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USE OF A TRANSFECTED AND AMPLIFIED DROSOPHILA HEAT SHOCK PROMOTER CONSTRUCTION FOR INDUCIBLE PRODUCTION OF TOXIC MOUSE C-MYC PROTEINS IN CHO CELLS

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Use of a transfected and amplified Drosophila heat shock promoter construction for inducible production of toxic mouse c-myc proteins in CHO cells.

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ABSTRACT

After transfection and selection with methotrexate, CHO cell lines were established which contained up to 2000 copies of an expression vector for c-myc protein. The vector contained the Drosophila heat shock protein 70 (hsp70) promoter fused with the coding region of the mouse c-myc gene. Incubation of cells for up to 3 hours at 43°C resulted in at least a 100-fold induction of recombinant c-myc mRNA. When cells were shifted back to 37°C, within 1 to 4 hours this RNA was translated into protein to yield about 250 μg per 10^9 cells. Cells died a few hours later, suggesting that high concentrations of intracellular c-myc are cytotoxic.

The gene of interest was introduced and amplified to high copy numbers in a silent form. The presence of so many Drosophila hsp70 promoters in the cells did not impair their induction. The method described here may provide a general tool for expression of proteins, especially those unsuitable for constitutive overproduction due to toxicity or other factors.

INTRODUCTION

A number of mammalian or viral proteins of pharmaceutical interest have been produced in Escherichia coli and yeast using inducible expression vectors (1,2). These vectors were chosen because the proteins, when expressed constitutively, proved to be toxic to the microbial host or inhibited its' growth rate.

In most transfected mammalian cells, production of proteins of interest has been accomplished by use of constitutive expression vectors, often having viral components (3-10). Inducible expresion systems, however, have certain advantages: Firstly, noninduced cells are expected to have higher growth rates because more of their metabolic activity can go into housekeeping functions. Secondly, there might be a substantial class of mammalian proteins whose high level expression is toxic to host cells. Thirdly, it might be desireable to initiate the formation of the product at will, especially in an industrial situation with hardware and working schedule constraints. Although of increasing interest, only a few inducible vectors have been used so far in mammalian cells (11-16). We describe here the Drosophila heat shock protein 70 promoter (hsp70 promoter) and its molecular biology in stably transfected cell lines. We believe it is the most effective inducible promoter

so far evaluated in mammalian cells. Its potential as a general expression vector in mammalian cells is discussed.

RESULTS AND DISCUSSION

General remarks

The Drosophila hsp70 promoter originates from a genomic fragment containing one of the multiple heat shock loci in Drosophila melanogaster (17,18). When used to drive the expression of a marker gene in mammalian cells, this promoter has an extremely low level of transcription at 37°C (19). Even in cells containing up to $4x10^4$ copies of a heat shock promoter, a basal level of transcription could not be detected (20). When cells were shifted to 43°C for 60 minutes or more, heat shock mRNA is induced at a rate equal to or greater than that of the strong SV40 promoter (19,20).

The product of the protooncogene c-myc is suspected to be toxic to mammalian host cells when overexpressed constitutively. This is based on the assumption that the expression of a protein with regulatory functions is controlled in a strict and sensitive way - so constitutive high level expression would create an imbalance in the cells' regulatory mechanisms. C-myc is highly conserved in vertebrates; several lines of evidence suggest that the gene and its products play an important role in mechanisms involved in control of cell growth and differentiation (21,22). In many instances the gene is responsible for the generation of malignant tumors after chromosomal rearrangements or when activated by retroviruses (23-26). The nature and function of the protein itself remains obscure due to its extremely low expression level in cells. Attempts to overexpress c-myc in mammalian cells using constitutive

promoters led either to very poorly growing refractile cells or to cell lines in which the gene was partially or totally deleted (27). In order to inducibly express the mouse c-myc protein we chose to transfect and stably integrate the c-myc gene under the control of the Dr. hsp70 promoter into dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary cells (CHO) (28). These cells are widely used for production of exogeneous mammalian or viral proteins (3-7). This is due to two major advantages of these cells over other systems. First, transfected DNA usually becomes integrated into the genome, thus enabling the establishment of stable cell lines (30). Second, using the gene for the enzyme dihydrofolate reductase as a selectable marker and treating cell populations with the DHFR antagonistic drug methotrexate, the integrated DNA can be amplified many times, generally leading to elevated expression levels (29, 30, 3-6).

