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# Mammary Tumorigenesis in Feral *Mus cervicolor popaeus*

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**A pedigreed breeding population of feral *Mus cervicolor popaeus* with a high incidence of mammary tumors, arising between 6 and 14 months of age, is described. These mice were chronically infected with a type B retrovirus which is distantly related to the mouse mammary tumor virus (MMTV) of inbred strains of *Mus musculus*. MMTV-induced mammary tumors in inbred mice frequently (80%) contained an insertion of the viral genome into the *int-1* or *int-2* loci of the tumor cellular genome. These two cellular genetic loci were also altered by viral insertion in 11 of 20 *M. cervicolor popaeus* mammary tumor cellular DNAs tested. Results of our study of mammary tumorigenesis in feral mice demonstrate that viral-induced rearrangement and activation of the *int* loci are not limited to the genetic background of inbred mice selected for highly infectious MMTV and a high incidence of mammary tumors.**

The study of mammary cancer in laboratory mice has focused on a few inbred strains of *Mus musculus* which have a high incidence of mammary adenocarcinomas (30). The development of mammary tumors in these mice is associated with a chronic infection of the mammary tissue by the mouse mammary tumor virus (MMTV). The inbred mouse strains with high incidences of mammary adenocarcinomas are the result of a continuous selection for highly virulent MMTV and a cellular genetic background which render the mouse more susceptible to mammary tumorigenesis.

MMTV, like other retroviruses, can act as an insertion mutagen on the cellular genome of infected cells. Recently, two cellular genetic loci (*int-1* and *int-2*) have been described which are frequently occupied by a MMTV genome in mammary tumor cellular DNA (18, 19). The *int-1* locus was identified in mammary tumors of the C3H strain, and the *int-2* locus was identified in mammary tumors of the BR6 (infected with MMTV [RIII]) strains of mice. The two *int* loci are unrelated by nucleotide sequence analysis and are located on different chromosomes (17, 20). The insertion of a MMTV genome at either loci activates the expression of a cellular gene within the affected locus (11, 17). It has been proposed that the unique expression of these cellular genes contributes to the development of mammary tumors in mice (18, 19).

Previous surveys of feral *Mus musculus* demonstrated the presence of poorly infectious MMTV in the milk of breeding females (2). In general, the incidence of mammary tumors in feral breeding females is low, and tumor development occurs late in life (3, 22). In this report we describe a pedigreed breeding colony of *Mus cervicolor popaeus* which has a high incidence of mammary tumors.The present study was undertaken to determine whether the involvement of the *int-1* and *int-2* loci in mammary tumors extends to feral species of mice which are reproductively separate from *M. musculus*. We have previously shown that *M. cervicolor popaeus* milk and mammary tumors contain MMTV-like particles (designated MC-MTV) and viral-associated proteins which are immunologically related to, but distinguishable from, the laboratory strains of MMTV (9, 14, 24, 28). Cellular DNA from these mammary tumors contains additional MMTV proviral genomes compared with normal cellular DNA from the corresponding individuals. Analysis of the genetic integrity of the *int-1* and *int-2* loci showed that, respectively, 10 and 1 of 20 mammary tumor cellular DNA contained an MMTV genome within these loci. These results demonstrate the existence of infectious MC-MTV in mammary tumors of the evolutionarily separate species *M. cervicolor* and provide support for the involvement of the cellular *int* loci in mammary tumor development.

## MATERIALS AND METHODS

**The *M. cervicolor popaeus* breeding colony.** The characteristics of *M. cervicolor popaeus* and its evolutionary relationship with other species of mice have been described previously in detail (4, 6, 15). Feral *M. cervicolor popaeus* were trapped in the Tak province of Thailand during 1976 by J. T. Marshall (Smithsonian Institution, Washington, D.C.) and subsequently sent to our laboratory for the establishment of a pedigreed breeding colony. The founder population of this colony was composed of 11 breeding pairs of mice. At present, 7 of the original maternal lineage groups are represented in the colony. The colony is being maintained in a conventional manner and is housed at the National Cancer Institute contract site at Litton Bionetics, Inc. For the first six generations the colony was outbred to preserve the genetic diversity of the founder population. At generations seven and eight one maternal lineage group, designated line D, was composed of first cousin matings. In generations 9 through 12 inbreeding by brothers and sisters was instituted in this line. Since the establishment of the colony, the mice have been observed regularly for the development of tumors. Tumor-bearing mice were sacrificed by cervical dislocation, and autopsies were performed. Samples of tumor and normal tissue were prepared for histopathology and were frozen in liquid nitrogen.

