Characterization of the DIM Series of BALB/c Preneoplasms for Mouse Mammary Tumor Virus-mediated Oncogenesis

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ABSTRACT

The DIM series of preneoplasms, developed from the BALB/c mammary cell line COMMA-D, and DIM-derived tumors were examined for evidence of mouse mammary tumor virus (MMTV) involvement in the tumorigenic process. DIM tissues were shown to be free of exogenous MMTV as much as Southern blot analysis of DNAs extracted from DIM preneoplasms and tumors showed the lack of a PstI-generated 4.2-kilobase MMTV gag-pol band. To examine the possibility of endogenous MMTV action via enhancer insertion, DNAs from DIM preneoplasms and tumors were restricted with EcoRI, BamHI, or SstI and subjected to Southern blot analysis using an MMTV-long terminal repeat repeat probe. The normal endogenous proviral content was detected in all DIM tissues assayed; no new proviruses were found. To examine the possibility of endogenous MMTV gene product oncogenesis, MMTV transcripts were quantified by slot blot analysis and MMTV envelope proteins were assayed by immunohistochemical staining of DIM tissue sections. While all DIM tissues expressed MMTV-long terminal repeat transcripts, the level of expression did not correlate with tumorigenicity. Most frequently, MMTV-env-containing sequences were more abundant than the 1.6-kilobase long terminal repeat transcript, the latter being the most promising candidate for a MMTV gene product mediating mammary tumorigenesis. However, preneoplasms and tumors of only the DIM 4 line contained levels of MMTV M, 52,000 or 36,000 glycoproteins detectable by immunoperoxidase staining. Electron microscopy did not reveal any virus particles in DIM 4 tissues. These data do not substantiate MMTV as a causative agent in the formation of the DIM preneoplasms or tumors. Thus while MMTV may confuse the interpretation of causative events in the formation of other preneoplasms and neoplasms, the DIM preneoplasms represent a model system for the study of MMTV-independent mechanisms.

INTRODUCTION

Preneoplastic mammary epithelial lesions, which are at greater risk of neoplastic transformation than the normal mammary epithelium, have served as a model for the study of early events in tumorigenesis (1-5). In the mouse, preneoplastic lesions are amplified by transplantation of preneoplastic cells or tissues into gland-free fat pads of syngeneic mice (1-4). The genetic integrity of preneoplastic lines can be maintained for tumor potential, ovarian hormone dependence, and response to DMBA (7).

In an attempt to identify the mechanism(s) of preneoplastic and neoplastic transformation, we examined the DIM tissues for MMTV expression and proviral copy number. The proposed mechanisms of viral oncogenesis are enhancer insertion and gene product oncogenesis. Enhancer insertion predicts that a provirus inserted within the genome can regulate the expression of adjacent cellular genes (9). A number of common proviral integration sites have been identified in different mammary precancers and tumors (10-14). Of these, proviral integration within the int-1 (10) and int-2 (11) domains are the best characterized. MMTV gene product oncogenesis predicts that a product of MMTV is directly involved in tumorigenesis. The most popular candidate for this role is the product of the MMTV-LTR open reading frame for the following reasons: (a) the nucleotide sequence of the open reading frame is highly conserved among virus strains (15); (b) a 1.6-kilobase transcript containing predominantly LTR sequences, including the open reading frame, has been identified in different mammary tissues (16, 17); (c) the function of the LTR open reading frame gene product is unknown; and (d) an oncogenic function has been implied by the following observations: the 1.6-kilobase LTR transcript was selectively increased in C3H/Sm mammary tumors relative to normal glands (18); in vitro translation of cloned LTR sequences produced, among other proteins, a M, 36,000 protein (14); and antisera raised against a mammary tumor cell line precipitated a M, 36,000 protein in mammary tumor cells (19).

Exogenous MMTV is undisputed as an oncogenic agent (5, 19); however, the role of endogenous MMTV sequences in oncogenesis is still unclear. Inbred mouse strains contain varied combinations of at least 15 endogenous proviral loci (20). Of these only three loci, Mtv-1, Mtv-2 (21) and Mtv-4 (22), have been implicated in virus production and tumor formation. Mammary tumors from mouse strains harboring these loci contained extra proviral copies of Mtv-1, -2, or -4 exclusively (12, 21, 22), supporting enhancer insertion as the mechanism of MMTV action. Endogenous MMTV proviruses other than Mtv-1, -2, or -4 may indirectly cause tumors by mediating the effects of oncogenic agents. Exposure to carcinogens could result in increased levels of MMTV RNA, resulting in a greater probability of proviral integration or increased levels of gene products.

