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Elements Controlling the Expression and Induction of the Skin Hyperproliferation-associated Keratin K6*

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The suprabasal keratin 6 (K6) is remarkable among the keratins as, in addition to being constitutively expressed in different stratified epithelia, it is induced in epidermis under hyperproliferative conditions, such as benign or malignant tumors, psoriasis, and wound healing. In addition, this keratin is also induced in skin treated with 12-*O*-tetradecanoylphorbol-13-acetate or retinoic acid (RA). These characteristics make the study of K6 regulatory elements an especially interesting issue, in particular because these elements could be useful in designing gene constructs for the therapy of skin diseases. We have analyzed by mobility shift and footprinting experiments the cell type-specific enhancer of the bovine K6β gene (Blessing, M., Jorcano, J. L., and Franke, W. W. (1989) *EMBO J.* 8, 117–126) and have identified an AP-2-like element, two AP-1 elements (one of them composite), and a retinoic acid-responsive element (RARE). Mutagenesis experiments and cotransfections with retinoic acid receptors show that the RARE mediates enhancer activation by RA. Chloramphenicol acetyltransferase assays show that under normal culture conditions, the AP-1 element retains most of the enhancer transcriptional activity, while the RARE and AP-2 are weakly active. However, following RA treatment, the AP-1 element is repressed and the RARE is activated, resulting in an overall stimulation of the enhancer by RA in the BMGE+H cells used in our study. These results explain in part the complex and sometimes contradictory response of keratin 6 to hyperproliferative stimuli.

In the last few years, much attention has been drawn toward keratins, since this broad family of cytoskeletal proteins specifically expressed in epithelia has been found to be related to a series of epidermal hereditary diseases (for a recent review on this topic, see Ref. 1). The keratin family is subdivided into acidic (type I) and basic (type II) proteins. Keratin intermediate filaments are formed by heterodimers containing one molecule of each type (see Ref. 2 for a recent review on keratin structure and function). Particular pairs of type I and type II keratins have been correlated with the different routes of epithelial differentiation (3) in such a way that each epithelial cell type expresses a characteristic combination of keratin pairs. This accurately regulated expression pattern suggests that correct keratin expression is necessary to achieve structural integrity and/or correct function of a given epithelium.

Keratin K6[1] is particularly interesting, because it seems to be regulated in two different ways. (i) It is constitutively expressed in several internal stratified epithelia, such as those of the oral cavity (tongue and palate), esophagus, and genital tract (exocervix and vagina) (4, 5). It is also expressed in some areas of the epidermis such as footsoles (4) and in the hair follicle outer root sheath (6, 7). (ii) In addition, this keratin is induced in interfolicular epidermis under hyperproliferative situations, such as wound healing (8), psoriasis, tumors, and, *in vitro*, in cultured epidermal cells (see, *e.g.*, Refs. 9 and 10). It is also induced *in vivo* by agents provoking epidermal hyperplasia, such as TPA and RA (11–14). Thus, the expression of this keratin seems to be regulated both in a constitutive and in an inducible manner. It is precisely this capability to be induced by topical treatments that makes the K6 gene attractive for the development of gene therapy protocols for skin diseases. Elucidation of the mechanisms that mediate K6 induction would allow activation and deactivation at will of the expression of genes under the control of the K6 regulatory region.

We have shown previously that the enhancer of the bovine keratin K6β gene (BK6β, formerly referred to as BK IV*), located between −247 and −647 from the translation start, is able to drive the cell type-specific expression of a heterologous reporter gene (15), indicating that sequences within the enhancer play an important role in the tissue-specific expression of this gene. Here, we report the identification and characterization of several transcription factor binding sites in the BK6β enhancer, some of which may mediate its response to RA, TPA, and hyperproliferation.

MATERIALS AND METHODS

Plasmid Constructs and DNA Sequencing—The nucleotide sequence of the BK6β 5′-upstream region has been published previously (15) and can be obtained under GenBank/EMBL accession number X14478. All constructs used were made by cutting plasmid pBLCAT2-*Eco*RI-*Xmn*I (650 bp; called here EXm-CAT2) or pBLCAT3-*Bgl*II-*Bgl*II (2.2 kilobase pairs) (15), with appropriate restriction enzymes and cloning the desired fragments into the pBLCAT2 vector (16). The obtained plasmids were designated *X*-CAT2, where *X* represents the corresponding restriction fragment from the BK6β enhancer present in the plasmid. All constructs were sequenced (17) to verify the presence of a correct insert and purified twice on a cesium chloride gradient. Chemical DNA sequencing was done according to Chupilo and Kravchenko (18). Glucocorticoid receptor and RARα expression plasmids were gifts from Dr. M. Beato (Universität Marburg, Germany) and Dr. A. Aranda (Instituto de Investigaciones Biomedicas, Madrid, Spain), respectively. Reporter plasmid pRSVLacZ contains the gene for β-galactosidase under the

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[1] The abbreviations used are: K, keratin; BK, bovine keratin; HK, human keratin; CAT, chloramphenicol acetyltransferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EMSA, electrophoretic mobility shift assay; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; bp, base pair(s); PCR, polymerase chain reaction.