**Restriction endonuclease digestion and agarose gel electrophoresis of high-molecular-weight DNA samples.** Cellular DNA was extracted from tumors and livers as described previously (13). Conditions used for restriction endonuclease digestion of DNA were those recommended by the suppliers (Bethesda Research Laboratories, Gaithersburg, Md., and New England Biolabs, Inc., Beverly, Mass.). The conditions for electrophoresis of the samples in 0.8% agarose gels, ethidium bromide staining, and transfer to

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TABLE 1. Mammary tumor incidence in *M. cervicolor popaeus* line D females

| Generation | Breeding females[a] | | | Virgin females[b] | | |
|---|---|---|---|---|---|---|
| | No. of mice | No. of mammary tumors | Average age at time of tumor development (mo)[c] | No. of mice | No. of mammary tumors | Average age at time of tumor development (mo)[c] |
| 1 | 1 | 1 | 8.6 | | | |
| 2 | 2 | 2 | 16 (15–21) | 4 | 0 | |
| 3 | 5 | 2 | 15.9 (15–16.8) | 13 | 1 | 24 |
| 4 | 8 | 3 | 13.3 (6.2–16.9) | 10 | 0 | |
| 5 | 5 | 5 | 15.1 (11–23.7) | 30 | 10 | 18.3 (8–20) |
| 6 | 22 | 9 | 21 (13.8–35.1) | 24 | 2 | 18.1 (17.3–18.5) |
| 7 | 11 | 6 | 16.9 (13.1–28.3) | 92 | 3 | 18.2 (13.3–25) |
| 8 | 4 | 4 | 16.6 (12–22.7) | 38 | 0 | |
| 9 | 12 | 5 | 13.1 (9–20.5) | | | |
| 10 | 10 | 5 | 9.6 (7.8–12.5) | | | |
| 11 | 6 | 5 | 11.9 (6–18) | | | |
| 12 | 3 | 2 | 11.9 (10–13.2) | | | |

[a] Breeding females over 12 months of age, unless they had a mammary tumor.
[b] After the generation eight, virgin or nonbreeding females were not maintained to determine spontaneous tumor incidence.
[c] The numbers in parentheses represent the age range, in months.

nylon membranes (Genatran 45; Plasco Inc., Woburn, Mass.) have been described previously (26).

**Extraction of RNA and selection of the poly(A)$^+$ fraction.** Total cellular RNA was isolated from tissues by the guanidium thiocyanate method (7). Poly(A)$^+$ RNA was prepared by binding to poly(rU) Sephadex (Bethesda Research Laboratories) in a buffer containing 0.5 M LiCl, 0.01 M Tris hydrochloride (pH 7.8), 0.001 M EDTA, and 0.1% sodium dodecyl sulfate (SDS). The samples were heated at 65°C for 10 min before they were loaded. The columns were washed extensively first with the same buffer and then with buffer containing only 0.1 M LiCl. The poly(A)$^+$ RNA was eluted with 90% formamide–0.01 M Tris hydrochloride (pH 7.5)–0.01 M EDTA–0.2% SDS.

Samples of poly(A)$^+$ RNA (3 μg) were denatured by incubation at 65°C for 15 min in 65% formamide–2.2 M formaldehyde, 2× RNA running buffer (1× RNA running buffer is 0.02 M morpholine propanesulfonic acid [pH 7.0], 0.001 M EDTA, 0.005 M sodium acetate). The RNA samples were electrophoresed in a 1% agarose gel (containing 1× RNA running buffer and 2.2 M formaldehyde) and transferred to a nylon membrane (Genatran 45; Plasco) as described previously (29).

**Preparation of labeled DNA probes.** Recombinant DNA plasmids containing molecular clones of portions of the MMTV genome were generously provided by J. Majors and H. Varmus (University of California): MMTV long terminal repeat (LTR) [pMMTV (C3H) 8.19, which contains the 1.4-kilobase-pair (kbp) *Pst*I fragment of the C3H viral genome], MMTV *gag-pol* [pMMTV (C3H) 2.5, which contains the 3.9-kbp *Pst*I fragment of the C3H viral genome], MMTV *env* [pMMTV (C3H) 8.21, which contains the 1.7-kbp *Pst*I fragment of the C3H viral genome]. A MMTV *gag*-specific probe was prepared (*Pvu*II to *Xho*I fragment) from the pMMTV (GR) 7.1a recombinant plasmid kindly provided by N. Hynes. The *int-1* 2.4-kbp *Eco*RI-*Bam*HI fragment, as well as the 2.0-kbp *Sac*I and the 1.8-kbp *Sac*I-*Eco*RI fragments from the 5′ and 3′ ends, respectively, of the *int-2* locus, were generously provided by R. Nusse and C. Dickson.

The recombinant DNAs used as probes were labeled with [$^{32}$P]dCTP to a specific activity of $5 \times 10^7$ to $1 \times 10^8$ cpm/μg by the nick-translation method of Rigby et al. (21).

