleomycin belongs to a group of related glycopeptide antibiotics which are believed to cause DNA strand scission	204, 205
hisD (histidinol dehydrogenase)	Resistance to histidinol	Histidinol is cytotoxic; HD oxidizes histidinol to histidine	206
trpB (tryptophan synthase (β subunit))	Indole as the source for tryptophan synthesis		206
atpA (Na^+, K^+-ATPase α subunit)	Resistance to ouabain	Ouabain belongs to a group of related cardiac glycosides which block transport of Na^+ and K^+ by intact cell membranes	207, 208
ada (adenosine deaminase)	Resistance to Xyl-A[c]	Xyl-A is converted to Xyl-ATP, which damages nucleic acids	209, 210
codA (cytosine deaminase)	Resistance to PALA[d]	PALA blocks *de novo* synthesis of pyrimidines; CD converts cytosine in the medium to uracil	211, 212
	Negative selection		
codA (cytosine deaminase)	Cell death	CD converts 5-fluorocytosine to 5-fluorouracil	213
HSV-TK (Herpes simplex virus thymidine kinase)	Cell death	TK phosphorylates the selection drug ganciclovir which incorporates into DNA; ganciclovir is a poor substrate for mammalian TK	214
	Positive or negative selection		
Fusion: hyg–tk	Positive: resistance to hygromycin Negative: cell death		215
Fusion: tk–neo	Positive: resistance to G418 Negative: cell death		216
Fusion: tk–bsd	Positive: resistance to blasticidin S Negative: cell death		217
Fusion: pac–tk	Positive: resistance to puromycin Negative: cell death		218
Fusion: hyg–codA	Positive: resistance to hygromycin Negative: cell death		218
Fusion: codA–neo	Positive: resistance to G418 Negative: cell death		218
Fusion: codA–bsd	Positive: resistance to blasticidin S Negative: cell death		218
Fusion: pac–codA	Positive: resistance to puromycin Negative: cell death		218

[a] Methotrexate. Stable cell lines may be established using DHFR-deficient Chinese hamster ovary cells and a normal *dhfr* gene or wild-type cells and a mutant *dhfr* encoding an enzyme resistant to MTX.
[b] G418 is an aminoglycoside, similar in structure to neomycin.
[c] 9-β-D-Xylofuranosyl adenine.
[d] *N*-(phosphonacetyl)-L-aspartate.

taneous expression of the gene of interest and an artificial transcriptional activator to stimulate transcription through binding sites in the promoter. This "positive feedback loop" was achieved using a fusion transcription factor composed of the Herpes simplex virus VP16 transcriptional activation domain and the DNA-binding domain of LexA. It was shown that the transcriptional activity of two different promoters was increased 14- to 100-fold while maintaining cell type specificity (45).

Promoters can be divided into two classes, those that function constitutively and those that are regulated by induction or derepression. Inducible promoters are desirable for the production of proteins that may be toxic to the host cell, such as diptheria toxin (46), for the study of gene regulation during development in transgenic animals (13,47), and for experimental and therapeutic applications of gene transfer (48). Promoters used for high-level production of proteins in mammalian cells should be strong and, preferably, active in a wide range of cell types to permit qualitative and quantitative evaluation of the recombinant protein. Inducible promoters should exhibit a minimal level of basal transcriptional activity and be capable of substantial induction with a nontoxic inducer in a simple and cost-effective manner.

The widely used metallothionein promoter exhibits high basal expression level and a poor induction ratio (49). Moreover, heavy metals used to induce this promoter are cytotoxic. Two groups have addressed the high basal expression of the human MTIIA promoter. In one study, the substitution of multiple metal response elements for a region involved in basal expression caused up to 200-fold inducibility of the promoter (50). In the other study, mutation of specific nucleotides within the promoter resulted in low basal activity and a 13- to 35-fold induction ratio, depending on the cell line (51).

The functionality of the bacterial *lac* operator–repressor system in mammalian cells (52,53) has been exploited for the inducible expression of heterologous genes in mammalian cells. A potential advantage of this system over the use of endogenous cellular transactivators is that the *lac* operator, the recognition sequence for the *lac* repressor, occurs at low frequency in mammalian cells (54) and should facilitate high specificity in target gene regulation. In addition, the *lac* repressor has an extremely high affinity for the *lac* operator—the dissociation constant is about 10^{-13} M (55). Different versions of this system exhibited 1200-fold (56) or 10,000- to 20,000-fold (57) induction of gene expression, with no detectable expression in the absence of inducer and a high specificity for the gene under study. However, the inducer isopropyl β-D-thiogalactopyranoside (IPTG) is cytotoxic, albeit at high concentration (50 mM) (53). IPTG concentrations typically used for induction of mammalian expression systems range from 0.1 to 5 mM. The toxicity of IPTG has precluded its use in the large-scale production of therapeutic proteins. Several studies have described the use of temperature-sensitive *lac* repressors for use in mammalian cells (57) or in *Escherichia coli* (58–60). The bacterial thermoinducible *lac* repressor systems should be adaptable for use in mammalian cells. Recent advances in the construction of mammalian inducible expression systems have been reviewed (8–13).

