

HET/SAF-B Overexpression Causes Growth Arrest and Multinuclearity and Is Associated with Aneuploidy in Human Breast Cancer¹

Steven M. Townson, Toby Sullivan,
QingPing Zhang, Gary M. Clark,
C. Kent Osborne, Adrian V. Lee, and
Steffi Oesterreich²

Breast Center, Departments of Medicine and Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030 [S. M. T., Q. P., G. M. C., C. K. O., A. V. L., S. O.], and Texas Tech University Health Science Center, Lubbock, Texas 79430 [T. S.]

ABSTRACT

HET/SAF-B was originally cloned as a nuclear matrix protein that bound to matrix attachment regions and as a transcriptional repressor of the small heat shock protein hsp27. In addition, we have found recently that HET/SAF-B is also a corepressor of estrogen receptor activity. Estrogen receptor has a very well-described role in breast cancer, and aberrant expression of nuclear matrix and heat shock proteins has also been implicated in breast tumorigenesis. Therefore, we asked whether HET/SAF-B itself could be important in breast cancer. Toward this goal we examined its expression in breast cancer cell lines and asked whether HET/SAF-B can affect breast cancer cell proliferation. Finally, we studied HET/SAF-B expression in clinical breast cancer samples.

HET/SAF-B protein and mRNA were detected at varying levels in all of the eight breast cancer cell lines examined. Using a number of different approaches to modulate the level of HET/SAF-B protein in the cell, we found that HET/SAF-B levels are inversely correlated with cell proliferation. In addition, transfection of HET/SAF-B fused to the green fluorescent protein led to the formation of multinucleated cells not observed in cells transfected with green fluorescent protein alone, suggesting that this effect is a direct result of HET/SAF-B overexpression. Western blot analysis of HET/

SAF-B in 61 human breast tumors revealed widely varying levels of HET/SAF-B expression, with some tumors (16%) lacking any detectable HET/SAF-B. Statistical analysis showed that high HET/SAF-B expression in these tumors was associated with low S-phase fraction and with aneuploidy, consistent with our results from transfection experiments in tissue culture cells. We conclude that HET/SAF-B plays an important role in breast cancer, and we discuss possible mechanisms of the involvement of HET/SAF-B in cell proliferation and division.

INTRODUCTION

HET/SAF-B was originally cloned as a protein binding to matrix/scaffold attachment regions (1) and as a NMP³ binding to the hsp27 promoter in human breast cancer cells (2). Subsequently, it was shown to bind to the COOH-terminal domain of RNA polymerase II and to a subset of serine/arginine-rich RNA processing factors (SR proteins) and to function in mRNA splicing (3). This suggests that HET/SAF-B is involved in the formation of a “transcriptosomal” complex, bringing transcription and mRNA processing together. These macromolecular complexes have been shown previously to be associated with the nuclear matrix (4, 5).

The nuclear matrix consists of a protein-RNA network that is involved in structural organization of DNA within the nucleus, thereby controlling important regulatory processes such as transcription and DNA replication (reviewed in Ref. 6). Not surprisingly, many NMPs have been shown to be important in cell transformation. The NMP pattern of expression shows significant differences between normal and cancer tissue in bladder (7), colon (8), head and neck (9), prostate (10), and breast (11). Consistent with this, various NMPs were found to have potential as prognostic markers for cancer add (12, 13). Additionally, a role for the nuclear matrix in steroid hormone action was postulated many years ago (14–18) but only recently have specific NMPs been characterized that directly bind to hormone receptors and modulate their activity (19). For example, recently, the glucocorticoid receptor-interacting protein GRIP 120 has been identified as the NMP hnRNPU (20). We have shown recently that the NMP HET/SAF-B regulates the activity of the estrogen receptor (21).

HET/SAF-B binds to the ER and functions as an ER corepressor. In this way, HET/SAF-B is similar to several other

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² To whom requests for reprints should be addressed, at Department of Medicine, Breast Center, Baylor College of Medicine, One Baylor Plaza, MS:600, Houston, TX 77030. Phone: (713) 798-1623; Fax: (713) 798-1642; E-mail: steffio@bcm.tmc.edu.

³ The abbreviations used are: NMP, nuclear matrix protein; hsp, heat shock protein; ER, estrogen receptor; GFP, green fluorescent protein; FBS, fetal bovine serum; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMV, cytomegalovirus; RPA, RNase protection assay; FACS, fluorescence-activated cell sorter.

recently identified ER-interacting proteins, REA (repressor of estrogen receptor activity; Ref. 22), SMRT (the silencing mediator of retinoid and thyroid receptors; Refs. 23 and 24), and NcoR (nuclear receptor corepressor; Ref. 25), all of which also act as corepressors. Because estrogen is one of the most potent mitogens for breast cancer cells and is a known risk factor for breast cancer, a role of HET/SAF-B in estrogen action implies a role in ER-positive breast cancer cell growth control. Alternatively, it is also possible that HET/SAF-B can act as a transcriptional repressor independent of ER by interacting with other transcription factors. It has been shown that known steroid receptor-interacting proteins such as the coactivator SRC1 (26, 27), which was originally cloned as a steroid receptor cofactor, also mediates transactivation by other transcription factors including AP1 (28), serum response factor (28), nuclear factor- κ B (29), cyclic AMP-responsive element binding protein, and signal transducers and activators of transcription (30). More recently SRC1 has also been found to bind to p53 and potentiate its transactivation, whereas two other ER coactivators, amplified in breast cancer (AIB1) and *Xenopus* steroid receptor coactivator (xSRC-3), were found to repress p53-mediated transactivation (31). This suggests that these factors might have important and distinct roles in tumorigenesis independent of their function as a steroid hormone receptor regulator.

