[CANCER RESEARCH 54, 3092–3095, June 15, 1994]

*Advances in Brief*

# A Negative Regulatory Factor Is Missing in a Human Metastatic Breast Cancer Cell Line[1]

**Denise M. Stover, Indira Carey, Richard J. Garzon, and Zendra E. Zehner[2]**

*Department of Biochemistry and Molecular Biophysics, and the Massey Cancer Center, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298*

## Abstract

**The intermediate filament protein, vimentin, is differentially expressed in various tissues and stages of development and in metastatic *versus* nonmetastatic breast cancer cell lines. Previously, we have shown vimentin expression to be regulated at least in part by a silencer element which binds a $M_r$ 95,000 protein and an overriding, antisilencer element which binds a $M_r$ 140,000 protein. Southwestern blot (DNA-protein) analyses indicate that silencer protein binding activity is missing in the metastatic breast cancer cell line (MDA-MB-231), where vimentin is highly expressed, but is present in the nonmetastatic breast cancer cell line, MCF-7, where vimentin is not expressed. This suggests that the absence of a functional silencer protein may lead to expression of vimentin as well as other genes which contribute to the metastatic state.**

## Introduction

Although 5-year survival rates have improved, metastasis remains the leading cause of mortality in breast cancer. It is clear that crucial genetic controls must be altered in the metastatic cell. In order to understand metastases, we must determine what genetic alterations contribute to attaining the metastatic state. For this purpose, we have examined the regulation of the vimentin gene, which is highly expressed in the metastatic breast cancer cell line, MDA-MB-231, but not in the noninvasive MCF-7 cell line (1–3). Both of these cell lines have been developed from mammary gland carcinomas and are thought to represent cancer cells from various stages of tumor progression. From several studies, it has been suggested that vimentin expression is a marker for identifying a subset (10–25%) of hormone-independent breast carcinomas with a poor prognosis (4, 5). Only 36% of these patients survive 5 years compared to 82% with $VIM^-$ tumors. Because breast carcinomas probably progress through several stages of transformation ultimately leading to the malignant phenotype, one of the transforming events responsible for metastasis may also induce vimentin expression (1). An elucidation of that event will, therefore, contribute to understanding key steps leading to metastases in mammary gland carcinoma.

Previously, we have shown that at least two elements and factors contribute to the unique expression pattern of vimentin (6–8). One is a SE[3] which is found in multiple copies in the chicken and human genes and binds a $M_r$ 95,000 protein from chicken, mouse, or human nuclear extracts (6, 8). The second is an overriding ASE which binds a $M_r$ 140,000 protein (7). Here, we have further localized antisilencer binding to a 25-base pair element and examined the binding activity of these two regulatory proteins in the breast cancer cell lines. We see a direct correlation between vimentin expression and the lack of a functional silencer protein in the metastatic MDA-MB-231 breast cancer cell. In addition, binding activity of the antisilencer protein is high. In MCF-7 cells, binding activity for the silencer protein predominates. This suggests that these regulatory proteins work in concert to regulate expression of the vimentin gene and that the absence of a functional silencer protein in the metastatic breast cancer cell results in high expression of vimentin.

## Materials and Methods

**Cell Culture, DNA Transfections, and CAT Assays.** Mouse L929 cells were grown in Dulbecco's modified essential medium as described previously except that cells were seeded at $8 \times 10^5$ cells/dish prior to transfection (7). Transfection values are reported as the average of at least nine separate transfections and are expressed as a percentage *versus* the maximal CAT activity of the pcV-160 construct. The SEM was calculated and depicted as an error bar.

Human breast cancer cell lines, MCF-7 and MDA-MB-231, derived from mammary gland tumors were obtained from the American Type Culture Collection. MCF-7 cells were maintained in Eagle's minimal essential medium with nonessential amino acids, 1 mM sodium pyruvate, 10 $\mu$g/ml of bovine insulin (GIBCO), 10 $\mu$m estradiol, and 10% fetal bovine serum. MDA-MB-231 cells were maintained in L-15 medium and 10% fetal bovine serum according to the American Type Culture Collection.

