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Promoting Gene Therapy: Expression Systems for Transgenes and Post-transcriptional Gene Silencing

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Introduction

Gene therapy is an encompassing term that refers to the introduction of genetic information to transiently or stably alter gene expression within the targeted cells and tissues. The objective is to complement, inhibit, or replace the function of disease-related genes, whether they be an endogenous mutant gene or an introduced viral gene. Concepts of gene therapy were developed almost twenty years ago and have been transformed recently into clinical reality. In 1990, the first gene therapy study for the treatment of adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID) began (Blaese *et al.*, 1995), followed by a rapidly growing number of clinical gene therapy trials across diseases caused by genetic disorders, viral infections, and malignancies (Miller, 1992; Anderson, 1998). For gene therapy to be successful, an appropriate amount of the therapeutic gene must be delivered into the target tissue without excessive toxicity. The gene of interest should be able to persist in cells and should be efficiently expressed. Thus, gene therapy would involve exogenous delivery or expression of RNA/DNA-based inhibitors to inhibit gene expression, or an intact gene to complement a genetic defect. In both cases, a typical expression system would involve the use of a promoter, followed by the gene of interest, and then a transcriptional termination signal. Early gene therapy studies primarily involved the use of simple Pol II-based transcription cassettes for the expression of transgenes. On the other hand, expression of RNA-based antivirals like ribozymes, RNA decoys, siRNAs, etc., involved the use of both Pol II-based and Pol III-based expression systems. As the potential applications of gene therapy increased,

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Abbreviations: AP-1, activator protein-1; EF-1α, elongation factor 1alpha; IFN, interferon; LTR, long terminal repeat; NFκB, nuclear factor-kappaB; P-TEFb, positive transcription elongation factor B; RRE, Rev response element; YY1, Yin Yang 1.

Biotechnology and Genetic Engineering Reviews – Vol. 23, December 2006
0264–8725/07/23/71–91 $20.00 + $0.00 © Lavoisier/Intercept, 14 rue de Provigny, F-94236 Cachan cedex, France

so did the necessity to develop better expression systems that could be regulated in a temporal or tissue specific manner. This review focuses mainly on advances in promoter design and other novel expression systems used for transgene expression.

RNA Pol II-based expression systems

The choice of a promoter for transgene expression is governed by the transgene itself, as well as the cell type in which the transgene needs to be expressed. Gene therapy involving transgenes that deliver corrective genes may require constitutive expression, whereas an approach involving the delivery of suicide genes or foreign proteins often requires an inducible system under tight control.

One of the most commonly used promoters for transgene expression in cells is the cytomegalovirus (CMV) major immediate-early promoter/enhancer. The CMV promoter/enhancer is active in many cell culture systems and is considered to be one of the strongest promoters *in vitro*. The human CMV promoter consists of at least four types of repetitive sequence elements, referred to as the 17-, 18-, 19-, and 21-bp repeats, which are present three to five times within the promoter/enhancer region of the CMV promoter and which form complexes with nuclear proteins (Boshart *et al.*, 1985; Ghazal *et al.*, 1987). The 18- and 19-bp repeats contain consensus binding sites for NFκB and CREB/ATF, respectively, and were shown to mediate the enhancement of CMV promoter activity by these transcription factors (Hunninghake *et al.*, 1989; Sambucetti *et al.*, 1989; Stamminger *et al.*, 1990). The 17-bp repeat was suggested to bind to the transcription factor NF-1 (Niller and Hennighausen, 1991). The 21-bp repeat binds to a negative regulator specific for undifferentiated cells, as well as to YY1, and was suggested to repress CMV promoter-dependent transcription (Kothari *et al.*, 1991; Sinclair *et al.*, 1992). Other factors that bind to the CMV promoter are AP-1 (Sambucetti *et al.*, 1989), SP 1 (Lang *et al.*, 1992), and MDBP (Zhang *et al.*, 1991, 1995). Transcription factors of the NFκB/Rel family play a central role in the regulation of a variety of cellular and viral genes (Baeuerle and Henkel, 1994; Verma *et al.*, 1995; Baeuerle and Baltimore, 1996; Wulczyn *et al.*, 1996). Four NFκB consensus binding sites are present in the CMV promoter, and three of them are identical to the Igκ consensus binding site. Efficient transcription from the CMV promoter was dependent on these sites (Bellas *et al.*, 1995; Prosch *et al.*, 1995), although the effect of the κB binding site was strongly dependent on the cell type used (Niller and Hennighausen, 1991).

However, when this promoter was used *in vivo* for gene therapy, it was silenced in several organs, including the liver, within a few weeks (Loser *et al.*, 1998). The transcriptional silencing observed with this promoter was due to methylation of its CpG motifs (Brooks *et al.*, 2004). Reactivation of this promoter was often observed by treatment with lipopolysaccharide and infection with recombinant adenoviruses (Loser *et al.*, 1998), which is known to activate transcription factor NFκB. As alternatives to the CMV promoter, the albumin, methallonin, or the EF-1α promoter have been used for transgene expression (Najjar and Lewis, 1999). When the EF-1α promoter is used in place of the CMV promoter, a prolonged expression of reporter genes in stable transduced cells occurs (Teschendorf *et al.*, 2002). Nevertheless, most investigators still prefer to use the CMV promoter for transgene expression. Other promoters commonly used for constitutive transgene expression are from the

ubiquitin C promoter (Gill *et al.*, 2001), Rous Sarcoma Virus (RSV–LTR) (Voeks *et al.*, 2002; Nomoto *et al.*, 2003), the Simian Virus 40 (SV40) (Isola and Gordon, 1989), Murine Sarcoma Virus (MSV–LTR) (Sutrave *et al.*, 1990), and Mouse Mammary Tumour Virus (MMTV–LTR) (Lu and Steiner, 2000; Finkle *et al.*, 2004).

INDUCIBLE TRANSGENE EXPRESSION

Expression of certain genes needs to be controlled very tightly. The genes that fall under this class are mainly suicide genes that have been used extensively in cancer therapy studies, or genes encoding foreign proteins that might trigger an immunological response. A great deal of effort has been dedicated to the engineering of inducible systems. Promising results have been obtained in cell culture experiments (Furth *et al.*, 1994; Wyman *et al.*, 1999; Johansen *et al.*, 2002). Inducible systems also allow the generation of stable cell lines carrying the gene of interest where the expression of those genes would be toxic to cell growth. Indeed, inducible systems have been employed with some success to generate both stable cell lines and animal models (Romano *et al.*, 2001; Christen and von Herrath, 2002). In most cases, the design of the promoter governs inducibility at the transcriptional level. In very few instances, however, translation or splicing also has been used to regulate gene expression. One study involves the use of *trans*-splicing mRNA. This study showed that the intron of one pre-mRNA molecule can interact with another pre-mRNA molecule, giving rise to a chimeric mRNA molecule via *trans*-splicing. This approach can be used for corrective gene therapy (Puttaraju *et al.*, 1999). Another study involves the use of nutrient molecules to regulate translation by affecting the activity of eukaryotic initiation factor 2B (Proud, 2002). Some studies regulate gene expression at the level of both transcription and splicing. One such approach for conditional gene expression for HIV-1 gene therapy involves tight regulation of cytosine deaminase, diphtheria toxin, and interferon alpha2 using the Tat/Rev axis of HIV-1 (Ragheb *et al.*, 1999). In that study, the U3R region of the HIV-1 IIIB LTR provides the promoter and Tat-responsive element, a modified intron derived from the human c-*src* gene facilitates the splicing of inserted genes, and the HIV-1 RRE region enhances the transport of unspliced mRNAs. However, control at the level of transcription still remains the most commonly used approach for inducible transgene expression.

