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Basal Regulatory Promoter Elements of the hsp27 Gene in Human Breast Cancer Cells[1]

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The small human heat shock protein hsp27 has been shown to play important roles in diverse cellular processes such as actin polymerization, thermotolerance, growth, and chemotherapeutic drug resistance. Two breast cancer cell lines MCF-7 and MDA-MB-231 were used as a model to study the molecular mechanisms important for basal hsp27 promoter transcriptional activity. A genomic clone containing 1.1 kb of the hsp27 promoter was sequenced and the regulatory elements were characterized. The first 200 bp within this 5′-flanking region holds the majority of the transcriptional activity, according to transient transfection assays using a series of hsp27 promoter deletion fragments in luciferase reporter vectors. The basal activity of this fragment is largely confined to a G/C-rich region containing overlapping SP1 and AP2 transcription factor binding sites.

Hsp27 belongs to the family of small heat shock proteins (hsps) (1). Under normal conditions, high levels of hsp27 are found in normal estrogen target organs of the female reproductive tract, such as the uterus, vagina, cervix, and placenta (2,3). However, lower levels can be detected in several other normal cell types, including testis and smooth muscle tissues (for review see 1). High levels of hsp27 are also found in some human breast tumors and breast cancer cell lines, especially those that are positive for expression of the estrogen receptor (ER) (2). It is unclear why certain cell types express high levels of certain hsps, however it is well known that hsps are induced by a variety of stressful environmental conditions, such as exposure to heat and certain toxic substances (4). Some commonly used therapeutic agents also specifically induce expression of the small hsps (5). Likewise, a variety of different functions have been described for the small hsps, including roles in thermotolerance (6, 7), actin polymerization (8, 9), and protein chaperon activity (10).

It has long been thought that accumulation of hsps is an adaptive response to adverse environmental conditions. A number of studies suggest that hsp27 can protect cells from the cytotoxic effects of chemotherapeutic agents. The first evidence that hsp27 was directly involved in drug resistance was the early work of Huot et al. (11), who stably transfected a human hsp27 cDNA into Chinese hamster ovary cells. Hsp27-overexpressing hamster cells demonstrated increased cross resistance to doxorubicin, colchicine, and vincristine. In our experiments we have observed that heat shock treatment of human breast cancer epithelial cell lines increased their resistance to doxorubicin (12). More recently we have demonstrated that doxorubicin sensitivity of human breast cancer cells can be altered directly by modulating the level of hsp27 (13). These results have encouraged us to explore whether molecular approaches to modulate hsp27 expression could be used to inhibit hsp27-associated drug resistance.

To this aim, we have now used two breast cancer cell lines as a model to investigate the

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Abbreviations: ERE, estrogen-responsive element; ER, estrogen-receptor; HSE, heat shock element; HSF, heat shock factor; TK, thymidine kinase.

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mechanism(s) controlling basal hsp27 transcription. Approximately 1.1 kb of 5′-flanking region of the human hsp27 gene was sequenced, and the regulatory elements were characterized. This region of the hsp27 promoter contains several distinct features, including characteristics of housekeeping genes (such as SP1 binding sites), of regulated genes (TATA sequences), of heat-inducible genes (the heat shock element or HSE), and of estrogen-inducible genes (an imperfect estrogen response element or ERE). We have determined that the majority of the hsp27 promoter activity is contained within the proximal 200 bp of this 5′ region.

MATERIALS AND METHODS

Cell culture and transfection. The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells were originally obtained from Dr. Herbert Soule (Michigan Cancer Foundation). These cell lines were maintained as described in (13). For transient transfection assays, 8×10^4 cells were plated in 6-well plates. Transfections were carried out by the Lipofectamine method according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Cells were cotransfected with 1 μg of the different hsp27 promoter fragment-luciferase reporter constructions plus 0.2 μg of an pRSV-βgal control plasmid. The cells were incubated with DNA for 14 h, harvested 24 h later, and then analyzed for β-galactosidase and luciferase activities. Luciferase activity was determined using a commercial kit (Promega Corp., Madison, WS). β-galactosidase activity was assayed to correct for transfection efficiency, and was determined as described (14) using chlorophenolred β-galactopyranoside (CPRG; Boehringer Mannheim, Indianapolis, IN). Estrogen receptor (ER)-associated influence on the transcriptional activity was carried out by cotransfecting 20 ng of a CMV-driven ER expression vector into the ER-negative MDA-MB-231 cells line. For these experiments the cells were kept on phenol red-free Eagle's Medium containing 10% charcoal-treated FBS. Eight hours before harvesting, 10^{-8} M estradiol was added to the cells.

