Acid and Epidermal Growth Factor Responding Gene*

The Human Hyaluronan Synthase 2 Gene Is a Primary Retinoic Acid and Epidermal Growth Factor Responding Gene*

Received for publication, January 6, 2005

Katri Saavalainen‡, Sanna Pasonen-Seppänen§, Thomas W. Dunlop‡, Raija Tammi§, Markku I. Tammi, and Carsten Carlberg¶

From the Departments of ‡Biochemistry and §Anatomy, University of Kuopio, FIN-70211 Kuopio, Finland

Hyaluronan is an abundant and rapidly turned over matrix molecule between the vital cell layers of the epidermis and subject to large concentration changes associated with keratinocyte proliferation, migration, and differentiation induced by paracrine and endocrine factors like epidermal growth factor (EGF) and all-trans-retinoic acid (RA). We found that in REK cells EGF and all-trans-RA up-regulated hyaluronan synthase 2 (Has2) gene expression within 2 h 4-fold each and in HaCaT human immortal keratinocytes 8- and 33-fold, respectively. The first 10 kb of the human Has2 promoter were scanned in silico and in vitro for potential response elements of signal transducer and activator of transcription (STAT) or RA receptor (RAR) proteins. We identified a STAT-response element in the proximal promoter region and confirmed its functionality in response to EGF by chromatin immunoprecipitation (ChIP) assays. Direct in vitro binding of RARs to four RARE candidates within the Has2 promoter could not be observed at stringent gel shift conditions, but reporter gene assays demonstrated functionality of a complex of two of these RAREs located ~1200 bp upstream of the transcription start site. Moreover, ChIP assays using antibodies against nine nuclear proteins monitored all-trans-RA-dependent binding of RAR, retinoid X receptor, mediator protein, and RNA polymerase II and also histone 4 acetylation to a promoter region containing the complex RARE. Taken together, the human Has2 gene is a potent primary EGF and all-trans-RA responding gene with a complex regulation.

The glycosaminoglycan hyaluronan is a high molecular mass polysaccharide that is a key component of the vertebrate extracellular matrix and is involved in a wide range of cellular functions including migration, adhesion, and proliferation by its unique physicochemical properties and interactions with specific cell surface receptors (1). Hyaluronan is synthesized by the Has enzymes Has1, Has2, and Has3 at the plasma membrane (2). In skin epidermis, the narrow extracellular space surrounding keratinocytes contains a high concentration of hyaluronan, but it is found mainly between the basal and spinous cell layers of normal human epidermis and much less in terminally differentiated layers (3). Both in normal and diseased epidermis, keratinocyte growth and differentiation are regulated by paracrine and endocrine signaling molecules, such as EGF1 and the nuclear hormone all-trans-RA. Interestingly, hyaluronan synthesis rate is stimulated by EGF in epidermal keratinocytes in monolayer (4) and organotypic cultures (5) and by all-trans-RA in human skin organ cultures (3). Direct evidence for the biological role of hyaluronan in epidermal keratinocytes emerged by the finding that Has2-mediated hyaluronan synthesis controls the migration rate of keratinocytes in scratch-wounded monolayer cultures (6). Hyaluronan concentration is closely correlated with the proliferative activity and volume of the vital part of the epidermis and inversely related with the markers of differentiation, suggesting that hyaluronan synthesis regulated by Has2 and Has3 is an important component in the proliferative reactions of the epidermis and is also involved in the epidermal differentiation process (5).

EGF is one of the most powerful molecules influencing the behavior of keratinocytes (7). The growth factor binds to the EGF receptor (EGFR), which belongs to the erbB receptor tyrosine kinase family (8). Activated EGFRs dimerize, which promotes autophosphorylation of specific tyrosines located in their cytoplasmic domain. These phosphorylated residues serve as docking sites for a variety of signaling molecules, whose recruitment finally stimulates numerous transcription factors including members of the STAT protein family (9). In this way, activated EGFRs induce via the tyrosine kinase Src the phosphorylation of STAT proteins 1, 3, and 5 (10), which then translocate to the nucleus. STAT homodimers recognize REs within promoter regions of EGF-responding genes of the sequence TTCN2–4GAA with a preference for three spacing nucleotides (11).

Like most members of the nuclear receptor superfamily, the three RAR subtypes (α, β, and γ) contain two zinc finger motifs that form a characteristic DNA-binding domain (12) and a carboxyl-terminal ligand-binding domain (13). Ligand binding causes a conformational change within the ligand-binding domain leading to interactions with coactivator and mediator (MED) proteins (14). Coactivators link the ligand-activated

* This work was supported by Academy of Finland Grants 50319 and 54062, the Finnish National Technology Agency TEKES, and the Juselius Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland. Tel.: 358-17-163062; Fax: 358-17-2811510; E-mail: carlberg@messi.uku.fi.