Three types of controlled expression can be obtained: temporal, tissue specific, and conditional. Temporal control involves the expression of the transgene at a desired time and is generally achieved by adding an inducer to the growth milieu of cells. The most common system employing temporal control is the tetracycline system (Gossen and Bujard, 1992). Gene expression can be turned on by either adding or removing the antibiotic and is accordingly called either the Tet ON or the Tet OFF system (*Figure 5.1a*). The tetracycline system involves the Tet operator sequences of the *Escherichia coli* tetracycline resistance gene cloned upstream of a minimal promoter. The Tet repressor from *E. coli* (Tn10 Tc resistance gene product) is fused to the transactivation domain of the Herpes Simplex Virus (HSV) transactivator. Binding of the repressor/VP16 to Tet sequences upstream of the minimal promoter would activate expression of the gene in a tetracycline-dependent manner. A single mutation in the Tet repressor can allow the repressor to bind its sequence either in the

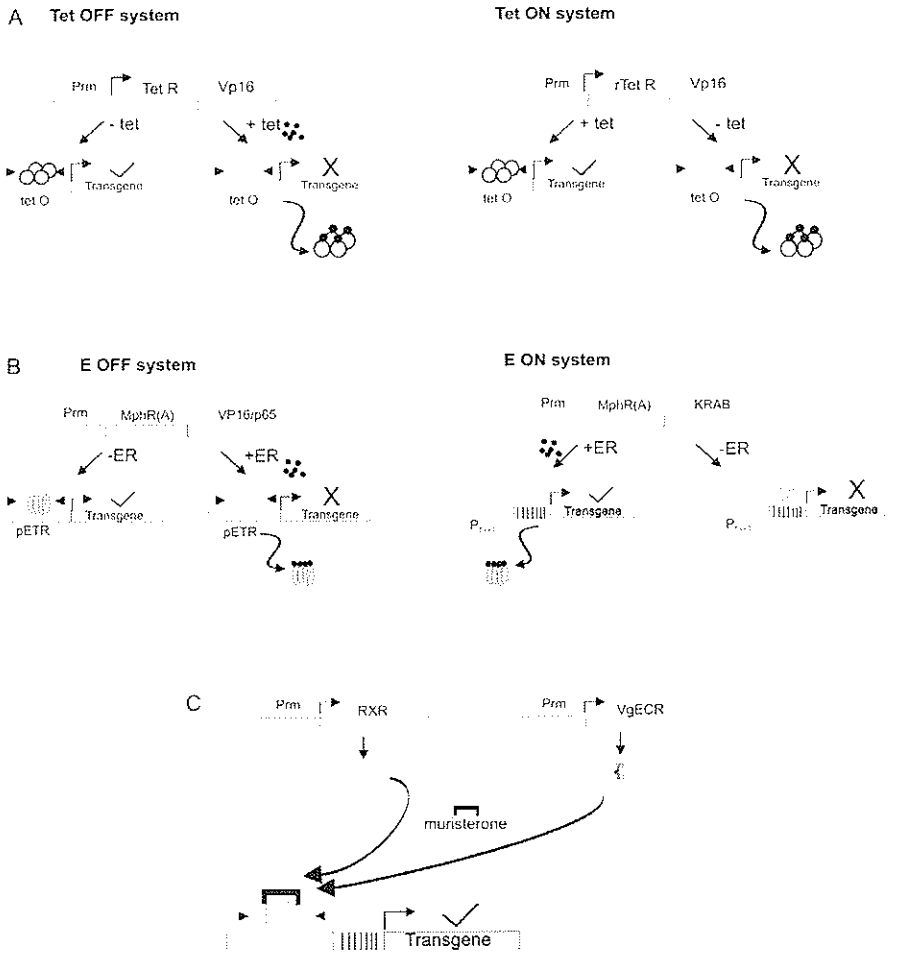

Figure 5.1. Schematic representations of inducible systems for transgene expression. (a) The tetracycline inducible expression system. In the case of the Tet OFF system, the transactivator binds to the Tet-O sequence in the absence of tetracycline, thus activating transcription by virtue of its VP16 transactivation domain. The addition of tetracycline causes the transactivator to dissociate from the promoter, thus turning OFF transcription. In the Tet ON system, a single mutation in the repressor–VP16 transactivator allows the repressor to bind in the presence of tetracycline and activate transcription. (b) The macrolide inducible system. The antibiotic-dependent interaction between the repressor (E) and the operator (ETR) is exploited to develop both E 'ON' and E 'OFF' systems. (c) The ecdysone inducible system developed by No *et al.* (1996). EcR is coupled to the transactivation domain of VP16 and hence the binding of the heterodimer to its binding sites upstream of a minimal *Drosophila* hsp70 promoter activates gene expression in the presence of the ecdysone muristerone.

presence or absence of tetracycline (Gossen *et al.*, 1995; Paulus *et al.*, 1996). There are, however, a few disadvantages in the Tet system as it relies only on control of transcription at the level of initiation. The minimal CMV promoter, mainly coupled to the Tet-O sequences by itself, can have a low level of expression, rendering the system slightly leaky. Alternately, after integration, the minimal CMV promoter may get activated by nearby enhancers, contributing to a higher background

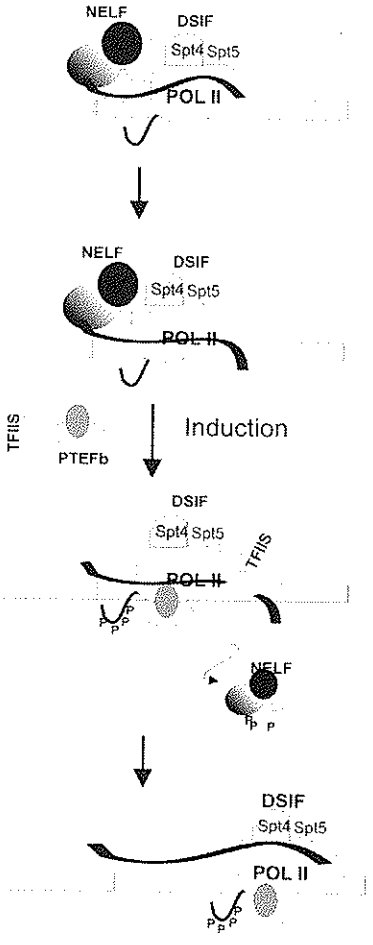

Figure 5.2. Schematic representation of elongation control. The NELF-E subunit binds to the nascent RNA emerging from the exit channel of the RNA Pol IIa, stalling the polymerase. The stall causes the RNA polymerase to reverse translocate, thereby misaligning the 3' end of the transcript. Recruitment of P-TEFb on induction leads to phosphorylation of NELF-E and the CTD of Pol II. Simultaneously, the transcript cleavage factor, TFIIS, cleaves the misaligned 3' end to restart the Pol II.

expression (van Craenenbroeck *et al.*, 2001). However, a number of studies have tried to optimize the Tet inducible system and eliminate background by developing a Tet repressor protein based on KRAB (Kruppel-associated Box), a zinc finger protein which mediates strong transcriptional suppression (Rossi *et al.*, 1998).

Moreover, at least one report demonstrates the presence of functional interferon-inducible response elements embedded within the Tet operator sequences. Gel shift analyses in these studies indicated the binding of IFN stimulated gene factors to these sequences (Rang and Will, 2000). A similar system, based on the macrolide group of antibiotics, has been employed for transgene control in mammalian cells and mice (*Figure 5.1b*). The antibiotic-dependent interaction between the repressor

(E) and the operator (ETR) is exploited to develop both E 'ON' and E 'OFF' systems. The E 'OFF' system consists of a chimeric erythromycin-dependent transactivator constructed by fusing the repressor to transactivation domains that bind to upstream ETR containing sites of synthetic promoters (Weber *et al.*, 2002). A number of other inducible systems have been developed along similar lines using DNA binding domains of transcription factors linked to transactivation domains of transcription factors.

One such approach involves the use of nuclear receptors. A report by No and colleagues takes advantage of a naturally occurring steroid-inducible system (No *et al.*, 1996). A pulse of steroid hormone, ecdysone, triggers metamorphosis in *Drosophila melanogaster*. Mediating this response is the functional ecdysone receptor, a heterodimer of the ecdysone receptor, EcR, and RXR. EcR is coupled to the transactivation domain of VP16, and hence the binding of the heterodimer to its binding sites upstream of a minimal *Drosophila* hsp70 promoter activates gene expression in the presence of the ecdysone muristerone (*Figure 5.1c*). The use of the *Drosophila* hsp70 promoter has an added advantage in that transcription is controlled not only at the level of initiation but also elongation (*Figure 5.2*). Transcription from the hsp70 promoter pauses within the first 20 to 45 nucleotides in a phenomenon called promoter proximal pausing. A number of functionally diverse promoters, including the c-myc promoter and the HIV long terminal repeat (LTR), are regulated at the level of promoter proximal pausing. The stalled Pol II is phosphorylated on Ser5 of the Pol II CTD (but not Ser2), and the nascent mRNA is partially capped and is not elongation competent. Also, shortly after initiation of transcription, RNAPII comes under the negative control of the DRB sensitivity inducing factor (DSIF) and the negative elongation factor (NELF). DSIF and NELF cause transcriptional pausing through physical association with Pol II (for a review, see Garriga and Grana, 2004). DSIF binds to, but does not significantly affect the catalytic activity of the Pol II (Yamaguchi *et al.*, 1999a). NELF binds to the complex of DSIF and Pol II to repress transcription (Wada *et al.*, 1998; Yamaguchi *et al.*, 1999b). Indeed, depleting salivary glands of NELF using RNA interference diminished the level of promoter proximal pausing occurring on hsp70 in these glands (Wu *et al.*, 2003). Chromatin immunoprecipitation analyses to detect NELF at the hsp70 promoter were found to crosslink to the hsp70 promoter region prior to heat shock induction. The NELF-E subunit has an RNA recognition motif (RRM), which is known to bind nascent transcripts about 30 nucleotides long, causing the polymerase to pause.

