MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3080–3087
0270-7306/88/083080-08$02.00/0

Vol. 8, No. 8

c-*myc* Can Induce Expression of G_0/G_1 Transition Genes

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Received 13 November 1987/Accepted 1 April 1988

The human c-*myc* oncogene was linked to the heat shock-inducible *Drosophila* hsp70 promoter and used to stably transfect mouse BALB/c 3T3 cells. Heat shock of the transfectants at 42°C followed by recovery at 37°C resulted in the appearance of the human c-*myc* protein which was appropriately localized to the nuclear fraction. Two-dimensional analysis of the proteins of density-arrested cells which had been heat shock treated revealed the induction of eight protein species and the repression of five protein species. All of the induced and repressed proteins were nonabundant. cDNA clones corresponding to genes induced during the G_0/G_1 transition were used as probes to assay for c-*myc* inducibility of these genes. Two anonymous sequences previously identified as serum inducible (3CH77 and 3CH92) were induced when c-*myc* was expressed. In response to serum stimulation, 3CH77 and 3CH92 were expressed before c-*myc* mRNA levels increased. However, in response to specific induction of c-*myc* by heat shock of serum arrested cells, 3CH77 and 3CH92 mRNA levels increased after the rise in c-*myc* mRNA. Therefore, we hypothesize that abnormal expression of c-*myc* can induce genes involved in the proliferative response.

A key role for the c-*myc* oncogene in cellular proliferation has long been postulated. However, circumstantial evidence relating c-*myc* to proliferation primarily includes the fact that c-*myc* expression is elevated in proliferating cells relative to that in quiescent cells and in cells stimulated by mitogens (4, 24). Attenuation of the c-*myc* protein with antisense oligodeoxynucleotide (20) or with anti-c-*myc* antibody (22) is reported to prevent cellular DNA replication. However, antibodies to c-*myc*, once purified of a DNA polymerase inhibitor, do not appear to affect replication (18). Therefore, a role for c-*myc* in DNA synthesis is unclear. In addition, c-*myc* can contribute toward neoplasia. It has been demonstrated that the c-*myc* gene, constitutively expressed after transfection into primary rat embryo cells, can immortalize primary cells (27). This immortalization function, in cooperation with the *ras* oncogene, can transform cells. In this way, c-*myc* is functionally similar to the adenovirus E1a protein (35). In many leukemias, elevated expression of the c-*myc* gene is observed (3, 33). In Burkitt's lymphoma, for example, both rearranged and unrearranged forms of the c-*myc* gene can be found in which c-*myc* is transcriptionally activated (11, 12, 14, 15). Other neoplastic tissues such as HL60 and COLO 320 cells contain amplified c-*myc* genes which are concomitantly overexpressed (2, 8, 13).

One obvious implication of the key role of c-*myc* is that it may serve to activate and/or repress other cellular genes to effect its role in proliferation or the neoplastic processes. That c-*myc* is a nuclear protein and has been reported to have DNA-binding ability (1, 19, 37) is consistent with such a hypothesis. Recently, c-*myc* has been reported to act as a positive regulator of the human hsp70 promoter and as a negative regulator of the mouse metallothionein I promoter in trans within transiently transfected mammalian cells (23). Thus, although it is not known whether c-*myc* acts directly on these genes, it is confirmed that c-*myc* could act as a regulator of cellular gene activity. However, the role of hsp70 and metallothionein I in proliferation or in neoplasia is not clear. We report here that the c-*myc* gene, under the inducible control of the *Drosophila* hsp70 promoter, is capable of inducing some genes previously identified as being part of the G_0/G_1 transition. The implications of this observation are discussed.

MATERIALS AND METHODS

Plasmid construction. The *Drosophila* hsp70 promoter in pVC18 was kindly provided by Victor Corces (Johns Hopkins University). Approximately 490 base pairs (bp) of this promoter (*Xba*I-*Bam*HI fragment) was positioned 3′ to a 285-bp *Eco*RI-*Xba*I fragment containing the simian virus 40 origin of replication but lacking the enhancer sequences (provided by E.S.P. Reddy). The composite plasmid pSVhsp contained the origin and hsp70 promoter between the *Eco*RI and *Bam*HI sites of pBR322. The 775-bp *Eco*RI-*Bam*HI fragment of pSVhsp was excised by partial *Bam*HI digestion after the *Eco*RI site was filled in with Klenow fragment. This fragment was inserted 5′ to the two-exon (lacks exon 1) and three-exon human c-*myc* genes in the plasmids pMC41/S2 and pMC41/S3, respectively. The insertion was ligated into the *Nru*I and *Bam*HI sites of those plasmids. The ligation of the filled-in *Eco*RI site and the blunt-end *Nru*I site created a new *Eco*RI site at the 5′ end of the simian virus 40 origin. Another *Eco*RI site 3′ to the c-*myc* third exon provided the ability to excise the entire construct and to insert it into the *Eco*RI site of the plasmid pAT153 (Amersham Corp.). The resulting two-exon and three-exon c-*myc* genes linked to the *Drosophila* hsp70 promoter (termed pATS2 and pATS3, respectively) were used to transfect BALB/c 3T3 cells. The *Eco*RI-*Bam*HI fragment of pSVhsp was also transferred to pAT153, resulting in plasmid pATSVhsp, which was used to transfect BALB/c 3T3 cells.

