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Inducible overproduction of the mouse c-myc protein in mammalian cells

(gene amplification/heat shock induction/DNA-mediated gene transfer/gene fusion/protooncogene)

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ABSTRACT **We have made Chinese hamster ovary (CHO) cell lines that contain up to 2000 copies of the coding region of the mouse c-*myc* gene fused to the promoter of the *Drosophila* gene (*hsp70*) encoding a M_r 70,000 heat shock protein. Incubation of these cells at 43°C results in an estimated 100-fold induction of c-myc mRNA. Translation of this mRNA occurs when the cells are returned to 37°C, and during the first 3 hr of recovery at 37°C, the c-myc protein is one of the most abundantly synthesized proteins in the cells. The products of the induced c-*myc* gene are phosphoproteins of apparent M_r 64,000, 66,000, and 75,000. Induced cells die, suggesting that elevated levels of c-myc are cytotoxic. Amplification of genes placed under control of the *Drosophila hsp70* promoter may provide a general method for inducibly overexpressing proteins in mammalian cells.**

Purification and characterization of a protein is greatly aided by starting with a cell population that overexpresses that protein. Strategies that allow overproduction of protein have been devised for bacterial, yeast, insect, and mammalian systems (1–4). Inducible expression systems have been used in bacteria and yeast to overproduce proteins that might prove toxic if expressed constitutively (5, 6). Overexpression of protein in mammalian systems has to date been accomplished either by amplifying the appropriate gene, resulting in constitutive overexpression (7–10), or by introducing the expressed gene on a viral genome to achieve transient overexpression (11, 12). The former technique requires long-term passage in tissue culture, and thus is not useful for proteins that are cytotoxic. The latter technique is relatively cumbersome and is inappropriate for potentially biohazardous proteins.

The protein product of the c-*myc* gene has been shown to play a causal role in tumor formation in several avian and mammalian systems (13–15). It is expressed at very low levels in normal cells, and inappropriate increases in the level of c-myc protein can apparently transform a normal cell to a tumor cell (14–19). It has not yet proved possible to establish mammalian cell lines that produce c-myc protein at levels greater than 20-fold above normal levels, suggesting that constitutive overproduction of the protein might prove cytotoxic (unpublished observations). Although overproduction of c-myc has been achieved in *Escherichia coli*, yeast, and insect systems (20–22), it is not clear whether it is possible to obtain a properly modified and properly functional protein in nonmammalian cells. We report here the establishment of mammalian cell lines that inducibly overproduce the c-myc protein. We have fused the coding region of the mouse c-*myc* gene to the *Drosophila hsp70* (M_r 70,000 heat shock protein) promoter, allowing integration and amplification of the recombinant c-*myc* construct while the gene is in an "off" state. Induction of the gene at 43°C and subsequent recovery at 37°C results in overexpression of the c-*myc* protein product.

MATERIALS AND METHODS

DNA Transfection and Amplification. Dihydrofolate reductase (DHFR)-deficient CHO cells (CHO-DUKX BII; ref. 23) were obtained from L. Chasin (Columbia University, New York) and were propagated by standard procedures (24). Cells were transfected by a modification (25) of the calcium phosphate coprecipitation technique of Graham and van der Eb (26). Six independent cell lines were established, from which four (4-HS-MYC, 5A-HS-MYC, 5B-HS-MYC, and 6A-HS-MYC) were grown in stepwise 4-fold increasing concentrations of methotrexate, starting with a concentration of 0.005 μM and ending with 320 μM. Cells were allowed to acclimate to each increased level of selection for 2–4 weeks.

Plasmids. Plasmid pCVSVEII has been described (25). Construction of pHS-myc was as follows. pSV2myc (27) was cut with *Xba* I, the ends were filled with Klenow polymerase, and the plasmid was cut with *Bam*HI. The c-*myc*-containing fragment was cloned into *Hinc*II/*Bam*HI-cut pSP6-HS-9 to obtain pHS-myc. pSP6-HS-9 contains a *Hin*dIII–*Pst* I fragment from p232.3 (28) that encodes a *Drosophila hsp70* promoter inserted into *Pst* I/*Hin*dIII-cleaved pSP65 (29).

