Identification of a Unique Mouse Mammary Tumor Virus in the BALB/cNIV Mouse Strain

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We examined the genetic structure, in terms of restriction endonuclease recognition sites, of the milk-transmitted, low-oncogenic mouse mammary tumor virus (MuMTV) of the BALB/cNIV mouse strain. An analysis with *EcoRI* documented the presence of acquired cNIV proviruses in the mammary tumor DNAs of BALB/cNIV animals. A comparison of tumor DNAs digested with *PstI* showed that both the cNIV MuMTV and C3Hf MuMTV proviruses lacked the 4.3- and 1.1-kilobase pair fragments characteristic of C3H MuMTV patterns. An examination of mammary tumor and normal, nonmammary tissue DNAs with *BamHI* supported the idea that the cNIV MuMTV is identical to the C3Hf MuMTV and demonstrated that these two low-oncogenic proviruses are identical to the high-oncogenic C3H MuMTV provirus with respect to a pair of *BamHI* sites which define a 1.3-kilobase pair fragment. For each of the three MuMTV strains, we also mapped DNAs generated in isolated virions by reverse transcription of their genomic RNAs. Our results showed that cNIV and C3Hf MuMTV are distinct entities by virtue of an additional *PstI* site within the cNIV long terminal repeat sequence. Another unique feature of cNIV MuMTV revealed by the analysis of virion-generated DNAs was the existence of a family of genomes within the cNIV population. We concluded that cNIV is distinct from its presumptive C3Hf MuMTV predecessor.

The exogenous mouse mammary tumor virus (MuMTV) of C3H mice is the highly tumorigenic milk agent described by Bittner. In its natural host, C3H MuMTV is responsible for a 90% mammary tumor incidence at about 8 months and is transmitted exclusively through the milk (17, 18).

The prevention of infection of newborn mice with C3H MuMTV by foster nursing results in the C3Hf strain in which mammary tumors also develop, but at a lower frequency (about 40%) and with a longer latent period (14 to 18 months) (16, 25). The tumorigenic agent is the genetically transmitted C3Hf MuMTV, which constitutes a specific locus of the C3H mouse genome, *Mtv-1* (25). C3Hf MuMTV expression is detected as viral antigens (16) and as mature, infectious B particles (13, 19).

To physically and biologically separate the high-oncogenic C3H MuMTV and low-oncogenic C3Hf MuMTV for comparative study, each was transmitted independently to the infectious virus-free BALB/c strain.

The C3H MuMTV was readily transmitted by foster nursing or virus injection. Subsequent milk transmission, tumor latency, and tumor

incidence characteristics were essentially identical for such BALB/cfC3H animals when compared to the C3H strain (17, 18). However, successful transfer of the C3Hf MuMTV (referred to as nodule-inducing virus [NIV] [6]) into BALB/c mice required the use of hybrid mice and tissue transplantation methods (20). The resultant BALB/cNIV strain harbors an exclusively milk-transmitted virus which otherwise behaves biologically in BALB/c mice as does the C3Hf MuMTV in the C3H strain (6). We refer to this virus as cNIV MuMTV.

Although the similarity of the nodule and tumor incidences of the BALB/cNIV and C3Hf strains remains stable in our colony (L. J. T. Young, K. B. De Ome, P. B. Blair, and D. R. Pitelka, submitted for publication), we have recently found that the BALB/cNIV milk virus is immunologically distinct from both C3H and C3Hf MuMTVs (24). To determine whether other differences exist between the C3Hf and cNIV MuMTVs at the genetic level, we used restriction endonuclease analysis to compare MuMTV-specific DNA sequences from both tissue and viral sources.

We document here the overall similarity of acquired cNIV and C3Hf proviruses in tumor DNAs and their common differences relative to C3H MuMTV. We also present evidence, ob-

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tained with virion-generated double-stranded (ds) DNA, that cNIV is a distinct form of MuMTV.

