Redistribution of Transcription Factor AP-2α in Differentiating Cultured Human Epidermal Cells

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Expression of the transcription factor AP-2α was examined in cultured human epidermal cells. Levels of AP-2α mRNA increased substantially after the cultures reached confluence, similar to the expression pattern of the differentiation markers involucrin and keratinocyte transglutaminase. The level of AP-2α protein in nuclear extracts declined markedly after confluence, however, along with its ability to form complexes with oligonucleotides containing the AP-2 response element. In contrast, the levels of AP-2α protein in cytoplasmic extracts increased dramatically after confluence, but these extracts had low DNA binding activity. Supershift experiments with specific antisera detected only AP-2α and not the β or γ isoforms. Examination of its localization by confocal microscopy revealed that AP-2α was primarily in the nucleus of basal cells and largely cytoplasmic in the most superficial cells. Localization was a dynamic phenomenon in that changing the medium resulted in accumulation of this transcription factor in the nucleus after several hours. Overall, the data indicate that AP-2α transcriptional activity is regulated in a differentiation-dependent manner in cultured keratinocytes and that this occurs by relocalization of the protein. Nuclear localization of the AP-2α protein in basal cells permits its accessibility to response elements in gene promoters, whereas sequestration in the cytoplasm as the differentiation program progresses curtails its transcriptional activity. This regulatory scheme may provide keratinocytes with the ability to restore AP-2 transcriptional activity rapidly by redistribution to the nucleus after receiving an appropriate growth signal, such as a medium change. ***Key words: growth/involucrin/TGM1/SCC-9/stratification. J Invest Dermatol 117:864–870, 2001***

AP-2α is a homodimeric 100 kDa transcription factor with a core recognition element sequence of 5′-GCCNNNGGC-3′ (Williams and Tjian, 1991). This factor was first purified from HeLa cells on the basis of its ability to bind to enhancer regions of SV40 and human metallothionein IIA promoters and to stimulate transcription from them *in vitro* (Mitchell *et al*, 1987). It is expressed in neural crest cell lineages during mouse embryogenesis (Mitchell *et al*, 1991) and, in view of the fatal defects that result in homologous gene knockout mice (Schorle *et al*, 1996; Zhang *et al*, 1996), has a critical role during development. The expression of AP-2α mRNA is stimulated upon differentiation of several cell lines, including NT2 human teratocarcinoma and P19 mouse embryonal carcinoma cells after treatment with retinoic acid (Williams *et al*, 1988; Phillip *et al*, 1994), suggesting that this transcription factor helps mediate differentiation events and even certain retinoid actions during development. On the other hand, AP-2α has also been reported in some cases to suppress transcription. This action has been attributed to the specific location of its response element, positioned so that protein binding interferes either with assembly of the transcription machinery (Gelman *et al*, 1995) or with binding by another, more critical transcription factor at an overlapping site (Chen *et al*, 1997).

AP-2α is now known to be a family of proteins, resulting from alternative splicing of transcripts from the same gene, that can form heterodimers (Buettner *et al*, 1993; Meier *et al*, 1995; Ohtaka-Maruyama *et al*, 1998). Five variants have been detected, two of which appear to act as dominant negatives, and at least four of which can be coexpressed in individual tissues. Moreover recent work has revealed two other related genes encoding transcription factors that bind to the same core response element (Moser *et al*, 1995; Bosher *et al*, 1996; Chazaud *et al*, 1996; Oulad-Abdelghani *et al*, 1996). AP-2β and AP-2γ are nearly identical to AP-2α in a segment of 96 residues in the C-terminal half that are critical for DNA binding and dimerization, and thus can form heterodimers, but are substantially divergent in the activation domain (N-terminal half), suggesting different interactions with the transcription machinery. They have overlapping but distinct patterns of expression in tissues, and in knockout mice they display at least some nonredundant function. Each is expressed in the stratified squamous epithelia. Although AP-2 has been implicated in expression of several epidermal keratins, a curious lack of effect of AP-2α deletion on keratin expression in mouse epidermis (Talbot *et al*,

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Abbreviations: hEp, human epidermal keratinocytes; TBST, 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 0.5% Tween-20; TGM1, keratinocyte transglutaminase.

1999) has been suggested to result from some overlap in function, as indicated in certain other systems (Maconochie *et al*, 1999).

The intricate maturation process of epidermal keratinocytes presumably occurs in concert with coordinated changes in transcription factor expression or activity, elucidation of which will be instrumental in understanding the normal program and its perturbation in disease. Potential AP-2 response elements have been noted by inspection in the promoters of a number of genes encoding keratinocyte differentiation markers, and some show functionality (Eckert *et al*, 1997). A recent report demonstrating the role of a critical AP-2 site for conferring epidermis-specific expression of the keratin 14 gene in transgenic mice (Sinha *et al*, 2000) has renewed interest in this transcription factor as an important regulator of keratinocyte genes. Our effort is directed to exploring the relation of AP-2 expression to keratinocyte programming in culture. For this purpose, serially passaged cultures, optimized for growth using 3T3 feeder layer support, are highly appropriate inasmuch as they are well stratified and display key features of terminal differentiation (Green, 1979). Our initial efforts have revealed the unexpected finding that keratinoyte maturation is accompanied by accumulation of AP-2 in the cytoplasm. Dynamic intracellular localization of proteins is becoming recognized as an important cellular mechanism for the regulation of activity. An example is the cytoplasmic sequestration of transcription factors NFkB and NFAT, precluding transcriptional activation until an incoming signal results in nuclear relocalization. Our findings suggest that, like NFkB and NFAT, AP-2 transcriptional activity in cultured keratinocytes is regulated in part by changes in its distribution between nucleus and cytoplasm. Translocation between nucleus and cytoplasm has also been reported recently for keratinocyte basonuclin (Iuchi *et al*, 2000).

MATERIALS AND METHODS

Cell culture Normal human epidermal keratinocytes (hEp) were grown in the presence of lethally irradiated 3T3 feeder cells in a mixture of Dulbecco–Vogt Eagle's and Ham's F-12 media (3:1) supplemented with 5% fetal bovine serum, 0.4 μg per ml hydrocortisone, 10 ng per ml epidermal growth factor (EGF), 10 ng per ml cholera toxin, 5 μg per ml insulin, 5 μg per ml transferrin, 20 pM triiodothyronine, and 0.18 mM adenine (Allen-Hoffmann and Rheinwald, 1984). Cells were inoculated at a density of 7×10^4 per 10 cm dish and reached approximately 25% confluence in 4 d, approximately 50% confluence in 5 d, and approximately 100% confluence in 8 d. Cultures from passages 3 through 5 were used for experiments. SIK spontaneously immortalized human epidermal cells (Rice *et al*, 1993) and SCC-9 malignant keratinocytes (Rheinwald and Beckett, 1981) were grown in the same way except that EGF was omitted from the medium for the latter. Swiss mouse 3T3 fibroblasts (Todaro and Green, 1963) used for feeder layers were grown in Dulbecco–Vogt Eagle's medium supplemented with 10% bovine serum and examined for AP-2 localization without irradiation.