† The abbreviations used are: EGF, epidermal growth factor; ARP0, acidic riboprotein P0; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DR5, direct repeat spaced by 5 nucleotides; EGF-R, epidermal growth factor receptor; FBS, fetal bovine serum; HABc, hyaluronan binding complex; Has, hyaluronan synthase; MED, mediator; RE, response element; Pol II, RNA polymerase II; pPol II, phosphorylated RNA polymerase II; pSTAT, phosphorylated STAT; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RER, rat epidermal keratinocyte; RXR, retinoid X receptor; STAT, signal transducer and activator of transcription; TSS, transcription start site; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

Published, JBC Papers in Press, February 18, 2005, DOI 10.1074/jbc.M500206200

This paper is available online at http://www.jbc.org
RARs to enzymes displaying histone acetyltransferase activity, such as CREB-binding protein (CBP), that cause chromatin relaxation. In a subsequent step, RARs interact with a MED complex that acts as a bridge to the basal transcription machinery having RNA polymerase II (Pol II) as its core (15). In this way ligand-activated RARs fulfill two tasks, remodeling chromatin and activating transcription. RARs bind to hexameric DNA motifs with the consensus sequence RGKTCA (R = A or G, K = G or T). The three RAR subtypes have a very similar functional profile, but the predominant subtype in epidermis is RARγ (16). RARs form preferentially heterodimers with the retinoid X receptor (RXR), another nuclear receptor family member, which also contacts DNA (17). Simple RAREs are often formed by a direct repeat of two hexameric DNA motifs spaced by five nucleotides (DR5). Every transcriptionally responsive primary RA target gene should contain at least one functional RARE in its promoter region and these RAREs are assumed to be located relatively close to the transcription start site (TSS) of these genes (18).

The major protein constituents of chromatin are the histones. Discrete covalent modifications of these nucleosome components, in particular acetylation and methylation at lysine residues of their tails, neutralize the positive charge of the lysines, and thus reduce their attraction for the negatively charged DNA. Histones in the active promoter regions have a high degree of acetylation, the chromatin is loosely packed, and the associated DNA is available for interaction with protein complexes involved in gene regulation. Therefore, the combined effect of these covalent modifications has a large impact on chromatin organization and accessibility of transcription factor binding sites (19). An essential prerequisite for the direct modulation of transcription via EGF or all-trans-RA is the location of activated STAT proteins or RARs, respectively, close to the transcription start site (TSS) of genes (18).

The proximal promoter region of all three Has genes show constitutive activity, of which Has2 has the lowest basal level (20), i.e. the Has2 gene seems to be the main candidate for modulating the hyaluronan synthesis rate. In this study, we observed that all-trans-RA up-regulated hyaluronan content almost as efficiently as EGF in long term organotypic cultures originated from REK cells and that Has2 gene expression was rapidly increased in monolayer culture. Has2 mRNA and hyaluronan production were up-regulated even more prominently in HaCaT human immortal keratinocytes. In silico screening of the first 1000 bp of the human Has2 promoter revealed the presence of putative binding sites of transcription factors Sp1, NF-Y, and NF-κB (20), but no further information about the promoter is yet available. Therefore, we performed in this study a combined in silico and in vitro scanning of the first 10 kb of the human Has2 promoter and identified an active STAT-RE in the proximal region. ChIP assays on a number of RE-containing promoter regions of the human Has2 promoter showed all-trans-RA-dependent histone 4 acetylation and binding of RAR, RXR, MED protein, and Pol II to a promoter region containing a complex RARE. The human Has2 gene is thus a potent primary EGF and RA responding gene with a complex regulation.

MATERIALS AND METHODS

Cell Culture—The REK cell line (derived from the epidermis of newborn rat skin) was cultured in minimal essential medium containing 5% fetal bovine serum (FBS) and 4 mM l-glutamine (21) and the human immortalized keratinocyte HaCaT cell line (22) was grown in Dulbecco’s modified Eagle’s medium with 10% FBS and 2 mM l-glutamine. For organotypic cultures, REK cells were cultured at the air-liquid interface on type I collagen support (from rat tail) as described previously (21). The organotypic REK cell cultures were grown for 2 weeks, with the medium changed daily for the first week and every 2 days thereafter. The treatment of the cultures with the test substances started 1 day after the cells were raised to the air-liquid interface and was continued for 10 days. Then the cultures were fixed overnight in Histochoice® (Amresco, Solon, OH), embedded in paraffin, cut into 3-μm thick vertical sections and stained with hematoxylin and eosin.

