Specific Organization of the Negative Response Elements for Retinoic Acid and Thyroid Hormone Receptors in Keratin Gene Family

Nadezda Radoja,*§ Danilo V. Diaz,* Todd J. Minars,* Irwin M. Freedberg,*† Miroslav Blumenberg,‡§ and Marjana Tomic-Canic*§

*The Ronald O. Perelman Department of Dermatology, †Department of Cell Biology, and ‡Department of Biochemistry, New York University Medical Center, New York, U.S.A.; §Institute Vinca, Yugoslavia

Retinoic acid and thyroid hormone are important regulators of epidermal growth, differentiation, and homeostasis. Retinoic acid is extensively used in the treatment of many epidermal disorders ranging from wrinkles to skin cancers. Retinoic acid and thyroid hormone directly control the transcription of differentiation-specific genes including keratins. Their effect is mediated through nuclear receptors RAR and T3R. We have previously identified the response element in the K14 gene, K14RARE/TRE, to which these receptors bind, and found that it consists of a cluster of five half-sites with variable spacing and orientation. To determine whether this specific structure is found in other keratin genes, we have mapped and analyzed the RARE/TRE elements in three additional epidermal keratin genes: K5, K6, and K17. We used three different approaches to identify these elements: co-transfection of promoter deletion constructs, gel-shift assays, and site-specific mutagenesis. We localized the RARE/TRE elements relatively close to the TATA box in all three promoters. All three RARE/TRE elements have a similar structural organization: they consist of clusters of 3–6 half-sites with variable spacing and orientation. This means that the clustered structure of the RARE/TREs is a common characteristic for keratin genes. RARE and TRE in the K5 promoter are adjacent to each other whereas in the K17 promoter they overlap. All three keratin REs bind specifically both RAR and T3R in gel-shift assays. Interestingly, addition of ligand to the receptor changes the binding pattern of the T3R from homodimer to monomer, reflecting the change in regulation from induction to inhibition. *Key words: clustered half-sites/epidermis/negative regulation/promoters. J Invest Dermatol 109:566–572, 1997*

Thyroid hormone (T3), steroids, and active metabolites of vitamins A and D3 belong to a large group of factors that contribute to vertebrate development and homeostasis. They serve as biologic signals that control cell growth and differentiation. These hormones and vitamins mediate their effects through nuclear receptors, transcription factors that directly regulate gene transcription. Nuclear receptors have several functions. They bind specific ligand, they recognize and bind DNA sequences in the target genes, and they either stimulate or inhibit expression of particular genes (Mangelsdorf *et al*, 1995). The specific DNA sequences are called response elements (RE). Naturally occurring REs contain hexamer sites homologous to the consensus palindrome AGGTCA, designated as a "core site" or "half-site" (Umesono *et al*, 1991; Perlman *et al*, 1993). Spacing and orientation between the half-sites determines the specificity of particular nuclear receptor binding (Umesono *et al*, 1991; Naar *et al*, 1991). Although not completely understood, the known function of the RE is to provide binding of the nuclear receptors to the promoter they regulate.

Epidermis is a major target tissue for hormone/vitamin regulation. Retinoic acid (RA) maintains the balance of epithelial differentiation (Darmon and Blumenberg, 1993). Hypervitaminosis A inhibits keratinization, causes hyperplasia, and blocks terminal keratinocyte differentiation. Hypovitaminosis A, conversely, does the opposite: it causes epidermal hyperkeratinization and keratinization of nonkeratinizing epithelia (Fuchs and Green, 1981; Rosenthal *et al*, 1992; Fisher *et al*, 1995). Although less studied, T3 also affects epidermal differentiation. Alterations of T3 levels cause changes in cornification and lipogenesis (Rosenberg *et al*, 1986; Isseroff *et al*, 1989). Almost all patients (90%) with hypothyroidism have epidermal changes (Blumenberg, 1996).

Nuclear receptors that are expressed in epidermis include steroid receptors, vitamin D3 receptor, RA and T3 receptors (Ponec, 1987; Pillai *et al*, 1988; Zelent *et al*, 1989; Blumenberg, 1996). We have used keratin genes to study the effects of RA/T3 on gene regulation in the epidermis. Keratins, the intermediate filament proteins of epithelial cells, are precisely controlled in various physiologic and pathologic stages of epidermis. Basal keratinocytes express keratins K5 and K14. As they undergo differentiation K5/K14 expression is shut down and the K1/K10 keratin pair is induced. During inflammation keratinocytes express K17, whereas during proliferative processes such as wound healing, when keratinocytes become activated, the K6 and K16 keratin pair is expressed (Blumenberg, 1996).

Previous studies, from our laboratory and others, have shown that

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Reprint requests to: Dr M. Tomic-Canic, Department of Biochemistry, New York University Medical Center, 550 First Avenue, New York, NY.

Abbreviations: CAT, chloramphenicol acetyl transferase; hRARα, human retinoic acid receptor alpha; LB, Luria Broth; PBS, phosphate-buffered saline; RA, retinoic acid; RARE, retinoic acid response element; RE, response element; T3, thyroid hormone; TRE, thyroid hormone response element; cT3Rα, chicken thyroid hormone receptor alpha; WT, wild-type.

