Malignant transformation of fibroblasts and epithelial cells is often accompanied by increased hyaluronan production and accumulation. Despite recent progress in the study of hyaluronan biosynthesis, the mechanisms underlying the transformation-induced overproduction of hyaluronan have not been elucidated. Here we report that activity and transcriptional levels of hyaluronan synthase (HAS) significantly increased after oncogenic malignant transformation of a rat 3Y1 fibroblast cell line. Of three HAS isoforms (HAS1, HAS2, and HAS3), only HAS2 gene expression was increased in the v-Ha-ras transformed 3Y1 cells, which show less malignancy. In contrast, both HAS1 and HAS2 expressions were elevated in the highly malignant cells transformed with v-src and/or v-fos. To assess the contribution of HAS expression to the oncogenic malignant transformation, we established stable cell transfectants expressing sense and antisense HAS genes. Antisense suppression of the HAS2 expression significantly decreased hyaluronan production in the cells transformed by the oncogenic v-Ha-ras and eventually led to a reduction in tumorigenicity in the rat peritoneum. The introduction of the HAS1 and HAS2 genes promoted the growth of subcutaneous tumors in a manner dependent on the levels of hyaluronan synthesis. Significant growth promotion was observed within a wide range of HAS1 expression. In contrast, the growth stimulation was only seen within a narrow range of HAS2 expression, and high levels of HAS2 expression even inhibited tumor growth. These results suggest that proper regulation of the expression of each HAS isoform is required for optimal malignant transformation and tumor growth.

Increased synthesis of hyaluronan (HA) is often associated with malignant progression in certain types of human tumors, including colon cancer, lung cancer, breast cancer, mesotheliomas, and gliomas, and the levels of HA in sera of some cancer patients are significantly elevated over those of normal individuals (1–3). Although increased HA synthesis is not a universal characteristic of tumors, there seems to be a general tendency for transformed cells to exhibit higher levels of HA production. For instance, infections with oncogenic viruses cause an enormous increase in the rate of HA synthesis as well as an abnormal acceleration of cell growth (4–7). Alterations in the HA synthesis may thus take part in the sequential events leading to cell proliferation and oncogenic transformation. Previous studies have suggested that the increased deposition of the HA-rich matrix provides a suitable environment for cell proliferation and migration during tumor development (8–11). Furthermore, tumor-associated HA is correlated with metastatic abilities of mouse mammary carcinoma and B16 melanoma cell lines (12–14).

HA is a linear polysaccharide composed of the repeating disaccharide N-acetyl-D-glucosamine-β(1→4)-D-glucuronic acid-β(1→3). This polysaccharide is not simply a structural component of the extracellular matrix but also regulates a variety of cell properties such as cell adhesion, motility, growth, and differentiation by acting on intracellular signaling pathways through interaction with cell surface receptors (15–17). The biosynthesis of HA, which is critical in establishing its biological form, is multiply regulated by three mammalian HA synthases; HAS1, HAS2, and HAS3 (18–20). Previous studies have suggested that the three HAS isoforms differ from each other in the expression profiles during embryonic development and in the enzymatic characteristics (21, 22). Furthermore, the respective HAS transfectants clearly differ in their ability to form the HA matrix and in the molecular masses of the HA produced by them. Because HA has a wide variety of biological and physiological roles, some of which are size-dependent, the existence of three different HAS isoforms implies that HA functions are diversely regulated through control of the activities.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AB097568 and AB097569.

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and expressions of the HAS isoforms. It is therefore plausible that the HAS isoforms are involved in different stages of malignant tumor progression. Molecular cloning of the HAS genes enabled us to control HA biosynthesis and investigate HA functions in malignant transformation (23–30). Overexpression of HAS2 enhanced both the anchorage-independent growth and tumorigenicity of a human fibrosarcoma cell line (24). Similarly, HAS3 overexpression promoted the growth of TSU prostate cancer cells (26). Moreover, the expression of HAS1 restored the metastatic potential of mouse mammary carcinoma tumors that had low levels of HA synthesis and metastatic ability (23). Our recent study demonstrated that an abnormal accumulation of HA matrix induced by the overexpression of HAS genes diminished contact inhibition of non-transformed cells (31). Although increasing evidence suggested the involvement of HA in the transformation, tumorigenesis, and malignant progression, the expression patterns and the respective roles of the three mammalian HAS isoforms during the oncogenic transformation had not been studied in the same cell systems.

The rat 3Y1 fibroblastic cell line has been utilized as a model system to study transforming activities of oncogenes (32–37). Transformed 3Y1 cells bearing adenosine A1 fragment or simian virus 40 show slow rate of tumorigenesis despite of the increases in both saturation cell density and anchorage-independent growth (33, 35). On the other hand, cell transformation driven by v-Ha-ras or v-src causes significantly high tumorigenesis or acquires metastatic potential (35–37). Here the transformed 3Y1 cell lines that have different degrees of transforming ability were examined the changes in HAS activity and transcriptional levels during the malignant transformation. Furthermore, we generated stable cell lines expressing sense or antisense HAS genes in ras-transformed 3Y1 cells to assess the contribution and role of the HAS expression in malignant transformation. We show here for the first time that oncogenic malignant transformation and subsequent tumor growth are crucially regulated by proper levels of HA concentration and HA synthesis and expression. This novel finding provides a new insight for understanding the physiological significance of HA production during tumor development. In addition, it would be an important knowledge about the molecular basis and regulatory mechanisms underlying oncogene-induced overproduction of HA.