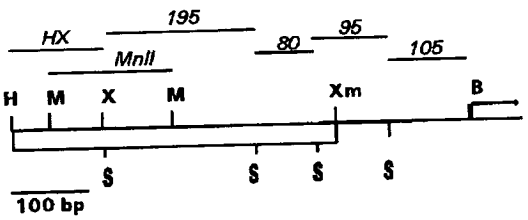

FIG. 1. **Scheme of the 5′-upstream region of the BK6β gene.** The *open box* represents the enhancer. The sequence of this region is presented in Fig. 7. The restriction sites are: *B*, *Bgl*II; *H*, *Hin*dIII; *M*, *Mnl*I; *S*, *Sau*3A; *X*, *Xho*II; *Xm*, *Xmn*I. The *lines* above indicate the DNA fragments used in this study, and the *numbers* their lengths (in bp). The *Bgl*II site lies at the cap site.

control of the Rous sarcoma virus long terminal repeat.

Cell Culture, Transfections, and CAT Assays—BMGE+H bovine mammary gland epithelial cells were cultured according to Schmid *et al.* (19). Four days before transfection, they were changed to Dulbecco's modified Eagle's medium plus 20% fetal calf serum. Under these conditions, no changes in the keratin pattern were observed. PB murine epidermal cells were obtained from Dr. S. Yuspa (National Institutes of Health, Bethesda, MD) and maintained in minimal Eagle's medium plus 8% fetal calf serum. Transfections and CAT assays were as described, including the use of β-galactosidase activity to normalize transfection efficiency (20). All transfections were performed in duplicate at least four times, with reproducible results. When necessary, delipidized serum obtained by treatment with activated charcoal and AG501-8X resin (Bio-Rad), followed by centrifugation and sterilization, was used.

DNA-Protein Interactions—Nuclear extracts were prepared by the method of Shapiro *et al.* (21), with the corrections described by Casatorres *et al.* (20). EMSA and footprinting were performed as described (20).

Mutagenesis—A deletion of the RARE of the BK6β enhancer was constructed by assembling two PCR fragments framing the sequence to be deleted, and using two separate rounds of PCR. In the first round, the 5′ fragment was PCR-amplified using a *Hin*dIII-containing direct primer (GCCAAGCTTGTTCTTATGCTGTAAA) and a *Pvu*II-containing reverse primer (GTCCAGCTGGATCTCCTGACTG), 5′-adjacent to the sequence to be deleted. Similarly, the 3′ fragment was amplified using a *Bgl*II-containing reverse primer (AGCTAAAGAACAGATCTATGA) and a *Pvu*II-containing direct primer (GACAGCTGCAAACATGGCCCAAAACC), 3′-adjacent to the sequence to be deleted. The two amplified fragments were *Pvu*II-digested and ligated. One-tenth of the ligation product was used as template in the second round of PCR, using only the external primers, generating a fragment in which the deleted sequences were replaced by a *Pvu*II site. The resulting product was restriction-digested with *Hin*dIII and *Xmn*I, and this fragment was used to replace the same wild-type fragment in EXm-CAT2. The presence of the deletion was confirmed by the apparition of a new *Pvu*II site and by DNA sequencing.

RESULTS

Dissection of the Enhancer—The sequence of the BK6β enhancer (a 427-bp *Hin*dIII/*Xmn*I fragment) has been published previously (15). For our studies, we have divided the enhancer region into several fragments, as shown in Fig. 1. Fragments were delimited by the restriction enzymes *Hin*dIII/*Xho*I (fragment HX, 120 bp), *Mnl*I (fragment *Mnl*I, 165 bp), or *Sau*3A (fragments 195, 80, and 95; the numbers indicate their length). Fragment *Mnl*I overlaps fragments HX and 195. Fragment 95 includes the last 25 bp of the enhancer 3′-end, as well as 70 bp of the proximal promoter. We also analyzed fragment 105, delimited by enzymes *Sau*3A and *Bgl*II, which consists of the sequences immediately 5′ to the cap site, also out of the enhancer. Together, all these fragments span 608 nucleotides, from the cap site to the 5′-end of the enhancer.

