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# A novel G<sub>1</sub>-specific enhancer identified in the human heat shock protein 70 gene

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## ABSTRACT

Expression of the human heat shock protein 70 gene (*hsp70*) is induced by various kinds of stress and by oncogenes. In the absence of stress, *hsp70* is mainly expressed in the G<sub>1</sub> and S phases of the cell cycle, but the elements contributing to cell cycle-dependent expression from the *hsp70* promoter remain elusive. We have previously reported that two elements, named HSP-MYCA and HSP-MYCB, located ~200 bp upstream (-200) from the transcription start site (+1) of human *hsp70*, are important for initiation of DNA replication at the *hsp70* locus. In this report we examine the effect of these two elements on transcriptional activity from the *hsp70* promoter, especially in terms of cell cycle-dependent expression. Various segments of the *hsp70* promoter region (up to -300) were linked to the luciferase gene and the constructs were transfected into mouse L cells to examine their transcriptional activity. A strong enhancer activity was defined in the HSP-MYCB element, but not in HSP-MYCA. Mutations introduced within HSP-MYCB abolished the transcriptional activation. In synchronized cells, pHB-Luc (a luciferase construct containing ~2.4 kb of the *hsp70* promoter region) as well as endogenous *hsp70* showed two peaks of expression; one in G<sub>1</sub> and the other in the S phase. Site-directed mutagenesis of HSP-MYCB in pHB-Luc abolished the expression peak in G<sub>1</sub>, but not that in the S phase. To test promoter specificity, wild-type and mutant HSP-MYCB elements were then linked to the luciferase gene in combination with the *hsp70*, the cyclin A or the PCNA promoter. Both in transient experiments and established cell lines, a strong peak of expression in mid-G<sub>1</sub> phase was observed with all the constructs containing wild-type HSP-MYCB, but not with the constructs containing the mutant sequence. These results suggest that the HSP-MYCB sequence is a G<sub>1</sub>-specific enhancer and is responsible for cell cycle-dependent expression of *hsp70*.

## INTRODUCTION

Expression of the heat shock 70 gene (*hsp70*) is induced by various stimuli, such as heat, serum or ionic stress. Various sequence motifs corresponding to the respective stimuli have been identified in the promoter region of human *hsp70*, including

a heat shock element (HSE) and a serum-responsive element (SRE), which is not classical (1–5). *hsp70* transcription is also regulated by a variety of oncogene products, such as T antigens of simian virus 40 and polyomavirus (6), adenovirus E1A (7–11), c-Myc (12,13), c-Myb (14) and wild-type p53 (15,16). Among them, E1A, Myb and p53 have recently been found to regulate *hsp70* expression by interacting with another protein(s) which directly binds to the respective DNA sequences in *hsp70*. As for regulation of *hsp70* by c-myc, two reports by Kingston and co-workers have shown that the sequences from -200 to -780 and from -120 to -1250 in *Drosophila* and human *hsp70* respectively are involved in regulation (12,13). Besides expression induced by stress or oncogene products, *hsp70* is constitutively expressed at the G<sub>1</sub>/S boundary and in the S phase of the cell cycle at both the RNA and protein levels (2,8,17). None of the regulatory elements, including the SRE and HSE, however, have been identified in terms of cell cycle-dependent expression of the gene.

We have reported that two sequences around -200 in human *hsp70*, termed HSP-MYCA (from -232 to -226) and HSP-MYCB (from -157 to -151), which are homologous to the putative DNA replication origin/transcriptional enhancer in the human c-myc gene, were bound by protein complexes including the c-Myc protein (18). The elements have also been found to be important for DNA replication. An initiation site of cellular DNA replication was mapped in the region containing the HSP-MYC elements of human *hsp70*. Moreover, the short segment containing, or oligonucleotides corresponding to, the elements showed autonomously replicating activity both in transient and stable systems (19).

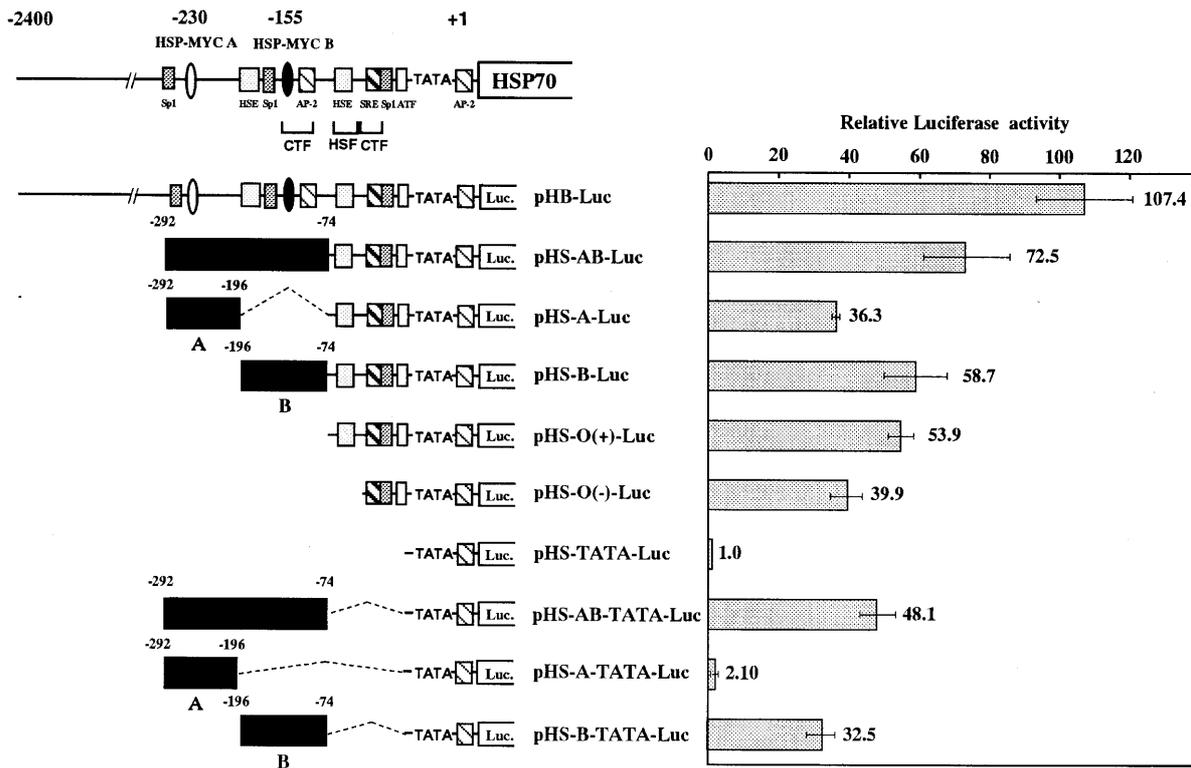
Here we examine the HSP-MYC sequences for transcriptional activity and reveal that HSP-MYCB, but not HSP-MYCA, has a strong enhancer activity on transcription from the *hsp70* promoter. Moreover, the HSP-MYCB element caused a peak of expression in G<sub>1</sub> of the cell cycle, not only for transcription from the *hsp70* promoter but also from the cyclin A and PCNA promoters. Introduction of mutations in the HSP-MYCB sequence abolished the expression peak in G<sub>1</sub>, but not that in the S phase. The results suggest that the HSP-MYCB sequence enhances transcription from various promoters specifically in G<sub>1</sub> and controls cell cycle-dependent expression of *hsp70*.