Northern blot analysis For isolation of total cellular RNA, hEp cultures were washed twice with 10 ml of isotonic phosphate-buffered saline (PBS) and lysed using Trizol reagent (Gibco BRL), a modification of the guanidinium thiocyanate/acid phenol solvent of Chomczynski and Sacchi (1987). Samples of RNA (30 μg per lane) were fractionated in 1.5% denaturing agarose gels (Sambrook *et al*, 1989). The fractionated RNAs were transferred to nitrocellulose membrane and fixed by baking. After transfer, the membrane was prehybridized at least 4 h at 42°C in a solution containing 50% formamide, 5 × sodium citrate/chloride buffer (SSC), 5 × Denhardt's solution, 50 mM sodium phosphate buffer (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg per ml denatured sheared salmon sperm DNA. The membrane was then hybridized sequentially with ^{32}P-labeled cDNA probes specific for AP-2α, human involucrin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) overnight at 42°C. The hybridization solution contained 50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 0.1 mg per ml denatured sheared salmon sperm DNA. After hybridization the membrane was washed sequentially in two changes each of 2 × SSC–0.1% SDS and 0.5 × SSC–0.1% SDS, and then once in 0.1 × SSC–0.1% SDS at 42°C for 20 min. Specific hybridization signals were visualized by autoradiography and quantitated using a Molecular Dymanics SI phosphorimager. The relative maximal levels of AP-2α and AP-2αB mRNA were estimated by comparing exposure times, phosphorimage intensities, and relative probe activities.

Two DNA probes for AP-2α were employed. The first was an EcoRI/NcoI fragment from a cDNA clone spanning nucleotides −45 to +1223 common to the various alternatively spliced forms (cDNA clone was kindly provided by Dr. Robert Tjian). The second, specific for AP-2αB (nucleotides 873–1500), was prepared by polymerase chain reaction (PCR) from human genomic DNA using the AP-2αB-specific primers 5′-AGCGAATCCACTTGCTAACT-3′ and 5′-GAGGCATTTGGAG-GGTCTAT-3′ (Buettner *et al*, 1993). The involucrin probe was an 850 bp PstI fragment from the involucrin coding region in the genomic subclone plambdaI-3H6B (Eckert and Green, 1986). The keratinocyte transglutaminase (TGM1) probe was a 2.5 kb EcoRI fragment from the cDNA clone hTG13 (Phillips *et al*, 1990). A 780 bp PstI/XbaI fragment of the human GAPDH coding region was used to normalize for the amount of RNA loaded in each lane. The probes were gel purified and labeled with [α-^{32}P]dCTP to specific activities of $8–10 \times 10^8$ cpm per μg using random primers (Ambion kit).

Preparation of nuclear and cytoplasmic extracts Preparations from hEp were performed by a modification (Chen *et al*, 1997) of the original method of Schreiber *et al* (1989). After removal of residual 3T3 and contaminating dermal fibroblasts, cells were harvested by trypsinization and collected by centifugation in 15 ml tubes. Cell pellets from individual cultures were rinsed twice in ice-cold PBS and resuspended in 0.5 ml of ice-cold hypotonic buffer [20 mM HEPES-HCl (pH 7.6), 1 mM ethylenediamine tetraacetic acid (EDTA), 10 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40] containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 1 μg per ml each of leupeptin, aprotinin, pepstatin) and phosphatase inhibitors (5 mM sodium pyrophosphate, 50 μM sodium vanadate). The cell suspension was transferred to a 1.5 ml microfuge tube, incubated on ice for 15 min, vortexed for 10 s, and centrifuged for 30 s at $16{,}000 \times g$. The supernatant (cytoplasmic extract) was transferred to a new tube. The pelleted material was washed twice with 1 ml of ice-cold hypotonic buffer and resuspended in 0.1 ml of extraction buffer (20 mM HEPES-HCl pH 7.6, 1 mM EDTA, 430 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40) containing the protease and phosphatase inhibitors. After 20 min on ice with occasional shaking, the suspension was centrifuged for 15 min at $16{,}000 \times g$ at 4°C, and the supernatant (nuclear extract) was transferred to a new tube. After determination of protein concentration (Bradford, 1976) using Coomassie G-250 (Bio-Rad), aliquots of each extract were stored at −80°C until use.

Immunoblot analysis Nuclear (12 μg) or cytoplasmic (40 μg) extracts were fractionated on 10% SDS polyacrylamide (30:0.8) gels after heating for 2 min at 100°C in the presence of SDS and 2-mercaptoethanol. Proteins were then transferred to nitrocellulose membranes (0.2 μm) in a buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine, and 20% methanol at 15 V for 30 min at room temperature utilizing a semidry electrotransfer cell (Bio-Rad). Nonspecific binding was prevented by pretreating the membrane in 5% nonfat milk in TBST buffer [20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 0.5% Tween-20] for 1 h at room temperature. The blots were then incubated overnight at 4°C with AP-2α-specific mouse monoclonal antibody 3B5 (hybridoma supernatant), which is directed against an epitope between residues 165–200, just N-terminal to the DNA binding domain (Turner *et al*, 1998). After three 15 min washes in TBST buffer, the blots were incubated 1 h with peroxidase-conjugated goat antimouse IgG (Amersham) diluted 1:5000 with TBST containing 5% nonfat milk. Blots were then given three 15 min washes in TBST buffer and developed using the ECL Plus system (Amersham).