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RA directly suppresses keratin gene expression at the transcriptional level, measuring both mRNA and promoter activity (Tomic *et al*, 1990; Stelmach *et al*, 1991). On the other hand, immunofluorescence studies of RA-treated skin have shown either no change or an increase of keratin protein levels (Rosenthal *et al*, 1992; Eichner *et al*, 1992). Regulation of keratin gene expression, however, is very complex and depends on inputs of multiple signalling pathways. For example, we have shown that growth factors and cytokines specifically induce subsets of keratin genes (Jiang *et al*, 1993, 1994). More importantly, the regulation of keratin genes by nuclear receptors is codominant with the regulation by growth factors and cytokines (Tomic-Canic *et al*, 1996b). Therefore, the suppression of keratin gene expression by RA has the important function of maintaining the balance with other factors that induce keratin gene expression.

We have shown that expression of keratin genes is regulated by RA and T3 receptors in a novel and specific manner: keratin genes are constitutively activated by unliganded T3R but they are inhibited by ligand-occupied T3R or RAR (Tomic *et al*, 1990). Regulation occurs through direct binding of the T3 and RA receptors to the REs in the keratin promoters (Tomic-Canic *et al*, 1996a). In addition, we have identified the RARE/TRE in K14 keratin gene, and found that it has a specific organization. It consists of a cluster of five half-sites in variable orientation and spacing (Tomic-Canic *et al*, 1992).

There are several questions that have arisen from that study, such as: Are all keratin RARE/TRE structurally the same? Are their sequences homologous to each other? Do they all bind the receptors in a similar manner? In this paper, we used three different approaches to answer these questions: co-transfection of promoter deletion constructs, gel-shift assays, and site-specific mutagenesis. We describe the identification of three new RARE/TRE, those in the K5, K6, and K17 keratin genes. The RARE/TRE elements in keratin genes share the same structural organization: they consist of a cluster of several half-sites with variable spacing and orientation. Although their structure is similar, the sequences of the RARE/TRE in keratin genes are not homologous. In gel-shift experiments all identified RARE/TRE bind specifically to purified T3 and RA receptors. Furthermore, addition of T3 decreases the homodimer binding and increases the monomer binding of the T3R. Site-specific mutagenesis of RARE/TRE in the K5 and K17 promoters confirms that the identified response elements are functional, because mutant promoters in which the RARE/TRE elements were altered were not regulated by RA and T3 receptors. Taken together, these results identify and characterize a new group of native negative response elements that are responsible for specific regulation of gene expression in epidermis.

MATERIALS AND METHODS

Plasmids, their growth and purification Plasmids pK5CAT, pK6CAT, and pK17CAT, which contain the promoters of the K5, K6, and K17 keratin genes, in front of the chloramphenicol acetyl transferase (CAT) gene and pRSVZ, which contains the RSV promoter driving the gene for β-galactosidase, have been previously described (Ohtsuki *et al*, 1992; Bernerd *et al*, 1993; Jiang *et al*, 1994). To mutagenize RARE/TRE in the K5 and K17 promoters we used a previously described method (Tomic-Canic *et al*, 1996c). We used standard conditions for polymerase chain reaction and cloning procedures previously described (Tomic-Canic *et al*, 1996c). Primers used in polymerase chain reactions are as follows:

1 For K5 promoter:

K5WTF 5' GGG*TCTAGA*GGATCCCCGGGTTCCTAAC (Xba I);

K5WTR 5' GGG*AAGCTT*CTTGTTCCTGGTGGAG (Hind III);

K5M1.2F 5' GGGG*GTACC*GGGCGGTTTTTTTTTTGCCCACTTAATCATCAT (Kpn I);

K5M1R 5' GGGG*GTACC*ACCCGCCCCTCGAGACCTGCACAG (Kpn I);

K5M2R 5' GGCGC*GTACC*TGAGCTCACCGGTCACACCTGCACAGTGGTGGG (Kpn I);

K5M3F 5' GGGC*TCGAG*GGGGCGGGTCAGCTGCCCCCCAGGCATGCCCA (Xho I);

K5M3F 5' GGGC*TCGAG*ACACCTGCACAGTGGTGTGGGGTGCAAA (Xho I);

2 For K17 promoter:

K17WTF 5' GGG*CTGCA*GCAACCCATTTCCCCCACCA (Pst I);

K17WTR 5' GGG*AAGCTT*CATGGTGGCGGCGGC (Hind III);

K17M1F 5' GGGC*TAGTTC*TAGAGCCCAGGGGAATGGAAA (Spe I);

K17M1R 5' GGG*ACTGTTG*GGCCACCTGCCAGCTCC (Spe I).

All mutants were designed to have a unique restriction site (indicated in parentheses) to be used for diagnostic purposes. To create K5-M4 we used K5-M1 as a template and a pre-existing PstI site positioned at −475 bp from ATG and deleted the DNA between this PstI site and an engineered Kpn I site of K5-M1. Therefore, K5-M4 contains an internal deletion in the background of K5-M1. Positive clones were diagnosed by specific restriction enzyme digestion and, subsequently, by sequencing. The plasmids expressing human RARα and RARγ nuclear receptors (pSV-RARα and pSV-RARγ) were gifts from Dr. P. Chambon and the plasmids expressing cT3Rα (pSV-cT3Rα) and GH-TRE were gifts from Dr. H. Samuels. Plasmids were grown in JM101 *Escherichia coli* host to saturation density in Luria Broth (LB) medium. DNA was extracted and purified using the Maxi Prep Kit from Promega (Madison, WI).

