Identification of the Retinoic Acid and Thyroid Hormone Receptor–Responsive Element in the Human K14 Keratin Gene

Marjana Tomic-Canic, Ivana Sunjevaric, Irwin M. Freedberg, and Miroslav Blumenberg

Ronald O. Perelman Department of Dermatology (MT-C, IS, IMF, MB) and Department of Biochemistry (MB), NYU Medical Center, New York, New York, U.S.A.

The promoter of human K14 keratin gene, specific for the basal layer of stratified epithelia, is regulated by nuclear receptors for retinoic acid and thyroid hormone. However, the DNA sequences responsible for this regulation have not yet been identified. To identify the retinoic acid–responsive site, we have devised a simple site-specific mutagenesis method and introduced mutations into the K14 keratin gene promoter. These mutations identify the retinoic acid–responsive site. The site consists of a cluster of consensus palindrome half-sites in various orientations. As shown previously, retinoic acid and thyroid hormone receptors can recognize and bind common sequences in regulated genes. Here, we describe mutations that abolish regulation by both receptors. Interestingly, the hormone-dependent and -independent regulatory sites of the thyroid hormone nuclear receptor can be separated. Clusters of half-sites that share structural organization with the K14 regulatory site were found in the K5 and K10 keratin gene promoters. Similar clusters may be responsible for retinoic acid–mediated transcription regulation in epidermis. *J Invest Dermatol 99:842–847, 1992*

The first evidence that retinoic acid (RA) controlled epidermal differentiation was reported in 1934 [1]. Since then, for over half of the century, the effects of RA on skin have been explored. RA modulates normal epidermal differentiation: RA deficiency induces keratinocytes to differentiate and leads to hyperkeratosis, whereas excess of RA prevents differentiation and preserves keratinocytes in a basal layer–like phenotype [2]. Retinoids have profound effects on disorders of keratinization, sebaceous gland function, even epithelial malignancies, and they are currently used in the treatment of a variety of epidermal diseases [3].

RA acts primarily by regulating gene expression through nuclear receptors. These receptors are proteins that specifically bind their cognate ligand; recognize and bind, through their zinc-finger motifs, to DNA sequences known as recognition elements (REs); and, in regulated genes, modify transcription [4]. RA receptors belong to the much larger family of steroid, thyroid hormone nuclear receptors [5,6]. Of the three known members of the retinoic acid receptor (RAR) family, α, β, and γ [7–9], RARγ is predominantly and specifically expressed in skin [10]. mRNAs for each of these three receptor may be differentially spliced, which results in at least seven different mRNAs for each receptor gene [11]. The recently discovered retinoid "X" receptor (RXR) family also has three known members: α, β, and γ [12,13]. The RXRs require significantly higher concentrations of RA for their function than the RARs *in vitro*. The cognate ligand for these receptors has been identified as 9-cis-RA [14]. RXRs can form heterodimers with other members of the nuclear receptor family, potentially modifying their action [13].

RA nuclear receptors can bind to a common recognition element in the DNA (RARE). RAREs have been identified in the promoter regions of growth hormone, laminin, osteocalcin, EGF receptor, and RARβ genes [15–19]. Receptors for RA, thyroid hormone (T3), vitamin D3, and estrogen can bind to identical sequences, such as the thyroid hormone responsive element (TRE) perfect palindrome [20]. Most natural RAREs can bind several receptors, and among the identified RAREs only RAREβ is specific for the RA receptors [19,21]. Studies with synthetic sequences indicate that the spacing and orientation of half-sites specify and distinguish receptor binding [22,23]. However, the native RE sequences that exist in regulated genes, even if they share homology with the consensus palindrome, demonstrate different, sequence-dependent, regulatory patterns [15–19].

During the past decade, several reports suggested that RA indirectly regulates keratin genes expression [24,25]. However, we have shown that RA and T3, through their receptors, directly regulate expression of epidermal keratin genes [26]. The expression of both the basal cell–specific keratin genes K5 and K14 and the differentiation-specific K10 keratin gene are suppressed by RA and T3. Therefore, we have proposed that RA has a dual effect on keratin expression in epidermis: indirect, by regulating epidermal differentiation so that a change of keratin expression is a consequence of

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Marjana Tomic-Canic's and Ivana Sunjevaric's permanent address: Institute Vinca, Yugoslavia.

Reprint requests to: Dr. Blumenberg, Ronald O. Perelman Department of Dermatology, NYU Medical Center, 550 First Avenue, New York, NY 10016.

