The synthesis of a major collagen-binding heat-shock protein of molecular weight 47,000 (hsp47) was shown previously to be decreased after transformation of chick embryo fibroblasts by Rous sarcoma virus (Nagata, K., and Yamada, K. M. (1986) J. Biol. Chem. 261, 7531-7536; and Nagata, K., Saga, S., and Yamada, K. M. (1986) J. Cell Biol. 103, 223-229). In this paper, further study demonstrated that the total amount and the synthesis of hsp47 are also decreased by a factor of three in BALB/3T3 cells transformed by simian virus 40 (SV40). Higher synthesis was observed for BALB/3T3 cells in the resting state compared to those in the proliferating state. The synthesis of hsp47 in SV40-transformed cells, however, was consistently lower than that in normal cells irrespective of the cell density. Pulse label and chase experiments revealed that hsp47 was stable in the cells for at least 6 h and that there was no difference between normal and transformed BALB/3T3 cells in terms of the half-life. Decreases in the amount and the synthesis of hsp47 by transformation apparently correlate with the decreased synthesis of collagen in transformed cells. Immunoprecipitation using rat monoclonal antibody against hsp47 demonstrated the association of hsp47 with intracellular procollagen. Immunofluorescence studies using anti-hsp47 monoclonal antibody and anti-collagen antibody confirmed the co-localization of hsp47 and procollagen in both nonshocked and heat-shocked cells. Furthermore, we determined the biochemical characteristics of hsp47 of heat-shocked cells.

A collagen binding membrane glycoprotein of $M_r = 47,000$ has been found to be a novel heat shock protein (1). The synthesis of this heat-shock protein (hsp47) decreases in chick embryo fibroblasts (CEF) transformed by Rous sarcoma virus (RSV), although the degree of phosphorylation of hsp47 increases by a factor of seven after transformation (2). hsp47 also increases markedly during the differentiation of mouse F9 teratocarcinoma cells after the addition of retinoic acid and dibutyryl cyclic AMP (Ref. 3). The induction of hsp47 by heat shock and the decrease of hsp47 synthesis in RSV-transformed CEF are regulated by the level of mRNA (4).

Simian virus 40 (SV40) is a small, DNA-containing tumor virus. One of its gene products, the large tumor antigen, is known to be essential for cell transformation (5), the mechanism of cell transformation by SV40 being different from that by RSV. On the other hand, collagen, a major extracellular matrix protein, is reported to be synthesized at a lower level in fibroblasts transformed by DNA tumor virus or by RNA tumor virus (6-9).

In this paper, we report that decreased synthesis of hsp47 has been found in BALB/3T3 cells transformed by simian virus 40 (SV40) and is apparently correlated to the decreased synthesis of collagen in transformed cells. hsp47 of normal BALB/3T3 cells is synthesized at a higher level during the resting state than during the proliferating state and was shown not to be a major phosphoprotein. The immunoprecipitation and immunofluorescence studies demonstrate the association of hsp47 and intracellular procollagen in the endoplasmic reticulum.

**EXPERIMENTAL PROCEDURES AND RESULTS**

Phosphorylation of hsp47 in Mouse Cells—As shown in Fig. 1, the decreased synthesis of hsp47 was observed after transformation of BALB/3T3 cells with SV40 (see also Figs. 2-4 in the Miniprint). In chick embryo fibroblasts, phosphorylation of hsp47 has been shown to increase after transformation by Rous sarcoma virus (2). In the present study, normal BALB/3T3 cells, SV40-transformed 3T3 cells, and CEK as a control were labeled with $[^{32}P]orthophosphate for 14 h. The lysates were purified by gelatin-Sepharose and analyzed by SDS-PAGE (Fig. 5A), or the total lysates were analyzed by two-dimensional gel electrophoresis. Unexpectedly, hsp47 of BALB/3T3 cells was not phosphorylated when analyzed on two-dimensional slab gels (data not shown). hsp47 from transformed as well as normal cells purified by gelatin Sepharose was found not to be phosphorylated by

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2K. Hirayoshi, A. Nakai, and K. Nagata, unpublished observation.

3Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 2-4) are presented in mini-print at the end of this paper. Mini-print is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the journal that is available from Waverly Press.

4The abbreviations used are: hsp, heat-shock protein; SV40, simian virus 40; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NPHGE, non-equilibrium pH gradient electrophoresis; PBS, phosphate-buffered saline; CEF, chick embryo fibroblast(s); PDI, protein disulfide isomerase.
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FIG. 1. Two-dimensional analysis by NEPHGE/SDS-polyacrylamide gel electrophoresis of 1% Nonidet P-40-soluble extract. Uninfected and SV40-transformed BALB/3T3 cells were labeled with [35S] methionine (0.1 mCi/ml) for 1 h. Aliquots of cell extracts containing equal amounts of trichloroacetic acid-precipitable radioactivity were analyzed by two-dimensional gel electrophoresis consisting of NEPHGE as the first dimension and SDS-PAGE as the second dimension. A, uninfected BALB/3T3 cells and B, SV40-transformed cells. Open arrows indicate hsp47. Thick arrows indicate collagenase-sensitive spots and thin arrows fibronectin. A, actin.

FIG. 5. Phosphorylation of hsp47. A, uninfected (lane 1) and SV40-transformed BALB/3T3 cells (lane 2) and CEF (lane 3) were labeled for 14 h with 0.3 mCi/ml [32P]orthophosphate. Gelatin-bound fractions were analyzed by SDS-10% polyacrylamide gel electrophoresis. B, BALB/3T3 cells (lanes 1 and 3) and CEF (lanes 2 and 4) were labeled with 0.1 mCi/ml [35S]methionine for 1 h with (lanes 3 and 4) or without (lanes 1 and 2) treatment of 1 µg/ml tunicamycin for 8 h. FN, fibronectin.

SDS-PAGE (Fig. 5A, lanes 1 and 2), although hsp47 of CEF was phosphorylated as reported previously (Fig. 5A, lane 3 and Ref. 2). We examined the phosphorylation of hsp47 in another cultured mouse cell line, F9 teratocarcinoma cells and also found no evidence of phosphorylation (data not shown). Besides the phosphorylation, the apparent molecular sizes of hsp47 were found to be different between murine and chick cells. Mouse hsp47 migrated faster than chick hsp47 irrespective of the treatment with tunicamycin, which suggested that the apparent difference in molecular size was not due to the difference in glycosylation (Fig. 5B). The major phosphorylated protein detected on lane 2 in Fig. 5A was identified to be grp78 on the basis of pi and molecular size of the protein (data not shown) and by the fact of being induced by the treatment with tunicamycin (Fig. 5B, lanes 3 and 4; see “Discussion”).

The Synthesis of hsp47 Treated by Heat Shock—Differences in the induction of hsp47 in normal and transformed cells by heat shock were examined. Normal and SV40-transformed cells were incubated at 42 °C for 80 min and then labeled with [35S]methionine at 37 °C for 1 h. Aliquots containing equal trichloroacetic acid-precipitable radioactivity were analyzed by two-dimensional gel electrophoresis (Fig. 6). hsp47 consists of one major spot (indicated by the thick arrows in Fig. 6) and an acidic satellite spot (indicated by the thin arrows in Fig. 6), which share a common epitope to hsp47 when examined by immunoblot analysis on two-dimensional slab gels using anti-hsp47 monoclonal antibody (data not shown). These two spots were separated only when the resolution of two-dimensional gel electrophoresis was good. In fact, we could not detect two spots in Fig. 1. The ratios of the synthesis of the major spot to that of the satellite spot were various, but the major spot was always synthesized in larger amounts than the satellite spot. After the heat shock, only the major spot of hsp47 was strongly induced in both cells, whereas the satellite spot disappeared (Fig. 6 C and D). Other stress proteins (70s, 90s) were also induced in both cells by heat shock (indicated by arrowheads). Low molecular weight hsp could not be detected because of the lack of methionine in this protein in mammalian cells (13).

