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Negative Response Elements in Keratin Genes Mediate Transcriptional Repression and the Cross-talk among Nuclear Receptors*

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Very little is known about the mechanisms responsible for the findings that binding of nuclear receptors (NR) to some promoter elements leads to transcriptional activation, whereas binding to others leads to repression. Case in point is the group of epidermal keratin genes and their DNA sequences responsible for repression by NR. Keratin response elements (KREs) interact with receptors for retinoic acid, thyroid hormone, and glucocorticoids. KREs, by their structure and sequence, direct the binding of retinoic acid and thyroid hormone as homodimers and glucocorticoids as monomers. Such specific DNA-receptor interactions are crucial for the repression signal of transcription. In this paper we have analyzed the interactions between the KREs and NR that lead to such repression. We have found that KREs are promoter-independent. They not only provide a docking platform for the receptors, but also play a key role in directing the receptors to bind into particular configurations and coordinating the interactions among different receptors. Both an intact KRE and an intact receptor DNA-binding domain are necessary for the regulation to occur, which emphasizes the importance of interaction between the DNA and NR for proper signaling. Furthermore, KREs allow simultaneous binding of multiple receptors, thus providing fine-tuning of transcriptional regulation. The DNA/DNA-binding domain interactions in keratin promoters exemplify tissue and gene specificity of hormone action.

Glucocorticoids (GC)[1] and retinoic acid (RA) are important regulators of development, differentiation, and gene expression in many tissues, including epidermis (1–3). They are extensively used in the treatment of many skin diseases ranging from psoriasis to skin cancers (4). GC, similar to thyroid hormone and retinoids, mediate their effect through nuclear receptors (NR) that regulate the transcription of specific genes. NR constitute a large family of transcription factors that act by binding to specific sequences, response elements (RE), in the regulated genes (5). Once bound to the DNA, the receptors interact with co-regulators and the transcriptional machinery to regulate transcription (6, 7).

Extensive studies over the years have shown that steroid receptors act as homodimers whereas nonsteroid receptors, such as T3R and RAR, act as heterodimers in regulating transcription (8–10). These studies led to the current dogma that heterodimers of T3R and RAR with RXR are the functional protein complexes involved in gene regulation. Indeed, very few exceptions to this rule have been found. The complex TRE in the *TSH* gene contains a palindrome that activates and an independent half-site that mediates binding of a monomer of T3R and repression of transcription (11, 12). Similarly the TRE in *thyrotropin-releasing hormone* and *apolipoprotein A1* genes contains a complex element that involves the T3R monomer and a T3R-RXR heterodimer (13, 14). Furthermore, formation of a monomer + homodimer of GR was found to inhibit transcription of the *POMC* gene (15). It has been shown recently that the sequence of a given RE and the context of the promoter region in which the RE is located play important roles in GR regulation and interactions with AP1 transcription factors (16). Keratin response elements (KREs), which we have identified in a family of epidermal keratin genes, also constitute exceptions to the dimerization rules. KREs mediate repression of *K5, K14, K6, K16*, and *K17 keratin* genes by binding of homodimers of RAR and T3R in addition to monomers of GR (17, 18).

The KREs comprise the first group of native negative regulatory elements identified in a gene family (19). KREs have a similar structure, with clusters of sites providing a number of possible combinations for binding of NR. Additionally, we have identified the "signature" sequences in the acidic keratin and basic keratin gene families (19). Signature sequences are highly conserved clusters (over 90% identical) of binding sites found within the acidic keratin and basic keratin gene group. Interestingly, they are found only in the group of keratin genes that are regulated by all three receptors: RAR, T3R, and GR, but not in the keratin or other epidermal genes that are regulated only by RAR or GR (19).[2]

In this paper we analyze the molecular function of such specific, complex response elements that mediate repression of transcription in a selected group of genes. We found that KREs are promoter independent. We also found that KREs, by positioning a receptor in a particular configuration, form a signal for repression. This does not arise from blocking or displacing

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[1] The abbreviations used are: GC, glucocorticoids; RA, retinoic acid; NR, nuclear receptor(s); KRE, keratin response element; RE, response element(s); DBD, DNA-binding domain; GRE, glucocorticoid receptor; ERE, estrogen response element; TK, thymidine kinase; ER, estrogen receptor; GR, glucocorticoid receptor; CAT, chloramphenicol acetyltransferase; BES, *N,N*-bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid; T3, thyroid hormone; T3R, thyroid hormone receptor; TRE, thyroid response element; RAR, retinoic acid receptor.

[2] Presland, R., Tomic-Canic, M., Lewis, S. P., and Dale, B. A. (2001) *J. Dermatol. Sci.* **27,** 192–205.