Plasmid constructions. The source of the human hsp27 promoter sequence was a 1105 bp Bgl II/Aat II fragment from lambda 2711 (15). This fragment contains both transcription start sites and extends into the 5′-untranslated leader region of the mRNA. The 3′ Aat II site was converted into a Hind III site by treatment with mung bean nuclease followed by insertion of a synthetic linker, and subcloning into BlueScript KS+ (Stratagene, La Jolla, CA). This fragment was then sequenced to determine an additional kb of upstream sequence beyond that reported (15). Four promoter 5′ deletion fragments terminating at the 3′ Hind III sites were generated by digestion with a 5′ Bgl II (−1090 bp), Bam HI (−722 bp), Pst I (−441 bp), and Eco RI (−210 bp). Each was introduced into the luciferase expression vector pLuc F3 replacing the SV40 promoter. To determine the specific elements in the most proximal region of the hsp27 promoter, three consecutive fragments designated E, F and G were amplified by polymerase chain reaction (PCR) using sense primers 6 (5′-CCTTAACGAGAGAAGGTTCCAGATGAGG-3′), 7 (5′-TGAGGGCTGAACCCTCTT-3′), 8 (5′-CTCAAA-CGGGTCATTGCCAT-3′), respectively, and antisense primer 5 (5′-TGCTCAGAAAAGTGCGG-3′) (the location of the primers is shown in Fig. 1). Hind III sites plus 2 additional nucleotides were added to the 5′ end of each primer for cloning purposes. The purified PCR products were Hind III digested and ligated into the Hind III site of the pGL2-Basic Luciferase vector (Promega). A mammalian expression vector for expressing ER was prepared by subcloning the Bam HI/Eco RI fragment from a previously described ER yeast expression plasmid, YEPE10 (16) into a similarly digested pcDNAI plasmid (Invitrogen, San Diego, CA). The reporter plasmid ERE-tk-luciferase was kindly provided by Donald McDonnell (Duke University) (17).

Western and Northern blot analysis. Cells were mechanically harvested, washed in phosphate-buffered saline, and extracted with 5% SDS as previously described (18). Equal amounts of protein (50 μg) were resolved on 12.5% polyacrylamide gels. Proteins were then transferred from the gel to nitrocellulose membranes and subjected to immunodetection with an hsp27-specific monoclonal antibody (19) and the Enhanced Chemiluminescence (ECL) system (DuPont, Boston, MA). Extraction of total RNA from cells and Northern blot analyses were accomplished by a previously described method (12). Hsp27 (20) and pGAD-28 cDNA clones (21) were used for hybridization probes.

Gel-retardation analyses. For the analysis of AP2 and SP1 binding sites, an hsp27 promoter fragment from −205 to −84 bp was used as a probe in gel-retardation assays. This fragment was amplified by PCR (22) using the following primers: sense 5′-ATTTGCTTTTCCTTAACG-3′ and antisense 5′-CAATGACCCGTTTGAG-3′. These two primers correspond to primers 1 and 3 in Fig. 1. Gel-retardation assays were performed as previously described (23) with nuclear extracts (24). Purified SP1 and AP2 proteins were obtained from Promega (Madison, WI). In some experiments a 100-fold excess of unlabeled oligonucleotides containing SP1-(5′-GATCGAACTGACCGCCCGCGGCCCGT-3′) consensus sequences were added as specific competitors. In other experiments, 50 ng, 250 ng or 1250 ng of antiserum specific for SP1 and AP2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the reaction was incubated for an additional 30 min. The SP1 oligonucleotides were obtained from Promega (Madison, WI), and all the other oligonucleotides were synthesized by Genosys (Houston, TX).

GeneBank accession number. The accession number for the human hsp27 promoter sequence reported in this paper is XO3900.

