

Human major HSP70 protein complements the localization and functional defects of cytoplasmic mutant SV40 T antigen in Swiss 3T3 mouse fibroblast cells

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The CT3 cytoplasmic localization mutant of SV40 T antigen is neither properly transported to the nucleus nor is it functional in rodent cells. Human precursors are able to complement this mutation, as they are fully transformed by CT3 with wild-type efficiency. The human-specific factors responsible for this species-specific difference in response to CT3 were localized to human chromosome 6 by synteny in a panel of six somatic cell hybrids. A major human HSP70 heat shock protein located on chromosome 6 is expressed constitutively in human cells. Hsp70 proteins have been reported to play a role in intracellular movement of newly synthesized proteins. To test whether human HSP70 played a role in the complementation by human cells of the defect of CT3, we constructed a series of mouse cell lines expressing human HSP70 and tested them for their ability to localize CT3 T antigen in the nucleus and for their ability to be transformed by CT3 DNA. Mouse cell lines expressing human HSP70 protein were able to translocate mutant CT3 T antigen into the nucleus and were transformed by CT3 at rates comparable with wild-type SV40. Mouse-inducible HSP70 protein was not able to translocate cytoplasmic T antigen in Swiss 3T3 mouse fibroblast cells, even after heat shock. Apparently human HSP70 is capable of complementing directly or indirectly the structural and functional alterations in SV40 T antigen introduced by the CT3 mutation.

[*Key Words*: CT3 cytoplasmic mutant; SV40 T antigen; Swiss 3T3 mouse cells; HSP70 protein]

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The phosphoprotein SV40 T antigen is normally transported to the nucleus by a short nuclear localization signal sequence composed of basic amino acids (Kalderon et al. 1984a,b; Colledge et al. 1986; Dingwall and Laskey 1986; Burglin and DeRobertis 1987), similar to that of other nuclear proteins (Dingwall and Laskey 1986; Wy-chowski et al. 1986; Burglin and DeRobertis 1987; Picard and Yamamoto 1987; Dang and Lee 1989; Roux et al. 1990). Components in the nuclear pore complex are thought to accomplish nuclear localization in two steps: rapid binding at the nuclear envelope, followed by slow transport through the nuclear pore (Richardson et al. 1988; Borer et al. 1989).

The relationship between localization and function has been reported for many oncogene products (Van Etten et al. 1989). In fully transformed cells, SV40 T antigen is uniquely localized both to the nucleus and to the outer cell surface (Santos and Butel 1985). In rat liver, SV40 T antigen transport to the nucleus is mediated by an ATP-dependent active transport system (Markland et al. 1987). Microinjection of antibodies raised against negatively charged peptides [Asp-Asp-Glu-Asp] inhibits nuclear transport of SV40 T antigen in a variety of cell lines

(Yoneda et al. 1988), suggesting that the cellular molecules involved in nuclear transport can be modulated experimentally. Deletion or mutation of the region of the T-antigen gene encoding the nuclear localization signal damages the capacity of mouse, hamster, and monkey cells to transport T antigen to the nucleus (Lanford and Butel 1982, 1984; Kalderon et al. 1984a,b; Alfano and McMacken 1989). For instance, the localization-signal mutant T antigen encoded by SV40 CT3, in which asparagine substitutes for lysine at amino acid 128, remains entirely in the cytoplasm of mouse cells (Lanford and Butel 1980, 1982, 1984).

Unlike the cells of mice, human precursors fibroblast cells can be transformed efficiently by CT3 T antigen. Translocation of cytoplasmic T antigen into the nucleus in human precursors fibroblast cells occurs with high efficiency as well (Chen et al. 1988). We have hypothesized that factors present in human fibroblasts function in a species-specific manner to complement the defect of CT3 T antigen, assisting transport of the antigen to the nucleus of human fibroblast cells, where CT3 T antigen can then bring about transformation (Chen et al. 1988).

The human heat shock multigene family encodes a set

of different proteins from genes located on at least three human chromosomes: 6, 14, and 21 (Harrison et al. 1987). The amino acid sequences of heat shock proteins are well conserved among different species (Hunt and Morimoto 1985). Their localization is cell cycle regulated; they are cytoplasmic in the absence of heat shock but accumulate in the nucleus in the S phase of the cell cycle (Milarski and Morimoto 1986; Milarski et al. 1989). The major constitutive human heat shock protein is encoded by a gene on chromosome 6, close to the major histocompatibility complex (MHC) (Harrison et al. 1987; Sargent et al. 1989). Human major HSP70 protein, as with other heat shock proteins, has a nuclear localization signal sequence and has been reported to be translocated into the nucleus upon heat shock (Welch and Feramisco 1984; Arrigo et al. 1988). Heat shock proteins have been reported to interact with oncogene products (Koskinen et al. 1991). In *ras/p53*-transformed rat fibroblast cells, the constitutive heat shock cognate protein hsc70 can complex with mutant p53 protein (Pinhasi-Kimhi et al. 1986; Finlay et al. 1988), whereas adenovirus E1A protein, which has been reported to increase transcription of human *HSP70* genes (Wu et al. 1986; Banerji et al. 1987), has been found colocalized with HSP70 protein in the nucleus (White et al. 1988). Variant-sized versions of SV40 T antigen have been reported to bind to heat shock proteins (Sawai and Butel 1989; May et al. 1991). In transformed mouse cells the complex formed between variant SV40 T antigen and cellular HSP70 does not require that any additional complex be formed between variant SV40 T antigen and cellular p53 protein (Sawai and Butel 1989). The mechanistic role, if any, of HSP70-variant T-antigen complex has not been elucidated.