Hyaluronan Assay—Hyaluronan content was measured from culture medium of the keratinocyte cultures with a sandwich-type ELISA (23). The tissue samples were extracted with acetic acid, digested with pepsin, and diluted with 1% bovine serum albumin in PBS before the assay as described previously (5). ELISA plates were coated by creating 96-well Maxisorb Plates (Nunc, Roskilde, Denmark) with the hyaluronan binding complex of the cartilage aggrecan G1 domain (HABC, 1 μg/ml) for 2 h at 37 °C, washing with 0.5% Tween in PBS and blocking with 1% bovine serum albumin for 1 h at 37 °C. The HABC probe was prepared as described (24). The dilutions of standard hyaluronan (Provisc, Algon Laboratories, Woburn, MA) alone or in combination.

Rearrangement of the Has2 Promoter

| Gene   | [MgCl2] | Cycling conditions | Primer sequences (5’-3’) | Product size |
|--------|---------|--------------------|--------------------------|-------------|
| Human Has2 | 3 mM    | 40 cycles: 30 s at 95 °C, 40 s at 56 °C, 30 s at 72 °C | CAGAATCCCCAACACGACAGTTT | 186 bp |
| Rat Has2  | 3 mM    | 40 cycles: 30 s at 95 °C, 25 s at 56 °C, 30 s at 72 °C | TAAGGTGTTGGTGTGGTACTG | 257 bp |
| Human ARP0 | 3 mM    | 40 cycles: 30 s at 95 °C, 40 s at 56 °C, 30 s at 72 °C | CATTGAGGACATCTTCAGTGAAG | 318 bp |
| Rat ARP0  | 3 mM    | 40 cycles: 30 s at 95 °C, 40 s at 56 °C, 30 s at 72 °C | AGATGCAGCAAGATCCGCGAT | 318 bp |

Gene Extraction and Real-time PCR—Total RNA and mRNA were extracted using TRI-reagent (Sigma) and Oligotex mini mRNA Kit (Qiagen, Hilden, Germany), respectively. One hundred ng of mRNA was used as a template in a cDNA synthesis reaction using 100 pmol of oligo(dT)18 primer in the presence of reverse transcriptase (Fermentas, Vilnius, Lithuania). The reaction was performed for 1 h at 37 °C. Real-time quantitative PCR was performed in an IQ-cycler (Bio-Rad) using the dye SYBR Green (Molecular Probes, Leiden, The Netherlands).

Fold inductions were calculated using the formula 2-ΔΔCt, where ΔΔCt is ΔCt (stimulant) - ΔCt (basal) and ΔCt is Ct (stimulant) - Ct (basal).

Gel Shift Analysis—In vitro translated RARγ and RXRa proteins were generated by coupled in vitro transcription/translation using their respective pSG5-based full-length cDNA expression constructs (25) and
rabbit reticulocyte lysate as recommended by the supplier (Promega, Madison, WI). Protein batches were quantified by test translation in the presence of [35S]methionine. The specific concentration of the receptor protein was 4 μg/ml (10% corresponds to approximately to 0.2 pmol) after taking the individual number of methionine residues per protein into account. Gel shift assays were performed with 10 ng of the appropriate in vitro translated proteins or 750 ng of recombinant

STAT1 protein (Lab Vision, Fremont, CA). The proteins were incubated for 15 min in a total volume of 20 μl of binding buffer (150 mM KCl, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-dC), 5% glycerol, 10 mM Hepes, pH 7.9).

Competition assays (1 ng) of [35S]-labeled double-stranded oligonucleotides (50,000 cpm, for core sequences see Fig. 3A) were then added and incubation was continued for 20 min at room temperature. Protein-DNA complexes were resolved by electrophoresis through 5 or 8% non-denaturing polyacrylamide gels in 0.5 Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3.

Transfection and Luciferase Reporter Gene Assays—HaCaT cells were seeded into 6-well plates (10% cells/ml) and grown overnight in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% charcoal-stripped FBS. Two copies of an idealized RARE (for sequence see Fig. 3C) and the RARE cluster (combination of RAREs 1 and 2, see Fig. 3A) of the Has2 promoter were fused with the thymidine kinase promoter driving the firefly luciferase reporter gene. Plasmid DNA containing liposomes were formed by incubating these reporter plasmids and an expression vector for human RAR α into a 1:1 ratio with 60 μg/ml poly(dI-dC), 5% glycerol, 10 mM Hepes, pH 7.9). Constructs were transfected into HaCaT cells. After centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (1 mM EDTA, 10 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 (1 mM EDTA, 10 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 (1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Chromatin solutions were incubated with 5 μl of the indicated antibodies overnight at 4 °C with rotation. The beads were pelleted by centrifugation for 1 min at 4 °C at 4 × g and the supernatant was collected and the cross-linking was reversed by adding NaCl to a final concentration of 200 mM and incubating overnight at 65 °C (26). The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubating 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 3 M 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 preincubation at 94 °C and then 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at primer specific temperatures 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 II. The PCR products were separated by electrophoresis through 2% agarose supplemented with 0.5 μg/ml ethidium bromide.