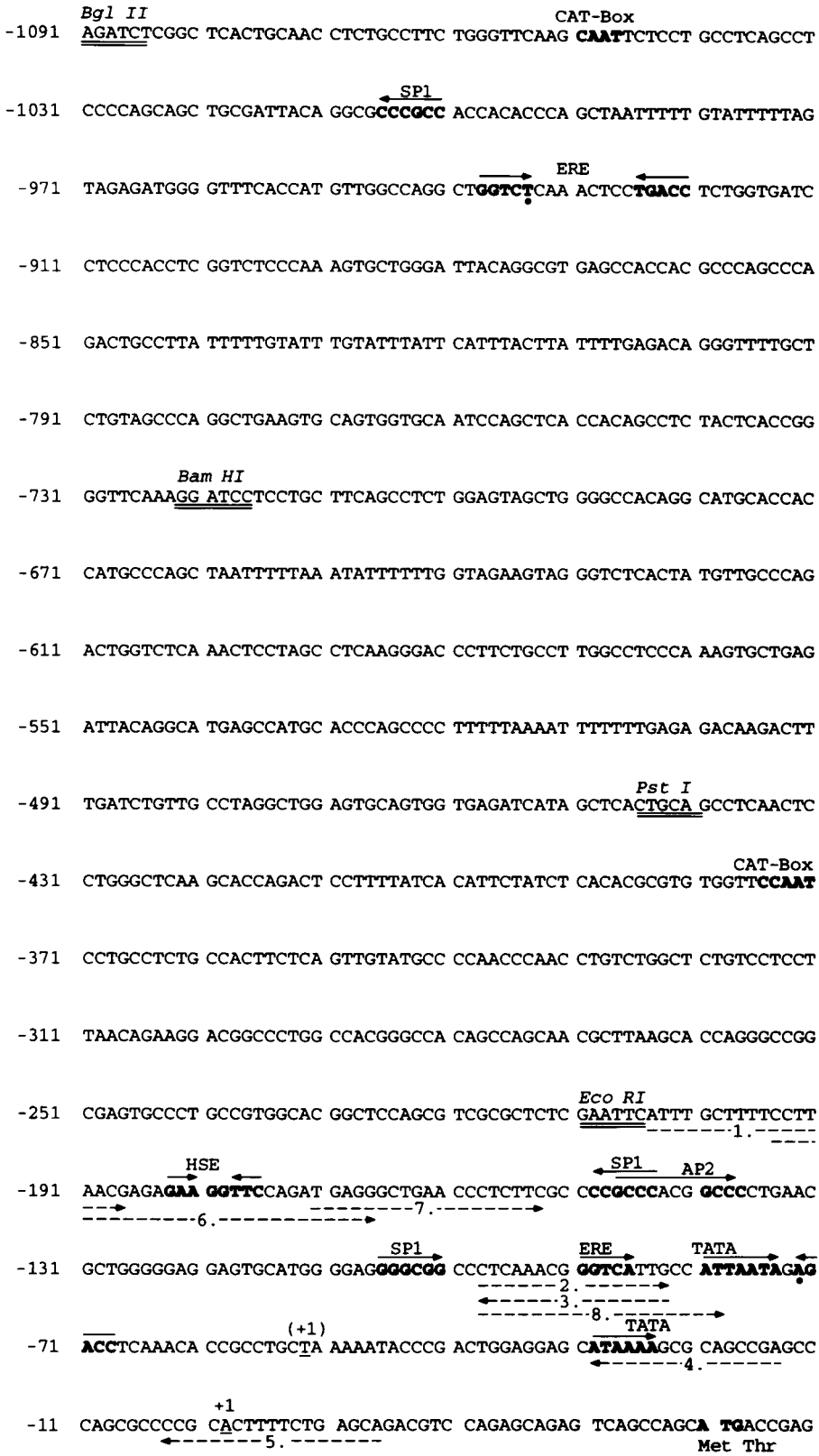
RESULTS

The hsp27 promoter contains multiple putative response elements. Only approximately 200 bp of the 5′-flanking region of the human hsp27 gene has been previously described (15). This 5′-proximal region contains a consensus HSE (GAAnnTTC at −177 to −184), (25, 26), two TATA sequences (ATAAAA and ATTAATA starting at −25 and −75, respectively) (27) upstream from the two mRNA transcription start sites, and finally a putative SP1 binding site (GGGCGG at −102 to −107). We have now determined additional hsp27 gene 5′-upstream sequences and this information along with the previously reported 5′-proximal region is shown in Fig. 1. The hsp27 promoter region contains two potential CAAT elements at −1048 (GCAAT) and at −372 (CCAAT). Approximately 150 bp upstream from the major transcription start site (15), there is an expanded G/C-rich region. This area contains two, partially-overlapping SP1 and AP2 sites in a tight cluster. The SP1 site starting at −145 bp (CCGCCC) is inverted, whereas the one previously reported at −102 bp (15) matches the consensus SP1 sequence (GGGCGG). The AP2 site (28, 29) located within this G/C-rich region is located at −145 to −153 (GCCCCGCCC). Finally, the hsp27 promoter contains elements which resemble ERE half-sites. The ERE half-sites are palindromic; however, the spacer regions between the two half-sites are longer in both cases than the 3 bp spacer found in a perfect consensus ERE (30). Furthermore, both the ERE at −921 (GGTCT-8 bp spacer-TGACC), as well as the ERE at −68 (GGTCA-13 bp-AGACC), have one mismatch each as compared to the consensus vitellogenin A1 ERE (GGTCANNNTGACC). Of interest is the inclusion of a TATA box within the 13 bp spacer of the most proximal ERE half-sites starting at −68. Thus, the promoter of the human hsp27 gene has a compound structure with many potential regulatory elements having features of both housekeeping and regulated genes.

