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Sequence and expression of the cDNA for MEP (major excreted protein), a transformation-regulated secreted cathepsin

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The major excreted protein (MEP) of malignantly transformed mouse fibroblasts is a secreted thiol proteinase. Sequencing of the MEP cDNA shows the coding region for the protein to be identical with the sequence for a mouse cysteine proteinase isolated from macrophages, but the MEP cDNA is polyadenylated at a different site in the 3′ non-coding region. Strong homology of MEP with human cathepsin L suggests that MEP is the mouse analogue of cathepsin L. Amino acid sequencing of the *N*-terminus of the secreted form of MEP indicates that, during secretion, the polypeptide is cleaved between amino acids 17 and 18. We have placed the MEP cDNA in a eukaryotic expression vector and demonstrated the production of the 39 kDa polypeptide form of mouse MEP in monkey CV-1 cells.

INTRODUCTION

The major excreted protein (MEP) of mouse fibroblasts cells is a 39 kDa secreted glycoprotein that is induced by malignant transformation, growth factors and tumour promoters (Gottesman, 1978; Gottesman & Sobel, 1980; Scher *et al.*, 1982). Kirsten-virus-transformed NIH 3T3 (KNIH) cells and 12-*O*-tetradecanoylphorbol 13-acetate- and platelet-derived-growth-factor-treated NIH 3T3 (NIH) cells synthesize and secrete severalfold more MEP than do non-transformed or untreated NIH cells. These increases in synthesis are due to increased mRNA levels (Gottesman & Sobel, 1980) resulting from increased transcription (Rabin *et al.*, 1986).

Several lines of evidence suggest that MEP is the precursor for a lysosomal thiol proteinase. Within cells, MEP is processed from a 39 kDa form to two non-secreted lower-molecular-mass forms (29 and 20 kDa) found in the Golgi and lysosomes (Gal *et al.*, 1985). The secreted 39 kDa form of MEP is an activatable acid proteinase that cleaves bovine serum albumin, oxidized insulin B-chain, fibronectin, collagen, laminin and the synthetic peptide benzyloxycarbonyl-Phe-Arg 4-methyl-coumarylamide and is also inhibited by leupeptin and iodoacetic acid, both cysteine-proteinase inhibitors (Gal & Gottesman, 1986*a*). With oxidized insulin B-chain (Gal & Gottesman, 1986*b*) or synthetic substrates (R. W. Mason, S. Gal & M. M. Gottesman, unpublished work) the biochemical specificity of mouse MEP and its profile of inhibition are the same as those of human cathepsin L (Mason *et al.*, 1986). Recent partial sequence data supports the idea that MEP is a member of the family of thiol proteinases (Denhardt *et al.*, 1986).

The full coding sequence reported here establishes that MEP is a thiol proteinase homologous with human cathepsin L (Mason *et al.*, 1986) and identical with mouse cysteine proteinase (Portnoy *et al.*, 1986), but contains a different polyadenylation site. When the MEP cDNA is cloned into a eukaryotic expression vector with a human metallothionein promoter and transfected into monkey CV-1 cells, authentic 39 kDa MEP is synthesized.

MATERIALS AND METHODS

Sequencing of MEP cDNA

The MEP cDNA, pMMEP-14, containing a 1450-bp insert in the restriction-endonuclease-*Pst*I site of pBR322, was cloned from RNA from Kirsten-virus-transformed NIH 3T3 cells (Doherty *et al.*, 1985). The *Pst*I fragment was subcloned into M13mp18 and deletion mutants were generated (IBI, New Haven, CT, U.S.A.) and a partial consensus sequence was determined with radiolabelled dideoxynucleotides (Sanger *et al.*, 1977). Additional dideoxynucleotide sequencing was performed using double-stranded supercoiled plasmids with the K/RT sequencing kit from Promega Biotec (Madison, WI, U.S.A.). In all, 1167 base-pairs of the cDNA were sequenced by subcloning either *Bam*HI or *Sau*3AI restriction fragments into the *Bam*HI site of pGEM-3. The large subclone containing base-pairs 694–1167 was partially digested with *Xmn*I, further digested with either *Sma*I or *Hin*cII, and then re-ligated to generate two subclones containing inserts of fewer than 300 bp. The sequence of the 3′ terminal non-coding region of the cDNA was determined by using synthetic oligonucleotides (Applied Biosystems 3A) with the original pMMEP-14 as template. The entire sequence was confirmed with multiple subclones, and both strands of the cDNA were sequenced. Analysis of DNA sequences was performed by using the DNA Inspector II program for the Macintosh computer and the DNA : Ideas package on the DEC-10 mainframe computer at the National Institutes of Health (NIH).