These data suggest that mammary tumors may be induced by a number of different molecular mechanisms, one of which may be the activation of endogenous MMTV proviruses. To determine if endogenous MMTV was involved in the progression from normal mammary epithelium to neoplasm, DIM preneoplasms and tumors were assayed for MMTV RNA, protein, and proviral integrations. The following data characterize a murine mammary tumor system which is apparently free of MMTV involvement.

MATERIALS AND METHODS

Tissues. Tissues were obtained from female BALB/cMed or BALB/cVMed mice that were bred and maintained in a closed colony in the...
Department of Cell Biology, Baylor College of Medicine. BALB/cMed mice do not contain exogenous MMTV; however, they express low levels of MMTV transcripts from endogenous proviruses and have a mammary tumor incidence of less than 1% in 15-month-old multiparous females (7). BALB/cVMed mice were derived from BALB/cMed mice and produce MMTV virions which result in a 50% mammary tumor incidence at 10 months of age in breeding females (23).

DIM tissues were derived from the in vivo transplantation of the COMMA-D cell line. The COMMA-D cell line originated from the in vitro culture of collagenase-digested mammary glands from midpregnancy BALB/c mice. The cells were passed on plastic 12-14 times prior to in vivo injection. The DIM HOG lines were propagated by serial transplantation in “cleared” mammary fat pads of syngeneic mice. HOG lines DIM 1, 2, and 3 were classical hyperplastic alveolar outgrowth lines. DIM 4 was initially a ductal outgrowth which progressed with transplantation to an alveolar outgrowth. Mammary adenocarcinomas arose from the HOGs at 4-10 months after transplantation depending on the individual line. The tumor incidence of DIM 4 outgrowths increased with morphological progression (7).

RNA and DNA Extraction. RNAs were extracted by a modification of the method of Chirgwin et al. (24). Briefly, tissues were homogenized in 10 volumes of 4.5 M guanidinium thiocyanate, 28 mM sodium citrate, 0.6% Sarkosyl, 0.1 M β-mercaptoethanol, and 0.1% Sigma antifoam A, adjusted to pH 7.0. For normal tissues and tumors, nucleic acids were precipitated at -20°C by the addition of 0.0286 volume of 1 M acetic acid, 0.125 volume of water, and 0.8438 volume of ethanol. The resulting pellet was dissolved in 7.5 M sodium hydroxide-25 mM sodium citrate-5 mM dithiothreitol, adjusted to pH 7.0, and RNA was selectively precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The precipitation step was repeated twice and then the RNA pellets were washed in absolute ethanol to remove all traces of guanidine hydrochloride. The RNA was dissolved in 50% dimethyl sulfoxide-10 mM Tris (pH 7.5)-1 mM EDTA (pH 7.5)-0.1% SDS, incubated for 5 min at 45°C, and finally precipitated with 0.1 volume of 2.75 M sodium acetate (pH 5.5) and 2 volumes of ethanol.

For hyperplastic outgrowths, the guanidine thiocyanate homogenate was layered over a 2.5-ml cushion of 5.7 M cesium chloride and then with phenol:chloroform:isoamyl alcohol (24:24:1) until the interface was clear. The resulting aqueous phase was dialyzed against sodium citrate-5 mM dithiothreitol, adjusted to pH 7.0, and RNA was selectively precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The precipitation step was repeated twice and then the RNA pellets were washed in absolute ethanol to remove all traces of guanidine hydrochloride. The RNA was dissolved in 50% dimethyl sulfoxide-10 mM Tris (pH 7.5)-1 mM EDTA (pH 7.5)-0.1% SDS, incubated for 5 min at 45°C, and finally precipitated with 0.1 volume of 2.75 M sodium acetate (pH 5.5) and 2 volumes of ethanol.

