Molecular Cloning, Occurrence, and Expression of a Novel Partially Secreted Protein "Psoriasin" That Is Highly Up-Regulated in Psoriatic Skin

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Analysis of the protein patterns of normal and psoriatic non-cultured unfractionated keratinocytes has revealed several low-molecular-weight proteins that are highly up-regulated in psoriatic epidermis. Here, we have cloned and sequenced the cDNA (clone 1085) for one of these proteins that we have termed psoriasin. The deduced sequence predicted a protein of molecular weight of 11,457 daltons and a pI of 6.77. The protein co-migrated with psoriasin as determined by two-dimensional (2D) gel analysis of [^{35}S]-methionine-labeled proteins expressed by RK13 cells transfected with clone 1085 using the vaccinia virus expression system. Analysis of the predicted sequence revealed a potential calcium-binding sequence of the EF-hand type, as well as the absence of a signal sequence at its amino terminal. Psoriasin is not related to other proteins that migrate closely in 2D gels (MRP 14, also known as calgranulin B, L1 and calprotectin; MRP 8, or calgranulin A and cystatin A or stefin A), and bears no significant sequence homology with any other protein of known primary structure. Increased expression of psoriasin mRNA in psoriatic keratinocytes was confirmed by Northern blotting and in situ hybridization.

Psoriasin showed a restricted occurrence in fetal human tissues as determined by 2D gel electrophoresis. Of 21 tissues analyzed, only ear, skin, and tongue showed significant levels of this protein. Psoriasin was not detected in normal human fibroblasts, lymphocytes, endothelial cells and transformed epithelial cells of keratinocyte origin. Granulocyte extracts contained this protein suggesting that its overexpression by psoriatic keratinocytes may be linked to the inflammatory stimuli. *J Invest Dermatol 97:701–712, 1991*

Psoriasis is a chronic skin disease of widespread occurrence characterized by epidermal hyperplasia, vascular alterations and inflammation with infiltration of polymorphonuclear leukocytes, activated T cells, Langerhans cells, and macrophages [1–3]. At present, we know relatively little about the molecular mechanisms underlying this disease and, in general, most studies have been directed towards the involvement of cytokines (INF-γ [4–6], IL-1 [7,8], IL-6 [7–9], IL-8 [7–8]), growth factors (TGF-α [10,11], TGF-β [10,12]), and proto-oncogenes (ras, c-myc, c-Ha ras, c-erb, c-Jun) [13,14] on the various manifestations of the disease. Recently, some of these results have been integrated in a model that hypothesized a molecular pathway for recruitment and intraepidermal trafficking of T cells [5,15,16]. The model, which involves several cell types (keratinocytes, T cells, macrophages, endothelial cells) and a network of cytokines and growth factors, emphasizes the importance of microenvironmental associations in the understanding of the pathophysiology of the disease [5,15,16].

To date, there have been few studies aimed at revealing changes in the overall pattern of gene expression of non-cultured psoriatic keratinocytes that may provide additional clues to which protein components contribute directly or indirectly to the hyperplasia and inflammation observed in psoriatic epidermis. To approach this problem systematically we have established a computer-accessible 2D gel protein database of unfractionated normal non-cultured epidermal keratinocytes [17], and have analyzed the protein patterns of epidermal keratinocytes both from normal (uninvolved) and psoriatic origin [17,18]. As a result of these studies, we have identified several low-molecular-weight proteins that are highly up-regulated in psoriatic epidermis [17,18]. We report here the molecular cloning and expression of one of these proteins that we have termed "psoriasin" (a partially secreted protein of approximate molecular mass of 11,000 daltons and pI of 6.21). This novel protein exhibits a restricted occurrence in fetal human tissues and human cell lines and

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Abbreviations:
2D: two-dimensional
EF-hand: calcium binding structure
EF-2: elongation factor 2
IEF: isoelectric focusing
SSP: sample spot number

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its overexpression may be linked to the inflammatory stimuli. We also report the identification of known proteins among other polypeptides that migrate close to psoriasin in 2D gels. These correspond to MRP 14 [19–21], also known as calgranulin B [22], L1 [21,23,24], and calprotectin [24]; MRP 8 [19–21] or calgranulin A [22], and cystatin A [25,26] or stefin A [27], a member of the cystatin superfamily [25,26].

MATERIALS AND METHODS

Patients and Skin Biopsies Twenty patients with plaque psoriasis on the limb or trunk that were resistant to all forms of treatment were studied. Dermatome shaving was carried out under local anesthesia in the outpatient clinic [28]. Serial shaving were done to a depth at which bleeding occurred from the dermal communicating vessels. At that level, the cut surface had the uniform white color of the reticular dermis, whereas in the more superficial layers there was patchy yellow staining, presumably confined to areas where the thick psoriatic parakeratotic epidermis remained [28]. This study was approved by the Aarhus University Hospital.

Preparation of Unfractionated Human Epidermal Keratinocytes from Psoriatic and Normal Skin Strips of skin (normal, or psoriatic, about 1g) were washed 3 times in Hanks' buffered saline solution (HBSS) and placed in 10 ml of 0.25% trypsin in HBSS (GIBCO, 1 : 250) at 4°C for 15–17 h. Following incubation, the strips were washed 3 times in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and the epidermis was detached from the dermis by using fine forceps [29]. The epidermal samples were then carefully washed 3 times in DMEM containing 10% sera and finally resuspended in 8 ml of the same solution. The samples were then shaken vigorously in a 10-ml plastic tube to detach basal and suprabasal cells. The suspension of epidermal cells was allowed to stand for a few minutes at room temperature and the upper 5 ml of the suspension, which contained single cells and small aggregates, was aspirated with a 10-ml pipette. Epidermal keratinocytes were labeled in suspension (noncultured) with [^{35}S]-methionine immediately after preparation (see below).

Preparation of Psoriatic Keratinocytes Enriched in Basal Cells Unfractionated epidermal keratinocytes obtained as described above were placed on microtiter wells (96 wells; NUNC, Denmark). After 40 min the medium was aspirated and the attached cells were washed 2–3 times with DMEM containing 10% fetal calf serum. Under these conditions mainly firmly attached round cells remained in the bottom of the well [29].

Labeling of Noncultured Keratinocytes with [^{35}S]-Methionine Unfractionated epidermal cells in suspension (0.3 ml) were pelleted by centrifugation (2000 × *g* for 3 min) and resuspended in 0.3 ml of laboratory-made DMEM (1 g/l, $NaHCO_3$) lacking methionine and containing 10% dialyzed fetal calf serum and 150 μCi of [^{35}S]-methionine (SJ 204, Amersham) [30]. After labeling for 14 h, the medium was removed with the aid of an elongated Pasteur pipette (secreted proteins) and the cells were resuspended in 200–300 μl of lysis solution [31]. Keratinocyte populations enriched in basal cells were labeled in microtiter wells.

2D Gel Electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% running gel; 5% stacking gel) was carried out essentially as described by Laemmli [32]. 2D gel electrophoresis with isoelectric focusing (IEF) in the first dimension [31] was carried out as described by Bravo et al [33] and Celis et al [34]. In short, the first dimension was performed for 18 h at 400 V in 130 mm × 1.2 mm 4% w/v polyacrylamide gels containing 2% w/v carrier ampholytes (1.6% pH 5–7, Serva; 0.4% pH 3.5–10, LKB). First-dimensional gels were equilibrated in 3 ml (3 min at room temperature) of equilibration solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT and 10% glycerol) [31]. Gels were then stored at −20°C until use. First-dimensional gels were applied to the second dimension with the aid of agarose solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT; 10% glycerol; 1% agarose and 0.002% Bromophenol Blue) [31]. Following fluorography [35] the dried gels were exposed to X-ray films for various periods of time.

Immunoblotting 2D gels for immunoblotting were placed for 5 min in TGM (Tris, glycine, methanol) prior to transfer (24 h at 130 mA) [34]. Following protein transfer (total cellular extracts from unfractionated psoriatic keratinocytes mixed with small amounts of [^{35}S]-methionine–labeled extracts) onto nitrocellulose, the sheet was dried and exposed to X-ray film. The amount of keratinocyte extract applied to the gels was carefully calibrated to obtain the maximum amount of protein transferred without affecting the quality of the 2D gels. Dry sheets could be kept at room temperature for extended periods of time (up to 3 months) without significant change in the reactivity of the proteins. For immunodetection of the antigen the following procedure was used. Nitrocellulose sheets were incubated for 16 h at room temperature in HBSS containing 1.5% bovine hemoglobin (Sigma, type II). The blots were then incubated at room temperature for 2 h with HBSS containing 2% hemoglobin and a 1 : 100 to 1 : 1000 dilution of the first antibody. After three washes with HBSS (3 × 30 min), the blot was incubated with peroxidase-labeled second antibody (Dakopatts; 1 : 100 dilution) for 2 h. It was then washed 3 times (3 × 30 min) with HBSS and finally stained with 4-chloro-1-naphthol (HRP color development solution, Bio-RAD). The stained sheets were dried, marked with radioactive ink and exposed to X-ray film.