We have isolated stable transfectants of Swiss 3T3 mouse fibroblast cells that express human major HSP70 protein constitutively under normal culture conditions. With these cell lines we have been able to show that this member of the human HSP70 heat shock protein family can complement the translocation defect of CT3 T antigen in Swiss 3T3 mouse fibroblast cells, thereby permitting mouse fibroblast cells to be transformed by the mutant viral protein at frequency comparable with wild-type SV40 T antigen. Heat shock studies showed that this effect is species specific, as inducible mouse HSP70 protein was not able to complement the nuclear localization defect of SV40 CT3 mutation.

Results

Translocation of CT3 T antigen into the nucleus in human fibroblast cells is due to human species-specific factors

CT3 T antigen is translocated into the nucleus as well as into the cytoplasm in human precrisis fibroblast cells, whereas it stays in the cytoplasm in Swiss 3T3 mouse fibroblast cells (Lanford and Butel 1984; Chen et al. 1988). To determine which human chromosomes encode the information for this species-specific difference, we

assayed localization of CT3 T antigen in a panel of six different human-mouse hybrid cell lines. Direct transformation of these hybrids was not possible owing to their partially transformed phenotypes; therefore, we transfected the hybrid lines with *neo^r* plasmids containing the gene for either CT3 or wild-type SV40 T antigen and selected *neo^r* subclones. *Neo^r* clones were subjected to Southern blot hybridization to check the integration of viral DNA sequences. All *neo^r* clones had integrated either CT3 or wild-type T antigen genes into many different sites (data not shown). Indirect immunofluorescence staining with monoclonal anti-T-antigen antibody (Pab416) was used to check the localization of either CT3 or wild-type T antigen in these clones.

Two human-mouse hybrid cell lines translocated CT3 T antigen into the nucleus as well as into the cytoplasm, and four did not (Table 1; Figure 1A-F). This result demonstrates that one, or a small number, of human gene products is capable of complementing at least a portion of the CT3 defect. Localization of CT3 T antigen and wild-type T antigen did not change with extensive subcloning of transformed hybrid lines. As expected, wild-type SV40 T antigen was localized into the nucleus exclusively in all six different hybrid cell lines (data not shown). We observed that chromosome 6 is the only human chromosome shared by the two hybrids that translocated CT3 T antigen to the nucleus. As shown by Harrison et al. (1987), the multiple copies of human heat shock protein gene on chromosome 6 exhibit a substantial amount of basal expression in the absence of heat shock. However, human chromosome 6 is also present in hybrid BDXE364a, which did not translocate CT3 T antigen.

Because no single human chromosome provided a complete correlation with CT3 translocation, we checked the presence of HSP70-specific sequences in the six hybrid cell lines by Southern hybridization using a 2.3-kb *HindIII-BamHI* fragment of plasmid Rous sarcoma virus (pRSV)-HSP70 gene as a probe. The two hybrid cell lines that translocated CT3 T antigen to the nucleus generated bands at the position of HSP70-specific sequences in human HeLa cells, as well as bands of mouse HSP70 sequences (Fig. 2). The three hybrid cell lines WAVR4dA19, BDA17b17-1, and BDA14b25, which did not translocate CT3 T antigen into the nucleus, did not contain any detectable human HSP70 sequences. Hybrid cell line BDXE364a, which also does not translocate CT3 T antigen into the nucleus, showed gross genomic rearrangement of human HSP70 sequences (Fig. 2). These data are consistent with the hypothesis that constitutively expressing major human heat shock protein from chromosome 6 of the human genome is capable of at least partial restoration of the defect in nuclear transport of CT3 T antigen.

Human major HSP70 protein is expressed stably in subclones of Swiss 3T3 mouse fibroblast cells

To test directly the hypothesis that human HSP70 protein is one of the proteins capable of translocating CT3 T

Table 1. Localization of CT3 T antigen in human-mouse hybrid cell lines carrying subsets of human chromosomes

	WAV4RdA19	41Pt2a	BDA10a3	BDA17b17-1	BDA14b25	BDXE364a
CT3 T antigen	cyto	nuc	nuc	cyto	cyto	cyto
human <i>HSP70</i>	-	+	+	-	-	rearranged
	<i>human chromosomes present</i>					
1	-	-	-	+	+	-
2	-	-	+	-	-	+
3	-	+	-	-	-	-
4	-	-	-	+	+	-
5	-	-	-	+	-	-
6 (*)	-	+	+	-	-	-
7	-	-	-	-	+	-
8	-	-	+	+	-	-
9	-	-	-	-	-	+
10	-	-	+	-	-	-
11	-	-	+	-	-	-
12	-	-	+	+	-	-
13	-	-	+	+	-	-
14	-	+	-	-	+	-
15	-	+	-	-	-	-
16	-	-	+	+	-	-
17	-	-	+	-	-	-
18	-	-	-	-	-	-
19	-	+	-	-	-	-
20	-	-	-	-	-	-
21	+	-	-	+	-	-
22	+	-	-	-	-	-
X	-	-	-	-	-	-

The localization of CT3 T antigen, determined by immunofluorescence with monoclonal antibody, was not changed upon extensive subcloning of transformed hybrid lines. (cyto) All cells had T antigen only in their cytoplasm; (nuc) a fraction (~50%) of cells had T antigen in their nuclei, as well as in their cytoplasm (see Fig. 1). The presence of human *HSP70* genes was determined by Southern blot analysis of the hybrid clones. Synteny (*) indicates that human chromosome 6 carries factors for translocation of CT3 T antigen to the nucleus of a mouse cell.

antigen to the nucleus of mouse cells, DNA encoding human major heat shock protein HSP70 was transfected into Swiss 3T3 mouse fibroblast cells together with the gene for neomycin resistance. To assure a high level of expression, the human *HSP70* gene was placed under the control of the RSV long terminal repeat (LTR) promoter. Two to three weeks after transfection, neo^r clones were picked randomly and expanded in medium selective for those clones. The stable integration of transfected human major HSP70 DNA in neo^r cells was checked by DNA hybridization with a 2.3-kb *Hind*III-*Bam*HI fragment of human major HSP70 sequences as probe. The probe detected four bands of homologous, endogenous mouse HSP70 DNA in untransfected as well as transfected 3T3 mouse cells, and additional different size inserts of exogenous human HSP70 DNA in transfected cells (data not shown). We chose to study further five HSP70-positive clones.

