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# Nuclear Colocalization of Cellular and Viral *myc* Proteins with HSP70 in *myc*-Overexpressing Cells

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**The c-*myc* oncogene and its viral counterpart v-*myc* encode phosphoproteins which have been located within cell nuclei, excluding nucleoli. We have expressed the c-*myc* gene under the simian virus 40 early promoter and studied the distribution of its protein product in transient expression assays in COS, HeLa, and 293 cells. We found three distinct patterns of c-*myc* immunofluorescence in the transfected cells: one-third of the c-*myc*-positive cells displayed a diffuse nuclear distribution, and in two-thirds of the cells the c-*myc* fluorescence was accumulated either in small amorphous or in large multilobed phase-dense nuclear structures. Unexpectedly, these structures also stained for the HSP70 heat shock protein in both heat-shocked and untreated cells. Our results indicate that both transient and stable overexpression of either the c-*myc* or v-*myc* protein induces translocation of the endogenous HSP70 protein from the cytoplasm to the nucleus, where it becomes sequestered in structures containing the *myc* protein. Interestingly, the closely related N-*myc* protein does not stimulate substantial nuclear expression of the HSP70 protein. Studies with chimeric *myc* proteins revealed that polypeptide sequences encoded by the second exon of c-*myc* are involved in colocalization with HSP70.**

The c-*myc* oncogene is deregulated in many types of neoplasia through the mechanisms of gene amplification, chromosomal translocation, or retroviral enhancer insertion (2, 6, 11, 31). In normal cells, enhanced c-*myc* expression is associated with transition of cells from the $G_0$ to the $G_1$ phase of the cell cycle (28), but throughout the cell cycle, the levels of the c-*myc* mRNA and protein synthesis stay constant (21, 57).

The c-*myc* protein as well as its viral counterpart v-*myc* is localized in the nuclei of cells (3, 18, 46), where it may be associated with nuclear matrix structures through specific metal interactions (14, 58). The protein is able to bind nonspecifically to DNA in vitro (46, 59). The c-*myc* protein has a short half-life (19, 48), but exposure of cells to elevated temperatures reduces the solubility and increases the synthesis and stability of c-*myc* and some other nuclear proteins (15, 36).

The closely related N-*myc* gene also encodes a DNA-binding nuclear phosphoprotein with a short half-life (24, 49, 52). In contrast to the wide spectrum of cells and tissues expressing c-*myc*, N-*myc* is expressed preferentially during embryogenesis, but only in few cell lines or adult tissues (13, 25). The c-*myc* and N-*myc* oncogenes have similar genomic structures containing three exons, which encode two polypeptides with different amino termini. The translation initiation codons for both of the N-*myc* polypeptides are located near each other in the second exon of the gene (37), while the translation of the longer c-*myc* polypeptide is initiated from a CTG codon in the first exon (20). Although the overall homology of the amino acid sequences of these two *myc* family members is only 32%, there are regions throughout the proteins that are nearly identical (4, 32, 55). These structural similarities suggest that the *myc* proteins might also have similar biological properties. The exact functions of the *myc*-encoded oncoproteins are not known, but evidence is accumulating that they could be involved in the regulation of transcription (26, 29, 44, 47), DNA synthesis (9, 10, 22, 23), and/or RNA processing (54). Both genes can transform Rat-1A cells (53) and complement c-*ras* oncogenes in transformation of primary rat embryo fibroblasts (51, 66).

Our earlier studies have shown that the v-*myc* protein is diffusely distributed throughout the cytoplasm of mitotic cells (3, 63). In interphase cells, the v-*myc* and c-*myc* proteins are relatively evenly distributed in the nuclei in a granular pattern excluding nucleoli (3, 18, 46, 54, 63). Here we report that in cells transiently or stably overexpressing c-*myc* or v-*myc* protein, these proteins accumulate in amorphous, phase-dense nuclear structures in codistribution with the HSP70 heat shock protein. Instead, overexpressed N-*myc* protein does not show significant nuclear accumulation or codistribution with HSP70.

## MATERIALS AND METHODS

**Molecular clones.** The different human c-*myc* DNA fragments were derived from the pHSR-1 genomic clone (1), human N-*myc* fragments were derived from the pNb-9 genomic clone (55), and simian virus 40 (SV40) viral sequences were derived from the pSVc*myc*1 plasmid (ATCC 41029). For the truncated pSV-c-*myc* construct, a 4.5-kb *Xba*I-*Eco*RI fragment containing the second and third exons of the c-*myc* gene was ligated into a vector carrying the SV40 early promoter. For the pSV-c/N-*myc* construct, an *Xba*I-*Bgl*II fragment containing the c-*myc* second exon and a *Bgl*II-*Eco*RI fragment containing the N-*myc* third exon were linked together into the vector carrying the SV40 early promoter; for the pSV-N/c-*myc* construct, this was done vice versa. For the full-length pSV-Tc-*myc* and pSV-Tc/N-*myc* constructs, a 780-bp *Ava*I-*Xba*I fragment containing part of the first exon of the c-*myc* gene was first inserted into the polylinker of the pGEM-3 plasmid and then transferred to the *Xba*I sites of the pSV-c-*myc* and pSV-c/N-*myc* con-

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structs. The construction of the pSV-N-*myc* plasmid has been described previously (37).

The pSV-*neo* and pSV-v-*myc* plasmids were obtained from ATCC (37149 and 45014, respectively). The c-*myc*(317-336)/pyruvate kinase vector (12) and the deletion mutants of the c-*myc* gene in a pSV vector (56) were kindly provided by William Lee, University of California, San Francisco.

**Antibodies.** Rabbit polyclonal anti-*myc* antisera were raised against the carboxy-terminal 32 amino acids of the human c-*myc* protein (48), against the bacterially expressed portions of the v-*myc* (3) or human N-*myc* (37) protein, or against a pan-*myc* peptide (42) which recognizes all *myc* proteins in various species. Two different mouse monoclonal anti-HSP70 heat shock protein antibodies were used: N27, which recognizes both the inducible and the constitutive HSP70 proteins, a kind gift from William Welch, University of California, San Francisco, and C92, which recognizes only the inducible HSP70 protein (RPN.1197; Amersham). Mouse monoclonal anti-Sm and anti-rRNA antibodies (34) were obtained from Joan Steitz, Yale University, New Haven, Conn., and rabbit anti-chicken muscle pyruvate kinase antiserum (27) was obtained from Bruce Roberts, Integrated Genetics, Framingham, Mass. Rhodamine-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (Cappel), fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Jackson Laboratories), and Texas red-conjugated sheep anti-mouse immunoglobulin G (Amersham) were used as second antibodies.

