Hyaluronan is an abundant and rapidly turned over matrix molecule between the vital cell layers of the epidermis. In this study, epidermal growth factor (EGF) induced a coat of hyaluronan and a 3-5-fold increase in its rate of synthesis in a rat epidermal keratinocyte cell line that has retained its ability for differentiation. EGF also increased hyaluronan in perinuclear vesicles, suggesting concurrent enhancement in its endocytosis. Cell-associated hyaluronan was most abundant in elongated cells that were stimulated to migrate by EGF, as determined in vitro in a wound healing assay. Large fluctuations in the pool size of UDP-N-acetylglucosamine, the metabolic precursor of hyaluronan, correlated with medium glucose concentrations but not with EGF. Reverse transcriptase-polymerase chain reaction (RT-PCR) showed no increase in hyaluronan synthases 1 and 3 (Has1 and Has3), whereas Has2 mRNA increased 2-3-fold in less than 2 h following the introduction of EGF, as estimated by quantitative RT-PCR with a truncated Has2 mRNA internal standard. The average level of Has2 mRNA increased from ~6 copies/cell in cultures before change of fresh medium, up to ~54 copies/cell after 6 h in EGF-containing medium. A control medium with 10% serum caused a maximum level of ~21 copies/cell at 6 h. The change in the Has2 mRNA levels and the stimulation of hyaluronan synthesis followed a similar temporal pattern, reaching a maximum level at 6 h and declining toward 24 h, a finding in line with a predominantly Has2-dependent hyaluronan synthesis and its transcriptional regulation.

Hyaluronan is a large glycosaminoglycan found in the extracellular space of most animal tissues. It forms a loose, highly hydrated, gel-like matrix that contributes to the maintenance of the extracellular space and facilitates nutrient diffusion. Furthermore, hyaluronan is involved in cell proliferation and differentiation, produces an environment favorable for migration (1), and stimulates cell locomotion (2, 3). Elevated tissue levels of hyaluronan occur during embryonic growth of tissues and organs (1), wound healing (4, 5), inflammation (6), and invasion of certain cancers (7-10).

In skin epidermis, the narrow extracellular space surrounding keratinocytes contains a high concentration of hyaluronan (11, 12), as do other stratifying squamous epithelia (13, 14). The half-life of labeled epidermal hyaluronan in human skin organ culture is ~1 day (15), indicating fast local turnover by keratinocytes. The importance of the strikingly high concentration and turnover of hyaluronan in the multilayered squamous epithelia is not completely understood, but we have hypothesized that the former is necessary to maintain an extracellular space for the nutritional needs of the more superficial cell layers, whereas the latter allows the dramatic modulation of cell shape that occurs during differentiation and for the high migratory potential of keratinocytes that is activated, e.g. in wound healing (16).

Unlike other glycosaminoglycans, hyaluronan is synthesized at the inner surface of the plasma membrane by hyaluronan synthase (Has) and is extruded through the plasma membrane into the extracellular space simultaneously with the ongoing synthesis (for review, see Ref. 17). Currently, three different Has genes have been identified in mammalian cells: Has1 (18, 19), Has2 (20-22), and Has3 (23). The three Has genes are highly homologous but appear to differ from each other in kinetic properties and product size (24, 25). Limited data are available on the factors that regulate the expression level and enzymatic activity of the different Has enzymes in various cells and tissues (20, 26-29), but a number of studies have suggested that the overall synthesis rate of hyaluronan is stimulated by some growth factors and cytokines (30-38).

Epidermal growth factor (EGF) is one of the most powerful agents that influences the behavior of keratinocytes. EGF transmits its information through the EGF receptor (EGF-R), which belongs to the erbB receptor tyrosine kinase family (39, 40). Signaling through EGF-R regulates many cellular processes, including cell adhesion, expression of matrix-degrading proteinases, and cell locomotion; these phenomena are all important in skin wound healing. Keratinocyte EGF-R expression is transiently elevated 5-7-fold within 2 days after wounding and returns nearly to baseline within 4 days (41). It is also important for keratinocyte proliferation and migration during reepithelialization (for review, see Ref. 42). Exogenously added EGF and overexpression of EGF-R result in enhanced ligand-mediated migration of keratinocytes and faster reepithelialization (43).

