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Repression of involucrin gene expression by transcriptional enhancer factor 1 (TEF-1)

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Abstract Involucrin is one of the precursor proteins of keratinocyte cornified envelope that is formed beneath the inner surface of the cell membrane during terminal differentiation. Although involucrin is specifically expressed in the upper squamous cells of the epidermis, the precise regulatory mechanism of involucrin gene expression remains unknown. Transcriptional enhancer factor 1 (TEF-1), which binds to SV40 enhancer, is a nuclear protein expressed in various types of cells including keratinocytes. Immunohistochemical study has revealed that TEF-1 protein is highly expressed on the basal cell layer of the epidermis. To examine the possible regulatory mechanism of involucrin gene expression by TEF-1 protein, we analysed involucrin promoter activity of the INV-CAT vector, which was constructed by connecting the 5′ upstream region of the involucrin gene (–801 bp upstream from the transcription start site and downstream including the untranslated first exon) to the chloramphenicol acetyltransferase (CAT) reporter gene. The INV-CAT vector was transfected to SV40-transformed human keratinocytes (SVHK). Cotransfection of the TEF-1 expression vector significantly repressed INV-CAT promoter activity in a dose-dependent manner. The repression was also observed by transfection of the GAL4-TEF-1 vector, which was constructed by replacement of the TEF-1 DNA binding domain by the GAL4 activator domain. This suggests that TEF-1-induced repression is due to interference/squelching of a limiting transcriptional intermediary factor that is essential for involucrin expression. Analysis of the deleted INV-CAT vector suggested that the region from –599 to –495 of the involucrin gene, which contains two possible TEF-1 binding sites, was critical for the repression of the involucrin gene by TEF-1. By gel retardation analysis, the specific DNA binding of SVHK cell nuclear extracts and the recombinant TEF-1 protein was confirmed. TEF-1-dependent repression of involucrin gene expression might explain the suprabasal involucrin expression in the epidermis.

Key words Involucrin · TEF-1 · Limiting transcriptional intermediary factor

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Introduction

The cornified envelope (CE) is a highly insoluble structure formed beneath the plasma membrane of keratinocytes during terminal differentiation [1–4]. One of the precursor proteins of CE, involucrin, is specifically expressed in the squamous tissues, and this protein is detected in the upper stratum spinosum and the stratum granulosum of normal epidermis.

The human involucrin gene has been cloned by Eckert and Green [5]. It is composed of two exons separated by an intron. The first 5′ exon is only 43 bp long and is not translated. The coding region in the 3′ exon contains a central portion, which is composed of 39 repeats of a homologous ten amino acid sequence. On average, each repeat contains three glutamine residues, each of which is a potential amine acceptor for transglutaminase-catalysed formation of crosslinks that stabilize the cornified envelope.

Transcriptional regulation of proliferation and differentiation is modulated by a complex between cell-specific nuclear protein(s) and the transcriptional regulatory sequence(s). Although cell-specific regulatory factors have been found in various target tissues, such as MyoD in striated muscle, or GHT/Pit-1 in the anterior pituitary [6-8], information regarding keratinocyte-specific regulatory factors is scarce [9]. Recently we reported that the human involucrin gene promoter that contains three possible TPA responsive elements is regulated by protein kinase C [10]. Despite the keratinocyte-specific expression of involu-