EXPERIMENTAL PROCEDURES

Cell Lines—Rat 3Y1 fibroblastic cell lines transformed by various oncogenes, EIA-3Y1, SV-3Y1, HR-3Y1, and SR-3Y1, were obtained from Riken Bioresource Center (Ibaraki, Japan). HR-3Y1 and SR-3Y1 cells were previously established as cell lines bearing v-Ha-ras and v-src, respectively. v-fos SR-3Y1 cells bearing v-src and v-fos was prepared as described previously (36). S17N SR-3Y1 cells bearing a dominant negative form of Ras substituted for Ser at position 17 of v-Ha-Ras were also prepared as described previously (38). All cell lines were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum and 2 mM glutamine (growth medium).

Particle Exclusion Assays—Fixed erythrocytes were reconstituted in phosphate-buffered saline (PBS) to a density of 5 x 10⁶ cells/ml and used for the particle exclusion assay as described previously (22, 39). HA matrices were visualized by adding 1 M UDP-[¹⁴C]GlcA (Nakalai Tesque, Kyoto, Japan), and 2 µCi of UDP-[^14]C(GlcA. Reactions were terminated by adding SDS at 2% (w/v). The incorporation of radioactivity into high molecular mass HA was measured by descending paper chromatography using Whatman 3MM paper developed in 1 M ammonium acetate, pH 5.5, and ethanol (35, 65, v/v). The amounts of radioactivity at the origins were measured by liquid scintillation counting. The HAS activity was determined by calculating the amounts of GlcA incorporated using known specific radioactivities.

Quantitative Analyses of the HAS Transcripts—Relative levels of HAS expression in oncogenically transformed cells were determined by real-time quantitated RT-PCR as described previously (23). Primers were designed to detect messenger RNA among different samples was made using the values that were selected for standard curve obtained using serial dilutions of standard HA.

HAS Synthase Assays—HAS activity was monitored using UDP-[¹⁴]CGlca (325.9 mCi/mmol, ICN Biomedicals, Irvine, CA) as described previously (22, 39). Briefly, the crude membrane fractions isolated from subconfluent 3Y1 cells and oncogenic transformants were resuspended and incubated at 37°C for 1 h in 0.2 ml of 25 mM Hepes-NaOH, pH 7.1, 1.5 mM dithiothreitol, 7.1 mM MgCl₂, 1 mM NaCl, and 2 µM of UDP-[¹⁴]CGlca. Reactions were terminated by adding SDS at 2% (w/v).

The determination of the HA content in the conditioned medium was recovered. The conditioned medium was also recovered from S17N SR-3Y1 cells incubated with or without 2 µM dexamethasone (Sigma) for 2 days. A mixture of conditioned medium and the histidine-linked HA-binding region of aggrecan (b-HABP, Seikagaku Corp., Tokyo, Japan) was added to 96-well plates precoated with HA-bovine serum albumin and then incubated for 1 h at 37°C. Horseradish peroxidase-conjugated streptavidin was used as secondary probe, and the enzymatic activity was measured using o-phenylenediamine dihydrochloride (Sigma) as a substrate. The HA contents were calculated from a standard curve obtained using serial dilutions of standard HA.

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structs, and the G418-resistant colonies were picked and further enriched five times by fluorescence-activated cell sorting. The clones stably expressing EGFP at the different levels were selected and used for the experiments. HA matrix formation, HA accumulation in the conditioned medium, and the HAS activity were determined as described in this section.

Estimation of the Amounts of FLAG-tagged Recombinant HAS Proteins—Amounts of FLAG-tagged HAS fusion proteins in HAS transfectants were estimated by immunoblotting as described previously (22). In this study, whole proteins of transfectants were solubilized, separated on SDS-PAGE, and then transferred onto nitrocellulose membranes. Blotting was performed according to the manufacturer's instructions using anti-FLAG peptide antibody M5 (Sigma) followed by anti-mouse IgG antibody conjugated with peroxidase. The FLAG-tagged protein bands were quantified by densitometric scanning of the digitized image using NIH Image (version 1.57) software. A standard curve for each measurement was created by immunoblotting of known amounts of FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP, molecular mass of 49.4 kDa). The amounts of recombinant HAS protein were expressed in arbitrary units with band intensity equivalent to 1 ng of FLAG-BAP.

Soft Agar Growth Assay—Six-well plates were coated with 1 ml of 0.6% agarose in growth medium. Then, 2 × 10^5 cells from each transfected colony were suspended in 0.2 ml of growth medium, mixed with 3.8 ml of 0.3% agarose in growth medium (52 °C), and divided into two agarose-coated wells. After solidification of the agarose, 3 ml of growth medium was added to each well. The medium was exchanged every 3 days for 1 month, and the colonies were counted every week.