Construction of expression vectors

To produce high yields of the c-myc protein, we transfected CHO-DUKX cells (28) with the vector pHS-Myc. This vector (see fig. 1) was made with a promoter containing a DNA fragment of 850 bp whose essential elements are - from 5' to 3': four "heat shock boxes", conserved sequences of 25 bp which have been found to be crucial for heat inducibility (19,33,34), the TATA box, the transciption start site and a stretch of 88 bp of the nontranslated leader sequence of the hsp70 gene. This part of the heat shock leader contains one of two "consensus sequences", which are conserved in various heat shock genes. The function of these sequences has not yet been elucidated (32). A 4.5 kb genomic mouse c-myc fragment consisting of exon 2 and 3 together with an intron and nontranslated 3'-sequences was linked to the promoter. The first exon of mammalian and avian c-myc genes contains multiple stop condons in all

reading frames and is regarded as noncoding (35, 36). In our construction, the first translation start codon following the transcription start site of the heat shock promoter is that of the c-myc gene (fig. 1). The selectable marker was provided by a second vector in which the expression of the c-DNA of a mouse DHFR gene was driven by the constitutive Adeno EII promoter (pCVSVEII-DHFR, 37). The plasmid pHS-Myc and pCVSVEII-DHFR were mixed in a ratio of 5:1 and transfected. Recombinant clones were picked after two weeks of growth in selective medium, propagated separately into 100 mm petri dishes, and then subjected to increasing concentrations of methotrexate. After six months 4 cell lines resistant to 320 μ molar concentrations of methotrexate were established.

Copy number and chromosomal location of transfected heat shock-c-myc construction

To test whether the transfected c-myc DNA had been integrated into the genome and amplified during methotrexate selection a Southern experiment was performed. Genomic DNAs were isolated, restricted, electrophoretically separated, and transferred to nitrocellulose. The filters were probed with *in vitro* transcribed P32-labelled c-myc specific RNA. As can be seen in fig 2, each of the 4 lines contained c-myc sequences, though to differing degrees. By comparing the intensities of the signals with those generated by dilutions of the plasmid pHS-Myc, the copy number of the construction in each of the four lines was estimated. The line 4HSMyc contained about 900 copies, 5AHSMyc 2000 copies, 5BHSMyc about 100 copies and 6HSMyc about 2500 copies. The additional bands generated by the latter line indicate DNA rearrangements which occurred probably early in the selective procedure and which were amplified together with the intact construction.

In the line 5AHSMyc the chromosomal location of the hsp70/c-myc construction has been investigated by using a biotinylated c-myc probe in in-situ hybridizations of metaphase chromosomes. The amplified heat shock c-myc construction was found to be located on the long arm of chromosome 2. (data not shown).

Inducibility and maintenance of recombinant c-myc transcripts

Cells were induced by feeding them with 43°C prewarmed medium and keeping them at that temperature for 1 to 2 hours. To measure the extent of the induction, RNA from induced and uninduced cells was isolated and hybridized to in vitro transcribed c-myc specific RNA. The hybrid molecules were digested with RNase to remove single stranded regions and subjected to polyacrylamide gel electrophoresis. The result of this experiment is shown in fig. 3a. Each of the cell lines exhibited a clear induction of the c-myc mRNA (lanes 1-10). It is important to note here that not a trace of a signal could be detected from cells kept at 37°C - even after prolonged exposures of the film. The induced concentrations of recombinant mRNA were proportional to the copy number of the introduced construction. Therefore there did not appear to be an insufficiency of cellular factors responsible for the induction of the hsp70 promoter region, even in the most highly amplified lines. This is shown in Fig. 3a, lanes 11-16, where the RNA from the line 5AHSMyc was analyzed using cells at different levels of methotrexate selection. We estimate that the degree of induction of the Drosophila hsp70 promoter in the amplified lines is at least 100 fold, because we could not detect a basal level of expression in uninduced lines. A similar kind of experiment was done to investigate how long after heat shock recombinant RNA remained detectable inside the cells.