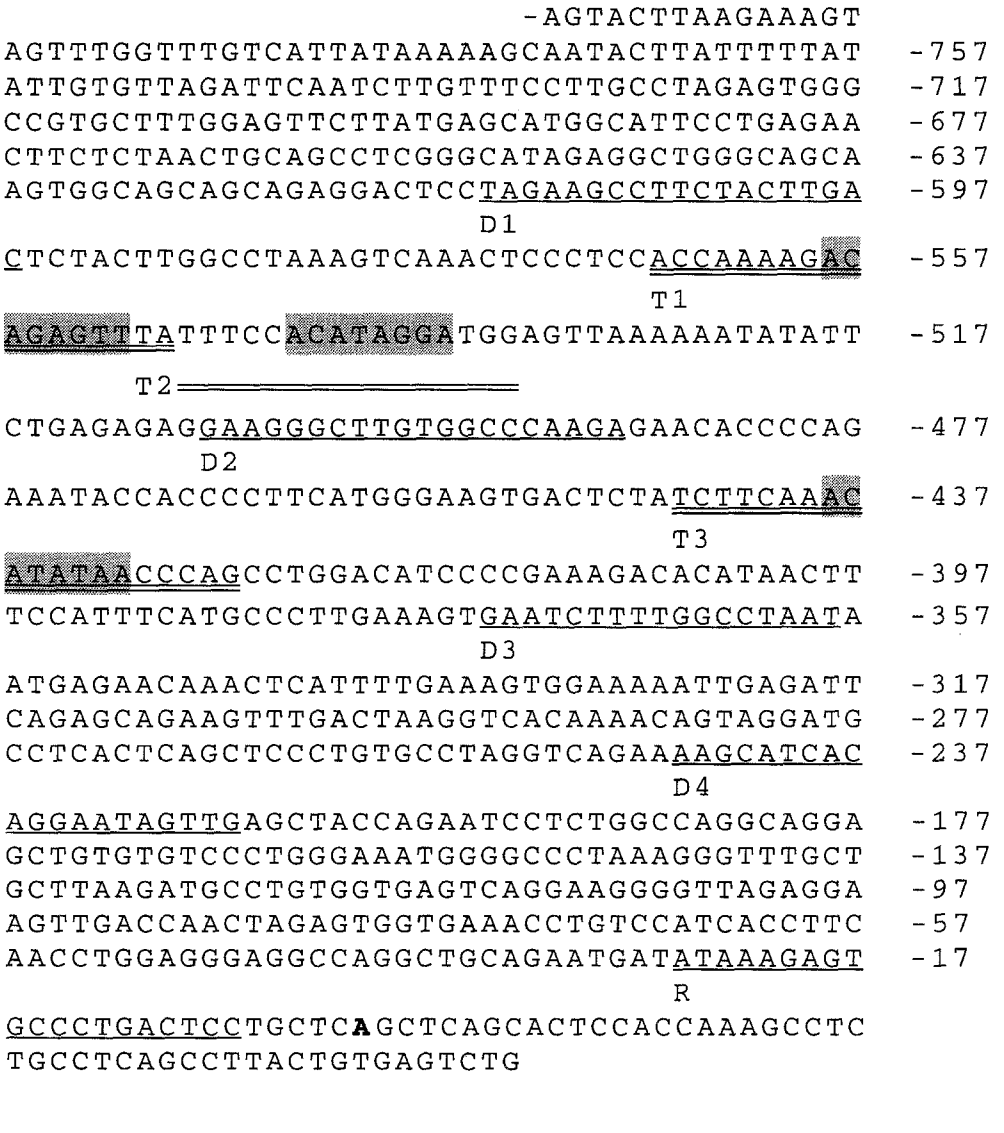

Fig. 1 Sequence of the 5′-upstream region of the human involucrin gene. *D1, D2, D3, D4* and *R* indicate the position of the primers. *T1, T2* and *T3* indicate the position of oligonucleotides for the gel shift analyses. Bold letter, **A**, indicates the transcriptional start site. The three hatched boxes indicate putative TEF-1 binding sites

crin, the tissue-specific *cis* element on the involucrin gene and its DNA binding factor have not been identified.

Transcriptional enhancer factor (TEF-1), which was originally purified from a HeLa cell nuclear extract, specifically binds the GT-IIC and Sph regions of the simian 40 (SV40) enhancer [11]. TEF-1 protein has a relative molecular mass of about 53 kDa and is expressed in keratinocytes, fibroblasts, hepatocytes, and kidney cells, but not in lymphocytes [12, 13]. Transcriptional activation by TEF-1 requires not only the TEF-1 protein itself, but also a cell-specific coactivator protein, a limiting transcriptional intermediary factor, which is required for activated but not for basal transcription [14]. Therefore, overexpression of TEF-1, by squelching the cofactor inhibits TEF-1-dependent transcription.