**Hybridizations conditions.** After transfer of the nucleic acids, nylon membranes were rinsed in 6× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) and baked for 2 h at 80°C. Prehybridization was carried out at 37°C for at least 2 h in 30% formamide–5× Denhardt solution (10)–1% SDS–3× SSPE (20× SSPE is 3 M NaCl, 0.2 M $NaH_2PO_4$ [pH 7.4], 0.02 M EDTA)–2.5% dextran sulfate–0.001 M sodium phosphate (pH 6.5). The nylon membranes were then hybridized for 48 h at 37°C in heat-sealed plastic bags containing the hybridization solution (identical to prehybridization solution) mixed with $3 \times 10^6$ cpm of $^{32}$P-labeled plasmid DNA per ml of solution. Unreacted probe was removed from the membrane by washing one time at room temperature with 3× SSC–0.5% SDS and three times 15 min with 0.3× SSC–0.5% SDS at 60°C. The membrane were then exposed for autoradiography for 15 to 48 h.

## RESULTS

**A pedigreed breeding colony of feral *M. cervicolor popaeus* with a high incidence of mammary tumors.** Pedigreed breeding colonies of reproductively separate Asian species of the genus *Mus* were established to study the evolution of endogenous retroviruses and their role in neoplastic diseases (6). Since the establishment of these colonies in 1975 to 1976, the incidence of spontaneous tumors in each has been monitored. With one exception, all of the colonies exhibited a frequency of spontaneous tumors of 2% or less (unpublished data). One *M. cervicolor popaeus* colony (see above) had an overall spontaneous tumor incidence of 10%. Over half of these tumors were mammary adenocarcinomas. By tracing the ancestry of these mice, it was determined that all were descendants of three of the founding females, two of which also developed mammary tumors. The development of mammary tumors could not be directly linked to their paternal ancestry. The tumor incidence in females of one of these maternal lineage groups (line D) is shown in Table 1. The female parent of this lineage group developed an organoid-type adenocarcinoma which was initially pregnancy dependent. She was the daughter of one of the founding breeders that died of other causes. During 12 generations the mammary tumor incidence in line D breeding females remained relatively high (40 to 100%). Examination of the histological features of these tumors demonstrated that they were primarily type B mammary adenocarcinomas (12). They were

also classified, on the basis of the timing of their development, as (Fig. 1A) (i) pregnancy-dependent tumors which appeared during pregnancy (6 to 12 months of age), regressed after parturition, reappeared in subsequent pregnancies, and finally, continued to develop in the absence of pregnancy, and (ii) pregnancy-independent tumors which arose in retired breeders or during pregnancy (13 to 20 months of age) and continued to develop (Fig. 1A). Virgin females that were monitored during the first six generations had a 7.5% incidence of mammary tumors which appeared at 13 to 25 months of age (Fig. 1B). We have previously described MMTV-like particles (MC-MTV) and viral-related proteins in *M. cervicolor popaeus* milk and mammary tumors (14, 24). The occurrence of maternal lineage groups of *M. cervicolor popaeus* with a high incidence of mammary tumors is consistent with the transmission of infectious MC-MTV in this population. Pregnancy-dependent and -independent mammary tumors in inbred strains of *M. musculus* are associated with specific laboratory strains of MMTV (i.e., GR or RIII and C3H strains, respectively). The results presented here suggest that infectious viruses with similar biological properties exist in natural populations of feral mice reproductively isolated from *M. musculus*.

**Endogenous MMTV genomes in normal *M. cervicolor popaeus* liver cellular DNA.** Earlier studies have demonstrated the presence of MMTV-related sequences in *M. cervicolor popaeus* cellular DNA (5, 16, 23). Thermal denaturation of the molecular hybrids showed that there is extensive base pair mismatch between the MMTV genomes of these two species (23). We used recombinant DNA probes containing different regions of the MMTV (strains GR and C3H) genome and reduced the stringency of blot hybridization conditions (see above) to determine the organization of related sequences in restricted *M. cervicolor popaeus* liver cellular DNA (Fig. 2). The three enzymes (*Eco*RI, *Pst*I, and *Bgl*II) used in this analysis appeared to cut within the LTR of a majority of the endogenous proviral genomes. Several restriction fragments which reacted strongly with the different probes probably correspond to proviral genomes that bear common internal restriction sites. A provisional restriction map identifying the location of restriction sites within the predominant family of endogenous MMTV-related genomes is shown in Fig. 2B. The position of the restriction sites within the LTR was suggested by the reaction of common restriction fragments with LTR and *gag* gene probes, but not with the *env* gene probe, and by analysis of relevant double restriction digests. Although the pattern of restriction sites in the *M. cervicolor popaeus* MMTV genome differs demonstrably from those found in the *M. musculus* proviral genomes (8), the *Eco*RI site (at 6.0 map units) and several *Pst*I sites (at 0.1, 1.5, 2.5, 6.6, 8.7, and 9.2 map units) appear to have been conserved.