The expression of human major HSP70 protein in clones carrying human major HSP70 DNA was determined by immunoblotting cell extracts with mouse mAb 3a3 directed against human HSP70 protein (gift of Dr. R.I. Morimoto). Three of five clones carrying human major HSP70 sequences (H1, H2, and H5) showed detectable levels of exogenous human major HSP70 protein in addition to a mouse hsc70 protein constitutively present

in Swiss 3T3 mouse fibroblast cells (Fig. 3A). Against this endogenous background, the antibody recognized a slightly faster-migrating human major HSP70 protein in control HeLa cells and in the three positive transfected

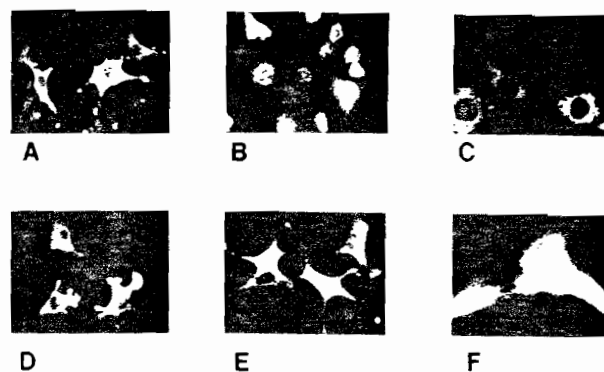


Figure 1. Immunofluorescence staining of SV40 T antigen in hybrid cell lines transfected by CT3 SV40 T antigen. Hybrid cells were stained with monoclonal antibody to SV40 T antigen. (A) Hybrid WAVR4dA19; (B) hybrid 41pt2a; (C) hybrid BDA10a3; (D) hybrid BDA17b17-1; (E) hybrid BDA14b25; (F) hybrid BDXE364a. All hybrid lines showed CT3 in the cytoplasm. In addition, nuclear localization of CT3 T antigen was seen in cells of hybrid lines 41pt2a and BDA10a3.

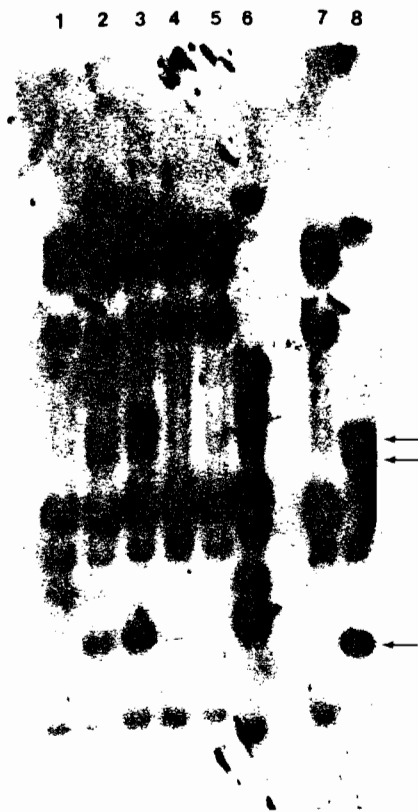


Figure 2. Presence of human HSP70 DNA in hybrid cell lines that can translocate CT3 T antigen into the nucleus. Southern blot hybridization of human HSP70 gene in human–mouse hybrid cell lines. Genomic DNAs of six hybrid cell lines were prepared, cut with *Pst*I, and hybridized with a 2.3-kb *Hind*III–*Bam*HI fragment of pRSV–HSP70 plasmid. (Lanes 1–6) DNAs from WAVR4dA19, 41pt2a, BDA10a3, BDA17b17-1, BDA14b25, and BDXE364a, respectively. Note the presence of additional bands, presumably human heat shock protein sequences, in 41Pt2a and BDA10a3 cell lines. In hybrid BDXE364a, human HSP70 sequences are rearranged and assumed to be inactive. Human and mouse species control DNAs were from mouse 3T3 cells (lane 7) and human HeLa cells (lane 8).

clones (Fig. 3A). The same antibody was used for immunofluorescence on coverslips of H1 or H5 (Fig. 3B). Untransfected 3T3 cells show only background fluorescence with this antibody. Constitutive, exogenous human HSP70 appears in H1 as a bright, completely cytoplasmic antigen (Fig. 3B). The cytoplasmic localization of exogenous human HSP70 in H1 cells did not change after subcloning and a second drug selection with hygromycin.

The expression of human major HSP70 protein had no detectable effect on the growth control of cell lines H1 and H5 (Table 2). Growth responses in both 10% and 1% FCS and in agarose were comparable for H1, H5, and control 3T3 mouse cells. In particular, neither control Swiss 3T3 nor clones H1 and H5 were able to grow in semisolid medium. Control Swiss 3T3 mouse fibroblast cells and clones H1 and H5 also did not show appreciable

differences in actin organization under normal cell culture conditions (Table 2). Subclones of H1 and H5 transfected with hygromycin-resistance plasmid and selected for secondary resistance to this drug did not differ from H1 or H5 in growth properties (Table 2).