Cell culture and immunoprecipitation of MEP

NIH cells and African-green-monkey kidney CV-1 cells (obtained from Dr. B. Howard of the NIH), were cultured as previously described (Gal *et al.*, 1985). For

Abbreviation used: MEP, major excreted protein; MCP, mouse cysteine proteinase.

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These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00314.

immunoprecipitation of MEP, 2.5×10^5 cells were plated in a 25 cm^2 flask and then, after either 2 or 3 days, incubated with [^{35}S]methionine (100 Ci/mmol; New England Nuclear) for 30 min at a concentration of 100 μCi/ml in methionine-free Dulbecco–Vogt medium (NIH media unit). Cells were lysed and immunoprecipitation and electrophoresis were performed as previously described on 12% (w/v) polyacrylamide gels (Bonner and Laskey, 1974; Gottesman & Cabral, 1981; Laemmli, 1970).

Construction of expression vector and DNA-mediated transformation

The 1167-bp *Bam*HI fragment of the MEP cDNA (which contains the complete open reading frame) was subcloned into a eukaryotic expression vector downstream from the human metallothionein IIA promoter (pMTP-3HL; obtained from Dr. Neil Rosen of the Medical Breast Cancer Section of this Institute). Subcloning involved filling in the *Bam*HI 5′ overhanging ends with the Klenow fragment of DNA polymerase I, blunt ligation to *Hin*dIII linkers, and ligation into the unique *Hin*dIII site just distal to the metallothionein promoter, thereby generating the pMTMEP-2 subclone. CV-1 cells (2.5×10^5) were transfected with 10 μg of DNA (Gorman *et al.*, 1983), and MEP expression was examined by [^{35}S]methionine labelling and immunoprecipitation of MEP after 48 h.

Amino acid sequencing

MEP (30–300 ng) was purified from conditioned medium from KNIH cells as described previously (Gal & Gottesman, 1986*a*), dialysed against 10 mM-sodium phosphate, pH 6.5, and then freeze-dried. Edman degradation was performed by Dr. Alan Smith (Protein Structure Laboratory, University of California at Davis, CA, U.S.A.) using a Beckman model 890M liquid sequencer.

RESULTS AND DISCUSSION

Nucleic acid and amino acid sequence

Approx. 1400 base-pairs were mapped by restriction-enzyme analysis, subcloned into M13 and pGEM, and sequenced by the dideoxy method of Sanger *et al.* (1977) (Fig. 1). The longest open reading frame consists of 1002 base-pairs and predicts a 334-amino-acid polypeptide with a molecular mass of 36287 Da. Although the observed molecular mass of the secreted form of MEP on SDS/polyacrylamide gels is 39 kDa, MEP contains approx. 5–10% neutral sugar by weight and the form of MEP translated *in vitro*, lacking these sugars, is 2–3 kDa smaller than mature MEP (Gottesman & Cabral, 1981).

The mouse fibroblast MEP cDNA sequence between base-pairs 9 and 1285 is identical with the sequence of a cDNA isolated from the mouse macrophage-like cell line J774 by Portnoy and his co-workers (Portnoy *et al.*, 1986). The one exception is base number 45. This may reflect a true polymorphism or may be an error in reverse transcription. The sequence between 333 and 970 base-pairs is also identical with a partial sequence of MEP reported by Denhardt and his co-workers (Denhardt *et al.*, 1986). Because the isolate of Portnoy *et al.* (1986) shared significant predicted amino acid similarity with five other cysteine proteinases, they termed their protein 'mouse cysteine proteinase' (MCP).