Tumor Formation—The HR-3Y1 cells stably expressing sense or antisense HAS were cultivated in the growth medium and harvested by trypsinization. After inactivation of trypsin by the addition of serum, the cells were washed with PBS and suspended to a single cell level with Hank's balanced salt solution. Then, 1 ml of 2.5 × 10^6 cells was injected into the peritoneal cavities of 8-week-old female Fisher 344 rats (Charles River Japan Inc., Kanagawa, Japan). After 1 month, the rats were sacrificed and examined for the formation of tumors. The number of macroscopic nodules (>1 mm in diameter) in the peritoneal cavity was counted.

The HAS transfectants were harvested and resuspended in Hank's balanced salt solution at 2.5 × 10^5 cells/ml as described above. The cell suspensions (200 μl) were subcutaneously injected in the left flanks of nude mice (6-week-old female BALB/c mice; SLC, Shizuoka, Japan). The appearance of visible tumors was monitored, and the tumor size in all of the mice was measured for 42 days using a dial caliper. The tumor volume (V) was determined by the following equation: V = (L × W) × 0.5, where L is the length and W is the width of a tumor. All animal experiments were performed according to the Aichi Medical University Guidelines for the care and use of laboratory animals.

Statistical Analysis—Statistical analyses were conducted using the Student's t test. Significantly different results were indicated at p values.

RESULTS

Enhanced HA Production and Matrix Formation in Oncogenic Malignant Transformation—We first assessed the levels of HA production and matrix formation in various oncogenic transformants. When the HA matrices were visualized by particle exclusion assay and by fluorescent staining with b-HABP, increased matrix formation was observed around the cell surfaces of both ras- and v-src-transformed rat 3Y1 cells, which were designated as HR-3Y1 and SR-3Y1 cells, respectively (Fig. 1). The HA production of the parental and the transformed cells was assessed by measuring the amounts of HA secreted into the culture medium. HA accumulation in the conditioned medium was ∼3-fold and 5-fold higher in the tumorigenic HR-3Y1 and the metastatic SR-3Y1 cells, respectively, than in parental 3Y1 cells (Fig. 2A). The highest level of HA production was detected in the highly metastatic v-fos SR-3Y1 cells. In contrast, the increase in HA production was not significant in the less malignant cells transformed with the adenovirus E1A fragment or simian virus 40. Therefore, the current results suggest a close association of the HA production with the malignancy of the transformed cells. To clarify the primary step responsible for the enhanced HA production, HAS activity was measured and compared with that of the parental cells. A good correlation was found between the HAS activity and the HA production (Fig. 2), suggesting that the HA production enhanced by oncogenic transformation was mainly due to the up-regulation of the HAS activity.

Several lines of evidence have demonstrated that the Ras-MAP kinase cascade is a major signaling pathway constitutively activated by v-src. We therefore investigated the contribution of the Ras signaling pathway to the Src-enhanced HA synthesis in culture systems using S17N SR-3Y1 cells, in which the dominant negative S17N ras is expressed in a dexamethasone-inducible manner. A previous study demonstrated that the relative amount of GTP-bound Ras was dramatically decreased by the expression of a dominant negative S17N Ras (37). As shown in Fig. 2, induction of the dominant negative S17N Ras in the presence of dexamethasone suppressed HA production by 50%, and the effect was time-dependent (Fig. 2C). These results suggest that constitutive activation of the Ras-signaling pathway in src-transformed cells may cause the up-regulation of HA biosynthesis. Because the HA synthesis is closely associated with HAS activity, we decided to investigate the transcriptional regulation of each HAS isoform in oncogenic malignant transformation.

Up-regulation of HAS Transcription by Oncogenic Malignant Transformation—We used a real-time RT-PCR method to examine whether enhancement of the HAS transcription accounts for the enhanced HA synthesis in oncogenic transformation. Equal amounts of mRNA from the parental and the transformed 3Y1 cell lines were used for the RT-PCR amplification of these HAS transcripts. Fluorescence intensity was monitored in real-time during PCR amplifications, and the expression levels of the HAS isoforms were calculated as a relative expression coefficient using standard curves that was generated by serial dilution of total RNA isolated from SR-3Y1 cells (Fig. 3). Variation in the efficiency of reverse transcription was normalized with a GAPDH housekeeping control mRNA. The transcriptional levels of HAS1 and HAS2 were relatively low in the parental 3Y1 cells, consistent with the low level of HA synthesis. The HAS2 transcript was five times more abundant in the HR-3Y1 cells than in the parental 3Y1 cells,
whereas there was no significant change in the mRNA levels of HAS1 and HAS3. Besides the similar levels of HAS2 expression in HR-3Y1 and SR-3Y1, a marked increase in the HAS1 expression was detected in the SR-3Y1 and v-fos SR-3Y1 cells, but there was no change in the HAS3 expression in these transformants. These comparative analyses suggest that the enhanced HA synthesis is attributable to the up-regulation of HAS2 transcription in the HR-3Y1 cells and those of HAS1 and HAS2 isoforms in the SR-3Y1 and v-fos SR-3Y1 cells. Induction of the dominant negative S17N Ras resulted in the suppression of HAS1 and HAS2 transcription in the SR-3Y1 cells, whereas the transcriptional level of HAS3 was not significantly affected.

