The Book of Opposites: The Role of the Nuclear Receptor Co-regulators in the Suppression of Epidermal Genes by Retinoic Acid and Thyroid Hormone Receptors

Sang H. Jho,* Constantinos Vouthounis,* Brian Lee,* Olivera Stojadinovic,* Mark J. Im,* Harold Brem,† Ankit Merchant,* Katherine Chau,* and Marjana Tomic-Canic*‡

*The Ronald O. Perelman Department of Dermatology, New York University School of Medicine, New York, New York, USA; †Department of Surgery, Wound Healing Program, Columbia University College of Physicians and Surgeons, New York, New York, USA; ‡Department of Microbiology, New York University School of Medicine, New York, New York, USA

Transcriptional regulation by nuclear receptors occurs through complex interactions that involve DNA response elements, co-activators/co-repressors, and histone modifying enzymes. Very little is known about how molecular interplay of these components may determine tissue specificity of hormone action. We have shown previously that retinoic acid (RA) and thyroid hormone (T3) repress transcription of a specific group of epidermal keratin genes through a novel mechanism that utilizes receptors homodimers. In this paper, we have analyzed the epidermal specificity of RA/T3 action by testing the role of co-repressors and co-activators in regulation of epidermal genes. Using transient co-transfections, northern blots, antisense oligonucleotides, and a histone deacetylase (HDAC) inhibitor, trichostatin A, we found that in the context of specific keratin RE (KRE), co-activators and histone acetylase become co-repressors of the RA/T3 receptors in the presence of their respective ligands. Conversely, co-repressors and HDAC become co-activators of unliganded T3Rα. The receptor–co-activator interaction is intact and occurs through the NR-box. Therefore, the role of co-activator is to associate with liganded receptors whereas the KRE–receptor interaction determines specific transcriptional signal, in this case repression. This novel molecular mechanism of transcriptional repression conveys how RA and T3 target specific groups of epidermal genes, thus exerting intrinsic tissue specificity.

Key words: co-activators/co-repressors/epidermal keratin genes/retinoids/thyroid hormone
J Invest Dermatol 124:1034–1043, 2005

Retinoic acid (RA) and thyroid hormone (T3) are important regulators of development, differentiation, and gene expression in many tissues, including the epidermis (Stellmach *et al*, 1991; Rosenthal *et al*, 1992; Fisher *et al*, 1995; Blumenberg, 1996; Fisher and Voorhees, 1996). The profound effects of retinoids on the epidermis have been known for more than 70 years (reviewed in Fisher *et al*, 1995). Both *in vivo* and *in vitro*, RA deficiency causes hyperkeratosis and promotes differentiation of epidermal keratinocytes, whereas an excess of RA prevents "keratinization" and keeps keratinocytes in a basal layer-like phenotype (Gilfix and Eckert, 1985; Lichti *et al*, 1985; Magnaldo *et al*, 1992). Retinoids are also used in the treatment of a large number of epidermal disorders ranging from ichthyoses to skin cancers (Epstein, 1981; Fisher *et al*, 1991; Kraemer *et al*, 1992; Steijlen *et al*, 1993; Futoryan and Gilchrest, 1994; Moon *et al*, 1995; Lotan, 1996). In addition, hypothyroidism causes numerous epidermal changes including scaly, dry, hyperkeratotic skin, eczema, and ichthyosis. Similarly, T3 deficiency *in vitro* results in increased expression of late differentiation markers (Holt and Marks, 1977; Rosenberg *et al*, 1986; Isseroff *et al*, 1989; Heymann, 1997). Topical T3 application stimulates proliferation, whereas it also stimulates proliferation inhibitory factor(s) in the skin (Safer *et al*, 2003).

RA and T3 mediate their effects through binding to specific nuclear receptors (NR), proteins that, on one hand, bind their ligand and, on the other, bind DNA at specific sites in the vicinity of regulated genes. Once bound to the DNA, the receptors interact with co-regulators and the transcriptional machinery to regulate transcription (Mangelsdorf *et al*, 1995; Fondell *et al*, 1996; Vom Baur *et al*, 1996; Hong *et al*, 1997; Gronemeyer and Miturski, 2001).

There are two possible pathways that lead to either repression or enhancement of transcription by NR, depending on the presence or absence of the ligand on the receptor. In the first pathway, the liganded receptor binds to the response element (RE) and recruits a group of proteins called co-activators (such as NCoA, SRC-1, or GRIP-1),

Abbreviations: CAT, chloramphenicol acetyl transferase; DR-4, a direct repeat of consensus sequence spaced by four nucleotides; EGF, epidermal growth factor; GH, growth hormone; HAT, histone acetylase; HDAC, histone deacetylase; KRE, keratin response element; NFκB, nuclear factor κB; NR, nuclear receptor; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RE, response element; T3, thyroid hormone; T3R, thyroid hormone receptor; TRE, thyroid hormone response element; TSA, trichostatin A

which interact with other co-integrators (p-CIP, CBP/p300) that bind histone acetylase (HAT), leading to the induction of transcription (Heinzel *et al*, 1997; Montminy, 1997; Nagy *et al*, 1997; Spencer *et al*, 1997; Torchia *et al*, 1997). In addition, CBP and p300 have the activity of a HAT and can induce transcription without further interaction with HAT (Ogryzko *et al*, 1996; Torchia *et al*, 1997). Conversely, the unliganded receptor binds to the RE and recruits a co-repressor (such as NCoR or SMRT), which then interacts with co-integrator proteins (such as mSin3A). The co-integrator binds to histone deacetylase (HDAC), leading to repression of transcription. Most co-regulators are found to be promiscuously expressed and seem to participate in many transcriptional pathways in addition to those involving NR (Khan and Nawaz, 2003).

One of the central questions is what determines the specificity of particular transcriptional regulation: how is the specificity of the regulation directed to a tissue and, further, to specific genes? To extrapolate this question to skin: how is this general system tailored to suit the purpose of epidermal physiology and epidermal gene expression? The complexity of transcriptional regulation depends on the selection of the appropriate combination of DNA RE and interacting transcription factors. Combined, they select, by resuming appropriate conformation, a particular group of co-regulators that mediate the signal to the transcriptional machinery. Therefore, the diversity and potential of the transcriptional regulation is limited by the number of possible combinations of numerous interacting factors (McKenna *et al*, 1999).

We have shown previously that RA, T3, and glucocorticoids (GC) regulate epidermal keratin genes though unique molecular mechanisms. Retinoic acid receptor (RAR) and T3R homodimers and four monomers of glucocorticoid receptor (GR), in the presence of their respective ligands, mediate repression of specific, disease-associated, epidermal keratin genes: K5, K14, K6, K16, and K17 (Tomic-Canic *et al*, 1992; Radoja *et al*, 1997; Lee *et al*, 2004). Furthermore, these three receptors all bind to the specific keratin RE (KRE) that are highly conserved among the regulated group of keratin promoters and it is the structure of the KRE that determines the specific receptor configuration and mediates the signal for repression.

Knowledge about co-regulators has been obtained from reporter genes that are either induced by liganded receptors or repressed by unliganded receptors. Very little is known about the function of these proteins in repression of transcription mediated by liganded receptors and activation mediated by unliganded receptors. Therefore, we used a family of genes that are negatively regulated by RA and T3 to answer directly the important questions of which co-regulators are specific for the regulation of keratin gene expression by liganded RAR and T3R; what is the mechanism involving those co-regulators and what is the role of the histone acetylation/deacetylation in this process? We found that co-activators act as co-repressors of liganded RAR and T3R in the context of KRE. Conversely, we found that co-repressors become co-activators of unliganded T3R in the context of the KRE. Furthermore, we also found that a HAT, CBP participates in repression, whereas HDAC participates in activation of keratin gene expression. Taken together, we conclude that the interaction between co-regulators and the appropriate receptors (liganded *vs* unliganded) is consistent and does not seem to depend on specific RE; however, it is the RE that probably initiate the transcriptional output. The liganded receptor interacts with a co-activator and in the case of a consensus element the signal enhances transcription, whereas in the case of KRE the signal is opposite and leads to repression.