The cells were induced at 43°C for 2 hours and then shifted back to 37°C for up to 3 hours. RNA isolated after 1,2, and 3 hours was hybridized and analyzed as described before. Recombinant mRNA was found even 3 hours after shifting the cells back to 37°C (Fig. 3b). We have not determined whether this was due to high stability of the recombinant c-myc RNA, continued high promoter activity or a combination of these effects. Recently, Simcox et al. (38) found that a deletion of the 3'end of hsp70 mRNA increased the stability of the mRNA during the recovery period after heat shock. Our construction did not have the hsp70 3'end either, hence the maintenance of the recombinant hsp70/cmyc mRNA may be due to the fact that it did not contain sequences responsible for rapid degradation of normal heat shock messages during recovery.

c-myc protein synthesis in induced recombinant cell lines

Initially, cell lines were investigated for c-myc protein synthesis immediately after a 2 hour heat shock at 43°C. At that time point no c-myc protein could be detected as judged by labelling with (35S) methionine or by immunoblotting. When cells were allowed to recover at 37°C for extended periods, however, c-myc protein was detected by both methods. The level of protein increased rapidly during the first 2-3 hours of recovery (fig. 4), but continued to increase only slightly over the next 6 hours. Usually cells were harvested for detection and isolation of c-myc after 3 hours of recovery.

Three protein bands with apparent molecular weights of 64,000, 66,000 and 74,000 were recognized by monoclonal and polyclonal antibodies raised against a portion of the human c-myc protein that is conserved in mouse c-myc (42).

The smaller two of these were seen in previous studies (39, 40). It is not clear whether the third species with a molecular weight of 74,000 is a form of c-myc present in normal cells or is an aberrant form. It is possible, for example, that overproduction of c-myc results in some modification, such as ubiquitination (43), not relevant to the normal biology of the c-myc protein. The cell line with the higher copy number (5AHSMyc) produced at least twice the amount of c-myc protein as the line 4HSMyc. In experiments using metabolic labelling of cells with 35S-methionine, it was found that protein synthesis in the recombinant lines during recovery after heat shock was reduced to about 20% of the values seen in the nonrecombinant parental line which was induced in the same way. This may have been due to the suspected c-myc toxicity on the cells metabolic pathways. In spite of this finding, the intensity of the c-myc band in SDS/polyacrylamide gels revealed that this protein was one of the most actively synthesized during the recovery period (fig. 4b, lane 4). In cells labelled with orthophosphate (32P), all three recombinant c-myc species were phosphorylated (fig 4b), indicating proper modification of these molecules. All avian and mammalian c-myc proteins analyzed so far have been found to be phosphorylated (41,42,44). It should be noted that the pure genetic information of the c-myc gene would produce an unmodified protein with a molecular weight of 48,000 D. It is not known why the c-myc proteins migrate in SDS/polyacrylamide gels at the molecular weight range of 62000 D to 68000 D (45). Whatever the basis of this anomalous migration, the CHO cells provided a means to produce a protein which by all known criteria appeared identical to authentic c-myc protein.

In immunoblot experiments we estimated the yields of recombinant c-myc produced by these cells relative to a standard of purified c-myc protein produced in insect cells (kindly donated by R. Chizzonite at Hoffman LaRoche). We estimated the amount of c-myc protein to be 200 to 300 μg per 10^9 cells. Theoretically, this corresponds with a specific productivity of about 2 fg per cell per day. We have developed a purification scheme for c-myc protein and are in the process of isolating larger amounts for further analysis.

Overproduction of c-myc protein is cytotoxic.

It was suspected that constitutive overproduction of c-myc is toxic. We tested, therefore, the effect of the induced overproduction of this protein on the viability of the cells. Parental and recombinant lines were plated at about 10% confluency, kept at 37^{o}C for 3 hours, then either heat shocked at 43^{o}C for 2 hours and then returned to 37^{o} for a period of 3 days, or, kept at 37^{o}C all the time. No living cells were left on heat shocked dishes of any of the recombinant lines, as well as both the heat shock and control dishes of the parental line, were confluent. An example of this experiment is shown in fig. 5a.

