Characterization of human involucrin promoter distal regulatory region transcriptional activator elements – a role for Sp1 and AP1 binding sites

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Human involucrin (hINV) is an important precursor of the keratinocyte cornified envelope that is specifically expressed in the suprabasal layers of stratifying epithelia. Previous truncation and mutagenesis experiments have shown that an activator protein 1 (Ap1) site, AP1-5, located 2100 bp upstream of the transcription start site, is required for optimal promoter activity. These previous studies suggest that AP1-5 is part of a distal regulatory region spanning nucleotides –2473 to –2088. In the present report, we study the distal regulatory region (DRR), which surrounds AP1-5. Our studies show that this region contains weak and strong activator elements spanning nucleotides –2473/–2216 and –2140/–2088, respectively. The strong activator element contains AP1-5 and an adjacent specificity protein 1 (Sp1) site. The AP1-5 site is absolutely required for DRR activity, as its mutation reduces transcription to basal levels. Mutagenesis studies of the AP1-5 and Sp1 sites in the

presence or absence of the weak activator element indicate that the Sp1 site and the weak activator element synergistically activate the AP1-5 site-dependent transcription. The cooperation between the Sp1 and AP1-5 sites is also observed in the context of the full-length promoter. Gel mobility shift and supershift studies show that Sp1, but not Sp2, Sp3 or Sp4 binds to the Sp1 site. When the Sp1 site is mutated or the distance between the AP1-5 and Sp1 site is increased, the binding of AP1 factors to AP1-5 is markedly reduced. Surprisingly, gel shift studies suggest that activation does not require the formation of a stable AP1/Sp1/DNA ternary complex. These studies suggest that the AP1-5 site is absolutely required for transcriptional activation, that the weak activator element and Sp1 sites serve to enhance this activation, and that the Sp1 site is required for optimal AP1 factor binding at the AP1-5 site.

INTRODUCTION

Human epidermis includes several distinct layers [1]. The proliferative keratinocytes are located in the basal layer adjacent to the dermis. As these keratinocytes differentiate, they migrate outwards to the surface of the skin. This process entails extensive morphological remodelling and biochemical change. Early in this process, the cells lose the ability to proliferate, and ultimately, they are transformed into corneocytes. The corneocyte is a dead cell that consists of a network of cytokeratin filaments surrounded by a cornified envelope, which consists of covalently crosslinked protein [1–9]. Several genes that are activated during differentiation encode proteins that are precursors of the corneocyte cornified envelope. These envelope precursor proteins, and transglutaminase, the enzyme activity responsible for assembly of the envelope, must be expressed at the correct time and appropriate level to permit proper envelope formation [10–14]. Involucrin is an important 68 kDa glutamine- and glutamic acid-rich precursor of the keratinocyte cornified envelope that is expressed early in keratinocyte differentiation [2–4,15–17]. It accumulates as a non-crosslinked precursor in granular and spinous layer cells [2,4] and becomes covalently crosslinked to other proteins as a participant in crosslinked envelope formation during the final stages in keratinocyte differentiation [17].

Transgenic mouse and cell culture transfection studies indicate that the promoter is expressed in a cell type-specific manner *in vivo* and *in vitro* [15,18–20]. The distal regulatory region (DRR) of the human involucrin (hINV) promoter, which includes the

nucleotides between –2473 to –2088 bp upstream of the transcription start site [19], is important for transcriptional activation. Our previous studies show that this region accounts for one-half of the basal and phorbol ester-induced activity of the hINV promoter [19]. This region contains binding sites for several known transcription factors, including specificity protein 1 (Sp1) and activator protein 1 (AP1). The AP1 (AP1-5) and Sp1 sites are separated by a single nucleotide. AP1 factors junB, junD and Fra-1 interact with the hINV promoter at the AP1-5 site [19]. This binding is important for maintenance of basal transcription and is also involved in mediating phorbol ester-dependent activation of the promoter [19]. AP1 factors have been shown to collaborate with Sp1 in other systems, but the role of the Sp1 site in the hINV promoter has not been studied. In the present study, we examine the function of the DRR region. Our studies show that the DRR can be divided into two discrete elements: a weak activator element that maps to nucleotides –2473/–2216, and a strong activator element that maps to nucleotides –2140/–2088. In the present manuscript, we study the strong activator element and show that an Sp1 site and the AP1-5 site are absolutely required for transcriptional activity. The AP1-5 site appears to function as an on/off switch, as when the AP1-5 is inactivated the entire DRR is silent. The weak activator element and the Sp1 site (which binds Sp1, but not Sp2, Sp3 or Sp4) cooperate with the AP1-5 site to maximally activate transcription. The Sp1 site appears to enhance AP1-5 site-dependent transcription by enhancing AP1 factor binding.

