

# EXHIBIT M

## Coamplification and Coexpression of Human Tissue-Type Plasminogen Activator and Murine Dihydrofolate Reductase Sequences in Chinese Hamster Ovary Cells

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**Expression of human tissue-type plasminogen activator (t-PA) at high levels has been achieved in Chinese hamster ovary (CHO) cells by cotransfection and subsequent coamplification of the transfected sequences. Expression vectors containing the t-PA cDNA gene and a dihydrofolate reductase (DHFR) cDNA gene were cotransfected into CHO DHFR-deficient cells. Transformants expressing DHFR were selected by growth in media lacking nucleosides and contained low numbers of t-PA genes and DHFR genes. Stepwise selection of the DHFR<sup>+</sup> transformants in increasing concentrations of methotrexate generated cells which had amplified both DHFR genes and t-PA genes over 100-fold. These cell lines expressed elevated levels of enzymatically active t-PA. To optimize both t-PA sequence amplification and t-PA expression, various modifications of the original procedure were used. These included alterations to the DHFR expression vector, optimization of the molar ratio of t-PA to DHFR sequences in the cotransfection, and modification of the methotrexate resistance selection procedure. The structure of the amplified DNA, its chromosomal location, and its stability during growth in the absence of methotrexate are reported.**

Many proteins of great interest are synthesized by biological systems in minute quantities, and this has greatly restricted progress towards understanding their biological function and their medical application since purification is both tedious and expensive. However, gene-cloning techniques are now capable of isolating genes encoding extremely rare proteins, and it should now be possible to synthesize large quantities of these proteins in surrogate cell systems. For a variety of reasons, including the glycosylation patterns and the secondary structures of some of these proteins, synthesis in a mammalian cell expression system is highly preferable. One particularly effective system which has been used to obtain permanent cell lines synthesizing large quantities of heterologous proteins exploits the ability to cotransfect and subsequently coamplify the copy number of the integrated DNA (5, 11, 12, 16, 19, 30, 32, 43). When cells are selected for growth in the presence of cytotoxic concentrations of methotrexate (MTX), a resistant population is obtained which contains multiple copies of the dihydrofolate reductase (DHFR) gene (2). Since MTX binds to and inhibits DHFR stoichiometrically, this amplification of the DHFR gene with a resultant increase in intracellular DHFR is responsible for the ability of the cells to grow in higher concentrations of MTX. Upon further sequential increases in the concentration of MTX in the culture medium, cells are selected which can harbor over 1,000 copies of the DHFR gene per cell (for a review, see reference 33). Chinese hamster ovary (CHO) cells which are deficient in DHFR (40) can be transformed with a DHFR gene in combination with any particular gene of interest. The DHFR<sup>+</sup> transformants thus produced can then be selected on the basis of their ability to grow in the absence of

nucleosides. The gene sequences cotransfected with the DHFR gene are frequently integrated adjacent to the DHFR gene, and thus subsequent amplification of the DHFR gene results in coamplification of the adjacent DNA. DNA which has been coamplified in this manner is also coexpressed. This approach has been successfully used to obtain CHO cells which express very high levels of proteins that are difficult to obtain from other sources (11, 12, 19, 32).

Two human plasminogen activators have been extensively characterized: urokinase plasminogen activator and tissue-type plasminogen activator (t-PA). These proteins have been sequenced and were found to be highly homologous serine proteases (23, 26). However, t-PA is thought to be the major activator of the extrinsic fibrinolytic system (1, 4, 15, 28) and has properties particularly suitable to its clinical use as a thrombolytic agent in various thrombotic disorders (3, 18). t-PA has a significant affinity for fibrin but not fibrinogen and is itself activated by association with fibrin (24, 29). It has been shown that, unlike less-specific plasminogen activators, t-PA produces little systemic activation of plasminogen, its action being efficiently localized to the site of the thrombus (3, 6, 18, 39). The scarcity of this protein from natural sources has precluded extensive investigation of its *in vivo* properties and its clinical application. t-PA has a molecular weight of 68,000, contains 35 cysteine residues, and is multiply glycosylated (41), making it most suitable for synthesis by secretion from a mammalian cell system in which appropriate secondary modification can be achieved. In this study we use DNA cotransfection and MTX selection to isolate CHO cells which express high levels of the human t-PA. We also describe methods of cotransfection and MTX selection which make the system more amenable to a wide variety of genes.

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## MATERIALS AND METHODS

**Isolation of full-length cDNA encoding t-PA.** Polyadenylated mRNA was isolated from the Bowes melanoma cell line (generously provided by D. Rifkin, New York University), used as a template for double-stranded cDNA synthesis, and introduced into bacterial plasmids by the addition of homopolymeric deoxyguanosine-deoxycytidine tails or the addition of *EcoRI* (at the 3' end) and *SalI* (at the 5' end) asymmetric synthetic linkers (17). The plasmid libraries were screened for t-PA encoding sequences by hybridization (10) to <sup>32</sup>P-radiolabeled oligonucleotide probes (a pool of 24 17-mers) prepared from the deduced amino acid sequence of the amino terminus of mature t-PA protein (purified from the Bowes melanoma cell conditioned medium). Three overlapping cDNA sequences that spanned the full coding sequence and portions of the untranslated 5' and 3' regions were ligated at two internal *EcoRI* sites to produce a contiguous t-PA encoding segment. This segment was introduced into a derivative of pBR322 so the *BglII* site at 391 base pairs (bp) 3' of the translation stop codon for t-PA was flush ended with the Klenow fragment of DNA polymerase I and ligated to a similarly flush-ended pBR322 *Clal* site. A *SalI* site present 125 bp 5' of the initiator ATG codon (introduced by the cloning procedure) was preserved in this cloning so that upon *SalI* and *HindIII* digestion a fragment of 2,210 bp encoding the full-length t-PA cDNA could be isolated. The entire coding region was sequenced to find an open reading frame of 563 amino acids in agreement with the published sequence (26).