Microscopic examination of the recombinant cell lines during the recovery period showed detachment and slow deterioration of cells and no further cell divisions (fig. 5b). This may have been the result of a general cytotoxic effect of the high c-myc protein levels. Alternatively, the recombinant

hsp70/c-myc constructions may block or slow the normal cellular response to heat. Two observations favor the former possibility: Firstly, in SDS-polyacrylamide gels containing samples from the recombinant cells, the normal heat shock proteins were observed at their usual intensity relative to the other proteins (fig. 4). Therefore, there was a proper induction of the endogeneous heat shock genes despite the presence of so many recombinant heat shock genes. Secondly, as mentioned earlier, recombinant lines containing high constitutive levels of c-myc are extremely difficult to establish in culture and have an abnormal and labile phenotype. Hence there may be a continuous selection against cells which express the c-myc protein at high levels, due to the toxicity of the protein itself.

A general method for inducible expression in mammalian cells?

We chose to overexpress the mouse c-myc protein in CHO cells for two reasons: Firstly, we believed that this approach would enable us to obtain this protein in an authentic form in sufficient quantities to allow detailed analysis. Secondly, it was hoped the experiments would show whether the Drosophila hsp70 promoter could be used as a general tool for obtaining proteins which interact adversely with the mammalian cell host. We have demonstrated that the Drosophila hsp70 promoter is highly inducible even when amplified. The control exerted by this promoter was so tight that even after a 2000 fold amplification no protein was detected without an inducing heat shock. The expression levels of other mammalian inducible promoters, like MMTV-LTR, human metallothionein and interferon promoter are lower than those of the Drosophila hsp70 promoter when induced and they have higher levels of basal transcription in the range of 1% to 30% of the induced activity (11-16).

This may present an obstacle for the expression of toxic proteins, especially after amplification. Amplification methods presently used can often lead to at least 50 copies of the introduced genes. Under these conditions, a promoter with only 1% of its maximal activity would, without induction, give moderately high mRNA levels constitutively, which could prohibit expression of toxic proteins of interest.

A third reason to favor the Drosophila hsp70 promoter was the observation that the growth of the CHO cells was not impaired by the introduction and amplification of the DNA constructs. This again is probably due to the tightness of the promoter at 37°C preventing diversion of cellular metabolic and housekeeping functions thus permitting maximal growth rates. In large scale situations, where mammalian cells are used for production of proteins of pharmaceutical interest (46), cell growth and medium consumption rates are very important factors and economic advantages will accrue where they can be influenced positively. Thus the Drosophila hsp70 promoter might also be valuable for non-toxic proteins. It should be emphasized that the use of this promoter is not restricted to CHO cells but is functional in various other mammalian substrates as previously shown (19, 33, 47).

In summary, we have described a promoter and its use for the induced production of a rare protein in mammalian cells. This protein is thought to play a key role in the metabolism of cells and seems to exert a cytotoxic effect when expressed to very high levels for prolonged periods. We think the tightness of this promoter at 37°C and its degree of inducibility in highly amplified lines as shown will prove applicable for inducible expression of a variety of other mammalian proteins as well.

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LEGENDS TO FIGURES

FIGURE 1

Plasmids used to establish recombinant myc lines:

A. Expression vector pHS-Myc: This plasmid contains a genomic mouse c-myc fragment from a unique XbaI site 48 bp upstream of the translation start codon in exon 2 to the unique BamHI site about 2.5 kb downstream of exon 3. The filled boxes denote the c-myc coding region and the light dotted boxes denote the remainder of mouse c-myc genomic sequences. The open box denotes the *Drosophila* hsp70 promoter region, and the arrow denotes the start site of transcription. The fusion between the promoter region and the c-myc gene is at +88 of the 5' untranslated region of the hsp70 promoter.

B. DHFR selection vector pCVSVEII-DHFR (37): The DHFR coding region is denoted by the solid box, splice and polyadenylation signals are denoted by the hatched box, and the adenovirus EII promoter and SV40 enhancer region are contained in the area denoted by the open box.