## MATERIALS AND METHODS

### Construction of plasmids

pH2.8, containing human heat-inducible type *hsp70*, and pHBCAT, possessing the *hsp70* upstream region linked to the

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**Figure 1.** Transcriptional enhancer domains in the region upstream from human *hsp70*. The region of ~2.4 kb upstream from human *hsp70* and its various segments were linked to the luciferase gene (shown in left panel) and the constructs were transfected into mouse L cells. Two days after transfection, the luciferase activities were assayed. Relative activities standardized to the activity due to pHS-TATA-Luc (set as 1), which contains only the minimal promoter of *hsp70* linked to the luciferase gene, are shown.

chloramphenicol acetyltransferase gene, were kindly supplied by R. Morimoto. Various segments of *hsp70* were cloned into pGVB containing the luciferase gene (see Fig. 1). For pHB-Luc, the *Bam*HI fragment of pH2.8 was inserted in the *Bgl*III site of pGVB. For pHS-O(+)-Luc, the *Bam*HI–*Sac*I fragment of pH2.8 was inserted between the *Bgl*III and *Sac*I sites of pGVB. For pHS-O(-)-Luc, pHS-O(+)-Luc was digested with *Xho*I, treated with exonuclease III and self-ligated. For pHS-AB-Luc, pHS-A-Luc and pHS-B-Luc, the *Hind*III–*Sac*I fragments of pHS-AB, pHS-A and pHS-B (19) respectively were inserted between the *Hind*III and *Sac*I sites of pGVB. The construct carrying the *hsp70* minimal promoter (containing the TATA box) linked to the luciferase gene in pGVB, designated pHS-TATA-Luc, was constructed as follows. PCR was carried out under the same conditions as in constructing pMuB2-HB-Luc below in a mixture of primer A (5'-CCCGGGCTTATAAAGCCAGGGG-3') and primer RL. The product was digested with *Sma*I and *Hind*III and inserted in the same sites of pGVB. For pHS-AB-TATA-Luc, pHS-A-TATA-Luc and pHS-B-TATA-Luc, the *Hind*III–*Sac*I fragments of pHS-AB, pHS-A and pHS-B respectively were inserted in the same sites of pGVB. For pwtA-TATA-Luc, pMuA-TATA-Luc, pwtB-TATA-Luc, pMuB-TATA-Luc, pSp1-TATA-Luc, pSp1-wtB-TATA-Luc, pwtA-wtB-TATA-Luc, pMuA-wtB-TATA-Luc and pwtA-MuB2-TATA-Luc, the nucleotide sequences of the oligonucleotides used for plasmid construction (18,19) were as follows (only plus strands are shown): HSP-MYCA, 5'-AGCTTCCTCTCAGGG-3'; HSP-MYCB, 5'-AATTCTGGCCTCTGATTG-3'; MuA, 5'-AGCTTCAGGTAGGG-3'; MuB2, 5'-AATTCTGGTCGCTGATTG-3'; Sp1,

5'-GACTTCGGGCGGAGTTAC-3'. The complementary oligonucleotides were annealed and ligated to *Sma*I sites of the *hsp70* minimal promoter in pHS-TATA-Luc. For pycA-Luc, the *Hind*III–*Sac*I fragment of the human cyclin A promoter (-516 to ~+245) (20) was inserted in the same sites of pGVB. For pwtB-cycA-Luc and pMuB2-cycA-Luc, the complementary oligonucleotides of wtB and MuB2 respectively were annealed and ligated to *Sac*I sites of pycA-Luc. For pPCNA-Luc, the *Sall*–*Hind*III fragment of the rat PCNA promoter (-240 to ~+78) (21) was inserted between the *Hind*III and *Xho*I sites of pGVB. For pwtB-PCNA-Luc and pMuB2-PCNA-Luc, the annealed wtB and MuB2 oligonucleotides respectively were inserted in the *Sac*I site of pGVB.

#### Site-directed mutagenesis

Mutation of HSP-MYCB in pHS-TATA-Luc was by polymerase chain reaction (PCR). Nucleotide sequences of the primers used were as follows: muB2-up, 5'-GtCgCTGATTGGTCCAAGGAG-3'; muB2-low, 5'-ATCAGcCaCCAGAGTGCCGCCC-3'; RL, 5'-GTTTTTGGCGTCAAC-3'; FL, 5'-GGTACTGTAAGTACTGAGCT-3'. RL and FL were complementary to the sequence upstream and downstream of HS-TATA-Luc in the vector respectively. First PCR was carried out in a mixture containing 60 mM Tris-HCl, pH 9.5, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 20 ng pHB-Luc, 50 ng each FL and muB2-low primers or muB2-up and RL primers and 2 U Taq polymerase (Expand High Fidelity; Boehringer) with pHS-TATA-Luc as template and reacted in a cycle of 94°C for 3 min and 30 cycles of 94°C for 1 min, 52°C for 2 min and 72°C for 3 min. One tenth volumes

of two PCR products were mixed and the second PCR was carried out in a mixture containing 50 ng each FL and RL primers and the same solutions as in the first PCR and reacted under the same conditions as the first PCR. The product was digested with *Xho*I and *Hind*III and inserted in the same sites of pGVB. The construct was named pMuB2-HB-Luc. Mutation in HSP-MYCB in pMuB-HB-Luc was confirmed by nucleotide sequencing.