**Plasmid Constructions.** The content of expression vector p8CAT as well as the vimentin constructs pcV-160, pcV-607, or pcV-1612 (originally labeled as pcV-1604) were described elsewhere (7, 8). Briefly, these constructs contain 160, 607, or 1612 nucleotides, respectively, 5′ to the transcriptional initiation site plus 45 nucleotides of 5′ untranslated region from the chicken vimentin gene fused to the reporter gene, CAT. The remaining vimentin 5′-end constructs were constructed via PCR methodology and a series of defined cloning steps as follows. Oligonucleotides which defined the 5′-end as pcV-1587 (primer 7, CTGAATTCAGCACTGACACAAACATATG); pcV-1535 (primer 1, CGCGAATTCCTGCTTCTTTACCAGAACAAACACC); and pcV-1412 (primer 2, CGCGAATTCATTAAGAGAAGCAGATGGAGCCAAG) containing an *Eco*RI site at the 5′-end plus 2 nucleotides (to ensure *Eco*RI cleavage following the PCR reaction) were synthesized. The 3′-end PCR primer was synthesized as the complement to the vimentin sequence at −714 to −694 (primer 6, CTGAATTCGAGCTCTTACAAAAAGGTCA), which contains the sole 5′-end vimentin *Sst*I site plus a 5′-*Eco*RI site as described above to facilitate cloning. In individual reactions, each pair of primers, *e.g.*, primers 7 plus 6, was used to prime synthesis of the appropriate fragment against a template containing the entire upstream region of the vimentin gene (−3500 to +1) subcloned in pUC18. PCR protocols were as suggested by Stratagene. Following *Eco*RI digestion, the appropriately sized fragment was excised from an agarose gel, eluted, precipitated in ethanol, and subcloned into *Eco*RI-digested pUC18. Each PCR product was isolated as an *Eco*RI/*Sst*I-digested fragment and fused to a *Sst*I-digested −694/pUC18 subclone containing the remaining 3′-half of the vimentin 5′-flanking region as described above. Next, the entire 5′-end fragment was digested with *Eco*RI, filled-in with the Klenow fragment of DNA polymerase, digested with *Bam*HI, and ligated into *Sma*I/*Bam*HI-digested p8CAT vector. Correct fusion of all fragments and sequence of the PCR products were confirmed by DNA sequencing (7).

Received 2/4/94; accepted 5/3/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by USPHS Grant HL45422 to Z. E. Z.

[2] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biophysics, Box 980614 MCV Station, Virginia Commonwealth University, Richmond, VA 23298.

[3] The abbreviations used are: SE, silencer element; ASE, antisilencer element; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; SW, Southwestern blot; HSV, herpes simplex virus; tk, thymidine kinase; cDNA, complementary DNA.



Downloaded from http://aacrjournals.org/cancerres/article-pdf/54/12/3092/2454007/cr0540123092.pdf by guest on 30 May 2024

**Preparation of Nuclear Extracts and Partial Protein Purification.** Nuclear extracts were prepared from HeLa cells (6, 9), and the silencer (8) and antisilencer proteins (7) were partially purified as described previously.

**Southwestern Blotting.** SW blots were performed with 50 $\mu$g portions of HeLa nuclear extract, ammonium sulfate, or column fractions as described previously (7, 8). Protein transfer and the approximate molecular weight of the hybridization bands was determined by comparison to prestained molecular weight markers (Sigma Chemical Co.). Nuclear extracts from the human breast cancer cell lines were similarly analyzed, and the hybridization signal was quantitated by densitometry.

**Northern Analysis.** Vimentin mRNA synthesis was analyzed in the breast cancer cell lines by Northern blots as described (7). Equal loading and RNA transfer was verified by visualization of ethidium bromide-stained gels prior to and after transfer to nitrocellulose.

