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13. ABSTRACT (Maximum 200 Words) Eukaryotic chromosomes are thought to be separated into topologically independent loop domains by periodic attachment onto an intranuclear frame known as the nuclear matrix. Specific DNA sequences that bind to the nuclear matrix are called matrix attachment regions (MARs), in which a specialized DNA context sequences exhibiting high base unpairing propensity (BUR) is typically found. Besides organization of eukaryotic DNA, BURs/MARs may also be important for various functions including replication, transcription and recombination. A strong BUR-binding activity, p114, previously reported in breast carcinoma cells, was identified to be a combined property poly ADP-ribose polymerase (PARP) and scaffold attachment factor A (SAF-A). PARP is upregulated in breast carcinoma SK-BR-3 cells as compared to normal mammary epithelial cells in culture. Additionally, HMG I(Y) was also found to be a BUR-binding protein, and its expression was well correlated with aggressive breast cancer cells. Thus, we have shown that the expression of several BUR-binding proteins correlates strongly with the aggressive phenotype of breast carcinoma cells. Specific binding of these proteins to BURs in cancers may contribute towards gene regulation to trigger or maintain the malignant phenotype in response to signals from extracellular matrix (ECM), or during progression of tumorigenesis and differentiation.				
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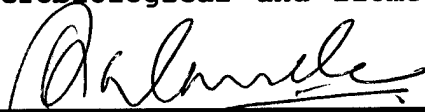
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INTRODUCTION

We identified a protein with 114 kDa (p114) from breast carcinoma cells that exhibit a strong binding activity to a specialized DNA context sequences that exhibit high base unpairing propensity. Such a base unpairing region (BUR) is typically found in nuclear matrix attachment regions (MARs); and are thus a hallmark thereof (reviewed in Kohwi-Shigematsu and Kohwi, 1997). BURs/MARs are thought to be important for functional organization of eukaryotic DNA (Gasser and Laemmli, 1987). The BUR-binding activity of this protein (p114) was detected in human breast tumor specimens, but it was virtually undetectable in normal breast counterparts (Yanagisawa et al., 1996). The activity correlated with progression of breast cancer: stronger activity was detected in more aggressive breast cancer than in more differentiated breast cancer. Previously, we identified P114 as poly ADP-ribose polymerase (PARP) (Galande and Kohwi-Shigematsu, 1999). This finding that PARP can recognize primary sequences such as BURs was totally unprecedented (Galande and Kohwi-Shigematsu, 1999). Using BUR-affinity chromatography to isolate BUR-binding proteins from breast cancer SK-BR-3 cells, we almost exclusively obtained a complex of poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK). Both PARP and DNA-PK are activated by DNA breaks and are implicated in DNA repair, recombination, DNA replication, and transcription. In contrast to the previous notion that PARP and Ku autoantigen, the DNA-binding subunit of DNA-PK, mainly bind to free ends of DNA, we found that both proteins individually bind BURs with high affinity and specificity in an end-independent manner using closed circular BUR-containing DNA substrates. We also demonstrated that PARP and Ku autoantigen form a molecular complex *in vivo* and *in vitro* in the absence of DNA, and as a functional consequence, their affinity and specificity to the BURs is synergistically enhanced. ADP-ribosylation of the nuclear extract abrogated the BUR-binding activity of this complex (Galande and Kohwi-Shigematsu, 1999). These results provide a mechanistic link towards understanding the functional overlap of PARP and DNA-PK and suggest a novel role for these proteins in the regulation of chromatin structure and function. We hypothesized that the interaction between various MAR-binding proteins including PARP and MARs in breast cancer cells may be critical for the onset and/or maintenance of the malignant phenotype.

Recently, I found that the p114 MAR-binding activity is attributed to one more protein other than PARP. This protein has a similar molecular weight, and migrates almost to the same position as that of PARP by SDS-polyacrylamide gel electrophoresis. I identified this protein to be scaffold associated factor A (SAF-A). SAF-A has strong affinity for scaffold or matrix attachment sequences (SARs/MARs) (Fackelmayer et al. 1994).

Additionally, Dr. W.-M. Liu, the previous recipient of this fellowship, purified a doublet of 20 kDa proteins by a BUR affinity column from human breast carcinoma cells. These proteins were identified as the high mobility group protein, HMG I, and its splicing variant, HMG Y, which specifically bind BURs. Dr. Liu systematically analyzed whether a substantial increase in HMG I(Y) expression is indeed correlated with breast cancer cells that have metastasizing ability (please see attached reprint of an article entitled "HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype", Liu *et al.* (1999), Cancer Research).

Thus, we have shown that the expression of several BUR-binding proteins correlates strongly with the aggressive phenotype of breast carcinoma cells. Specific binding of these proteins to BURs in cancers may contribute towards gene regulation to trigger or maintain the malignant phenotype in response to signals from extracellular matrix (ECM), or during progression of tumorigenesis and differentiation. Our research plan is aimed at providing an important insight into the fundamental mechanism underlying breast cancer development and/or maintenance, and may also provide a clue for breast carcinoma therapy in the future.

BODY

SPECIFIC AIM 1. To fully establish the correlation between p114 MAR-binding activity and the alteration of cell shape associated with either normal or malignant phenotype. To determine whether p114 MAR-binding activity is critical for the cause/or maintenance of the malignant phenotype.

PROGRESS 1:

We primarily focused on the characterization of p114 MAR-binding activity. We had identified one component of p114 MAR-binding activity as poly ADP-ribose polymerase (PARP) which, besides DNA ends and nicks, can also recognize primary sequence of BUR (Galande and Kohwi-Shigematsu, 1999). Additionally, we also found that PARP forms a complex with DNA-dependent protein kinase (DNA-PK) via its interaction with the Ku autoantigen (Galande and Kohwi-Shigematsu, 1999). We therefore also wished to monitor the expression level of DNA-PK catalytic subunit (cs) and found that it is also upregulated in cancer.

PROGRESS 2:

It has been well established that PARP contributes to some, if not all, MAR-binding activity of p114. However, depletion of PARP by immunoprecipitation revealed presence of another protein that also bound the MAR probe on southwestern blots. This protein was identified to be SAF-A based on its cross-reactivity towards anti-SAF-A.

PRELIMINARY RESULTS:

1. A high level of PARP (p114) expression in breast carcinoma cells is not simply because these cells have a high rate of proliferation. Human breast carcinoma SK-BR-3 cells showed similar proliferation as normal human mammary epithelial cells (HMEC) in plastic culture dishes (Fig. 1A).
2. PARP is expressed at least 20-fold higher in a human mammary carcinoma cell line SK-BR-3 as compared to the normal mammary epithelial cells (Fig. 1B). The p114 MAR-binding activity shows a similar pattern indicating a correlation between PARP expression and p114 activity (Fig. 1C). Interestingly, DNA-PKcs is also upregulated in SK-BR-3 cells. The two bands observed in the western blot correspond to the full length (460 kDa) and proteolyzed (250 kDa) versions of DNA-PKcs (Fig. 1D). This result suggests that the expression of these proteins may be regulated by a common mechanism, which may contribute towards the onset and/or maintenance of malignancy.

The immediate future experiment is to compare the expression levels of these proteins in breast tissue/tumor samples.

3. Immunoprecipitation experiments followed by Southwestern analysis to monitor MAR-binding activity clearly indicated that p114 is composed of two different proteins, namely SAF-A and PARP. If the SDS-PAGE conditions are optimized, p114 activity, which is usually noted as a single band appears as a doublet (Fig. 2). Anti-SAF-A selectively precipitates the top band (corresponding to SAF-A) and anti-PARP specifically precipitates the bottom band (corresponding to PARP). However, in typical gel electrophoresis, p114 appears as a single species. Thus, the p114 MAR-binding activity is a combined property of both PARP and SAF-A proteins.

Fig. 1 p114 MAR-binding activity correlates with the level of PARP expression. **A.** Growth curve for SK-BR-3 (top) and human mammary epithelial cells (bottom). Cells were grown in McCoy's 5A and mammary epithelial cell growth medium respectively. **B.** Monitoring PARP expression levels by immunoblot analysis. Forty μ g of cell extracts each from HMEC (lane 1) and SK-BR-3 (lane 2) were separated on a 7.5% SDS polyacrylamide gel and subjected to western blot analysis using anti-PARP (H-250, Santa Cruz Biotechnology). **C.** Southwestern analysis of p114 MAR-binding activity from HMEC (lane 1) and SK-BR-3 (lane 2) cells. After immunoblotting as described above, proteins were renatured *in situ* and incubated with 32 P-labeled MAR probe. After washing unbound probe, the blot was subjected to autoradiography. **D.** Monitoring DNA-PKcs expression level by immunoblot analysis. Cell extracts from HMEC (lane 1) and SK-BR-3 (lane 2) cells were subjected to SDS-PAGE and immunoblotted as described above. Anti-DNA-PKcs antibody 4 (Neomarkers) was used.

