*Proc. Natl. Acad. Sci. USA*
Vol. 85, pp. 2434–2438, April 1988
Biochemistry

# Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin $\beta_A$ chain

(THP-1 cells/λgt10/follicle-stimulating hormone/Chinese hamster ovary cells)

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*Communicated by Howard Green, November 24, 1987*

ABSTRACT **We have isolated a protein that exhibits a potent differentiation-inducing activity toward mouse Friend erythroleukemia (MEL) cells and human K-562 cells. The protein, designated erythroid differentiation factor (EDF), was found in the culture fluid of human THP-1 cells that had been treated with phorbol 12-myristate 13-acetate. EDF is a homodimer with a $M_r$ of 25,000; the $M_r$ of the monomer is 15,500. cDNA clones encoding the $M_r$ 15,500 subunit of EDF from THP-1 libraries were isolated and sequenced. Surprisingly, the sequence of EDF mRNA is identical to that for the $\beta_A$ subunit of inhibin, a gonadal protein that suppresses the secretion of pituitary follicle-stimulating hormone. Southern blot analysis indicates that only one gene for EDF/inhibin $\beta_A$ exists in the human genome. When the EDF subunit cDNA was linked to a simian virus 40 expression vector containing the dihydrofolate reductase gene and transfected into Chinese hamster ovary dihydrofolate reductase negative cells, the transformants began to secrete EDF, demonstrating that the cDNA actually encoded the EDF subunit.**

In the course of searching for antitumor compounds with fewer side effects, we discovered a protein that was excreted by several kinds of human transformed cell lines such as THP-1 (1) when stimulated by phorbol 12-myristate 13-acetate (PMA). This protein was capable of inducing Friend erythroleukemia (MEL) cells (2) and human K-562 cells (3) to differentiate into more mature erythroid cells and was designated erythroid differentiation factor (EDF) (4). EDF was purified to homogeneity, and its complete amino acid sequence was determined.

Surprisingly, the amino acid sequence of EDF was identical to that of the $\beta_A$ subunit of inhibin (5–8) and the follicle-stimulating hormone (FSH)-releasing protein (FRP) subunit (9). Inhibin has been purified from porcine follicular fluids and characterized as a heterodimeric protein consisting of $\alpha$ and $\beta_A$ polypeptides linked by disulfide bonds. FRP is a homodimeric protein consisting of two inhibin $\beta_A$ chains linked by disulfide bonds, and it stimulates the secretion of FSH by cultured anterior pituitary cells.

This paper describes the isolation of cDNAs encoding EDF and the determination of its nucleotide sequence together with the expression of EDF in Chinese hamster ovary (CHO) cells.§

## MATERIALS AND METHODS

**Bioassay of EDF.** The EDF assay was performed in a 96-well microtiter plate. Serially diluted samples were added to MEL cells at a cell density of $4.5 \times 10^4$ cells per well in 200 μl. After incubation for 5 days at 37°C, the percentage of differentiated cells was determined after staining with *o*-dianicidine. The units of activity of the sample were calculated at the highest dilution at which cells differentiated. MEL cells were grown in soft agar as described in a previous paper (10). The maximal response of the assay was about 40 colonies per well (40% colonizing efficiency).

**Construction of cDNA Libraries.** Total RNAs were isolated from THP-1 cells that were treated with PMA at 100 ng/ml for 48 hr by using the guanidinium thiocyanate/cesium chloride gradient method (11). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (12). cDNA was synthesized (13, 14) using oligo(dT) or random primer (6-mer, Pharmacia) as described by Gubler and Hoffman (15). Double-stranded DNA was treated with *Eco*RI methylase, ligated to *Eco*RI linker, digested with *Eco*RI, and size fractionated on a Sepharose 4B column (16). A 100-ng aliquot from fractions larger than 600 base pairs was ligated to 2 μg of *Eco*RI-cut dephosphorylated λgt10 DNA (17) and was packaged using Packagene (Promega Biotec, Madison, WI).