Promoter proximal pausing occurs when the nascent transcript emerges from the RNA exit channel of the Pol II and is grabbed by the NELF-E subunit. Tethering of the NELF-E to the elongation complex would generate a rigid body that could restrict the movement of the Pol IIa. This model is supported by several observations. The paused polymerase is in the Pol IIa state (O'Brien *et al.*, 1994), and NELF and DSIF only inhibit elongation by Pol IIa (Yamaguchi *et al.*, 1999a). *In vitro* transcription analysis indicates that the elongation complex is not receptive to inhibition by NELF and DSIF until the nascent transcript is circa 30 nucleotides long (Yamaguchi *et al.*, 1999a). This also causes the polymerase to reverse translocate. Backward movement misaligns the 3' end of the nascent RNA with the RNA polymerase active site, thereby prohibiting continued RNA synthesis, even when the negative factors are removed (Adelman *et al.*, 2005).

When induced, P-TEFb kinase, a heterodimer of CDK-9 and Cyclin T1, is recruited to the promoter, which phosphorylates the NELF-E subunit (Fujinaga *et al.*, 2004), which in turn causes the NELF to dissociate. P-TEFb also phosphorylates the Ser2 residues on the CTD heptapeptide repeats, thus making the polymerase elongation competent (Renner *et al.*, 2001). However, significant induction still occurs in the presence of flavipyridole (Ni *et al.*, 2004), which is a potent inhibitor of P-TEFb, suggesting that some activation still occurs even when P-TEFb is inactive and other cellular processes might be involved. The transcript cleavage factor, TFIIS, further cleaves the misaligned 3' end caused by reverse translocation of the polymerase and restarts the arrested RNA polymerase by creating a new 3' end that is properly aligned for catalysis (Adelman *et al.*, 2005).

Although temporal control of gene expression is widely applied in research-based applications, this method is limited for *in vivo* applications since administration of the drug will result in every cell that harbours the construct expressing the transgene. This would be a disadvantage in studies where expression is desired in a particular cell type. For such studies, depending on the tissue in which expression is desired, tissue specific promoters are used. Indeed, in the absence of a precise, targeted gene delivery mechanism, only the use of tissue specific promoters can restrict transgene expression to a particular cell type. A variety of tissue specific promoters have been employed for transgene expression. For instance, myelin basic promoter (Asipu and Blair, 1994; Wei *et al.*, 2003), tyrosine hydroxylase promoter (Kessler *et al.*, 2003), and human dopamine ß hydroxylase promoter (Hwang *et al.*, 2001) allow for a brain-associated gene expression. Similarly, myosin heavy chain promoter (Sanbe *et al.*, 2003), and other synthetic promoters, were designed for transgene expression in the heart and muscles (Li *et al.*, 1999). The osteocalcin promoter restricts gene expression to bone tissues (Hou *et al.*, 1999). Selective expression of transgene in prostrate adenocarcinoma cells has been achieved with the use of the prostrate-specific membrane antigen (PSMA) promoter/enhancer (Zeng *et al.*, 2005). Similarly, the progression-elevated gene-3 (PEG-3) promoter has been used for targeted gene expression in cancer cells (Su *et al.*, 2005). The promoter of PEG-3 displays robust expression in a broad spectrum of human cancer cell lines, with marginal expression in normal cellular counterparts. In like manner, the rat insulin promoter conferred selective gene expression in human pancreatic cancer cells (Wang *et al.*, 2004). These promoters can be particularly useful for suicide gene-directed enzyme pro-drug strategies for cancer (Yazawa *et al.*, 2002).

DUAL/MULTIPLE GENE EXPRESSION FROM SINGLE TRANSCRIPTION UNITS

The expression of a single transgene is often sufficient for gene therapy of simple Mendelian disorders. Nevertheless, a number of other diseases like cancer may require simultaneous targeting of different defective genes. For viral disorders like HIV infection, multiple pathways of the viral life cycle need to be targeted to prevent the emergence of escape mutants. Dual or multiple gene expression is often commonly observed in viruses. Due to the restriction placed by the size of their genome, many viruses encode more than one protein from a single transcript. These viruses employ a number of strategies to express multiple genes. There are three main approaches to multiple gene expression. Of potential significance to gene therapy

are the internal ribosome entry sites (IRES) to express two genes from the same transcript. IRES are *cis*-acting RNA sequences able to mediate internal entry of the 40S ribosome subunit on some eukaryotic and viral messenger RNAs upstream of a translation initiation codon, thus serving as a ribosome launching pad for efficient internal initiation of translation (*Figure 5.3a*) (for review see Komar and Hatzoglou, 2005). IRES elements were earlier believed to be restricted to the picornaviruses group but now appear to be far more extended than previously described, and are known to exist in flaviviruses, plant viruses, retroviruses, DNA viruses, and even mammalian genomes. Currently, the most commonly used IRES for transgene expression is the encephalomyocarditis virus (EMCV). One of the drawbacks with using the EMCV IRES is that the expression of the downstream gene is significantly less efficient than the upstream gene (Mizuguchi *et al.*, 2000). However, IRESs have been used widely for transgene expression in cell cultures and in mice (Sokolic *et al.*, 1996; Wagstaff *et al.*, 1998; Garton *et al.*, 2002; Wong *et al.*, 2002; Wang *et al.*, 2005). At least one study has involved the stable expression of three genes from a tricistronic retroviral vector. In that study, a 9 nt segment of a cellular mRNA IRES was used along with a picornavirus IRES, since the two elements have minimal sequence homology and would eliminate recombination (Li and Zhang, 2004). Another study employed an IRES for tissue specific gene expression where the IRES in the 5' transcript leader of the mRNA of Scamper, a putative intracellular calcium channel activated by sphingosylphosphocholine, is used to drive tissue-specific transgene expression in the kidney epithelial cells (de Pietri Tonelli *et al.*, 2003).

A completely different approach for dual gene expression involves polyproteins that self-process co-translationally into separate components. Such proteins have been generated using self-cleaving 2A-like peptides of the foot-and-mouth disease virus (FMDV) and other picornaviruses (Furler *et al.*, 2001). However, application of this technology has been limited, as it requires molecular engineering of both transgenes and introduces sequence changes that may affect the activity, stability, and immunogenicity of their protein products. A study by Amendola and colleagues has instead exploited synthetic bidirectional promoters designed by fusing minimal core promoters in opposite orientation to full promoters of ubiquitously expressed genes, namely phosphoglycerate kinase promoter, or the human ubiquitin C promoter (Amendola *et al.*, 2005) (*Figure 5.3b*).

The rationale was that the enhancer elements of the full promoter would be sufficient to initiate transcription from the minimal promoter, which also was oriented in the opposite direction. Using this approach, the authors have demonstrated that, unlike IRES elements where the downstream gene is not efficiently expressed, efficient dual gene expression was observed. However, the disadvantage of this technique vis-à-vis the IRES is that one is required to clone an additional polyadenylation signal sequence, thereby increasing the size of the construct. This would be a disadvantage in vector systems that are constrained by size, as in the case of adeno-associated virus vectors.

RNA Pol III promoter cassettes drive expression of siRNA-like molecules to accomplish post-transcriptional gene silencing: post-transcriptional gene silencing by RNA interference was first observed in the nematode, *Caenorhabditis elegans* (Fire *et al.*, 1998), and subsequently shown to be conserved in higher eukaryotes (Hannon, 2002; Montgomery, 2004). The phenomenon called RNA interference