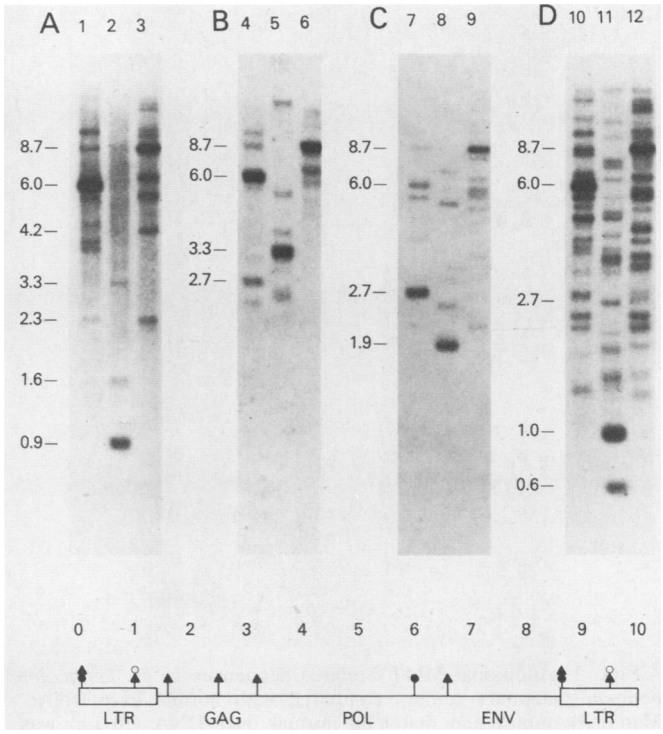


FIG. 2. Organization of MMTV-related sequence in *M. cervicolor popaeus* cellular genome. Normal liver cellular DNA (10 μg) was digested with *Eco*RI (lanes 1, 4, 7, and 10), *Pst* I (lanes 2, 5, 8, and 11), and *Bgl*II (Lanes 3, 6, 9, and 12). The restricted DNA was run on a 0.8% agarose gel and transferred to a nylon membrane. Membranes were hybridized with MMTV *gag* (A), *gag-pol* (B), *env* (C), and LTR (D) probes (see text). Numbers to the left of the gels are in kilobase pairs. Below the gels is shown a provisional restriction map of the predominant family of *M. cervicolor popaeus* endogenous MMTV-related genomes. The restriction sites are indicated as follows: ●, *Eco*RI; ○, *Bgl*II, ▲, *Pst*I.

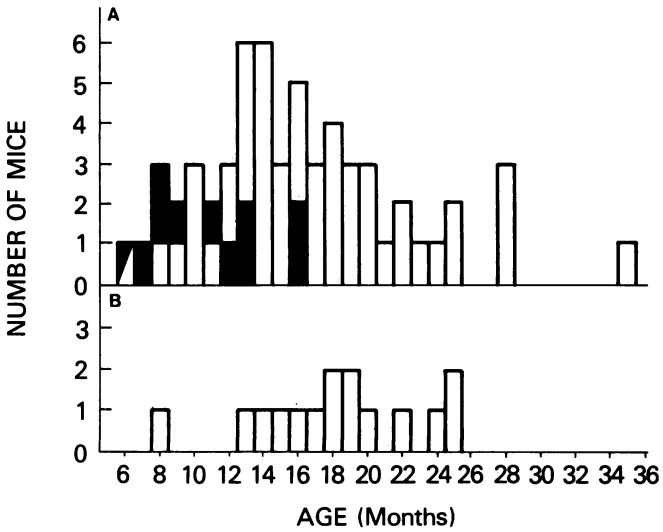


FIG. 1. Classification of mammary tumor development as a function of age in the *M. cervicolor popaeus* line D mice in breeding (A) and virgin (B) females. Solid columns represent the pregnancy-dependent mammary tumors, and open columns represent the pregnancy-independent mammary tumors. The age at which the tumor was first observed is given in months.