Basal transcription is controlled by sequences in the proximal 200 bp region of the Hsp27 promoter. We chose for study two breast cancer cell lines with different steady state levels of hsp27 protein and RNA. Fig. 2 shows that there are 4 to 5-fold higher levels of hsp 27 protein (panel A) as well as hsp27 RNA expression (panel B) in the MCF-7 cell line as compared to the MDA-MB-231 cell line. First we undertook conventional promoter deletion analyses: Four consecutive hsp27 promoter deletion mutants were generated by restriction digestion and cloned into a luciferase reporter vector (Fig. 3A). We transfected identical amounts of each plasmid into MDA-MB-231 and MCF-7 cells. The results of a representative transient expression assay with these hsp27 constructs is shown in Fig. 3B. In both of the cell lines, the majority of the transcriptional activity was contained within the shortest fragment, the Eco RI (D) fragment. These results suggest that the most proximal region of the hsp27 promoter is the most important region involved in basal regulation.

The results shown in Fig. 3B suggest that a potential negative regulatory element may be contained within the Pst I (C) fragment. We observed that transcriptional activity in MCF-7 cells decreased 2.5-fold when the most proximal hsp27 promoter region was extended by an additional 231 bp from the EcoRI to the PstI site. This result suggests that this region, −210 to −441, may harbor a silencer element. However, there is no similarity between this region and any other region yet described to be active as a silencer element (for review see (31)). To confirm that this region does indeed contain inhibitory activity, we have cloned the Pst I to Eco RI fragment upstream of an SV40 promoter-driven luciferase reporter gene, and found a two-fold reduction of transcriptional activity (results not shown). Inhibition was found to be independent of orientation.

One possible explanation for the differences in hsp27 levels in these two cell lines is the fact that MCF-7 cells contain abundant levels of the estrogen receptor (ER) transcription factor, whereas MDA-MB-231 cells do not. Thus the difference in hsp27 transcriptional activity we observed could be due to ER-mediated transcriptional up-regulation in MCF-7 cells. We therefore tested this hypothesis by transiently cotransfecting MDA-MB-231 cells with an ER expression vector, along with the A through D hsp27 promoter fragments, however no significant increase in activity was


Bgl II
CAT-Box
-1091 AGATCTCGGC TCACTGCAAC CTCTGCCTTC TGGGTTCAAG CAATTCTCCT GCCTCAGCCT
SP1
-1031 CCCCAGCAGC TGCGATTACA GGCGCCCGCC ACCACACCCA GCTAATTTTT GTATTTTTAG
ERE
-971 TAGAGATGGG GTTTCACCAT GTTGGCCAGG CTGGTCTCAA ACTCCTGACC TCTGGTGATC
-911 CTCCCACCTC GGTCTCCCAA AGTGCTGGGA TTACAGGCGT GAGCCACCAC GCCCAGCCCA
-851 GACTGCCTTA TTTTTGTATT TGTATTTATT CATTTACTTA TTTTGAGACA GGGTTTTGCT
-791 CTGTAGCCCA GGCTGAAGTG CAGTGGTGCA ATCCAGCTCA CCACAGCCTC TACTCACCGG
Bam HI
-731 GGTTCAAAGG ATCCTCCTGC TTCAGCCTCT GGAGTAGCTG GGGCCACAGG CATGCACCAC
-671 CATGCCCAGC TAATTTTTAA ATATTTTTTG GTAGAAGTAG GGTCTCACTA TGTTGCCCAG
-611 ACTGGTCTCA AACTCCTAGC CTCAAGGGAC CCTTCTGCCT TGGCCTCCCA AAGTGCTGAG
-551 ATTACAGGCA TGAGCCATGC ACCCAGCCCC TTTTTAAAAT TTTTTTGAGA GACAAGACTT
Pst I
-491 TGATCTGTTG CCTAGGCTGG AGTGCAGTGG TGAGATCATA GCTCACTGCA GCCTCAACTC
CAT-Box
-431 CTGGGCTCAA GCACCAGACT CCTTTTATCA CATTCTATCT CACACGCGTG TGGTTCCAAT
-371 CCTGCCTCTG CCACTTCTCA GTTGTATGCC CCAACCCAAC CTGTCTGGCT CTGTCCTCCT
-311 TAACAGAAGG ACGGCCCTGG CCACGGGCCA CAGCCAGCAA CGCTTAAGCA CCAGGGCCGG
Eco RI
-251 CGAGTGCCCT GCCGTGGCAC GGCTCCAGCG TCGCGCTCTC GAATTCATTT GCTTTTCCTT
1.
HSE
SP1
AP2
-191 AACGAGAGAA GGTTCCAGAT GAGGGCTGAA CCCTCTTCGC CCCGCCCACG GCCCCTGAAC
6.
7.
SP1
ERE
TATA
-131 GCTGGGGGAG GAGTGCATGG GGAGGGCGG CCCTCAAACG GGTCATTGCC ATTAATAGAG
2.
3.
8.
(+1)
TATA
-71 ACCTCAAACA CCGCCTGCTA AAAATACCCG ACTGGAGGAG CATAAAAGCG CAGCCGAGCC
4.
+1
-11 CAGCGCCCCG CACTTTTCTG AGCAGACGTC CAGAGCAGAG TCAGCCAGCA TGACCGAG
5.
Met Thr