Abbreviations used: hINV, human involucrin; DRR, distal regulatory region; AP1, activator protein 1; HBSS, Hanks balanced salt solution; KSFM, keratinocyte serum free culture medium; PRR, proximal regulator region; Sp1, specificity protein 1.

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MATERIALS AND METHODS

Chemicals and reagents

Keratinocyte serum-free culture medium (KSFM), trypsin, Hanks balanced salt solution (HBSS), gentamicin, and Lipofectin reagent were obtained from Life Technologies, Inc. The pGL2-Basic plasmid, oligonucleotides containing AP1, Sp1, AP2 consensus sequences, and the luciferase assay system were from Promega. Dispace was obtained from Boehringer-Mannheim. Chemiluminescence was measured using a Berthold luminometer. Oligonucleotides for gel shifts and construction of mutant promoter sequences, were synthesized using an Applied Biosystems DNA synthesizer.

Plasmid construction

Construction of pINV-2473, pINV-2216, pINV-2100 (formerly pINV-2136) [19], pINV-1336 and pINV-41 has been previously described [19]. pINV-2473 was used as a template to produce the additional plasmids used in this study. Point and block mutations of the AP1-5 and Sp1 sites were created by digesting the promoter with restriction enzymes and replacing this region with an oligonucleotide containing the desired mutation. Plasmid sequences were confirmed by restriction mapping and/or DNA sequencing.

Tissue culture, cell transfection and luciferase assay

Primary human keratinocytes were cultured from human foreskin samples and prepared for transfection exactly as previously described [19,21]. Third passage keratinocytes were transfected in 60 mm diam. dishes when 60% confluent. Sixteen μ g of Lipofectin reagent and 4.5 μ g of test plasmid were mixed, added to the cells in 3 ml of KSFM, and incubated for 4 h at 37 °C. At 5 h, 3 ml of KSFM was added and the incubation continued for an additional 19 h. Fresh KSFM was then added and the incubation continued an additional 48 h with addition of fresh medium every 24 h.

Transfected keratinocytes were washed twice with phosphate-buffered saline, dissolved in 250 μ l of cell culture lysis reagent (Promega), and harvested by scraping. Luciferase assays were performed immediately using a Berthold luminometer and the Promega luciferase assay. Cell extracts (20 μ l) and 100 μ l of luciferin mixture were reacted for 4 s and light output was monitored over the next 20 s. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Luciferase activity was normalized per μ g of extract protein. This method of correction proved to be reliable and more convenient than the more elaborate procedure utilized previously [19].

Preparation of nuclear extracts

Keratinocytes (third passage) were grown in KSFM until 80% confluent. Nuclear extracts were prepared at approx. 1.0 mg protein/ml as previously described [19] according to the method of Dignam et al. [22,23].

Gel mobility shift assay

For mobility shift assays, a 20 μ l reaction containing 15% glycerol, 75 mM KCl, 0.375 mM dithiothreitol, 0.375 mM PMSF 12.5 mM NaCl, 0.1 mg/ml poly(dI-dC), 2.5 μ g of nuclear extract, and 0.3 ng radiolabelled DNA was incubated for 5 min at room temperature. The samples were immediately electrophoresed at 250 V for 1.5 h on a 5% non-denaturing acrylamide

gel using $\frac{1}{4} \times$ TBE running buffer, dried and autoradiographed. For competition studies, radioinert DNA competitor was added at 0- to 200-fold molar excess. For gel supershift assays, the complete gel mobility shift assay mixture, without the 32 P-labelled oligonucleotide, was incubated at 4 °C for 2 h in the presence of antibodies specific for Sp1, Sp2, Sp3 or Sp4 (SC-59X, SC-643X, SC-644X, and SC-645X, respectively, Santa Cruz) at 1 μ g rabbit IgG per reaction. The [32 P]DNA was then added to the mixture and incubated at room temperature for 5 min. The resulting complexes were electrophoretically separated and characterized as outlined above.

