A novel G₁-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 G₁ 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 G₁ and the other in the S phase. Site-directed mutagenesis of HSP-MYCB in pHB-Luc abolished the expression peak in G₁, 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-G₁ 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 G₁-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 G₁/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 autonomous 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 stronger enhancer activity on transcription from the hsp70 promoter. Moreover, the HSP-MYCB element caused a peak of expression in G₁ 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-MYC sequence abolished the expression peak in G₁, but not the S phase. The results suggest that the HSP-MYC sequence enhances transcription from various promoters specifically in G₁ 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
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 pH2.8-Luc, the BamHI fragment of pH2.8 was inserted between the BglII and SacI sites of pGVB. For pH-O(+)-Luc, the BamHI–SacI fragment of pH2.8 was inserted between the BglII and SacI sites of pGVB. For pH-O(−)-Luc, pH-O(+)-Luc was digested with XhoI, treated with exonuclease III and self-ligated. For pH-B-Luc, pH-A-Luc and pH-B-Luc, the HindIII–SacI fragments of pH2.8, pHS-A and pHS-B (19) respectively were inserted between the HindIII and SacI sites of pGVB. The construct carrying the hsp70 minimal promoter (containing the TATA box) linked to the luciferase gene in pGVB, designated pH2-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′-CCCCGGGCTTATAAAAGCCCGAGG-3′) and primer RL. The product was digested with Smal and HindIII and inserted in the same sites of pGVB. For pH2.8-Luc, pH2-TATA-Luc and pHS-B-TATA-Luc, the HindIII–SacI fragments of pH2.8, pHS-A and pHS-B respectively were inserted between the HindIII and SacI sites of pGVB. For pHS-TATA-Luc, the construct carrying the hsp70 minimal promoter (containing the TATA box) linked to the luciferase gene in pGVB, was designed as follows. PCR was carried out under the same conditions as in constructing pMuB2-HB-Luc below in a mixture of primer A (5′-CCCCGGGCTTATAAAAGCCCGAGG-3′) and primer RL. The product was digested with Smal and HindIII and inserted in the same sites of pGVB. For pH2.8-Luc, pH2-TATA-Luc and pHS-B-TATA-Luc, the HindIII–SacI fragments of pH2.8, pHS-A and pHS-B respectively were inserted between the HindIII and SacI sites of pGVB. For pHS-TATA-Luc, the construct carrying the hsp70 minimal promoter (containing the TATA box) linked to the luciferase gene in pGVB, was designed as follows. 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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 XhoI and HindIII 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 µg CMV-β-gal, a β-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 Trition 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 β-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 µg pHB-Luc, pMu-HB-Luc, pHS-TATA-Luc, pwtB-TATA-Luc or pMuB2-TATA-Luc together with 0.25 µg 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 µg/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.

**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 32P-labeled cDNAs of hsp70 and glyceraldehyde 3′-phosphodehydrogenase (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-MYC 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 A (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.

**Run-on assay**

B-wtB cells cultured in two 10 cm dishes were synchronized in G0 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 MgCl2 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 MgCl2, 0.1 mM EDTA and 40% glycerol. In vitro transcription was carried out in a 100 µl reaction mixture containing 10 mM Tris–HCl, pH 8.0, 5 mM MgCl2, 300 mM KCl, 0.5 mM each ATP, CTP and GTP, 100 µCi [α-32P]UTP (3000 Ci/mmol) and the nuclei at 30°C for 30 min. The reaction mixture was then treated with 20 µg/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 µg/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 µg each cDNA of hsp70, luciferase and G3PDH.


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'-TGGCCCTCTGATT-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 pH5-O(+)-Luc lacking region B (Fig. 1, value 53.9). Moreover, site-directed mutagenesis of the HSP-MYCB element in pH5-Luc covering the whole promoter region of hsp70 hardly affected the level of expression (see Figs 6 and 7, data for pH5-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 G1-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 G1/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, pwtA-TATA-Luc, MuB2-TATA-Luc, 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 $G_0/G_1$, S and $G_2/M$ phases of the cell cycle are indicated.
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 G1/G0 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 G2 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 G1 phase of the second round of the cell cycle respectively. B-Wt B1 and B-Wt B2, 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 of activity 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 32P-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 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-G1 phase of the cell cycle (Fig. 3). Between the S and G2/M phases (12–24 h after serum addition) the activity was lower than in the G1/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 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 G1-specific expression of
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 (wIB, wIB-1 and wIB-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.

In Figure 6, the luciferase activities were assayed for a longer period after synchronization, over the second round G1 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 G1 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 G1 phase. The results suggest that the contribution of the HSP-MYCB element to *hsp70* expression is a G1-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 G1 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 G1 (Fig. 7A, Mu-HB). The HSP-MYCB element directly linked to the *hsp70* minimal promoter yielded a single peak in G1 (Fig. 7A, wIB). Neither basal enhancement nor a peak in G1 was observed for the mutated HSP-MYCB sequence linked to the minimal promoter (Fig. 7A, MuB-TATA and TATA).

**HSP-MYCB yields a G1-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 G1 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. 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 G1 (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 G1, 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 G1-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 G1 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 G1 and the other in the S phase of the cell cycle, consistent with former reports that hsp70 was expressed in the G1/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 G1 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-G1, 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 G1 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 G1-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 G0 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 $G_1$-specific enhancement of $hsp70$ expression. HSP-MYCB enhanced transcription specifically in $G_1$ 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 $G_1$-specific enhancer and may thereby be called a $G_1$RE ($G_1$-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 $G_1$ 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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