In the present study, we subcloned various sized 5′ regions upstream of the human involucrin gene that was connected to the chloramphenicol acetyltransferase (CAT) gene. Using these expression vectors, we investigated the regulation of the involucrin promoter activity by TEF-1 protein and analysed the interaction between this protein and the involucrin promoter DNA sequence.

Materials and methods

Plasmid constructs

The ScaI-digested fragment from the genomic involucrin gene was inserted into the XbaI site of the promoterless 0-CAT plasmid, that had been blunt-ended by a Klenow fragment (INV-CAT) [10]. The pXJ40-TEF-1A, -GAL-TEF-1 and -GAL expression vectors were generous gifts from Dr. P. Chambon (Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, France) [12]. β-Galactosidase expression vector was kindly supplied by Dr. T. Watanabe (Medical Institute of Bioregulation, Kyushu University, Japan). Each deleted fragment was generated by polymerase chain reaction (PCR) using oligonucleotides (D1, 5′-TAGAAGCTTTCTACTTGAC-3′; D2, 5′-GAAAAGCTTGTGGCCCAAGA-3′; D3, 5′-GAAGCTTTTGGCCTAATAAT-3′; D4, 5′-AAGCTTCACAGGAATAGTTG-3′; R, 5′-GGAGTCAGGGCACTCTTTAT-3′). The positions of these sequences are indicated in Fig. 1. Each PCR product, which contained a *Hin*dIII and *Pst*I site, was digested by *Hin*dIII and *Pst*I restriction enzymes and were subcloned into the *Hin*dIII/*Pst*I sites of pGEM3Zf(+) and was confirmed by sequence analysis. The INV-CAT vector contained polylinker sites, which included the *Hin*dIII site upstream of the involucrin gene. The INV-CAT vector was digested by *Hin*dIII and *Pst*I restriction enzymes and the deleted INV-CAT vector was ligated to PCR products.

Cell culture

SV40-transformed human keratinocytes (SVHK) [14] were a generous gift from Dr. M. L. Steinberg (Department of Chemistry, City College of the City University of New York). Cos7 and HeLa cells were obtained from the Japanese Cancer Research Bank (JCRB). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37° C in air containing 5% CO_2.

Immunohistochemical studies

Immunohistochemistory was performed on 5-μm thick frozen normal skin sections. Following incubation with normal goat serum for 30 min, the sections were incubated with anti-TEF-1(P2) antibody for 1 h. The sections were then treated with biotinylated goat antirabbit immunoglobulin for 10 min, and then with streptavidin-peroxidase complex (Histofine SAB-PO(M), Nichirei, Japan) for 10 min. The peroxidase reaction was developed using 3-amino-9-ethylcarbazole (AEC) and H_2O_2 as substrates. The sections were counterstained with Mayer's haematoxylin and mounted in Enteran. As the control, normal rabbit IgG was used instead of the primary antibody. P2 antibody was kindly provided by Dr. P. Chambon (Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, France) [12].

Transfection and CAT assay

Transfection of plasmid DNA into these cells was performed using the liposome method with lipofectin [16]. Typically, 5 μg reporter plasmid and 2 μg β-galactosidase plasmid were transfected into 10^5 cells. The β-galactosidase plasmid was used as the internal standard to normalize each transfection efficacy. After 48 h, the cells were collected and the CAT assay was performed according to the method of Neuman et al. [17]. The enzyme activity of β-galactosidase in the transfected cell extracts was measured spectrophotometrically according to the method described by Glover [18].

Nuclear extract preparation

Cells (5×10^6) were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 400 μl buffer A (10 m*M* HEPES, pH 7.8, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, and 1 m*M* phenylmethylsulphonyl fluoride). After 15-min, Nonidet P-40 was added to a final concentration of 0.6%. Nuclei were pelleted and resuspended in 50 μl buffer C (20 m*M* HEPES pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA and 1 m*M* EGTA). After 30 min at 4° C, the lysates were centrifuged and stored at –70° C.