Mouse cell lines H1 and H5 can be transformed by SV40 cytoplasmic mutant T antigen CT3 at high efficiency

The cytoplasmic localization mutant CT3 T antigen transforms Swiss 3T3 mouse cells, but at a very low frequency compared with wild-type T antigen (Lanford and Butel 1984). In contrast, CT3 can transform human fibroblasts with the same high efficiency as wild-type T antigen (Chen et al. 1988). To demonstrate directly the capacity of human major HSP70 protein to restore function while complementing the nuclear localization defect of CT3 T antigen, we assayed the appearance of foci with transformed morphology in 1% serum after CT3 transfection of clones H1 and H5 with a plasmid carrying the gene for SV40 mutant T antigen CT3. Confirming earlier reports (Lanford and Butel 1984; Chen et al. 1988), we found the transformation frequency of CT3 in control mouse fibroblast cells to be very low compared with that of wild-type T antigen (Table 3). In contrast, CT3 was able to transform clones H1 and H5 with an efficiency similar to that of wild-type SV40 (Table 3). We conclude that in the presence of human HSP70 protein CT3 T antigen has reacquired the capacity to transform mouse cells stably at high efficiency.

Subclones of H1 were isolated directly as dense foci after transfection with either CT3 or wild-type T-antigen gene. Such clones were found to be transformed stably, by the criteria of increased growth rate in regular and low serum, increased anchorage independence, and decreased actin cytoskeletal organization (Table 2). We also isolated and characterized a rare CT3-transfected 3T3 clone. Such clones arise with a frequency ~1% of that obtained with wild-type T antigen (Table 3). These cells have partially lost growth control but are not fully transformed (Table 2). We conclude that CT3 can transform H1 and H5 mouse cells completely at high efficiency.

To determine whether CT3 could generate transformed subclones of H1 or H5 in the absence of direct selection for the transformed phenotype, we introduced T antigen into the *neo^r* clones H1 and H5 without requiring it to act as a transforming agent, by using hygromycin as a second selective drug. Two to three weeks after cotransfection of hygromycin-resistance gene with either the CT3 T antigen or the wild-type T-antigen gene, we isolated hygromycin-resistant clones H1–CT3–hyg, H5–CT3–hyg, H1–WT–hyg, and H5–WT–hyg. These clones were all more transformed than WT–3T3 and as transformed as H1–CT3 or H5–CT3 (Table 2), suggesting that the selectively isolated complete transformants H1–CT3 and H5–CT3 were not products of rare events requiring selection.

Human major HSP70 protein is still produced in H1 subclones transformed by either wild-type or CT3 T an-

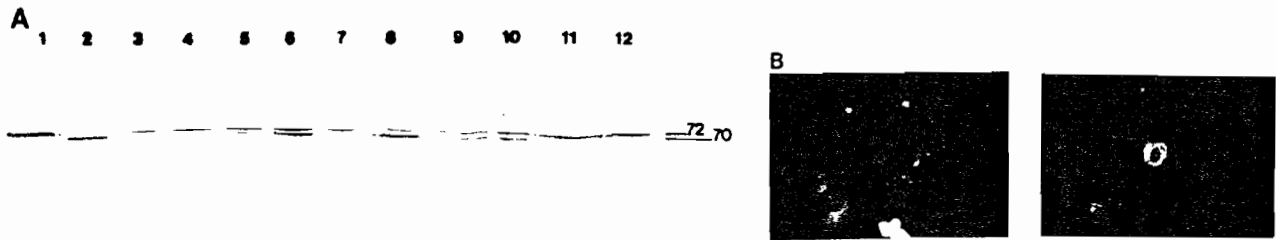


Figure 3. Human HSP70 protein expression in Swiss 3T3 clones carrying integrated human HSP70 gene. (A) Western blot of cell lysates from HSP70 DNA positive clones, reacted with mouse anti-human HSP70 mAb 3a3. (Lane 1) Control 3T3 cells, showing mouse hsc70 as a slow-migrating band. Mouse HSP70 does not appear unless cells are heat shocked. (Lanes 2–6) Human heat shock protein 3T3 lines H5, H4, H3, H2, and H1, respectively. (Lane 7) 3T3–neo control cells. (Lane 8) A 1 : 1 mixture of extracts from H5 and HeLa cells, showing coincidence of HeLa and H5 human fast bands. (Lane 9) A 1 : 1 mixture of extracts from HeLa and 3T3 cells. (Lane 10) H5 extract. (Lane 11) 3T3 extract. (Lane 12) HeLa extract. The slower-migrating human band is a 72-kD member of the heat shock protein family, which is constitutively expressed in HeLa cells (Milarski and Morimoto 1986). We conclude that the fast band in clones H1, H2, and H5 that is not seen in untransfected mouse cells is exogenous human major HSP70 protein. (B) Immunofluorescence assay with mAb 3a3 showing background fluorescence with no detectable mouse antigen in 3T3 cells (left), and cytoplasmic human HSP70 in subline H1 (right).

Table 2. Growth control of 3T3 and sublines expressing human HSP70 protein

Mouse cell line	Saturation density (cells/dish $\times 10^{-4}$)		Anchorage-independent colonies	Doubling time in 10% serum (hr)	Actin cables
	10% serum	1% serum			
Normal					
3T3–neo	79	no growth	–	22.9	++++
H1	72	no growth	–	20.9	++++
H5	69	no growth	–	23.9	++++
H1–hyg	80	no growth	–	20.6	ND
H5–hyg	72.5	no growth	–	22.8	ND
Transformed					
3T3–WT	208	122	++	13.5	–
3T3–CT3	190	23.5	–	17	++
H1–WT	254	199	+++	16	–
H1–CT3	251	71	+++	16	–
H1–CT3–hyg	230	57	+++	16	–
H5–CT3–hyg	199	73	+++	16.2	–
H1–WT–hyg	200	114	ND	ND	–
H5–WT–hyg	204	135	ND	ND	–

Neomycin-resistant 3T3 and descendent cell lines were assayed for growth control and cytoskeletal actin organization. H1, H5, and their hygromycin-resistant subclones did not lose any detectable degree of growth control as a result of expressing exogenous human HSP70 protein. Cell growth assays are described in Materials and methods.

tigen (Fig. 4). No genomic rearrangement of human major HSP70 gene was detected by Southern blot in these subclones (data not shown). We conclude that the human HSP70 protein synthesized by a transfected gene in clones H1 and H5 enables these mouse cells to translocate continuously CT3 cytoplasmic mutant T antigen into their nuclei and thereby to give rise to transformed clones after transfection.