**Cells and transfections.** COS cells expressing the SV40 T antigen (16), HeLa cells (ATCC CCL 2), and the human kidney cell line 293 expressing the E1A antigen (ATCC CRL 1573) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. QT6 (ATCC CRL 1708) and MC29 virus-transformed Q8 (7) quail fibroblasts were grown as described by Bunte and coworkers (8). All cells were routinely screened for mycoplasma contamination by using the Hoechst DNA fluorochrome 33258 (50), with negative results. COS cells were transfected by the DEAE-dextran method (38), and HeLa and 293 cells were transfected by the calcium phosphate precipitation technique (17). In general, the transfected cells were analyzed within 48 h.

**Metabolic labeling and immunoprecipitation.** Two days after transfection, the cells were labeled for 1 h with [$^{35}$S]methionine (250 μCi/ml; Amersham) and lysed in a buffer containing 10 mM Tris-hydrochloride, pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40, and 100-U/ml aprotinin. Cell lysates were immunoprecipitated by incubating them at 4°C with protein A-Sepharose (Pharmacia) particles together with antibodies. After 1 to 2 h, the precipitates were washed several times with the lysis buffer, twice with phosphate-buffered saline (PBS), and finally once with water. Thereafter, they were dissolved in electrophoresis sample buffer, boiled for 3 min, and analyzed in a 10% SDS-polyacrylamide gel (33). After electrophoresis, the gels were fixed in 10% acetic acid, impregnated with Amplify (Amersham), dried onto filter paper, and fluorographed with X-Omat AR film (Eastman Kodak).

**Immunofluorescence.** For immunofluorescence studies, the cells were transfected on coverslips. Two days after transfection, they were fixed with 4% (wt/vol) paraformaldehyde in PBS for 30 min at room temperature or with methanol for 2 min at −20°C. After three washes with PBS, the paraformaldehyde-fixed cells were permeabilized with 0.2% (vol/vol) Nonidet P-40 (BDH Chemicals Ltd.) in PBS for 5 min and then washed again with PBS. For trypan blue exclusion assay, the cells were stained with 0.2% (wt/vol) trypan blue in PBS for 30 min at 37°C before fixation. For in situ RNase digestion, the methanol-fixed cells were incubated with RNase A (Sigma; 100 μg/ml in PBS) for 15 min at room temperature. The different staining steps with primary antibodies and fluorochrome-conjugated secondary antibodies were carried out consecutively with three PBS washes between each step. During the final PBS washes, Hoechst DNA fluorochrome 33258 (50) was added when needed for the staining of DNA. Before double-labeling experiments, it was carefully checked that the different antibodies and species-specific fluorochromes did not cross-react with one another. Stained samples were mounted in 50% glycerol and photographed on Agfa Pan 400 film by using Dialux 20 EB (Leitz) or Axiophot (Zeiss) immunofluorescence microscopes with appropriate fluorochrome-specific filters.

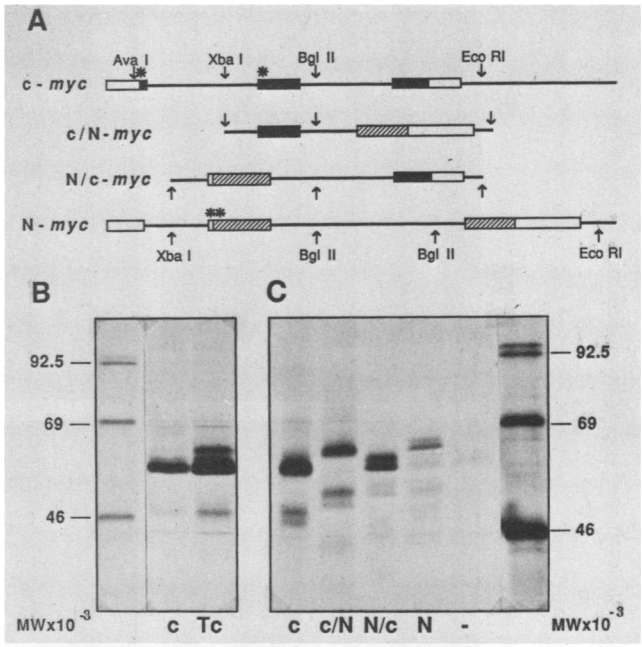


FIG. 1. Construction and analysis of the *myc* expression vectors. (A) The different *myc* vectors were constructed as described in Materials and Methods by using the restriction sites indicated above the schematic diagrams of the respective genes. Symbols: ■, protein-coding regions of the c-*myc* gene; ▨, protein-coding regions of the N-*myc* gene; □, nontranslated sequences; *, translation initiation sites of the *myc* polypeptides. The constructs were transfected into COS cells, and 2 days after transfection, the cells were labeled with [$^{35}$S]methionine and lysed. (B) The pSV-c-*myc* and pSV-Tc-*myc* lysates were immunoprecipitated with anti-c-*myc* antibodies and analyzed in an SDS-polyacrylamide gel. (C) The pSV-c-*myc*-, pSV-c/N-*myc*-, pSV-N/c-*myc*-, pSV-N-*myc*- and mock-transfected (−) lysates were immunoprecipitated with anti-pan-*myc* antibodies and analyzed as in panel B.