## Results and Discussion

Previously, we (6–8, 10, 11) as well as others (12–14) have shown that expression of the vimentin gene depends upon multiple *cis*-acting elements and *trans*-acting factors. In the case of the chicken vimentin gene, these are localized to the 5′-end of the gene and include: (*a*) a proximal promoter element (−160 to +1) comprised of functional GC boxes, a CAAT and a TATA box; (*b*) a proximal enhancer element (−321 to −161) which appears to be tissue-specific; (*c*) a SE (−607 to −567) which is present in multiple copies, at least two of which are required to repress transcription by 90% (6, 8, 11); and (*d*) a positive acting, ASE (−1612 to −1535) which restores vimentin gene expression in some cells (7). We can find comparable elements in the 5′-end of the human vimentin gene (8, 12).

In addition to the promoter of the vimentin gene, the SE is capable of suppressing expression of the heterologous promoter, HSVtk, when fused to the reporter gene CAT (tkCAT). Interestingly, increasing the SE copy number from 1 to 4 results in additive repression to the tkCAT construct (6), just as it does to the vimentin gene (8).

Previously, we have found that the SE binds a $M_r$ 95,000 protein which is present in nuclear extracts from a variety of cell types, including fibroblasts, myoblasts, or HeLa cells, where the vimentin gene is known to be expressed (6, 7). Therefore, a mechanism must exist which restores vimentin expression in those cells where the gene is expressed despite the presence of an active silencer protein. To explore this possibility, we searched for a DNA element which when fused to StkCAT would restore reporter gene activity (7). In this case, we found a 77-base pair fragment (−1612 to −1535) which could restore CAT activity but only when the SE was present. By itself, the 77-base pair element had no effect on tk promoter activity; hence, we named it an ASE. When multiple copies of the SE were present, *i.e.*, 2StkCAT or 4StkCAT, multiple copies of the ASE were required to fully restore gene activity (7). We were anxious to confirm the importance of this ASE within the context of the vimentin gene and to assess factor binding in various cell types where the gene is differentially expressed.

**Transient Transfections of Various 5′-end:CAT Constructs.** Using PCR technology, we generated several vimentin 5′-end:CAT constructs which began at precise positions within the −607 to −1612 region and maintained the normal 5′-end sequence of the vimentin gene fused to the reporter gene. The activity of each construct was assessed by multiple (at least nine) transfections in mouse L929 cells where vimentin is highly expressed (Fig. 1). The maximal activity of the basal promoter element (pcV-160) was set at 100%. Inclusion of the three SEs within pcV-607 repressed transcription 92% as noted previously (6, 8). Including 5′-flanking DNA out to −1535 had little effect on restoring gene activity, *i.e.*, only a 2-fold increase over pcV-607. Construct pcV-1587 divides the original 77-base pair fragment into two regions, one of which contains a putative AP-1 site (a 7/8 match to the AP-1 consensus sequence) at −1574 to −1582 (12).

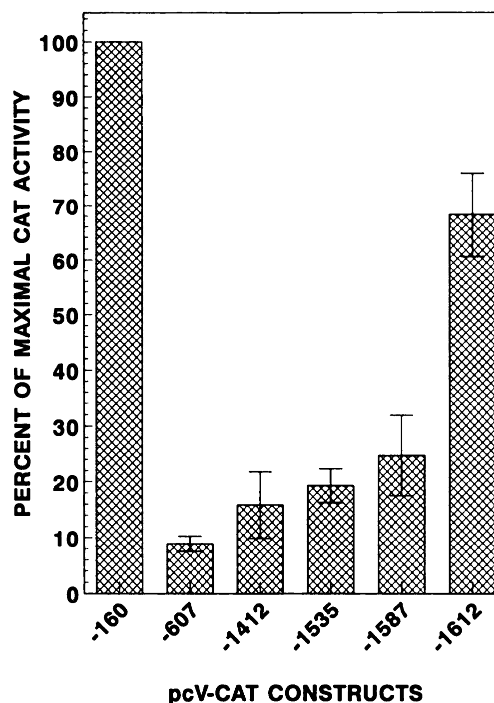


Fig. 1. Transient transfections of various chicken 5′-flanking chimeric CAT constructs in mouse L929 cells. Each construct is identified by the length of 5′-flanking DNA that it contains from the transcriptional start site at +1 to −160, *i.e.*, pcV-160. CAT activity is expressed as a percentage relative to the activity of the promoter element (pcV-160) set as 100%. Results are an average of at least nine separate transfections. *Bars,* SEM.