Nuclear Proteins Binding to the Keratin K6β Enhancer—To identify the different nuclear factors that interact with the BK6β enhancer, we performed EMSA experiments with BMGE+H cells nuclear extracts using the different DNA fragments as probes. Several fragments rendered a retarded band and were further analyzed by DNase I footprinting.

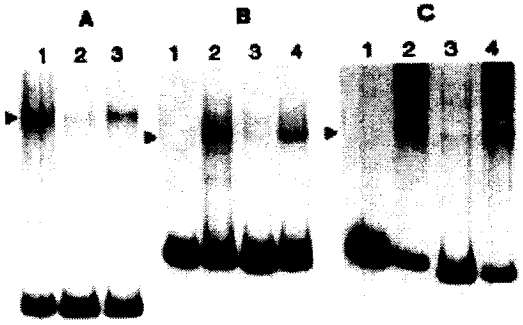

FIG. 2. **Binding of nuclear factors to fragments of the BK6β gene enhancer.** 10 μg of BMGE+H nuclear extract were used in each lane. *Arrowheads* indicate retarded bands. *A*, fragment 80. *Lane 1*, no competitor DNA added; *lane 2*, addition of a 100-fold molar excess of fragment *Pst*I/*Xmn*I from BK5, containing the AP-1 site found in the enhancer of this gene (20); *lane 3*, addition of a 100-fold molar excess of an unrelated DNA fragment. *B*, fragment HX. *Lane 1*, no nuclear extract added; *lane 2*, no competitor DNA added; *lane 3*, addition of a 100-fold molar excess of unlabeled probe; *lane 4*, addition of a 100-fold molar excess of an unrelated DNA fragment. *C*, fragment *Mnl*I. *Lane 1*, no nuclear extract added; *lane 2*, no competitor DNA added; *lane 3*, addition of a 100-fold molar excess of unlabeled probe; *lane 4*, addition of a 100-fold molar excess of an unrelated fragment.

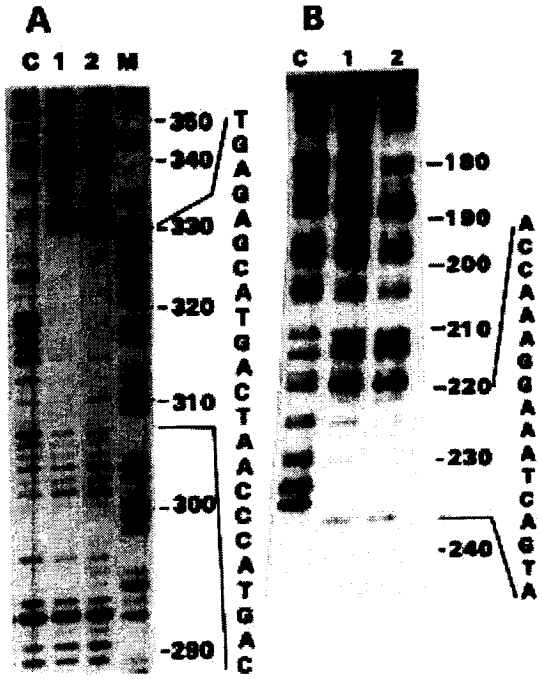

FIG. 3. **Identification of protein binding sites in fragments 80 (*A*) and 95 (*B*) of the BK6β gene enhancer by DNase I footprinting.** End-labeled fragments 80 or 95 were incubated with BMGE+H nuclear extracts and treated with DNase I as described under "Materials and Methods." Although only one strand is shown, similar results were obtained with the opposite strand. The protected sequences and their positions from the translation start are shown. *Lanes C*, no nuclear extract added; *lanes 1* and *2*, addition of different amounts of BMGE+H nuclear extract; *lane M*, Maxam and Gilbert sequence markers.

Fragment 80 was able to produce a single, specific retarded band. Fig. 2*A* shows that this band is totally competed by an oligonucleotide containing the AP-1 element found to play an essential role in the BK5 gene enhancer (20), but not by an unrelated competitor. DNase I footprints using this fragment gave a 23-nucleotide-long protected region whose sequence is 5′-TGAGAGCATGACTAACCCATGAC-3′, between positions −309 and −331 (Fig. 3*A*). This region includes a TGACTAA