Next, we examined any differences between normal and
FIG. 6. Two-dimensional analysis by NEPHGE/SDS-PAGE of extracts from cells treated at high temperature. Cells were preincubated at 42 °C for 80 min and then labeled at 37 °C for 1 h with 0.1 mCi/ml of [³⁵S]methionine. Aliquots containing equal amounts of trichloroacetic acid-precipitable radioactivity were analyzed by two-dimensional gel electrophoresis consisting of NEPHGE as the first dimension and SDS-10% polyacrylamide slab gel electrophoresis as the second dimension. A and C, uninfected BALB/3T3 cells and B and D, SV40-transformed cells. A and B, 37 °C and C and D, 42 °C. The basic major spots and the acidic satellite spots of hsp47 are indicated by thick and thin arrows, respectively. The large and the small arrowheads indicate the hsp70s and the hsp90, respectively.

transformed cells in inducibility of this protein by heat shock. Both cells were incubated at 42 °C for various times and then labeled with [³⁵S]methionine for 1 h at 37 °C. To quantitate the increased synthesis of hsp47, gelatin binding fractions purified from the cell lysates containing equal trichloroacetic acid-precipitable radioactivity were examined using SDS-polyacrylamide slab gels (Fig. 7A) and quantitated with a densitometer (Fig. 7B). SV40-transformed cells synthesized hsp47 at a lower level than normal cells before heat shock, and induction was observed 30 min after heat shock in both cell types. Although the slopes of the graphs of hsp47 induction plotted against the period of heat treatment were almost equal, the maximum level of the induction was lower in transformed cells than in normal cells.

The Association of hsp47 with Intracellular Procollagen—We have demonstrated that hsp47 bound to collagen and gelatin (denatured types I and III collagen) and that hsp47 localized in the endoplasmic reticulum in chick embryo fibroblasts. Next we performed a double-label staining experiment to identify the co-localization of hsp47 with intracellular procollagen (Fig. 8, A-F). BALB/3T3 cells were treated with 0.1% collagenase for 30 min to remove surface collagen, fixed, permeabilized, and then incubated with the mixture of the anti-hsp47 and the anti-type I collagen antibodies. As to hsp47, intense fluorescence of fine granular staining at the perinuclear region and weak staining at the...
After heat shock, the staining pattern changed to a much brighter granular staining at the peripheral region (indicated by thin arrows in Fig. 8, lanes 1–5, 0 h; lanes 6–10, SV40-transformed BALB/3T3 cells. Lanes 1 and 6, 0 h; lanes 2 and 7, 0.5 h; lanes 3 and 8, 1 h; lanes 4 and 9, 2 h; and lanes 5 and 10, 4 h. FN, fibronectin. Using a densitometer, the relative density of each band was estimated by arbitrarily normalizing the value of lane 1 to 1 unit (B). Uninfected BALB/3T3 cells (O); SV40-transformed BALB/3T3 cells (●).}

periphery (indicated by thick arrows in Fig. 8, B and H) were observed in BALB/3T3 cells incubated at 37 °C (Fig. 8). After heat shock, the staining pattern changed to a much brighter granular staining at the peripheral region as well as the perinuclear region (indicated by thin arrows in Fig. 8 F; Refs. 17–20). As was expected, the staining of intracellular procollagen (type I) was exactly the same as that of hsp47 (Fig. 8, C and F). No exceptional staining was observed. These staining patterns were consistent with that of the endoplasmic reticulum (14–18). When we performed a double-label staining using anti-hsp47 antibody and anti-protein disulfide isomerase polyclonal IgG, which is known to localize in the endoplasmic reticulum, the staining patterns by the two antibodies were exactly the same. These results suggest that hsp47 and procollagen localize diffusely in the endoplasmic reticulum and that the staining pattern represents that of the endoplasmic reticulum.