Cell culture HeLa cells were maintained in Dulbecco-modified Eagle's medium supplemented with 10% calf serum at 37°C in a 5% CO_2 atmosphere in media containing penicillin and streptomycin as described (Tomic *et al*, 1990). The day before transfection cells were plated onto 60-mm dishes.

Normal human foreskin epidermal keratinocytes were a generous gift from Dr. M. Simon. The cultures were initiated using 3T3 feeder layers as described (Randolph and Simon, 1994) and then frozen in liquid N_2 until used. Once thawed, the keratinocytes were grown without feeder cells in defined serum-free keratinocyte medium supplemented with epidermal growth factor and bovine pituitary extract (GIBCO BRL, Gaithersburg, MD). Cells were expanded through two 1:4 passages before transfection.

Transfection using $Ca_3(PO_4)_2$ We have generally followed published procedures for transfecting HeLa cells that were at 80% confluence (Chen and Okayama, 1987). Four hours before transfection the medium was changed to DMEM supplemented with 10% calf serum depleted of RA and T3 as described (Tomic *et al*, 1990). At the time of transfection 3 μg of the keratin-CAT construct, 1 μg of the nuclear receptor expression vector plasmid, 1 μg pRSVZ reference plasmid, and a sufficient amount of carrier to bring the total to 10 μg of DNA were added into each dish. The cells were harvested 48 h after transfection by scraping into 5 ml of phosphate-buffered saline (PBS), washed once more in PBS, and resuspended in 150 μl of 0.25 M Tris buffer pH 7.8. CAT and β-galactosidase assays were performed as described (Tomic *et al*, 1990; Jiang *et al*, 1991). All transfections are performed in duplicate plates, and each transfection experiment was repeated two to five times.

Transfection using polybrene with DMSO shock We used this method to transfect primary keratinocytes at 90–100% confluency, as previously described (Jiang *et al*, 1991). Each transfection contained 10 μg per dish of keratin-CAT constructs, 3 μg of the nuclear receptor expression vector plasmid, and 3 μg per dish of RSVZ. The cells were then incubated with or without 10^{-6} M RA (10^{-3} M stock in DMSO) or 10^{-6} T3 (10^{-3} M stock in 0.1 N NaOH). Both all-trans-RA and T3 were purchased from Sigma (St. Louis, MO). Forty-eight hours after transfection cells were washed twice with PBS and harvested by scraping as described above. The cell disruption by repeated freeze-thaw cycles, as well as CAT and β-galactosidase assays, has also been described (Jiang *et al*, 1991). All CAT values were normalized for transfection efficiency by calculating the ratio of CAT activity to β-galactosidase in each transfected plate. Each transfection experiment was separately performed in duplicate plates, three or more times.

Purification of hRARα and cT3Rα receptor *E. coli*-expressed hRARα and cT3Rα were purified as previously described (Forman *et al*, 1992). Briefly, an overnight culture of the bacteria containing the receptor expression vector was grown in LB containing 250 μg ampicillin per ml and 30 μg chloramphenicol per ml. Two milliliters of the overnight culture was transferred into 200 ml LB containing 250 μg ampicillin per ml and 30 μg chloramphenicol per ml and incubated at 37°C with shaking at 250 rpm until OD 600 of 1.0. The cells were then induced with 1 mM isopopylthiogalactoside (IPTG) for 30 min after which rifampicin was added to a final concentration of 50 μg per ml and cultures were grown for an additional hour. The cells were then chilled on ice and centrifuged at 5000 × *g* for 10 min. The pellet was washed once with PBS and frozen in GTETD–400 containing 15% glycerol, 25 mM Tris-HCl, 0.05% Triton X-100, 10 mM EDTA, 1 mM PMSF, and 400 mM KCl. The cells were thawed on ice and the lysed bacterial suspension was sonicated to shear the DNA, and centrifuged for 15 min at 17000 × *g* to remove bacterial debris. Nucleic acid was precipitated with 0.2% polyethyleniminne and removed by centrifugation for 15 min at 17000 × *g*. Further purification was achieved by ammonium sulphate precipitation between 0 and 35% saturation. The ammonium sulphate pellet was resuspended in GTETD-200 (same as GTETDT-400 except the KCl is 200 mM) and applied to a heparin-agarose column. The column was washed with GTETD-225 (containing 225 mM

KCl) and receptor was eluted in GTETD-375 (containing 375 mM KCl). The volume was reduced in an Amicon-100 microconcentrator. The quality of the purified receptor was evaluated by the standard protein gel electrophoresis.