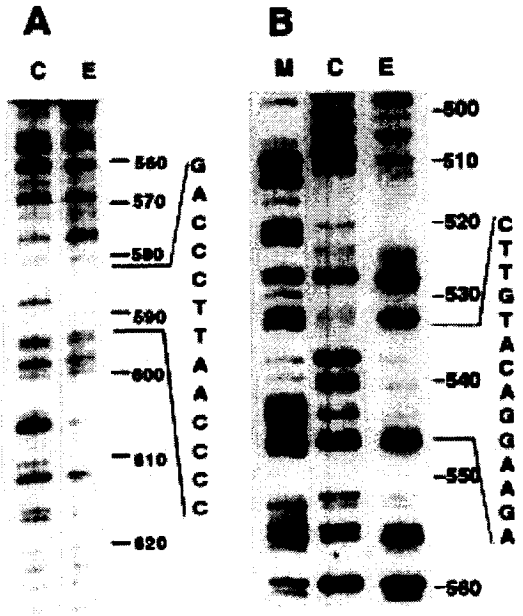

FIG. 4. **Identification of protein binding sites in fragments HX (*A*) and *MnlI* (*B*) of the BK6β gene enhancer by DNase I footprinting.** End-labeled HX (*A*) or *MnlI* (*B*) fragments were incubated with BMGE+H nuclear extracts and treated with DNase I as described under "Materials and Methods." Although only one strand is shown, similar results were obtained with the opposite strand. The protected sequences and their positions from the translation start are shown at the *right*. *Lanes C*, no extract added; *lanes E*, addition of BMGE+H nuclear extract; *lane M*, Maxam and Gilbert sequence markers.

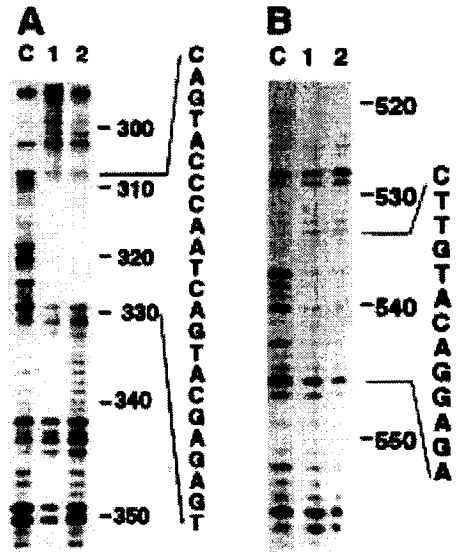

FIG. 5. **DNase I footprinting of fragments of the BK6β gene enhancer using PB nuclear extracts.** End-labeled 80 (*A*) or *MnlI* (*B*) fragments were incubated with PB nuclear extracts and treated with DNase I as described under "Materials and Methods." The protected sequences and their positions from the translation start are shown at the *right*. *Lanes C*, no nuclear extract added. *Lanes 1* and *2*, addition of different amounts of PB nuclear extract.

sequence very similar to the consensus for AP-1 (22). These results indicate a role for the Fos-Jun complex in the BK6β gene regulation.

Fragment 95 also produced a specific retarded band when assayed with BMGE+H nuclear extracts (not shown). The nucleotide sequence of fragment 95 displays an AAACCAAA motif, which matches the consensus AAPuCCAAA (referred to as "epidermal box" in Ref. 23), that has been found preceding the TATA box in several genes expressed in epidermis, in particular keratins (23) and papillomavirus (24). In spite of being located outside the enhancer, this sequence shows a footprinting protected region, 5′-ATGACTAAAGGAAACCA-3′, between positions −222 and −238 (Fig. 3*B*). This protected region includes a TGACTAA sequence, identical to the AP-1 element found in fragment 80, as well as the first 6 bp of the "epidermal box," suggesting that this element could be a "composite AP-1 site" similar to others found in a number of genes (see, for instance, Ref. 25).

Fragment HX also rendered a specific retarded band (Fig. 2*B*). DNase I footprinting of this fragment showed a protected region, 5′-CCCCAATTCCCAG-3′, between positions −594 and −582 (Fig. 4*A*). Examination of the protected sequence and the adjacent nucleotides allowed the identification of a TTCCCAGGC sequence (most of which is contained in the footprint protected region) that is highly homologous to the sequences that have been found to bind transcription factor AP-2 in SV40 and in several keratin genes (26–30). Although other putative protein binding sequences can also be found in the protected region (see "Discussion"), as AP-2 has been involved in the regulation of several keratin genes, it is probable that the protected element binds AP-2, although further work will be necessary to confirm it. Interestingly, there is a TCTGCAGGC sequence, between positions −135 and −143 (outside the enhancer), highly homologous to the AP-2 also found upstream of the TATA box of the human K14 keratin gene (27). However, EMSA and DNase I footprinting experiments with fragment 105, which includes this sequence, were negative (not shown). These results suggest that the AP-2-like sequence at −135 is not functional, which is supported by the fact that deletion of this AP-2-like site does not alter the proper expression pattern of a K6β promoter/*lacZ* construct in transgenic mice.[2] In some footprint experiments with fragment HX, we have also observed another protected, almost palindromic region, 5′-GTGTCATGTCCC-3′, between nucleotides −604 and −615 (see Fig. 4*A*). We have not studied the sequences protected in the HX fragment in greater detail due to the low level of activity shown in CAT assays (see below).