DNA transfections. BALB/c 3T3 cells were used to create permanent cell lines bearing the hsp-*myc* constructs. Cells were transfected by the calcium phosphate coprecipitation method (17). Briefly, 10 μg of the construct plasmid DNA was cotransfected with the pSV2neo plasmid. At 48 h after transfection, selection in 400 μg of G418 per ml was started. Individual G418-resistent clones were picked about 2 weeks later. Expanded clones were screened by Southern blot hybridization analysis for the presence of the construct and

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by Northern blot hybridization analysis for heat shock-inducible expression of the transfected c-*myc* gene. Clone A3-1 contains the two-exon *myc* gene (pATS2), clone A5-9 contains the three-exon *myc* gene (pATS3), and clone A7-4 contains the plasmid pATSVhsp. Cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum and antibiotics. Serum-arrested cells were maintained in 0.5% serum.

Heat shock conditions. Transfectant clones were heat shocked for 2 h precisely at 42°C in a slowly shaking water bath. Cells were in tightly closed 75-cm^2 flasks. Post-heat shock recovery was at 37°C in a 5% CO_2 atmosphere for 2 h before harvest, unless otherwise indicated.

DNA and RNA analysis. Genomic DNA was isolated and analyzed by standard techniques. RNA was isolated by harvesting cells in guanidine isothiocyanate (Bethesda Research Laboratories, Inc.) and pelleting the RNA through a CsCl cushion (5). RNAs were fractionated on agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose in 20× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate [pH 7.4]) (31). Equal amounts were loaded in each lane as observed by ethidium bromide staining. Gel-purified DNA fragments corresponding to the indicated probes were labeled by nick translation (34). Hybridizations were carried out in 50% formamide, 4× SSPE (1× SSPE is 180 mM NaCl, 10 mM, NaH_2PO_4, 1 mM EDTA, [pH 7.4]), 5× Denhardt solution (1× Denhardt solution is 0.02% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 100 μg of denatured DNA per ml, 0.1% sodium dodecyl sulfate (SDS), and 1×10^6 to 2×10^6 cpm of probe per ml. (All probes were gel-purified fragments devoid of vector sequences.) Washes were done in 0.2× SSPE–0.1% SDS at 42 to 55°C followed by autoradiography of the filters at −80°C.

Protein analysis. For analysis of protein, cells were labeled in vivo with [^{35}S]methionine. A 75-cm^2 flask of cells was heat shocked for 2 h at 42°C and then allowed to recover at 37°C. During the last 30 min of the 2-h recovery period the medium was changed to include 1 mCi of [^{35}S]methionine. For immunoprecipitation, cells were harvested and washed twice in cold phosphate-buffered saline and then lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 2% aprotinin, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM EDTA]. Trichloroacetic acid-insoluable material (10^7 cpm) was immunoprecipitated with monoclonal antibody to c-*myc* peptide in the presence or absence of unlabeled competing peptide. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (26) followed by fluorography (28). For two-dimensional gel electrophoresis, cells were lysed in hot 3% SDS and 5% 2-mercaptoethanol. Isoelectric focusing in the first dimension was performed with ampholines at pH 3 to 10. The second dimension was performed in a 12.5% polyacrylamide slab gel. Analysis was performed by Protein DataBases, Inc. (Huntington Station, N.Y.).

Cell fractionation was performed by a modification (Fujiwara, unpublished observations) of the procedure of Evan and Hancock (16) in which isotonic conditions were employed. All steps were carried out at 4°C, and all reagents except phosphate-buffered saline included protease inhibitors (1% aprotinin, 0.4 mM phenylmethylsulfonyl fluoride, 0.01 mM *N*-tosyl-L-lysine chloromethyl ketone, 0.1 mM tolylsulfonyl phenylalanyl chloromethyl ketone). Labeled cells were washed twice in cold phosphate-buffered saline and Dounce homogenized 30 times in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.8)–5 mM KCl–5 mM $MgCl_2$–0.05% Nonidet P-40–0.25 M sucrose and then centrifuged at 1,000 × *g* for 5 min. The crude nuclear pellet was saved for further processing (see below). The supernatant was centrifuged at 100,000 × *g* for 60 min. The supernatant constituted the cytoplasmic fraction. The pellet was lysed in RIPA buffer and centrifuged at 100,000 × *g* for 35 min. The supernatant constituted the membrane fraction. The crude nuclear pellet was suspended in 20 mM HEPES (pH 6.8)–5 mM KCl–5 mM $MgCl_2$–0.05% Nonidet P-40–0.1% sodium deoxycholate–0.25 M sucrose and centrifuged at 1,000 × *g* for 5 min. The supernatant constituted the nuclear wash. The pellet was lysed in RIPA and centrifuged at 100,000 × *g* for 30 min. The supernatant constituted the nuclear fraction. Before immunoprecipitation, the cytoplasmic and nuclear wash fractions were adjusted to 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 0.5% deoxycholate (pH 7.5).

RESULTS

Creation of stably transfected cell lines which express c-*myc* under heat shock promoter control. The *Drosophila* hsp70 promoter was linked to the human c-*myc* genes containing *myc* exons 1 through 3 or only exons 2 and 3 (Fig. 1). These constructs were cotransfected with pSV2neo into BALB/c 3T3 cells by the calcium phosphate precipitation method (17). G418-resistant clones were expanded and tested for heat shock-inducible c-*myc* RNA expression. Clones derived from the two-exon construct (clone A3-1) and the three-exon construct (A5-9) were selected for further analysis because they were shown to express heat shock-inducible *myc* RNA (data not shown). In addition, a third construct containing the hsp70 promoter but devoid of any c-*myc* gene was used to create cell line A7-4, which was used as a negative control.

