Hyaluronan (HA) is a polysaccharide of the vertebrate extracellular matrix, produced by three related HA synthases (HASs) that influence numerous physiological processes. We screened the first 2250 bp of the HAS2 promoter for transcription factor response elements (REs) in silico and found 1 cluster of 2 retinoic acid (RA) REs, 3 discrete NF-κB factors, and 12 Sp1 REs. In parallel, we scanned nine overlapping promoter regions in HaCaT human immortalized keratinocytes using chromatin immunoprecipitation assays to identify binding of mediator, coactivator, and corepressor proteins and Sp1 transcription factor in response to all-trans-RA and tumor necrosis factor-α (TNF-α). We found that all-trans-RA modulated the binding of the RA receptor and several coregulators to the region containing the RARE cluster at position −1230. The importance of this region is supported in reporter gene assays by the all-trans-RA induction of the respective promoter region. Similarly, we showed by chromatin immunoprecipitation assays as well as by gel-shift assays with nuclear extracts that TNF-α induced NF-κB binding to regions at positions −380, −1420, and −1890, demonstrated its association with RNA polymerase II and cofactor proteins, and confirmed the functionality of the respective promoter regions in vivo. These findings partially explain the induction of HAS2 mRNA by all-trans-RA and TNF-α and provide an example how the action of different transcription factor families can use the same cofactors.

The glycosaminoglycan HA is a high molecular mass linear non-sulfated polysaccharide that is a key component of the vertebrate extracellular matrix and has a variety of functions during and after development (1, 2). Its unique physiochemical properties and its interactions with specific cell surface receptors provide HA with a central role in cellular migration, adhesion, and proliferation (3). HA is synthesized by the enzymes HAS1, HAS2, and HAS3, located at the plasma membrane (4). Modified or aberrant HA metabolism is important in diseases such as malignancies, osteoarthritis, and pulmonary and vascular disorders along with other immune and inflammatory diseases (5). In the skin epidermis the narrow extracellular space surrounding keratinocytes contains a high concentration of HA (6). Keratinocyte growth and differentiation in both the normal and diseased epidermis are regulated by endocrine and paracrine signaling molecules such as the nuclear hormone all-trans-RA and epidermal growth factor (7). Direct evidence for the biological role of HA in epidermal keratinocytes emerged by the finding that HAS2-mediated HA synthesis controls the migration rate of keratinocytes in scratch-wounded monolayer cultures (8).

It is known that a number of transcription factors such as RAR, signal transducers and activators of transcription 3, and Sp1 are involved in the regulation of HAS2 mRNA synthesis (7, 9); however, a systematic analysis of the promoter region has not been performed to compare the mechanisms of activation utilized by these transcription factors on this particular gene promoter.

The three RAR subtypes, α, β, and γ, have very similar functional profiles, but the predominant subtype in epidermis is RARγ (10). Like most members of the nuclear receptor superfamily, RARs contain two zinc finger motifs that form a characteristic DNA binding domain (11) and a carboxyl-terminal ligand binding domain (12). Ligand binding causes a conformational change within the ligand binding domain that leads to the displacement of corepressor proteins, such as nuclear receptor corepressor (NCoR) (13) and subsequent interaction with coactivator proteins, such as steroid receptor coactivator 1 (SRC-1) (14). Coactivators link the ligand-activated RARs to enzymes displaying histone acetyltransferase activity, such as CREB-binding protein (CBP)
(15). The combined effect of these covalent modifications has a large impact on chromatin organization and accessibility of transcription factor binding sites (16). In a subsequent step RARs interact with proteins of the mediator (MED) complex, such as MED1 (17) or poly (ADP-ribose) polymerase family, member 1 (PARP1) (18), which act as a bridge to the basal transcription machinery having Pol II as its core (19). Preferentially, RARs form heterodimers with the retinoid X receptor, another nuclear receptor family member that also contacts DNA (20) with hexameric DNA motifs containing the consensus sequence RGKTCA (R = A or G, K = G or T), arranged as a direct repeat with five intervening nucleotides (DR5) (21). An essential prerequisite for the direct modulation of transcription via all-trans-RA is the location of activated RARs close to the basal transcriptional machinery. Therefore, the presence of RAREs is predicted within the activated chromatin regions in the promoters of RA target genes.

NF-κB is a ubiquitously expressed transcription factor that plays a pivotal role in the expression of various inducible target genes that regulate cell proliferation, differentiation, apoptosis, and immune and inflammatory responses. The transcription factor is a member of the Rel protein family, of which the most common member is formed by a p50/p65 heterodimer (22). In non-stimulated cells, the heterodimeric NF-κB complex is sequestered in the cytoplasm of most cell types by inhibitory proteins of the 1κB family (23). These inhibitors mask the NF-κB nuclear localization domain and inhibit its DNA binding activity. In response to a large variety of stimuli, such as TNF-α, the 1κB inhibitor is rapidly phosphorylated and degraded. This allows NF-κB nuclear translocation, DNA binding to REs of the consensus sequence GGGRNNYYCC (Y = any base), and contact with coactivator and MED proteins (24).

In contrast to nuclear receptors and NF-κB factors, the Sp1-like proteins are constitutively active transcription factors (25). Sp1 is ubiquitously expressed and binds to GC-rich REs and, therefore, regulates a large number of “housekeeping” genes that have GC-rich promoters whose products take part in nearly all cellular functions, including cell proliferation, apoptosis, differentiation, and neoplastic transformation (26). The members of the Sp1-like family regulate transcription by interacting with coactivators, such as CBP, involving histone acetylation (27, 28).

