Transfer, by Selective Breeding, of the Pathogenic *Mtv-2* Endogenous Provirus from the GR Strain to a Wild Mouse Line Free of Endogenous and Exogenous Mouse Mammary Tumor Virus

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The GR laboratory mouse strain has five endogenous proviral copies of the mouse mammary tumor virus (MMTV). One of these, *Mtv-2*, is unique because it causes mammary carcinomas in virtually 100% of breeding GR females prior to 1 year of age. To facilitate studies of this locus in particular, and mammary tumorigenesis in general, we genetically tailored a new mouse line, WXG-2, which bears *Mtv-2* as its only endogenous MMTV provirus. The WXG-2 line was constructed by making hybrids between the GR strain and a wild mouse line free of both endogenous and exogenous MMTV, backcrossing to the MMTV-free line, and fixing the *Mtv-2* locus in a population with the desired genotype. Mammary tumors were observed in 5 of the 20 hybrid females carrying the endogenous *Mtv-2* provirus. The WXG-2 line represents a new model system for studying MMTV-induced mammary tumorigenesis in the absence of multiple endogenous proviruses.

Multiple endogenous proviral copies of the mouse mammary tumor virus (MMTV) genome are present in the germ line of all tested inbred mouse strains (43). For example, the GR strain carries five endogenous MMTV proviruses (29). These proviruses complicate studies of the exogenous proviruses observed in mammary hyperplasias and tumors, particularly when methods such as Southern blot analysis (5-7, 10, 12, 13, 15, 19, 24, 30) and genomic cloning (34, 35) are used. The objective of the experiments reported here was to develop a new mammary tumorigenesis system that is more amenable to molecular studies by transferring the pathogenic *Mtv-2* endogenous provirus from the inbred GR strain to an endogenous and exogenous MMTV-free wild mouse line (8).

The *Mtv-2* locus was first defined genetically (2) as a stable dominant Mendelian locus responsible for high titers of milk-borne virus at the first lactation and a high frequency of mammary tumors at a relatively young age (45) and has been localized to mouse chromosome 18 (28). Southern blot comparisons of genomic DNAs between the high-tumor-incidence GR strain (31) and the congenic low-tumor-incidence GR-*Mtv-2*⁻ strain (44) revealed that the congenic strain is missing one of the five endogenous MMTV proviruses that is present in GR (13, 28). The *Mtv-2* provirus is found in heterologous cells infected with virus from a GR mammary tumor cell line (14). In addition, the *Mtv-2* provirus is present at increased copy number in GR mammary tumors and hyperplasias as well as in infected normal mammary glands (13, 15). The *Mtv-2* locus, therefore, harbors a single endogenous provirus that is both expressed and pathogenic.

Transfer of the *Mtv-2* locus from GR to the MMTV-free wild mouse line was accomplished by selective breeding for a hybrid wild mouse line carrying only the *Mtv-2* provirus. MMTV genotypes of the hybrids were determined by Southern blot analysis of DNAs from peripheral blood by using

MMTV probes and checking for characteristic *Mtv-2* restriction fragments. This method, called genetic tailoring, was initially proposed by Cohen et al. (8). The new mouse line is named WXG-2.

MATERIALS AND METHODS

Mice. The GR mouse strain, also called GR/A and GRS/A (31, 41), was obtained from The Netherlands Cancer Institute in 1980 and has been bred for 16 generations in our laboratory. The ev⁻ wild mouse line is an outbred population from an endogenous MMTV-free breeding stock of *Mus musculus domesticus* discovered near Lake Casitas, Calif. (8, 9). This line has no detectable MMTV antigens in normal mammary tissues, hyperplastic alveolar nodules, or DMBA-induced mammary tumors (16). No spontaneous mammary tumors have been observed in our colony of more than 200 breeding females. MMTV proviral DNA has not been detected in ev⁻ DNAs from normal tissues or DMBA-induced mammary tumors (17).

