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# Competitive Inhibition of *hsp70* Gene Expression Causes Thermosensitivity

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**A novel method has been developed for modulating the expression of an endogenous chromosomal gene in a higher eukaryote, by competitive inhibition at the level of gene transcription. The gene studied was the *hsp70* gene, which encodes a 72-kilodalton (kD) heat shock protein that is synthesized after thermal stress. The 5′ control region of the *hsp70* gene was inserted on a plasmid containing the eukaryotic gene for dihydrofolate reductase. The hybrid plasmid was then introduced into a Chinese hamster ovary cell line and elevated in copy number approximately 20,000-fold by selection of cells with methotrexate. Heat-inducible expression from the intact *hsp70* gene was reduced by at least 90% in the modified cells when compared with the induction in control cells, and the modified cells also displayed elevated thermosensitivity. The change in heat shock protein synthesis is presumably caused by competition among the increased number of binding sites for the heat-shock transcription factor, leading to altered expression from the native heat shock gene. These results support a role for heat shock protein in the recovery of mammalian cells from acute thermal stress.**

EUKARYOTIC GENE EXPRESSION IS modulated by interactions among cis-acting control sequences that are topologically contiguous with the regulated genes, and trans-acting regulatory proteins that bind to the control sequences (*1*). It is presumed that changes in activity or abundance of the regulatory proteins are sufficient to explain most aspects of the control of gene expression, but direct tests of these concepts are difficult to construct. In one approach, expression from reporter genes on plasmids transiently introduced into cells can be modulated by cointroduction of another plasmid that contains genes or gene fragments that share control sequences with the first (*2, 3*). When expression from the first plasmid is reduced in such an experiment, it is inferred that the control sequences on the two plasmids are competing for binding to a positive-acting regulatory protein that is present in limiting abundance in the host cell. With excess competing plasmid, fewer regulatory proteins are available for binding to the reporter gene's control region, and reporter gene transcription is correspondingly diminished.

This approach has not been successful, however, in demonstrating modulation of expression from endogenous chromosomal genes. Even in cases where competition among introduced genes is clear, expression from endogenous genes with shared control sequences has remained unaffected (*3*). Several explanations may be offered for this puzzling result. (i) The introduced genes may reside too briefly within cells before degradation to compete effectively with endogenous genes, or they may reside within an intracellular compartment (such as micronuclei) that is isolated from the endogenous genes. (ii) Newly introduced genes may compete effectively with one another during chromatin assembly, whereas the preexisting chromatin configuration of endogenous genes may be stable against such transient disruptions. (iii) Only a small fraction of cells may incorporate transfected DNA (*3*), and the majority of cells may not experience effective competition. (iv) Cells may be transiently damaged during DNA transfection, and this may alter the cell's normal regulatory mechanisms during the course of the assay. (v) Cells may vary the abundance or localization of regulatory proteins in response to transient changes in the number of binding sites for the proteins.

The method of directed gene coamplification provides an opportunity to circumvent these difficulties. To achieve coamplification, genes or gene fragments are transfected into cells together with the gene for dihydrofolate reductase (*4*); the genes may then become stably integrated into the cell's chromosomes at random sites of insertion. By varying the level of methotrexate selection to which transfected cells are subsequently exposed, one can also vary the copy number