Abbreviations:
- bp: base pairs
- CAT: chloramphenicol acetyl transferase
- dNTP: deoxynucleotide-triphosphate
- LB: Luria broth
- PBS: phosphate-buffered saline
- PCR: polymerase chain reaction
- RA: retinoic acid
- RAR: retinoic acid receptor
- RARE: retinoic acid recognition element
- RE: recognition element
- RXR: retinoid "X" receptor
- T3: thyroid hormone
- T3R: thyroid hormone receptor
- TRE: thyroid hormone responsive element
- WT: wild type

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phenotypic change of the keratinocytes, and direct by direct interaction of nuclear receptors with keratin genes.

The cell type-specific regulation of the K14 keratin gene is determined at the 5′ upstream sequences [27]. The site recognized by a general transcription factor AP-2 has been shown to play a role in transcription, and both proximal and distal sequences seem important for correct expression [28,29]. Although the complete constellation of transcription factors is the subject of intense investigation, in this report we specifically focus on the sites of action, i.e., the recognition elements for nuclear receptors that modify the level of expression of K14 gene, namely, RARE and TRE.

To identify the RE for the RA and T3 nuclear receptor within the K14 keratin gene promoters, we have developed a site-specific mutagenesis method [30] and used it to mutagenize the putative RE in the K14 promoter. The mutations introduced into the putative RE alter the normal RA/T3 regulatory pattern of the K14 promoter. The RE consists of a tight cluster of degenerate consensus half sites, and such clusters have also been identified in the promoters of other RA-regulated keratin genes. Our results thus identify RA/T3 RE in the K14 promoter and confirm direct regulation of keratin promoters by RA and T3 nuclear receptors.

MATERIALS AND METHODS

Plasmid Growth and Purification Plasmids pK14CAT and pRSVZ have been previously described [27]. The expression vectors containing the genes encoding the T3 nuclear receptor and the RARα nuclear receptor were kind gifts from Drs. H. Samuels and P. Chambon, respectively [8,31]. They were grown in JM101 *Escherichia coli* host to saturation density in LB medium. DNA was extracted and purified through two equilibrium banding centrifugations in CsCl-ethidium bromide, as described [32].

Oligonucleotide Primers The oligonucleotide primers were synthesized on Pharmacia Gene Assembler Plus synthesizer. Sequences of primers used for amplification are presented in Fig 1. All primers are designed to have from their 5′ ends two or three nucleotides to facilitate restriction, followed by a restriction site for a specific enzyme, and additional 25-16 nucleotides complementary to the template DNA. The K14R oligonucleotide contains the restriction site for Hind III; and K14F as well as D1F, D2F, D3F, D4F, and D5F oligonucleotides contain Pst I restriction sites [27]. MR, M1F, M2F, and M3F contain restriction sites for Bsp MI and four spacer nucleotides.

Polymerase Chain Reaction The *T. aquaticus* DNA polymerase as well as dNTPs were purchased from Perkin Elmer Cetus (Ampli-Taq Kit). The reaction of amplification was performed following the protocol from the kit. Ten nanograms of the template DNA (K14CAT plasmid) and 200 nM of each primer were used. Thirty-five temperature cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min were performed.

Ligation and Cloning The products of the PCR were purified on agarose gels. They were digested with Hind III and Pst I in a 40-μl mixture with buffer recommended by the manufacturer (New England Biolabs). After the digestion, the DNA segments were phenol extracted and ethanol precipitated. In addition, the segments amplified with M-series of oligonucleotides were digested with Bsp MI, phenol extracted, and ethanol precipitated [30]. All segments were ligated into the pGCAT-C plasmid DNA that was previously digested with Hind III and Pst I and purified on preparative agarose gel [27,32,33]. The ligation mixtures were used to transform *E. coli* JM101 and the *amp*^{r} colonies were expanded. The plasmid DNAs were purified as described, using two consecutive CsCl-ethidium bromide equilibrium gradient centrifugations [32].

DNA Sequencing We used the di-deoxy chain termination method [34] as modified for double-stranded templates [35]. The sequences of all constructs obtained from PCR reactions were confirmed by sequencing. No differences between the amplified and template sequences were seen.

A.