**DHFR and t-PA expression vectors.** pAdd26SVp(A)3 (13) contains a mouse DHFR cDNA transcription unit which utilizes the adenovirus type 2 major late promoter (MLP). To derive pCVSVL2, a derivative of pAdd26SVp(A)1 which contains the simian virus 40 (SV40) enhancer and origin of replication, SV40 DNA was digested with *AvaII*, *XhoI* linkers (Collaborative Research, Inc., Waltham, Mass.) were applied, and the D fragment (680 bp) was isolated after agarose gel electrophoresis. This fragment was ligated into the *XhoI* site of pAdd26SVp(A)1 (13) which is 259 bp upstream from the adenovirus type 2 major late cap site. The resultant plasmid pCVSVL2 contains a single direct repeat of the SV40 *AvaII* D fragment such that the SV40 late promoter is in the same orientation as the adenovirus MLP. pCVSVL2 was then converted by a three-step process to pB2L2 to make it convenient for the introduction of the t-PA cDNA. First, one of the two *PstI* sites in pCVSVL2 (the *PstI* site located 3' of the SV40 polyadenylation site) was deleted by partial *PstI* digestion, treatment with Klenow fragment of DNA polymerase I, ligation, and transformation of *Escherichia coli*. The correct resultant plasmid from this step was digested with *BglII*, treated with Klenow fragment of DNA polymerase I, ligated, and used to transform *E. coli* to destroy the *BglII* site present within the intron of the DHFR cDNA transcription unit. Finally, the *PstI* site was converted to a *BglII* site by *PstI* digestion, treatment with Klenow fragment of DNA polymerase I, and addition of *BglII* linkers (Collaborative Research). After complete *BglII* digestion and gel electrophoresis to isolate the linear vector, the DNA was ligated and used to transform *E. coli* to tetracycline resistance to generate a unique *BglII*-containing vector, pB2L2. The full-length t-PA cDNA described above was digested with *HindIII* and *SalI* and treated with Klenow fragment of DNA polymerase I. *BamHI* linkers (Collaborative Research) were ligated to the 2.1-kilobase (kb) fragment with subsequent *BamHI* digestion, gel purification, and

ligation into the *BglII* site of pB2L2. Ligated DNA was used to transform *E. coli* strain HB101 to tetracycline resistance, and colonies were screened by the hybridization (10) of <sup>32</sup>P-labeled probe derived from the t-PA cDNA insert by end labeling with T4 DNA polymerase (9). pLDSG is a plasmid isolated which contains the t-PA cDNA in the proper orientation with respect to the adenovirus type 2 MLP.

**Cell culture and DNA transfection.** The DHFR-deficient CHO DUKX-B11 cell line has been described (12, 40). For DNA transfection, 25 µg of total DNA [pLDSG and pAdd26SVp(A)3 in proper ratio as described below] was mixed and ethanol precipitated with 0.3 M sodium acetate (pH 4.5); the DNA pellets were then suspended in 2XHEBSS (0.5 ml), mixed vigorously with 0.25 M CaCl<sub>2</sub> (0.5 ml), and used to transfect logarithmically growing CHO DUKX-B11 cells (12). After transfection, the cells were fed with alpha-medium containing 10% fetal bovine serum and thymidine, adenosine, deoxyadenosine, penicillin, and streptomycin (10 µg/ml each). Two days later the cells were subcultured into alpha-medium with 10% dialyzed fetal bovine serum, penicillin, and streptomycin, but lacking nucleosides. Cells were fed again with the selective media after 4.5 days, and colonies appeared 10 to 12 days after subculturing.

Plasminogen-free serum was obtained by passing heat-inactivated serum over a column of lysine-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.).

**t-PA assay.** t-PA activity was monitored by two assays which measure the catalyzed conversion of plasminogen to plasmin in the presence of fibrin. The appearance of plasmin was detected by the cleavage of the chromogenic substrate S2251, obtained from Kabi Diagnostics, Inc., Greenwich, Conn. (8). To harvest samples for t-PA assay, cells were first rinsed with serum-free medium (5 ml per 10-cm-diameter plate) and incubated with 4 ml of serum-free medium for 20 h at 37°C, and then the conditioned medium was taken for assay. Cell number was determined with a Coulter Counter. Quantitation of the t-PA activity was obtained by a comparison to a standard curve utilizing urokinase obtained from Calbiochem-Behring, La Jolla, Calif. A unit (CTA; Committee on Thrombolytic Agents) of activity is defined by comparison to the World Health Organization standard reference preparation of urokinase. Measurements of activity were expressed as the number of milliunits (mU) per cell per day. Samples of conditioned medium from cells lacking t-PA expression exhibited a low background of proteolytic activity which did not change upon elimination of fibrin from the standard assay. In contrast, the activity from t-PA-producing CHO cell lines exhibited fibrin dependence very similar to t-PA isolated from the Bowes melanoma cell line.

The synthesis of t-PA was monitored by labeling cells (2 × 10<sup>6</sup>/10 cm) with 1 ml of methionine-free medium containing 1 m Ci of [<sup>35</sup>S]methionine (specific activity greater than 800 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Cells were incubated for 4 h at 37°C, and the conditioned medium was assayed by immune precipitation with rabbit anti-human t-PA by using *Staphylococcus aureus* as the immunoadsorbant. For pulse labeling, 2 × 10<sup>6</sup> cells were labeled for 30 min with 0.5 mCi of [<sup>35</sup>S]methionine in 0.5 ml of methionine-free medium, and cell lysates were prepared with RIPA buffer (12). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide (6%) gel electrophoresis. Rabbit anti-human t-PA immunoglobulin G was generously provided by D. Rifkin.

**Cytogenetics.** Cells were arrested in metaphase by the addition of Colcemid (0.3 µg/ml). After 2 h the cells were

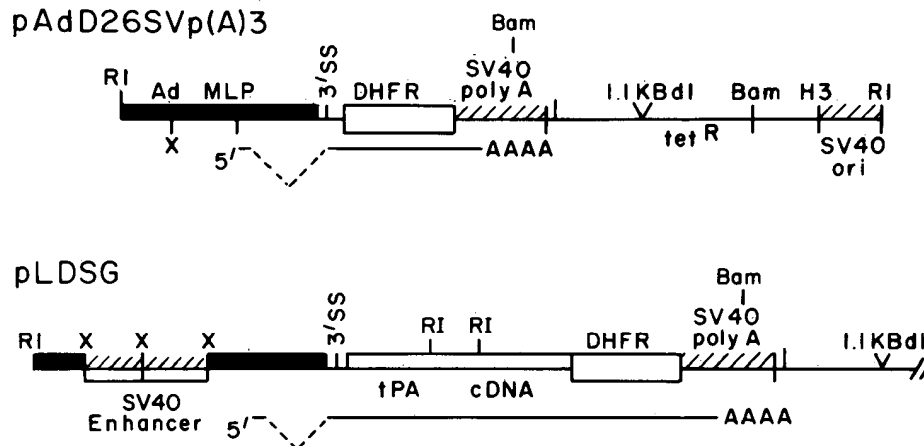


FIG. 1. t-PA and DHFR expression vectors. The DHFR expression vector, pAdD26SVp(A)3 (13), contains the adenovirus MLP including the first late leader and 5' splice site. The leader exon from the RNA transcript is spliced properly to an introduced 3' splice site. The vector contains the SV40 early polyadenylation site, 2.7 kb derived from pSV0d (21) which contains the ColE1 origin of replication, a pBR322 derivative lacking sequences detrimental to replication in mammalian cells, tetracycline resistance, and the SV40 origin of replication. The t-PA expression vector pLDSG is identical to the DHFR expression vector except that it contains the SV40 enhancer upstream from the AdMLP and the t-PA coding region upstream of the DHFR coding region. *EcoRI* (RI), *BamHI* (Bam), and *XhoI* (X) restriction sites are indicated.

