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Novel Nuclear Matrix Protein HET Binds to and Influences Activity of the HSP27 Promoter in Human Breast Cancer Cells

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Abstract Since the small heat shock protein hsp27 enhances both growth and drug resistance in breast cancer cells, and is a bad prognostic factor in certain subsets of breast cancer patients, we have characterized the transcriptional regulation of hsp27, with the long-term goal of targeting its expression clinically. The majority of the promoter activity resides in the most proximal 200 bp. This region contains an imperfect estrogen response element (ERE) that is separated by a 13-bp spacer that contains a TATA box. Gel-shift analysis revealed the binding of a protein (termed HET for Hsp27-ERE-TATA-binding protein) to this region that was neither the estrogen receptor nor TATA-binding protein. We cloned a complete cDNA (2.9 kb) for HET from an MCF-7 cDNA library. To confirm the identity of the HET clone, we expressed a partial HET clone as a glutathione S-transferase fusion protein, and showed binding to the hsp27 promoter fragment in gel-retardation assays. The HET clone is almost identical to a recently published scaffold attachment factor (SAF-B) cloned from a HeLa cell cDNA library. Scaffold attachment factors are a subset of nuclear matrix proteins (NMP) that interact with matrix attachment regions. Analyzing how HET could act as a regulator of hsp27 transcription and as a SAF/NMP, we studied its subnuclear localization and its effect on hsp27 transcription in human breast cancer cells. We were able to show that HET is localized in the nuclear matrix in various breast cancer cell lines. Furthermore, in transient transfection assays using hsp27 promoter-luciferase reporter constructs, HET overexpression resulted in a dose-dependent decrease of hsp27 promoter activity in several cell lines. J. Cell. Biochem. 67:275–286, 1997. © 1997 Wiley-Liss, Inc.

Key words: hsp27 expression; breast cancer; nuclear matrix protein; DNA-binding; promoter; repressor

We have previously established that the small heat shock protein hsp27 plays a role in both growth and drug resistance of human breast cancer cells in culture [Oesterreich et al., 1993]. Supporting this observation, hsp27 has been found to contribute to increased drug resistance in Chinese hamster ovary (CHO) cells [Lavoie et al., 1993], colon cancer cells [Garrido et al., 1996], and testis cancer cells [Richards et al., 1996]. More recently, we have found that elevated hsp27 levels also correlate with increased invasion of human breast cancer cells [Lemieux et al., 1996]. Although many contradictory studies have been published on the prognostic value of hsp27 in various tumors, by analyzing samples from 425 breast cancer patients by immunohistochemistry and 788 samples by Western blot analysis we have found that hsp27 is not an *independent* prognostic marker [Oesterreich et al., 1996b] in breast cancer. However, this large study did show that hsp27 predicts a significantly worse outcome in a subset of estrogen receptor (ER)-positive/

Abbreviations: NMP, nuclear matrix protein; S/MAR, scaffold/nuclear matrix attachment region; hsp, heat shock protein; GST, glutathione S-transferase; ERE, estrogen response element; ER, estrogen receptor; ORF, open reading frame; IF, intermediate filament; ECL, enhanced chemiluminescence

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untreated breast cancer patients. Furthermore, in this study we confirmed previous findings [Dunn et al., 1993; Tandon et al., 1990] that the expression of hsp27 is strongly correlated with the expression of ER in breast tumors. The correlation of hsp27 and ER expression agrees with the finding of estrogen induction of hsp27, which has been described in breast tumors [Seymor et al., 1990] and in breast cancer cell lines [Moretti-Rojas et al., 1988].

Several groups have tried to decrease the expression of hsps in order to circumvent drug resistance in tumors. For example, the antiestrogen toremifene [Mahvi et al., 1996], and the bioflavonoid quercetin [Sliutz et al., 1996], both decrease hsp expression and have been successfully used as chemosensitisers in tissue culture. We have decided to choose a different approach, e.g., by characterizing the hsp27 promoter, with the long-term goal of decreasing expression by targeting its promoter, which might lead to a novel therapeutic strategy in the treatment of breast cancer. While analyzing the hsp27 promoter in detail, we found that most basal transcriptional activity resides in the most proximal region [Oesterreich et al., 1996a]. In this fragment, there is an interesting region of DNA, 70 bp upstream from the start site, consisting of an imperfect palindromic estrogen response element (ERE) that, instead of being separated by the normal 3-bp spacer, is separated by a 13-bp spacer containing a TATA box. Another TATA box 40 bp downstream of this region is the major transcriptional start site, however transcription can also occur from the upstream TATA box [Hickey et al., 1986]. Here we describe the cloning of a protein (HET) binding to this ERE-TATA region (termed HET—Hsp27-ERE-TATA-binding protein), its identification as a nuclear matrix protein, and characterization of its effect on transcriptional activity of the hsp27 promoter in transient transfection assays.