Mutant CT3 T antigen and human HSP70 protein are both found in the nuclei of H1–CT3- and H5–CT3-transformed cells

Because we had found that mAb 3a3 gave only back-

Table 3. Transformation frequencies of wild-type and CT3 T-antigen genes in Swiss 3T3 mouse fibroblast cells and in sublines H1 and H5

Cell line	Human HSP70	SV40 T antigen gene	Transformation frequency (dense foci/ 10^5 infected cells)
3T3	–	CT3	0.25 ± 0.4
	–	wild type	20 ± 7
H1	+	CT3	16 ± 5
	+	wild type	10 ± 6

Transformation was assayed as dense foci appearing per 10^5 transfected cells, after 3 weeks in 1% serum.

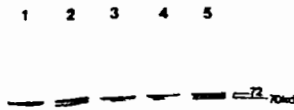


Figure 4. Immunoblot of human HSP70 protein in H1-CT3 and H1-WT cells. [Lane 1] Control neo^r 3T3 cells; [lanes 2-4] H1 subclones transformed by CT3 mutant T antigen; [lane 5] H1 subclone transformed by wild-type T antigen. Human HSP70 is present in all transformed subclones of H1.

ground fluorescence in 3T3 cells or their transformants (Fig. 3B), we used this antibody to localize specifically human HSP70 in H1 and H5, and their wild-type- and CT3-transformed subclones. The localization of T antigen in transformed lines was assayed on sister coverslips. Figure 5 shows the results of these assays for H1 and its subclones. 3T3 cells transformed by either wild-type or CT3 T antigen showed only nonspecific background fluorescence (Fig. 5C,D) and localized their T antigens to the nucleus and cytoplasm (Fig. 5A,B). H1-wild-type-transformed cells localized T antigen to the nucleus and human HSP70 to the cytoplasm, as expected in the absence of any interaction (Fig. 5E,G). H1-CT3-transformed cells, however, localized both CT3 T antigen and human HSP70 to the nuclei of some cells (Fig. 5F,H). H5 and its CT3 subclones gave similar results.

Four of five H1-wild-type-transformed clones behaved as in Figure 5, E and G; seven of eight CT3-transformed H1 or H5 subclones showed human HSP70 in the nucleus as well as in the cytoplasm, and all eight showed CT3 T antigen in the nucleus as well as in the cytoplasm (Table 4). These data are consistent with a specific inter-

action between CT3 mutant T antigen and human HSP70 proteins in mouse cells, either directly or through unknown mediating molecules. This hypothesis predicts that human skin fibroblasts (HSFs), which are transformed by CT3 T antigen with high efficiency (Chen et al. 1988), should also show a movement of human HSP70 from cytoplasm to nucleus after CT3 transformation. The human heat shock family of proteins visualized by mAb 3a3 in human cells was distributed evenly throughout HSFs transformed either by CT3 or wild-type T antigen, a result consistent with the hypothesis but otherwise uninformative (Table 4).

Interaction between exogenous human HSP70 protein and T antigen in 3T3 mouse cells

Variant molecular weight versions of SV40 T antigen bind to mammalian HSP70 proteins, including mouse hsc70 protein (Sawai and Butel 1989; May et al. 1991). We used immunoprecipitation with monoclonal mouse anti-T antigen (Pab108) and monoclonal mouse anti-human-HSP70 antibody (C92F3A5) to search for specific complex formation between human HSP70 and CT3 T antigen in mouse cells. Although C92F3A5 detects inducible mouse HSP70, as well as constitutive human HSP70, it does not detect constitutive mouse hsc70 or any other heat shock proteins in an uninduced mouse cell. Although we detected coprecipitation of mouse p53 with antibody to T antigen, we failed to detect coprecipitation of either CT3 or wild-type SV40 T antigen or mouse p53 protein in immunoprecipitates by using C92F3A5 antibody to HSP70 (data not shown). We conclude that complex formation between human HSP70 and CT3 T antigen, if any, cannot be as stable as the complex formation between T antigen and p53.