Electrophoretic mobility shift assay Synthetic single strand DNA oligonucleotides were purified in a denaturing 10% polyacrylamide gel and annealed to their complementary oligonucleotides (Sambrook *et al*, 1989). Probes were end-labeled by filling in the recessed 3′ ends with [α-^{32}P]dCTP using the Klenow fragment of *Escherichia coli* PolI. Approximately 3 μg of nuclear or 8 μg of cytoplasmic extract were preincubated on ice for 15 min with 0.5 μg of poly dI:dC in binding buffer (20 mM HEPES–HCl pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 120 mM NaCl, 10% glycerol). The labeled probe (0.02 pmol, 2×10^5 cpm) was then added and the incubation was continued for an additional 45 min on ice in a final volume of 20 μl. For competitive studies, the reaction mixtures were preincubated for 15 min on ice with various amounts of unlabeled competitor oligonucleotide before addition of the labeled DNA. DNA–protein complexes were resolved in a 6% nondenaturing polyacrylamide (29:1)

gel in 0.25 × TBE buffer (pH 8.3) containing 22.5 mM Tris, 22.5 mM borate, and 0.5 mM EDTA. The gels were dried, exposed to film, and quantified by phosphorimaging. For supershift analysis, reaction mixtures were prepared as above without the radioactive DNA probe and preincubated with 1 μl of specific antibody for 1 h on ice. The ^{32}P-labeled DNA probe was added and incubation on ice was continued for an additional 45 min. DNA–protein–antibody complexes were then electrophoresed and detected as above. Specific antibodies were mouse monoclonal 5E4 (AP-2α) and rabbit polyclonal epitope-selected β94 (AP-2β) and γ96 (AP-2γ). Each of these antibodies is effective in specifically recognizing only a single form of AP-2 (Turner *et al*, 1998; T. Williams, unpublished).

Oligonucleotides used in this study, shown below, have a single G overhang at the 5′ end of one or both strands to allow labeling by filling in with [^{32}P]dCTP. In oligonucleotide (1) a 27-mer containing the AP-2 response element spans positions −653 to −628 in the human involucrin promoter plus an added G overhang at each 5′ end. The consensus binding site is shown in bold, where underlined bases are those mutated in the binding site in oligonucleotide (2). Oligonucleotide (3), a 24-mer spanning positions −132 to −110 in the involucrin proximal promoter (plus an added G overhang at the proximal end) contains an AP-1 response element shown in bold that is known to be active in transcription (Welter *et al*, 1995). As an indication of specificity, binding of nuclear extract protein to (1) was prevented by a 25–50-fold excess of the unlabeled oligonucleotide but was not affected by either (2) or (3) (data not shown). The former oligonucleotide, with three point mutations in strategic locations, did not form a protein complex when radiolabeled and incubated with nuclear extract.

(1) 5′-GAACTGCA**GCCTCGGGC**ATAGAGGCT-3′ 27-mer
3′-TTGACGT**CGGAGCCCG**TATCTCCGAG-5′
(2) 5′-CTCTAACTGCA**AACTCGTGC**ATAGAGGCTGGG-3′ 33-mer
3′-GAGATTGACGT**TTGAGCACG**TATCTCCGACCCG-5′
(3) 5′-GCCTGTGG**TGAGTCA**GGAAGGGG-3′ 24-mer
3′-GGACACC**ACTCAGT**CCTTCCCCG-5′

Immunocytochemistry Cultures were grown in small glass or plastic culture chamber slides and fixed at various times in PBS containing 2% p-formaldehyde for 24 h. The slides were rinsed three times in PBS, incubated in permeabilizing buffer [PBS with 10 mg per ml bovine serum albumin (BSA) and 1% Triton X-100] for 1 h, incubated in blocking solution (PBS with 10 mg per ml BSA and 0.5% Tween-20) with two changes (15 min each), and finally incubated in 3B5 monoclonal anti-AP2α antibody or control ascites overnight at 4°C in a moist chamber. The slides were then rinsed twice with blocking solution (15 min each with agitation), and blocked in goat serum (1:50 in blocking solution) for 30 min. Slides were then incubated in goat antimouse Alexa Fluor 488 or goat antimouse Texas Red (Molecular Probes, Eugene, OR) for 1 h, and rinsed in PBS with 0.5% Tween-20 for 1 h with agitation. Slides stained with Alexa Fluor 488 were counterstained with the DNA-specific stain BO-PRO-3 iodide (Molecular Probes) for 10 min and rinsed in PBS. Slides were mounted in mounting medium (90% glycerol/10% PBS with 0.2% n-propyl gallate) and examined using scanning laser confocal microscopy. A Bio-Rad MRC 600 scanning laser confocal microscope was used for all image collections (Cherr *et al*, 1999). Either a 20× or a 60× ultraviolet-corrected objective lens was used, and Kalman averaging in the slow scan mode was employed for most samples. Both the 488 nm and 568 nm laser lines were employed to observe Alexa Fluor 488 and BoPro fluorescence simultaneously. Samples that had only Alexa 488 or BoPro were observed to ensure there was no bleedthrough of signal between the different channels. Control samples (control ascites or no primary antibody) were observed first for each experiment and black level and gain were set based on whatever background fluorescence was present. All subsequent images were collected at exactly the identical settings to ensure direct comparability between treatments.

RESULTS

Time course of AP-2α, involucrin, and TGM1 mRNA expression Levels of AP-2α mRNA were measured in hEp cultures at various degrees of confluence. A probe that detected the various alternatively spliced forms showed that the mRNA level increased greatly starting at confluence and was maintained at maximal values, 4–5-fold higher than in preconfluent cells, for approximately 2 wk (**Fig 1**). A probe specific for AP-2αB, a splice variant serving as a negative regulator (Buettner *et al*, 1993), gave a distinct pattern, low in log phase cultures, reaching a maximum

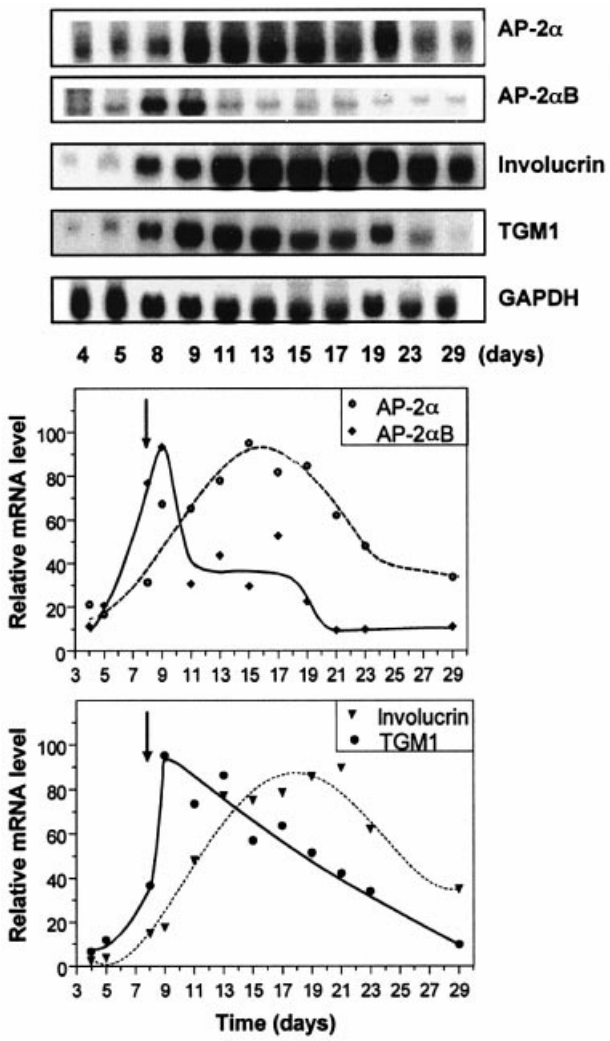

Figure 1. Expression of AP-2α, involucrin, and TGM1 mRNA as a function of time. Illustrated at the top are representative phosphorimages of hybridization signals from the mRNA at the times indicated. The bottom two panels show the relative amounts of mRNA normalized to the GAPDH signal for each lane. Each value is the mean of two independent experiments. The vertical arrow at day 8 shows when the cultures reached confluence.