Analysis of RNA and DNA. Isolation and analysis of cytoplasmic RNA was performed as described elsewhere (30, 31), using radiolabeled RNA probes prepared with SP6 polymerase. Analysis of genomic DNA from cell lines was done by standard techniques (32).

Induction of Recombinant Cell Lines. Recombinant cells were plated the day before induction at 60–80% confluence in 10-cm tissue culture dishes. They were heat-shocked by feeding with 43°C medium and were incubated at that temperature for 1–2 hr. Medium at 37°C was then added and the cells were allowed to recover at 37°C for the indicated time before harvest, usually 3–4 hr.

Analysis and Identification of c-myc Proteins. For lysis, 2 ml of RIPA buffer (0.01 M Tris·HCl, pH 7.2/0.15 M NaCl, 1% Triton X-100/0.1% $NaDodSO_4$/1% sodium deoxycholate/0.02% NaN_3) was added to 50–60%-confluent cells in 10-cm dishes. The highly viscous solution was sonicated three times for 5 sec on ice. $NaDodSO_4$/PAGE and immunoblot analysis were performed using 12% gels (33, 34). Immunoprecipitations were performed by a modification of the standard procedure (35). For immunodetection, polyclonal and monoclonal antibodies raised against a portion of the human c-myc protein that is conserved from human to mouse were used. These antibodies were a generous gift from R. Chizzonite (Hoffmann-La Roche).

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Abbreviation: DHFR, dihydrofolate reductase.

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RESULTS

Amplification of the c-*myc* Gene in CHO Cells. Initial attempts to amplify the c-*myc* gene fused to a constitutive promoter were unsuccessful (data not shown), leading us to make plasmid pHS-myc (Fig. 1). This plasmid links a *Drosophila hsp70* promoter region, which has an extremely low basal level of expression, and the second and third exons of a mouse c-*myc* gene isolated from a plasmacytoma (14). In pHS-myc, the first ATG 3′ of the *hsp70* transcription initiation site is that of the c-myc protein. Plasmids pHS-myc (5 μg) and pCVSVEII-DHFR (1 μg) (Fig. 1) were introduced into DHFR-deficient CHO-DUKX BII cells. Ten days after the cells were placed in selection, individual colonies were cloned and subsequently expanded into cell lines. Six tested cell lines all contained the pHS-myc plasmid, as determined by RNA analysis (see below). Four of these lines were cultivated in selective media containing stepwise increasing concentrations of methotrexate, an antagonist of DHFR function. This selection pressure results in amplification of the transfected DHFR gene and associated DNA (7, 8).

After establishment in 320 μM methotrexate, the copy number of the introduced c-*myc* gene in each cell line was determined by Southern blot hybridization. Genomic DNA, cleaved with *Hin*dIII and transferred to nitrocellulose, was hybridized with a mouse c-*myc*-specific RNA probe (Fig. 2, lanes 6–9). In comparison to reconstructions made using the plasmid pHS-myc, the recombinant cell lines contained approximately 900 (line 4), 2700 (5A), 90 (5B), and 1500 (6A) copies of the introduced c-*myc* gene (Fig. 2). These levels of DNA were the result of amplification of copy number during selection, as demonstrated for line 5A in Fig. 2, lanes 13–16. One of the four lines (6A) contained additional rearranged c-*myc* genes as well, as evidenced by the extra bands in Fig. 2, lane 9.

Induction of c-myc mRNA in Recombinant Lines. To determine whether transcription of the amplified c-*myc* gene in the recombinant cell lines could be induced, RNA was isolated from growing cells incubated for 2 hr at either 37°C or 43°C. RNA was hybridized to an internally labeled RNA probe, digested with RNase, and analyzed by electrophoresis in polyacrylamide gels (Fig. 3). In both the original isolates of the cell lines (lanes 1–10) and the amplified cell lines (lanes 11–16), the amount of appropriately initiated message from the *hsp70*-c-*myc* fusion gene was substantially increased after heat shock. The level of RNA after induction increased as DNA copy number increased (Fig. 3*A*, lanes 11–16). The recombinant genes remained highly inducible, even in the amplified cell lines (lanes 5, 6, 15, and 16). c-myc RNA levels continued to increase with increasing time of heat shock through 3 hr (data not shown) and remained relatively constant for 3 hr after the temperature shift (Fig. 3*B*).