MATERIAL AND METHODS

Cell Culture and Transfection

The human breast cancer cell line MCF-7/MG was originally obtained from Dr. Herbert Soule (Michigan Cancer Foundation) and has been maintained in our laboratory for the past 14 years. MDA-MB-231 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). MCF-7/MG, MDA-MB-231, and COS cells were maintained as described in [Oesterreich et al., 1996a]. For HeLa cells, the media was not supplemented with insulin or gentamicin sulfate. T47DE and ZR-75 human breast cancer cell lines were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 1% (v/v) L-glutamine, 1% (v/v) glucose, 1% (v/v) penicillin/streptomycin and 5% fetal bovine serum (FBS). Transient transfections were performed as previously described [Oesterreich et al., 1996a], except that Renilla luciferase plasmid (pRL-TK) (Promega, Madison, WI) (50 ng), instead of pRSV-βgal, was cotransfected to correct for transfection efficiency. Briefly, the cells were plated at 2×10^5 cells/well in triplicate in six-well plates, the next day cells were transfected using Lipofectamine (Life Technologies, Grand Island, NY) and OPTIMEM (Gibco-BRL). Cells were transfected with 0.5 μg CF (hsp27 promoter fragment; see below) and with 100 ng HET (MDA-MB-231 and HeLa) and 250 ng HET (T47D, MCF-7/MG and COS cells), respectively. Cells were exposed for 16 h and then incubated in MEM plus 10% FBS for a further 24 h. Cells were lysed and the Dual Luciferase assay (Promega) performed according to the manufacturer's protocol; the values are expressed as relative luciferase units (RLU). The RLU were compared between co-transfection with pcDNAI and HET, respectively, and statistical analysis performed using a two-way analysis of variance (ANOVA) test.

Materials and Plasmids

All materials and chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated. The source of the human hsp27 promoter fragment containing the nonconsensus ERE and the TATA box was a clone originally described in [Fuqua et al., 1989]. The fragment (−99 to −15 bp) was amplified by polymerase chain reaction (PCR), using the following primers: sense 5′-CTCAAACGGGTCATTG-3′ and antisense 5′-TCGGCTGCGCTTTTAT-3′. *Hin*dIII sites plus 2 additional nucleotides (GC) were added to the same primers for cloning purposes. The purified PCR product was digested with HindIII and ligated into pGL2-Basic (Promega) to create the fragment CF. The vector pRLTK (Renilla luciferase gene under thymidine kinase promoter) was also obtained from Promega.

Cloning of HET

An MCF-7 expression library was screened as described [Moretti-Rojas et al., 1988], using a random prime-labeled (Boehringer Mannheim, Indianapolis, IN) hsp27 ERE-TATA promoter fragment. The following modifications were also included in the protocol: the filters were first immersed in 10 mM IPTG for 30 min, followed by denaturation/renaturation steps, including incubation in decreasing concentrations (30 min in 6, 3, 1.5, 0.75, 0.375, and 0.19 M, respectively) of GnHCl in HEPES-buffer (25 mM HEPES, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT). Positive phage DNA was purified, analyzed by dideoxy sequencing of both strands, using the USB (Cleveland, OH) sequencing kit. This partial cDNA (position 680–1,202 bp in the HET sequence) was expressed as a GST-fusion protein to confirm binding to the hsp27 promoter by cloning it into the *Eco*RI site of pGEX-1λT (Pharmacia, Piscataway, NJ). The pGEX-1λT vector clone was used as a negative control in all experiments. GST-HET and pGEX-1λT only were transformed into DH5α and protein was isolated according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). Briefly, single clones were grown overnight in LB (50 μg/ml ampicillin), the next day an aliquot was grown for 4 h, followed by the addition of 100 mM IPTG, for an additional 1.5 h. The bacteria were pelleted, resuspended in phosphate-buffered saline (PBS) and sonicated for 10 s. After the insoluble material was removed by centrifugation, the supernatant was incubated with glutathione Sepharose 4B for 10 min at room temperature. The beads were then extensively washed with PBS, followed by a 10-min incubation with 5 mM glutathione in order to elute the bound proteins. Aliquots were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were also used for gel-retardation assays.

The same partial cDNA clone was then used as a probe to screen another MCF-7 cDNA library (λZap), a kind gift of Dr. Jeffrey Marks (Duke University, NC). Bluescript subclones were excised according to the manufacturer (Stratagene, La Jolla, CA), and analyzed by dideoxy sequencing of both strands using the USB sequencing kit. Since the library was originally constructed by cloning cDNAs into the *Eco*RI site of λZap vectors we excised HET with *Eco*RI and ligated the insert into the eukaryotic expression vector pcDNAI (Invitrogen, Carlsbad, CA).

Southwestern Blotting

Nuclear extracts from MCF-7 cells were separated on a 12.5% SDS–PAGE gel, transferred onto nitrocellulose and the proteins were denatured/renatured by incubating the membrane in decreasing concentrations (10 min in 6, 3, 1.5, 0.75, 0.375, and 0.19 M, respectively) of GnHCl in Z-buffer (25 mM HEPES, pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP40, 100 mM KCl, 1 mM DTT, and 10 μM $ZnSO_4$) at 4°C. After blocking for 30 min in Z-buffer and 3% nonfat dry milk, the membrane was incubated with random prime-labeled hsp27 ERE/TATA fragment (1–2 $\times 10^6$ cpm/ml) in Z-buffer and 0.25% nonfat dry milk with 10 μg/ml sonicated calf thymus DNA. Finally, the membrane was washed three times in Z-buffer in 250 mM KCl for a total time of 15 min and exposed to a film.

Gel-Retardation Assay

Gel-retardation assays were carried out as published [Fuqua et al., 1994]. The hsp27 promoter fragment was dephosphorylated and 5′-end labeled using [α-^{32}P]ATP and T4 polynucleotide kinase. The end-labeled fragment was incubated in the presence of 20 μg whole cell extracts [Fuqua et al., 1994], and after a 30-min incubation at room temperature the samples were loaded onto a 5% polyacrylamide gel and electrophoresed for 2.5 h. For supershift experiments, ER-specific antibody mAb H222, which was kindly provided by Dr. G. Greene (Ben May Institute, University of Chicago, IL) was added (40-ng/reaction), and the reaction mixtures were incubated on ice for an additional 2 h.