The inclusion of this sole AP-1 site contributed little to overriding the SE, unlike what was seen for the human gene (12). However, the addition of the remaining 25 base pairs restored gene activity to 70%. Moreover, this region contains a DNA sequence (−1587 to −1612) which exhibited protection in previous DNA footprinting experiments with HeLa nuclear extracts (7). No DNA footprint was observed over the sole AP-1 site (7). These results corroborate the importance of the 25-base pair sequence in functional transfection assays and negate any contribution from the single Ap-1 site (contained within the original 77-base pair fragment) to overriding the SE.

**Characterization of the Antisilencer and Silencer Protein.** In order to determine how the antisilencer and silencer proteins work in regulating vimentin gene expression, we needed to purify these proteins. Because we have found comparable transfection results in HeLa cells as well as in mouse L cells (6) and HeLa can be easily grown in large quantities for the purification of transcription factors, we initiated purification of the silencer and antisilencer proteins from HeLa nuclear extracts. SW blot analysis of crude extracts revealed that a major protein (approximately $M_r$ 140,000) plus several minor proteins bind to the 77-base pair ASE (Fig. 2*A*). This protein localizes to the 30–70% ammonium sulfate fraction and is missing from the 0–30% (data not shown) and 70–90% ammonium sulfate fractions. Therefore, the 30–70% ammonium sulfate fraction was desalted and applied to a phosphocellulose column (7, 9). Proteins were eluted with increasing KCl step gradients and assayed for the antisilencer protein by SW blot analysis with $^{32}$P-labeled 77-base pair DNA element. The antisilencer protein eluted in the 0.3 M KCl fraction. Band shift assays showed several shifted bands in the crude and 30–70% ammonium sulfate fraction but a single band in the 0.3 M KCl fraction (7). Therefore, two completely independent DNA protein binding assays, *i.e.*, SW blots and band shift assays, independently confirmed the presence of the antisilencer protein in the 0.3 M KCl fraction from the



Downloaded from http://aacrjournals.org/cancerres/article-pdf/54/12/3092/2454007/cr0540123092.pdf by guest on 30 May 2024