MATERIALS AND METHODS

Animals. Mice of the following strains were used: BALB/c Crgl (Cancer Research Lab, University of California, Berkeley, Calif.), BALB/cfC3H/Si (Simonsen Laboratories, Gilroy, Calif.), C3H Crgl, and BALB/cNIV. C3Hf mice were obtained from the Institute for Cancer Research (Philadelphia, Pa.) and from J. Schlom (National Institutes of Health, Bethesda, Md.). All C3H mice were of the C3H/He substrain with respect to the endogenous MuMTV proviral sequences they contained (4).

Virus. C3H virions from the Mm5mt/c1 cell line were obtained from Frederick Cancer Research Center, Frederick Md., through the Research Resources, Biological Carcinogenesis Branch, National Cancer Institute, and were further purified by equilibrium sedimentation in sucrose gradients. C3Hf virions and cNIV virions were isolated from skim milk pools with a final gradient step as above.

Nucleic acid extraction. Tissue nucleic acids were isolated as described previously (11).

Viral ds MuMTV DNA. Virus (0.1 to 10 mg) was permeabilized by suspension at 1 to 5 mg of viral protein per ml in 5 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–0.1 mM EDTA–5 mM dithiothreitol–0.04% (vol/vol) Nonidet P-40 and incubation at 0°C for 20 min. This suspension was then diluted one to four times into a concentrated solution containing the following components to give the following final concentrations: 50 mM Tris-hydrochloride (pH 8.1), 50 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, and 1 mM each dATP, dCTP, dGTP, and dTTP. The mixture was incubated at 37°C overnight. The reaction was stopped by addition of EDTA, yeast RNA, sodium dodecyl sulfate, and proteinase K to concentrations of 20 mM, 100 µg/ml, 0.5%, and 200 µg/ml, respectively, and incubation was continued for 30 to 60 min at 37°C before phenol extraction. The nucleic acids were ethanol precipitated three to four times and dissolved in 5 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–0.1 mM EDTA.

By use of this modification of the method of Rothenberg et al. (21), we have documented de novo DNA synthesis by demonstrating the incorporation of radiolabeled deoxynucleotide triphosphate into ds MuMTV DNA. Although all viral ds DNAs analyzed for this report were incubated at 37°C overnight, we have been able to show, for the C3H virus, that some ds DNA exists in the virions before permeabilization (unpublished data).

Radiolabeled MuMTV-specific DNAs. Radiolabeled cDNA (³²P)cDNA representative of the entire MuMTV genome was synthesized as previously described (11). DNA from the long terminal repeat (LTR) sequences of C3H MuMTV was kindly provided by J. E. Majors and H. E. Varmus (14). The LTR DNA and the pBR322 plasmid into which it was inserted were radiolabeled by nick translation as follows: DNA (50 to 100 ng) was incubated in a 20-µl reaction mixture containing 50 mM Tris-hydrochloride (pH 7.4), 10 mM MgCl₂, 1 mM dithiothreitol, 50 µM each dCTP, dGTP,

and dTTP, 50 µCi of [α -³²P]dATP (New England Nuclear Corp., Boston, Mass; 2,000 to 3,000 Ci/mmol), and 20 µg of DNase I (Sigma Chemical Co., St. Louis, Mo.) for 1 min at 25°C. Ten units of DNA polymerase (Boehringer Mannheim Corp., New York, N.Y.) was added, and the incubation was continued at 10°C for 15 min. The reaction was stopped by the addition of EDTA and sodium dodecyl sulfate to 100 mM and 0.5%, respectively, and the sample was passed over a Sephadex G-50 column and further processed as for the cDNA described above.

Phage λ DNA and ϕ X174 RF DNA were nick translated as described above, except 1 to 3 µg of each DNA was used per reaction.

Restriction enzyme digests and blotting. Restriction endonucleases were purchased from New England Biolabs, Beverly, Mass. Digestion mixtures contained 5 to 20 µg of cellular DNA or empirically determined amounts of viral ds DNA and 1 µg of phage λ DNA in 50 mM Tris-hydrochloride (pH 7.4)–50 mM NaCl–5 mM MgCl₂–5 mM dithiothreitol–50 µg of lysozyme per ml. After the addition of enzyme, the reactions were incubated at 37°C and stopped by the addition of EDTA to 25 mM, and the products were electrophoresed into 1% agarose. The gels were stained with ethidium bromide and examined under short UV illumination. The completeness of the reaction was assessed by the appearance of the λ DNA fragment pattern. The transfer of DNA from the agarose gels to nitrocellulose filters (Schleicher & Schuell Co., Keene, N.H.) and further processing were performed as described previously (11).