The proximal promoter region of all three HAS genes show constitutive activity, of which HAS2 has the lowest basal level (29, 30), and therefore, the HAS2 gene seems to be the main candidate for modulating HA synthesis rate by external stimuli. In this study we screened the first 2250 bp of the HAS2 promoter for transcription factor REs in silico and identified a cluster of 2 putative RAREs and 3 possible NF-κB REs as well as 12 candidate Sp1 REs. In parallel, we scanned nine overlapping promoter regions by ChIP assays in HaCaT cells for the binding of MED, coactivator, corepressor, and Sp1 proteins. We demonstrated all-trans-RA-induced binding of RARY to a promoter region that contains the RARE cluster, showed its association with Pol II and several cofactor proteins, and confirmed the induction of the respective promoter region by all-trans-RA. Similarly, we showed NF-κB binding to three regions of the promoter, demonstrated its association with Pol II and cofactor proteins, and confirmed the functionality of the respective promoter regions. These findings partially explain the induction of human HAS2 gene mRNA by all-trans-RA and TNF-α and provide an example of how the action of different transcription factor families can use the same cofactors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human immortalized keratinocyte HaCaT cell line (31) was cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 2 mM l-glutamine. Before mRNA extraction, nuclear extract preparation, or ChIP assays, HaCaT cells were treated at a density of 50–60% confluence for 0–240 min with solvent, 0.1 μM all-trans-RA (Sigma), 20 or 30 ng/ml recombinant TNF-α (Calbiochem), and 10 μM IKK-2 inhibitor (Calbiochem) alone or in combination. The medium was then supplemented with stock solutions of the compounds and was not changed at the moment of cell stimulation.

**ChIP Assays**—Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 10 min at room temperature. Cross-linking was stopped by adding glycerol to a final concentration of 0.15 M and incubating for 5 min at room temperature on a rocking platform. The medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline supplemented with a protease inhibitor mixture (Roche Applied Science). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1), and the lysates were sonicated to result in DNA fragments of 300–1000 bp in length. Cellular debris was removed by centrifugation, and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors, 16.7 mM Tris-HCl, pH 8.1). Chromatin solutions were incubated with 5 μl of the indicated antibodies overnight at 4 °C with rotation. The antibodies against RARy (sc-773), SRC-1 (sc-8995), MED1 (sc-5334), phosphorylated (p) RNA polymerase (pPol II; sc-13583), CBP (sc-369), NCoR (sc-8994), Sp1 (sc-14027), NF-κB p65 (sc-372), PARP1 (sc-25780), and control IgG (sc-2027) were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). The immunocomplexes were collected with 60 μl of protein A-agarose slurry (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4 °C with rotation. The beads were pelleted by centrifugation for 1 min at 4 °C with 100 × g and washed sequentially for 4 min by rotation with 1 ml of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris-HCl, pH 8.1), and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with 1 ml of TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0). For re-ChIP, the immunocomplexes were eluted by adding 100 μl of re-ChIP elution buffer (10 mM dithiothreitol) for 30 min at room temperature with rotation, the supernatant was diluted 1:40 in ChIP dilution buffer, and the antibody against the second protein of interest
was added. The new immunocomplexes were allowed to form by incubating overnight at 4 °C on a rocking platform, collected by incubating with 120 μl of protein A-agarose slurry for 3 h at 4 °C on a rocking platform, and finally, were washed as indicated above. The immunocomplexes were then eluted by adding 500 μl of elution buffer (1% SDS and 100 mM NaHCO3) and incubating for 30 min at room temperature with rotation. The cross-linking was reversed by adding NaCl to a final concentration of 200 mM and incubating overnight at 65 °C. The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubation for 1 h at 45 °C. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extractions and precipitated with 0.1 volume of 3M sodium acetate, pH 5.2, and 2 volumes of ethanol using glycogen as a carrier. Immunoprecipitated DNAs were then used as a template for PCR with the following profile: 5 min of preincubation at 94 °C and then 35 cycles (45 in case of primers for region 4) of 30-s denaturation at 95 °C, 30-s annealing at 60 °C, and 30-s elongation at 72 °C, with one final incubation for 10 min at 72 °C. The PCR primers and the location of the investigated chromatin regions are listed in Table 1. The PCR products were separated by electrophoresis through 2.0% agarose gels and quantified on a FLA-3000 reader (Fuji, Tokyo, Japan) using ScienceLab99 software (Fuji).