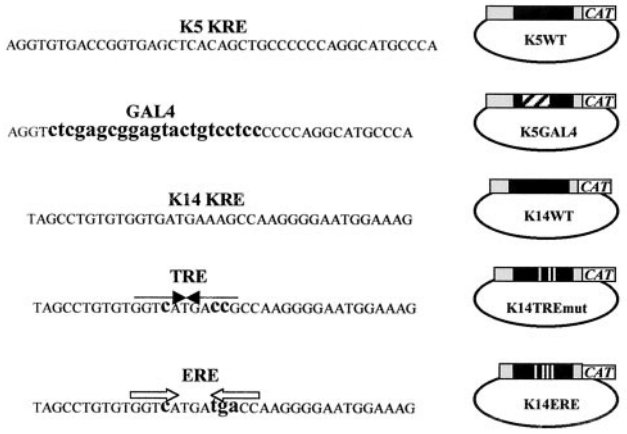

FIG. 1. **Plasmids used in the experiments.** The corresponding sequences of the KRE and introduced changes (*bold lowercase letters*) are shown on the *left*. *Arrows* are marking the position and orientation of the half-sites. Plasmid diagrams are shown on the *right*. *Black box* represents the KRE (note that the size is not proportional to the promoter size). *Striped box* represents the GAL-4-binding site. *White lines* represent single base pair substitutions.

an activator. Both an intact KRE and a receptor's DBD are necessary for repression. Furthermore, KREs allow simultaneous binding of at least two receptors (T3R and GR), and T3R dominates over GR in transcriptional regulation. Therefore, the role of KREs is to instruct the receptors to bind in a specific configuration and to create a relative order of binding among the receptors. This means that in addition to interacting with NRs in a very specific manner, KREs introduce a new level of transcriptional regulation allowing simultaneous interplay of multiple receptors.

EXPERIMENTAL PROCEDURES

Plasmids, Their Growth and Purification—Plasmids pK14CAT, pK5CAT, pK14TREmut, pK14EREmut, pKRE-TK, and pRSVZ have been described previously (19, 20). Briefly, pK14CAT and pK5CAT contain the 2 kilobases of a 5′ upstream regulatory region of the K14 and 2.5 kilobases of the 5′ upstream regulatory region of the *K5* gene. pK14TREmut was created by introducing 3-base pair mutations into the KRE of the K14 promoter thus converting the core of the KRE into the consensus TRE (Fig. 1 and Ref. 21). Similarly, using the same method (21) pK14EREmut was created to contain the perfect ERE in the core of the KRE (Fig. 1). Plasmids containing human GR nuclear receptor, RAR/ER chimeric receptor, GRE-CAT, and ERE-CAT were gifts from Dr. P. Chambon (22, 23), plasmids containing DR-4, T3R-GAL4, RAR-GAL4, and VP116 were gifts from Dr. H.H. Samuels (24). GRE-CAT, ERE-CAT, DR-4, and VP16 plasmids contain consensus response elements for glucocorticoid (GRE), estrogen (ERE), thyroid/retinoid receptor (TRE), or GAL-4, respectively, and are used as established positive controls in co-transfection experiments. RAR/ER chimeric receptor is a retinoic acid receptor containing the DNA-binding domain (DBD) of the estrogen receptor (ER). Plasmids were grown in JM101 *Escherichia coli* host to saturation density in LB medium. DNA was extracted and purified using the Magic Mega Prep Kit from Promega.

Cloning and Mutagenesis—We have used a previously described method (25) to change the KRE site into the GAL-4-binding site in the K5 promoter and create K5GAL-4. Primers used for polymerase chain reactions were: K5GAL4F, 5′-GGGCTCGAGCGGAGTACTGTCCTCCGCCCAGGCATGCCCA; K5GAL4R, 5′-TTTCTCGAGCACAGTGGTGTGGGGTGCAA; K5outF, 5′-GGGTCTAGAGGATCCCCGGGTTCCTAAC; K5outR, 5′- GGGAAGCTTCTTGTTCCTGGTGGAG. We used *Xho*I for the inside restriction enzyme and the insert was introduced into *Xba*I and *Hin*dIII restriction sites. Using this method we have created plasmid K5GAL-4, which instead of the KRE contains a gal-4-binding site in the same position (Fig. 1).

Cell Growth—HeLa cells were maintained in Dulbecco's 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 (26). The day before transfection cells were plated onto 60-mm dishes in low density (10–15% confluency). Four hours before transfection the medium was changed to phenol red-free Dulbecco's modified Eagle's medium supplemented with charcoal pretreated, depleted 10% calf serum, as described (26).

Normal human foreskin epidermal keratinocytes were a generous gift from Dr. M. Simon. The cultures were initiated using 3T3 feeder layers as described (27) 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 (keratinocyte-SFM, Life Technologies, Inc.). Cells were expanded through two 1:4 passages before transfection and transfected at 100% confluency.

