which lead to formation of arterial foam cells.

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Inactivation of the Retinoblastoma Susceptibility Gene in Human Breast Cancers

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Mutational inactivation of the retinoblastoma susceptibility (RB) gene, a recessive cancer gene, has been implicated in the genesis of retinoblastoma and certain other human neoplasms. This gene is now shown to be inactivated in two of nine human breast cancer cell lines examined. The RB gene of one cell line had a homozygous internal duplication of a 5-kilobase region containing exons 5 and 6. The RB messenger RNA transcript was correspondingly lengthened, and its translation was probably terminated prematurely due to a shifted reading frame. The other cell line had a homozygous deletion of the RB gene that removed the entire gene beyond exon 2. The RB gene product, pp110^{RB}, was not detectable in either cell line by immunoprecipitation with specific antibodies. These findings are significant in relation to proposed genetic mechanisms of breast cancer formation.

SEVERAL APPROACHES HAVE BEEN APplied to identify genetic elements involved in tumorigenesis. Oncogenes were initially defined in tumor-inducing retroviruses and in tumor DNA capable of transforming nonneoplastic cells in culture (*1*). Most oncogenes are activated homologs of proto-oncogenes that exist in normal cells (*2*). Another class of cancer genes has been proposed for which loss of gene function is associated with oncogenesis (*3*). The existence of such genes was first indirectly suggested by studies with restriction fragment length polymorphisms that indicated a loss of specific chromosomal regions in tumor DNA compared to somatic DNA from the same patients. This "loss of heterozygosity" has been observed in many tumor types including retinoblastoma (*4*), osteosarcoma (*5*), Wilms's tumor (*6*), hepatoblastoma, and rhabdomyosarcoma (*7*). Genes giving rise to tumors by loss of function have been termed "recessive oncogenes" or "cancer suppressor genes," since the presence of one or more normal alleles is sufficient to prevent expression of the cancer phenotype. As a model system for recessive oncogenesis, retinoblastoma has been most intensely studied; recently, the presumptive cancer suppressor gene (RB) that controls retinoblastoma formation has been identified (*8, 9*). Evidence of RB gene inactivation has been found in most retinoblastomas as well as some osteosarcomas and soft tissue sarcomas even without prior retinoblastoma (*10, 11*), indicating a broader role for this gene in oncogenesis than might have been anticipated.

In contrast, the search for specific genetic features in more common human tumors, such as breast cancer, has been hampered by marked genetic and biological heterogeneity in tumor cells and cell lines. Although a specific 1q marker chromosome is sometimes present (*12*), most breast cancer cells demonstrate multiple, complex genetic rearrangements that are refractory to further categorization (*13*). Two recent studies have identified a loss of heterozygosity in breast cancer DNA by means of probes for polymorphic loci on several different chromosomes. In one study of ductal breast carcinomas from men and premenopausal women, loss of heterozygosity was found for markers on chromosome 13, but not on chromosome 11, in four of ten cases (*14*). Another study demonstrated loss of chromosome 11 heterozygosity in 20% of 56 stage II and III breast carcinomas (*15*), but probes on chro-

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mosome 13 were not examined. These results are not necessarily inconsistent, since more than one cancer suppressor gene might be involved in the formation of different types of breast cancer.

The loss of chromosome 13 heterozygosity in some breast tumors, a higher risk of breast cancer in mothers of children with osteosarcoma and soft tissue sarcomas (*16*), and the location of the RB gene on chromosome 13 (*9*) suggested the possibility that inactivation of the RB gene has a causative role in breast cancer formation. We had previously characterized an affinity-purified antibody that immunoprecipitated the RB gene product, $pp110^{RB}$, in many cultured cells but not in five of five retinoblastoma cell lines (*17*). This antibody was used to examine nine human breast cancer cell lines for absence of the RB protein (Fig. 1A). Cells were metabolically labeled with [^{32}P]phosphoric acid and their lysates were immunoprecipitated with immunoglobulin G (IgG) antibody to RB (anti-RB IgG). LAN-1 cells that express a large amount of RB protein (*17*) were used as a positive control. RB protein of 110 kD was found in seven breast cancer cell lines (Fig. 1A). However, RB protein was not detected in tumor lines MDA-MB-436 and MDA-MB-468, even after prolonged autoradiography. Two other antisera recognizing synthetic peptides based on the RB sequence were also unable to precipitate specific proteins from these two cell lines (*18*). Since RB protein is found routinely in most cultured cells (*17*), its absence from these two cell lines suggested mutational inactivation of the RB gene.