**Screening of the cDNA Library.** A single oligodeoxynucleotide (48-mer; 5′-ATTCTGGATGTCCTTCTTGATGATATTCTGGCCATCATCGTAATACAG-3′) was predicted from a 16 amino acid sequence of EDF (Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn), based on the codon-usage rules of Lathe (18). The purified oligodeoxynucleotide was 5′ end-labeled and used as a hybridization probe to screen the cDNA library. Hybridization was performed at 42°C in 0.75 M NaCl/50 mM sodium phosphate, pH 6.5/20% formamide/5× Denhardt's (1× Denhardt's = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) containing denatured calf thymus DNA at 50 μg/ml. Filters were washed twice in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% $NaDodSO_4$ at 37°C for 30 min and washed once in 0.2× SSC/0.1% $NaDodSO_4$ at 37°C for 30 min.

**DNA Sequencing.** cDNA inserts from positive phages were subcloned into the *Eco*RI site of plasmid pUC13. Nucleotide sequences of cDNA inserts were determined by the chain-termination method of Sanger in both directions (19–21).

**Blot-Hybridization Analysis.** Poly(A)$^+$ RNA was denatured for 15 min at 65°C in electrophoresis buffer (20 mM Mops/5 mM sodium acetate/1 mM EDTA, pH 7.0) containing 6.6% formaldehyde and 50% formamide and was fractionated on a 1.2% agarose/6.6% formaldehyde gel (22–23). RNA was transferred to nitrocellulose using 20× SSC, fixed by baking in a vacuum oven at 80°C for 2 hr, and hybridized to cDNA inserts labeled by nick-translation using [α-

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Abbreviations: EDF, erythroid differentiation factor; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; FSH, follicle-stimulating hormone; FRP, FSH-releasing protein; DHFR, dihydrofolate reductase.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03634).

$^{32}$P]dCTP (24). Hybridization was performed in 0.75 M NaCl/50 mM sodium phosphate, pH 6.5/50% formamide/10% dextran sulfate/1× Denhardt's containing denatured calf thymus DNA at 100 μg/ml. Filters were washed twice in 2× SSC/0.1% $NaDodSO_4$ at 42°C for 30 min and twice in 0.1× SSC/0.1% $NaDodSO_4$ at 42°C for 30 min.

**Genomic DNA Analysis.** High molecular weight genomic DNA was purified from the nuclear fraction of THP-1 cells according to the procedure of Marmur (25). The DNA was digested with restriction enzymes, fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized to cDNA inserts labeled by nick-translation. Washing conditions were the same as those used for blot-hybridization analysis.

**Transfection into CHO Dihydrofolate Reductase Negative (DHFR$^-$) Cells.** CHO DHFR$^-$ cells were plated at a density of $5 \times 10^5$ cells per 60-mm dish and were grown for 24 hr in minimal essential medium (MEM) alpha (GIBCO; no. 410-1900) containing 10% fetal calf serum. Calcium phosphate precipitates (26) were formed with 10 μg of pSD(X)/EDF (see Fig. 5) and were added to the cells. After standing 4 hr at 37°C, the monolayer of cells was incubated with 20% dimethyl sulfoxide in MEM alpha (containing 10% fetal calf serum) for 2 min, washed with phosphate-buffered saline twice, and cultured in MEM alpha (containing 10% fetal calf serum) for 48 hr at 37°C. Then transformants were selected for 2 weeks at 37°C in a selection medium that did not contain ribonucleotides and deoxynucleotides (MEM alpha; GIBCO no. 410-2000). Medium was changed every 3 days.

