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Biosynthesis of peptide precursors and protease inhibitors using new constitutive and inducible eukaryotic expression vectors

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A series of expression vectors has been constructed as based on the pML derivative of pBR322. The eukaryotic transcription units employ various promoters followed by polycloning sites for 3–9 commonly used restriction enzymes and are completed by the SV40 polyadenylation sequence. In 4 of the vectors, designed for co-transfection or transient expression studies, only a single transcription unit containing either a constitutive or an inducible promoter was incorporated. The human ubiquitin (UbC) promoter was used as a strong constitutive promoter, while the mouse metallothionein promoter and the promoter of the long terminal repeats of the mouse mammary tumor virus were used as inducible promoters. Another vector contained an additional transcription unit encoding a eukaryotic selection marker, the neomycin resistance encoding gene. The vectors were used in CHO cells and in neuroendocrine CA77 cells to synthesize peptide precursors, protease inhibitors and a protease. It is shown that these vectors are very efficient for the constitutive and inducible expression of nucleotide sequences in both transient and stable transfections of eukaryotic cells.

Ubiquitin promoter; Polylinker; Transfection; CA77 cell; Recombinant DNA; Simian virus 40

1. INTRODUCTION

Biosynthesis of biologically active eukaryotic proteins frequently requires posttranslational modifications [1]. Many of these are not performed by, for example, bacterial cells. Therefore, biosynthesis of functional eukaryotic proteins encoded by cloned nucleotide sequences often has to be performed in eukaryotic cells. This is the case, for example, in the biosynthesis of many eukaryotic secretory proteins for structure-function studies [2,3]. The synthesis of exogenous protein in eukaryotic cells requires transfection of the exogenous DNA integrated in an eukaryotic expression vector [4].

As the vectors have to be multiplied in bacteria prior to transfection of the eukaryotic cells, any eukaryotic expression vector must contain sequences which facilitate the prokaryotic propagation along with one or more eukaryotic transcription units. The prokaryotic sequences usually include a bacterial resistance gene and an origin of DNA replication which functions in bacteria. The eukaryotic transcription unit should consist of a powerful eukaryotic promoter (inducible or constitutive), a polyadenylation signal, and finally a polylinker for insertion of nucleotide sequences encoding the protein in question [4]. The early eukaryotic expression vectors (see e.g. [5–7]) all contained constitutive virus promoters. The polylinker of otherwise useful vectors like pMSG (Pharmacia), pMT2 [8] or pUANL [9] are not very useful in general. We describe the construction and function of a series of small versatile eukaryotic expression vectors containing strong constitutive or inducible promoters and an extensive polylinker.

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2. MATERIALS AND METHODS

2.1. *Vector construction*

The basic vector contains a 53 bp fragment from the pIC19R [10] polylinker (*Eco*RI to *Bam*HI) ligated into pML [11] between *Eco*RI and *Sal*I, 2344 bp. Prior to ligation the *Sal*I site had been converted to a *Bam*HI site by *Bam*HI linkers. The simian virus 40 (SV40) polyadenylation signal, a 237 bp *Bam*HI to *Bcl*I fragment corresponding to position 2451–2688 of the SV40 genome [12], was inserted into the *Bam*HI site of this vector in two different orientations, thereby regenerating one *Bam*HI site. This fragment contains two polyadenylation signals on one strand and one on the other [12]. In the vector where the regenerated *Bam*HI site was located between the polylinker and the polyadenylation signal, the polylinker was extended with *Eco*RI and *Sma*I by insertion of a 24 bp *Bam*HI/*Bgl*II fragment from the polylinker of pSP6/T3 (Pharmacia) into the *Bam*HI site. Prior to insertion, the original *Eco*RI site was removed. This vector (pSV-LIC) was the basic vector used for construction of the pTEJ-series, except for pTEJ-3A.

pTEJ-3A which contains the constitutive human ubiquitin (UbC) promoter [13,14], was based on the vector where the 237 bp *Bam*HI/*Bcl*I SV40 fragment was inserted in the orientation which regenerated the *Bam*HI site at the 3′ end. This vector contains a unique *Hpa*I site between the polylinker and the polyadenylation signal [12]. The human ubiquitin promoter, as an approximately 1500 bp *Hin*dIII fragment, was excised from p753 [13] (obtained from Dr E.