Results

To establish the role of the co-regulators in transcriptional regulation of genes in the epidermis, we have used co-transfection experiments and tested how specific co-regulators affect regulation of keratin gene expression by RA and T3 receptors. We have shown previously that these epidermal genes are suppressed by RAR and T3R homodimers and therefore, we tested first common co-repressors: NCoR and SMRT. Interestingly, we found that NCoR and SMRT had no effect. Neither of the two co-repressors affected suppression of K5, K14, and K17 promoters by either RAR or T3R in the presence of their ligands (Fig 1). Both positive controls, a direct repeat of consensus sequence spaced by four nucleotides (DR-4) (containing consensus TRE) and retinoic acid response element (RARE) (containing consensus RARE) were induced by RAR and T3R in the presence of their ligands and, as expected, NCoR and SMRT did not significantly affect their activation (Fig 1). To confirm proper NCoR and SMRT activity in our experimental system, we tested their activity in the presence of unliganded T3R (Fig 2*A*). As expected, both NCoR and SMRT acted as co-repressors of the T3R in the absence of its ligand in the regulation of positive control, DR-4. We found that both NCoR and SMRT enhanced the activation of K5, K14, and K17 by the unliganded T3R, suggesting that in the context of these specific KRE they act as co-activators rather than co-repressors (Fig 2*A*). To further confirm this, we tested their activity in the presence of constitutive repressor, v-erbA receptor, a T3R mutant unable to bind its ligand (Tomic-Canic *et al*, 1996). As expected, NCoR and SMRT enhanced repression of DR-4 by the v-erbA (Fig 2*B*). Consistent with our initial findings, both NCoR and SMRT enhanced v-erbA-mediated activation of K5, K14, and K17 promoters, thus confirming that they are indeed co-activators in the context of these promoters (Fig 2*B*).

If the co-repressors behave as co-activators, the logical question is how co-activators behave in the context of KRE. To establish the role of co-activators, we tested NCoA and GRIP-1 in co-transfection experiments. Interestingly, we found that both enhanced suppression of K5, K14, and K17 promoters by RAR and T3R in the presence of their respective ligands. As expected, they enhanced activation of DR-4 and RARE controls by liganded T3R and RAR (Fig 3*A*). Therefore, we conclude that NCoA and GRIP-1 act as co-repressors in the context of KRE. To test how the receptor and co-regulator interact in the context of a KRE, we used the GRIP-1 NR box mutant in co-transfection experiments. The GRIP-1 NR box mutant carries a mutation in the region responsible for interaction with the receptor (NR box) and is

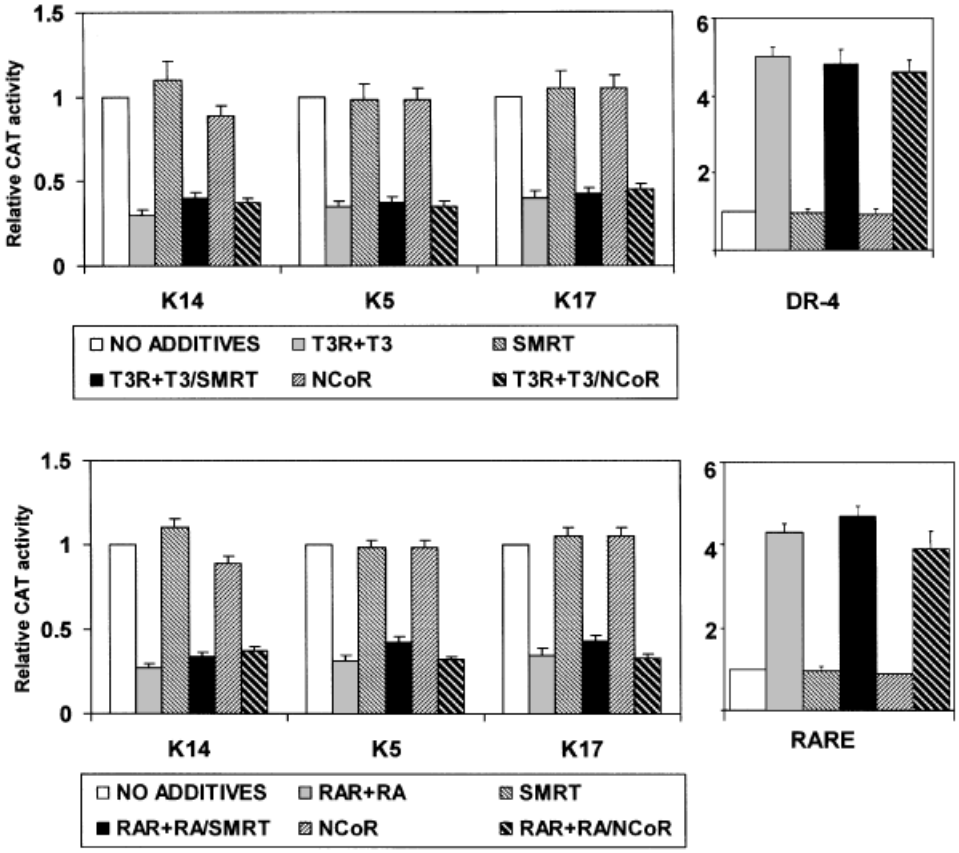

Figure 1
Co-repressors NCoR and SMRT do not participate in repression of keratin genes by retinoic acid (RA) and thyroid hormone (T3). Effects of co-transfection of NCoR and SMRT on keratin gene expression in the presence of the T3 and thyroid hormone receptor α (T3R) is shown on the top left graph. There is no change either on the basic promoter activity or on the repression of keratin genes by T3 in the presence of co-repressors. As expected, NCoR and SMRT did not have any effect on a positive control, DR-4, in the presence of the liganded T3R (*top right*). Similarly, NCoR and SMRT did not significantly affect repression of keratin promoters by retinoic acid receptor (RAR) in the presence of RA (*bottom left panel*). As expected, the activation of retinoic acid response element (RARE) was not affected by NCoR and SMRT (*bottom right*).