RESULTS

All-trans-RA and EGF Increase Epidermal Thickness and Hyaluronan Content—Organotypic cultures of REK cells resulted within 10 days in a relatively thin, normally organized vital epidermis and multiple layers of well differentiated corneocytes (Fig. 1A). Cultures, which were treated for the whole 10-day period with 0.1 μM all-trans-RA, 20 ng/ml EGF, or the combination of both, showed increased numbers of vital cells, often in a bit of disorganized layers (Figs. 1, B–D). In line with previous reports (5, 21), the epidermal hypertrophy and slightly defective differentiation patterns were associated with increased hyaluronan content in the all-trans-RA- and EGF-treated cultures (Fig. 1G). The EGFR kinase inhibitor AG1478 alone had only a minor effect on the thickness of the epidermis (Fig. 1E), although it markedly reduced the all-trans-RA-induced epidermal hypertrophy (Fig. 1F). The effects of AG1478 on hyaluronan content closely corresponded to those on epidermal thickness, i.e., relatively small inhibition on unstimulated cultures and a more substantial decrease of the hyaluronan deposition induced by all-trans-RA (Fig. 1G).

Has2 mRNA Expression in REK Cells Is Up-regulated by All-trans-RA and EGF—Because the Has2 enzyme is a major producer of hyaluronan in the epidermis (4), we next assessed by quantitative real-time PCR the expression level of the Has2 gene in response to the same set of stimuli as in the organotypic culture. However, because we were interested in characterizing the primary responses of the Has2 gene, the stimulation period was only 2 h and the REK cells were cultured as regular monolayers (Fig. 2A). Despite these significant differences in treatment time and culture conditions, we found that the Has2 mRNA levels in regular REK cell culture (Fig. 2A) roughly correlated with the hyaluronan production in organotypic REK cell culture (Fig. 1G). Normalized to the expression of the reference gene acidic riboprotein P0 (ARP0, also known as 36B4), both all-trans-RA and EGF stimulated the Has2 mRNA level ~4-fold and the combination of both stimuli resulted in a more than 8-fold induction. AG1478 application resulted in a slight but not significant induction of Has2 expression, whereas the combined treatment with all-trans-RA and AG1478 showed a minor if any reduction of the all-trans-RA response of the gene.

| Region No. | Location | Primer sequences (5′-3′) |
|------------|----------|-------------------------|
| 1 | −227 to −501 | CTTCTCTGAGCTACAGG CAACCTTTCTCTCCTTG |
| 2 | −878 to −1264 | CCTCTGCGAAATTTGACAGG |
| 3 | −1504 to −2001 | GTGGGAAACCTACCTTG |
| 4 | −1866 to −2217 | GTGAGAGGTAGTGAAGTCTC |
| 5 | −2451 to −2806 | CATTGTTGACCATTGTTGC |
| 6 | −6751 to −7077 | CTTCTAGCTGACTTTGTC CATAAGCACTGATCCTAC |
All-trans-RA and EGF Up-regulate Has2 mRNA Expression in HaCaT Cells—We next shifted to a human system and tested Has2 expression to the same set of stimulation conditions in HaCaT human immortal keratinocytes (Fig. 2B). Interestingly, in this cellular system all stimuli showed a more prominent effect than in REK cell culture. All-trans-RA and EGF stimulated human Has2 mRNA levels already 8.3- and 33-fold, respectively, after 2 h of treatment, and the combined treatment resulted in a 63-fold induction. In contrast, AG1478 reduced Has2 mRNA expression to 18% of solvent-treated cultures. This reduced level could be stimulated 9.3-fold by all-trans-RA, but the net stimulation by a combined treatment with all-trans-RA and AG1478 was only 1.6-fold, when compared with non-treated cultures. A time course of the RA induction of Has2 mRNA demonstrated a maximal induction of ∼19-fold after 5 h stimulation, but hardly any induction was left after 24 h (data not shown).