In Vitro Transcription/Translation

TNT rabbit reticulocyte lysate (Promega) was used according to the manufacturer's recommendation with 2 μg of HET cloned into pcDNAI as a template, and 1 μl of T7 polymerase was added last to the reaction mixture followed by a 90-min incubation at 30°C.

Preparation of HET-Specific Antibodies

Antigenicity and hydrophobicity plots were generated to predict immunogenic peptides for the generation of polyclonal antibodies to HET. A 14-amino acid peptide (A156: C-PEARDSKEDGRKF) was synthesized, coupled to KLH,

and injected into rabbits (Alpha Diagnostic, San Antonio, TX). The resulting antiserum was purified by affinity chromatography. Briefly, the immunogenic peptide was cross-linked through the N-terminal cysteine onto an activated sepharose column (Sulfolink, Pierce, Rockford, IL) according to the manufacturer's protocol; the column was washed with low- and high-salt buffer. After elution of the antibody with 100 mM glycine, at pH 2.5, the antibody was dialyzed overnight against PBS/0.02% NaN_3.

Western Blotting

Protein extracts were resolved on polyacrylamide gels as previously described [Tandon et al., 1989], transferred from the gel to nitrocellulose membranes and subjected to immunodetection [Tandon et al., 1989] with an HET-specific polyclonal antibody (see below) and the enhanced chemiluminescence (ECL) system (DuPont, Boston, MA). Both the HET-specific antibody as well as the antirabbit Ig HRP-conjugated secondary antibody (Amersham, Arlington Heights, IL) were used at a dilution of 1:1,000.

Purification of Nuclear Matrix Proteins

Nuclear matrices were prepared according to methodologies as previously described [Fey and Penman, 1988; Samuel et al., 1997]. Briefly, the samples were resuspended in 10 ml of ice-cold TNM (100 mM NaCl, 300 mM sucrose, 10 mM Tris pH 8.0, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol) with 100 mM PMSF. The cell suspension was homogenized with a Teflon pestle on ice with 0.5% (v/v) Triton X-100 and the nuclei collected by centrifugation. Following centrifugation, the nuclei were resuspended to a concentration of 20 A_{260}/ml in cold digestion buffer (DIG) (50 mM NaCl; 300 mM sucrose; 10 mM Tris–HCl, pH 7.4; 3 mM $MgCl_2$; 1% (v/v) thiodiglycol; 0.5% (v/v) Triton X-100) and digested with DNase I (D5025, Sigma) 20 min at room temperature. Ammonium sulfate (final concentration of 0.25 M) was added and the nuclear matrix (NM-IF) pellet was obtained by centrifugation. The NM-IF pellet was then resuspended in ice-cold DIG buffer with 1 mM PMSF, extracted by adding 4 M NaCl to a final concentration of 2 M, and incubated on ice for 30 min. The NM2-IF pellet was collected by centrifugation and once again resuspended in ice cold DIG buffer, reextracted with 2 M NaCl and centrifuged. The NM2-IF was then resuspended in Disassembly Buffer (8 M urea, 20 mM 2-[N-morpholino]ethane sulfonic acid, pH 6.6; 1 mM EGTA; 1 mM PMSF; 0.1 mM $MgCl_2$; 1% (v/v)-mercaptoethanol), and dialyzed overnight at room temperature against 2 L of Assembly Buffer (0.15 M KCl; 25 mM imidazole, pH 7.1; 5 mM $MgCl_2$; 2 mM DTT; 0.125 M EGTA; 0.2 mM PMSF). The IFs were removed by ultracentrifugation and the resulting supernatant containing NMPs removed. The NMP containing supernatant was then dialyzed against ddH2O for 8 h to reduce the salt concentration and then lyophilized. Lyophilized samples were resuspended in appropriate volumes of 8 M urea, aliquoted and frozen at −20°C. The protocol for Western blot analysis was slightly modified, in that the Tween concentration was increased from 0.1% to 0.2%, and the secondary antibody was used at a dilution of 1:5,000.

GENEbank Accession Number

The accession number for the human HET (Hsp27 ERE-TATA-binding protein) is U72355.

RESULTS

HET Binds to the hsp27 Promoter

To analyze the most transcriptionally active region of the hsp27 promoter [Oesterreich et al., 1996a] in more detail, we first investigated whether protein factor(s) bound to this region. Gel-retardation analysis with an 84-bp promoter fragment containing the ERE-TATA box (−99 bp to −15 bp) and whole cell extracts from MCF-7/MG cells revealed binding of a protein complex (Fig. 1A, lane 3). Since this fragment contains an ERE, separated by a TATA box, we named this protein HET (Hsp27 ERE-TATA-binding protein). As hsp27 has been described as an estrogen-inducible protein, we first excluded the possibility that the factor was the ER by doing supershift experiments using ER-specific antibodies. As a positive control we used a consensus ERE as a probe, and we saw retardation of the probe by ER, which was then further upshifted by adding antibodies to ER (lanes 1 and 2). However, ER antibodies did not upshift the protein complex bound to the hsp27 promoter fragment (lanes 3 and 4). Southwestern blotting of MCF-7/MG nuclear extracts with the same hsp27 promoter fragment (Fig. 1B) indicated binding of two proteins with molecular weights of approximately 120 kDa and 45 kDa. The 45-kDa protein has a molecular weight