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The abbreviations used are: Has, hyaluronan synthase; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; REK, rat epidermal keratinocyte; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bHABC, biotinylated hyaluronan binding complex; DAB, 3,3'-diaminobenzidine.
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Aberrant expression or activation of the EGF-R is common and has been proposed to have a role in epithelial tumor progression (44, 45). As in wound healing, the EGF-R may provide an important contribution to the migratory and invasive potential of carcinomas. The migration induced by EGF requires the actin binding domain of EGF-R (46), and recent studies have shown an important role of matrix metalloproteinases (MMPs) in keratinocyte migration as well as in their ability to invade other tissues (47, 48).

Whereas both increased hyaluronan and EGF signaling have been observed in migrating cells and in wound healing, neither activation of Has nor production of hyaluronan in response to EGF has been investigated in keratinocytes. In this study, we establish that EGF specifically increases the mRNA level of Has2, resulting in enhanced synthesis of hyaluronan that correlates with enhanced keratinocyte migration in a wound assay. Interestingly, a large proportion of the newly synthesized hyaluronan of EGF-treated cells resides in intracellular vesicle-like structures, suggesting that a significant proportion of Has2-directed hyaluronan is endocytosed immediately and recycled back into the cell.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A newborn rat epidermal keratinocyte (REK) cell line was developed by MacCallum and Lillie (49) from neonatal rat epidermal cells originally isolated by Baden and Kubilus (50). REKs were cultured in Dulbecco's modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (HyClone, Logan, UT), streptomycin (50 μg/ml), penicillin (50 units/ml) and 1–2 mM l-glutamine (all from PAA Laboratories, Linz, Austria) at 37 °C in a humidified atmosphere containing 5% CO2. Cells were trypsinized when they reached confluency using 0.02% EDTA (w/v), 0.025% trypsin (w/v) (Sigma). For biochemical assays and radiolabeling, the cells were grown close to confluency in 6-well plates and incubated in the presence of Has2-directed hyaluronan is endocytosed immediately and recycled back into the cell.

**Chemical Quantitation of Hyaluronan with Double Labeling**—For radiolabeled hyaluronan, Healon®, Amersham Pharmacia Biotech) was added to each sample to improve the recovery of radiolabeled hyaluronan during the purification procedures and gel filtration. Each cell fraction was suspened in 500 μl of 50 mM sodium acetate containing 5 mM EDTA and 5 mM cysteine, pH 6. Cysteine and EDTA were added to each of the medium and trypsinate fractions (5 mM final concentrations). Each sample was treated with papain (Sigma) (200 μg/ml final concentration) at 60 °C for 1.5 h. Papain was inactivated in a boiling water bath (10 min). After cooling, cetylpyridinium chloride (1% in water, 1.2 ml) was added to each sample followed by incubation at room temperature for 10 min. After centrifugation at 13,000 × g for 15 min, each supernatant was carefully removed by aspiration and discarded. Samples were washed with 1 ml of water, centrifuged, and the supernatants were discarded as above. Each cetylpyridinium chloride precipitate was dissolved in 50 μl of 4 M guanidine HCl, and 900 μl of absolute ethanol was added. Samples were kept at −20 °C for 30 min and then centrifuged. Supernatants were discarded as above, and pellets were dissolved in 30 μl of water.

**Isolation of Secreted and Cell-associated Glycosaminoglycans**—Cells were grown in 6-well plates (9.6 cm2/well) in 1 ml of medium and subsequently washed with 400 μl of Hank's solution (HyClone). For each culture, the medium and wash were combined and designated as “medium.” Each cell layer was trypsinized, the resulting suspension removed, and the well was washed with 250 μl of medium essential medium. Each cell suspension, combined with the washes was centrifuged. The resulting supernatant and two subsequent 250-μl washes of the cell pellet with serum-free medium were combined and designated as “trypsinate.” The resulting cell pellet was designated as the “intracellular” fraction.