Introns

Most genes from higher eukaryotes contain introns which are removed during RNA processing. Genomic constructs have been shown to be expressed more efficiently in transgenic animals than identical constructs lacking introns (61,62). Although many cDNA constructs lacking introns can be expressed efficiently in mammalian cells, Buchman and Berg (63) showed that the inclusion of introns leads to a 10- to 20-fold increase in expression, and some sequences, such as the β-globin cDNA, show a virtual requirement for the presence of an intron. The placement of introns at the 3′ end of the transcription unit has been reported to lead to aberrant splicing (64,65); therefore, it is preferable to place introns at the 5′ end of the open reading frame. Another caveat involves the use of the SV40 19S late mRNA intron, which appears to be inappropriate for the production of antibodies (66). A synthetic intron SIS generated by the fusion of an adenovirus splice donor site and an immunoglobulin G splice acceptor site was very active in a variety of cell types (67). In addition to their ability to increase gene expression, introns have been used in plasmid constructions in order to facilitate gene expression in both mammalian and yeast (68) or mammalian and bacterial cells (69).

Polyadenylation Signals

Most eukaryotic nascent mRNAs possess a poly(A) tail ($n \approx 200$) at their 3′ ends, which is added during a complex process that involves cleavage of the primary transcript and a coupled polyadenylation reaction (70). The poly(A) tract is important for mRNA stability and translatability (71,72). The signals for polyadenylation of mammalian mRNAs are well defined: One component consists of a highly conserved AAUAAA sequence which is located about 20–30 nucleotides upstream of the 3′ end of the mRNA, and the other element consists of an unconserved GU-rich sequence immediately downstream of the polyadenylation site (73,74). There are several efficient poly(A) signals to use in mammalian expression vectors, including those derived from bovine growth hormone (75), mouse β-globin (76), the SV40 early transcription unit (77), and the Herpes simplex virus thymidine kinase gene (78).

Transcription Terminators

Continued transcription from an upstream promoter through a second transcription unit inhibits the function of the downstream promoter, a phenomenon known as promoter occlusion or transcriptional interference. This event has been described in both prokaryotes (79) and eukaryotes (80,81). The proper placement of transcriptional termination signals between two transcription units can prevent promoter occlusion (81). Prokaryotic transcription terminators are well characterized, and their incorporation in expression vectors has been shown to have multiple beneficial effects on gene expression (reviewed in 82). In eukaryotes, a consensus sequence consisting of ATCAAA(A/T)TAGGAAGA has been identified in the termination region of nine genes (83).

4. TRANSLATIONAL CONTROL ELEMENTS

The optimal expression of eukaryotic cDNAs requires the careful consideration of several structural features, including the 5′ and 3′ untranslated sequences and the nucleotide context around the translation initiation codon (the Kozak sequence). In addition, codon usage may have a substantial impact on the translation efficiency of some genes in mammalian cells.

Kozak Sequence

Using systematic mutagenesis of specific genes as well as comparison of eukaryotic mRNA sequences, Kozak (84) defined the optimal translation initiation sequence in eukaryotic mRNAs. CC(A/G)CCaugG emerged as the consensus sequence for initiation in higher eukaryotes. The purines A or G in position −3 (i.e., three nucleotides upstream from the AUG codon) and G immediately following the AUG codon are the most influential in facilitating optimal translation initiation.

5′ Untranslated Region

In eukaryotic cells translation of most mRNAs is initiated according to the "scanning model" (85). The initiation complex, consisting of the 40S ribosomal subunit and cap-binding proteins, forms at the mRNA 5′ terminal cap (m^7GpppN) followed by movement of the ribosome to the "correct" initiating AUG codon in a favorable sequence context (84). The presence of AUG codons in the 5′ untranslated region (5′ UTR) of the transcript can severely depress translational initiation at the "authentic" start codon, although the extent of inhibition depends on sequences surrounding the upstream AUG (86,87). Such inhibition can be minimized by the presence of a translation termination codon in-frame with the upstream AUG (86,87). An additional concern involves the potential of the 5′ UTR to form extensive secondary structure. Thus, GC-rich regions have the potential to form stable hairpin structures which can inhibit translation initiation, a phenomenon that has been extensively documented in eukaryotic (88–90) and prokaryotic expression systems (reviewed in 82). One solution to these potential problems is the removal of the 5′ UTR prior to the insertion of cDNAs into expression vectors, with the caveat that the 5′ UTR may contain translational enhancer elements, such as the SP163 element of the vascular endothelial growth factor mRNA (91). The SP163 sequence has been shown to enhance the translation of different mRNAs 25- to 40-fold in several mammalian cell types (91).