When we performed EMSA with fragment *MnlI* (which overlaps with fragments HX and 195, see Fig. 1), we found a major specific retarded band (Fig. 2*C*). DNase I footprinting experiments rendered two protected areas. One, spanning from nucleotide −582 to −594, is the same sequence protected at the equivalent position in fragment HX and therefore corresponds to the putative AP-2 site (not shown). The other protected region, 5′-AGAAGGACATGTTC-3′, extends from −536 to −549 and is shown in Fig. 4*B*. A detailed analysis of this sequence, in which a RARE is found, is presented below. This sequence was also the only protected region found in fragment 195 (not shown).

Given the complex tissue-specific expression of K6, we also tested the pattern of protected regions in footprinting experiments using nuclear extracts from PB cells, a murine epidermal keratinocyte cell line which expresses K6. We obtained the same protected sequences as described above for BMGE+H cells (Fig. 5 and data not shown), suggesting that similar transcription factors bind the BK6β enhancer both in bovine mammary and murine epidermal cell lines.

Dissection of the Enhancer Activity—To study whether the protein binding sequences identified were able to activate transcription on their own when isolated from the rest of the enhancer and to determine the contribution of each element to the activity of the enhancer, we linked the fragments HX (contain-

[2] A. Ramírez and J. L. Jorcano, unpublished results.

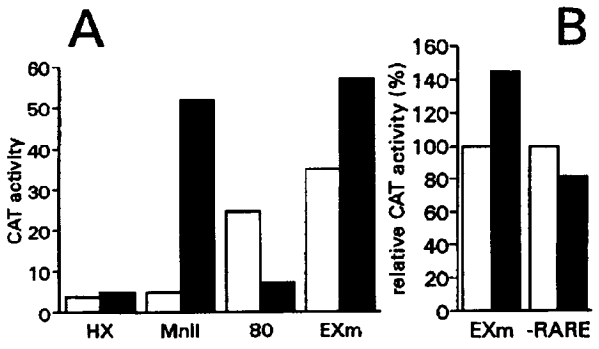

FIG. 6. **Analysis of the transcriptional activity of fragments of the BK6β gene enhancer.** *A*, BMGE+H cells were either transfected (*open bars*) with plasmids containing fragments HX, *MnlI*, or 80 cloned 5′ to the plasmid pBLCAT2, as well as with construct EXm-CAT2 (containing the BK6β whole enhancer), or cotransfected (*solid bars*) with these same constructs and an expression plasmid carrying the RARα. 10^{-6} M RA was added to the plates cotransfected with RARα. 48 h after transfection, cell extracts were collected and CAT activity assayed as described under "Materials and Methods." CAT activity is represented as -fold stimulation over background (pBLCAT2 activity). *B*, Construct EXm-CAT2 (*EXm*) and a similar construct in which the RARE has been deleted (*−RARE*) were transfected (*open bars*) or cotransfected with an expression plasmid carrying the RARα (*solid bars*), as in *A*. 10^{-6} M RA was added to the plates cotransfected with RARα. Both constructs were similarly active in the absence of RA and RARα.