In vitro transcription and translation

PXJ40-TEF-1A plasmid was linearized with the restriction enzyme, *Bgl*II. The 1 μg linear plasmid was transcribed with T7 RNA polymerase and the 1 μg RNA product was translated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The translation products were subjected to electrophoresis on a 12.5% SDS-PAGE gel, dried, and autoradiographed overnight.

Western blotting

Nuclear extracts were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto nitrocellulose for 1 h in a buffer containing 25 m*M* Tris-HCl, pH 8.3, 192 m*M* glycine and 20% methanol. The blots were blocked with 5% nonfat milk in PBS for 1 h at room temperature, and then incubated at 37° C for 1 h with P2 antibody that had been diluted 500-fold in Tris-buffered saline (TBS, pH 7.6). After washing at room temperature with 0.1% Tween-20 in TBS, a blotting detection kit for rabbit antibody (Amersham International, UK) was used for immunodetection.

Gel retardation analyses

Gel retardation analysis was performed essentially according to the method described by Davidson et al. [11]. Briefly, binding was performed in a 30 μl reaction buffer with 2 μl in vitro translation mixture or 4 μl nuclear extract and 10–20 fmol ^{32}P-labelled oligonucleotide probe ($2–4 \times 10^4$ cpm); the position and orientation of the probe are indicated in Fig. 1. The incubation was performed for 30 min at 20° C. DNA-protein complexes were then separated on 4% nondenaturing polyacrylamide gel and autoradiographed overnight.

Materials

Dulbecco's modified Eagle's medium were purchased from Gibco (Grand Island, N.Y., USA). Penicillin and streptomycin were obtained from M. A. Bioproducts (Walkersville, Md., USA) [γ-^{32}P]ATP, [^{3}H]acetyl coenzyme A and [^{35}S]methionine were purchased from Amersham (Tokyo, Japan). Lipofectin was obtained from BRM (Bethesda, Md., USA). T7 RNA polymerase and the in vitro reticulocyte lysate system were purchased from Promega (Madison, Wis., USA).

Results

Immunohistochemistry revealed a significant expression of TEF-1 protein in the cytoplasm of basal cells (Fig. 2). Western blot analyses showed that a 53-kDa and a 51-kDa TEF-1 protein, which were derived from two transcriptional starting points [12], were expressed in the SVHK cells (Fig. 3, lane 3). Cos7 cells derived from monkey kidney cells, showed a single band with a molecular mass slightly different from SVHK cells (Fig. 3, lane 2). The TEF-1 protein was not detected in Ky cells, a human B lymphoid cell line (Fig. 3, lane 1).

To examine the effects of TEF-1 protein on the involucrin promoter, we cotransfected the INV-CAT vector with TEF-1A vector into SVHK. The TEF-1A expression vec-

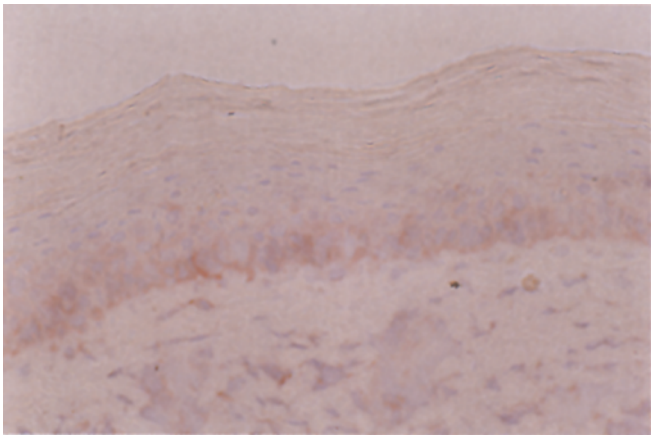

Fig. 2 TEF-1 protein expression of normal epidermis. Basal cells are stained by anti-TEF-1 antibody (× 200)