Microsequencing Protein spots from several Coomassie brilliant blue–stained, dried 2D gels were cut and combined. Protein extracts were routinely mixed with [^{35}S]-methionine–labeled proteins from psoriatic keratinocytes in order to assign to the microsequenced polypeptides its corresponding number in the master keratinocyte database. The numbering system used in this study differs from that previously published [17] due to changes in computer hardware.* The procedures for protein recovery, electrotransfer, membrane in situ protease cleavage, peptide-separation by reversed phase HPLC, and amino acid sequencing have been described in detail elsewhere [36–38].

Computer Search for Identity or Similarity The amino acid sequence homology searches were carried out using the FASTA or the TFASTA computer program of Genetics Computer Group [39] for screening protein or nucleic acid databases. The protein database were the PIR International database: MIPSX (release 18.0, November 1990) a merged database including NBRF-PIR protein (release 26.0), NBRF-PIR new, EM trans, Los Pro, and Swiss-prot. The nucleotide databases were the EMBL database including GENBANK (release 24.0) and the NYEDATA database (the daily updates of the EMBL database since release).

Preparation of cDNA Libraries, Screening, and Sequencing RNA was prepared from unfractionated noncultured psoriatic keratinocytes by the guanidinium thiocyanate/CsCl method. mRNA was purified using poly dT "push columns" (Stratagene) and double-stranded cDNA was prepared according to Gubler [40]. The cDNA was size fractionated on low melting agarose gels in fractions ranging from 0 to 300, 300 to 800, 800 to 1600, and > 1600 basepairs (bp). The poly dT_{15} primed cDNA libraries were constructed in λgt11 and amplified as described by Sambrook et al [41].

A 15-mer deoxyribonucleotide with a degeneration of 256 was synthesized. Following 5′ labeling the oligonucleotides were used to screen replica filters using a modification of the tetramethylammoniumchloride salt method [42]. Positive clones were subcloned into M13 and sequenced by the dideoxy method [43].

Northern Analysis of mRNA Poly $(A)^+$ RNA was purified from total RNA and subjected to gel electrophoresis on a horizontal 1% formaldehyde/agarose gel [41]. The Poly $(A)^+$ RNA was trans-

* A revised and up-to-date database will be published in Electrophoresis, November 1991.

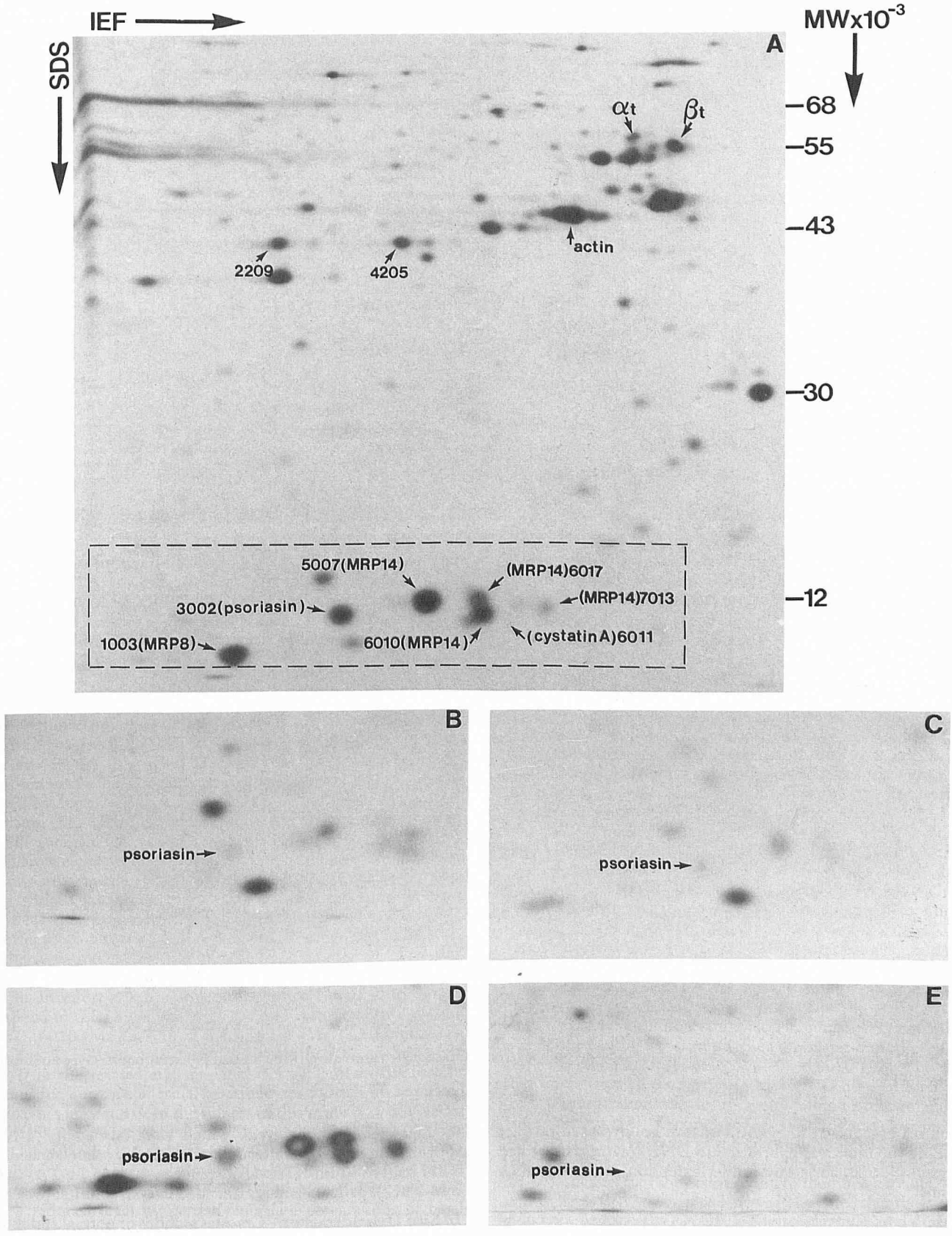

Figure 1. Localization of psoriasin in 2D gels of proteins from psoriatic and normal keratinocytes. *(A,B)*, 2D gels (IEF) of [^{35}S]-methionine–labeled proteins from total non-cultured keratinocytes from psoriatic *(A)* and normal origin *(B)*. *(C)* [^{35}S]-methionine–labeled proteins from psoriatic keratinocytes enriched in basal cells. *(D,E)* Coomassie brillant blue–stained 2D gels (IEF) of epidermis (D) and dermis (E) of psoriatic patients. Low–molecular-weight proteins microsequenced in this study (enclosed in a *box*) are indicated with their corresponding number in the up-dated keratinocyte 2D gel protein database. Other up-regulated proteins (IEF 2209 and 4205) as well as known reference proteins (α- and β-tubulin, actin) are indicated in *(A)*.