immediately after confluence, and then dropping sharply to half or less of its maximal value. The maximal value of AP-2αB, around day 9, was estimated as 10% of the maximal value of total AP-2α detected with the general probe (judging by autoradiography and phosphorimaging), but it was usually expressed at a much lower level. Such a relatively low level may have prevented its detection in a developmental study of AP-2α in mouse skin, where it was found only in the dermis (Byrne *et al*, 1994).

During the transition from log phase growth to slower growth after confluence, a number of differentiation markers are known to increase in expression. As indicators of this process, mRNA levels were measured for involucrin and TGM1, which are expressed suprabasally in culture and in upper spinous cells in the epidermis *in vivo* (Thacher and Rice, 1985). As is evident in **Fig 1**, expression of both markers was very low during log phase growth. Levels of TGM1 mRNA increased at least 10-fold and reached a maximum a day after confluence, whereas levels of involucrin mRNA increased at least 30-fold, reaching maximal values a week after confluence. As shown in **Fig 1** the time course of AP-2α mRNA expression resembled that of the two differentiation markers.

Electrophoretic mobility shift analysis of AP-2 levels and isoforms To complement the time course of AP-2 mRNA expression, nuclear extracts from cultures at various stages of growth were compared by mobility shift assay. As shown in **Fig 2**(*A*), AP-2 response element binding activity was relatively high in subconfluent cultures but dropped greatly after confluence to 6%–20% of maximal values. By contrast, cytoplasmic extracts prepared from the same cells contained low AP-2 DNA binding activity at any stage of growth (**Fig 3**). In the experiment illustrated, the degree of complex formation was 20-fold higher using nuclear extracts from half-confluent cultures than from any of the cytoplasmic extracts. Addition of cytoplasmic extract from postconfluent cells did not reduce DNA binding activity of nuclear extract from preconfluent cells.

To identify the AP-2 isoforms in complexes observed in band shift experiments, nuclear extracts were incubated with antibodies specific for the α, β, or γ family members. As seen in **Fig 4**, a supershifted band was evident in the extracts of preconfluent through postconfluent cultures, but only using antibodies reacting with AP-2α. No supershifts, regardless of cell growth state, were observed using antibodies specific for AP-2β or AP-2γ. AP-2β mRNA has been detected in adult mouse skin, but the levels were much lower than of AP-2α (Moser *et al*, 1995).

Distribution of AP-2α protein between nuclear and cytoplasmic extracts The relative amounts of AP-2 protein were examined by immunoblotting using a monoclonal antibody specific for the α isoform. As shown in **Fig 5**, the relative amount of this protein in nuclear extracts decreased markedly within 2 d after confluence in a striking parallel to the DNA binding activity evident in mobility shift experiments. By contrast, the protein in cytoplasmic extracts increased markedly after confluence, correlating well with the increasing mRNA level. No difference was evident in mobilities of the major band of immunoreactive protein in the two types of extract.

Localization of AP-2α by laser confocal microscopy As shown in **Fig 6**(*A*), AP-2 immunoreactivity in rapidly growing preconfluent human epidermal cells, which exhibited little stratification, was indeed concentrated in the cell nuclei. By contrast, in cultures that recently reached confluence, a gradient of AP-2 localization was evident. Basal cells (**Fig 6*B***) appeared similar to the preconfluent cells, with prominent staining in the nucleus and relatively little in the cytoplasm. The superficial cells often exhibited uniform staining, however, with little additional stain visible in the nucleus (**Fig 6*C***). In several experiments, linear wounds were created in confluent cultures, and the cells migrating into the wound were examined. The AP-2 staining pattern was essentially identical to that observed in cells of the basal layer from which the migrating cells were derived.

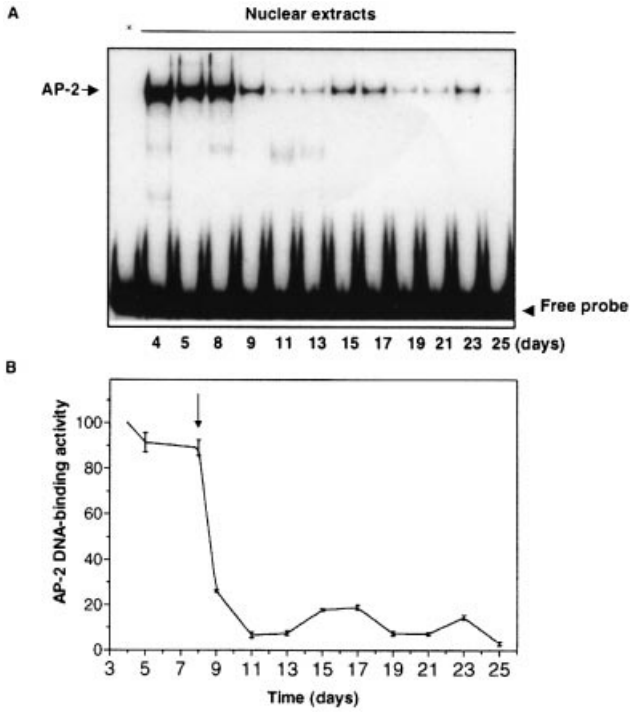

Figure 2. AP-2 DNA binding activity in nuclear extracts. Binding activities were measured using nuclear extracts from cultures harvested on the days indicated. (*A*) A representative phosphorimage of a resulting gel; (*B*) relative AP-2 DNA binding activity in nuclear extracts normalized to the binding in the day 4 extract. The asterisk (*) denotes a lane without added extract. Values in the graph represent the means and standard deviations from three independent experiments. The vertical arrow at day 8 shows when the cultures reached confluence.