FIGURE 2

Copy number of c-myc constructs in recombinant CHO cell lines:

Genomic DNA isolated from four different recombinant cell lines growing at 320 μM methotrexate (lanes 6-9, 2 μg each), or from the cell line

5A HS MYC growing at different levels of methotrexate (lanes 13-16, 0.5 µg each) was restricted to completion with HindIII and together with HindIII restricted dilutions of the plasmid pHSmyc (lanes 1-4): 20ng, 6ng, 2ng, 0.6ng and lanes 10-12: 4ng, 1.3ng, 0.45ng) subjected to agarose gel electrophoresis and Southern transfer to Genescreen Plus membranes (NEN). The membranes were then hybridized to SP6 polymerase in-vitro synthesized ^{32}P-RNA consisting of the sequences of the PstI-HindIII fragment containing c-myc exonxx 2, intron 2 and exon 3. The last lane on the right contains radiolabelled 1kb-ladder DNA (BRL).

FIGURE 3

A and B: Presence, inducibility and stability of c-myc mRNA in recombinant CHO cells.

Cytoplasmic RNA from uninduced (-) and heat shock induced (2 hours at 43 C, +) recombinant cells was analyzed using an internally labelled RNA probe (shown in B) on a 10% denaturing polyacrylamide gel (lanes 1-10) or on 6% non-denaturing polyacrylamide gels.

A. Lanes 1-10; RNA samples (10 µg) from recombinant cell lines (as indicated) in 0 methotrexate were analyzed. Lanes 11-16; RNA samples (5 µg) from the line 5A HS MYC growing at different levels of methotrexate were analyzed.

B. RNA samples (3 μg) from line 4 HS-MYC (lanes 1-4), 5A HS-MYC (lanes 5-8) and CHO-DUKX (lanes 9 and 10) were analyzed as in A. RNA was isolated from cells growing at 37°C (lanes 1,5, and 9), or from cells that had been incubated at 43°C for 2 hours and allowed to recover at 37°C for 1 hour (lanes 2 and 6), 2 hours (lanes 3, 7 and 10) or 3 hours (lanes 4 and 8). An SP6 RNA polymerase generated transcript containing the sequences of the hsp 70 promoter from PstI (+88) to HindIII (-700) was used as probe. The bands labelled 5' end migrate at 90 bases in denaturing gels.

FIGURE 4

A and B: Time course of induction of c-myc protein and analysis of protein levels in induced cells.

Levels of c-myc protein in cell lines growing at 37°C (A: lanes 1, 5, 9, 11; B: lanes 1, 3, 7,) and after heat shock induction for 2 h at 43°C and varying time periods of recovery at 37°C (A: lanes 2 and 6, 1 hour recovery; lanes 3, 7, and 10, 2 hours recovery; lanes 4 and 8, 3 hours recovery. B: lane 4, 1 hour recovery; lanes 2 and 5, 2 hours recovery; lane 6, 3 hours recovery). RIPA buffer lysates (2 ml) were prepared from $2x10^7$ cells. Samples (20 μl) were analyzed by immunoblotting using anti c-myc monoclonal antibodies (R. Chizzonite, Hoffmann-La Roche) and ^{125}Iodine labelled Protein A (NEN). In B, lanes 8 and 9 contain purified c-myc (lane 8;10 ng lane 9; 50 ng) dervied from recombinant insect cells (G. Ju, Hoffmann-La Roche).

C. The c-myc protein is one of the major proteins synthesized in the recombinant lines during recovery and is made as a phosphoprotein.

RIPA buffer lysates of ^{35}S methionine (lanes 1-4:400 000cm/lane) and ^{35}phosphate labelled proteins (lanes 5-10): 400 000cpm/lane, immunopreciptiation: lanes 11-17) were separated on 12% SDS-PAGE. Protein isolates were from the indicated cell line either growing at 37°C (-) or induced for 2 hours at 43°C (+) and allowed to recover at 37°C for either 2 hours (lanes 2, 4, 6, 9, 12 and 15) or hours (lanes 7, 10, 13, 16, and 17). Lane 17 is a light exposure of lane 16.

FIGURE 5

Overexpression of c-myc is cytotoxic.

A) 100 mm petridishes seeded with parental or recombinant CHO cells at about 10% confluency were heat shocked for 2 hours at 43°C and recovered for 3 days at 37°C (b,d) or left at 37°C all the time (a,c). Petridishes were stained with alcoholic cristalviolett solution (1%).

B. Micrographs from approximately 50% confluent CHO-DUKH B1 cells (a-d) and from recombinant cell line 4 HS MYC (e-h) before heat shock and after different periods of recovery after heat shock as indicated.