### Cell culture and transfection

Mouse L or Balb3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Two micrograms of a reporter plasmid and 1  $\mu$ g CMV- $\beta$ -gal, a  $\beta$ -galactosidase expression vector, were transfected into ~60% confluent cells in a 6 cm dish by the standard calcium phosphate method (22). Two days after transfection, whole cell extracts were prepared by addition of the Triton X-100-containing solution from the Pica Gene Kit (Wako Pure Chemicals Co. Ltd, Kyoto, Japan) to the cells. An ~20% volume of the extract was used for  $\beta$ -galactosidase assay to normalize the transfection efficiency as described previously (23) and luciferase activity due to the reporter plasmid was determined using the Pica Gene Kit and a luminometer (Luminocounter ATP300; Advantec Toyo Co. Ltd, Tokyo). The same experiments were repeated five to 10 times.

### Establishment of cell lines

Subconfluent mouse Balb3T3 cells in a 10 cm dish were transfected with 2  $\mu$ g pHB-Luc, pMu-HB-Luc, pHS-TATA-Luc, pwtB-TATA-Luc or pMuB2-TATA-Luc together with 0.25  $\mu$ g pSV2bsr, an expression vector of the blasticidin S resistance gene linked to the SV40 promoter, by the standard calcium phosphate precipitation method. Two days after transfection, the cells were replated in medium containing 6  $\mu$ g/ml blasticidin S and the medium was changed every 3 days. Approximately 2 weeks after transfection, cell colonies resistant to blasticidin S appeared. The colonies were isolated and cell lines established. Total cellular DNA was extracted from the cell lines and digested with several restriction enzymes to confirm the intact form of the *hsp70*-derived sequence linked to the luciferase gene integrated in chromosomal DNA.

### Synchronization of the cells and analysis of DNA by flow cytometry

Cells were cultured under low serum conditions (0.2% calf serum) for 60 h to induce the G<sub>0</sub> phase of the cell cycle. The cells were harvested at various time after addition of serum to 10%. The cells were fixed with 70% ethanol, treated with 20  $\mu$ g/ml RNase A for 10 min at 37°C, stained with 25  $\mu$ g/ml propidium iodide for 10 min at room temperature and analyzed by FACSsort (Becton Dickinson). For transiently transfected cells, the serum in culture medium was reduced to 0.2% at 15–16 h after transfection and the cells were thus cultured for 36 h before addition of serum to 10% (24).

### Northern blot analysis

Total RNA was extracted from cells by the standard guanidine thiocyanate method. Twenty micrograms of the RNA was separated in a 1.4% agarose gel containing formaldehyde and blotted onto a nitrocellulose filter. Filters were hybridized under highly stringent conditions with <sup>32</sup>P-labeled cDNAs of *hsp70* and glyceraldehyde 3'-phosphodehydrogenase (G3PDH).

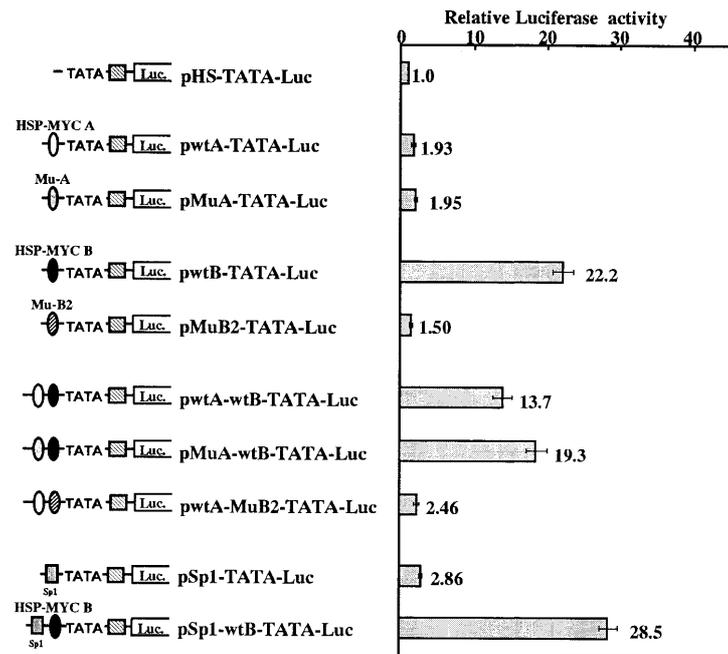
### Run-on assay

B-wtB cells cultured in two 10 cm dishes were synchronized in G<sub>0</sub> by serum starvation as above. Cells were harvested at various times after serum addition, washed with phosphate-buffered saline (PBS), treated in 0.5 ml lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub> and 0.1% NP-40 at 0°C for 10 min. Nuclei were prepared by centrifugation of the cells at 3000 r.p.m. for 5 min, washed with lysis buffer and suspended in storage buffer containing 50 mM Tris-HCl, pH 8.3, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 40% glycerol. *In vitro* transcription was carried out in a 100  $\mu$ l reaction mixture containing 10 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 300 mM KCl, 0.5 mM each ATP, CTP and GTP, 100  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP (3000 Ci/mmol) and the nuclei at 30°C for 30 min. The reaction mixture was then treated with 20  $\mu$ g/ml DNase I at 30°C for 10 min and stop solution containing 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 2% SDS, 100  $\mu$ g/ml proteinase K added. After incubation at 42°C for 30 min, RNA was extracted from the reaction mixture by phenol extraction, precipitated with ethanol and used for hybridization with a nitrocellulose filter containing 20  $\mu$ g each cDNA of *hsp70*, luciferase and G3PDH.