3′ Untranslated Region

mRNA destabilization can be effected by specific sequences present in the 3′ UTR. This topic is discussed in section 5 (mRNA Stability). In addition, translational regulation of certain mRNAs is mediated by protein-binding AU-rich elements located in the 3′ UTR (92).

Termination Codon

Experimental evidence indicates that translational termination in mammalian genes may be modulated by nucleotides additional to those of the trinucleotide stop codon. Statistical analysis of the context of termination codons in 5208 mammalian genes showed a highly significant bias in the position immediately following the stop codon (Fig. 1 in 93). The significance of this bias in translational termination was tested in both *in vivo* and *in vitro* assays, and it was determined that the base following the stop codon influences the efficiency of translation termination. Thus, tetranucleotides with a purine in the fourth position are more effective as termination signals than those with a pyrimidine (93).

Codon Usage

Both prokaryotic and eukaryotic genes exhibit a nonrandom usage of synonymous codons (94,95). In general, highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones, and the frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs (96). Most studies of codon optimization for gene expression have been carried out in *E. coli* (reviewed in 82,97). *E. coli* exhibits a highly biased codon usage and, therefore, the possibility exists that heterologous genes enriched with codons that are rarely used by *E. coli* (98) may not be expressed efficiently in *E. coli*. Similarly, it is possible that mammalian codon usage may affect transla-

tion efficiency of heterologous genes, as documented below.

As part of gene transfer studies for the correction of human genetic disorders, Wheeler *et al.* (99) studied the mitochondrial enzyme ornithine transcarbamylase (OTC). The OTC gene was synthesized by PCR using codons optimized for mammalian mitochondrial as well as for universal codon usage. The synthetic OTC gene was successfully expressed in *E. coli.* However, transient transfections of COS-7 cells failed to produce enzymatic activity or immunoreactive OTC protein, despite the detection of mRNA specific for the synthetic OTC gene and the successful transient expression of an unmodified human OTC gene (99). It is likely that OTC mRNA could not be translated by the mammalian cytoplasmic tRNA pool, a supposition that potentially could be proved by the use of a mitochondrial *in vitro* translation system. The jellyfish *Aequorea victoria* green fluorescent protein (GFP) is widely used as a reporter in many gene transfer applications, including gene therapy. Different versions of the GFP gene optimized for human codon usage have been shown to exhibit significantly higher expression levels (4- to 10-fold) and increased fluorescence intensity in mammalian cells (100,101). It is possible, however, that the altered codon content may have stabilized the GFP mRNA, in addition to enhancing its translational efficiency. In another example of codon optimization, a GFP gene modified to contain synonymous codons from highly used human genes showed a 20-fold higher expression level in maize leaf cells than in the original GFP sequence (102).

Codon optimization may also have a significant impact in vaccination studies. A sequence from the human immunodeficiency virus type 1 gp120 gene was optimized using codons from highly expressed human genes, resulting in higher expression levels (103,104). It was shown that the increase in efficiency of expression was not due to enhanced mRNA stability (103). The difference in expression levels between the codon-optimized and wild-type constructs depended on the vector/host combination used. Thus, in 293T cells transiently transfected with the vector pCdm7, there was a 10- to 50-fold increase in expression levels with the synthetic gene. Moreover, immunization of BALB/c mice with the same codon-optimized DNA resulted in significantly increased antibody titer and cytotoxic T-lymphocyte reactivity, suggesting a correlation between expression levels and the immune response (104). Similar observations have been obtained with a different pathogen, *Listeria monocytogenes.* Codon-optimized plasmid DNA sequences showed substantially higher expression levels in mammalian cells and conferred partial protection against listerial infection in mice (105).

5. mRNA STABILITY

The turnover of mRNA is an important posttranscriptional mechanism for the physiological control of gene expression (106). The short half-life of some mRNAs, such as cytokines, cell cycle control factors, and oncogenes, is thought to permit the rapid cessation of protein production in response to rapidly changing physiological conditions. Conversely, the high degree of stability of some mRNAs, such as the globins, collagens, and crystallins, ensures their accumulation to high steady-state levels following an increase in transcription (107). Thus, the recognition that the metabolic stability of mRNA can have profound effects on gene expression has led to specific suggestions for potential therapeutic interventions (107). The potential ability to extend significantly the half-life of transcripts offers an attractive means of enhancing protein production in mammalian expression systems.