**Purification of Radiolabeled Hyaluronan—**Carrier (6 μg of hyaluronan, Healon®, Amersham Pharmacia Biotech) was added to each sample to improve the recovery of radiolabeled hyaluronan during the purification procedures and gel filtration. Each cell fraction was suspened in 500 μl of 50 mM sodium acetate containing 5 mM EDTA and 5 mM cysteine, pH 6. Cysteine and EDTA were added to each of the medium and trypsinate fractions (5 mM final concentrations). Each sample was treated with papain (Sigma) (200 μg/ml final concentration) at 60 °C for 1.5 h. Papain was inactivated in a boiling water bath (10 min). After cooling, cetylpyridinium chloride (1% in water, 1.2 ml) was added to each sample followed by incubation at room temperature for 10 min. After centrifugation at 13,000 × g for 15 min, each supernatant was carefully removed by aspiration and discarded. Samples were washed with 1 ml of water, centrifuged, and the supernatants were discarded as above. Each cetylpyridinium chloride precipitate was dissolved in 50 μl of 4 M guanidine HCl, and 900 μl of absolute ethanol was added. Samples were kept at −20 °C for 30 min and then centrifuged. Supernatants were discarded as above, and pellets were dissolved in 30 μl of water.

**RNA Isolation and Northern Blot**—Keratinocytes were cultured in ~28 cm2 dishes until confluence and scraped into TRIzol®-reagent (Life
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**FIG. 3.** The specific activity of GaINac in chondroitin sulfate synthesized by confluent keratinocyte cultures. a, the specific activities were measured every 6 h, each after a 6-h labeling period (horizontal bars). Culture medium was changed at 0 h. The 0-h control represents the specific activity in control cultures before the change of medium, 2 days after the previous change. The specific activities were calculated from the double-label data as described under “Experimental Procedures.” b, time course of the glucose concentration in culture medium during an experiment similar to that in a. The shaded area shows the level of glucose in the medium before addition to the cultures (range of 3 assays). All cultures were treated with or without EGF (20 ng/ml), and the vertical bars show the range of duplicate cultures.

Technologies, Inc.) for total RNA isolation according to the instructions of the manufacturer. RNA was dissolved in a small amount of distilled H2O and quantitated with a spectrophotometer at 260 nm.

RNA was analyzed by electrophoresis on 1% formaldehyde/agarose gels and transferred onto Hybond-N\(^+\) nylon membranes (Amersham Pharmacia Biotech). A Has2-specific probe (1200 base pairs) was obtained from human mRNA by RT-PCR using the primers 5'-GAA CAGCCCCACGCAAAGAC-3' and 5'-CTCCCCCACCCTACCTCAACC-3' and labeled with [\(^{32}\)P]dCTP by PCR. Hybridization was done by following the ULTRAhyb\(^+\) hybridization protocol for DNA probes to RNA blots (Ambion, Austin, TX).

**RT-PCR with Has1, Has2, Has3, and GAPDH Primers—**

Keratinocyte RNA was isolated with the TRIzol\(^\text{\textregistered}\)-reagent (Life Technologies, Inc.) for total RNA isolation according to the instructions of the manufacturer. RNA was dissolved in a small amount of distilled H2O and quantitated with a spectrophotometer at 260 nm.