It was important to demonstrate that lack of RB protein in these cell lines was due to mutations of the RB gene rather than a nonspecific loss of gene expression. We performed RNA blotting analysis using polyadenylated RNA from four breast tumor cell lines as well as a cell line (HBL-100) derived from nonneoplastic human mammary epithelium (Fig. 1B). Normal-sized RB transcripts of 4.7 kb were found in the mammary epithelial cell line (lane 1) and in two breast tumor cell lines, MDA-MB-361 and MDA-MB-435S (lanes 4 and 5), that expressed intact RB proteins. However, a slightly larger RB transcript (100 to 200 added nucleotides) was found in MDA-MB-436 (lane 2), whereas no RB transcript was detectable in MDA-MB-468 (lane 3). Expression of the esterase D gene was demonstrated in all cell lines as a control for mRNA quality and suggested that chromosome region 13q14 was generally intact (*9*). Lack of RB protein in these two breast tumor cell lines therefore reflected characteristic alterations in RB gene expression (absence of RB mRNA or changes in its size) similar to those seen in retinoblastomas (*9*).

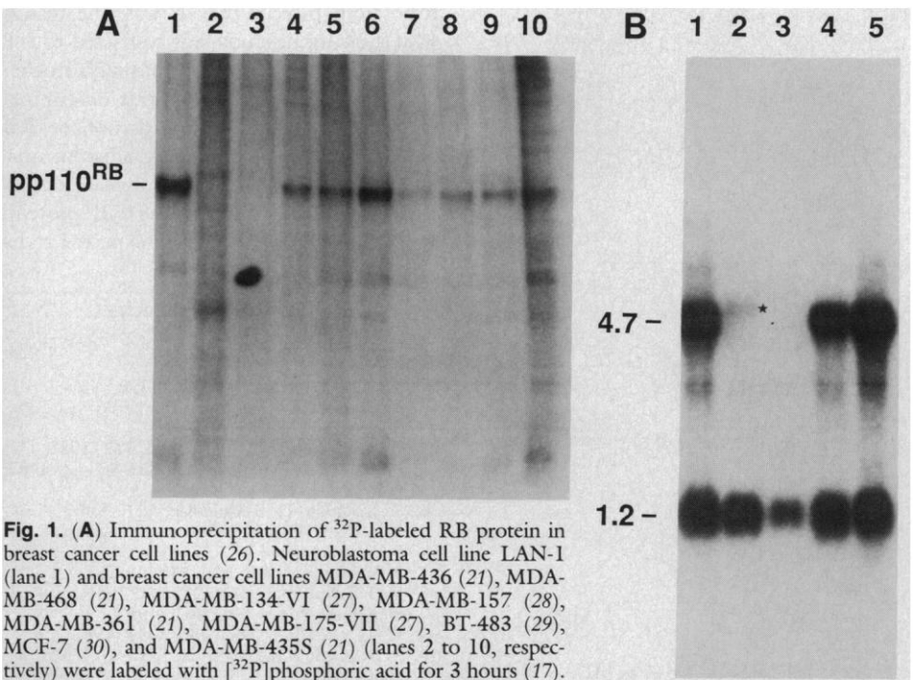

Fig. 1. (**A**) Immunoprecipitation of ^{32}P-labeled RB protein in breast cancer cell lines (*26*). Neuroblastoma cell line LAN-1 (lane 1) and breast cancer cell lines MDA-MB-436 (*21*), MDA-MB-468 (*21*), MDA-MB-134-VI (*27*), MDA-MB-157 (*28*), MDA-MB-361 (*21*), MDA-MB-175-VII (*27*), BT-483 (*29*), MCF-7 (*30*), and MDA-MB-435S (*21*) (lanes 2 to 10, respectively) were labeled with [^{32}P]phosphoric acid for 3 hours (*17*). Cell lysates were immunoprecipitated with an affinity-purified IgG directed against the RB protein (*17*). Immunoprecipitates were separated in 7.5% SDS-polyacrylamide gels and autoradiographed overnight. (**B**) RNA blotting analysis of RB and esterase D transcripts in breast tumor cell lines. Five micrograms of polyadenylated RNA prepared from a mammary epithelial cell line HBL-100 (*31*) (lane 1) and breast cancer lines MDA-MB-436 (lane 2), MDA-MB-468 (lane 3), MDA-MB-361 (lane 4), and MDA-MB-435S (lane 5) were separated by electrophoresis in 1% formaldehyde-agarose gels and transferred to nitrocellulose filters with 20× standard saline citrate (SSC). Filters were hybridized with cDNA probes for RB (*9*) and esterase D (*32*) genes labeled with ^{32}P by the random primer method (*33*). Filters were washed once in 2× SSC plus 0.1% SDS at room temperature for 30 min and twice in 0.2× SSC plus 0.1% SDS at 65°C for 30 min. Autoradiographic exposure was for 2 days. *, abnormal RB transcript in cell line MDA-MB-436.