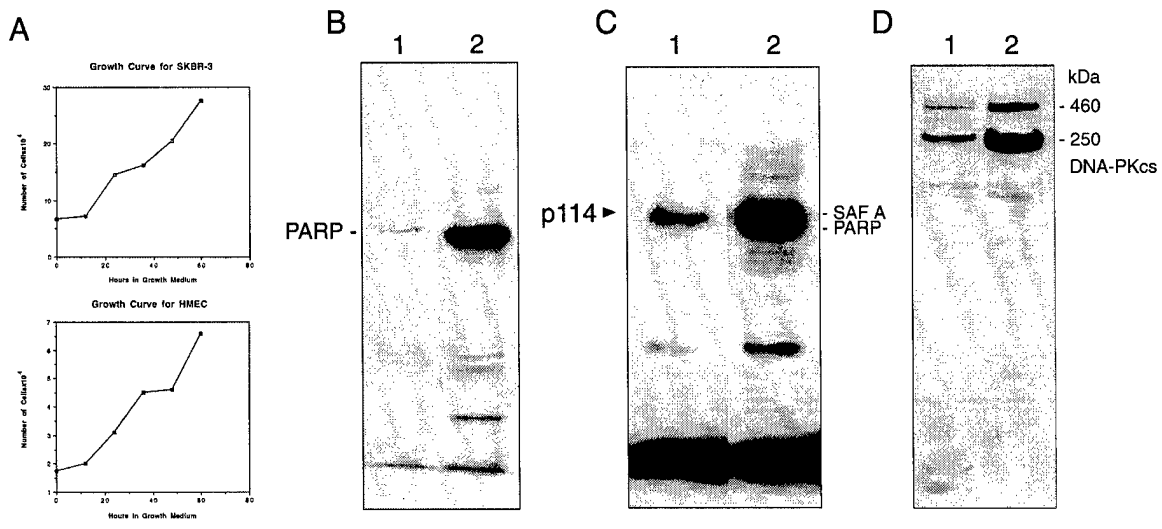
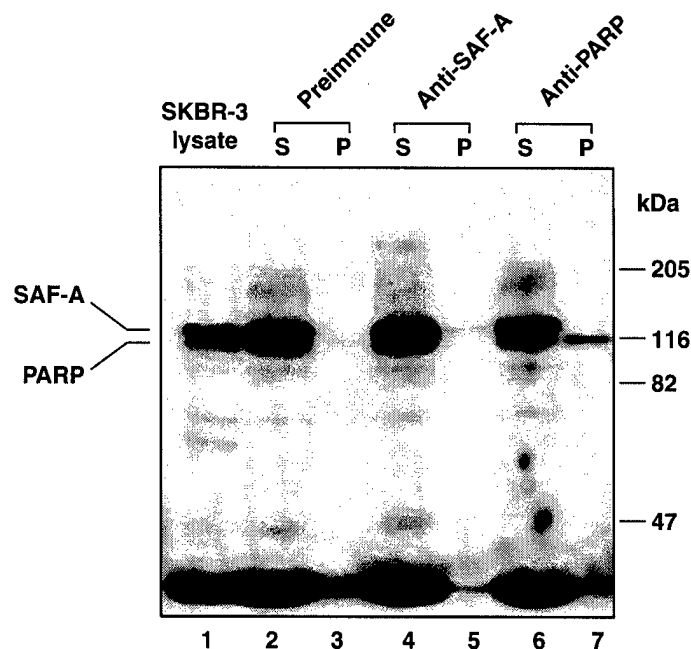


Fig. 2 Molecular dissection of p114: the p114 MAR-binding activity is a combined property of PARP and SAF-A. The components of the p114 MAR-binding activity were analyzed by immunoprecipitation followed by Southwestern analysis. Lane 1, Forty μ g of SK-BR-3 extract without any treatment. For each immunoprecipitation reaction, 100 μ g of SK-BR-3 cell extract was incubated either with preimmune serum (lanes 2-3) or Anti-SAF-A (lanes 4-5) or Anti-PARP (lanes 6-7). Protein A/G plus beads were then added, and the immune complexes were recovered by centrifugation. Supernatants (lanes 2,4,6) and immunoprecipitates (lanes 3,5,7) were suspended in SDS-PAGE loading buffer and subjected to Southwestern blot analysis using a 32 P-labeled MAR probe. Positions of protein molecular weight standards in kDa are indicated on right, while the positions of PARP and SAF-A are depicted on the left.



Summary: My preliminary results shown here strongly suggest that p114 MAR-binding activity is a combined property of PARP and SAF-A, both of which may be key players in the maintenance of the aggressive phenotype of breast malignancy.

SPECIFIC AIM 2. A set of genomic sequences bound to p114 *in vivo* in T4-2 cells grown in EHS matrix will be obtained. The association of these sequences with nuclear matrix prepared from T4-2 cells either grown in EHS matrix (tumor phenotype) or where treated with β 1-integrin antibody (non-malignant phenotype) will be compared.

N/A for this period.

SPECIFIC AIM 3. For selected genomic sequences bound to p114 in T4-2 cells grown in EHS culture, specific genes will be identified nearby such genomic sequences. We will determine whether these specific genes expression is regulated by p114 MAR-binding activity altered by ECM signalling.

N/A for this period.

LITERATURE CITED

Fackelmayer, F.O., Dahm, K., Renz, A., Ramsperger, U., and Richter, A. Nucleic-acid-binding properties of hnRNP-U/SAF-A, a nuclear-matrix protein which binds DNA and RNA *in vivo* and *in vitro*. *Eur. J. Biochem.*, 221: 749-757, 1994.

Galande, S. A., and Kohwi-Shigematsu, T. Poly (ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. *J. Biol. Chem.*, 274: 20521-20528, 1999.

Gasser, S. M., and Laemmli, U. K. A glimpse at chromosomal order. *Trends Genet.*, 3: 16-22, 1987.

Kohwi-Shigematsu, T., and Kohwi, Y. *In*: R. Curtis Bird (ed.) *Nuclear Structure and Gene Expression: High unwinding capability of matrix attachment regions and ATC-sequence context-specific MAR-binding proteins*, pp. 111-144. San Diego: Academic Press, 1997.

Liu, W.-M., Guerra-Vladusic, F. K., Kurakata, S., Lupu, R., and Kohwi-Shigematsu, T. HMG I(Y) recognizes Base-Unpairing regions (BURs) of matrix attachment regions and is directly linked to metastatic breast cancer phenotype. *Cancer Res.*, 59:5695-5703, 1999.

Roskelley, C. D., Srebrow, A., and Bissell, M. J. A hierarchy of ECM-mediated signaling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.*, 7: 736-47, 1995.

Yanagisawa, J., Ando, J., Nakayama, J., Kohwi, Y., and Kohwi-Shigematsu, T. A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast carcinomas and not in normal and benign breast disease tissues. *Cancer Res.*, 56: 457-462, 1996.

SUMMARY

Research accomplishments

- a. One publication in Cancer Research (1999) "HMG I(Y) Recognizes Base-Unpairing Regions of Matrix Attachment Sequences and Its Increased Expression is Directly Linked to Metastatic Breast Cancer Phenotype". Reprint is attached.
- b. Important preliminary results demonstrating that p114 MAR-binding activity is actually composed of two different proteins namely PARP and SAF-A. Our previous results have already indicated that PARP could be a key player in the maintenance of the aggressive phenotype of breast malignancy.
- c. We have shown that DNA-PKcs is upregulated in breast carcinoma cell line SK-BR-3. Ku Autoantigen, the DNA binding subunit of DNA-PK, binds to PARP and to MARs. Previously, we have shown that expression levels of the MAR binding protein HMG (I/Y) also correlate with the degree of malignancy. Thus, the activities of various MAR binding proteins may be a critical determinant of malignancy.

HMG-I(Y) Recognizes Base-unpairing Regions of Matrix Attachment Sequences and Its Increased Expression Is Directly Linked to Metastatic Breast Cancer Phenotype¹

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ABSTRACT

Base-unpairing regions (BURs) contain a specialized DNA context with an exceptionally high unwinding propensity, and are typically identified within various matrix attachment regions. A BUR affinity column was used to purify a doublet of *M*_r 20,000 proteins from human breast carcinoma cells. These proteins were identified as the high-mobility group (HMG) protein, HMG-I, and its splicing variant, HMG-Y. We show that HMG-I(Y) specifically binds BURs. Mutating BURs so as to abrogate their unwinding property greatly reduced their binding affinity to HMG-I(Y). Numerous studies have indicated that elevated HMG-I(Y) expression is correlated with more advanced cancers and with increased metastatic potential. We studied whether the expression of HMG-I(Y) responds to signaling through the heregulin (HRG)-*erbB* pathway and the extracellular matrix. HMG-I(Y) expression was increased in MCF-7 cells after stable transfection with an HRG expression construct that led cells to acquire estrogen independence and metastasizing ability. A high level of HMG-I(Y) expression was detected in metastatic MDA-MB-231 cells, but the expression was virtually diminished, and the metastasizing ability was lost after cells were stably transfected with an antisense HRG cDNA construct. HMG-I(Y) was also decreased in MDA-MB-231 cells when treated with a chemical inhibitor for matrix metalloproteinase-9 that led to a reduction of invasive capability *in vitro*. The level of HMG-I(Y) expression, therefore, is dynamically regulated in human breast cancer cells in response to varying types of signaling that affect metastatic ability, including the HRG-*erbB* pathway and those from the extracellular matrix.

INTRODUCTION

Specialized genomic DNA sequences that have a high affinity to the isolated nuclear matrix *in vitro* have been designated as MARs/SARs⁴ (1-6). MARs were thought to tether chromatin onto the nuclear matrix, thus separating genomic DNA into topologically independent loop domains, which may be important for various functions including transcription and replication. For instance, MARs have been shown to be directly linked to biological activity such as cell type-specific transcription (7), demethylation (8, 9), and chromatin accessibility (9).

MARs typically contain a region with a specialized context that possesses an exceptionally high propensity to unwind by contiguous

base-unpairing when subjected to negative supercoiling (10, 11). Such a region is referred to as a BUR and typically contains clusters of ATC sequence stretches, a specific sequence context in which one strand exclusively consists of mixed A, T, and C nucleotides (ATC sequences; reviewed in Ref. 12). BURs, when they are in a double-stranded form, are specific targets of cell type-specific factors such as special AT-rich sequence-binding protein 1 (SATB1), predominantly expressed in thymocytes (13) and a B-cell factor, Bright (14). Recently, genomic DNA sequences that bind *in vivo* to SATB1 were isolated, and these sequences were shown to be localized at the bases of chromatin loops *in vivo* (15). BURs are also preferential targets of other proteins including nucleolin (16), mutant p53 (17), and p114, which is found in breast carcinoma cells (18). When a BUR is mutated to abrogate the unwinding capability, its affinity to SATB1 is abolished (13), and its affinity to the nuclear matrix is greatly reduced (11).