Samples to be S1 nuclease digested before application to gels were incubated in 30- to 50-µl reaction mixtures containing 30 mM sodium acetate (pH 4.5), 300 mM NaCl, 3 mM ZnSO₄, and 20 U of S1 nuclease (Miles Laboratories, Inc., Elkhart, Ind.) for 45 to 60 min at 37°C. The reactions were stopped by the addition of EDTA to 50 mM. Yeast RNA was added to 50 µg/ml, and the nucleic acids were precipitated by the addition of two volumes of ethanol. The resultant pellets were suspended in the restriction nuclease buffer described above and were electrophoresed.

RESULTS

Acquired proviruses in BALB/cNIV tumor DNAs. Virus-induced mammary tumors in high-incidence C3H and GR strains have additional copies of MuMTV DNA (3, 11). We first asked whether the mammary tumor DNAs of the low-tumor-incidence strains C3Hf and BALB/cNIV also contain additional MuMTV-specific sequences suitable for comparative restriction endonuclease mapping. All full-length MuMTV proviruses studied so far have a single *EcoRI* site, and digestion with this enzyme produces two fragments per provirus, each containing MuMTV sequences covalently linked to host DNA (4, 5). Thus, additional proviruses were detected as extra bands superimposed on the endogenous MuMTV pattern.

A BALB/c liver DNA digest (Fig. 1A, lane 7) illustrates the *EcoRI* pattern expected of the endogenous MuMTV proviruses of this strain

FIG. 1. Acquired proviruses in tumor DNAs from BALB/cNIV mice (A) and C3Hf mice (B). Tissue DNAs (5 to 10 μ g) were digested with *Eco*RI. The products were subjected to agarose gel electrophoresis, and MuMTV-specific fragments were visualized by the technique of Southern (23) as previously described (13). (A) Lanes 1, 3, and 5 contain BALB/cNIV liver or spleen DNAs, and lanes 2, 4, and 6 contain animal-matched tumor DNAs. Lane 7 contains BALB/c liver DNA, and lane 8 contains BALB/cfC3H tumor DNA. (B) Animal-matched C3Hf liver (lane 1) and tumor (lane 2) DNAs. Arrows adjacent to each lane 1 identify fragments of endogenous MuMTV proviruses, and the accompanying numbers refer to the fragment sizes in kbp. Arrows to the right of lanes 6 and 8 locate the positions of faint bands of fragments of acquired MuMTV sequences. One of the additional bands of the tumor DNA in lane 2 comigrates with the 9.2-kbp endogenous fragment.

(2). A BALB/cfC3H tumor DNA digest (lane 8) showed extra bands of the acquired, high-oncogenic C3H provirus at approximately 6.0, 8.5, and 13 kilobase pairs (kbp). The remainder of Fig. 1A shows digests of animal-matched liver (or spleen) and tumor DNA pairs from BALB/cNIV mice. The nontumor DNA samples exhibited only the endogenous BALB/c bands, as expected, but each tumor had at least two extra bands in addition to endogenous proviral fragments. All eight BALB/cNIV tumor DNAs examined to date have acquired MuMTV proviral sequences.

Fig. 1B shows the same analysis with C3Hf liver DNA (lane 1) and matched tumor DNA (lane 2). The liver pattern is identical to that previously reported (4, 5). The tumor DNA had several extra bands, and therefore it contained additional MuMTV sequences.

Internal structure of the cNIV provirus. Next we examined the proviral internal organization to determine whether cNIV could be considered a distinct MuMTV. We chose the enzymes *Pst*I and *Bam*HI for the comparison, since each differentiates among other MuMTV proviral variants (2, 3). A summary of our findings is presented in Fig. 2 and 3.

Tissue DNA containing C3H MuMTV yielded the 4.3- and 1.1-kbp *Pst*I fragments (Fig. 2, arrows) routinely used to denote exogenous infection of tissues by this virus (3). Tissue DNAs containing either cNIV or C3Hf MuMTVs did not have these bands. Thus, C3H

and cNIV MuMTVs are different, but *Pst*I did not distinguish between C3Hf and cNIV MuMTVs.