harvested by trypsinization, swollen in 0.075 M KCl for 8 to 12 min, and fixed in two changes of 3:1 methanol-acetic acid. Staining protocols included Giemsa-trypsin banding (34) and quinacrine banding (7). Metaphases used for autoradiography were stained with quinacrine, photographed, and then prepared for in situ hybridization. In situ hybridizations were carried out as described previously (25). <sup>3</sup>H-labeled pLDSG was prepared by nick translation (27) to a specific activity of 10<sup>7</sup> cpm/μg by using [<sup>3</sup>H]dCTP. Hybridization slides were then dipped in Kodak NTB-2 emulsion, exposed at 4°C for 2 to 10 weeks, developed in Dektol (Eastman Kodak Co., Rochester, N.Y.), stained with 8% Giemsa, and rephotographed.

**RNA analysis.** RNA was prepared from confluent cells with 20 mM vanadyl adenosine complex as the ribonuclease inhibitor (13). Total RNA (25 μg) was denatured with glyoxal, electrophoresed on a 1.2% agarose gel (20), and transferred to nitrocellulose (38). Filters were baked, hybridized to a <sup>32</sup>P-radiolabeled t-PA probe prepared by nick translation (27) of the t-PA cDNA, and autoradiographed (13).

**Genomic DNA analysis.** High-molecular-weight DNA was isolated (37), and 10 μg was digested to completion (12) and electrophoresed on 1.1% agarose gels for Southern blot transfer (36). Filters were hybridized (13) to either DHFR or t-PA probes prepared by nick translation (27) of isolated DNA fragments.

## RESULTS

**Cotransfection of DHFR and t-PA into CHO DHFR-deficient cells.** The expression vectors used for DHFR and t-PA expression are diagramed in Fig. 1. pAdD26SVp(A)3 (13) is a DHFR transcription unit which contains (in a 5'-to-3' polarity) the adenovirus type 2 MLP (AdMLP), the first leader and 5' splice site from adenovirus type 2 late mRNAs, an introduced 3' splice site, the 800-bp coding region of murine DHFR, and the SV40 early polyadenylation signal. pAdD26SVp(A)3 contains an SV40 origin fragment, but it was constructed to lack an enhancer element, which is required for the efficient expression from the AdMLP in CHO cells (13).

The t-PA transcription unit is carried in the plasmid pLDSG, which is similar to pAdD26SVp(A)3 except for the insertions of (i) the SV40 enhancer upstream from the AdMLP and (ii) the t-PA coding region immediately upstream from the DHFR coding region (Fig. 1). The t-PA coding region is transcribed to yield a hybrid mRNA which contains the first late leader of adenovirus at the 5' end and the mouse DHFR sequence at the 3' end. Although this hybrid mRNA might yield a polycistronic transcript encoding both t-PA and DHFR, very few DHFR<sup>+</sup> colonies arose when pLDSG was introduced into CHO DHFR<sup>-</sup> cells (Table 1). Since the DHFR<sup>+</sup> colonies that arose upon transfection of pLDSG alone did not express detectable levels of t-PA, the transformants likely resulted from rearrangements in the t-PA coding region to yield DHFR expression. Thus, the DHFR coding region in pLDSG likely acts as a 3' untranslated sequence on the t-PA mRNA. The t-PA transcription unit in pLDSG was shown to be functional by its ability to direct expression of secreted t-PA activity upon DEAE-dextran-mediated transfection (35) of COS-7 monkey cells (data not shown).

Subsequently, pLDSG was mixed in different molar ratios with pAdD26SVp(A)3, which encodes an active DHFR transcription unit, and the DNA transfected into CHO

TABLE 1. Cotransfection of DHFR and t-PA cDNA genes<sup>a</sup>

Plasmid(s) (ratio)	Efficiency (no. of transformants/no. of transfected cells)
pLDSG	2 × 10 <sup>-5</sup>
pAdD26SVp(A)3	3 × 10 <sup>-5</sup>
pLDSG + pAdD26SVp(A)3 (1:1)	7.5 × 10 <sup>-4</sup>
pCVSVL	4.8 × 10 <sup>-4</sup>
pLDSG + pAdD26SVp(A)3 (20:1)	6 × 10 <sup>-5</sup>
pLDSG + pAdD26SVp(A)3 (100:1)	4 × 10 <sup>-5</sup>

<sup>a</sup> Plasmid DNAs (25 μg total) were mixed as indicated and transfected into DUKX-B11 cells (2.5 × 10<sup>6</sup> cells) as described in the text. After 48 h the cells were subcultured into alpha-medium lacking nucleosides, and colonies were scored 2 weeks later. In the absence of added plasmid DNA, the transformation efficiency was less than 10<sup>-7</sup>.



TABLE 2. t-PA activity in clones selected for MTX resistance and the stability of t-PA production upon growth in the presence and absence of MTX selection<sup>a</sup>

Clone	t-PA activity (mU/cell per day) in MTX ( $\mu$ M) concns of:					Minus MTX (no. of cell doublings)
	0	0.02	0.05	0.1	0.5	
<b>A.</b>						
1E2	0.02	0.015		0.005	0.005	
4D2	0.015	0.0015		0.003	0.002	
2D5	0.01	0.04		0.04	0.025	
4C1	0.04	0.03		0.035	0.03	
4C1 Subclones						
H3B	0.09	0.26		1.0		
B10A	0.025	0.09		0.19		
H8B	0.02	0.045		0.035		
H12B	0.05	0.15		0.005		
D8B	0.08	0.095				
<b>B.</b>						
4 $\rightarrow$ $\alpha$ pool	0.03	0.09	1.9			
Subclones						
3			4.25			
4			0.07			
5			1.80			
9			0.40			
12			0.60			
13			1.55			
16			0.10			
18			0.03			
20			6.00			
<b>C.</b>						
4			0.81		0.38 (17)	
9			0.35		0.23 (20)	
12			0.42		0.07 (20)	
5			3.5		3.2 (38)	
4F10			1.3		1.1 (30)	
H3B			0.35		0.26 (30)	