The RT-PCR reactions were done with the RNA PCR Core Kit (PerkinElmer Life Sciences, Branchburg, NJ). To obtain rat Has1- and Has3-specific primers, cDNA sequences were amplified from rat keratinocyte RNA with mouse Has1 and Has3 specific primers using RT-PCR. The PCR products were cloned into a pSport1 (Life Technologies, Inc.) plasmid and sequenced. Primers for Has1 and Has3 were 5'-GC TCTATGGGCGGTTCCTC-3' and 5'-CACACAATAAGTGGCAGGGTCC-3', 5'-ACTCTGATCAGTCTTCCTAC-3' and 5'-ACGACTCTTGCCGTC-3', respectively. Rat Has2- and GAPDH-specific primers (5'-TCCGAAACACATCTGGTGATG-3' and 5'-CCAGATGTAAGTGACT-GATTTGTCCTCC-3') and 5'-GACATCTGGTGCTGAGTATG-3' were designed from GenBank\(^\text{TM/EBI}\) sequences AF008291 and M17701, respectively. For quantitation of Has2 mRNA, a shortened (internal standard) Has2 cDNA containing the primer binding sites identical to those in the wild-type Has2 cDNA was prepared by PCR. A poly(T)\(_n\) sequence was tagged into this cDNA at its 3'-end through an appropriately designed Has2-specific downstream primer. Thus after in vitro transcription treatment, the shortened Has2 RNA strand contained a poly(A) tail. The shortened Has2 cDNA was prepared with FastTrack\(^\text{TM}\) mRNA isolation kit (Invitrogen BV, Leek, The Netherlands), dissolved in a small amount of H2O and quantitated with a spectrophotometer at 260 nm. RT-PCR was done with constant amounts of the wild type and different concentrations of the shortened Has2 RNAs. The resulting products were run on an agarose gel, digitized by a BioDocII\(^\text{TM}\) Video Documentation System (Biometa, Göttingen, Germany), and quantitated by Ethic fluorescence by using Image software (Wayne Rasband, NIH, Bethesda).

Assay of Keratinocyte Migration and Proliferation—

Keratinocytes were cultured until they just reached confluency. Two lines (70 mm wide) crossing each other at right angles were drawn with a 250-μl disposable pipette tip. The migration of keratinocytes to the cleared area was inspected under a microscope. The areas covered with cells were measured before and 18 h after the treatment. The change in the area was counted in pixels using the NIH Image software and converted to mean migration distance (μm) of the cell front.

To determine the proliferation rate, about 20,000 REKs were seeded in 400 μl of medium into the wells of a 24-well plate, and grown for 3 days. Then medium was changed and supplemented with 0–200 ng/ml EGF. For each EGF concentration, 2 wells were harvested by trypsin digestion after 24 and 48 h. The cells were then collected by centrifugation, resuspended, and counted in a hemocytometer.

**Microscopic Detection of Hyaluronan—**

Fixation, incubation with bHABC, and the DAB reaction were done as above. The cells were then dehydrated in graded ethanol and embedded in Spurr's resin. Semi-thin sections were cut perpendicular to the cell layer and stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed with a Jeol EX1200 electron microscope.

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Fermentas, Vilnius, Lithuania), and a rat Has2 full-length cDNA (4172 base pairs, GenBank™/EBI AF008201) was ligated into the multiple cloning site of pCl-neo. After transformation, the plasmid sequences were confirmed, and the REK cells were transfected with Has2 anti-sense plasmids according to the manufacturer’s instructions with FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals). Transfected cells were cultured in the presence of 500 μg/ml of G418 (Calbiochem-Novabiochem Corp., La Jolla, CA) until separate colonies about 0.5 cm in diameter were found. The colonies were reseeded and grown in the presence of 250 μg/ml G418 except during the experiments. The presence of the Has2 antisense construct was verified with Southern blotting.

RESULTS

Induction of Hyaluronan Secretion in Epidermal Keratinocytes by EGF—Confluent monolayer rat keratinocyte cultures were labeled with [3H]glucosamine and [35S]sulfate for 6 h. The amounts of newly synthesized [3H]hyaluronan, [3H,35S]chondroitin sulfate, and chondroitinase-resistant glycosaminoglycans (mainly heparan sulfate) in medium were determined using the double label method described under “Experimental Procedures.” The total amount of newly synthesized hyaluronan and that associated with either the trypsinate or the intracellular compartment are shown. The 3-h labeling periods are indicated by horizontal bars. The vertical bars show the range of duplicate cultures.