We observed significant variation in the pattern of MMTV-related restriction fragments in normal liver cellular DNA from different line D mice. (Fig. 3, lanes B, D, and F, and data not shown). The unique restriction fragments primarily represent different host-viral junction sequences detected with the MMTV LTR probe. Although the gene pool within the line D mice was severely restricted by our breeding program, there was still a significant amount of heterozygosity in this population of mice. The sporadic appearance of mutants affecting coat color and development is consistent with this conclusion. In our view, the unique restriction fragments reflect the segregation of endogenous MC-MTV genomes that accumulated at numerous sites

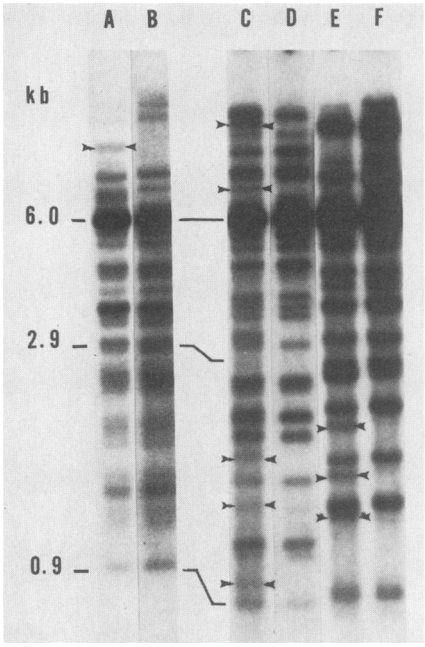


FIG. 3. Additional MMTV-related sequences in *M. cervicolor popaeus* mammary tumors compared with normal liver DNAs. Mammary tumor and matching normal liver DNA (10 μg) was digested with *Eco*RI, run on a 0.8% agarose gel, and transferred to a nylon membrane. The cellular DNAs were from mammary tumors CL87 (lane a), CL1 (lane c), CL91 (lane e); the corresponding normal liver DNAs were in lanes b, d, and f, respectively. The membranes were hybridized with the MMTV LTR probe (see text). Arrows indicate the position of tumor-specific MMTV-related restriction fragments.

within the cellular genome during the evolution of the species. The genetic burden of endogenous MC-MTV proviral genomes in *M. cervicolor popaeus* cellular DNA was estimated by the number of host-viral junction fragments detected in restricted normal cellular DNA (Fig. 2 and 3). The observed frequency of 15 to 20 copies of MMTV proviral DNA per cellular genome is significantly higher than the one observed in feral and inbred *M. musculus*.

**Evidence for common integration regions for infectious MC-MTV in *M. cervicolor popaeus* mammary tumor cellular DNA.** Attempts to transmit the *M. cervicolor popaeus* milk-borne MC-MTV or tumor-associated MC-MTV to the BALB/c or NIH Swiss inbred strains of *M. musculus* by foster nursing or injection of concentrated virus have not been successful. At the molecular level, one characteristic of retroviral infection is the presence of additional integrated proviral genomes in tumor DNA which are not observed in cellular DNA from normal tissue (16). We compared matched sets of restricted normal liver and mammary tumor cellular DNA from different tumor-bearing mice. Each restricted tumor cellular DNA contained MMTV LTR-related fragments which were absent in the maching normal liver cellular DNA (Fig. 3). This represents strong evidence for the presence of infectious MC-MTV in these mice. Our inability to transmit MC-MTV to *M. musculus* may reflect a limited host range or low infectivity of the virus.

Previous studies of MMTV-induced mammary tumors in inbred strains of *M. musculus* have demonstrated the existence of two cellular genetic loci (designated *int-1* and *int-2*) which are frequently occupied by an MMTV genome in tumor cellular DNA (15, 18). The *int-1* locus in *M. cervicolor popaeus* is defined by two overlapping 8.0-kbp *Bgl*II and *Eco*RI restriction fragments which span 14 kbp of cellular DNA. The *Bgl*II fragment is composed of the *int-1* transcription region and the 5′-flanking sequences. The *Eco*RI fragment also extends through the transcription region and into 3′-flanking cellular DNA. A comparison of *Bgl*II-restricted *M. cervicolor popaeus* mammary tumor and normal cellular DNA from inidividual mice showed that 6 of 20 tumors tested contained novel *int-1* fragments in the tumor cellular DNA (Fig. 4, lanes a to l). A similar analysis of *Eco*RI-restricted cellular DNA revealed five tumors with a novel *int-1*-related fragment (Fig. 4, lanes m to v). In one of these tumors a rearrangement appears to have occurred within both the *Bgl*II- and *Eco*RI-related *int-1* fragments (Fig. 4, lanes i and q). The novel fragments did not appear to be the result of a partial digestion, because they were reproduced in several independent experiments (data not shown). In several of the tumor cellular DNAs, the *int-1* probe reacted less intensively with the novel fragments than with the germ line gene. This probably reflects an heterogeneity in the tumor cell population with respect to the altered *int* locus.