Transfection and Reporter Gene Assay—HaCaT cells were seeded into 6-well plates (105 cells/ml) and grown overnight in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal bovine serum. The generation of a nested series of HAS2 promoter constructs was described previously (29). Plasmid DNA-containing liposomes were formed by incubating these reporter plasmids and pcDNA3-based expression vectors for human p50 and p65 (1 μg each) with 10 μg of N-[1-(2,3-dioleoyloxy)propyl]-N,N,Ntrimethylammonium methyl sulfate (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl of phenol red-free Dulbecco’s modified Eagle’s medium, the liposomes were added to the cells. Phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 500 μl of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time either 0.1 μM all-trans-RA, 10 μM IKK-2 inhibitor, or 30 ng/ml TNF-α or solvent were also added. In combined treatments, IKK-2 inhibitor was added 30 min before the other stimulant. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Applied Science), and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (PerkinElmer Life Sciences). The luciferase activities were normalized with respect to protein concentration, and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

Gel-shift Analysis—Gel-shift analysis was performed with extracts from HaCaT cells. Cells were harvested by mechanical scraping into 5 ml of phosphate-buffered saline. Cell pellets were resuspended in 0.5 ml of low salt buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 50 mM KCl, 0.5 mM dithiothreitol, proteinase inhibitors). Cells were then resuspended in 2–3 packed volumes of the same low salt buffer with 0.5% Nonidet P-40 and homogenized by pipetting on ice. The nuclear pellet was then resuspended in one packed cell volume of high salt buffer (10 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, proteinase inhibitors). Extraction was performed for 30 min on ice. Gel-shift assays were performed with 10 μg of the nuclear extracts. The core sequence of the consensus NF-κB binding site was GGGACTTTCC. The core sequences of the putative NF-κB REs on HAS2 promoter are given in Fig. 1. Double-stranded oligonucleotides were labeled by a Klenow fragment DNA polymerase (Fermentas, Vilnius, Lithuania)-mediated filling-in reaction in the presence of a nucleotide mixture containing radiolabeled [-α-32P]dCTP. Constant amounts (1 ng) of 32P-labeled oligonucleotides (50,000 cpm) were incubated with the nuclear extracts for 20 min at room temperature. As a specificity control anti-p65 antibody was added 15 min after the nuclear extract to the DNA. Protein-DNA complexes were resolved by electrophoresis through 8% nondenaturing polyacrylamide gels in 0.5 × Tris-buffered EDTA (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and detected on a FLA-3000 reader.

RNA Extraction and Real-time PCR—Total RNA was extracted using the Mini RNA Isolation II kit (HiSS Diagnostics, Freiburg, Germany), and cDNA synthesis was performed for 1 h at 37 °C using 1 μg of total RNA as a template, 100 pmol of oligo(dT)18 primer and 40 units of reverse transcriptase (Fermentas). Real-time quantitative PCR was performed in an IQ-cycler (Bio-Rad) using the dye SybrGreen I (Molecular Probes, Leiden, The Netherlands). Per reaction, 1 unit of Hot Start Taq polymerase (Fermentas) and 3 mM MgCl2 were used, and the PCR cycling conditions were 40 cycles of 30 s at 95 °C, 30 s at 56 °C (HAS2 and ribosomal protein, large, P0, RPLP0, also known as 36B4) or 60 °C (matrix metalloproteinase 13 (MMP13)) and 30 s at 72 °C. -Fold inductions were calculated using the formula 2^{-[(ΔCt)_{sample}−(ΔCt)_{solvent}]}, where ΔCt is Ct(stimulus) − Ct(solvent). ΔCt is Ct(HAS2/MMP13) − Ct(RPLP0) and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs (and product sizes) were as follows: HAS2 gene forward, CAGAATCCAAACAGACAGTTC, and reverse, CAGCAGATCCGCAT, and reverse, GTGGTGATACCTAAAAG; MMP13 gene forward, GAGGAAAGCCTCAGTTTG, and reverse, CAGAGGAGTTACATCGGACC (357 bp); RPLP0 gene forward, AGATGCAGCAGACTGGCAT, and reverse, GTGGTGATACCTAAAGGCTGT (318 bp). PCR product quality was monitored using post-PCR melt curve analysis.

RESULTS

The Human HAS2 Promoter Contains Several Putative RAR, NF-κB, and Sp1 Binding Sites—The first 2250 bp of the human HAS2 promoter were screened in silico for putative RAR, NF-κB, and Sp1 binding sites (Fig. 1). For RARE identification, the promoter was screened for two copies of the RGKTA consensus sequence in a DR5 arrangement. By restricting the maximal deviation from the consensus sequence to one position we identified a cluster of two overlapping RAREs located between positions −1208 to −1237 relative to the transcription start site (TSS). The use of the net-based program ConSite with a transcription factor score cutoff of 85% identified three putative NF-κB REs located at positions −375 to −384, −1417 to
Patterns of Transcriptional Regulator Recruitment to the Human HAS2 Promoter during Stimulation by RA and TNF-α—

The association of the MED protein MED1, the coactivators CBP and SRC-1, the corepressor NCoR, and the transcription factor Sp1 with the first 2250 bp of the human HAS2 promoter was monitored by ChIP assays with antibodies specific for the respective proteins (Fig. 2). For this purpose chromatin was extracted from HaCaT cells that had been treated for 0, 60, and 240 min with 0.1 μM all-trans-RA (Fig. 2A) or for 0 and 60 min with 30 ng/ml TNF-α (Fig. 2B). The nine genomic ChIP primer pairs allowed the monitoring of the respective promoter regions (see Fig. 1). The resulting PCR products were quantified and expressed as % of input control, i.e. compared with the amount of PCR products obtained from non-immunoprecipitated chromatin templates. The specificity of the results was determined in relation to the maximal level of PCR products (2% of input) obtained on chromatin unspecifically binding to IgG, and therefore, we consider all signals above 2% of input value as being specific.

