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Suppression of mouse mammary tumor proviral DNA and protooncogene expression: Association with nutritional regulation of mammary tumor development

(mouse mammary tumor virus/chronic energy intake restriction)

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ABSTRACT **Chronic energy intake restriction (CEIR) reduces mouse mammary tumor virus (MMTV)-induced mammary tumors in C3H/Ou mice. Fewer than 10% of C3H/Ou mice developed mammary tumors during 88 wk of study when subjected to CEIR regardless of calorie source (fat vs. carbohydrate). By contrast, 100% of mice fed ad libitum diets relatively high in fat or carbohydrate or a commercial diet developed tumors by 35–40 wk. MMTV proviral DNA transcription was shown to be activated in spleen, liver, lung, kidney, small intestine, and mammary gland of mice consuming these diets ad libitum. By contrast, these messages were suppressed by CEIR in all tissues analyzed except spleen. MMTV proviral messages in liver and mammary gland increased with age in full-fed mice and were suppressed by CEIR. These findings suggest that the nutritional regulation of MMTV proviral DNA expression is tissue-specific. In CEIR mice the suppressed MMTV proviral DNA transcripts in mammary gland and liver increased with time in association with the delayed onset of mammary tumors. Mammary tumorigenesis in C3H mice is associated with integration of MMTV proviral DNA, which appears to activate a putative mammary tumor protooncogene, *int-1*. CEIR apparently decreases the frequency of viral reintegration adjacent to the *int-1* gene and thus inhibits expression of *int-1* and probably an initiation step in mammary tumorigenesis. Expression of other putative protooncogenes, *int-2* and *ras*, in liver tissue was also reduced by CEIR. These findings indicate that both initiation and promotion of mammary tumorigenesis are influenced by CEIR in C3H/Ou mice.**

Milk-transmitted mouse mammary tumor virus (MMTV) is an etiological agent of mammary tumors (MTs) in mice (1). In C3H/Ou mice such tumors occur after a period of incubation, with median tumor incidence achieved at 30–35 wk of age. However, the pathogenic mechanism(s) underlying induction of mammary tumorigenesis remains unclear. Evidence suggests that integration into the host genome of MMTV at a proviral DNA stage can activate a putative MT protooncogene (*int-1*) in C3H mice. The presence of MMTV proviral DNA integration in association with *int-1* activation is found in 69% of spontaneous MTs (2), supporting an insertional mutagenesis model of MMTV action and suggesting that MMTV-induced MTs occur by clonal growth following insertion of provirus in the vicinity of the *int-1* locus. Other reports involving transgenic mice suggest that putative cellular protooncogenes such as c-*myc* (3) and c-Ha-*ras* (4) driven by the long terminal repeat of MMTV may play a role in malignant transformation of mammary epithelial cells. In addition to the MT-related protooncogenes, hormones (5), genetic makeup (6), diet (7), immunological status (8), and the presence of MMTV play important roles in initiation and/or development of MTs.

Prior studies revealed that restricted diets drastically reduce mammary tumor development, decrease production of MMTV A and B particles (9, 10), and reduce development of hyperplastic nodules of mammary glands but do not greatly influence levels of somatotropin or thyrotropin or the onset or phases of estrous cycles (9). Similarly, chemically induced MTs in rats are inhibited by decreased calorie consumption (11). Herein we present studies on the influence of calorie intake and calorie source on spontaneous murine MT and on MMTV-related molecular events and demonstrate that MMTV proviral DNA expression and activation of putative cellular protooncogenes are regulated by calorie intake.