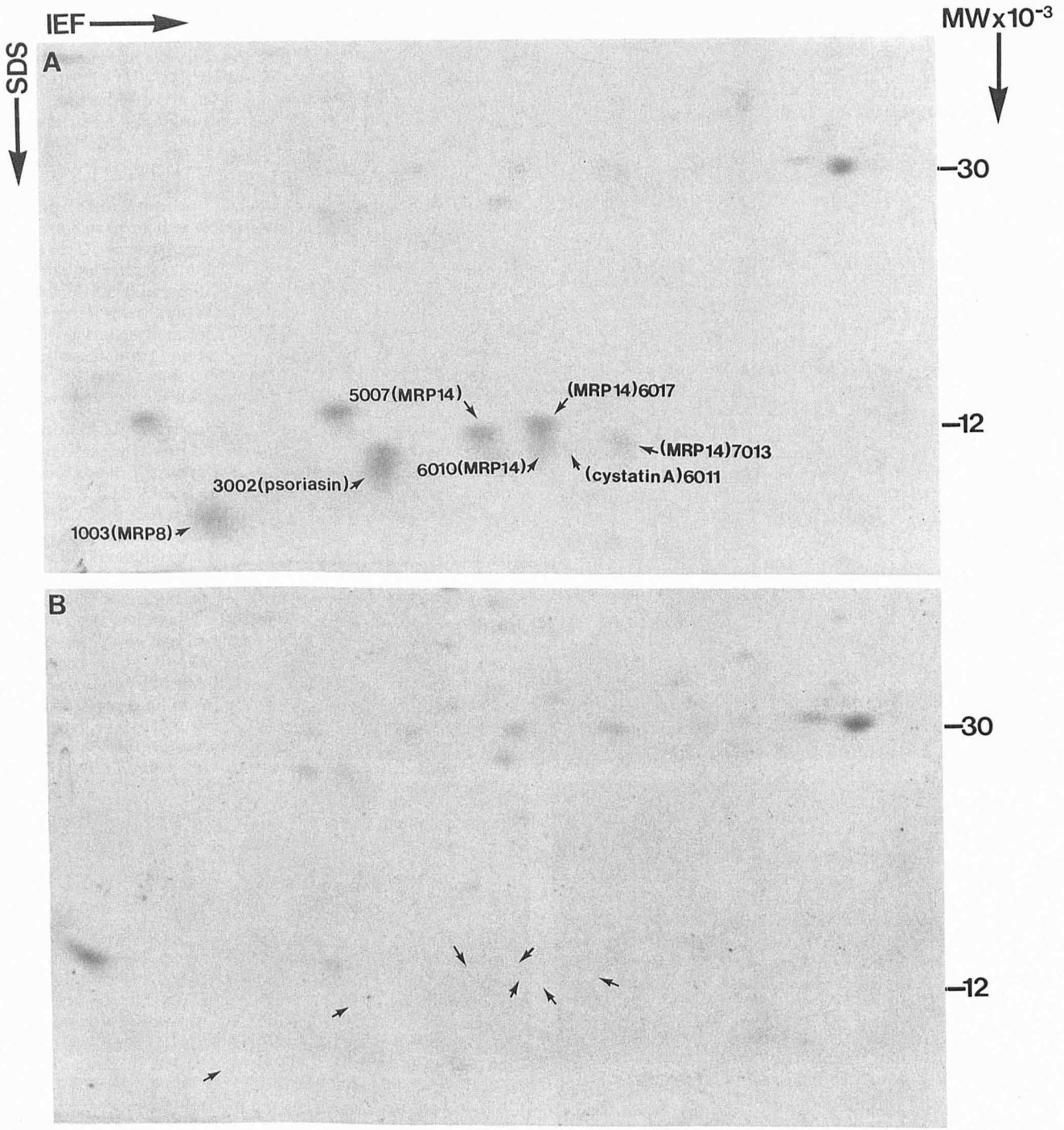

Figure 2. 2D gel patterns of Coomassie brillant blue–stained proteins from unfractionated psoriatic *(A)* and normal *(B)* keratinocytes lyzed immediately after preparation.

ferred onto a Hybond-N filter (Amersham). Following ultraviolet crosslinking the filter was hybridized to DNA fragments that had been strand-specific labeled by primer extension of cloned DNA fragments.

Vaccinia Virus Expression The cDNA was cloned directionally in plasmid Δ6 and recombined in vivo into vaccinia virus essentially as described in a manual prepared by B. Moos and H. Stunnenberg (personal communication).† The recombinant virus was used to infect RK13 monolayers, which were labeled for 1 h with [^{35}S]-methionine, 18 h after infection. Cells were detached using TEN buffer (40 mM Tris-HCl, pH 7.5; 10 mM EDTA; and 150 mM NaCl), pelleted and resuspended in lysis solution [31].

In Situ Hybridization Cryostat sections of normal and psoriatic skin placed on polylysine coated coverslips were fixed in HBSS containing 4% formaldehyde for 1 h. Following fixation, the sections were treated with protease K, acetylated, and dehydrated as described by Angerer et al [44]. The coverslips were then mounted in pairs on glass slides. The 1085 cDNA clone was inserted into a M13 vector containing a T7 promotor and a [^{35}S]-ATP labeled antisense RNA probe was transcribed. The probe was purified on a low melting agarose gel. Hybridization was carried out in a solution

† The manual was used at the EMBO course on "Expression of recombinant proteins using vaccinia virus" held at the EMBL in Heidelberg, September 2–13, 1990. The manual is available from H. Stunnenberg.

Table I. Partial Amino Acid Sequences of Abundant Low-Molecular-Weight Proteins Present in Total Psoriatic Keratinocytes

IEF SSP	Apparent Molecular Weight	pI	Partial Peptide Sequences[a]	Residues in Identified Protein	Protein Name
1003	9,400	7.11	XLETE(C)PQYIR	45-57	MRP-8
			GADVXFK	50-56	
3002	11,000	6.21	SIIGMIDMFHK	9-19	Psoriasin
			ENFP(N)FLSAXD	38-48	
			KGTNYLADVFEK	50-61	
			(K)IDFSEFLSLLGDIA(T)(D)(Y)(H)	69-87	
			QSHGAAPXSGGSQ	89-101	
5007	11,700	5.67	NIETIINTFXQY	11-22	
			XGHPDTLNQGEF(K)	26-38	MRP-14
			M(H)EGXXXP	94-101	
6010	11,700	5.43	NIETIINTF	11-19	
			LG(H)PDTLNQ(G)	26-35	MRP-14
			MXEGDEGP	94-101	
6011	11,300	5.27	(S)LP(G)QNEDLVLXXY	72-85	Cystatin A, stefin A
			XESEN	[b]	
6017	12,500	5.48	LGHPDTLNQGEF(K)	26-38	MRP-14
			MHEGDE	94-99	
			MHEGDEGPGHHHXP	94-107	
7013	11,700	5.13	LGHPDTLNQGEF	26-37	MRP-14
			MXEGDEXP	94-101	

[a] The amino acid residues are given in the one-letter notation. X means that no PTH-amino acid could be detected. Amino acids in parantheses represent the most probable assignment.
[b] This peptide sequence may be derived from a contaminant protein.

containing 50% formamide; 0.3 M NaCl; 20 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1 × Denhardt's; 500 μg RNA/ml; 10% dextran sulfate; and 100 mM DTT [44]. Washing was carried out essentially as described by Angerer [44], except that the RNAse treatment was omitted. Slides were dipped in Amersham's emulsion, exposed for 8 d, developed, and analyzed using conventional light microscopy.

RESULTS

Microsequencing of Psoriasin and Other Low-Molecular-Weight Proteins That Migrate Closely in 2D Gels: Protein Identity Figure 1 shows 2D gel patterns (IEF) of [^{35}S]-methionine-labeled cellular proteins from non-cultured unfractionated epidermal keratinocytes from psoriatic (Fig 1*A*) and normal origin (Fig 1*B*; only the relevant region of the gel is shown). The 2D patterns were quite reproducible (at least for the low-molecular-weight proteins described in this article) from patient to patient. Also, similar results were observed when comparing Coomassie brillant blue-stained proteins obtained from unfractionated psoriatic (Fig 2*A*) and normal (Fig 2*B*) keratinocytes lyzed immediately after preparation. For reference, we have enclosed in a box (Fig 1*A*) the low-molecular-weight proteins that migrate closely to psoriasin (IEF SSP 3002) and that have been microsequenced in this study in an effort to assess their relation to this protein. These correspond to IEF SSP 1003 (Mr 9,400, pI 7.11), 5007 (Mr 11,700, pI 5.67), 6010 (Mr 11,700, pI 5.43), 6011 (Mr 11,300, pI 5.27), 6017 (Mr 12,500, pI 5.48), and 7013 (Mr 11,700, pI 5.13) (see Table I) in the revised and up-dated 2D gel protein database of non-cultured human epidermal keratinocytes.‡ Other up-regulated proteins that have not been the subject of this study (IEF SSP 4205, Mr 40,100, pI 5.91; IEF SSP 2209, Mr 39,500, pI 6.58) [17,18] are also indicated in Fig 1*A* for reference. The numbers assigned to the spots differ from those published earlier [17] due to changes in the computer hardware. As shown in Fig 1*C*, psoriasin is synthesized at a much reduced level by partially purified preparations of basal psoriatic keratinocytes, and is barely detected in the dermis of psoriatic patients (Fig 1*E*; compare with Fig 1*D*, epidermal fraction).