DNA extractions. DNAs from peripheral blood were isolated by a modification of the method described by Kunkel et al. (23). Tail incisions were made; and 10 to 20 drops of blood were collected in heparinized tubes, brought to 3 ml with cold 10 mM Tris (pH 7.6)-0.32 M sucrose-5 mM MgCl₂-1% Triton X-100, and disrupted in a Dounce homogenizer. Nuclei were pelleted for 1 min in a Beckman microfuge, and the pellets were suspended in 0.2 ml of 10 mM Tris (pH 8.0)-10 mM NaCl-10 mM EDTA. Proteinase K was added to 0.5 mg/ml, and sodium dodecyl sulfate was added to 0.5%. The mixtures were incubated for 30 min at 37°C and then extracted once with buffer-equilibrated phenol, once with buffer-equilibrated phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1). After extraction, the DNAs were ethanol precipitated twice and suspended by vortexing in 0.1 ml of 10 mM Tris (pH 8.0)-10 mM NaCl-0.1 mM EDTA. This procedure yielded sufficient DNA for two to four Southern blot analyses.

Tissue DNAs were isolated by mincing frozen tissue with

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a razor blade and homogenizing in a Dounce homogenizer in 10 mM Tris (pH 8.0)–10 mM NaCl–10 mM EDTA. The cell suspensions were lysed in 0.5% sodium dodecyl sulfate, extracted as described above, and dialyzed for 48 h in the cold against several changes of 10 mM Tris (pH 8.0)–10 mM NaCl–0.1 mM EDTA. DNA concentrations were determined by absorbance at 260 nm or empirically by agarose gel electrophoresis, ethidium bromide staining, and comparison with a known standard (27).

Restriction enzyme digestion and Southern blot analysis. Restriction enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). Cellular DNA (5 to 10 µg) was mixed with 0.5 µg of bacteriophage lambda DNA and digested with a 5- to 10-fold excess of enzyme in 100 mM Tris (pH 7.4)–10 to 100 mM NaCl–5 mM MgCl₂–50 µg of lysozyme per ml for 2 to 3 h at 37°C. Digested DNAs were electrophoresed in horizontal 1% agarose gels containing 50 mM Tris base, 20 mM sodium acetate, 2 mM sodium EDTA, 22 mM NaCl, and 30 mM acetic acid. After electrophoresis, the gels were stained with ethidium bromide, and the lambda restriction patterns were checked to verify the completeness of digestion. The restriction fragments were transferred to nitrocellulose by the method of Southern (40) in 20× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) (42). Hybridization was in 2 ml of 45% deionized formamide–3× SSC–3× Denhardt solution–100 µg of yeast RNA per ml–50 µg base-sheared calf thymus DNA per ml at 37°C for 3 days with probe at a concentration of 2×10^6 to 3.5×10^6 cpm/ml. The blots were washed at room temperature once in 2× SSC for 10 min, twice in 0.2× SSC for 10 min, and once in 2× SSC for 30 min at 65°C. Three- to five-day autoradiographic exposures with Kodak XAR-5 film at –70°C with a Dupont Cronex Lightening-Plus intensifying screen were routinely required (27).

Preparation of radiolabeled DNAs. Insert DNAs from various plasmids were purified by digestion with the appropriate enzymes, polyacrylamide gel electrophoresis, and electroelution of the desired fragments. Purified insert DNAs (100 ng) were nick translated with 50 µCi of [α -³²P]dATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in a 10-µl reaction mixture containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 50 µg of bovine serum albumin BSA per ml, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 10 pg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.5 U of DNA polymerase I (Bethesda Research Laboratories) per µl at 15°C for 0.5 to 2 h (36). Unincorporated [³²P]dATP was removed by gel filtration through Sephadex G-50. Probes routinely had a specific activity of 2×10^8 to 4×10^8 cpm/µg.

