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CHANGES IN CASEIN SEQUENCE ABUNDANCE IN DNA OF MOUSE MAMMARY TUMORS

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SUMMARY

The casein gene content of mouse mammary tumor virus-induced mammary tumors was found to be elevated, apparently in proportion to the increase in MTV proviral copy number. Conversely, mammotropic hormone-induced mammary tumor DNA was deficient in casein genes in 2 of 3 tumors. Globin gene abundance, however, in both normal and malignant mammary tissues was unchanged, regardless of changes in casein gene content. These results suggest that perturbations have occurred in a common region of DNA from mammary tumors with 2 different etiologies.

INTRODUCTION

Mouse mammary tumor virus (MTV) is a potent oncogenic agent for mouse mammary epithelium, though little is known about the molecular basis for this action or its functional relationship with mammary gland morphogenesis. However, the expression of MTV genes in mouse mammae parallels that of the milk protein casein in many respects. The induction of both occurs only in mammary tissue, requires similar concentrations of insulin and glucocorticoids (1-4), and during pregnancy and lactation, both show a similar temporal correlation with mammary gland development and lactogenic activity (5,6). Thus MTV expression evidently relates directly to differentiated parenchymal function. We and others have previously found alterations in the restriction patterns of MTV proviral DNA which appear to be linked to transformation in that they are found only in neoplastic tissues (7-9), and have therefore analyzed casein DNA sequences in the same tissues to determine whether DNA alterations involve differentiated mammary specific functions as well. We report here an increase in the abundance of casein gene sequences in MTV-induced mammary adenocarcinomas that parallels the increase in MTV genes, while two (of three) hormone-induced mammary tumors studied were deficient in casein sequences.

METHODS AND MATERIALS

Mammary tumors: MTV-induced mammary tumors were obtained from BALB/cfC3H mice, maintained as isolated stocks in the Michigan Cancer Foundation animal facility. Tumor incidence in force-bred females is approximately 90% by 9 months

Abbreviations used: HAN, hyperplastic alveolar nodules; EDTA, ethylenediaminetetraacetic acid; SDS, socium dodecyl sulfate; cDNA, complementary DNA; Cot, moles of deoxynucleotides X liter^{-1} X seconds; Crot, moles of ribonucleotides X liter^{-1} X seconds.

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(average parity of 5). Hormone-induced mammary tumors were obtained from transplantable D2 mammary hyperplastic alveolar nodules (HAN) (10) maintained in cleared mammary fat pads (11) of virgin BALB/c female mice, either with ("primed") or without ("unprimed") 2 to 3 hypophyses implanted in the renal capsules. These implants release high concentrations of prolactin and greatly enhance tumorigenesis in D2 HANs (12).

Purification of DNA: DNA was purified from tissues using guanidine thiocyanate (13). Minced tissues were homogenized on ice in 10 vol. of 4M guanidine thiocyanate (Tridom Fluka), 0.05 M Tris-HCL (pH7.4), 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.1% β-mercaptoethanol, 0.5% sodium lauroyl sarcosinate (Pfaltz and Bauer) using either a Polytron (Brinkman Instruments) or a loose fitting Teflon pestled Potter-Elvehjem homogenizer. The homogenates were made 2 M in LiCl and 50% in ethanol, and incubated at least 12 hours at -20°C. The precipitates were recovered by centrifugation, dissolved in 6 M guanidine-HCl, 0.05 M Tris-HCl (pH 7.4), 0.01 M EDTA, 0.1% sodium lauroyl sarcosinate, reprecipitated with LiCl and ethanol as before, washed in 70% ethanol, 0.08 M sodium acetate (pH 5.0) dissolved in 0.05 M Tris-HCl (pH 9.2), 0.01 M EDTA, 0.075 M NaCl, 0.5% sodium dodecyl sulfate, 100 µg protease K (EM Biochemicals) per ml, incubated at 37°C for 1 hour, then extracted once with an equal volume of chloroform, phenol, isoamyl alcohol (24/24/1 v/v/v), then twice with chloroform. To the aqueous phase were added dropwise with rapid stirring at 25° C, 0.5 volume of ethanol, 0.3 volume of 15 M LiCl, and 0.05 volume of 2.75 M sodium acetate (pH 5.0). Samples were then incubated at -20° C. Under these conditions, 4s and larger RNA is precipitated, while DNA remains completely soluble. After incubation at -20° C for at least 24 hours, the samples were centrifuged until clarified. The DNA was recovered from the supernatant by ethanol precipitation, sonicated, alkali digested and ethanol precipitated as described previously (14).