## RESULTS

### The HSP-MYCB element in *hsp70* has a strong enhancer activity

Various regulatory elements for transcription have been identified in the promoter region of human *hsp70*, up to -300 from the transcription start site (+1). In addition to the HSE, the SRE and binding sites for Sp1, AP-2 and ATF, we have previously identified two sequences, termed HSP-MYCA (at -230) and HSP-MYCB (at -155) respectively, as binding sequences for protein complexes containing c-Myc protein (18; see Fig. 1, upper panel). The HSP-MYCA and HSP-MYCB elements were suggested to be important for DNA replication at the *hsp70* locus (19). To examine the HSP-MYCB elements for transcription activity, various segments of the *hsp70* promoter region, with or without deletions, were linked to the luciferase gene. The constructs were transfected into mouse L cells and enzyme activity was assayed. The activity due to each construct was standardized by that for pHS-TATA-Luc, which contains the minimal promoter sequence of *hsp70*, including the TATA box (Fig. 1). The strong luciferase activity from pHS-AB-Luc containing the sequences up to -292 (value 72.5) was decreased by 50% by deleting region B (from -196 to -74), including HSP-MYCB (36.3 for pHS-A-Luc), while deletion of region A (from -292 to -196), including HSP-MYCA, had less effect on the activity (58.7 for pHS-B-Luc). The region between the minimal promoter and regions A and B, where various elements including the HSE, SRE, Sp1 and ATF binding sites exist, showed strong activation of transcription. To see the effect of the A and B regions on *hsp70* expression without the influence of the other transcriptional elements, regions A and B were directly linked to the TATA box in pHS-TATA-Luc and luciferase activity was examined. Both regions A and B (pHS-AB-TATA-Luc) and B alone (pHS-B-TATA-Luc) dramatically stimulated luciferase activity, while region A alone (pHS-A-TATA-Luc) showed little effect. The results suggest that a strong enhancer activity is present in region B, but not in A.



**Figure 2.** Transcriptional enhancer activity of HSP-MYCA and HSP-MYCB elements in the region upstream from human *hsp70*. Several oligonucleotides were synthesized corresponding to HSP-MYCA, HSP-MYCB and the Sp1 binding sequence in the *hsp70* promoter region, with or without mutations, and were cloned into pHS-TATA-Luc, which contains only the minimal promoter of *hsp70* linked to the luciferase gene, as shown on the left. The constructs were transfected into mouse L cells. Two days after transfection, the luciferase activities were assayed. Relative activities standardized to the activity due to pHS-TATA-Luc (set as 1) are shown.

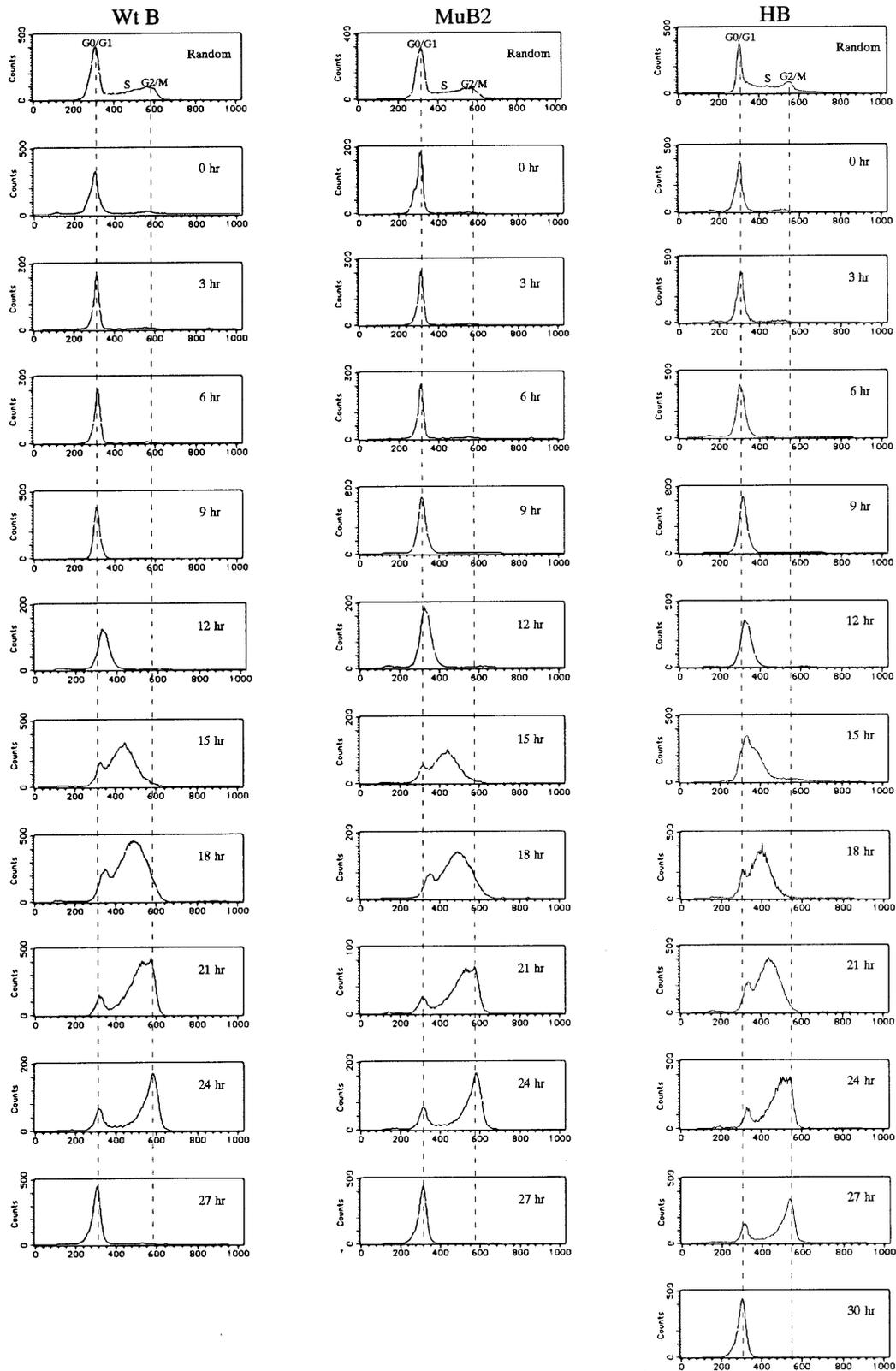
Since region B contains the HSP-MYCB element, oligonucleotides corresponding to the element with or without mutations were linked to the TATA box in pHS-TATA-Luc and luciferase assays carried out. As shown in Figure 2, the wild-type HSP-MYCB sequence (pwtB-TATA-Luc) highly activated transcription. Introduction of a mutation within the sequence (from 5'-TGGCCTCTGATT-3' to 5'-TGGtCgCTGATT-3') suppressed transcription activation (pMuB2-TATA-Luc). Only a slight enhancement of transcription was observed with either the wild-type or mutated HSP-MYCA sequences (pwtA-TATA-Luc and pMuA-TATA-Luc). Since region B contains the Sp1 binding site in addition to HSP-MYCB, reporter plasmids containing the Sp1 site alone or both the Sp1 and HSP-MYCB sequences linked to the TATA box (pSp1-TATA-Luc and pSp1-wtB-TATA-Luc) were constructed and assayed. The results indicate that activation of the *hsp70* TATA box due to the HSP-MYCB element was much stronger than that due to the Sp1 binding site. Among the constructs containing HSP-MYCA and HSP-MYCB in tandem (pwtA-wtB-TATA-Luc, pMuA-wtB-TATA-Luc and pwtA-MuB2-TATA-Luc), mutation in HSP-MYCB decreased enhancer activity, while mutation in HSP-MYCA had little effect. These results hence suggest that the HSP-MYCB element is a major element responsible for transactivation due to region B.