Fig 1

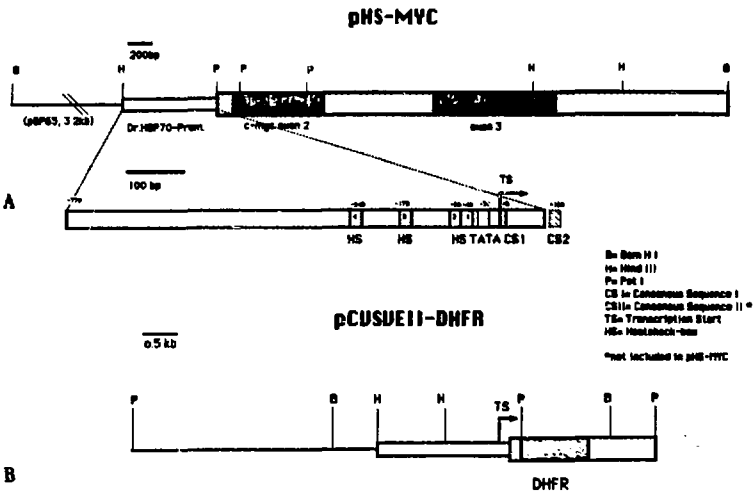

pHS-MYC
200bp
B
H
P
P
P
H
H
B
(pBR63, 3.2kb)
Dr.HSP70-Prom.
c-myc exon 2
exon 3
100 bp
TS
A
HS
HS
HS TATA CS1
CS2
B= Bam H I
H= Hind III
P= Pst I
CS I= Consensus Sequence I
CSII= Consensus Sequence II *
TS= Transcription Start
HS= Heatshock-box
*not included in pHS-MYC
pCUSVEII-DHFR
0.5 kb
P
B
H
N
TS
P
B
P
B
DHFR

Fig 2

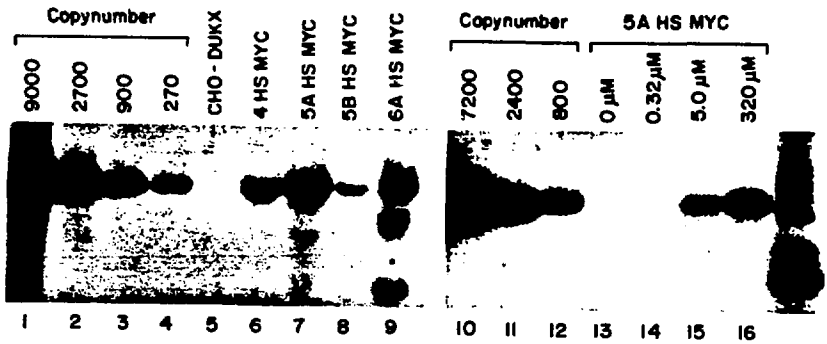
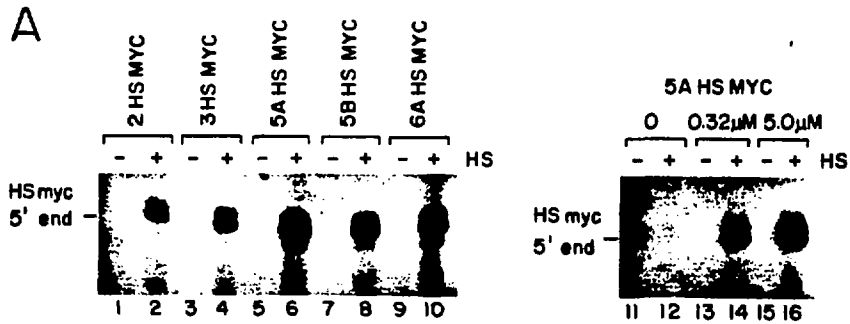

Copynumber
9000
2700
900
270
CHO - DUKX
4 HS MYC
5A HS MYC
5B HS MYC
6A HS MYC
Copynumber
7200
2400
800
5A HS MYC
0 µM
0.32µM
5.0 µM
320µM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig 3