A group of proteins may exist that bind specifically to BURs in cancer cells and participate in gene regulation to trigger or maintain the malignant phenotype. Here we searched for a BUR-binding protein, the expression of which is well correlated with the aggressive phenotype of breast carcinoma cells that have the metastasizing ability. We detected two proteins that migrate as a doublet on SDS-PAGE at approximately *M*_r 20,000, the BUR-binding activity of which is dramatically elevated in highly metastatic breast carcinoma cell lines but is much less so in nonmetastatic breast carcinoma cell lines. The *M*_r 20,000 proteins were identified as HMG-I(Y), a member of the HMG nonhistone chromatin proteins. HMG-I and HMG-Y are splice variants of the same gene located on human chromosome 6p21 (19), and these proteins will be referred to collectively as HMG-I(Y). HMG-I(Y) has been demonstrated to strongly bind SARs/MARs (20). Here, we found that HMG-I(Y) exhibits a strict preference for double-stranded BUR sequences, the key structural element of MARs, over its mutated version that is still AT-rich but has lost the unwinding propensity.

In general, most normal differentiated mammalian cells and adult tissues express extremely low levels of HMG-I(Y) mRNAs and proteins (reviewed in Ref. 21). Overexpression of HMG-I(Y) was first shown in HeLa cells (22) and, since then, in various types of tumors in mice and rats (23-28). Interestingly, an antisense construct for HMG-I-C, another AT-hook containing HMG protein, has been shown to prevent neoplastic transformation of rat thyroid cells by retroviral oncogenes, and this led to the elimination of the endogenous level of HMG-I(Y) (29). Human thyroid neoplasias and thyroid carcinoma cell lines express HMG-I(Y) at a high level, whereas benign follicular adenomas, goiters, and normal thyroid cells do not (30). HMG-I(Y) expression has also been observed to increase in various types of human cancer, including prostate cancer (31, 32), colorectal carcinomas (33), uterine leiomyomata (34), and squamous intraepithelial and invasive lesions of the uterine cervix (35). Apparently, high HMG-I(Y) expression associates with the malignant phenotype of human cells and tissues.

Numerous studies have indicated that growth factors and their

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⁴ The abbreviations used are: MAR, matrix attachment region; SAR, scaffold attachment region; BUR, base-unpairing region; HMG, high-mobility group; HMG-I(Y), high-mobility group protein I(Y); EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin; ECM, extracellular matrix; MMP, matrix metalloproteinase; E₂, estradiol; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; RT, reverse transcription; ER, estrogen receptor; GST, glutathione-S-transferase.

receptors play an important role in cancer biology. The EGFR family is a group of transmembrane tyrosine kinases that is frequently overexpressed in a variety of carcinomas (36–39), and high EGFR levels are associated with poor clinical prognosis (reviewed in ref. 40). Holth *et al.* (41) have observed an EGF-induced overexpression of HMG-I(Y) in the highly metastatic Hs578T but not in the nonmetastatic MCF-7 cells. It suggested that HMG-I(Y) may have an important implication concerning the cellular mechanisms involved in the progression of mammary epithelial tumors. Besides EGFR, amplification and overexpression of one of the *erbB*-receptors, *erbB-2*, also correlates with a poor prognosis in some adenocarcinomas, most notably breast cancer (36, 42–48). The activator for *erbB-2*, HRG, originally called gp30 (49), has been suggested to play an important role in breast tumor development (50). HRG is a growth factor originally isolated from hormone-independent and invasive breast cancer cells (51, 52). MCF-7 cells can be converted to a more aggressive phenotype and rendered tumorigenic and metastatic *in vivo* merely by transfecting them with a HRG expression construct (53). On the other hand, transfecting highly metastatic MDA-MB-231 cells with an antisense-HRG construct led to an inhibition of tumorigenic phenotype.⁵ To further address the role of HMG-I(Y) in carcinogenesis and to examine the effect of HRG on HMG-I(Y) expression, we have undertaken studies in these two cell models of human breast cancer progression. In both cases, the higher level of HMG-I(Y) proteins was found to be strictly correlated with the high expression of HRG and the metastasizing capability of cells.

Proteases that degrade the ECM, including the serine proteases and MMPs gelatinase A (MMP-2) and B (MMP-9), have been implicated in various pathological states of growth, invasion, and metastasis (54, 55). Inhibition of MMP-9 expression using a ribozyme has been shown to block metastasis in the rat sarcoma model system (56). We report here that blocking MMP-9 activity in MDA-MB-231 cells with a MMP-9 chemical inhibitor resulted in a loss of invasive phenotype *in vitro* and a decrease of the HMG-I(Y) protein levels. Our observations suggest that the expression of HMG-I(Y) can be regulated dynamically in response to various types of signaling that affect metastatic ability, including HRG and ECM.

MATERIALS AND METHODS

Cell Lines and Cell Extract Preparation. Breast carcinoma cell lines MDA-MB-453, SK-BR-3, BT-474, MCF-7, Hs578T, BT-549, and MDA-MB-231, were obtained from American Type Culture Collection (Rockville, MD). These cell lines were maintained in improved MEM containing 10% fetal bovine serum (Life Technologies, Inc.). MCF-7/HRG cells were maintained as described previously (53). Improved MEM phenol red-free media and charcoal-stripped serum were used for experiments looking at the effect of 17 β -estradiol in MCF-7 cells. Cells were stimulated for 48 h with E₂ (10⁻⁹ M; Ref. 53), tamoxifen (Tam, 10⁻⁷ M; Ref. 53), or antiestrogen ICI 164,384 (ICI, 10⁻⁷ M; Ref. 57). MDA-MB-231/AS cell lines were maintained as MDA-MB-231 cells except for the addition of 200 μ g/ml G418 (Geneticin, Sigma) into the medium. The MMP-9 inhibitor (*N*-methyl-(3S)-S-[(2R)-2-hydroxyaminocarbonylmethyl-I-oxoundecyl]-hexahydropyridazine-3-carboxamide, a gift from Sankyo Pharmaceutical Company, Tokyo, Japan) was added to MDA-MB-231 cell cultures at the indicated concentrations (2.5, 5, and 10 μ M) for six days. *In situ* cell death detection kit (Boehringer Mannheim) was used for TUNEL assay. Cell extracts were prepared as described previously (16). Briefly, the cell pellet was resuspended in approximately 5 \times volume of Dignam's extraction buffer [0.42 M NaCl, 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each leupeptin and aprotinin]. Cells were lysed in a Dounce homogenizer (50 strokes with pestle A) and followed by a brief sonication. The

extract was centrifuged for 20 min in a TLA-100 rotor in a Beckman TL-100 ultracentrifuge at 100,000 rpm, and the supernatant was either assayed directly using Southwestern and Western analyses or used for affinity purification of MAR-binding proteins.

Boyden Chamber Assay. Methodology for the Boyden chamber assay has been described extensively in a previous publication (57). A chemoinvasion analysis was conducted with MDA-MB-231 cells in the Boyden chamber assay. Cells were plated at 10,000 cells/well (in quadruplicate) onto polycarbonate filters (12 μ m pore, PVP free, Nucleopore, Pleaston, CA) coated with matrigel (Collaborative Biochemical Products, Bedford, MA, 1:20 dilution of stock) for the chemoinvasion assays. MMP-9 inhibitor (0, 0.1, 0.5, 1, 5, 10, 15, and 20 μ M) was added to the upper chamber and lower chambers. NIH3T3 fibroblast conditioned media was used as a chemoattractant in the lower chambers and was prepared according to previously established protocols (57). Cells were incubated at 37°C, 5% CO₂/95% air for 6 h; filters were then fixed and stained with hematoxylin, and cells on the top surface were removed. Filters were mounted onto glass slides, and the number of cells that had migrated through the pores were assessed for each treatment group by image analysis systems or by microscopy.

DNA Affinity Purification of MAR-binding Proteins and Identification. The MAR-affinity column was prepared exactly as described previously (16). Briefly, the wild-type complementary oligonucleotides, 5'-TCTTAAATTCTAATATATTAGAAAttc-3' and 5'-TTCTAAATATATTAGAAATTAAGAGaa-3', were annealed, phosphorylated at 5' ends, and ligated to form concatemers.⁶ Routinely, 200 μ g of the double-stranded oligomers was coupled to 1 ml of cyanogen bromide-activated Sepharose 6 MB (Pharmacia). The oligonucleotide 5'-TCTTAAATTCTACTGCTTTAGAAAttc-3' and its complementary strand 5'-TTCT-AAAGCAGTAGAAATTAAGAGaa-3' were used for generating a mutated MAR DNA-affinity column. Cell extract was prepared from approximately 10⁹ cells, incubated with salmon sperm DNA competitors, centrifuged, and diluted with buffer Z [25 mM HEPES (pH 7.9), 1.25 mM MgCl₂, 1 mM DTT, 20% glycerol, and 0.1% (v/v) NP40] as described previously (18). Cell extract was readjusted to 0.1 M KCl in buffer Z, loaded on a mutated MAR DNA-affinity column, and washed with the same buffer. The bound proteins were eluted with buffer Z containing 0.2, 0.3, 0.4, 0.6, and 1.0 M KCl, and this MAR-binding protein was eluted at 0.3 M KCl. The eluted fraction was readjusted to 0.1 M KCl in buffer Z, loaded on a wild-type MAR DNA-affinity column, and washed with buffer Z containing 0.1 and 0.2 M KCl. The bound proteins were eluted at 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 M KCl, and this MAR-binding protein was eluted at 0.4–0.6 M KCl. The purified p20 was sent to the Protein Structure Laboratory at University of California at Davis for partial amino acid sequencing analysis.