The Has2 mRNA stimulation or reduction levels after 2-h treatments correlated with the amounts of hyaluronan in culture medium of HaCaT cells after a 24-h ligand treatment (Fig. 2C). All-trans-RA and EGF increased hyaluronan amounts 2.2- and 5.8-fold, respectively, and the combined treatment resulted in a 7.9-fold induction. As observed on the level of Has2 mRNA, AG1478 treatment reduced hyaluronan to 33% of control levels within 24 h and in the presence of AG1478 all-trans-RA had no significant stimulatory effect. The latter discrepancy between Has2 mRNA levels and hyaluronan production may be related...
to the different kinetics of EGF and RA signaling, i.e. that the blocking of EGFR may be more long lasting than the induction of Has2 gene expression by all-trans-RA. In conclusion, in rat and human keratinocytes all-trans-RA and EGF created a significant induction of Has2 mRNA levels and hyaluronan production. The combination of both stimuli had an additive or even synergistic effect on Has2 mRNA. Blocking EGFR signaling greatly reduced the basal Has2 mRNA level and hyaluronan synthesis in human keratinocytes, but did not prevent the induction of Has2 by all-trans-RA even though the absolute levels of the mRNA remained low in the presence of AG1478. In contrast to the human cells, the Has2 mRNA levels of REK cells in monolayer showed relatively minor responses to AG1478.

**REs for STAT and RAR within the Has2 Promoter**—The significant response of the Has2 gene both to all-trans-RA and EGF already after 2 h stimulation suggested that the gene is a primary retinoid and EGF responding gene and that its promoter might contain binding sites for RARs and STAT proteins. Therefore, we performed in silico screening of the first 10 kb of the human Has2 promoter for nuclear receptor binding motifs of the sequence RKRCTA in a DR5 arrangement. By restricting the maximal deviation from this consensus sequence to one position we identified only four DR5-type REs (Fig. 3A). Allowing between two and six spacing nucleotides between a pair of the trimeric STAT recognition motif TTC, we also found four putative STAT-REs.

We performed gel shift assays with these four putative STAT-REs using recombinant STAT protein (Fig. 3B). Under highly stringent conditions, identical to our previous comparative gel shift studies (27, 28), and in reference to an idealized STAT-RE, only the first RE, located at position −379 upstream of the TSS, showed a significant binding of STAT protein. The binding of in vitro translated RARγ and RXRs, either alone or in combination, was assessed on the four putative RAREs in reference to an idealized DR5-type RARE (Fig. 3C). Although the reference RARE demonstrated a clear RAR-RXR heterodimer complex formation, on none of the four tested REs could binding of RAR-RXR heterodimers, RAR homodimers, or RXR homodimers be detected. The same observation was made with RARα-RXR heterodimers and nuclear extracts from all-trans-RA-treated HaCaT cells (data not shown). RAREs 1 and 2 (Fig. 3A) are parts of the same complex RE, but even with this extended sequence, containing all three hexameric nuclear receptor binding motifs, we were not able to detect RAR-RXR heterodimer complex formation in vitro (data not shown).

However, when the RARE cluster was fused to the thymidine kinase promoter, it mediated a significant all-trans-RA-dependent induction of luciferase reporter gene activity, which is comparable with that of the idealized DR5-type RARE in the same heterologous promoter context (Fig. 3D). The complete sequence of the rat Has2 promoter is not yet known, but it is already obvious that the human and rat gene promoters are only moderately conserved. In particular, none of the eight investigated putative transcription factor binding sites are sufficiently conserved between the two species to allow an extension of the results from human to rat. Taken together, our combined in silico and in vitro scanning for STAT protein and retinoid receptor binding sites within the human Has2 promoter resulted in one
STAT-RE within the proximal promoter and a functional RARE cluster located -1200 bp upstream of the TSS.

Recruitment of RARs and STAT Proteins to the Human Has2 Promoter—To investigate whether retinoid receptors and STAT proteins associate in live cells with the human Has2 promoter, we performed ChIP assays with all-trans-RA and EGF-stimulated HaCaT cells. For this purpose we designed primer pairs for the detection of the five human Has2 promoter regions that contain the putative RAREs and STAT-REs (regions 1, 2, 3, 5, and 6 in Fig. 3A). In addition, one region (number 4) was selected as negative control for the specificity of the antibodies. To minimize the number of primers in repetitive sequences, we employed the web-based CENSOR server screening service (29) to identify repetitive sequences in the Has2 promoter sequence.

Chromatin was extracted from HaCaT cells that had been treated for 0 and 60 min with 0.1 μM all-trans-RA (A) and 20 ng/ml EGF (B). ChIP assays using antibodies against RARγ, STAT3, and pSTAT3 were performed. On reverse cross-linked chromatin the binding of the three proteins was detected on the five selected regions of the Has2 promoter for both time points. Promoter region 4 served as a negative control. Representative PCR products of at least three independent cell treatments are shown. The input lane confirms the comparable strength of the primer pairs specific for the six promoter regions.