To determine whether the novel *int-1* restriction fragments resulted from the insertion of an MC-MTV genome near this locus, blots of the restricted tumor cellular DNAs were probed with MMTV proviral probes. The novel *int-1*-related fragments in tumors CL87, CL91, and CL311 each comigrated with the MMTV *gag* gene-related *Eco*RI or *Bgl*II restriction fragments which were not present in matching liver cellular DNA (Fig. 5). In each of these tumor cellular DNAs the novel *int-1* restriction fragments were larger than the germ line fragments. Similarly, in one *Bgl*II-digested tumor DNA (CL188), the novel *int-1* fragment (Fig. 4, lane e) comigrated with a tumor-specific MMTV LTR-related fragment which was larger than the germ line band (data not shown). This suggests to us that either the viral infectious genomes were partially deleted or they lacked the *Eco*RI or *Bgl*II sites in their LTRs. In other tumor cellular DNAs, our ability to correlate the novel *int-1* fragments with specific MMTV-related restriction fragments was complicated by the presence of endogenous MMTV-related sequences. Although analysis of recombinant DNA clones of these novel *int-1* fragments will be required to establish the nature of the genetic alteration, we speculate that these rearrangements are also the result of MMTV insertional mutagenesis. Some of the tumors contained two or three novel *int-1* fragments. These fragments could result either from the insertion of an MMTV within the 2.4-kbp region of the cellular genome represented by the *int-1* probe or from multiple viral insertion events within the *int-1* locus in different tumor cell populations.

The *int-2* locus is defined by a 12-kbp *Hin*dIII fragment adjacent to a 5′-flanking 10-kbp *Eco*RI restriction fragment of cellular DNA. With two probes representing different regions of this locus, only one tumor was observed in which the *int-2* locus was rearranged (data not shown). Thus, 55% (11 of 20) of the mammary tumors tested contained a viral-associated rearrangement of either the *int-1* or *int-2* locus.

**Evidence for the unscheduled expression of the *int-1* locus in MMTV-induced mammary tumors.** Insertion of an MMTV genome into the *int* loci of *M. musculus* mammary tumor cellular DNA leads to the expression of flanking cellular sequences (11, 17). We compared the expression of *int-1*-related RNA in a *M. cervicolor popaeus* mammary tumor containing an *int-1* locus occupied by MC-MTV and a tumor containing an unaltered *int* locus. The CL87 tumor which