Probes. *Mtv-2* 3'-flanking DNA (probe 4A) was isolated from the plasmid p4S-D by digestion with *Sst*I and *Fnu*4H-I and gel purification of the 2.0-kilobase (kb) flanking DNA fragment (see Fig. 3C). p4S-D is a plasmid clone of the 2.3-kb 3' host genome-virus genome junction *Sst*I fragment of the endogenous *Mtv-2* provirus which was subcloned from lambda genomic clone λGRT4. λGRT4 is a 11.0-kb *Mbo*I partial digestion restriction fragment from GR cloned in λL47.1 that contains the 3' 7.8 kb of the endogenous *Mtv-2* provirus and 3.2 kb of flanking DNA (D. W. Morris, H. D. Bradshaw, Jr., and R. D. Cardiff, unpublished data).

pBR322 clones of three of the four internal *Pst*I restriction fragments from unintegrated exogenous C3H-MMTV provirus were provided by Majors and Varmus (26) and used to prepare MMTV-specific probes. In this report an equal-mass mixture of the inserts from the three plasmids is termed rep probe.

RESULTS

Breeding. The breeding strategy is illustrated in Fig. 1. For the sake of clarity, only the lineage leading directly to the WXG-2 line is shown. Numerous other lineages were established and abandoned subject to the vagaries of genotype, breeding success, and animal death. To avoid transmitting any MMTV variants that were potentially present in the GR milk-borne virus population to the WXG-2 line, females for the initial cross and two backcrosses were from the *ev*[–] wild mouse line.

The GR strain carries five endogenous MMTV proviruses, *Mtv-2*, *Mtv-3*, *Mtv-8*, *Mtv-19*, and *Mtv-20*, all on different chromosomes (4, 29, 33). Random segregation of the different proviral loci eliminated three of the four unwanted loci in male 3922 (Fig. 1) from the first backcross generation (BC₁). The remaining unwanted locus was missing from female 4109 (Fig. 1) from the second backcross generation (BC₂). A male with a similar genotype was not found due to low numbers of progeny from the 1091 × 3922 cross (Fig. 1), delaying by one generation a cross to fix the *Mtv-2* locus. The colony is now in the F₄ generation.

Female 4109 was the only animal from the pedigree chart to develop a mammary tumor. Several other tumors developed in *Mtv-2*-bearing animals from lineages not shown.

Genotyping. Mice were genotyped directly by Southern blot analysis of *Sst*I-cleaved DNAs from peripheral blood using MMTV probes. Blood samples could be collected at as early as 6 weeks of age. The ability to determine the genotype at an early age and the lack of ambiguity inherent in the methods used in this study were significant advantages over the standard biological assays for the *Mtv-2*-associated phenotype (44, 45).

An example of the genotyping data is shown in Fig. 2. All DNAs were from the progeny of male 3922 (Fig. 1). The *Mtv-2* provirus, detectable in lanes 3, 4, 5, and 7 (Fig. 2A), has four characteristic *Sst*I restriction fragments of 6.1, 2.5, 2.3, and 1.2 kb. These bands were assigned to the *Mtv-2* locus by Southern blot comparisons of GR and GR-*Mtv-2*[–] DNAs (13). The second provirus present in this group of animals was identified as *Mtv-3* on the basis of its *Eco*RI restriction pattern determined with DNAs from animals carrying only this provirus (fragments of ~21, 6.9, and 0.9 kb; data not shown). These fragments were previously shown to cosegregate (25). They have been assigned to the *Mtv-3* locus based on concordance between the presence of the fragments and p27 expression in a group of recombinant inbred lines between BALB/c and STS/A (29). A low level of p27 expression is characteristic of the *Mtv-3* provirus (33) which is present in the STS/A strain. The unique low-molecular-weight band in lane 4 (Fig. 2A) is unexplained.

Homozygotes at the *Mtv-2* locus were selected in the F₂ generation by Southern blot analysis with host DNA probe 4A from unique sequences flanking the endogenous *Mtv-2* provirus (Fig. 3). DNAs digested with *Bgl*II from animals homozygous for *Mtv-2* and those without *Mtv-2* were clearly distinguished when they were probed with probe 4A (Fig. 3A). Probe 4A recognized host *Bgl*II restriction fragments of 4.2 and 2.6 kb. Interruption of the 4.2-kb fragment by the *Mtv-2* provirus resulted in a 3.1-kb host genome-virus genome junction fragment. As expected, DNAs from animals known to be heterozygous for *Mtv-2*, based on their parentage, had a composite of both restriction patterns (data not shown). The major band recognized by probe 4A in animals carrying the *Mtv-2* provirus comigrated with the 3' host genome-virus genome junction fragment, as predicted