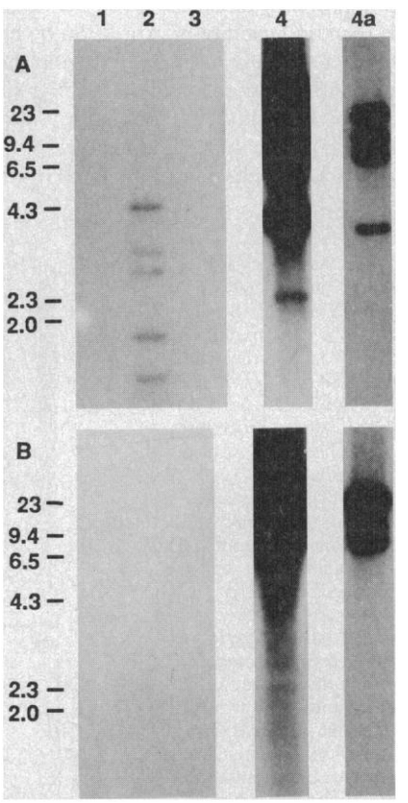


**Fig. 1.** Coamplification of *dhfr* and *hsp70* control region. DNA was isolated from CHO $K_1$, *dhfr*$^-$, and CHO $DH_f$ cells, and from an independently derived CHO $K_1$ line [resistant to 500 n*M* methotrexate and designated $B_{11}$ (*9*)] in which the native hamster *dhfr* gene is amplified 50-fold. The DNA samples were digested with Eco RI, and 5 μg of each sample was separated by electrophoresis in 0.7% agarose, capillary-blotted to nitrocellulose, and probed with radiolabeled ($0.5 \times 10^9$ cpm/μg) *dhfr* (**A**) or *hsp70* control region (**B**) gene fragments (*9*). Lanes 1, CHO $K_1$; lanes 2, $B_{11}$; lanes 3, *dhfr*$^-$; and lanes 4 and 4a, CHO $DH_f$. Exposure to x-ray film was for 24 hours (lanes 1 to 4) or 45 min (lanes 4a). Molecular size markers are indicated at left in kilobases.

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of the introduced DNA sequences. The structures of the corresponding endogenous chromosomal genes are not altered in such an experiment. The model presented above provides a prediction that is subject to experimental test: as the copy number of a particular positive-acting control sequence is progressively increased, the relative expression from an intact native gene that possesses a similar control sequence should diminish as required regulatory proteins become limiting.

We have selected for our study a gene whose expression pattern is tightly regulated and whose protein product apparently is not essential to cell survival at 37°C, namely, the gene for the heat shock protein hsp70. We reasoned that in this way we could manipulate chromosomal content without immediate adverse cellular effects and that such effects should only be manifest after the exposure of cells to thermal stress. The heat shock proteins constitute a diverse group that share inducibility of expression by heat and other stresses, and that contain in their gene control regions multiple binding sites for the heat-shock transcription factor (HSF) (*5*). The best characterized of the heat shock proteins is hsp70, which is actually a member of a small family of closely related proteins from 70 to 73 kD (some of which are constitutively expressed, not highly inducible by heat shock, and lack multiple binding sites for HSF). The precise role of these proteins remains controversial and many different potential activities have been suggested. Recent evidence, however, indicates that at least one important function of the hsp70 family is in adenosine triphosphate (ATP)–dependent protein folding (*6*). At normal temperatures this function may be carried out by products of the constitutively expressed members of this family (which in hamster cells have an apparent molecular mass of 73 kD). Restoration of normal protein structure after thermal denaturation is likely to be an important event in the recovery of cells from heat stress, and apparently requires the additional participation of the heat-inducible form of hsp70 (apparent molecular mass of 72 kD).

To initiate these tests, we have constructed a plasmid, designated pDH, which includes both a modified dihydrofolate reductase (*dhfr*) gene with its normal promoter and polyadenylation signals, and a fragment of an *hsp70* gene isolated from *Xenopus laevis* (*7*) located downstream of the *dhfr* gene. This fragment contains the 5′ control sequence with three binding sites for HSF and 400 bp of the transcribed portion of the *hsp70* gene, including the AUG start codon, but it lacks the majority of the *hsp70* coding sequence. Although the *hsp70* control sequence is derived from an amphibian source, the consensus binding site for HSF is highly conserved and the *Xenopus* promoter functions properly in mammalian cells (*5*). The pDH plasmid was transfected into a $dhfr^-$ strain (*8*) of the Chinese hamster ovary (CHO $K_1$) cell line, thereby restoring the cells to a $dhfr^+$ phenotype. Individual $dhfr^+$ colonies were isolated and grown with stepwise increases in methotrexate concentration (*9*), resulting in increasingly drug-resistant cell populations. Methotrexate inhibits the enzyme dihydrofolate reductase and is therefore cytotoxic. The rare cells that survive methotrexate selection do so by overproduction of dihydrofolate reductase, and this overproduction is achieved in turn by amplification of the dihydrofolate reductase gene. In CHO cells the extra gene copies typically reside on the chromosome and are generally not found extrachromosomally (*4*). Because the *hsp70* gene fragment is adjacent to the *dhfr* gene on the pDH plasmid, amplification of the *dhfr* gene in the host cells is accompanied by the passive coamplification of the *hsp70* control sequence.