RT-PCR and DNA Constructs. Total cellular RNA was extracted from MDA-MB-231 cells by a one-step acid guanidinium isothiocyanate-phenol procedure using Tri Reagent (Sigma), precipitated with ethanol, and quantitated by spectrophotometry. Five μ g total RNA was used for RT-PCR as instructed by the manufacturer (Life Technologies, Inc.). Two hundred units of Super Script II, Rnase H⁻ Reverse Transcriptase, was added in a typical 20- μ l reaction (50 ng specific primer, 5 μ g total RNA, 1 \times First Strand Buffer, 10 mM DTT, and 0.5 mM dNTP), and incubated 1 h at 42°C. The reaction was inactivated by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, RNase H was added and incubated at 37°C for 30 min. The purified cDNA was used as a template for amplification in PCR. The primers, including *Bam*HI linker for amplification of HMG-I(Y) cDNA, are 5'-gaaggatccATGAGTGAGT-CGAGCTCGAAGT-3' and 5'-caggatccAAGGAAGT-GCTCCTCCAGTGA-3'. PCR was then performed in a 50- μ l volume including 2.5 units of Taq polymerase, 10 \times buffer (Promega), 1.5 mM MgCl₂, 200 μ M dNTPs, and 0.2 μ M each primer under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s; followed by 5 min at 72°C. The 372-bp *Bam*HI fragment of HMG-I cDNA and the 339-bp *Bam*HI fragment of HMG-Y cDNA were cloned into the pGEX-4T-1 vector (Stratagene) to express GST-HMG-I(Y) proteins. GST-fusion proteins were synthesized in *Escherichia coli* and purified as described previously (58). Protein concentrations were determined by using a protein assay kit (Bio-Rad) and by comparing to BSA standards on SDS-PAGE gel stained with Coomassie Blue.

⁵ L. Shamon, C. K. Tang, M. Cardillo, E. A. Vladusic, and R. Luptu. Heregulin is a modulator of tumor growth, manuscript in preparation.

⁶ Lowercase type indicates single-strand overhangs.

Southwestern and Western Analyses. Southwestern and Western blots were done basically as described previously (16) with some modifications. For Southwestern analysis, protein samples were adjusted to 1× SDS-PAGE loading buffer and incubated at 37°C for 10 min before being applied to a 15% polyacrylamide minigel layered with 3.75% stacking gel. After electrophoresis, separated proteins were transferred electrophoretically at 45 V/cm at 0.75 mA for 1 h onto Immobilon P membrane (Millipore). The membrane was blocked with 5% BSA in the binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.25% BSA] at room temperature for 1–2 h. To 20 ml of binding solution, heat-denatured salmon sperm DNA to a final concentration of 50 µg/ml was added, and the membrane was preincubated at room temperature for 30 min. ³²P-labeled probe (approximately 10⁸ cpm/µg DNA) was added and further incubated for 40 min at room temperature. The membrane was washed four times for 10 min in binding buffer. It was then exposed to XAR film. For Western blot analysis, proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon P membranes (Millipore) in 20 mM sodium phosphate buffer (pH 6.8). Prestained broad range protein marker (Bio-Rad) was included for internal molecular mass standards. The filter was blocked in 5% BSA in TST [20 mM Tris-HCl (pH 7.6), 0.5 M NaCl, and 0.05% Tween 20], washed in TST buffer minus BSA, and incubated at room temperature for 1.5 h with either polyclonal antibody to HMG-I(Y) (from Santa Cruz Biochemicals or a generous gift obtained from Dr. Vincenzo Giancotti, Università degli Studi di Trieste, Trieste, Italy) at a 1:200 dilution, or monoclonal anti-β-actin antibody (Sigma). After being washed in TST, the filter was incubated with a 1:10,000 dilution of rabbit antigoat, goat antirabbit, or mouse IgG horseradish peroxidase conjugate. After extensive washing in TST, the blots were incubated with ECL (enhanced chemiluminescence) reagent solutions (PIERCE) and exposed to XAR film for visualization of protein bands. Quantitation of Southwestern and Western blots were done individually by PhosphorImager and densitometer (Storm 860, Molecular Dynamics).

Gel-Mobility Shift Assay. The experiment was performed basically as described previously (16). Binding reactions were done in 20 µl total volume containing 10 mM HEPES (pH 7.9), 1 mM DTT, 50 mM KCl, 2.5 mM MgCl₂, 10% glycerol, 1 µg of double-stranded poly (dI-dC), 10 µg of BSA, and 2.5–120 ng GST-HMG-I or Y protein. Samples were preincubated at room temperature for 5 min before radiolabeled DNA probe was added. The *Bam*HI-*Hind*III fragments of wild-type (25)₇ and mutated (24)₈ (13), or the *Eco*RI fragments of wild-type and mutated IgH 3'MAR (5), were end-labeled by the klenow fragment of DNA polymerase and used as DNA probes in the present study.

RESULTS

Increased MAR-binding Activity of Small Proteins (M_r 20,000) in Metastatic Breast Cancer Cells. To investigate BUR-binding proteins that might be correlated with the aggressive phenotype of breast carcinomas, we chose to examine seven human breast carcinoma cell lines (listed in Table 1). High levels of HRG expression have been observed in aggressive breast cancer cells (Hs578T, BT-549, and MDA-MB-231) that are vimentin-positive/ER-negative, invasive on matrigel outgrowth assay, and metastatic *in vivo* in nude mice (50, 59, 60). In contrast, MDA-MB-453, SK-BR-3, BT-474, and MCF-7 are representatives of nonmetastatic breast cancer cell lines with no detectable level of HRG (50, 59, 60) or vimentin proteins (57). Using whole cell extracts from these cells, we performed South-

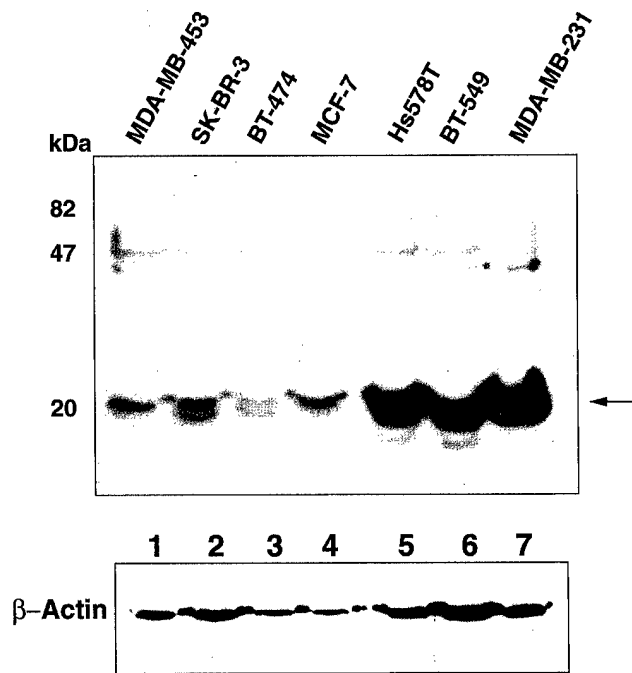


Fig. 1. The high level of MAR-binding activity due to small molecular-weight proteins (around M_r 20,000) occurs in human metastatic breast carcinoma cells (Lanes 5–7) but not in nonmetastatic cells (Lanes 1–4). Southwestern blot analysis was performed to investigate MAR-binding activity. Twenty-five µg of proteins in cell extracts were loaded on a 15% SDS-polyacrylamide gel. After electrophoresis, the proteins were renatured; then the gel was blotted and hybridized with the radiolabeled wild-type (25)₇ probe. The same amount of proteins used in the Southwestern blot was subjected to Western blot analysis using anti-β-actin antibody.

western analysis using the synthetic MAR probe, wild-type (25)₇. This multimer contains the 25-bp sequence that was derived from a MAR 3' of the IgH enhancer and contains the core unwinding element (10). Wild-type (25)₇ has a base-unpairing property (thus, it is a BUR) and binds to the nuclear matrix with high affinity (11). Interestingly, the BUR-binding activity of small proteins (Fig. 1; M_r 20,000, 20 kDa, arrow) is remarkably higher in aggressive metastatic cells (Fig. 1, Lanes 5–7) as compared with nonmetastatic cells (Fig. 1, Lanes 1–4). Three bands were seen at small protein range, a doublet p20(s) and a very faint band around (M_r 15,000, 15 kDa). Larger proteins on the same Southwestern blot exhibited an overall similar and relatively weaker BUR-binding activity (Fig. 1). This result suggests a possible correlation between increased BUR-binding activity of small proteins and the progression of breast cancer cells to a metastatic phenotype. The same Southwestern blot was directly subjected to Western blot analysis using anti-β-actin antibody (Fig. 1, bottom). Each sample was precisely quantitated for its protein amount before loading. There was some variation in the β-actin expression depending on different cell lines. However, the strong MAR-binding activity detected in metastatic cells, especially MDA-MB-231, is apparently not due to an excess protein loading (compare Fig. 1, Lanes 1 and 2 to Lanes 5 and 7, for example).

Southwestern blot analysis using a mutated (24)₈ probe was performed for MDA-MB-231 cells. This mutated DNA was derived from the original 25-bp sequence by specifically mutating the core unwinding element to abrogate the unpairing propensity (10). The M_r 20,000 proteins bound poorly to the mutated (24)₈ on the Southwestern blot (data not shown), which suggested that these proteins are BUR-binding proteins similar to SATB1 (13) and nucleolin (16).

Purification and Identification of a M_r 20,000 MAR-binding Protein from MDA-MB-231 Cells. To purify 20 kDa MAR-binding proteins, wild-type and mutated MAR DNA affinity columns were

Table 1 Nonmetastatic versus metastatic human breast cancer cell lines

Cell line	HRG	ER	Vimentin	Invasive <i>in vitro</i>	Metastatic <i>in vivo</i>
1 MDA-MB-453					NT ^a
2 SK-BR-3					NT
3 BT-474		+			
4 MCF-7		++			
5 Hs578T	++		++	++	++
6 BT-549	+		++	+	++
7 MDA-MB-231	++		++	++	++

^a NT, nontumorigenic; blank, no expression activity detected; +, medium expression activity; ++, strong expression activity.