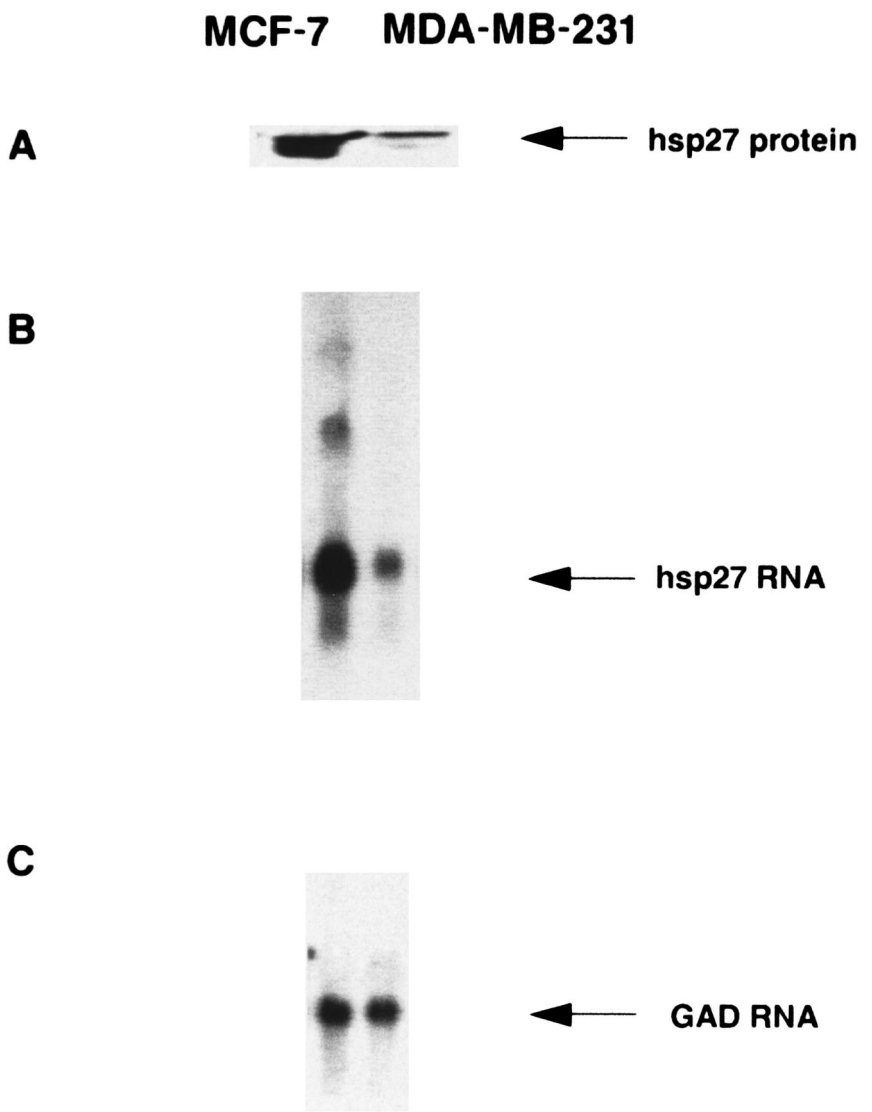


FIG. 2. Hsp27 expression levels in breast cancer cell lines MCF-7 and MDA-MB-231. A) Western blot analysis. B) Northern blot hybridization (hsp27). C) The nylon membrane from B) was reprobed with an pGAD-28 probe.

seen (data not shown). Thus, ER does not appear to be capable of directly transactivating this portion of the hsp27 gene promoter.