HET/SAF-B is involved in a number of cellular processes that are associated with tumorigenesis. These include its role in the repression of hsp27, which has been shown to positively regulate breast cancer cell proliferation (32), as well as its role as a NMP and as an ER corepressor. Therefore, we have set out to analyze whether HET/SAF-B plays a role in breast cancer. Here we report that overexpression of HET/SAF-B causes growth inhibition and multinuclearity in cultured cells. Consistent with these findings from tissue culture, HET/SAF-B expression is associated with lower proliferation but also with aneuploidy in human breast tumor specimens. Thus, as predicted, HET/SAF-B plays a role in breast tumor behavior. Possible mechanism(s) will be discussed in more detail.

MATERIALS AND METHODS

Plasmid Constructs and Chemicals. The cloning of the HET/SAF-B expression construct in pcDNA1 has been described previously (2). To generate an antisense construct, the full-length *EcoRI*-digested HET/SAF-B construct was cloned into pcDNA1 in the antisense direction, which was verified by sequencing. For the RNase protection assay, an *ApaI-EcoRV* HET/SAF-B fragment (99–443 bp) was cloned into pGEM5zf(+) (Promega Corp., Madison, WI) and restriction-digested with *XhoI* (200 bp), and the probe was made using a T7 polymerase. The 36B4 probe has been described previously (33). A GFP-HET/SAF-B fusion protein with GFP positioned at the COOH-terminal of HET/SAF-B was cloned by ligating the full-length HET/SAF-B into the *EcoRI* site of pEGFP-C3 (Clontech, Palo Alto, CA). To generate an inducible HET/SAF-B construct, HET/SAF-B cDNA1 was subcloned from pcDNA1 using *EcoRI* and cloned into the unique *EcoRI* site in pUHD10–3 (34) to generate pUHDHET. The orientation was confirmed by sequencing. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cell Lines and Tumors. Breast cancer cell lines MCF-7/MG, MDA-MB-468, MCF-7, MDA-MB-231, MCF-7/BK, MDA-MB-330, ZR-75, and T47D, along with CHO-K1 (Chinese hamster ovary) cells and T24 (human bladder carcinoma), were maintained in IMEM supplemented with 10% FBS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin. NIH3T3 (mouse embryo) and 293 (transformed human embryonic kidney) cell lines were kept in DMEM (Life Technologies, Inc., Grand Island, NY), with the same supplements as IMEM. SFM consisted of IMEM + 10 mM HEPES (pH 7.4), 1 μ g/ml transferrin, 1 μ g/ml fibronectin, 2 mM glutamine, 50 IU/ml penicillin, 50 μ g/ml streptomycin, and trace elements (Biofluids, Rockville, MD). The clinical breast tumor specimens for the Western blot study were obtained from the National Tissue Resource maintained by our Breast Cancer Specialized Program of Research Excellence. These specimens were originally sent by hospitals throughout the United States to Nichols Institute Research Laboratories in San Juan Capistrano, CA, for routine measurements of steroid receptors and cell cycle analyses by flow cytometry. The flow cytometric assays were performed using methods described previously (35).

Transfections, Cell Growth, and Cell Cycle Analysis. All transfections were performed using Lipofectamine (Life Technologies, Inc.) or Fugene (Roche Molecular Biochemicals, Indianapolis, IN). Transient transfections were analyzed 48 h after transfection. To establish stable cell lines, NIH3T3 cells were cotransfected with pcDNA1 only or HET/SAF-B-pcDNA1 (2) and pSVneo, and transfected clones were selected in 1000 μ g/ml G418.

For growth analysis, cells were plated in quadruplicate at 2500 cells/well in a 96-well plate. The next day (day 0), cell number was assessed by MTT assay as described previously by us (36). Cells were then incubated in SFM or medium with 10% FBS, and cell number was determined at days 2, 4, and 6.

For colony formation assays, MCF-7/MG cells were transfected with 20 μ g of pCDNA1 vector control or HET/SAF-B-pcDNA1 sense and antisense, respectively, along with 1 μ g of pSVneo. After 3 weeks incubation in 400 μ g/ml G418, colonies were stained with 1% crystal violet.

293 cells, which display very high transfection efficiency, were used for proliferation assays measuring [3 H]thymidine incorporation into DNA. Cells (8×10^4) were plated in triplicate in six-well plates and transfected on day 2 with increasing amounts of pcDNA1 or HET/SAF-B-pcDNA1 antisense constructs. On day 4, the cells were incubated for 1 h with 1 μ l/ml [3 H]thymidine (Amersham; 1 mCi/ml). After washing in cold PBS and cold 5% trichloroacetic acid, the cells were kept on ice for 30 min in the presence of 5% trichloroacetic acid and finally lysed in 0.5 M NaOH.

For generation of inducible HET/SAF-B-expressing cells, we used the tetracycline inducible expression system, which has been described in detail previously (34, 37). The tetracycline inducible MDA-MB-453rtTA cells were given to us by Dr. Douglas Yee (University of Minnesota). Briefly, cells were stably transfected with a plasmid (pUHD172–1-neo) expressing a protein termed rtTA (VP16 linked to a tetracycline binding protein). Stable clones were selected in 1000 μ g/ml G418, expanded, and then tested for expression of rtTA by transient transfection with a reporter plasmid (pUHD16–3) consisting of

seven tetracycline operator sequences upstream of a luciferase gene. Treatment of cells with doxycycline (an analogue of tetracycline) at 1 $\mu\text{g/ml}$ for 24 h indicated inducible luciferase expression (2–10-fold) in a number of clones. We used the clone with the highest inducibility (MDA-435rtTA1) for transfection with an expression plasmid containing HET/SAF-B under the control of a tetracycline-inducible CMV promoter (pUHDHET). After transfection, these cells were selected in 600 $\mu\text{g/ml}$ hygromycin and analyzed for inducible HET/SAF-B expression by Western blot analysis.