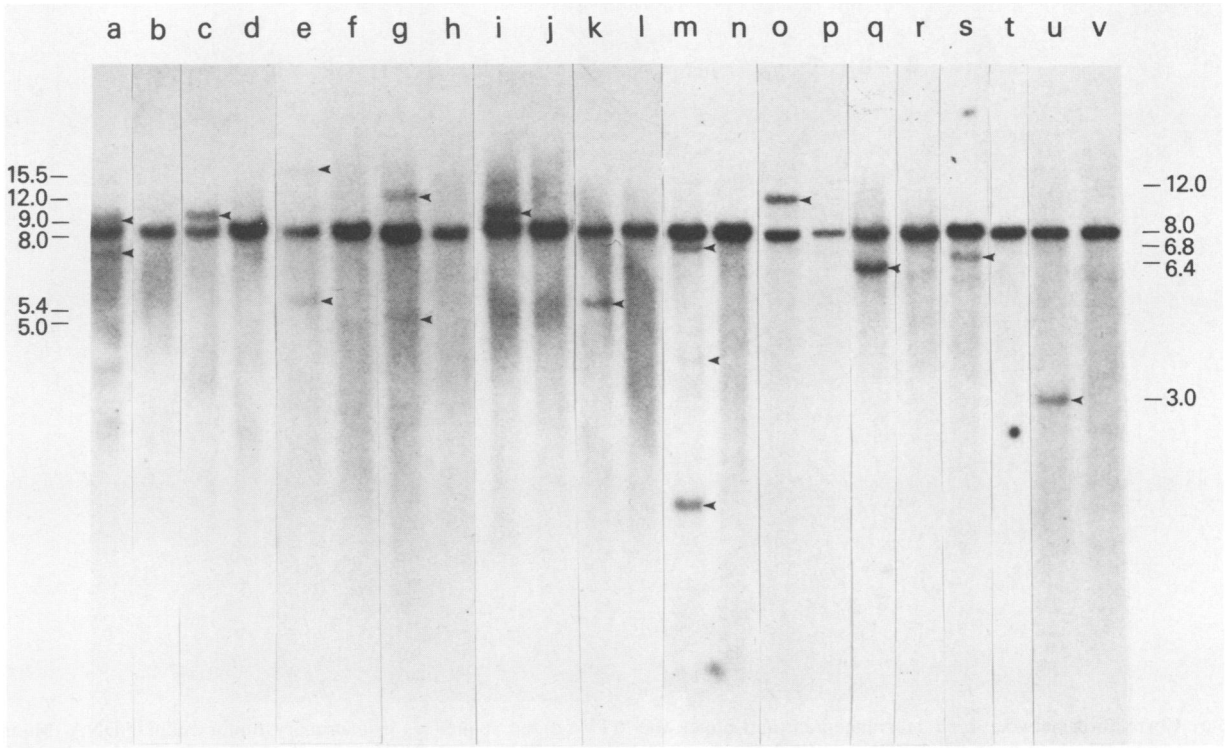


FIG. 4. Rearrangement of *int-1* in mammary tumor cellular DNA. Mammary tumor and normal matching liver cellular DNA (10 μg) was digested with *Bgl*II (lanes a through l) or *Eco*RI (m through v), run on a 0.8% agarose gel, and transferred to a nylon membrane. The cellular DNAs were from mammary tumors CL676 (lane a), CL91 (lane c), CL188 (lane e), CL311 (lane g), CL9 (lane i), CL1045 (lane k), CL286 (lane m), CL87 (lane o), CL9 (Lane q), CL1041 (lane s), and CL1072 (lane u); the corresponding normal liver were in lanes b, d, f, h, j, l, n, p, r, t, and v, respectively. The membrane was then hybridized with the *int-1* probe (see text). Arrows indicate the position of the *int-1*-specific rearranged restriction fragments. Molecular weights on the left correspond to lanes a through l, and those on the right side correspond to lanes m through v.

contained a MC-MTV-occupied *int-1* locus (Fig. 5) expressed a 2.6-kbp *int-1*-related RNA (Fig. 6, lane b). This RNA species was absent from the mammary tumor RNA (CL95) in which the *int-1* locus was intact (Fig. 6, lane a). The MMTV LTR probe did not hybridize to a 2.6-kbp species of RNA (data not shown); thus, the mechanism of activation of *int-1* in this tumor is probably similar to that proposed for the inbred strains of mice (11, 17).

## DISCUSSION

We have described a maternal lineage group (line D) of breeding females within a pedigreed breeding colony of feral *M. cervicolor popaeus* which had a high incidence of mammary tumors. Previously, we have shown that the milk and mammary tumors in these mice contain an MMTV-like virus (MC-MTV) (14, 24). The feral origin of MC-MTV is suggested by (i) the unique immunologic cross-reactivity of the virus in a group-specific radioimmunoassay for p29 and gp52 proteins of laboratory strains of MMTV (9, 24), (ii) the low level of nucleic acid sequence homology between the genome of milk-borne MC-MTV and laboratory MMTV (23), and (iii) the inability to transmit MC-MTV to inbred strains of *M. musculus* by foster nursing or injections of tumor extracts. The presence of additional integrated proviral genomes observed in mammary tumor cellular DNA relative to matching cellular DNA from normal tissue strongly suggests that MC-MTV is infectious in *M. cervicolor popaeus*.

The *int-1* and *int-2* loci were identified in mammary tumors of the C3H, BALB/cfC3H, and BR6 inbred strains of *M. musculus*. These mouse strains were selectively bred for a high incidence of mammary tumors. This breeding program resulted in highly virulent strains of MMTV and in strains of mice that were sensitive to MMTV infection. We were interested in determining whether this selection process may also have affected the inbred mouse *int* loci in such a manner that the consequences of their activation in mammary tumors is relevant only to these or closely related strains of mice. The results presented here extend the previous findings (i) by demonstrating an association between MMTV-induced rearrangement or activation of the *int-1* and *int-2* loci and mammary tumor development in the distantly related feral species *M. cervicolor popaeus* and (ii) by the fact that activation of these cellular genes in mammary tumors does not appear to be limited to highly infectious laboratory strains of MMTV.

MC-MTV induces both pregnancy-independent and pregnancy-dependent mammary tumors. It is probable that MC-MTV is a mixture of viruses, some of which, like MMTV (C3H), induce pregnancy-independent tumors and others, like MMTV (RIII) and MMTV (GR), induce pregnancy-dependent tumors. It is striking that in the C3H [MMTV (C3H)] mouse strain, 80% of the mammary tumors contained a viral-induced rearrangement of the *int-1* locus, while in the mammary tumors of the BR6 [MMTV (RIII)] mouse strain the *int-2* locus was affected in 50% and the *int-1* locus was affected in 25% of the cases. In the present study 8 of 15 pregnancy-independent tumors contained a rearranged *int-1* locus, and 1 of 15 had altered an *int-2* locus. In the pregnancy-dependent tumors the *int-1* locus was affected in two of six cases. These observations point to a potential corre-