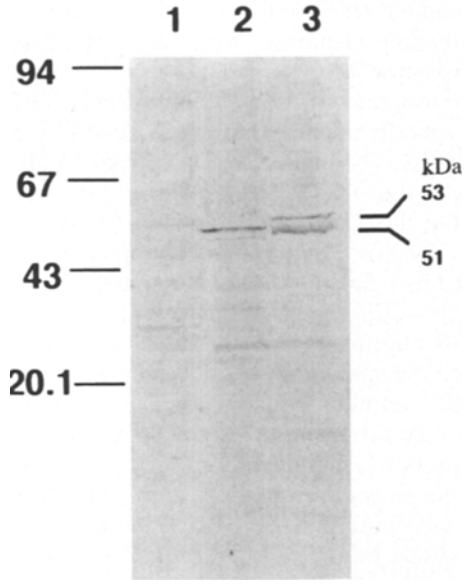

Fig. 3 Western blot analysis of endogenous TEF-1 protein from various cells. *1* Ky cells (human B cell line), *2* Cos7 cells (monkey kidney cell line), *3* SVHK cells. SVHK cells show 53-kDa and 51-kDa bands. Cos7 cells show a single band at the position slightly different from the 51-kDa band of the SVHK cells

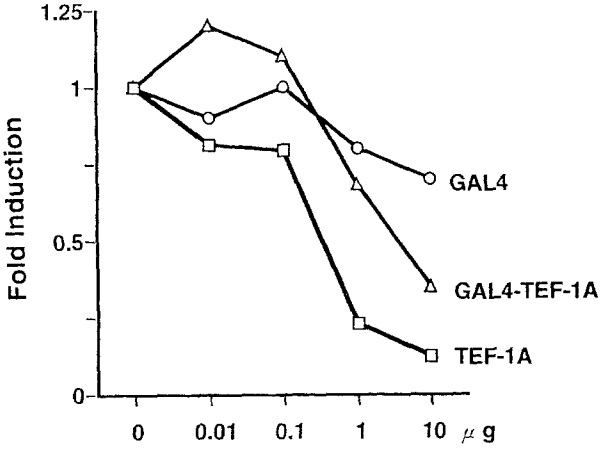

Fig. 4 Effect of TEF-1A, GAL4-TEF-1A, and GAL4 vectors on INV-CAT-transfected SVHK. SVHK were transfected with INV-CAT (5 μg/10^5 cells) and various concentrations of TEF-1A, GAL4-TEF-1A and GAL4 vectors. The relative CAT activities are shown. The activity of cells transfected with INV-CAT alone was designated as 1.0. The data shown are representative of three independent experimental series with duplicated assays

tor significantly suppressed the promoter activity in a dose-dependent manner (Fig. 4). Although the GAL4 vector had no effect, the GAL4-TEF-1A chimeric vector, that was constructed by replacement of the TEF-1A DNA binding domain by the GAL4 activator domain, inhibited the promoter activity (Fig. 4).

Sequence analysis of the involucrin promoter region of the INV-CAT vector detected three possible TEF-1 binding sites (5′-ACAGAGTT-3′, 5′-ACATAGGA-3′ and 5′-

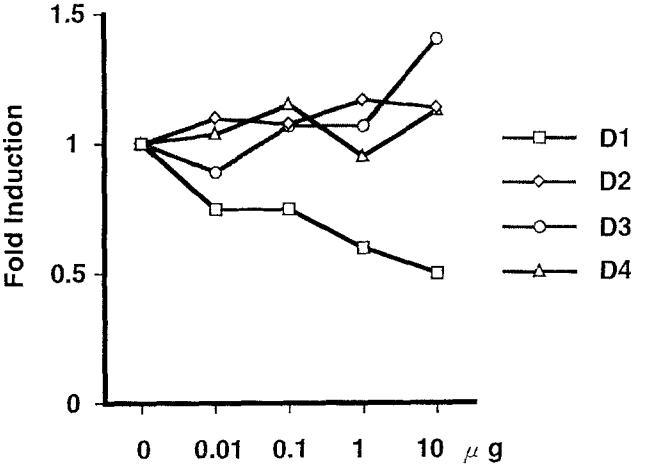

Fig. 5 Effect of TEF-1A vector on D1-, D2-, D3- and D4-INV-CAT-transfected SVHK. Each deleted INV-CAT vector was transfected with various concentrations of TEF-1A vector and incubated for 24 h. The relative CAT activities of the transfected cells are shown. The activity of cells transfected with INV-CAT alone was designated as 1.0. The data shown are representative of three independent experimental series with duplicated assays