For DNA extraction, tissues were digested with proteinase K (200 µg/ml) in the presence of 10 mM EDTA and 0.1% SDS at 37°C until the tissue was dissolved. The solution was extracted first with phenol, then with phenol:chloroform:isoamyl alcohol (24:24:1) until the interface was clear. The resulting aqueous phase was dialyzed against sodium citrate-5 mM dithiothreitol, adjusted to pH 7.0, and RNA was selectively precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The precipitation step was repeated twice and then the RNA pellets were washed in absolute ethanol to remove all traces of guanidine hydrochloride. The RNA was dissolved in 50% dimethyl sulfoxide-10 mM Tris (pH 7.5)-1 mM EDTA (pH 7.5)-0.1% SDS, incubated for 5 min at 45°C, and finally precipitated with 0.1 volume of 2.75 M sodium acetate (pH 5.5) and 2 volumes of ethanol.

For slot blots, RNAs were denatured with glyoxal (26), separated by electrophoresis through a 1.6% agarose gel, and transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) according to the method of Thomas (27). Prior to prehybridization, the blots were rinsed in boiling Tris (pH 8.0).

For slot plots, RNAs were denatured with formaldehyde and applied to nitrocellulose paper using a Minifold II apparatus according to manufacturer's directions (Schleicher & Schuell).

DNAs for Southern blot analysis were digested to completion by the appropriate restriction enzymes (EcoRI, BamHI, SstI, or PstI) as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, MD). DNA fragments (8-10 µg) were separated by electrophoresis through a 0.7% agarose gel, visualized with ethidium bromide, and denatured in 1.5 M NaCl-0.5 M NaOH. After neutralization with 1 M Tris (pH 7.0)-1.5 M NaCl, the DNA was transferred to nitrocellulose paper (28).

All blots were baked at 80°C in a vacuum for 2 h and then placed in individual sealed bags and prehybridized with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 250 µg/ml sonicated and denatured salmon sperm DNA, and 0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll, at 42°C overnight. Hybridizations were carried out in the same buffer with 105 cpm/ml of α-32P-labeled (3000 Ci/mmol; ICN Radiochemicals, Irvine, CA) nick-translated probe (nick translation kit from Bethesda Research Laboratories) for 20 h for Northern and slot blots or 48 h for Southern blots. The blots were first washed in four changes (15 min each) of 2x SSC-0.1% SDS-0.1% sodium pyrophosphate at room temperature, then in two changes (15 min each) of 0.1x SSC-0.1% SDS-0.1% sodium pyrophosphate at 50°C, and finally in 0.1x SSC for 10 min at room temperature. The blots were air dried and exposed to either Kodak XAR or XRP film at -80°C with Dupont Cronex lightning-plus intensifying screens.

 Autoradiograms generated from slot plots were scanned with a Quick Scan densitometer (Helena Labs, Beaumont, TX) and areas under curves were quantified using an electronic digitizer (model 1224 from Numeronics Corp. (Landsdale, PA).

 Probes. Plasmids containing (C3H)MMTV sequences were a generous gift from Dr. Harold Varmus (29). The MMTV inserts were isolated by PstI digestion of the plasmid followed by agarose gel electrophoresis and electrolution as described by Maniatis et al. (25). The subgenomic MMTV probes are located on the map in Fig. 4.

 Immunohistochemistry. The avidin-biotin-peroxidase method as described by Hogan and Smith (30) was used to visualize MMTV envelope gp52/gp36. The antisera, a gift from Dr. Janet Butel and Dr. Betty Slagle, was prepared in rabbits against affinity-purified (C3H)MMTV gp52/gp36 and has been characterized previously (31).

 Transmission Electron Microscopy. Tissues were fixed for 2 h at

Fig. 1. MMTV RNAs in DIM preneoplasms and tumors. (A) slot blot analysis and (B) Northern blot analysis of total cellular RNA from DIM tissues hybridized to a 32P-MMTV-LTR probe and (C) slot blot analysis using a 32P-MMTV-env probe. Procedures were as described in “Materials and Methods.” 2H, 3H, 4H indicate RNAs from pooled outgrowths of DIM HOG lines 2, 3, and 4, respectively. 1T, 2T, 3T, and 4T indicate RNAs extracted from different, individual DIM tumors from DIM HOG lines 1, 2, 3, and 4, respectively. The sequence of samples was identical for A and C such that the first IT represents the (A) LTR and (C) envelope transcripts from the same tumor, and so on. Control samples are BALB/c liver (LIV), midpregnant (M), and lactating (L) mammary glands or BALB/cV lactating (VL) mammary glands. For slot blots RNAs were spotted in 2-fold dilutions from left to right beginning with 2.5 µg for VL and 5 µg for all other samples. For Northern analysis, 10 µg of midpregnant mammary gland RNA (a high LTR expressor) and 20 µg each of the DIM HOG RNAs were electrophoresed. kb, kilobase.