A
2 HS MYC
3HS MYC
5A HS MYC
5B HS MYC
6A HS MYC
- + - + - + - + - + HS
HS myc
5' end
1 2 3 4 5 6 7 8 9 10
5A HS MYC
0 0.32μM 5.0μM
- + - + - + HS
HS myc
5' end
11 12 13 14 15 16

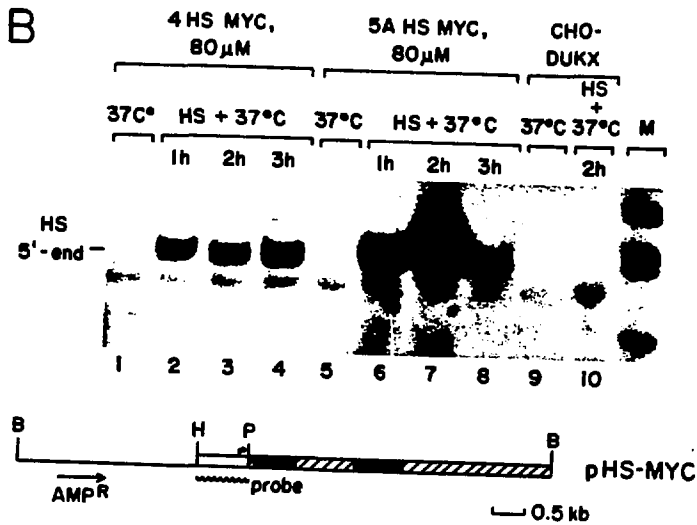
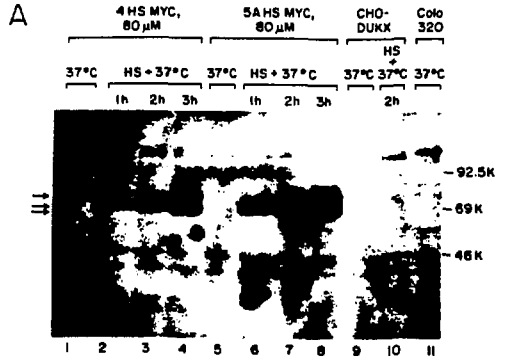
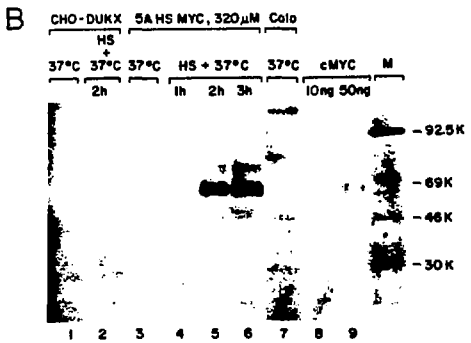
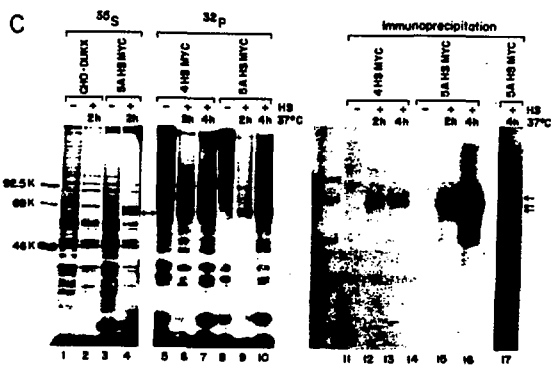
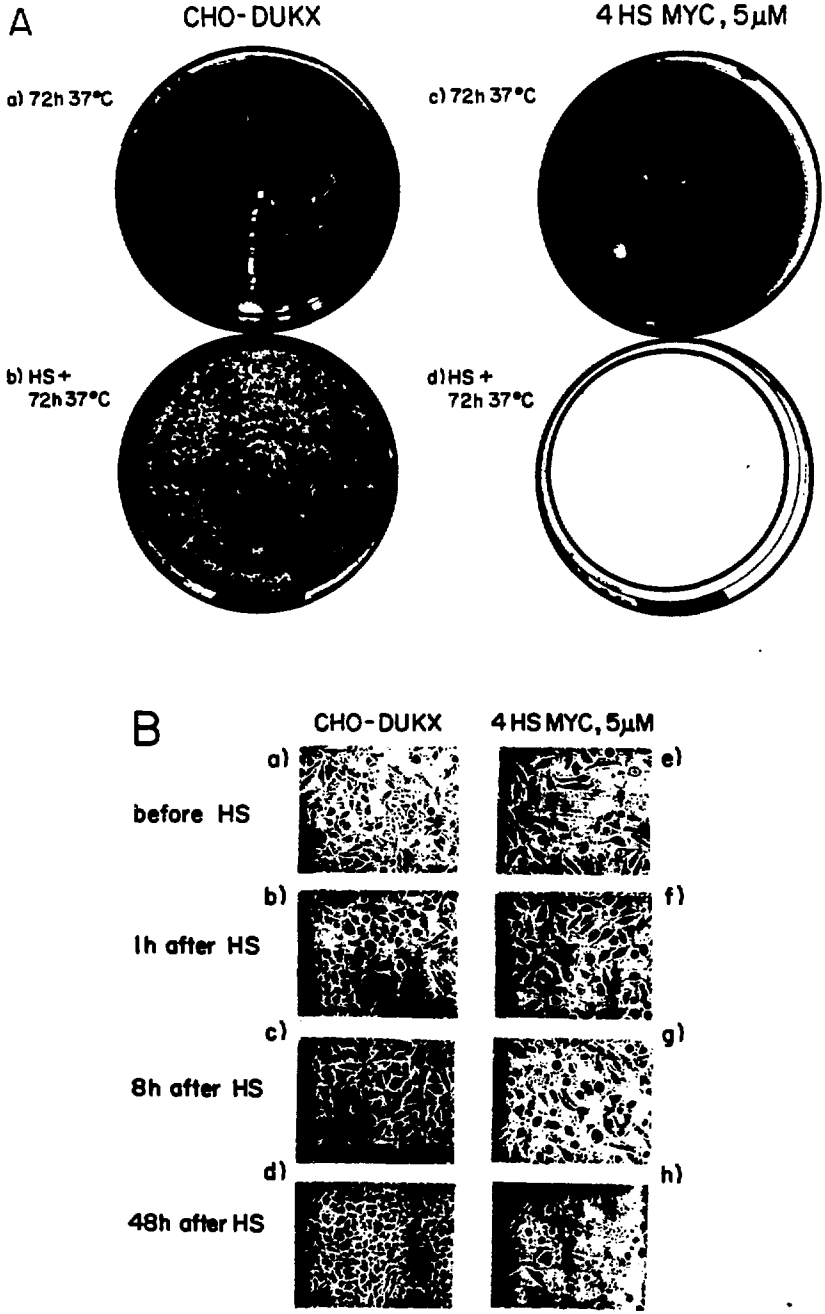