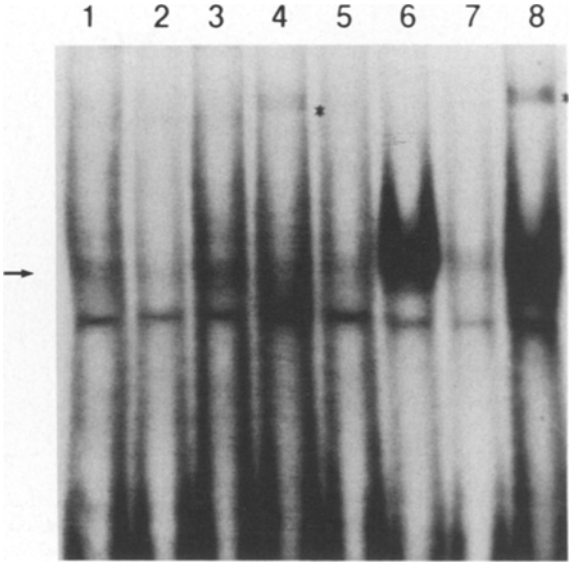

Fig. 6 Gel shift analysis with nuclear extracts from SVHK cells and HeLa cells. The nuclear extracts (5 μl) were reacted with T1 oligomer (see Fig. 1). The DNA-protein complex was separated on 4% polyacylamide gel under nondenaturing conditions. The *arrow* indicates the specific TEF-1 protein and nuclear DNA complex. The *asterisk* indicates the supershift band, which indicates specific binding of T1 with TEF-1 and anti-TEF-1 antibody. *Lane 1* nuclear extract from SVHK cells, *lane 2* nuclear extract from SVHK cells plus 50X unlabelled probe, *lane 3* nuclear extract from SVHK cells plus unrelated DNA oligomer, *lane 4* nuclear extract from SVHK cells plus 1 μl anti-TEF-1 antibody, *lane 5* nuclear extract from SVHK cells plus anti-c-Jun antibody, *lane 6* nuclear extract from HeLa cells, *lane 7* nuclear extract from HeLa cells plus 50X unlabeled probe, *lane 8* nuclear extract from HeLa cells plus 1 μl anti-TEF-1 antibody

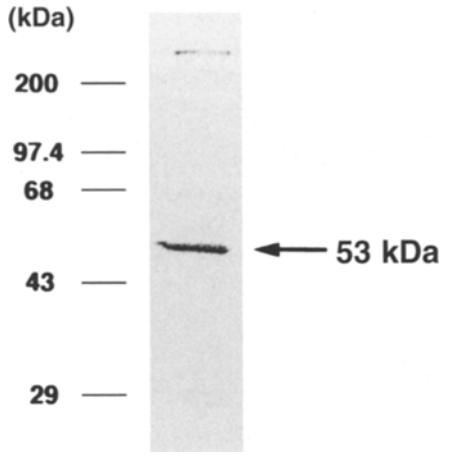

Fig. 7 In vitro transcription-translation of TEF-1A. TEF-1A RNA was synthesized by using T7 RNA polymerase. The RNA was translated using a rabbit reticulocyte lysate system and 5 µl of the translation product was analysed on a denatured 12.5% SDS polyacrylamide gel. The faint line at the top is the gel front