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Boel, Novo-Nordisk, Copenhagen), and inserted into the *Hin*dIII site of this vector (Fig. 1). The promoter in the λHUb13 clone [14], was originally excised as a *Xho*I fragment, covering the region from position approximately −1450 to +43 in the ubiquitin gene. Putative TATA box and cap site are located at position −74 and −44, respectivelyly [14].

pTEJ-4 was constructed by insertion of the *Hin*dIII fragment of the human ubiquitin promoter [13] into the *Hin*dIII site of pSV-LIC, followed by a selective repair of the staggered ends of the *Hin*dIII site located at the 5′ end of the ubiquitin promoter, in order to preserve the *Hin*dIII site as a unique site in the polylinker (Fig. 1).

pTEJ-5 contains the inducible promoter of the mouse metallothionein I gene [15] (Fig. 1). The promoter was excised as a 250 bp *Bgl*I-*Bgl*II fragment corresponding to position −185 to +65 of the mouse metallothionein 1 gene [15] (obtained from Dr R. Palmiter, University of Washington, Seattle) and converted to a blunt-ended fragment by Klenow polymerase. The fragment was then ligated into the *Eco*RV site of pSV-LIC.

pTEJ-7 was constructed by inserting the long terminal repeat of mouse mammary tumor virus [16] into pSV-LIC. The promoter was excised from pMSG (Pharmacia) as a 1471 bp *Hin*dIII to *Xho*I fragment, corresponding to position 6182–18, which was converted to blunt end and ligated into the *Eco*RV site of pSV-LIC (Fig. 1).

pTEJ-8 is a derivative of pTEJ-4 containing the neomycin phosphotransferase encoding gene, neo [17] under control of SV40 early promoter (Fig. 2). The SV40 early and late promoters, enhancer, and origin of DNA replication were excised as a 326 bp *Hin*dIII to *Pvu*II fragment corresponding to position 5085 to 189 of the SV40 genome [12]. This fragment was converted to blunt ends and ligated into the blunt end converted *Bgl*II site of pTEJ-4. This new plasmid has the SV40 early promoter operating in the opposite direction to the ubiquitin promoter (see Fig. 2). The neo gene was inserted into the *Eco*RV site, under the control of the SV40 early promoter, as a blunt end converted 1238 bp *Bam*HI to *Dra*I fragment. This fragment excised from p753, also contains the SV40 polyadenylation signal at the 3′ end of the neo gene [13] (Fig. 2).

2.2. *Transfection*

CHO cells for transfection were seeded in 80 cm^2 flasks (Costar) at a density of 10^4 per cm^2. After 24 h the cells were transfected with 20 μg of DNA using the calcium phosphate precipitate procedure of Graham and Van der Eb [20]. Cotransfection experiments were performed with 19 μg of the specific transfection plasmid and 1 μg of pSV2-neo [5]. CHO cells were grown at 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin, 100 IU and 100 mg/ml, respectively. CHO clones transfected with pTEJ-8 or cotransfection with pSV2-neo were selected with 0.8 mg/ml G418 (Gibco). CA77 cells could not be successfully transfected by the calcium phosphate procedure; therefore these cells were transfected by the scrape loading method as modified from Fechheimer et al. [21]. DNA, 60 μg, was dissolved in 200 μl PBS and added to 4×10^6 cells, which had been seeded 3 days

