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Development of a Mouse Mammary Tumor Virus-Negative Mouse Strain: a New System for the Study of Mammary Carcinogenesis

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All inbred strains of mice transmit one or more copies of mouse mammary tumor virus (MMTV) DNA integrated as proviral sequences. This complicates efforts to define viral-induced mammary carcinogenesis. Here we report the use of surgical nonlethal splenectomy in tissue typing mice and the development of an MMTV-negative mouse strain. The MMTV-negative strain allows study of the involvement of non-MMTV genes in mammary carcinogenesis. In addition, it can be used as a sterile background into which MMTV variants can be introduced. Through the techniques described here, mice containing single MMTV loci or specific combinations can be specially chosen and rapidly developed. In this manner, the oncogenecity of particular MMTV variants may be assessed.

Mouse mammary tumor virus (MMTV) is a retrovirus which may be transmitted either exogeneously as a milk-borne virus or as a genetically transmitted (endogenous) provirus, i.e., a viral genome integrated and covalently linked to the host genome (3, 6). When milk-borne infection occurs, tumors develop at an early age (6 to 9 months). However, even in the absence of any infectious virus, mammary tumors occur in inbred mouse strains, although usually at a lower incidence and later age (12 to 18 months) (1, 7). Thus, in considering the mechanism of tumor induction by these viruses, one must evaluate the role of both endogenously and exogenously acquired proviral sequences.

A major obstacle in defining viral-induced mammary carcinogenesis is the observation that all inbred mouse strains are known to harbor endogenous MMTV sequences (4, 6). Characterization of these proviruses by biochemical means has revealed at least 14 separate virus-specific loci (4, 10) among various inbred strains of mice. Thus, the participation of any one of these loci, the interaction of multiple loci, and the coordinate expression of endogenously and exogenously acquired proviruses cannot be determined with the strains of inbred mice presently available.

During studies of endogenous, MMTV-specific proviruses of inbred and feral mice (*Mus musculus domesticus*) (4), it was observed that two individual animals of the feral population at a squab farm near Lake Casitas, Calif., did not have any endogenous MMTV. All other feral *Mus musculus* tested were found to have one or more endogenous proviruses. These results, along with characterization of endogenous MMTV proviruses of inbred mouse strains, led to the conclusion that these sequences were acquired by multiple independent infections of the germ line and that MMTV proviruses are not essential to the development of mice. From these data, the idea of developing a line of mice devoid of endogenous MMTV sequences was conceived. In the work here reported, a procedure for the selection and breeding of animals with defined genotypes was used in the development of a line of mice lacking all endogenous MMTV sequences.

MATERIALS AND METHODS

DNA extraction and restriction endonuclease digestion. DNA was extracted from the livers of mice which fasted overnight as described previously (3). Briefly, liver homogenates were incubated with sodium dodecyl sulfate and proteinase K to deproteinate the sample. Next, phenol-chloroform (2:1) extraction and ethanol precipitation by spooling on a glass rod were performed. DNAs were suspended in 5 mM Tris-hydrochloride (pH 7.4)–0.1 mM EDTA for storage. DNA was cleaved with a restriction endonuclease, either *Eco*RI or *Pst*I, under conditions recommended by the supplier (New England Biolabs, Beverly, Mass.). Reactions were judged complete on the basis of lambda bacteriophage DNA included in the reac-

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tions and visualized by ethidium bromide staining and UV irradiation.

Gel electrophoresis, DNA transfer, and hybridization. After restriction endonuclease digestion, DNA samples were electrophoresed in horizontal slab gels of 0.8% agarose in buffer containing 0.04 M Tris-acetate (pH 8.15), 0.02 M sodium acetate, 0.018 M NaCl, and 0.002 M EDTA. DNA was transferred to nitrocellulose sheets by a modification of the procedure originally described by Southern (8). Virus-specific fragments were detected by hybridization with 1×10^6 to 2×10^6 cpm of ^{32}P-labeled MMTV representative cDNA synthesized from cloned MMTV DNA (a generous gift from J. Majors, University of California, San Francisco) with calf thymus oligomers used as primers (3). The annealing buffer included 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 0.05 M *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0), 200 μg of yeast RNA per ml, 50 μg of alkali-sheared and denatured salmon sperm DNA per ml, and Denhardt solution (0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone) (5). After preannealing in the above buffer without cDNA for 12 h at 41°C, filters were annealed with cDNA for 48 h. After incubation, filters were sequentially washed in 2× SSC at room temperature, 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C, and 0.1× SSC at room temperature. Filters were air dried and exposed to Kodak XAR film at −70°C in the presence of Dupont Cronex Lightning Plus intensifying screens (9).