Although many types of mutations might lead to aberrant gene expression, gross genomic rearrangements may be detected by DNA blotting analysis. Genomic DNA was extracted from MDA-MB-436 and MDA-MB-468 cell lines; digested with restriction endonucleases Bam HI, Hind III, and Msp I; and analyzed with probes derived from RB cDNA (*9*). In MDA-MB-436 DNA digested with Hind III, an extra 5-kb fragment was present in addition to five expected bands with probe RB0.8 (lane 2, Fig. 2A). Furthermore, the largest Msp I fragment in this cell line was displaced from the normal 7.5 to 12.5 kb. All other bands detected by RB cDNA probes appeared unchanged (Fig. 2B). Comparison with the normal RB genomic map (*19*) indicated duplication of exons 5 and 6 in both RB alleles, generating an extra Hind III site and creating a larger Msp I fragment (Fig. 2C). Duplication of exons 5 and 6 would add 107 nucleotides to the mRNA transcript (*20*), as suggested by RNA blotting analysis (Fig. 1B). Moreover, the reading frame of this transcript would be shifted with premature termination at nucleotides 659 to 661 (*17*), consistent with the observed lack of intact RB protein. Confinement of the duplicated region to within the RB gene reinforced our conclusion of specific RB gene involvement in the genesis of this tumor line.

In cell line MDA-MB-468, all three restriction digestions demonstrated homozygous deletion of most of the RB gene (Fig. 2, A and B). The 12-kb Bam HI fragment that contained exon 2 was shortened to 8 kb, whereas a 19-kb Hind III fragment that contained exons 1 and 2 was intact; therefore the deletion junction was located within intron 2 as shown (Fig. 2C). The deletion in MDA-MB-468 extended an unknown distance beyond the 3′ end of the RB gene. This cell line has a hypodiploid karyotype, identical to a malignant pleural effusion sampled from the original patient (*21*). A visible deletion of region 13q14 was not noted. However, normal expression of the esterase D gene indicated that the deletion did not include this closely linked gene.

Breast cancer, one of the most prevalent of all cancers, is characterized by marked heterogeneity in pathological features. These include variation in morphology, physiology (for example, hormone dependence for cell growth), and genetic composition of tumor cells (*13*). Most breast cancers