c-myc Protein Synthesis in Recombinant Lines. c-myc protein was not detectable in recombinant cell lines immediately after a 2-hr heat shock at 43°C, as judged by metabolic labeling with [^{35}S]methionine or by immunoblotting. In contrast, c-myc protein was detectable by immunoblotting when cells were allowed to recover at 37°C, and the level of protein increased with increasing time of recovery (Fig. 4*A*). Three bands with relative mobilities corresponding to M_r 64,000, 66,000, and 75,000 were recognized by either monoclonal or polyclonal antibodies raised against a portion of the human c-myc protein that is conserved in mouse c-myc (22). The smaller two of these have been seen in previous studies (18, 36, 37). Cell line 5A produces at least twice as much c-myc protein as line 4, the latter having a lower copy number of the c-*myc* construct. By comparing levels of protein with a purified protein produced in an insect expression vector (22), we estimate that 10^9 CHO cells produced approximately 1 mg of c-myc protein (data not shown). These lines produce substantially more c-myc protein than the human tumor line COLO 320 HSR (18), which contains approximately 20 copies of an endogenous c-*myc* gene (Fig. 4*A*). Metabolic labeling of cells with [^{35}S]methionine between 1 and 3 hr after heat shock revealed that c-myc was one of the most actively synthesized proteins in line 5A during this period (Fig. 4*B*, lane 4). The mammalian c-myc protein has been shown to be phosphorylated (36, 38, 39), as is the c-myc protein produced in recombinant lines 4 and 5A (Fig. 4*B*). Immunoprecipitation revealed that all three species of the c-myc protein appear to be phosphorylated.

Viability of Cell Lines After Induction of c-*myc*. Initial experiments raised the possibility that high levels of c-myc are cytotoxic. To test this hypothesis, we investigated the viability of our recombinant lines after induction of c-*myc*. Parental and recombinant lines were plated at approximately 10% confluence and either were heat-shocked for 2 hr at 43°C and returned to 37°C or were left at 37°C. After 2 days at 37°C, no living cells were left on heat-shocked dishes of any of the four recombinant lines, while the control dishes of the recombinant lines, as well as both the heat shock and control dishes of the parental line, were confluent (data not shown). Microscopic examination of the cells during this recovery period showed slow deterioration and no further cell division after induction of c-*myc* (Fig. 5).

DISCUSSION

Amplification of an Inducible Gene. In order to fully understand the function of the gene product of the cellular oncogene c-*myc*, it will be necessary to purify and characterize the protein. As an initial step toward this goal, we have made mammalian cell lines that overproduce the mouse c-myc protein. Our strategy, creation of an amplified heat-inducible c-*myc* gene, was chosen to circumvent potential problems resulting from amplification of constitutive genes expressing toxic products. Using DHFR-mediated amplification of a silent *Drosophila hsp70* promoter/c-*myc* fusion, we were able to establish stable cell lines containing approximately 2000 copies of the recombinant c-*myc* gene (Fig. 2).

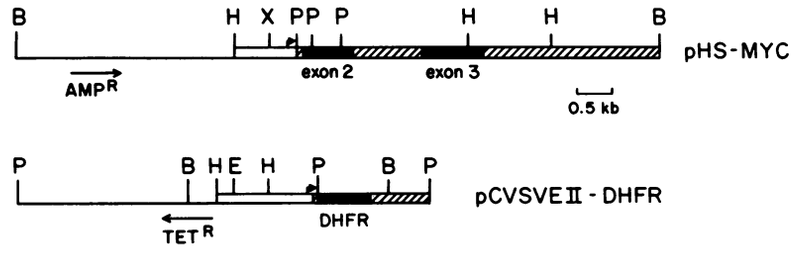

FIG. 1. Plasmids used to establish recombinant *myc* lines. Black boxes denote coding regions, and hatched boxes denote processing signals. In pHS-myc, open box denotes a *Drosophila hsp70* promoter (bases −780 to +88, ref. 28); in pCVSVEII-DHFR, open box denotes the adenovirus EII promoter and simian virus 40 enhancer (26). Restriction sites: B, *Bam*HI; H, *Hin*dIII, P, *Pst* I; X, *Xba* I; E, *Eco*RI. Positions and orientations of genes conferring ampicillin resistance (Amp^R) and tetracycline resistance (Tet^R) are shown. kb, Kilobase.