We have previously reported that both the intracellular forms of newly synthesized MEP and the form of MEP synthesized by translation *in vitro* show complex charge and size heterogeneity (Gottesman & Cabral, 1981), suggesting the possibility of multiple genes or gene products. Additionally, MEP is expressed in many different mammalian tissues (S. Gal & M. M. Gottesman, unpublished work). Despite these observations, the complete identity between the cDNAs from two different cell types suggests that there may be only one gene for MEP. This conclusion has been proved by isolating the unique mouse MEP gene that contains all of the DNA fragments detected by probing mouse genomic DNA on Southern blots with radiolabelled MEP cDNA (B. R. Troen, D. Ascherman, D. Atlas & M. M. Gottesman, unpublished work).

Polyadenylation of the mouse fibroblast MEP mRNA is different from polyadenylation of mouse macrophage mRNA. The 3′ non-coding region in the fibroblast cDNA is 89 bases longer than in the cDNA from the mouse macrophage J774 cell and contains the consensus AATAAA polyadenylation signal 20 bases upstream from the poly(A) tail. In many genes, such as β-tubulin (Lee *et al.*, 1983) and dihydrofolate reductase (Setzer *et al.*, 1982), there are alternative polyadenylation sites resulting in multiple mRNAs that differ only in the 3′ non-coding region. The presence of only one canonical polyadenylation signal in the MEP cDNA and the absence of other non-standard motifs such as ATTAAA, AATACA, and AACAAA (Boardman *et al.*, 1985; Swimmer & Shenk, 1985; Mason *et al.*, 1985) suggest that there may be other sequence determinants leading to polyadenylation of the MEP mRNA in macrophages.

Homology with cathepsin L

The *N*-terminal sequence of the secreted 39 kDa form of MEP is the same as the sequence predicted from the cDNA nucleotide sequence, confirming that the cDNA clone pMMEP-14 encodes authentic MEP (Fig. 2*a*). When the predicted MEP amino acid sequence is compared with published *N*-terminal sequences of the heavy and light chains of human cathepsin L reported by Mason *et al.* (1986), 80% of the residues are identical and half of the non-identical amino acids are conservative substitutions. Since human cathepsin L and mouse MEP also have very similar enzymic properties (Gal & Gottesman, 1986*a*,*b*; R. W. Mason, S. Gal & M. M. Gottesman, unpublished work), we believe that MEP is the precursor to the mouse analogue of human cathepsin L. As also noted by Portnoy *et al.* (1986), significant homology was found with the thiol proteinases rat liver cathepsin H, papaya plant papain, chinese-gooseberry actinidin, and rat liver cathepsin B. However, the homology between MEP and these proteins is no greater than 50%.

Processing sites in MEP

The *N*-terminal amino acid sequence of the secreted 39 kDa form begins with the eighteenth predicted amino acid in the open reading frame of the MEP cDNA. This 17-amino-acid prepeptide fragment has a hydrophobic core preceded by a positively charged asparagine residue and is similar to other signal sequences in both eukaryotic and prokaryotic proteins (Von Heijne, 1983; Perlman &

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MEP       CGGCGACCTC CGGGGATCCG AGTTTGCAGA CTTCTTGTGC GCACGTAGCC GCCTCAGGTG TTTGAACC    68
                  :: :::::::::: :::::::::: :::::::::: :::: ::::: :::::::::: ::::::::
MCP               TC CGGGGATCCG AGTTTGCAGA CTTCTTGTGC GCAC-TAGCC GCCTCAGGTG TTTGAACC