RESULTS

The human involucrin gene promoter drives transcription in human keratinocytes

Transfection of human keratinocytes with pINV-2473, a plasmid containing the full-length hINV promoter fused to the luciferase reporter gene (Figure 1A), produces strong transcriptional activation (Figure 1B). Compared to the full-length promoter, truncation to positions -2216 and -2100, respectively, consistently produces a 30 and 50% reduction in activity. No further reduction in activity is observed upon truncation to nucleotide -1336. The basal promoter, pINV-41, shows minimal activity. These results are consistent with our previous report [19], except

Figure 1 Deletion analysis of hINV promoter

The plasmids shown in (A), pINV-2473, pINV-2216, pINV-2100, pINV-1336 and pINV-41, were transfected into normal human keratinocytes and luciferase activity (B) was determined as described in the Materials and methods section. The results are expressed as luciferase activity (light units)/ μ g protein. The numbers at the top in (A) indicate the distance upstream of the transcription start site [19,39]. The filled circles, numbered 1-5, represent the five AP1 sites present in the promoter [19]. The sole Sp1 site is indicated by a filled box. The filled rectangle with the overlying arrow represents the luciferase coding region. The result is representative of three independent experiments. In this and other figures, promoter activity results are expressed as the ratio of luciferase activity/ μ g protein. The actual activity is obtained by multiplying this ratio by 1000.

Figure 2 Functional characterization of the distal regulatory region

Plasmids pINV(-2473/-2088), pINV(-2216/-2088), pINV(-2473/-2216) are schematically shown in (A). Each of the DNA segments indicated by a solid line were fused (indicated by a dashed line) to the hINV basal promoter, pINV-41. The AP1-5 site and Sp1 site are indicated in each construct by a solid circle and square, respectively. (B) The results of a functional analysis of activity for each construct, expressed as luciferase activity/ μ g protein. Similar results were observed in each of four experiments. Plasmids pINV-2473 and pINV-41 encode the intact full-length promoter and basal promoter, respectively.

that we now describe the reduction in activity that accompanies removal of the segment from -2473/-2216. These results suggest that the -2473/-2216 segment and the -2216/-2100 segment contain element(s) that combine to produce one-half of the activity of the hINV promoter. Point mutagenesis studies, described in our previous report, demonstrate that the most distal activator protein 1 site, AP1-5, shown in Figure 1(A), is necessary for this activity [19]. However, this site does not account for all of the activity. The goal of this paper is to characterize this upstream region, the distal regulatory region (DRR), in greater detail.

The distal regulatory region (DRR)

To study the DRR, the DNA segment from -2473/-2088 was fused to the hINV minimal promoter construct, pINV-41, to produce plasmid pINV(-2473/-2088). This plasmid produces a high level of transcriptional activity (i.e. six times more activity than the full-length promoter) (Figure 2). pINV(-2216/-2088), which lacks the -2473/-2216 segment, is less active than pINV(-2473/-2088), but is four times more active than pINV-2473. Thus, the sequence within -2216/-2088 drives 60% of the activity of the -2473/-2088 segment. These results are consistent with the results shown in Figure 1. Plasmid pINV(-2473/-2216) also drives transcription, but is less active. These results suggest that the -2216/-2088 segment, which contains the AP1-5 and Sp1 sites, is a strong transcriptional

Figure 3 Synergistic activation of hINV promoter activity by AP1-5 and Sp1

(B) The structure of the three plasmids, pINV(-2473/-2088), pINV(-2216/-2088) and pINV(-2140/-2088) tested in this experiment. The AP1-5 and Sp1 sites are indicated by a filled circle and square, respectively. The length of 100 bp is shown. (A) The sequence of the various AP1-5 and Sp1 mutations. The wild-type sequence is shown in the top line, mutated bases in the other sequences are underlined. For AP1-5, 'm' indicates a point mutation and 'mm' indicates a more drastic, block mutation (see Table 1). (C) The results from functional analysis of activity for each construct. The key indicates the identity of each construct. Similar results were observed in each of three experiments.

activator and that a less active activator is present in the -2473/-2216 segment.

The boundaries of the activator elements

The experiment shown in Figure 2 suggests that a weak transcriptional activator exists in the -2473/-2216 segment and that a strong activator exists in the -2216/-2088 segment. This hypothesis is tested as part of the experiment shown in Figure 3. Our results show that addition of the region from -2216/-2140 to plasmid pINV(-2140/-2088) to make pINV(-2216/-2088) does not change activity (AP1-5/Sp1, Figure 3C). However, subsequent addition of the -2473/-2216 segment increases activity by 40% (AP1-5/Sp1, Figure 3C). These results, combined with the results shown in Figure 2, strongly suggest that the -2473/-2140 region contains an independent element that modulates the activity of the -2140/-2088 segment and that this element is located between -2473 and -2216.