Pooled spots excised from Coomassie brilliant blue-stained gels of proteins from unfractionated psoriatic keratinocytes were microsequenced using a simple and reproducible procedure recently described by us [36-38]. In short, dry Coomassie brilliant blue-stained spots were rehydrated, concentrated by stacking into a new gel, electroblotted onto inert membranes, and digested in situ with trypsin. Peptides eluting from the membranes were then separated by reversed-phase HPLC and sequenced.

A comparison of the peptide sequences presented in Table I showed that psoriasin is different to the other low-molecular-weight proteins that migrate closely in the 2D gels (Fig 1*A*). Furthermore, trypic peptide patterns from this polypeptide (Fig 3*A*) were quite different to those of the other low-molecular-weight proteins (compare with Figs 3*B-E*). Peptide sequences from psoriasin showed no significant homology with any other protein of known sequence reported in Databases (see *Materials and Methods*). Also, 2D immunoblotting experiments using a rabbit polyclonal antibody raised against psoriasin extracted from Coomassie brilliant blue-stained 2D gels showed that this protein is not related to other up-regulated proteins (Fig 4*A*; compare Figs 4*A,B*). At least two variants of this protein were detected and these correspond to IEF SSP 5023 (Mr 11,000, pI 5.56) and 5022 (Mr 11,000, pI 5.52) (Fig 3*A*). Other spots detected in the 2D immunoblots (Fig 4*A*) may be due to crossreactivity and or contaminant antibodies.

Further analysis of the partial peptide amino acid sequences listed in Table I indicated that polypeptides IEF SSP 5007, 6010, 6017, and 7013 share common amino acid sequences. Their relatedness was confirmed by 2D immunoblotting using a mouse polyclonal antibody raised against IEF SSP 6017 + 6010 [17]. The antibody that reacts with all four polypeptides in 2D blots of total proteins from psoriatic keratinocytes (Fig 4*C*) failed to recognize IEF SSP 1003, 3002, and 6011 (compare Figs 4*C,D*). Their close mobility in 2D gels (Fig 1*A*) as well as the fact that the HPLC profiles of the tryptic peptides of all four proteins are very similar, but distinct (Figs 3*B-E*), suggest that they correspond to modified variants. A search in Databases (see *Materials and Methods*) revealed that these polypeptides are related to the calcium binding protein MRP 14 (migration inhibitory factor related protein) [19-21], also known as calgranulin B [22], L1 [21,23,24], and calprotectin [24]. These proteins, which have been termed R:6 and R:7 by Gemmell and Anderson [45], have been found to be expressed in human leukocytes from patients with rheumatoid arthritis. MRP 14 has been shown to be expressed by infiltrated macrophages during inflammatory reactions [19]. Interestingly, four variants of this protein have been reported so far by Gemmell and Anderson [45] and two of them,

‡ To be published in Electrophoresis, November 1991.

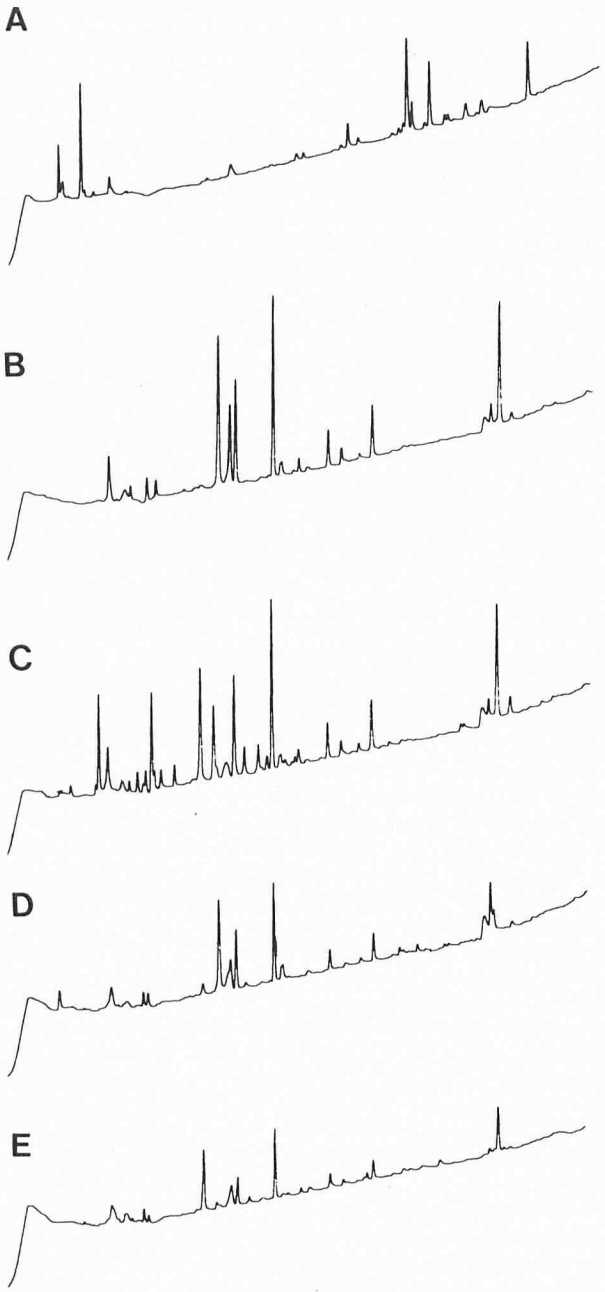

Figure 3. Tryptic peptides obtained by membrane in situ cleavage of IEF SSP 3002 *(A)*, 5007 *(B)*, 6017 *(C)*, 6010 *(D)*, and 7013 *(E)*. Peptides were separated on a C_4 (0.46 × 25 cm) reversed-phase column (Vydac Separation Group, USA). Solvent A consisted of 0.1% TFA, and solvent B was 70% acetonitrile in 0.1% TFA. A gradient from 0% solvent B to 100% solvent B was applied in a linear mode over 70 min. The eluate (1 ml/min) was recorded by absorbance at 214 nm (0.1 AUFS).

most likely corresponding to IEF SSP 6017 and 7013, are phosphorylated [45].

Comparison of the peptide sequences of IEF SSP 1003 with those of known proteins showed that it matches amino acid sequences present in the calcium binding protein MRP 8 [19–21], also known as calgranulin A [22]. This protein has been observed in blood serum of patients with cystic fibrosis [19], and like MRP 14 is synthesized by cells of myeloid origin such as granulocytes, monocytes, and macrophages [19]. A similar comparison of the peptide sequence of IEF SSP 6011 with those in databanks revealed complete homology to cystatin A or stefin A (Mr 11,000, pI 5.27), a member of a superfamily of proteinase inhibitors known as cystatins [25,26]. This protein, which is abundant in epidermis [26,46], is regulated in inflammatory diseases [46,47] and is stimulated in macrophages [48]. The identity of IEF SSP 6011 as cystatin A was further confirmed by immunoblotting using antibodies specific for cystatin A kindly provided by Dr. A. Takeda (results not shown).

Occurrence of Psoriasin in Fetal Human Tissues and Human Cell Lines To determine the normal human tissue distribution of psoriasin we carried out a visual analysis of silver stained gels of 21 fetal tissues that include adrenal gland, brain, cerebellum, ear, eye, heart, hypophysis, liver, lung, meninges, mesonephric tissue, striated muscle, pancreas, skin, spleen, submandibular gland, small intestine, thymus, thyroid gland, tongue, and ureter. The results showed that with the exception of skin (Fig 5*A*), ear, and tongue, which showed low levels of psoriasin, all other tissues exhibited undetectable levels of this protein. The position of psoriasin was determined by mixing the tissue extracts with [^{35}S]-methionine–labeled extracts enriched in psoriasin (results not shown). So far, we have analyzed many adult human tissues and have obtained essentially the same results (results not shown).