The doublet of high-molecular-weight *Pst*I fragments (5.4 and 5.1 kbp) in BALB/c tissues (Fig. 2, lanes 1 to 4) represents virtually identical regions of each of the two full-length BALB/c endogenous MuMTV proviruses (2). For the BALB/cNIV tumor shown (lane 3) and for others we have analyzed, the intensity of the 5.4-kbp fragment was increased relative to that of the 5.1-kbp fragment, suggesting that the acquired cNIV MuMTV proviruses have *Pst*I sites similar to those of the unit II endogenous MuMTV (2, 4).

Figure 3 shows the results of *Bam*HI digestion of the same DNAs shown in Fig. 2. The 1.3-kbp fragments expected in C3H tumor DNA (lane 7) and BALB/cfC3H tumor DNA (lane 4) were present. At least one of the endogenous C3H proviruses also has the two *Bam*HI sites defining this fragment, since it also appeared in C3Hf liver DNA (lane 5). Tumor DNA from C3Hf mice (lane 6) had the 1.3-kbp fragment that is contributed definitely by endogenous MuMTV sequences and presumably also from amplified proviruses (see below).

All BALB/cNIV tumor DNAs examined had the 1.3-kbp fragment shown in lane 3. BALB/cNIV spleen DNA (lane 2) and the BALB/c liver DNA (lane 1) lacked this band.

These results provide support for the notion that the expressed and tumorigenic C3Hf

FIG. 2. Demonstration of the lack of C3H MuMTV sequences among the acquired proviruses of BALB/cNIV tumor DNA. Tissue DNAs were digested with *Pst*I and analyzed as described in the legend to Fig. 1. The sources of DNA were: lane 1, BALB/c liver; lane 2, BALB/cNIV spleen; lane 3, BALB/cNIV tumor (from animal of lane 2); lane 4, BALB/cfC3H tumor; lane 5, C3Hf liver; lane 6, C3Hf tumor (from animal of lane 5); lane 7, C3H tumor. The arrows denote the 4.2-kbp (upper) and 1.1-kbp (lower) fragments characteristic of the C3H MuMTV provirus (5).

MuMTV is also the added provirus in BALB/cNIV tumor DNAs.

Comparison of viral ds DNAs. To avoid the difficulties in interpreting fragment patterns of added provirus superimposed on endogenous patterns, we analyzed ds DNA copies of virion RNA to directly map and compare the C3Hf and cNIV MuMTV genomes.

Undigested DNAs from C3H MuMTV (Fig. 4, lane 4) and cNIV MuMTV (lane 2) migrated as would be expected for 10 to 11-kbp linear ds DNA. Each was resistant to RNase digestion and totally hydrolyzed by DNase (data not shown). Treatment of these DNAs with S1 nuclease yielded the products shown in lane 5 (C3H) and lane 3 (cNIV). A small amount of single-stranded DNA apparently existed, but it did not interfere with restriction nuclease digestion (see below). We interpreted the sensitivity to S1 nuclease as a manifestation of gaps introduced during reverse transcription of the viral

RNA, as has been documented in other retroviral systems (9, 12, 26).

The results of *Pst*I digestion of C3H, C3Hf, and cNIV ds DNAs were as shown in Fig. 5. The pattern for C3H MuMTV ds DNA was that expected from previous data (22). The patterns for C3Hf (lane 4) and cNIV (lane 3) ds DNAs were clearly distinguishable from one another, and each was distinct from that of the C3H (lane 2) ds DNA digest. The C3Hf and cNIV genomes lacked the *Pst*I site defining the junction of the 4.3 and 1.1-kbp fragments of the C3H ds DNA digest (see Fig. 6).