Gel-shift assays Oligonucleotides were synthesized on a Gene Assembler Plus Synthesizer (Pharmacia). The sequences of oligonucleotides containing GGG 5′ overhangs are as follows:
K5-RE 5′ GGGTGACCGGTGAGCTCACAGCTGCCCCCCAGG-CATGCCA;
K6-RE 5′ GGGTGCAAGCTCACCTTCCAGGACTAGGGCCAGCCCA-TGCT;
K17-RE 5′ GGGTGGGAGCTGGCAGGTGGCCAGTGGTGATGAAAGC-CCAGGGG;
K14-RE 5′ GGGTAGCCTGTGGTGATGAAAGCCAAGGGGAAAGGAA
TRE-pal 5′ GGGAGGTCATGACCT;
K5-Site A (used as NS) 5′ GGGCCACTTAATCATCATCAGCTC.
Double stranded oligonucleotides corresponding to the keratin-RE and TRE palindrome were labeled with [α-^{32}P]dCTP, using the Klenow Fill In Kit (Boehringer, Mannheim, Germany). Thirty thousand cpm of the resulting probe was mixed with 2.5 fmol of purified receptor proteins and incubated first for 30 min at room temperature and then for 10 min at +4°C. The incubation was carried out in a 30 µl volume in 25 mM Tris, pH 7.8; 560 µM EDTA; 88 mM KCl; 10 mM 2-β-mercaptoethanol; 0.1 µg of aprotinin; 0.1 µg of poly(dIdC); 0.05% Triton X-100 (vol/vol); 10% glycerol (vol/vol). Samples were loaded on 4% polyacrylamide gel and separated by electrophoresis (20–25 mA) at +4°C for 2 h with a buffer containing 10 mM Tris, 7.5 mM acetic-acid, and 40 µM EDTA pH 7.8. Gels were dried and analysed by autoradiography.

Competition experiments were performed as follows: a 100 molar excess of the competitor DNA was incubated with protein at room temperature for 15 min prior to addition of the radioactively labeled DNA probe. Binding reactions were further incubated at room temperature for 15 min and then at +4°C for an additional 10 min.

RESULTS

Identification of the RARE/TRE in the K5 promoter We used a series of deletion constructs of the K5 promoter to identify a segment of the promoter responsible for regulation by RA and T3. In addition to the full size promoter (905 bp), three deletion constructs 700 bp, 560 bp, and 300 bp in length were cotransfected into primary human keratinocytes with hRARγ and cT3Rα vectors. Keratinocytes are stratified epithelial cells that express K5, K6, and K17 *in vivo*. Keratinocyte physiology is strongly affected by both RA and T3, and they express both receptors *in vivo*. We used hRARγ in these experiments because it is predominantly expressed in keratinocytes. A GH-CAT construct was used as positive control representing a native RARE/TRE that is induced by both hRARα and cT3Rα receptors in the presence of their ligands. The cells were incubated in the presence or absence of their respective ligands. Results are presented in **Fig 1**. All deletion constructs, including the smallest one (300 bp), were regulated as the wild-type K5 promoter (K5-WT): they were inhibited by hRARγ in the presence of RA and by cT3Rα in the presence of T3. In addition, RA or T3 alone had a small effect on both the K5 promoter and a positive control, due to the endogenous receptors. Their effect was enhanced by co-transfection of the receptors. Therefore the RARE/TRE element is located in the 300-bp segment of the K5 promoter. We have repeated the same experiment in HeLa cells (data not shown). We found similar results in HeLa cells, i.e., all constructs were normally regulated, they were inhibited by both hRARα and cT3Rα receptors in the presence of their ligands, confirming the location of the RARE/TRE within the 300-bp sequence.

We looked for sequences that are homologous to the TRE half-site, AGGTCA, similar to the previously identified K14RARE/TRE, within the 300-bp segment of the K5 promoter, the region between −220 and −183 that has high homology to the consensus half-site and is similar to the K14 RARE/TRE (see **Fig 9** and Tomic-Canic *et al*, 1992). In this case, the identified region has a cluster of six half-sites with variable spacing and orientation.

Identification of the RARE/TRE in the K6 promoter Using a similar approach, testing deletion constructs of the promoter, combined with sequence analysis, we identified the RARE/TRE in the K6 promoter. Two deletion constructs, 158 bp and 114 bp, in addition to the full size K6 promoter, were tested in primary keratinocytes. Results are presented in **Fig 2**. Interestingly, the 114-bp deletion construct was not regulated by hRARγ or cT3Rα receptors in the presence of their ligands, whereas the 158-bp deletion construct was regulated as the WT promoter. These results localized the RARE/TRE between −158 and −114 bp of the K6 promoter. Sequence analysis of this region identified a cluster of four sites homologous to the TRE half-sites, thus identifying the RARE/TRE in the K6 promoter (see **Fig 9**). In this case, two of the half-sites overlap.

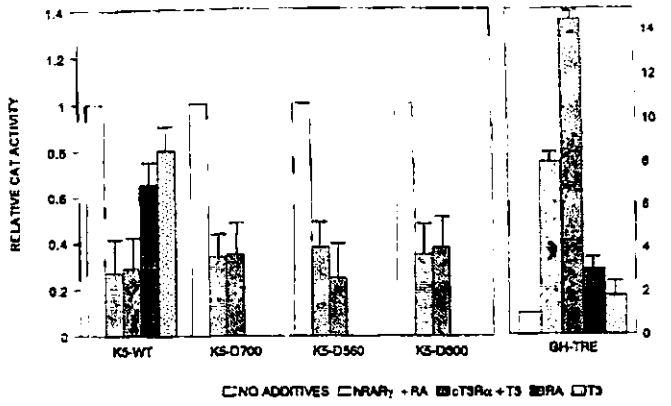

Figure 1. Regulation of the K5 deletion constructs by RA and T3 receptors in keratinocytes. The K5 deletion constructs were co-transfected along with constructs expressing hRARγ or cT3Rα into normal human epidermal keratinocytes. Effects of ligands alone (without co-transfection of the receptors) were tested with K5WT and GH-TRE constructs. Cells were harvested 48 h after transfection and CAT and β-galactosidase activities were determined. The basic activity of each CAT construct is designated as 1. Numbers on the left of the positive control plasmid represent relative CAT activities of K5 deletion constructs, and those on the right GH-TRE. *Error bars*, mean ± SD.