For the analysis of cell cycle distribution, cells were harvested, washed with PBS, fixed in 70% ethanol, and stored at -20°C . Immediately before analysis on a FACS STAR PLUS (Becton Dickinson, San Jose, CA), propidium iodide and RNase were added to the cell pellet to final concentrations of 0.1 and 0.5 mg/ml, respectively. Data were analyzed using CellQuest software.

The expression of HET/SAF-B throughout the cell cycle was investigated in T24 bladder carcinoma cells grown in IMEM containing 5% FBS by first growing the cells to confluence and then leaving them for 3 days to arrest in G_0 - G_1 (37). The cells were then subcultured into 10-cm plates and plated at a density of 1×10^6 per well. Cells lysates were produced at different time points after subculture by first washing the cells in PBS and then lysing them in high salt buffer [50 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 0.4 M NaCl, 10% glycerol, and 1% NaPO_4] containing Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals, Indianapolis, IN) used at the concentration suggested by the manufacturer. T24 cells were blocked in G_2 -M using nocodazole. Cells were grown in 15-cm culture dishes and blocked by the addition of nocodazole at 40 ng/ml for 18 h. Cell lysates were produced as described above. HET/SAF-B protein levels in the cell lysates were determined by Western blotting using 50 μg of total protein and our monoclonal HET/SAF-B antibody (21).

RNA and Protein Analysis. The RPAs were performed as described previously (32) using a HET/SAF-B-specific probe as well as a probe for 36B4 as a loading control. For Western blot analysis, cell pellets were resuspended in 5% SDS or high salt buffer and sonicated, and 50 μg of total protein were analyzed by Western blotting using HET/SAF-B antibody as well as a polyclonal antibody against the p85 subunit of PI3K (Upstate Biotechnology, Lake Placid, NY) as a loading control. For the quantitative Western blot analysis of human tumors, each gel contained 50 μg of MCF-7 SDS extract as an internal standard. The HET/SAF-B bands were quantitated by densitometric scanning using NIH Image 1.6 software, and the levels were calculated in arbitrary units by the ratio of the integrated densitometry signal in the tumor sample relative to the internal standard on each gel. For the detection of GFP-HET/SAF-B fusion protein, an anti-GFP antibody was used at a 1:1000 dilution (Clontech, Palo Alto, CA).

Statistical Analysis. All statistical analyses were performed using SAS (Version 6.11; SAS Institute, Cary, NC) running on a Sun Microsystems SparcServer 1000. Relationships between HET/SAF-B expression and S-phase fraction and between HET/SAF-B and ER expression were analyzed using Spearman's rank correlation coefficients. The relationship be-

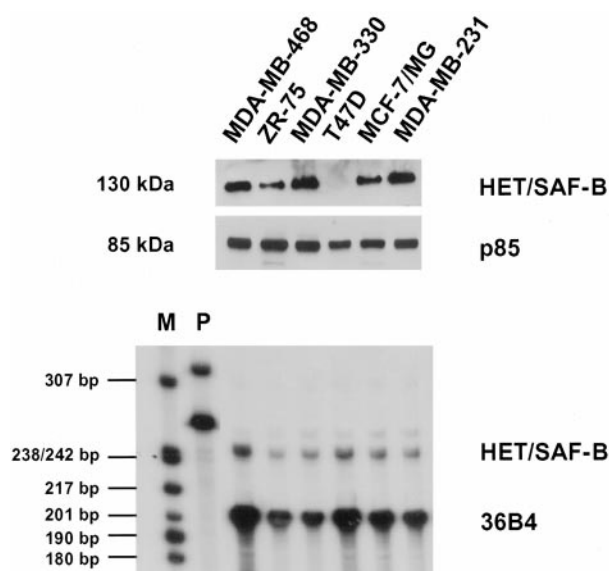


Fig. 1 HET/SAF-B expression in breast cancer cell lines. For the Western blot (A), 50 μg of SDS-protein extracts were loaded onto 6% SDS-PAGE, transferred to nitrocellulose, and blotted with HET/SAF-B- and p85-specific antibodies. For the RPAs (B), 20 μg of RNA from the cell lines used in the Western blot were used, and the protected HET/SAF-B fragment of 240 bp is shown. 36B4 was used as a loading control.

tween HET/SAF-B expression and ploidy was analyzed using a *t* test.

RESULTS

Expression of HET/SAF-B in Breast Cancer Cell Lines.

Renz and Fackelmayer (1) have shown previously that *HET/SAF-B* is a ubiquitously expressed gene. Northern blot analysis using a variety of different human cancer cell lines and different tissues detected HET/SAF-B mRNA in all analyzed samples. To see how it is expressed in various breast cancer cell lines, we performed Western blot analysis using the HET/SAF-B antibody and a p85 antibody as a loading control (Fig. 1). HET/SAF-B protein levels varied between cell lines, with the highest expression in MDA-MB-231, ranging to almost undetectable levels in ZR-75 cells. The subsequent RPA (Fig. 1B) indicated that ZR-75 cells do express HET/SAF-B mRNA. Thus, although *HET/SAF-B* is a ubiquitously expressed gene, the levels in breast cancer cell lines vary.

Overexpression of HET/SAF-B in Tissue Culture Cell Lines and Effect on Cell Growth.