Similar 2D gel analysis of various human cell types labeled with [^{35}S]-methionine has failed to reveal this protein (at least at the level of detection of the current technology) in normal [total peripheral blood mononuclear cells (Fig 5*B*) [49], $CD4^+$ helper cells, $CD8^+$ suppressor T cells, $CD20^+$ B cells, N901 NK cells, embryonal fibroblast (lung, skin [Fig 5*C*], meninges, kidney, MRC-5, W138), amnion, endothelial cells] and transformed cells [K14 (SV40 transformed keratinocytes) (Fig 5*D*) [50], A431 (Fig 5*E*), HeLa, transformed human amnion cells (AMA), W138 SV40 (SV40 transformed W138 fibroblasts), CaCo-2 cells, FL-amnion, T47D, WISH, Molt-4, HT 1080, HL60], of lymphoid, fibroblast, and epithelial origin. Also, erythrocytes that are found in low numbers in psoriatic epidermis do not express this protein (P. Gromow and J.E. Celis, submitted for publication). Extracts of granulocytes, however, contain significant levels of this protein (Fig 5*F*).

Visual analysis of 2D gels published by other groups indicate that psoriasin is not expressed by monocytes [45].

Primary Structure of Psoriasin Partial amino acid sequences obtained by microsequencing were used to derive oligodeoxynucleotides to clone the psoriasin cDNA. Five tryptic peptides separated by reversed-phase HPLC were sequenced with the following results: peptide 1, SIIGMIDMFHK; peptide 2, ENFPNFLSAXD; peptide 3, KGTNYLADVFEK; peptide 4, KIDFSEFLSLLGDIATDYH; and peptide 5, QSHGAAPXSGGSQ. Oligonucleotide probes derived from a part of the amino acid sequence of peptide 3 were used to screen a λg11 cDNA library prepared from unfractionated epidermal keratinocytes obtained from psoriatic plaques. The nucleotide and deduced amino acid sequences of the fragment contained in one of the cDNA clones (clone 1085) is shown in Fig 6. The open reading frame codes for a protein composed of 101 amino acids that contains all the tryptic peptides sequenced from psoriasin and has a molecular weight of 11,457 daltons and a calculated pI of 6.77. These results are in close agreement with the apparent molecular weight and pI calculated from the 2D gels (Table I). Further evidence indicating that the predicted protein is the same as psoriasin was obtained by 2D gel analysis of [^{35}S]-methionine–labeled proteins expressed by RK 13 cells (rat kidney) transfected with clone 1085 using the vaccinia virus expression system (B. Moss and H. Stunnenberg, unpublished). The *arrow* in Fig 7*A* indicates the [^{35}S]-methionine–labeled protein that co-migrates with psoriasin. A control using a nonrelevant clone is shown in Fig 7*C*.

At present, we have evidence indicating that psoriasin is partially secreted to the medium even though it lacks a signal sequence at its amino terminal (Fig 6). Figure 8 shows IEF 2D gels of [^{35}S]-methionine–labeled cellular (Fig 8*A*) and secreted (Fig 8*B*) proteins from unfractionated psoriatic keratinocytes. Clearly, the presence of psoriasin in the medium (Fig 8*B*) is not due to cell death and lysis as most cellular proteins, including known extractable proteins such as α- and β-tubulin, EF-2 and Lipocotin V, are not present in the

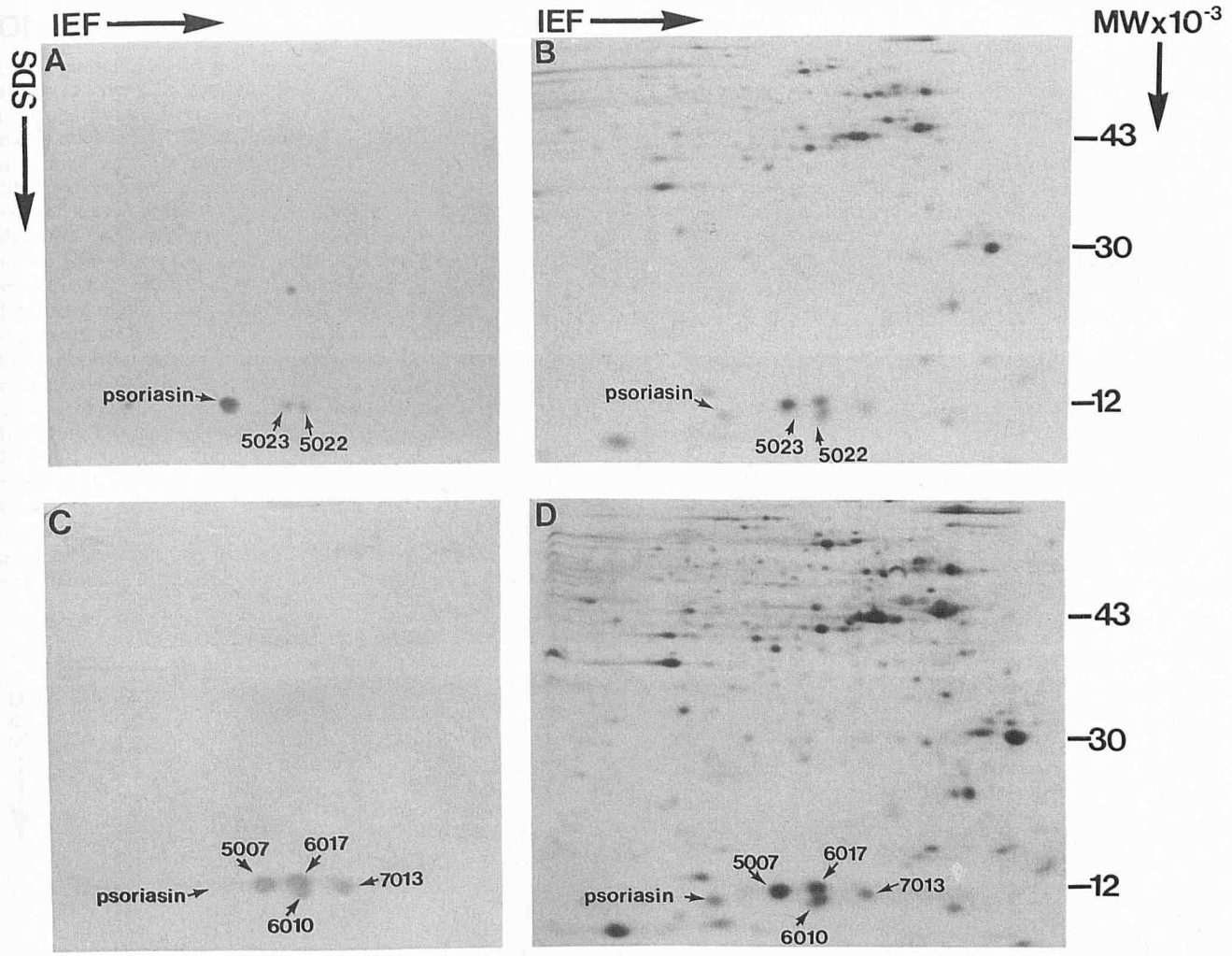

Figure 4. 2D immunoblotting of proteins from psoriatic keratinocytes reacted with a rabbit polyclonal antibody raised against psoriasin *(A)* or a mouse polyclonal antibody raised against polypeptides IEF SSP 6017 + 6010 *(C)*. *(A,C)* immunoblot. *(B,D)* autoradiograms of the same blot. The identity of the antigen(s) was determined by superimposing both images with the aid of radioactive marks.

media [34]. At present it is not clear why there is actin in the media. Taken together these results suggest that psoriasin is transported to the exterior through a pathway distinct from the classical route involving the endoplasmic reticulum and the Golgi apparatus [51].

Close inspection of the predicted sequence revealed a putative calcium-binding sequence of the EF-hand type that spanned from positions 54 to 82 (Fig 6*A*). Thirteen of 16 essential amino acids required to form a calcium-binding EF-hand [52,53] are present in this sequence as indicated in Fig 6*B*. Comparison of the deduced amino acid sequence of the cloned psoriasin with sequences available in the EMBL and Genbank Databases revealed no significant homology with known proteins, including the calcium-binding proteins S100 [54], the thin intestinal calcium-binding protein [55], calcyclin [56], p9ka [55,57], calgranulins A and B [58], and the p11 regulatory subunit of lipocortin II [59]. Only a very weak homology to bovine and mouse p11 was observed.