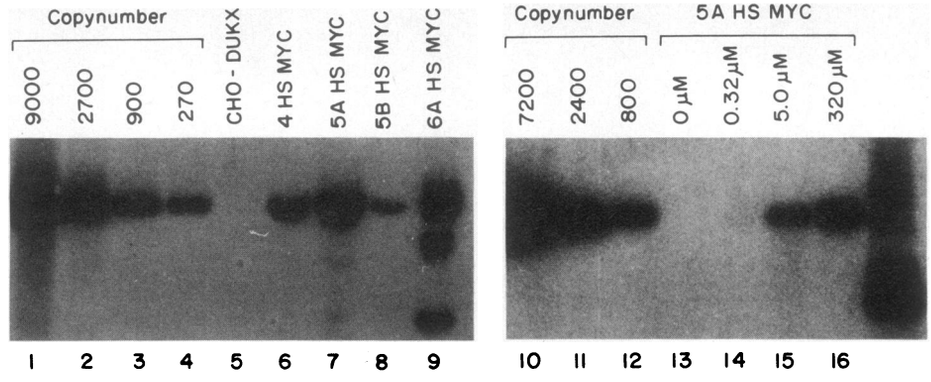

FIG. 2. Copy number of c-*myc* constructs in recombinant CHO cell lines. Genomic DNA isolated from the indicated cell lines growing at 320 μM methotrexate (lanes 6–9, 2 μg of DNA per lane) or at different levels of methotrexate (lanes 13–16, 0.5 μg of DNA per lane) was digested to completion with *Hin*dIII and analyzed by Southern transfer. Dilutions of *Hin*dIII-digested pHS-myc (lanes 1–4, 20 ng, 6 ng, 2 ng, and 0.6 ng; lanes 10–12, 4 ng, 1.3 ng, and 0.45 ng) were included for comparison. The ^{32}P-labeled RNA probe contained the *Pst* I–*Hin*dIII fragment of c-*myc* exons 2 and 3. Lane at far right: 1-kilobase "ladder" (Bethesda Research Laboratories).

One potential problem was that cellular factors responsible for induction of the *hsp70* promoter region might be limiting, resulting in an inability to effectively induce the amplified *hsp70*/c-*myc* construction. However, we could detect no substantial limitation in the inducibility of the amplified cell lines as compared to the starting cell lines (Fig. 3). Final induced levels of RNA increase as gene copy number increases, and we estimate that the degree of induction of the *Drosophila hsp70* promoter in these lines is at least 100-fold.