## RESULTS

**Distinct patterns of c-*myc* distribution in transfected COS cells.** To study the properties of the c-*myc* protein, we constructed two expression vectors carrying parts of the human c-*myc* gene under the constitutive SV40 early promoter (Fig. 1A; see also Materials and Methods). The constructs were transfected into COS cells, in which plasmids possessing the SV40 origin are efficiently replicated

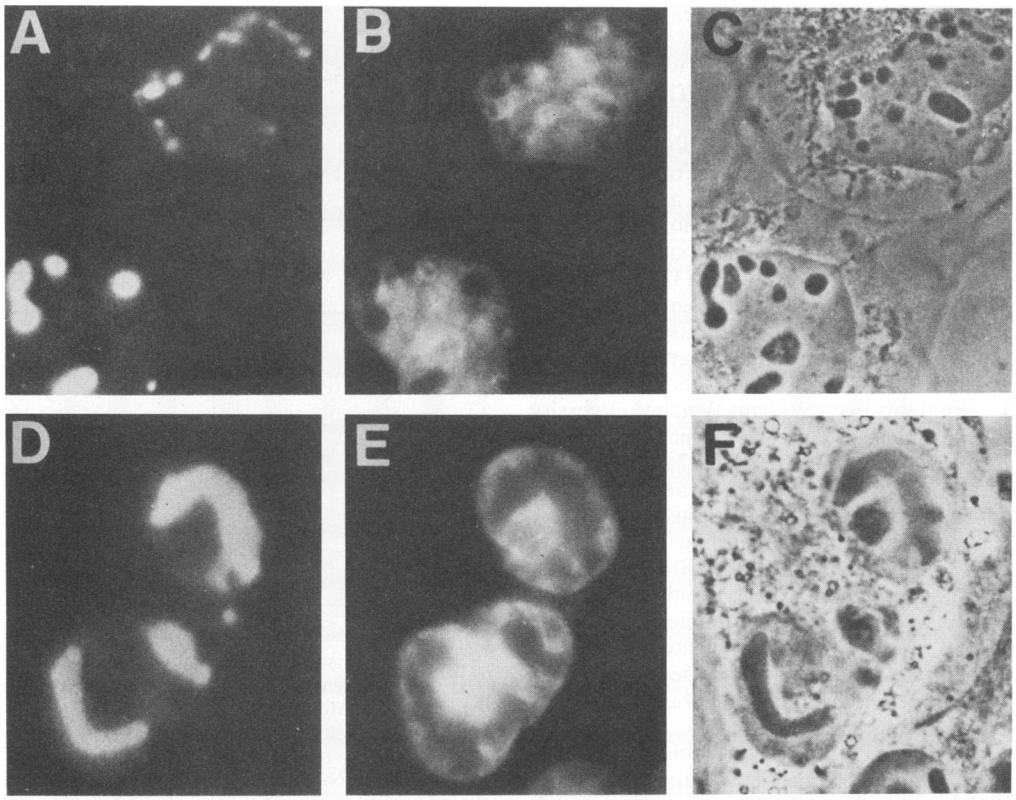


FIG. 2. Distribution of the c-*myc* protein in phase-dense nuclear structures in pSV-Tc-*myc*-transfected COS cells: c-*myc* (A and D) and DNA fluorescent (B and E) as well as phase-contrast (C and F) micrographs of the COS cells fixed and stained 2 days after transfection.

(16). The full-length pSV-Tc-*myc* construct encodes both of the two c-*myc* polypeptides, while the truncated pSV-c-*myc* construct encodes only the shorter one (Fig. 1B).

Two days after transfection, the cells were fixed and stained with anti-c-*myc* antibodies. Maximally 10% of the cells expressed the transfected c-*myc* gene in their nuclei, while the endogenous c-*myc* levels were too low to be detected. In one-third of the c-*myc*-positive cells transfected with the full-length pSV-Tc-*myc* construct, c-*myc* staining was diffusely distributed in the nuclei, excluding nucleoli, as has been reported in earlier studies (3, 63; see also Fig. 5A). Besides this, two other patterns of c-*myc* distribution were found in the transfected cells: small amorphous phase-dense structures (Fig. 2A to C) and larger, confluent areas of c-*myc* fluorescence (Fig. 2D to F). In the phase-contrast micrographs (Fig. 2C and F), these c-*myc*-positive structures resembled nucleoli. However, in contrast to the few nucleoli normally seen in COS cells, these nucleoluslike structures were devoid of chromatin in DNA staining (Fig. 2B and E), as if the c-*myc*-containing material had displaced DNA from these structures. Also, when the transfected cells were double stained with anti-c-*myc* and anti-rRNA antibodies, it was confirmed that the c-*myc*-positive structures and rRNA-containing nucleoli localized to distinct phase-dense structures (data not shown). In cells transfected with the pSV-c-*myc* construct encoding only the shorter c-*myc* polypeptide, similar patterns of c-*myc* fluorescence were observed. All c-*myc*-positive cells excluded trypan blue when stained before fixation, indicating that the expression of c-*myc* was not toxic for the cells (data not shown).

Spector and coworkers (54) have shown that the c-*myc* and v-*myc* proteins colocalize with small nuclear ribonucleoprotein particles (snRNPs) in an RNase-sensitive speckled pattern. However, when we double stained pSV-Tc-*myc*-transfected, methanol-fixed cells with anti-c-*myc* antiserum and anti-Sm antibodies against snRNPs, only a partial colocalization was detected, mainly in small clusters visible also by phase-contrast microscopy (Fig. 3A). Comparison of the effects of different fixatives showed that methanol, which affected the cell morphology more drastically than paraformaldehyde, gave a speckled pattern of c-*myc* fluorescence similar to that observed by Spector and coworkers (54) (compare methanol-fixed cells in Fig. 3 with cells fixed with paraformaldehyde in the other figures). Furthermore, treatment of the transfected cells with RNase A had no effect on the intensity of c-*myc* fluorescence, although the snRNP fluorescence was greatly reduced (Fig. 3B).

**Overexpression of the c-*myc* or v-*myc* protein induces nuclear translocation of the endogeneous HSP70 protein.** Several reports have demonstrated that heat treatment of cells induces a rapid migration of the HSP70 heat shock protein into the nucleus, where it resides predominantly in nucleolar structures (45, 60, 61). During the recovery period after heat shock, increasing amounts of HSP70 are seen in other parts of the nucleus before the protein returns to the cytoplasm. The expression of HSP70 is cell cycle regulated, being maximal in early S phase (40). In heat-shocked cells, the distribution of HSP70 also appears to be cell cycle dependent, as in $G_2$ no nucleolar HSP70 can be detected by monoclonal antibodies, presumably because of its association with some other cellular proteins (41).