Figure 4 Effect of AP1-5 and Sp1 mutations in the context of the full-length hINV promoter

(B) The structure of pINV-2473 with the five AP1 sites indicated by filled circles and the sole Sp1 site identified by a filled square. (A) The sequence of the various AP1-5 and Sp1 mutations. The conventions are the same as in Figure 3. (C) The results of functional analysis of each construct, expressed as luciferase activity (light units)/ μ g protein. The results are representative of five experiments.

Role of the weak activator, and the Sp1, and AP1-5 sites

Previous studies suggest that Sp1, a ubiquitously expressed general transcription factor, can cooperate with other transcription factors to modulate transcription [24–29]. We sought to determine the contribution of the AP1-5 and Sp1 sites in the

hINV DRR. We constructed point or block mutations in AP1-5, Sp1 and in both sites simultaneously in each of three constructs (Figures 3A and 3B). Figure 3(C) shows that simultaneous mutation of the AP1-5 and Sp1 sites (AP1-5m/Sp1m) reduces transcription to near background levels. Activity is not restored by the wild-type Sp1 site (AP1-5m/Sp1, AP1-5mm/Sp1). Thus, both point and block mutations in AP1-5 reduce activity to basal levels. Compared to the double mutant, restoration of the AP1-5 site (AP1-5/Sp1m) produces a 15-fold increase in activity. Subsequent restoration of the Sp1 site produces a 60-fold increase in activity (AP1-5/Sp1). Restoration of the weak activator element in pINV(–2473/–2088) also increases transcription (cf. solid bars vs. cross-hatched bars). Moreover, based on its activity when tested alone, the weak activator element produces a stronger than expected activation when coupled to the strong activator. These results show that this region contains strong (–2140/–2088) and weak (–2473/–2216) activator elements and that combination of these elements produces a stronger activation than expected from their individual activities. These results also show that activity of the DRR is completely dependent upon the presence of an intact AP1-5 site.

AP1-5 and Sp1 synergistically activate the full-length hINV promoter

The above experiments were performed using constructs containing different lengths of the –2473/–2088 fragment fused to the involucrin minimal promoter. It is possible that this artificial arrangement might alter the ability of AP1-5 and Sp1 to synergistically activate the promoter. We therefore tested the effect of the AP1-5 and Sp1 mutants and the double mutant in the context of the full-length promoter. As shown in Figure 4, elimination of the Sp1 site reduces activity to 50 % and mutation of the AP1-5 site reduces activity to 25 %. Double mutants also displayed 25 % activity. Thus, the two sites produce what appears to be a weak synergistic activation. However, if the background promoter activity is subtracted (i.e. the 25 % of residual activity that remains when both the AP1-5 and Sp1 sites are mutated) the synergism is pronounced. Moreover, in the full-length promoter, as with the constructs shown in Figure 3, Sp1 produces no activation in the absence of an intact AP1-5 site. Thus, the AP1-5 site appears to be absolutely required for DRR activity.

Transcription factor binding to the AP1-5 and Sp1 sites

The above experiments suggest that the AP1-5 and Sp1 sites are positive regulators of hINV promoter activity. To characterize

Table 1 Sequence of hINV AP1 and Sp1 site oligonucleotides

The sequences in bold are the AP1-5 and Sp1 sites, respectively. The underlined bases are those that have been modified from the wild type sequence. Each oligonucleotide is named based on the mutation (i.e. AP1-5mm/Sp1 has an intact Sp1 site and a block mutation in the AP1-5 site). The consensus AP2 binding site is indicated in bold in the AP2c oligonucleotide.