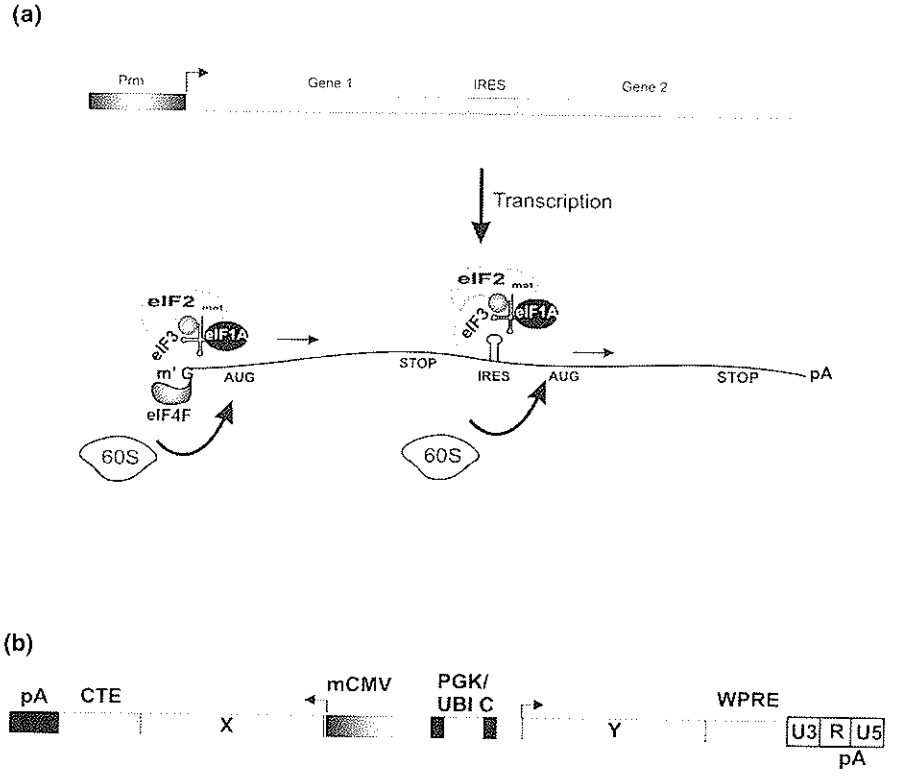

Figure 5.3. (a) Schematic representation of IRES-based internal translation initiation. Note that this scheme is a simplified presentation of the pathway and omits additional initiation factors participating in the process. The internal ribosome entry site serves as a launching pad for internal initiation of translation, allowing expression of two or more genes from a single transcript. (b) Bidirectional promoter system developed by Amendola *et al.* (2005). A minimal core promoter (mCMV) is fused in the opposite orientation to full promoters of ubiquitously expressed genes, namely phosphoglycerate kinase promoter or the human ubiquitin C promoter. Since enhancer elements can function in either orientation, elements of the full promoter can activate transcription from the core promoter in the opposite direction.

(RNAi) has been investigated by both biochemical and genetic approaches. The effector molecules of RNAi are short 21–23-mer double-stranded RNAs that are processed from longer precursors by Dicer (DCR), a multidomain enzyme of the RNase III family (Zamore *et al.*, 2000; Bernstein *et al.*, 2001) (*Figure 5.4*). Two classes of short RNA molecules, small interfering RNAs (siRNA) and microRNAs (miRNA), have been identified as sequence-specific post-transcriptional regulators of gene expression (for a review, see Sontheimer and Carthew, 2005). siRNAs are small double-stranded RNA that are processed from a longer double-stranded RNA precursor to 21-mers, while microRNAs are 22 nucleotide regulatory RNAs derived from longer RNA transcripts. Though the specific functions of most miRNAs are unknown, as a class they are involved in regulation of a number of cellular processes via post-transcriptional gene silencing (PTGS). Both siRNAs and miRNAs use common molecular pathways to achieve sequence-specific gene inhibition. In the

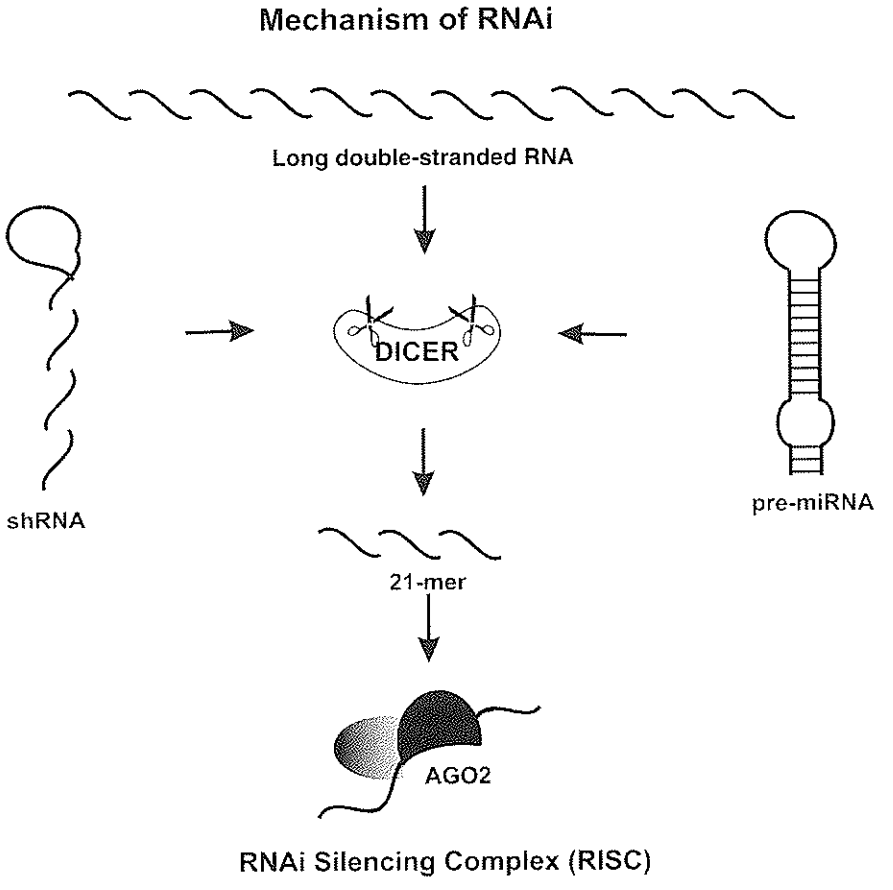

Figure 5.4. Mechanism of RNAi. Long double-stranded RNA, pre-miRNA, or shRNA act as substrates of the RNAse III enzyme, DICER, which processes them to functional 21-mer siRNA or miRNA molecules. These molecules are incorporated in the RISC complex to mediate either mRNA cleavage (siRNA) or translational inhibition (miRNA).

case of siRNAs, sequence-specific cleavage of cognate RNA is observed, while miRNAs mediate translational repression. Both siRNAs and miRNAs are generated by DCR siRNAs, and miRNAs are incorporated into related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively. *In vitro* and *in vivo* biochemical studies have shown that under the appropriate conditions of complementarity to a target mRNA, a siRISC can function as a miRISC to repress translation of the target mRNA, and vice versa (Doench *et al.*, 2003).

RNA interference has been used extensively as a tool for pathway validation in functional genomics (Silva *et al.*, 2004), and is also being exploited for therapeutic applications. RNAi mediated gene silencing in mammalian cells has been achieved by either transfecting synthetic siRNAs (Caplen *et al.*, 2001; Elbashir *et al.*, 2001) or plasmids (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Miyagishi and Taira, 2002b; Paul *et al.*, 2002) that encode Pol III promoter expressed individual sense and antisense strands or short hairpin RNAs (shRNAs) that act as substrates for Dicer (Xia *et al.*, 2002; An *et al.*, 2003).

Since a typical suppression by transfecting short double-stranded RNA molecules can last only for 5–7 days, most approaches for functional genomics or therapy rely upon expressing siRNAs within the cells as individual sense and antisense strands or as short hairpins. The most commonly used system for expressing si/shRNAs are type III Pol III promoters. Pol III promoters encode structural or catalytic RNAs including the 5S ribosomal RNA, 7SL RNA, U6 small nuclear RNA, and the RNA subunit of RNAse P(H1 RNA) (Baer *et al.*, 1990). They each have a precise transcription start, compact organization with minimal *cis*-acting elements, and accurate termination at a simple cluster of four or more T residues.

A number of early studies have been reported on using either a U6 or an H1 promoter that translated into a robust expression and an efficient knockdown of gene expression (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002). A range of Pol III expression systems have been reported. The simplest systems, where both the strands are individually expressed from two tandem transcription units, were reported by two groups (Lee *et al.*, 2002; Miyagishi and Taira, 2002b). In one study (Lee *et al.*, 2002), siRNAs directed against different target sites in HIV-1 genome were expressed either individually as sense and antisense strands on two plasmids or as both cassettes on one plasmid (*Figure 5.5a*). Efficient inhibition of HIV-1 gene expression was observed in transient transfection assays using these expression cassettes.

Subsequent proof of concept experiments for RNAi have involved expression of short hairpin molecules with a stem–loop structure to forego the need to express the two strands individually from different cassettes (Paddison *et al.*, 2002). It was presumed that the stem length of 21 nucleotides or more would serve as efficient substrates for DCR and would be processed to functional siRNA molecules (*Figure 5.5b*). Indeed, efficient expression and processing to siRNA-sized molecules and a concomitant inhibition of target gene expression was observed with these studies (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002).

Pol III promoter was again the promoter of choice for most functional genomics studies involving reverse genetics. A number of groups have constructed first generation RNAi libraries of shRNA expression vectors (Berns *et al.*, 2004; Paddison *et al.*, 2004). One study involves creating gene specific siRNA libraries and expressing them from an opposing promoter vector system involving two convergent U6 and H1 promoters modified to accommodate the termination signals of the opposing promoter (Seyhan *et al.*, 2005) (*Figure 5.5c*).