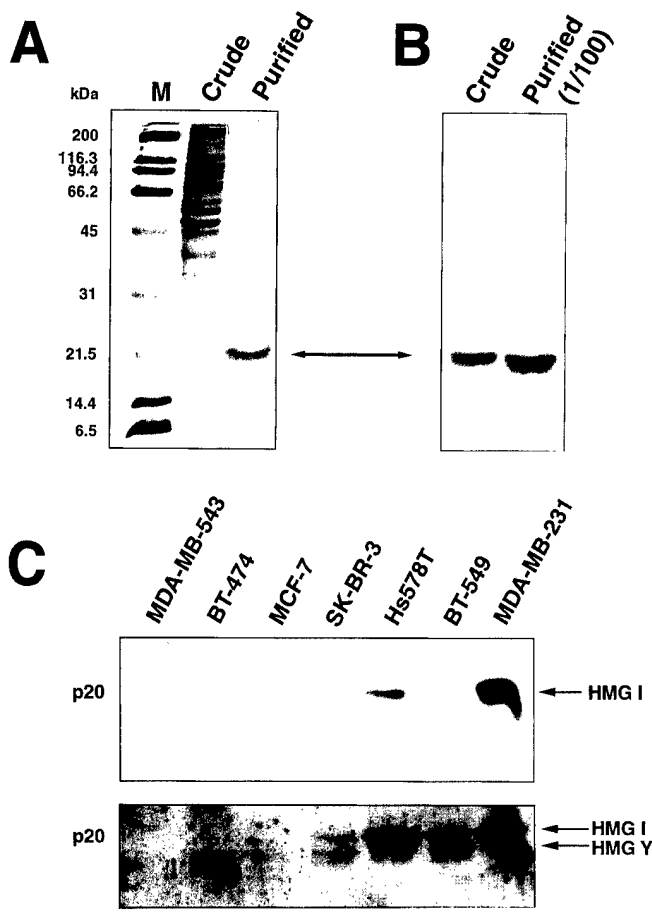


Fig. 2. The M_r 20,000 protein was identified as HMG-I(Y). A, affinity purification of a MAR-binding protein from a breast carcinoma cell line, MDA-MB-231. Coomassie Blue staining analysis was performed for 40 μ g of protein from a crude cell extract prepared from the MDA-MB-231 cells (*Crude*) and column-purified p20 from the MDA-MB-231 cell extract (*Purified*). M, molecular size marker. Arrow, p20. B, Southwestern blot analysis using radiolabeled wild-type (25)₇ as the probe was performed with the same crude extract and purified fraction (1/100) from A. C, Western blot analysis using anti-HMG-I(Y) antibody was performed for 25 μ g of protein from cell extracts prepared from human breast carcinoma cell lines. M, molecular size marker; top panel, shorter exposure; bottom panel, longer exposure.

prepared by coupling multimers of either the wild-type 25 mer oligonucleotide or the mutated 24 mer oligonucleotide to activated Sepharose as described in "Materials and Methods." The strategy for purifying this p20(s) from MDA-MB-231 cell extracts is first to allow p20(s) to bind the multimerized mutated (24)_n DNA affinity column by passing through the column three times under low salt conditions. Then, the eluted fractions were applied onto the multimerized wild-type (25)_n DNA affinity column. Following this protocol, a major band of 20 kDa was purified by BUR-affinity chromatography and stained with Coomassie Blue (Fig. 2A). The purified protein and proteins in the crude extract were analyzed side-by-side by Southwestern blot analysis, confirming that the 20 kDa BUR-binding protein was purified (Fig. 2B). Previously identified p114 (18) were purified from the same column at higher salt concentrations beyond 0.6 M. Sequencing of two peptide fragments obtained by tryptic digestion of the gel-purified 20 kDa protein, KQPPVSPGTALVGSOK and EPSEVPTPK, revealed their identity with the published sequence of human HMG-I (61). HMG-I (11 kDa) migrates as a 20 kDa protein in a 15% SDS-PAGE gel. Western blot analysis using anti-HMG-I(Y) antibody indicated that increased MAR-binding activity detected in human metastatic breast cancer cells (Fig. 1, Lane 5–Lane 7) was due to the elevated HMG-I (see Fig. 2C). An additional

protein that migrates slightly faster than HMG-I is also recognized by anti-HMG-I(Y) antibody after longer exposure (see Fig. 2C, bottom panel). This is most likely an isoform of HMG-I, the HMG-Y, which is produced by alternative splicing of mRNA transcripts from a single gene. This may explain the appearance of a doublet on the Southwestern blot around M_r 20,000 (Fig. 1). We therefore conclude that the two MAR-binding proteins that migrate close to 20 kDa from MDA-MB-231 cells are HMG-I and HMG-Y.

GST-HMG-I(Y) Fusion Proteins Selectively Bind BURs. To prepare GST-fused HMG-I and HMG-Y proteins, HMG-I(Y) cDNAs were cloned by RT-PCR strategy using RNA from MDA-MB-231 cells. The 372-bp fragment of HMG-I cDNA and the 339-bp fragment of HMG-Y cDNA were separately cloned into the pGEX-4T-1 vector (Stratagene). GST-HMG-I and GST-HMG-Y fusion proteins, purified from bacteria, were examined by gel mobility shift assay using wild-type (25)₇ and mutated (24)₈ (Fig. 3A). Both GST-HMG-I and GST-HMG-Y bound with strong affinity to wild-type (25)₇, with an estimated dissociation constant (kDa) in the range of 4×10^{-9} M (Fig. 3A, upper panel) under conditions of protein excess (16). In contrast, these proteins exhibited dramatically reduced binding affinity, by at least two orders of magnitude, to the mutated (24)₈ (Fig. 3A, bottom

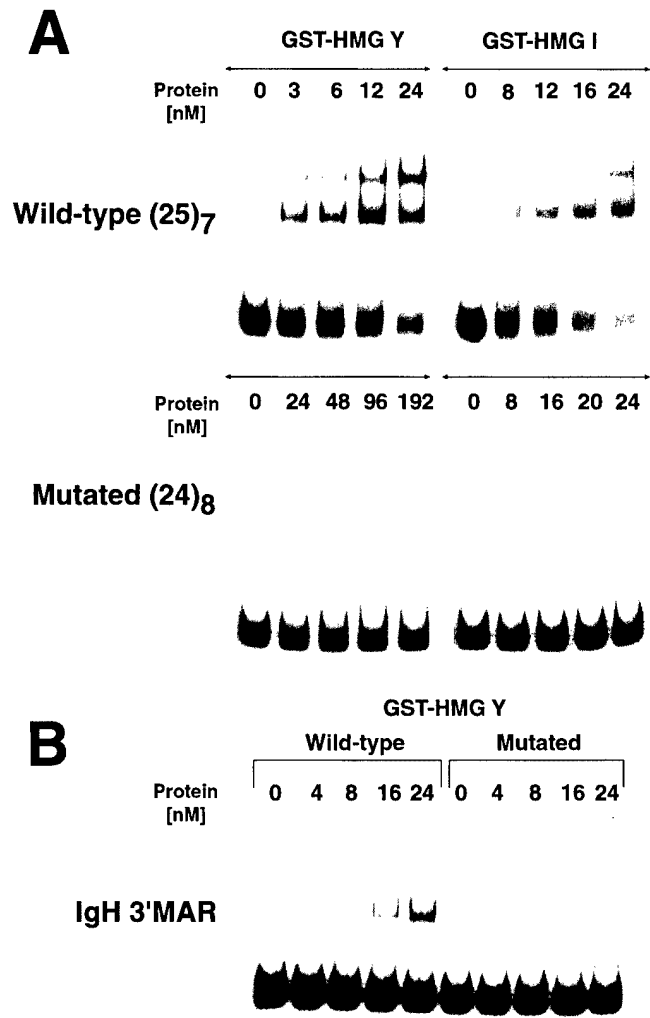
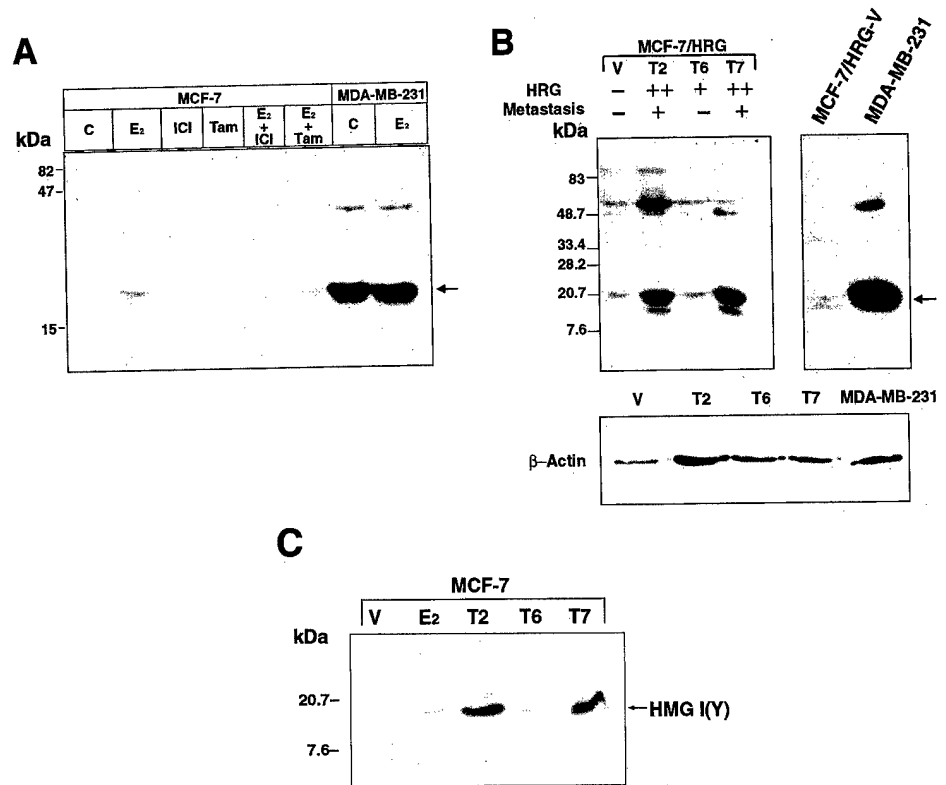


Fig. 3. Gel-mobility shift assay was performed for GST-HMG-I and GST-HMG-Y with a radiolabeled wild-type (25)₇ probe (A, top), a mutated (24)₈ probe (A, bottom), a wild-type or a mutated IgH 3'MAR probe (B, left and right). The DNA probes were incubated with varying amounts of protein in 20 μ l of binding reaction mixture and electrophoresed on a 6% native polyacrylamide gel. [nM], units of protein concentrations.