K#14F	CGCCTGCAGGAATCACGCCTGGCGGGAC
K#14R	GCGAAGCTTGGTGCCGAGGAGGGAG
M1F	CGCACCTGCAAAAGGTCATGACCGCCAAGGGGAATGGA
M2F	CGCACCTGCAAAAGGTGATGAAATTTAAGGGGAATGGA
M3F	CGCACCTGCAAAAGGTCATGATGACCAAGGGGAATGGA
MR	CGCACCTGCAAAAGACCCACAGGCTAGCG
D1F	CGCTGCAGTGAATCACGCCTGGCGGGAC
D2F	CCCTGCAGCCCCAAACAATGAGTTT
D3F	CGCTGCAGGTGGAGGAGAGGAGG
D4F	CGCTGCAGGACCTGGCTGGGAGTT
D5F	CGCTGCAGTGGCGCTAGCCTGTGGG

B.

WT	GCTAGCCTGTG GGTGATGAAAGCC AAGGGGAATG CGATCGGACAC CCACTACTTTCGG TTCCCCTTAC
M1	GCTAGCCTGTG GGTCATGACCGCC AAGGGGAATG CGATCGGACAC CCAGTACTGGCGG TTCCCCTTAC
M2	GCTAGCCTGTG GGTGATGAAATTT AAGGGGAATG CGATCGGACAC CCACTACTTTAAA TTCCCCTTAC
M3	GCTAGCCTGTG GGTCATGATGACC AAGGGGAATG CGATCGGACAC CCAGTACTACTGG TTCCCCTTAC

Figure 1. Oligonucleotide sequences. The sequences of primers used in the PCR to create deletions and mutations of the K14 promoter are presented in *A*. All oligonucleotides are written 5′ to 3′ and the restriction sites are *underlined*. The sequences of the resulting mutagenized segments are presented in *B*. Position and orientation of the TRE half-sites are indicated with *black arrows*. The segment that was mutagenized is *boxed*. Introduced mutations are marked with *asterisks*.

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 [27,33]. The day before transfection cells were plated onto 100-mm dishes. Four hours before transfection the medium was changed.

Transfection using $Ca_3(PO_4)_2$ We have generally followed our published procedure for cells that were at 80% confluence [33]. At the time of transfection into each dish were added 5 μg of the CAT plasmid, 2.5 μg of the nuclear receptor expression vector plasmid, 1.5 μg pRSVZ reference plasmid, and a sufficient amount of carrier to bring the total to 20 μg of DNA. The next day the medium was changed. The cells were harvested 48 h after transfection by scraping into 15 ml of phosphate-buffered saline (PBS), collected by centrifugation and then washed once more in PBS. Each cell pellet was resuspended in 150 μl of 0.25 M Tris buffer pH 7.8.

Enzyme Assays Cells were disrupted by ultrasonication for 45 seconds using an ultrasonic homogenizer sonicator (Cole-Parmer Instrument Co.). Cell debris was removed by centrifugation for 7 min at 10,000 rpm in the bench-top centrifuge, and 50 μl of the supernatant was saved for the β-galactosidase assay. The remainder of the supernatant was heated at 65°C for 10 min, clarified by

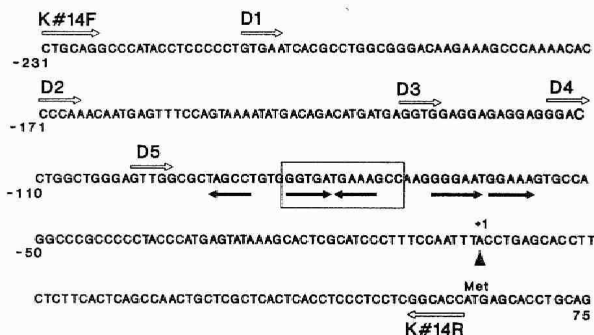
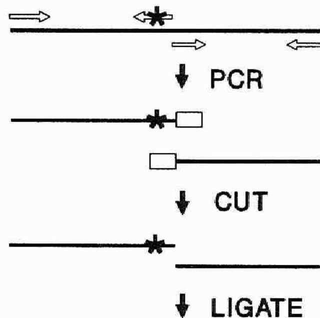


Figure 2. The sequence of the K14 promoter. *Open arrows*, position and orientation of the primers used in PCR. *Solid arrows*, position and orientations of the TRE half-palindrome sequences. *Box*, the sequence that has been mutagenized. *Triangle*, the transcription start; *Met*, the translation initiation codon.