To investigate the effects of RA stimulation on the human HAS2 promoter, in the first panel of ChIP assays (Fig. 2A) we found in the absence of ligand that the histone acetyltransferase CBP was already associated with promoter regions 6 (which contains the RARE cluster), 7 (where NF-κB RE2 resides), and 8 (that contains the NF-κB RE3). After 60 min of all-trans-RA treatment, CBP is found in regions 1 (TSS), 4, 5, and 6 (RARE cluster), whereas after 240 min it can be detected in regions 4, 6 (RARE cluster), 7, and 8 and 9 (both NF-κB RE3). Constitutive binding of the mediator protein MED1 was found only in region 3 (NF-κB RE1), whereas after 60 min all-trans-RE treatment it can be detected also in regions 1 (TSS), 2, 3 (NF-κB RE1), and 8 and 9 (both NF-κB RE3). The same binding pattern was still observed after 240 min, except for binding to region 9, which was now absent. The coactivator SRC-1 showed ligand-independent binding to regions 1 (TSS), 5, 7 (NF-κB RE2), and 8 (NF-κB RE3). After 60 min of all-trans-RA stimulation SRC-1 associated with regions 2, 5, and 7 (NF-κB RE2), and after 240 min it was still in contact with regions 2, 5, 7 (NF-κB RE2) and 8 (NF-κB RE3). Before ligand treatment the corepressor NCoR was found in regions 1, 2, 3 (NF-κB RE1), 7 (NF-κB RE2), and 8 and 9 (both contain the NF-κB RE3 sequence). After 60 min of all-trans-RA treatment NCoR remained associated with regions 7 (NF-κB RE2), and 8 and 9 (both NF-κB RE3), but after 240 min it was found nowhere. Constitutive binding of Sp1 could be detected in regions 1 (TSS) and 3 (NF-κB RE1), whereas after 60 min of all-trans-RA treatment it was found in regions 3 (NF-κB RE1), 6 (RARE cluster), and 8 (NF-κB RE3). Finally, after 240 min this transcription factor was associated with regions 1 (TSS), 3 (NF-κB RE1), 4, 6 (RARE cluster), and 8 (NF-κB RE3).

In a similar fashion in the second ChIP panel (Fig. 2B) where we investigated the effects of TNFα-mediated HAS2 gene promoter activation, we could confirm the ligand-independent association of CBP with regions 6 (RARE cluster) and 8 (NF-κB RE3) and obtained in addition an indication for an association with regions 1 (TSS), 2, and 3 (NF-κB RE1). At 60 min after

**TABLE 1**

Genomic PCR primers

Sequence and location of the PCR primer pairs used to detect 9 genomic regions covering the first 2250 bp of the HAS2 gene promoter. For all nine primer pairs the first sequence represents the sense primer, and the second represents that of the anti-sense primer. Annealing temperature was 60°C for all primer pairs.

| Region | Location | Primer sequence (5’ to 3’) |
|--------|----------|-----------------------------|
| 1      | −32 to +57 | GGAGGCGAAGTGCGACACCAAGC    |
| 2      | −246 to −12 | GGTTCAAAGGCGCTCTCTGGCAAGCC |
| 3      | −481 to −244 | GGGCCTGGTCTAAATCTCCTCAAGT |
| 4      | −675 to −460 | GGGGCTGACCATCTGATCCAGGAG |
| 5      | −1048 to −655 | CTGAGTCATGCAGCTGCGGTTT |
| 6      | −1338 to −1027 | CGCTGCAAGCTAGTGGCAAGAG |
| 7      | −1613 to −1318 | CACCCATGCCGAGTTCCAAACAG |
| 8      | −1896 to −1554 | GAT TGCTGCTGCTGCTGCTGCTG |
| 9      | −2250 to −1874 | CTCTGCTGCTGCTGCTGCTGCTG |

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**FIGURE 1.** Overview of the human HAS2 promoter. The first 2250 bp of the human HAS2 promoter were screened in silico for putative RAREs (DR5-type REs), putative NF-κB REs (consensus sequence GGGACTTTCC), and putative Sp1 REs by using our own software a modified hexamer binding site search (38) and the net-based program ConSite (applying the transcription factor binding search cutoff 85%). The locations of the nine genomic regions used in ChIP assays are also indicated. Please note that to avoid false positives, we consider only signals above 2% of input control. **TABLE 1** Gene-specific primers designed for PCR amplification. The primer pairs allowed the monitoring of the respective promoter regions (see Fig. 1). The resulting PCR products were quantified and expressed as % of input control, i.e. compared with the amount of PCR products obtained from non-immunoprecipitated chromatin templates. The specificity of the results was determined in relation to the maximal level of PCR products (2% of input) obtained on chromatin unspecifically binding to IgG, and therefore, we consider all signals above 2% of input value as being specific. To investigate the effects of RA stimulation on the human HAS2 promoter, in the first panel of ChIP assays (Fig. 2A) we found in the absence of ligand that the histone acetyltransferase CBP was already associated with promoter regions 6 (which contains the RARE cluster), 7 (where NF-κB RE2 resides), and 8 (that contains the NF-κB RE3). After 60 min of all-trans-RA treatment, CBP is found in regions 1 (TSS), 4, 5, and 6 (RARE cluster), whereas after 240 min it can be detected in regions 4, 6 (RARE cluster), 7, and 8 and 9 (both NF-κB RE3). Constitutive binding of the mediator protein MED1 was found only in region 3 (NF-κB RE1), whereas after 60 min all-trans-RE treatment it can be detected also in regions 1 (TSS), 2, 3 (NF-κB RE1), and 8 and 9 (both NF-κB RE3). The same binding pattern was still observed after 240 min, except for binding to region 9, which was now absent. The coactivator SRC-1 showed ligand-independent binding to regions 1 (TSS), 5, 7 (NF-κB RE2), and 8 (NF-κB RE3). After 60 min of all-trans-RA stimulation SRC-1 associated with regions 2, 5, and 7 (NF-κB RE2), and after 240 min it was still in contact with regions 2, 5, 7 (NF-κB RE2) and 8 (NF-κB RE3). Before ligand treatment the corepressor NCoR was found in regions 2, 3 (NF-κB RE1), 7 (NF-κB RE2), and 8 and 9 (both contain the NF-κB RE3 sequence). After 60 min of all-trans-RA treatment NCoR remained associated with regions 7 (NF-κB RE2), and 8 and 9 (both NF-κB RE3), but after 240 min it was found nowhere. Constitutive binding of Sp1 could be detected in regions 1 (TSS) and 3 (NF-κB RE1), whereas after 60 min of all-trans-RA treatment it was found in regions 3 (NF-κB RE1), 6 (RARE cluster), and 8 (NF-κB RE3). Finally, after 240 min this transcription factor was associated with regions 1 (TSS), 3 (NF-κB RE1), 4, 6 (RARE cluster), and 8 (NF-κB RE3).