                                                                        ↓
    ATG AAT CTT TTA CTC CTT TTG GCT GTC CTC TGC TTG GGA ACA GCC TTA GCT ACT CCA AAA   128
    MET Asn Leu Leu Leu Leu Leu Ala Val Leu Cys Leu Gly Thr Ala Leu Ala Thr Pro Lys
     1                                                                  18      20
    TTT GAT CAA ACC TTT AGT GCA GAG TGG CAC CAG TGG AAG TCC ACG CAC AGA AGA CTG TAT   188
    Pho Asp Gln Thr Phe Ser Ala Glu Trp His Gln Trp Lys Ser Thr His Arg Arg Leu Tyr
                                                                            40
    GGC ACG AAT GAG GAA GAG TGG AGG AGA GCG ATA TGG GAG AAG AAC ATG AGA ATG ATC CAG   248
    Gly Thr Asn Glu Glu Glu Trp Arg Arg Ala Ile Trp Glu Lys Asn MET Arg MET Ile Gln
                                                                            60
    CTA CAC AAC GGG GAA TAC AGC AAC GGG CAG CAC GGC TTT TCC ATG GAG ATG AAC GCC TTT   308
    Leu His Asn Gly Glu Tyr Ser Asn Gly Gln His Gly Phe Ser MET Glu MET Asn Ala Phe
                                                                            80
    GGT GAC ATG ACC AAT GAG GAA TTC AGG CAG GTG GTG AAT GGC TAT CGC CAC CAG AAG CAC   368
    Gly Asp MET Thr Asn Glu Glu Phe Arg Gln Val Val Asn Gly Tyr Arg His Gln Lys His
                                                                           100
                                                          ↓
    AAG AAG GGG AGG CTT TTT CAG GAA CCG CTG ATG CTT AAG ATC CCC AAG TCT GTG GAC TGG   428
    Lys Lys Gly Arg Leu Phe Gln Glu Pro Leu MET Leu Lys Ile Pro Lys Ser Val Asp Trp
                                           114                              120
    AGA GAA AAG GGT TGT GTG ACT CCT GTG AAG AAC CAG GGC CAG TGC GGG TCT TGT TGG GCG   488
    Arg Glu Lys Gly Cys Val Thr Pro Val Lys Asn Gln Gly Gln Cys Gly Ser Cys Trp Ala
                                                                           140
    TTT AGC GCA TCG GGT TGC CTA GAA GGA CAG ATG TTC CTT AAG ACC GGC AAA CTG ATC TCA   548
    Phe Ser Ala Ser Gly Cys Leu Glu Gly Gln MET Phe Leu Lys Thr Gly Lys Leu Ile Ser
                                                                           160
    CTG AGT GAA CAG AAC CTT GTG GAC TGT TCT CAC GCT CAA GGC AAT CAG GGC TGT AAC GGA   608
    Leu Ser Glu Gln Asn Leu Val Asp Cys Ser His Ala Gln Gly Asn Gln Gly Cys Asn Gly
                                                                           180
    GGC CTG ATG GAT TTT GCT TTC CAG TAC ATT AAG GAA AAT GGA GGT CTG GAC TCG GAG GAG   668
    Gly Leu MET Asp Phe Ala Phe Gln Tyr Ile Lys Glu Asn Gly Gly Leu Asp Ser Glu Glu
                                                                           200
    TCT TAC CCC TAT GAA GCA AAG GAC GGA TCT TGT AAA TAC AGA GCC GAG TTC GCT GTG GCT   728
    Ser Tyr Pro Tyr Glu Ala Lys Asp Gly Ser Cys Lys Tyr Arg Ala Glu Phe Ala Val Ala
                                                                           220
    AAT GAC ACA GGG TTC GTG GAT ATC CCT CAG CAA GAG AAA GCC CTC ATG AAG GCT GTG GCG   788
    Asn Asp Thr Gly Phe Val Asp Ile Pro Gln Gln Glu Lys Ala Leu MET Lys Ala Val Ala
                                                                           240
    ACT GTG GGG CCT ATT TCT GTT GCT ATG GAC GCA AGC CAT CCG TCT CTC CAG TTC TAT AGT   848
    Thr Val Gly Pro Ile Ser Val Ala MET Asp Ala Ser His Pro Ser Leu Gln Phe Tyr Ser
                                                                           260
    TCA GGC ATC TAC TAT GAA CCC AAC TGT AGC AGC AAG AAC CTC GAC CAT GGG GTT CTG TTG   908
    Ser Gly Ile Tyr Tyr Glu Pro Asn Cys Ser Ser Lys Asn Leu Asp His Gly Val Leu Leu
                                                                           280
                                                ↓
    GTG GGC TAT GGC TAT GAA GGA ACA GAT TCA AAT AAG AAT AAA TAT TGG CTT GTC AAG AAC   968
    Val Gly Tyr Gly Tyr Glu Gly Thr Asp Ser Asn Lys Asn Lys Tyr Trp Leu Val Lys Asn
                                           291                              300
    AGC TGG GGA AGT GAA TGG GGT ATG GAA GGC TAC ATC AAA ATA GCC AAA GAC CGG GAC AAC  1028
    Ser Trp Gly Ser Glu Trp Gly MET Glu Gly Tyr Ile Lys Ile Ala Lys Asp Arg Asp Asn
                                                                           320
    CAC TGT GGA CTT GCC ACC GCG GCC AGC TAT CCT GTC GTG AAT TGA TGGGTAGCGG          1083
    His Cys Gly Leu Ala Thr Ala Ala Ser Tyr Pro Val Val Asn  •
                                                    334
MEP TAATGAGGAC TTATGGACAC TATGTCCAAA GGAATTCAGC TTAAAACTGA CCAAACCCTT ATTGAGTCAA  1153