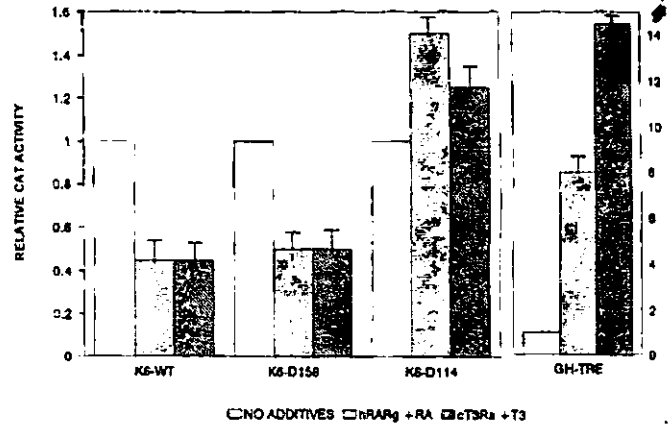

Figure 2. Regulation of the K6 promoter deletion constructs in keratinocytes. The K6 deletion constructs were co-transfected along with constructs expressing hRARγ or cT3Rα into normal human epidermal keratinocytes in the presence or absence of RA and T3, respectively. The basic, unregulated activity of each CAT construct is designated as 1 to show regulation by RA and T3 (left). Regulation of the positive control plasmid, GH-TRE, is shown on the right. *Error bars*, mean ± SD.

Identification of the RARE/TRE in the K17 promoter Because the identified RARE/TRE elements in K14, K5, and K6 keratin genes are very similar in their structure, containing a cluster of half-sites, we proceeded to identify the RARE/TRE in the K17 gene directly from the promoter sequence. Detailed analysis of the sequence revealed a region located at −180 to −158, which has a cluster of three half-sites. This region has striking similarity in structure and sequence to K14, K5, and K6 RARE/TRE elements (see **Fig 9**), and therefore we hypothesized that it is the K17RARE/TRE. This hypothesis was confirmed in subsequent experiments (see below).

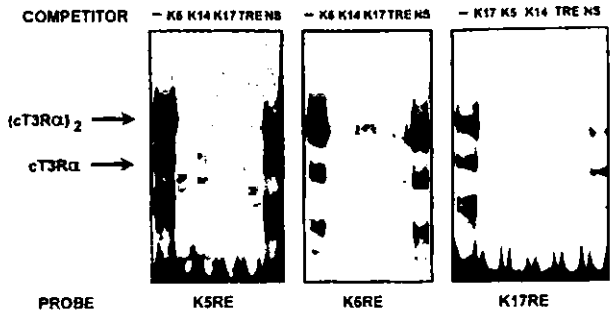

Figure 3. Purified cT3Rα specifically binds to the keratin RE. Autoradiograms of the gel-shift assays with K5RE, K6RE, and K17RE are presented. RE from keratin gene promoters were used as radioactively labeled probes and mixed with purified cT3Rα either alone (*first lanes*) or in the presence of 100-molar excess of nonlabeled competitor DNA. Competitor DNA used in each experiment are indicated on the top. DNA-protein complexes were resolved on nondenaturing polyacrylamide gel. →, binding of the receptor monomer, indicated as cT3Rα, and receptor homodimer $(cT3R\alpha)_2$. The lower unlabeled bands originate from the binding of receptor degradation products. NS, nonspecific DNA.

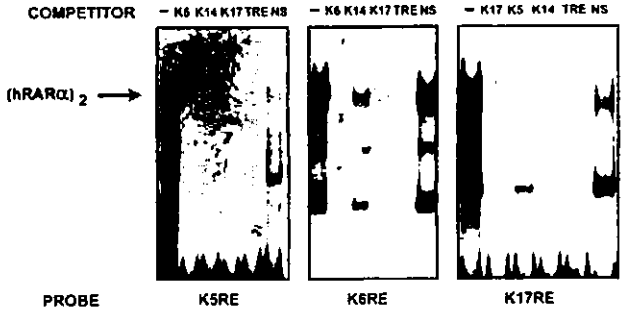

Figure 4. Purified hRARα specifically binds to the keratin REs. Autoradiograms of the gel-shift assays with K5RE, K6RE, and K17RE are presented. The RE were used as radioactively labeled probes and mixed with purified hRARα either alone (*first lanes*) or in the presence of 100-molar excess of nonlabeled competitor DNA. The competitor DNA used in each experiment are indicated on the top. The DNA-protein complexes were resolved on nondenaturing polyacrylamide gel. →, binding of the receptor homodimer $(hRAR\alpha)_2$. NS, nonspecific DNA.