In a similar fashion in the second ChIP panel (Fig. 2B) where we investigated the effects of TNFα-mediated HAS2 gene promoter activation, we could confirm the ligand-independent association of CBP with regions 6 (RARE cluster) and 8 (NF-κB RE3) and obtained in addition an indication for an association with regions 1 (TSS), 2, and 3 (NF-κB RE1). At 60 min after
stimulation with TNF-α, CBP was still found in regions 2, 3, (NF-κB RE1), and 8 (NF-κB RE3). In the absence of stimulation MED1 binding was confirmed in region 3 (NF-κB RE1), and additional association with regions 4, 5, and 8 (NF-κB RE3) was suggested. TNF-α treatment led to binding of MED1 also to regions 1 (TSS), 4, 6 (RARE cluster), and 8 (NF-κB RE3). Ligand-independent binding of SRC-1 was confirmed in regions 1 (TSS) and 8 (NF-κB RE3), and additional binding was suggested in regions 2, 3 (NF-κB RE1), 4, and 8 and 9 (8 and 9 contain both NF-κB RE3). The ligand-independent binding of NCoR to regions 2, 3 (NF-κB RE1), and 8 (NF-κB RE3) could be confirmed. Interestingly, TNF-α treatment stimulated NCoR binding to regions 2, 3 (NF-κB RE1), and 8 (NF-κB RE3) could be confirmed. Constitutive binding of Sp1 to region 3 (NF-κB RE1) was confirmed, and additional association was suggested with regions 2, 4, and 8 (NF-κB RE3). In the presence of TNF-α, Sp1 remained bound to all of the four regions, 2, 3 (NF-κB RE1), 4, and 8 (NF-κB RE3), and an additional association with region 7 (NF-κB RE2) was detected.

In summary, different types of cofactors, such as MED1, CBP, SRC-1, and NCoR as well as the constitutive active transcription factor Sp1 each have an individual pattern for their stimulation-dependent and -independent association with HAS2 promoter regions. All five proteins were involved in association with NF-κB REs 1 and 3, whereas only CBP and Sp1 showed effective interaction with the region containing the RARE cluster. Treatment with all-trans-RA increased the association of CBP with the RARE cluster but also the binding of Sp1 to NF-κB RE1, that of CBP and SRC-1 to NF-κB RE2, and that of CBP, MED-1, NCoR, and Sp1 to NF-κB RE3. At the same time, TNF-α treatment alone stimulates the association of SRC-1 to NF-κB RE1, CBP to NF-κB RE2, and Sp1 to NF-κB RE3 and Sp1 to NF-κB REs 2 and 3 as well as the binding of MED1 to the RARE cluster.

RAR Locates Specifically and Recruits Coregulators Selectively to the HAS2 Promoter—Promoter region 6 contains a cluster of two RAREs (Fig. 1) and demonstrates all-trans-RA-dependent recruitment of cofactors (Fig. 2A). To induce transcription, this region must move closer to the TSS of the HAS2 gene, which is part of region 1, by DNA looping. Therefore, we investigated the chromatin composition on both regions in further detail (Fig. 3A). Chromatin was extracted from HaCaT cells that were stimulated for 0, 60, and 240 min with 0.1 μM all-trans-RA, and ChIP assays were performed with antibodies against pPol II, RAR, and a known coregulator involved in RA-mediated transcriptional responses, PARP1. Furthermore, re-ChIP assays were performed with anti-
Regulation of the HAS2 Promoter