MATERIAL AND METHODS

Diet Regimen and Feeding. The dietary regimens used in this study were designed to provide isocaloric consumption of semipurified diets either relatively high in carbohydrate or high in fat (10, 12). Virgin female 6- to 8-week-old C3H/Ou mice (13) obtained from H. C. Outzen (Prehn Laboratory, San Jose, CA) were maintained according to Public Health Service/National Institutes of Health publication 86-23. Chronic energy intake restriction (CEIR) mice were fed twice weekly and weighed weekly. Groups of 36 C3H/Ou virgin female mice (group N) or 20 C3H/Ou virgin mice foster-nursed on C57 black dams (group F) were fed a standard commercial diet ad libitum. Mice in groups A_1 or A_2 were fed ad libitum the diets described in Table 1. A_1 mice consumed a diet in which energy (calories) was derived largely from carbohydrate, except for the protein, with 4.5% of calories from safflower oil to provide essential fatty acids. A_2 mice consumed a diet in which fat was the principal calorie source. B_1 and B_2 mice (CEIR mice) consumed $\approx$60% of the energy (calories) consumed by mice fed the A_1 or A_2 diets. The protein and essential amino acids of the CEIR diets were adequate and proportional to calories consumed. Equal amounts of essential vitamins and minerals were consumed by mice of all groups (10).

Mammary Tumors and Tissues. MTs induced by milk-transmitted MMTV were identified by palpation. Upon euthanasia at various intervals, MTs or specific organs were collected from animals of each experimental group and were

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Abbreviations: CEIR, chronic energy intake restriction; MMTV, mouse mammary tumor virus; MT, mammary tumor.

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Table 1. Composition of diets

Constituent	A_1		B_1		A_2		B_2	
	g	kcal	g	kcal	g	kcal	g	kcal
Sucrose	47.25	189.0	26.51	106.05	—	—	—	—
Glycerol	16.0	64.0	8.98	35.91	—	—	—	—
Lard	—	—	—	—	28.1	252.9	15.77	141.93
Casein	29.4	117.6	17.64	70.56	29.4	117.6	17.64	70.56
Methionine	0.6	2.4	0.36	1.44	0.6	2.4	0.36	1.44
Safflower oil	2.0	18.0	2.0	18.0	2.0	18.0	2.0	18.0
AIN vitamin mix	1.0	3.95	1.0	3.95	1.0	3.95	1.0	3.95
AIN mineral mix	3.5	1.652	3.5	1.652	3.5	1.652	3.5	1.652
Inositol	0.05	0.2	0.05	0.2	0.05	0.2	0.05	0.2
Choline bitartate	0.2	0.8	0.2	0.8	0.2	0.8	0.2	0.8
Total	100.0	397.6	60.24	238.56	64.85	397.5	40.52	238.53
Energy, kcal/g	3.98		3.96		6.13		5.89	
kcal ratio								
Prot/total	0.302		0.302		0.302		0.302	
CHO/total	0.636		0.595		—		—	
Fat/total	0.045		0.075		0.681		0.670	

CHO, carbohydrates; Prot, protein; 1 cal = 4.184 J.

processed immediately or snap frozen. Age-matched control tissues were obtained from foster-nursed mice.

RNA Isolation and Northern Blot Analyses. Fresh or snap-frozen tissues were rapidly homogenized for 1 min in 5 M guanidinium isothiocyanate in a Tissuemizer homogenizer (VirTis) by using a one-step method of total cellular RNA isolation (14). Briefly, homogenized tissues were deproteinized with phenol/chloroform, 1:1 vol/vol, and RNA was selectively precipitated with ethanol. Polyadenylylated RNA (mRNA) was isolated by oligo(dT)-cellulose affinity chromatography and run on agarose gel after denaturing with glyoxal and dimethyl sulfoxide. mRNA was transferred by capillary blotting to a Nytran filter (15) and applied either to a slot-blot apparatus (Schleicher & Schuell) or a dot-blot apparatus (Bethesda Research Laboratories) under gentle suction.