Region B showed a strong enhancer activity when isolated and directly linked to the minimal promoter, as described above. In the presence of other transcriptional elements, including the HSE and SRE between region B and the minimal promoter, however, additional enhancement of expression due to region B was not so significant: expression of pHS-B-Luc (Fig. 1; value 58.7) was comparable with that of pHS-O(+)-Luc lacking region B (Fig. 1, value 53.9). Moreover, site-directed mutagenesis of the HSP-MYCB element in pHB-Luc covering the whole promoter region

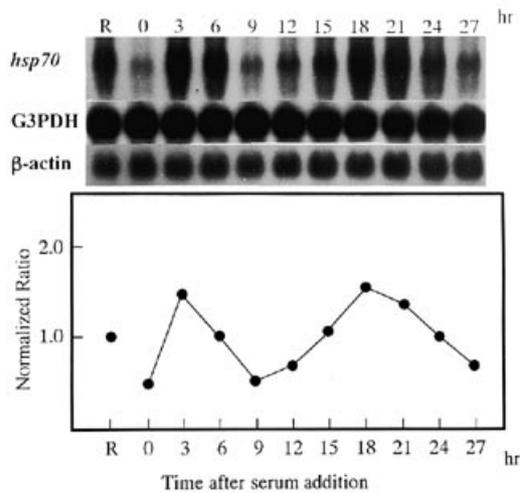
of *hsp70* hardly affected the level of expression (see Figs 6 and 7, data for pHB-Luc and pMuHB-Luc). The contribution of the HSP-MYCB element to *hsp70* transactivation with the whole promoter region was thus suggested to be insignificant, at least concerning general levels of expression in exponentially growing cells, although HSP-MYCB by itself strongly enhanced transcription.

### HSP-MYCB contributes to a G<sub>1</sub>-specific enhancement of transcription of *hsp70*

The question was what is the role of HSP-MYCB, a potentially strong enhancer, in regulation of *hsp70* expression. Since previous reports described higher constitutive expression of *hsp70* at the G<sub>1</sub>/S boundary and in the S phase than in other phases of the cell cycle (1,8,17), we examined the element for cell cycle-dependent transcription. Various luciferase constructs containing the *hsp70* promoter region or corresponding oligonucleotides (namely pHB-Luc, pMuHB-Luc, pwtB-TATA-Luc, pMuB2-TATA-Luc and pHS-TATA-Luc) as well as pSV2-Luc were co-transfected with pSV2bcr, a blasticidin S resistance gene expression vector, into mouse Balb3T3 cells and the cells cultured in the presence of blasticidin S. The cells resistant to blasticidin S were then isolated. Total cellular DNA was extracted from the resistant cells and analyzed by digestion with various combinations of restriction enzymes to select cell lines which harbored the introduced luciferase gene and adjacent sequences in intact form. All cell lines containing one copy of the intact form of the respective luciferase gene and adjacent sequences, examined by Southern blotting (data not shown), were used in further experiments. Allowing for clonal differences, at least three clones



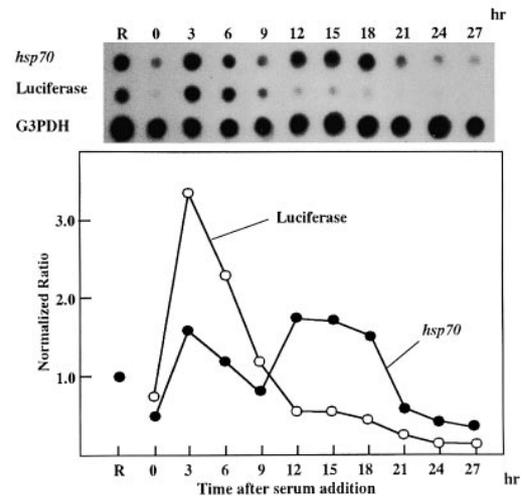
**Figure 3.** Cell cycle analyses of B-WtB, B-MuB2 and B-HB cells by flow cytometry. The cell lines B-WtB, B-MuB2 and B-HB, harboring pwtB-TATA-Luc, pMuB2-TATA-Luc and pHB-Luc respectively, were synchronized by serum depletion for 60 h. At various times after addition of serum the cells were stained with propidium iodide and analyzed by flow cytometry as described in Materials and Methods. Random indicates the cells from random culture without synchronization. Times after serum addition and the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle are indicated.



**Figure 4.** Northern blot analysis of endogenous *hsp70* expression. Total RNA was extracted from various cell lines harvested at various times after synchronization, blotted to a nitrocellulose filter and hybridized with labeled *hsp70*,  $\beta$ -actin and G3PDH cDNA as probes. The results for the cell line B-WtB-2 are shown. Numbers above the figure show the times (h) after serum addition to the cells after starvation. R indicates sample using randomly cultured cells. Normalized ratios of *hsp70* expression to that of G3PDH are plotted in the lower panel after quantitation of the intensities of signals by the imaging analyzer (Fuji BAS 2000).

of cell lines transfected with the same plasmid were similarly examined and the results for a clone are shown.