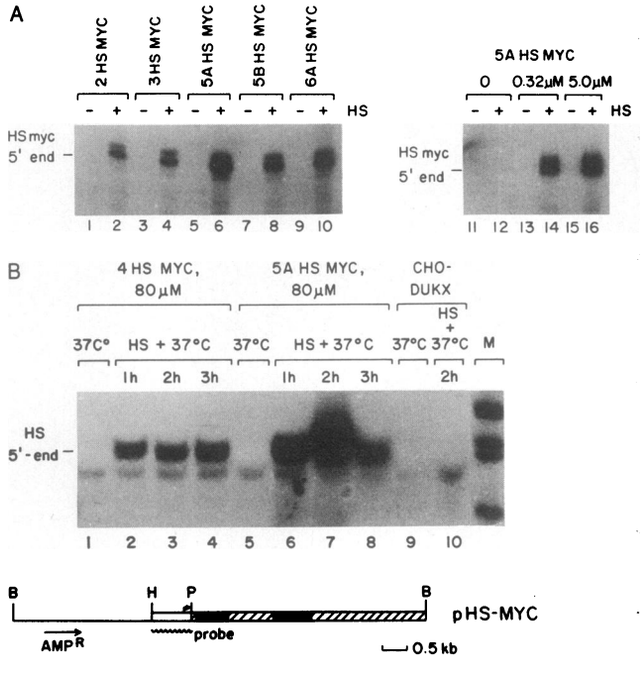

FIG. 3. Presence, inducibility, and stability of c-myc mRNA in recombinant cells. Cytoplasmic RNA from uninduced (−) and heat-shock (2 hr at 43°C, +) recombinant cells was hybridized with an internally labeled RNA probe (shown in *B*), digested with RNase, and electrophoresed in polyacrylamide gels. (*A*) Lanes 1–10: RNA (10 μg) from the indicated cell lines in the absence of methotrexate. Lanes 11–16: RNA (5 μg) from line 5A-HS-MYC growing in medium containing methotrexate as indicated. Lanes 11 and 12 show identical induction to that seen in lanes 5 and 6, when equivalent autoradiographic exposures are compared. (*B*) RNA (3 μg) from lines 4-HS-MYC (lanes 1–4), 5A-HS-MYC (lanes 5–8), and CHO-DUKX (lanes 9 and 10) were analyzed. Cells were grown at 37°C (lanes 1, 5, and 9) or were incubated at 43°C for 2 hr and allowed to recover at 37°C for 1 hr (lanes 2 and 6), 2 hr (lanes 3, 7, and 10) or 3 hr (lanes 4 and 8). Bands labeled 5'-end migrate at 90 bases in denaturing gels. Lane M: 1-kilobase "ladder" (Bethesda Research Laboratories).

A second potential problem results from observations that heat shock adversely affects RNA processing and translation (40, 41). Indeed, we could not detect any synthesis of c-myc protein immediately after heat shock despite the presence of high c-myc message levels. However, synthesis of c-myc protein occurred after the cells were allowed to recover at 37°C. c-myc protein levels increased rapidly during the first 3–4 hr of recovery and continued to increase slightly over the next 6 hr (data not shown). We have used the shorter recovery period because of the cytotoxic effects that cause loss of cellular integrity after longer periods of recovery (Fig. 5).

One apparent reason that c-myc protein levels increase throughout recovery is that the level of c-myc message remains relatively constant and high (Fig. 3*B*). We have not determined whether this is due to high stability of the recombinant c-myc message, continued high promoter activity, or a combination of these effects. By comparison to known amounts of purified c-myc protein produced in insect cells, we estimate tht the recombinant lines produce approximately 1 mg of c-myc protein per 10^9 cells. It is not clear whether insect- and mammalian-cell-produced proteins are equally reactive with the antibody preparations used, and thus this value is subject to verification once purification of the recombinant c-myc protein is completed. It is clear, however, that c-myc is one of the most abundantly synthesized proteins in the cell during the period of recovery from heat shock (Fig. 4).

The protein produced from these recombinant cell lines has the characteristics described previously for the endogenous mouse c-myc protein (18, 36, 37). These earlier studies demonstrated that the mouse c-myc protein is a phosphoprotein that runs in NaDodSO$_4$/PAGE as two species of apparent M_r 64,000 and 66,000. In addition to these two species, we see a third, minor species of apparent M_r ≈75,000. It is not clear whether this is a form of c-myc present in normal cells or is a byproduct of the overproduction. It is possible, for example, that overproduction of c-myc results in some modification, such as ubiquitination, not relevant to the normal biology of the c-myc protein. All three forms of c-myc are phosphorylated (Fig. 4).

Overproduction of c-myc Protein Is Cytotoxic. Induction of c-*myc* in these recombinant lines leads to cell death (Fig. 5). This may result from a general cytotoxic effect of the high c-myc protein levels; alternatively, the recombinant *hsp70*/c-*myc* gene may prevent the normal functioning of the cellular response to heat. The observation that recombinant lines containing high constitutive levels of c-myc are difficult to produce and have an extremely abnormal phenotype argues in favor of the former possibility. Cell lines that contain high