Fig. 4. HMG-I(Y) expression is increased in MCF-7 cells on estrogen treatment or HRG cDNA transfection. Southwestern blot analysis using radiolabeled wild-type (25)₇ as the probe was performed with A and B. Western blot analysis of HMG-I(Y) was performed with C. A, 25 μ g of proteins extracted from MCF-7 and MDA-MB 231 cells with various treatments for 48 h were used for Southwestern analysis; C, control; E₂, estradiol (10⁻⁹ M); ICI, antiestrogen ICI 164,384 (10⁻⁷ M); Tam, tamoxifen (10⁻⁷ M). B, 25 μ g of proteins extracted from MCF-7 and MDA-MB-231 cells were used for Southwestern analysis; V in left upper panel, MCF-7 cells transfected with the pRC/CMV vector only; T2, T6 and T7, three MCF-7 clones transfected with HRG- β 2 cDNA. The same amount of protein used in the Southwestern blot was subjected to Western blot analysis using anti- β -actin antibody. C, 50 μ g of proteins were extracted from MCF-7 cells, MCF-7 cells were treated with E₂, and MCF-7/T cells were used in the Western blot analysis using anti-HMG-I(Y) antibody.



panel). This indicates that HMG-I(Y) has a remarkable specificity toward the wild-type (25)₇ probe as opposed to the mutated (24)₈ probe, although these probes are both AT-rich (see "Material and Methods").

To further examine whether it is a general phenomenon that HMG-I(Y) selectively binds BURs, a gel-mobility shift assay was performed using the native MAR of 300 bp located 3' of the enhancer and the mutated version in which the core unwinding element, ATATAT, is specifically mutated to CTGTCT (Fig. 3B). The 300-bp MAR 3' of the IgH enhancer is 70% AT-rich and contains a BUR; and the three nucleotide mutations within the BUR do not significantly alter the overall AT content; yet the mutation completely abrogates the unwinding property of this MAR (10). As shown in Fig. 3B, GST-HMG-Y specifically bound wild-type IgH 3' MAR but not the mutated IgH 3' MAR. Similar results were also found for GST-HMG-I (data not shown). Russnak *et al.* (62) has demonstrated that, under the appropriate conditions, HMG-I exhibits different affinities to some AT-rich sequences. Here, our data showed that HMG-I(Y) has a high affinity to BURs and specifically recognizes the core unwinding element within these regions.

HMG-I(Y) Expression Is Increased in MCF-7 Cells on Estrogen Treatment or HRG cDNA Transfection. Estrogen is known to increase cellular proliferation and activation of estrogen responsive genes in MCF-7 cells, an ER-positive cell line (63). Because HMG-I(Y) is expressed at elevated levels in proliferating and undifferentiated cells (61), it was of interest to determine whether estrogen had any effect on HMG-I(Y) expression. Holth *et al.* (41) have shown that estrogen has no significant effect on up-regulating HMG-I(Y) expression determined by Northern blot analysis. However, by Southwestern and Western analyses, we observed reproducibly a small induction of MAR-binding activity of HMG-I(Y) in MCF-7 cells when they were exposed to E₂ (Fig. 4, A and C). Two antiestrogen drugs, tamoxifen (Tam) and antiestrogen ICI 164,384 (ICI), completely abolished this

induction, indicating that estrogen stimulation of MAR-binding activity was mediated through the ER pathway (Fig. 4A).

HRG is a growth factor expressed in about 30% of invasive breast cancers, and it activates the *erbB* receptor pathway (reviewed in Ref. 60). As shown in Table 1, HRG expression correlates with the invasive and metastatic phenotypes of breast cancer cells *in vitro* and *in vivo* (50). HRG is inversely correlated with ER expression. To study the role of HRG in breast cancer tumor progression, Tang *et al.* (53) developed a breast cancer progression model by transfecting an HRG expression construct to an ER-positive cell line, MCF-7. MCF-7/HRG-transfected cell clones, which expressed a relatively high level of HRG, developed estrogen independence and resistance to antiestrogen drugs *in vitro* and *in vivo* (53, 64). This is consistent with a more aggressive hormone-independent phenotype. Wild-type MCF-7 cells are known to be nontumorigenic in the absence of estrogen, and these cells never metastasize in nude mice, even in the presence of estrogen. However, all MCF-7/HRG clones having similar proliferation rates; for example, T2, T6, and T7, can form large tumors in nude mice in the absence of an estrogen supplement (64). Tumors generated from T2 and T7 cells (but not T6) became metastatic and developed lymphatic invasion (64). The MCF-7/HRG cell series, therefore, provides a system to study the aggressive tumor progression in breast cancer. Southwestern and Western analyses of HMG-I(Y) were performed for T2, T6, T7, and control MCF-7 cells transfected with vector only. As expected, two metastatic clones, T2 and T7, had significantly increased BUR-binding activity (Fig. 4B) attributed to the elevated level of HMG-I(Y) protein (Fig. 4C). In contrast, MCF-7 cells treated with estrogen and T6 cells expressed the same low level of HMG-I(Y) as the control MCF-7 cells (Fig. 4C).

Previously, HMG-I(Y) expression was shown to be induced by EGF in metastatic Hs578T cells but not in nonmetastatic MCF-7 cells (41). Without induction, no difference of HMG-I(Y) mRNA was detected between Hs578T and MCF-7 cells by primer extension and

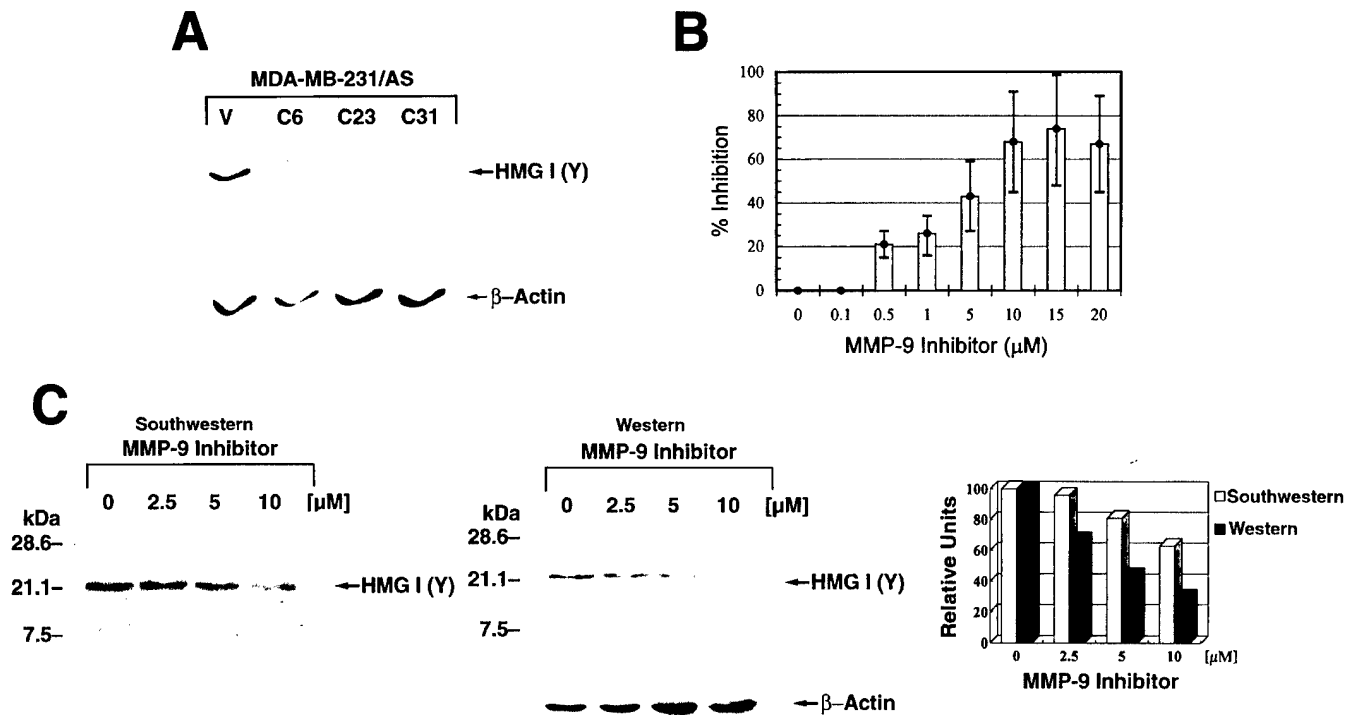


Fig. 5. HMG-I(Y) expression is reduced in MDA-MB-231 cells on antisense-HRG transfection and MMP-9 inhibitor treatment. **A**, 20 μg of proteins, extracted from a series of MDA-MB-231/AS cells, were used for Western blot analysis of HMG-I(Y) and β -actin. **V**, MDA-MB-231 cells transfected with the pRC/CMV vector only; **C6**, **C23**, and **C31**, three MDA-MB-231/AS clones transfected with antisense-HRG. **B**, Boyden chamber assay. MDA-MB-231 cells were tested in the Boyden chamber assay in the presence or absence of increasing concentrations (0–20 μM) of the MMP-9 inhibitor for 6 h. Chemoinvasion was measured according to the number of cells transverting matrigel-coated filters. *Data points*, the average of four independent assays from a representative experiment. SD was calculated for each data point and indicated by a *bar*. **C**, 20 μg of proteins extracted from MDA-MB-231 cells with MMP-9 inhibitor treatments for 6 days at indicated concentration (0, 2.5, 5, and 10 μM) were used for Southwestern analysis (*left*), using a radiolabeled wild-type (25)₇ probe, and for Western blot analysis (*center*) of HMG-I(Y) and β -actin. Quantitation of Southwestern blot and Western blot were done individually by PhosphorImager and densitometer. *Right panel*, summary of their relative units.