While the constitutive expression from Pol III promoters sufficed for most proof-of-concept experiments to study gene knockdown, the need was felt for inducible siRNA expression systems for potential applications of RNAi in gene therapy, and also in functional genomics, where knockdown of a particular gene was desired and

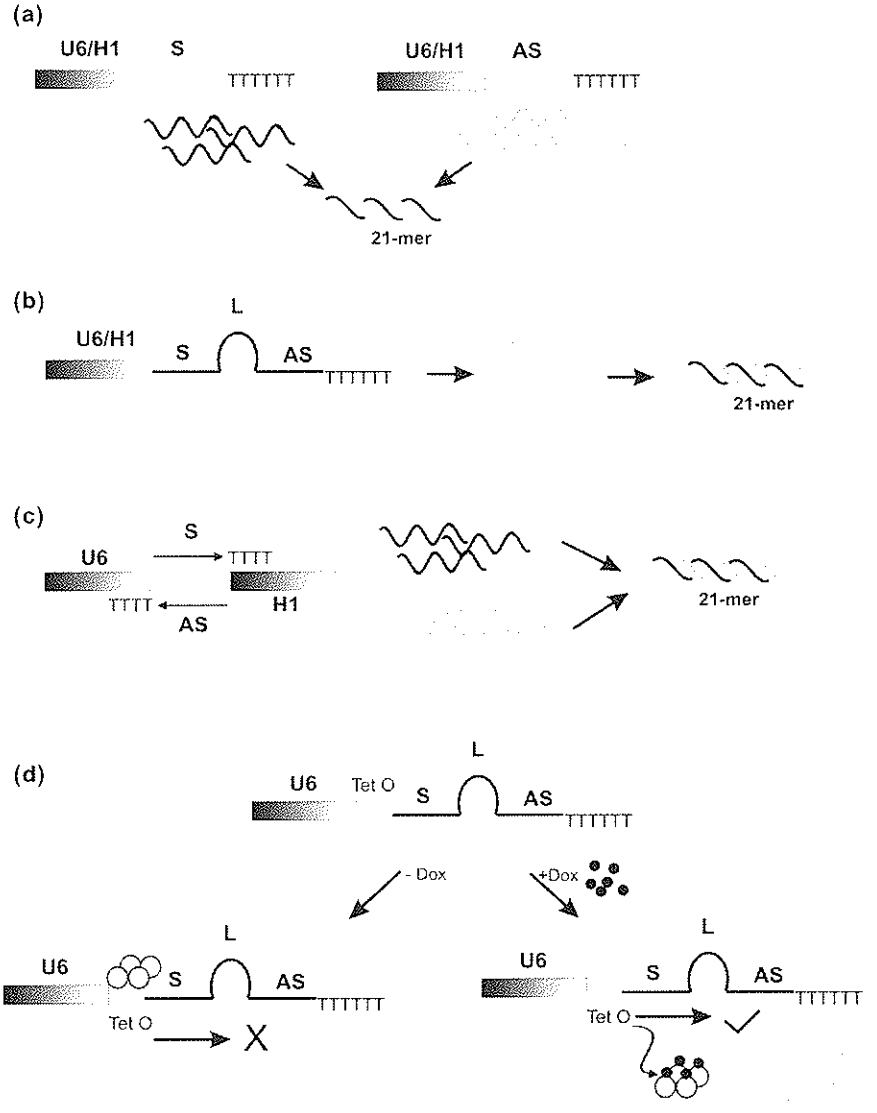

Figure 5.5. Expression systems for post-transcriptional gene silencing. (a) siRNAs can be expressed either individually as sense and antisense strands on two plasmids, or from a single plasmid from Pol III promoters. The sense and antisense strands then hybridize to generate 21-mer double-stranded siRNA molecules. (b) Another approach for siRNA expression exploits the ability of DICER to process hairpin stems expressed from Pol III promoters to functional 21-mer siRNA molecules. shRNA have also been expressed from Pol II promoters with a minimal polyadenylation signal for transcription termination. (c) Pol III expression system reported by Seyhan *et al.* (2005) using an opposing promoter vector system involving two convergent U6 and H1 promoters modified to accommodate the termination signals of the opposing promoter. (d) Tetracycline inducible shRNA expression involves cloning the Tet operator sequence close to the TATA box of Pol III promoters. The binding of Tet repressor to Tet-O in the absence of tetracycline interferes with transcription from the promoter. Addition of tetracycline/doxycycline causes the repressor to dissociate, thereby allowing transcription.

could be lethal, especially genes essential for cell survival, cell cycle regulation, and cell development. Also, recent reports indicate that even partial complementarity can result in some RNAi effect, leading to non-specific knockdown of gene expression. Some reports have even mentioned an upregulation of a non-specific interferon response in cells expressing siRNA. Moreover, it is believed that siRNAs compete with cellular microRNAs for export using the exportin-5 pathway. Thus, it becomes imperative to tightly regulate siRNA expression only in desired cell types or under specific conditions.

Inducible siRNA expression systems

Since Pol III promoters were the promoters of choice for shRNA expression but are inherently constitutive, attention has focused on making these promoters inducible. The first generation inducible systems involved creating a transcriptional block to Pol III transcription using prokaryotic operator–repressor interactions. One approach was to create a tetracycline inducible siRNA system by cloning Tet-R specific operator modules adjacent to the TATA boxes of the H1 or U6 promoters (*Figure 5.5d*). Tet-R binding to Tet-O interfered with H1 driven siRNA transcription in a tetracycline dependable manner (Miyagishi and Taira, 2002a; Matsukura *et al.*, 2003; van de Wetering *et al.*, 2003). Another approach by Gupta and colleagues, to develop an ecdysone inducible U6 promoter driven shRNA system, replaced the natural U6 enhancer elements with Gal4 binding sites (Gupta *et al.*, 2004). A Gal4-Oct2Q fusion protein was then expressed from an ecdysone inducible system. In the presence of the inducer muristerone A, the Gal4-Oct2Q fusion protein was expressed, which bound to Gal4 binding sites upstream of the U6 promoter, thereby activating the shRNA expression. The RNAi mediated effect was reversible in that, upon removal of the inducer, there was partial recovery of the targeted p53 mRNA levels at 48 hours, and full recovery at 96 hours.

Although Pol II systems are the promoters of choice for transgene expression, since they are often naturally regulated at the levels of both transcriptional initiation and elongation, comparatively little attention has been paid in developing them for shRNA expression. Also, minimal Pol II promoters commonly employed for transgene expression, like the CMV promoter or the *Drosophila* hsp70 promoter, can respond to *cis*-acting sequences placed upstream of the TATA elements, and hence would be attractive alternatives to Pol III-based systems if conditional shRNA expression is desired.

The first proof of concept experiments with a Pol II promoter system for shRNA expression was developed by Xia and colleagues (Xia *et al.*, 2002). Functional siRNAs require minimal to no 5' and 3' extensions (Nykanen *et al.*, 2001). Therefore, a model siRNA was placed within 6-bp of the transcription start of a modified human cytomegalovirus immediate early promoter (mPhCMV) and terminated by a synthetic minimal polyadenylation site.

This expression configuration enabled sustained constitutive transgene silencing *in vitro* and *in vivo* (Xia *et al.*, 2002). In our lab, we have developed a conditional siRNA expression system targeting HIV-1 Rev where we have used the minimal *Drosophila* hsp70 promoter for siRNA expression (Unwalla *et al.*, 2004) (*Figure 5.6*). The minimal promoter is devoid of its regulatory sequences both upstream and