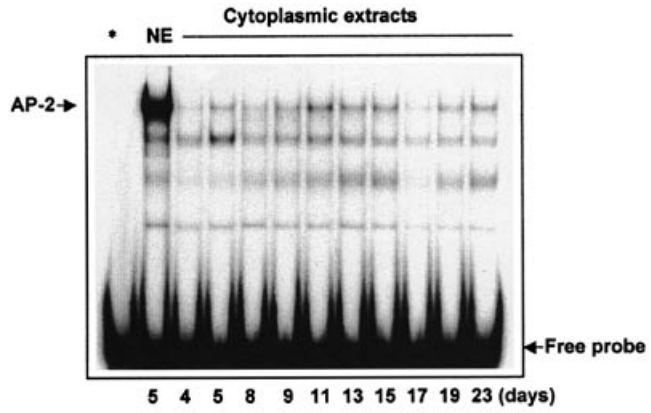

Figure 3. AP-2 DNA binding activity in cytoplasmic extracts. Binding activities were measured using cytoplasmic extracts from cultures harvested on the days indicated. The asterisk (*) denotes a lane without added extract. NE denotes a lane in which nuclear extract from half-confluent cultures was examined in parallel for comparison.

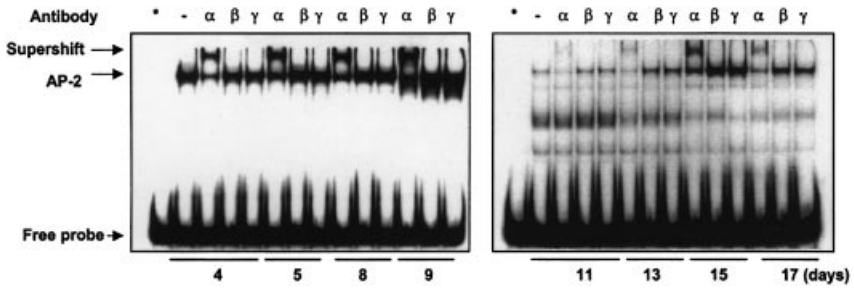

Figure 4. Identity of AP-2 isoforms interacting with the AP-2 response element. Protein complexes were analyzed using nuclear extracts from cultures harvested on the indicated days. Prior to the electrophoresis, incubations were performed in the presence of antibodies specific for the α, β, or γ AP-2 isoforms as shown above the lanes. The asterisk (*) denotes lanes without added extract, and the minus sign (–) denotes samples prepared without added antiserum.

AP-2 localization was influenced markedly in the superficial cells of confluent cultures by changing the medium. This manipulation induced an increase in nuclear staining evident after 3–8 h and largely gone after a day. **Figure 6** shows the striking contrast in staining pattern obtained 3 d (*panel C*) *vs* 8 h (*panel D*) after a medium change. Several other manipulations of physiologic conditions had minor if any effect. For example, cells grown in serum-free medium containing low levels of calcium (90 μM) exhibited some cytoplasmic but stronger nuclear staining, a pattern that was not much affected by raising the calcium level to 0.5 mM for 3 d. Similarly, treating the cultures with 12-O-tetradecanoyl-phorbol-13-acetate or forskolin for periods up to several hours did not induce a clear change in distribution.

Cultures of the spontaneously immortalized human epidermal keratinocyte line (SIK), which is minimally deviated from normal in most properties (Rice *et al*, 1993), exhibited nearly the same AP2 localization patterns as normal human epidermal cells with respect to growth, stratification, and medium change. The presence or absence of EGF in the medium, to which the cell growth is quite sensitive, had little if any effect. In contrast to the normal and SIK cultures, which showed considerable cytoplasmic staining at confluence, the malignant SCC-9 cells (which remain as a monolayer) displayed a high concentration of staining in the nucleus regardless of cell density (**Fig 7*A***). The striking contrast between the SCC-9 pattern and the extreme cytoplasmic staining attainable in normal cells confluent for 3 wk is shown in **Fig 7(*B*)**. For further comparison, 3T3 cells were examined, as it was evident in feeder layer cultures that their pattern was distinct. This distribution, which also did not appear to be dependent upon cell density, was characterized by clear cytoplasmic staining and a striking perinuclear concentration of the immunoreactivity (**Fig 7*C***).

DISCUSSION

Befitting a role of AP-2α in differentiation, stimulatory response elements for this transcription factor have been described in numerous gene promoters. In particular, its expression during development in ectodermally derived lineages, including epidermis (Mitchell *et al*, 1991), led to the recognition of a role for AP-2α in the appearance in fetal mouse skin of keratins 5 and particularly 14

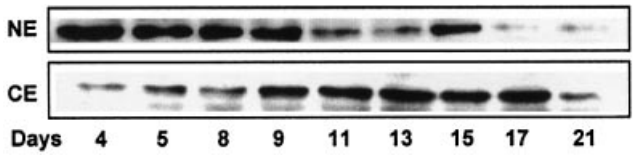

Figure 5. Distribution of AP-2α protein between nuclear and cytoplasmic extracts. Nuclear or cytoplasmic extracts (12 μg or 40 μg of protein, respectively) prepared from cultures harvested on the indicated days were resolved by 10% SDS polyacrylamide gel electrophoresis. The relative amounts of AP-2 protein were examined by immunoblotting using a monoclonal antibody specific for the α isoform.

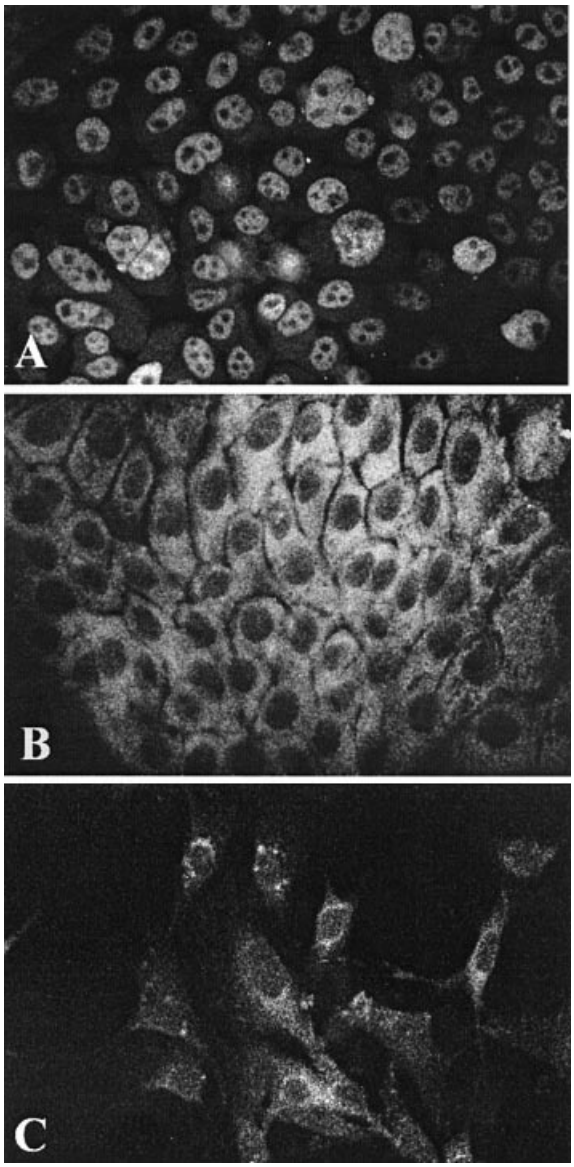

Figure 7. Contrast in localization between hEp, malignant keratinocytes, and 3T3 cells. (*A*) SCC-9 squamous carcinoma cells, showing predominant nuclear labeling. (*B*) Postconfluent hEp with almost exclusively cytoplasmic labeling. (*C*) 3T3 fibroblasts, showing labeling that is cytoplasmic and perinuclear.