Induction of Hyaluronan Secretion in Epidermal Keratinocytes by EGF—Confluent monolayer rat keratinocyte cultures were labeled with [3H]glucosamine and [35S]sulfate for 6 h. The amounts of newly synthesized [3H]hyaluronan, [3H,35S]chondroitin sulfate, and chondroitinase-resistant glycosaminoglycans (mainly heparan sulfate) in medium were determined using the double label method described under “Experimental Procedures.” Hyaluronan synthesis rate was highest when keratinocytes were cultured in medium containing 20 ng/ml EGF (Fig. 1). When the concentration of EGF was further increased, the stimulation of hyaluronan synthesis decreased somewhat but remained ~2-fold higher than the basal level. The synthesis of other glycosaminoglycans (heparan sulfate and chondroitin sulfate) was not altered appreciably by EGF treatment. Subsequent experiments used the optimal concentration of 20 ng/ml EGF.

Because serum also stimulates hyaluronan synthesis, we examined the interactive effects of EGF and serum. In the control cultures, 6 h after the medium change, 10% serum increased the rate of hyaluronan synthesis by ~70% when compared with cells cultured with 0.5% serum (Fig. 2). In EGF-treated cultures, the presence of 10% serum showed an approximately additive increase in hyaluronan synthesis (compare the Δ increases). This suggested that factors in serum, such as IGF-1 and PDGF that might contribute to increased synthesis, are additive with EGF and that stimulation by EGF is obvious in all serum concentrations. Because the vitality of cells may suffer in extended cultures with low serum concentration, we decided to use 10% serum in later experiments.

UDP-[3H]N-acetylhexosamine Specific Activity with Time After Replacing the Medium—Previous studies have shown that it is important to correct for precursor dilution by endogenous substrates, as indicated by the marked changes in specific activity of [3H]glucosamine in newly synthesized glycosaminoglycans (Fig. 3a), to correctly interpret [3H] incorporation with
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Time Course of EGF-induced Hyaluronan Synthesis—To monitor the rate of hyaluronan synthesis at different times between 0 and 24 h following introduction of EGF, we used 3-h labeling windows by adding small aliquots of the radiolabeled precursors into the medium at the different times indicated in Fig. 4. In confluent keratinocyte cultures the medium change alone caused a 2–3-fold increase of total hyaluronan synthesis by 3–9 h (Fig. 4a). Nevertheless, EGF-treatment showed an additional ∼3–6-fold increase of newly synthesized total hyaluronan above the control in the 3–9-h labeling windows (Fig. 4a). The stimulatory effect of EGF decreased thereafter, but the total synthesis at 21–24 h was still more than twice that in the control cultures (Fig. 4a).

Even if most of the hyaluronan synthesized in extended labeling periods were found in the culture medium, a significant fraction (∼50% at max, 3–6 h) remained associated with the cell layer in the 3-h labeling windows, either with the trypsinate (Fig. 4b) or the intracellular (Fig. 4c) fractions. The newly synthesized hyaluronan in these fractions was substantially increased by EGF with similar kinetics, peaking in the 3–9-h labeling windows. The amount of newly synthesized trypsinate hyaluronan increased 2–3-fold in maximum during the 3–9-h labeling windows (Fig. 4b). The highest (6–10-fold) increase by EGF was detected in the newly synthesized intracellular hyaluronan pool (Fig. 4c). The trypsinate hyaluronan represents molecules bound to the receptors, mainly CD44, whereas the intracellular hyaluronan probably represents endocytosed material destined for lysosomal degradation and perhaps those under synthesis and still bound to the hyaluronan synthase (52).