<sup>a</sup> t-PA activities were determined as described in the text and are given in milliuunits per cell per day. Section A shows the results from four original DHFR<sup>+</sup> transformants before and after selection for resistance to 0.5  $\mu$ M MTX. Before MTX selection, five subclones of clone 4C1 were assayed and then propagated in sequentially increasing concentrations of MTX. Section B shows the assay results from a pool of approximately 100 DHFR<sup>+</sup> transformants before and after mass selection for growth in 0.02 and 0.05  $\mu$ M MTX. Nine subclones were isolated from this mass-selected population and assayed. Section C shows the stability of various clones propagated either in the presence of 0.05  $\mu$ M MTX or in the absence of MTX for the indicated number of cell doublings. Clones 4, 5, 9, and 12 are the same as those described in section B, H3B was described in section A, and 4F10 is an independent clone isolated as described for the clones in section B.

DHFR<sup>-</sup> cells. Table 1 shows the efficiency for generating transformants capable of growth in nucleoside-free medium (i.e., DHFR<sup>+</sup> transformants). Neither pLDSG nor pAdD26SVp(A)3 alone transformed DHFR<sup>-</sup> cells to the DHFR<sup>+</sup> phenotype efficiently. Inefficient transformation by pAdD26SVp(A)3 was due to the absence of an enhancer element which is important for efficient transcription from the AdMLP (13). When pLDSG was mixed with pAdD26SVp(A)3 in a 1:1 molar ratio, there was a 10- to 20-fold increase in transformation efficiency compared to that with either plasmid alone. This may be attributed to *cis*

activation of pAdD26SVp(A)3 by the SV40 enhancer from pLDSG after plasmid recombination *in vivo*, since the frequency of transformation with the two plasmids combined was equivalent to that observed with a DHFR cDNA gene which contained the SV40 enhancer (pCVSVL). When the amount of pAdD26SVp(A)3 was decreased during cotransfection, the transformation efficiency correspondingly decreased. When similar dilutions of pAdD26SVp(A)3 were made with plasmids which do not contain an SV40 enhancer, the transformation efficiency also correspondingly decreased from 3/10<sup>6</sup> cells to less than 1/10<sup>7</sup> cells. Similar results were obtained by transfecting plasmids which were first digested with the restriction endonuclease *Cla*I (which linearizes each plasmid in the pBR322 region) and then ligated *in vitro*. These results suggest that the enhancer from the t-PA cDNA gene stimulates expression of the DHFR gene. This can only occur with a *cis*-acting enhancer element if cotransfection of an enhancer-deficient DHFR gene with an enhancer-containing t-PA gene generates transformants which contain the t-PA gene linked to the DHFR gene. Indeed, this type of integrated gene structure was found in CHO clone 13 (see below) when genomic DNA was analyzed by restriction enzyme digestion and Southern blot analysis. In this clone the t-PA gene was oriented in the opposite direction to that of the DHFR gene and on the same 12-kb *Bam*HI restriction fragment. Thus, pLDSG had recombined with pAdD26SVp(A)3 so that the SV40 enhancer lay upstream of both transcription units.

**Amplification of the transforming DNA.** After DHFR<sup>+</sup> transformants were obtained by growth in selective medium (alpha-medium lacking nucleosides), two protocols for selection and MTX amplification were followed. In the first protocol, single cloned DHFR<sup>+</sup> transformants were grown in stepwise increasing concentrations of MTX. In the second protocol, a pool of 100 DHFR<sup>+</sup> transformants was propagated in increasing concentrations of MTX, and then individual clones were isolated from the pooled population and analyzed for t-PA expression; clones which exhibited the highest levels of t-PA expression were then grown in additional increasing concentrations of MTX.

Using the first protocol, we found that approximately 25% of the DHFR<sup>+</sup> transformants also expressed t-PA activity. This percentage did not increase consistently with molar ratios of input DNA of pLDSG to pAdD26SVp(A)3 from 5:1 to 100:1. However, DNA blot analysis of 12 cloned transformants showed a close correlation between the level of t-PA expression and the number of copies of the t-PA sequences integrated into the initial transformant (data not shown). Four clones (1E2, 4D2, 2D5, and 4C1) exhibiting high t-PA activity before MTX selection were chosen for MTX resistance selection by using, sequentially, 0.02, 0.1, and 0.5  $\mu$ M MTX. Surprisingly, none of the four clones synthesized higher levels of t-PA activity as the MTX concentration was increased (Table 2).

To understand why t-PA activity did not increase with MTX resistance, clone 4C1 was subcloned in nucleoside-free medium before MTX selection to generate five subclones, and these subclones were selected for MTX resistance. Only subclones H3B and B10A expressed higher levels of t-PA when selected for MTX resistance (Table 2). The variation in responses of the different subclones to coamplification could have resulted from the integration of multiple DHFR genes at different sites, only some of which were linked to the t-PA gene, and upon MTX selection some subclones amplified DHFR sequences not linked to t-PA sequences. Alternatively, the variation could have resulted from rearrange-