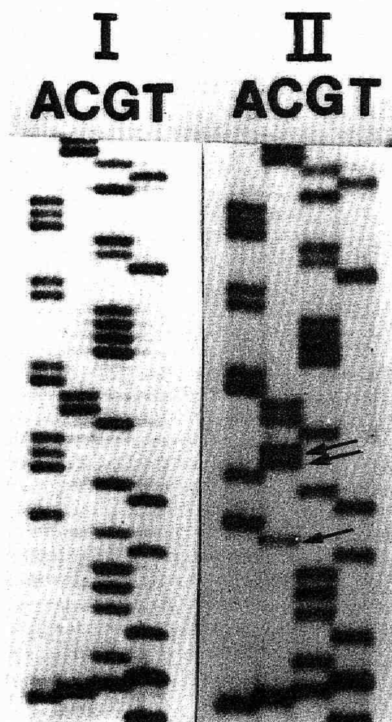


Figure 4. Mutagenesis of the K14 promoter. *A*) Design of the experiment. *Open arrows*, primers used in PCR; *open boxes*, restriction site for Bsp MI. Introduced mutations are marked with *asterisks*. *B*) Comparison of the sequence in the mutagenized region. I, sequence of the WT K14 promoter. II, sequence of the M1 mutagenized promoter. *Arrows*, introduced mutations.

centrifugation, and stored at −20°C until used in the CAT assay as described [33]. Final values of CAT activity presented in our figures are results from normalizing the CAT activity with the β-galactosidase activity that serves as internal control for efficiency of transfection.

RESULTS

Deletions of the K14 Promoter To localize the segment of the K14 promoter responsible for receptor binding we have constructed six promoter deletions using polymerase chain reaction (PCR). The same reverse primer, K14R, was used with K14F and five forward primers designated D1–D5 (Fig 2). The six resulting segments were cloned in front of the CAT gene. Each of the six constructs was co-transfected into the HeLa cells with RA and T3 nuclear receptor vectors in the presence or absence of RA and T3, respectively. We have specifically chosen the HeLa cells as recipients because they have very low levels of endogenous receptor and thus the regulation by ligands depends on the co-transfected receptor vectors. The basal, i.e., unregulated levels of activity of all six constructs vary considerably as we have shown before [27], but the basal levels were sufficient to allow us to determine whether the constructs are regulated by RA and T3. All six constructs, including the smallest one, were regulated the same as the full-length K14 promoter; they were down-regulated approximately tenfold by RA and T3 with their receptors, and upregulated two- to fourfold by T3 receptor in the absence of its ligand (Fig 3). Smaller constructs than those shown were nonfunctional, i.e., the basal levels of activity were not above the background and therefore we could not assess their regulation (data not shown). Therefore, we have determined that the RA/T3

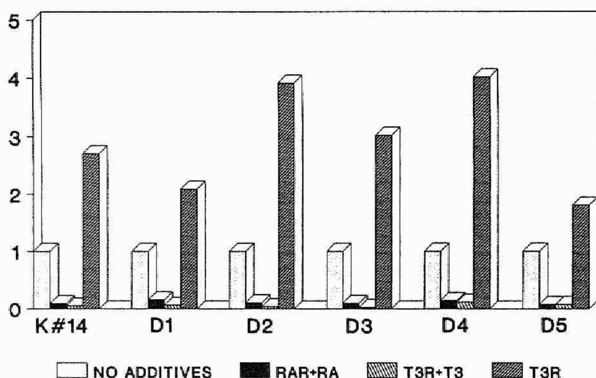


Figure 3. Regulation of the K14 promoter and its deletion constructs by RA and T3. F.R., fold regulation. The basic, unregulated activity of each CAT construct is designated as 1 to show regulation by RA and T3. The basic activities of the full-length and the five deletion constructs are 22, 33, 16.1, 1.5, 3.1, and 4.4 CAT units, respectively.