are sporadic, but familial aggregations are sometimes found. The familial form shows significantly earlier age of onset, excess of multiple primary tumors, and a pattern of inheritance consistent with transmission of dominant gene or genes (*22*). From epidemiological studies, the risk of breast cancer in the mothers of children with osteosarcoma and soft tissue sarcoma is three times higher than expected, suggesting a common heritable component in the etiology of these cancers (*16*). The loss of heterozygosity of chromosome 13 or 11 in some ductal breast carcinomas (*14, 15*) also suggests that a recessive cancer gene or genes may be involved in these tumors. Our finding of specific inactivation of the RB gene in two of nine breast cancer cell lines, as well as that of specific RB gene inactivation in about 50% of randomly selected osteosarcomas and 30% of soft tissue sarcomas (*23*), provides a molecular framework to interpret these phenomena. Furthermore, loss of chromosome 13 heterozygosity in certain breast tumors, specific RB gene inactivation in breast cancer cell lines, and the familial association of breast cancer and certain RB-related sarcomas (*24*) indirectly suggest that familial predisposition to certain kinds of breast cancer may be mediated by mutated RB alleles. These results provide a clear direction for further investigation of cancer family syndromes, particularly those involving breast carcinomas, sarcomas, and retinoblastomas as described by Li and Fraumeni (*24*).

Our results with cell lines must be interpreted with caution until they are confirmed in fresh breast tumor specimens. Only about 20% of breast tumor cell lines show evidence of RB gene inactivation, and this prevalence may be biased by selection for tumors that are successfully cultured. One earlier study noted three primary breast tumors with karyotypic deletions of 13q14 that removed one allele of the RB gene, but internal rearrangements of the RB gene were not observed in any of 14 breast tumors (*10*). However, RB gene inactivation does not necessarily require gross genomic rearrangements (*9*), and some DNA rearrangements are easily missed (*19, 25*). Furthermore, since whole tumors may contain substantial numbers of intermixed nonneoplastic cells, detection of normal restriction fragments in tumor DNA may incorrectly imply that the RB gene is intact in cancer cells. The same artifact will affect data on immunoprecipitation of RB protein from fresh tumors, which was one reason why the current study was restricted to cell lines. We anticipate that immunohistochemistry will allow unambiguous determination of the presence or absence of RB protein in tumors cells, using adjacent normal cells as controls. Monoclonal antibodies with higher affinities for the RB protein, now under development, are expected to be useful in this regard.