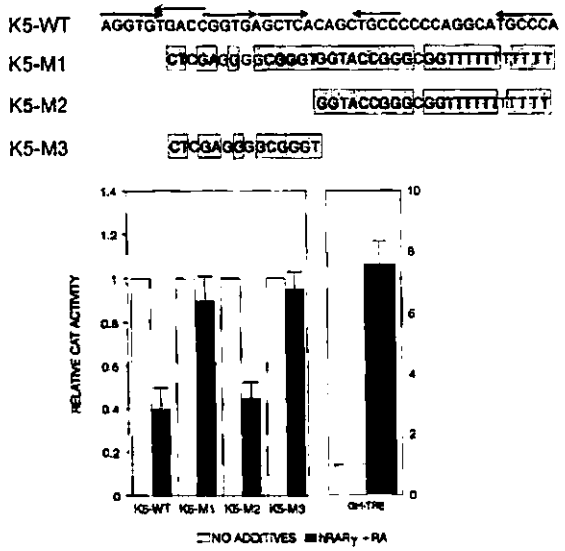

Figure 6. Identification of the K5RARE by site-specific mutagenesis. The sequence of the K5-WT is shown on the top and the sequences of the mutants are shown below. The mutated nucleotides are enclosed in the *grey boxes*. The results from co-transfections of K5M1, K5M2, and K5M3 along with hRARγ into the keratinocytes are presented on the left by *black bars*. The regulation of the positive control, GH-TRE, is shown on the right.

All identified keratin RARE/TRE elements bind RAR and T3R RARE/TRE elements from K5, K6, and K17 promoters were used as probes in gel-shift assays with purified hRARα and cT3Rα. Each of the three probes, K5 RE, K6 RE, and K17 RE, forms two complexes (noted with *arrows*) with the cT3Rα, identified as monomers and homodimers (Fig 3). To determine whether the binding is specific, we used 100 molar excess of unlabeled RARE/TRE elements of K5, K14, and K17, the perfect TRE palindrome and a nonspecific DNA K5-SiteA, which is a binding site for another transcription factor but not the nuclear receptor (Ohtsuki *et al*, 1992). cT3Rα binds specifically to all three elements, because the binding is efficiently competed by excess of specific competitors but it is not affected by excess of nonspecific competitors (NS) (Fig 3). Therefore, we conclude that the identified TREs in the K5, K6, and K17 promoters specifically bind purified cT3Rα receptor.

hRARα predominantly binds as a homodimer (*arrow*) to the K5, K6, and K17 RARE/TRE elements (Fig 4), which is similar to what

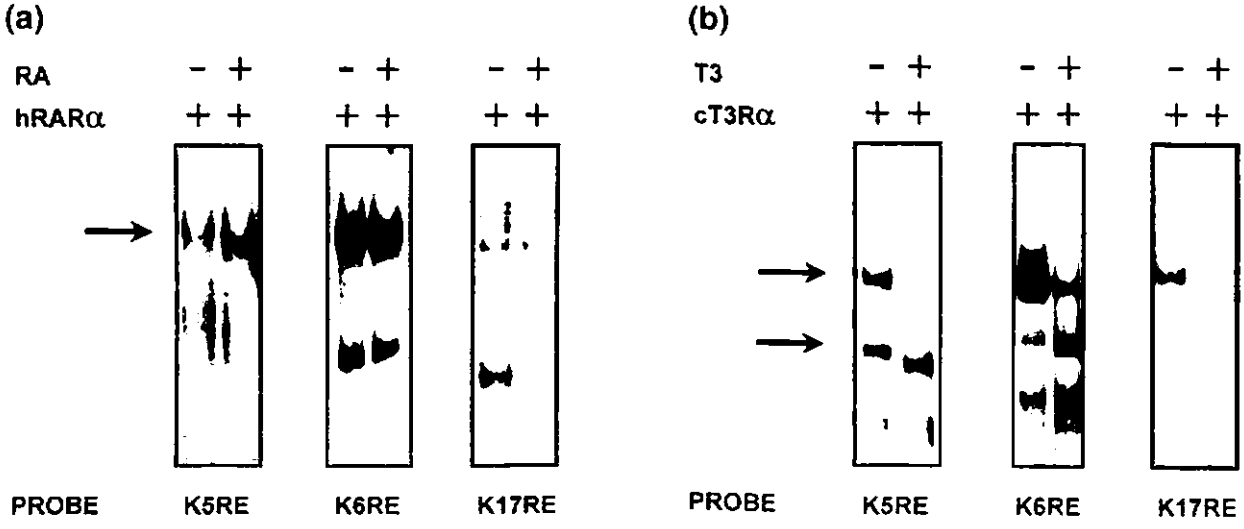

Figure 5. The effects of ligands on the binding of the receptors to the keratin RE. Autoradiograms of the gel-shift assays with all three keratin RE as probes and purified receptors in the absence and presence of their respective ligands are presented. (*a*) The binding pattern of the hRARα to keratin RE does not significantly change in the presence of RA; (*b*) the binding pattern of cT3Rα to keratin REs significantly changes in the presence of T3.