Northern Blotting and in situ Hybridization To determine the level of 1085-specific transcript in normal and psoriatic keratinocytes we performed Northern blot analysis using cDNA clone 1085 as a probe. The expression of 1085 transcript was found to be very high in unfractionated psoriatic keratinocytes (Fig 9*A, lane 3*) as compared to their normal counterpart *(lane 1)*. Interestingly, the Northern blot analysis of the 1085 transcript showed a more pronounced difference between normal and psoriatic keratinocytes than that revealed by the 2D gel analysis of the [^{35}S]-methionine–labeled protein [17,18]. This, we believe, is due to the fact that overnight incubation of unfractionated normal keratinocytes in [^{35}S]-methionine–labeling media (MEN) may partially and temporarily induce the synthesis of psoriasin, a situation that has been also observed in the case of oncogenes [13]. This was confirmed by Northern analysis of poly (A)$^+$ RNA obtained from total normal keratinocytes cultured overnight in DMEM containing 10% fetal calf serum *(lane 2)*. The level of 1085 gene expression was below the limit of detection in SV40-transformed human keratinocytes (K14, *lane 4*); A431 cells *(lane 5)*; AMA cells (transformed human amnion cells) *(lane 6)*; MRC-5 V2 (SV40 transformed embryonal human lung fibroblasts) *(lane 7)*, and MRC-5 (embryonal human lung fibroblasts) *(lane 8)* (Fig 9).

The localization of psoriasin mRNA in sections of normal (Fig 10*A*) and psoriatic skin (Fig 10*B*) was determined by in situ hybridization using a [^{35}S]-labeled antisense cRNA probe. In line with the 2D gel and Northern blot experiments, the results showed that psoriasin mRNA is present in elevated levels in psoriatic epidermis (Fig 10*B*) as compared to its normal counterpart (Fig 10*A*). The grains were abundant in the suprabasal layers and often occurred in clusters. Only a few silver grains were observed in the dermis in line with the 2D gel analysis shown in Fig 1*E*.

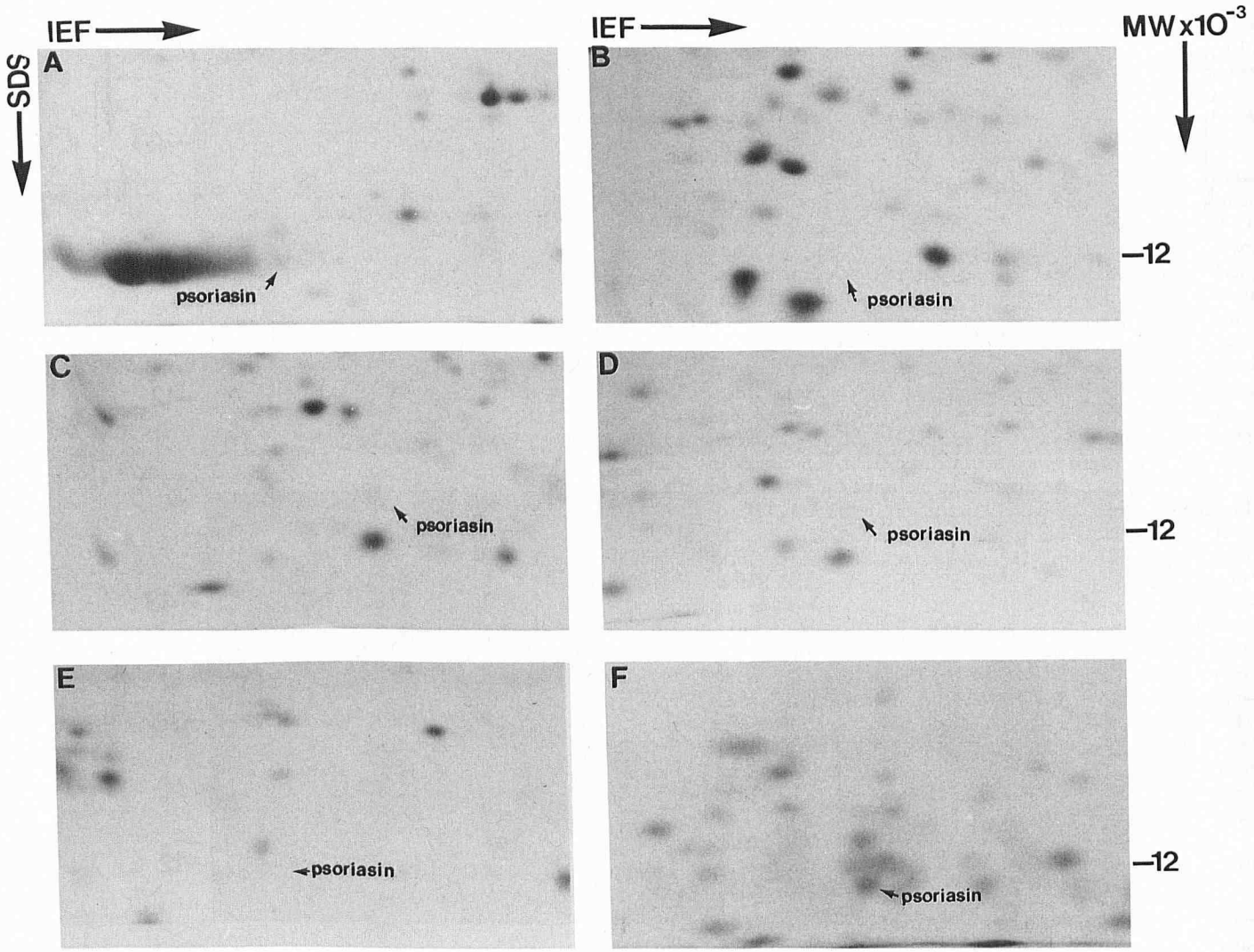

Figure 5. Occurrence of psoriasin in fetal human tissues and human cell lines. *(A,F)* Silver-stained 2D gels (IEF) of proteins from fetal human skin *(A)* and granulocyte extracts [61] *(F)*. *(B,E)* fluorograms of 2D gels (IEF) of [^{35}S]-methionine–labeled proteins from total peripheral blood mononuclear cells *(B)*, embryonal skin fibroblasts *(C)*, SV40 transformed keratinocytes K14 *(D)*, and A431 epidermoid carcinoma cells *(E)*. Due to cell heterogenecity silver staining was preferred to [^{35}S]-methionine labeling in the case of tissue samples. Only the relevant area of the gels is shown.