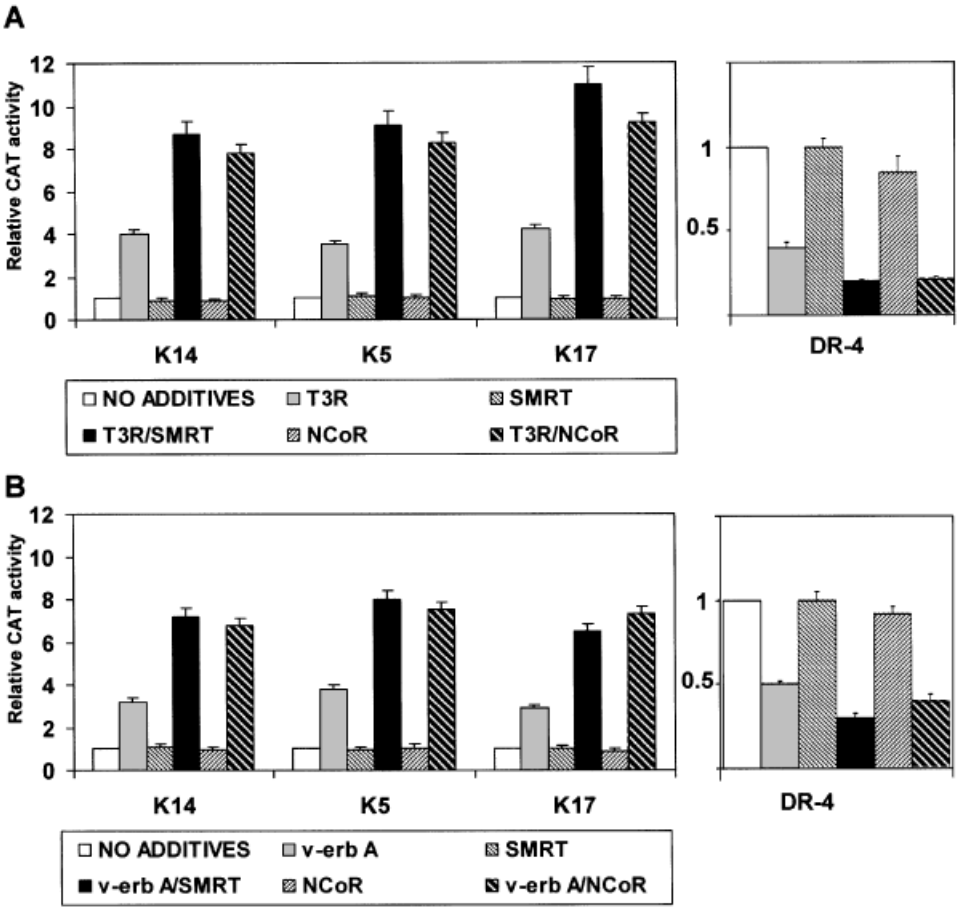

Figure 2
Co-repressors NCoR and SMRT become co-activators of unliganded thyroid hormone receptor α (T3R) in the context of keratin promoters. (*A*) NCoR and SMRT enhanced the activation of keratin promoters by unliganded T3R 2-fold in co-transfection experiments (*left*), thus acting as co-activators. As expected, NCoR and SMRT enhanced repression of a positive control, a direct repeat of consensus sequence spaced by four nucleotides (DR-4), thus acting as co-repressors. (*B*) NCoR and SMRT were co-repressors of v-erbA receptor (a mutant T3R receptor unable to bind its ligand) in repression of keratin promoters (*left*) whereas they were co-repressors of v-erbA in repression of DR-4 (*right*).

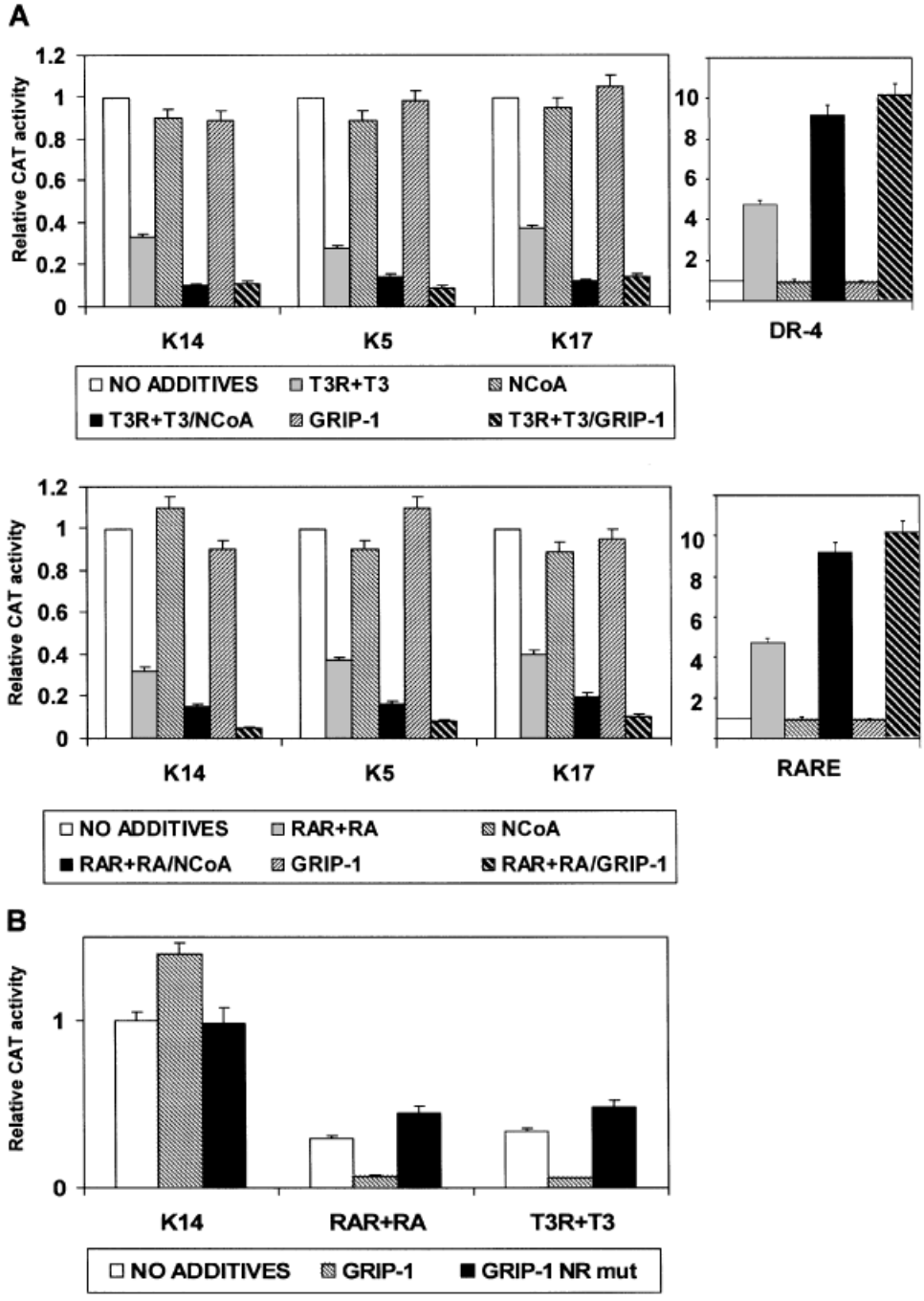

Figure 3
Co-activators, GRIP and NCoA become co-repressors of thyroid hormone receptor α (T3R) and retinoic acid receptor (RAR) in the presence of their ligands. (*A*) GRIP and NCoA enhance repression of keratin promoters mediated by the T3R in the presence of the T3 (*top left*) and RAR in the presence of retinoic acid (RA) (*bottom left*). As expected, GRIP and NCoA enhance activation of positive controls DR-4 (a direct repeat of consensus sequence spaced by four nucleotides) by T3R in the presence of T3 (*top right*) and RARE by RAR in the presence of RA (*bottom right*). (*B*) In a contrast to GRIP wild-type, a GRIP mutant unable to bind nuclear receptors, GRIP nuclear receptor (NR) mut, did not affect repression of keratin promoter by RAR and T3R receptors in the presence of their respective ligands.

unable to bind the receptor through this site. We found that the mutation in the NR box was sufficient to abolish the co-repressive function of GRIP-1, as GRIP-1 NR box mutant had no effect on suppression by either liganded RAR or T3R (Fig 3*B*). This suggests that the interaction between the NR and co-activator occurs through a common site in the context of KRE and is not affected by the KRE.

If a co-activator becomes a co-repressor in the context of the KRE, the question is do they further interact with HDAC or HAT? To further test the role of HAT in repression of keratin genes, we tested CBP. We used four different concentrations of CBP: 0.3, 1, 3, and 9 μg, whereas the receptor amount was kept constant. We found that CBP acted as a repressor by enhancing the repression of the K14 promoter by liganded T3R and RAR in a concentration-dependent manner (Fig 4). The highest dose decreased the basic activity of the promoter. Furthermore, we tested the effect of the small dose of 0.3 μg of CBP on two different amounts of the receptor: 0.5 and 1 μg. We found that CBP enhanced repression of K14 by liganded RAR in a receptor concentration-dependent manner (Fig 5*A*). We also found that it had similar effects on liganded T3R—it enhanced its repression of K14 promoter (Fig 5*B*). Thus, CBP also behaves as a co-repressor of liganded RAR and T3R. Interestingly, we found that it reduced the activation of unliganded T3R when 1 μg of the receptor was used (Fig 5*C*). This indicates the possible squelching of endogenous co-activators. CBP is known to interact with co-activators

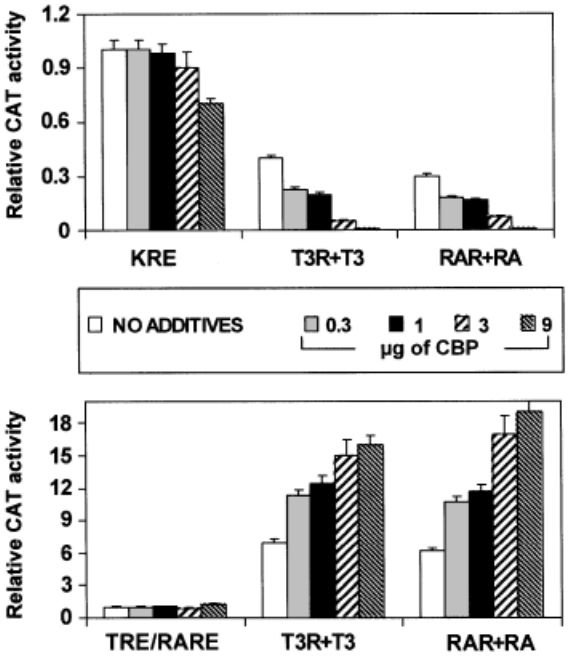

Figure 4
CBP also becomes a co-repressor of liganded thyroid hormone receptor α (T3R) and retinoic acid receptor (RAR) in the context of keratin promoters. CBP, in a concentration-dependent manner, enhanced repression of K14 keratin promoter by T3R and RAR in the presence of their ligands (*top panel*). It enhanced activation of a positive control TRE/retinoic acid response element (RARE) (*bottom panel*).

and in this context it may preclude their binding to unliganded T3R, which may further lower the level of K14 promoter activation.