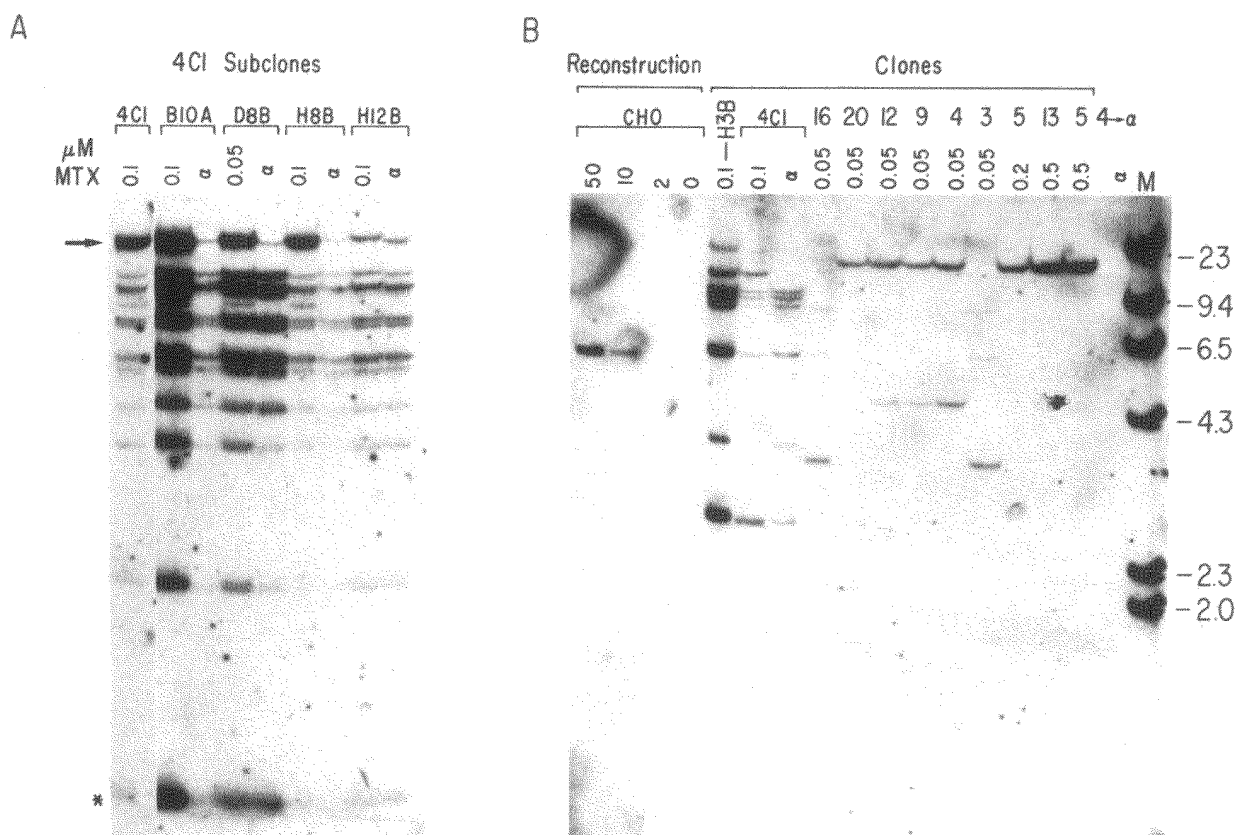


FIG. 2. Southern blot analysis of DHFR<sup>+</sup>, MTX-resistant clones. DNA was isolated from the cell lines indicated, and 10 μg was digested to completion with either *Eco*RI (panel A) or *Bam*HI (panel B) and electrophoresed on 1.1% agarose gels for Southern blot transfer. Filters were hybridized to a <sup>32</sup>P-labeled probe prepared by nick translation of the t-PA cDNA insert. Clones analyzed here are the original DHFR<sup>+</sup> 4C1 clone before (α) and after selection for 0.1 μM MTX resistance and its subclones H3B, B10A, D8B, H8B, and H12B before (α) and after selection for 0.05 to 0.1 μM MTX resistance. Also shown is the original 4→α pool before MTX selection (α) and eight subclones (i.e., 3, 4, 5, 9, 12, 13, 16, and 20) selected for growth in 0.05, 0.2, and 0.5 μM MTX as indicated. A reconstruction of pLDSG with CHO DNA is depicted and represents 2, 10, and 50 copies of pLDSG per genome of CHO DNA. *Hind*III λ molecular weight markers are indicated at the right. The arrow shows a restriction fragment frequently amplified in the 4C1 subclones upon MTX selection. The asterisk shows the position of a 475-bp internal *Eco*RI fragment present in the t-PA cDNA. Its abundance correlates with levels of t-PA expression.

ments around the site of amplification which separated the t-PA gene from the DHFR gene. To examine these possibilities in more detail, Southern blot analysis of the original and MTX-resistant transformants derived from clone 4C1 was performed. The results showed that most subclones amplified a similar, if not identical, restriction fragment (35-kb band; Fig. 2A, arrow). However, subclone B10A amplified all restriction fragments containing t-PA sequences. Since B10A had amplified both the 35-kb band and the other restriction fragments, they were probably closely linked. In other subclones the copy number of the 35-kb fragment did not correlate with the copy number of the various t-PA restriction fragments (for example, compare subclones D8Bα and H8Bα), but the 35-kb band was preferentially amplified in all subclones. These results indicate that the coamplification variability between subclones probably results from DNA rearrangements which dissociate the DHFR gene from the t-PA gene during the MTX selection. One subclone (H12B) exhibited no DNA amplification upon MTX selection, implying that in this subclone MTX resistance probably resulted from a mechanism other than gene amplification.

The results of the second amplification protocol are shown in Table 2. A total of 100 colonies (from four independent

plates) obtained by selection in medium lacking nucleosides were pooled and selected for MTX resistance by using, sequentially, 0.02 and 0.05 μM MTX. The original pooled transformants expressed t-PA activity at a rate of 0.03 mU per cell per day and, upon culture in media containing first 0.02 and then 0.05 μM MTX, expressed activities of 0.9 and 1.9 mU per cell per day, respectively. Individual clones within the population growing in 0.5 μM MTX were isolated and found to vary as much as 60-fold in the level of t-PA expression (from 0.1 to 6 mU per cell per day). As a result, this second protocol allowed for more rapid identification of clones expressing high levels of t-PA at elevated MTX concentrations.

The success in utilizing the second protocol for coamplification may have resulted from either the selection in MTX of a subset of transformants which had taken up more DNA in the initial transfection or, alternatively, the selection of a subset of transformants which had integrated the t-PA and DHFR genes in a chromosomal location susceptible to high expression or frequent DNA amplification. This subset may be only a small proportion of the total number of transformants and thus would be found infrequently by the first protocol. Southern blot analysis of the initial pool of transformants and MTX-resistant clones sub-