FIG. 1. Breeding scheme leading to the WXG-2 line. The MMTV genotypes of males 1360, 3826, and 3922 are given in parentheses, and the genotypes of the other animals are indicated in the legend. A female from the endogenous MMTV-free Lake Casitas *ev*⁻ wild mouse line was crossed with a GR male, and the male progeny were backcrossed twice to the *ev*⁻ line. Brother-sister mating was started with the progeny of the second backcross, designated the parental (P) generation. The desired *Mtv-2*-carrying line was established in the F₂ generation of inbreeding.

(Fig. 3). Note that the GR strain has 10 junction fragments from five proviruses detectable with long terminal repeat (LTR) probe. The WXG-2 line retains two fragments, while the *ev*⁻ line is completely free of provirus (Fig. 3B). The two WXG-2 *Bgl*II fragments recognized by the LTR probe are present in the GR strain but missing from the congenic GR-*Mtv-2*⁻ strain (data not shown).

The *Sst*I and *Bgl*II restriction maps of the *Mtv-2* locus and the *Sst*I restriction map of the *Mtv-3* locus (Fig. 2B and 3C) were established by Southern blot analyses with kidney DNA from animals from the breeding program in this study that carried only the provirus of interest. The samples were probed with LTR and probe 4A DNAs and various DNAs from specific internal regions of MMTV. Whenever possible, the sites were verified by restriction mapping of lambda clones containing portions of these loci.

Mammary tumors. Twenty females carrying the *Mtv-2* locus were identified in the parental through F₂ generations. Of these, five developed palpable mammary tumors at an average age of 9 months. One mammary tumor was also observed in an actively breeding male from the BC₁ generation at an age of 10 months. All were A- or B-type adenocarcinomas (11, 38) and were expressing MMTV antigens, as determined by the immunoperoxidase technique (39). In addition, four animals developed splenic lymphomas at an average age of 14 months. We assume that these lymphomas were caused by exogenous murine leukemia viruses transmitted from the progenitor *ev*⁻ line (18). Studies have not yet been performed to rigorously determine the tumor incidence or frequencies of hyperplastic alveolar nodules and hormone-dependent tumors in the colony. As inbreeding proceeds, the colony is likely to change with

respect to these characteristics. Lineages with mammary tumor-bearing females are preferentially selected for inbreeding in an effort to increase the tumor incidence of the colony.

DISCUSSION

In this report the construction of a new mouse line for mouse mammary tumor virus studies has been described. The line was genetically tailored for use in MMTV-induced tumorigenesis experiments which would be facilitated by a simpler MMTV-genetic background than is available in inbred strains. An example, which is currently being pursued in numerous laboratories, is the cloning of host DNA sequences that flank exogenous MMTV proviruses (34, 35). In contrast to the GR strain, working with the WXG-2 line (i) makes it easy to assess the total number of exogenous proviruses in tumor samples; (ii) decreases the number of MMTV-positive clones that must be restriction mapped to identify host genome-virus genome junction clones from any given exogenous provirus; and (iii) allows direct restriction mapping of exogenous proviruses and their flanking sequences with genomic DNA (from samples with single, newly acquired proviruses) because of a decrease in the background of endogenous proviral fragments on Southern blots. In addition, the level of exogenous provirus in WXG-2 tumors is low. At least two tumors have already been identified with single, newly acquired proviruses. Neither has an interrupted *int-1* (34) or *int-2* (35) locus D. W. Morris, R. Strange, R. D. Cardiff, unpublished data).