A Southern analysis of clones A3-1 and A5-9 is shown in Fig. 2. Genomic DNA from parental cell line BALB/c 3T3 and transfectants A3-1 and A5-9 along with their respective constructs were cleaved with *Eco*RI (Fig. 2A) or *Pst*I (Fig. 2B). The DNAs were transferred to nitrocellulose and hybridized with a human c-*myc* probe under high stringency. In both the two-exon and three-exon transfectants, the majority of the integrated construct appears to be unaltered. Approximately 10 to 50 copies per genome have been integrated. A similar analysis of the negative control cell line A7-4 shows that the construct containing hsp70 promoter but lacking a c-*myc* gene was also stably integrated (data not shown).

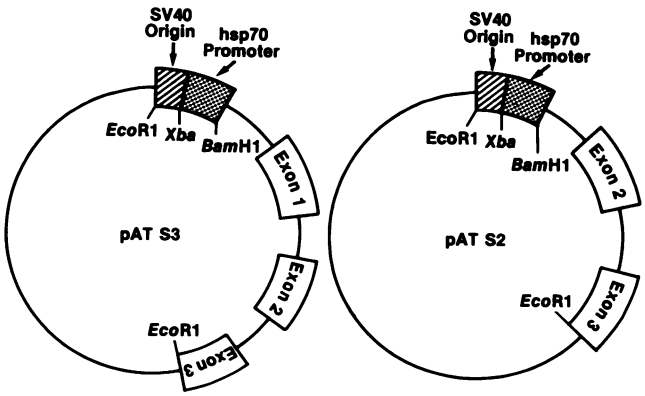

FIG. 1. Schematic representation of the three-exon and two-exon hsp-*myc* constructs used to make the heat shock-inducible cell lines. SV40, Simian virus 40.

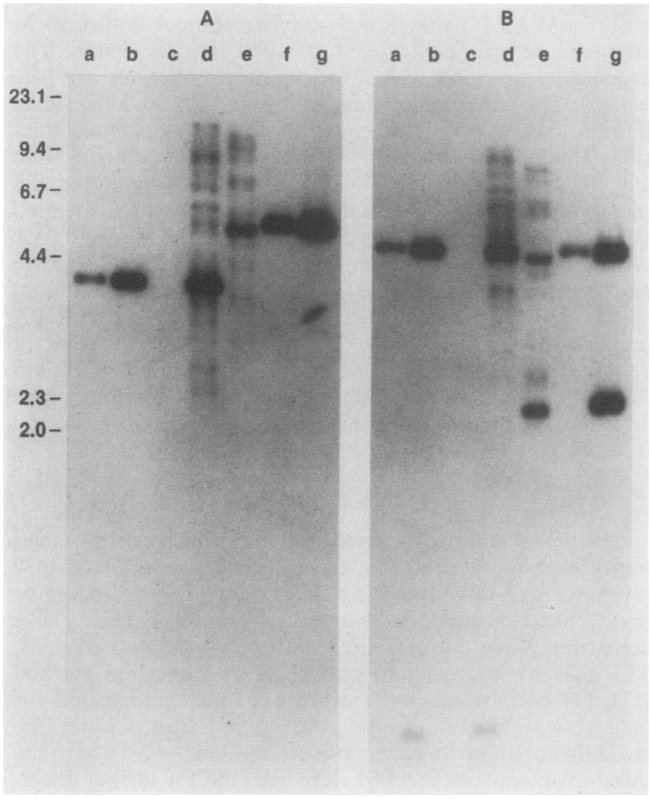

FIG. 2. Southern blot hybridization analysis of cell lines harboring hsp-*myc* constructs. (A) *Eco*RI digest; (B) *Pst*I digest. Lanes: a and b, pATS2 plasmid (10 and 50 gene copy equivalents, respectively); c, parental cell line BALB/c 3T3 genomic DNA; d, cell line A3-1 genomic DNA (BALB/c 3T3 cells transfected with pATS2); e, cell line A5-9 genomic DNA (BALB/c 3T3 cells transfected with pATS3); f and g, pATS3 plasmid (10 and 50 gene copy equivalents, respectively). Samples of 15 μg of genomic DNA per lane were used. The blot was probed with a 5.87-kbp DNA fragment containing the human c-*myc* gene, including exons 1, 2, and 3. Molecular size markers are in kilobase pairs.

Heat shock inducible expression of c-*myc* protein. The two heat shock-inducible lines A3-1 and A5-9 were originally identified by assaying for c-*myc* mRNA expression (see Fig. 7). Subsequent analysis revealed that *myc* RNA was detectable immediately at the conclusion of a 2-h heat shock at 42°C and persisted at a high level for at least 4 h, declining to undetectable levels by 8 h post-heat shock (data not shown). Endogenous hsp70 mRNA expression exactly paralleled this time course (data not shown). To assess the expression of the c-*myc* protein, cells were labeled with [^{35}S]methionine, and samples were immunoprecipitated during the post-heat shock recovery period with monoclonal antibody to c-*myc* peptide. c-*myc* protein from cell line A3-1 was readily detected by 1 h after heat shock and persisted for at least 4 h (Fig. 3). The immunoprecipitation of the induced c-*myc* protein is specifically inhibited by the inclusion of c-*myc* peptide. Another protein migrating at M_r 75,000 may share epitopes with c-*myc*, but it is not induced upon heat shock. In fact, it appears to be sensitive to heat shock. The M_r 75,000 protein might also form a complex with c-*myc*. However, since M_r 75,000 fractionates with the cytoplasmic fraction (Fig. 4), whereas c-*myc* is in the nuclear fraction, such a complex is likely to be nonphysiological.