Purification of RNA: RNA was isolated from cultures of Swiss-3T3 cells by treatment with SDS and protease (15) phenol extraction and DNAse 1 digestion (14). Rauscher leukemia virus (RLV, obtained from Frederick Cancer Research Center) was isolated by the same procedure, but omitting DNAse treatment.

[^{14}C]unique sequence Mouse DNA: DNA purified from a BALB/c mouse mammary tumor cell line MCF-8 (16) incubated with [^{14}C]thymidine (New England Nuclear; 2.5 µCi/ml of medium for 24 hours in log phase of growth) was hybridized to a Cot value of 800. Double stranded DNA so formed (middle and highly repetitive DNA) was removed by hydroxylapatite chromotography essentially as described by Morris, et al (17), yielding unique sequence DNA with a specific activity of 1.2×10^4 cpm/µg.

Preparation of cDNAs: 1) MTV cDNA. MTV 70s RNA was purified by velocity sedimentation from RNA isolated by phenol extraction from MTV-S (obtained from Frederick Cancer Research Center) produced by Mm5MT/Cl cells (18). This RNA template was copied into [^{3}H]cDNA essentially as described by Myers and Spiegelman (19), but using 2 mg of calf thymus oligodeoxynucleotides per ml of reaction mixture for priming cDNA synthesis (20). This cDNA protected greater than 90% of [^{125}I]MTV 70s RNA at a cDNA:RNA ratio of 5:1, and did not hybridize to Rauscher leukemia virus (RLV) or Swiss 3T3 RNAs (Table 1). 2) Globin cDNA. Mouse globin mRNA, the generous gift of Dr. J. Lingrel, was purified from mouse reticulocytes by oligo dT-cellulose chromotography and sedimentation velocity. cDNA was prepared from this RNA as described above, but primed with 5 µg of oligo dT_{12-18} (P-L Biochemicals) per ml of reaction mixture. 3) Mouse casein[^{3}H]cDNA was generously provided by Dr. J. Rosen. This full length cDNA represents predominantly α and β casein mRNAs (21). It did not hybridize to MTV RNA, nor did similar preparations hybridize to polyadenylated liver RNA (Table 1). Additional mouse casein cDNA was also generously provided by Drs. R. Ganguly and M. Banerjee. This cDNA, used in Fig. 1 D, had very similar properties (4) and didn't hybridize to mouse liver RNA (Table 1).

Hybridization: Time variable DNA hybridization was performed by incubating DNA at 15 mg/ml with cDNA at 5 ng/ml in 0.5 M NaCl, 0.025 M HEPES (pH 7.2), 0.003 M EDTA (NHE) at 64° C for variable time intervals as indicated (Results). Con-

centration variable RNA excess hybridizations were performed at 64° C for 48 hours in NHE. Concentration of DNA or RNA in moles nucleotide x sec. x L^{-1} corrected for equivalent hybridization rates at 0.18 M Na^+ (22), was expressed as equivalent Cot (ECot) or Crot (ECrot). [^{14}C]-unique sequence DNA was included as a single gene complexity internal standard during hybridizations. Percent hybrid formation was determined by nuclease S1 (Miles Laboratories) digestion (23).