These results suggest that the HAS2 transcription is mainly controlled by the Ras signaling pathway and that the HAS1 gene is regulated by both Ras-dependent and -independent signaling pathways located downstream of Src.

Establishment of Stable Transfectants Expressing Sense and Antisense HAS2 in HR-3Y1 Cells—As an approach to assess the contribution of HA to tumor malignancy, we generated various stable transfectants (HR HAS2-S and HAS2-AS) ex-
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pressing sense and antisense HAS2 genes in the HR-3Y1 cells and investigated changes in their malignant phenotypes. More than 10 clones were isolated by limiting dilution of neomycin-resistant colonies arising from single cells transfected with sense or antisense HAS2 constructs. We chose two HR Mock, three HR HAS2-S, and three HR HAS2-AS clones with different degrees of HA matrix formation and production (Fig. 4 and Table I). When the transcriptional levels of endogenous HAS isoforms were measured by real-time quantitative RT-PCR analysis using primers and probes specific for the respective rat isoforms, the levels of HAS2 transcription in HR AS-4, 6, and -10 clones were 91%, 72%, and 66%, respectively, of the levels in parental HR-3Y1 cells. Although there were no significant changes in the levels of the other HAS genes (data not shown). In addition, the expressions of all endogenous HAS genes were not affected by the overexpression of HAS2 transgene (data not shown). When the formation of HA matrices was assessed by particle exclusion assay, all HR HAS2-S clones (HR HAS2–2, -3, and -11) formed massive HA pericellular coats (Fig. 4 and Table I). HA accumulation in the conditioned medium was 2.8- to 5.2-fold higher in the HR HAS2-S cells than in control cells (Table I). No or thin HA pericellular coats were detected around the HR AS-6 and AS-10 cells, and the HA production and HAS activity levels of these cells were significantly lower than those of Mock transfectants (Table I). In contrast, HR AS-4, having a level of HA synthesis similar to that of HR Mock cells, had viable pericellular coats (Fig. 4).

Anchorage-independent Growth of HR-3Y1 Cells Expressing Sense and Antisense HAS2—A previous study has shown that overexpression of HAS2 enhanced both anchorage-independent growth and in vivo growth of tumor cells (24). We therefore examined the effect of overexpression or antisense suppression of the HAS2 gene on anchorage-independent growth of HR-3Y1 cells. Different clones of HR Mock, HAS2-S, and HAS2-AS cells were cultured in soft agar and scored for colony formation. Antisense suppression of HAS2 reduced approximately by one-third the colony formation of HR-3Y1 cells in soft agar (Table II), supporting the previous observations (24) that HA produced by HAS2 is important for promotion of the anchorage-independent growth and tumor growth. However, no significant increase in the anchorage-independent growth was observed in the HR-3Y1 cells when an excess amount of HA was produced by HAS2 overexpression (Table II). Therefore, it is likely that the increase in HA production by ras transformation already had caused maximal colony growth.

Expression of Sense and Antisense HAS2 Reduced Peritoneal Tumorigenesis of the HR-3Y1 Cells—To evaluate the contribution of HA to the malignant phenotypes of ras-transformed cells, we transplanted HR HAS2-S and HAS2-AS transfectants into the peritoneal cavities of syngeneic rats. The tumor formation was assessed after 1 month by counting the number of the macroscopic peritoneal nodules in the mesentery and peritoneal wall (Fig. S5 and Table II). Injection of HR Mock cells caused massive tumor formation in 100% of the injected animals. The incidence of tumor nodules of the HR Mock cells was similar to that obtained using the parental HR-3Y1 cells (data not shown). Antisense suppression of HAS2 transcription in HR-3Y1 cells mostly decreased the number of the nodules in the mesentery and peritoneum (Table II), suggesting that HA plays key roles in the development of tumors in the peritoneal cavity. We also tested additional HR HAS2-AS clones and obtained similar results. Consistent with the results of the anchorage-independent growth, no significant difference was observed in the ability to form tumors between the control HR Mock cells and the HR AS-4 cells, in which there was a slight suppression of HA biosynthesis (Table II). Unexpectedly, overexpression of HAS2 markedly decreased the ability to form nodules in the peritoneal cavity (Table II). Taken together,