hsp47 has been demonstrated to bind to collagen and gelatin when mixed in vitro. To establish the association of hsp47 to endogenous intracellular procollagen, immunoprecipitation was performed using rat anti-hsp47 monoclonal antibody (7C8B1) and rabbit anti-type I collagen polyclonal antibody (Advance Co., Ltd.). When hsp47 was immunoprecipitated with anti-hsp47 antibody, procollagen were co-precipitated (Fig. 9, lane 3). The identity of procollagen was confirmed by being digested with collagenase (Fig. 9, lanes 7 and 8). The co-precipitation of hsp47 by anti-type I collagen was, however, not clear in our experimental condition (Fig. 9, lane 4). In addition, as was expected from the fact that the binding of hsp47 to gelatin was disrupted in the low pH buffer (14), the amount of procollagen co-precipitated with hsp47 decreased by lowering the pH of the reaction buffer to 6.0 (Fig. 9, lane 5).

**FIG. 7.** Time course of hsp47 induction after treatment at 42 °C. Subconfluent cultures were preincubated at 42 °C for various time periods and then labeled with 0.1 mCi/ml [35S]methionine at 37 °C for 1 h. Aliquots containing equal trichloroacetic acid-precipitable radioactivity were applied to gelatin-Sepharose and the gelatin-bound fractions analyzed by SDS-10% polyacrylamide gel electrophoresis (A). Lanes 1–5, uninfected BALB/3T3 cells and lanes 6–10, SV40-transformed BALB/3T3 cells. Lanes 1 and 6, 0 h; lanes 2 and 7, 0.5 h; lanes 3 and 8, 1 h; lanes 4 and 9, 2 h; and lanes 5 and 10, 4 h. FN, fibronectin. Using a densitometer, the relative density of each band was estimated by arbitrarily normalizing the value of lane 1 to 1 unit (B). Uninfected BALB/3T3 cells (O); SV40-transformed BALB/3T3 cells (●).

**DISCUSSION**

Our major conclusions in this study are: 1) the synthesis of hsp47 decreases after SV40 transformation of BALB/3T3 cells, although mouse hsp47 is not a major phosphoprotein; 2) the synthesis of hsp47 in BALB/3T3 cells increases as the cell density becomes higher; 3) double-label experiments with anti-hsp47 and anti-collagen antibodies revealed the co-localization of hsp47 with intracellular procollagen in the endoplasmic reticulum; 4) immunoprecipitation demonstrated the co-precipitation of hsp47 with procollagen; and 5) only the main spot of hsp47 on two-dimensional slab gels is induced after heat shock, whereas the more acidic satellite spot disappears.

We found that the quantity as well as the synthesis of this collagen-binding 47,000-dalton protein decreases to one-third the value of normal BALB/3T3 cells in SV40-transformed cells also similar to the case of RSV-transformed CEF. We have also recently found that synthesis of this protein is decreased in NIH/3T3 cells transformed by activated c-H-ras oncogene.6 Cates et al. (21, 22) reported decreased synthesis of gp46, which is probably identical to hsp47, after RSV transformation of L6 rat myoblasts. Taken together, these data suggest that the decrease in the synthesis of hsp47 is not restricted to the case of CEF and may be a universal phenomenon induced by virus transformation or by transformation with oncogenes. One of the most striking effects of viral transformation on the biosynthetic program of cultured cells is the reduced synthesis of extracellular matrix proteins such as collagen (6, 23–26). On the other hand, hsp47 is strongly induced during the differentiation of mouse teratocarcinoma F9 cells (Ref. 3). Interestingly, type IV collagen is simultaneously induced during the differentiation of F9 cells. Considering these results, the synthesis of hsp47 seems to correlate with that of collagen.