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MMTV IN DIM MAMMARY PRENEOPLASMS

Table 1 Levels of MMTV transcripts in DIM series mammary HOGs and tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissue</th>
<th>MMTV-LTR*</th>
<th>MMTV-env*</th>
<th>env/LTR</th>
<th>Incidence (at 1 yr) (%)</th>
<th>Latency (mo)</th>
<th>Transplant generation</th>
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<tr>
<td>DIM 1</td>
<td>HOG</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>87</td>
<td>4.7</td>
<td>2-7</td>
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<td></td>
<td>Tumor</td>
<td>19</td>
<td>148</td>
<td>7.8</td>
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<td></td>
<td>Tumor</td>
<td>1480</td>
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<tr>
<td></td>
<td>Tumor</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>0</td>
<td>32</td>
<td></td>
<td></td>
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<tr>
<td>DIM 2</td>
<td>HOG</td>
<td>24</td>
<td>62</td>
<td>2.6</td>
<td>80</td>
<td>6.8</td>
<td>10</td>
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<tr>
<td></td>
<td>Tumor</td>
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<td>Tumor</td>
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<td>3738</td>
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<td>DIM 3</td>
<td>HOG</td>
<td>215</td>
<td>626</td>
<td>2.9</td>
<td>75</td>
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<td>Tumor</td>
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<td>Tumor</td>
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<td>Tumor</td>
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<td>599</td>
<td>0.9</td>
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<tr>
<td></td>
<td>Tumor</td>
<td>55</td>
<td>263</td>
<td>4.8</td>
<td></td>
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<tr>
<td>DIM 4</td>
<td>HOG</td>
<td>944</td>
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<td>6.0</td>
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<td>Tumor</td>
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<td>4030</td>
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<tr>
<td></td>
<td>Tumor</td>
<td>98</td>
<td>3120</td>
<td>31.8</td>
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* Values are expressed as percentage of lactating where lactating = 100%.
ND, not determined.
Not detectable.

Table 2 MMTV envelope protein expression in DIM lines (anti-gp52/gp36)

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissue</th>
<th>No. of positive samples</th>
<th>No. of total samples</th>
<th>% positive</th>
<th>Degree of response</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIM 2</td>
<td>HOG</td>
<td>0/8</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DIM 3</td>
<td>HOG</td>
<td>0/7</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>0/4</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>DIM 3 CV*</td>
<td>HOG</td>
<td>1/1</td>
<td>100</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>1/1</td>
<td>100</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>DIM 4</td>
<td>HOG</td>
<td>7/10</td>
<td>70</td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>4/7</td>
<td>57</td>
<td>2.8</td>
<td></td>
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</table>

ND, not determined.
DIM 3 HOGs were transplanted into virus-positive hosts (BALB/cV).
The degree of MMTV envelope protein expression was graded according to the percentage of alveoli which exhibited positive staining to antibody raised against MMTV gp52/gp36. The scale was: 0 = no staining; 1+ = 6-25%; 2+ = 26-50%; 3+ = 51-75%; 4+ >75% positive alveoli.

RESULTS

Endogenous MMTV expression can be regulated at transcriptional, posttranscriptional or posttranslational levels (5). Thus DIM preneoplasms and tumors were examined for MMTV RNA, proteins, and virus particles.

MMTV RNA. MMTV transcript levels in DIM tissues were compared with levels in normal lactating glands. Fig. 1 shows that the three DIM HOG lines examined (2H, 3H, and 4H) expressed MMTV RNA. Slot blot hybridization using an MMTV-LTR probe (Fig. 1A), which detects all MMTV transcripts (genomic, envelope, and LTR), showed that DIM 2 HOGs contained one-fourth the level of MMTV transcripts detected in lactating glands, DIM 3 HOGs twice as many, and DIM 4 HOGs nearly 10 times as many. The three individual control BALB/c lactating mammary glands varied in their level of LTR expression by at most one-fifth of their averaged value. Thus the level of MMTV RNA in DIM 2 HOGs was less than that seen in lactating controls and the levels in DIM 3 and DIM 4 HOGs were higher.