Transfection using $Ca_3(PO_4)_2$ and BES Co-precipitation—We have generally followed published procedures for cells that were at 15–20% confluence (28). At the time of transfection, to each dish were added 3–5 μg of the CAT plasmid, 1 μg of the nuclear receptor expression vector plasmid, 1 μg of pRSVZ reference plasmid, and a sufficient amount of carrier to bring the total to 10 μg of DNA. The DNA mixture was prepared with 50 μl of 2.5 M $CaCl_2$ and filled to 500 μl of dH_2O and subsequently added to 500 μl of BES buffer followed by vigorous shaking. After 10 min incubation at room temperature the precipitate mixture was aliquoted to the duplicate plates. After transfection, cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-pretreated depleted calf serum in the presence or absence of 0.1 or 1 μM dexamethasone (Sigma) in ethanol or T3 (Sigma) in 0.1 N NaOH. The cells were harvested 48 h after transfection by scraping into 5 ml of phosphate-buffered saline, washed once more in phosphate-buffered saline, and resuspended in 150 μl of 0.25 M Tris buffer, pH 7.8. All transfections were performed on duplicate plates, and each transfection experiment was repeated two to five times. CAT and β-galactosidase assay were performed as described (29).

Transfection by Polybrene with Me_2SO Shock—Transfections using Polybrene with Me_2SO shock were used to transfect the DNA into the 100% confluent keratinocytes as previously described (29). On the day of transfection, cells were washed and incubated in the basal medium without epidermal growth factor and bovine pituitary extract. Each transfection contained 10–15 μg/dish of keratin-CAT construct and 3 μg/dish of RSVZ construct. The cells were then incubated with or without 0.1 or 1 μM dexamethasone (Sigma) dissolved in ethanol or T3 (Sigma) in 0.1 N NaOH. 48 h after transfection cells were washed twice with phosphate-buffered saline and harvested by scraping. The cell disruption by repeated freeze-thaw cycles, as well as CAT and β-galactosidase assays have also been described (29).

Enzyme Assays—Briefly, the substrate solution contained 6 mg of *o*-nitrophenyl-D-galactoside (Sigma) freshly dissolved in PM buffer (66 mM Na_2HPO_4, 33 mM NaH_2PO_4, 40 mM mercaptoethanol, 2 mM $MgSO_4$, and 0.1 mM $MnCl_2$). The reaction mixture contained 100 μl of substrate solution, 300 μl of PM buffer, and 50 μl of keratinocyte cell extract or 20–30 μl of HeLa cell extract. It was incubated at 37 °C until development of yellow color was obvious, usually 10–30 min. The time of the reaction was recorded and the reaction stopped by addition of 0.4 ml of 1 M Na_2CO_3. OD_{420} was measured on a spectrophotometer (Gilford).

The CAT reaction mixture contained 69 μl of 1 M Tris HCl, pH 7.8, 1 μl of ^{14}[C]chloramphenicol (Cm, 40–50 mCi/mmol, PerkinElmer Life Science), 20 μl of 4 mM acetyl-CoA solution, 30–60 μl of cell extract, and enough water to bring the total reaction volume to 150 μl. After incubation at 37 °C for 30 min, the mixture was extracted into 1 ml of ethyl acetate, phases were separated by brief centrifugation, the organic layer was transferred to a new tube, and the solvent evaporated. The residue was dissolved in 30 μl of ethyl acetate and separated by thin layer chromatography on silica gel in chloroform:methanol, 95:5. The plates were exposed to x-ray film for 12 to 24 h, and the intensity of radioactive spots determined using Ambis Radioanalytic System (Ambis, Inc., San Diego, CA). The conversion of chloramphenicol to its monoacetylated derivative was kept below 50% by varying the amount of extract or the duration of the reaction.

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 three or more times, with each data point resulting from duplicate or triplicate transfections.

Electrophoretic Gel Mobility Shift Assays and Footprinting—The *E. coli*-expressed DNA-binding domain portion of hGR was a gift from Dr. H. H. Samuels and previously described (30). Purification of recombinant cT3Ra was done following a previously published procedure (17, 18). Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus Synthesizer.

1 μg of primer K14ft (5′-AGGGGGCGGGCCTGGCACTTTCCA) was labeled by polynucleotide kinase (Promega) and [γ-^{32}P]dATP (Amer-

sham Pharmacia Biotech). 1.5×10^6 cpm of each primer is used in the primer extension reaction using K14ft template (5′-GACCTGGCTGGGAGTTGGCGCTAGCCTGTGGGTGATGAAAGCCAAGGGGAATGGAAAGTGCCAGGCCCGCCCCCT) and Klenow endonuclease (Roche Molecular Biochemicals). The product was purified from the 2.5% agarose gel. The band corresponding to the size of the probe is cut out of the gel and eluted overnight in TE buffer, pH 8, at +4 °C, which was followed by ethanol precipitation. Double-stranded TRE oligonucleotide AGGTCATGACCT, flanked by *Hin*dIII overhangs (5′-AGCTT-3′) was labeled with [α-^{32}P]dATP, using the Klenow fragment of *E. coli* DNA polymerase I. 30,000 cpm of each resulting probe was mixed with 2.5 fmol of purified receptor proteins and incubated first for 30 min at room temperature then for 10 min at +4 °C. The incubation was done in a 30-μl volume in 25 mM Tris, pH 7.8, 500 μM EDTA, 88 mM KCl, 10 mM 2-mercaptoethanol, 0.1 μg of aprotinin, 0.1 μg of poly(dI-dC), 0.05% Triton X-100 (v/v), 10% glycerol (v/v). Samples were either loaded on 4% polyacrylamide gels 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, or used in DNase I footprinting experiments (see below). Gels were dried and exposed to x-ray film for 4 h at −70 °C.