## RESULTS

**Biological Activity of EDF.** THP-1 cells were cultured to a cell density of $5 \times 10^5$ cells per ml; then PMA was added to a final concentration of 100 ng/ml. After 3 days, an EDF activity of 130 units/ml was detected in the conditioned medium. EDF was purified to homogeneity, and its complete amino acid sequence was determined (will be reported elsewhere). Purified EDF was used to assay its biological activities (Figs. 1 and 2). EDF induced the differentiation of MEL cells at low concentrations (minimum concentration of 0.5 ng/ml). EDF strongly suppressed the growth of MEL cells in a soft agar medium; the half maximal inhibitory dose ($ID_{50}$) was 1.5 ng/ml (60 pM). MEL cells differentiated by EDF could not grow in soft agar. The differentiation-inducing activity of EDF was extremely high, as indicated by the fact that the protein at as low a concentration as 3 ng/ml (120 pM) was able to induce differentiation (Fig. 2). It had no cytotoxic effects up to 0.8 μg/ml, which was the highest concentration studied.

**Screening and Sequencing EDF Subunit cDNA Clones.** cDNA libraries were constructed in λgt10 using PMA-treated THP-1 cell mRNA. First, one clone (CL-10) was isolated from a random-primed cDNA library by screening $5 \times 10^5$ plaques with the labeled synthetic oligodeoxynucleotide (48-mer) whose sequence was based on the amino acid sequence determined for purified EDF. CL-10 had a 600-base-pair *Eco*RI fragment, which included the nucleotide sequence of the synthetic probe. Next, three positive clones (CL-7, CL-21, and CL-65) were isolated from an oligo(dT)-primed cDNA library by screening $6 \times 10^5$ plaques using the cDNA insert of CL-10 as a probe. Restriction endonuclease mapping showed that they represent the same mRNA (Fig. 3*a*). CL-65 had the longest cDNA insert (1300 base pairs). CL-68 was further isolated from the random-primed cDNA library using an *Eco*RI/*Hin*dIII fragment of CL-65 as a probe. A complete nucleotide sequence of the EDF subunit precursor was determined by sequencing CL-7, CL-10, CL-65, and CL-68 (Fig. 3*b*). The total nucleotide sequence of the EDF subunit precursor cDNA represented by three overlapping clones is 1.9 kilobases long. A methionine codon at nucleotide 119–121 initiates a long open reading frame specifying a protein with 426 amino acids ($M_r$ = 47,442), of which the C-terminal 116 residues represent the EDF subunit proper. The EDF subunit is preceded by five consecutive arginines, at which it is presumably cleaved proteolytically from the precursor. Protein sequence analysis of EDF revealed that it did not contain any sugar chains (data not shown). The nucleotide sequence of the EDF subunit precursor mRNA agrees completely with that of human inhibin $\beta_A$ mRNA. It strongly suggests that the mRNA of the EDF subunit and the inhibin $\beta_A$ subunit is transcribed from the same gene.