To establish the relationship between HET/SAF-B expression and cell proliferation, we attempted to transfect HET/SAF-B into cell lines. A number of initial efforts to isolate stable breast cancer cell lines that constitutively overexpressed HET/SAF-B were unsuccessful; after transfection with HET/SAF-B-pcDNA1, some drug-resistant clones formed, but none survived further passaging in culture. In contrast, we were able to select several hundred control clones transfected with pcDNA1 alone. These results indicate that HET/SAF-B either inhibits proliferation or is toxic to the cells. To circumvent this problem, we used a tetracycline-inducible

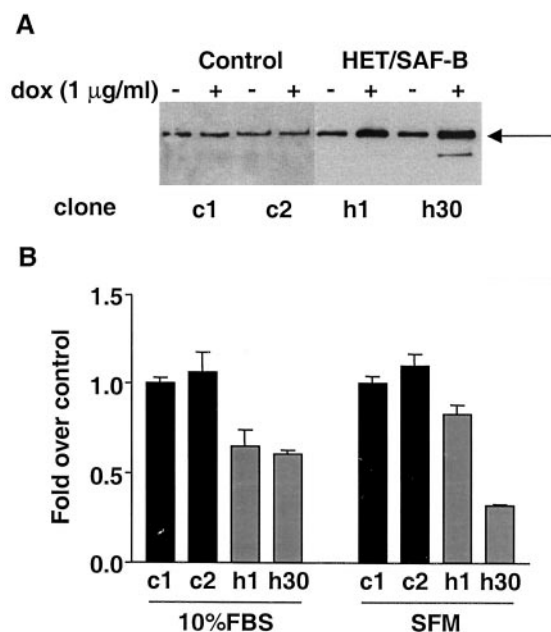


Fig. 2 Inducible expression of HET/SAF-B and growth inhibition in MDA-MB-435rtTA cells. **A**, MDA-MB-435rtTA cells containing a HET/SAF-B plasmid under control of a tet-inducible CMV promoter were induced with doxycycline for 24 h, lysed in 5% SDS, and analyzed by Western blotting using HET/SAF-B-specific antibodies. **B**, cells were plated in 96-well plates in quadruplicate in 10% serum (■) and in SFM (□), and doxycycline was added the next day. On day 5, MTT assay was performed, and the data are presented as percentage growth inhibition as compared with control cells; bars, SD.

system in MDA-MB-435 cells (MDA-435 rTA1). These cells were transfected previously with an inducible transactivator (etoposide linked to a tetracycline binding protein) and show 5–10-fold induction of reporter gene activity in the presence of the inducer doxycycline. We therefore cloned the HET/SAF-B cDNA downstream of a tetracycline-regulated CMV promoter and generated inducible HET/SAF-B clones. We were able to isolate two inducible clones, which showed increased HET/SAF-B expression when cells were stimulated with doxycycline for 24 h (Fig. 2A). We performed MTT growth assays to measure proliferation rate in these clones as compared with control clones and detected a significant decrease in cell number when cells were stimulated with doxycycline (Fig. 2B). The HET/SAF-B overexpressing cells showed growth inhibition in 10% serum as well as in serum-free medium. However, further passaging of those cells resulted in loss of inducibility of HET/SAF-B expression. Because we detected a slight leakiness of the system in transient assays, *i.e.*, expression of HET/SAF-B in the absence of inducer (data not shown), we suggest that the clones were lost because of a low overexpression of HET/SAF-B, even in the absence of doxycycline.

Because NIH3T3 cells are known to be less sensitive to overexpression of exogenous genes as compared with breast cancer cells, we attempted to generate stable HET/SAF-B transfectant overexpression clones with these cells. We could not detect any HET/SAF-B by Western blot in parental NIH3T3 cells, which could be attributable either to very low expression

or to the inability of the antibody raised against human HET/SAF-B to recognize murine HET/SAF-B. Just as in breast cancer cells, drug-resistant colonies formed after transfection in NIH3T3 cells, but most did not survive passaging, although we were able to select a high number of control clones transfected with the empty vector pcDNA1. Finally, we were able to generate one HET/SAF-B overexpressing clone (#25), as confirmed by Western blotting (Fig. 3A). Anchorage-dependent growth assays (MTT assay) showed that the HET/SAF-B-overexpressing clone grew much slower than two vector-alone control clones, either in SFM or in 10% FBS (Fig. 3B). Furthermore, cell cycle analysis confirmed that the slower growth of the HET/SAF-B-overexpressing clone was accompanied by a decrease in S-phase (15.6–4.0%). Thus, HET/SAF-B overexpression results in growth inhibition. This growth inhibition seems to be independent of ER, because it can be observed in ER-negative cells.

Overexpression of a GFP-HET/SAF-B Fusion Protein in Tissue Culture Cell Lines. As a final approach to generate HET/SAF-B-overexpressing clones, we used a GFP-HET/SAF-B fusion protein for our transfection studies. We hypothesized that using the fluorescently tagged HET/SAF-B would improve our screening procedure, because only fluorescent clones would be picked, expanded, and analyzed for overexpression. GFP-HET/SAF-B is functional, because it was able to corepress ER activity (data not shown), similar to our findings using the HET/SAF-B construct (21).

We transfected MDA-MB-435 cells using the GFP-HET/SAF-B construct and GFP only as a control. Of 20 fluorescent clones that were transfected with GFP alone, all 20 were still brightly fluorescent after keeping them in culture for 4–6 weeks (data not shown). However, of 120 fluorescent GFP-HET/SAF-B clones that we originally isolated, only 4 were still fluorescent after expanding them (2–3 weeks). In two of those clones, HET/SAF-B localized to the cytoplasm (data not shown), which has not been described before and which might represent an “escape mechanism” from the growth-inhibitory and/or toxic effects of HET/SAF-B overexpression. The other two clones showed only very faint fluorescence, in only approximately 1–3% of the cells, and HET/SAF-B was not detectable by Western blotting using the HET/SAF-B antibody, presumably because of the low level of expression in a small number of cells. However, a very weak signal could be detected using an anti-GFP antibody on Western blots (data not shown). We did not perform any growth assays with these clones because the expression of the exogenous HET/SAF-B was considerably lower than endogenously expressed HET/SAF-B, so that significant effects were unlikely to be observed. Thus, although the use of a GFP-HET/SAF-B fusion protein allowed us to perform a more efficient primary screen of the colonies, subsequently we were again unable to keep HET/SAF-B-overexpressing cells in culture.