Radiolabeling of Probes and Blot Hybridization. Probes obtained from the appropriate recombinant plasmids carried in *Escherichia coli* included: p203, an *env* fragment of MMTV proviral DNA (C3H strain) (16); pSVc-myc1, a subfragment of mouse c-*myc* gene (17); and HB-11, a subfragment of Harvey murine sarcoma virus oncogene v-Ha-*ras* (18) (American Type Culture Collection). Plasmids were generously provided as follows: S621, a mouse int-1 cDNA clone, from H. E. Varmus (2); int-2f, a subfragment of mouse *int-2* gene, from C. Dickson (19); C7V, a subfragment of mouse *int-3* gene, from R. Callahan (20); and actin, representing *Drosophila* actin gene, from A. Sodja (21). DNA probes were radiolabeled by using an oligonucleotide-labeling method (22). We used hexadeoxyribonucleotide as primer with [α-^{32}P]dCTP. Filters with the transferred DNA or RNA species were incubated with 1–2 × 10^7 cpm of radioactive probe in a mixture containing 50% formamide, 3× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate), 200 μg of sonicated denatured salmon sperm DNA per ml, 100 μg of transfer RNA per ml, 0.5% sodium dodecyl sulfate (SDS), 0.05% sodium pyrophosphate, and 1× Denhardt's buffer (0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll) for 24–48 hr at 42°C. After hybridization, the filters were washed three times for 30 min each in 0.1× SSC/0.1% SDS/0.01% sodium pyrophosphate at 52°C, dried, and exposed to Kodak x-ray film with intensifying screens at −70°C.

RESULTS

Influence of CEIR and Dietary Composition on Breast Cancer in C3H/Ou Mice. As expected for the C3H/Ou strain, median tumor incidence of mice fed a commercial diet was reached by 35 wk, and 100% incidence occurred at 46 wk (Fig. 1). By contrast, development of MTs was greatly delayed in CEIR mice regardless of whether fat provided 7.5% or 67.0% of calories. During 84 wk of study, only five MTs occurred among the CEIR mice (B_1 or B_2 diets), whereas mice fed the purified diets ad libitum (A_1 or A_2) developed MTs as early as 25 wk, with median tumor incidence achieved at 45 wk and 41 wk in the mice fed diets A_1 or A_2 respectively.

Presence of MMTV Proviral, int-1, and int-2 Transcripts in MTs. Table 2 summarizes observations on the MMTV proviral *env* expression and *int-1* and *int-2* protooncogene expressions by RNA blot-hybridization (Northern) analysis in MTs of mice on different diets. MT tissue regularly expressed *env* gene regardless of the dietary regimen consumed, but expression of *int-1* and *int-2* varied among individual mice. In mice fed ad libitum a diet wherein calories came largely from carbohydrate (A_1), 50% of MTs expressed only *int-1* gene and 50% expressed both *int-1* and *int-2* genes. In mice fed the fat-based A_2 diet ad libitum, 85.7% of MTs expressed only *int-1* and 14.2% expressed both *int-1* and *int-2*. These data are consistent with previous reports of *int-1* and *int-2* expression associated with MT development (2).

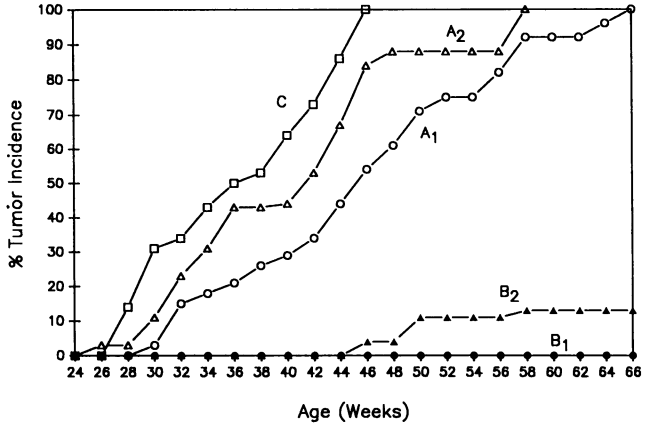

FIG. 1. Influence of CEIR and dietary composition on breast cancer in C3H/Ou mice. Incidence of MTs among C3H/Ou mice fed diets A_1 or A_2 ad libitum, a commercial diet (C), or CEIR diets B_1 or B_2.