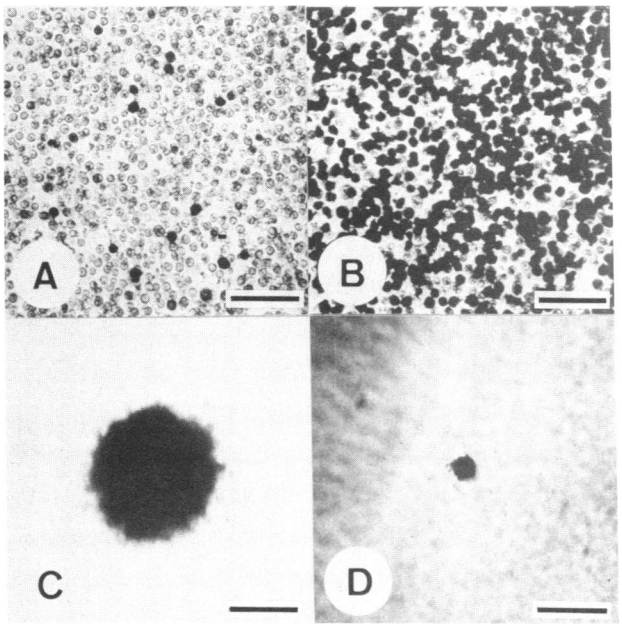


FIG. 1. Differentiation of mouse Friend (MEL) cells with EDF as seen by hemoglobin production and decreased colony formation in soft agar. The differentiation of MEL cells was assayed by dianisidine staining (*A* and *B*) and growth in a soft agar medium (*C* and *D*). (*A* and *B*) A cell suspension (0.2 ml) was mixed with 0.02 ml of a freshly prepared dianisidine solution (10:1 mixture of 2% *o*-dianisidine in 0.5 M acetic acid/30% hydrogen peroxide). Cells that stained dark brown indicate the presence of hemoglobin. (*A*) Cells not treated with EDF. (*B*) Cells treated with EDF (50 ng/ml) for 5 days. MEL cells were grown in soft agar as previously described (10) in the absence (*C*) or presence (*D*) of EDF at 50 ng/ml. The maximal response of the assay was about 40 colonies per well (40% colonizing efficiency). (Bars = 100 μm.)

**RNA and DNA Blot Analysis.** The expression of EDF subunit precursor in PMA-treated THP-1 cells was studied by RNA blot-hybridization analysis. THP-1 cells, which had

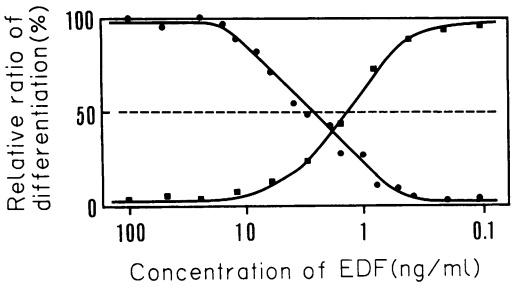


FIG. 2. Dose–response curves of differentiation as exemplified by hemoglobin production and colony-forming ability. The percentages of dianisidine positive cells (differentiated cells) (●—●) and the numbers of colonies greater than 50 μm in diameter per 100 cells inoculated (■—■) were plotted as a function of the EDF concentration. EDF had no cytotoxic effects up to 800 ng/ml, which was the highest concentration examined.