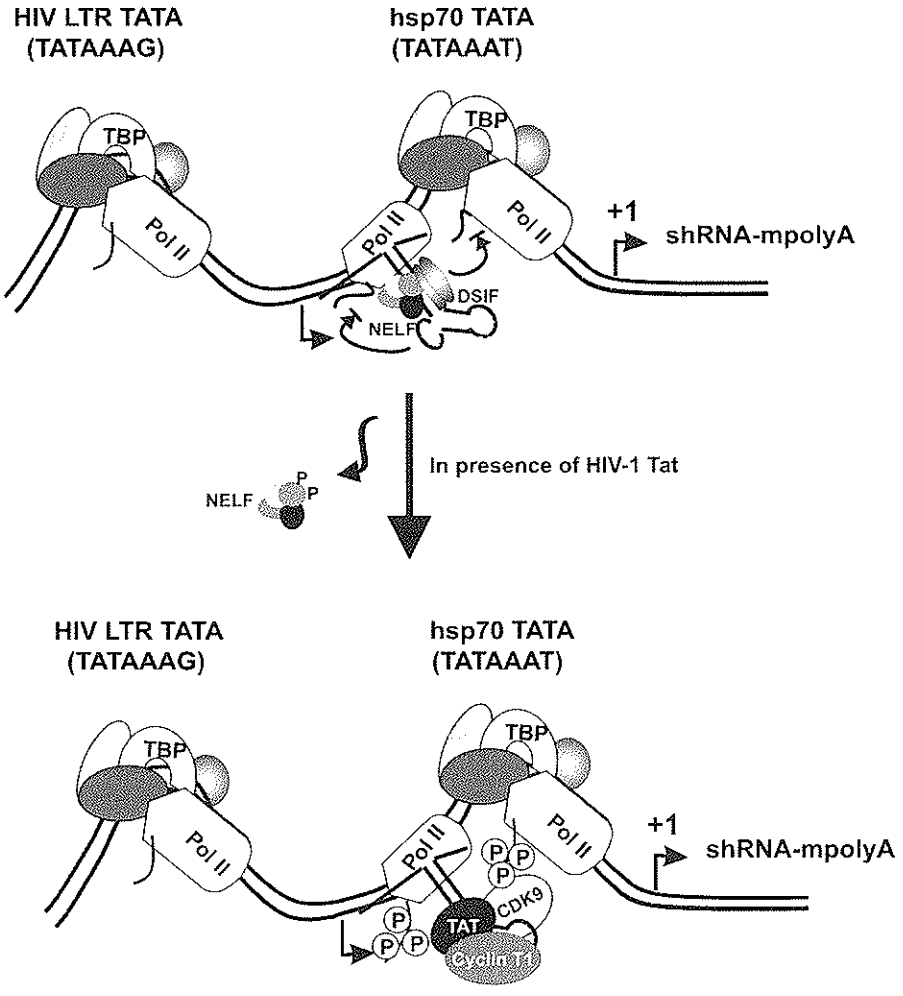

Figure 5.6. HIV-1 Tat inducible siRNA expression system as reported by Unwalla *et al.* (2004). Both components of the fusion promoter system, namely the HIV-1 LTR and the *Drosophila* hsp70 promoter, are regulated by elongation control. In the absence of Tat, NELF and DSIF are recruited in close proximity of the minimal *Drosophila* hsp70 to suppress basal transcription. When cells harbouring this construct are supplied with TAT in *trans* either from a plasmid or by an HIV-1 infection, P-TEFb kinase, a heterodimer of CDK-9 and Cyclin T1, is recruited to the TAR element, which phosphorylates the NELF-E subunit of NELF, causing its dissociation, with a concomitant phosphorylation of the CTD of RNA Pol II to release the elongation block and activating shRNA expression.

downstream of the promoter where promoter proximal pausing occurs. This abolishes the pause and makes the expression constitutive. To reconstitute this pause, the HIV-1 LTR, up to and including the transactivation response element (TAR), was placed upstream of the hsp70 promoter. The fusion promoter exploits the significant similarities of both the promoter elements, namely that both the promoters are regulated by promoter proximal pausing and that basal transcription in both these promoters is arrested by NELF and DSIF (Ping and Rana, 2001; Fujinaga *et al.*,

2004). In both these promoters, the positive transcription elongation factor-b (P-TEFb), a heterodimer of CDK-9 and Cyclin T1, is recruited to the promoter to release the elongation block. Basal transcription from the HIV-1 LTR forms the TAR loop, which in the absence of HIV-1 Tat, recruits NELF and DSIF.

These negative factors repress transcription from both the LTR as well as the hsp70 promoter, thereby reconstituting the pause. When cells are infected with HIV-1, TAT is expressed, which in turn recruits P-TEFb kinase, a heterodimer of CDK-9 and Cyclin T1. This complex phosphorylates the NELF and the spt5 subunit of DSIF, causing the dissociation of NELF, with a concomitant phosphorylation of the C-terminal domain of RNA Pol II, thereby releasing the elongation block. Using this approach, an 85–90% inhibition of HIV-1 gene expression was observed in CEM cells and primary cells infected with HIV-1. Moreover, transient transfection assays demonstrated that the shRNA was expressed only in the presence of HIV-1, thus establishing a negative feedback loop.

Summary

In this review, we have discussed expression systems employed for both transgene expression and post-transcriptional gene silencing. Although constitutive expression systems can function well for gene therapy protocols for simple Mendelian disorders, other protocols may require carefully designed systems that either ensure targeting to cells of interest or alternatively, transcription that is tightly regulated and conditionally activated. Indeed, it is of utmost importance to regulate expression of potentially immunogenic proteins, such as those encoded by suicide genes, to limit their immunogenic potential.

Meanwhile, RNAi may be employed for therapeutic silencing of aberrant cellular or viral genes, but the potential for off-target effects due to activation of double-stranded RNA response pathways or undesired targeting of partial complementary sequences may necessitate carefully controlled and regulated expression of short hairpin RNAs. As we exhaust the current repertoire of simple promoter systems for transgene expression or for RNAi, we need to develop newer systems that combine the high activity of viral promoters with cell type specificity or conditionally active *cis*-elements. Novel combinations of promoter elements should be one approach for creating such regulated promoters.

Acknowledgements

This work was supported by NIH grants AI29329, AI42552, AI061389, and HL07470.

References

ADELMAN, K., MARR, M.T., WERNER, J. *ET AL.* (2005). Efficient release from promoter-proximal stall sites requires transcript cleavage factor TFIIS. *Molecular Cell* **17**, 103–112.

AMENDOLA, M., VENNERI, M.A., BIFFI, A., VIGNA, E. AND NALDINI, L. (2005). Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. *Nature Biotechnology* **23**, 108–116.

AN, D.S., XIE, Y., MAO, S.H., MORIZONO, K., KUNG, S.K. AND CHEN, I.S. (2003). Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Human Gene Therapy* **14**, 1207–1212.

ANDERSON, W.F. (1998). Human gene therapy. *Nature* **392**, 25–30.

ASIPU, A. AND BLAIR, G.E. (1994). Regulation of myelin basic protein-encoding gene transcription in rat oligodendrocytes. *Gene* **150**, 227–234.

BAER, M., NILSEN, T.W., COSTIGAN, C. AND ALTMAN, S. (1990). Structure and transcription of a human gene for H1 RNA, the RNA component of human RNase P. *Nucleic Acids Research* **18**, 97–103.

BAEUERLE, P.A. AND BALTIMORE, D. (1996). NF-kappa B: ten years after. *Cell* **87**, 13–20.

BAEUERLE, P.A. AND HENKEL, T. (1994). Function and activation of NF-kappa B in the immune system. *Annual Review of Immunology* **12**, 141–179.

BELLAS, R.E., LEE, J.S. AND SONENSHEIN, G.E. (1995). Expression of a constitutive NF-kappa B-like activity is essential for proliferation of cultured bovine vascular smooth muscle cells. *Journal of Clinical Investigation* **96**, 2521–2527.

BERNS, K., HIJMANS, E.M., MULLENDERS, J. *ET AL.* (2004). A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* **428**, 431–437.

BERNSTEIN, E., CAUDY, A.A., HAMMOND, S.M. AND HANNON, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.

BLAESE, R.M., CULVER, K.W., MILLER, A.D. *ET AL.* (1995). T lymphocyte-directed gene therapy for ADA–SCID: initial trial results after 4 years. *Science* **270**, 475–480.

BOSHART, M., WEBER, F., JAHN, G., DORSCH-HASLER, K., FLECKENSTEIN, B. AND SCHAFFNER, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521–530.

BROOKS, A.R., HARKINS, R.N., WANG, P., QIAN, H.S., LIU, P. AND RUBANYI, G.M. (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *Journal of Gene Medicine* **6**, 395–404.

BRUMMELKAMP, T.R., BERNARDS, R. AND AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553.

CAPLEN, N.J., PARRISH, S., IMANI, F., FIRE, A. AND MORGAN, R.A. (2001). Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 9742–9747.

CHRISTEN, U. AND VON HERRATH, M.G. (2002). Transgenic animal models for type 1 diabetes: linking a tetracycline-inducible promoter with a virus-inducible mouse model. *Transgenic Research* **11**, 587–595.

DE PIETRI TONELLI, D., MIHAILOVICH, M., SCHNURBUS, R., PESOLE, G., GROHOVAZ, F. AND ZACCHETTI, D. (2003). Translational control of Scamper expression via a cell-specific internal ribosome entry site. *Nucleic Acids Research* **31**, 2508–2513.

DOENCH, J.G., PETERSEN, C.P. AND SHARP, P.A. (2003). siRNAs can function as miRNAs. *Genes and Development* **17**, 438–442.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K. AND TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.

FINKLE, D., QUAN, Z.R., ASGHARI, V. *ET AL.* (2004). HER2-targeted therapy reduces incidence and progression of midlife mammary tumours in female murine mammary tumor virus huHER2-transgenic mice. *Clinical Cancer Research* **10**, 2499–2511.