RESULTS

The kinetics of hybridization of casein cDNA to normal BALB/c mammary gland DNA are shown in Fig. 1A. A lactating gland preparation and mammary DNA from a midpregnant animal both hybridized at approximately the same rate to this cDNA. The average rate difference between these and mouse unique sequence DNA (single gene complexity) reassociation (Fig. 1A) indicates approximately 3 gene equivalents per haploid genome for sequences complementary to the casein cDNA. When

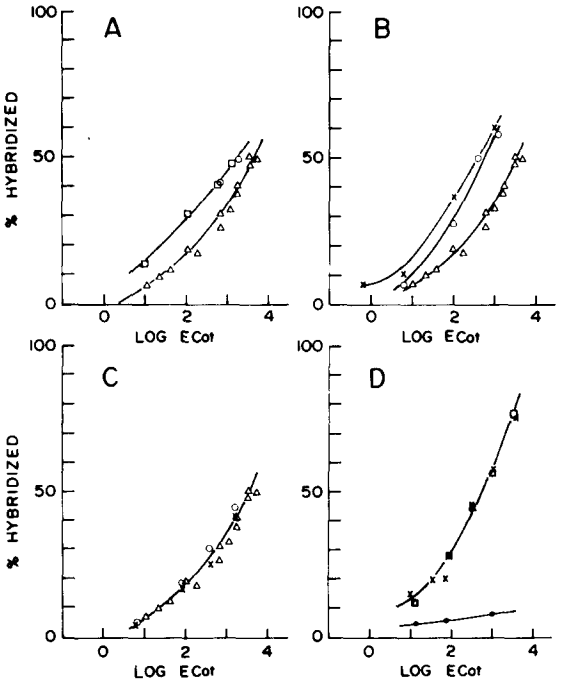

Figure 1. Hybridization of casein cDNA to normal and mammary tumor DNAs. Casein cDNA was hybridized to the indicated DNAs as described in Methods and Materials. △—△, [^{14}C]-unique sequence mouse DNA. A. ○—○, midpregnant BALB/c mouse mammary gland DNA: □—□, 8 to 10 day lactating BALB/c mammary gland DNA. B. MTV-induced mammary tumor DNA: ×—×, tumor #1; ○—○, tumor #2. C. Hormone-induced mammary tumor DNA: ○—○, tumor #1 (unprimed); ×—×, tumor #2 (primed). D. □—□, Hormone-induced mammary tumor DNA, tumor #3 (primed); ×—×, 8 to 10 day lactating BALB/c mouse mammary gland DNA; ●—●, calf thymus DNA.

the casein cDNA was hybridized to MTV-induced mammary tumor DNA, however, a substantial rate increase was observed (Fig. 1B). The Cot curves for DNA from 2 different viral-induced tumors indicate 2 to 4 fold increases in casein gene equivalents. Two hormone-induced mammary tumors contained fewer casein sequences than normal mammary gland DNA (Fig. 1C) though a Cot curve for a third such tumor was coincident with that for Balb/c lactating mammary gland DNA (Fig. 1D).

The MTV proviral content in these DNAs was determined as shown in Fig. 2. Consistent with previous reports (14,24), 4 to 5 proviral equivalents were found in normal BALB/c mammary gland DNA. The same number were detected in hormone-induced tumor DNA. Infection of mammary epithelium by exogenous MTV typically results in the appearance of many additional copies of provirus in the DNA of derivative tumors (14). BALB/cfC3H mammary tumors 1 and 2 were found to contain 17 to 20 and 10 to 12 MTV genome equivalents respectively (Fig.2). When correlated with the casein DNA abundance data (Table 2), it became evident that in viral-induced mammary tumors the ratio of casein to MTV DNA remained fairly constant regardless of the degree of increase in MTV DNA, i.e., the increases were coordinate.

To determine whether DNA sequences not expressed in the mammary epithelial genome remained unaltered in concentration, mouse globin cDNA was hybridized to these DNAs. The results, shown in Fig. 3, indicate that all the mouse DNAs, both normal and malignant, hybridized with the same kinetics to globin cDNA.

DISCUSSION

The increase in casein sequence abundance in DNA in MTV-induced tumors could have resulted from at least four types of change in host cell DNA, 1) an increase in the relative number of chromosomes containing casein genes, 2) an intrachromosomal increase in DNA at a casein gene locus, 3) the induction of extrachromosomal forms of DNA containing casein sequences, or 4) the introduction of exogenous casein sequences by MTV during infection.