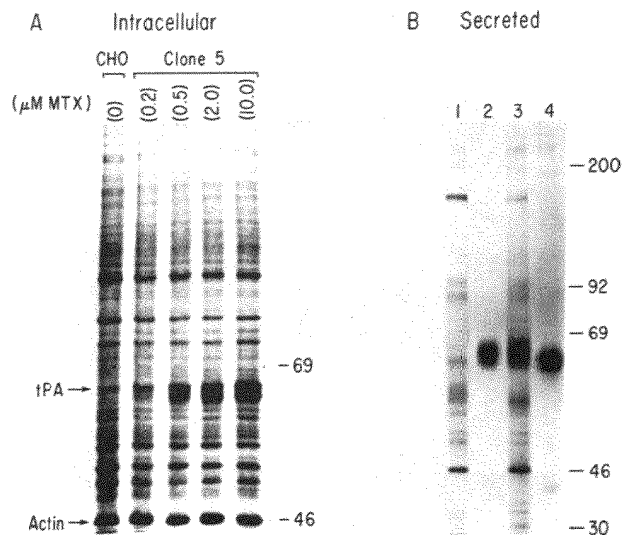


FIG. 3. Polyacrylamide gel electrophoresis of intracellular and secreted proteins from transformed CHO cells. Cells were labeled with [<sup>35</sup>S]methionine for 30 min (panel A) or 4 h (panel B), and cell extracts and conditioned media were prepared and analyzed as described in the text. Panel A represents approximately equal numbers of counts from cell extracts isolated from the original DHFR<sup>-</sup> CHO cell line, and from the DHFR<sup>+</sup> t-PA-producing clone 5 selected in 0.2, 0.5, 2.0, and 10.0  $\mu$ M MTX. Panel B represents total labeled secreted protein from the original DHFR<sup>-</sup> CHO cells (lane 1) and the t-PA-producing clone H3B propagated in 0.1  $\mu$ M MTX (lane 3). Also shown are results from immune precipitations with rabbit anti-human t-PA clone H3B in 0.1  $\mu$ M MTX and with the original Bowes human melanoma cell line conditioned medium (lanes 2 and 4, respectively).

sequently derived favored the latter alternative (i.e., selection of a subset of transformants which frequently amplify the transfected DNA) (Fig. 2). The initial pool of transformants ( $4 \rightarrow \alpha$ ) did not generate any common *Bam*HI restriction fragments, indicating heterogeneity in the integration of the t-PA gene in the mass population. However, eight independent clones isolated from the  $4 \rightarrow \alpha$  pool after selection in MTX exhibited only three different classes of restriction patterns for the integrated DNA. Subclones 3 and 16, subclones 4, 9, and 12, and subclones 5, 13, and 20 could be grouped into the three different classes of transformants based on these integration patterns. Thus, upon MTX resistance selection of the pooled transformants, only a limited set of transformants outgrew the population. Rather than containing multiple integrations, these transformants appeared to have efficiently amplified a single integrated t-PA sequence as much as 100-fold (for example, clones 5 and 13 [Fig. 2]). By comparison, clone H3B, selected by the first protocol, contained multiple integrations of the t-PA gene. We conclude that subjecting a large population of independent transformants to growth in MTX will select for transformants which efficiently coamplify the DHFR and t-PA genes. Presumably, if enough clones from the original transformation were individually selected in MTX, these coamplifying transformants would be found, but this would require an inordinate amount of work.

The stability of t-PA expression upon propagation of cells in the absence of MTX is presented in Table 2. Several clones lost high-level t-PA expression, whereas other clones

maintained the ability to express t-PA for over 30 cell doublings.

**t-PA synthesis in transformed CHO cells.** As described above, t-PA synthesized by transformed CHO cells was initially analyzed by measuring the rate of accumulation of plasminogen-activating activity in conditioned medium on a per cell per day basis. t-PA synthesis was also analyzed by [<sup>35</sup>S]methionine labeling of CHO cells in culture by using either a 30-min pulse to examine intracellular protein or a 4-h pulse to examine secreted protein. Gel electrophoresis of the total and immunoprecipitable t-PA protein was performed (see above). Intracellular t-PA ran as a doublet with a molecular size of approximately 68,000 daltons, whereas secreted t-PA ran as a more diffuse band though of similar molecular size (Fig. 3). This heterogeneity can be attributed to glycosylation patterns, since t-PA synthesized in the presence of tunicamycin shows a single discrete band at 58,000 daltons (data not shown).

The rate of intracellular t-PA synthesis in clone 5 grown at increasing MTX concentrations was analyzed by 30-min pulse-labeling with [<sup>35</sup>S]methionine, and it was found that, as expected, the rate of synthesis increased with increasing MTX resistance consistent with an increase in the t-PA gene copy number. At the highest MTX concentration, approximately 5% of the total incorporated label was found in t-PA (severalfold higher than in actin, a major cellular protein). In 4-h labeling studies, t-PA represented more than 50% of the total protein secreted by several lines (Fig. 3, lane 3), was immune precipitated with a polyclonal antibody directed towards Bowes melanoma t-PA (Fig. 3, lane 2) and comigrated with t-PA synthesized by the human Bowes melanoma cell line (Fig. 3, lane 4).

Purified t-PA, isolated from transformed-CHO-cell conditioned medium, has been further characterized to determine its enzyme kinetic parameters. These are indistinguishable from those of human t-PA (data not shown).

**t-PA mRNA in amplified CHO cells.** Figure 4 depicts the Northern blot analysis of total cytoplasmic RNA isolated from t-PA-producing CHO transformants. The blot was hybridized to a t-PA-specific <sup>32</sup>P-labeled probe. All t-PA-producing cell lines exhibited a single 3.1-kb mRNA species, as expected from the transcription unit used. The relative amounts of t-PA mRNA found in various lines was in direct proportion to the copy number of the t-PA sequences in each line. Primer extension by using reverse transcriptase (13) and S1 nuclease mapping (13) with 5'- and 3'-end-labeled probes confirmed that the mRNA structure was precisely as depicted in Fig. 1 (data not shown). When the same RNA blot was hybridized with a DHFR <sup>32</sup>P-labeled probe, an additional 1-kb mRNA in equal abundance to the t-PA mRNA was observed, indicating that the t-PA mRNA was expressed equally as well as the DHFR mRNA.

**Chromosomal location of amplified DNA sequences.** The chromosomal location of the amplified DNA in clones isolated by the second protocol was examined by *in situ* hybridization. Two independent subclones of clone 5, both containing approximately 100 copies of the t-PA gene, were found to have very similar karyotypes (Fig. 5). When compared to the original DUKX-B11 cell line (14), three new marker chromosomes (M1, M2, and M3) were typically seen (Fig. 5A). The amplified DNA was associated with a homogeneously staining region in M1 and, in some cells, with a submetacentric chromosome slightly smaller than M2 (Fig. 5B). When clone 5, which was resistant to 0.5  $\mu$ M MTX and contains two regions having multiple copies of the transfected sequences (Fig. 5A), was propagated in the



absence of MTX for more than 30 cell doublings, no change was observed in the karyotype (data not shown). When clone 5 was propagated in a higher concentration of MTX (2  $\mu\text{M}$ ), the karyotype tended to be tetraploid (not shown) and additional sites of probe hybridization could be detected (Fig. 5C). In contrast, another amplified clone (4F10), isolated from an independent transfection and also amplified to approximately 100 copies per cell, contained a single region of amplified DNA (Fig. 5D) without any other major change in its karyotype. It is interesting that the location of the amplified DNA in clone 4F10 was also on a chromosome corresponding to M1.