Because the overexpressed c-*myc* protein has been shown

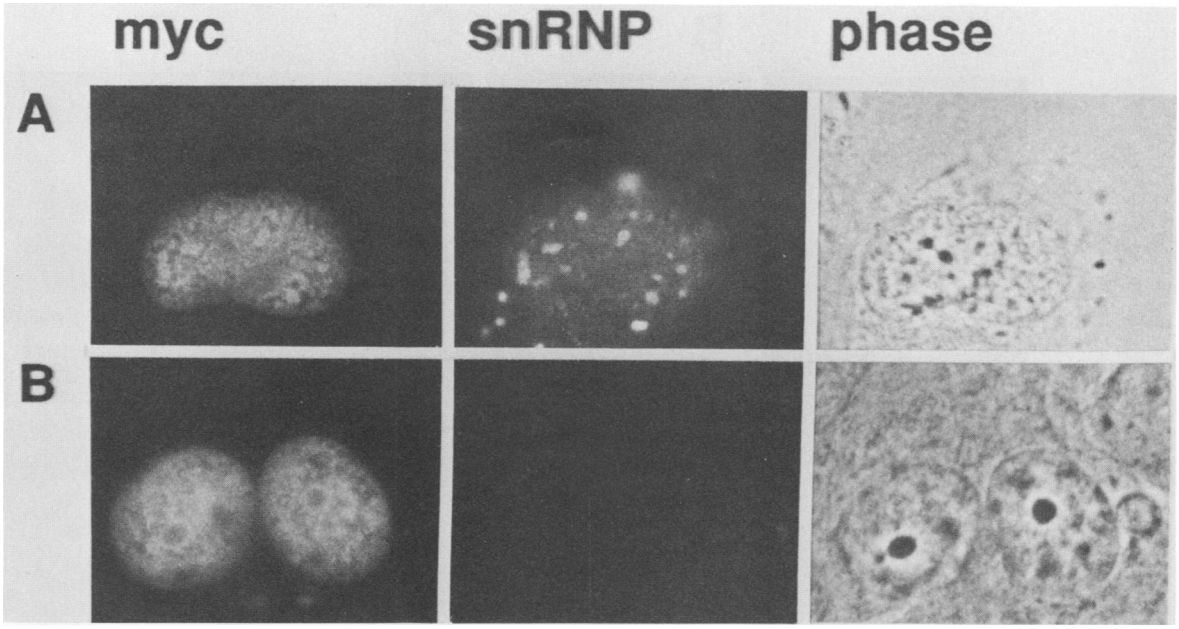


FIG. 3. Comparison of the distribution of c-*myc* and snRNPs in pSV-Tc-*myc*-transfected COS cells. The cells were fixed with methanol and double stained with anti-c-*myc* antiserum and anti-Sm antibodies. Before staining, the cells shown in panel B were treated with RNase A. Phase, Phase-contrast micrographs.

to stimulate transcription from transfected HSP70 promoters (26, 29), we were interested in examining the possible effects of the transiently expressed c-*myc* protein on the subcellular distribution of the endogenous HSP70. Therefore, the transfected cells were stained with two different monoclonal anti-HSP70 antibodies (N27 and C92), which gave similar results. Only a few nuclei of COS cells transfected with the pSV-*neo* control plasmid were positive for HSP70, but to our surprise, even 5% of the pSV-Tc-*myc* transfected cells displayed intense nuclear HSP70 fluorescence patterns similar to those seen with the c-*myc* antibodies in these cells (Fig. 4A to C). When the transfected cells were heat shocked before staining with the anti-HSP70 antibodies, a typical nucleolar fluorescence was obtained in most of the cells (Fig. 4D). However, the atypical distributions of HSP70 fluorescence described above were seen in heat-induced, pSV-Tc-*myc*-transfected cells with the same frequency as in the uninduced cells. Also, heat treatment had no effect on the distribution of c-*myc* fluorescence, as could be seen by comparison of c-*myc* staining in cells with or without prior heat treatment (data not shown).

This unexpected finding prompted us to study by double immunostaining the possible codistribution of c-*myc* and HSP70 antigens in the nuclei of the transfected cells. In about one-half of the cells displaying a diffuse c-*myc* fluorescence, the two antigens were distributed independently of each other also after heat induction, while in the other half, the c-*myc* and HSP70 proteins were found to colocalize (Fig. 5A). More strikingly, in almost all cells displaying either of the two c-*myc* accumulation patterns, HSP70 colocalized in phase-dense nuclear structures (Fig. 5B) whether the cells were heat shocked or not. Several control experiments, including a comparison of the frequencies of the distinct patterns stained by either one of the antibodies, confirmed that these patterns were not caused by antibody cross-reactions (data not shown). Also, when the anti-c-*myc* antibody was incubated with the corresponding c-*myc* peptide prior to staining, c-*myc* fluorescence was abolished while HSP70 fluorescence was unaffected (Fig. 5C). Similar results of colocalization were also obtained in COS cells transfected with the pSV-v-*myc* construct expressing the viral counterpart of c-*myc* (data not shown). By contrast, when COS cells were transfected with a construct expressing the closely related N-*myc* gene (Fig. 1A and C), most of the nuclei of N-*myc*-positive cells remained negative for HSP70 (Fig. 5D).

For examination of the generality of the codistribution of the c-*myc* and HSP70 proteins, two other cell lines, HeLa and 293, were used. Although fewer c-*myc*-positive cells were found than in COS cells, three similar patterns of c-*myc* fluorescence were observed. Double immunostaining of c-*myc*-transfected HeLa and 293 cells revealed that the c-*myc* and HSP70 proteins colocalized in these cells also. Furthermore, we observed frequencies of different fluorescence patterns similar to those in COS cells (Fig. 5E and data not shown). This indicates that the efficient replication of the SV40-driven c-*myc* constructs in transfected COS cells was not responsible for the nuclear translocation of HSP70 in the c-*myc*-positive cells.

**The major determinant for HSP70 translocation is encoded by the second exon of c-*myc*.** Because of the dissimilar results obtained for the c-*myc* and N-*myc* proteins, we wanted to study which domain of the c-*myc* protein mediates its colocalization with HSP70. Therefore, we prepared chimeric *myc* constructs by exchanging either of the two coding exons of human c-*myc* and N-*myc* genes with each other (Fig. 1A; see also Materials and Methods). When transfected into COS cells, the chimeric polypeptides were expressed at levels similar to those of their parent proteins (Fig. 1C). As expected, the electrophoretic mobilities of the chimeras were intermediate between those of the parental ones. Both c/N-*myc* and N/c-*myc* also localized to cell nuclei (data not shown).