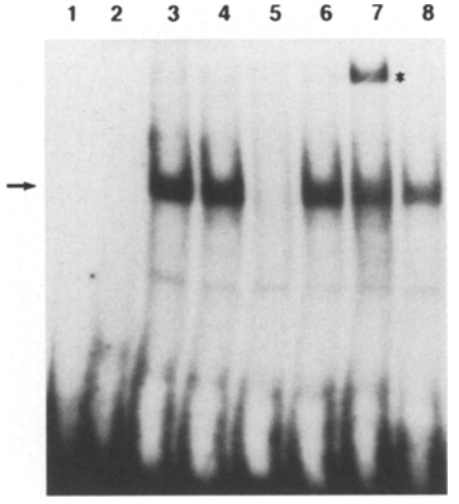

Fig. 8 Gel shift analysis with the in vitro transcription-translation product of TEF-1A. The 2 µl translation product was reacted with T1 oligomer. The DNA-protein complex was separated on 4% polyacrylamide gel under nondenaturing conditions. The *arrow* indicates the specific complex of TEF-1 protein and nuclear DNA. The *asterisk* indicates the supershift band, which indicates the specific binding of T1 DNA oligomer and TEF-1 protein and anti-TEF-1 antibody. *Lane 1* no extract, *lane 2* reticulocyte lysate with no TEF-1 RNA, *lane 3* translation product, *lane 4* translation product plus 5X unlabelled probe, *lane 5* translation product plus 50X unlabelled probe, *lane 6* translation product plus unrelated DNA oligomer, *lane 7* translation product plus 1 µl anti-TEF-1 antibody, *lane 7* translation product plus anti-c-Jun antibody

ACATATAA-3′; Fig. 1). To determine the precise TEF-1 responsive region, we constructed four deleted INV-CAT vectors (D1-, D2-, D3 and D4-INV-CAT vectors) and analysed the effect of the TEF-1A vector on these deleted INV-CAT vectors. Although the inhibitory effect of TEF-1A on the involucrin promoter was observed by cotransfection of the D1-INV-CAT vector which conserved three TEF-1 binding sites, only 50% inhibition was detected. The D2-INV-CAT vector, which contained only one TEF-1 binding site, showed no inhibitory effect of the TEF-1A vector, and the D3- and D4-INV-CAT vectors, that had no TEF-1 binding sequence, were also not affected by the TEF-1A vector (Fig. 5).

We investigated the interaction between TEF-1 protein and the specific nuclear DNA sequence(s) by gel retardation analysis. The nuclear extracts from SVHK cells and HeLa cells specifically bound ^{32}P-labelled oligonucleotide containing the TEF-1 binding site (T1) (Fig. 6, lanes 1 and 6). Specific DNA-protein complex was efficiently competed by a 50-fold excess of unlabelled oligomer containing the TEF-1 binding site (Fig. 6, lanes 2 and 7). Anti-TEF-1 antibody added together with nuclear extracts decreased the specific band, and a higher molecular mass super-shift complex appeared near the top of the lane (Fig. 6, lanes 4 and 8). Similar results were observed with T2 and T3 probes (data not shown).

In order to confirm that this nuclear factor was TEF-1 protein, we synthesized TEF-1 protein in an in vitro reticulocyte lysate translation system. A 53-kDa recombinant protein was synthesized that was consistent with the expected molecular mass of TEF-1 protein [12] (Fig. 7). Immunoblot analysis showed that this product specifically reacted with anti-TEF-1 antibody (data not shown). The recombinant TEF-1 protein also formed a specific DNA-protein complex (Fig. 8, lane 3) and the addition of anti-TEF-1 antibody decreased this DNA-protein complex and the super-shift band was also detected (Fig. 8, lane 7).

Discussion

Our results indicate that the human involucrin promoter is negatively regulated by TEF-1. Overexpression of TEF-1 as well as GAL4-TEF-1A inhibited INV-CAT promoter activity, suggesting that the effect of TEF-1 and GAL4-TEF-1 is due to interference/squelching of a limiting transcriptional intermediary factor [14]. The question of whether the effect of TEF-1 is a direct effect on the involucrin gene itself or an indirect effect with one or several steps in between remains to be determined: mutation analyses of putative TEF-1 binding site(s) would be required to resolve this question.