Figure 6. Nucleotide and corresponding amino acid sequence of the cDNA clone for psoriasin. The cDNA codes for a protein of 101 amino acids starting at nucleotide position 49 and ending with a termination codon at position 352. The protein has a molecular weight of 11,457 and a calculated pI of 6.77. These values are in agreement with the parameters observed by 2D gel electrophoresis. The five partial peptide sequences obtained by microsequencing (underlined) are identical to the predicted amino acid sequence *(A)*. The cysteine residues in peptides 2 and 5 were not detected in amino acid sequence analysis. The peptide sequence NYLAD (from peptide three) was backtranslated and fully degenerated oligodeoxy ribonucleotides were synthesized and used for screening. The putative calcium binding EF-hand motif is enclosed by a *box*. In *B*, the EF-hand sequence has been aligned with the consensus motif of the EF calcium binding hand [62] (**n** stands for residues with hydrophobic side chains: L, I, V, F, M; **d** stands for residues having an oxygen as a calcium ligand: D, N, E, Q, S, T; **x** for any residue). *Asterisks*, residues that obey the consensus sequence [53,55,62].

```
A
                                                    M  S  N  T    4
ATTCTTCTACTCGTGACGCTTCCCAGCTCTGGCTTTTTGAAAGCAAAGATGAGCAACACT   60

 Q  A  E  R  S  I  I  G  M  I  D  M  F  H  K  Y  T  R  R  D    24
CAAGCTGAGAGGTCCATAATAGGCATGATCGACATGTTTCACAAATACACCAGACGTGAT  120

 D  K  I  D  K  P  S  L  L  T  M  M  K  E  N  F  P  N  F  L    44
GACAAGATTGACAAGCCAAGCCTGCTGACGATGATGAAGGAGAACTTCCCCAACTTCCTT  180

 S  A  C  D  K  K  G  T  N  Y  L  A  D  V  F  E  K  K  D  K    64
AGTGCCTGTGACAAAAAGGGCACAAATTACCTCGCCGATGTCTTTGAGAAAAAGGACAAG  240

 N  E  D  K  K  I  D  F  S  E  F  L  S  L  L  G  D  I  A  T    84
AATGAGGATAAGAAGATTGATTTTTCTGAGTTTCTGTCCTTGCTGGGAGACATAGCCACA  300

 D  Y  H  K  Q  S  H  G  A  A  P  C  S  G  G  S  Q  END       101
GACTACCACAAGCAGAGCCATGGAGCAGCGCCCTGTTCCGGGGGCAGCCAGTGACCCAGC  360

CCCACCAATGGGCCTCCAGAGACCCAGGAACAATAAAATGTCTTCTCCCACCAGAAAAAA  420

AAAAAAAAAAAAAAAAA                                           437

B
E n x x n n x x n D x d x d G x I d x x E n x x n n x x n
  *   * *     *   *   *     * *     * *     * *     *
Y L A D V F E K K D K N E D K K I D F S E F L S L L G D I
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DISCUSSION

We have presented evidence showing that psoriatic keratinocytes overexpress a novel low-molecular-weight partially secreted protein termed psoriasin that exhibit a restricted occurrence in fetal human tissues and human cell lines. The fact that this protein has been termed psoriasin should not be taken to imply that it is a specific marker for psoriasis. The mRNA seems to be enriched in suprabasal keratinocytes suggesting that its overexpression is the result of the altered differentiation program followed by these cells. Whether this protein is up-regulated in other inflammatory skin disorders is at present unknown and will be the subject of future studies using monospecific antibodies.

At present, we have no firm information as to the role of psoriasin in the pathophysiology of psoriasis. All available information, however, indicates that it is not directly involved in the hyperplastic phenotype of the psoriatic epidermis. We have shown that normal proliferating keratinocytes exhibit low levels of psoriasin and that SV40 transformed keratinocytes (K14) [50] and A431 epidermoid carcinoma cells do not synthesize significant levels of this protein. Furthermore, proliferating normal human fetal skin shows only low levels of psoriasin. Thus, it would seem likely that the overexpression of psoriasin may be linked to the immune activation and tissue inflammation caused by hyperproliferation and abnormal differentiation. Experiments are underway to express and purify the recombinant protein for biochemical studies.