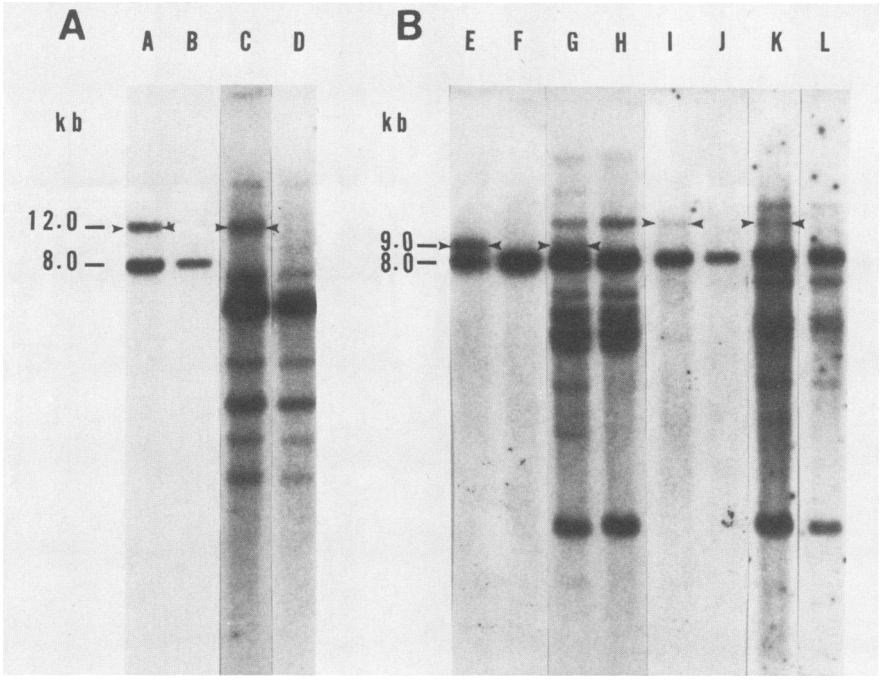


FIG. 5. Correlation between *int-1* rearrangement and novel MC-MTV-related sequences in mammary tumor cellular DNA. Mammary tumor and normal matching liver cellular DNA (10 μg) was digested with *Eco*RI (A) and *Bgl*II (B), run on a 0.8% agarose gel, and transferred to a nylon membrane; the membranes were then hybridized with the *int-1* probe (lanes A, B, E, F, I, and J) and dehybridized for 0.5 h in 0.4 N NaOH, neutralized in 0.1× SSC–0.2 M Tris (pH 7.5)–0.5% SDS for 1 h at 42°C. Membranes were then rehybridized with the MMTV-*gag* probe (lanes C, D, G, H, K, and L) (see text). The cellular DNAs were from mammary tumors CL87 (lanes A and C), CL91 (lanes E and G), and CL311 (lanes I and K); the corresponding normal liver DNAs were in lanes B, D, F, H, J, and L, respectively.

lation between activation of the *int-1* locus and the development of pregnancy-independent mammary tumors.

Not all of the *M. cervicolor popaeus* mammary tumors (50%) contained insertions in the *int-1* or *int-2* locus. It is possible that some occurred near the *int* loci but outside the regions defined by the restriction enzymes used in this study. Another possibility is that there are still other *int* loci which have not been identified as yet. Potential candidates could include other reported proto-oncogenes. Activation of the *myc* and *ras* proto-oncogene families by amplification or rearrangement has been implicated in the development of solid tumors of other tissues (1, 25). In other studies, transgenic mice containing recombinant c-*myc* linked to the MMTV LTR were found to have a high incidence of mammary tumors (27). However, restriction enzyme analysis of the N-*ras*, K-*ras*, H-*ras*, N-*myc*, and c-*myc* oncogenes in *M. cervicolor popaeus* mammary tumor cellular DNA provided no evidence for rearrangements of these proto-oncogenes (data not shown). Evidence for a new *int* locus has been obtained in MMTV-induced mammary tumors of feral *M. musculus musculus* (D. Gallahan and R. Callahan, manuscript in preparation). This locus is unlrelated to *int-1* or *int-2* or to other known retroviral oncogenes. Direct evidence for the oncogenic potential of activated *int* loci is not available at present. However, the frequent association between the activation of *int* loci by different strains of MMTV and mammary tumor development in different species of mice, as well as the conservation of the loci in distantly related species such as humans, promotes confidence in the view that the *int* loci represent a new family of tumor-associated cellular genes.

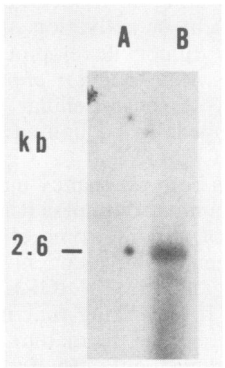


FIG. 6. Expression of *int-1* in *M. cervicolor popaeus* mammary tumors. Poly(A)$^+$ RNA (3 μg) prepared (see text) from two independent mammary tumors was denatured in the presence of formaldehyde and run on a 1% agarose gel containing formaldehyde. The RNA samples were then transferred to a nylon membrane and hybridized with the nick-translated *int-1* probe. Tumor CL87 (lane b) was previously found to have an altered *int-1* locus; in tumor CL95 (lane a) the *int-1* locus was not rearranged.

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