Northern analysis showed that the increased MMTV expression in DIM 3 and DIM 4 HOGs was largely due to the expression of the 3.8-kilobase envelope transcript, rather than the 1.6-kilobase LTR transcript (Fig. 1B). Because slot blot analysis using an MMTV-LTR probe did not distinguish among the three MMTV transcripts, a method for the comparison of levels of the 1.6-kilobase LTR transcript in different DIM tissues was necessary. Thus slot blot hybridization was also done using an MMTV-env probe (Fig. 1C). Only 72 bases (4%) of the MMTV-env probe are homologous to the 1.6-kilobase LTR transcript, while 100% of the probe (1.7 kilobases) is homologous to the genomic and envelope transcripts. The 1.6-kilobase LTR transcript contains almost no MMTV-envelope sequences (16, 17). The extent of expression of the 1.6-kilobase LTR transcript was determined by the relative expression of MMTV-envelope-containing transcripts divided by the relative expression of MMTV-LTR-containing transcripts. Levels of MMTV-env-containing transcripts in DIM tissues are expressed relative to levels in lactating glands in Table 1. BALB/c lactating mammary glands contain predominantly 1.6-kilobase LTR transcripts and little (32) or none (16, 17) of the other MMTV-LTR transcripts. In our experiments levels of env-containing transcripts in lactating mammary glands were extremely low (Fig. 1C). The ratio of MMTV-env-containing transcripts to MMTV-LTR-containing transcripts [env/LTR (Table 1)] is also expressed relative to levels in lactating mammary glands. DIM tissues exhibiting an env/LTR ratio >1 had comparatively more envelope-containing transcripts than did lactating glands. Similarly, those tissues exhibiting an env/LTR ratio of <1 had a preponderance of the 1.6-kilobase LTR.
transcript relative to lactating glands. Finally, an env/LTR ratio of 1 indicates that envelope-containing transcripts:LTR containing transcripts were present in the same proportion as found in lactating glands. The three DIM HOG lines examined each contained env/LTR ratios greater than 1 reflecting an asymmetrical increase in MMTV transcripts other than the 1.6-kilobase LTR.

Although the DIM outgrowth lines 2H, 3H, and 4H expressed different levels of MMTV transcripts, their tumor incidences and latency periods were essentially the same (Table 1). Thus MMTV RNA levels did not correlate with tumorigenicity. If MMTV expression is involved in the preneoplastic to neoplastic transition, MMTV RNA levels may have been elevated only in those cells which gave rise to tumors. This should be reflected by increased levels of MMTV RNA in tumors compared with preneoplasms. Slot blot analysis of RNA populations extracted from four DIM tumor lines (1T, 2T, 3T, and 4T) demonstrated a significant variability in MMTV transcript levels, even among tumors of the same DIM line (Fig. 1A; Table 1). MMTV-LTR transcript levels varied more than 80-fold in DIM 1 tumors, 25-fold in DIM 2 tumors, 12-fold in DIM 3 tumors, and 10-fold in DIM 4 tumors. There was at least one tumor from each of the HOG lines which expressed MMTV transcripts at levels comparable to or lower than those of the control lactating glands. In three of four DIM 1 tumors, MMTV-LTR transcript levels were less than 20% of the level in lactating glands. MMTV transcripts were also qualitatively different in individual tumors. Northern analysis of a series of DIM 4 tumors showed that individual tumors expressed only the 1.6-kilobase LTR transcript, both the 1.6-kilobase LTR and the 3.8-kilobase envelope transcripts, or all three MMTV transcripts (data not shown). We could not determine whether this heterogeneity in transcription was also obvious at the preneoplastic stage as HOGs were pooled for RNA extraction.

MMTV Protein. Because translational blocks in the expression of MMTV have been demonstrated (33), we examined the
production of MMTV proteins to determine if this correlated with RNA expression. Although it would have been desirable to examine the 1.6-kilobase LTR RNA translation product, good antibodies are not readily available. Thus the production of the envelope glycoproteins gp52/gp36 were monitored as a marker for MMTV protein synthesis.

While all three of the DIM lines transcribed the 3.8-kilobase envelope message, DIM 2 and DIM 3 HOGs and DIM 3 tumors did not produce detectable levels of gp52/gp36 (Table 2; Fig. 2). By contrast 70% of DIM 4 HOGs and 57% of DIM 4 tumors produced gp52/gp36. Although immunocytochemical reaction product was present in both the cytoplasm and lumen in DIM 4, it was most easily seen in the lumen (Fig. 2). Mature virus particles were found in DIM 4 tissues only when grown in hosts which were infected with exogenous MMTV (Fig. 3).