In addition to the novel protein psoriasin we have identified three known polypeptides among the low-molecular-weight proteins that migrate closely to this protein in 2D gels (Fig 1*A*, Table I). These correspond to MRP 14 [19-21], also known as calgranulin B [22], L1 [21,23,24], and calprotectin [24]; MRP 8 [19-21] or calgranulin A [22], and cystatin A [25,26] or stefin A [27], a member of the cystatin superfamily of proteinase inhibitors [25,26]. Both MRP 14 and 8 are expressed by infiltrate macrophages in chronic inflammations [19]. These proteins are also expressed by granulocytes and monocytes [19,22,58]. High levels of MRP 8 have also been reported in blood serum of patients with cystic fibrosis [58]. The function of these calcium-binding proteins is presently unknown, although they have been implicated in macrophage activation and defense mechanisms [24]. In line with our published results, Gabrielsen et al [60] have shown that the L1 antigen(s) is overexpressed in psoriatic keratinocytes, although from our studies it is difficult to assess how much of these proteins is contributed by the keratinocytes. This protein(s) was also shown to be present in keratinocytes of a variety of skin diseases (Lichen planus, dermatitis herpetiformis, erythema multiforme, Rosacea, and sarcoidosis) and overall, the results suggest a general role for these proteins in inflammatory skin diseases. The increased levels of the proteinase inhibitor cystatin A

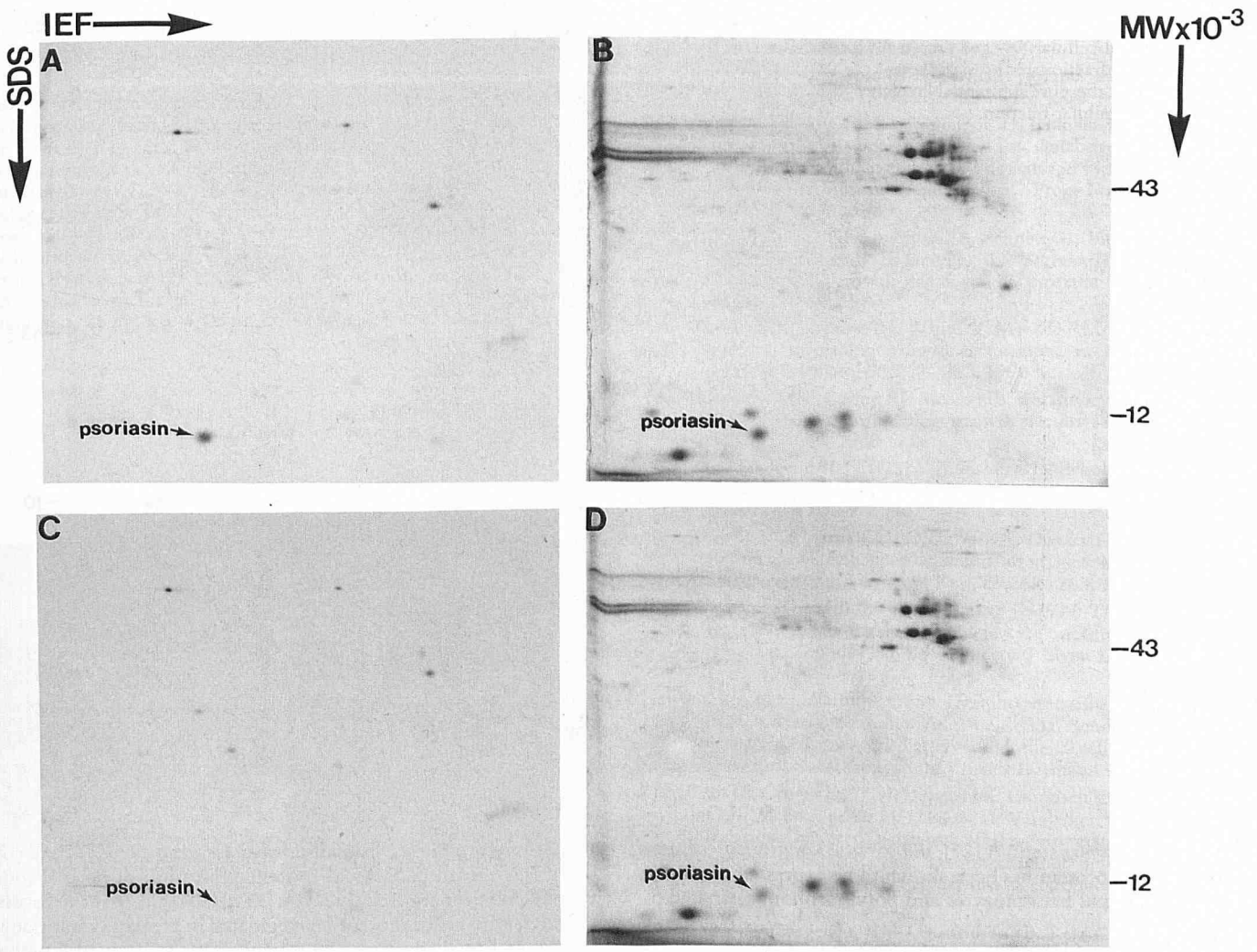

Figure 7. 2D gel electrophoresis of [^{35}S]-methionine-labeled proteins synthesized by RK 13 monolayer cells infected with vaccinia virus carrying the 1085 clone *(A)* and a non-relevant clone *(C)*. The preparations were mixed with unlabeled extracts of psoriatic epidermis enriched in psoriasin in order to facilitate the identification. *(B)* and *(D)* correspond to the Coomassie-stained gels shown in *(A)* and *(C)*, respectively. The identity of the radioactively labeled psoriasin was assessed by superimposing the images.

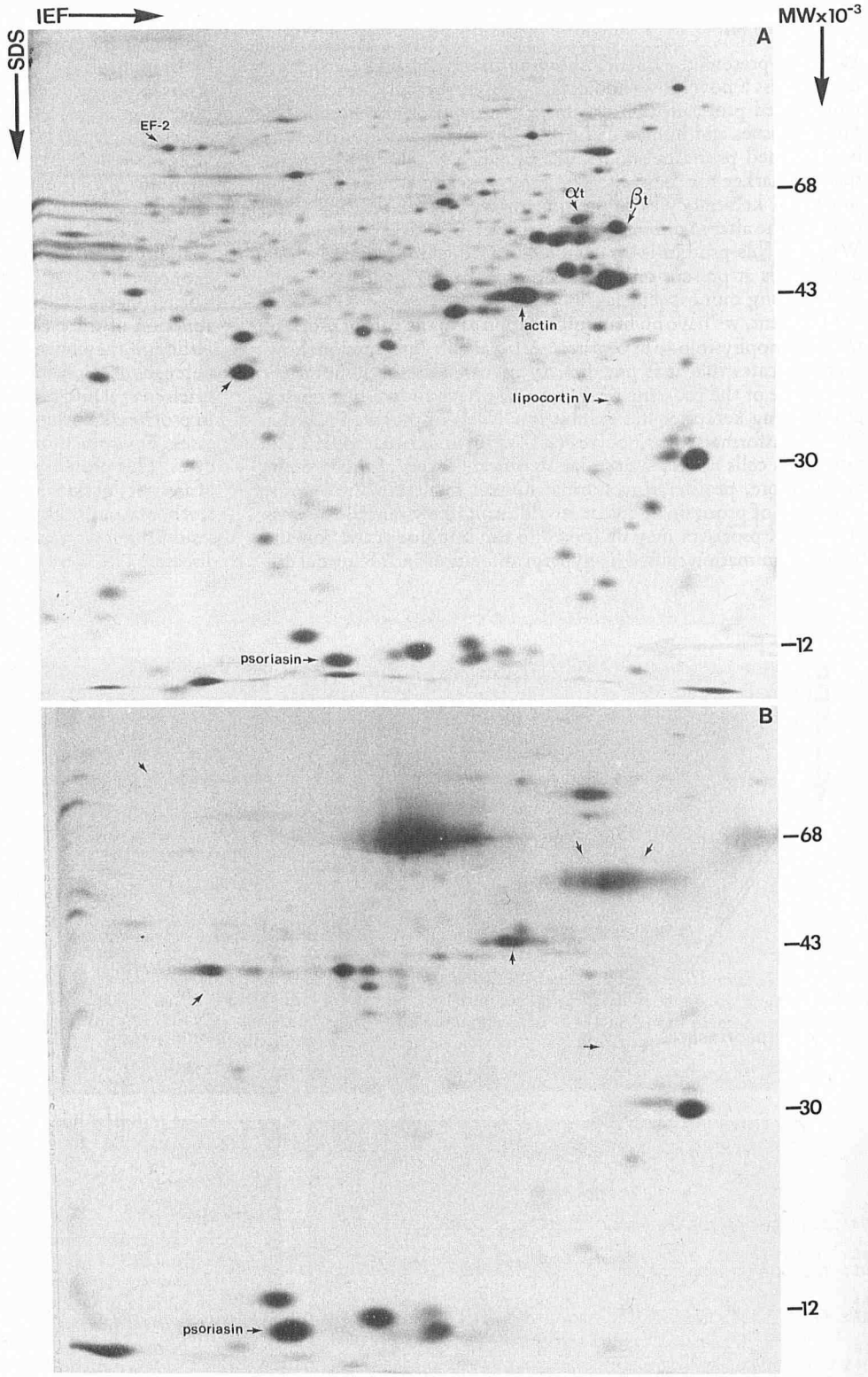

Figure 8. 2D gel patterns of [^{35}S]-methionine-labeled cellular *(A)* and secreted proteins *(B)* from unfractionated psoriatic keratinocytes. Note that there are substantial levels of psoriasin in the cellular fraction.

[25,26], also known as stefin A [27], in psoriatic keratinocytes is not surprising as this protein has been shown to be expressed predominantly by epidermal keratinocytes and polymorphonuclear leukocytes [26,47].

It should be stressed that our approach to the study of psoriasis has been systematic and directed towards the identification of protein markers that may be used to dissect the various stages of differentiation in both normal and psoriatic keratinocytes [17,18,29]. As a first approximation we established a computer-accessible comprehensive 2D gel protein database of normal keratinocytes [17] and compared the overall patterns of protein expression of normal (uninvolved) and psoriatic keratinocytes [17,18]. So far, we have focussed our studies on major proteins up-regulated in psoriatic keratinocytes but research will be eventually expanded to the study of less-abundant proteins. Once a phenotype-specific protein is identified, it will be possible to microsequence it, to store the information in the database, to search for homology with previous characterized proteins, and to clone the cDNA. Using this comprehensive approach

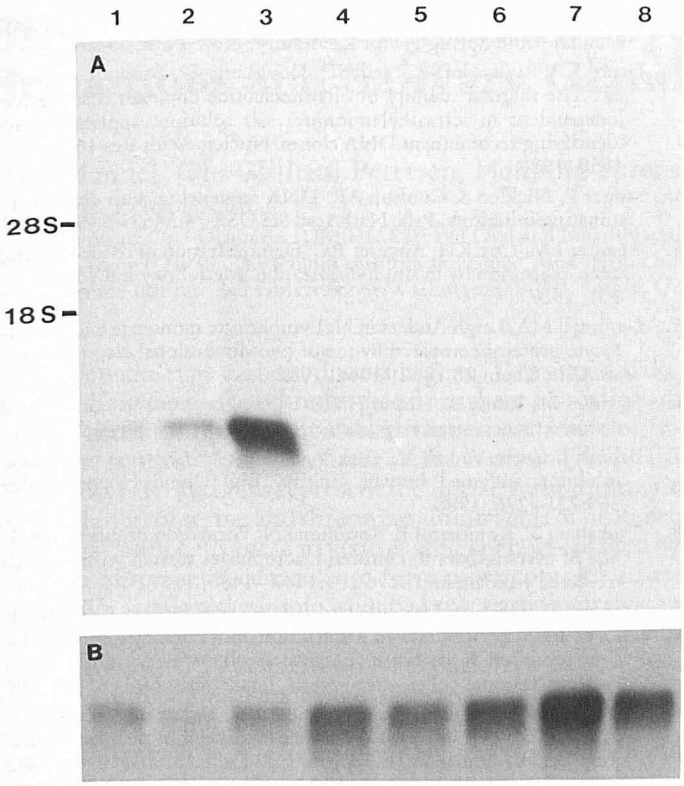

Figure 9. Northern analysis of mRNA from *(lane 1)* normal keratinocytes; *(lane 2)* normal keratinocytes cultured overnight; *(lane 3)* psoriatic keratinocytes; *(lane 4)* K14 cells (SV40 transformed keratinocytes); *(lane 5)* A431 cells (epidermoid carcinoma); *(lane 6)* AMA cells (transformed amnion cells); *(lane 7)* MRC-5V2 cells (SV40 transformed MRC-5 cells); *(lane 8)* MRC-5 cells (embryonal lung fibroblast cells). The filter was hybridized *(A)* to a 350-bp antisense 3′ fragment of the cDNA 1085 clone. *(B)* the same filter hybridized to a 550-bp *sal* I/*xho* II antisense fragment of the human β-actin cDNA. Poly $(A)^+$ RNA purified from the following amounts of total RNA was loaded on the gel *(lane 1,* 40 μg; *lane 2,* 50 μg; *lane 3,* 44 μg; *lane 4,* 19 μg; *lane 5,* 25 μg; *lane 6,* 25 μg; *lane 7,* 13 μg; *lane 8,* 50 μg).

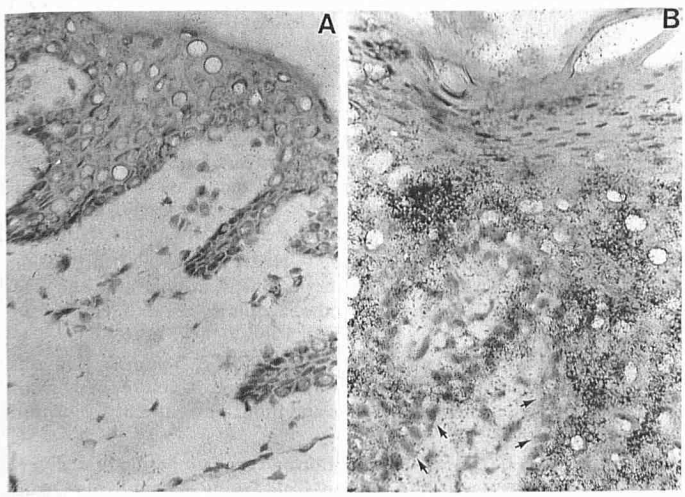

Figure 10. In situ hybridization of psoriasin mRNA in *(A)* normal and *(B)* psoriatic skin. *Arrows,* basal cell layer.

it should be possible to build up an integrated picture of the expression levels, properties, and function of the thousands of keratinocyte proteins that may be directly or indirectly involved in properties associated with differentiation, proliferation and disease.

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