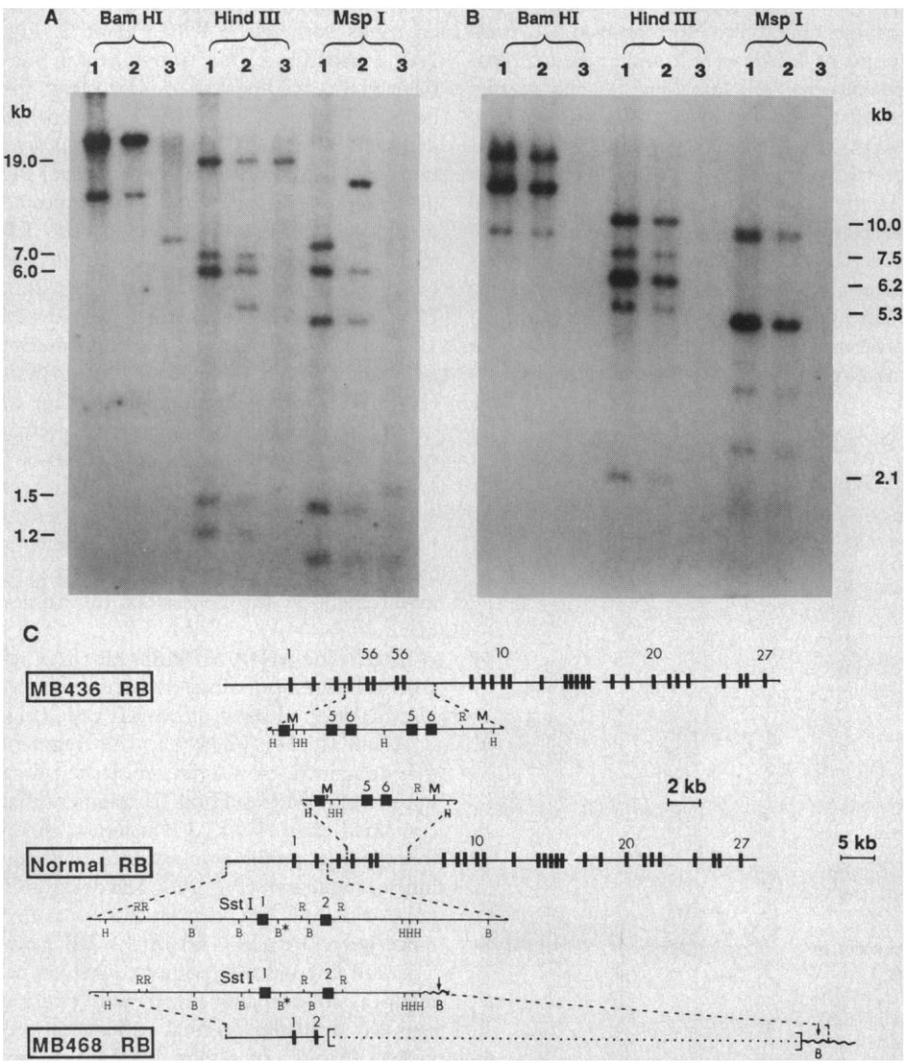

Fig. 2. DNA blotting analysis of the RB gene in breast tumor cell lines MDA-MB-436, MDA-MB-468, and normal cells. Equal amounts of DNA (3 μg per lane) from normal lymphocytes (lane 1), MDA-MB-436 (lane 2), and MDA-MB-468 (lane 3) were digested with restriction endonucleases Bam HI, Hind III, and Msp I and analyzed by DNA blotting (washing conditions as in Fig. 1B) with cDNA probes RB0.8 (**A**) and RB3.8 (**B**) from the RB gene. RB0.8 and RB3.8 are the 5′ and 3′ portions, respectively, of an Eco RI–cut 4.5-kb cDNA segment reconstructed from RB cDNA clones RB-1 and RB-5 (*9*). We inferred the structure of mutant RB genes (**C**) from the normal RB gene map (*19*) (selected Msp I sites added) and data from (A) and (B). In MDA-MB-436, the RB gene contains two extra exons 5 and 6, resulting from duplication of a 5-kb region. In MDA-MB-468, a large deletion (dotted line) extends from intron 2 to the 3′ end of the RB gene. The deletion junction was located between Hind III and Bam HI sites in intron 2. Exons (solid vertical bars) are not to scale. R, Eco RI; H, Hind III; B, Bam HI; M, Msp I; and *, polymorphic site.

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Isoform-Specific Modulation of Na^+,K^+-ATPase α-Subunit Gene Expression in Hypertension

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Sodium, potassium–adenosine triphosphatase (Na^+,K^+-ATPase) is hypothesized to be involved in systemic vascular hypertension through its effects on smooth muscle reactivity and myocardial contractility. By means of RNA blot analyses of cardiac, aortic, and skeletal muscle RNAs in two rat hypertensive models, Na^+,K^+-ATPase α-subunit messenger RNA isoforms (α2 and α3) were shown to be deinduced in response to increased intravascular pressure. The changes were observed after 48 hours or more of experimental hypertension. Under these conditions, there is coordinate induction of another α isoform (α1) and of β-subunit messenger RNAs, probably in response to alterations in sodium flux rather than to elevated blood pressure.

THE NA-, K-DEPENDENT ADENOSINE triphosphatase (Na^+,K^+-ATPase) maintains the Na^+ and K^+ electrochemical gradient across the cell membrane to which is coupled other vectorial transport mechanisms important for cell homeostasis and specialized functions (*1*). Because Na^+,K^+-ATPase maintains the Na^+ gradient, this enzyme has been hypothesized to be involved in the pathogenesis of hypertension through its effects on vascular smooth muscle reactivity (*2–4*) and myocardial contractility (*5*). Conflicting data have been reported regarding changes in Na^+,K^+-ATPase activity in various types of experimental and human hypertension (*6, 7*). At least three α-subunit isoforms, α1, α2, and α3 (α, α+, αIII) (*8*) have been characterized in rats (*8, 9*) and humans (*10*). These isoforms have a complex pattern of expression (*9, 11*) and significant functional diversity (*12, 13*). Identification of isoform-specific differential regulation of expression during hypertension could give insight into the roles of the isoforms and the molecular mechanisms involved in hypertension.