Extra Proviral Copies. To demonstrate that MMTV expression in DIM tissues was not a product of exogenous virus infection, we exploited differences in the restriction maps of endogenous and exogenous MMTV sequences (Fig. 4). BALB/c contain three endogenous proviruses, two of which consist of complete MMTV genomes (Mtv-8 and Mtv-9), the third containing predominantly LTR sequences (Mtv-6) (34). Restriction
MMTV IN DIM MAMMARY PRENEOPLASMS

Fig. 4. Restriction maps of MMTV endogenous to BALB/c (Mtv-8 and -9) and C3H and GR exogenous MMTV (29, 35, 46). Restriction enzyme cut sites are: B, BamHI; E, EcoRI; F, PstI; S, SstI; Pst, viral DNA; C, LTR DNA; —, cellular DNA. Specific hybridization probes are: 1, LTR; 2, gag-pol; 3, env kb; and 4, respectively, and VT, BALB/cV mammary tumor. Arrowhead, 4.2-kilobase

Fig. 5. Exogenous MMTV DNA in DIM tissues. PstI-restricted DNAs (8-10 µg) were electrophoresed through 0.7% agarose, transferred to nitrocellulose paper, and hybridized to a nick-translated 32P-MMTV-gag-pol probe. DNAs were extracted from: L, BALB/c lactating mammary gland; 1H, 2H, 4H, DIM HOGs 1, 2, and 4, respectively; 1T, 2T, 3T, 4T, DIM tumors from HOG lines 1, 2, 3, and 4, respectively, and VT, BALB/cV mammary tumor. Arrowhead, 4.2-kilobase (kb) restriction fragment characteristic of exogenous virus (see Fig. 4). Molecular weight size markers are from HindIII-restricted λDNA.

Fig. 6A is an autoradiogram

DISCUSSION

The mammary epithelium is one of the few systems where preneoplastic lesions, intermediate between normal epithelium and tumors, can be identified and studied (1-4). The molecular mechanisms involved in the generation of such preneoplasms are not known. MMTV, an etiologic agent in murine breast cancer, is believed to be capable of action in either the normal to preneoplastic or preneoplastic to neoplastic transition (4, 5). Two mechanisms for MMTV action have been proposed, enhancer insertion and gene product oncogenesis. To determine if MMTV was acting via enhancer insertion, MMTV proviral copy number was examined in the DIM series preneoplasms and tumors. Similarly, to determine if MMTV was acting through gene product oncogenesis, MMTV expression was measured at the RNA and protein levels.

First the DIM tissues were shown to be free of exogenous virus by the absence of a 4.2-kilobase PstI gag-pol fragment characteristic of C3H and GR milk-transmitted viruses. This means that MMTV-mediated oncogenesis in the DIM tissues would have been the result of activation of endogenous proviral templates.

To test the hypothesis that increased MMTV gene expression may have contributed to preneoplasms or tumor formation, levels of MMTV transcripts were examined in normal tissues and in DIM preneoplasms and tumors. Previous results had shown increased levels of MMTV RNA from endogenous proviral templates in some tumors from low mammary tumor incidence mouse strains when exposed to X-irradiation, chemical carcinogens, or increased levels of hormones (reviewed in Ref. 5). When increased levels of MMTV RNA were detected in such BALB/c tumors, the RNA was enriched in polyadenylate-adjacent sequences (36, 37). This suggests increased levels of the 1.6-kilobase LTR transcript (5). Of unknown function, this transcript is a candidate RNA for the mam gene, a putative MMTV gene the product of which would function in oncogenesis (19). In order to adequately test the hypothesis of MMTV gene product oncogenesis in DIM tissues, it was therefore necessary to examine the levels of the 1.6-kilobase LTR transcript specifically. This was difficult because all three of the MMTV transcripts contain the sequences which comprise the 1.6-kilobase LTR transcript and Northern analysis using a MMTV-LTR probe resulted in autoradiograms which were too light for accurate quantification. Therefore Northern analysis was combined with slot blot analysis using both MMTV-LTR and -env probes. Examination of DIM tissues for general MMTV transcripts showed that increased transcript levels were random and independent of tumor incidence or latency in

pattern and lack of additional bands in DNAs from BALB/c normal lactating mammary glands (L) and DIM preneoplasms (2H, 4H) and tumors (1T, 2T, 3T, 4T). Extra proviral copies were detected in the BALB/cV mammary tumor DNA (VT).