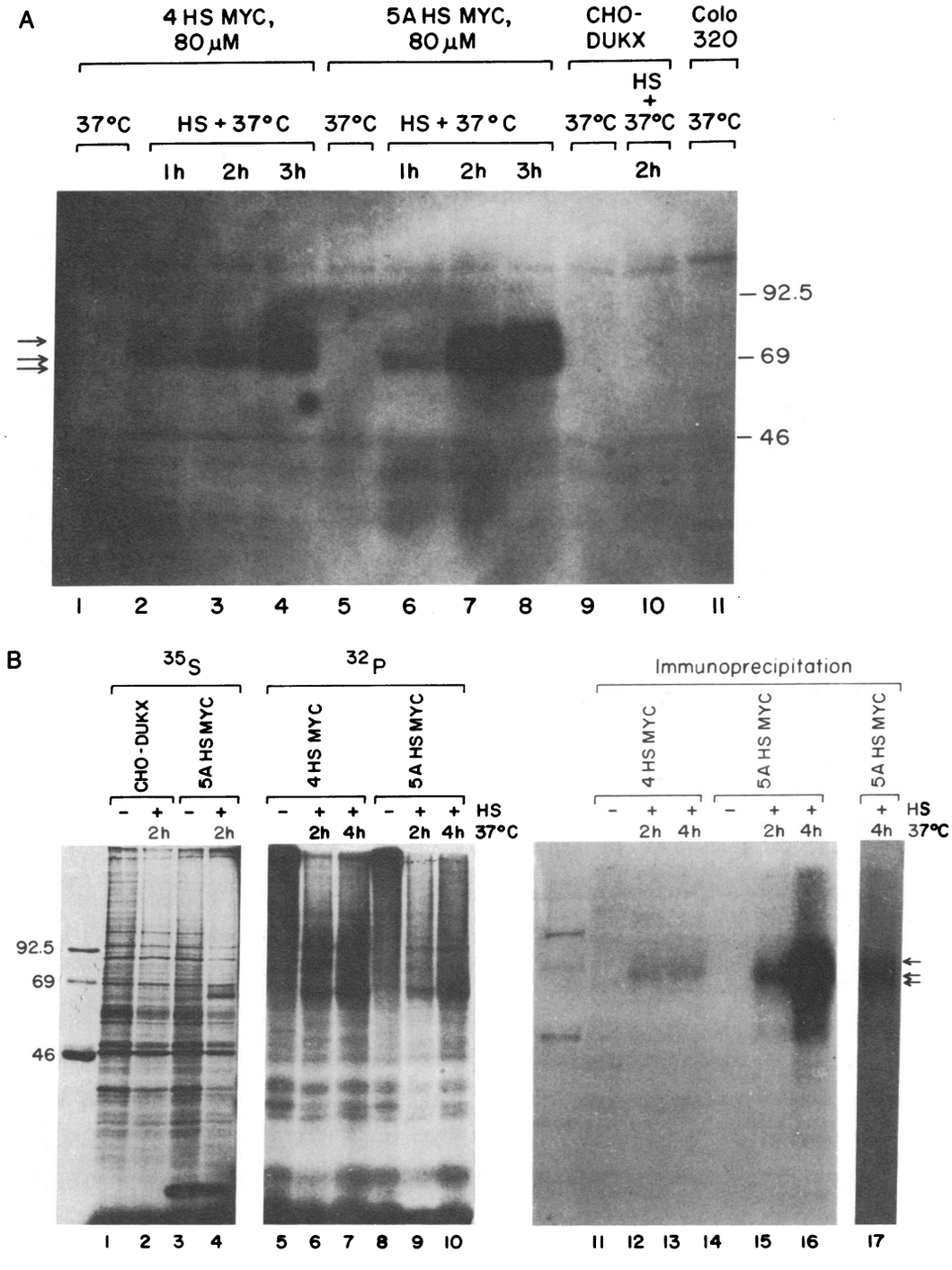

FIG. 4. Induction and characterization of c-myc protein in amplified cell lines. (*A*) Levels of c-myc protein in cell lines growing at 37°C (lanes 1, 5, 9, and 11) or heat-induced for 2 hr at 43°C and then returned to 37°C for 1 hr (lanes 2 and 6), 2 hr (lanes 3, 7, and 10), or 3 hr (lanes 4 and 8). Lines 4-HS-MYC and 5A-HS-MYC were grown in the presence of 80 μM methotrexate. Samples (from 2×10^5 cells) were analyzed by immunoblotting using anti-c-myc monoclonal antibodies (R. Chizzonite, Hoffmann–La Roche) and ^{125}I-labeled protein A (New England Nuclear). Positions and $M_r \times 10^{-3}$ of marker proteins are at right. (*B*) RIPA buffer lysates (see *Materials and Methods*) of [^{35}S]methionine- (lanes 1–4, 400,000 cpm per lane) and [^{32}P]phosphate-labeled proteins (lanes 5–10, 400,000 cpm per lane; lanes 11–17, immunoprecipitates) were analyzed by NaDodSO$_4$/12% PAGE and autoradiography. Protein isolates were from the indicated cell lines either growing at 37°C (−) or induced for 2 hr at 43°C (+) and allowed to recover at 37°C for 2 hr (lanes 2, 4, 6, 9, 12, and 15) or 4 hr (lanes 7, 10, 13, 16, and 17). Lane 17 is a light exposure of lane 16. Lane M: molecular weight markers ($M_r \times 10^{-3}$ at left). Unnumbered lane to left of lane 11: markers (M_r 92,500, 69,000, and 46,000).

levels of c-myc protein produced from a constitutive promoter tend to have an inactive resident c-*myc* gene, an observation that has led to the theory that c-myc protein autoregulates its expression (42). If c-myc is cytotoxic at high levels, a second explanation exists for this phenomenon: cells whose resident c-*myc* gene remains active in the presence of an amplified or rearranged allele will die due to overexpression of the protein, causing a selection for cells that have mutated to turn off the resident gene.