Microscopic Assay of Total Cell-associated and Intracellular Hyaluronan—The EGF-induced increase of hyaluronan associated with the cell layer was also shown by staining the keratinocyte cultures with the hyaluronan-specific probe (bHABC) (Fig. 5, a and b). This visual impression was confirmed by determination of the total optical densities as described under “Experimental Procedures," resulting in higher values for EGF-treated cultures at all the time points (4–20 h) studied (data not shown). The proportion of intracellular hyaluronan was specifically measured in cultures fixed and digested with Streptomyces hyaluronidase to remove cell surface hyaluronan, then permeabilized and stained with bHABC. The assay of

this precursor (53, 54). The metabolic activity of the cells gradually depletes essential nutrients in the growth medium, such as glucose (Fig. 3b) and glutamine that are utilized in the intracellular synthesis of glucosamine. As glucose was depleted, the proportion of the exogenous radiolabeled [3H]glucosamine that contributes to the intracellular pool, i.e. its specific activity, increased. This was particularly pronounced after 12 h (Fig. 3a). However, whereas the specific activity of the hexosamines (UDP-GlcNAc and UDP-GalNAc are in an equi-
Fig. 9. Hyaluronan synthase mRNA expression in EGF-treated cultures. a, total RNA isolated from equal numbers of keratinocytes treated with EGF for 3 h (E) and controls (C) were reverse transcribed and amplified with 35 PCR cycles for the different Has types and GAPDH, an internal control. b, Northern blot analysis of Has2 mRNA at the indicated time points in control (C) and EGF-treated cultures (E). The two Has2 transcripts and GAPDH as a loading control are indicated. c, an example of the standard curves used for the determination of the Has2 mRNA copy numbers. The points indicate band fluorescence intensity ratios of native Has2 mRNA (nt) and the truncated internal standard Has2 cRNA (ts). d, assay of Has2 mRNA at different time points following change into EGF containing (unfilled circles) and control medium (filled circles), utilizing the above standardization. The average RNA copy numbers per cell were calculated without an attempt to correct for the recovery in RNA isolation.

O.D. values for these stainings showed consistently higher intracellular hyaluronan values already by 1 h after the addition of EGF (Fig. 6).

Morphological Changes of Keratinocytes—The keratinocytes were quite flattened in sub confluent cultures and covered a large area of the substratum (Fig. 5a). Shortly after introduction of EGF, keratinocytes began to round up and form membrane ruffles and microspikes (Fig. 5, b–e), which was followed by cell elongation and the appearance of lamellipodia (Fig. 5, c and e). These changes in morphology were apparent in a few of the cells even after 1 h, and most cells showed the altered morphology after a 60-h EGF treatment.

Localization of Cell Surface Hyaluronan in EGF-treated Keratinocytes—The distribution of hyaluronan in control cultures was similar to that described previously (52), with most of the hyaluronan residing in plasma membrane patches (Figs. 5a and 7c). In EGF-treated cells, the hyaluronan signal intensity was generally increased (Figs. 5, b, c, e and 7d). Hyaluronan covered the membrane ruffles and microspikes in cells undergoing rounding (Fig. 5, b–e). In elongating cells the midportion (around the nucleus) and the trailing edge showed an intense hyaluronan signal (Fig. 5, c and e). The lamellipodia, instead, appeared almost negative or showed only localized spots (Fig. 5, c and e, arrows). The amount of hyaluronan that accumulated in response to EGF treatment was sufficient to exclude sedimenting red blood corpuscles on keratinocyte surfaces, a frequently used test of hyaluronan coat formation and hyaluronan synthesis (Fig. 7, a and b). More hyaluronan was also found on the underside of the EGF-treated cells than in control cells as seen in Fig. 7, c and d (arrows), and in confocal images (not shown). This was particularly evident in the rounded, apparently migrating cells. Electron microscopy of ultrathin vertical sections suggested that in sites where hyaluronan was deposited under the cell, the distance between plasma membrane and the substratum increased (Fig. 7e).

EGF-induced Changes in Intracellular Hyaluronan—Intracellular hyaluronan was specifically detected by removing cell surface hyaluronan with Streptomyces hyaluronidase (Fig. 5f). The intracellular localization was also confirmed by confocal analysis (not shown) and in semi-thin vertical sections (Fig. 7, c and d, arrowheads). Hyaluronan signal was present in cytoplasmic structures conforming to vesicles of various sizes and appeared to line their membrane (Figs. 5, c and f, short arrows and 7, c and d, arrowheads). No nuclear hyaluronan signal was found. The accumulation of intracellular hyaluronan in response to EGF was most conspicuous in rounding cells, whereas cells retaining a more flattened morphology contained less.