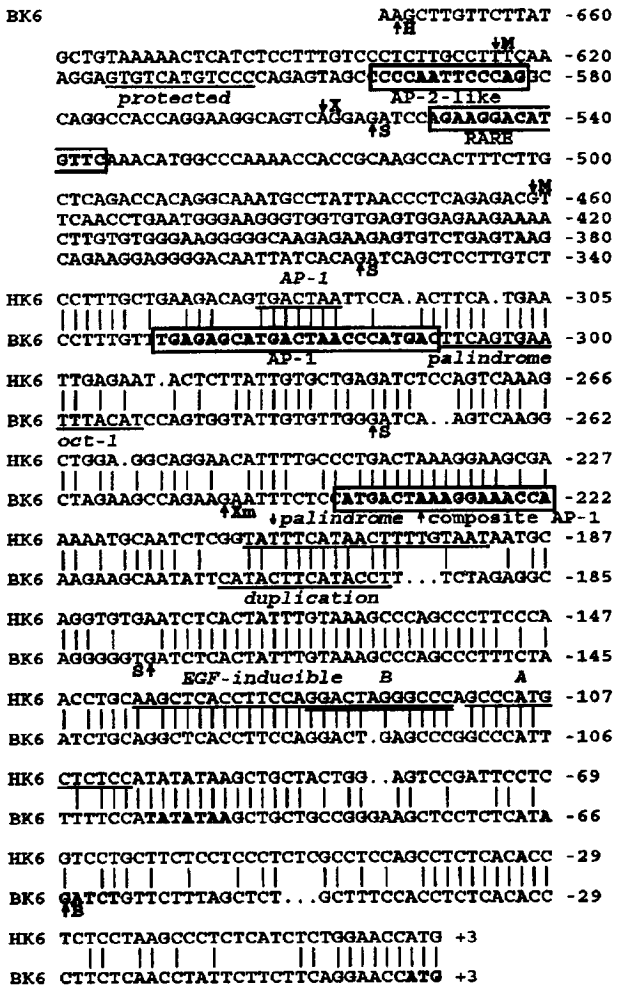

FIG. 7. **Sequence comparison of the 5′-upstream regions of HK6 and BK6β genes.** Sequences protected in the BK6β enhancer are *boxed* and labeled below. Sequences identified in the HK6 promoter by other authors (29, 40) are *underlined* and labeled above. Tentative regulatory sequences in the BK6β regulatory region are *underlined* and labeled below. The TATA box, cap site, and ATG codon in the BK6β are shown in *boldface type*. Restriction sites used to delimit the BK6β enhancer fragments are indicated with *arrows* and *initials* (*B*, *Bgl*II; *H*, *Hin*dIII; *M*, *Mnl*I; *S*, *Sau*3A; *X*, *Xho*II; *Xm*, *Xmn*I).

ing the AP-2-like site), *MnlI* (containing the AP-2 and RARE-like sites), and 80 (containing the AP-1 site) to the plasmid pBLCAT2 (16). As a reference, we used a similar construct, EXm-CAT2, bearing the whole BK6β enhancer in the same pBLCAT2 vector (15). Transient transfections in BMGE+H cells showed (Fig. 6, *open bars*) that fragment 80 retained about 70% of the activity of the whole enhancer, while fragments HX and *MnlI* displayed little activity. Therefore, at least in BMGE+H cells, AP-1 is the only significantly active element when isolated from the rest of the enhancer, while the other two sites (AP-2 and RARE) may collaborate with AP-1 in activation, but are unable to activate by themselves. This suggests that under normal culture conditions, Fos and Jun are the main factors driving the activity of this enhancer.

Fragment MnlI Contains a Retinoic Acid-responsive Element—BMGE+H cells were originally isolated and are routinely maintained in medium containing several hormones, including hydrocortisone (19). It is therefore possible that glucocorticoids could have an effect on BK6β gene expression in these cells. This possibility is reinforced by the existence, in the protected region, 5′-AGAAGGACATGTTCA-3′, of the *MnlI* fragment, of a degenerated palindromic repeat of the TGTTCT motif that mediates interaction with the glucocorticoid receptor element (31). Consequently, we investigated the possible effect of glucocorticoids on the *MnlI* fragment activity. For this, we transfected BMGE+H cells with plasmid *MnlI*-CAT2, and some plates were cotransfected with an expression plasmid coding for the glucocorticoid receptor. Addition of hydrocortisone to the transfected cells had no effect on CAT activity, either in the non-cotransfected plates or in those with the glucocorticoid receptor (not shown). These results were identical in cells cultured in medium with or without hormones, in the presence of normal or delipidized serum (not shown), indicating that the sequences protected in the *MnlI* fragment do not behave as a glucocorticoid response element.

Response to vitamin D, RA, or thyroid hormone is mediated by the orientation and spacing of variations of the AGGTCA consensus (32, 33). Such elements can be found in the region protected in the *MnlI* fragment, and also in the sequences preceding it, either in direct or in palindromic repeats (Fig. 7), suggesting that this region could be a target for regulation by retinoids. As it is known that K6 expression responds to RA treatment (13, 34–37), we studied whether or not this response to RA was mediated by fragment *MnlI*. For this, we cotransfected BMGE+H cells with the *MnlI*-CAT2 plasmid and an expression plasmid coding for RARα. Addition of 10^{-6} M RA to the cotransfected plates increased CAT activity more than 10-fold (Fig. 6), indicating that the element found in the *MnlI* fragment is indeed a RARE. Similar results were obtained with RARβ or RARγ (not shown). Cotransfection with RARα, without addition of RA, had only a slight activating effect in CAT activity. Similarly, addition of 10^{-6} M RA to cells not transfected with the RARα plasmid increased CAT activity 2-fold (not shown).