Sites	Sequence
AP1-5	5'-CTTAAGGCTCTTATTATGCCG TGAGTCAGAGGGCGGG AGGCAGGATCTGGCAGATA-3'
AP1-5	5'-CTTAAGGCTCTTATTATGCCG TGAGTCAGAGTCAAGG AGGCAGGATCTGGCAGATA-3'
AP1-5m	5'-CTTAAGGCTCTTATTATGCCG TGAGCCAGAGGGCGGG AGGCAGGATCTGGCAGATA-3'
AP1-5m	5'-CTTAAGGCTCTTATTATGCCG TGAGCCAGAGTCAAGG AGGCAGGATCTGGCAGATA-3'
AP1-5mm	5'-CTTAAGGCTCTTATTATGCCG AGCTCCAGAGGGCGGG AGGCAGGATCTGGCAGATA-3'
Sp1c	5'-ATTCGATCG GGGCGGG GCGAGC-3'
AP1c	5'-CGCTTGAT TGAGTCAG CCGGAA-3'
	 - - - - -
	AP1-5 Sp1
AP2c	5'-GATCGAACTGACCG CCCGCGGCC CGT-3'

Figure 5 Gel mobility shift characterization of protein interaction with the AP1-5/Sp1 region of the hINV promoter

Nuclear extracts were prepared as outlined previously [19] and incubated with [32 P]AP1-5/Sp1 (Table 1) in the presence of a 0-, 20- or 200-fold molar excess of AP1-5/Sp1, AP1-5/Sp1m, AP1-5m/Sp1m, Sp1c, SP1c, AP1c or AP2c. Complexes were separated by electrophoresis on a 6% acrylamide gel. The 'N' lane shows the migration of radiolabelled probe alone (all other lanes contained nuclear extract). The arrows indicate the migration of the major Sp1 and AP1 shifted bands. The arrowhead indicates the migration of free probe. (B) [32 P]AP1-5m/Sp1 oligonucleotide was incubated in the absence (N) or presence (all other lanes) of nuclear extract in the presence of a 0- or 200-fold molar excess of radioinert oligonucleotide, Sp1c, containing an Sp1c consensus sequence.

transcription factor binding to these sites, we performed gel mobility shift experiments. When nuclear extracts were incubated with radiolabelled wild-type oligonucleotide, [32 P]AP1-5/Sp1 (Table 1), radioinert wild-type oligonucleotide efficiently competed for the bands indicated by the arrows (Figure 5A). The AP1-5/Sp1m oligonucleotide competed for binding to the AP1-5 site, but did not compete at the Sp1 site. The double mutant, AP1-5m/Sp1m, did not compete for binding. The Sp1c oligonucleotide, which contains a canonical Sp1 site [30], competes efficiently for binding at the [32 P]AP1-5/Sp1 Sp1 site, and the AP1 consensus site oligonucleotide, AP1c, competes for AP1 binding. As expected, AP2c, which encodes an activator protein 2 binding site, did not compete for binding.

As seen in Figure 5(A), the Sp1 complex is partially obscured by the AP1 complex. To provide a more distinct visualization of Sp1 binding we incubated nuclear extract with [32 P]AP1-5m/Sp1, an oligonucleotide that is identical to the wild-type sequence except for a mutation in the AP1 site. This oligonucleotide does not bind AP1. As shown in Figure 5(B), [32 P]AP1-5m/Sp1 shifts a single band that is competed by the Sp1c oligonucleotide. Thus, these gel shift results demonstrate protein complex formation at the hINV promoter Sp1 site.

Gel supershift identification of Sp1 as part of the binding complex

The above experiments have assumed that the slower mobility complex, which is specifically competed by the Sp1c oligonucleotide, includes the Sp1 transcription factor. To provide evidence that Sp1 protein is present in this complex, we performed gel mobility supershift experiments. As AP1 binding can obscure

Figure 6 Identification of transcription factors binding to Sp1 site

Nuclear extract was incubated with [32 P]AP1-5m/Sp1 oligonucleotide (Table 1) in the absence (N) or presence (all other lanes) of nuclear extract. The control lane shows the shifted complex in the absence of radioinert competitor or anti-Sp protein antibody. Extract electrophoresed in the lane labelled '200 Sp1c' contains a 200-fold molar excess of radioinert Sp1c. Lanes labelled anti-Sp1, anti-Sp2, anti-Sp3 and anti-Sp4 contain nuclear extracts that were preincubated with antibodies specific for Sp1, Sp2, Sp3 and Sp4, respectively, in the absence of radioinert competitor. The samples were then electrophoresed on a 6% non-denaturing acrylamide gel. The Sp1 arrow indicates the major complex shifted in the absence of Sp protein-specific antibody. The *Sp1 arrow indicates migration of the Sp1 supershifted complex. The open arrow indicates the migration of free probe.