c-*myc* has been widely reported to be localized to the nucleus (1, 19). The induced c-*myc* protein was present only

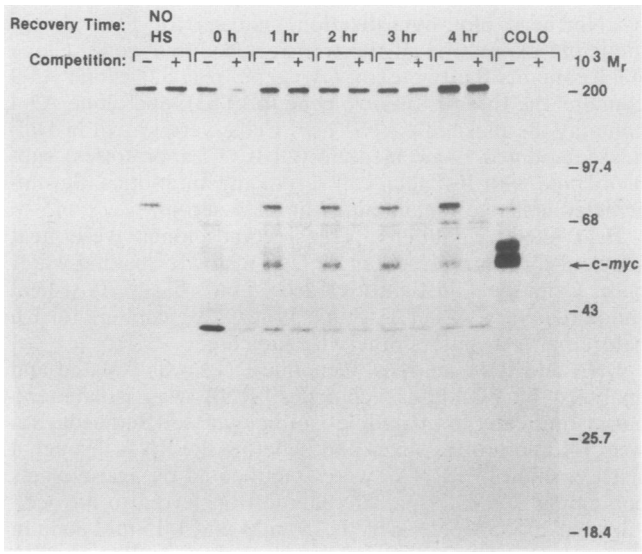

FIG. 3. Time course of c-*myc* protein expression after a 2-h heat shock at 42°C. Cells were labeled with 1 mCi of [^{35}S]methionine during the final 30 min of the heat shock or recovery period. [^{35}S]methionine-labeled A3-1 cells were lysed in RIPA buffer, and samples (10^7 trichloroacetic acid-insoluable cpm) were immunoprecipitated with monoclonal antibody to c-*myc* peptide. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Competition was with (+) or without (−) c-*myc* peptide. COLO refers to human colon carcinoma cell line COLO 320. Molecular size markers are M_r 200,000, 97,400, 68,000, 43,000, 25,700, and 18,400.

in heat shocked cells containing the transfected c-*myc* gene; it was localized exclusively to the nuclear fraction (Fig. 4). Again, the c-*myc* peptide specifically competed with immunoprecipitation.

c-*myc* expression and induction and repression of other proteins. Since c-*myc* is known to be localized to the nucleus (1, 19) and to have the ability to bind DNA (37), it has been postulated that the function of c-*myc* is to regulate the expression of other genes. As a way of assessing, broadly, changes in gene expression which accompany heat shock induction of c-*myc*, two-dimensional gel electrophoresis of in vivo [^{35}S]methionine-labeled clone A3-1 cells was performed. Three samples were run for comparison: A3-1 clone (heat shock induced), A3-1 clone (uninduced), and A7-4 clone (heat shock treated). Figure 5 shows the complete gel of the heat shock-induced A3-1 clone. Boxed areas highlight the labeled protein spots whose expression was increased or decreased concomitantly with c-*myc* expression compared with uninduced clone A3-1. We were not able to identify a specific candidate protein spot as being the heat shock-induced c-*myc* protein, probably because it is a very rare species. Alterations due to heat shock alone were determined by comparison to the A7-4 clone sample and were not scored. Figure 6 shows a direct comparison of the boxed areas in uninduced and induced clone A3-1. Again, differences attributable to heat shock alone were not scored. From this analysis, it is clear that some proteins were coinduced with *myc* (eight), whereas others were repressed (five). Table 1 shows a quantitation of the levels of induction or repression for each protein spot. All of the detectable proteins whose expression was altered upon c-*myc* induction were nonabundant (about 0.1 to 0.01% of the protein synthesized during the labeling period). None of the altered

TABLE 1. Quantitation of induced and repressed proteins

Spot no.	M_r	pI	37°C (ppm)[a]	HS (ppm)
36	17,000	4.00	409.6	46.6
1137	26,400	4.97	293.3	639.5
1217	40,700	5.20	319.2	158.1
1635	78,700	5.09	139.6	18.5
2612	85,200	5.47	169.2	392.9
3118	29,700	5.82	226.8	550.5
4445	55,200	5.87	966.3	474.5
5425	59,600	6.35	77.4	253.4
5441	54,800	6.23	1,666.5	346.2
5442	54,800	6.31	77.7	405.7
7524	77,000	>7.00	69.0	318.8
8120	30,300	>7.00	76.3	285.0
8553	77,500	>7.00	45.3	95.7

[a] ppm, Parts per million (in arbitrary units).

protein species corresponded to a previously identified protein in an NIH 3T3 cell protein data bank (Protein Data-Bases, Inc.). It has been recently shown that c-*myc* can induce the heat shock locus itself (23, 25). However, the abundant hsp70 protein is not counted in this method of assay, since heat shock itself is the mode of induction and therefore is also induced in the negative control cell line A7-4.