We did, however, notice an obvious morphological change in the GFP-HET/SAF-B-expressing MDA-MB-435 cells as compared with the GFP-expressing cells (Fig. 4A). Many GFP-HET/SAF-B cells were polynucleated, with some cells having as many as 20 nuclei. We did not detect any polynucleated cells in the control GFP-transfected cells. We confirmed this result in transiently transfected CHO-K1 cells (data not shown), where

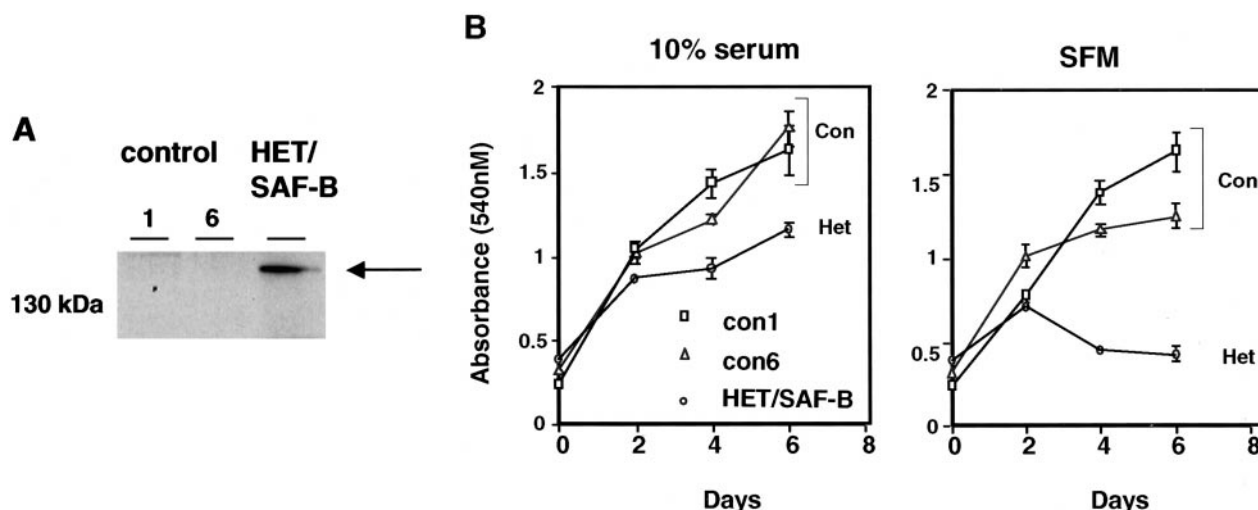


Fig. 3 Overexpression of HET/SAF-B and growth inhibition in NIH3T3 cells. **A**, for the Western blot, 50 μ g of SDS-protein extracts were loaded onto 6% SDS-PAGE, transferred to nitrocellulose, and blotted with HET/SAF-B-specific antibodies. **B**, cells were plated in quadruplicate in 96-well plates in 10% serum or SFM, and MTT assays were performed on the next day (day 0) and on day 2, 4, and 6. Bars, SD.