For the DNase I footprinting experiments we have followed previously described protocol (18). Two different reactions were performed in parallel: A/G Maxam-Gilbert sequencing (following the standard protocol) and DNase I footprinting. For the footprinting reaction our protocol for gel shifts was used to allow binding of the protein to the DNA (see above). After 20 min incubation at +4 °C, 50 μl of solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added and incubated 1 min on ice. Next, 3 μl of the 1:25 dilution of the DNase I (5 units/ml stock), which we have found optimal for our conditions, was added and incubated exactly 1 min on ice. The reaction was stopped by adding 90 μl of "stop" solution containing 20 mM EDTA, pH 8.0, 1% SDS, 0.2 M NaCl, and 100 μg/ml yeast RNA. DNA was purified by phenol extraction, followed by ethanol precipitation. The pellet was resuspended in 1.4 μl of 9 M urea, 1% Nonidet P-40 and, after mixing, 4.6 μl of formamide loading buffer (commercial from U. S. Biochemical Corp.) was added. All samples were heated at 90 °C for 5 min, chilled on ice, and loaded on the 12% sequencing polyacrylamide gel together with samples with A/G Maxam-Gilbert sequencing reactions of the same DNA. Gels were dried on the gel-dryer and exposed to x-ray film. The footprint localization was determined by the bands that are "protected" by the bound protein from cleavage by DNase I, which appears on the film as "disappeared" bands when the footprinted sample lane on the gel is compared with the sample that had no protein in the mixture. The protected bands are then compared with the A/G sequence lane on the same gel, revealing the nucleotides involved in binding of protein.

RESULTS

The KREs Mediate Transcriptional Regulation by Active Repression—The complex structure of KREs provides binding for all three receptors, RAR, T3R, and GR. To establish the binding patterns of these receptors to the KRE we performed footprinting experiments with K14RE and purified T3R, RAR, and GR-DBD. We found that the binding sites for each of these three receptors overlap (Fig. 2). RAR occupies the largest portion of the KRE, from −50 to −92. The T3R site is significantly smaller, occupying 16 base pairs, from −62 to −78. Last, the primary GR-binding site is located at −51 to −57. As we increased the concentration of GR we found that up to four GR monomers bind to the KRE (19).

The immediate question is what is the function of the KREs? There are three possible models: 1) *docking*, where KREs only provide a binding sites for the receptors, while the negative regulation occurs through interactions with additional auxiliary repressor protein(s); 2) *de-activation*, where the receptor displaces a positive regulator(s) from keratin DNA, or forms inactive complexes with the positive regulators that convert activation to inhibition; and 3) *active repression*, where KREs, by their structure and sequence, instruct the receptor to act as a negative regulator. This last model implies that the information for negative regulation is inherent in the sequence of KREs, and that this information is conveyed to the receptor through specific DNA-protein interactions, causing the receptor to repress transcription.

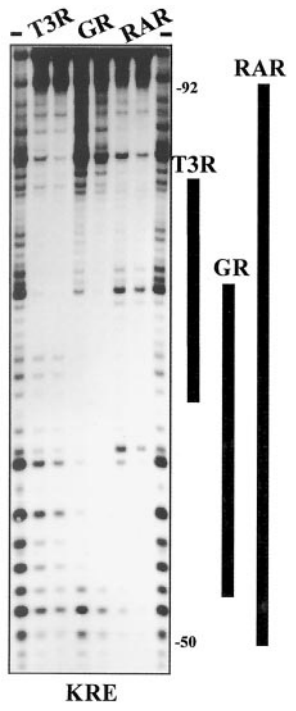

FIG. 2. **Footprinting of the purified T3R-, GR-DBD-, and RAR-binding sites on the KRE.** The DNA surface occupied by T3R is marked with the *gray rectangle*; GR-DBD with *black,* and RAR with *white. Numbers* indicate the position of the KRE relative to ATG.

First, we tested the docking hypothesis by converting the K5RE into a GAL4-binding site. This mutagenesis introduced a 17-nucleotide long binding site, which completely altered the sequence and structure of the KRE but still providing a docking site to the receptor through the GAL4-binding site. If the KRE only functions to provide a docking site for the receptor, the GAL4-binding site would also provide this function, allowing the receptor to bind and repress transcription. If, however, the binding of the receptor is not the only function of the KRE, repression should not occur.