We therefore have analyzed Na^+,K^+-ATPase α- and β-subunit mRNAs in different tissues during hypertension in two rat models—rats uninephrectomized and treated with deoxycorticosterone (DOC)-salt (*14*) and rats infused with angiotensin-II (A-II) for short periods (*15*) (Table 1). RNA blot analyses of Na^+,K^+-ATPase mRNA were done with RNA from aortic, cardiac left ventricular, and skeletal muscle from hypertensive and control rats. In aorta, a two- to threefold increase (*16*) in amounts of α1-subunit mRNA was noted only in rats treated with DOC-salt (Fig. 1A) and persisted from 2 to 8 weeks of DOC-salt administration. The amounts of aortic α1 mRNA in normotensive, uninephrectomized rats treated with DOC–low salt (DOC-LS) were equivalent (*16*) to those in uninephrectomized controls, thus eliminating the possibility that our results were due to an up regulation of α1 mediated by DOC. The α1 mRNA in aortas from the hypertensive rats treated with A-II for short periods, however, did not show any up regulation (*16*) as compared to aortas from control normotensive rats infused with A-II medium (acetic acid, 0.01*N*) or with subpressor doses of A-II (Fig. 1B, C1 and C2, respectively). These results along with the twofold increase (*16*) in α1 mRNA in the skeletal muscle of hypertensive rats treated with DOC-salt (Fig. 2) demonstrate that the α1 up regulation is not a direct response to increased intravascular pressure. This α1 up regulation is not a nonspecific phenomenon, since the amounts of mRNA of other

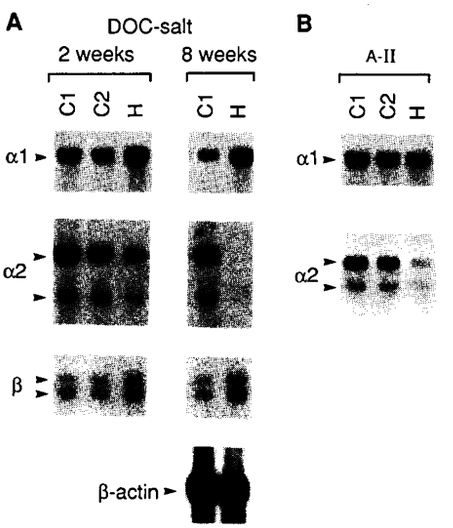

Fig. 1. Na^+,K^+-ATPase α- and β-subunit mRNAs in aortas from hypertensive rats. (**A**) The mRNA of Na^+,K^+-ATPase subunits α1, α2, and β (α3, none detected) were analyzed in separate RNA blots with equivalent amounts of total cellular RNA derived from pooled aortas from hypertensive, uninephrectomized rats treated with DOC-salt (H), control uninephrectomized rats (C1), and control normotensive DOC-LS rats (C2). As a control, β-actin mRNA was also detected. The specific hybridizing bands to the respective probes are indicated by arrowheads. Two hybridizing bands are detected in α2 and β, representing two sizes of mRNAs with different lengths of 3′UT generated by differential utilization of polyadenylation signals (*8, 21*). (**B**) Na^+,K^+-ATPase α-subunit mRNAs, α1 and α2, were analyzed in aortic total cellular RNA from rats made hypertensive by 1-week intraperitoneal A-II infusion. C1, control normotensive rats with pump implanted to infuse medium. C2, control normotensive rats with subpressor dose of A-II infused (75 ng/min). H, hypertensive rats with pressor dose of A-II infused (200 ng/min). Specific hybridizing bands to respective probes are noted with arrowheads. Total cellular RNA was isolated from pooled aortic samples ($n = 3$) and RNA blots were done (*9*). Random-primed ^{32}P-labeled cDNA probes were prepared (*22*) and represented comparable amino acid coding regions: α1, 5′ untranslated region (UT)–3′UT; α2, amino acid 27 to 3′UT; α3, amino acids 10 to 940 (*23*); β, 5′UT–3′UT (*24*); β-actin 5′UT–3′UT. Under stringent hybridization and washing conditions (*9*), the α-subunit cDNA probes used do not cross-hybridize with each other.

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