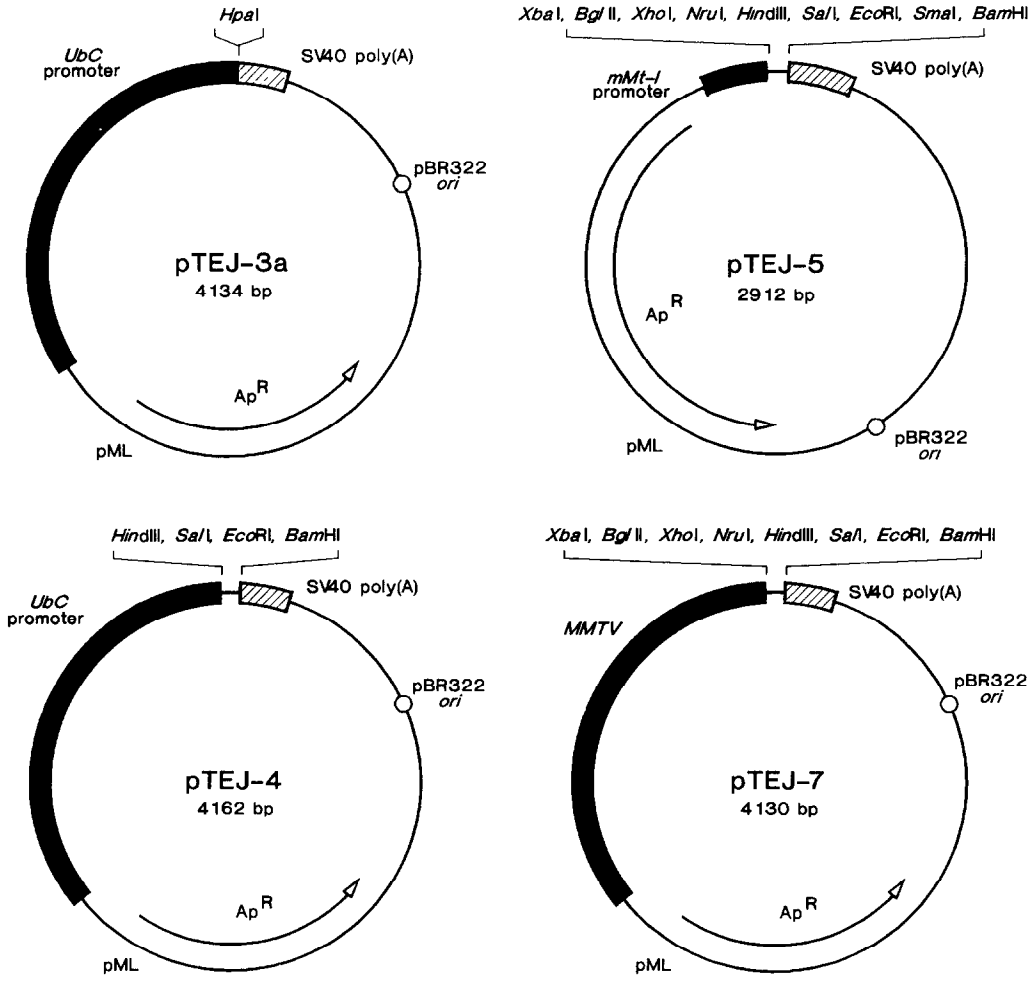


Fig. 1. A schematic representation of four pTEJ vectors designed for cotransfection or transient expression studies. Only the unique restriction sites of the polylinker are indicated. mMt-1, mouse metallothionein I; MMTV LTR, long terminal repeat of mouse mammary tumor virus; Poly(A), polyadenylation signal; Ap^R, ampicillin resistance.

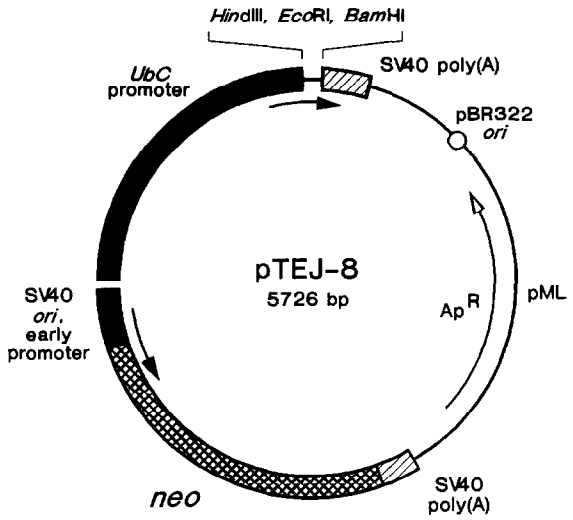


Fig. 2. A schematic representation of the pTEJ-8 vector. pTEJ-8 is identical to pTEJ-4 except for the additional eukaryotic transcription unit containing the SV40 early promoter, neomycin phosphotransferase gene (neo), and the SV40 polyadenylation signal. Only the unique restriction sites of the polylinker are indicated.