The distinction between cNIV and C3Hf ds DNAs was twofold. First, the cNIV ds DNA had an extra *Pst*I site within the LTR region. As a result, the cNIV DNA digest lacked the 1.3- and 1.4-kbp LTR fragments seen in the other two viral ds DNA samples. Lower-molecular-weight bands, unique to cNIV MuMTV, appeared concomitantly at 0.95 kbp and at 0.5 and 0.4 kbp. Second, the cNIV ds DNA *Pst*I digest contained an 8.0-kbp component, suggesting that this preparation of cNIV contained a second, unique viral genome. The 8.0-kbp fragment

FIG. 3. Shared *Bam*HI sites among C3H, C3Hf, and cNIV MuMTVs. The same samples of Fig. 2 were digested with *Bam*HI and analyzed as described in the legend to Fig. 1. The lane orientations are identical to those of Fig. 2. Arrows denote the 1.3-kbp *Bam*HI fragment considered diagnostic of C3H MuMTV but found also in C3Hf MuMTV (lanes 5 and 6) and in cNIV MuMTV (lane 3).

FIG. 4. Analysis of the ds DNAs from cNIV MuMTV (lanes 2 and 3) and from C3H MuMTV (lanes 4 and 5). ds DNAs were synthesized in vitro, extracted from virions, and electrophoresed in agarose gels, and MuMTV-specific sequences were visualized as described in the text. Samples were either undigested (lanes 2 and 4) or digested with single-strand-specific S1 nuclease (lanes 3 and 5) before electrophoresis. Lane 1 shows the four largest products of a *Hind*III digest of λ [32 P]DNA. The sizes of the λ DNA fragments, in kbp, are given at the left of the figure.

apparently lacked other internal *Pst*I sites (denoted by dots in Fig. 6B) but retained the remaining sites of the other cNIV component. A detailed examination of this material is in progress.

Lanes 5 to 7 of Fig. 5 show *Bam*HI digests of the three viral ds DNAs. The viral genomes were identical by this criterion, and the pattern was as expected from published C3H MuMTV maps (22). The smallest fragment was the virion DNA counterpart of the 1.3-kbp *Bam*HI fragment detected in tissue DNA digests and discussed above. These results are summarized in Fig. 6.

DISCUSSION

The C3Hf MuMTV has been considered the prototype low-oncogenic MuMTV (16, 17, 18, 25). Thus, comparisons of its biological and biochemical properties with those of its high-oncogenic counterpart, C3H MuMTV, have been undertaken in attempts to elucidate those factors critical for tumorigenesis (1, 13, 16, 24; Young et al, submitted for publication). Since both MuMTVs reside in the same mouse strain, analysis of each was obscured by the presence

of the other. Therefore, each MuMTV was transferred independently to the infectious virus-free BALB/c strain. C3H MuMTV was readily transferred. C3Hf MuMTV was apparently first successfully transferred, as the infectious virus of (C3Hf $\times$ BALB/c) F1 hybrids, into a transplanted BALB/c normal mammary gland. The MuMTV subsequently detected in BALB/c mice was designated cNIV (nodule inducing virus from BALB/c), and the line harboring it was termed BALB/cNIV (6).

The newly established BALB/cNIV strain contained an infectious, low-oncogenic virus. By this and other biological criteria, the cNIV MuMTV appeared to be identical to C3Hf MuMTV except that it was transmitted exclusively via milk. Thus, it was concluded that the endogenous virus of the C3H mouse strain had been transferred to BALB/c mice in which it acted as an exogenous MuMTV (6).

FIG. 5. Restriction nuclease analysis of viral ds DNAs. Viral ds DNAs from C3H virions (lanes 2 and 5), cNIV virions (lanes 3 and 6), and C3Hf virions (lanes 4 and 7) were digested with *Pst*I (lanes 2 to 4) and *Bam*HI (lanes 5 to 7) and analyzed as described in the legend to Fig. 1. Markers in lanes 1 and 8 were a mixture of *Hind*III-digested λ [32 P]DNA and *Hae*III-digested ϕ X174 RF [32 P]DNA. The sizes of the marker fragments are given in kbp to the left of lane 1. Arrows in lane 3 denote the positions of faint bands thought to be products of *Pst*I cleavage within the cNIV LTR sequence.