c-*myc* induction and the expression of some G_0/G_1 transition genes. c-*myc* expression has been shown to increase in cells which are stimulated to mitogenic activity (24). c-*myc* is one of a class of several genes, including c-*fos* and other, as yet uncharacterized, genes, whose expression is induced upon stimulation of resting cells with serum or platelet-derived growth factor (6, 7). It was of interest, therefore, to test directly whether c-*myc* induction could cause expression of other G_0/G_1 transition genes. Cell line clones A7-4, A3-1, and A5-9 were grown to confluence and then shifted down to 0.5% serum for 3 days. Cells were heat shock treated at 42°C for 2 h or left at 37°C. After a 2-h post-heat shock recovery period at 37°C, total cellular RNA was isolated and analyzed by Northern (RNA) hybridization. Of eight G_0/G_1 transition genes tested (3CH61, 3CH77, 3CH92, 3CH96, and 3CH134 [29]; 2F1, 4F1, and 2A9 [21]), two were expressed in response to c-*myc* induction. Figure 7 shows a Northern hybridization analysis probed with the two sequences which respond to c-*myc*. We conclude that 3CH77 and 3CH92 are expressed in response to c-*myc* induction and not in response to heat shock because the negative control cell line, A7-4, did not express or only marginally expressed these mRNAs in response to heat shock (Fig. 7, lanes a). On the other hand, only where c-*myc* was induced were 3CH77 and 3CH92 also induced, and there was an appropriate correlation between the amount of 3CH77 and 3CH92 mRNAs and the amount of c-*myc* mRNA expressed. Probes 3CH77 and 3CH92 hybridized to mRNAs of 3.6 and 2.2 kilobases, respectively (Fig. 7A and B); Fig. 7C shows the induction of c-*myc* in response to heat shock.

Lau and Nathans (29, 30) had previously explored the temporal expression of the G_0/G_1 transition genes. Interestingly, they demonstrated that expression of 3CH92 and 3CH77 precedes c-*myc* expression when arrested BALB/c 3T3 cells are stimulated with serum. The Northern analysis in Fig. 8 demonstrates the temporal appearance of 3CH77, 3CH92, and the endogenous c-*myc* gene in response to serum stimulation. This result is in agreement with that of Lau and Nathans but is in apparent conflict with the concept that these genes can be induced by c-*myc*. Figure 9 shows the temporal expression 3CH77, 3CH92, and the transfected c-*myc* mRNAs in response to heat shock. Under these conditions, c-*myc* expression did precede that of 3CH77 and 3CH92. (Again, as in Fig. 8, 3CH92 showed a slight induction in response to heat shock alone.) The significance of this is discussed below. Another difference between serum in-

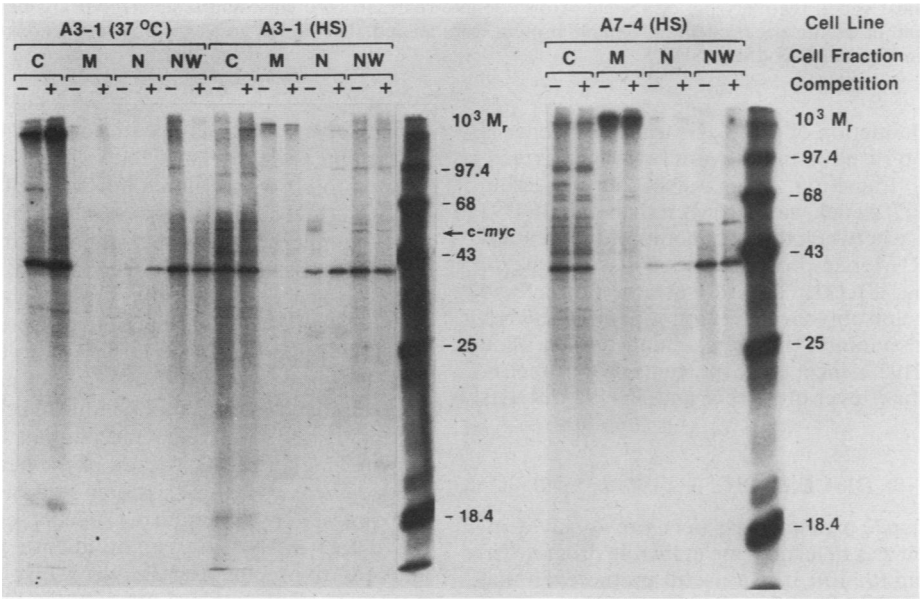

FIG. 4. Subcellular localization of heat shock (HS)-induced c-*myc* protein. Cells were labeled with 1 mCi of [^{35}S]methionine during the final 30 min of the 2-h post-heat shock recovery period. [^{35}S]methionine-labeled cells (A3-1 and A7-4 as indicated) were fractionated (10^7 trichloroacetic acid-insoluable cpm), and samples were immunoprecipitated with monoclonal antibody to c-*myc* peptide. Cells were either heat shocked at 42°C or left at 37°C as indicated. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Competition was with (+) or without (−) c-*myc* peptide; subcellular fractions are cytoplasmic (C), membrane (M), nuclear (N), and nuclear wash (NW). Molecular weight markers are M_r 97,400, 68,000, 43,000, 25,700, and 18,400.