MEP ACCATGGTAC TTGAATCATT GAGGATCCAA GTCATGATTT GAATTCTGTT CCCATTTTTA CATGGGTTAA  1223

MEP ATGTTACCAC TACTTAAAAC TCCTGTTATA AACAGCTTTA TAATATTGAA AACTTAGTGC TTAATTCTGA  1293
    :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::
MCP ATGTTACCAC TACTTAAAAC TCCTGTTATA AACAGCTTTA TAATATTGAA AACTTAGTGC TTAAAAAAAA

MEP GTCTGGAATA TTTGTTTTAT ATAAAGGTTG TATAAAACTT TCTTTACCTC TTAAAAATAA ATTTTAGCTC  1363

MCP AAAAAAAAAA

MEP AGTGTGTGTG TAAAAAAAAA                                                        1383
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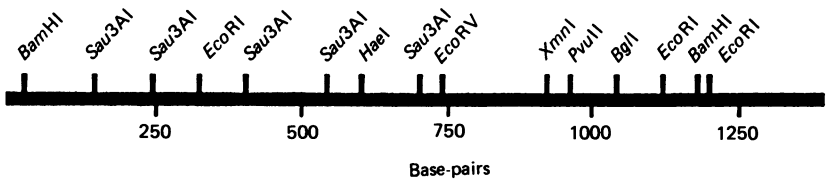

Fig. 1. Nucleotide and predicted amino acid sequence of MEP cDNA compared with that of MCP

The nucleotide sequence of MEP is almost identical with that of mouse cysteine proteinase (MCP), which is shown only in those regions lacking homology with MEP. Potential glycosylation sites are in bold type. Arrows point to putative cleavage sites. The polyadenylation signal is underlined. A restriction endonuclease map of MEP is shown at the bottom of the figure.