### Table I

| Clone     | HA synthesis | HAS activity | HA pericellular coat/ cell<sup>a</sup> |
|-----------|--------------|--------------|---------------------------------------|
|           | µg HA/mg protein | pmol/mg protein |                                         |
| HR M-2    | 3.21 ± 0.21 | 2.03 ± 0.08 | 0.52 ± 0.11 |
| HR M-4    | 3.50 ± 0.07 | 2.03 ± 0.02 | 0.47 ± 0.07 |
| HR HAS2-2 | 16.67 ± 0.42<sup>b</sup> | 8.95 ± 0.76<sup>b</sup> | 1.04 ± 0.14<sup>b</sup> |
| HR HAS2-3 | 10.10 ± 0.67<sup>b</sup> | 6.43 ± 0.61<sup>b</sup> | 1.59 ± 0.20<sup>b</sup> |
| HR HAS2-11 | 9.79 ± 0.21<sup>b</sup> | 10.91 ± 0.55<sup>b</sup> | 1.24 ± 0.37<sup>b</sup> |
| HR AS-4   | 2.84 ± 0.07 | 1.88 ± 0.11 | 0.28 ± 0.07 |
| HR AS-6   | 2.24 ± 0.11<sup>c</sup> | 1.44 ± 0.02<sup>c</sup> | 0.11 ± 0.07<sup>c</sup> |
| HR AS-10  | 1.42 ± 0.10<sup>c</sup> | 1.03 ± 0.03<sup>c</sup> | 0.08 ± 0.05<sup>c</sup> |

<sup>a</sup>Ratio of matrix area to cell area (n = 10).
<sup>b</sup>p < 0.01.
<sup>c</sup>p value < 0.06.

### Table II

Anchorage-independent growth and the peritoneal tumor formation of various transfectants expressing sense and antisense HAS2

Five or six mice were used in each group.

| Clone     | Colony assay | Tumor incidence Tumor nodules |
|-----------|--------------|-------------------------------|
|           | colony/cm²   | >200, >200, >200, >200, >200 |
| HR M-2    | 438 ± 31     | 5/5 (100%)                    |
| HR M-4    | 563 ± 35     | >200, >200, >200, >148, 2     |
| HR HAS2-2 | 238 ± 15     | 2/5 (40%)                     |
| HR HAS2-3 | 441 ± 90     | 37, 8, 0, 0, 0                |
| HR HAS2-11 | 533 ± 40    | 1/5 (20%)                     |
| HR AS-4   | 233 ± 28     | 0, 0, 0, 0, 0                 |
| HR AS-6   | 98 ± 15      | 2/5 (40%)                     |
| HR AS-10  | 128 ± 12     | 5, 1, 0, 0, 0                 |
| HR AS-1   | ND<sup>d</sup> | 2/5 (40%)                     |
| HR AS-3   | ND<sup>d</sup> | 8, 2, 0, 0, 0                 |
| HR AS-18  | 45/1 (60%)   | 32, 23, 0, 0, 0               |
|           | ND<sup>d</sup> | 3, 2, 1, 0, 0                 |

<sup>d</sup>ND, not determined.
these results suggest that a certain level of HA synthesis optimizes oncogenic malignant transformation by v-Ha-ras.

**A Certain Level of HA Production Is Associated with the Maximum Promotion of Tumor Formation**—To further investigate the role of HA synthesis in the malignant properties of the transformed cells, we transplanted HR HAS2-S and HAS2-AS transfectants subcutaneously into BALB/c nude mice. The mean values of tumor size were measured for 42 days after injection. The tumors in nude mice receiving HR Mock transfectants were detectable at about 2 weeks after injection, and grew rapidly thereafter, reaching ~25 mm in diameter at 42 days (Fig. 6). HR HAS2-AS transfectants also developed visible tumors within 15 days in 100% of animals tested. Inconsistent with the growth of peritoneal tumors, that of subcutaneous tumors was almost equal between the Mock and AS transfectants. Requirements of HA for intraperitoneal and subcutaneous seeding and growth are very likely to be different. Since the amounts of HA produced by the AS transfectants are still higher than that by the non-transformed 3Y1 cells, there could be sufficient HA by these AS cells to support subcutaneous seeding and growth but not intraperitoneal. On the other hand, tumor growth rates of all HR HAS2-S cells markedly reduced, suggesting that the reduction in the incidence of peritoneal dissemination may be partly due to the suppression of the *in vivo* growth.

Previous studies by others have shown stimulatory effects of HAS expression on tumor growth of fibrosarcoma and prostate cancer cells. Our current study, however, demonstrated that overexpression of HAS2 in the HR3Y1 cells caused a reduction in the peritoneal and subcutaneous tumor formation. To further assess the effects of HAS2 expression on the tumor growth, we established HR-HAS2 transfectants that stably express various levels of HAS2. HR-3Y1 cells received mouse HAS2 cDNA in the pIRE2-EGFP bicistronic expression vector, which allows both HAS and EGFP genes to be translated from a single bicistronic mRNA. Positive cells were primarily selected by G418, enriched by fluorescence-activated cell sorting based on the expression of EGFP, and then characterized by their capacities for HA synthesis and matrix formation. We also established stable transfectants expressing various levels of the HAS2 mutant, which contains the amino acid substitution of Trp-354 to Tyr at the catalytic site. These control transfectants showed no or less increase in the HA production compared with that of parental HR-3Y1 cells (Fig. 7A). Nude mice inoculated with these HAS transfectants developed subcutaneous tumors during the observation period, and the mean tumor sizes were measured at 32 days after injection. The expression of the HAS2 resulted in aggressive tumor growth in a manner dependent on the levels of HA synthesis, but at high levels, the growth was inhibited as observed above (Fig. 7A). In contrast, the expression of HAS2 mutant had no such stimulatory or inhibitory effects on the tumor growth, suggesting that the HAS activity is responsible for the modulation of the *in vivo* growth by HAS2 expression. The results also support the idea that proper levels of HAS2 expression may optimize *in vivo* growth of the ras-transformed cells.