The G/C-rich region of the Hsp27 promoter is necessary for activity. As discussed in the previous section, the most proximal hsp27 promoter region (the D fragment in Fig. 3) contained the majority of the transcriptional activity in both MCF-7 and MDA-MB-231 cells. To localize in more detail the specific regulatory elements within this proximal region, we further subcloned +17 to

FIG. 1. Nucleotide sequence of the 1.1 kb 5′ region of hsp27. Bold sequences represent consensus recognition sequences (mismatches are marked with dots), and the arrow denotes the direction of the recognition site. Double underlined sequences are restriction sites used for subcloning of the different promoter regions. Primers used for PCR are indicated by arrows below the sequence.

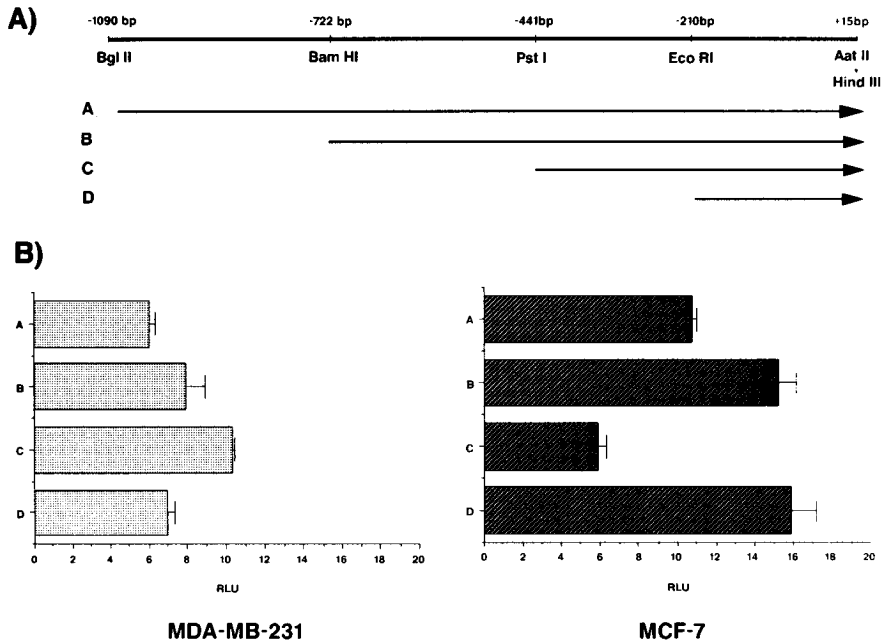


FIG. 3. Transcriptional activity of 5′ deletion mutants of the hsp27 promoter. A) Diagram of the hsp27 promoter deletion constructs. B) Relative luciferase activities (RLU) from experiments performed in duplicate. All transfections were repeated three times and yielded equivalent results.