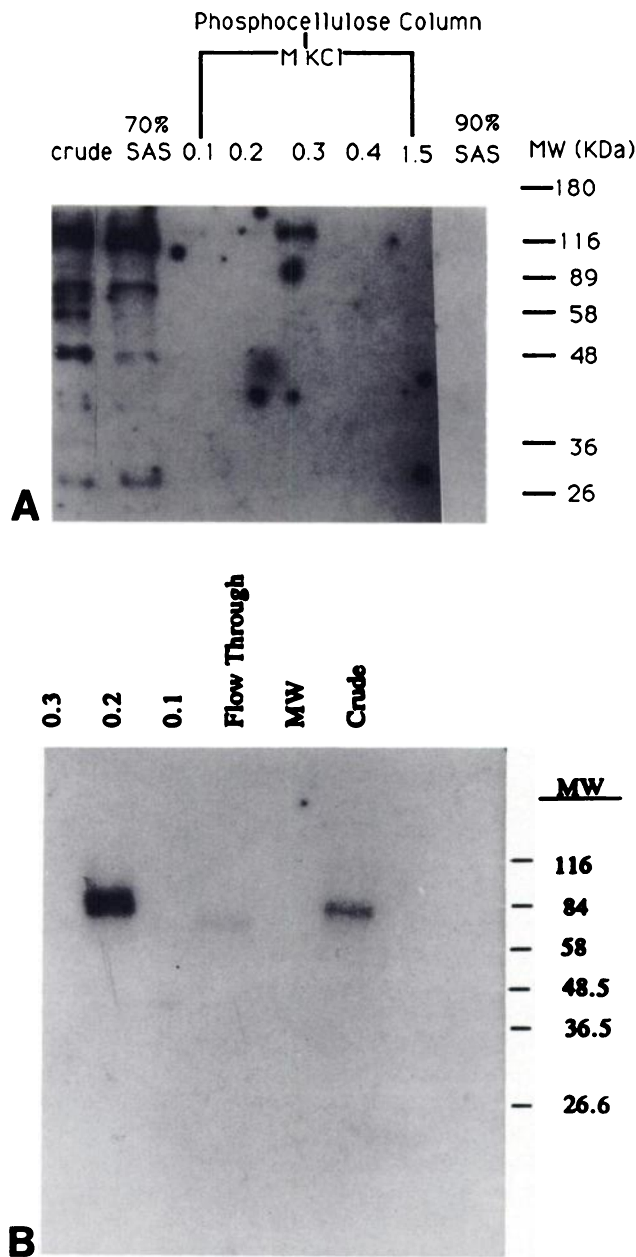


Fig. 2. SW blot analyses during partial purification of the $M_r$ 140,000 antisilencer and $M_r$ 95,000 silencer protein. *A*, a 50-$\mu$g portion of crude nuclear extract, ammonium sulfate precipitate, or salt-eluted column fractions was separated on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose filters as described in "Materials and Methods." Filters were hybridized with the $^{32}$P-labeled 77-base pair ASE. The position of prestained molecular weight markers are indicated. *B*, as in *A* but filters were hybridized with the $^{32}$P-labeled 40-base pair SE.

phosphocellulose column and verified the size of the protein to be approximately $M_r$ 140,000.

Similar partial purification of the silencer protein revealed that binding activity was located in the 0.2 M KCl fraction from the phosphocellulose column (Fig. 2*B*). Band shift assays corroborated the presence of the silencer protein in this fraction (8). Therefore, we have shown that, even at early stages of purification, it is possible to separate the antisilencer and silencer proteins. In addition, we have confirmed the relative size of these proteins and our ability to detect these proteins even in crude extracts by SW blot analyses.

**Vimentin Expression in Breast Cancer Cell Lines.** Because of the putative antagonism between silencer and antisilencer binding, we proposed to investigate the binding activity of these two regulatory factors in cells where the vimentin gene is differentially expressed. Previously, we have shown that there is a direct correlation between the binding activity of these proteins and vimentin gene expression in muscle development (7). During myogenesis, the binding activity of the silencer protein increases, while the antisilencer protein decreases. This result correlates with the down-regulation of the vimentin gene during this developmental transition. Here, we wish to examine the mechanism of vimentin expression in breast cancer cell lines where vimentin is differentially expressed.

Northern blot analysis confirmed that the metastatic breast cancer cell line, MDA-MB-231, contains a considerable amount of vimentin mRNA (Fig. 3*A*) as was seen previously (1). However, no vimentin mRNA could be detected in the nonmetastatic, MCF-7 counterpart, even though the Northern blot was greatly overexposed in order to detect minor amounts of vimentin mRNA. It has been shown that the presence or absence of vimentin protein as measured by immunofluorescence, isoelectric focusing, or Western blots correlates with the lack of vimentin mRNA in this cell line (1, 2). In fact, a vimentin cDNA was originally isolated by screening an MDA-MB-231 cDNA library with a cDNA probe subtracted against mRNA from the MCF-7 cell line (1).

SW blot analyses was used to compare the binding activity of the silencer and antisilencer proteins in nuclear extracts from the two breast cancer cell lines. These experiments were conducted on equivalent amounts of nuclear extracts, separated on the same gel, and