Because extra proviral copies may comigrate with endogenous proviral sequences, DIM and control DNAs were restricted with SstI or BamHI and similarly analyzed (Fig. 6, B and C). In all cases extra proviral copies were detected only in the BALB/cV mammary tumor DNA. Thus neither endogenous nor exogenous extra proviral copies were detected in the DIM tissues. Using this approach we would not have detected extra endogenous proviruses integrated at random within the genome. However, the model of enhancer insertion predicts clonal dominance of cells harboring virus in a critical integration site; random integrations should be of no consequence.
preneoplasms and were random in tumors. Both Northern and slot blot analyses indicated that the predominant MMTV transcript in most DIM tissues was the 3.8-kilobase envelope transcript, not the 1.6-kilobase LTR transcript. The exact levels of the 1.6-kilobase LTR transcript were not determined. Increased levels of this transcript did not correlate with tumorigenicity.

In addition, MMTV protein production did not correlate with MMTV RNA expression or with tumorigenicity. The MMTV envelope glycoproteins gp52/gp36 were detected only in DIM 4 tissues which, of the four HOG lines, expressed the highest level of MMTV transcripts with the highest env/LTR ratio. It is possible that the level of MMTV proteins in DIM 2 and DIM 3 tissues was below the sensitivity of the immunoperoxidase assay as Durban et al. detected very low levels of MMTV proteins in control tissues using Western blotting. However, even if MMTV protein production did correlate with MMTV RNA expression, it did not correlate with tumorigenicity. The DIM 4 HOGs which were assayed for gp52/gp36 were taken from transplant generations 2–4, which exhibited a lower tumor incidence (40%) and a longer latency (>12 months) than HOGs from the DIM 2 or DIM 3 line (75%, 6.2 months; and 74%, 7.8 months, respectively). Thus MMTV RNA and protein expression did not support the notion of a directly oncogenic MMTV gene product active in DIM tissues.

The possibility of MMTV oncogenesis through enhancer insertion was examined by Southern analysis of DIM tissue DNA. Of BALB/c origin, DIM tissues contained endogenous MMTV proviruses characteristic of BALB/c, Mtv-6, -8, and -9. Other HOGs and tumors of low mammary tumor incidence mouse strains have been found to contain extra proviral copies of MMTV which presumably arose from one of these endogenous proviral templates. Gray et al. (13) found a new proviral copy in three different BALB/c preneoplastic lines induced by chemicals and hormones and Knepper et al. (38) reported a DMBA-induced BALB/c mammary tumor with a new proviral copy. Also, Svec (39) identified Mtv-8 as the source of an extra proviral copy. In BALB/c mammary gland, int-2, int-3, and int-H. The results of several studies suggest that int-1 and int-2 are not likely candidates for DIM-associated oncogenes. The particular int locus implicated seems to have some dependence upon both the MMTV strain and the mouse strain. For example, 100% (9 of 9) of C3H spontaneous mammary tumors had a MMTV provirus integrated in int-1. This decreased to 40% (7 of 18) when the C3H exogenous virus was used to infect BALB/c mice (BALB/cfC3H) (42). Similarly, the GR virus was found integrated in int-2 in 44% (12 of 27) of GR spontaneous mammary tumors and in only 11% (1 of 9) of BALB/c-GR tumors (42). Additionally, Gray et al. (13) found that extra endogenous proviral copies were integrated in domains other than int-1 or int-2 in three different BALB/c HOG lines. Knepper et al. (38) reported that BALB/c DMBA-induced tumors did not express int-1 or int-2 transcripts regardless of extra proviral integrations. The int-3 locus remains a candidate presumptive protooncogene because it has recently been identified in Czech II mice (14) and has not been as thoroughly investigated. The int-H locus is currently the most intriguing int for BALB/c as it was identified in BALB/c...
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preneoplasms (13). However, a transcript has not yet been identified for this region.

In addition to the introns, a number of protooncogenes which were originally identified in other systems have been implicated in breast cancer [e.g., myc (43), ras (44), neu (45)]. The DIM tissues provide an excellent murine model system to examine the potential function of these protooncogenes in preneoplasms and neoplasms inasmuch as MMTV is not involved in their inception.

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