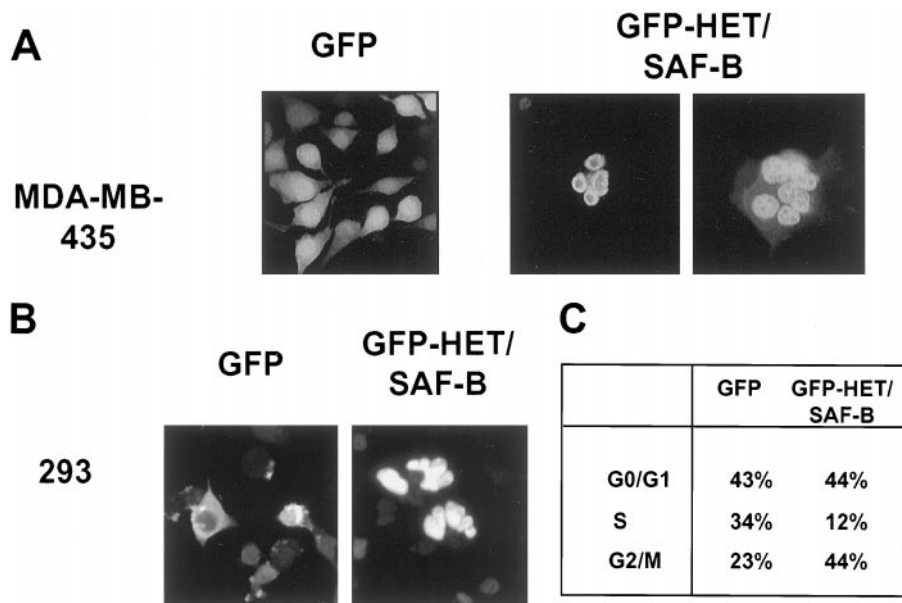


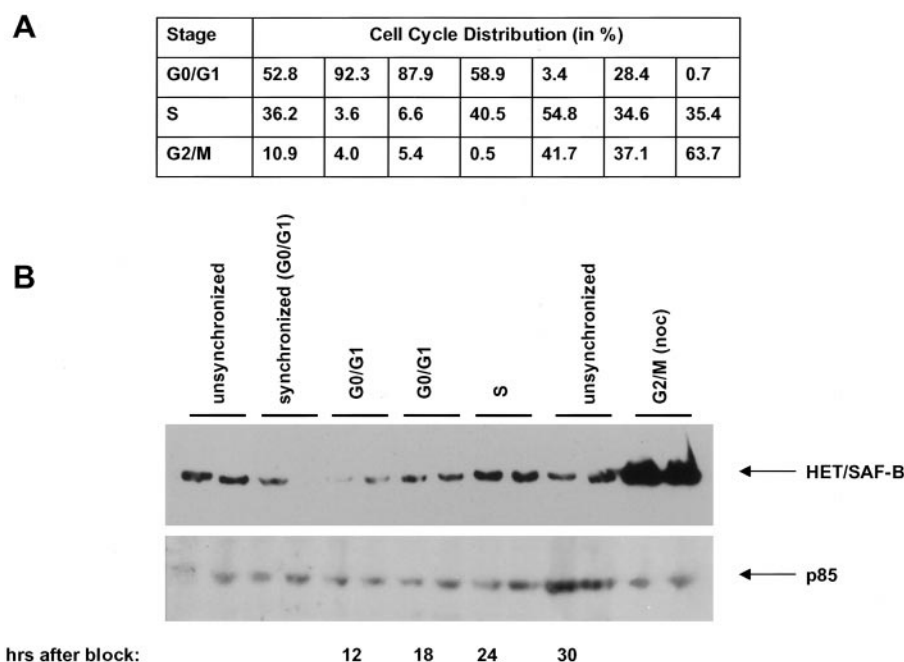
Fig. 4 Generation of multinucleated cells after GFP-HET/SAF-B transfection. **A**, MDA-MB-435 cells were transfected with GFP only or with GFP-HET/SAF-B. Cells were observed using a FITC filter on an Olympus CK40 fluorescence microscope. Representative pictures of one control clone (GFP) and two GFP-HET/SAF-B clones are shown. **B**, 293 cells were transiently transfected with GFP or GFP-HET/SAF-B, and representative pictures were taken. **C**, transfected 293 cells were sorted on a FACS, and DNA histogram analysis was performed on fluorescent cells only.

again we detected many polynucleated cells in the GFP-HET/SAF-B-overexpressing cells but not in the GFP cells. Finally, we repeated the transient transfection in 293 cells, which display very high transfection efficiency. As seen in MDA-MB-435 and CHO-K1 cells, we again observed many polynucleated cells among the HET/SAF-B-overexpressing cells. Depending on the cell line used, we detected multinucleated cells in 1–5% of the cells. We analyzed the cell cycle distribution of the transfected 293 cells by sorting the fluorescent cells and subjecting them to DNA histogram analysis (Fig. 4D). As shown previously (in the NIH3T3 transfection in Fig. 3), the number of cells in S-phase was decreased, from 34% in control cells to 12% in GFP-HET/

SAF-B-overexpressing cells. We also observed a block in G₂-M in the HET/SAF-B-overexpressing cells (23–44%). Thus, overexpression of GFP-HET/SAF-B was associated with multinuclearity and significant changes in cell cycle.

Because overexpression of HET/SAF-B seemed to block cells in G₂-M, we asked whether HET/SAF-B protein levels vary through the cell cycle. To answer this question, we used T24 human bladder carcinoma cells that can be easily synchronized by contact inhibition as described previously (38). They reenter the cell cycle upon replating at a lower dilution. Breast cancer cells do not synchronize upon confluence but can be synchronized by withdrawal of serum. However, reentry into the

Fig. 5 Cell cycle-dependent expression of HET/SAF-B. **A**, the cell cycle distribution for the cell populations used in the Western blot in **B**. *Columns* correspond to the *lanes* on the Western blot below. **B**, Western blot of HET/SAF-B in T24 cells either synchronized or blocked in G₂-M by nocodazole. The lanes represent extract from duplicate plates. T24 cells were synchronized, and samples were taken at 8, 12, 24, and 30 h after synchronization to investigate HET/SAF-B expression. T24 cells were also blocked in G₂-M using nocodazole at 40 ng/ml. HET/SAF-B protein levels were determined using our HET/SAF-B antibody. p85 was used as a loading control.



cell cycle by serum stimulation may produce artifactual results because serum stimulation may affect HET/SAF-B levels or phosphorylation. Thus, we used synchronized T24 cells. In addition, we analyzed T24 cells that were blocked in G₂-M by treatment with nocodazole. Cell cycle analysis was performed by FACS, and as expected, we were able to obtain cells synchronized in G₀-G₁, G₁-S, and G₂-M (Fig. 5A). Subsequent Western blot analysis demonstrated that HET/SAF-B was expressed throughout the cell cycle, but the levels of HET/SAF-B protein increased during S-phase and peaked in G₂-M (Fig. 5B). The expression of HET/SAF-B in nocodazole-treated cells confirmed the increase of HET/SAF-B expression in G₂-M. Thus, HET/SAF-B protein levels are at their highest in G₂-M, which is the stage at which overexpressed HET/SAF-B blocks cells.