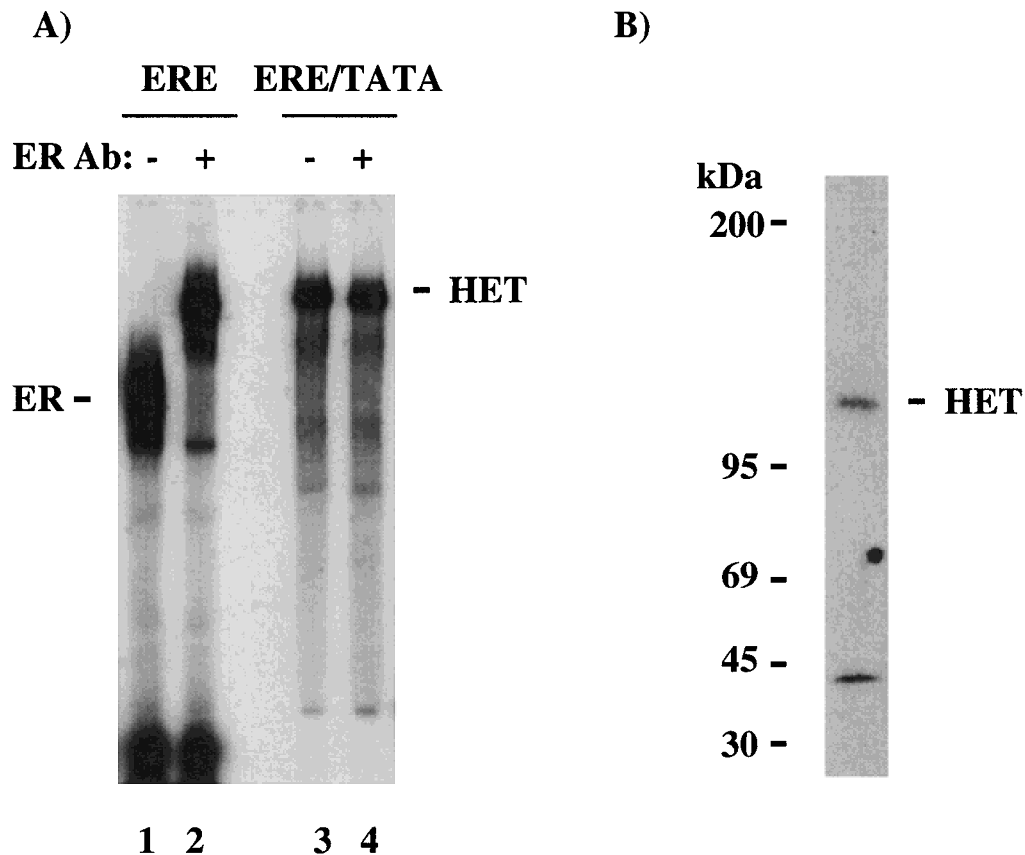


Fig. 1. Identification of HET as an hsp27 promoter binding protein with a molecular weight of approximately 120 kDa. **A:** Gel-retardation assay using a ^{32}P-labeled consensus ERE (**lanes 1 and 2**) and the hsp27 promoter fragment (−99 to −15 bp) containing the ERE/TATA site (**lanes 3 and 4**), respectively, as probes and whole cell extracts from MCF-7/MG cells. ER-antibodies were added to lanes 2 and 4. **B:** Southwestern blot of MCF-7/MG nuclear extract (25 µg) separated by SDS–PAGE, transferred to nitrocellulose, renatured, and probed with the ^{32}P-labeled hsp27 promoter fragment.

consistent with a TATA-binding protein. The difference in size of HET (the 120-kDa protein) as compared to the ER (65 kDa) again indicates that HET is distinct from the ER. Furthermore, we detected binding of HET using extracts of MDA-MB-231 cells (data not shown), which are ER-negative breast cancer cells. We therefore pursued cloning of this hsp27 promoter binding protein.

Cloning of HET as an hsp27 Promoter-Binding Protein

We first isolated a partial cDNA clone (900 bp) by screening an MCF-7 expression library [Moretti-Rojas et al., 1988] with the hsp27 promoter fragment from −99 bp to −15 bp. We then expressed this partial cDNA as a GST fusion protein (Fig. 2A, lane 3), using GST only (Fig. 2A, lane 2) as a negative control. After purification of GST only (Fig. 2A, lane 4) and GST-HET fusion proteins (Fig. 2A, lane 5), we used these extracts in a gel-retardation assay. There we saw specific binding of purified GST-HET to the hsp27 promoter fragment (Fig. 2B, lanes 3 and 4) in a dose-dependent manner, whereas purified GST-alone failed to bind (Fig. 2B, lanes 1 and 2).

We then used this partial clone to screen another MCF-7 breast cancer cell cDNA library (kindly provided by Dr. Jeff Marks, Duke University, NC) and identified an HET clone of 3040 bp in length (GENEbank accession number U72355). The original partial cDNA clone we isolated is identical to position 680–1202 bp in the full-length HET sequence. We confirmed the identity of the HET sequence within the full-length clone by sequencing another four positive clones, which were all overlapping with the sequence from the full-length HET cDNA. The original, partial cDNA clone thus might represent a portion corresponding to a mRNA splicing variant of HET, a hypothesis supported

Fig. 2. The GST-HET fusion protein binds to the hsp27 promoter. **A:** Coomassie-stained SDS–PAGE of extracts from *Escherichia coli* expressing GST-HET and GST only (pGEX-1λT) as a negative control, before (**lanes 2 and 3**) and after purification (**lanes 4 and 5**) on glutathione beads. Molecular-weight markers were loaded in **lane 1**. **B:** 2 and 10 μl of purified GST (**lanes 1 and 2**) and GST-HET (**lanes 3 and 4**), respectively, were then used in a gel-retardation assay using the hsp27 promoter fragment as a probe.

by the appearance of minor bands on Northern blots (data not shown). There is a putative initiation codon at nucleotide 54 in our full-length HET cDNA clone, which shows a weak homology to the translation initiation consensus sequence [Kozak, 1983]. There is also a translation stop codon at nucleotide 2799, which is followed by a polyadenylation signal and a poly(A) tract. A potential open reading frame (ORF) coding for 916 amino acids is present, which results in a predicted molecular weight of approximately 100 kDa for the HET protein. Using PROSITE, we identified several potential phosphorylation, glycosylation, and mysristylation sites, furthermore we learned that the protein contains an unusually high number of charged amino acids (221 negatively charged and 164 positively charged; theoretical pI = 5.05). No classic DNA-binding motif was identified using PROSITE.