We tested the K5GAL4 mutant in co-transfection experiments with the GAL4-RAR and GAL4-T3R in primary human keratinocytes. Both GAL4-RAR and GAL4-T3R induced rather than repressed the K5-GAL4 promoter in the presence of their respective ligands (Fig. 3*A*), whereas the wild-type K5 promoter was not regulated by GAL4 constructs. The control plasmid containing the GAL-4-binding site, VP116, was induced by both GAL4 receptors, as expected. We obtained similar results from co-transfections in HeLa cells (data not shown). This result clearly eliminates the simple docking hypothesis. It further suggests that the basic function of the receptor simply docked to a promoter is to induce transcription.

To test the deactivation hypothesis, we altered K14RE into the consensus TRE palindrome. The resulting mutant promoter had only three nucleotides changed, while the orientation and spacing between the half-sites remained intact. It is important to note that the introduced mutations are substitutions, not insertions or deletions, therefore they altered neither the size nor the position of the RE. If the deactivation model is correct, the mutation would still allow the receptor to bind and perform its function of blocking a positive regulator. Therefore, the mutated promoter should be normally repressed by the receptors. However, if the function of the KRE is not simply de-activation, the mutated keratin promoter should not be repressed by the receptors. This is exactly what we found. First, we wanted to establish the binding properties of the K14TRE mutant. We used the consensus TRE, K14RE, and K14TRE

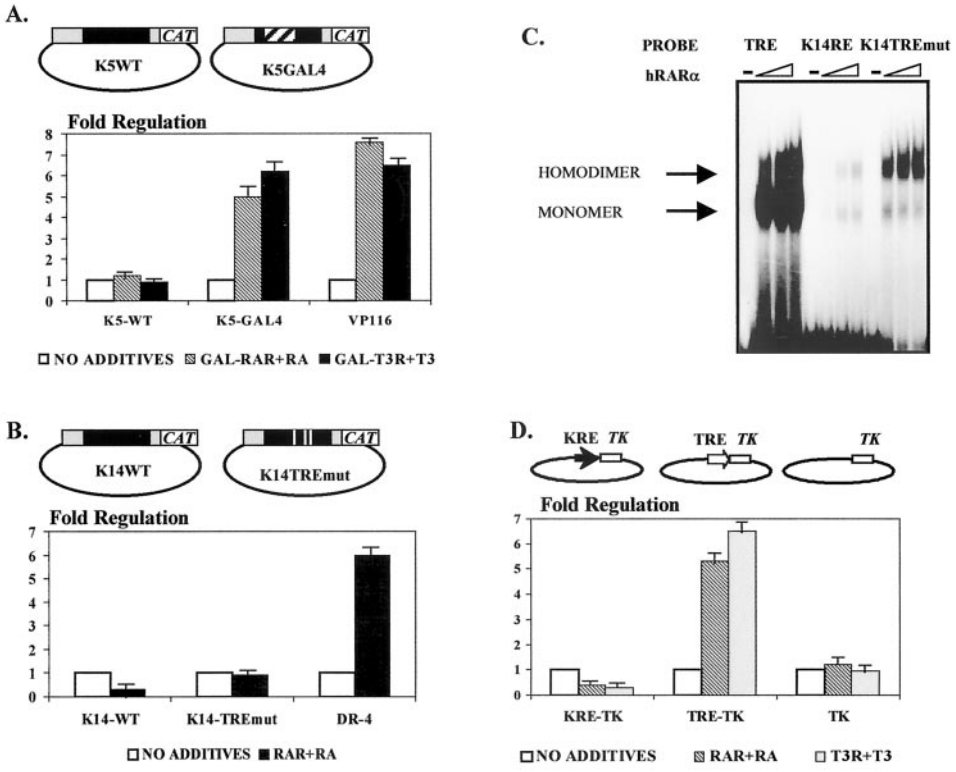

FIG. 3. **KREs are true negative response elements.** *A,* docking of the receptors to the promoter using GAL4 is not sufficient for repression to occur. Results from co-transfection experiments in primary human keratinocytes with K5WT and K5GAL4 mutant promoter are presented. The change in K5 promoter is shown in the diagram and in Fig. 1. *B,* de-activation is not the model of receptor function on KREs. In co-transfection experiments K14-WT, containing intact KRE, is repressed whereas K14TREmut, containing mutated KRE, which is converted to the consensus TRE, is not regulated by RAR in the presence of its ligand. Control plasmid, DR-4 (consensus TRE), contains two direct repeats of half-sites (AGGTCA), spaced by four nucleotides, and it is induced by RAR, as expected. *C,* K14TREmut efficiently binds the receptor although it does not mediate repression of the K14 promoter. In gel-shift experiments, purified RAR receptor binds similarly to all three probes, the TRE consensus palindrome (a positive control), K14RE, and K14TREmut. The intensity of the bands corresponds to the affinity of the binding. *D,* KREs are not promoter context-dependent. KRE was cloned into the minimal TK promoter and tested for regulation in co-transfection experiments. KRE-TK was repressed by both RAR and T3R in the presence of their ligands. This means that KRE confers repression to heterologous, TK promoter. TRE-TK (a positive control that contains consensus TRE) was induced by both receptors whereas TK (negative control that contains no response elements) was not regulated, as expected.