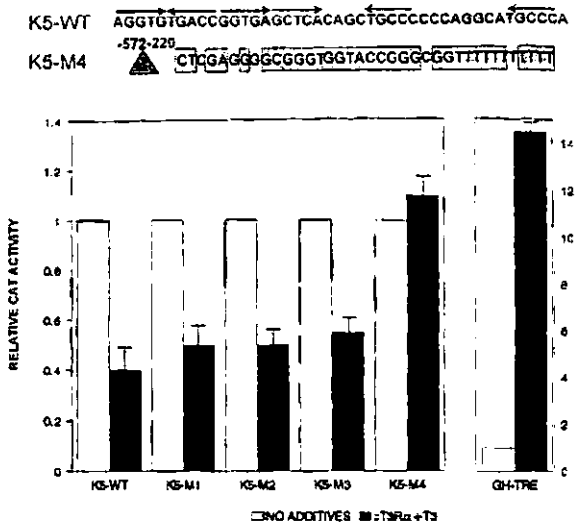

Figure 7. Identification of the K5TRE by site-specific mutagenesis. Internal deletion of the half-site responsible for cT3Rα regulation is indicated by a *triangle*. Results from co-transfections of all four K5 promoter mutants along with cT3Rα into the keratinocytes are presented on the left by *black bars*. The positive control, GH-TRE, is shown on the right.

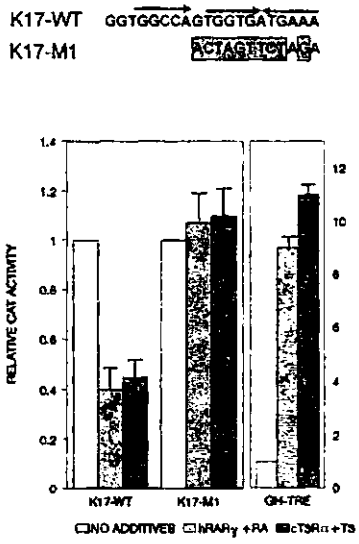

Figure 8. Identification of the K17RARE/TRE by site-specific mutagenesis. The sequence of the K17-WT is shown on the top and the sequence of the mutant is shown below. The mutated nucleotides are enclosed in *grey boxes*. The bar graphs represent co-transfection experiments in keratinocytes. Regulation of the K17WT and K17M1 by RAR or T3R in the presence of their respective ligands are shown on the left. Regulation of the positive control, GH-TRE, is shown on the right.

we found with K14RARE/TRE (Tomic-Canic *et al.* 1996a). Binding is specific because it is competed with the specific but not with the nonspecific DNA competitors (NS) (**Fig 4**). We conclude that the identified RAREs in K5, K6, and K17 promoters specifically bind purified hRARα as well.

Next, we analyzed the effects of ligand in our gel-shift experiments. We found that addition of RA causes a small change in mobility, which occurs due to a conformational change of the receptor (**Fig 5*a***). Importantly, however, the addition of T3 essentially changed the binding of the cT3Rα to the DNA (**Fig 5*b***). We found a significant increase of monomer binding and a decrease of homodimer binding due to addition of the ligand (**Fig 5*b***). This means that the addition of the hormone promotes the binding of the receptor monomer at the expense of the homodimer. Similar results were found with K14RARE/TRE (Tomic-Canic *et al.* 1996a).

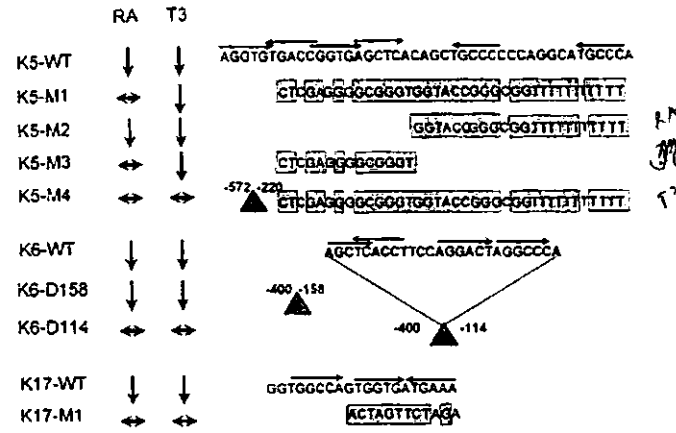

Figure 9. Characterization of the RARE/TREs in keratin genes. The summary of the results is presented. The orientations of the *arrows* indicate the type of regulation. *Grey boxes* indicate the introduced mutations in the promoters; *triangles* with numbers indicate the positions of the deletions.

Site-specific mutagenesis of the K5 and K17 RARE/TRE promoters To characterize the RARE/TRE sites in keratin promoters, we performed site-specific mutagenesis of the K5 and K17 promoters. We choose K5 as representative of the basic keratins and K17 as representative of the acidic keratins. The complete sequence of the five half-sites (40 bp) in the K5 promoter was changed, which resulted in mutant K5-M1 (**Fig 6**). Mutant K5-M1 is not regulated by hRARγ in the presence of RA (**Fig 6**), thus confirming that −220 to −183 contains a functional RARE. To determine which of these five half-sites are responsible for RA regulation, we have created two additional mutants of K5 the promoter: K5-M2, in which the three distal half-sites are altered, and K5-M3, in which the two proximal half-sites are altered (**Fig 6**). Interestingly, K5-M2 was not regulated by RARγ in the presence of RA, whereas K5-M3 was (**Fig 6**). Similar results are obtained in HeLa cells with the hRARα receptor (data not shown). This means that within the identified RARE/TRE cluster of K5 promoter, the region responsible for RA regulation is located in the first three half-sites, between −220 and −206 bp.