Immunofluorescence staining with anti-HSP70 antibodies

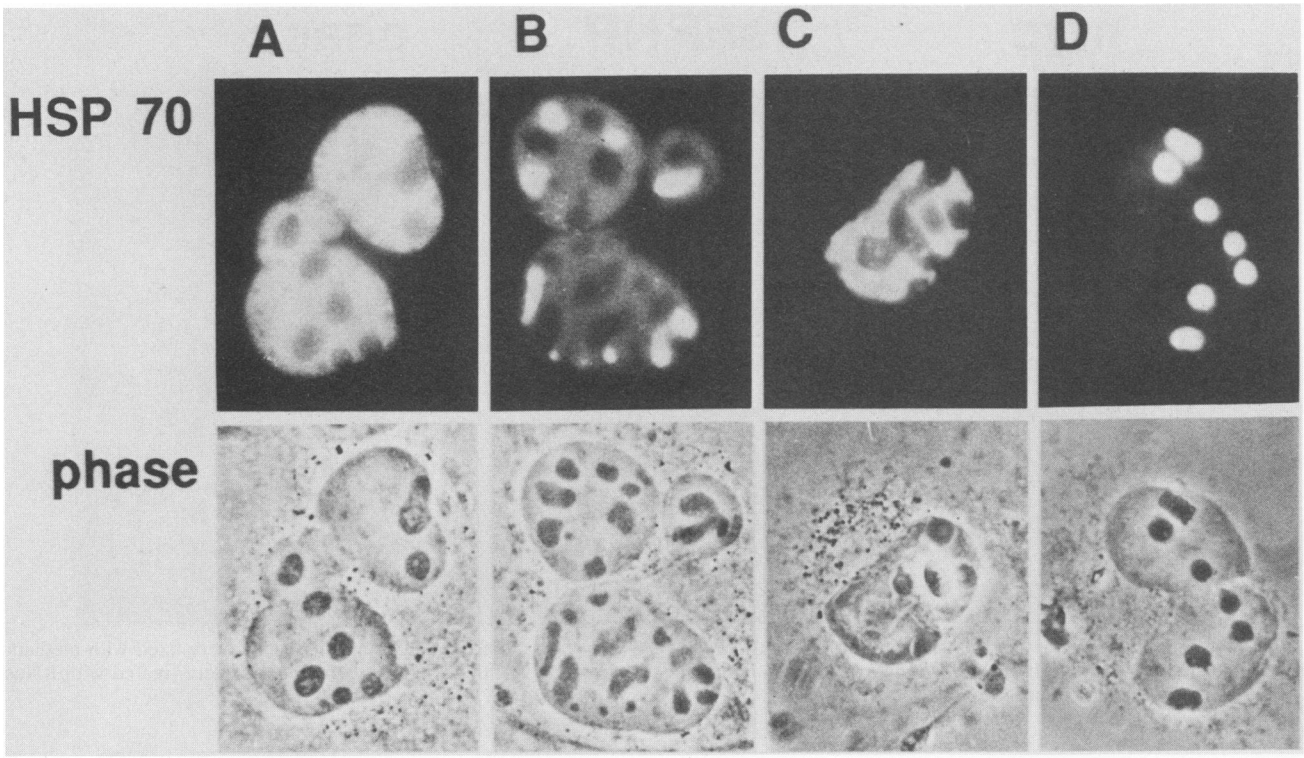


FIG. 4. Distribution of HSP70 in pSV-Tc-*myc*-transfected COS cells: HSP70 fluorescence and phase-contrast micrographs of COS cells fixed and stained with the C92 antibody 2 days after transfection. The cells shown in panel D were heat shocked at 42°C for 1 h and allowed to recover at 37°C for 2 h before fixation; cells in the other panels were grown at 37°C. The distinct atypical types of fluorescence were diffuse staining (A), small inclusions (B), and large inclusions (C). The nucleolar fluorescence typical for heat-shocked cells is shown in panel D.

revealed that the c/N-*myc* protein induced the nuclear translocation of HSP70, although not as efficiently as c-*myc*, while the N- and N/c-*myc* proteins showed much less activity. A graphic summary of the different patterns of HSP70 distribution in the nuclei of *myc*-transfected cells is shown in Fig. 6. These results indicate that the polypeptide sequences encoded by the second exon of the c-*myc* gene are involved in the redistribution of HSP70.

Figure 7 shows a summary of the frequencies of the different patterns of *myc* fluorescence in c-*myc*- or N-*myc*-transfected COS cells. Examination of the transfected cells for immunofluorescence showed that 18 h after transfection, the c-*myc* protein was mostly diffusely distributed, but thereafter, an increasing fraction of the c-*myc*-positive cells acquired small, phase-dense c-*myc* inclusions which later increased and coalesced to form large, less-phase-dense c-*myc*-containing structures (Fig. 7A). In contrast, the N-*myc* protein accumulated with a slower kinetics, which may explain the differences between the *myc* proteins in HSP70 staining. No large, confluent inclusions were seen in N-*myc*-transfected cells, even when they were analyzed 56 h after transfection (Fig. 7B), when the transient expression of both *myc* genes was maximal. HSP70 accumulated into nuclei with kinetics similar to those of the *myc* proteins, being most evident in cells with *myc* inclusions (compare the patterns of HSP70 and *myc* fluorescence 48 h after transfection in Fig. 6 and 7).

As an additional control, we transfected into COS cells a chimeric pyruvate kinase construct containing the sequences responsible for the c-*myc* nuclear translocation (12). The c-*myc*/pyruvate kinase fusion protein was efficiently expressed and translocated into the nucleus, but unlike in the case of the c-*myc* protein, the number of HSP70-positive nuclei was not increased (data not shown). The results with both N-*myc* and c-*myc*/pyruvate kinase suggest that the effect of c-*myc* on the distribution of HSP70 is not a general consequence of nuclear overexpression of any protein.

**HSP70 is detected also in nuclei of cells stably transformed by v-*myc* virus.** The nuclear translocation of HSP70 in response to overexpression of c-*myc* resembled the previously reported effect of the adenoviral E1A protein, which was shown to colocalize with HSP70 in heterogeneous nuclear structures, including nucleoli (62). Since in this report nuclear colocalization was seen in cells newly infected with adenovirus but not in cells stably expressing E1A, we were interested in comparing the effects of transient and stable *myc* overexpression. For this purpose, we used two lines of MC29 myelocytomatosis virus-transformed Q8 quail cells (7) stably expressing a *gag*-v-*myc* fusion protein (3). One of the cell lines did not contain a helper virus, while the other cell line had been superinfected with the ring-necked pheasant virus (RPV) known to enhance v-*myc* expression (7).