Table 2. Presence of MMTV proviral, *int-1*, and *int-2* gene transcripts in MTs detected by Northern blot analysis

		env		int-1		int-2		int-1/int-2	
Diet	Tumors	*n*	(%)*	*n*	(%)*	*n*	(%)*	*n*	(%)*
A_1	6	6	(100)	3	(50.0)	0	(0)	3	(50.0)
A_2	7	7	(100)	6	(85.7)	0	(0)	1	(14.2)
B_2	1	1	(100)	1	(100)	0	(0)	0	(0)
N	5	5	(100)	4	(80)	0	(0)	1	(20.0)

Poly(A)$^+$ RNA preparations (1 μg) of MT tissues from mice fully fed diets A_1 or A_2 or fed CEIR diet B_2 or a commercial (N) diet were hybridized with *env*, *int-1*, and *int-2* probes as described.
*Incidence of tumors expressing respective transcripts (*n*) is shown in parentheses.

Tissue Distribution of MMTV Proviral DNA Transcripts As Influenced by Calorie Intake. Next, we compared expression of MMTV proviral DNA transcripts by Northern blot analysis using mRNA extracts of muscle, heart, spleen, kidney, lung, liver, small intestine, and mammary gland from 34-wk-old tumor-bearing mice fed the A_1 diet ad libitum (Fig. 2, even numbered lanes) to MMTV expression distributions of age-matched non-tumor-bearing CEIR mice fed diet B_1 (Fig. 2, odd numbered lanes). As shown, a 3.8-kilobase-pair (kbp) *env* gene-specific transcript of MMTV proviral DNA was expressed in spleen, kidney, lung, liver, intestine, and mammary gland (lanes 6, 8, 10, 12, 14, and 16) of full-fed mice. In contrast, CEIR dramatically reduced expression of *env* gene of MMTV proviral DNA in kidney, lung, liver, small intestine, and mammary gland (lanes 7, 9, 11, 13, and 15). Neither muscle nor heart expressed proviral messages (lanes 1–4). *env* gene expression was observed in spleens of full-fed and CEIR mice. Actin gene, used as an internal control, was not altered in any of the tissues despite great ranges in energy intake. Identical findings were obtained for each tissue site for at least two pairs of age-matched mice at each calorie level.

Time-Course Investigation of MMTV-Specific mRNA Appearance in Mammary Gland, Liver, and Spleen as Influenced by Calorie Intake. We determined the earliest expression of both endogenous and exogenous MMTV by slot-blot and dot-blot analyses in mammary gland, liver, or spleen in mice full-fed a commercial diet (group F) or mice fed diets A_1 or B_1. Mammary glands of foster-nursed, MMTV-free mice were also evaluated at 19 wk (group F). Mice were sacrificed at intervals from 2 to 84 weeks after imposition of diets. For each tissue sample, a 1:1 serial dilution (three dilutions) of mRNA starting with the highest concentration of 1 μg of mRNA was used. By 16 wk, mammary tissue from C3H/Ou mice fed a commercial diet or foster-nursed mice fed a commercial diet expressed the MMTV *env* gene (Fig. 3), indicating that, whereas endogenous MMTV provirus is expressed only in group F mice, exogenous as well as endogenous MMTV messages are expressed by 16 wk of age in mammary tissue of mice fed a commercial diet. Furthermore, viral expression reached a maximum by 25 wk in mammary tissue of group N or diet A_1 mice. By contrast, mammary tissue of CEIR mice expressed low levels of the *env* gene at 25, 35, and 45 wk, with full expression delayed until 65 wk of age, when a few MTs had appeared. The delay in MMTV *env* expression parallels the increased latency to tumor formation in CEIR mice. Similar findings were observed with respect to the appearance of MMTV message in liver, except that full expression in liver tissue peaked at 65 or 85 wk in A_1 or B_2 mice, respectively. In spleen, MMTV expression reached a maximum at 25–35 wk, and no differences in *env* gene expression were observed as a function of diet.