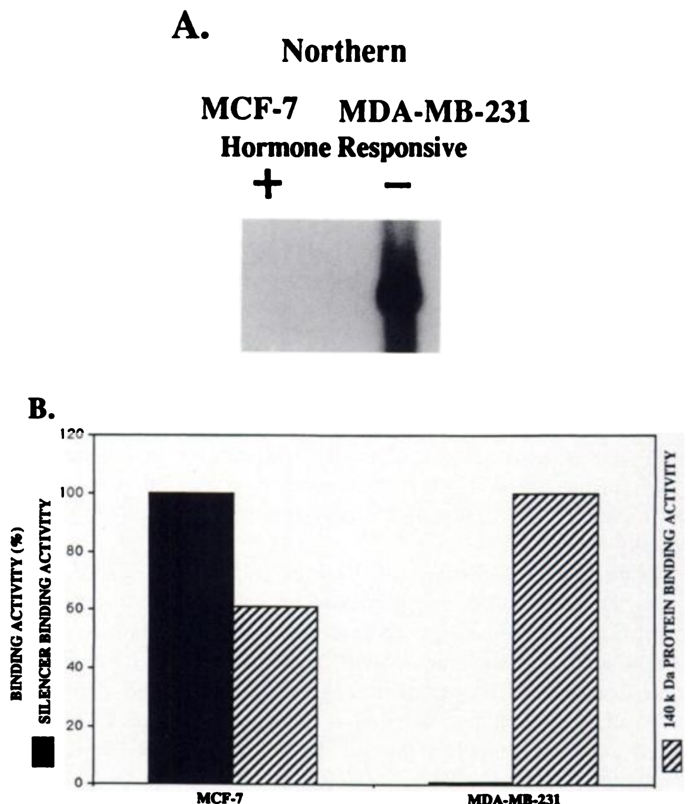


Fig. 3. Northern and SW blot analyses of two breast cancer cell lines, MCF-7 and MDA-MB-231. *A*, total RNA (3 $\mu$g) was analyzed on formaldehyde gels, transferred to nitrocellulose and hybridized with a $^{32}$P-labeled human vimentin cDNA as described in "Materials and Methods." *B*, crude nuclear extracts (50 $\mu$g) from both breast cancer cell lines were hybridized with $^{32}$P-labeled 40-base pair SE and the 77-base pair ASE of equal radiospecific activity. The resulting SW blots were quantitated using scanning densitometry.



Downloaded from http://aacrjournals.org/cancerres/article-pdf/54/12/3092/2454007/cr0540123092.pdf by guest on 30 May 2024

transferred to nitrocellulose. The resulting nitrocellulose strips were probed with either $^{32}$P-labeled 40-base pair SE or 77-base pair ASE of equal radiospecific activity. The resulting autoradiogram was scanned by densitometry, and the results were quantitated (Fig. 3*B*). Although substantial activity was noted for the positive-acting antisilencer protein, silencer protein binding activity was virtually absent in the MDA-MB-231 nuclear extract. On the other hand, in MCF-7 cells, there was roughly 2-fold more binding activity for the $M_r$ 95,000 silencer protein than the antisilencer. Although considerable antisilencer binding activity remains, the vimentin gene remains off. Either the amount of silencer protein activity in MCF-7 cells is sufficient to keep the vimentin gene off or other mechanisms such as methylation, chromatin condensation, and/or nucleosome structure contribute to total repression of the vimentin gene in the epithelial cell. Likewise, van de Klundert *et al.* have detected no expression of a hamster vimentin 5′-end CAT construct when transfected into MCF-7 cells (14). In addition, these authors could detect binding activity to at least one of the negative elements within the hamster gene. Therefore, it would appear from both of our studies that vimentin is not expressed in MCF-7 cells, probably due to the presence of functional silencer protein(s) and perhaps other levels of gene control as mentioned above.

There is no doubt that in some human metastatic breast cancer, as represented by the MDA-MB-231 cell line, vimentin is highly expressed but not in the more epithelial-like, nonmetastatic tumor cell (1–3). The absence of functional silencer protein in these cells could greatly contribute to expression of other genes as well as vimentin leading to tumor progression. A search of the genomic data base shows that a number of genes contain SE homology. A partial list includes human *IGF1-R*, insulin, *HPRT,* 2-adrenergic receptor, v-*erb*A-related *ear*-2 and *ear*-3, cytomegalovirus $M_r$ 25,000 protein gene, and major immediate-early gene; mouse alpha-fetoprotein and c-*jun*; and rat growth hormone, interleukin-2 receptor, and *L-TAT* genes. Interestingly, many of these genes code for metabolic enzymes, growth factors, receptors, or oncogenes, *i.e.,* proteins which could clearly play a role in neoplasia. A number of these genes also contain a sequence homology to the ASE. Therefore, these regulatory factors could orchestrate the expression of many genes. Absence of functional silencer factor could permit escape synthesis of a number of normally repressed genes whose products contribute to metastatic behavior. If this is the case, will vimentin expression correlate with metastases in other types of tumors? Interestingly, vimentin has been found to be expressed (via subtractive hybridization) in a highly aggressive, rat prostatic tumor derived from the Dunning R-3327-H adenocarcinoma but not in the relative benign counterpart (15). This result is analogous to what is seen in breast cancer and suggests that the absence of the $M_r$ 95,000 silencer factor correlates with aberrant gene expression in a number of metastatic tumor types.