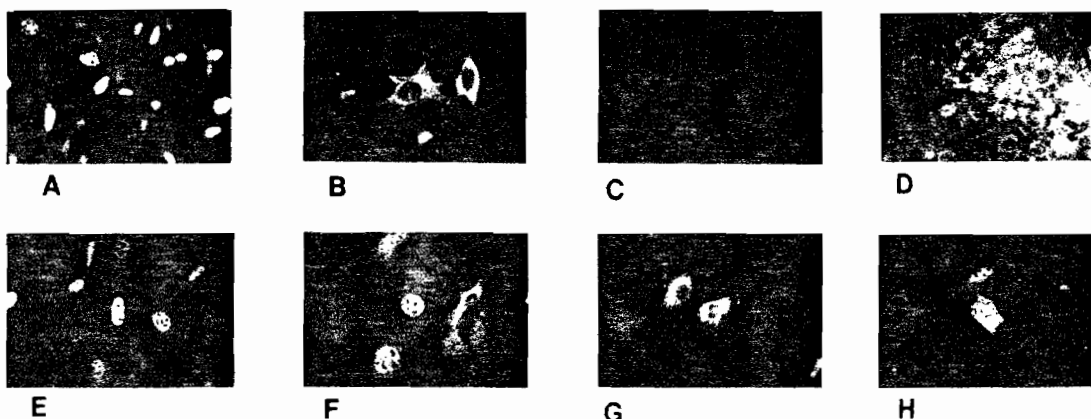


Figure 5. Human HSP70 and CT3 T antigen both localize in the nucleus of H1 mouse cells. Immunofluorescent detection of T antigen [A,B,E,F] and human HSP70 [C,D,G,H] in 3T3 and H1 cells transformed by SV40 wild-type or CT3 T antigen. [A] Nuclear T antigen in 3T3-WT-transformed cells. [B] Cytoplasmic T antigen in 3T3-CT3-partially transformed cells. [C (3T3-WT) and D (3T3-CT3)] Background, nonspecific fluorescence in these cells. [E] Nuclear T antigen in H1-WT-transformed cells. [F] Nuclear as well as cytoplasmic T antigen in H1-CT3 cells. [G] Cytoplasmic human HSP70 in H1-WT-transformed cells. [H] Nuclear as well as cytoplasmic human HSP70 in H1-CT3-transformed cells.

Table 4. Localization of SV40 T antigen and HSP70 in mouse and human cell lines

Cell line	Subclones	T antigen		HSP70	
		nuc	cyto	nuc	cyto
Mouse lines					
3T3-neo	1	-	-	-	-
3T3-CT3	1	-	+	-	-
3T3-WT	1	+	-	-	-
H1	1	-	-	-	+
H5	1	-	-	-	+
H1-hyg	1	-	-	-	+
H5-hyg	1	-	-	-	+
H1-CT3	1	+	+	+	+
H5-CT3	1	+	+	-	+
H1-CT3-hyg	2	+	+	+	-
H5-CT3-hyg	4	+	+	+	+
H1-WT-hyg	1	+	-	+	-
H5-WT-hyg	2	+	-	-	+
H1-WT	1	+	-	-	+
H5-WT	1	+	-	-	+
Human lines					
human skin fibroblast	1	-	-	-	-
HeLa	1	-	-	+	+
HSF-CT3	2	+	+	-	-
HSF-WT	2	+	-	-	-

Cells were grown on coverslips and stained with antibody to either human HSP70 or SV40 T antigen. Localization was determined by inspection at 500 \times . [nuc +, cyto -] Antigen was seen in the nuclei of cells examined and not seen in the cytoplasm; [nuc -, cyto +] antigen was seen in the cytoplasm of cells examined and not in their nuclei [nuc + cyto +] antigen was seen in the cytoplasm of $\geq 10\%$ of cells examined and also in the nuclei of $\geq 10\%$ of cells examined. Typically, HSP70-transfected, CT3-transformed cell lines contained nuclear T antigen in $\geq 50\%$ of cells.

Mouse-inducible heat shock protein does not complement CT3 mutant T antigen

Human HSP70 is constitutively produced at 37°C by H1 and H5 cells and by the human tumor line HeLa (Table 4). Mouse cells subject to heat shock at 42°C will produce elevated levels of mouse-inducible HSP70 protein (Fig. 6A). We reasoned that if the interaction between

CT3 T antigen and human HSP70 was not species specific, the excess of mouse HSP70 produced after heat shock might be able to complement the localization of CT3 T antigen in 3T3-CT3 cells. We found, however, that inducible mouse HSP70 protein does not have the capacity to complement the nuclear localization defect of CT3 T antigen (Fig. 6B). CT3-transformed 3T3 mouse cells were incubated at 42°C for 2, 4, or 6 hr and then shifted to 37°C to allow them to accumulate mouse-inducible HSP70 protein. Cell lysates were prepared at each time point and subjected to immunoblot analysis for mouse-inducible HSP70 protein. After 2 hr at 42°C mouse-inducible HSP70 protein was increased. The level of constitutively expressed mouse heat shock cognate protein remained the same for ≤ 6 hr (Fig. 6A). The localization of CT3 T antigen in heat-shocked 3T3 mouse fibroblast cells was assayed on coverslips from duplicate plates by indirect immunofluorescence staining with mouse monoclonal anti-T-antigen PAb416. CT3 T antigen was not transported to the nucleus in heat-shocked mouse cells, despite the increase in mouse HSP70 protein (Fig. 6B). Heat shock does not interfere with the translocation of CT3 T antigen localization to the nucleus in H1-CT3 or H5-CT3 cells (data not shown). This suggests that induced mouse HSP70 does not synergistically interact with transfected human HSP70 protein in these cells.

We conclude that mouse-inducible HSP70 protein, unlike human major HSP70 protein, does not have the ability to translocate SV40 CT3 T antigen to the nucleus, even after induction by heat shock, and that the interaction of human HSP70 with CT3 T antigen is specific.

Discussion

In human cells the family of heat shock proteins includes both constitutive and inducible genes located on at least three different human chromosomes—6, 14, and 21 [Harrison et al. 1987]. Their cellular localization is regulated in part by the cell cycle [Milarski and Morimoto 1986; Milarski et al. 1989]. The family of human HSP70 proteins has a variety of functions, including supporting protein folding and assembly [Ostermann et al.



Figure 6. Mouse HSP70 proteins induced by heat shock do not complement the localization or functional defect of CT3 T antigen. (A) Western blot of HSP70 induced by heat shock in 3T3-CT3 mouse cells and detected by mAb 3a3. The lowest of three immunoreactive bands is the mouse-inducible HSP70 protein. The middle band is the constitutively expressed mouse hsc70 protein. The top band is not identified but is present in mouse cells at both temperatures. (Lane 1) 6 hr at 42°C, followed by 6 hr at 37°C; (lane 2) 4 hr at 42°C, followed by 4 hr at 37°C; (lane 3) 2 hr at 42°C, followed by 2 hr at 37°C; (lane 4) no heat shock. Two hours of heat shock is sufficient to generate a maximal production of mouse-inducible HSP70. (B) Cytoplasmic localization of CT3 T antigen in 3T3-CT3 mouse cells after heat shock. Cells were grown on coverslips, fixed, and stained with monoclonal mouse-anti-SV40-T antigen. (Left) CT3 T antigen in the cytoplasm under normal culture conditions at 37°C; (right) CT3 T antigen remains in the cytoplasm after 2 hr of heat shock at 42°C.