Transient Modulation of HET/SAF-B Levels in Tissue Culture Cell Lines. As shown in the above experiments, it was difficult to stably integrate HET/SAF-B into the genome of various cell lines. Thus, as a final approach to prove the effect of HET/SAF-B on proliferation, we used a transient tissue culture assay in breast cancer cells commonly used by other investigators (39) to demonstrate negative effects of genes on growth. In this assay, cells are transfected with the gene of interest and a selection marker and then grown in selection media until colonies can be stained and counted. We transfected MCF-7/MG cells with pSV-neo plasmid and empty vector only (pcDNA1) or HET/SAF-B cDNA in sense or antisense orientation. Transfection of the antisense construct resulted in decreased HET/SAF-B expression at both RNA and protein levels (data not shown). The cells were selected for resistance to G418, and after 3 weeks, colonies were stained with crystal violet (Fig. 6A). The number of colonies was dramatically reduced after transfection of HET/SAF-B sense cDNA, whereas antisense transfection had no significant effect compared with pcDNA1

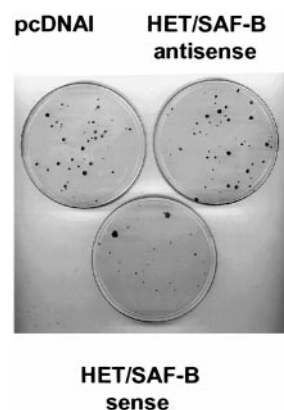


Fig. 6 Transient modulation of HET/SAF-B levels in MCF-7 cells. Colony formation assay in MCF-7 breast cancer cells is shown. MCF-7/MG cells were transfected with pcDNA1 vector control or with pcDNA1-HET/SAF-B in antisense or sense orientation, along with pSVneo, and incubated in G418 for 3 weeks. After staining with crystal violet, pictures were taken.

only. The inhibition of colony growth after HET/SAF-B overexpression confirms its growth inhibition. We were, however, surprised by the finding that antisense transfection did not increase colony formation in this experiment, and this could have several reasons. For instance, it is possible that we did not decrease the endogenous levels enough for generation of a phenotype in the transfected MCF-7 cells. Therefore, we repeated the transfection of the antisense DNA in 293 cells, which are known to display very high transfection efficiency. Determining [³H]thymidine incorporation into DNA as a direct measurement of cell proliferation, we were able to detect a dose-

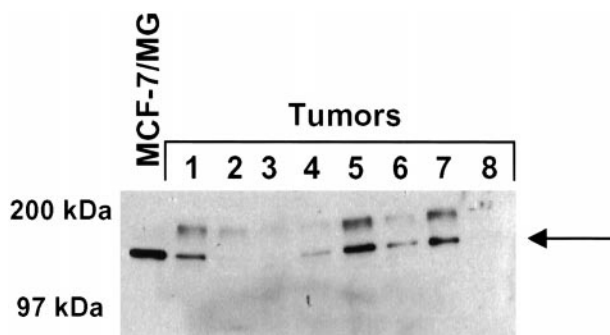


Fig. 7 Expression of HET/SAF-B in clinical breast cancer samples. A Western blot using 50 μ g of SDS extracts from breast tumors and HET/SAF-B-specific antibodies is shown. In the left lane, an SDS-extract from MCF-7 breast cancer cells was loaded as a positive control. Arrow, position of HET/SAF-B.

dependent increase in the proliferative index after transfection of the antisense construct (data not shown), thus again supporting our other data describing HET/SAF-B as a negative growth regulator.

Expression of HET/SAF-B in Clinical Breast Cancer Specimens. Lastly, we asked whether in tumor samples we could detect an association of HET/SAF-B with proliferative markers in a way reflective of our findings from tissue culture experiments. Therefore, we analyzed HET/SAF-B expression in human breast cancer specimens and correlated this with proliferation rate as measured by S-phase fraction. We measured HET/SAF-B levels in 61 primary breast tumors by Western blot (a representative blot is shown in Fig. 7) and found that, as in the cell lines, HET/SAF-B protein content varied widely. Some tumors expressed high amounts (e.g., no. 5), some moderate (e.g., no. 6), and in 10 tumors (16%), no HET/SAF-B could be detected (e.g., no. 8), even after prolonged exposure of the film. The same extracts were analyzed for histone H3 levels,⁴ and samples that were HET/SAF-B negative still showed abundant histone H3 expression. Thus, we can exclude artifacts such as nonspecific protein degradation. We quantified HET/SAF-B protein levels by densitometry, and statistical analysis revealed a trend toward a negative correlation with S-phase fraction. The correlation was only borderline significant (Spearman's rank correlation, -0.22 ; $P = 0.08$), but the sample size was relatively small, and a larger analysis is planned to verify this correlation. We also detected an association between HET/SAF-B and ploidy; high HET/SAF-B levels were associated with increased aneuploidy ($P = 0.021$). HET/SAF-B levels did not correlate with ER levels (Spearman's rank correlation, -0.064 ; $P = 0.62$), although as shown in a number of previous studies, we were able to detect a correlation between high ER levels and low S-phase (Spearman's rank correlation, -0.23 ; $P = 0.025$). Thus, in human breast tumors, HET/SAF-B protein expression varied widely, and higher levels were associated with aneuploidy. Also, we detected a trend toward a negative association

with proliferation, which is consistent with our findings from tissue culture experiments.

DISCUSSION

HET/SAF-B is a NMP that was cloned in our laboratory as a negative regulator of hsp27 expression (2) and in the laboratory of Renz and Fackelmayer (1) as a scaffold/matrix attachment site binding protein. The nuclear matrix was postulated many years ago to interact with nuclear hormone receptors (14–18), but only recently have specific NMPs been identified that bind directly to hormone receptors and modulate their activity (19). Because ER, like HET/SAF-B, is also involved in regulating hsp27 expression (40), we asked whether HET/SAF-B interacts with this receptor and modulates its activity and found that HET/SAF-B acts as an ER corepressor. Here we show more generally that HET/SAF-B is a growth inhibitor independent of its interaction with ER, blocking the cell in G₂-M, and in some situations causing multinuclearity.