mutant DNA as probes in gel-shift experiments. The consensus TRE palindrome used as a control probe binds RARα, as monomer and homodimer. A similar binding pattern was found with K14RE. Interestingly, we found that the K14TRE mutant binds RARα with an affinity even higher than the wild-type K14RE (Fig. 3*C*). The introduced mutations did allow the efficient binding of the receptor and therefore the receptor should block the binding of a positive regulator, if de-activation was the mechanism. However, RARα did not regulate the K14TREmut in the presence of its ligand (Fig. 3*B*), which indicates that the deactivation model does not hold. Please note that RARα and its ligand, as expected, induced the DR-4, a positive control containing a consensus positive TRE cloned into the TK promoter. This means that the de-activation is not the function of the NR bound to KREs.

An alternative explanation could be that the introduced mutations changed the binding of a positive regulator. If a positive regulator is not able to bind the mutant, a decrease in basic promoter activity should be detected. On the other hand, if the introduced mutations increased the binding of the positive regulator so that RARα can no longer block its binding, the basic promoter activity should be increased. However, there was no significant difference in the basic level of transcription between the WT and the mutated promoter (data not shown). This argues against the existence of a displaceable positive regulator, strengthening our conclusion that the function of the KREs is not de-activation.

This leaves us with the third hypothesis that KREs are true negative elements that mediate active repression through direct binding of the receptors. To test this hypothesis we have removed the KRE from its promoter and cloned it into a heterologous promoter. The TK-promoter has been used extensively to test heterologues DNA binding elements (31, 32). If the active repression model is correct, the KRE should still function as a negative RE even in the context of the TK promoter. This is precisely what we found: the KRE cloned into the TK promoter mediated repression by RAR and T3R (Fig. 3*D*) and GR (18). This means that KREs are "self-contained negative REs," *i.e.* they contain all the information necessary to instruct the receptor to repress transcription, independently of the background of the promoter. This result is very important because it proves that KREs are not promoter-context dependent and that they mediate regulation of keratin gene expression by active repression.

The DNA-binding Domain of the NR "Reads" the Instruction from the KRE Sequence—Binding of the receptor to the KREs mediates repression of transcription. This means that KREs instruct the receptor to repress through specific interactions with the receptor DBD. To test this instructive model, we altered the DBD of the RAR to eliminate the specific interactions between the RAR DBD and the KRE. We used a chimeric RAR that has the DBD of the ER. ER does not regulate keratin gene expression, *i.e.* its DBD has no ability to communicate with KREs. If our hypothesis is correct, the chimeric ER/RAR should not regulate the keratin promoter. Indeed, while ER/RAR normally regulates the ERE-CAT positive control, the

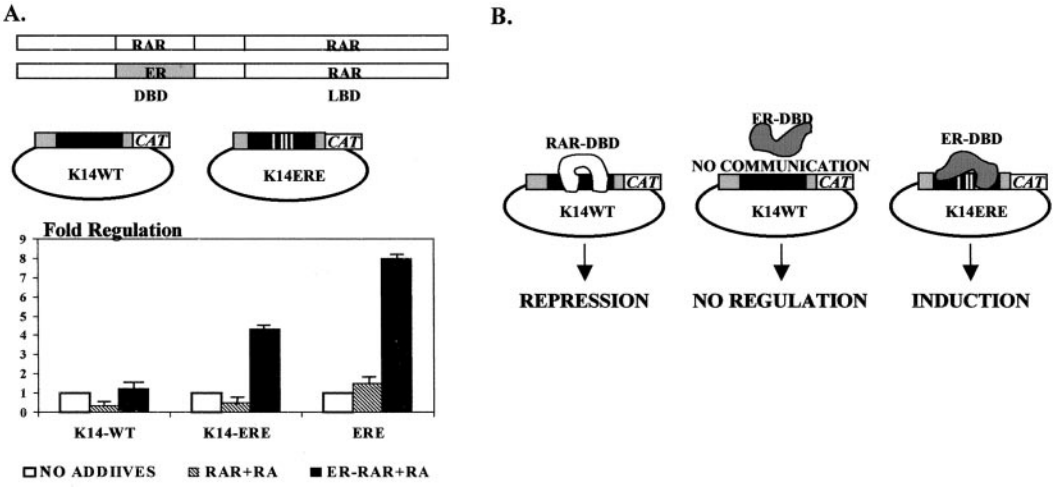

FIG. 4. **Intact KRE and the receptor's DBD are necessary for repression of keratin gene expression.** *A,* K14WT is not regulated by ER/RAR chimera receptor in co-transfection experiments because there is no communication between ER-DBD and KRE. Conversely, changing the KRE to ERE restores the communication and the K14EREmut is regulated. Schematic presentations of the receptors used in the experiments are shown on the *top* followed by the diagrams of the plasmids, shown *below*. Results from co-transfection experiments are shown at the *bottom*. *B*, cartoon illustrates the conclusion that intact KRE and receptor's DBD are necessary for the repression.

chimera does not regulate the K5 and K14 promoters (Fig. 4). This means that an intact DBD of the RAR receptor is necessary for communication with the KRE and repression of *keratin* gene expression.