Figure 7 Absence of simultaneous Sp1 and AP1 binding to response elements

Nuclear extract prepared from normal human keratinocytes was incubated with 32 P-labelled probes (*AP1c, *Sp1c and *AP1-5/Sp1) in the absence (—) or presence (Sp1c, AP1c) of a 200-fold molar excess of radioinert competitor. The samples were then separated on a 6% acrylamide gel. The arrows indicate the major Sp1 and AP1 complexes.

Sp1 binding (e.g. Figure 5A), we used a mutant oligonucleotide, [32 P]AP1-5m/Sp1, for the Sp1 supershift experiments. This oligonucleotide contains a mutated AP1-5 site (Table 1). However, it is important to note that identical results were observed with the wild-type oligonucleotide, [32 P]AP1-5/Sp1 (not shown). As shown in Figure 6, addition of nuclear extract to [32 P]AP1-

Figure 8 An intact Sp1 site is necessary for optimal AP1 binding

Nuclear extract was incubated with [32 P]AP1-5/Sp1 or [32 P]AP1-5/Sp1m (*AP1-5/Sp1, *AP1-5/Sp1m) in the absence (—) or presence (AP1c) of a 200-fold molar excess of radioinert Sp1c. The lane labelled N shows the migration of free probe in the absence of nuclear extract. The samples were then separated on a 6% acrylamide gel. The arrows indicate the major Sp1 and AP1 complexes.

5m/Sp1 results in the appearance of a low mobility band (Sp1 arrow) which is specifically competed by a 200-fold molar excess of radioinert Sp1c. To identify the Sp factors binding at the Sp1 site, [32 P]AP1-5m/Sp1 was incubated with nuclear extract in the presence of antibodies specific for the known family members. Addition of Sp1-specific antibody (anti-Sp1) results in the appearance of a supershifted band (*Sp1 arrow). In contrast, addition of antibodies specific for Sp2, Sp3 or Sp4 does not produce a supershift. These results suggest that Sp1 or an Sp1-like protein is the major Sp factor interacting at the hINV Sp1 site.

The Sp1 site is required for optimal AP1 factor binding

One possible mechanism whereby AP1 and Sp1 could produce a synergistic activation is via formation of a ternary complex where Sp1 and AP1 are simultaneously bound to [32 P]DNA. However, this possibility appears unlikely since we can find no evidence for ternary complex formation in gel shift experiments. For example, the experiment shown in Figure 7 compares the mobility shift observed with [32 P]AP1c, [32 P]Sp1c and a mixture of these binding site consensus oligonucleotides, to the mobility shift observed with [32 P]AP1-5/Sp1. AP1-specific and Sp1-specific binding are detected when [32 P]AP1-5/Sp1 is incubated with an excess of Sp1c or AP1c, respectively. The Sp1 band co-migrates with a band shifted by [32 P]Sp1c (*Sp1c), while the AP1

Figure 9 Increasing the distance between the AP1-5 and Sp1 sites reduces promoter activity

(A) presents the sequence of the wild-type AP1-5/Sp1 element and a mutant element containing a 20 bp spacer inserted between the AP1-5 and Sp1 sites (SP20). The AP1-5 and Sp1 sites are indicated in bold and the intervening sequence is shaded. The wild-type and spacer-containing pINV-2473 (B) and pINV(-2216/-2088) (C) constructs were tested for activity in the wild-type and spacer format and the results are presented as a percent of activity of the wild-type construct.

band co-migrates with a band shifted by [32 P]AP1c. Combining the [32 P]AP1c and [32 P]Sp1c oligonucleotides results in a shift that is the addition of the pattern observed when these oligonucleotides are shifted alone. These results show that oligonucleotides containing only AP1 or Sp1 consensus binding sites ([32 P]AP1c, [32 P]Sp1c) shift complexes that migrate at the same position as complexes shifted by [32 P]AP1-5/Sp1, suggesting that AP1 and Sp1 do not simultaneously form a stable complex with [32 P]AP1-5/Sp1.