RESULTS AND DISCUSSION

Since the majority of endogenous proviruses are phenotypically silent, it was necessary to use biochemical means to assess the MMTV genotype of animals before breeding. The method developed involved the nonlethal surgical splenectomy of mice followed by the extraction of DNA for analysis by restriction endonucleases (3). In this manner a large number of animals were screened, and breeding pairs were established. Since the Lake Casitas population was known to harbor MMTV-negative animals, individual animals from this group were biochemically genotyped as an initial step in developing this new line of mice. From a total of more than 60 animals, two successful breeding pairs were established from presumptive negative animals. The results of this selected breeding are presented in Fig. 1.

The data in Fig. 1 are from a representative sample of animals from progeny obtained from the F4 generation. At the time of analysis, all samples were identified by number only so that any bias would be avoided. As can be seen from data of the progeny from the mating of feral animals, five of nine animals were found to be completely negative for all endogenous MMTV sequences (Fig. 1). Controls for this negative result include: (i) the ethidium bromide staining of the gel before transfer to nitrocellulose paper (Fig. 1, bottom panel), showing that digested

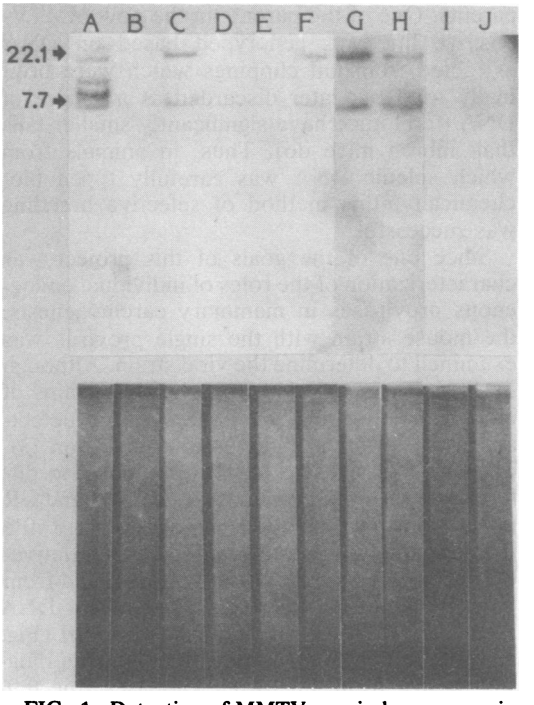

FIG. 1. Detection of MMTV proviral sequences in feral mice. DNA from the livers of either inbred BALB/c mice (lane A) or selectively bred feral mice (lanes B through J) was digested with the restriction endonuclease *Eco*RI and was electrophoresed on 0.8% agarose gels as described previously (3). After electrophoresis, the gel was stained with ethidium bromide for photographing with a UV transilluminator (lower panel), and then the DNA was transferred to nitrocellulose sheets for hybridization with virus-specific, ^{32}P-labeled, representative cDNA (1, 3). After hybridization, virus-specific bands were detected by autoradiography (upper panel). The size in kilobases of selected fragments (indicated on the left) was determined by including in a parallel lane (not shown) a *Hin*dIII digestion of ^{32}P-labeled lambda bacteriophage DNA.

DNA was present in all lanes; (ii) the *Eco*RI digestion of DNA from the BALB/c strain (Fig. 1, lane A), demonstrating both the specificity and sensitivity of the hybridization reaction; and (iii) radiolabeled *Hin*dIII-digested lambda bacteriophage DNA (not shown), indicating both the successful transfer of DNA and the molecular weight of virus-specific fragments.

When the results of biochemical analysis were compared with the breeding history of the two original breeding pairs, it was found that the four animals positive for MMTV (Fig. 1, lanes C, F, G, and H) were derived from one of the pairs, whereas the five negative animals were progeny of the remaining pair. An explanation for this provirus escaping detection was obtained by examining the original genotyping data on the

parents. One of the parents in the now MMTV-positive line was genotyped based on DNA extracted from tail clippings which were originally tried but later discarded as a source of DNA (feral mice have significantly smaller tails than inbred mice do). Thus, in animals from which splenic DNA was carefully typed biochemically, this method of selective breeding was successful.

Since one of the goals of this project was characterization of the roles of individual endogenous proviruses in mammary carcinogenesis, the mouse strain with the single provirus was examined to determine the viral strain. Although MMTV strains isolated from various strains of inbred mice are related, differences are detectable by restriction endonuclease digestion (3). The enzyme *Pst*I was shown previously to differentiate the milk-borne viruses of C3H and GR mice from those found endogenous to the BALB/c and other strains and from the relatively less oncogenic milk-borne virus isolated from the C3Hf mouse strain (3). Digestion of DNA from the MMTV-positive strain with *Pst*I (Fig. 2) resulted in four virus-specific fragments ranging in size from 3.8 to 0.9 kilobases (the 0.9-kilobase fragment was difficult to detect since part of it was deleted from the clone we used in synthesizing radiolabeled cDNA). The 3.8-kilobase fragment was found previously to be unique to the highly oncogenic strains of MMTV transmitted in the milk of both C3H and GR mouse strains. Therefore, the MMTV-positive strain has a single endogenous provirus that is closely related, if not identical, to the milk-borne virus in these two inbred mouse strains.