Taken together, our results suggest that co-activators act as co-repressors and co-repressors act as co-activators in the context of keratin gene promoters. All results described above were, however, obtained from co-transfection of the expression plasmids, i.e., an exogenous source of the co-regulators. To block the endogenous NCoR, SMRT, and NCoA, we used antisense technology. The oligonucleotides were designed to bind the initiation codon and the sequences immediately upstream of human SMRT, NcoR, and NCoA. They were synthesized as phosphorothioates and added both to the DNA transfection mixture and subsequently to the medium. As a control for non-specific antisense oligonucleotide effects, we have used a nuclear factor κB (NFκB) antisense oligonucleotide (AS-NFκB), which should not affect keratin regulation by RAR/T3R. We chose AS-NFκB because it has been reported to successfully block, specifically, another transcription factor, NFκB (Reuning *et al*, 1995). We used GH-TRE as a positive control, because this RE originates from the growth hormone (GH) gene, i.e., a native promoter that is induced by RAR and T3R. We found that AS-SMRT and AS-NCoR block the activation of keratin genes by unliganded T3R, whereas they block the suppression of GH-TRE. Conversely, AS-NCoA blocks the ligand-mediated suppression of keratin gene expression by both RAR and T3R, whereas it blocks the induction of GH-TRE (Fig 6*A*). AS-NFκB had no effect, although it specifically blocked the NFκB mediated regulation of NFκB-CAT (Fig 6*B*), a well-characterized positive-response construct (Vietor *et al*, 1996). Furthermore, co-

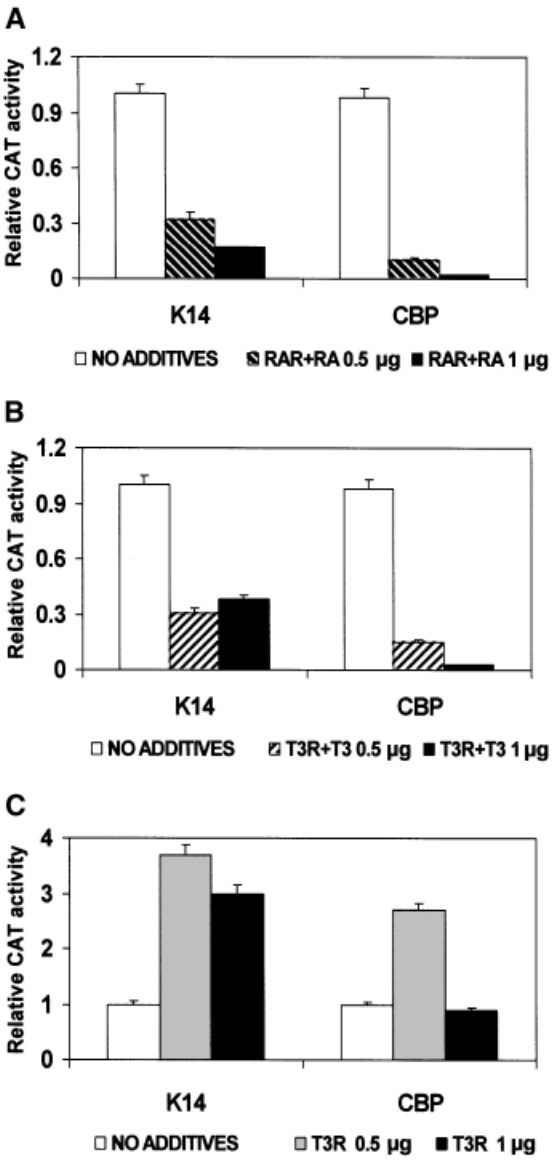

Figure 5
Co-repression of CBP is consistent with different receptor concentrations. The effects of a small dose of CBP (0.3 µg) were tested with increasing amounts of the receptors. (*A*) CBP enhanced repression of K14 by liganded retinoic acid receptor (RAR) in the receptor concentration-dependent manner. (*B*) Similarly, CBP enhanced repression of K14 by liganded thyroid hormone receptor α (T3R). (*C*) CBP reduced the activation of unliganded T3R when 1 µg of the receptor was used.

transfection of exogenous cDNA of NCoR partially overcomes the block of regulation by AS-NCoR, whereas co-transfection of NCoA cDNA partially overcomes the block of regulation by AS-NCoA (data not shown). Taken together, all these results indicate that that SMRT and NCoR are co-activators, whereas NCoA is a co-repressor of keratin gene regulation by RAR/T3R. Furthermore, a HAT, CBP also acts as a co-repressor in the presence of the liganded receptors, suggesting that it also participates in repression. Conversely, co-repressors act as co-activators of unliganded receptors. This raises the question of the role of HDAC in the context of keratin genes. To test the role of HDAC, we used trichostatin A (TSA), a specific inhibitor of HDAC (Garcia-Villalba *et al*, 1997; Jenster *et al*, 1997; Wong *et al*, 1997). If co-activators and HAT participate in repression and co-