Figure 6. Mouse HSP70 proteins induced by heat shock do not complement the localization or functional defect of CT3 T antigen. (A) Western blot of HSP70 induced by heat shock in 3T3-CT3 mouse cells and detected by mAb 3a3. The lowest of three immunoreactive bands is the mouse-inducible HSP70 protein. The middle band is the constitutively expressed mouse hsc70 protein. The top band is not identified but is present in mouse cells at both temperatures. (Lane 1) 6 hr at 42°C, followed by 6 hr at 37°C; (lane 2) 4 hr at 42°C, followed by 4 hr at 37°C; (lane 3) 2 hr at 42°C, followed by 2 hr at 37°C; (lane 4) no heat shock. Two hours of heat shock is sufficient to generate a maximal production of mouse-inducible HSP70. (B) Cytoplasmic localization of CT3 T antigen in 3T3-CT3 mouse cells after heat shock. Cells were grown on coverslips, fixed, and stained with monoclonal mouse-anti-SV40-T antigen. (Left) CT3 T antigen in the cytoplasm under normal culture conditions at 37°C; (right) CT3 T antigen remains in the cytoplasm after 2 hr of heat shock at 42°C.

1989; Parsell and Sauer 1989; Beckman et al. 1990), assisting in translocation of secretory and precursor proteins (Chirico et al. 1988; Deshaies et al. 1988), DNA replication (Alfano and McMacken 1989), uncoating of clathrin vesicles (Chappel et al. 1986), recovery of nucleolar morphology after heat shock (Pelham 1984; Welch and Suhan 1985), and modulation of developmental pathway (Kothary et al. 1987). We have shown that human-specific factors located on human chromosome 6 are capable of complementing the nuclear localization and the function of the CT3 cytoplasmic localization mutant of SV40 T antigen. We have also shown that the human major HSP70 protein located on chromosome 6 is sufficient to restore completely CT3 to normal function in mouse 3T3 cells, both translocating CT3 into the nucleus of mouse fibroblast cells and restoring it to functionality as a transforming protein. Although Hwang and Kaguni (1991) have shown that the bacterial heat shock protein *dnaK* can complement *dnaA*⁻ mutants, we have found the first example of a human heat shock protein complementing an amino acid substitution mutation in another protein, restoring its localization and function.

CT3-transformed cell lines H1-CT3 and H5-CT3, which translocated CT3 T antigen into the nucleus, showed typical increased growth rate in low serum and capacity to grow in the absence of anchorage. Because of the high frequency of transformation by CT3 of H1 and H5 cells, and because H1-CT3 and H5-CT3 subclones all remained stable mixtures of cells expressing T antigen in the cytoplasm as well as the nucleus rather than evolving toward populations with T antigen only in their nuclei, we consider it unlikely that CT3 T antigen reverted to wild type in these cells.

The effect of human HSP70 on CT3 translocation in mouse cells is species specific. We were able to induce mouse HSP70 protein synthesis by heat shock of 3T3-CT3 cells. CT3 T antigen remained entirely cytoplasmic in these cells, despite the presence of induced mouse HSP70. It is not clear how human major HSP70 protein translocates CT3 T antigen into the nucleus. It remains to be seen whether the change in localization of human HSP70 protein and restoration of transforming activity in H1 and H5 cells are the results of continuous molecular interaction between CT3 T antigen and human HSP70 protein, or whether complexes such as p53-CT3 T antigen play a necessary role in CT3-human HSP70 interaction.

Antibodies against negatively charged polypeptides block the transport of SV40 T antigen into the nucleus in various cells, including mouse fibroblasts (Yoneda et al. 1988). This suggests that wild-type SV40 T antigen may be transported to the nucleus by a charge-sensitive receptor molecule that recognizes a nuclear signal sequence on the T-antigen molecule. We are currently testing the hypothesis that CT3 T antigen is not properly recognized by these receptor molecules in mouse cells and that human major HSP70 protein can somehow modify CT3 T antigen so that its mutant signal sequence is recognized and the molecule is thereby transported to the nucleus by mouse nuclear localization molecules,

perhaps while it is still complexed with other mouse proteins.

Human HSP70 and CT3 T antigen might have a sufficient interaction short of stable binding to allow cryptic nuclear localization signals to be detected by mouse transport proteins. In this context it is useful to consider that HSP70 might suppress a number of conformation-related mutant phenotypes (Hwang and Kaguni 1991).

Materials and methods

Cells and antibodies

Swiss 3T3 mouse fibroblast cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. Drug-selected cell lines were grown in either 10% FCS/G418 (300 µg/ml) or 10% FCS/hygromycin (75 µg/ml) medium. Mouse anti-human HSP70 mAb 3a3 was kindly provided by Dr. Richard Morimoto. This antibody recognizes the HSP70 family of proteins, including the heat shock cognate proteins of human and mouse cells. A 1 : 20 dilution of mAb 3a3 was used in both immunoblot and immunofluorescence staining for the presence of HSP70 proteins. Another antibody with somewhat more restricted specificity, C92F3A5, was kindly provided by Dr. William Welch. C92F3A5 recognizes inducible mouse HSP70 protein and human HSP70 protein but not mouse hsc70 cognate protein. This antibody was used at 1 : 250 dilution in 1% BSA solution.