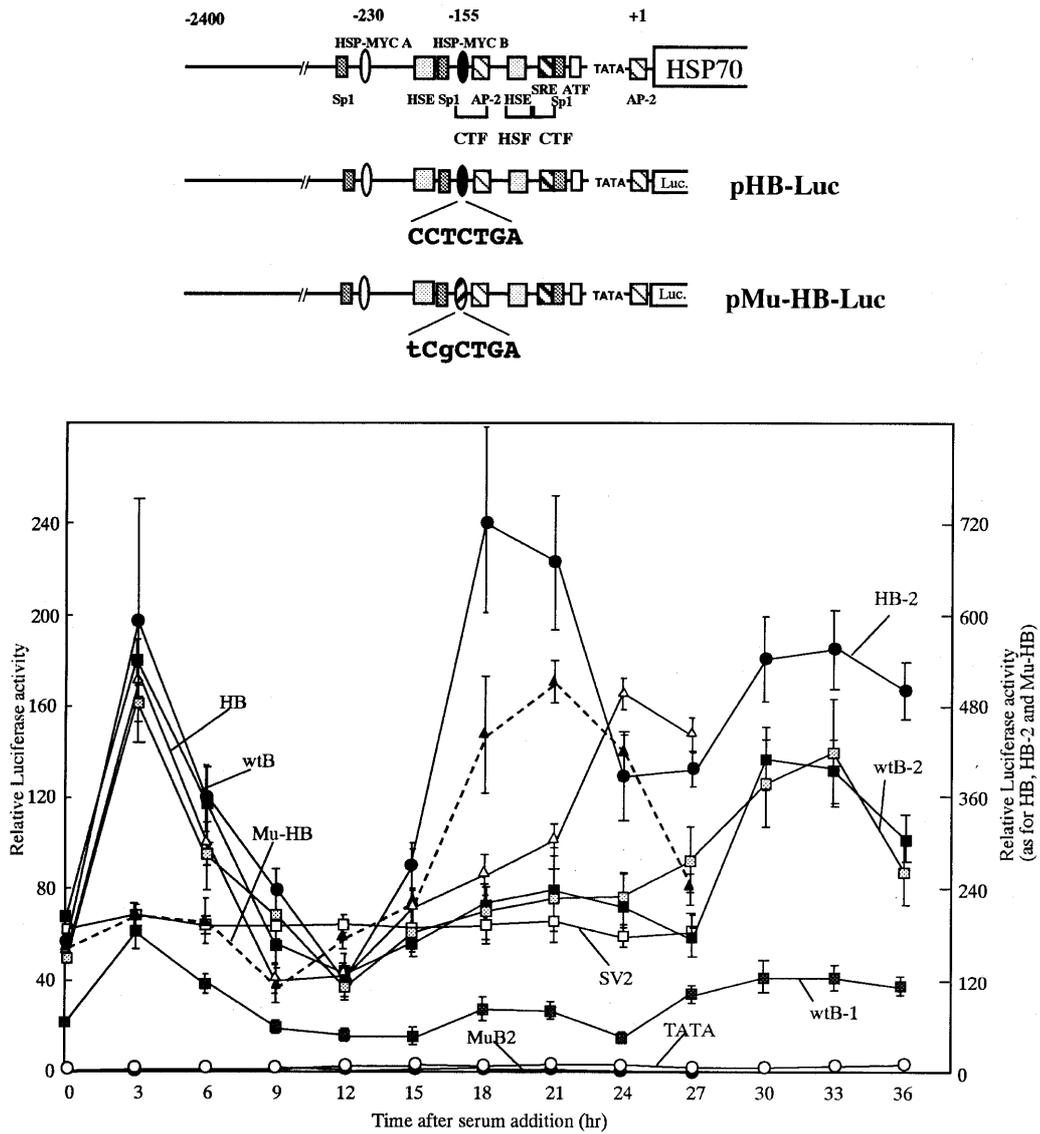
The cells were synchronized in the  $G_1/G_0$  phase by serum depletion (in the presence of 0.2% serum) for 60 h and analyzed by flow cytometry of the cells after staining with propidium iodide. The results for the B-WtB, B-MuB2 and B-HB cells, transfected with pwtB-TATA-Luc, pMuB2-TATA-Luc and pHB-Luc respectively, are shown as examples (Fig. 3). After the addition of serum, all the cells entered S phase together and then the  $G_2$  and M phases. Twelve hours after serum addition, the cells began to enter the S phase and the peak of S phase appeared between 15 and 18 h in B-WtB and B-MuB2 cells and between 18 and 21 h in B-HB cells. Twenty seven or 30 h after serum addition, B-WtB and B-MuB2 cells or B-HB cells entered the  $G_1$  phase of the second round of the cell cycle respectively. B-WtB1 and B-WtB-2, transfected with pwtB-TATA-Luc, and B-HB-2 cells, transfected with pHB-Luc, showed similar cell cycle movements to those of B-WtB and B-MuB2 cells in flow cytometry analyses (see Fig. 6). To verify expression of endogenous *hsp70* in the cell lines during the cell cycle, total RNA was extracted from the synchronized cells at various times after serum addition and analyzed by Northern blotting. In all the cell lines endogenous *hsp70* transcription showed two peaks during the cell cycle; a peak at 3 h and a strong one at 15–24 h, as reported previously (2,17; Fig. 4). Furthermore, to determine transcriptional activity of the cells at a given time, nuclear run-on assays were carried out. Nuclei were prepared from the cells at various times after serum addition and *in vitro* transcription was carried out in the presence of  $^{32}P$ -labeled UTP. Labeled RNAs were used as probes in a hybridization reaction with the cDNAs for *hsp70*, luciferase and G3PDH. The results for RNA from B-WtB cells, transfected with pwtB-TATA-Luc, are shown in Figure 5 as a typical example: two peaks of expression for *hsp70*



**Figure 5.** Nuclear run-on assay using synchronized cell extracts. Nuclei were prepared from B-WtB cells harvested at various times after synchronization and RNAs were synthesized *in vitro* in the presence of  $^{32}P$ UTP. The cDNAs of the *hsp70*, luciferase and G3PDH genes blotted on filters were hybridized with the labeled RNAs synthesized *in vitro*. Numbers above the figure show the times (h) after serum addition to the cells and R indicates sample using randomly cultured cells. Normalized ratios of *hsp70* or luciferase expression to that of G3PDH are plotted in the lower panel after quantitation of the intensities of signals by the imaging analyzer (Fuji BAS 2000).

appeared 3 and 12–18 h after serum addition, in comparison with a single peak at 3 h for luciferase. Similar results were obtained in more than three experiments, consistent with the luciferase activities observed in B-WtB cells (see Figs 6 and 7).