previously in 100 mm diameter petri dishes (Costar). The cells were loaded with DNA by scraping with a rubber policeman. After 10 min at room temperature, the cells were seeded in fresh medium and incubated at 37°C in 10% CO_2. Stably transfected cells were selected after 48 h by changing to medium containing 0.2 mg/ml G418, 1% horse serum and 0.5% fetal calf serum and transferred to 10% CO_2. The medium for CA77 cells was a (1:1, v/v) nutrient mixture F-10 (Ham)/Dulbecco's modified Eagle medium 1885 supplemented with 10 μg/ml Insulin, 5 μg/ml transferrin, 30 nM selenous acid and penicillin/streptomycin, 100 IU and 100 mg/ml, respectively. Transfection generally gave rise to 30–50 clones per 4×10^6 cells.

3. RESULTS AND DISCUSSION

3.1. *Basic elements of the vectors*

Based on the pML derivative of pBR322, a series of eukaryotic expression vectors has been developed by incorporation of different eukaryotic transcription units. In addition to the common sequence for prokaryotic propagation, all the vectors contain the SV40 polyadenylation sequence in their transcriptional unit, which has been shown to be an efficient signal for the processing of RNA hybrids [7,22]. All vectors, except pTEJ-3a which is designed for expression of blunt-ended fragments, have a polylinker in front of the polyadenylation signal containing at least 3 sites of commonly used restriction enzymes, producing cohesive protruding 5′ termini (Figs 1, 2 and 3). We have chosen not to include any splice signal in the pTEJ vectors, in order to obtain small vectors which will be able to contain genes with a size greater than 10000 bp. Early studies suggested that splicing was required for the production and sorting of mRNA. We now know that many genes do not have introns and furthermore, many cDNAs have been efficiently expressed from vectors which lack splice signals (see e.g. [23,24]).

3.2. *Vectors containing constitutive promoter*

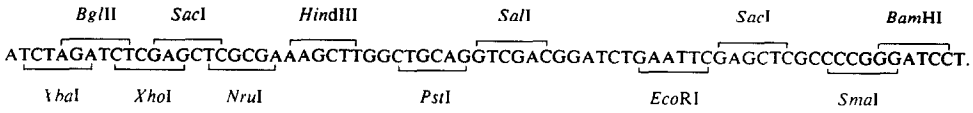
Four of the vectors (see Fig. 1) contain only a single eukaryotic transcription unit with a polylinker; accordingly these vectors are used in cotransfection experiments or in transient expression studies. To achieve constitutive and ubiquitous expression of the inserted cDNA, we have used the human ubiquitin promoter (UbC) [14], as the transcriptional control sequence, in 3 of the vectors, pTEJ-3a, pTEJ-4 and pTEJ-8. Ubiquitin is one of the most well-conserved proteins examined so far. The amino acid sequence is unaltered from insect to man, and the protein is expressed in all cells in all eukaryotic organisms tested [25]. In transfection experiments the UbC promoter has been used to drive expression in a variety of cell lines (Dr B.S. Wulff, personal communication). This makes the ubiquitin promoter an excellent tool as a constitutive promoter in a universal eukaryotic expression vector.

As shown in Table I, tPA and pro-glucagon have been synthesized by CHO cells transfected with pTEJ-3a containing the respective cDNA. pTEJ-4 derivatives containing cDNAs encoding PSTI, pro-NPY, and cystatin C have been expressed in CHO cells. Similarly, the ubiquitin promoter was used to drive the expression of pro-NPY and cystatin C in CA77 cells, a well-differentiated, neuroendocrine cell line [19]. The median expression of protein from the ubiquitin promoter in populations of stably transfected clones was between 1 and several hundred pmol per 10^6 cells per 24 h. The difference in the expression level is conceivably due to differences in the cellular handling of the different mRNAs in question, and/or the stability of the protein in the tissue culture medium. The maximal biosynthesis observed in transfected CHO cells was 620 pmol cystatin C per 10^6 cells per 24 h (Table I). This high secretion level, which corresponds to 10 mg cystatin C/liter of culture medium, can possibly be explained by the fact that the cystatin C cDNA contains both the optimal Kozak initiation consensus ACCATGG and its own polyadenylation signal, AATAAA [26]. It should also be noted that protease inhibitors are more stable than hormone precursors, which are in fact enzyme substrates. The ubiquitin promoter gave a higher yield of the exogenous protein in CHO cells as compared to CA77 cells. This could be due to the different transfection procedures or to basic differences between the two mammalian cell lines. It should be noted that, in the CA77 cells, the expression levels of protein correspond to the amount of endogenously produced peptide hormone, calcitonin gene related peptide (CGRP), 20–25 pmol/10^6 cells/24 h.