Based on the above experiments, it is possible that Sp1 assists AP1 binding at the adjacent AP1-5 site and simultaneously dissociates. This mechanism predicts that AP1 binding should be decreased when the Sp1 site is inactivated by mutation. We tested this hypothesis by mutating the Sp1 site and determining whether AP1 binding is reduced. The wild-type oligonucleotide, *AP1-5/Sp1, shifts AP1 and Sp1 bands (Figure 8). In contrast, when the Sp1 site is modified so that Sp1 cannot bind (*AP1-5/Sp1m) AP1 binding is reduced. This result suggests that the Sp1 site is required for optimal binding of AP1 to the hINV AP1-5 binding site. This idea was again tested in an additional experiment which involved changing the spacing between the AP1-5 and Sp1 sites. In the wild-type sequence, these sites are separated by a single nucleotide (Figure 9A). If retaining the position of the Sp1 site is required for AP1 binding, increasing the spacing between the sites should reduce transcriptional activity. To test this we placed a 20 nucleotide spacer between the AP1 and Sp1 sites (Figure 9A). As shown in Figure 9(B), the full-length promoter containing the spacer (pINV-2473SP) displayed a 50% reduction in activity compared to the intact wild-type plasmid (pINV-2473). A similar reduction in activity was observed when the spacer is inserted into the pINV(-2216/-2088) construct where the AP1-5/Sp1 element is positioned adjacent the hINV TATA box (Figure 9C). The results shown in Figures 9(B) and 9(C) demonstrate that the important interaction is between the Sp1 and AP1-5 sites and not between these sites and the hINV TATA box region.

Figure 10 The hINV promoter upstream regulatory region

Experiments outlined in this paper identify a DRR spanning nucleotides -2473 to -2088 of the hINV promoter. This DNA segment contains weak and strong activator segments. The strong activator segment includes the AP1-5 and Sp1 sites. The proximal regulatory region (PRR), which contains a functionally important AP1 site (AP1-1) [19], is also shown. The five AP1 sites (closed circles) are indicated and nucleotides are numbered relative to the transcription start site (arrow) [19,39]. Intact AP1-5 (filled circle, 5) and Sp1 (filled square) sites and the presence of the weak activator segment are required for maximal activity.

DISCUSSION

The distal regulatory region contains transcriptional activator elements

AP1 is an important positive regulator of both basal and phorbol ester-dependent hINV promoter activity. Mutagenesis and DNA binding studies indicate that 2 of the 5 AP1 binding sites in the hINV promoter upstream region, AP1-1 and AP1-5, are required for activity (Figure 10) [19]. Our present studies identify a functional region ($-2473/-2088$), surrounding the AP1-5 site, that we call the DRR. Functional studies indicate that the DRR can be divided into weak and strong activator elements (Figure 10). The strong transcriptional activator element ($-2140/-2088$) includes the AP1-5 site and an Sp1 site. The weak activator element is located further upstream ($-2473/-2216$) and has less transcriptional activity. Linking the weak and strong activator regions produces a synergistic increase in activity, suggesting that they cooperate and that context of the sites is important for activity. Thus, regulation of hINV gene expression during development and/or differentiation may result from an interplay among multiple transcriptional regulators, including AP1 and Sp1.

AP1 and Sp1 sites are required for optimal activation of the hINV promoter

The $-2473/-2088$ segment of the hINV promoter is highly active in keratinocytes. Simultaneous mutation of the AP1-5 and Sp1 sites contained in this region reduces activity to near basal levels. When the activity of the double mutant is set at 1, restoration of the AP1-5 site increases activity to 15 and restoration of both AP1-5 and Sp1 sites increases activity to 60. This pattern of increase is retained in the context of the full-length promoter. However, the synergy is not as pronounced in this context, since downstream regulatory elements increase the basal activity. Truncation studies show that this synergism is completely contained within the 52 bp $-2140/-2088$ segment and that the activity can be completely accounted for by the contribution of the Sp1 and AP1-5 sites. Our functional studies indicate that the Sp1 site alone does not promote activity above basal levels in the absence of an intact AP1-5 site. Likewise, the weak activator element is not active in the absence of an intact

AP1-5 site. These results suggest that AP1-5 provides an on/off switch regulating transcription and that the Sp1 site and the weak activator element potentiate the AP1-dependent activation.

Sp1 is a zinc finger-containing, sequence-specific DNA binding protein [30] that binds to the so-called GC box consensus sequence, 5'-GGGCGG-3', that is functional in either orientation [30]. Sp1 can act alone [31,32], but can also participate as a co-regulator of gene expression with non-Sp1 transcription factors including ets-1 [29], NF- κ B [28], Stat1 [24], GBF factors [26], C/EBP β [25] and Egr-1 [33]. Our results suggest that cooperation between Sp1 and AP1 is required to regulate hINV gene expression.