To enable further analysis of HET we developed a rabbit polyclonal antiserum. This antiserum recognized HET in whole cell extracts; however, other nonspecific bands were seen (data not shown). After affinity purification (see under Material and Methods), the polyclonal antibody recognized a single band of approximately 120 kDa (data not shown), in agreement with our Southwestern blot analysis (Fig. 1B). The discrepancy between the predicted and the approximate molecular weight by SDS–PAGE, may be a result of post-translational modification. We then used this purified antibody preparation to determine whether it would recognize the HET protein prepared by coupled in vitro transcription/translation. HET cDNA was in vitro transcribed and translated in the presence (Fig. 3A) and absence of [^{35}S]methionine (Fig. 3B), and the protein products were analyzed by SDS–PAGE. [^{35}S]methionine in vitro transcription/translation of HET cDNA resulted in expression of a single high-molecular-weight protein (Fig. 3A, lane 1), whereas no protein was detected with control DNA (vector only) (Fig. 3A, lane 2). The protein product from the unlabeled in vitro transcription/translation were exposed to Western blot analysis using the HET-specific purified polyclonal antibody and, as expected, we detected a band comparable in size to the product from the in vitro transcription/translation in the presence of [^{35}S]methionine (Fig. 3B, lane 1). Again, no band was detected when control DNA was used (Fig. 3B, lane 2). Taken together, these facts strongly indicate that the cloned cDNA encodes the full-length HET protein.

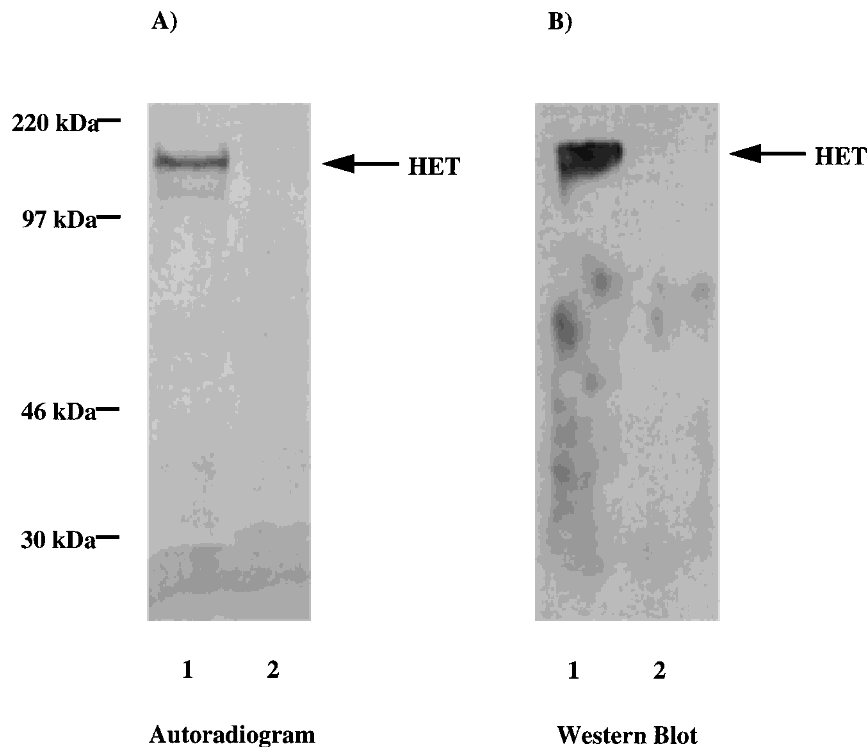
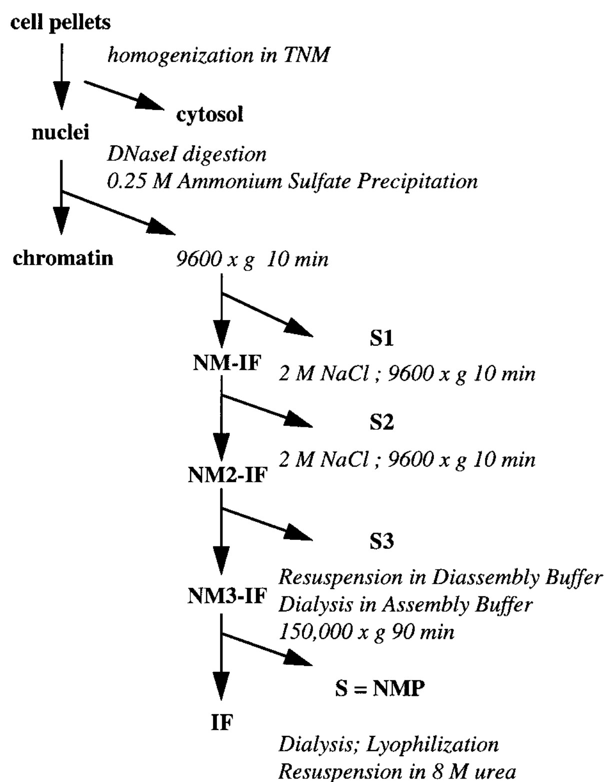