Luciferase activities in the synchronized cell lines were assayed using extracts prepared at various times (Fig. 6). In B-TATA cells, harboring the luciferase gene linked to the *hsp70* minimal promoter, enzyme activity was very weak at all times examined. A control cell line harboring the luciferase gene linked to the SV40 promoter/enhancer (B-SV2) yielded a moderately high activity independent of the cell cycle. B-WtB cells, transfected with pwtB-TATA-Luc, as well as B-HB and B-HB-2 cells, transfected with pHB-Luc, gave rise to rather high activity and a strong peak of activity appeared at 3 h after serum addition. B-HB and B-HB-2 cells yielded another peak of activity at 24 or 21 h respectively, as strong as that at 3 h. In B-WtB cells another slight peak was also observed at 21 h, but much weaker than that at 3 h. The timing of the two peaks corresponded to the early/mid- $G_1$  phase of the cell cycle (Fig. 3). Between the S and  $G_2/M$  phases (12–24 h after serum addition) the activity was lower than in the  $G_1/S$  phase. Two other cell lines transfected with pwtB-TATA-Luc, B-WtB-1 and B-WtB-2 showed the same or a similar pattern to that of B-WtB cells (Fig. 6). In B-MuB2 cells, transfected with pMuB2-TATA-Luc, carrying a mutation in HSP-MYCB of pwtB-TATA-Luc, activity was very weak at all times examined, comparable with that due to pHS-TATA-Luc containing only the *hsp70* minimal promoter, and no peak of activity was detected. In B-MuHB cells, transfected with pMuHB-Luc, a mutant of pHB-Luc containing mutation MuB2 in the HSP-MYCB element, enzyme activity was generally comparable with that in B-HB and B-HB-2 cells, but the peak at 3 h was not observed. To assess the activity of the HSP-MYCB sequence in  $G_1$ -specific expression of



**Figure 6.** Transcription from the *hsp70* promoter during the cell cycle in various cell lines. Stable cell lines harboring pHB-Luc (HB and HB-2), pMu-HB-Luc (Mu-HB), pwtB-TATA-Luc (WtB, WtB-1 and WtB-2), pMuB2-TATA-Luc (MuB2), pHS-TATA-Luc (TATA) and pSV2-Luc (SV2) were synchronized by serum depletion for 60 h. At various times after addition of serum the cells were harvested and luciferase activity was assayed. Relative activities from the average of three experiments, standardized to the activity due to pHS-TATA-Luc at time 0 (set as 1), are shown.

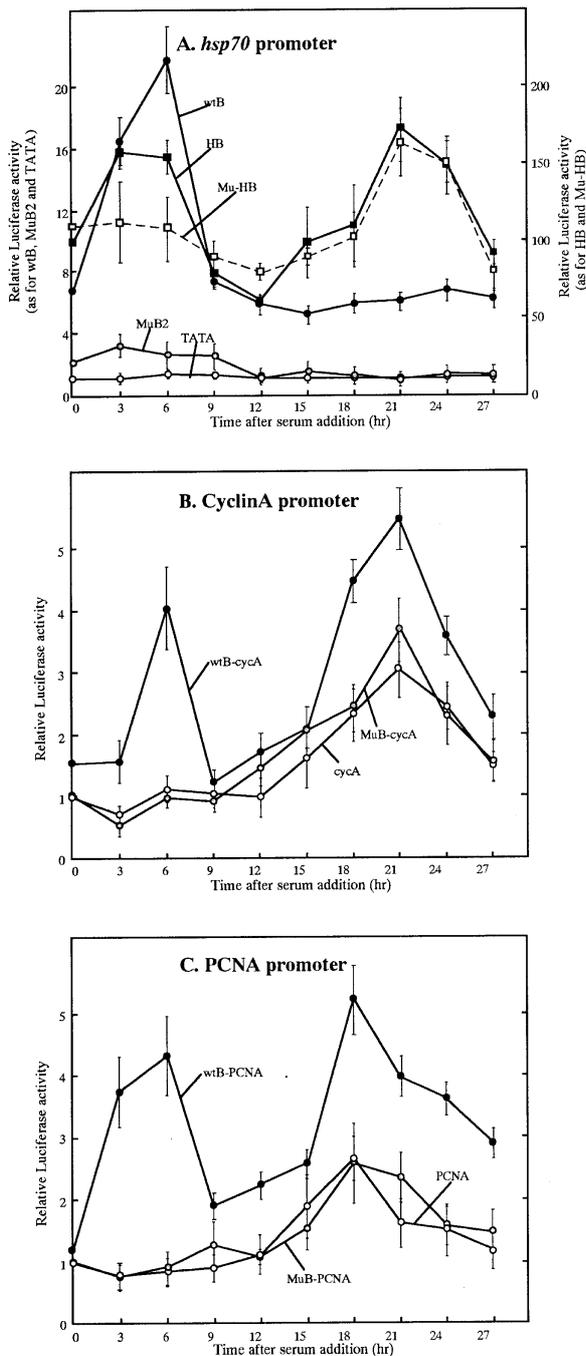
*hsp70*, the luciferase activities were assayed for a longer period after synchronization, over the second round G<sub>1</sub> phase of the cell cycle (Fig. 6). All the cell lines carrying pwtB-TATA-Luc or pHB-Luc showed activity peaks in both the first and the second G<sub>1</sub> phases, 3 and 30–33 h after serum addition. Only B-MuHB cells, on the other hand, showed no peaks, either in the first or the second G<sub>1</sub> phase. The results suggest that the contribution of the HSP-MYCB element to *hsp70* expression is a G<sub>1</sub>-specific, rather than general, enhancement of expression, although the element by itself strongly enhanced transcription from the *hsp70* minimal promoter.

Similar results were also obtained in transient experiments (Fig. 7). The whole promoter region of *hsp70* gave rise to two peaks of expression in the G<sub>1</sub> and the S phases of the cell cycle (Fig. 7A, HB), while the same region carrying mutations within

the HSP-MYCB sequence yielded a similar expression peak in the S phase but not in G<sub>1</sub> (Fig. 7A, Mu-HB). The HSP-MYCB element directly linked to the *hsp70* minimal promoter yielded a single peak in G<sub>1</sub> (Fig. 7A, wtB). Neither basal enhancement nor a peak in G<sub>1</sub> was observed for the mutated HSP-MYCB sequence linked to the minimal promoter (Fig. 7A, MuB-TATA and TATA).