One determinant of eukaryotic mRNA lability is an AU-rich sequence in the 3′ UTR of many unstable mammalian mRNAs (108–110). The insertion of an AU-rich element into the 3′ UTR of a stable mRNA destabilizes the chimeric transcript (109,111). The optimal sequence for this destabilizing determinant is believed to be UUAUUUAUU (111) or UUAUUUA(U/A)(U/A) (112). The removal of these sequences from the 3′ UTR of unstable mRNAs is desirable for maximal protein production.

Synthetic 5′ secondary structures have been shown to increase mRNA half-lives in *E. coli* (113). In seeking to maximize transcript stability and protein production in mammalian cells, investigators have substituted the UTRs of stable mRNAs, such as β-globin, for the UTRs of transcripts of interest (e.g., 114,115). This strategy, effective in specific cases, may not have universal application, as mRNA degradation is effected by multiple pathways in mammalian cells (116,117). Thus, in addition to exonucleolytic activity at both the 5′ and 3′ termini, determinants of mRNA half-life have been mapped to the coding regions of several mRNA species (106,117,118). In this case, the addition of a stabilizing UTR probably will have no effect on transcript stability. Furthermore, mRNA stability is modulated by a variety of cell-specific proteins that act *in trans* to destabilize (119,120) or stabilize transcripts (121–123). The use of a specific UTR for the purpose of stabilizing a heterologous transcript in mammalian cells assumes the presence of the cognate UTR-binding proteins in the same cells. At present, our knowledge of the distribution of such proteins in different mammalian cell lines used for protein production is incomplete (107,120).

It would be a significant omission not to mention that levels of heterologous proteins are also affected by protein degradation pathways. This is an important topic, beyond the scope of this review. Strategies for

TABLE 5

Fusion Moieties for Gene Expression in Mammalian Cells[a]

Fusion partner (source)	Ligand/substrate	Detection	Application	Reference[b]	Commercial source of expression vector[c]
FLAG peptide	Anti-FLAG monoclonal antibodies M1 and M2	Antibody	Purification, detection	219, 220	Stratagene (www.stratagene.com)
$(Histidine)_6$	Ni^{2+}-nitrilotriacetic acid	Antibody	Purification, detection	221, 222	Invitrogen (www.invitrogen.com) Qiagen (www.qiagen.com)
Glutathione *S*-transferase (*Schistosoma japonicum*)	Glutathione	Biochemical, antibody	Purification, detection	223, 224, 225	Amersham Pharmacia (www.apbiotech.com)
c-myc epitope	Antibody	Antibody	Purification, detection		Invitrogen
Calmodulin-binding peptide	Calmodulin		Purification		Stratagene
Fc-Hinge	Protein A		Purification, protein dimerization, higher protein yield, longer protein half-life	226, 227	
IgG1 and IgM heavy chain constant regions			Longer protein half-life	228	
Streptococcal protein G	Serum albumin		Purification, longer protein half-life	229	
Serum albumin			Longer protein half-life	229, 230[d]	
Viral glycoprotein transmembrane domain			Surface expression for vaccination	231	
Platelet-derived growth factor receptor (PDGFR) transmembrane domain			Surface expression for ligand-binding interactions		Invitrogen
Herpes simplex virus glycoprotein D (gD) domain	Antibody	Antibody	Purification	232	
Epstein-Barr virus nuclear antigen 1 GGAGAGAG			Longer protein half-life	125	
Growth hormone (human, rat)	Antibody	Immunoassay	Detection, monitor promoter activity	233, 234	
Alkaline phosphatase (mammalian/bacterial)	*p*-Nitrophenyl phosphate	Electrochemical, chemiluminescence, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 235, 236	Clontech (www.clontech.com)
β-Galactosidase (*Escherichia coli*)	β-Galactosides	Electrochemical, chemiluminescence, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 237	Clontech
Chloramphenicol acetyltransferase (*E. coli*)	Chloramphenicol or its derivatives	Radioisotope, fluorescence	Detection, monitor promoter activity, biosensors	143, 234, 238, 239	Promega (www.promega.com)
Luciferase (*Photinus pyralis*) (*Luciola mingrelica*)	Firefly luciferin	Bioluminescence	Detection, monitor promoter activity, biosensors	143, 234, 240–242	Promega
Luciferase (*Vibrio harveyi*)	*n*-Decyl aldehyde	Bioluminescence	Detection, monitor promoter activity, biosensors	143, 240[e]	
Luciferase (*Renilla reniformis*)	Coelenterazine	Bioluminescence	Detection, monitor promoter activity, biosensors	243	
Green fluorescent protein and its variants (*Aequorea victoria*)		Fluorescence	Detection, monitor promoter activity, higher protein yield, biosensors	143, 144, 244, 245, 246	Clontech, Invitrogen
Aequorin (*A. victoria*)	Coelenterazine	Bioluminescence	Immunoassay, hybridization assay, Ca^{2+} reporter	143	