Staining of v-*myc*-overexpressing Q8 cells with anti-pan-*myc* or anti-v-*myc* antibodies revealed a predominantly diffuse nuclear distribution for the v-*myc* protein. However, in RPV-superinfected cells producing higher levels of v-*myc*, the protein accumulated in phase-dense nuclear inclusions similar to those seen in cells transiently transfected with c-*myc* (compare Fig. 8A and 5B). When Q8 cells were stained also with the C92 anti-HSP70 antibody, no reaction was obtained (Fig. 8A). Instead, when stained with the N27

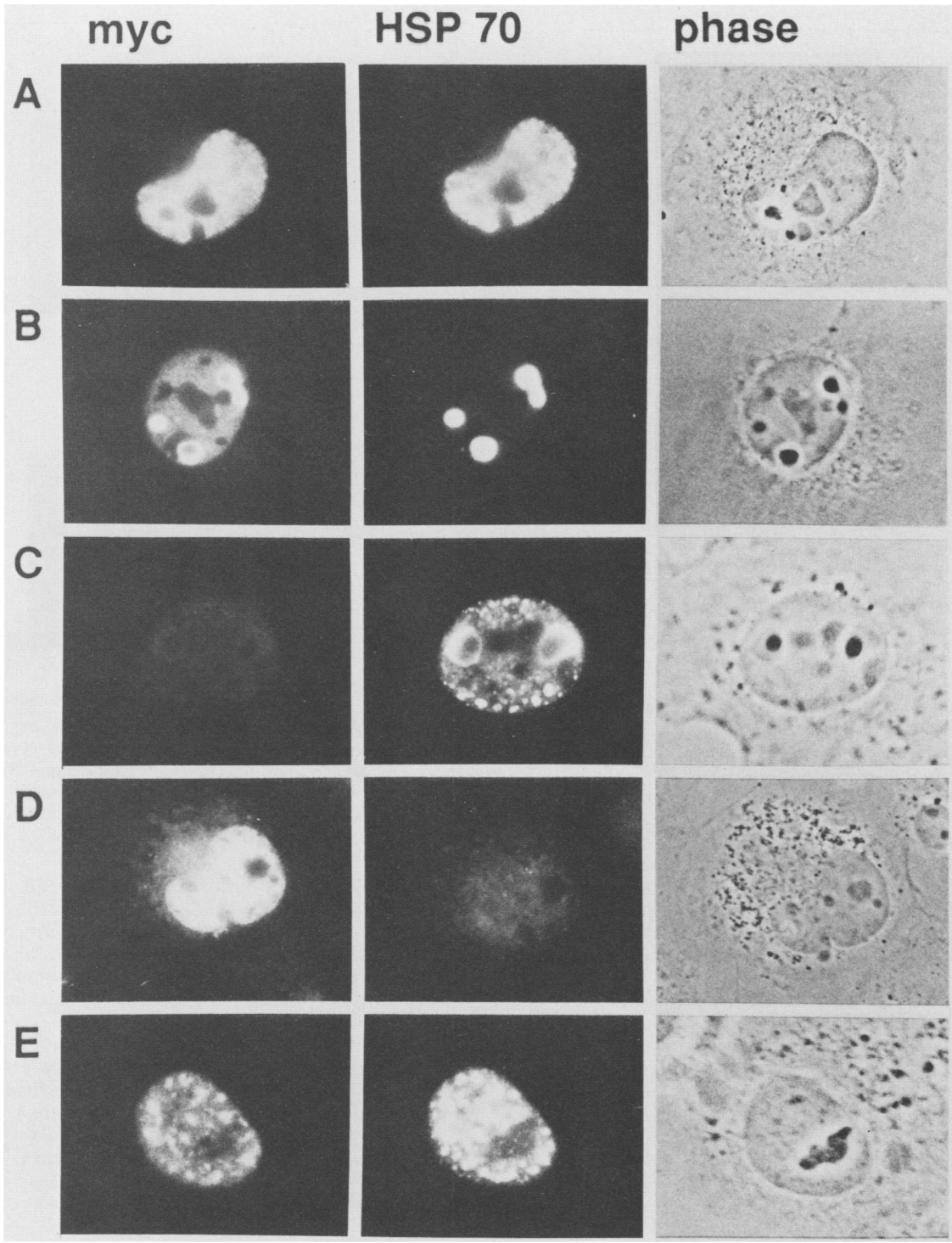


FIG. 5. Comparison of the distribution of c-*myc* and HSP70 proteins. COS (A to D) or HeLa (E) cells were transfected with either the pSV-Tc-*myc* (A to C and E) or the pSV-N-*myc* construct (D), fixed 2 days later, and double stained with anti-*myc* and anti-HSP70 (C92) antibodies. Prior to staining of cells in panel C, the anti-c-*myc* antibody was incubated with the corresponding c-*myc* peptide. Phase, Phase-contrast micrograph.

antibody, cells of both Q8 lines displayed a nuclear HSP70 fluorescence colocalizing with v-*myc* (Fig. 8B). The nuclear levels of HSP70 fluorescence varied depending on the amount of v-*myc* protein expressed and were most intense in RPV-superinfected cells with v-*myc* inclusions. The reactivity of the N27 antibody with quail samples was further confirmed by incubating uninfected QT6 quail cells at 45°C for 2 h, after which a nucleolar staining typical for heat-shocked cells was observed (Fig. 8C). Again, no cross-reactivity between the antibodies was detected. Thus, both

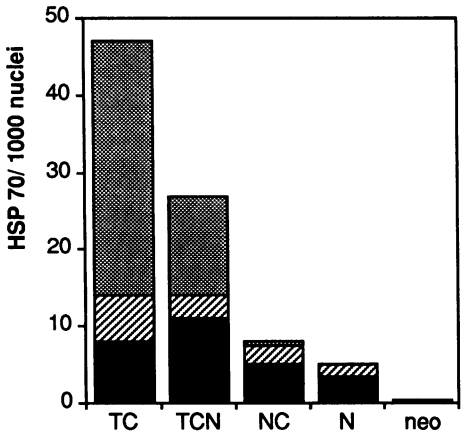


FIG. 6. Distribution of distinct patterns of HSP70 fluorescence among 1,000 COS cells transfected with the pSV-*neo* or pSV-*myc* constructs and fixed after 48 h. Symbols: ■, cells displaying a diffuse HSP70 fluorescence; ▨, cells displaying small inclusions; ▩, cells displaying large inclusions.

transient and stable overexpression of c-*myc* or its viral counterpart results in translocation of HSP70 into nuclear *myc*-containing structures.