FIGURE 3. Responsiveness of the HAS2 promoter-associated co-regulators to all-trans-RA. A, chromatin was extracted from HaCaT cells that had been treated with all-trans-RA (0, 60, and 240 min, 0.1 μM). Regular ChiP experiments were performed using anti-pPol II, anti-RARγ, and anti-PARP1 antibodies and IgGs as specificity control. For re-ChiP assays the first precipitation was with an anti-RARγ, and the second was with anti-pPol II, anti-SRC-1, anti-MED1, anti-CBP, and anti-NCoR antibodies. PCR was performed with primers specific for region 1 (TSS) and region 6 (RARE cluster); PCR on input chromatin template served as a positive control (100% reference), and that on IgG-precipitated template served as a specificity control (all below the 2% threshold indicated by the dashed horizontal line). Bars represent the mean ± S.D. of at least three experiments. B, reporter gene assays were performed with extracts from HaCaT cells that were transiently transfected with luciferase reporter constructs carrying the indicated 12 different HAS2 promoter fragments. Cells were treated for 16 h with either solvent (0.1% Me2SO) or all-trans-RA (0.1 μM). Relative luciferase activity was determined, and -fold inductions are calculated in reference to solvent-treated controls. Bars represent the mean ± S.D. of at least three experiments. Two-tailed paired Student’s tests were performed to determine the significance of the luciferase inductions in reference to solvent controls (*, p < 0.05; **, p < 0.01).

RARγ antibodies in combination with antibodies against pPol II, SRC-1, MED1, CBP, and NCoR. Although the binding of pPol II to the TSS (region 1) remained relatively constant and was only marginally detectable on the RARE cluster (region 6), RAR binding to the TSS and the RARE cluster clearly increased after all-trans-RA treatment (Fig. 3A). Additionally, some weaker but ligand-dependent association of PARP1 with both promoter regions was detected. In addition, we demonstrated all-trans-RA-dependent association of RAR with pPol II and with SRC-1 on the RARE cluster. Furthermore, the preassembled RAR-NCoR complex on this promoter region dissociated after ligand addition. Moreover, complexes of RAR with SRC-1, MED1, CBP, and NCoR were detectable on the TSS, but they showed no significant sensitivity to treatment with all-trans-RA.

The all-trans-RA-dependent recruitment of RAR-associated cofactors to HAS2 promoter regions suggested functionality in containing the putative NF-κB REs (regions 3, 7, 8, and 9) showed responsiveness to TNF-α in the recruitment of transcriptional regulators, we investigated the chromatin composition on all four regions and on the TSS (region 1) in further detail (Fig. 4A). Chromatin was extracted from HaCaT cells that were stimulated for 0 and 60 min with 30 ng/ml TNF-α, and ChiP assays were performed with antibodies against NF-κB p65 and pPol II. Furthermore, re-ChiP assays were performed with anti-NF-κB p65 antibodies in combination with antibodies against pPol II, SRC-1, MED1, CBP, and NCoR. We could detect prominent constitutive binding of pPol II to the TSS (region 1) and to region 3 (that contains NF-κB RE1) but not to the three other regions. Moreover, we found strong, TNF-α-inducible binding of NF-κB to the NF-κB RE1, weaker but inducible association to region 7 (comprising NF-κB RE2), and more prominent but not inducible contact with region 8 response to retinoids, which we aimed to support by reporter gene assays. For that purpose a nested set of HAS2 promoter constructs was created in the luciferase reporter gene-containing vector, pGL-3 (29). The 3′ end of each promoter fragment was located at position +43, and the longest insert extended to position −2118 bp relative to the TSS (Fig. 3B). The RARE cluster was contained in promoter fragments 11 and 12, which provided a 5.4- and 1.7-fold response to all-trans-RA, respectively. Shorter promoter fragments showed a drastic loss of basal activity and all-trans-RA inducibility. However, promoter fragment 10 is an exception in that it gave a 2.3-fold induction of luciferase activity, although it lacked the characterized RARE. However, it must be noted that both the basal and the stimulated absolute luciferase activity for this promoter fragment were both below that of the basal luciferase activity value of the constructs containing the RARE cluster.

Taken together, the all-trans-RA-dependent recruitment of cofactors of RAR to regions 1 (encompassing the TSS) and 6 (containing the RARE cluster) as shown by ChiP assays and the functionality of the RARE cluster-containing HAS2 promoter fragments in reporter gene assays suggests that RAR is a critical transcription factor for this promoter.

Role of NF-κB in the TNFα-mediated Activation of the HAS2 Promoter—Because the regions...
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A chromatin was extracted from HaCaT cells that had been treated with TNF-α (0 or 60 min, 30 ng/ml). Regular ChIP experiments were performed using anti-NF-kB p65 and anti-pPol II antibodies. For re-ChIP assays the first precipitation was with anti-NF-kB p65 antibodies, and the second was with anti-pPol II, anti-SRC-1, anti-MED1, anti-CBP, and anti-NCoR antibodies. PCR was performed with primers specific for the TSS (region 1) and regions 3 (NF-kB RE1), 7 (NF-kB RE2), and 8 and 9 (NF-kB RE3); PCR on input chromatin template served as a positive control (100% reference), and that on IgG-precipitated template as specificity control (all below the 2% threshold indicated by the dashed horizontal line). Bars represent the mean ± S.D. of at least three experiments.

B Reporter gene assays were performed with extracts from HaCaT cells that were transiently transfected with luciferase reporter constructs carrying the indicated 12 different HAS2 promoter fragments and pcDNA3 empty vector or with expression vectors for human p50 and p65 in the absence or presence of IKK-2 inhibitor (10 μM). Relative luciferase activity is shown, and fold inductions are calculated in reference to the respective untreated sample transfected with empty expression vector. Bars represent the mean ± S.D. of at least three experiments. Two-tailed paired Student’s t tests were performed to determine the significance of the luciferase inductions in reference to the respective controls (*, *p < 0.05; **, *p < 0.01). Gel-shift experiments were performed with nuclear extracts of HaCaT cells that had been treated either with solvent or with TNF-α (60 min, 20 ng/ml) or with IKK-2 inhibitor (90 min, 10 μM) in the presence of 32P-labeled REs. As a specificity control anti-p65 antibody was added 15 min after the nuclear extract to the DNA. Protein-DNA complexes were resolved from free probes through non-denaturing 8% polyacrylamide gels, and a representative gel is shown.