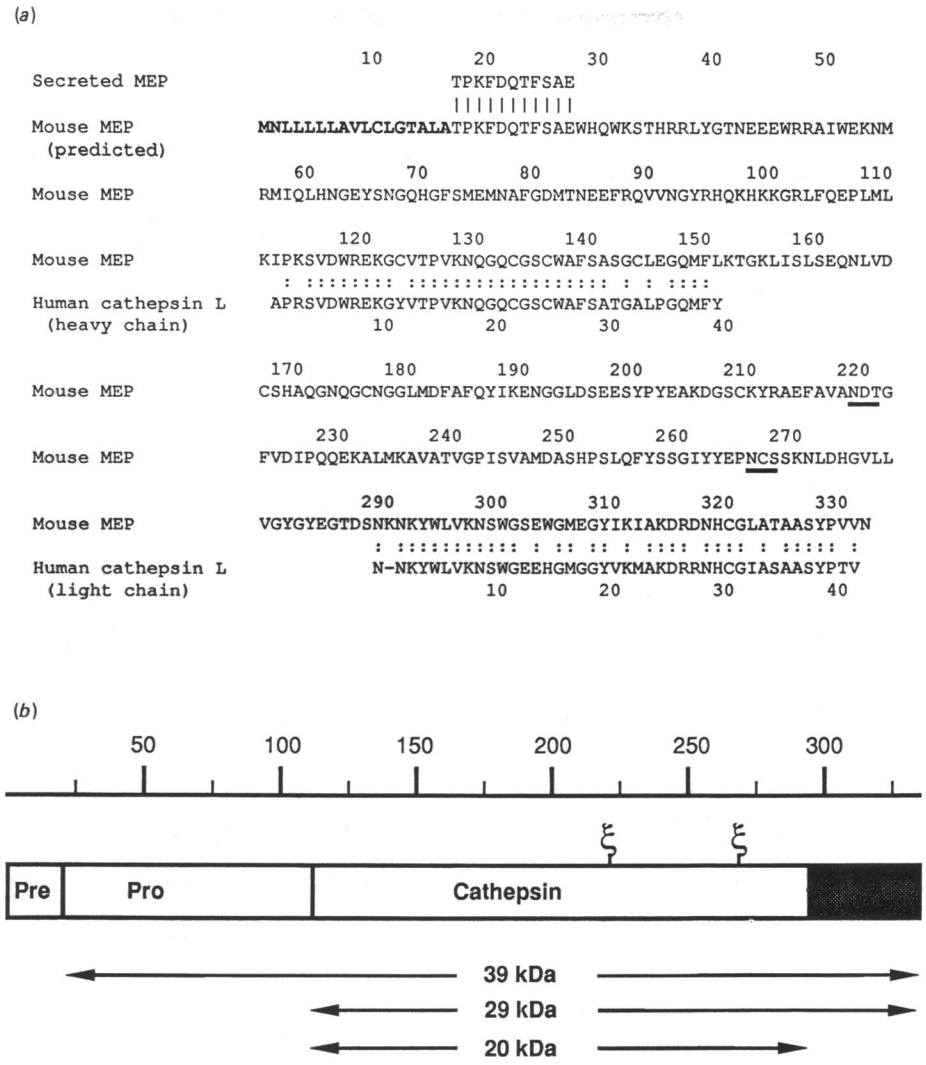

Fig. 2. Homology of MEP with cathepsin L: cleavage sites

(*a*) The derived amino acid sequence of MEP compared with the amino acid sequences of secreted MEP and human cathepsin L. The presecretory signal sequence is shown in bold type and the potential glycosylation sites are underlined. (*b*) Structure and proposed processing of MEP. ξ symbols denote glycosylation sites. The molecular masses shown are those of the forms of MEP seen on SDS/polyacrylamide gels.

Halvorson, 1983). In addition, the cleavage between alanine and threonine is preceded by Ala-Xaa-Ala, the sequence found most frequently at the cleavage sites. It is not possible to assign, with certainty, cleavage sites for the generation of the intracellular 29 and 20 kDa forms of MEP from these data, since *N*-terminal sequences of these fragments of the mouse protein are not known and the published human cathepsin L sequences are not 100% identical with the mouse sequence. However, on the basis of the general homology of MEP with cathepsin L (see Fig. 2), the putative cleavage sites would occur between residues 113 and 114 and residues 290 and 291 (Fig. 1). The fragment from residues 114 up to and including 334, with a predicted non-glycosylated molecular mass of 23.010 kDa, corresponds to the glycosylated 29 kDa form of MEP (Fig. 2*b*). The structure of the MEP cDNA and its subsequent post-translational processing are very similar to the recently cloned cDNAs of mouse and human pre-pro-cathepsin B (Chan *et al.*, 1986). Both MEP and pre-pro-cathepsin B have a presecretory signal sequence, an *N*-terminal propeptide extension, a mature chain greater than 150 residues and a *C*-terminal peptide (Fig. 2*b*).