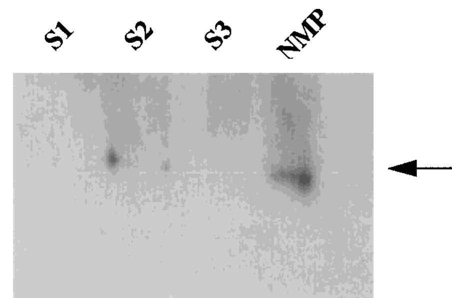
Fig. 3. The HET cDNA encodes for a 120-kDa protein recognized by a purified polyclonal HET-antibody. A) After in vivo excision with helper phage, HET cDNA was recovered in a Bluescript plasmid, which could then be directly used for coupled in vitro transcription/translation analysis. Two µg DNA (HET, **lane 1** and vector only, **lane 2**) were in vitro transcribed and translated using the TnT coupled Reticulocyte Lysate system (Promega) in the presence (**A**) or absence (**B**) of ^{35}S-methionine. The products were then separated by SDS–PAGE, dried, and exposed to a film (**A**), or transferred to nitrocellulose and incubated with 1:1,000 dilution of HET-specific antibody (**B**). The signal was developed using ECL.

HET Is Localized in the Nuclear Matrix

Recently, searching of sequences within GENbank has led to the discovery that HET is almost identical to the scaffold attachment factor SAF-B (GENbank accession number L43631). As a result of a sequencing error at position 344 bp in SAF-B (position 548 bp in HET), a frameshift occurred in the first 100 amino acids, and the 5′ end is apparently missing in the published SAF-B sequence [Renz and Fackelmayer, 1996]. Our HET cDNA has an additional 205 bp at the 5′ end, of which 152 bp code for amino acids. Furthermore, there are 15 mismatches in the 3′ untranslated region comparing HET to SAF-B. SAF-B was recently cloned from a HeLa cell cDNA library [Renz and Fackelmayer, 1996] based on its ability to bind to scaffold/matrix attachment regions (S/MAR), that are AT-rich DNA regions shown to be involved in attaching the base of chromatin loops to the nuclear matrix. Scaffold attachment factors are a specific subset of nuclear matrix proteins (NMP) that specifically bind to S/MAR. We hypothesize that HET is a scaffold attachment factor localized in the nuclear matrix that might also influence hsp27 promoter activity in breast cancer cells. We next tested this hypothesis with the following experiments.

To determine whether HET is localized within the nuclear matrix, various subnuclear fractions from ZR-75, an ER-positive human breast cancer cell line, were isolated as described under Materials and Methods and outlined in Figure 4A. Briefly, cells were homogenized in buffer containing 0.5% Triton X-100 to release lipids and soluble proteins. The nuclei was then digested with DNase I and precipitated with 0.25 M ammonium sulfate to facilitate chromatin removal. The resulting nuclear matrix-intermediate filament (NM-IF) pellet containing nuclear matrix and associated intermediate filaments was then subjected to sequential salt extractions with 2 M NaCl for the further removal of residual histones [Coutts et al., 1996]. The NM2-IF pellet was resuspended in disassembly buffer containing 8 M urea and dialyzed





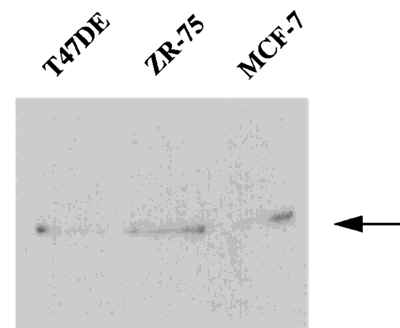


Fig. 4. HET localized in the nuclear matrix. **A:** Diagrammatic representation of the subcellular fractionation scheme. **B:** Twenty μg of protein from various fractions (as indicated) were resolved in each lane by SDS–PAGE, and exposed to Western blot analysis, using HET-antibodies. **C:** Twenty μg of nuclear matrix proteins from various breast cancer cells (as indicated) was resolved in each lane by SDS–PAGE and exposed to Western blot, using HET-antibodies.

against assembly buffer. Dialysis allowed for removal of the urea and for reassembly of IF. The IFs were collected by centrifugation, and the resulting supernatant contained nuclear matrix proteins. Western blotting was then done on all fractions using HET-specific antibodies (Fig. 4B). While there was some HET protein detected in the NM2-IF fraction (S2) (lane 2), most of it was observed to be localized in the nuclear matrix (lane 4). Similar fractions were also prepared from two other ER-positive human breast cancer cell lines (T47DE and MCF-7) to assess whether HET was also localized to the nuclear matrix in these cell lines. Both cell lines were observed to contain similar levels of HET as compared to ZR-75 (Fig. 4C). By contrast, lower levels of HET were detected in the nuclear matrix of MDA-MB 231 cells, an ER-negative human breast cancer cell line (data not shown).