B
4 HS MYC, 80μM
5A HS MYC, 80μM
CHO-DUKX
37C° HS + 37°C 37°C HS + 37°C 37°C HS + 37°C M
1h 2h 3h 1h 2h 3h 2h
HS 5'-end
1 2 3 4 5 6 7 8 9 10
B H P B
AMP^R
probe
pHS-MYC
0.5 kb

Fig 4


A
4 HS MYC, 80 μM
5A HS MYC, 80 μM
CHO-DUKX
Colo 320
HS + 37°C
37°C
HS + 37°C
37°C
HS + 37°C
37°C
37°C
37°C
1h 2h 3h
1h 2h 3h
2h
92.5K
69K
46K
1 2 3 4 5 6 7 8 9 10 11


B
CHO-DUKX
5A HS MYC, 320μM
Colo
HS + 37°C
37°C
37°C
HS + 37°C
37°C
cMYC
M
2h
1h 2h 3h
10ng 50ng
92.5K
69K
46K
30K
1 2 3 4 5 6 7 8 9


C
35S
32P
Immunoprecipitation
CHO-DUKX
5A HS MYC
4 HS MYC
5A HS MYC
4 HS MYC
5A HS MYC
HS 37°C
2h
4h
92.5K
69K
46K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


A
CHO-DUKX
4 HS MYC, 5µM
a) 72h 37°C
b) HS +
72h 37°C
c) 72h 37°C
d) HS +
72h 37°C
B
CHO-DUKX
4 HS MYC, 5µM
a)
before HS
b)
1h after HS
c)
8h after HS
d)
48h after HS
e)
f)
g)
h)