Stimulation of Migration but Not Proliferation by EGF Treatment—The cells became elongated in EGF-treated cultures (Fig. 5), a common finding in cells with enhanced mobility. A stimulation in the migration of the keratinocytes by EGF was confirmed by artificial wounding of the cell layer and quantitation of the speed at which the cells migrated into the cleared area (Fig. 8). The migratory activity peaked at the same EGF concentration as for the synthesis of hyaluronan (compare Figs. 1 and 8a), and corresponded to that reported earlier (55).

The cell number almost doubled by 24 h, independent of the presence of EGF (2–200 ng/ml) (Fig. 8b). Further increases at 48 h were also independent of EGF (2–20 ng/ml) with some inhibition at the 200 ng/ml level. Thus, EGF had no significant effect on the proliferation rate of the keratinocytes in conditions that showed the highest stimulation in hyaluronan synthesis and migration.

EGF-induced Increase in Hyaluronan Synthase 2 mRNA—To reveal changes in the expression of the different hyaluronan synthases responsible for the enhanced hyaluronan synthesis by EGF, we estimated the mRNA levels of rat Has1, Has2, and Has3 using RT-PCR. This comparative analysis suggested that Has1 and Has3 mRNA levels were not markedly changed by EGF treatment, nor contributed to the hyaluronan synthesis stimulation (Fig. 9a). In contrast, Has2 level was increased by EGF (Fig. 9a). However, the basal level of Has2 mRNA in the keratinocytes was so low that the level of increase in its two transcripts was difficult to estimate by Northern blot (Fig. 9b). To approximate the increase in Has2 mRNA level in keratinocytes, we used quantitative RT-PCR with an internal, truncated cRNA standard, and compared the sample band densities with a set of standards as shown in Fig. 9c. These analyses for control keratinocyte cultures indicated a low copy number before the change to fresh medium (~6/cell), a detectable increase of Has2 mRNA even after 1 h, a peak at 6 h (~54/cell), and a decrease toward basal level by 24 h (Fig.
correlation between migration and keratinocytes as described under “Experimental Procedures.” The migration (a) and the quantities of newly synthesized hyaluronan on cell surface (trypsinate) and growth medium (b) were determined in control cells containing an empty transfection vector (C) and the antisense gene (AS). The bars in a show the standard error of 8 wounding assays in separate dishes, whereas those in b show the range of duplicate dishes.

Whereas the level of HAS2 mRNA also increased in control cultures following change into fresh medium, the number of HAS2 mRNA copies was 1.5–8 times higher in the EGF-treated cultures at all time points examined (Fig. 9). The Has2 antisense cells showed a clearly reduced migration compared with its mock-transfected controls (Fig. 10a), indicating that Has2 has an important role in the migration process.

**DISCUSSION**

Has2 mRNA Levels—Hyaluronan synthase mRNAs are presumed to occur in low numbers but, as far as we know, there is no published data on its copy numbers per cell, estimated with internal RNA standards. Whereas the actual amounts, not corrected for recovery in RNA isolation, may be slightly higher than those in Fig. 9, the data demonstrate a 10-fold increase of HAS2 mRNA level and the maximum of at least ~54 copies/cell. This increase in the mRNA corresponded to about 30-fold enhancement of hyaluronan production from the basal synthesis rate. The changes in mRNA and hyaluronan synthesis levels also showed a temporal correlation, strongly suggesting a tight transcriptional regulation of hyaluronan synthesis. According to our unpublished data, the peak level of HAS2 mRNA in the cumulus oophorus cells during the preovulatory hyaluronan synthesis reaches ~400 copies per cell, about eight times that in EGF-treated keratinocytes. However, the corresponding hyaluronan synthesis rates in the EGF-treated keratinocytes (~0.2 pg/cell/12 h) and cumulus cells (~3 pg/cell/12 h) (56), show a similar ratio between the peak HAS2 mRNA levels and hyaluronan production.