We also tested the response of other enhancer elements to RA treatment. Fragments HX and 80 cloned in pBLCAT2 were cotransfected with the RARα expression plasmid into BMGE+H cells. Fig. 6*A* shows that, upon addition of 10^{-6} M RA, fragment HX remained inactive, while fragment 80 lost most of its activity, probably due to AP-1 down-regulation produced by cross-talk interactions between Jun/Fos and RAR

(38). However, the construct EXm-CAT2, bearing the whole enhancer, was activated to a level similar to that obtained with fragment *MnlI*. These results suggest that upon RA treatment, the AP-1 element is inhibited and the transcriptional activity of the enhancer is due fundamentally to the RARE located in the *MnlI* fragment.

Since the only element in the BK6β enhancer that is activated by RA seems to be the RARE, we eliminated it from the enhancer by means of a two-step PCR (see "Materials and Methods"). The mutated and the wild-type enhancers were active at similar levels when transfected in BMGE+H cells, demonstrating that, as suggested above, the RARE does not play a significant role in BK6β regulation under normal conditions. However, when cotransfected with RARα, in contrast to the wild-type enhancer, the mutant enhancer failed to be stimulated by RA to appreciable levels (Fig. 6*B*), suggesting that the RARE found in the *MnlI* fragment mediates the RA induction of the enhancer.

Comparison of the 5'-Upstream Sequences of Human and Bovine K6 Genes—Recently, Bernerd *et al.* (39) have identified, by means of EMSA, four nuclear protein binding sites in the 381 bp 5'-upstream of the translation start of the HK6 gene. A comparison of the human and bovine sequences is displayed in Fig. 7. Some of the elements that we have identified in BK6β (AP-2-like and RARE) are outside the region studied in HK6 and cannot be compared. The AP-1 element found in fragment 80 of BK6β at position −309 is also present in HK6 and binds factors from HeLa cells and human keratinocytes (39). Interestingly, from the DNase I-protected region in BK6β, only the AP-1 consensus is conserved in HK6. The bovine composite AP-1 (position −222) is well conserved in HK6 (14 of 17 nucleotides), but no nuclear factor has been found to bind this element in human cells (39). A similar situation holds true for the A, B, and epidermal growth factor-responsive elements of HK6 (39, 40), which are well conserved in BK6β, although we were not able to detect any factor which binds to them. The differences observed in the pattern of nuclear proteins bound by the 5'-upstream regions of the bovine and human gene are not due to the type of tissue or organism used in each study, since we have obtained the same results with BMGE+H (from bovine mammary gland) and PB (murine epidermal keratinocyte) cells. These differences could in part be due to the fact that HK6 and BK6β may represent different genes, since keratin 6 is coded by more than one gene in humans and bovids (23, 41–43). In fact, we have isolated a third BK6 gene (BK6γ), whose promoter region has a closer similarity to the HK6 gene.[3] In particular, the protected sequences in the HK6 gene are better conserved in the BK6γ gene than in BK6β.

In addition to the identified elements, a number of potential binding sequences can be found in the BK6β 5'-upstream region (Fig. 7). For instance, there is a palindrome TTCAGTGAA at position −308, immediately downstream of the AP-1 site, and a duplication of the CATACTT motif at position −208, which coincides with the palindrome sequence of HK6 known to bind a nuclear factor (39). Finally, at position −149, there is a stretch of 28 nucleotides perfectly conserved between the two genes. The significance of these sequences is at present unknown.

DISCUSSION

Keratin genes, due to their complex pattern of epithelial tissue expression, constitute very attractive systems for the study of gene regulation. K6 regulation seems to be particularly complex (see Introduction). We have identified in BK6β a tissue-specific enhancer located between positions −180 and −605 from the cap site (15), which is necessary to provide a correct tissue-specific as well as inducible expression of a reporter (44). To understand BK6β gene regulation, we have studied the nuclear transcription factors binding to the region comprising this enhancer and the proximal promoter zone. Some of the sequences found (two AP-1, one of them composite, and a RARE) and their interactions permit an explanation, at least in part, of the induction of this gene in hyperproliferative situations and the complex behavior of K6 when epidermal keratinocytes are treated with RA.