Northern blot analysis (41). However, our Southwestern (Fig. 1) and Western analyses (Fig. 2C) clearly showed that the protein level and BUR-binding activity of HMG-I(Y) are both remarkably higher in metastatic cell lines (including Hs578T) than in nonmetastatic cell lines (including MCF-7; see Fig. 2C). On the basis of our data, there is an association between HMG-I(Y) protein levels, expression of HRG, and malignant phenotype of human breast cancer cells.

It is noteworthy that the protein level and BUR-binding activity of HMG-I(Y) in T2 or T7 cells that can metastasize are still much lower than the activity in MDA-MB-231 cells (Fig. 4B). Levels of HMG-I(Y) vary dramatically between cell types; for instance, three metastatic cell lines—Hs578T, BT-549, and MDA-MB-231—express different levels of HMG-I(Y) even under similar culture conditions (Fig. 1 and 2C). Therefore, an absolute amount of HMG-I(Y) or its DNA-binding activity, per se, is apparently not the determinant factor for metastasis. However, in a given cell line, the level of HMG-I(Y) consistently increases as it becomes more aggressive (due to the overexpression of HRG, for example) and decreases as it becomes less aggressive (reduction of HRG expression, see below).

HMG-I(Y) Expression Is Reduced in MDA-MB-231 Cells Transfected with a HRG Antisense Construct. Because MDA-MB-231 cells are highly metastatic and express high levels of HRG (50, 53), we studied the effect of reducing the HRG levels on HMG-I(Y) expression by stably transfecting these cells with antisense HRG construct. A series of stably transfected MDA-MB-231 cell clones were prepared. Cells harboring pRV/CMV vector only (a control called AS-V) and three other cell clones (AS-C6, AS-C23, and AS-C31) transfected with an antisense HRG construct showing much reduced levels of HRG expression, were isolated for further analysis. These MDA-MB-231 cells, transfected with HRG antisense HRG-

construct, have lost metastasizing ability in nude mice.⁴ Western blot analysis using anti-HMG-I(Y) antibody has shown that HMG-I(Y) protein levels in all three of the antisense-HRG cells, AS-C6, AS-C23, and AS-C31, were either dramatically decreased or undetectable (Fig. 5A). This result suggests that the HMG-I(Y) level decreased concomitantly with the loss of metastatic phenotype because of the disruption of HRG expression. This result is consistent with our previous results obtained from the studies with MCF-7/HRG cells (Fig. 4C) that the metastatic potential of breast cancer cells is associated with elevated HRG and HMG-I(Y) levels.

Treatment of MDA-MB-231 Cells with a Chemical Inhibitor for MMP-9 Reduced the Levels of HMG-I(Y). It is generally believed that one key element of the metastatic process is the enhanced proteolysis of both basement membrane and stromal ECM. Among the proteinase capable of degrading these barriers are the MMPs. The gelatinase A (MMP-2) and B (MMP-9) are two members of the MMP family that have been implicated in breast cancer tumor progression (54, 55). MDA-MB-231 cells seem to secrete a significantly high level of MMP-9 activity as compared with MCF-7 cells.⁷ The inhibition of MMP-9 expression using a ribozyme has been shown to block metastasis in the rat sarcoma model system (56). Consistently, confluent MDA-MB-231 cells, treated with a chemical inhibitor for MMP-9—*N*-methyl-(3S)-2-[(2R)-2-hydroxyaminocarbonylmethyl-1-oxoundecyl]hexahydropyridazine-3-carboxamide—in the range of 0.1–20 μM concentration, resulted in a significant reduction in the invasive phenotype *in vitro* determined by the Boyden chamber assay (Fig. 5B). At a 10- μM concentration of the inhibitor,

⁷ R. Lupu, unpublished results.

the effect became saturated and exhibited approximately 70% inhibition of the invasive activity. The morphology of MDA-MB-231 cells changed from stellar-like shape to steroid-like shape after treatment with the MMP-9 inhibitor as revealed by the matrigel outgrowth assay.⁶ The chemical inhibitor used in this study has an IC_{50} value of 38 nM for gelatinase A and an IC_{50} value of 1.2 nM for gelatinase B and, therefore, has a high specificity for the latter (65). To examine the effect of the MMP-9 inhibitor on HMG-I(Y) expression in MDA-MB-231 cells, these cells in culture at different densities were treated with this MMP-9 specific inhibitor (see "Materials and Methods"), and their HMG-I(Y) expression was measured by Southwestern and Western blot analyses. When subconfluent cells were treated with the MMP-9 inhibitor for 6 days at varying concentrations (1, 2.5, 5, and 10 μ M), no significant changes in the level of HMG-I(Y) were detected (data not shown). However, when confluent cells were treated with the MMP-9 inhibitor, we observed the gradual decay of HMG-I(Y) with the increasing concentration of the MMP-9 inhibitor (Fig. 5C). The MMP-9 inhibitor treatment (10 μ M for six days) reduced HMG-I(Y) protein levels down to 30% of the original level based on Western analysis (Fig. 5C). Although Southwestern analysis is not as quantitative as Western blot analysis, both assays independently showed an inverse correlation between the concentration of the MMP-9 inhibitor and expression of HMG-I(Y). In parallel, the apoptotic cell death were quantitated by TUNEL assay because some of the effect could be due to induction of apoptosis in cells treated with the MMP-9 inhibitor. However, there is no difference on cell viability observed under the conditions used (data not shown). These data suggest that an alternation in the ECM by inhibiting MMP-9 resulted in the down-regulation of HMG-I(Y) expression. Our results show that, similar to the effects of growth factor such as HRG, HMG-I(Y) expression is sensitive to signaling from ECM.

DISCUSSION

Identification of BUR-binding and Metastasis-associated Proteins as HMG-I(Y). We detected proteins of $M_r \sim 20,000$ in aggressive human breast carcinoma cells that bind strongly to BURs with a specialized ATC sequence context with high base-unpairing propensity. Using DNA affinity chromatography, a M_r 20,000 protein was purified and identified as HMG-I. Another protein with a slightly smaller mass that also confers similar binding specificity was found to be the splicing variant, HMG-Y. We demonstrate that HMG-I(Y) specifically recognizes BURs; when mutated at a core unwinding element sites within BURs to abrogate this propensity, HMG-I(Y)—binding to BURs—was either abolished or greatly reduced. This binding specificity of HMG-I(Y) for double-stranded BURs is remarkably similar to that of the previously cloned MAR-binding protein SATB1, which binds along the minor groove with very little contact with the bases (13).

There are several reports describing the binding specificity of HMG-I(Y) (62, 66–68). These studies have shown that HMG-I(Y) preferentially binds to the minor groove of AT-rich B-form DNA (66, 67), and yet it does not bind to all of the stretches of A+T-rich DNA with equal affinity (62). One study suggests that structure of DNA, rather than the primary sequence per se, is a dominant factor that controls HMG-I(Y)-binding specificity (68). The present study, which has demonstrated that HMG-I(Y) specifically binds to BURs, the key structural elements of MARs, may have an important implication in cancer. BURs are the *in vivo* targets of a cell type-specific MAR-binding protein (15), and ablation of the MAR-binding protein caused

major dysregulation of multiple genes.⁸ Furthermore, our most recent study has shown that at least one such BUR is an origin of replication in the mouse tissue culture system.⁹ In addition to the case for HMG-I(Y), there is another BUR-binding protein, p114, that increases as breast cancer becomes more aggressive (18). These results suggest that BURs are important genomic loci at which many regulatory events may take place. Any alternation at these loci, including the level of binding proteins, could lead to malignancy or is necessary to maintain the malignant phenotype.

Association between HMG-I(Y) Expression and Malignant Phenotype in Human Breast Tumor Cells. We examined the changes in HMG-I(Y) levels using directly comparable systems using the same cell lines that either express or do not express HRG. Specifically, we compared: (a) nonmetastatic MCF-7 cells with MCF-7 cells containing an HRG cDNA construct that became metastatic; and (b) metastatic MDA-MB-231 cells with MDA-MB-231 cells containing an antisense HRG construct that became nonmetastatic. This series of experiments allowed us to systematically examine how HMG-I(Y) expression varies with malignant progression in human breast cancer cells. Our results were consistent: HRG overexpression that promoted metastasizing ability resulted in an increase of HMG-I(Y) expression, and, conversely, the reduction of HRG that blocked metastasis led to a reduction in HMG-I(Y) expression. MDA-MB-231/antisense HRG cells have a similar growth rate on plastic culture as compared with vector control cells,⁴ which suggests that the net expression of HMG-I(Y) is not merely a factor of proliferating activity of cells. This demonstrates that there is a direct correlation between an increased HMG-I(Y) protein level and high HRG expression that is linked to metastatic potential in human breast cancer cells, independent of their relative rates of cell proliferation, at least under the experimental condition used.

We also examined the effect of a MMP-9 chemical inhibitor on the expression of HMG-I(Y) in MDA-MB-231 cells. The gelatinase B (MMP-9) is a member of the MMPs, which are zinc-dependent endopeptidases implicated in cancer invasion and metastasis (55). MMP-9 is known to cleave native collagens of type IV, V, and XI, and elastin (reviewed in Ref.69). Blocking MMP-9 activity in MDA-MB-231 cells with the MMP-9 chemical inhibitor resulted in a loss of invasive phenotype *in vitro* and a decrease in the HMG-I(Y) protein levels (Fig. 5, B and C). It is known that the blocking function of ECM proteinases can decrease tumor growth (70, 71). HMG-I(Y) could be down-regulated as a result of reduced aggressiveness of cancer cells by this treatment. Any alternations in ECM such as blocking the function of metalloproteinases may affect signaling pathways of cells that ultimately regulate nuclear events such as the HMG-I(Y) expression.

On the basis of our results, it seems that the absolute amount of HMG-I(Y) is not a determining factor for the metastatic phenotype. For example, MMP-9 inhibitor-treated cells still retained a high level of HMG-I(Y), although it was reduced in comparison with the original levels. Therefore, changes in the level of HMG-I(Y) expression seem to correlate with, or reflect the changes in, the status of malignancy of given breast carcinoma cells.