**Altered cell morphology associated with increased t-PA expression.** As t-PA expression from the CHO cells increased with increasing MTX concentrations, a clear change in cell morphology could be observed (Fig. 6). The cells became more round, had irregular, ruffled plasma membranes, and attached poorly to the tissue culture dish. This

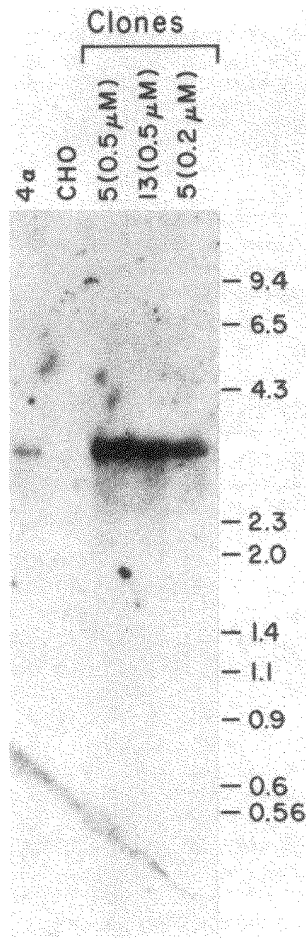


FIG. 4. Northern analysis of t-PA mRNA in amplified cell lines. Approximately 25  $\mu\text{g}$  of total cytoplasmic RNA isolated from clone 5 grown in 0.2 and 0.5  $\mu\text{M}$  MTX, clone 13 grown in 0.5  $\mu\text{M}$  MTX, the original pooled DHFR<sup>+</sup> transformants grown in nucleoside-free medium (4 $\alpha$ ), and the original DHFR-deficient CHO cells (CHO) were electrophoresed on a glyoxal agarose gel, transferred to nitrocellulose, hybridized to a <sup>32</sup>P-labeled probe prepared by nick translation of the isolated t-PA cDNA fragment, and prepared for autoradiography. The numbers on the right indicate the molecular weight markers (in kilobases).

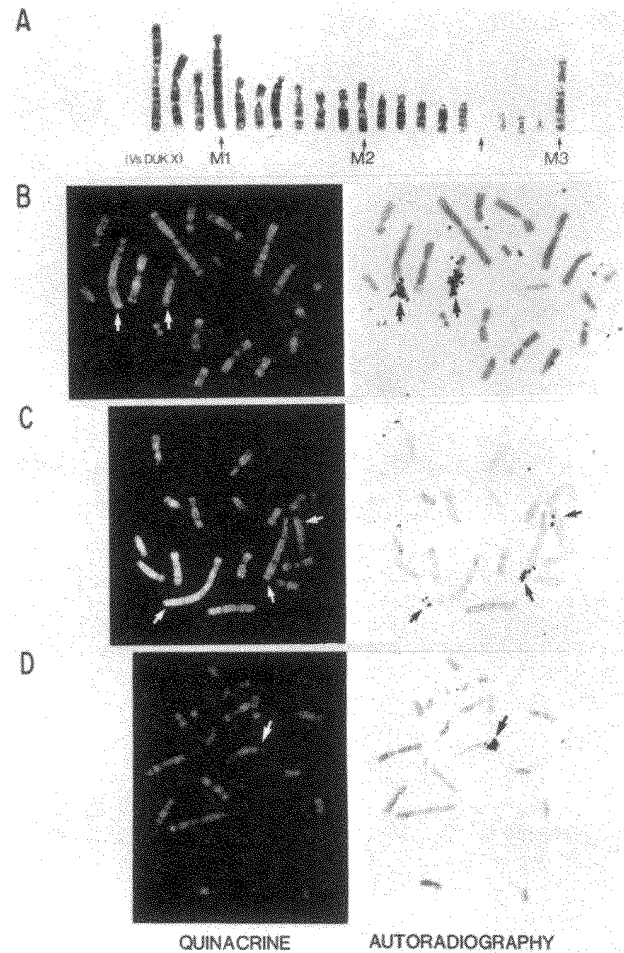


FIG. 5. Localization of amplified DNA. Panel A depicts Giemsa-trypsin banded chromosomes from clone 5 grown in 1.0  $\mu\text{M}$  MTX. Chromosomes have been arranged according to comparison to the CHO DHFR<sup>-</sup> DUKX cell line (14). The changes from DUKX (arrows) include marker chromosomes (M1, a larger long arm; M2, a larger short arm; M3, a new chromosome; arrow alone, the loss of a small chromosome). Metaphase cells from two independent subclones of clone 5 selected for growth in 0.5  $\mu\text{M}$  MTX (panel B) and 2.0  $\mu\text{M}$  MTX (panel C) and from an independent clone (4F10) selected for growth in 1.0  $\mu\text{M}$  MTX (panel D) were stained with quinacrine, destained, subjected to in situ hybridization, and autoradiographed. Slides were then stained with Giemsa, and the same metaphases were rephotographed. Regions of hybridization are indicated by arrows.

may have resulted from either the increased secretion activity of the cells or from an activity associated specifically with t-PA expression. The altered morphology could be reversed by growing the cells in the presence of 0.5% aprotinin or a serine protease inhibitor or in serum which was depleted of plasminogen (by passage of serum over a lysine-Sepharose column). We conclude that high-level expression of t-PA probably converts serum plasminogen (present at 100  $\mu\text{g}/\text{ml}$ ) to plasmin, a broad-spectrum protease. The plasmin then acts to proteolyze the cells in much the same way as does trypsin. Long-term propagation of the cells in medium containing aprotinin effectively overcomes the altered morphology without affecting the secretory properties of the cells.