3.3. *Vectors containing inducible promoters*

The mouse metallothionein I promoter and the promoter in the long terminal repeat from the mouse mammary tumor virus were used as inducible promoters. The mouse metallothionein I promoter and 5′ flanking

Polylinker for pTEJ-5and 7 :



Polylinker for pTEJ-4and 8 :

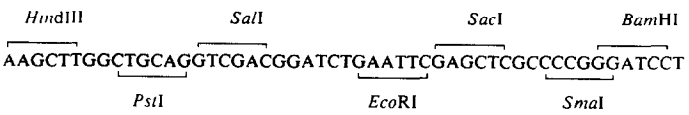


Fig. 3. The nucleotide sequence of the polylinkers from the pTEJ vectors. The cleavage site for restriction enzymes, which recognize 6 bases, are indicated. The unique restriction sites in the polylinker of the different vectors are shown in Figs 1 and 2.

sequences have previously been used to confer metal responsiveness to genes or cDNAs transfected into mammalian cells [15]. The metal responsiveness of this promoter used in pTEJ-5 in a 12 bp DNA motif which is repeated 5 times [15]. CHO and CA77 cells were transfected with pTEJ-5 including cDNA encoding PAI-I and pro-NPY. In the basal uninduced state the median expression levels were below 0.5 pmol/10^6

Table I

Biosynthesis and secretion of specific protein by stable clones of CHO and CA77 cells transfected with plasmids derived from the pTEJ series

Transfection plasmids[a]	Vector type	Promoter	cDNA	Cell line	Secretion[b]		Induction[c]		*n*
					Median	Maximal	Median	Maximal	
pTUB 191	pTEJ-3A	*UbC*	*tPA*	CHO	0.9	1.2	–	–	6
pTUB 239	pTEJ-3A	*UbC*	*pro-glucagon*	CHO	2.3	7.8	–	–	8
pTUB 196	pTEJ-4	*UbC*	*PSTI*	CHO	18.4	48.7	–	–	6
pTUB 196	pTEJ-4	*UbC*	*PSTI*	CA77	3.4	20.4	–	–	15
pTUB 214	pTEJ-4	*UbC*	*pro-NPY*	CHO	27.3	106.3	–	–	16
pTUB 214	pTEJ-4	*UbC*	*pro-NPY*	CA77[d]	6.5	20.4	–	–	8
pTUB 236	pTEJ-4	*UbC*	*cystatin C*	CHO	240.5	490.0	–	–	12
pTUB 213	pTEJ-5	*mMt-I*	*pro-NPY*	CHO	1.1	5.6	310	1220	16
pTUB 223	pTEJ-5	*mMt-I*	*PAI-1*	CA77	1.8	16.5	350	850	13
pTUB 223	pTEJ-5	*mMt-I*	*PAI-1*	CHO	2.4	13.4	470	1230	10
pTUB 237	pTEJ-7	*MMTV*	*cystatin C*	CHO	1.6	5.4	510	800	14
pTUB 237	pTEJ-7	*MMTV*	*cystatin C*	CA77	0.2	1.0	130	180	8