The functionality of the three putative NF-kB binding sites was tested by reporter gene assays in HaCaT cells using the same set of luciferase reporter gene constructs as described above (Fig. 3B). Reporter gene assays traditionally accumulate signals and are not very suited for the measurement of transient stimulations, such as a treatment with TNF-α. Therefore, we focused in these assays on the effects of NF-kB on the basal activity of the HAS2 promoter by overexpressing p50 and p65, two subunits of NF-kB (Fig. 4B). In addition, we applied the IKK-2 inhibitor to detect possible repressive effects of basal NF-kB binding. Promoter fragment 12 containing all three NF-kB REs was induced 7.3-fold when p50 and p65 were overexpressed. Under the same conditions promoter fragment 11 (lacking NF-kB RE3) showed only a 4.5-fold induction, and fragment 10 (lacking both NF-kB RE3 and 2) showed no induction. When NF-kB RE1 was isolated from putative upstream regulatory elements (fragment 5), a drop of basal activity was observed that could be restored by p50/p65 overexpression.
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resulting in a 3.0-fold induction. Interestingly, the IKK-2 inhibitor treatment had in most cases neutral effects on the basal activity of the HAS2 promoter fragments, but in the case of fragment 5 it showed a stimulatory effect when p50 and p65 were overexpressed. This could be an indication of a repressing effect of NF-κB binding to RE1, which is normally modulated by unknown elements upstream of this site. Finally, the shortest promoter fragments 4 to 1, which did not contain any NF-κB RE, showed no significant response to p50/p65 overexpression.

To assess whether NF-κB binds directly to three potential NF-κB REs of the HAS2 promoter, we performed gel-shift assays with nuclear extracts from HaCaT cells, which had been stimulated for 60 min with 20 ng/ml TNF-α with and without 90-min co-treatment with 10 μM IKK-2 inhibitor (Fig. 4C). An idealized NF-κB RE served as positive control. On the latter RE, TFN-α stimulation induced two complexes, which were previously identified as p50-p65 heterodimers (lower complex) and p65 homodimers (upper complex) (32). Faint p65 homodimer binding was also observed on RE2 of the HAS2 promoter, whereas on RE1 and RE3 only p50-p65 heterodimer binding could be detected. IKK-2 inhibitor treatment of the cells as well as incubation of the nuclear extract with anti-p65 antibody could reduce the binding of both types of NF-κB complexes to all four, isolated NF-κB REs. In this direct comparison in vitro NF-κB RE2 seems to be the strongest binding element of the HAS2 promoter. However, in the functional ChIP assay, NF-κB RE2 did not associate with p65, indicating that greater complexities exist in the chromatin environment in vivo.

In summary, in living cells NF-κB in complex with pPol II and cofactor proteins associates with the TSS of the HAS2 promoter as well as with the promoter regions 3, 7, and 8/9, which contain the three NF-κB REs, 1, 2 and 3, respectively. The functionality of these three NF-κB REs was supported by reporter gene assays, and the direct binding of NF-κB to these elements was shown by gel-shift assays.

HAS2 mRNA Expression Profile—The basal mRNA expression levels of the three HAS genes were monitored by real-time quantitative PCR, in relation to the control gene RPLPO in HaCaT cells (Fig. 5A). The data demonstrated that in this cell line, HAS2 was the predominantly expressed member of the HAS gene family, since it was found to be 30-fold more highly expressed than HAS3 and 10,000-fold more than HAS1. This confirmed our assumption that in HaCaT cells the regulation of the HAS2 gene is of larger impact than that of its family members. The basal HAS2 mRNA expression was increased 7.4-fold by all-trans-RA treatment, but only 1.8-fold through TNF-α stimulation (Fig. 5B). As expected, the co-treatment with IKK-2 inhibitor abolished the TNF-α response of the HAS2 gene, but interestingly, it also reduced the all-trans-RA induction of the gene down to 3.2-fold. In comparison, a co-treatment with all-trans-RA and TNF-α provided no additive effect (data not shown). The rather modest response of the HAS2 gene to TNF-α raised the question of whether HaCaT cells are capable of more potent TNF-α signaling. Therefore, we evaluated the expression of the known NF-κB target gene MMP13 under identical conditions (Fig. 5C). This gene showed only a very faint response to all-trans-RA but a 7.6-fold response to TNF-α, which was reduced to 3.7-fold because of cotreatment with the IKK-2 inhibitor. No significant response to a cotreatment of all-trans-RA and IKK-2 inhibitor was observed with this gene.

Taken together, HAS2 is the dominant HAS gene in HaCaT cells, and a primary target of both all-trans-RA- and TNF-α-stimulated cells. Interestingly, a co-treatment of all-trans-RA and IKK-2 inhibitor suggested a HAS2-specific interference of retinoid and NF-κB signaling.