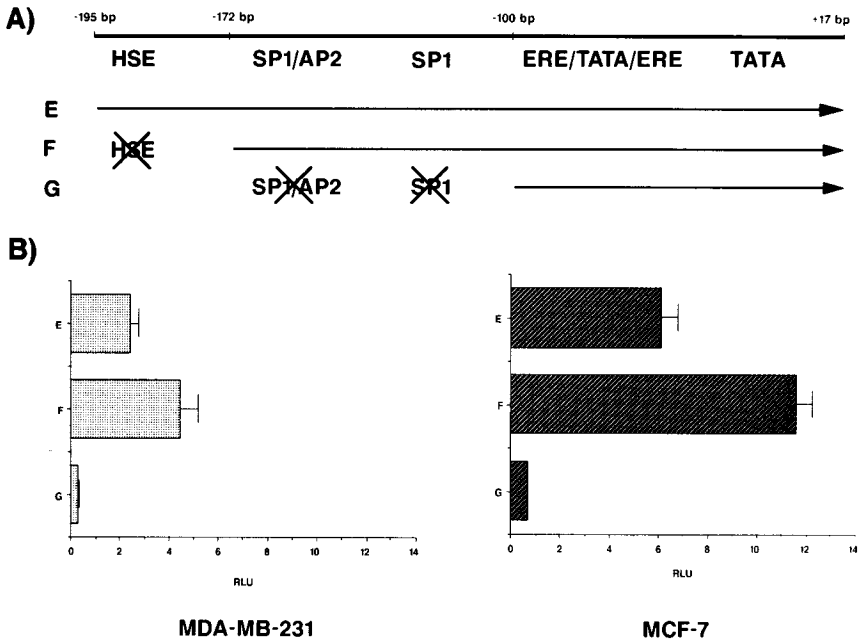


FIG. 4. Transcriptional activity of deletion mutants of the most proximal hsp27 promoter fragment. A) Diagram of the deletions made in the most proximal promoter region. B) Relative luciferase activities from experiments done in triplicate.

−195 bp (Fig. 4A). Three consecutive constructs were prepared: fragment E that essentially corresponds to fragment D in Fig. 3; fragment F in which the HSE has been deleted; and fragment G which eliminates the G/C-rich region containing the putative SP1 and AP2 elements. Interestingly, deletion of the HSE from the hsp27 promoter resulted in an increase in transcriptional activity (compare fragments E versus F in Fig. 4B). What is most apparent from this experiment, however, is that the majority of hsp27 transcriptional activity is lost upon deletion of the region containing the putative SP1 and AP2 sites. To determine whether the putative sites within this G/C-rich region could bind authentic AP2 and/or SP1, we next utilized gel-retardation analyses (Fig. 5) with a probe prepared from this region of the hsp27 promoter. First, commercially-available AP2 and SP1 proteins were used to determine the location of the hsp27 promoter-AP2 (lane 1) and the hsp27 promoter-SP1 (lane 2) retarded complexes in the gel-retardation assay. Nuclear extracts were then prepared from MDA-MB-231 (lanes 3–5) or MCF-7 cells (lanes 6–8), and were found to contain both SP1 and AP2 binding activity. SP1 binding (lanes 4,5, 7,8) was competed with excess nonradioactive SP1 consensus oligonucleotides. These results have been further substantiated with DNase I footprinting of this region (results not shown) and gel-retardation analyses using SP1 and AP2-specific antibodies (Fig. 5, lanes 9–14). The addition of increasing amounts of these antibodies resulted in further upshifts of the specific DNA-protein complexes. These results indicate that this region of the hsp27 promoter is competent for binding of these transcription factors, and furthermore that both factors are expressed in MDA-MB-231 and MCF-7 cells.

DISCUSSION

In this paper we have focused upon the transcriptional regulation of hsp27 in human breast cancer cells. In breast cancer, the emergence of drug resistance during treatment with chemothera-