[a] *pTUB 191*, human *tPa* was excised from a pBR322 clone (obtained from Dr S. Degen, Department of Pediatrics, University of Cincinnati, OH) as a 2182 bp fragment obtained by partial *Sac*I and *Bgl*II double digestion, where the *Bgl*II in position 311 and *Sac*I in position 1541 were left undigested. This fragment was ligated into pIC19R, re-excised as a blunt-ended *Eco*RV and *Hin*dIII fragment and ligated into the *Hpa*I site of pTEJ-3A. *pTUB 239*, hamster *pro-glucagon* was excised from psh-Glu [31] (obtained from Dr G.I. Bell, University of Chicago) as a 992 bp *Dde*I fragment and inserted into the *Hpa*I site of pTEJ-3A. *pTUB 196*, human *PSTI* was excised from pTIC11 [32] (obtained from Drs N. Tomita, A. Hori and K. Matsubara, Osaka University, Japan) as an *Eco*RI fragment and ligated into the polylinker of pTEJ-4. *pTUB 214*, human *NPY* [33] (originally obtained from Drs C.D. Minth and J.E. Dixon, Perdue University, IN) was excised as a 387 bp *Bam*HI fragment from p753 [13] and inserted into pTEJ-4. *pTUB 236*, human *cystatin C* was excised from p6C [26] (obtained from Dr M. Abrahamson, University of Lund) as an *Eco*RI fragment and cloned into the *Eco*RI site of pTEJ-4. *pTUB 213*, was constructed as pTUB 214, but with pTEJ-5 as the recipient vector. *pTUB 223*, human *PAI-1* was excised from pPAI-1-A1 [34] (obtained from Drs P.A. Andreasen and K. Danø, Finsen Laboratory, Rigshospitalet, Copenhagen), as a 1314 bp *Eco*RI to *Bgl*II fragment and ligated into pTEJ-5. *pTUB 237* was constructed as pTUB 236, but with pTEJ-7 as the recipient vector

[b] The values of the specific proteins secreted in the cell culture medium are given in pmol/10^6 cells/24 h. The specific proteins were measured in medium collected after 24 h. Glucagon [35], NPY [36], PSTI [37], and cystatin C (Vogel et al., in preparation) were analyzed by radioimmunoassays. PAI-1 and tPA were determined by ELISA [38]

[c] The levels of secreted proteins seen with the *mMt-I* and *MMTV* promoters correspond to maximal induction obtained with 5×10^{-6} M cadmium chloride and with 2×10^{-6} M dexamethasone respectively

[d] Transient expression, 72 h after transfection

cells/24 h. The NPY and PAI-I synthesizing clones selected for the highest secretion level, synthesized between 5.6 and 16.5 pmol/10^6 cells/24 h, in the induced state (Table I). As shown in Fig. 4 and Table I, the secretion of NPY from the pTEJ-5 vectors could be increased by 12.3-fold, compared to the uninduced level, by treatment of the cells with cadmium. Among the different populations of clones, the median induction was 3.1–4.7-fold. Maximal induction was achieved with 0.5×10^{-5} M $CdCl_2$ with a rather narrow working range of stimulatory concentrations, due to the toxic effect of the cadmium ions at high concentrations (Fig. 4). The mouse metallothionein promoter can also be induced with other divalent cations [27]; however, this was not tested in the present study. The long terminal repeat of the mouse mammary tumor virus DNA has proven to be useful for inducible expression of cloned genes or cDNAs in mammalian cells [16]. This promoter contains the viral transcription promoter signals as well as the DNA sequences that confer glucocorticoid induction of expression. The mouse mammary tumor virus promoter of pTEJ-7 was induced by dexamethasone, and showed similar characteristics as the mouse metallothionein I promoter, both in respect of basal protein production and degree of induction (Fig. 4, Table I). However, in this case, a very broad range of concentrations of the inducer, dexamethasone, could be used. Almost maximal induction was achieved with concentrations of dexamethasone from 10^{-9} to 10^{-5} M (Fig. 4). Interestingly, the mouse mammary tumor virus promoter appeared to function poorly in the CA77 cells. In the basal state, the amount of cystatin C synthesized was only around one-tenth of that synthesized with the other vectors in these cells (Table I). Furthermore, even in the most inducible clones, the secretion of protein only increased by a factor of 1.8. It should be noted that the subclone of CA77 cells used in the present study synthesized large amounts of CGRP rather than calcitonin as in the case of most CA77 cells. The differential RNA splicing which leads to the generation of either calcitonin or CGRP from the same transcript is in fact regulated by corticosteroids [28]. It is therefore possible that for example the glucocorticoid receptor in this particular CA77 subclone does not function appropriately, neither in the regulation of the RNA splicing nor in the stimulation of the mouse mammary tumor virus promoter in the transfection vector.