**HSP-MYCB yields a G<sub>1</sub>-specific enhancement of transcription from various promoters, including the cyclin A and PCNA promoters**

The HSP-MYCB element thus enhances transcription from the *hsp70* promoter specifically in G<sub>1</sub> of the cell cycle. To test promoter specificity of the transactivation due to HSP-MYCB, the element with or without mutations was linked upstream of



**Figure 7.** Transcription from the *hsp70*, cyclin A or PCNA promoters during the cell cycle in transient transfected systems. Balb3T3 cells were transfected with various plasmids carrying the luciferase gene linked to several promoters in combination with the wild-type or mutated HSP-MYCB element and synchronized by serum depletion for 60 h. At various times after serum addition the cells were harvested and luciferase activity was assayed. The averages of results from three experiments are shown as relative activities to that of pHS-TATA-Luc (A), pCycA-Luc (B) or pPCNA-Luc (C) at time 0. (A) The luciferase gene was linked to the promoter of *hsp70* in the plasmids used for transfection. HB, pHB-Luc; Mu-HB, pMu-HB-Luc; wtB, pwtB-TATA-Luc; MuB2, pMuB2-TATA-Luc; TATA, pHS-TATA-Luc. (B) The luciferase gene was linked to the human cyclin A promoter in the plasmids used for transfection. wtB-cycA, pwtB-cycA-Luc; MuB-cycA, pMuB-cycA-Luc; cycA, pCycA-Luc. (C) The luciferase gene was linked to the rat PCNA promoter in the plasmids used for transfection. wtB-PCNA, pwtB-PCNA-Luc; MuB-PCNA, pMuB-PCNA-Luc; PCNA, pPCNA-Luc.

other promoters, namely the human cyclin A and the rat PCNA promoters, in combination with the luciferase gene. The constructs were transfected into Balb3T3 cells synchronized by serum depletion. The luciferase activity was assayed at various times after serum addition (Fig. 7B and C). Transcription from either the cyclin A or PCNA promoters showed a single strong peak at ~21 h, corresponding to the S phase, but another peak was hardly observed in G<sub>1</sub> (Fig. 7B, cycA; Fig. 7C, PCNA). Cells transfected with constructs containing wild-type HSP-MYCB yielded an obvious peak at 3–9 h, corresponding to G<sub>1</sub>, in addition to the peak in the S phase (Fig. 7B, wtB-cycA; Fig. 7C, wtB-PCNA). In cells transfected with constructs containing mutated HSP-MYCB sequence, in contrast, such induction of a G<sub>1</sub>-specific peak of expression was not observed (Fig. 7B, MuB-cycA; Fig. 7C, MuB-PCNA). The results suggest that the HSP-MYCB element enhances transcription specifically in G<sub>1</sub> not only from *hsp70* but also from various promoters.

## DISCUSSION

In this report we have examined the HSP-MYCA and HSP-MYCB elements in the human *hsp70* promoter region for transcriptional activity. Various segments of the *hsp70* promoter region or synthetic oligonucleotides corresponding to the HSP-MYCA and HSP-MYCB elements were linked to the luciferase gene and the constructs were transfected into mouse L cells to test transcriptional activity. HSP-MYCB, but not HSP-MYCA, showed a strong enhancer activity when the elements were isolated and ligated to the minimal promoter of *hsp70*. Mutations introduced within HSP-MYCB abolished transcriptional enhancement. The contribution of HSP-MYCB to the general level of *hsp70* expression, however, was not significant, although the element by itself highly transactivated expression. We further examined the element for cell cycle-dependent expression. Transcription of endogenous *hsp70* showed two peaks; one in G<sub>1</sub> and the other in the S phase of the cell cycle, consistent with former reports that *hsp70* was expressed in the G<sub>1</sub>/S or S phase at the RNA and protein levels respectively (2,8,17). The luciferase construct pHB-Luc containing the whole promoter region of *hsp70* also yielded two peaks of expression in the G<sub>1</sub> and S phases, similarly to endogenous *hsp70*. Luciferase activity due to pwtB-TATA-Luc, containing the HSP-MYCB element directly linked to the *hsp70* minimal promoter, on the other hand, showed a strong peak in mid-G<sub>1</sub>, as did pHB-Luc, but the peak in the S phase was almost missing. Neither basal transactivation nor cell cycle-specific peaks of expression were observed for pMuB2-TATA-Luc, a pwtB-TATA-Luc variant carrying mutations in the HSP-MYCB sequence. Moreover, site-directed mutagenesis of the HSP-MYCB element in pHB-Luc covering the whole promoter region of *hsp70* hardly affected the level of expression, but resulted in loss of the expression peak in G<sub>1</sub> of the cell cycle. Similar results were obtained in both transient experiments and stable cell lines. We hence conclude that the HSP-MYCB element defines G<sub>1</sub>-specific enhancement of *hsp70* expression.

The HSP-MYCB element strongly enhances transcription by itself, but the contribution of the element to general expression of *hsp70* is not significant. In addition to stress-responsive elements, such as HSE and SRE, a number of transcriptional elements exist in the *hsp70* promoter region and constitutive expression of the gene is fairly high, even in G<sub>0</sub> of the cell cycle. To induce an

expression peak over such a high background, the responsible element should be a powerful enhancer. A potentially strong transactivation activity of HSP-MYCB may thus allow the element to regulate G<sub>1</sub>-specific enhancement of *hsp70* expression. HSP-MYCB enhanced transcription specifically in G<sub>1</sub> not only from the *hsp70* promoter but also from various other promoters, including the cyclin A and PCNA promoters. The HSP-MYCB element is thus suggested to be a G<sub>1</sub>-specific enhancer and may thereby be called a G<sub>1</sub>RE (G<sub>1</sub>-responsive element). The *myc*(H-P) core sequence in an enhancer of the *c-myc* gene shares homology with the HSP-MYCB sequence and was also recognized *in vitro* by protein complexes including the c-Myc protein (25). The *c-myc* gene is expressed from the G<sub>1</sub> to the S phases and the *myc*(H-P) core sequence homologous to HSP-MYCB might contribute to cell cycle-dependent expression of *c-myc*.

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