HET Decreases hsp27 Promoter Activity in a Dose-Dependent Fashion

To analyze whether HET would not only bind to the hsp27 promoter but would also influence its transcriptional activity, we performed transient transfection assays with an hsp27 promoter-luciferase reporter construct (called CF) and an expression plasmid containing full-length HET (Fig. 5). CF contains the imperfect ERE and the TATA box region from the hsp27 promoter initially used to clone HET (see under Materials and Methods) and, as shown above, harbors the HET-binding site. The CF fragment displays relatively low basal activity; however, it is about 12-fold higher than that of the pGL2 basic vector alone. We measured the relative luciferase activity of the CF fragment in the presence of either vector alone (pcDNAI) or HET (Fig. 5). In all cell lines analyzed (MDA-

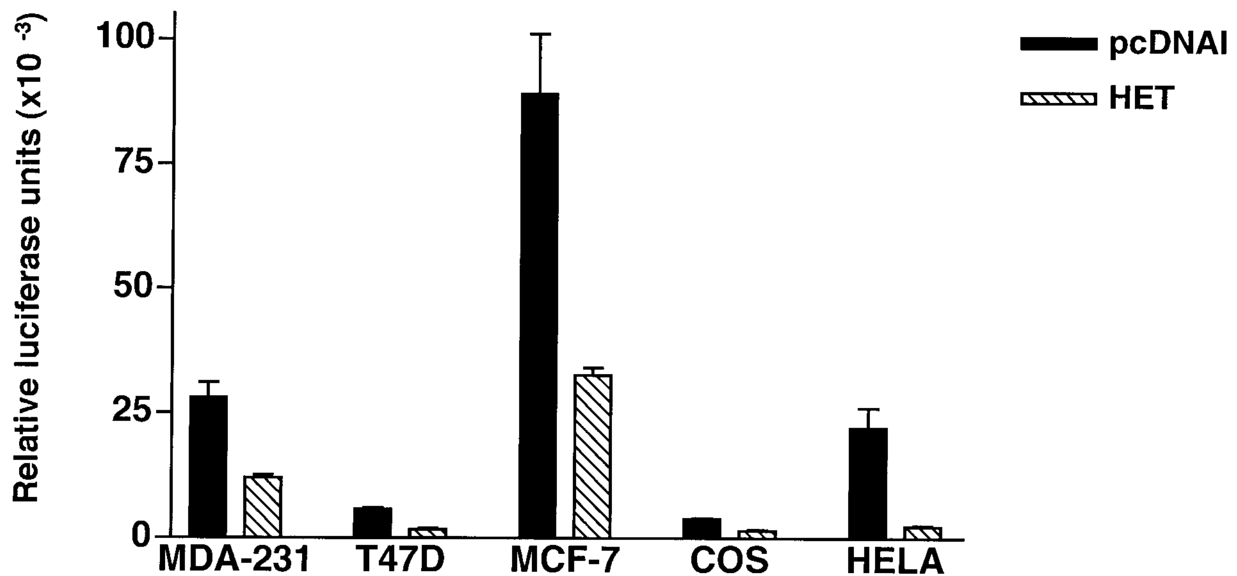


Fig. 5. HET overexpression results in a decrease of hsp27 promoter activity. In transient transfection assays HET was cotransfected with an hsp27 promoter-luciferase construct containing the ERE/TATA site. The luciferase values were corrected for transfection efficiency. Values represent the mean of triplicate wells ± SEM. Significance was calculated by ANOVA.

231, T47D, MCF-7/MG, COS, and HeLa), the co-transfection with HET significantly reduced transcriptional activity of the hsp27 promoter fragment ($P < 0.05$). By contrast, the activity of the Renilla-luciferase construct, which contains a thymidine kinase promoter, and which we used to correct for transfection efficiency, did not decrease after adding HET. To address the question of specificity further, we performed a transient assay using a 1.5-kb IGF-1 gene promoter construct [Adamo et al., 1993], kindly provided by Dr. M. Adamo (UTHSCSA, San Antonio), and pGL2 control, which contains an SV-40 promoter. The activity of the IGF-1 promoter was not affected by HET, and the SV40-promoter was only affected after adding higher concentrations of HET. Therefore, we conclude that HET is able to specifically decrease the activity of the hsp27 promoter in breast cancer cells, however, it may have other targets as described for other NMPs.

DISCUSSION

The small heat shock protein hsp27 is involved in the phenomenon of drug resistance in breast cancer cells [Oesterreich et al., 1993]. Furthermore, overexpression of hsp27 results in increased proliferation in vitro and increased invasiveness in vitro and in vivo [Lemieux et al., 1996]. In accordance with these results, we recently found that hsp27 is a bad prognostic marker in certain ER-positive/untreated breast cancer patients [Oesterreich et al., 1996b]. These recent findings prompted us to study the transcriptional regulation of hsp27 with the long-term goal of intervening with its expression. In a previous report we were able to show that the majority of the promoter activity is in the most proximal 200-bp region of hsp27 [Oesterreich et al., 1996a]; therefore, we have focused first on this region. This region contains an imperfect ERE in which the palindromic half-sites are separated by 13 bp, including a TATA box. In this communication we describe the cloning and characterization of a factor binding to this region, which we have named HET (for Hsp27ERETATA binding).

We first identified HET in gel-retardation assays using the hsp27 promoter fragment as a probe. We then used the same probe to screen an MCF-7 breast cancer cell library, and cloned a 3-kb cDNA coding for HET. This cDNA is almost identical to a recently described human scaffold attachment factor (SAF-B), which was cloned from a HeLa cell cDNA library [Renz and Fackelmayer, 1996]. However, in SAF-B, the 5′ end is missing as a result from a sequencing mistake at position 344 bp, which leads to a frameshift in the first 100 amino acids. Furthermore, our HET cDNA has an additional 205 bp

at the 5′ end. In conclusion, the high sequence similarity between HET and the recently published SAF-B indicates that they are most probably coded for by the same gene.