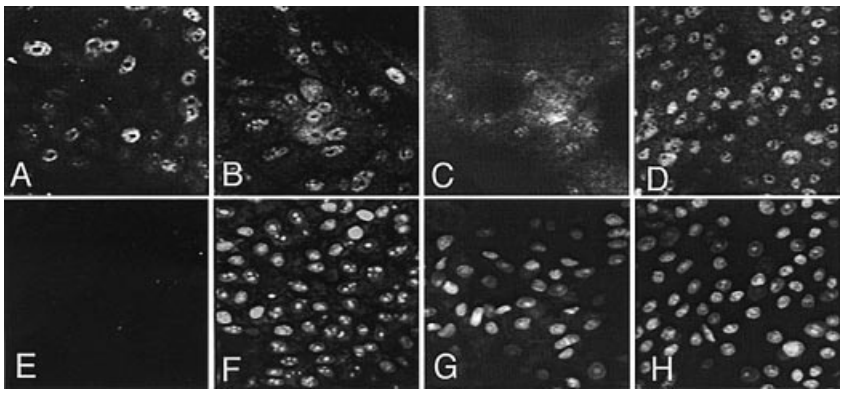

Figure 6. AP-2 localization in cultured hEp. (*A*) Preconfluent culture showing localization primarily in nuclei. (*B*) Confluent culture showing localization primarily in nuclei of basal cells. (*C*) Confluent culture 3 d after medium change, showing diffuse cytoplasmic labeling in the most superficial layer of cells. (*D*) Confluent culture 8 h after medium change, showing marked nuclear staining in the most superficial layer of cells. (*E*) Control preconfluent culture processed in parallel with (*A*) but lacking primary antibody. (*F*), (*G*), (*H*) Same fields as (*B*), (*C*), and (*D*), respectively, counterstained with nuclear-specific BoPro stain.

(Byrne *et al*, 1994), which have AP-2 response elements in their promoters and are expressed primarily in basal keratinocytes. In cotransfections, AP-2α stimulates the expression of reporters driven by the keratin promoters in cell lines such as 3T3 and HepG2, where these keratins are not normally seen, but it is clear that AP-2α alone is insufficient for expression *in vivo* (Magnaldo *et al*, 1993; Byrne *et al*, 1994). On the other hand, a decrease in AP-2α protein or DNA binding has been associated with later stages of differentiation. For example, in the case of corneal epithelial cells, the reduction in AP-2 response element binding observed after confluence in nuclear extracts correlated with induction of keratin 3 (Chen *et al*, 1997). AP-2 has recently been found to regulate expression of keratin 10 indirectly, by suppressing C/EBPα, a stimulator of keratin 10 expression, in basal cells where AP-2 immunostaining was evident but not in superficial cells where staining was much reduced (Maytin *et al*, 1999). Our results provide insight into one mechanism to achieve selective AP-2 activity in basal cells. In these cells, we hypothesize that AP-2 protein is translated and imported into the nucleus where it contributes to transcription of basal cell keratins 5 and 14 and suppresses keratin 10 expression. As cells move superficially, AP-2 activity is down-regulated, contributing to loss of keratin 5 and keratin 14 expression and commencement of keratin 10 gene transcription. We suggest that, at least under certain conditions, such as the hyperproliferative state that occurs in cell culture, AP-2 down-regulation is achieved by relocalization to the cytoplasmic compartment. This may well be a reversible process so that, when the cell receives the appropriate signal, AP-2 can be rapidly re-imported into the nucleus and resume transcriptional activity. Our finding that a medium change in culture stimulates nuclear relocalization suggests that this mechanism may allow cells to respond rapidly to a growth stimulus.

The mechanism regulating AP-2 localization remains to be explored. Other examples of transcription factors exhibiting this phenomenom may provide insight. One is the p65 subunit of NFkB, which resides in the cytoplasm of resting cells in a complex with the anchoring protein, IkB. When an incoming signal targets IkB for proteolysis, NFkB is freed to enter the nucleus where it exhibits transcriptional activity (reviewed in Mercurio and Manning, 1999). Localization of another transcription factor, NFAT, is regulated by the phosphorylation state of critical serine residues, which results in masking of its nuclear localization signal and exposing a nuclear export signal (Okamura *et al*, 2000). Activation of calcineurin, a Ca^{2+} activated phosphatase, dephosphorylates these sites, resulting in rapid nuclear import.

Evidence has accumulated that the transactivating ability of the AP-2 protein can be altered both by interaction with other proteins and by post-translational modification. AP-2α is capable of forming complexes with a number of proteins including several transcriptional coactivators (Kannan *et al*, 1999), the Myc and RB proteins (Gaubatz *et al*, 1995; Batsche *et al*, 1998; Wu and Lee, 1998), and SV40 T antigen (Mitchell *et al*, 1987). Binding to other protein or post-translational modification has also been invoked to explain the observation that AP-2αB inhibits the DNA binding of AP2αA in cell extracts but not when the latter is expressed by bacteria (Buettner *et al*, 1993). Initial characterization of AP-2 transactivating activity in HeLa cells revealed stimulation by agents increasing activity of protein kinases A or C (Imagawa *et al*, 1987) without affecting AP-2 mRNA or protein levels (Luscher *et al*, 1989). A dramatic increase in AP-2 response element binding, observed upon treatment of hepatic stellate cells with a 5-lipoxygenase inhibitor, has been attributed to post-translational modification of the protein (Chen *et al*, 1996). Our results are thus highly compatible with the loss of DNA binding activity and cytoplasmic relocalization of AP-2α protein in postconfluent keratinocyte cultures being due to altered post-translational modification or binding to a protein not yet identified. A plausible scenario would involve sequestering AP-2 in an inactive state in the cytoplasm in differentiating keratinocytes from which it is released by a phosphorylation or dephosphorylation event that alters either the factor itself or a binding protein. Although AP-2α is known to be phosphorylated by protein kinase A *in vitro* and upon cAMP treatment of adipocytes (Park and Kim, 1993; Garcia *et al*, 1999), treatment of keratinocytes with activators of protein kinases A or C did not alter its localization in this work.