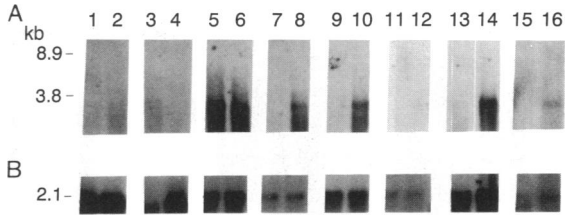

FIG. 2. Tissue distribution of MMTV proviral DNA transcripts as influenced by calorie intake. Poly(A)$^+$ RNA (2 μg) from each organ of age-matched (34 wk) mice fed diet A_1 or B_1 was collected on a Nytran filter and analyzed with *env* gene probe A and actin gene probe B as described. Lanes: 1 and 2, muscle; 3 and 4, heart; 5 and 6, spleen; 7 and 8, kidney; 9 and 10, lung; 11 and 12, liver; 13 and 14, small intestine; 15 and 16, mammary gland. Even numbers represent fully fed A_1 mice; odd numbers represent calorie-restricted B_1 mice. Each panel represents a 5-day exposure.

Expression of MMTV-Specific mRNA in Mammary Tumor, Mammary Gland, or Liver Tissues of Mice on Different Diets. Fig. 4 presents representative data for both envelope (3.8 kbp) and full-length (8.9 kbp) MMTV proviral messages expressed in individual mammary tumors (Fig. 4*A*), pooled mammary gland (Fig. 4*B*), and individual livers (Fig. 4*C*) of mice fed different dietary regimens. env RNA and full-length MMTV messages were expressed in MTs taken from mice 36–58 wk of age fed a commercial diet or diets A_1 or A_2. No significant differences were detected in MMTV message in tumor tissue among the full-fed groups of mice (Fig. 4*A*). Similarly, in nontumorous mammary tissue taken from older mice (58–65 wk), no significant difference could be attributed to calorie intake or source (Fig. 4*B*). As expected, low

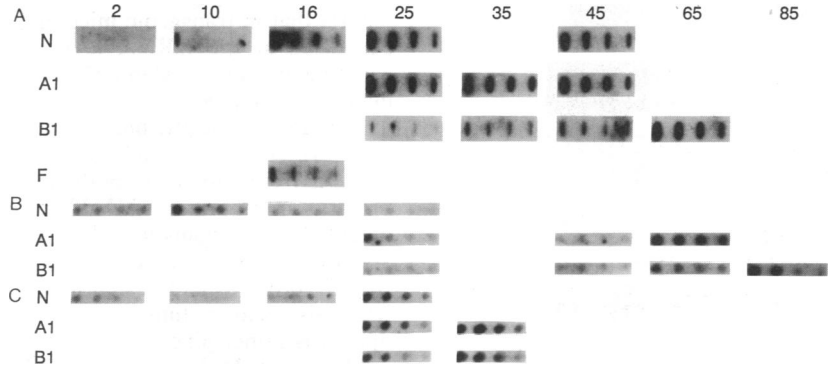

FIG. 3. Time-course study of MMTV-specific mRNA in mammary gland, liver, and spleen tissue under different dietary regimens. Slot-blot (*A*), or dot-blot (*B* and *C*) hybridizations of poly(A)$^+$ RNA of pooled mammary gland (*A*), individual liver (*B*), and individual spleen (*C*) from age-matched mice (2–85 wk, shown at the top) fed a commercial diet (N), diets A_1 or B_1, or foster-nursed and fed a commercial diet (F, age 19 wk). Serial 1:1 dilution of 1 μg of mRNA from left to right in each blot was applied to a Nytran filter through a slot or dot apparatus. The bound mRNA was hybridized to *env* probe as described. Each slot or dot represents a 5-day exposure.