By increasing at 2-week intervals the concentration of methotrexate in the cell culture media, we obtained after 2 to 5 months clonal populations that were resistant to high levels of methotrexate and that exhibited high levels of gene amplification. Both the transfected *dhfr* gene and the *hsp70* gene fragment were highly amplified in one of the cloned cell populations (designated CHO $DH_f$) that developed resistance to 5 $\mu M$ methotrexate (Fig. 1). We estimate the degree of amplification in this line as approximately $15 \times 10^3$ to $25 \times 10^3$ times for both the transfected *dhfr* gene and for the passively coamplified *hsp70* control region, on the basis of densitometric scanning of different exposures of x-ray films and hybridization to a dilution series of CHO $K_1$ and CHO $DH_f$ DNA (*10*). Microscopic examination of chromosomes prepared from these cells (*10*) demonstrated that extrachromosomal elements were absent and that the amplified genes reside intrachromosomally, as expected. The level of amplification we observed in the CHO $DH_f$ cells was greater than expected (several hundredfold amplification in response to 5 $\mu M$ methotrexate would be typical) and may reflect an unusual property of the site of chromosomal insertion in this cell line.

Cells from CHO $K_1$ and CHO $DH_f$ cell lines were labeled with [$^{35}$S]methionine for

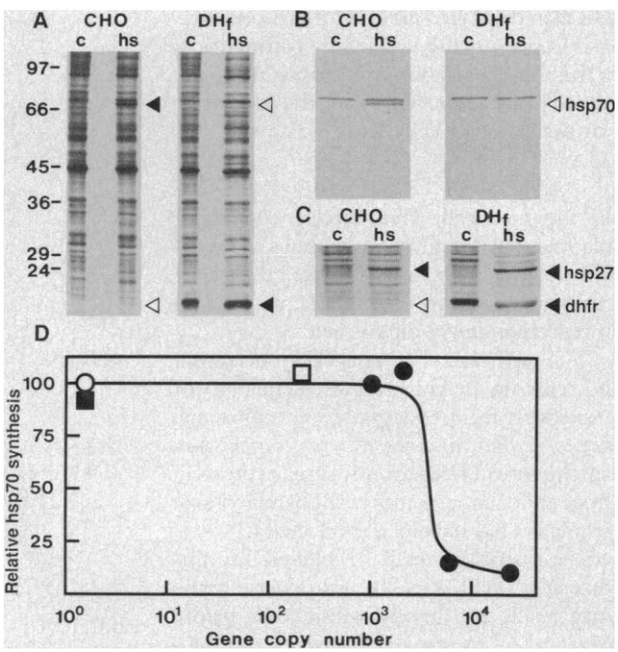


**Fig. 2.** Synthesis of heat shock proteins. (**A**) The incorporation of [$^{35}$S]methionine was determined for the CHO $K_1$ and the coamplified CHO $DH_f$ cells, before (c) and after (hs) a 30-min heat shock. Procedures for heat shock and isolation of proteins were as described (*11*). Proteins were loaded so that there were equal counts per minute per lane. Proteins were separated by electrophoresis on a 10% denaturing polyacrylamide gel, and synthetic patterns were determined by fluorography. Molecular sizes are indicated at left in kilodaltons. (**B**) The identity of the hsp70 proteins was confirmed by immunoblot analysis. Nonradioactive proteins were prepared in an experiment parallel to that shown in (A), electrophoretically separated, transferred to nitrocellulose, and then probed with antibody specific for hsp70 proteins (*12*) in an immunoperoxidase reaction (*13*). (**C**) Synthesis of additional heat shock proteins was monitored by incorporation of [$^{14}$C]leucine after a 20-min heat shock. A portion of the resulting fluorogram is shown, indicating synthetic patterns of low molecular size proteins, including a heat-inducible protein of 27 kD. The lanes in (B) and (C) are vertically aligned with the molecular size markers in (A). Bands corresponding to the positions of hsp70, hsp27, and dhfr are shown by filled or open arrowheads, indicating, respectively, the presence or absence of each protein in heat-shocked samples. (**D**) Variation in hsp70 synthetic capacity with gene copy number. Incorporation of [$^{35}$S]methionine into hsp70 protein was measured by densitometric scanning of fluorograms prepared as in (A) from heat-shocked $dhfr^-$ (■), CHO $DH_c$ (□), and CHO $DH_f$ cells (●), and compared with the synthesis by CHO $K_1$ cells (○), which was given an arbitrary value of 100. The degree of amplification was measured for each cell type as in Fig. 1.