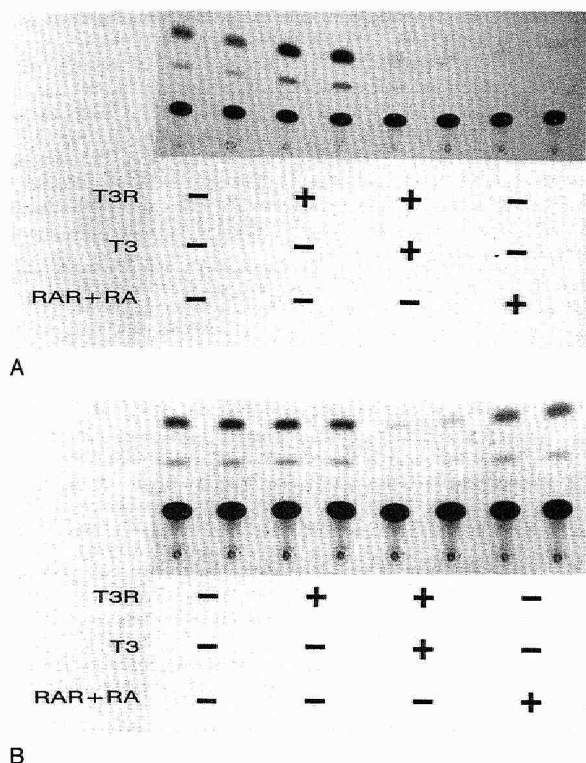


Figure 5. CAT assay of the WT K14 promoter and M1 mutant regulation by RA and T3. Each of the four DNA transfections was done in duplicate, and identical amounts of extract from the eight plates were used to assay the CAT activity. *A)* WT K14 promoter. *B)* M1 mutant. The mutated sequence is no longer regulated by T3R and RAR + RA, but is still down-regulated by T3R in the presence of T3.

receptor binding site is located within the proximal 165 bp of the K14 promoter, between the translation start and primer D5 (Fig 2).

Mutagenesis of the K14 Promoter The consensus responsive element recognized by both RAR and T3R is the palindromic sequence GGTCA-TGACC, and, although this perfect palindrome has not been found in any gene, the regulated genes have variants of this sequence with palindrome half-sites in various orientations and spacings [15–23].

To localize the recognition site, we have analyzed the proximal 165-bp sequence of the K14 promoter in great detail, paying special attention to the potential half-sites. We have found in the region −56 to −91 a cluster of four sequences similar to the half palindromes, which we believe are potential recognition sites (boxed in Fig 2). In this region the pseudogene sequence that we are using is identical to the sequence of the functional gene [36]. In order to test the −56 to −91 region for receptor recognition we have developed a method for site-specific mutagenesis [30]. The advantage of our method is that only the desired mutations are incorporated into the final product, whereas the remaining sequences remain unaltered. This method is based on PCR and restriction enzymes that cut the DNA at a short distance away from their recognition sites. The sites for each enzyme are amplified during the PCR, but have a convenient property of self-elimination from the final product. Thus, the target DNA is perfectly recreated except for the mutations designed into the experiment (Fig 4*A*).

Using this procedure we have mutagenized the putative recognition site for RA and T3 nuclear receptors in the K14 promoter. We focused our mutagenesis on the middle two half-sites because we expected that alterations in this area would have a more significant effect on regulation than alterations of the terminal half-sites. The primers for the PCR reactions used in creation of the mutated sequences, as well as the sequence of mutated regions, are presented in Fig 1. After PCR, the fragments were purified on agarose gel. Following our experimental design, the desired sequences were obtained. Except for the mutations we deliberately introduced into the DNA, the sequence of the promoter (template) and of the final product are identical (Fig 4*B*). After ligation this mutated DNA is properly positioned in front of the CAT reporter gene. Following the described procedure we have successfully produced three different mutants of the K14 promoter (Fig 1*B*).

Regulation of K14 Promoter Mutants by RA and T3 To test whether the mutated site is truly an RE for RA/T3 nuclear receptors, we have introduced the mutant K14-CAT constructs into HeLa cells along with RA and T3 nuclear receptor genes in the presence of their ligands.

The construct containing the mutation M1 is no longer down-regulated by the RA receptor and its ligand. This mutation, therefore, identifies the recognition element of the RAR. The M1 mutant is no longer up-regulated by the T3R receptor in the absence of T3, which identifies a site recognized by the T3R. The M1 mutant can still be down-regulated by the T3 receptor in the presence of T3 (Fig 5), which means that additional sites, not affected by the M1 mutation, are components of the TRE.

Exactly the opposite effects on regulation were obtained with the mutant M2. The construct containing the M2 mutation is no longer down-regulated by T3R in the presence of T3, which also identifies a site recognized by T3R. However, the M2 mutation did not affect regulation by RAR or by T3R in the absence of T3 (Fig 6).

The M3 mutation did not alter the normal pattern of K14 promoter regulation. The M3 mutated promoter is regulated as is the wild type: it is down-regulated by RAR and T3R in the presence of their ligands and up-regulated by the T3R receptor alone (Fig 6).

Taken together, these combinations of the introduced mutations in M1 and M2 identify the RA and T3 regulatory sites in the K14 promoter.