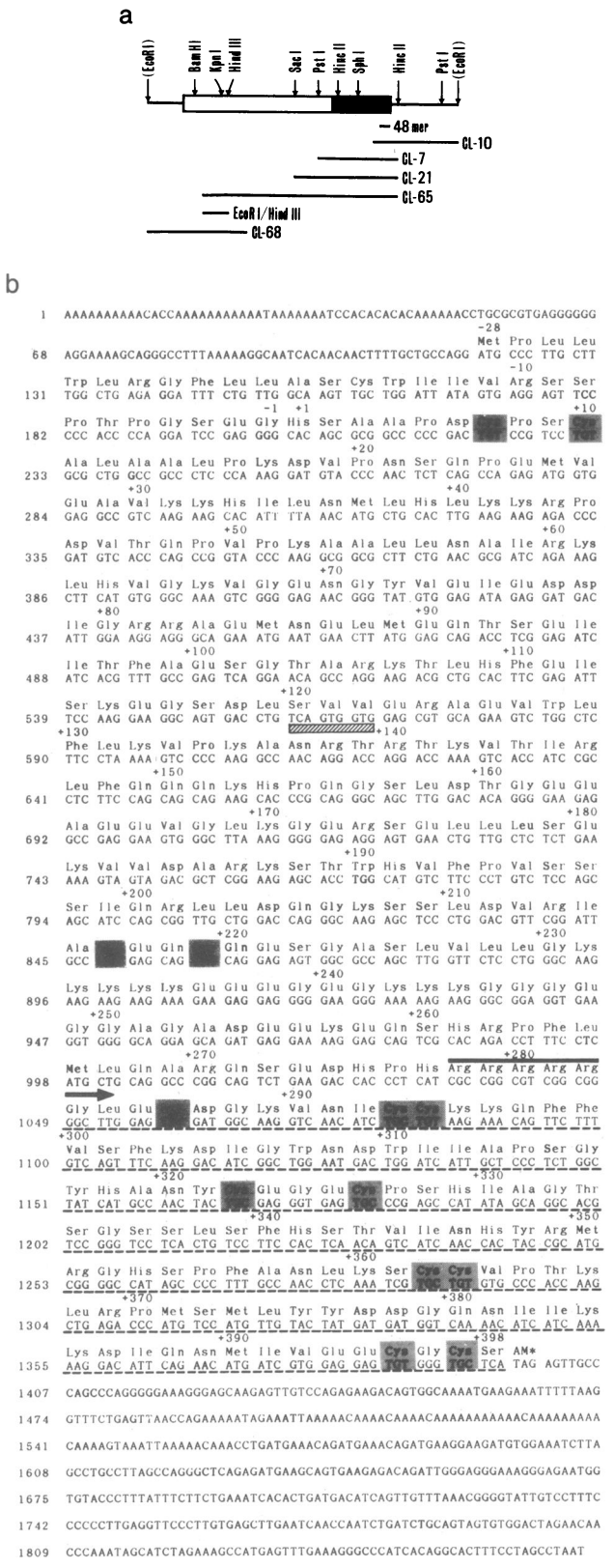


FIG. 3. (*a*) cDNA clones of EDF mRNA and the restriction map of the composite cDNA. The top line and bar represent the composite cDNA sequence predicted from the individual cDNA clones CL-7, CL-10, CL-21, CL-65, and CL-68. Restriction sites are indicated. The open box indicates the protein coding region, and EDF subunit coding region is indicated by the solid area in the box. The positions and sizes of the lines below the composite DNA restriction map indicate the relative sizes and locations of cDNA clones obtained. The *Eco*RI sites in parentheses were derived from the linkers used for cloning. The probe used for screening (synthetic 48-mer and the *Eco*RI/*Hin*dIII fragment of CL-65) are also indicated. (*b*) Nucleotide sequence and deduced amino acid sequence of EDF subunit precursor cDNA. The sequence was derived from overlapping cDNA clones (CL-7, CL-10, CL-65, and CL-68). The basic processing site and a potential glycosylation site in the precursor are indicated by black and hatched bars above the sequence, respectively. The region encoding mature EDF is underlined. Cysteine residues are shaded. The arrow indicates the N terminal of the EDF subunit.

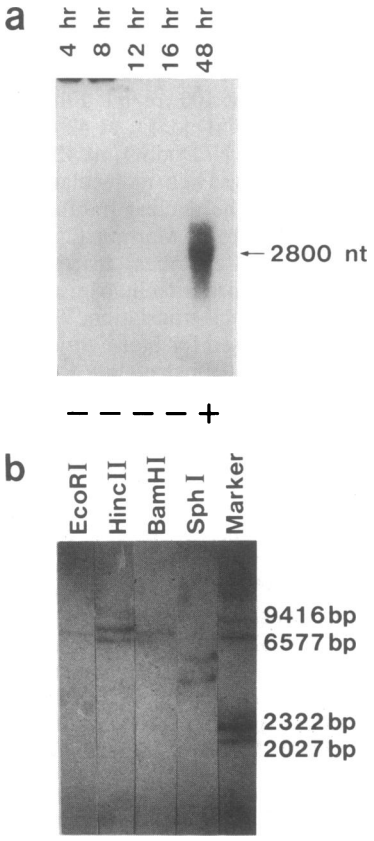


FIG. 4. (*a*) Blot-hybridization analysis of human EDF subunit mRNA. Each lane contained 10 $\mu$g of poly(A)$^+$ RNA from THP-1 cells that had been treated with PMA for various periods (4, 8, 12, 16, or 48 hr). The probe was the cDNA insert of CL-21. After removal of cells, each conditioned medium was assayed for differentiation-inducing activity. The arrow indicates the size of 2800 nucleotides (nt). (*b*) Genomic Southern blot analysis of the EDF subunit gene. High molecular weight DNA was isolated from THP-1 cells and digested with *Eco*RI, *Hin*cII, *Bam*HI, or *Sph* I. All lanes were hybridized with the cDNA insert of CL-21. bp, Base pairs.