## References


1. Sommers, C. L., Walker-Jones, D., Heckford, S. E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S., and Gelmann, E. P. Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. Cancer Res., *49:* 4258–4263, 1989.
2. Sommers, C. L., Heckford, S. E., Skerker, J. M., Worland, P., Torri, J. A., Thompson, E. W., Byers, S. W., and Gelmann, E. P. Loss of epithelial markers and acquisition of vimentin expression in Adriamycin- and vinblastine-resistant human breast cancer cell lines. Cancer Res., *52:* 5190–5197, 1992.
3. Thompson, E. W., Paik, S., Brunner, N., Sommers, C. L., Zugmaier, G., Clarke, R., Shima, T. B., Torri, J., Donahue, S., Lippman, M. E., Martin, G. R., and Dickson, R. B. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J. Cell. Physiol., *150:* 534–544, 1992.
4. Raymond, W. A., and Leong, A. S-Y. Vimentin—a new prognostic parameter in breast carcinoma. J. Pathol., *158:* 107–114, 1989.
5. Domagala, W., Lasota, J., Bartkowiak, J., Weber, K., and Osborn, M. Vimentin is preferentially expressed in human breast carcinomas with low estrogen receptor and high Ki-67 growth fraction. Am. J. Pathol., *136:* 219–227, 1990.
6. Farrell, F. X., Sax, C. M., and Zehner, Z. E. A negative element involved in vimentin gene expression. Mol. Cell. Biol., *10:* 2349–2358, 1990.
7. Stover, D. M., and Zehner, Z. E. Identification of a *cis*-acting DNA antisilencer element which modulates vimentin gene expression. Mol. Cell. Biol., *12:* 2230–2240, 1992.
8. Garzon, R. J., and Zehner, Z. E. Multiple silencer elements are involved in regulating the chicken vimentin gene. Mol. Cell. Biol., *14:* 934–943, 1994.
9. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res., *11:* 1475–1489, 1983.
10. Sax, C. M., Farrell, F. X., Tobian, J. A., and Zehner, Z. E. Multiple elements are required for expression of an intermediate filament gene. Nucleic Acids Res., *16:* 8057–8076, 1988.
11. Sax, C. M., Farrell, F. X., and Zehner, Z. E. Down-regulation of vimentin gene expression during myogenesis is controlled by a 5′-flanking sequence. Gene (Amst.), *78:* 235–242, 1989.
12. Rittling, S. R., Coutinho, L., Amram, T., and Kolbe, M. Ap-1/jun binding sites mediate serum inducibility of the human vimentin promoter. Nucleic Acids Res., *17:* 1619–1632, 1989.
13. Salvetti, A., Lilienbaum, A., Li, Z., Paulin, D., and Gazzolo, L. Identification of a negative element in the human vimentin promoter: modulation by the human T-cell leukemia virus type I Tax protein. Mol. Cell. Biol., *13:* 89–97, 1993.
14. van de Klundert, F. A. J. M., van Eldik, G. J., Pieper, R. F., Jansen, H. J., and Bloemendal, H. Identification of two silencers flanking an AP-1 enhancer in the vimentin promoter. Gene (Amst.), *122:* 337–343, 1992.
15. Bussemakers, M. J. G., Verhaegh, G. W. C. T., van Bokhoven, A., Debruyne, F. M. J., and Schalken, J. A. Differential expression of vimentin in rat prostatic tumors. Biochem. Biophys. Res. Commun., *182:* 1254–1259, 1992.


Downloaded from http://aacrjournals.org/cancerres/article-pdf/54/12/3092/2454007/cr0540123092.pdf by guest on 30 May 2024