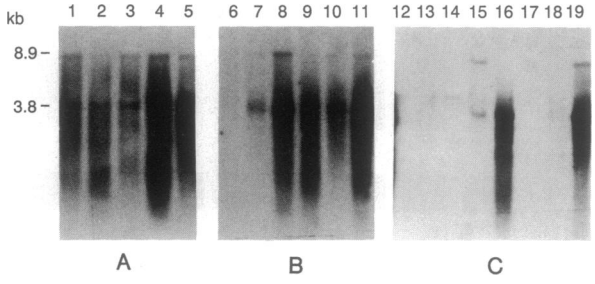

FIG. 4. Comparison of MMTV-specific transcription in MT, mammary gland, or liver tissue under different dietary regimens. Poly(A)$^+$ RNA preparation from individual MTs (*A*), pooled mammary glands (*B*), or individual liver specimens (*C*) of C3H/Ou mice under different diets analyzed by Northern blot-hybridization with *env* probe as described. (*A*) Lanes: 1, 1 μg of mRNA preparation of MT from 36-wk-old mice fed a commercial diet; 2 and 3, 36- to 59-wk-old mice fed diet A_2; 4 and 5, 36- to 59-wk-old mice fed diet A_1. Each lane represents a 2-hr exposure. (*B*) Lanes: 6, 2 μg of mRNA of pooled mammary gland tissue from four 19-wk-old group F mice; 7, 19-wk-old mice fed a commercial diet; 8, 58- to 65-wk-old mice fed diet B_2; 9, 30- to 50-wk-old mice fed diet A_2; 10, 58- to 65-wk-old mice fed diet B_1; 11, 30- to 50-wk-old mice fed diet A_1. Exposure time was 3 days. (*C*) Lanes: 12, 2 μg of mRNA of liver tissue from tumor-free, 19-wk-old group F mice; 13, 19-wk-old tumor-free mice fed a commercial diet; 14, 39-wk-old tumor-free mice fed diet B_2; 15, 59-wk-old non-tumor-bearing mice fed diet A_2; 16, 36-wk-old tumor-bearing mice fed diet A_2; 17, 39-wk-old non-tumor-bearing mice fed diet B_1; 18, 36-wk-old non-tumor-bearing mice fed diet A_1; lane 19, 46-wk-old tumor-bearing mice fed diet A_1. Each lane represents a 5-day exposure; 3.8 kbp of *env* gene and 8.9 kbp of full-length transcripts are indicated to the left.

expression was observed in pooled mammary tissue of young, foster-nursed mice fed a commercial diet (Fig. 4*B*, lane 6).

Distinct differences in levels of virus mRNA were observed in livers of older tumor-bearing and non-tumor-bearing mice fed different diets. mRNA expression in liver was significantly decreased in full-fed mice that had not yet developed MTs (Fig. 4*C*). Hepatic MMTV provirus expression thus appears to reflect the concurrent presence of a mammary malignancy. Reduced viral expression was observed in liver tissue of non-tumor-bearing mice, including foster-nursed mice fed a commercial diet (lane 12), Group N mice fed a commercial diet (lane 13), or mice fed the CEIR diets (lanes 14 and 17).

Expression of Protooncogenes in Mammary Tumors, Mammary Glands, and Liver Tissues of Mice on Different Diets.

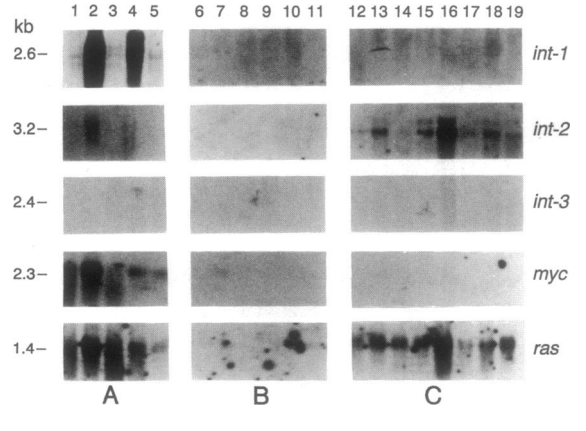

FIG. 5. Dietary influence on protooncogene expression in mammary tumor, mammary gland, or liver tissue. Tissues and methods were as in Fig. 4 except for probes, which are indicated. Each represents a 3-day exposure, except for *myc* and *ras* probes (*A*), *ras* probe (*B*), and (*C*) probes *int-2* and *ras*, which show 7-day exposure.