Deletion analysis of the INV-CAT vectors, however, suggested that two sequences of TEF-1 binding motifs [5′-AAGTATGCA-3′] on –559 bp to –536 bp are significantly involved in the TEF-1-induced repression. The inhibitory effect on the D1-INV-CAT vector, however, was significantly less than that on the whole INV-CAT vector. Since the gel retardation analysis revealed that TEF-1 protein specifically bound not only two TEF-1 binding motifs in the position –[–559–536], but also one TEF-1 binding motif in the position –[–438–431], the latter TEF-1 binding motif might also be required for the complete expression of TEF-1 function, although the TEF-1A vector showed no inhibitory effect on D2-INV-CAT promoter activity. TEF-1 protein synthesized in an in vitro reticulocyte lysate translation system showed a similar gel shift

pattern to that induced by SVHK nuclear extracts, indicating that TEF-1 is actually associated with the specific DNA sequences of the involucrin promoter.

Previous studies have shown that the transfection of the TEF-1 expression vector completely suppresses the activity of the SV40 enhancer and the HPV 16 P97 promoter [11, 12]. The amount of TEF-1 expression vector required for the suppression in those experiments was only about one-tenth of that required in our system. This might reflect the different efficacy of transfection or differences in host cell endogenous TEF-1 and/or transcriptional intermediary factor content. Affinity between TEF-1 protein and the specific DNA sequence(s) might also be a factor. Davidson et al. have reported that the binding affinity of TEF-1 protein for the GT-IIC motif is about four times higher than for the Sph motifs [10].

As well as in keratinocytes, the TEF-1 protein is expressed in hepatocytes, fibroblasts and kidney cells, but not in lymphocytes. This was confirmed by Western blot analysis (Fig. 3). Thus TEF-1 is by no means specific to keratinocytes. Transfection of the INV-CAT vector to these cells (kidney cells, fibroblasts, hepatocytes) but not to lymphoid cells revealed a similar activation of the involucrin promoter, suggesting that the INV-CAT promoter is driven by nuclear factor(s) of various cells sharing TEF-1.

Despite the keratinocyte-specific expression of involucrin, the strict cell-specific *cis* element has not been identified up to about 800 bp upstream of the involucrin gene. It may reside in the more expanded upstream region of the involucrin gene [9, 19, 20]. Although the octamer motif [5′-AANCCAAA-3′] that has been reported in type II keratin [21], and recently in a 230-kDa bullous pemphigoid antigen [22], is detected in the involucrin promoter at the position –[–575–582] [5′-AAGTCAAA-3′] (Fig. 1), keratinocyte-specific expression was not detected in our system (data not shown).

Keratinocyte transcription factor 1 (KTF-1), which interacts with the 5′CCCTGAGG-3′ sequence on the *Xenopus* epidermal keratin gene, is a relatively specific *trans*-activating factor, that has been shown to be identical or closely related to AP-2 [23–26]. KTF-1/AP-2 shows a similar tissue distribution to TEF-1. These proteins are highly expressed in keratinocytes, fibroblasts and kidney cells, but not in lymphocytes [11, 12, 23, 24]. It is interesting to note that a possible KTF-1/AP-2 binding site was found on the involucrin gene in the position –[–662–654] (Fig. 1). Whether AP-2 or its related protein actually binds to the region and regulates involucrin gene expression is under investigation.

Our results indicate that TEF-1 that is highly expressed in the basal cell layer of the epidermis shows an inhibitory effect on involucrin expression by keratinocytes. Because TEF-1 is apparently repressed in suprabasal keratinocytes, TEF-1-dependent regulation might have a physiological relevance to keratinocyte differentiation. Involucrin expression might be operative only after keratinocytes leave the TEF-1-rich basal cell layers. Although this is consistent with the suprabasal expression of involucrin in the epidermis, immunohistochemical study indicates that involucrin expression is delayed until the upper spinous or granular cell layers [27]. Thus the regulatory mechanism of involucrin gene expression appears to be under multifactorial control, that includes TEF-1, AP-1, calcium, etc. [4, 10, 28].

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