been treated with PMA for 48 hr, expressed EDF subunit precursor mRNA of about 2.8 kilobases (Fig. 4*a*). This result was consistent with the results that the EDF activity was also detected 48 hr after PMA treatment. These results indicate that the expression of EDF is regulated, for the most part, at the transcriptional level. Southern blot analysis of the DNA from THP-1 cells is shown in Fig. 4*b*. A single hybridizing band was detected with the probe for all the DNA samples except for those digested with *Sph* I or *Hin*cII, which showed two bands. In fact, these enzymes cut the CL-21 cDNA into two fragments. These data strongly suggest that the EDF gene is present in the human genome as a single gene and establish that EDF and inhibin $\beta_A$ are encoded by the same gene.

**Expression of EDF in CHO Cells.** An expression plasmid pSD(X)/EDF was constructed (Fig. 5). This plasmid contained a *DHFR* gene and the EDF subunit cDNA. The expression of both genes was regulated under the simian

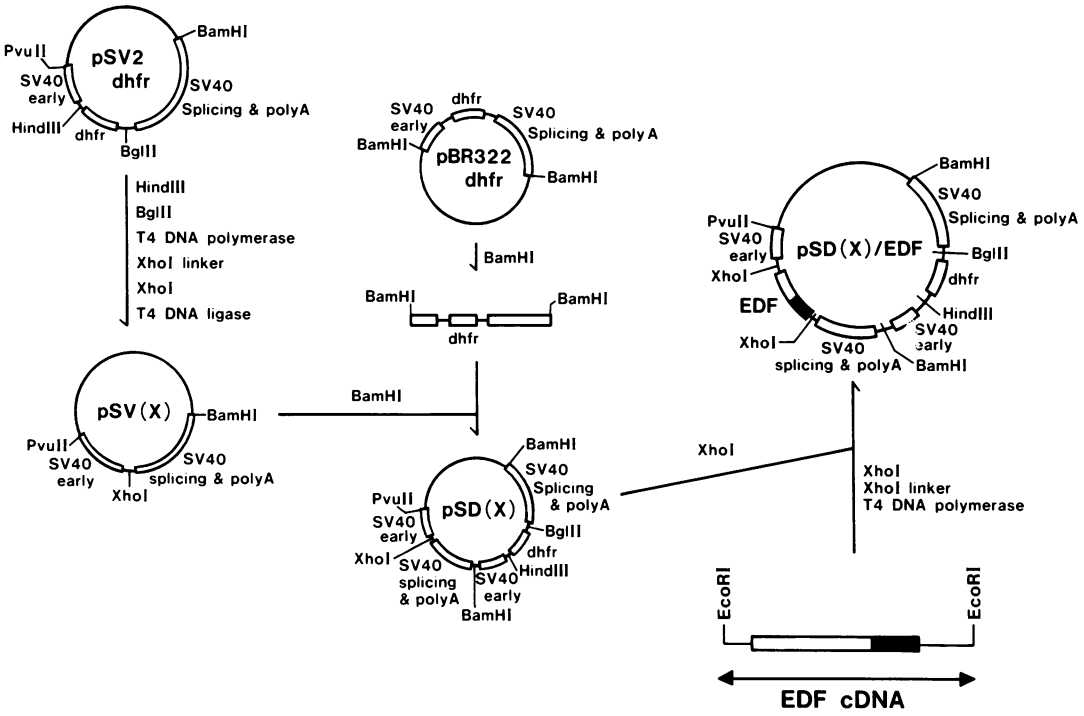


FIG. 5. Plasmid DNA constructed for the expression of EDF subunit cDNA. pSD(X) was constructed from pSV2dhfr as follows. pSV2dhfr was cleaved by *Hin*dIII and *Bgl* II, treated with T4 DNA polymerase to fill in the ends, and ligated to *Xho* I linkers (CCTCGAGG). pSV(X) had a single *Xho* I site just downstream of the simian virus 40 early promoter. The *Bam*HI fragment of pBR322-dhfr was then introduced into the *Bam*HI site of pSV(X). The resulting plasmid was named pSD(X). The *Eco*RI fragment was treated with T4 DNA polymerase, ligated to *Xho* I linkers, and then cleaved by *Xho* I. The resulting fragment was introduced into the *Xho* I site of pSD(X) in the normal orientation. Thus, pSD(X)/EDF is an expression vector for the EDF subunit cDNA under control of the simian virus 40 early promoter.

virus 40 early promoter and was expected to be constitutive in CHO cells.

pSD(X)/EDF was introduced into CHO DHFR$^-$ cells by a calcium phosphate method, and transformants (DHFR$^+$ cells) were isolated after growing in a selection medium. Then, the conditioned medium was assayed for EDF activity (Table 1).

EDF activity (30 ng/ml) was detected only in the conditioned medium of transformants that had been transfected with pSD(X)/EDF. In the case of pSD(X) transfection or mock transfection, EDF activity was not detected. The results shown in Table 1 indicate that the cDNA clone contains a necessary sequence for the expression of EDF activity in mammalian cells.