[a] This table does not include heterologous signal sequences or antibody variable regions used for protein targeting.
[b] References indicate application of fusion moieties in mammalian cells and do not necessarily reflect the original development of said fusion partner.
[c] No effort has been made to provide a complete list of commercial suppliers.
[d] Expression of albumin–CD4 fusion in yeast.
[e] Low expression of bacterial luciferase in mammalian cells, with an increase of more than 10-fold when cells were grown at 30°C.

minimizing protein degradation in prokaryotes have been reviewed (82,124). In contrast, less light appears to shine on this complex issue with regard to mammalian protein production. It is worthwhile to point out an interesting recent study on ubiquitinated proteins (125,126) that has implications for the modulation of protein degradation in eukaryotes. The insertion of a minimal eight-residue glycine–alanine repeat into a protein that is targeted for proteolysis via the ubiquitin–proteasome pathway inhibited its degradation.

6. POLYCISTRONIC MESSAGES

The scanning model of translation initiation (see section 4) does not apply to many viral (127,128) and apparently, some cellular messages (91,129). These are translated in a cap-independent manner at internal

sites known as internal ribosome entry sites (IRES). It is believed that cellular *trans*-acting proteins bind to the IRES element and facilitate ribosome binding and translational initiation (130). However, the precise mechanism of IRES-mediated translation is unclear. In a critical examination of published studies on IRES elements, Kozak (130a) concluded that recent concepts about the mechanism of internal translation initiation at putative IRES complexes are premature. Moreover, the experimental evidence for the presence of IRES elements in cellular mRNAs from mammals has been challenged (130a). Similarly, reports of internal initiation in yeast have been questioned (130b). The reader is referred to Kozak (130a) for a detailed discussion of this topic.

Earlier designs of polycistronic constructs for the expression of two or more genes from a single transcript had several limitations, discussed previously (131,132). Robust polycistronic vectors now utilize IRES elements that facilitate internal ribosome binding to the second and subsequent transcription units (128,133,134). Vectors containing IRES elements have a variety of applications: (1) establishment of stable mammalian cell lines which requires coexpression of the gene of interest and a selectable marker (135,136); (2) efficient gene amplification in the generation of stable cell lines (137); (3) clonal selection of cells expressing inducible gene products (138); (4) characterization of antibody responses in DNA immunization protocols (139); and (5) coexpression of genes for positive–negative (suicide) selections in gene therapy. For example, the multidrug resistance gene *MDR1* has been coexpressed with the Herpes simplex virus thymidine kinase (TK) gene (140). The TK gene acts both as a selectable marker in TK-deficient cells and as a suicide gene. Thus, cells expressing the TK gene can be selected against using the nucleoside analog ganciclovir. Additional applications of IRES elements include (6) gene trapping for the identification of developmentally regulated genes (134); (7) gene targeting (134,141); and (8) coordinated constitutive or adjustable high-level expression of three genes in mammalian cells (142).

7. FUSION MOIETIES

Their wide range of applications makes fusion components valuable tools in both prokaryotic and eukaryotic gene expression systems (82). Fusion moieties (Table 5) can be used as affinity handles for the facile isolation and purification of proteins, as reporter genes for the study of promoter activity or localization of proteins in cellular compartments, as protein dimerization domains, to increase expression, solubility, and secretion of proteins, or to display polypeptides on the surface of cells for vaccine development, protein–protein interactions, drug screening, and other potential applications. Fusion constructs have also been used to increase the half-life of target proteins for potential therapeutic applications (Table 5). In recent years, the fusion of reporter genes to heterologous promoters is being actively pursued for the engineering of bacterial biosensors in analytical, environmental, and clinical research (143,144). Many of the reporter genes used in bacterial biosensors should have applications in mammalian expression systems.

The design of protease cleavage sites between the fusion moiety and the target protein facilitates the separation of the two components. Technical issues pertaining to site-specific proteolysis of fusion proteins have been reviewed (145). The design of fusions for protein targeting to specific cellular compartments has been reviewed (146,147).

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