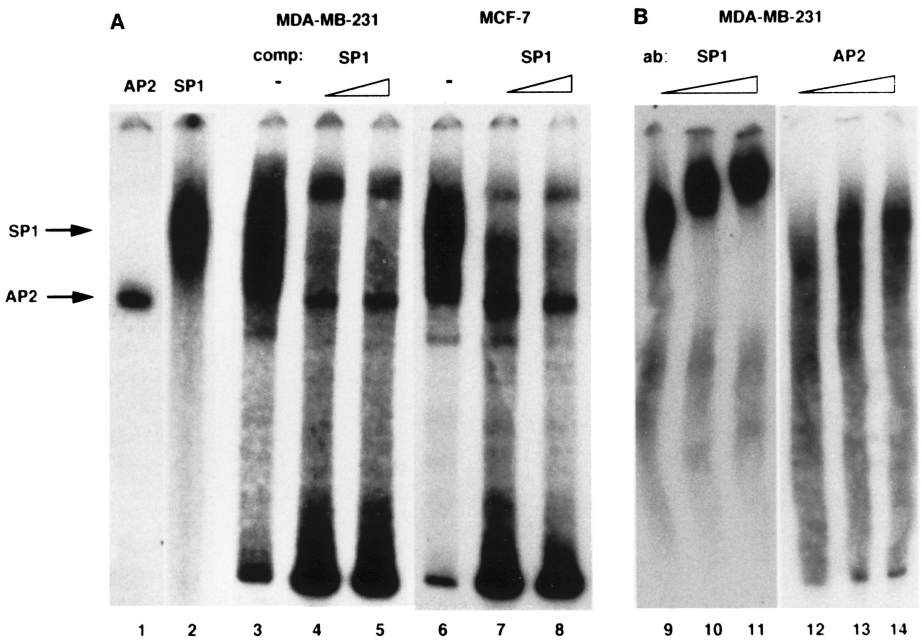


FIG. 5. AP2 and SP1 DNA-binding sites within the proximal hsp27 promoter. An hsp27 promoter fragment from −205 to −84 bp was used as a probe in gel retardation assays. A) In lane 1, purified AP2 protein (25 ng) and in lane 2, purified SP1 protein (30 ng) were added to the reactions. In lanes 3 to 5, MDA-MB-231 nuclear extract was incubated with the probe in the absence (lane 3) or the presence of a 25- and 100-fold excess of unlabeled oligonucleotides. Similar experiments were carried out as described in lanes 6–8, except nuclear extract from MCF-7 cells was utilized. B) MDA-MB-231 extracts were incubated with the probe in the presence of increasing concentrations of SP1 (lanes 9–11) or AP2 (lanes 12–14) specific antibodies.

peutic agents is a major clinical problem. Since hsp27 overexpression confers doxorubicin resistance on breast cancer cells (13), our eventual goal is to identify mechanisms by which we can interfere with this overexpression. We have been using two breast cancer cell lines, an ER-positive MCF-7 cell line and an ER-negative MDA-MB-231 cell line, to study regulation of hsp27 expression. Our previous studies suggest that breast cancer cells can utilize a number of regulatory mechanisms, including estrogen-dependent (32), heat shock transcription factor-dependent, or alternative pathways which are incompletely understood (23), to influence hsp27 levels. Thus the hsp27 promoter is regulated in response to a variety of stimuli in breast cancer cells. Because of the complexity of studying interacting transcriptional mechanisms, we have initially focused on *basal* hsp27 transcriptional activity.

It is known that many regulated promoters are frequently transcribed at basal levels, and we have determined that the hsp27 promoter has a significant level of basal expression in certain breast cancer cell lines. The majority of the hsp27 promoter activity is contained within the 210 bp region adjacent to the transcription start site. Interestingly, this region displays several similarities to elements found in the human hsp70 gene promoter that have been defined to bind specific factors important for basal hsp70 transcription. The proximal 150 bp of the hsp70 promoter contains these elements as the proximal hsp27 promoter, along with two sites which can bind CAAT transcription factor (CTF) (33). In rodent cells the elements required for basal hsp70 expression are the proximal CAT box, an SP1 site, and the TATA box; whereas in human cells, these proximal sequences and additional upstream sequences containing an HSE, an SP1, a CAAT sequence, and an AP2 site are required for basal transcription (34).

The presence of negative regulatory elements in the human hsp27 promoter might explain the observation that the human gene is expressed constitutively when introduced into rodent cells (7). It is possible that the heterologous inhibitory elements are not recognized by the equivalent rodent transcription factors.

The organization of the human hsp27 HSE is very similar to the mouse hsp25 gene, except that the mouse promoter has two palindromic HSE's (35). We can obtain heat shock induction of transcriptional activity in reporter constructions containing the hsp27 HSE (results not shown), thus this single HSE can confer heat inducibility. Interestingly, when the HSE is deleted from the hsp27 promoter, and this HSE-deleted construction is then tested for transcriptional activity, a two-fold increase in basal activity is seen (as shown in Fig. 4). This suggests that an HSE, or factors bound to the HSE, can influence the binding of downstream transcription factors involved in basal promoter activity. A repressing element was also found in the yeast hsp26 gene promoter and it has been proposed that heat-induced derepression plays an important role in activation of the gene during stress (36).

The G/C-rich region containing overlapping SP1/AP2 sites, and an additional downstream SP1 site, is the region most important for basal hsp27 transcription. Little is known about the role of AP2 in specific gene regulation (37, 38), but it is interesting to note that the AP2 transcription factor is induced by retinoic acid treatment in teratocarcinoma cells (39). Since it has been reported that mouse hsp25 can also be induced by retinoic acid treatment (40), it is tempting to speculate that the retinoic acid induction of mouse hsp25 might involve increased AP2 levels. It has also been shown that SP1 can bind to the first SP1 site in the hsp70 promoter, and that this binding stimulates hsp70 transcription *in vitro* (41). Thus, it would not be surprising if SP1 serves a similar important role in hsp27 basal transcription. Since other investigators have successfully targeted an SP1 site to decrease gene transcription in the human Ha-ras promoter, this region might prove to be a similarly useful target to modulate hsp27 expression in breast cancer (42).

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