2 hours at 37°C, either before or 12 hours after heat shock (*11*). The CHO $K_1$ cells synthesized a prominent protein of 72 kD (the expected product of the endogenous hamster *hsp70* gene) during recovery from heat shock, whereas the CHO $DH_f$ cells did not make detectable quantities of this protein (Fig. 2A). Densitometric scanning of the fluorograph indicates that synthesis of the 72-kD protein by heat-shocked CHO $DH_f$ cells was 10% or less of its synthesis in heat-shocked CHO $K_1$ cells. In other cell lines that were tested, in which either the native hamster *dhfr* gene or the transfected *dhfr* minigene (without the *hsp70* control region) were amplified, the capacity to synthesize the inducible hsp70 protein after heat shock was maintained (*10*). In further experiments, CHO $K_1$ and *dhfr*$^-$ cells, as well as CHO $DH_c$ cells (an independently transfected and coamplified cell line) and intermediates of the CHO $DH_f$ series that were resistant to varying levels of methotrexate and therefore variably amplified, were similarly compared for their capacity to synthesize hsp70 (Fig. 2D). Heat-inducible synthesis of this protein is maintained up to approximately $10^3$-fold amplification of the *hsp70* control region, but its inducibility declines rapidly at higher levels of amplification. We estimate from this result that the HSF regulatory protein is present with an abundance of at least several thousands of molecules per cell, a value consistent with other estimates (*5*).

To confirm the identity of the 72-kD protein, we tested its immunoreactivity with a monoclonal antibody prepared against hsp70 protein (*12*). Both CHO $K_1$ and CHO $DH_f$ cells contained a 73-kD protein that we identify as a constitutively synthesized cognate (*5, 6, 12*) of hsp70 (Fig. 2B). This protein is retained and may even increase in its abundance 12 hours after heat shock. The immunoperoxidase staining also revealed a 72-kD protein that was abundant in CHO $K_1$ cells after heat shock, but that was nearly absent from the CHO $DH_f$ cells. Faint traces of the 72-kD protein were sometimes detectable in the heat-shocked CHO $DH_f$ cells, indicating that inhibition of its synthesis in these cells may not be complete. Synthesis of at least one other heat shock protein was maintained, however, in the CHO $DH_f$ cells. When CHO $K_1$ or CHO $DH_f$ cells were heat-shocked for 20 min, then labeled 12 hours later with [$^{14}$C]-leucine, a methionine-poor 27-kD protein was inducibly synthesized in both cell types (Fig. 2C), even when synthesis of hsp70 protein was selectively inhibited in the CHO $DH_f$ cells. This result indicates that this member of the heat shock family of genes may be activated by mechanisms different from that of the *hsp70* gene, or that the affinity of its promoter region for HSF may be greater than that of *hsp70* (*5*), permitting its heat-inducible expression even when HSF is reduced in its availability.

We also examined the ability of experimental and control cells to survive the relatively severe heat stress employed in these experiments. When the CHO $K_1$ and *dhfr*$^-$ cells were heat-shocked, at least 50% of the cells survived for 24 to 72 hours, and approximately 1% of the population could resume division and form colonies during the following 7 to 10 days (Fig. 3). In the coamplified CHO $DH_f$ cell line, however, cell death began within 6 to 12 hours after heat treatment, and subsequent colony formation was further reduced to less than 5% of that observed for the control cells (Fig. 3). The rapid death of the coamplified cells after heat shock was accompanied by extensive vacuolation and lysis and transition from a flattened to a rounded morphology. Thus, those cells that were unable to manufacture significant amounts of the heat-inducible hsp70 protein also showed exaggerated thermosensitivity. From this result we infer that the inducible form of the hsp70 protein contributes substantially to the recovery from acute thermal stress, though we cannot exclude a role for other heat shock proteins in this process.

Together with the data presented in a recent report (*13*), these results support a functional role for a major heat shock protein in the recovery from thermal stress by higher eukaryotic cells. Although this relation has previously been argued from correlative data, from work with lower eukaryotes (*14*), and from experiments with cells in which the heat shock genes are developmentally regulated (*15*), attempts to experimentally verify this prediction in higher eukaryotes by direct manipulation of heat shock protein synthesis have until now been unsuccessful (*16*). Although we do not yet have direct proof, we infer that the inhibition of expression that we observed was achieved through competition among binding sites for the HSF, present in limiting abundance within cells.

Our method of competitive inhibition of gene expression constitutes a novel approach to the construction of "dominant negative mutations" (*17*) and will likely be most effective when competition is directed against less abundant transcription factors. This approach should provide a method for varying gene expression that is distinct from, but complementary to, the widely studied method of inhibition by synthesis of antisense RNA (*16*).