The endogenous *Mtv-2* provirus, rather than just the exogenous milk-borne virus, was transferred to the MMTV-free wild mouse line for several reasons. First, the milk-

FIG. 2. Genotyping data. (A) Southern blot analysis of DNAs from peripheral blood from BC₂ progeny of the 1091 × 3922 cross (Fig. 1) to determine MMTV genotypes. The samples were digested with *Sst*I and probed with the MMTV rep probe. The provirus assignments of the various restriction fragments are indicated. The film was overexposed to verify the presence of the weaker bands. The lanes contain DNAs from female 3981 (lane 1), female 3983 (lane 2), female 4104 (lane 3), male 3980 (lane 4), male 4105 (lane 5), female 4106 (lane 6), female 4109 (lane 7), and male 4108 (lane 8). Descendants of female 4109 and male 4108 were used to establish the WXG-2 line. The other animals were from lineages that were terminated. (B) *Sst*I restriction maps of the two proviruses shown in part A. The 6.1-, 2.5-, 2.3-, and 1.2-kb fragments from *Mtv-2* are characteristic of this provirus and can be used to distinguish it from the other four proviruses endogenous to GR.

borne virus is often lost from inbred strains that are resistant to MMTV (22). It is not known whether the *ev*⁻ line can support the long-term replication and transmission of exogenous GR virus. The introduction of endogenous *Mtv-2* into the *ev*⁻ line allows for a continuous replenishing of the exogenous infection. Second, the role of the *Mtv-2* locus in mammary tumorigenesis is not completely understood (20, 21, 32). The WXG-2 line provides an opportunity to study the effect of this locus and its products without the potential of interference from the other four GR proviruses, at least one of which, *Mtv-3*, is also expressed (33). Third, the presence of the *Mtv-2* locus in the germ line allows study of the hormone-regulated provirus (37) in normal mammary tissues as well as in nonmammary tissues during embryogenesis, development, and differentiation.

The tumor incidence in the P through F₂ generations confirms the observation that wild mice are relatively resistant to MMTV-induced tumorigenesis. Mammary tumors were observed in only 25% of the P through F₂ mice carrying the *Mtv-2* locus, which is considerably less than would be expected in the parental GR strain with a tumor incidence of > 95% at 12 months of age. While the WXG-2 mice are not strictly wild mice, but rather hybrids, the majority of their genetic composition is derived from the *ev*⁻ line. Gardner and Rasheed (18) were able to infect wild mice from Lake Casitas, Calif., with C3H/HeJ virus by foster nursing, but

only 20% of the animals developed tumors at 1 to 2 years of age. In earlier work, Andervont (1) infected wild mice from the Rockefeller Institute (New York, N.Y.) with C3H virus, also by foster nursing, but observed mammary tumors in only 11% of the females at an average age of 16 months. The tumor incidence of the uninfected line was <3%.

The results from this work demonstrate that the natural resistance of wild populations is not simply a resistance to viral infection (because every cell harboring *Mtv-2* is infected), but rather resistance at other levels in the process of tumorigenesis. These data support the Bittner three-factor hypothesis of mammary tumorigenesis: that complex interactions involving the milk factor (MMTV), hormones, and various host genetic factors affect or control tumor formation (3). Only in highly susceptible strains such as BALB/c and GR is the tumorigenic phenotype of the *Mtv-2* locus fully penetrant.

Despite the strong attenuation of the pathogenic effects of *Mtv-2* by the wild mouse genome, the tumor incidence of the WXG-2 line is sufficient to make tumorigenesis studies practical. The WXG-2 line therefore represents a new model system for studying MMTV-induced tumorigenesis. This system simplifies a variety of studies currently complicated by the presence of multiple endogenous proviruses in other

FIG. 3. Southern blot analysis of DNAs from the WXG-2 line and its progenitors, the GR strain and the *ev*⁻ line. (A) Analysis of homozygosity at the *Mtv-2* locus. DNAs were cleaved with *Bgl*II and probed with unique sequence *Mtv-2*-flanking DNA probe 4A. The WXG-2 line has the same probe 4A restriction pattern as the GR strain, whereas the *ev*⁻ line has a different pattern due to the absence of the *Mtv-2* provirus. (B) Verification of the MMTV genotypes. A replicate filter was hybridized with the LTR probe which recognizes two host genome-virus genome junction fragments per provirus. (C) *Bgl*II restriction map of the *Mtv-2* locus and the locations of probe 4A and the LTR probe. The restriction sites used to isolate the probes are also indicated: P, *Pst*I; F, *Fnu*4HI; and S, *Sst*I.

mouse systems. The line will also be useful to investigators studying the basic virology of MMTV.

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