A General Method for Inducible Overexpression? Proteins involved in regulatory events in mammalian cells, such as c-myc, the transcription factor SP-1, or the glucocorticoid

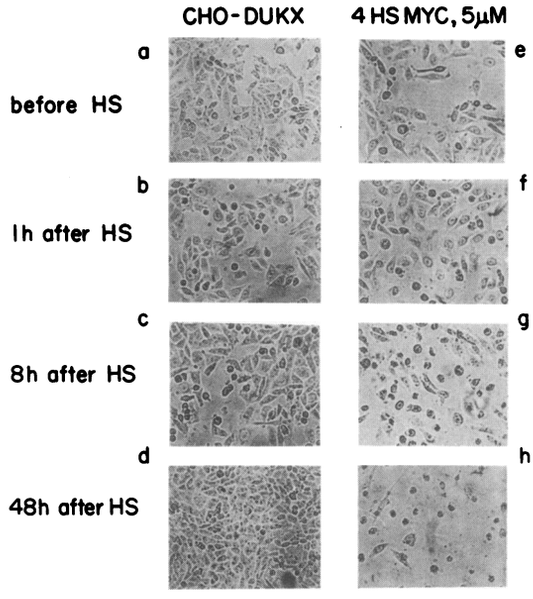

FIG. 5. Overexpression of c-*myc* is cytotoxic. Micrographs of ≈50%-confluent CHO-DUKX B1 cells (*a–d*) and recombinant cell line 4 HS MYC (in the presence of 5 μM methotrexate; *e–h*) before heat-shock (HS) induction (90 min, 43°C) and after different periods of recovery after heat-shock induction, as indicated. (×140.)

receptor, tend to be present at very low levels. Purification of these proteins is therefore problematic due to both the difficulty of obtaining enough starting material and the necessary degree of purification. We have shown that it is possible to inducibly overexpress such a protein in an appropriate mammalian cell type. This procedure would seem to have a greater likelihood of producing a properly modified, and therefore properly functional, protein than overexpression in a distantly related organism. We have demonstrated that the *Drosophila hsp70* promoter is highly inducible even when highly amplified, and that it is possible to obtain significant levels of protein using this promoter. The promoter has an extremely low basal level of transcription, allowing amplification of genes whose product is cytotoxic. Because the level of protein production in the recombinant c-*myc* lines may result from the continued high level of c-myc RNA that persists in these lines, it is not possible to guarantee that a similar fusion expressing a different mammalian protein would result in the same degree of overexpression. The degree of inducibility of the introduced *hsp70* promoter in these amplified lines suggests, however, that the procedure we have used for c-myc will prove applicable for inducible overexpression of other mammalian proteins.

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