FIRE, A., XU, S., MONTGOMERY, M.K., KOSTAS, S.A., DRIVER, S.E. AND MELLO, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.

FUJINAGA, K., IRWIN, D., HUANG, Y., TAUBE, R., KUROSU, T. AND PETERLIN, B.M. (2004). Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. *Molecular and Cellular Biology* **24**, 787–795.

FURLER, S., PATERNA, J.C., WEIBEL, M. AND BUELER, H. (2001). Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantia nigra neurons. *Gene Therapy* **8**, 864–873.

FURTH, P.A., ST. ONGE, L., BOGER, H. *ET AL.* (1994). Temporal control of gene expression in

transgenic mice by a tetracycline-responsive promoter. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 9302–9306.

GARRIGA, J. AND GRANA, X. (2004). Cellular control of gene expression by T-type cyclin/CDK-9 complexes. *Gene* **337**, 15–23.

GARTON, K.J., FERRI, N. AND RAINES, E.W. (2002). Efficient expression of exogenous genes in primary vascular cells using IRES-based retroviral vectors. *Biotechniques* **32**, 830, 832, 834 passim.

GHAZAL, P., LUBON, H., FLECKENSTEIN, B. AND HENNIGHAUSEN, L. (1987). Binding of transcription factors and creation of a large nucleoprotein complex on the human cytomegalovirus enhancer. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 3658–3662.

GILL, D.R., SMYTH, S.E., GODDARD, C.A. *ET AL.* (2001). Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1 alpha promoter. *Gene Therapy* **8**, 1539–1546.

GOSSEN, M. AND BUJARD, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 5547–5551.

GOSSEN, M., FREUNDLIEB, S., BENDER, G., MULLER, G., HILLEN, W. AND BUJARD, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**, 1766–1769.

GUPTA, S., SCHOER, R.A., EGAN, J.E., HANNON, G.J. AND MITTAL, V. (2004). Inducible, reversible, and stable RNA interference in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1927–1932.

HANNON, G.J. (2002). RNA interference. *Nature* **418**, 244–251.

HOU, Z., NGUYEN, Q., FRENKEL, B. *ET AL.* (1999). Osteoblast-specific gene expression after transplantation of marrow cells: implications for skeletal gene therapy. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7294–7299.

HUNNINGHAKE, G.W., MONICK, M.M., LIU, B. AND STINSKI, M.F. (1989). The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T lymphocyte stimulation and contains functional cyclic AMP-response elements. *Journal of Virology* **63**, 3026–3033.

HWANG, D.Y., CARLEZON, W.A., JR., ISACSON, O. AND KIM, K.S. (2001). A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Human Gene Therapy* **12**, 1731–1740.

ISOLA, L.M. AND GORDON, J.W. (1989). Expression of a methotrexate resistant dihydrofolate reductase gene in transgenic mice. *Developmental Genetics* **10**, 349–355.

JOHANSEN, J., ROSENBLAD, C., ANDSBERG, K. *ET AL.* (2002). Evaluation of Tet-on system to avoid transgene down-regulation in *ex vivo* gene transfer to the CNS. *Gene Therapy* **9**, 1291–1301.

KESSLER, M.A., YANG, M., GOLLOMP, K.L., JIN, H. AND IACOVITTI, L. (2003). The human tyrosine hydroxylase gene promoter. *Brain Research Molecular Brain Research* **112**, 8–23.

KOMAR, A.A. AND HATZOGLOU, M. (2005). Internal ribosome entry sites in cellular mRNAs: mystery of their existence. *Journal of Biological Chemistry* **280**, 23425–23428.

KOTHARI, S., BAILLIE, J., SISSONS, J.G. AND SINCLAIR, J.H. (1991). The 21bp repeat element of the human cytomegalovirus major immediate early enhancer is a negative regulator of gene expression in undifferentiated cells. *Nucleic Acids Research* **19**, 1767–1771.

LANG, D., FICKENSCHER, H. AND STAMMINGER, T. (1992). Analysis of proteins binding to the proximal promoter region of the human cytomegalovirus IE-1/2 enhancer/promoter reveals both consensus and aberrant recognition sequences for transcription factors Sp1 and CREB. *Nucleic Acids Research* **20**, 3287–3295.

LEE, N.S., DOHJIMA, T., BAUER, G. *ET AL.* (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nature Biotechnology* **20**, 500–505.

LI, T. AND ZHANG, J. (2004). Stable expression of three genes from a tricistronic retroviral vector containing a picornavirus and 9-nt cellular internal ribosome entry site elements. *Journal of Virological Methods* **115**, 137–144.

LI, X., EASTMAN, E.M., SCHWARTZ, R.J. AND DRAGHIA-AKLI, R. (1999). Synthetic muscle

promoters: activities exceeding naturally occurring regulatory sequences. *Nature Biotechnology* **17**, 241–245.

LOSER, P., JENNINGS, G.S., STRAUSS, M. AND SANDIG, V. (1998). Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. *Journal of Virology* **72**, 180–190.

LU, Y. AND STEINER, M.S. (2000). Transcriptionally regulated adenoviruses for prostate-specific gene therapy. *World Journal of Urology* **18**, 93–101.

MATSUKURA, S., JONES, P.A. AND TAKAI, D. (2003). Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Research* **31**, e77.

MILLER, A.D. (1992). Human gene therapy comes of age. *Nature* **357**, 455–460.

MIYAGISHI, M. AND TAIRA, K. (2002a). Development and application of siRNA expression vector. *Nucleic Acids Research* Suppl., (2) 113–114.

MIYAGISHI, M. AND TAIRA, K. (2002b). U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnology* **20**, 497–500.

MIZUGUCHI, H., XU, Z., ISHII-WATABE, A., UCHIDA, E. AND HAYAKAWA, T. (2000). IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Molecular Therapy* **1**, 376–382.

MONTGOMERY, M.K. (2004). RNA interference: historical overview and significance. *Methods in Molecular Biology* **265**, 3–21.

NAJJAR, S.M. AND LEWIS, R.E. (1999). Persistent expression of foreign genes in cultured hepatocytes: expression vectors. *Gene* **230**, 41–45.

NI, Z., SCHWARTZ, B.E., WERNER, J., SUAREZ, J.R. AND LIS, J.T. (2004). Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. *Molecular Cell* **13**, 55–65.

NILLER, H.H. AND HENNIGHAUSEN, L. (1991). Formation of several specific nucleoprotein complexes on the human cytomegalovirus immediate early enhancer. *Nucleic Acids Research* **19**, 3715–3721.

NO, D., YAO, T.P. AND EVANS, R.M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 3346–3351.

NOMOTO, T., OKADA, T., SHIMAZAKI, K. *ET AL.* (2003). Distinct patterns of gene transfer to gerbil hippocampus with recombinant adeno-associated virus type 2 and 5. *Neuroscience Letters* **340**, 153–157.

NYKANEN, A., HALEY, B. AND ZAMORE, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321.

O'BRIEN, T., HARDIN, S., GREENLEAF, A. AND LIS, J.T. (1994). Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. *Nature* **370**, 75–77.

PADDISON, P.J., CAUDY, A.A., BERNSTEIN, E., HANNON, G.J. AND CONKLIN, D.S. (2002). Short hairpin RNAs, (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes and Development* **16**, 948–958.

PADDISON, P.J., SILVA, J.M., CONKLIN, D.S. *ET AL.* (2004). A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428**, 427–431.

PAUL, C.P., GOOD, P.D., WINER, I. AND ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. *Nature Biotechnology* **20**, 505–508.

PAULUS, W., BAUR, I., BOYCE, F.M., BREAKEFIELD, X.O. AND REEVES, S.A. (1996). Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *Journal of Virology* **70**, 62–67.

PING, Y.H. AND RANA, T.M. (2001). DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates P-TEFb-mediated phosphorylation of RNA polymerase II and DSIF during transcription elongation. *Journal of Biological Chemistry* **276**, 12951–12958.

PROSCH, S., STAAK, K., STEIN, J. *ET AL.* (1995). Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFalpha is mediated via induction of NF-kappaB. *Virology* **208**, 197–206.

PROUD, C.G. (2002). Regulation of mammalian translation factors by nutrients. *European Journal of Biochemistry* **269**, 5338–5349.

PUTTARAJU, M., JAMISON, S.F., MANSFIELD, S.G., GARCIA-BLANCO, M.A. AND MITCHELL, L.G. (1999). Spliceosome-mediated RNA trans-splicing as a tool for gene therapy. *Nature Biotechnology* **17**, 246–252.

RAGHEB, J.A., COUTURE, L., MULLEN, C., RIDGWAY, A. AND MORGAN, R.A. (1999). Inhibition of human immunodeficiency virus type 1 by Tat/Rev-regulated expression of cytosine deaminase, interferon alpha2, or diphtheria toxin compared with inhibition by transdominant Rev. *Human Gene Therapy* **10**, 103–112.