**Regulation of Different Has Genes**—Whereas it is obvious that the synthesis rate of hyaluronan in various cell types is controlled by cytokines and growth factors, including EGF (30, 34), the contribution of the three known Has genes in this regulation remains uncertain. Like in keratinocytes, the hyaluronan synthesis of preovulatory ovarian follicle is up-regulated by Has2 mRNA levels (20) whereas Has1 and Has3 are not affected. Keratinocytes were reported to up-regulate Has1 mRNA as a response to TGFβ, but its functional importance, or the synthesis of hyaluronan were not studied (57). Among the cytokines and growth factors TGFβ seems exceptional because Has2 is the main target when hyaluronan synthesis is up-regulated by TNFα and IFNγ in renal tubular epithelial cells (28), by IL-1 in orbital fibroblasts (27), by FGF (58) or wound- (59) in mesothelial cells, and by osteogenic protein 1 in chondrocytes (29).

**Intracellular Hyaluronan in EGF-treated Keratinocytes**—The rapid and marked intracellular accumulation of endogenous hyaluronan in perinuclear vesicles by EGF treatment was quite striking and unexpected, although it is known that intracellular hyaluronan synthesis is enhanced upon stimulation of quiescent fibroblasts (60). At least a part of the intracellular hyaluronan resulted from enhanced receptor-mediated uptake because our unpublished data show that the EGF-enhanced intracellular hyaluronan synthesis is reduced by incubation with hyaluronan deacetylases, known to displace the CD44-bound hyaluronan in the keratinocytes (52). The very short half-life of hyaluronan in the epidermis of human skin organ cultures (15) and in organotypic keratinocyte cultures of the present cells (53) also suggests that hyaluronan was catabolized shortly after synthesis. This rapid catabolism has not been demonstrated previously in cell cultures. Whereas the biological importance of the hyaluronan turnover in keratinocytes is not currently known, it correlates with the enhanced motility of the cells as seen in the wound healing assay and is likely involved in somatosensory process (61).

**Biological Implications**—The data in this study establish that hyaluronan, a major extracellular matrix molecule in stratified epithelia such as dermis, is specifically increased in keratinocytes by EGF and therefore likely contributes to such biological consequences of EGF as stimulated keratinocyte migration after wound healing. Whereas hyaluronan synthesis rates higher than that induced here by EGF have been reported in other cells, it is obvious that the Has2 regulation described here could induce rapid and dramatic changes in the cellular environment of epidermal keratinocytes, considering the small extracellular space where hyaluronan accumulates. Indeed, transfection of Has2 antisense cDNA into the present keratinocytes demonstrates that hyaluronan synthesis is one of the factors that control the migration rate of the cells.

The ability of epidermal keratinocytes to rapidly cover an open wound is a biologically crucial motility response of those cells. Healing of skin wounds involves transient up-regulation of EGF receptors (41) and is aided by EGF-like growth factors (43). Hyaluronan is abundant in the frontline keratinocytes migrating into a wound (5). Furthermore, hyaluronan synthesis and Has2 expression are elevated at the edges of an in vitro wound in mesothelial cell cultures (59). Mice with targeted inhibition of the epidermal hyaluronan receptor CD44 (62) show delayed wound healing. Taken together, these studies indicate that stimulated EGF receptor signaling and enhanced hyaluronan metabolism are intimately connected with each other and with the epithelial wound healing process.

Acknowledgments—We thank Alpo Pelttari for the facilities of the Dept. of Electron Microscopy. Expert technical help by Arja Venālainen, Riikka Tiikonen, and Päivi Perttula is gratefully acknowledged.

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2 C. Fulop, unpublished data.

![Fig. 10. Effect of reduced Has2 expression on keratinocyte migration.](image-url)

The figure shows the effect of reduced Has2 expression on keratinocyte migration in EGF-treated cultures. The bars in a show the standard error of 8 wounding assays in separate dishes, whereas those in b show the range of duplicate dishes.

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