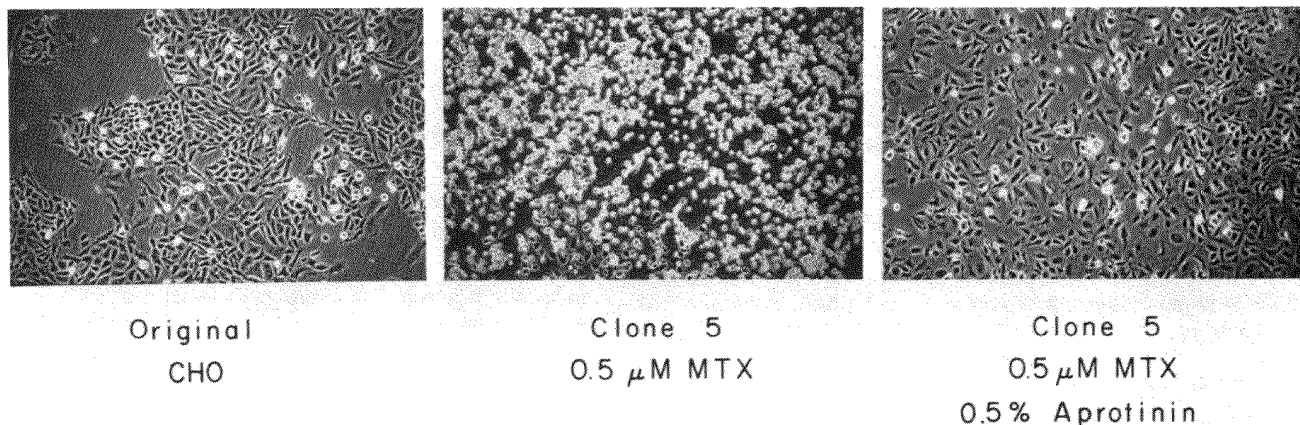


FIG. 6. Phase-contrast photomicrographs of t-PA-producing CHO cell lines. The original CHO DUKX-B11 cell line and the t-PA-producing clone 5 propagated in  $0.5 \mu\text{M}$  MTX were photographed after growth in medium containing 10% fetal calf serum. The panel on the right depicts clone 5 after similar growth for 48 h in the presence of 0.5% aprotinin.

### DISCUSSION

The purpose of this investigation was to apply gene amplification to the development of a mammalian cell line which produces high levels of t-PA, a potential thrombolytic agent. Since a direct selection method for the amplification of the t-PA gene was not available, a cotransfection method was used which yields cells containing the t-PA sequences closely linked to the DHFR sequences. Thus, selection for DHFR gene amplification by increasing MTX resistance provided for the coamplification of t-PA sequences. The DHFR transcription unit was constructed without an enhancer element. As a result, transformation of DHFR-deficient CHO cells to a DHFR<sup>+</sup> phenotype was inefficient with this DNA alone. However, cotransfection of the DHFR unit with a t-PA transcription unit containing an enhancer dramatically increased the efficiency of DHFR transformation. This appeared to occur by *in vivo* ligation of the two separate transcription units, resulting in the enhancement of DHFR expression by the t-PA-associated enhancer element. Efficient DHFR expression was thus dependent on continued association with the enhancer from the t-PA transcription unit.

The manner in which cells were selected for MTX resistance influenced the ability to isolate cells which had coamplified t-PA sequences. When individual cloned DHFR<sup>+</sup> transformants were assayed for t-PA expression, there was a correlation between the amount of transfected DNA incorporated and the level of t-PA expression. However, when the transformants expressing the highest t-PA levels were selected for MTX resistance, t-PA expression did not increase. DNA blot analysis of MTX-resistant subclones from one of these transformants indicated that only a small subset of the transfected t-PA sequences was amplified. Furthermore, all subclones amplified a common restriction fragment. Infrequently, all of the transfected DNA was amplified, and in these cell lines the level of t-PA expression correspondingly increased. These results are consistent with the existence of a gradient of DNA amplification as proposed by Roberts et al. (31). Under this hypothesis, transfected DNA sequences at the center of the gradient would be frequently amplified in all MTX-resistant clones, whereas more distal transfected DNA sequences would be less likely to be amplified.

In contrast to the results with individual DHFR<sup>+</sup> trans-

formants, when DHFR<sup>+</sup> transformants were pooled and mass selected for MTX resistance before being subcloned, cell lines were frequently obtained which amplified and expressed high levels of t-PA. Cloned lines from the populations mass selected for MTX resistance contained very few sites of DNA integration, but the transfected DNA was amplified to a high degree. This mass selection did not select for clones which had received large amounts of DNA in the transfection process, but rather selected for clones which efficiently amplified or expressed the newly acquired DNA. This finding is consistent with the observation that the frequency of amplification of transfected DNA can vary dramatically between different transformants (12, 42). This variation may result from proximity to a particular DNA sequence (possibly an origin of replication) or to the general location of transfected DNA in a chromosome (14). We presume that only a very small percentage of the initial transformants contain DHFR sequences integrated into sites where gene amplification is efficient. It was possible to select for this subset by selection of the total pool of cotransformants for MTX resistance. Since the continued presence of the enhancer element from the t-PA cDNA gene was necessary for efficient DHFR expression, a high percentage of this subset is expected to have coamplified t-PA sequences and to thus express t-PA at high levels. Our results are consistent with this scheme.

Previous studies have indicated that transfected and amplified DNA in CHO cells is chromosomally associated, very frequently as extensive, homogeneously staining regions (14, 16, 22). Karyotype analysis of MTX-resistant clones derived by the mass population MTX selection shows a similar pattern to those presented by others (14, 22) in which amplification occurred by MTX selection of clonal lines. In both cases, large homogeneously staining regions are frequently associated with the amplified DNA, although in a small number of clones the amplified DNA may be present with little cytological perturbation. It is interesting that two independent clones isolated here contain the amplified genes associated with a specific region of a single, elongated, telocentric marker chromosome (M1; Fig. 5A). Whether this results from some unique DNA sequence (possibly t-PA) in the vectors that were used or from the nature of the selection protocol (selection for amplifiable transfected DNA) is not known.

The linearity observed between MTX resistance, DHFR

expression, and t-PA expression indicates that synthesis and secretion of large amounts of t-PA is not a limiting factor in these cells. To date we have not observed any disparities between the level of gene amplification and the amount of protein secreted into the medium. CHO cells clearly have the capacity to accommodate the synthesis, glycosylation, and secretion of much larger quantities of protein than they do normally; this is substantiated by the fact that gene amplification has been used to obtain cell lines which express high levels of gamma interferon (11, 32), beta interferon (19), hepatitis B surface antigen (5), and now human t-PA. It appears that this approach will prove generally applicable to the development of cell lines producing high levels of a variety of proteins which may prove therapeutically useful but which have previously been difficult to obtain in sufficient quantities.

A limited analysis of the t-PA synthesized by CHO cells shows that it has approximately the same specific activity as native t-PA and is glycosylated in a similar but not identical manner. We are currently studying the properties of this t-PA in more detail. Amino acid sequence data show that the N-terminal sequence CHO-cell-produced t-PA is identical to that of native human melanoma t-PA.

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