Potential Role of HMG-I(Y) in Breast Cancer. Transcriptional control has become a major focus in current cancer research. Transcription of the *HMG-I(Y)* gene is predicted to be tightly regulated, and has been shown specifically to be induced in human cells by phorbol esters (72), calcium ionophores, (73) and EGF (41), and to be

⁸ J. D. Alvarez, D. H. Yasui, H. Niida, T. Joh, D. Y. Loh, and T. Kohwi-Shigematsu. The MAR-binding protein, SATB1, orchestrates temporal and spatial expression of multiple genes during T-cell development, submitted for publication.

⁹ W.-M. Liu and T. Kohwi-Shigematsu, unpublished results.

up-regulated by human papillomavirus type 16 E6 protein (74) and tumor promoter (28) in mouse cells. The transcriptional regulation and functional role of *HMG-I(Y)* in cancer development are, however, essentially unknown.

Several functions for HMG-I(Y) have been proposed, including nucleosome phasing (75), involvement in the 3' end processing of mRNA transcripts (62, 76), and the amplification of autonomously replicating sequences (77). HMG-I(Y) has been demonstrated to be involved in both positive and negative regulation of genes containing AT-rich regions of DNA, possibly by functioning as accessory "architectural transcription factors" (78–84). HMG-I(Y) also seems to bind specifically to regions of putative mammalian cell origins of replication (85) and to G/Q and C-bands of metaphase chromosomes (86). More recently, three distinct subnuclear populations of HMG-I(Y) have been demonstrated (87), suggesting they may be required for chromosome structural change during cell cycle. An increase in *HMG-I(Y)* gene expression associated with progression in the malignant phenotype may have effects on any combination of these molecular interactions.

The nuclear matrix is believed to play critical roles in regulating many key biological reactions in the nucleus such as gene transcription, DNA replication, DNA organization, and RNA splicing and processing (reviewed in Ref. 88). Our data suggest that HMG-I(Y) interacts with BURs, the key structural element of MARs. HMG-I(Y) may assemble specific three-dimensional transcription and/or replication complexes at BUR sites. If so, this may cause detachment of the sites from the nuclear matrix by successfully competing BURs sites from other MAR-binding proteins and, subsequently, alter the loop domain structure of chromatin. It would be interesting to explore this possibility in the future.

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REFERENCES

- Mirkovitch, J., Mirault, M.-E., and Laemmli, U. K. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell*, **9**: 223–232, 1984.
- Cockerill, P. N., and Garrard, W. T. Chromosomal loop anchorage of the κ immunoglobulin gene occurs next to the enhancer in a region containing topoisomerases II sites. *Cell*, **44**: 273–282, 1986.
- Gasser, S. M., and Laemmli, U. K. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. *Cell*, **46**: 521–530, 1986.
- Gasser, S. M., and Laemmli, U. K. The organization of chromatin loops: characterization of a scaffold. *EMBO J.*, **5**: 511–518, 1986.
- Cockerill, P. N., Yuen, M.-H., and Garrard, W. T. The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. *J. Biol. Chem.*, **262**: 5394–5397, 1987.
- Gasser, S. M., and Laemmli, U. K. A glimpse at chromosomal order. *Trends Genet.*, **3**: 16–22, 1987.
- Forrester, W. C., van Genderen, C., Jenuwein, T., and Grosschedl, R. Dependence of enhancer-mediated transcription of the immunoglobulin μ gene on nuclear matrix attachment regions. *Science (Washington DC)*, **265**: 1221–1225, 1994.
- Kirilov, A., Kistler, B., Mostoslavsky, R., Cedar, H., Wirth, T., and Bergman, Y. A role for nuclear NF- κ B-cell-specific demethylation of the *Igk* locus. *Nat. Genet.*, **13**: 435–441, 1996.
- Jenuwein, T., Foresster, W. C., Fernandez-Herrero, L. A., Laible, G., Dull, M., and Grosschedl, R. Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature (Lond.)*, **385**: 269–272, 1997.
- Kohwi-Shigematsu, T., and Kohwi, Y. Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. *Biochemistry*, **29**: 9551–9560, 1990.
- Bode, J., Kohwi, Y., Dickinson, L. A., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T. Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science (Washington DC)*, **255**: 195–197, 1992.
- Kohwi-Shigematsu, T., and Kohwi, Y. High unwinding capability of matrix attachment regions and ATC-sequence context-specific MAR-binding proteins. In: R. C. Bird (ed.), *Nuclear Structure and Gene Expression*, pp. 111–144. San Diego, CA: Academic Press, 1997.
- Dickinson, L. A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T. A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell*, **70**: 631–645, 1992.
- Herrscher, R. F., Kaplan, M. H., Lelsz, D. L., Das, C., Scheuermann, R., and Tucker, P. W. The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific *trans*-activator that describes a new DNA-binding protein family. *Genes Dev.*, **9**: 3067–3082, 1995.
- de Belle, I., Cai, S., and Kohwi-Shigematsu, T. The genomic sequences bound to special AT-rich sequence-binding protein 1 (SATB1) *in vivo* in Jurkat T cells are tightly associated with the nuclear matrix at the bases of the chromatin loops. *J. Cell Biol.*, **141**: 335–348, 1998.
- Dickinson, L. A., and Kohwi-Shigematsu, T. Nuclolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. *Mol. Cell Biol.*, **15**: 456–465, 1995.
- Will, K., Warnecke, G., Wiesmuller, L., and Deppert, W. Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing. *Proc. Natl. Acad. Sci. USA*, **95**: 13681–13686, 1998.
- Yanagisawa, J., Ando, J., Nakayama, J., Kohwi, Y., and Kohwi-Shigematsu, T. A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast carcinomas and not in normal and benign breast disease tissues. *Cancer Res.*, **56**: 457–462, 1996.
- Johnson, K. R., Lehn, D. A., and Reeves, R. Alternative processing of mRNA encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. *Mol. Cell Biol.*, **9**: 2114–2123, 1989.
- Zhao, K., Kas, E., Gonzalez, E., and Laemmli, U. K. SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*: HMG-I/Y is enriched in H1-depleted chromatin. *EMBO J.*, **12**: 3237–3247, 1993.
- Wunderlich, V., and Bottger, M. High-mobility-group proteins and cancer—an emerging link. *J. Cancer Res. Clin. Oncol.*, **123**: 133–140, 1997.
- Lund, T., Holtlund, J., Fredriksen, M., and Laland, S. G. On the presence of two new high mobility group-like proteins in HeLa S3 cells. *FEBS Lett.*, **152**: 163–167, 1983.
- Giancotti, V., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., and Crane-Robinson, C. Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sarcoma retrovirus. *Cancer Res.*, **45**: 6051–6057, 1985.
- Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R. H., Wright, C. A., and Goodwin, G. H. Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with *v-ras* and *v-mos* oncogenes and by co-transfection with *c-myc* and polyoma middle T genes. *EMBO J.*, **6**: 1981–1987, 1987.
- Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco, A., and Goodwin, G. H. Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. *Exp. Cell Res.*, **184**: 538–545, 1989.
- Bussemakers, M. J. G., van de Ven, W. J. M., Debruyne, F. M. J., and Schalken, J. A. Identification of high mobility group protein I(Y) as potential progression marker for prostate cancer by differential hybridization analysis. *Cancer Res.*, **51**: 606–611, 1991.
- Ram T. G., Reeves, R., and Hosick, H. L. Elevated high mobility group-I(Y) gene expression is associated with progressive transformation of mouse mammary epithelial cells. *Cancer Res.*, **53**: 2655–2660, 1993.
- Cmarik, J. L., Li, Y., Ogram, S. A., Min, H., Reeves, R., and Colburn, N. H. Tumor promoter induces high mobility group HMG-Y protein expression in transformation-sensitive but not -resistant cells. *Oncogene*, **16**: 3387–3396, 1998.
- Berlingieri, M. T., Manfioletti, G., Santoro, M., Bandiera, A., Viscconti, R., Giancotti, V., and Fusco, A. Identification of HMG-I-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. *Mol. Cell Biol.*, **15**: 1545–1553, 1995.
- Chiappetta, G., Tallini, G., De Biasio, M. C., Manfioletti, G., Martinez-Tello, F. J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V., and Fusco, A. Detection of high mobility group I HMGI(Y) protein in the diagnosis of thyroid tumors: HMGI(Y) expression represents a potential diagnostic indicator of carcinoma. *Cancer Res.*, **58**: 4193–4198, 1998.
- Tamimi, Y., van der Poel, H. G., Denyn, M.-M., Umbas, R., Karthaus, H. F. M., Debruyne, F. M. J., and Schalken, J. A. Increased expression of high mobility group protein I(Y) in high grade prostatic cancer determined by *in situ* hybridization. *Cancer Res.*, **53**: 5512–5516, 1993.
- Tamimi, Y., van der Poel, H. G., Karthaus, H. F. M., Debruyne, F. M., and Schalken, J. A. A retrospective study of high mobility group protein I(Y) as a progression marker for prostate cancer determined by *in situ* hybridization. *Br. J. Cancer*, **74**: 573–578, 1996.
- Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Vignetto, G., Manfioletti, G., Gasamassimi, A., Santoro, M., Giancotti, V., and Fusco, A. Human colorectal carcinomas express high levels of high mobility group HMGI(Y) proteins. *Cancer Res.*, **56**: 1896–1901, 1996.
- Williams, A. J., Powell, W. L., Collins, T., and Morton, C. C. HMGI(Y) expression in human uterine leiomyomata. *Am. J. Pathol.*, **150**: 911–918, 1997.
- Bandiera, A., Bonifacio, D., Manfioletti, G., Mantovani, F., Rustighi, A., Zancanati, F., Fusco, A., Bonito, L. D., and Giancotti, V. Expression of HMGI(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix. *Cancer Res.*, **58**: 426–431, 1998.
- Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science (Washington DC)*, **235**: 177–182, 1987.
- Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. Studies of the *HER-2/neu*