SAF-B was cloned as one of four proteins (SAF-A through D) that bind to S/MARs. S/MARs are DNA fragments of about 300–3,000 bp, which are AT rich; however, no consensus sequence seems to exist [Gasser et al., 1989]. These regions are involved in the fixation of chromatin to the nuclear matrix and are critical for the organization and function of nuclear chromatin in replication, transcription, and mitosis. Classic AT-rich S/MARs have been proposed to anchor the core enhancers and core origins complexed with low abundancy transcription factors to the nuclear matrix by the cooperative binding of abundant nuclear matrix proteins to S/MARs. This creates a unique nuclear microenvironment rich in regulatory proteins able to sustain transcription, replication, repair, and recombination. S/MARs have also attracted considerable interest in cancer research during the last decade. For instance, cancer researchers have been trying to understand why AT-rich regions are the preferable target for many highly cytotoxic drugs [Woynarowski et al., 1995]. It has become clear that these regions coincide with S/MARs [for review, see Boulikas, 1995] and that the binding of AT-specific drugs to these regions impedes important regulatory functions. Although the hsp27 fragment we used to identify/clone HET is much smaller than the average S/MAR, it is possible that this region is part of a S/MAR. As described for classic S/MAR, this region is very AT rich and harbors a site that is hypersensitive against DNase I, possibly indicating an altered chromatin structure site (S. Oesterreich and S.A.W. Fuqua, unpublished data). Future experiments will be directed at determining whether the hsp27 promoter is indeed a S/MAR and whether it has an appreciable affinity for the nuclear matrix.

S/MAR binding proteins are generally thought to be found within the nuclear matrix. To determine whether HET is localized in the nuclear matrix, biochemical cell fractionation was performed. Subsequently, these fractions were analyzed for the presence of HET using Western blot analysis. As expected, the majority of HET was partitioned in the nuclear matrix with a much lesser amount detected in the NM2-IF fraction. The NM2-IF fraction, obtained after sequential high salt extractions, is considered a nuclear matrix fraction prior to the removal of intermediate filaments [Fey et al., 1984].

Our observation that HET is localized in the nuclear matrix is not in agreement to the study conducted by Renz and Fackelmayer [1996], in which SAF-B was found to be a chromatin protein, but not part of the nuclear matrix. Renz and colleagues explained this observation by suggesting that there are two types of S/MAR DNA binding proteins, which differ in their partitioning upon biochemical fractionation. While we are not in disagreement with this potential explanation, it is also possible that differences in nuclear matrix isolation protocols between Renz and co-workers and our studies have led to this discrepancy. It is important to note that several studies [Dworetzky et al., 1992; Guo et al., 1995; Yanagisawa et al., 1996], suggest that the original nuclear matrix isolation protocol described by Fey et al. [1986] must be modified in order to isolate nuclear matrix from transformed cell lines. This may be one reason why Renz's group failed to detect SAF-B in the nuclear matrix. It is also possible that there are cell type-specific differences in the subcellular localization of HET/SAF-B.

The importance of higher-order nuclear structures, such as NMPs and chromatin, in gene regulation has become increasingly clear; in fact, many NMPs have been shown to be directly involved in transcriptional regulation [Guo et al., 1995; Merriman et al., 1995]. An excellent example is the nuclear matrix protein YY1 [Guo et al., 1995], which can increase or decrease transcriptional activity depending on the target gene and the cell context [Seto et al., 1991; Shi et al., 1991]. The recent discovery that the transcriptional co-activator CBP exhibits histone acetyl transferase activity [Bannister and Kouzarides, 1996] further supports the idea of a direct involvement of higher order nuclear structures in transcriptional regulation. In transient transfection assays, we found that HET decreased the activity of an hsp27 promoter construct, whereas we did not observe a change in activity of a thymidine kinase promoter and IGF-1 promoter in the same assay. However, we did observe an effect with the SV40 promoter (pGL2) and, as with other factors influencing transcription, it is unlikely that HET has only one target gene.

Of specific interest is that the hsp27 promoter region containing the HET binding site and possibly representing a part of an S/MAR also harbors an imperfect ERE. The expression of hsp27 correlates with the expression of ER in both breast cancer cell lines and in breast tumors, and hsp27 has been described as an estrogen-inducible protein. Recently Porter et al. [1996] showed that ER and Sp1 bind cooperatively to the distal half-site of this imperfect ERE, which is in proximity to an Sp1 consensus sequence. This reported binding appears to be very weak, and we would like to hypothesize that HET might stabilize this complex. A similar situation has been described for the vitellogenin B1 promoter, where the relatively weak estrogen response is potentiated through chromatin assembly [Schild et al., 1993]. We are currently testing the hypothesis that in the absence of estrogen, HET leads to a decrease in hsp27 promoter activity; for example, as a result of squelching of the ubiquitous transcription factor Sp1, and in the presence of estrogen, it might lead to an increase in hsp27 promoter activity as a result of stabilization of the Sp1–ER transcription factor complex.

In summary, we have cloned a nuclear matrix protein named HET, which binds to the hsp27 promoter and decreases transcription of hsp27. Before hsp27 was recognized to be involved in drug resistance in breast cancer, the mdr1 gene was characterized as a major player in cellular resistance [Cornwell, 1991]. Recent work by Ince and Scotto [1996] elegantly showed that the promoter activity of the mdr1 gene is strongly influenced by chromatin structure. Nonetheless, the study of higher order structures, such as NMPs, in cancer has been limited to date. However, if indeed the regulation of genes important in breast cancer progression are controlled at least in part by higher order nuclear structures, much more focus should be directed into this area of research.

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