## DISCUSSION

In several previous studies, the intranuclear distribution of the c-*myc* and v-*myc* proteins has been described as a diffuse or finely granular fluorescence which excludes nucleoli (3, 18, 54). However, in transiently transfected COS, HeLa, or 293 cells expressing large amounts of the c-*myc* protein, we also see two other staining patterns: small, amorphous, phase-dense inclusions or large, coalescent, less-phase-dense areas, both devoid of chromatin. Similar phase-dense nuclear inclusions containing the v-*myc* protein are present in MC29 myelocytomatosis virus-transformed quail cells superinfected with RPV. These structures may function as sites of accumulation of the excess *myc* protein. From the frequencies of the different staining patterns at various times after transfection, it can also be concluded that the development of the granular inclusions results from the accumulation of the c-*myc* protein in the transfected cells. However, since unsynchronized cells were used, the possibility of cell cycle-dependent variations in the distribution of *myc* protein cannot be excluded. Although very high levels of the c-*myc* protein have been shown to be cytotoxic (65), during our experiments the cells transfected with the SV40 early promoter-driven c-*myc* gene remained alive, as determined by trypan blue exclusion.

The *myc* proteins have been suggested to function as transcriptional regulators, in DNA synthesis, and in oncogenic transformation. Therefore, the *myc* proteins probably associate, directly or indirectly, with proteins involved in transcription or DNA replication. Yet very little is known about the proteins interacting with the *myc* proteins. Our double-label immunofluorescence results of cells expressing transiently introduced c-*myc* constructs revealed a previously unrecognized feature of c-*myc* overexpression. In many but not all c-*myc*-positive cells, the HSP70 heat shock protein was translocated into nuclear structures containing c-*myc* protein. The intranuclear distribution of HSP70, induced by c-*myc* in the absence of exogenous stress, was

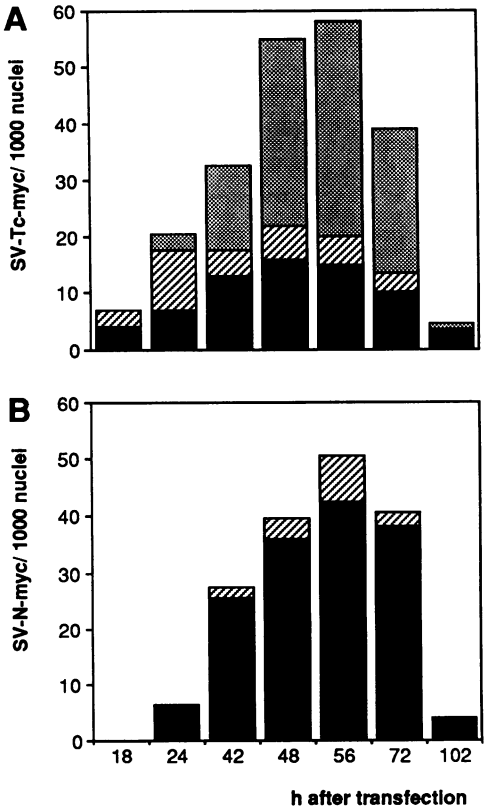


FIG. 7. Frequencies of distinct patterns of *myc* fluorescence among 1,000 COS cells transfected with pSV-Tc-*myc* (A) or pSV-N-*myc* (B) and fixed at various times after transfection. Symbols: ■, cells displaying a diffuse *myc* fluorescence; ▨, cells displaying small inclusions; ▩, cells displaying large inclusions.

clearly distinct both from the cell cycle-dependent interaction of HSP70 with the nuclei of unstressed cells during the S phase (40) and from the intranucleolar pattern obtained by heat shock treatment (45, 60, 61). Heat treatment of the transfected cells did not affect the distribution of the c-*myc* protein or its colocalization with HSP70. This observation, together with the recently published results on increased thermosensitivity of c-*myc*-transfected cells (35), suggests that overexpressed c-*myc* interferes with the normal heat shock response by preventing HSP70 from binding to its normal nuclear targets during the cellular stress response. Furthermore, treatment of methanol-fixed cells with RNase A had no effect on the distribution or intensity of either c-*myc* or HSP70 fluorescence in the nuclei. This observation was in agreement with the results of Eisenman and coworkers (14) but in conflict with the results of Spector and coworkers (54), who reported that the v-*myc* protein resides in RNase-sensitive nuclear structures in colocalization with snRNPs.

Thus, our results provide the first evidence that overproduction of a nuclear protein can cause translocation of a stress protein into the nucleus. The redistribution of HSP70 was seen when the two c-*myc* polypeptides were expressed together, as well as by the viral *myc* protein or the shorter c-*myc* polypeptide alone. Instead, the closely related N-*myc* protein did not significantly accumulate into c-*myc*-like inclusions or induce nuclear translocation of HSP70. The results obtained with the two chimeric *myc* constructs sug-