**Fig. 4. Recruitment of RARs and STAT proteins to the human Has2 promoter.** Chromatin was extracted from HaCaT cells that had been treated for 0 and 60 min with 0.1 μM all-trans-RA (A) and 20 ng/ml EGF (B). ChIP assays using antibodies against RARγ, STAT3, and pSTAT3 were performed. On reverse cross-linked chromatin the binding of the three proteins was detected on the five selected regions of the Has2 promoter for both time points. Promoter region 4 served as a negative control. Representative PCR products of at least three independent cell treatments are shown. The input lane confirms the comparable strength of the primer pairs specific for the six promoter regions.

| Region | Input | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|-------|---|---|---|---|---|---|
| **RARγ** |       |   |   |   |   |   |   |
| **STAT3** |       |   |   |   |   |   |   |
| **pSTAT3** |       |   |   |   |   |   |   |

Chromatin templates generated from HaCaT cells treated with and without EGF (Fig. 4B) confirmed the association of STAT3 to region 1 even without EGF stimulation. The five other Has2 promoter regions neither bound STAT nor pSTAT3 in the absence of growth factor stimulation. EGF treatment clearly increased the association of STAT3 to region 1 and also induced pSTAT3 binding to this region. In addition, a weak induction of the binding of STAT3 and pSTAT3 to regions 2 and 3 could be observed, but not to regions 4–6. Stimulation with EGF did not show any effect on the binding of RARs (data not shown). In summary, on the chromatin level five regions of the human Has2 promoter showed a broad response to stimulation with all-trans-RA not only affecting RARs but also the mediators of EGF signaling. In contrast, EGF treatment was more specific, because a significant induction of STAT3 binding only occurred to region 1. The latter result fits well with in vitro binding of STAT protein to STAT-RE1 in promoter region 1 (Fig. 3B).

All-trans-RA-induced Recruitment of Nuclear Proteins to the Human Has2 Promoter—In contrast to the straightforward EGF-induced up-regulation of the human Has2 gene via STAT-RE1 being located in the proximal promoter region, the retinoid response of the gene turned out to be more complex. To identify additional components associated with the activation of the Has2 promoter, we investigated by ChIP assays six other proteins that are usually involved in signal transduction of retinoid receptors (Fig. 5). These were the RAR partner receptor RXR, the coactivator CBP harboring histone acetyltransferase activity, the mediator protein TRAP150/MED23, Pol II, pPol II, and also acetylated histone 4 as a marker of activated, open chromatin. Because promoter region 5 showed a low responsiveness in the first screening (Fig. 4), we concentrated on regions 1, 2, 3, and 6. Promoter region 4 served again as a control for the specificity of the used antibodies.
Chromatin templates were isolated from all-trans-RA-stimulated HaCaT cells. The control region showed no association to the proteins except a constant, retinoid-independent binding of acetylated histone 4. RXR associated only to regions 2 and 3 and the binding increased on both regions following all-trans-RA treatment. In contrast, CBP binding was found to all four candidate regions of the promoter, of which region 3 showed the weakest association. MED23 bound constantly to regions 3 and 6, but associated in a retinoid-dependent fashion with promoter regions 1 and 2. Pol II showed a weak but constant binding to regions 2 and 3 and a retinoid-dependent association with regions 1 and 6. However, region 1 associated much stronger with Pol II than region 6 and had a clear maximum after 60 min of treatment with all-trans-RA. However, probably more functionally important was the association with pPol II, which for region 1 showed the same clear maximum after 60 min stimulation with all-trans-RA than with Pol II. Also region 6 displayed a clearly induced binding of pPol II at the 60-min time point and a slight decline at 240 min. Interestingly, regions 2 and 3 showed a constant binding of Pol II, whereas pPol II, absent at time point 0 min, increased over time. Finally, the overall activation status of the chromatin, which was monitored by acetylated histone 4 association, was found to be high for all the regions studied, but was all-trans-RA-induced only in the case of region 2.

Taken together, the association panel of nuclear proteins with the four RE-containing regions of the Has2 promoter remains complex despite the investigation of six additional nuclear proteins. Following the current model of retinoid signaling (30), the lack of RXR association to regions 1 and 6 disqualifies them as nucleation points for the action of all-trans-RA and RARs. Region 3, however, shows only a rather weak, retinoid-dependent association of pPol II but no other responses to the all-trans-RA treatment. This leaves region 2 as the most likely promoter region mediating the action of all-trans-RA on Has2 gene induction.