3.4. *Vector containing selective marker*

The eukaryotic expression vector, pTEJ-8, is identical to pTEJ-4, except for an additional transcription unit containing the selective marker, neomycin phosphotransferase gene (Figs 1, 2). About 300 G418-resistant clones per 10^6 cells/μg pTEJ-8 were obtained following transfection with pTEJ-8 or pTEJ-8 derivatives. The neomycin phosphotransferase encoding gene

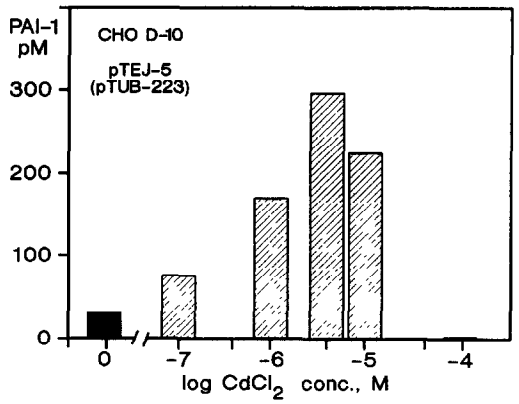


Fig. 4. Cadmium induction of a stable CHO clone (CHO D-10) transfected with pTEJ-5 containing human PAI-1 cDNA. PAI-1 was measured in 24 h medium collected after treatment with increasing concentrations of cadmium chloride.

is driven by the SV40 early promoter. In addition to the promoter, the 326 bp *Hin*dIII-*Pvu*II SV40 fragment also contains the origin of DNA replication [12,22,29]. This should allow this vector to replicate to a level greater than 10^5 copies per cell when transfected into e.g. COS cells [30]. The COS cell line produces high levels of large T antigen and is therefore permissive for SV40 DNA replication [30]. This high copy number of plasmids should ensure high level expression of the protein in transient experiments.

3.5. *Conclusion*

The pTEJ vectors are shown to be efficient for the expression of cloned nucleotide sequences in eukaryotic cells. Although they have elements in common with other previously described eukaryotic expression vectors, the pTEJ vectors have several advantages which make them particularly useful. (i) The human ubiquitin (UbC) promoter is a very powerful constitutive promoter, which gives ubiquitous expression. (ii) The two different inducible promoters of the series can be in-

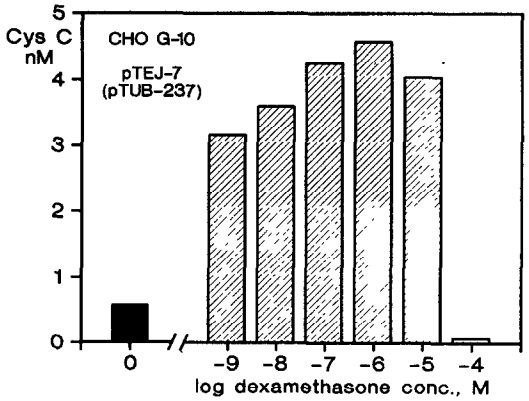


Fig. 5. Dexamethasone induction of a stable CHO clone (CHO G-10) transfected with pTEJ-5 containing human cystatin C cDNA. Cystatin C was measured in medium collected after 24 h, following treatment with increasing concentrations of dexamethasone.

duced up to 12-fold. (iii) The polylinker of the pTEJ vectors, except pTEJ-3a which has a single *Hpa*I blunt end site, contain at least 3 sites for the most commonly used restriction enzymes, *Hin*dIII, *Eco*RI and *Bam*HI. (iv) The neomycin phosphotransferase gene is included in the series as an eukaryotic selection marker. (v) The vectors, especially pTEJ-5, are small. We believe that the pTEJ series of vectors will have numerous applications, for example in structure-function studies of eukaryotic proteins.

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