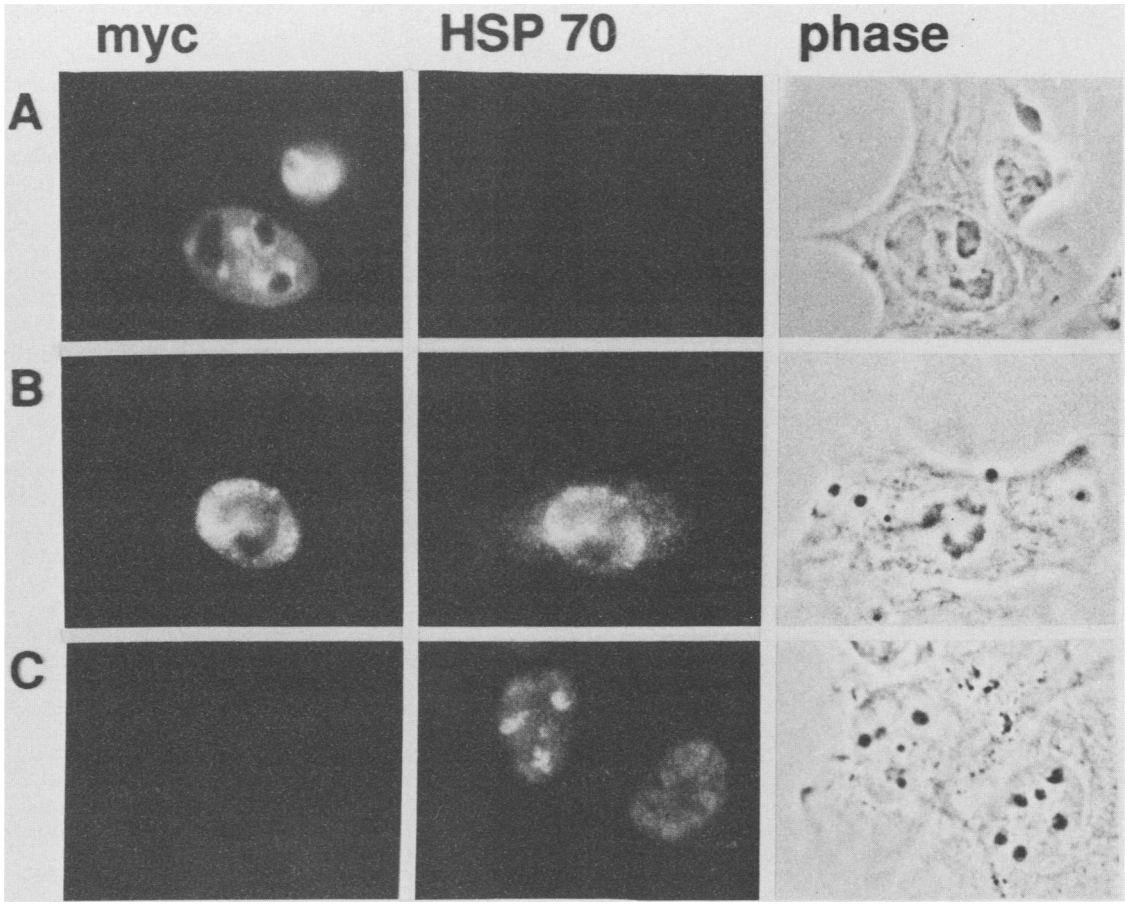


FIG. 8. Comparison of the distribution of v-*myc* and HSP70 proteins in quail fibroblasts. MC29 virus-transformed cells superinfected with RPV (A and B) or QT6 cells heat shocked at 45°C for 2 h (C) were double stained with anti-pan-*myc* antiserum and either C92 (A) or N27 (B and C) anti-HSP70 antibodies. Phase, Phase-contrast micrograph.

gest that the differences in the distribution of the c-*myc* and N-*myc* proteins and their distinct abilities to affect the subcellular localization of HSP70 result from differences in the polypeptide structures encoded by the second exons of the *myc* genes. Furthermore, when we tested various c-*myc* deletion mutants (56) by transient transfection, we found that large deletions within the second but not the third exon of c-*myc* resulted in greatly reduced accumulation of the c-*myc* protein into nuclear inclusions and accordingly in very few HSP70-positive nuclei (32a). Since comparable amounts of c-*myc* protein are produced from the different constructs in COS cells (56), the accumulation of c-*myc* together with HSP70 is apparently dependent on polypeptide sequences encoded by the second exon of c-*myc*.

The HSP70 protein binds to denatured cellular proteins; it seems to catalyze their resolubilization through an ATP-dependent mechanism (45, 61). HSP70 has also been shown to transiently interact with nascent polypeptides, facilitating protein folding and assembly (5). Furthermore, HSP70 may bind proteins which have not reached their normal cellular compartments (39). However, in our study a recombinant pyruvate kinase carrying the nuclear translocation signal of the c-*myc* protein (12) did not affect the distribution of the HSP70 protein, although this normally cytoplasmic protein was translocated into the nucleus. This suggests that overexpression of any protein per se does not lead to increased synthesis and/or translocation of the HSP70 protein.

Transfection of cells with c-*myc*, adenovirus E1A, or polyomavirus middle T expression vectors or infection of cells with viruses carrying these genes has been shown to stimulate transcription from the HSP70 promoter (29, 30, 43, 64). According to Kaddurah-Daouk and coworkers (26), the stimulatory effects of elevated c-*myc* expression on the HSP70 promoter are transmitted through sequences further upstream of the heat- and serum-responsive elements, suggesting that the transactivation of the HSP70 promoter does not simply represent a stress response to the overexpression of the c-*myc* protein.

The colocalization of c-*myc* and HSP70 in phase-dense nuclear structures raises the interesting possibility that cells have a natural mechanism to modulate the levels of functional c-*myc* protein. Thus, only a certain amount of the c-*myc* protein may be maintained in a soluble form, and the rest is stored in nuclear structures in association with HSP70. This kind of regulation may be important if cells can tolerate only modest fluctuations in c-*myc* levels. If this is the case, an interesting problem is whether HSP70 is involved in the accumulation of excess c-*myc* protein into nuclear inclusions or whether it is the c-*myc* inclusions that act as substrates for HSP70. It is also possible that c-*myc* with its nuclear translocation signal interacts with HSP70 already in the cytoplasm and then brings the putative complex into the nucleus. Although slightly increased levels of HSP70 protein synthesis were detected in c-*myc*- and v-*myc*-

overexpressing cells but not in cells overexpressing N-*myc*, no evidence for a direct complex between *myc* and HSP70 proteins was obtained in our immunoprecipitation conditions (unpublished results).

The colocalization of c-*myc* and HSP70 presented here developed through specific patterns during the transient transfection experiments, being most evident in cells displaying c-*myc* protein-containing inclusions. Similar interactions have also been shown to occur between the adenoviral E1A antigen and HSP70: in some but not all HeLa cells infected with adenovirus, the newly synthetized E1A antigens colocalize with HSP70 in nucleoli and in other phase-dense nuclear structures (62). Instead, constitutive expression of E1A in 293 cells does not affect the cellular distribution of HSP70 (62). This is in contrast to our results with MC29 virus-transformed quail cells, in which stably expressed v-*myc* protein colocalizes with HSP70 in the nuclei. The nuclear levels of HSP70 clearly depend on the amount of v-*myc* protein expressed, being again highest in cells with v-*myc* inclusion. This observation raises the interesting possibility that chronic overexpression of the c-*myc* protein in many tumors also is associated with increased nuclear levels of HSP70. It remains to be determined whether nuclear expression of HSP70 has a role in tumors induced by c-*myc* and v-*myc*.

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