It is well known that the growth of BALB/3T3 cells is inhibited in confluent culture, the cells being in a resting

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state. The growth of SV40-transformed cells, however, is not inhibited even after the culture becomes confluent. The possibility of the reduced synthesis of hsp47 being due to the difference between proliferating and resting states was ruled out (Fig. 4). In normal BALB/3T3 cells, however, the synthesis of hsp47 per equal total protein synthesized increases in the resting state. Two recent reports indicate that many high molecular weight heat-shock proteins are preferentially synthesized in the resting state (26, 27). These hsp47s were speculated to play roles in the regulation of the cell cycle. It remains to be clarified whether hsp47 has any correlation with such regulation.

hsp47 is a phosphoprotein in CEF and that phosphorylation increases by a factor of seven after RSV transformation (2). However, hsp47 of mouse BALB/3T3 cells, SV40-transformed 3T3 cells (Fig. 5A), and mouse teratocarcinoma F9 cells* was not phosphorylated. Cates et al. (21) reported that gp46 is a phosphoprotein in L6 rat myoblasts, although phosphorylation does not increase in RSV-transformed myoblasts, although phosphorylation does not increase in RSV-transformed myoblasts. As is the case with hsp47, Welch et al. (20) described that a 100-kDa stress protein is phosphorylated in SV40-transformed rat-1 cells and in L6 myoblasts but not in normal rat embryo fibroblasts (REF-52) or mouse 3T3 cells. Thus, it seems that phosphorylation of hsp47 might be influenced by differences in the species or cell origins. These data suggest that phosphorylation of hsp47 does not directly reflect cell transformation. Not only phosphorylation, but the molecular size of hsp47 is different in mouse and chick cells even when the glycosylation of hsp47 is inhibited by the treatment with tunicamycin. These results are thought to reflect some minor differences in amino acid sequence of hsp47 in both cell types.

hsp47 is detected as two spots on two-dimensional gel electrophoresis and only the basic spot was induced after heat shock (Fig. 6). The identity of these two spots were examined by immunoblotting and peptide mapping (data not shown). Two spots were also detected when glycosylation was blocked by the treatment with tunicamycin (data not shown). A pulse-chase experiment (labeled for 10 min) failed to detect the change in the ratio of these spots even after a 3-h chase period. Both spots were found to bind to gelatin irrespective of the glycosylation of the protein. It should be crucial to clarify whether the satellite spot is due to post-translational modifications.

We found that the phosphorylation of the ~80-kDa protein which bound to gelatin-Sepharose increased after SV40 transformation (Fig. 5). To identify this protein, we analyzed on two-dimensional gel electrophoresis the total cell lysates and gelatin binding fractions after cells were labeled with [35S]methionine or [32P]orthophosphate. We identified the ~80-kDa protein (upper band of the doublet in Figs. 3–5) to be grp78 by the position on the two-dimensional gels and by
the fact that the protein was induced by the treatment with tunicamycin or calcium ionophore, A23187 (data not shown). We also identified the lower band of the doublet appearing in those figures to be p72 (hsc70) by the position on the two-dimensional gels and by the immunoprecipitation using anti-hsp70 antibody (data not shown). We also identified the lower band of the doublet appearing in those figures to be p72 (hsc70) by the position on the two-dimensional gels and by the immunoprecipitation using anti-hsp70 antibody (data not shown).

Some precipitates were exposed to low pH buffer (pH 6.0, lanes 5 and 6) or digested with 0.1 unit/ml collagenase for 30 min at 37°C (lanes 7 and 8) as described in detail under "Experimental Procedures." Lanes 1 and 2 are the precipitates with rat IgG (mock) coupled Sepharose 4B and rabbit nonimmune serum, respectively. The positions of hsp47 and procollagen were indicated. Asterisk, procollagen digested with collagenase.

Double staining of hsp47 and cellular procollagen demonstrated the co-localization of these proteins, and immunoprecipitation revealed the association of hsp47 with procollagen and the disruption of the association by exposure to pH 6.0 buffer. It is of interest to note that similar or even lower pH values are observed in certain intracellular compartments involved in protein processing or translocation (28, 29). In the processing of endocytosis, for example, acidification of endosomes causes the disruption of the complexes of receptors and ligands which bind tightly to each other on the cell surface at neutral pH (30). The biological significance of the binding and dissociation of hsp47 to procollagen which were regulated by the change of pH should be elucidated in terms of the processing or transportation of procollagen.