**Roles of HAS1 and HAS2 Expression on Tumor Growth**—The transcription of both HAS1 and HAS2 genes was up-regulated in the metastatic SR-3Y1 cells, but only the HAS2 gene was up-regulated in the less malignant HR-3Y1 cells. Because the SR-3Y1 cells are more tumorigenic than the HR-3Y1 cells, it is possible that the elevated expression of HAS1 may be respon...
expression in tumorigenesis remain to be elucidated in the same cell system.

To evaluate the roles of HAS1 and HAS2 in the modulation of tumor growth, the *in vitro* and *in vivo* growth were compared between the stable transfectants expressing various levels of HAS1 and HAS2. As for the anchorage-independent growth, no significant differences were observed between these HAS transfectants and the control cells (data not shown), suggesting that the increase in HA production by ras transformation had already caused maximal colony growth, as described above. On the other hand, a good correlation (*r* = 0.82) was observed between the subcutaneous tumor growth and *in vitro* HA synthesis of HAS1 transfectants (Fig. 7A). A weak correlation (*r* = 0.75) between the rate of tumor growth and the ability to form the HA-rich matrix was found in the HAS1 transfectants but not in the HAS2 transfectants (Fig. 7B). When the expression levels of HAS proteins were evaluated by immunoblotting of FLAG-tagged fusion proteins, HAS1 expression was associated with a significant growth promotion at a wide range of protein expression (0.06–0.12 unit/mg total protein; Fig. 7C). In contrast, the stimulatory effects of HAS2 were only observed within a low and narrow range of protein expression (Fig. 7C). The expressions of HAS2 protein above the narrow range perturbed tumor growth. These results suggest that proper regulation of the expression of each HAS isoform is required for optimal malignant transformation and tumor growth.

**DISCUSSION**

Abnormalities in HA synthesis are considered to be closely associated with malignant transformation and tumor progression. It is now evident that the three HAS isoforms exhibit characteristic gene expression patterns corresponding to the degree of malignant cell transformation. Of the three HAS isoforms, only HAS2 gene expression was increased in the less malignant HR-3Y1 cells transformed with v-Ha-ras. In the highly malignant cells transformed with v-src and/or v-fos both HAS2 and HAS1 transcriptions were up-regulated. Together with the finding that the respective HAS isoforms exhibited different gene expression patterns in response to growth stimulation (40–47), our current observation suggests that divergent mechanisms control the expression of each HAS gene. Through the use of src-transformed SR-3Y1 cells expressing the dominant negative Ras, we demonstrated that the expression of the HAS2 gene was mainly regulated by the Ras signaling pathway, whereas the expression of the HAS1 gene was regulated by both Ras-dependent and -independent signaling pathways located downstream of Src. Analysis of the HAS1 promoter region has demonstrated the presence of various cis-elements, including potential binding sites for AP-2, CREB, IRF-1, IRF-2, and p53 (48). Therefore, the elevated HAS1 expression in src-transformed cells may be attributed to the binding of transcription factors to these cis-elements in the HAS1 promoter region. It is of interest to conduct similar analyses of the other HAS genes to elucidate how oncogene products regulate the expression of the genes.

The elevated HAS2 expression in HR-3Y1 cells reminded us of the contribution of HA synthesized by HAS2 to ras-induced malignant transformation. In HR HAS2-AS cells, where HA synthesis and matrix formation were suppressed by the expression of antisense HAS2, intraperitoneal tumor formation was significantly inhibited, suggesting that the acquisition of malignant phenotypes involves HA, whose synthesis is promoted by transformation. It should be noted that the antisense suppression in the subcutaneous tumor was less effective than that in the intraperitoneal tumor. This difference in the promotion of the tumor formation may reflect the different requirements for HA of the two systems. Because the amounts of HA pro-
duced by the AS transfectants are still higher than that by the non-transformed 3Y1 cells, there could be sufficient HA by these AS cells to support subcutaneous seeding and growth but not intraperitoneal seeding.

The previous finding that overexpression of HAS2 in a fibrosarcoma cell line promoted subcutaneous tumor formation in nude mice (24) prompted us to investigate whether overexpression of HAS2 also promotes tumor formation by HR-3Y1 cells. Unexpectedly, when HA synthesis and matrix formation were increased by overexpression of HAS2, both intraperitoneal and subcutaneous tumor formation were markedly inhibited. To confirm this finding, we then generated stable transfectants expressing various levels of HAS2 and examined their tumorigenicity in nude mice. The expression of the HAS2 resulted in aggressive tumor growth in a manner dependent on the levels of HA synthesis; but at the high levels, the growth was inhibited. Considered together with the results of antisense HAS2 suppression, these findings suggested that HA is the effector of tumor cell behavior and that both less and excess amounts of the HA inhibited the in vivo cell growth, although it should be noted that the inhibitory levels of HA amounts are much higher than those produced by the ras-, src-, and fos-transformed cells. This conclusion may help us state generally that the physiological significance of changes in HA concentration with tumor grade or stage, because HA accumulation in clinical samples varies and occasionally shows little statistically change with tumor grade. A recent study using glioma cells has shown that overexpression of HAS2 inhibited tumor formation and suggested the importance of a balance between the HA synthesis and the degradation by hyaluronidase (29). Considering that the degradation of HA may be involved in controlling the HA accumulation (49), excess HA may also have suppressed tumor growth in glioma cells. Analogous effects of over- and under-production have also been observed for transforming growth factor-β1, whose overproduction reduces the rate of tumor growth, whereas opposite effects were observed with underproduction (50).