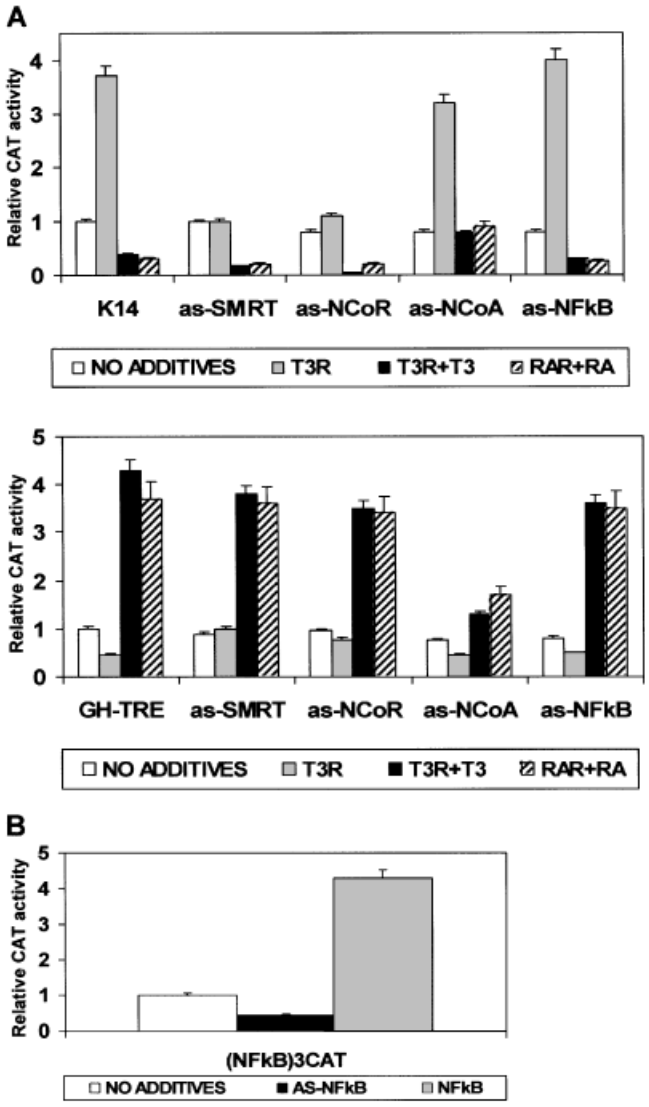

Figure 6
Co-activation by SMRT and NCoR as well as co-repression by NCoA can be blocked by specific antisense oligonucleotides. Specific oligonucleotides targeting the initiation codon and the sequences immediately upstream of human SMRT, NcoR, and NCoA were used in co-transfection experiments. AS-SMRT and AS-NCoR block the activation of keratin genes by unliganded thyroid hormone receptor (T3R), whereas they block the suppression of GH-TRE (a positive control containing native TRE from growth hormone (GH) promoter). Conversely, AS-NCoA blocks the ligand-mediated suppression of keratin gene expression by both retinoic acid receptor (RAR) and T3R, whereas it blocks the induction of GH-TRE. As a control for non-specific antisense oligonucleotide, we have used the nuclear factor κB (NFκB) antisense oligonucleotide (AS-NFκB), which should not affect keratin regulation by RAR/T3R. As expected, AS-NFκB had no effect (*B*). Control for antisense oligonucleotides. AS-NFκB specifically blocked the NFκB-mediated regulation of (NFκB)3-CAT. Co-transfection of expression plasmids containing NFκB-enhanced (NFκB)3-CAT.

repressors participate in activation, one may speculate that HDAC may participate in the activation of keratin genes. We tested two different concentrations of HDAC inhibitor, TSA, 10^{-7} M and 10^{-8} M. If our hypothesis is correct (HDAC participates in activation), we would expect that HDAC inhibitor blocks the activation by the unliganded T3R and does not affect the repression by the T3R in the presence of its ligand. This is exactly what we found. Both concentrations of TSA blocked the induction by unliganded T3R, but

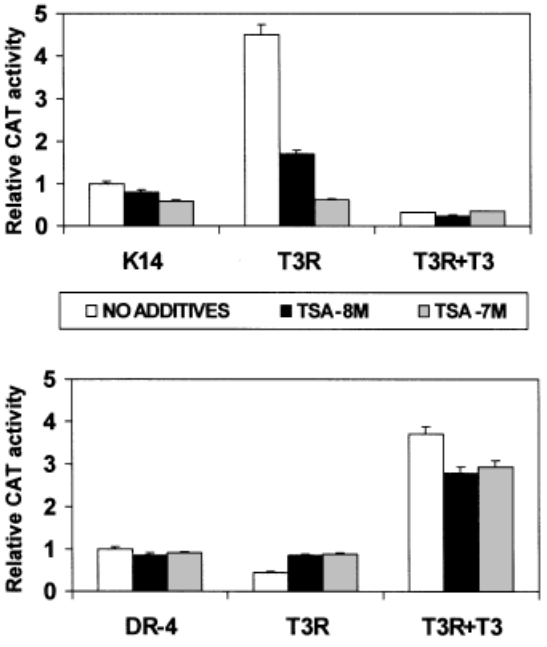

Figure 7
Histone deacetylation is contributing to activation of keratin gene transcription by unliganded thyroid hormone receptor α (T3R). Trichostatin A (TSA), a specific inhibitor of histone deacetylase, blocked the induction by unliganded T3R, but did not affect the regulation by liganded receptors in the context of keratin K14 promoters. TSA blocked repression of DR-4 (a direct repeat of consensus sequence spaced by four nucleotides) by unliganded T3R whereas the activation by liganded receptor was not affected. Two different concentrations were used, 10^{-7} M and 10^{-8} M, and both had a similar effect.

did not affect the regulation by liganded receptors (Fig 7; see also Fig 8).

Lastly, to confirm our results at the level of the endogenous keratin genes, we tested K14 messenger RNA (mRNA) levels using northern blot analyses. To test the effects of co-activator GRIP-1, we transfected keratinocytes with the expression plasmid-containing GRIP-1 cDNA and incubated them in the presence or absence of either T3R + T3 (Fig 8*A*) or RAR + RA (Fig 8*B*) and measured the changes in K14 mRNA levels using northern blot. As expected, we found that GRIP-1 indeed enhanced repression of K14 mRNA by both T3 and RA and their corresponding receptors. The levels of co-repression are perhaps not as high as in co-transfection experiments, as we anticipated. We believe that this difference originates from the background created by the cells that did not receive the transfected expression vector. Furthermore, we also tested the effects of the inhibitor of HDAC, TSA, in the presence of T3 and RA and found that the repression of K14 mRNA is maintained even when HDAC is inhibited (Fig 8). Taken together, our results have shown that the induction of keratin gene expression by the unliganded T3R involves NCoR and HDAC, whereas the suppression by the liganded T3R and RAR involves NCoA and CBP.

Discussion

Cooperative interactions of multicomponent transcriptional complexes are known to initiate and maintain many biological processes. Mechanisms for their tissue specificity,

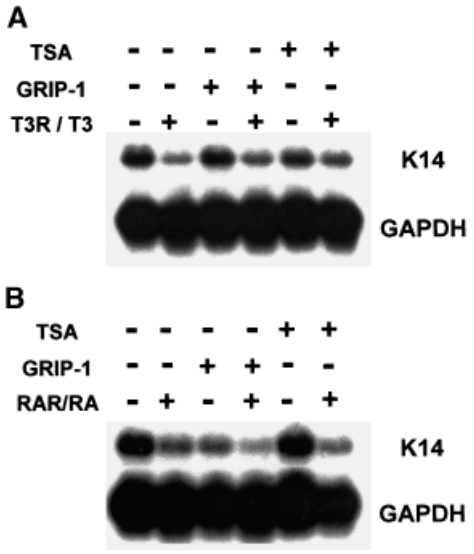

Figure 8
Endogenous K14 repression by thyroid hormone receptor α (T3) and retinoic acid (RA) is enhanced by co-activator GRIP-1 and occurs in the presence of histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). Northern blots with messenger RNA isolated from primary human keratinocytes transfected with co-activator GRIP-1 or incubated in the presence or absence of TSA. (*A*) Regulation by T3R in the presence of thyroid hormone (T3); (*B*) regulation by RARγ in the presence of RA. K14 is repressed by both T3 and RA, and this repression is enhanced in the presence of GRIP-1, thus confirming that GRIP-1 participates in repression. Furthermore, the repression is maintained in the presence of HDAC inhibitor TSA, confirming that HDAC does not participate in this repression.

however, are not well understood. In this paper, we show how various co-regulators whereas mediators of hormone signaling target specific epidermal genes. We found that in the context of epidermal keratin genes, NR co-activators become co-repressors, and vice versa (Fig 9). These findings are important because they change the current paradigm of the role of co-activators and co-repressors. Their function is to associate with a particular configuration of the receptor, determined by the DNA and the presence of the ligand. The "producers" of the signal for transcriptional regulation are the DNA and the receptor. Co-regulators, however, are necessary for transmitting that signal. Several important points derive from these findings. First, these results are consistent with our previous findings (Radoja *et al*, 1997, 2000; Jho *et al*, 2001) and they reinforce the proposed model of the DNA as a specific "ligand for a receptor" (Pearce *et al*, 1998; Yamamoto *et al*, 1998; Van Tilborg *et al*, 2000; Claessens *et al*, 2001). It is interesting that none of the co-regulators we have tested affect the KRE in the way initially described for the positive RE (Fig 9). The specific details of molecular interaction between these components within this KRE-specific complex remain to be elucidated and are the subject of these experiments. The results presented with various co-regulators, however, emphasize the point that regulation of keratin gene expression by NR occurs through a specific, novel mechanism.