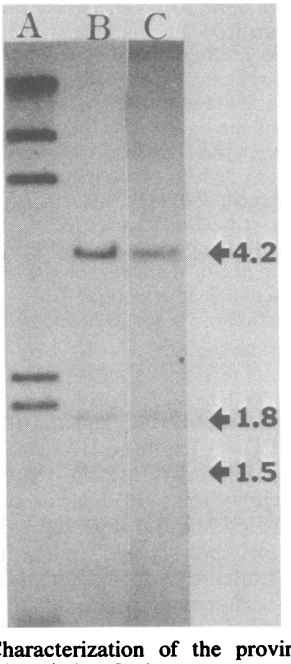

FIG. 2. Characterization of the provirus in the MMTV-positive Lake Casitas mouse strain. DNA from the livers of generation 5 mice was digested with the endonuclease *Pst*I and was analyzed as described in the text. The size of MMTV-specific fragments is indicated on the right in kilobases. (A) *Hin*dIII-digested ^{32}P-labeled lambda bacteriophage DNA molecular weight marker; (B and C) *Pst*I-digested DNA from MMTV-positive mice.

In this report a procedure is described by which mice of a specific genotype may be produced in a very short interval of time. Biochemical genotyping of a feral outbred population of mice before breeding permitted the production of a line of mice completely devoid of endogenous MMTV sequences in a single generation. This procedure was termed genetic tailoring since it designs animals to suit a particular purpose.

In this case, a mouse line was established which is ideal for studying the involvement of nonviral genes in mammary tumorigenesis. Furthermore, a line was produced which has only one endogenous MMTV sequence. Therefore, it is now possible to study the oncogenic potential of this virus in the genetically transmitted state and to ask questions concerning the modulation of an isolated provirus by hormone therapy and treatment with chemical carcinogens. In addition, the MMTV-negative line can be used as a sterile background into which other MMTV proviruses can be introduced either singly or in combination with other endogenous loci or the milk-borne virus. By the use of our genetic tailoring procedure, crosses between the MMTV-negative and established inbred mouse strains can be genotyped in the progeny of the F1 backcross to the MMTV-negative parent. All individuals in this population will be heterozygous for the MMTV loci derived from the inbred parent. However, all unlinked MMTV loci in the inbred parent will have segregated randomly (7). Biochemical genotyping will show which individuals to breed to obtain a new line which is homozygous for MMTV loci and of a defined genotype. Therefore, by selected crosses of the F1 backcross progeny, the next generation may have any desired array of endogenous MMTV proviruses. In this manner, it will be possible to obtain mouse strains which can then be used in experiments to evaluate carefully the role and interaction of individual MMTV-specific loci in mammary carcinogenesis.

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LITERATURE CITED

1. **Bentvelzen, P.** 1974. Host virus interactions in murine mammary carcinogenesis. Biochim. Biophys. Acta **355**:236–259.
2. **Cohen, J. C., J. E. Majors, and H. E. Varmus.** 1979. Organization of mouse mammary tumor virus-specific DNA endogenous to BALB/c mice. J. Virol. **32**:483–496.
3. **Cohen, J. C., P. R. Shank, V. L. Morris, R. Cardiff, and H. E. Varmus.** 1979. Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. Cell **16**:333–345.
4. **Cohen, J. C., and H. E. Varmus.** 1979. Endogenous mammary tumor virus DNA varies among wild mice and segregates during inbreeding. Nature (London) **278**:483–496.
5. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:631–646.
6. **Morris, V. L., E. Medeiros, G. M. Ringold, J. M. Bishop, and H. E. Varmus.** 1977. Comparison of mouse mammary tumor virus-specific DNA in inbred, wild and Asian mice and in tumors and normal organs from inbred mice. J. Mol. Biol. **114**:73–91.
7. **Nandi, S., and C. M. McGrath.** 1973. Mammary neoplasia in mice. Adv. Cancer Res. **17**:353–414.
8. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
9. **Swanstrom, R., and P. R. Shank.** 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioisotopes ^{32}P and ^{125}I. Anal. Biochem. **86**:184–192.
10. **Traina, V. L., B. A. Taylor, and J. C. Cohen.** 1981. Genetic mapping of endogenous mouse mammary tumor viruses: locus characterization, segregation, and chromosomal distribution. J. Virol. **40**:735–744.