Southern blot hybridization

Fifteen micrograms of genomic DNAs was prepared from neo^r clones and was digested by restriction enzymes (4 U/µg) overnight at 37°C. Restricted genomic DNAs were then electrophoresed through an 0.8% agarose gel and transferred onto nylon membrane. Southern hybridization was done under high-stringency conditions. The hybridization solution contains 50% deionized formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl. After hybridization overnight at 42°C, the filter was washed successively by 2 × SSC, 2 × SSC, 1% SDS, 0.1 × SSC. The filter was then exposed on Kodak XAR-5 film with an intensifying screen at -80°C.

Western blot analysis

Cells (2 × 10⁶) were washed twice with ice-cold PBS buffer harvested at 4°C, and centrifuged at 240g for 10 min. NP-40 lysis buffer [20 mM Tris (pH 8.3) buffer, 145 mM NaCl, 1% NP-40, 0.1 mM PMSF, 10 mM DTT] was added to cell pellets, and the mixture was incubated on ice for 30 min. After incubation, extracellular materials were removed by centrifugation at 35,000g for 40 min. Loading buffer was added to the supernatant, and this was boiled for 5 min and centrifuged at 17,360g for 10 min and immediately loaded for 15% SDS-PAGE overnight at 50 V.

Electrophoresed proteins were transferred onto nitrocellulose membrane on a semidry gel transfer system. After 2-3 hr transfer at room temperature, membrane with transferred proteins was treated with blocking solution [2% nonfat, dried milk (wt/vol), 0.2% Tween 20 (vol/vol)] for 1 hr at room temperature and washed for 20 min with TBS buffer. Mouse anti-human HSP70 mAb 3a3 was then added to the membrane at a ratio of 1 : 20 in blocking solution. Reaction was continued for 2 hr at room temperature, and the membrane was washed with TBS for 20 min at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG (1 : 3,000 dilution in blocking buffer) was

added to the membrane, and reaction continued for 1 hr at room temperature. After reaction, membrane was washed with TBS for 20 min and placed in developing solution [50 mg/ml of NBT in 70% (vol/vol) dimethyl formamide, 50 mg/ml of bromochloroindolyl phosphate (BCIP) in 100% dimethyl formamide] to visualize antigenic HSP70 protein.

Immunofluorescence staining

The day before staining, cells were plated out at a density of 2×10^5 cells/35-mm plate. The next day, cells were fixed by a 3.7% formaldehyde (vol/vol) solution for 20 min at room temperature and washed with $1 \times$ PBS several times; 1% (vol/vol) NP-40 was then added to permeabilize cell membrane for 10 min. Monoclonal antibody to human HSP70 or to SV40 T antigen was added for 1 hr in a humidified incubator at 37°C. Coverslips were then washed with PBS several times, and FITC-conjugated anti-mouse second antibody [1 : 100 or 1 : 200 dilution] was added for 1 hr. Coverslips were washed, mounted, and stored at 4°C until examination. Stained samples were photographed on Kodak Tri-X film with a Zeiss immunofluorescence microscope with fluorochrome specific filters.

Cell growth assays

Growth assays were carried out by conventional methods [Brown et al. 1986; Chen et al. 1988]. Cells were plated out at 5×10^4 cells/35-mm plate. Dishes were trypsinized and cells were counted every 2 days for 10 days by using the trypan blue exclusion method to count only viable cells. For the anchorage-independence assay, either 5×10^4 or 5×10^5 cells in medium containing 10% FCS, $1 \times$ Dulbecco's modified Eagle medium (DMEM), and 0.33% Seaplaque agarose were plated out onto 60-mm plates that contained a bed of 2 ml of 10% FCS, $1 \times$ DME, and 0.5% Seaplaque agarose. Cells were fed once a week with soft agar. After 2–3 weeks, colonies were scored with the assistance of a dissecting microscope. Scoring was as follows: (–) no detectable colonies of 50 cells or more; (+) 1–10 small colonies per plate; (++) 10–100 colonies per plate, some of them larger than 50 cells; and (–++) >100 colonies per plate, many large colonies.

DNA transfection

Each DNA was mixed with 125 mM CaCl_2 , $1 \times$ BBS buffer [25 mM *N,N*-bis (2-hydroxyethyl)-2-aminoethane sulfonate (BES) at pH 6.95, 140 mM NaCl, 0.75 mM Na_2HPO_4] while bubbling gently, and incubated at room temperature for 30–45 min. Transfection solutions were added drop-wise to cell plates and were incubated overnight in a humidified incubator (37°C, 3–5% CO_2). After 12–16 hr of transfection, medium was removed and cells were fed with fresh medium. Two to three days after transfection, cells were split in a ratio of 1 : 3 and placed in selection medium (300 $\mu\text{g}/\text{ml}$ of G418 or 75 $\mu\text{g}/\text{ml}$ of hygromycin).

Immunoprecipitation

Cell monolayers at 80% of confluency were washed several times with methionine-free DMEM, fed with the same medium, and incubated for 30 min at 37°C in a humidified incubator. The medium was removed, and cells were refed with methionine-free DMEM supplemented with 2% dialyzed FCS containing 150 μCi of ^{35}S . After 4 hr of incubation in a humidified incubator, cell lysates were prepared from each plate in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% deox-

ycholate, 1% Triton X-100, 0.1% SDS], and antibody—either C92F3A5 or PAb108—was added to the cell lysates. After 2 hr of reaction at 4°C, immune complexes were precipitated by reaction with protein A solution and centrifugation. Immunoprecipitates were suspended in RIPA buffer, centrifuged three times, boiled for 5 min, and resolved by 15% SDS-PAGE. The PAGE gel was treated with En³Hance [New England Nuclear] and autoradiographed at –80°C on Kodak XAR-5 film.

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