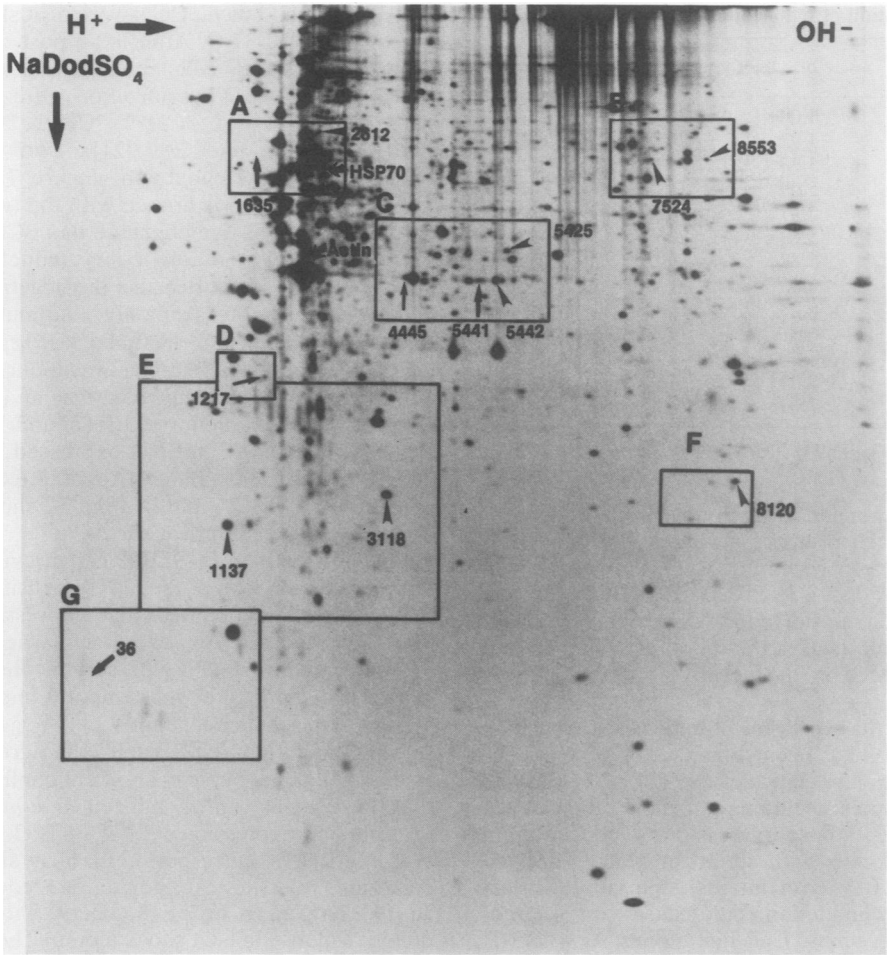

FIG. 5. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled A3-1 cells induced by heat shock. Cells were labeled for the last 30 min of a 2-h post-heat shock recovery period. Small arrows denote repressed proteins, and large arrowheads denote induced proteins. Samples of 300,000 cpm of trichloroacetic acid-insoluable protein were loaded. The boxed areas (A through G) are compared in uninduced versus induced cells in Fig. 6. $NaDodSO_4$, SDS.

duction and c-*myc* induction of the G_0/G_1 transition genes is that serum induction resulted in an overall stronger expression of the G_0/G_1 transition genes; also, during serum induction the 3CH77 signal was stronger than the 3CH92 signal. One similarity between the two modes of induction is that the 3CH92 mRNA rise precedes the respective rise of 3CH77 mRNA (Fig. 8) (31, 32). It was not possible to determine whether anisomycin inhibition of c-*myc* mRNA translation would also inhibit the c-*myc*-induced expression of 3CH77 and 3CH92, since these reagents also superinduced the steady-state level of 3CH77 and 3CH92 mRNAs (data not shown).

DISCUSSION

The *Drosophila* hsp70 promoter has been previously demonstrated to function as a heterologous inducible promoter in a mammalian system (9, 10). It is a useful promoter in that linked genes can be highly induced, whereas there is no detectable basal expression. We have produced stably transfected cell lines in which the transfected c-*myc* gene can be specifically induced in response to heat shock. Under the conditions employed (confluence or serum arrest or both), transcripts from the endogenous c-*myc* gene are not detected. *myc* protein is produced from the exogenous gene and is appropriately localized to the nucleus.

myc protein expression follows the induction of its mRNA by 1 to 2 h. This is most probably due to the fact that heat shock temporarily disrupts ongoing mRNA translation in favor of translation of endogenous heat shock mRNAs (32, 36). Non-heat shock mRNAs are again translated during the post-heat shock recovery period, and presumably this is also the first opportunity for translation of the heat shock-induced c-*myc* mRNA. The levels of c-*myc* protein immunoprecipitated by heat shock-induced cells is less than would be anticipated relative to the amount of mRNA. It is not known whether this is a result of the heat shock stress itself or represents posttranscriptional or translational attenuation of expression by other means such as secondary structure of the c-*myc* mRNA. It is not due to the inability of the antibody to precipitate all of the c-*myc* protein, since larger quantities of protein are precipitated from COLO 320 cells (Fig. 3). One way of assessing the effect of c-*myc* expression is to look for changes in the array of cellular proteins as a function of c-*myc* induction. Two-dimensional gel electrophoresis (Fig. 5 and 6; Table 1) reveals that the protein species whose expression is modulated by c-*myc* are rare