Expression of cloned MEP

To confirm that the MEP cDNA contains a functional open reading frame for MEP, we constructed a eukaryotic expression vector containing the human metallothionein IIA promoter upstream to the *Bam*HI fragment of MEP cDNA and then transfected CV-1 cells with the recombinant plasmid pMTMEP-2. Immunoprecipitation of cell lysates using mouse MEP antisera demonstrates that the cDNA codes for the 39 kDa mouse MEP. (Fig. 3) Control CV-1 cells (transfected with calf thymus DNA or with a control vector, pMTPDE) produce a 44 kDa primate analogue of MEP (B. R. Troen, D. Ascherman, D. Atlas & M. M. Gottesman, unpublished work), but produce no detectable amounts of 39 kDa protein (lanes

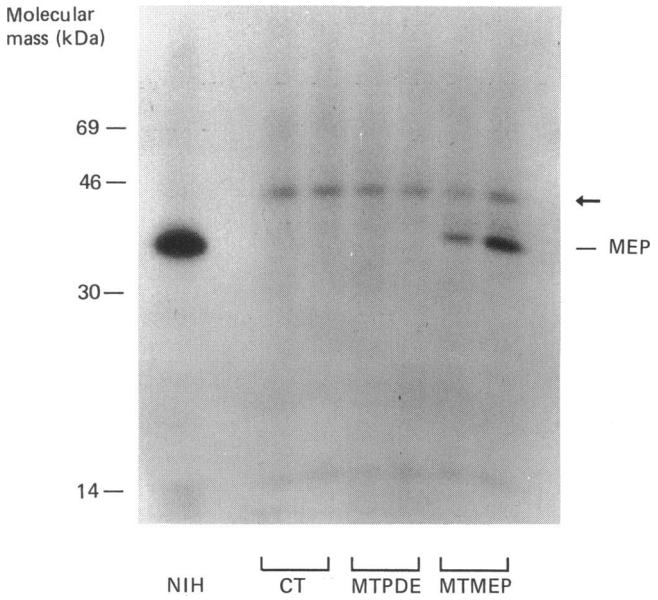

Fig. 3. Transient MEP cDNA expression in CV-1 transfectants

Duplicate flasks of 2.5×10^5 cells were transfected with either 10 μg of calf thymus (CT) DNA, pMTMEP-2 DNA or pMTPDE DNA (the metallothionein vector containing a cDNA for a phosphodiesterase gene). NIH cells (2.5×10^5) were also plated at the same time. At 48 h later, all cells were labelled with [^{35}S]methionine for 30 min and the cell lysates were subjected to immunoprecipitation, gel electrophoresis and fluorography. The arrow points to the 44 kDa primate analogue of mouse MEP.

2–5). However, CV-1 cells transfected with the MEP expression vector (pMTMEP) produce the 39 kDa MEP protein seen in NIH 3T3 cells (cf. lane 1 and lanes 6 and 7).

Conclusions

The deduced amino acid sequence for mouse MEP and enzymic data suggest that MEP is mouse cathepsin L. As judged by comparison with the sequence of MCP, MEP mRNA can have different 3′ non-coding regions depending upon its cellular origin. Finally, MEP has a signal sequence that is not detected in the mature secreted form of the protein. Expression of cloned MEP in cells not normally expressing large amounts of this protein will make it possible to study the function and secretion of this transformation regulated thiol proteinase.

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