Sp1 and AP1 interaction with the transcriptional activator element

Gel mobility shift/competition experiments indicate that both Sp1 and AP1 bind at their respective sites within the strong activator element. Fra-1, junB and junD bind at the AP1-5 site [19]. Supershift analysis shows that the Sp1 site binding activity is Sp1, and not Sp2, Sp3 or Sp4. This is important, as Sp1 and Sp3, for example, have distinct functions in regulating gene transcription (Sp1 is generally a transcriptional activator), and these factors appear to be differentially regulated during keratinocyte differentiation [34,35]. However, it should be noted that Sp1 binds to the hINV promoter Sp1 site with a 20-fold lower affinity compared to its binding affinity for the Sp1c consensus Sp1 element (not shown). Thus, the hINV site would be expected to have a moderate affinity for Sp1 factors [30]. *In vivo*, this may limit, but is not likely to prohibit, Sp1 binding at this site. An alternative situation exists in the CYP11A gene [31]. In this gene, AP1 and Sp1 consensus binding sites overlap, but the predominant protein that binds at these sites is Sp1. The CYP11A gene AP1 site appears to have no functional significance, as its mutation does not change the gel shift profile or modify promoter activity [31]. In contrast, in the hINV promoter mutation of either the AP1-5 or Sp1 sites modifies transcriptional activation and changes the gel mobility shift profiles.

Synergistic activation does not require simultaneous Sp1 and AP1 binding to the activator element

Our gel shift experiments demonstrate that Sp1 and AP1 form complexes with [32 P]AP1/Sp1, but, surprisingly, we can find no evidence for formation of a ternary complex consisting of AP1, Sp1 and [32 P]AP1/Sp1. This is unlike some of the systems where Sp1 forms stable ternary complexes with other transcription factors and DNA [27,28]. One model to explain our results is that Sp1 and AP1 need not simultaneously occupy their respective binding sites to synergistically activate the hINV promoter. It is possible that AP1 and Sp1 may form a short-lived higher order complex in which both factors transiently contact DNA. Along these lines, Sp1 has been reported to bend DNA in a DNA binding site-dependent manner [36,37]. Therefore, it is possible that the major role of Sp1 is to induce a conformational change in the DNA that contributes to the activation, perhaps by enhancing AP1 binding. This model is consistent with the fact that the AP1 site appears to be the primary driver of transcription within the strong activator element, and that AP1 binding is reduced when the Sp1 site is mutated or the Sp1 site is moved. This model is also consistent with a proposed mechanism of action of Sp1 in the CD11c leukocyte integrin gene where Sp1 facilitates AP1 binding [38] and with studies of the human immunodeficiency virus long terminal repeat where activation is highly dependent upon the spacing between functionally important Sp1 and NF- κ B binding sites [28].

Because the AP1 and Sp1 elements are closely juxtaposed (separated by a single nucleotide), our results are also consistent with an alternate mechanism of activation. In this model activation would depend solely on AP1. The primary site of AP1 contact with DNA would be the canonical AP1 response element, but AP1 contact with bases within the Sp G/C box element would also be necessary to optimally increase transcription. This would explain why the activity is partially reduced when the Sp1 site is mutated and further reduced when both the AP1 and Sp1 sites are mutated. Although formally possible, this mechanism appears unlikely for two reasons. First, previous DNase I protection studies of similar closely spaced AP1/Sp1 binding sites in the CD11c leucocyte integrin gene indicate that purified jun protein protects the AP1 site but does not protect the adjacent Sp1 site [38]. Secondly, this mechanism does not easily explain why the weak activator element, which is separated from the AP1-5 site by > 75 nucleotides, displays the same activity pattern as the Sp1 site (i.e. like the Sp1 site, it displays no activity when the AP1-5 site is mutated). It appears unlikely that AP1 factors would protect a segment of DNA spanning nearly 400 nucleotides.

In summary, we show that the involucrin DRR consists of weak and strong activator elements. The active factor that binds the weak activator is not presently known, but the strong activator element contains functionally important Sp1 and AP1 sites. It is increasingly appreciated that gene regulation depends upon the simultaneous presence of multiple transcription factors, used in a specific context, to produce activation in a tissue-specific and differentiation-appropriate manner. The present studies suggest that at least three independent transcription factors, operating in the correct context, are required for activation of the hINV DRR in keratinocytes.

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