The question remains of how the DNA–receptor interaction determines whether a gene will be induced or repressed. We believe that the sequence of the KRE, by determining the receptor configuration(s), homodimer *versus* heterodimer, or monomer *versus* heterodimer, plays a

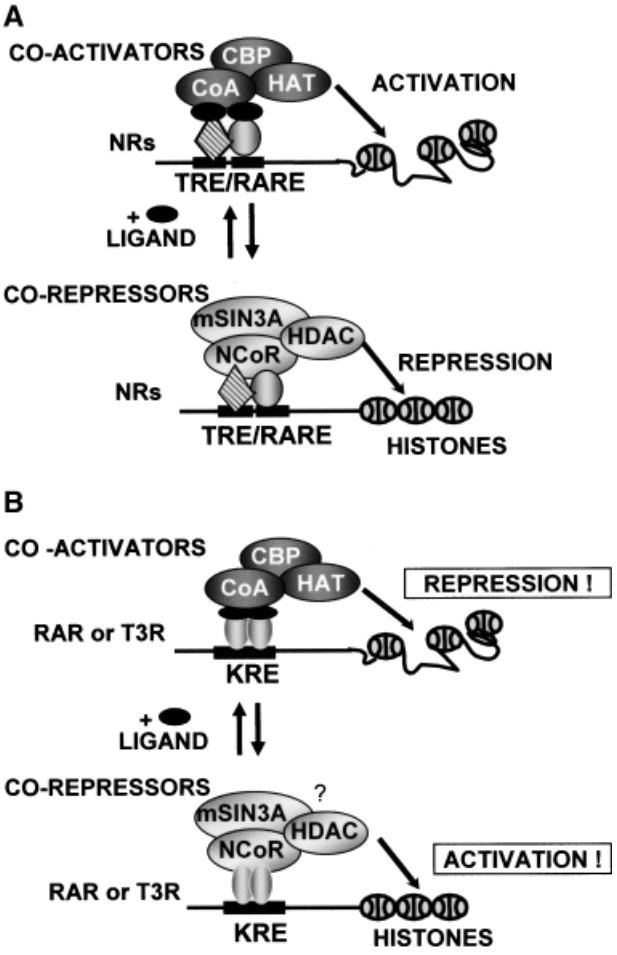

Figure 9
The summary of the keratin specific regulatory pathway. (*A*) A cartoon summary of the nuclear receptor regulatory pattern described for consensus, positive element is shown. These elements convey activation of transcription by heterodimers of receptors in the presence of their ligands and co-activators whereas they are repressed by unliganded receptors and co-repressors. (*B*) Our data indicate that the keratin response elements (KRE) convey repression by homodimers of receptors, and in this context of the DNA and receptor configuration, the co-activators become co-repressors of liganded retinoic acid receptor (RAR) and thyroid hormone receptor (T3R). Conversely, KRE convey activation by unliganded receptors, and in such a context, co-repressors become co-activators of unliganded T3R.The question mark indicates that further analyses are necessary to confirm the involvement of mSin3A and specific HDAC in co-activation by unliganded T3R.

crucial role in such signaling. Interestingly, we found that the GR, which also targets the same epidermal promoters, binds in a unique four monomer formation (Radoja *et al*, 2000) and as such does not interact with either co-activators or co-repressors that RAR/T3R interact with in the context of KRE (Radoja *et al*, 2000; Lee *et al*, 2004).[1] This supports the notion that DNA determines the receptor configuration that further transmits the appropriate transcriptional signal. Lastly, in this context, we found that GR dominates over RAR and T3R in the transcriptional regulation of epidermal keratin genes (Jho *et al*, 2001; Lee *et al*, 2004).

Another intriguing question of how HAT participates in the repression and how HDAC can participate in the induc-

[1]Stojadinovic *et al*, submitted.

tion of transcription. Although the mechanisms are not well understood, there are several reports in the literature that indicate such possibilities. For example, HAT activity was found to play an important role in the repression of ARG1 gene transcription and a decrease in the level of acetylated histones at the ARG1 promoter correlated with increased ARG1 expression (Ricci *et al*, 2002). Furthermore, a functional coordination between the promoter and enhancer through shared co-activators in the androgen receptor transcription complex discriminates between activation and repression. This means that there is a distinct function of co-activator complexes determined by their binding to two different DNA segments—a repression complex involves factors bound only at the promoter and not the enhancer, whereas formation of an activation complex involves AR, co-activators, and RNA polymerase II recruitment to both the enhancer and promoter (Shang *et al*, 2002). Conversely, it has also been shown that co-repressors may participate in the transcriptional activation. For example, the presence of N-CoR can enhance TR-β-mediated transcriptional activation (Li *et al*, 2002). HDAC also play a role in transcriptional activation of certain endogenous CREB target genes (Fass *et al*, 2003). Lastly, recent findings showed that HDAC plays a crucial role in interferon-stimulated transcriptional activation (Chang *et al*, 2004; Klampfer *et al*, 2004; Sakamoto *et al*, 2004). Taken together, the exact mechanisms through which HAT participate in transcriptional repression and HDAC in activation remains to be elucidated. However, examples of such activities in the specific promoter contexts or particular conditions support our finding that the complexes targeting a specific group of epidermal genes in which co-activators and HAT participate in repression, and co-repressors and HDAC participate in activation of transcription, is an excellent example of tissue specificity of hormone action.

The question is how these regulatory mechanisms affect epidermal biology, i.e., what is their role in the context of epidermal function? Epidermal keratins are important participants of epidermal function and are often used as markers of particular keratinocyte pathways. Therefore, their expression is tightly controlled by multiple mechanisms. For example, K6/K16/K17, hallmarks of the activated keratinocyte phenotype can be induced by epidermal growth factor (EGF)/transforming growth factor (TGF)-α, tumor necrosis factor-α (THF-α), interleukin-1 (IL-1) and interferon (IFN)-γ, all of which activate their transcription. Similarly, K5/K14, hallmarks of basal keratinocytes, are activated by TGF-β. As we presented in this paper, these promoters are repressed by liganded RAR and T3R complexed with co-activators. All these hormones, growth factors and cytokines act in concert to provide epidermal homeostasis. This means that under certain physiological conditions, such as during wound healing, they are not only simultaneously present, but may also target epidermal keratin genes at the same time. In such situations, they may either compete for the co-activators or perhaps share them. We have recently shown that when both EGF and RA are present simultaneously, the level of expression of K6/K16 is intermediate; a product of both signals (Lee *et al*, 2004). Similar co-dominant effects were found on K5/K14 and K17 keratin genes using IFN-γ, TGF-β, and thyroid hormone signaling molecules. These co-dominant effects are specific only for genes that are regulated by both pathways (Tomic-Canic *et al*, 1996). We have shown that the lack of a single copy of one NR co-activator (NRC-1) disturbs epidermal homeostasis, impairs EGF response and leads to a defect in epidermal wound repair and to the development of chronic wounds (Mahajan *et al*, 2004). Taken together, our results suggest that endogenous levels of all components may play a crucial role in determining the level of particular keratin gene activity. Furthermore, such complex molecular mechanisms may provide very refined tuning of gene dosages that may be fundamental for epidermal functions. For example, it has been shown that either excess or lack of K6 may cause similar effects on keratinocyte migration, suggesting that a particular amount of K6/K16 allows for cytoskeleton flexibility and provides for adequate migration capabilities (Coulombe and Omary, 2002; Wong and Coulombe, 2003). In this context, fine transcriptional tuning of keratin genes may be accomplished through the concerted effort of growth factors/cytokines (induction) and hormones/vitamins (repression) made possible by the molecular mechanisms we presented in this paper. Lastly, these results emphasize the need for understanding such tissue and context-specific transcriptional regulation, especially when new therapeutic modalities for various skin disorders are being developed.

Materials and Methods

Plasmids, their growth and purification Plasmids pK14CAT, pK5CAT, pK17, RAR, T3R, and v-erbA have been described previously (Tomic-Canic *et al*, 1992; Radoja *et al*, 1997). Plasmids containing DR-4, RARE (an inverted repeat of consensus sequence), and GH-TRE (GH RE) are gifts from Dr Herbert Samuels. Plasmids containing NCoR and NCoA were a gift from Dr Christopher Glass, a plasmid-containing SMRT was a gift from Dr Ronald Evans, a plasmid containing SRC-1 was a gift from Dr Bert O'Maley and plasmids containing GRIP-1 and GRIP-1 NR box mutant were a gift from Dr Michael Stallcup. Plasmids were grown in JM101 *Escherichia coli* host to saturation density in Luria Broth medium. DNA was extracted and purified using the Magic Mega Prep Kit from Promega (San Luis Obispo, California).