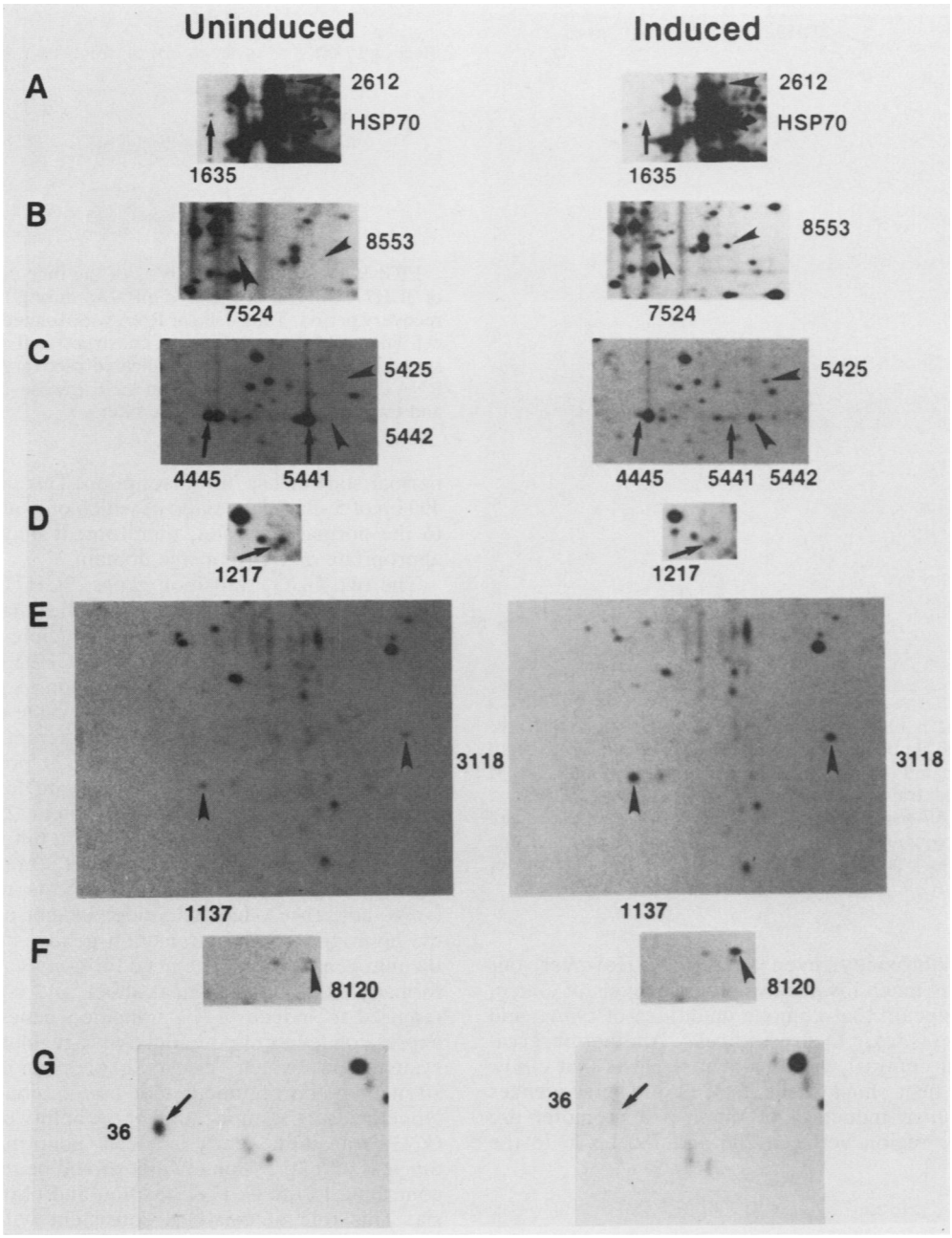

FIG. 6. Comparison of changes in protein expression in uninduced versus induced cells. The comparable areas from two-dimensional gels of uninduced and induced A3-1 cells are directly compared. Small arrows denote repressed proteins; large arrowheads denote induced proteins.

species (0.01 to 0.1% of the protein synthesized during the labeling period) implying that the effect of c-*myc* may be subtle in terms of levels of gene expression. In addition, we show that c-*myc* specifically activates the transcription of two genes previously identified as serum responsive genes. However, none of the *myc*-regulated protein species shown in the two-dimensional gel (Fig. 6) is a candidate for being either of the serum responsive genes 3CH77 or 3CH92, because the two-dimensional gel analysis was performed on density-arrested rather than serum-arrested cells. Northern blot analysis indicates similar quantities of 3CH77 and 3CH92 mRNA in confluent cells maintained in 10% serum before and after heat shock (data not shown). Furthermore, c-*myc* protein itself is not detectable on the two-dimensional gel because it is of insufficient abundance to generate a signal (only about 3×10^5 cpm was loaded on the two-dimensional gel in Fig. 5 and 6; in contrast, 10^7 cpm was used for immunoprecipitation to generate the signal in Fig. 3). The protein species marked in Fig. 6 do not correspond to any previously identified proteins. Preparative isolation from two-dimensional gels and partial amino acid sequencing may make it possible to generate anti-peptide antibodies or oligonucleotide probes useful for characterization of these proteins.

Recently, Wurm et al. (39) reported that overproduction of c-*myc* was cytotoxic to CHO cells within 8 h. We did not