Overexpression of the control HAS2 mutant had no stimulatory or inhibitory effects on the tumorigenicity, and overexpression of HAS1 enhanced tumor growth, even though the protein expression was much higher than the highest HAS2 expression. Therefore, artificial effects such as aberrant subcellular compartmentalization are not likely to be responsible for the tumor growth altered by HAS overexpression. Although we could not verify at the present time whether all of the effects of HAS expression were due to the HA production, a good correlation was observed between HA production and tumor growth. As demonstrated in Fig. 7, the maximum amount of HA produced by HAS1 transfectants was equivalent to the amount giving optimum tumor growth in the HAS2 transfectants, suggesting that the important factor for tumor promotion is a certain level of HA itself and that HAS1 is less efficient in producing HA than HAS2.

Although the increased HA production in the tissue from cancer patients has been well investigated, the biological relevance of the HAS isoforms has not been elucidated during malignant transformation. Our findings of characteristic gene expression patterns may suggest that malignant progression is associated with a switch in the HAS1 expression. Multiple regulation by HAS isoforms may allow HA synthesis to be optimized for tumor growth and malignant progression. As we demonstrated previously, HAS transfectants expressing different isoforms synthesize different sizes of HA and form matrices of different sizes (22). These differences arise from the enzymatic characteristics of the HAS isoforms, and these might influence the properties of cancer cells in different ways. Because it has been reported that the expression of HAS1 promotes metastasis (23), the HAS1 expression in SR-3Y1 cells might also accelerate the process of malignant tumor progression. From this point of view, it will be necessary to reveal the relationship between HAS expression and prognosis by statistical analysis using clinical samples. Our recent study using clinical samples of human colon cancer tissue indicated that elevated HAS1 expression correlates with poor prognosis (51). During malignant transformation and progression, the different transcriptional regulation of the three HAS genes may enable the cells to remodel the extracellular environment and make it suitable for the survival and spreading of cancer cells. Elucidation of how each HAS is involved in tumor progression may be rapidly made in the near future using animal models such as knockout and transgenic mice.