FIG. 6. Map of restriction nuclease sites in several MuMTV DNAs. A genetic map (A) adapted from Massey and Schochetman (15) orients the viral RNA to the DNA maps. The question mark is placed over a region of the genome with an open reading frame (10) which has yielded polypeptides in vitro (7, 8). The role of these proteins is unknown. *Pst*I sites (B) and *Bam*HI sites (C) are denoted by vertical lines for C₃Hf and cNIV viral ds DNAs and for unit II proviral DNA (2). Rectangles denote LTR sequences, and sawtooth lines represent attached cellular DNAs. Sizes of observed fragments are given in kbp.

Originally, our restriction endonuclease analysis supported this conclusion at the DNA level. For example, a 1.3-kbp fragment was found in *Bam*HI digests of virion ds DNAs from C₃H, C₃Hf, and cNIV MuMTVs. The same fragment was found in C₃Hf tumor and liver DNAs and in BALB/cNIV tumor DNA but not in BALB/cNIV liver DNA. This is consistent with genetic transmission in C₃Hf and infectious transmission of a similar virus in BALB/cNIV.

Mammary tumor DNAs from both C₃Hf and BALB/cNIV sources were characterized by additional copies of MuMTV proviruses. Additional proviral copies are characteristic of all MuMTV-induced tumor systems studied (3, 5, 11), and our results suggest that low- and high-oncogenic MuMTV variants share this feature of tumorigenesis.

Recently, immunological differences between cNIV and C₃Hf MuMTVs have been detected by a whole virion radioimmunoassay (24). These data suggest that genetic differences exist be-

tween cNIV and C₃Hf MuMTVs. However, digests of tumor DNAs failed to clearly distinguish between the C₃Hf and cNIV MuMTVs. The major problems in such a comparison were the ambiguities caused by the endogenous proviruses and the presence of host flanking sequences attached to the acquired proviruses.

These problems were alleviated by generating full-genome-length ds DNAs transcribed from viral RNA templates. By this method, MuMTV DNAs that were free of host flanking DNA or endogenous MuMTV sequences were obtained. Our study of ds DNA revealed that cNIV MuMTV is unique in two respects.

First, cNIV MuMTV was easily distinguished from the other two MuMTVs by the presence of an extra *Pst*I site within the LTR region. A single base pair substitution in the vicinity of this site (about 0.4 kbp from the 3' end) in the C₃H LTR could produce a *Pst*I site (10). Thus, assuming equivalence of the C₃H and C₃Hf LTR sequences, cNIV could have been generated

from C3Hf MuMTV by a single base pair substitution. Alternatively, since the endogenous unit II MuMTV has two *Pst*I sites in the LTR, cNIV MuMTV could have arisen from an activated unit II genome or as a recombinant between unit II and C3Hf MuMTV.

Although the distinctions between the two low-oncogenic MuMTVs are clear, they do not necessarily account for the antigenic differences in the envelope proteins (24). We are currently attempting to document additional sequence differences in this region. On the other hand, the proteins recently shown to be coded within the LTR sequence may ultimately be identified as important immunological determinants (7, 8, 10).

The second distinctive feature of the cNIV ds DNA was the appearance of other genomic species besides that yielding the unit II-like set of *Pst*I fragments. The example illustrated here was the 8.0-kbp *Pst*I band. We do not believe this fragment to be an artifact of incomplete digestion or an RNA-DNA hybrid because of the total digestion of added control λ phage DNA and the complete digestion of cNIV ds DNA with *Bam*HI.

Another cNIV virion preparation yielded ds DNA containing only the unit II-like *Pst*I pattern (5.4-, 1.9-, 0.95-, 0.5-, and 0.4-kbp fragments), whereas a third preparation appeared to consist solely of a variant with an internal 1- to 2-kbp deletion. Several C3H and two C3Hf ds DNA preparations have maps that are identical to their respective types shown above. Possibly, cNIV MuMTV is more prone to the generation of substrain variants than are the other C3H MuMTVs. These "mutant" forms apparently range from point substitutions to large deletions and may have arisen from faulty reverse transcription.

The studies reported here have utilized full-length ds DNA transcripts to document that cNIV MuMTV is distinct from either of the C3H MuMTVs in at least two respects: (i) existence of a characteristic *Pst*I digestion pattern of the LTR region and (ii) existence of a family of genome types. Further investigation will be required to determine the biological significance of these observations.

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