To determine whether the same elements are responsible for T3 regulation, we co-transfected all K5 mutants with the cT3Rα into keratinocytes. Interestingly, K5-M1, K5-M2, and K5-M3 were normally regulated by cT3Rα in the presence of T3. This suggested the possibility that the only half-site left unaltered in K5-M1 is the one responsible for the T3 regulation. To test this hypothesis we created an internal deletion of that site, resulting in construct K5-M4 (**Fig 7**). Indeed, K5-M4 construct was not regulated by T3Rα in the presence of T3 (**Fig 7**). This means that in K5RARE/TRE sites, RARE and TRE can be separated.

Using a similar approach we have mutagenized K17RARE/TRE. We altered the sequence of the middle inverted repeat (**Fig 8**), which resulted in the construct K17-M1. In co-transfection experiments the K17-M1 construct was no longer regulated by hRARα or hRARγ receptors in the presence of RA in either of the cell types we tested, HeLa and keratinocytes (**Fig 8** and data not shown, respectively). Therefore, we have localized a functional RARE in the K17 promoter to the region between −170 and −158 bp.

We also tested K17-M1 for regulation by T3. Interestingly, K17-M1 was not regulated by the cT3Rα receptor in the presence of T3 (see **Fig 8**). Because the same mutation in the K17 RARE/TRE promoter abolished regulation by both receptors, we tested this mutated K17 promoter for regulation by IFNγ. K17-M1 was normally induced by IFNγ (data not shown), as previously shown for the K17-WT promoter (Jiang *et al.* 1994). This means that the introduced mutation did not alter the general response to all stimuli of the K17 promoter, but specifically the ones responsible for RA and T3 regulation. We find it very interesting that in the K17 promoter RARE and TRE co-localize whereas in the K5 promoter they are separated.

why test K5-M4 & not just the mutant I made, or the deletion itself in wild type background.

where is glucocorticoid receptor RE? (K5-M3

DISCUSSION

In this paper we have identified and characterized the response elements for RA and T3 receptors in three different human epidermal keratin genes K5, K6, and K17 (Fig 9). This group of response elements, together with previously described RARE/TRE in keratin K14 promoter, has several unique characteristics: (i) it is the first group of native, negatively regulated RARE/TREs identified in the same gene family; (ii) all four RARE/TRE elements bind specifically both RAR and T3R; (iii) the elements are organized in clusters of 3–6 half-sites that vary in orientation and spacing; (iv) these clustered elements can convey regulation by at least two different nuclear receptors, RAR and T3R.

Localization of all four RARE/TREs (K5, K6, K14, and K17) is in close proximity of the TATA box in each promoter (within 250 bp), unlike in other genes where the response elements can be localized as far as 6–7 kb from the TATA box (Kato *et al*, 1995; Bulens *et al*, 1995). The close localization of the response elements to the TATA box in keratin genes may reflect the specificity of the mechanism by which nuclear receptors regulate keratin genes.

Although all four keratin RARE/TREs are alike, their sequences are not homologous. It is not possible to create any "consensus" keratin RE. In all four keratin RARE/TREs an inverted repeat is found, sometimes even overlapping as is the case in K5 and K6. In addition, a direct repeat separated by various numbers of nucleotides can be found in each of the four elements. This clustered organization of the half-sites within the elements provides several possible combinations of binding sites, which allows many different nuclear receptors to regulate through these elements. In the K5 promoter the sites are adjacent, in K17 they overlap, whereas in K14 they also overlap but can be distinguished by different mutations (Fig 9). Therefore, although very similar in structure, each response element has a specific sequence and configuration, possibly reflecting the strict regulatory pattern of every keratin gene.

Because of the combinatorial capabilities of the keratin RARE/TREs, it is very difficult to determine the importance of the spacing between the half-sites. There are two types of elements identified in various promoters: one that follows the 1–5 rule, and is a "perfect" direct repeat for a specific receptor, and the second that has spacings that vary from 7 to 15 bp, or even more (Kato *et al*, 1995). Interestingly, there are promoters that have combinations of two or more sites that belong to both types and contribute equally to the regulation by a given nuclear receptor (Liu *et al*, 1994; Pikarsky *et al*, 1994; Suzuki *et al*, 1994; Bulens *et al*, 1995). A RARE has been reported in the bovine K6b promoter, where it is a part of a composite enhancer that includes AP1 and AP2 sites (Navaro *et al*, 1995). Human keratin RARE/TREs do not have such combinations of two distant RE or composite structure. Instead, they are clustered in the same region.

Results from gel-shift experiments with T3R binding reveal a significant decrease of the homodimer binding and, at the same time, an increase of the monomer binding due to the addition of the hormone to the receptor. Importantly, the binding of the RAR is not similarly affected by addition of the ligand. These results correlate well with the previously described ones involving K14RARE/TRE. We have shown previously, using different mutants of T3R, that regulation of K5, K14, and K17 genes by unliganded T3R does not require heterodimerization with RXR receptors (Tomic-Canic *et al*, 1996a). Interestingly, similar results were recently found in two different negative TREs (Hollenberg *et al*, 1995; Toyoda *et al*, 1995). Taken together, these findings suggest that this novel mechanism of negative regulation of keratin genes by T3R functions in other systems as well.

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