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REFERENCES

1. Laurent, T. C., and Fraser, J. R. (1992) FASEB J. 6, 2397–2404
2. Toole, B. P., Wight, T. N., and Tammi, M. I. (2002) J. Biol. Chem. 277, 4593–4596
3. Rinnonen, K., Tammi, M., Parkkinnen, J., Esselin, M., Tammi, R., Lipponen, P., Agren, U., Alhava, E., and Kosma, Y.-M. (1998) Cancer Res. 58, 342–347
4. Hamerman, D., Todaro, G. J., and Green, H. (1965) Biochim. Biophys. Acta 101, 543–551
5. Ishimoto, N., Temin, H. M., and Strominger, J. L. (1966) J. Biol. Chem. 241, 2052–2057
6. Hopwood, J. J., and Dorfman, A. J. (1977) J. Biol. Chem. 252, 4777–4785
7. Leonard, J. G., Hale, A. H., Reid, D. E., Conrad, E. H., and Weber, M. J. (1978) Cancer Res. 38, 185–188
8. Turley, E. A. (1992) Cancer Metastasis Rev. 11, 21–30
9. Kazdoun, W. (1996) Am. J. Pathol. 148, 1721–1726
10. Dick, S. J., Macieci, B., Papazoglou, S., Oldfield, E. H., Kornblith, P. L., Smith, B. B., and Gately, M. K. (1983) Science 220, 739–742
11. Toole, B. P., Biwas, C., and Gross, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6299–6303
12. Kimata, K., Honma, Y., Okayama, M., Oguri, K., Hozumi, M., and Suzuki, S. (1983) Cancer Res. 43, 1347–1354
13. Turley, E. A., and Trettak, M. (1985) Cancer Res. 45, 5098–5105
14. Zhang, L., Underhill, C. B., and Chen, L. (1995) Cancer Res. 55, 428–433
15. Lee, J. Y., and Spicer, A. P. (2000) Curr. Opin. Cell Biol. 12, 581–586
16. Toole, B. P. (2001) Semin. Cell Biol. 12, 78–87
17. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) J. Biol. Chem. 277, 4589–4592
18. Weigel, P. H., Hascall, V. C., and Tammi, M. (1997) J. Biol. Chem. 272, 13997–14009
19. Itano, N., and Kimata, K. (1998) Trends Glycosci. Glycotechnol. 10, 23–38
20. Spicer, A. P., and Nguyen, T. K. (1999) Biochem. Soc. Trans. 27, 109–115
21. Simpson, M. A., and McDonald, J. A. (1998) J. Biol. Chem. 273, 1923–1932
22. Itano, N., Sawai, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shimomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spicer, A. P., McDonald, J. A., and Kimata, K. (1999) J. Biol. Chem. 274, 6085–20569
23. Itano, N., Sawai, T., Miyashita, O., and Kimata, K. (1999) Cancer Res. 59, 2499–2504
24. Kosaki, R., Watanabe, K., and Yamaguchi, Y. (1999) Cancer Res. 59, 1141–1145
25. Li, Y., and Heldin, P. (2001) Br. J. Cancer 85, 600–607
26. Liu, N., Gao, F., Han, Z., Xu, X., Underhill, C. B., and Zhang, L. (2001) Cancer Res. 61, 5207–5214
27. Simpson, M. A., Wilson, C. M., and McCarthy, J. B. (2002) Am. J. Pathol. 161, 849–857
28. Simpson, M. A., Wilson, C. M., Furcht, L. T., Spicer, A. P., Oegema, T. R., Jr., and McCarthy, J. B. (2002) J. Biol. Chem. 277, 10550–10557
29. Eneged, B., King, J. A., Styfl, S., Paradiso, L., Kaye, A. H., and Novak, U. (2002) Neurosurg 50, 1311–1314
30. Jacobsen, A., Rahmanian, M., Rubin, K., and Heldin, P. (2002) Int. J. Cancer 102, 212–219
31. Itano, N., Atsumi, F., Sawai, T., Yamada, Y., Miyashita, O., Senga, T., Hamaguchi, M., and Kimata, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3609–3614
32. Kimura, G., Itoh, I., and Summers, J. (1975) Int. J. Cancer 15, 684–706
33. Shimoura, H., Ohtsu, M., Matsuura, A., Mitsuomi, T., Onodera, K., and Kimura, G. (1988) Cancer Res. 48, 578–583
34. Tanaka, H., Zaitus, H., Onodera, K., and Kimura, G. (1988) J. Cell. Physiol. 136, 421–430
35. Zaitus, H., and Kimura, G. (1984) J. Cell Biol. 119, 82–88
36. Taniguchi, S., Tatsuka, M., Nakamatsu, K., Inoue, M., Sadano, H., Okazaki, H., Iwamoto, H., and Baba, T. (1989) Cancer Res. 49, 6738–6744
37. Thani, A. A., Sein, T. T., Liu, E., Machida, K., Kikkawa, F., Koike, T., Selki, M., Matsuura, S., and Hamaguchi, M. (1999) Oncogene 18, 6555–6563
38. Wang, X., Thani, A. A., Machida, K., Hiraiwa, Y., Iwata, H., Matsuura, S., and...
39. Hamaguchi, M. (1996) Int. J. Oncol. 12, 1097–1101
40. Itano, N., and Kimata, K. (1996) J. Biol. Chem. 271, 9875–9878
41. Sugiyama, Y., Shimada, A., Sayo, T., Sakai, S., and Inoue, S. (1998) J. Invest. Dermatol. 110, 116–121
42. Ohkawa, T., Ueki, N., Taguchi, T., Shindo, Y., Adachi, M., Amuro, Y., Hada, T., and Higashino, K. (1999) Biochem. Biophys. Acta 1448, 416–424
43. Jacobson, A., Brinck, J., Briskin, M. J., Spicer, A. P., and Heldin, P. (2000) Biochem. J. 348, 29–35
44. Usui, T., Amano, S., Oshika, T., Suzuki, K., Miyata, K., Araie, M., Heldin, P., and Yamashita, H. (2000) Invest. Ophthalmol. Vis. Sci. 41, 3281–3287
45. Pienimaki, J. P., Rilla, K., Fulop, C., Sironen, R. K., Karvinen, S., Pasonen, S., Lammi, M. J., Tammi, R., Hascaill, V. C., and Tammi, M. I. (2001) J. Biol. Chem. 276, 20428–20435
46. Recklies, A. D., White, C., Melching, L., and Roughley, P. J. (2001) Biochem. J. 354, 17–24
47. Sayo, T., Sugiyama, Y., Takahashi, Y., Ozawa, N., Sakai, S., Ishikawa, O., Tamura, M., and Inoue, S. (2002) J. Invest. Dermatol. 118, 43–48
48. Yamada, Y., Itano, N., Zako, M., Yoshida, M., Lenas, P., Niimi, A., Ueda, M., and Kimata, K. (1998) Biochem. J. 330, 1223–1227
49. Csoka, T. B., Frost, G. I., and Stern, R. (1997) Invasion Metastasis 17, 297–311
50. Ashley, D. M., Kong, M., Bigner, D. D., and Hale, L. P. (1998) Cancer Res. 58, 302–309
51. Yamada, Y., Itano, N., Narimatsu, H., Kudo, T., Morozumi, K., Hirohashi, S., Ochiai, A., Ueda, M., and Kimata, K. (2004) Clin. Exp. Metastasis, in press
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