Comparison of published physical chemical characteristics of bovine α, β, and κ caseins (25,26), 3 major rat caseins (27), and mouse caseins (28), indicates that there are likely a minimum of 3 casein genes in the mouse genome. The DNA hybridization data presented in Fig. 1 and Table 2 are consistent with this likelihood. The casein sequence increase could therefore have resulted either from a coordinate multiplication of a set of these genes, or the selective increase of certain casein gene species. The DNA isolation procedure used in the studies reported here copurifies high and low molecular weight DNA (60% of 5 to 10s linear duplex DNA is included) so the casein DNA detected could have been either in chromosomes or extrachromosomal.

Trisomy of chromosome 13 is a frequent correlate in C3H mouse (MTV-associated mammary cancer (29). Whether casein genes are located on chromosome 13 isn't

known at present, but if so a net increase would result. However, trisomy alone would at most account for only a 50% increase in casein (or any other) sequences, not the 2 to 4-fold enrichment found in MTV-induced tumors. Conversely, several clinical correlates of selective losses of, or deletions within, chromosome arms and particular malignancies have been documented (30,31). Thus the losses of casein sequences in D2 mammary tumors (Fig.1C and Table 2) could have resulted from selective deletions leading to mammary neoplasia of a specific chromosome region near or containing casein genes. In this context it is interesting that Rosen, et al, reported (21) that casein gene abundance in normal and DMBA transformed rat mammary DNA were the same.

It has been reported that a substantial tandem amplification in the ribosomal genes has been found in rat hepatoma and sarcoma cell lines (32,33). Gene amplifications of aspartyltranscarbamylase (34) and dihydrofolate reductase (35) have been documented, which in some cases are found in the form of "double minute" chromosomes (35). Evidently these selected-for gene increases also amplify flanking sequences (36) of unknown (and possibly unrelated) function.

Direct mediation of casein sequence abundance increase by MTV would presumably require either the introduction of exogenous casein sequences into mammary cells by MTV during infection, or viral-mediated host cell DNA rearrangements.

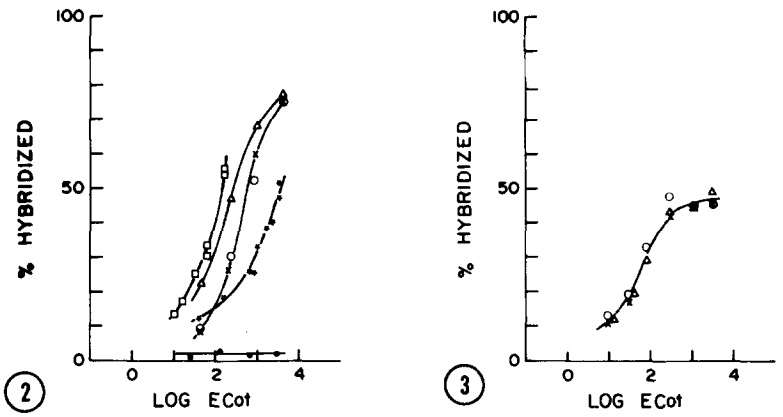

Figure 2. Hybridization of MTV cDNA to normal and mammary tumor DNAs. MTV cDNA was hybridized to the indicated DNAs as described in Methods and Materials. ✕—✕, 8 to 10 day lactating BALB/c mouse mammary gland DNA. ○—○, hormone-induced mammary tumor DNA, tumor #2. □—□, MTV-induced mammary tumor DNA, tumor #1. △—△, MTV-induced tumor DNA, tumor #2. ★—★, [^{14}C]-unique sequence mouse DNA. ●—●, rat liver DNA.

Figure 3. Hybridization of globin cDNA to normal and mammary tumor DNAs. Globin cDNA was hybridized to the indicated DNAs as described in Methods and Materials. ✕—✕, 8 to 10 day lactating BALB/c mouse mammary gland DNA. ○—○, MTV-induced mammary tumor DNA, tumor #2. △—△, hormone-induced mammary tumor DNA, tumor #2 (primed).