Cell growth 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, 1993) 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, GIBCO, Carlsbad, California). Cells were expanded through two 1:4 passages before transfection and transfected at 100% confluency. HeLa cells were maintained in Dulbeco-modified Eagle's medium (DMEM) 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 in low density (10%–15% confluency). Four hours before transfection, the medium was changed to phenol-red free DMEM supplemented with charcoal-pre-treated, depleted 10% calf serum, as described (Tomic *et al*, 1990). Preliminary experiments aimed to establish the effects of co-regulators and their appropriate concentration curves were performed initially in HeLa cells and, subsequently, results were confirmed in primary human keratinocytes.

Transfection using $Ca_2(PO)_4$ and N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) co-precipitation We have generally followed the published procedures for cells that were at 15%–20% confluence (Chen and Okayama, 1988). At the time of

transfection, 3–5 μg of the CAT plasmid, 1 μg of the NR expression vector plasmid, 1 μg pRSVZ reference plasmid, and a sufficient amount of carrier to bring the total to 10 μg of DNA in each dish were added. DNA mix was prepared with 50 μL of 2.5 M $CaCl_2$ and filled up to 500 μL dH_2O and subsequently added to the 500 μL of the BES buffer followed by vigorous shaking. After 10 min of incubation at room temperature, the precipitate mix was aliquoted to the duplicate plates. After transfection, cells were incubated in DMEM supplemented with charcoal-pre-treated 10% depleted calf serum in the presence or absence of 0.1 or 1 μM of dexamethasone (Sigma, St. Louis, Missouri) 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 (PBS), washed once more in PBS, and resuspended in 150 μL of 0.25 M Tris buffer pH 7.8. All transfections were performed in duplicate plates, and each transfection experiment was repeated two to five times. CAT and β-galactosidase assay were performed as described (Jiang *et al*, 1991).

Transfection by polybrene with dimethyl sulfoxide (DMSO) shock Transfections using polybrene with DMSO shock were used to transfect the DNA into 100% confluent keratinocytes, as previously described (Jiang *et al*, 1991). At the day of transfection, cells were washed and incubated in the basal medium without EGF and bovine pituitary extract. Each transfection contained 10–15 μg per dish of keratin–CAT construct and 3 μg per dish of RSVZ construct. The cells were then incubated with or without 0.1 μM dexamethasone (Sigma) dissolved in ethanol or T3 (Sigma) in 0.1 N NaOH. Forty-eight hours after transfection, cells were washed twice with PBS and harvested by scraping. The cell disruption by repeated freeze–thaw cycles, as well as CAT and β-galactosidase assays have also been described (Jiang *et al*, 1991).

Use of antisense oligonucleotides We used oligonucleotides with the sequences CATCTAGAAGAGATTATG for human SMRT (AS-SMRT), CATTATCAGTAAAGAAGTCC for human NCoR (AS-NCoR), CATCTTGAACACATATCAGC for human NCoA (AS-NCoA), and as a control, TGGATCATCTTCTGCCATTCT for NF-κB mRNA. They were synthesized as phosphorothioates to prolong their half-lives in the cells. These sequences were designed to bind the initiation codon and the sequences immediately upstream, sites that commonly confer the most efficient antisense blocking. We followed a protocol we successfully used previously (Tomic-Canic *et al*, 1992; Radoja *et al*, 1997). The keratinocytes were incubated in basal keratinocyte medium (Gibco) without bovine pituitary extract, EGF, hydrocortisone or thyroid hormone, and HeLa cells were incubated in 1% delipidized medium from the beginning of transfection. The antisense DNA were added to the transfected DNA mixture and subsequently to the medium of the cells transfected with the GR-responsive CAT constructs. Including the antisense DNA in the transfection mix has the advantage of ensuring that the cells that received the transfected DNA also received the antisense oligonucleotides. We added 5 mM concentrations of the oligonucleotides to the medium immediately after transfection and again 18 h later. Cells were harvested 36 h after transfection, and enzyme assays were performed.

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, Oberlin, Ohio).

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 per mMole, New England Nuclear, Boston Massachusetts), 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 ethylacetate, 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 12–24 h, and the intensity of radioactive spots determined using the Ambis Radioanalytic System (Ambis, San Diego, California). 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.

Northern blot Primary human keratinocytes were transfected with expression vectors for GRIP-1, T3R, and RAR, as described above. The cells were incubated in the presence or absence of corresponding ligands, 0.1 μM of either T3 or RA, and harvested 48 h later. Similarly, for the TSA experiment, cells were incubated in the presence or absence of 0.1 μM TSA and corresponding hormones. RNA isolation and purification was performed using Triazol (Invitrogen, Carlsbad, California) extraction and subsequently Quiagen RNeasy Kit column purification (Qiagen, Valencia, California) followed by northern blot as described (Radoja *et al*, 2000). K14 and glyceraldehyde-3-phosphate dehydrogenase probes were generated as described (Radoja *et al*, 2000).

Our research is supported by the National Institutes of Health grants AR45974, NR08029, DK59424, T3207190, and the American Diabetes Association.

DOI: 10.1111/j.0022-202X.2005.23691.x

Manuscript received September 10, 2004; revised December 3, 2004; accepted for publication December 28, 2004

Address correspondence to: Marjana Tomic-Canic, The Ronald O. Perelman Department of Dermatology, New York University School of Medicine, 550 First Avenue, TH100, New York, New York 10016, USA. Email: tomicm01@med.nyu.edu

References

Blumenberg M: Keratinocytes biology and differentiation. In: Arndt KP (ed). Cutaneous Medicine and Surgery, Vol. 1. Philadelphia: W.B. Saunders, 1996; p 58–74

Chang HM, Paulson M, Holko M, Rice CM, Williams BR, Marie I, Levy DE: Induction of interferon-stimulated gene expression and antiviral responses require protein deacetylase activity. Proc Natl Acad Sci USA 101:9578–9583, 2004

Chen CA, Okayama H: Calcium phosphate-mediated gene transfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques 6:632–638, 1988

Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombauts W: Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol 76:23–30, 2001

Coulombe PA, Omary MB: "Hard" and "soft" principles defining the structure, function and regulation of keratin intermediate filaments. Curr Opin Cell Biol 14:110–122, 2002

Epstein JH: Effects of retinoids on ultraviolet-induced carcinogenesis. J Invest Dermatol 77:144–146, 1981

Fass DM, Butler JE, Goodman RH: Deacetylase activity is required for cAMP activation of a subset of CREB target genes. J Biol Chem 278:43014–43019, 2003

Fisher C, Blumenberg M, Tomic-Canic M: Retinoid receptors and keratinocytes. Crit Rev Oral Biol Med 6:284–301, 1995

Fisher GJ, Esmann J, Griffiths CE, *et al*: Cellular, immunologic and biochemical characterization of topical retinoic acid-treated human skin. J Invest Dermatol 96:699–707, 1991

Fisher GJ, Voorhees JJ: Molecular mechanisms of retinoid actions in skin. FASEB J 10:1002–1013, 1996

Fondell JD, Ge H, Roeder RG: Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci USA 93:8329–8333, 1996

Futoryan T, Gilchrest BA: Retinoids and the skin. Nutr Rev 52:299–310, 1994

Garcia-Villalba P, Jimenez-Lara AM, Castillo AI, Aranda A: Histone acetylation influences thyroid hormone and retinoic acid-mediated gene expression. DNA Cell Biol 16:421–431, 1997

Gilfix BM, Eckert RL: Coordinate control by vitamin A of keratin gene expression in human keratinocytes. J Biol Chem 260:14026–14029, 1985

Gronemeyer H, Miturski R: Molecular mechanisms of retinoid action. Cell Mol Biol Lett 6:3–52, 2001

Heinzel T, Lavinsky RM, Mullen TM, *et al*: A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression [see comments]. Nature 387:43–48, 1997

Heymann WR: Advances in the cutaneous manifestations of thyroid disease. Int J Dermatol 36:641–645, 1997

Holt PJ, Marks R: The epidermal response to change in thyroid status. J Invest Dermatol 68:299–301, 1977

Hong H, Kohli K, Garabedian MJ, Stallcup MR: GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol 17:2735–27344, 1997