RANG, A. AND WILL, H. (2000). The tetracycline-responsive promoter contains functional interferon-inducible response elements. *Nucleic Acids Research* **28**, 1120–1125.

RENNER, D.B., YAMAGUCHI, Y., WADA, T., HANDA, H. AND PRICE, D.H. (2001). A highly purified RNA polymerase II elongation control system. *Journal of Biological Chemistry* **276**, 42601–42609.

ROMANO, G., REISS, K., TU, X. *ET AL.* (2001). Efficient *in vitro* and *in vivo* gene regulation of a retrovirally delivered pro-apoptotic factor under the control of the *Drosophila* HSP70 promoter. *Gene Therapy* **8**, 600–607.

ROSSI, F.M., GUICHERIT, O.M., SPICHER, A. *ET AL.* (1998). Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. *Nature Genetics* **20**, 389–393.

SAMBUCETTI, L.C., CHERRINGTON, J.M., WILKINSON, G.W. AND MOCARSKI, E.S. (1989). NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *EMBO Journal* **8**, 4251–4258.

SANBE, A., GULICK, J., HANKS, M.C., LIANG, Q., OSINSKA, H. AND ROBBINS, J. (2003). Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. *Circulation Research* **92**, 609–616.

SEYHAN, A.A., VLASSOV, A.V., ILVES, H. *ET AL.* (2005). Complete, gene-specific siRNA libraries: production and expression in mammalian cells. *RNA* **11**, 837–846.

SILVA, J., CHANG, K., HANNON, G.J. AND RIVAS, F.V. (2004). RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene* **23**, 8401–8409.

SINCLAIR, J.H., BAILLIE, J., BRYANT, L.A., TAYLOR-WIEDEMAN, J.A. AND SISSONS, J.G. (1992). Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line. *Journal of General Virology* **73** (Pt 2), 433–435.

SOKOLIC, R.A., SEKHSARIA, S., SUGIMOTO, Y. *ET AL.* (1996). A bicistronic retrovirus vector containing a picornavirus internal ribosome entry site allows for correction of X-linked CGD by selection for MDR1 expression. *Blood* **87**, 42–50.

SONTHEIMER, E.J. AND CARTHEW, R.W. (2005). Silence from within: endogenous siRNAs and miRNAs. *Cell* **122**, 9–12.

STAMMINGER, T., FICKENSCHER, H. AND FLECKENSTEIN, B. (1990). Cell type-specific induction of the major immediate early enhancer of human cytomegalovirus by cyclic AMP. *Journal of General Virology* **71** (Pt 1), 105–113.

SU, Z.Z., SARKAR, D., EMDAD, L. *ET AL.* (2005). Targeting gene expression selectively in cancer cells by using the progression-elevated gene-3 promoter. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 1059–1064.

SUI, G., SOOHOO, C., AFFAR EL, B., GAY, F., SHI, Y. AND FORRESTER, W.C. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 5515–5520.

SUTRAVE, P., KELLY, A.M. AND HUGHES, S.H. (1990). ski can cause selective growth of skeletal muscle in transgenic mice. *Genes and Development* **4**, 1462–1472.

TESCHENDORF, C., WARRINGTON, K.H., JR., SIEMANN, D.W. AND MUZYCZKA, N. (2002). Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumour cell lines using recombinant adeno-associated virus. *Anticancer Research* **22**, 3325–3330.

UNWALLA, H.J., LI, M.J., KIM, J.D. *ET AL.* (2004). Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. *Nature Biotechnology* **22**, 1573–1578.

VAN CRAENENBROECK, K., VANHOENACKER, P., LEYSEN, J.E. AND HAEGEMAN, G. (2001). Evaluation of the tetracycline- and ecdysone-inducible systems for expression of neurotransmitter receptors in mammalian cells. *European Journal of Neuroscience* **14**, 968–976.

VAN DE WETERING, M., OVING, I., MUNCAN, V. *ET AL.* (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Reports* **4**, 609–615.

VERMA, I.M., STEVENSON, J.K., SCHWARZ, E.M., VAN ANTWERP, D. AND MIYAMOTO, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes and Development* **9**, 2723–2735.

VOEKS, D., MARTINIELLO-WILKS, R., MADDEN, V. *ET AL.* (2002). Gene therapy for prostate cancer delivered by ovine adenovirus and mediated by purine nucleoside phosphorylase and fludarabine in mouse models. *Gene Therapy* **9**, 759–768.

WADA, T., TAKAGI, T., YAMAGUCHI, Y. *ET AL.* (1998). DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologues. *Genes and Development* **12**, 343–356.

WAGSTAFF, M.J., LILLEY, C.E., SMITH, J., ROBINSON, M.J., COFFIN, R.S. AND LATCHMAN, D.S. (1998). Gene transfer using a disabled herpes virus vector containing the EMCV IRES allows multiple gene expression *in vitro* and *in vivo*. *Gene Therapy* **5**, 1566–1570.

WANG, X.P., YAZAWA, K., YANG, J., KOHN, D., FISHER, W.E. AND BRUNICARDI, F.C. (2004). Specific gene expression and therapy for pancreatic cancer using the cytosine deaminase gene directed by the rat insulin promoter. *Journal of Gastrointestinal Surgery* **8**, 98–108; discussion 106–108.

WANG, Y., IYER, M., ANNALA, A.J., CHAPPELL, S., MAURO, V. AND GAMBHIR, S.S. (2005). Noninvasive monitoring of target gene expression by imaging reporter gene expression in living animals using improved bicistronic vectors. *Journal of Nuclear Medicine* **46**, 667–674.

WEBER, W., FUX, C., DAOUD-EL BABA, M. *ET AL.* (2002). Macrolide-based transgene control in mammalian cells and mice. *Nature Biotechnology* **20**, 901–907.

WEI, Q., MISKIMINS, W.K. AND MISKIMINS, R. (2003). The Sp1 family of transcription factors is involved in p27(Kip1)-mediated activation of myelin basic protein gene expression. *Molecular and Cellular Biology* **23**, 4035–4045.

WONG, E.T., NGOI, S.M. AND LEE, C.G. (2002). Improved co-expression of multiple genes in vectors containing internal ribosome entry sites (IRESes) from human genes. *Gene Therapy* **9**, 337–344.

WU, C.H., YAMAGUCHI, Y., BENJAMIN, L.R. *ET AL.* (2003). NELF and DSIF cause promoter proximal pausing on the hsp70 promoter in *Drosophila*. *Genes and Development* **17**, 1402–1414.

WULCZYN, F.G., KRAPPMANN, D. AND SCHEIDEREIT, C. (1996). The NF-kappa B/Rel and I kappa B gene families: mediators of immune response and inflammation. *Journal of Molecular Medicine* **74**, 749–769.

WYMAN, T., ROHRER, D., KIRIGITI, P. *ET AL.* (1999). Promoter-activated expression of nerve growth factor for treatment of neurodegenerative diseases. *Gene Therapy* **6**, 1648–1660.

XIA, H., MAO, Q., PAULSON, H.L. AND DAVIDSON, B.L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnology* **20**, 1006–1010.

YAMAGUCHI, Y., TAKAGI, T., WADA, T. *ET AL.* (1999a). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. *Cell* **97**, 41–51.

YAMAGUCHI, Y., WADA, T., WATANABE, D., TAKAGI, T., HASEGAWA, J. AND HANDA, H. (1999b). Structure and function of the human transcription elongation factor DSIF. *Journal of Biological Chemistry* **274**, 8085–8092.

YAZAWA, K., FISHER, W.E. AND BRUNICARDI, F.C. (2002). Current progress in suicide gene therapy for cancer. *World Journal of Surgery* **26**, 783–789.

YU, J.Y., DERUITER, S.L. AND TURNER, D.L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6047–6052.

ZAMORE, P.D., TUSCHL, T., SHARP, P.A. AND BARTEL, D.P. (2000). RNAi: double-stranded

RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.

ZENG, H., WU, Q., LI, H. *ET AL.* (2005). Construction of prostate-specific expressed recombinant plasmids with high transcriptional activity of prostate-specific membrane antigen (PSMA) promoter/enhancer. *Journal of Andrology* **26**, 215–221.

ZHANG, X.Y., INAMDAR, N.M., SUPAKAR, P.C., WU, K., EHRLICH, K.C. AND EHRLICH, M. (1991). Three MDBP sites in the immediate-early enhancer-promoter region of human cytomegalovirus. *Virology* **182**, 865–869.

ZHANG, X.Y., NI, Y.S., SAIFUDEEN, Z., ASIEDU, C.K., SUPAKAR, P.C. AND EHRLICH, M. (1995). Increasing binding of a transcription factor immediately downstream of the cap site of a cytomegalovirus gene represses expression. *Nucleic Acids Research* **23**, 3026–3033.