Regulatory Sites in Other Keratin Promoters Deletion analysis of the human K5 keratin gene promoter indicated that the RARE is located in the 300-bp proximal to the translation start [37],

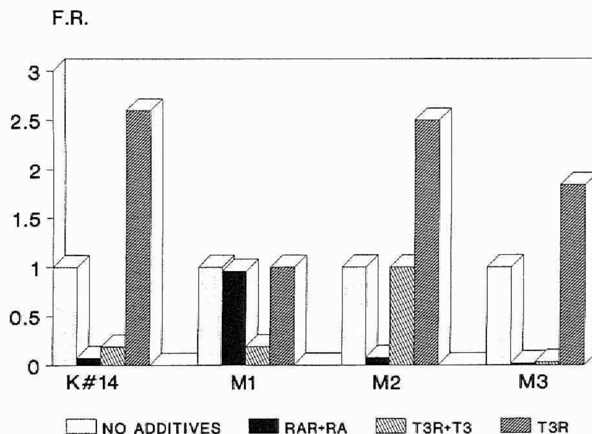


Figure 6. Regulation of the K14 mutants by RA and T3. F.R., fold regulation. The unregulated basic activities, which are not significantly different among the CAT constructs, are designated as 1.

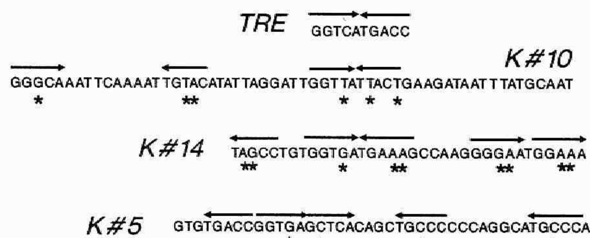


Figure 7. Comparison of the K14 RE with analogous sequences in K10 and K5 promoters. *Arrows*, positions and orientations of the TRE half-palindrome sequences. *Asterisks*, differences between the native sequence and perfect TRE palindrome half-site sequences shown on the top.

whereas in the K10 gene it is in the proximal 200 bp (Tomic-Canic, unpublished). Because the K14 promoter RARE consists of a cluster of sequences homologous to TRE half-palindromes, in both orientations, we searched for equivalent arrangements in other keratin-promoter sequences. We found similar regions in the K10 and K5 promoters (Fig 7). These segments have structural organizations similar to the K14 promoter RARE: they contain a cluster of several palindromic half-sites in various orientations, which indicates potential RE in these promoters.

DISCUSSION

The results presented in this paper identify the RA/T3 RE in the K14 promoter. We have located a cluster of four TRE half-palindrome–like sequences. Using site-specific mutagenesis we have altered these sequences, which resulted in disrupted regulation by RAR and T3R of the K14 promoter transcription.

Interestingly, by introducing different mutations we were able to separate the various effects of the receptors RAR and T3R. These results indicate that the parallelism of regulation by RAR and T3R is not always mediated through identical sequences even when their REs overlap and they are regulated similarly.

Several recent reports have suggested that spacing and orientation of half-palindrome sites determine RE for the T3, RA, vitamin D3, or estrogen receptors. The RE that we identified in the K14 promoter contains a cluster of four half-palindrome–like sequences. The central two create an inverted palindrome with no spacing between them. The proximal and distal half-sites are four and three bp apart from the central pair, respectively. In the M1 mutation we kept the orientation and spacing the same while introducing three changes within the central two half-sites, which abolished regulation by both RAR and T3R. Our results from the M2 mutations indicate that surrounding sequences are also important, because, although we kept the half-sites intact, we changed three bases in the proximity of the right half-site. Spacing between half-sites may play a role in regulation of the K14 promoter because the M3 mutation, which differs from the M1 mutation only because of a 3-bp insertion between two middle half-sites, restored both RA and T3 regulation patterns. Taken together, these results demonstrate that the functionality and specificity of REs in native genes depend more on the context of sequences than simply on the orientation and spacing of the half-sites. They also indicate that regulation by T3R in the presence or absence of its ligand can be two distinct and separate events, mediated through different sequences.

Comparison of the K14 promoter RE with the sequences of K5 and K10 keratin gene promoters indicates that all three have a similar structure consisting of a cluster of consensus half-sites in various orientations and spacings. We expect that all retinoid-regulated epidermal proteins may contain such clusters, and that the analysis of RAR interaction with the clusters will be essential for our understanding of the epidermal effects of retinoids in normal and pathologic conditions.

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