Conversely, we modified the K14RE into a consensus ERE. If the KRE plays a role in instructing the receptor, this mutant promoter should instruct the ER/RAR chimera to induce, rather than repress transcription. Results from co-transfection experiments confirm this hypothesis. The ER/RAR chimera, in a ligand-dependent manner, induced the K14EREmut (Fig. 4). In other words, if RAR is bound to KRE via RAR-DBD it represses, but if it is bound to ERE via ER-DBD it induces. This means that specific interactions between the intact DBD and KRE are necessary "key players" in the mechanism of repression of keratin gene expression.

KREs Mediate Nuclear Receptor "Cross-talk"—To determine how the simultaneous presence of multiple receptors modulates the binding pattern to KREs and regulation of keratin gene expression, we have performed footprinting, gel-shift, and co-transfection experiments with a combination of T3R and GR. We found that T3R and GR can simultaneously bind the KRE in gel-shift and footprinting experiments (Fig. 5). T3R occupies its two binding sites, which overlap the middle two of the four binding sites for GR. Interestingly, the binding of the T3R does not interfere with the binding of the GR to the outlying two binding sites. The binding of the T3R is "supershifted" by the presence of the GR, and the supershift "grows" due to the increased amount of the GR (Fig. 5*A*). These results were confirmed in footprinting experiments. Small amounts of GR present simultaneously with T3R allow the binding of the T3R to its sites and of the GR to its primary binding site. As the concentration of GR increases, the quaternary GR-binding site, which was unoccupied, binds to another monomer of the GR. This means that the middle two binding sites can be occupied by T3R and the outer two sites by two monomers of GR.

Furthermore, the T3R and GR interact when they are simultaneously bound to the KREs. In co-transfection experiments, T3R blocks keratin gene regulation by dexamethasone when both are present at the same time (Fig. 5*B*). Conversely, dexamethasone does not affect the induction of keratin gene expression mediated by unliganded T3R. We conclude that T3R dominates over GR in regulating keratin gene transcription. We have shown previously that RAR dominates over T3R in regulating keratin genes (17). These findings are important because they illustrate how the complex REs mediate multiple, simultaneous regulatory signals (Fig. 5*C*).

DISCUSSION

In this study we show that the molecular function of the complex response elements in keratin genes (KREs) is to determine a specific configuration of the bound receptors and cause them to repress transcription. This means that the KREs create the message of repression by instructing the receptors how to bind. In addition, KREs, by providing simultaneous binding and interaction of multiple receptors create a hierarchy among the receptors, a new level of transcriptional regulation. KREs serve as an excellent example of sequences customized to select for specific receptor configuration, and to allow simultaneous receptor binding to provide a gradient of hormone action. We found that the KREs function independently of the promoter context and that sequence of the KREs and receptors DBD are necessary for the regulation to occur.

The concept of DNA as a regulator of transcriptional signal is not novel (for review, see Refs. 33–35). For example, studies by Lefstin and Yamamoto included a variety of positive GREs and suggested that REs may act as "ligands for regulators" (34). Similarly, we found that in the case of KREs, DNA creates a signal to shut down the transcription of its own promoter, *i.e.* the DNA acts as an "antagonist" for the receptor. However, KREs do even more than that. They allow the interplay of multiple nuclear receptors, thus creating a gradient of hormonal action. The sequences of KREs, supplying the signal for repression, allow three receptors with their respective ligands to respond to a large variety of physiological conditions under which the repression of keratin genes is required. This is extremely important in the epidermis because it is a target tissue that is exposed to and depends on complex hormonal regulation. The purpose of the receptor interplay is to provide the fine-tuning of transcriptional regulation of keratin genes in a large variety of physiological and pathological conditions.

The hierarchy in receptor regulation of keratin genes is the following: RAR dominates over T3R (17) and T3R dominates over GR, although both receptors can simultaneously bind to the KRE. The footprinting results with these receptors on KRE suggest such a hierarchy. The RAR footprint is the largest, dominating the space and occupying almost the entire KRE, whereas the T3R, although overlapping, occupies a much smaller segment. Furthermore, GR binds as four monomer