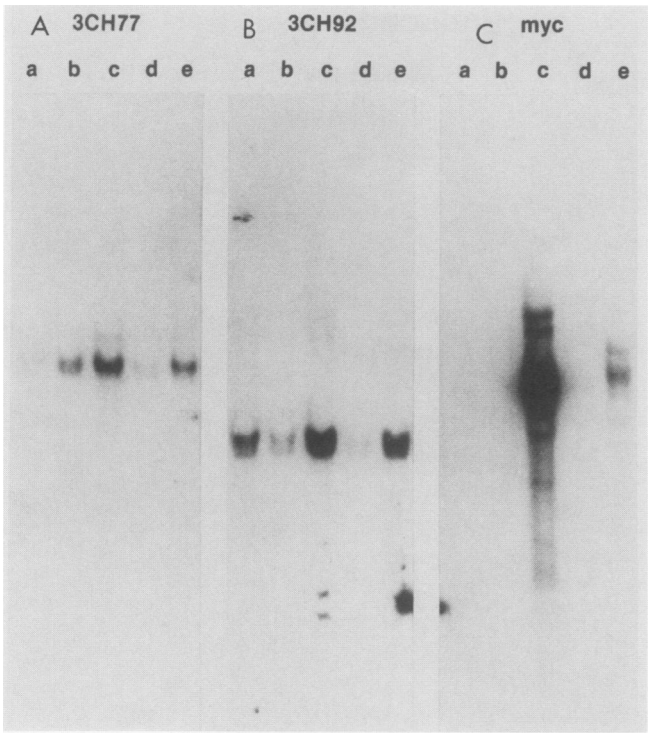

FIG. 7. Northern blot analysis of 3CH77, 3CH92, and c-*myc* mRNAs upon heat shock induction of c-*myc*. Total cellular RNA (10 μg) was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose filters. Lanes: a, induced cell line A7-4 (no *myc* gene transfectant); b, uninduced cell line A3-1 (two-exon *myc* gene transfectant); c, induced cell line A3-1; d, uninduced cell line A5-9 (three-exon *myc* transfectant); e, induced cell line A5-9.

observe any cytotoxicity, even after 48 h. However, our system produces much lower physiological levels of c-*myc*. Therefore, it appears that copious quantities of c-*myc* and not c-*myc* per se is toxic to stressed cells. Another observation in apparent contrast with our own suggests that c-*myc* can induce the heat shock locus itself (25). The sequences required for c-*myc* induction of the hsp70 promoter are localized to the region between 200 and 780 bp 5′ to the

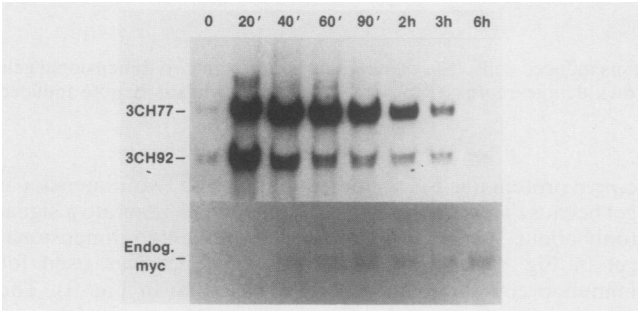

FIG. 8. Northern blot analysis of expression of 3CH77, 3CH92, and the endogenous c-*myc* (probed with mouse exon 1, a gift of M. Dean) in response to serum stimulation. A3-1 cells were arrested (0.5% serum) for 3 days and then stimulated with 20% serum. Cells were harvested at the indicated time points (′, minutes) after serum stimulation. Total cellular RNA (10 μg) was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose filters.

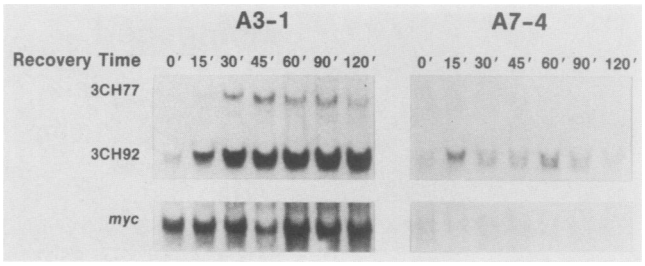

FIG. 9. Northern blot analysis of the time course of expression of 3CH77, 3CH92, and c-*myc* mRNAs during the post-heat shock recovery period. Total cellular RNA was isolated from heat-shocked cell lines A3-1 (contains c-*myc* construct) and A7-4 (contains construct lacking c-*myc*) at the indicated recovery times (′, minutes) RNA (10 μg) was fractionated on 1.2% agarose–2.2 M formaldehyde and transferred to nitrocellulose filters.

normal start of hsp70 transcription. Our construct contains 490 bp of 5′-flanking region of which only about 250 bp are 5′ to the normal start site; therefore, it may not contain the appropriate *myc* responsive domain.

The two G_0/G_1 transition genes, 3CH77 and 3CH92, are inducible by c-*myc*. Previous studies have shown these genes to be serum or mitogen inducible and that serum-induced expression of 3CH77 and 3CH92 precedes (temporally) serum-induced c-*myc* expression. A possible explanation for this is that these genes may have distinct promoter domains which are responsive to different stimuli, much in the same way that the human hsp70 promoter has been shown to be composed of three separate domains responsive to heat shock, serum, and c-*myc* protein (25, 38). This could also explain the recent report (20) that mitogen-stimulated T lymphocytes can still undergo G_0/G_1 transition in spite of attenuation of c-*myc* protein synthesis with a 5′-specific c-*myc* antisense oligonucleotide. In that case, induction of the appropriate G_0/G_1 transition genes is accomplished by the mitogen obviating the need for c-*myc*. Also, it cannot be formally ruled out that heat shock and c-*myc* together are required to induce G_0/G_1 transition genes. The testing of expression levels of potential c-*myc*-regulated genes in other systems in which c-*myc* expression modulates may strengthen the argument that c-*myc* alone is responsible. Nonetheless, the implication of the ability of c-*myc* to induce G_0/G_1 transition genes is clear: abnormal expression of c-*myc* (i.e., expression in what should be resting cells) could contribute to the G_0/G_1 transition and ultimately to neoplasia. This role of c-*myc* is consistent with the previously established ability of c-*myc* to immortalize primary tissue culture cells (27). Here, we have made the first demonstrable link between c-*myc* protein and specific G_0/G_1 transition genes. It is not yet known whether c-*myc* protein acts directly on the promoters of these genes or by an indirect mechanism.

ACKNOWLEDGMENT

C. W. Schweinfest was a fellow of the Leukemia Society of America.

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