We provide several lines of evidence that HET/SAF-B affects proliferation: (a) breast cancer cells with inducible HET/SAF-B expression grew significantly slower than their appropriate control clones; (b) NIH3T3 cells overexpressing HET/SAF-B also showed a significant decrease in growth; and (c) transient overexpression and underexpression of HET/SAF-B results in decreased colony formation and increased cell proliferation, respectively. Although interpretation of results from a single overexpressing NIH3T3 clone is limited because of the possible unpredictable effect of integration, the parallel evidence from HET/SAF-B-inducible MDA-MB-435rtTA cells and additional results from transient transfection assays in MCF-7 and 293 cells allow us to conclude that HET/SAF-B overexpression results in growth inhibition. In addition, in cultured cells as well as in clinical breast tumors, HET/SAF-B protein levels were inversely correlated with S-phase fraction, which is a direct measure of proliferation rate. In the present relatively small sample of breast tumors ($n = 61$), this correlation did not quite reach statistical significance ($P = 0.08$), and we are currently designing a larger study to address how well HET/SAF-B correlates with S-phase and other prognostic factors and whether HET/SAF-B levels could predict clinical outcomes of breast cancer patients. We have also shown that down-regulation of endogenous HET/SAF-B can lead to a higher growth rate, consistent with HET/SAF-B being a negative growth regulator, the absence of which may lead to excessive growth in tumors. Indeed, we have found that some breast tumors did not express HET/SAF-B protein at a detectable level.

The growth-inhibitory effects of overexpressed HET/SAF-B in ER positive cells can be readily explained by HET/SAF-B being an ER corepressor. It is feasible that HET/SAF-B suppresses estrogen-dependent transcriptional pathways related to breast epithelial cell proliferation, thus resulting in growth arrest. It is of interest to mention that the breast cancer susceptibility gene *BRCA1* was shown recently to inhibit ER activity in transient transfection assays (41).

The exact mechanism of estrogen-induced proliferation is yet to be defined, but we think that HET/SAF-B could play a role. When cells are primed to respond to estrogen in G₀-G₁ and in G₁-S-phase transition (42), HET/SAF-B levels are at their

⁴ C. K. Osborne, unpublished results.

lowest. In contrast, when cells do not respond to estrogen (in M phase), HET/SAF-B levels are high. Overexpression of HET/SAF-B, for instance as a result of our transfection studies, results in high HET/SAF-B levels at all parts of the cell cycle, including the phases when cells are primed to respond to estrogen. This presumably leads to inappropriate ER corepression, thus resulting in growth arrest.

As described by us (21) and others (1, 3), HET/SAF-B clearly has other functions, independent of its ER corepressor activity. These characteristics or other functions of HET/SAF-B yet to be defined might explain the growth-inhibitory effect of HET/SAF-B in ER-negative cells. For instance, hsp27 has clearly been shown to be associated with increased breast cancer cell growth, so that its down-regulation by HET/SAF-B could result in growth inhibition. It is also likely, just as discovered for other steroid receptor cofactors (42), that HET/SAF-B does not interact exclusively with ER. Indeed, our preliminary data⁵ indicate that HET/SAF-B can also inhibit the activity of other members of the steroid receptor as well as the retinoic acid/thyroid receptor families. Again, this regulation of other proteins besides the ER could explain the observed ER-independent effects.

HET/SAF-B has also been shown to bind to the COOH-terminal domain of RNA polymerase II and to a subset of serine/arginine-rich RNA processing factors (SR proteins; Ref. 3). This suggests that HET/SAF-B is involved in the formation of a transcriptosomal complex, bringing transcription and pre-mRNA processing together. The role of HET/SAF-B in this complex might be to prevent processing of mRNA transcripts, which would be consistent with the function of HET/SAF-B as a transcriptional repressor. Such an activity has been shown for CstF-50, which also binds to RNA polymerase II and is thought to prevent the processing of mRNA transcripts containing error (43). Overexpression of HET/SAF-B may disrupt the balance in the interaction with these splicing proteins, leading to a decrease in RNA processing and possibly resulting in growth arrest.

Finally, the attachment of HET/SAF-B to the nuclear matrix should be mentioned. The nuclear matrix organizes DNA into loop domains, the bases of which contain the S/MAR DNA sequences. It is the scaffold attachment factors such as HET/SAF-B that bind these sequences, thus connecting the chromatin to the NMP structures. The high levels of HET/SAF-B seen in G₂-M may also reflect its role in the packaging of chromatin for mitosis. When cells are in G₂-M, most transcription is repressed, and there is a dramatic increase in HET/SAF-B protein levels, which suggests that HET/SAF-B could be a major factor in the general repression of transcription at this phase of the cell cycle. The effect on cell proliferation when HET/SAF-B is overexpressed in both ER-positive and ER-negative cell lines may be the result of high intracellular HET/SAF-B levels throughout the cell cycle, leading to disruption of the organization of the transcriptionally active chromatin normally seen in G₀-G₁ and S-phase, thus producing transcriptionally inactive chromatin as seen in G₂-M. This repression then leaves cells without the

appropriate protein machinery to continue through the cell cycle and they become blocked.

In addition to the growth-inhibitory effects of overexpressed HET/SAF-B, our experiments with GFP-HET/SAF-B show that overexpression causes cells to be multinucleated. Furthermore, in breast tumors, HET/SAF-B was strongly associated with aneuploidy. Multinuclearity and aneuploidy are two of the most common features of tumor cells, but the exact molecular basis for these phenotypes is unknown (44, 45). Because HET/SAF-B overexpression results in a block in G₂-M, one could imagine that overexpressed HET/SAF-B somehow disrupts mitosis, either directly by altering the condensation of chromatin or indirectly by repressing genes involved in spindle formation and cytokinesis.

In summary, we have provided evidence that the NMP HET/SAF-B plays several roles in human breast cancer. Our current studies are aimed at further identifying the mechanism(s) of HET/SAF-B-mediated growth inhibition and multinuclearity.

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