Isseroff RR, Chun KT, Rosenberg RM: Triiodothyronine alters the cornification of cultured human keratinocytes. Br J Dermatol 120:503–510, 1989

Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai MJ, O'Malley BW: Steroid receptor induction of gene transcription: A two-step model. Proc Natl Acad Sci USA 94:7879–7884, 1997

Jho SH, Radoja N, Im MJ, Tomic-Canic M: Negative response elements in keratin genes mediate transcriptional repression and the cross-talk among nuclear receptors. J Biol Chem 276:45914–45920, 2001

Jiang CK, Connolly D, Blumenberg M: Comparison of methods for transfection of human epidermal keratinocytes. J Invest Dermatol 97:969–973, 1991

Khan OY, Nawaz Z: Nuclear hormone receptor co-regulators. Curr Opin Drug Discov Dev 6:692–701, 2003

Klampfer L, Huang J, Swaby LA, Augenlicht L: Requirement of histone deacetylase activity for signaling by STAT1. J Biol Chem 279:30358–30368, 2004

Kraemer KH, DiGiovanna JJ, Peck GL: Chemoprevention of skin cancer in xeroderma pigmentosum. J Dermatol 19:715–718, 1992

Lee B, Vouthounis C, Stojadinovic O, Brem H, Im MJ, Tomic-Canic M: From an enhanceosome to a repressosome: Molecular antagonism between glucocorticoids and EGF leads to inhibition of wound healing. J Mol Biol 345:1083–1097, 2005

Li X, Kimbrel EA, Kenan DJ, McDonnell DP: Direct interactions between corepressors and coactivators permit the integration of nuclear receptor-mediated repression and activation. Mol Endocrinol 16:1482–1489, 2002

Lichti U, Ben T, Yuspa SH: Retinoic acid-induced transglutaminase in mouse epidermal cells is distinct from epidermal transglutaminase. J Biol Chem 260:1422–1426, 1985

Lotan R: Retinoids and their receptors in modulation of differentiation, development, and prevention of head and neck cancers. Anticancer Res 16:2415–2419, 1996

Magnaldo T, Bernerd F, Asselineau D, Darmon M: Expression of loricrin is negatively controlled by retinoic acid in human epidermis reconstructed *in vitro*. Differentiation 49:39–46, 1992

Mahajan MA, Das S, Zhu H, Tomic-Canic M, Samuels HH: The nuclear hormone receptor co-activator NRC is a pleiotropic modulator affecting growth, development, apoptosis, reproduction, and wound repair. Mol Cell Biol 24:4994–5004, 2004

Mangelsdorf DJ, Thummel C, Beato M, *et al*: The nuclear receptor superfamily: The second decade. Cell 83:835–839, 1995

McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ, O'Malley BW: Nuclear receptor coactivators: Multiple enzymes, multiple complexes, multiple functions. J Steroid Biochem Mol Biol 69:3–12, 1999

Montminy M: Transcriptional activation. Something new to hang your HAT on. Nature 387:654–655, 1997

Moon TE, Levine N, Cartmel B, *et al*: Design and recruitment for retinoid skin cancer prevention (SKICAP) trials. The Southwest Skin Cancer Prevention Study Group. Cancer Epidemiol Biomarkers Prev 4:661–669, 1995

Nagy L, Kao HY, Chakravarti D, *et al*: Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380, 1997

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y: The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87: 953–959, 1996

Pearce D, Matsui W, Miner JN, Yamamoto KR: Glucocorticoid receptor transcriptional activity determined by spacing of receptor and nonreceptor DNA sites. J Biol Chem 273:30081–30085, 1998

Radoja N, Diaz DV, Minars TJ, Freedberg IM, Blumenberg M, Tomic-Canic M: Specific organization of the negative response elements for retinoic acid and thyroid hormone receptors in keratin gene family. J Invest Dermatol 109:566–572, 1997

Radoja N, Komine M, Jho SH, Blumenberg M, Tomic-Canic M: Novel mechanism of steroid action in skin through glucocorticoid receptor monomers. Mol Cell Biol 20:4328–4339, 2000

Randolph RK, Simon M: Characterization of retinol metabolism in cultured human epidermal keratinocytes. J Biol Chem 268:9198–9205, 1993

Reuning U, Wilhelm O, Nishiguchi T, Guerrini L, Blasi F, Graeff H, Schmitt M: Inhibition of NF-kappa B-Rel A expression by antisense oligodeoxynucleotides suppresses synthesis of urokinase-type plasminogen activator (uPA) but not its inhibitor PAI-1. Nucleic Acids Res 23:3887–3893, 1995

Ricci AR, Genereaux J, Brandl CJ: Components of the SAGA histone acetyltransferase complex are required for repressed transcription of ARG1 in rich medium. Mol Cell Biol 22:4033–4042, 2002

Rosenberg RM, Isseroff RR, Ziboh VA, Huntley AC: Abnormal lipogenesis in thyroid hormone-deficient epidermis. J Invest Dermatol 86:244–248, 1986

Rosenthal DS, Griffiths CE, Yuspa SH, Roop DR, Voorhees JJ: Acute or chronic topical retinoic acid treatment of human skin *in vivo* alters the expression of epidermal transglutaminase, loricrin, involucrin, filaggrin, and keratins 6 and 13 but not keratins 1, 10, and 14. J Invest Dermatol 98:343–350, 1992

Safer JD, Crawford TM, Fraser LM, *et al*: Thyroid hormone action on skin: Diverging effects of topical versus intraperitoneal administration. Thyroid 13:159–165, 2003

Sakamoto S, Potla R, Larner AC: Histone deacetylase activity is required to recruit RNA polymerase II to the promoters of selected interferon stimulated early response genes. J Biol Chem 279:40362–40367, 2004

Shang Y, Myers M, Brown M: Formation of the androgen receptor transcription complex. Mol Cell 9:601–610, 2002

Spencer TE, Jenster G, Burcin MM, *et al*: Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194–198, 1997

Steijlen PM, Reifenschweiler DO, Ramaekers FC, *et al*: Topical treatment of ichthyoses and Darier's disease with 13-cis-retinoic acid. A clinical and immunohistochemical study. Arch Dermatol Res 285:221–226, 1993

Stellmach V, Leask A, Fuchs E: Retinoid-mediated transcriptional regulation of keratin genes in human epidermal and squamous cell carcinoma cells. Proc Natl Acad Sci USA 88:4582–4586, 1991

Tomic M, Jiang CK, Epstein HS, Freedberg IM, Samuels HH, Blumenberg M: Nuclear receptors for retinoic acid and thyroid hormone regulate transcription of keratin genes. Cell Regul 1:965–973, 1990

Tomic-Canic M, Freedberg IM, Blumenberg M: Codominant regulation of keratin gene expression by cell surface receptors and nuclear receptors. Exp Cell Res 224:96–102, 1996

Tomic-Canic M, Sunjevaric I, Freedberg IM, Blumenberg M: Identification of the retinoic acid and thyroid hormone receptor-responsive element in the human K14 keratin gene. J Invest Dermatol 99:842–847, 1992

Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG: The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 387:677–684, 1997

Van Tilborg MA, Lefstin JA, Kruiskamp M, Teuben J, Boelens R, Yamamoto KR, Kaptein R: Mutations in the glucocorticoid receptor DNA-binding domain mimic an allosteric effect of DNA. J Mol Biol 301:947–958, 2000

Vietor I, Oliveira IC, Vilcek J: CCAAT box enhancer binding protein alpha (C/EBP-alpha) stimulates kappaB element-mediated transcription in transfected cells. J Biol Chem 271:5595–5602, 1996

Vom Baur E, Zechel C, Heery D, *et al*: Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. EMBO J 15:110–124, 1996

Wong J, Shi YB, Wolffe AP: Determinants of chromatin disruption and transcriptional regulation instigated by the thyroid hormone receptor: Hormone-regulated chromatin disruption is not sufficient for transcriptional activation. EMBO J 16:3158–3171, 1997

Wong P, Coulombe PA: Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. J Cell Biol 163:327–337, 2003

Yamamoto KR, Darimont BD, Wagner RL, Iniguez-Lluhi JA: Building transcriptional regulatory complexes: Signals and surfaces. Cold Spring Harb Symp Quant Biol 63:587–598, 1998