Despite a progressive loss of nuclear AP-2 DNA binding activity as cultured epidermal keratinocytes differentiate, our results show a striking increase in AP-2α mRNA at confluence in keratinocyte cultures, nearly in parallel with increases in mRNA for two prominent differentiation markers. This emphasizes that high expression levels of the mRNA are not necessarily accompanied by high levels of transactivation at AP-2 response elements. In addition, the overall AP-2 immunofluorescence intensity was reduced in the superficial cells, suggesting that the protein level also decreased with differentiation. A similar observation has been made upon transformation of PA-1 human teratocarcinoma cells by an N-ras oncogene, which led to greater than a 6-fold increase in AP-2α mRNA but also to a dramatic reduction in AP-2-dependent transactivation (Kannan *et al*, 1994).

In some cells, increased AP-2 expression is associated with increased growth, which may result from autocrine or paracrine stimulation by factors it induces such as transforming growth factor α (Berkowitz *et al*, 1997), insulin-like growth factor II (Zhang *et al*, 1998), insulin-like growth factor binding protein 5 (Duan and Clemmons, 1995), ERBB-2 (Bosher *et al*, 1995), and perhaps insulin-like growth factor I receptor (Turner *et al*, 1998). On the other hand, AP-2 can also mediate reduction of growth by stimulating $p21^{WAF/CIP1.}$ expression (Zeng *et al*, 1997) or complex formation with Myc, impairing the latter's transactivating ability (Gaubatz *et al*, 1995). Efforts have been made to link AP-2 expression to the neoplastic state and prognosis of human tumors, but results of tumor surveys so far are mixed, with loss of expression associated with invasiveness in breast cancer (Gee *et al*, 1999) and malignant melanoma (Karjalainen *et al*, 1998) but with no prognostic value in colorectal (Ropponen *et al*, 1999) or prostate cancer (Lipponen *et al*, 2000).

AP2α has recently been detected cytoplasmically in certain neoplasms, suggesting that intracellular localization needs to be considered in judging prognosis. Indeed, in the case of ovarian tumors, nuclear localization combined with low cytoplasmic expression is associated with poorer survival (Anttila *et al*, 2000). This observation is compatible with our findings that hEp exhibit cytoplasmic localization associated with keratinocyte differentiation, whereas malignant SCC-9 cells display predominantly nuclear localization at all times. Whether or not the neoplastic behavior of tumor cells is a direct consequence of the localization of AP-2, perturbation of its translocation from nucleus to cytoplasm is of considerable interest. Keratinocytes promise to be a good model system for elucidating the molecular basis of this phenomenon.

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REFERENCES

Allen-Hoffmann BL, Rheinwald JG: Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. *Proc Natl Acad Sci USA* 81:7802–7806, 1984

Anttila MA, Kellokoski JK, Moisio KI, Mitchell PJ, Saarikoski S, Syrjanen K, Kosma V-M: Expression of transcription factor AP-2α predicts survival in epithelial ovarian cancer. *Brit J Cancer* 82:1974–1983, 2000

Batsche E, Muchardt C, Behrens J, Hurst H, Cremisi C: RB and c-myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Molec Cell Biol* 18:3647–3658, 1998

Berkowitz EA, Hecht CP, Azizkhan JC, Chen X, Lee DC: Transcription factor AP2 is required for expression of the rat transforming growth factor-α gene. *Oncogene* 14:2229–2238, 1997

Bosher JM, Williams T, Hurst HC: The developmentally regulated transcription factor AP-2 is involved in c-erbB-2 overexpression in human mammary carcinoma. *Proc Natl Acad Sci USA* 92:744–747, 1995

Bosher JM, Totty NF, Hsuan JJ, Williams T, Hurst HC: A family of AP-2 proteins

regulates c-*erb*B-2 expression in mammary carcinoma. *Oncogene* 13:1701–1707, 1996

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976

Buettner R, Kannan P, Imhof A, *et al:* An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. *Molec Cell Biol* 13:4174–4185, 1993

Byrne C, Tainsky M, Fuchs E: Programming gene expression in developing epidermis. *Development* 120:2369–2383, 1994

Chazaud C, Oulad-Abdelghani M, Bouillet P, Decimo D, Chambon P, Dolle P: AP2.2, a novel gene related to AP-2, is expressed in the forebrain, limbs and face during mouse embryogenesis. *Mech Dev* 54:83–94, 1996

Chen A, Beno DWA, Davis BH: Suppression of stellate cell type I collagen gene expression involves AP-2 transmodulation of nuclear factor-1-dependent gene transcription. *J Biol Chem* 271:25994–25998, 1996

Chen T-T, Wu R-L, Castro-Munozledo F, Sun T-T: Regulation of K3 keratin gene transcription by Sp1 and AP-2 in differentiating rabbit corneal epithelial cells. *Molec Cell Biol* 17:3056–3064, 1997

Cherr GN, Yudin AI, Li M-W, Vines CA, Overstreet JW: Hyaluronic acid and the cumulus extracellular matrix induce increases in intracellular calcium in macaque sperm via the plasma membrane protein PH-20. *Zygote* 7:211–222, 1999

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Duan C, Clemmons DR: Transcription factor AP-2 regulates human insulin-like growth factor binding protein-5 gene expression. *J Biol Chem* 270:24844–24851, 1995

Eckert RL, Green H: Structure and evolution of the human involucrin gene. *Cell* 46:583–589, 1986

Eckert RL, Crish JF, Banks EB, Welter JF: The epidermis. Genes on – genes off. *J Invest Dermatol* 109:501–509, 1997

Garcia MA, Campillos M, Marina A, Valdivieso F, Vasquez J: Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation. *FEBS Lett* 444:27–31, 1999

Gaubatz S, Imhof A, Dosch R, Werner O, Mitchell P, Buettner R, Eilers M: Transcriptional activation by Myc is under negative control by the transcription factor AP-2. *EMBO J* 14:1508–1519, 1995

Gee JM, Robertson JF, Ellis IO, Nicholson RI, Hurst HC: Immunohistochemical analysis reveals a tumor suppressor-like role for the transcription factor AP-2 in invasive breast cancer. *J Pathol* 189:514–520, 1999

Gelman DK, Mutero A, Inoue K, Taylor P: Transcription factor repression and activation of the human acetylcholinesterase gene. *J Biol Chem* 270:23511–23519, 1995

Green H: The keratinocyte as differentiated cell type. *Harvey Lect* 74:101–138, 1979