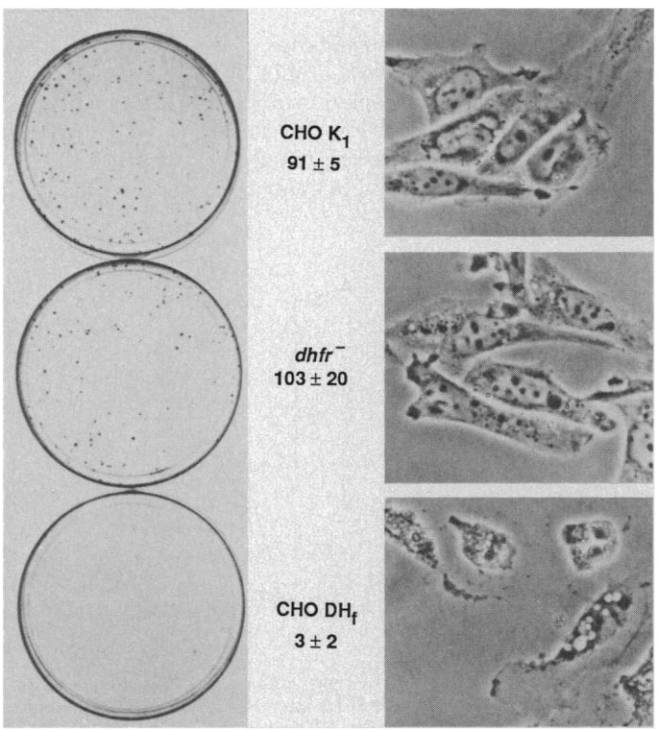


**Fig. 3.** Cell survival after heat shock. (**Left**) CHO $K_1$, *dhfr*$^-$, and CHO $DH_f$ cells were plated at $5 \times 10^4$ cells per plate. The cells were then heat-shocked for 30 min and subsequent colony formation evaluated in fixed and stained cultures after 7 to 10 days. Relative colony formation is indicated at center as mean ± SD × $10^{-4}$ with results from 6 or 7 plates pooled from three separate experiments. (**Right**) Cells were photographed 24 hours after heat shock.


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7. The pDH plasmid contains 2.9 kb of a pGEM3 (Promega Biotec)–derived vector sequence that confers ampicillin resistance and provides a bacterial origin of replication, a 4.0-kb dihydrofolate reductase minigene as a eukaryotic selectable marker (MG4) [C. S. Gasser and R. T. Schimke, *J. Biol. Chem.* **261**, 6938 (1986)], and a fragment (nucleotides −250 to +470) of an *hsp70* gene (hsp70A) (*5*). The plasmid was grown in *Escherichia coli* strain RR1.
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10. R. N. Johnston and B. L. Kucey, unpublished data.
11. Heat shock conditions used in these experiments were adapted from those described by G. C. Li and A. Laszlo [in *Changes in Eukaryotic Gene Expression in Response to Environmental Stress*, B. G. Atkinson and D. B. Walden, Eds. (Academic Press, New York, 1985), pp. 227–254]. Culture plates (100 mm in diameter, containing 10 ml of medium) with exponentially growing cells were sealed with Parafilm and placed in a 45°C circulating water bath for 20 or 30 min, as indicated; the culture medium was replaced after the heat treatment. For radioactive labeling, cells were incubated for 2 hours in the presence of 15 μCi of [$^{35}$S]methionine or [$^{14}$C]leucine per milliliter. The cells were then rinsed four times in phosphate-buffered saline, collected by scraping with a rubber policeman in 10 m*M* tris and 1 m*M* EDTA, *p*H 7.4, lysed by freezing and thawing and mixing in a vortex mixer, then centrifuged for 10 min at 12,000*g*. The trichloroacetic acid–precipitable supernatants were then analyzed. Incorporation of radioactivity in the heat-shocked samples varied from 5% to 50% of controls, with inhibition being greatest after heat shock of the CHO $DH_f$ cell line. The lanes of heat shock proteins were therefore loaded with larger amounts of total protein to equalize radioactivity per lane.
12. The mouse monoclonal antibody used in these experiments is designated C92 [W. J. Welch and J. P. Suhan, *J. Cell Biol.* **103**, 2035 (1986)] and is reactive against both the constitutive (73-kD) and inducible (72-kD) products of the *hsp70* gene family in hamster cells. Binding of secondary antibody linked to horseradish peroxidase was detected by reaction with diaminobenzidine and with color enhancement by $CoCl_2$. Equal aliquots of soluble protein were loaded per lane.
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# Unexpectedly High Levels of HIV-1 RNA and Protein Synthesis in a Cytocidal Infection