DISCUSSION

Altered synthesis and concentration of HA are associated with keratinocyte proliferation and differentiation and are disturbed in pathologic situations such as wounding and psoriasis. This makes it important to understand the regulation of HA synthesis in epithelial cells. Although it is widely acknowledged that various growth factors and hormones strongly influence HA synthesis, and some of their intracellular signaling pathways are known, virtually no data have been available on the control of HA synthesis at chromatin level (29). This missing information has become even more crucial with the accumulation of reports indicating that the concentration of HA in different cells and tissues is best correlated with HAS gene transcription, in particular with HAS2 mRNA levels (1, 8, 33, 34). This study extends and integrates previous reports on the binding of the inducible transcription factor RARγ and the constitutively active factor Sp1 to the HAS2 promoter (7, 9). Impor-
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tantly, we identified three NF-κB REs within the first 2250 bp of the promoter and demonstrated their functionality in living HaCaT cells. NF-κB RE1 at position −380 partially overlaid a previously reported binding site for the transcription factor signal transducers and activators of transcription 3 (7) and may contribute to the known strong response of the HAS2 gene to treatment with epidermal growth factor. Additionally, the two other NF-κB REs at positions −1420 and −1890 showed association with the NF-κB component p65 in living cells, but RE1 showed the strongest response to TNF-α stimulation. However, the most prominent recruitment of pPol II, MED, and coactivator proteins was observed at the TSS, where, probably via DNA looping, the activity of the three NF-κB REs is integrated. Reporter gene assays confirmed the functionality of all three NF-κB REs but also suggested that the promoter region between −640 and −512 contains a binding site for a factor with a modulatory effect on NF-κB activity on RE1. Interestingly, on this region (No. 4) ChIP assays demonstrated strong TNF-α-dependent recruitment of the corepressor NCoR (Fig. 2B). In contrast to nuclear hormones, which have a defined nuclear receptor target, the actions of the non-membrane-permeable stimulus TNF-α are more pleiotropic and result in both positive and negative effects on HAS2 gene expression. This may explain why, despite three functional NF-κB REs within the HAS2 promoter, TNF-α induces HAS2 mRNA expression by only a factor of 1.8-fold.

The response of the HAS2 promoter to all-trans-RA is similarly complex. A 2.1-kilobase HAS2 promoter fragment showed in reporter gene assays a 5.4-fold induction after all-trans-RA stimulation, which parallels the 7.5-fold induction of HAS2 mRNA. This observation was the basis of our decision to limit the scanning of the HAS2 promoter by overlapping regions for ChIP assays to the first 2250 bp, although we previously found evidence for additional weaker RAREs at positions −6990 and −2460 (7). Our in silico screening indicated the RARE cluster at position −1230 as the strongest putative RARE within the first 10 kilobases of the HAS2 promoter (this study and Ref. 7), but we failed to demonstrate in vitro binding of RAR-retinoid X receptor heterodimers in the RARE cluster. In the present study, however, we demonstrated the binding of RAR to the respective promoter region in living cells. Moreover, we also showed all-trans-RA-dependent recruitment of CBP and Sp1 proteins and dissociation of NCoR from the same promoter region. However, the average size of the chromatin fragments of 300–1000 bp as used in the ChIP assays limits the resolution of the promoter scanning. For this reason we also observed some effects in regions flanking the RARE cluster. In addition, we cannot exclude that RAR is also associating with sites in the vicinity of the RARE cluster, maybe even without direct DNA contact but in conjunction with other transcription factors. The puzzling reporter gene assay result that the region between positions −1129 and −930 mediated all-trans-RA responsiveness supports this possibility.

We identified within 2250 bp of the HAS2 promoter 12 putative Sp1 binding sites. We did not aim to prove the functionality of each of these sites, but Sp1 appears to act not only as constitutive transcription factor, on its RE1 close to the TSS, but also as a cofactor to both retinoid and NF-κB signaling. Therefore, we can assume Sp1 contributes both to the basal mRNA expression of the HAS2 gene as well as to its super-induction by all-trans-RA and TNF-α. The latter is supported by the observation that Sp1 interacts with CBP (27, 28). Because CBP interacts with numerous transcription factors, including nuclear receptors such as the RARs and NF-κB (35), it is well suited to integrate the actions of these regulatory proteins. A similar, but not as promiscuous role could be played by coactivator, corepressor, and MED proteins, since their short receptor interaction domains, composed of the sequence LXXLL in the case of coactivators (36) and LXXLXXX(I/L) in the case of corepressors (37), do not require very specific target recognition. A costimulation of TNF-α and all-trans-RA had no additional effect on HAS2 mRNA expression (data not shown), but the reduction of the all-trans-RA stimulation by the application of a NF-κB inhibitor inhibition provided an indication for interactions between NF-κB and RAR signaling on the HAS2 and the MMP13 gene promoters (Fig. 5, B and C, respectively). This interference may not only be on the level of cooperative action of both transcription factors on the chromatin organization of same promoter region and the sharing of common cofactors, as suggested in this study, but also on the level of modulation of target gene mRNA stability and the communication of NF-κB and RAR signaling with other signal transduction pathways. Further experiments are necessary to clarify these aspects.

In conclusion, we found that the human HAS2 gene promoter is under the control of the inducible transcription factors NF-κB and RAR and the constitutively active factor Sp1. These regulatory proteins share common cofactors, which provide numerous possibilities for functional interactions between the signaling pathways, of which they are the end point.

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