**DISCUSSION**

The structure and function of epidermis depends on a controlled balance between keratinocyte proliferation and differentiation, disturbed in pathologic situations, such as wounding and psoriasis. It has turned out that altered synthesis and concentration of hyaluronan are associated with these processes and regulate epidermal keratinocyte behavior. Moreover, malignant transformation of epithelial cells is frequently accompanied by increased hyaluronan production and accumulation, the level of which can be a strong, independent predictor of unfavorable prognosis, and the reduction of which reduces tumor spread in experimental animals (31). It is thus important to understand the regulation of hyaluronan synthesis in epithelial cells. Whereas it is widely acknowledged that various growth factors and hormones strongly influence hyaluronan synthesis and some of their intracellular signaling pathways are known, virtually no data has been available on the hyaluronan synthesis control at the chromatin level (20). This missing information has become even more crucial with the accumulation of reports indicating that the concentration of hyaluronan in different cells and tissues is best correlated with Has gene transcription, in particular with the levels of Has2 mRNA (4, 32).

This study extends the correlation between EGF- and all-trans-RA-induced hyperproliferation of REK cells in organotypic culture and the accumulation of tissue hyaluronan. In the HaCaT human keratinocyte cell line we demonstrated that the human Has2 gene is also a primary EGF and all-trans-RA responding gene. Although retinoids are applied in the therapy of various skin diseases, so far only a few primary RA responding genes have been established in human keratinocytes (33). Therefore, the 8-fold induction of Has2 mRNA after only 2 h treatment with all-trans-RA (Fig. 2B) is a remarkable response being comparable with that of the RARβ gene (18), which is the most responsive primary RA target gene currently known. This suggests that the Has2 gene could be used as a marker for monitoring the efficacy of synthetic RAR ligands in pre-clinical trials (34).

Because the responsiveness to EGFR and RAR ligands was more prominent in HaCaT cells than in REK cells, and because of the fact that the human but not the rat genome is sequenced in completeness, we screened the first 10 kb of the human Has2 promoter for STAT-REs and DR5-type RAREs. Based on *in vitro* gel shift analysis we identified an effective STAT-RE with the optimal number of three spacing nucleotides ~380 bp in front of the TSS (250 bp according to Monslow *et al.* (20)), i.e. in
the proximal candidate of the Has2 promoter. In contrast, the third other candidate STAT-REs failed in the in vitro screening. This in silico scanning result was confirmed by ChIP analysis of five RE-containing Has2 promoter regions. In non-stimulated HaCaT cells only region 1, spanning from positions −227 to −501 relative to the TSS, associated weakly with non-phosphorylated STAT3. However, after a 60-min treatment with EGF, STAT3 bound the promoter region more effectively. Most importantly, promoter region 1 was the only investigated region of the Has2 promoter that associated with activated pSTAT3 after EGF stimulation. This observation strongly suggests that the novel STAT-RE is functional and the mediator of EGFR activation on the Has2 promoter. Although the phosphorylation of STAT proteins by activated EGFRs seems to be the most straightforward EGF signaling pathway, it should not be ignored that EGFR initiates also other signaling cascades, such as those involving RAS and phospholipase Cγ (35). This can also lead to NF-κB activation. Because the novel STAT-RE is overlaid by a putative NF-κB binding site (20), it cannot be excluded that both STAT and NF-κB proteins act coordinately from this possible composite RE.

Our in silico screening for possible DR5-type RAREs resulted in four candidate REs, which, however, did not show any significant RAR-RXR heterodimer binding in vitro. This failure is because of the fact that the candidate REs are composed of non-perfect hexameric motifs, the in vitro gel shift conditions are very stringent, and comparably low protein amounts were used. However, in reporter gene assays the RARE cluster formed by RAREs 1 and 2 was able to mediate an all-trans-RA-dependent reporter gene induction that was comparable with that of the idealized DR5-type RARE.

We found all-trans-RA-induced association of the receptor to all five investigated RE-containing Has2 promoter regions, i.e. even to regions that do not carry any obvious RARE candidate. This surprising result was confirmed by using a different RARγ-specific antibody (sc-550 from Santa Cruz, data not shown). However, based on current models of retinoid signaling (30) RARs are only functional in complex with RXR. Therefore, it is important that only promoter regions 2 and 3 showed association with RXR, which leave only these two regions as sites of possible functional retinoid signaling through the Has2 promoter. The direct comparison of both regions concerning the association with other proteins of importance in retinoid signaling demonstrated that only the RARE cluster containing region 2 showed a significant, all-trans-RA-induced association with MED protein and pPol II, i.e. activated Pol II. Moreover, only at promoter region 2 could a retinoid-induced opening of chromatin be observed. Taken together, this evidence suggests that promoter region 2, spanning from positions −878 to −1264 of the human Has2 promoter, is the effective region for mediating the retinoid response of the gene. This fits with the presence of the RARE cluster composed of three hexameric nuclear receptor binding motifs (RAREs 1 and 2, Fig. 3A) within this promoter region, located −1220 bp in front of the TSS (970 bp according to Monslov et al. (20)).