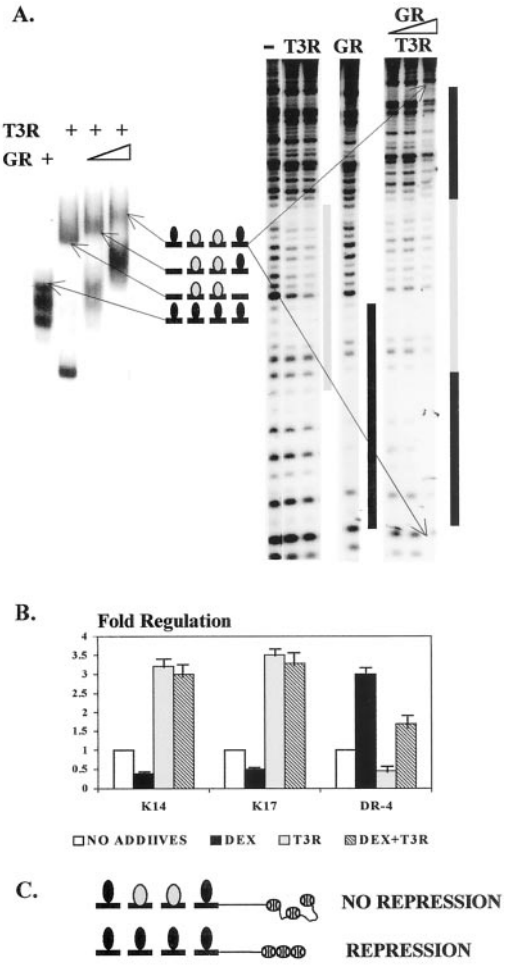

FIG. 5. **KREs provide receptor cross-talk.** *A,* simultaneous binding of the T3R and GR to the KRE in gel shift (*left*) and footprinting (*right*) experiments. The *first lane* in the gel-shift panel represents the binding of the GR-DBD to KRE in the formation of four monomers, as previously shown (18). The *second lane* shows the binding of the purified T3R in the formation of homodimer, as previously shown (17). The *last two lanes* represent the binding patterns when both receptors are simultaneously present. Binding of the T3R homodimer is shifted in the presence of the GR. This change corresponds to the binding of the GR monomer to its primary binding site, as shown in the footprint. Further increase of the GR leads to another shift in T3R binding, corresponding to the binding of the GR monomer to its quaternary site, as shown in the footprint. *Arrows* and the *cartoon* represent possible combinations of the binding pattern of the two receptors, which depend on the amount of the receptors present. Black *ovals* represent monomers of GR; white ovals represent homodimers of T3R. *B,* T3R dominates over GR in regulation of keratin gene expression in co-transfection experiments. K14 and K17 are repressed by GR in the presence of its ligand and induced by unliganded T3R. When both are simultaneously present, T3R is dominant over the GR and K14 and K17 are induced as with T3R alone. DR-4, a positive control containing consensus TRE, is induced by GR, repressed by unliganded T3R. However, when both receptors are simultaneously present neither of the receptors are dominant. *C,* cartoon represents the two alternative mechanisms of keratin gene regulation resulting from the T3R and GR cross-talk.

units and all four must be bound for regulation to occur. Therefore, one can envision multiple regulatory levels that are being introduced. The first is the concentration of the hormones that reach and are produced in epidermis (36, 37). For example, if there is a high concentration of RA in the epidermis, the differentiation process is inhibited and keratinocytes become "basal-like" by changing their phenotype and keratin gene expression pattern (1). Furthermore, the simultaneous presence of two hormones multiply the possibilities of regulatory patterns. For example, if RA and T3 are present, RAR will dominate over the T3R in regulating keratin genes (17). The presence of T3 will allow GC regulation whereas its absence will block it.

Furthermore, the type and amount of the hormones become irrelevant if there are no receptors. Therefore, the next regulatory level is the presence and a relative amount of a particular receptor. Keratinocytes express multiple NRs, such as RARγ, RXRα, T3R, GR, ER, progesterone receptor, peroxisome proliferator-activated receptor, and farnesoid X receptor *etc*. (1, 38–42). In the case of the presence of multiple receptors, their relative amounts become an important factor. Combined, the type and amount of receptors with the type and the amount of respective hormones determine the next regulatory level: which co-regulators will interact and participate in transcriptional regulation. For example, if the GC are present in high amounts GR binds KRE as four monomers. In this context, none of the common co-regulators (SRC-1, GRIP-1, and NCoA) are involved due to a specific conformation of the GR (18). Furthermore, when the liganded RAR or T3R interact with KRE, NCoA/SRC-1 become co-repressors rather than co-activators.[3]

Obviously, there is a large variety of possible scenarios, all examples of fine-tuning necessary for maintenance of the target tissue, epidermis. It is no longer "only" the DNA-receptor and its ligand co-regulators integrators and transcriptional machinery. The complex elements, such as KREs, provide numerous combinations of possible interactions among three receptors independently and simultaneously, their ligands and their interactive proteins. The important point is that such complicated, fine-tuning is made possible by very specific DNA elements, *e.g.* the chaos is well organized by the KREs. By providing the binding of a specific configuration of the receptors and multiple receptor binding, KREs introduce a new dimension in the transcriptional regulatory systems. Although these DNA elements are uniquely present in the keratin gene family, we believe that as we focus our studies more on native hormone elements in their physiological contexts, simultaneous multiple transcriptional signals maintained by specific DNA elements will probably become a general phenomenon.

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Negative Response Elements in Keratin Genes Mediate Transcriptional Repression and the Cross-talk among Nuclear Receptors

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