TABLE 1

RNA	cDNA	ECrot	%HYBRID	COMPLEMENTARITY TO cDNA
CASEIN mRNA	CASEIN[a]	0.0022	50. (21)	(≡1)
"	"	0.012	90. (21)	
MTV RNA	"	5.0	4.3	10^{-5}
LIVER RNA	"	1000.	7.5 (21)	10^{-7}
CASEIN mRNA	CASEIN[b]	0.0032	50. (22)	(≡1)
"	"	0.046	90. (22)	
LIVER RNA	"	1500.	4.0 (22)	10^{-8}
MTV RNA	MTV	0.0011	50.	(≡1)
"	"	0.0085	90.	
SWISS 3T3	"	1000.	5.0	10^{-7}
RLV RNA	"	500.	5.0	10^{-5}

Table 1: Hybridization specificities of CDNAs. The extent of hybrid formation between the indicated RNAs and cDNAs is given for the respective ECrot values (hybridization performed as described in Methods and Materials, or referenced if published data), and is followed by the calculated extent of cDNA complementarity to the RNAs, where the extent of hybridization to their respective RNA templates is taken as 1. a) obtained from J. Rosen, b) obtained from R. Ganguly and M. Banerjee.

Although copackaging of retroviral and host cell messenger RNA would not be unprecendented (globin mRNA has been detected in virions produced by Friend erythro leukemia cells induced for hemoglobin synthesis (37), it appears improbable that MTV itself could have acted as a vector for exogenous casein sequences, since hy-

TABLE 2

DNA	cDNA	AV. SINGLE GENE EQUIV.	RELATIVE GENE EQUIV. MALIGNANT / NORMAL	RELATIVE GENE EQUIV. CASEIN / MTV
MIDPREGNANT MAMMARY GLAND	CASEIN	3		0.6 - .75
LACTATING MAMMARY GLAND	"	3		0.6 - .75
HORMONE-INDUCED MAMMARY TUMOR #1	"	1	0.3	0.2 - .25
#2	"	1	0.3	0.2 - .25
#3	"	3	1.0	0.6 - .75
MTV-INDUCED MAMMARY TUMOR #1	"	11	3.7	0.5 - .6
#2	"	6	2.0	0.5 - .6
LACTATING MAMMARY GLAND	MTV	4 - 5		
HORMONE-INDUCED MAMMARY TUMOR #1	"	4 - 5		
MTV-INDUCED MAMMARY TUMOR #1	"	17 - 20		
#2	"	10 - 12		
HORMONE-INDUCED MAMMARY TUMOR #2	GLOBIN		1.0	
MTV-INDUCED MAMMARY TUMOR #2	"		1.0	

Table 2: The single gene equivalents of casein, MTV and globin sequences in normal and mammary tumor DNAs. The average single gene equivalents for sequences complementary to the casein, MTV and globin cDNAs (calculated from the data in Fig. 1, taking unique sequence DNA reassociation as 1) are given for the indicated normal and malignant DNAs. Under Relative Gene Equivalents are listed 1) the ratios of average single gene equivalents of casein or globin sequences in tumor v.s. normal DNA, and 2) for each DNA, the ratios of average single gene equivalents for casein v.s. MTV sequences.

bridization of casein cDNA to MTV 70s RNA was undetectable to the level of 1 part in 10^5 (Table 1).

The selectivity of the alterations reported here is suggested by the finding that the concentration of globin sequences was unchanged in DNAs that showed either an increase or a decrease in the rate of hybridization to the casein probe (Fig. 3, Table 2).

Distinguishing among such possibilities for the alterations in casein sequence abundance will be facilitated by restriction analysis of casein DNA sequences (now in progress), chromosomal assignments of mouse casein genes, and the use of cDNAs prepared from cloned casein genes.

An alteration in casein DNA per se would seem a priori to be of little consequence regarding carcinogenesis, but if part of a domain of developmental genes regulating proliferation and morphogenesis, this could be a marker for other changes of fundamental import. The findings presented in this report may offer a lead in understanding the oncogenic relationship between MTV and the regulation of mammary epithelial cell function.

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