## DISCUSSION

The sequence analysis of human EDF cDNA described here has revealed that the mRNA of the EDF subunit is identical with that of the inhibin $\beta_A$ subunit (5–8) and the FRP subunit from human ovary (9), and its amino acid sequence is also the same among porcine (7), bovine (27), and rat (28) inhibin $\beta_A$. The high degree of conservation of EDF (FRP) peptide among different species indicates the strong selective pressure imposed upon this particular gene and suggests the physiological importance of this peptide, which has multiple functions.

Table 1. Expression of EDF in CHO DHFR$^-$ cells

| Cells | Transfected DNA | EDF activity, ng/ml |
|---|---|---|
| CHO DHFR$^-$ | None | <1 |
| | pSD(X) | <1 |
| | pSD(X)/EDF | 30 |
| PMA-treated THP-1 | None | 130 |

Cells were grown in selection medium for 3 days, and then the conditioned medium was assayed for EDF activity using the dianisidine staining method. The conditioned medium of THP-1 cells treated with PMA (100 ng/ml) for 3 days was assayed as a positive control. The limit of EDF detection is 1 ng/ml.

Recent studies showed that EDF stimulated the secretion of FSH by cultured anterior pituitary cells (29). No apparent similarity in pathways can be formulated between the two biological functions of EDF and FRP, one erythrocyte maturation and the other stimulation of excretion of a peptide hormone. However, many lymphokines, including the interleukins, for example, are known to work on different target cells in a considerably different manner. It may not be surprising, then, if this peptide exhibits diverse functions depending upon the situations and types of cells involved. Thus, EDF (FRP) may be used to elicit more different biological reactions than those already described. EDF has substantial homology (≈35%) with type $\beta$ transforming growth factor (TGF-$\beta$), suggesting that these genes are derived from a common ancestral gene. Indeed, the effect of EDF on the growth of a number of cultured tumor cell lines was similar, though not always the same, to that of TGF-$\beta$ (unpublished observations). It is tempting to speculate that this peptide is a member of a family of genes that regulate growth and differentiation of a variety of cells.

Differentiation of MEL cells by EDF was induced at a concentration as low as 120 pM, suggesting a receptor-mediated nature of EDF. An analogous human cell line, K-562, with a similar property to differentiate into more mature erythroid cells, required 7 times more EDF to elicit the same extent of differentiation (data not shown). The reason for this different sensitivity is not known at present.

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