AP-1 is a regulatory element that has been found in an increasing number of keratin genes: HK18 (45), HK1 (46), BK5 (20), HK19 (47), HK6 (39), and here, in BK6β. However, the AP-1 sequence, found once in HK6 (39) and twice in BK6β, is not the perfect palindromic consensus TGACTCA (22), but TGACTAA. Since this sequence is identical to the AP-1 sequences found in the upstream regulatory regions of certain papillomaviruses (48–50) that are active in the suprabasal layers of stratified epithelia where K6 is also expressed, it is tempting to speculate that this particular AP-1 variant may be especially relevant mediating Fos/Jun action in these types of suprabasal cells. It has been found that different AP-1 sequences bind preferentially to different Jun and Fos proteins (51, 52), and perhaps the combination of Jun/Fos that exists in these suprabasal layers binds better to the variant form TGACTAA. The two AP-1 elements that we have found are very likely involved in the suprabasal K6 induction by TPA and hyperproliferative stimuli. Another similarity between HPV18 and BK6β is that in HPV18, tissue-specific expression is mediated by a cooperation between AP-1 and a close factor (KRF-1), and Oct-1 blocks this cooperation by binding to a KRF-1 overlapping binding site (50). Strikingly, in the BK6β enhancer, and at the same distance from the AP-1 site as in HPV18 (although in an inverted orientation; see Ref. 48 for a map of HPV18 regulatory elements), there is an Oct-1 sequence and a palindrome lies between these two elements (see Fig. 7). Although we have not detected any protein binding to these elements, they could play a role in BK6β regulation. In this respect, Oct-related proteins have been recently identified as regulators of keratin gene expression (53, 54).

It is difficult to ascertain the implication of AP-2 in BK6β gene regulation. Although the sequence protected in the HX fragment fits the AP-2 consensus well (26) and agrees well with AP-2 sequences found in several keratin genes (see Refs. 27 and 55), some other protein binding sequences can be found in this region, such as a CCAAT box, as well as binding sites for H-APF-1 (TTYCCAG, Ref. 56) and the adenovirus transcription factor E4F (AATTCCCA, Ref. 57). However, the fact that none of these factors has been related previously to keratins, together with the repeated involvement of AP-2 in keratin regulation (27–30), suggests that this sequence is a binding site for AP-2, although more work will be necessary to establish this point.

Retinoic acid exerts profound and complex effects on epidermal differentiation (58). The action of RA on keratin expression is also complex, and probably dependent on concentration and environmental conditions. Thus, the K1/K10, K5/K14, and K6/K16 pairs have been reported to be down-regulated by 10^{-6} M RA *in vitro* at the transcriptional level (36, 59). However, not only is the synthesis of the first two pairs not decreased in RA-treated skin *in vivo*, the K6/K16 pair is induced both in human and rodent skin (13, 14, 35, 37). An important result of our work has been the identification of a RARE in the BK6β enhancer, which is strongly stimulated *in vitro* by RA, and whose deletion eliminates the response of the enhancer to RA. These results are at variance with those of Tomic-Canic *et al.* (60), who found an element responding negatively to RA in the

[3] J. M. Navarro and J. L. Jorcano, unpublished results.

HK14 promoter. However, since there is no significant identity between the BK6β and HK14 RAREs, these two elements could be functionally different.

Our results explain the behavior of the BK6β enhancer in a relatively straightforward manner. On the one hand, under normal culture conditions the activity of the enhancer seems to be due mainly to the AP-1 element, which accounts for 70% of the total enhancer activity. There is no apparent synergism between AP-1 and the other identified elements (AP-2, RARE), since the activity of the total enhancer is the sum of the activities of the component elements (see Fig. 6). On the other hand, the presence of RARα and RA induces a positive response of the RARE element. At the same time, the activity of fragment 80, containing the AP-1 element, is drastically diminished due to negative cross-talk between the AP-1 and RAR, as has been described in other systems (38), and even in HPV18 (61). The effect of RA on the enhancer may be interpreted as a combination of the two effects, AP-1 inhibition and RARE stimulation, with the net effect of enhancer activation in BMGE+H cells. The finding in the BK6β enhancer of elements that are either activated (RARE) or inhibited (AP-1) by RA could help explain the complex and sometimes apparently contradictory response of K6 to RA both *in vivo* and *in vitro*. The induction or inhibition of this keratin may be the result of a delicate balance between isoforms of Fos and Jun, type and amount of RARs, and the effective concentration of RA. These elements would depend on cell type and treatment conditions, explaining, for instance, why K6 has been shown to be induced *in vivo* and repressed *in vitro* by RA (13, 14, 35–37), why the degree of RA-induced K6 inhibition is much stronger in SCC13 cells than in primary epidermal keratinocyte cultures (34, 36), or why different retinoids may stimulate or inhibit K6 expression (62). However, when considering K6 induction by RA or hyperproliferative conditions, one should consider that K6 is a minigene family. So far, three K6 variants have been identified in bovids and two in humans, and different isoforms could be regulated in different ways.

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