Imagawa M, Chiu R, Karin M: Transcription factor AP-2 mediates induction by two different signal-transduction pathways. Protein kinase C and cAMP. *Cell* 51:251–260, 1987

Iuchi S, Easley K, Matsuzaki K, Weiner L, O'Connor N, Green H: Alternative subcellular locations of keratinocyte basonuclin. *Exp Dermatol* 9:178–184, 2000

Kannan P, Buettner R, Chiao PJ, Yim SO, Sarkiss M, Tainsky MA: N-*ras* oncogene causes AP-2 transcriptional self-interference, which leads to transformation. *Genes Dev* 8:1258–1269, 1994

Kannan P, Yu Y, Wankhade S, Tainsky MA: Poly ADP-ribose polymerase is a coactivator for AP-2-mediated transcriptional activation. *Nucl Acids Res* 27:866–874, 1999

Karjalainen JM, Kellokoski JK, Eskelinen MJ, Alhava EM, Kosma VM: Downregulation of transcription factor AP-2 predicts poor survival in stage I cutaneous malignant melanoma. *J Clin Oncol* 16:3584–3591, 1998

Lipponen P, Aaltomaa S, Kellokoski J, Ala-Opas M, Kosma V-M: Expression of activator protein 2 in prostate cancer is related to tumor differentiation and cell proliferation. *Europ Urol* 37:573–578, 2000

Luscher B, Mitchell PJ, Williams T, Tjian R: Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. *Genes Dev* 3:1507–1517, 1989

Maconochie M, Krishnamurthy R, Nonchev S, Meier P, Manzanares M, Mitchell PJ, Krumlauf R: Regulation of Hoxa2 in cranial neural crest cells involves members of the AP-2 family. *Development* 126:1483–1494, 1999

Magnaldo T, Vidal RG, Ohtsuki M, Freedberg IM, Blumenberg M: On the role of AP2 in epithelial-specific gene expression. *Gene Expression* 3:307–315, 1993

Maytin EV, Lin JC, Krishnamurthy K, Bathvarova N, Ron D, Mitchell PJ, Habener JF: Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP2. *Dev Biol* 216:164–181, 1999

Meier P, Koedood M, Philipp J, Fontana A, Mitchell PJ: Alternative mRNAs encode multiple isoforms of transcription factor AP-2 during murine embryogenesis. *Dev Biol* 169:1–14, 1995

Mercurio F, Manning AM: Multiple signals converging on NF-kB. *Curr Opin Cell Biol* 11:226–232, 1999

Mitchell PJ, Wang C, Tjian R: Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50:847–861, 1987

Mitchell PJ, Timmons PM, Hebert JM, Rigby PWJ, Tjian R: Transcription factor AP-2 is expressed in neural crest lineages during mouse embryogenesis. *Genes Dev* 5:105–119, 1991

Moser M, Imhof A, Pscherer A, *et al:* Cloning and characterization of a second AP-2 transcription factor: AP-2β. *Development* 121:2779–2788, 1995

Ohtaka-Maruyama C, Hanaoka F, Chepelinsky AB: A novel alternative spliced variant of the transcription factor AP2α is expressed in the murine ocular lens. *Dev Biol* 202:125–135, 1998

Okamura H, Aramburu J, Garcia-Rodriguez C, *et al:* Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 6:539–550, 2000

Oulad-Abdelghani M, Bouillet P, Chazaud C, Dolle P, Chambon P: AP-2.2. A novel AP-2-related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp Cell Res* 225:338–347, 1996

Park K, Kim KH: The site of cAMP action in the insulin induction of gene expression of acetyl-CoA carboxylase is AP-2. *J Biol Chem* 268:17811–17819, 1993

Phillip J, Mitchell PJ, Malipiero Fontana A: Cell type-specific regulation of expression of transcription factor AP-2 in neuroectodermal cells. *Dev Biol* 165:602–614, 1994

Phillips MA, Stewart BE, Qin Q, Chakravarty R, Floyd EE, Jetten AM, Rice RH: Primary structure of keratinocyte transglutaminase. *Proc Natl Acad Sci USA* 87:9333–9337, 1990

Rheinwald JG, Beckett MA: Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous carcinomas. *Cancer Res* 41:1657–1663, 1981

Rice RH, Steinmann KE, deGraffenried LA, Qin Q, Taylor N, Schlegel R: Elevation of cell cycle control proteins during spontaneous immortalization of human keratinocytes. *Molec Biol Cell* 4:185–194, 1993

Ropponen KM, Kellokoski JK, Lipponen PK, Pietilainen T, Eskelinen MJ, Alhava EM, Kosma VM: p22/WAF1 expression in human colorectal carcinoma. Association with p53, transcription factor AP-2 and prognosis. *Br J Cancer* 81:133–140, 1999

Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1989

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ: Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* 381:235–238, 1996

Schreiber E, Matthias P, Muller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. *Nucl Acids Res* 17:6419, 1989

Sinha S, Degenstein L, Copenhaver C, Fuchs E: Defining the regulatory factors required for epidermal gene expression. *Molec Cell Biol* 20:2543–2555, 2000

Talbot D, Loring J, Schorle H: Spatiotemporal expression pattern of keratins in skin of AP-2α-deficient mice. *J Invest Dermatol* 113:816–820, 1999

Thacher SM, Rice RH: Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* 40:685–695, 1985

Todaro GJ, Green H: Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* 17:299–313, 1963

Turner BC, Zhang J, Gumbs AA, *et al:* Expression of AP-2 transcription factors in human breast cancer correlates with the regulation of multiple growth factor signalling pathways. *Cancer Res* 58:5466–5472, 1998

Welter JF, Crish JF, Agarwal C, Eckert RL: Fos-related antigen (Fra-1), jun B, and jun D activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. *J Biol Chem* 270:12614–12622, 1995

Williams T, Admon A, Luscher B, Tjian R: Cloning and expression of AP-2, a cell type-specific transcription factor that activates inducible enhancer elements. *Genes Dev* 2:1557–1569, 1988

Williams T, Tjian R: Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev* 5:670–682, 1991

Wu F, Lee AS: Identification of AP-2 as an interactive target of Rb and a regulator of the G1/S control element of the hamster histone H3.2 promoter. *Nucl Acids Res* 26:4837–4845, 1998

Zeng Y-X, Somasundaram K, El-Deiry WS: AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nature Genet* 15:78–82, 1997

Zhang J, Hagopian-Donaldson S, Serbedzija G, *et al:* Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* 381:238–241, 1996

Zhang L, Zhan S, Navid F, *et al:* AP-2 may contribute to IGF-II overexpression in rhabdomyosarcoma. *Oncogene* 17:1261–1270, 1998