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Transformation-sensitive Heat-shock Protein

EXPERIMENTAL PROCEDURES

Chemicals and Reactants - G-actin, bovine serum albumin, and trypsin were obtained from Miles Laboratories, Inc., Shawnee Mission, Kansas. Dibutyl-rhodamine-5-carboxaldehyde, rhodamine-phalloidin, and fluorescein-phalloidin were obtained from Molecular Probes, Inc., Eugene, Oregon. 1,10-Phenanthroline was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Missouri. G-actin was obtained from Molecular Probes, Inc., Eugene, Oregon. 1,10-Phenanthroline was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Missouri.

Cell Culture and Metabolic Labeling - HeLa (clone 5) cells in exponential phase were maintained in DMEM supplemented with 10% FCS as described previously. 

Cell lysates were obtained by homogenization in a Dounce homogenizer. The lysates were centrifuged at 100,000 x g for 30 min. The supernatant was used as the input cell lysate.

RESULTS

Decrease in Hsp70 Synthesis in HSF1-Transformed Cells - Decreased HSF1 levels in transformed cells were determined by Western blot analysis. The cells were cultured in the presence of 10% FCS for 24 h, and the HSF1 levels were determined by Western blot analysis. The results showed that the HSF1 levels were decreased in the transformed cells.

Fig. 2. The synthesis rate of heat-shock protein in transformed cells.

Cell isolation and metabolic labeling - HeLa cells were cultured in DMEM supplemented with 10% FCS as described previously. 

Cell lysates were obtained by homogenization in a Dounce homogenizer. The lysates were centrifuged at 100,000 x g for 30 min. The supernatant was used as the input cell lysate.

Fig. 2. The synthesis rate of heat-shock protein in transformed cells.
**Transformation-sensitive Heat-shock Protein**

Fig. 3. Chase experiments of hsp70.
A) After preculture for 24 hr in methionine-free medium, synchronized and SF90-transformed 37A5/273 cells were labeled for 30 min with 10 μCi/ml [35S]methionine. chase for the indicated times in medium containing excess unlabeled methionine (10 μg/ml). Equal volumes of cell extracts were applied to polyacrylamide gel electrophoresis. Lanes 1 and 2, gelatin-binding proteins recovered immediately after labeling period; and lanes 3-5 and 6-8, gelatin-binding proteins recovered after indicated chase periods (lanes 3 and 6, lanes 4 and 7, lanes 5 and 8). SDS-PAGE was performed for the 37A5/273 cells. The autoradiographic film was developed for 4 days. The results were evaluated as relative amounts. Hsp70 of uninfected (○) and SF90-infected cells (■); Fibronectin of uninfected (□) and SF90-infected cells (△).

B) Chase period (h)

Fig. 4. Dependence of hsp70 synthesis on cell density.
A) suspension and SF90-transformed 37A5/273 cells were counted at a density of 1 x 10^6 cells per 100 mm dish. 1, 2, and 3 days after subculturing of cells, cultures were labeled with 10 μCi/ml [35S]methionine for 1 hr. Aliquots containing equal incorporation radioactivity were applied to gelatin-precipitate and the gelatin-binding fractions analyzed by SDSPAGE. Lanes 1-3, uninfected cells; and lanes 4-6, SF90-transformed cells. Lanes 1 and 4, day 1; lanes 2 and 5, day 2; and lanes 3 and 6, day 3, 37A5/273 cells. B) Using a densitometer, the density of hsp70 bands were measured by normalizing the density of hsp70 to lane 1 as 1 unit. The results correspond to the lanes in A.
Transformation of BALB/3T3 cells by simian virus 40 causes a decreased synthesis of a collagen-binding heat-shock protein (hsp47).

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