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**The expression of a laboratory strain of HIV-1 (HTLV-IIIB) has been studied in mitogen-stimulated peripheral blood lymphocytes (PBLs) and in two lymphoid cell lines (CEM cells and C8166 cells). HIV-expressing cells contained from 300,000 to 2,500,000 copies of viral RNA per cell. Near-synchronous expression of an active infection could be achieved in C8166 cells. In these cells, the high copy numbers of viral RNA used as much as 40% of total protein synthesis for the production of viral *gag* protein, with high levels of viral RNA and protein synthesis preceding cell death by 2 to 4 days.**

STUDIES OF HIV-1–INDUCED CELL killing have shown that HIV-1–envelope glycoproteins can kill cells by inducing the formation of syncytia (*1*). Recently we reported that HIV-1–induced cell killing does not necessarily involve syncytium formation, because mitogen-stimulated peripheral blood lymphocytes (PBLs) as well as certain lymphoid cell lines undergo HIV-1–induced cytolysis as single, mononucleated cells (*2*). The current studies were undertaken to determine whether the HIV-1 life cycle might influence the cytopathicity of this virus. The life cycles of cytopathic viruses typically result in infections using 30% or more of the protein synthesis of the host cell (*3*). This domination of host protein synthesis is achieved by viruses such as adenovirus or poliovirus actively disrupting the production or translation of host messenger RNA (mRNA) or by viruses such as vesicular stomatitis producing levels of viral RNAs (several hundred thousand copies per cell) that compete with host mRNA for translation. In contrast, typical retrovirus infections produce relatively low copy numbers of viral mRNAs (several thousand copies per cell) and use only 1 to 2% of host protein synthesis (*4*). These moderate levels of expression have been consistent with retrovirus-infected cells continuing normal growth and differentiation while producing virus.

HIV-1 can undergo latent as well as active phases of infection (*5*). In most commonly used cell cultures, simultaneously infected cells produce viral proteins and undergo cytolysis at staggered times after infection. The early stages of a typical retrovirus life cycle (adsorption and penetration, uncoating, reverse transcription, and proviral integration) require between 12 and 24 hours in growing cells (*6*). However, in growing H9 cells, CEM cells, and mitogen-stimulated PBLs, very few infected cells express viral proteins by 48 hours after infection (*2*).

Because it is difficult to study an infection in which virus expression is not synchronized, a series of cultures [H9 cells (*7*),

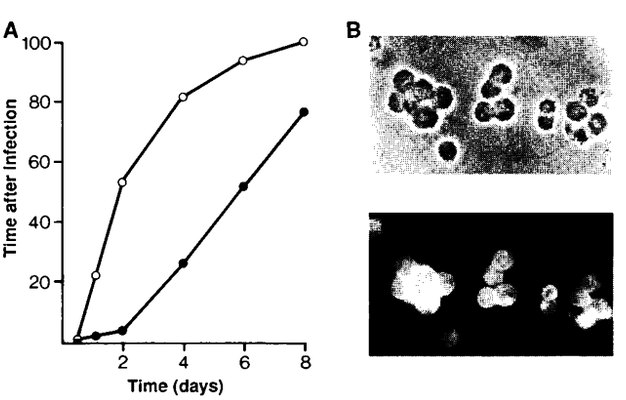


**Fig. 1.** Near synchronous expression of HTLV-IIIB in C8166 cells. (**A**) Time course of appearance of HTLV-IIIB protein-expressing cells (○) and dead cells (●). (**B**) Photomicrographs at 3 days after infection: phase (top) and fluorescence (bottom) microscopy. C8166 cells were infected at a multiplicity of infection of ≥0.2 infectious units of HTLV-IIIB. Leu3a (240 ng/ml) (Becton Dickinson) was added 3 hours after infection. Cultures were maintained at densities of 0.5 to 1.5 × $10^6$ cells per milliliter in RPMI 1640 supplemented with 15% fetal bovine serum and 240 ng/ml of leu3a by changing the culture medium at 2-day intervals. HTLV-IIIB–expressing cells were detected by indirect immunofluorescence on fixed cells (*2*). Dead cells were defined as cells that failed to exclude trypan blue.

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