Although the RARE cluster seems not to be perfect and did not show under stringent gel shift conditions any significant in vitro binding of retinoid receptors, its functionality in reporter gene assays makes it very likely that the retinoid response of the human Has2 gene is mediated via a larger protein complex containing RARγ, RXRa, CBP, MED proteins, and activated Pol II associated with this RARE cluster. Similar clusters form prominent REs of other nuclear receptor target genes, such as the CYP2B6 (36) or the osteocalcin (37) genes.

In REK cells, treatment with the EGFR kinase inhibitor AG1478 did not result in any significant effect on the short term induction of Has2 mRNA levels, but in HaCaT cells it caused a more than 5-fold reduction of the basal expression of the gene. This suggests that EGFR ligands that were either produced by HaCaT cells themselves or components of the FBS caused some basal activation of the Has2 gene, which was then blocked by the inhibitor. Our real-time PCR data suggested that the basal activity of the rat Has2 gene is significantly higher than that of the human Has2 gene, a finding in line with the low conservation between human and rat concerning transcription factor binding sites within the first 10 kb of the Has2 promoter. The lower basal activity of the human Has2 gene could be responsible for its higher inducibility by EGF and all-trans-RA. Taken together, although the rat and the human Has2 gene are both inducible by EGF and all-trans-RA, they seem to differ significantly in the details of their regulation.

The analysis of the all-trans-RA-dependent occupation of five RE-containing regions of the Has2 promoter with several nuclear proteins indicated that region 2 was not the only site that responded to retinoids. We found that all-trans-RA induced the recruitment of STAT3 and pSTAT3 to their binding sites in region 1 (Fig. 4A), although this region does not contain any obvious RARE (Fig. 3A). In addition, the ChIP assay results showed that STAT and/or pSTAT associated also in a RA-dependent fashion with regions 2, 3, 5, and 6, which do not carry any functional STAT-REs. All-trans-RA is a highly specific ligand to retinoid receptors, so that RARs have to be the core of these effects. Because the formaldehyde cross-linking in the ChIP assay is not only fixing proteins to DNA, but also proteins to other proteins, it is tempting to speculate that one (or several) large protein complex is formed on the Has2 promoter. This protein complex may have Pol II or pPol II as its core, linked to transcription factors, such as STAT3 and RAR, coactivator, and MED proteins. Moreover, this complex may also contain enzymes with chromatin modulating activity, such as CBP and others. The chromatin-complexed promoter DNA may loop around this protein complex and may be fixed at different positions. Such DNA fixation points are classically considered as transcription factor REs. In this way RA-activated RARs may influence other proteins in the complex without the need of directly contacting DNA or the respective protein partner. CBP could be such a linking partner between RARs and STAT proteins, because it can interact with both of them (38). It is obvious that more experiments are necessary to support this model of promoter activation.

In conclusion, we found that both the rat and human Has2 genes are primary EGF and RA responding genes, but that there are species-specific differences in the details of their regulation. The human Has2 gene contains a functional STAT-RE in its proximal promoter and a RARE cluster −1.2 kb upstream of the TSS mediates the retinoid response of the gene.

REFERENCES
1. Tammi, M. I., Day, A. J., and Turley, E. A. (2002) J. Biol. Chem. 277, 4581–4584
2. Weigel, P. H., Hascall, V. C., and Tammi, M. (1997) J. Biol. Chem. 272, 13997–14000
3. Tammi, R., Rimellino, J. A., Margolis, R. U., Maibach, H. I., and Tammi, M. (1989) J. Investig. Dermatol. 92, 326–332
4. Pienimäki, J. P., Rilla, K., Fulp, C., Sironen, R. K., Karvinen, S., Pasonen, S., Lamm, M. J., Tammi, R., Hascall, V. C., and Tammi, M. I. (1998) J. Biol. Chem. 273, 20428–20435
5. Pasonen-Steppan, S., Karvinen, S., Torroinen, K., Hyttinen, J. M., Jokela, T., Lamm, M. J., Tammi, M. I., and Tammi, R. (2003) J. Investig. Dermatol. 120, 1038–1044
6. Rilla, K., Lamm, M. J., Sironen, R., Torroinen, K., Lounkkonen, M., Hascall, V. C., Midura, R. J., Hyttinen, M., Pelkonen, J., Tammi, M., and Tammi, R. (2002) J. Cell Sci. 115, 3633–3643
7. Piepke, M., Pfitteko, M. R., and Cook, P. W. (1998) J. Investig. Dermatol. 111, 715–721
8. Holbro, T., and Hynes, N. E. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 195–217
9. Grandis, J. R., Drenning, S. D., Chakraborty, A., Zhou, M. Y., Zeng, Q., Pitt, A. S.,...