int-1, *int-2*, *int-3*, *myc* and *ras* gene expression was studied by using Northern blot analysis in the tissues described in Fig. 4 *A–C*. As shown, tissue from MTs regularly expressed *int-1*, *myc*, and *ras* protooncogene messages (Fig. 5*A*) regardless of dietary manipulation. *int-2* was not expressed independently, but was expressed in a small percentage of tumors in association with *int-1* (see also Table 2). No protooncogenes were detected in mammary tissue of non-tumor-bearing mice subjected to the different dietary regimens (Fig. 5*B*). Hepatic protooncogene expression was limited to *int-2* and *ras* expression (Fig. 5*C*). Hepatic *int-2* expression was reduced by CEIR, whereas hepatic *ras* expression was not as clearly influenced by calorie intake, and *myc* expression was not detected in liver tissue.

DISCUSSION

This study establishes that MT in C3H/Ou mice is influenced by total calorie intake with relative indifference to calorie source (Fig. 1). Although fat-based diets encourage earlier development of the first palpable tumor in mice and shortened the time to median tumor incidence, the overall tendency to develop mammary malignancy as measured by Survival Analysis using Weibull distribution did not differ between groups fully fed isocaloric diets, regardless of whether the diets derived calories largely from fat or carbohydrate. A 40% reduction of calories greatly delayed or inhibited MTs altogether, lowered mean body weight, and reduced circulating prolactin levels by a factor of 4 (9, 10).

The current investigations define the MMTV proviral and cellular protooncogene expression in various tissues under different dietary regimens. This approach seemed pertinent since virgin female C3H/Ou mice, a useful model of spontaneous mammary tumorigenesis and milk-transmitted exogenous MMTV, also may serve as a model for study of endogenous MMTV. Except for the mouse *Mtv-1* locus, the endogenous MMTVs (*Mtv-6*, *-8*, *-11*, and *-14*) are generally silent. *Mtv-1* is expressed as an infectious virus and is associated with late occurring MTs in C3H mice (23, 24). However, transcripts of MMTV provirus at mRNA level have been reported to occur primarily in the lactating mammary gland (25).

In the present study, a high level of MMTV *env* gene-specific transcripts was found in spleen, kidney, lung, liver, small intestine, and mammary gland tissue of tumor-free, ad libitum-fed virgin C3H/Ou mice at 34 wk of age. These MMTV-specific transcripts were dramatically suppressed by CEIR in all tissues except spleen. Full-length proviral messages were also detected in the two tissues studied in this regard (mammary gland and liver, Fig. 4 *B* and *C*), supporting reports that in transgenic mice endogenous MMTV may be expressed at the mRNA level in organs other than the lactating mammary gland (26). This expression was also inhibited by CEIR.

The MMTV-specific messages present in tissues of breast-fed C3H/Ou mice on all dietary regimens may have been derived from either or both endogenous and exogenous provirus. Furthermore, MMTV provirus messages were not detected in all organs tested (Fig. 2), suggesting that tissue-specific transcriptional control mechanism(s) exist for MMTV provirus expression in specific host tissues. Others have also shown limited tissue expression of the c-*myc* and/or *ras* oncogene messages in transgenic mice under the control of an MMTV promoter (the long terminal repeat of MMTV) (4).

In the present studies, high levels of MMTV messages were detected as early as 2 weeks of age in mammary gland, spleen, and liver in C3H/Ou mice. Furthermore, proviral expression increased rapidly with age in mice fed a commer-

cial diet—a finding consistent with the relatively short latency for MT development in C3H/Ou mice (13).

MMTV proviral transcription was demonstrated in multiple tissues in C3H/Ou mice. Mammary and hepatic expression of MMTV transcript occurred earlier in tissues of mice fed ad libitum and was clearly delayed by CEIR. This delay in proviral expression parallels increased latency in tumor development that characterizes CEIR mice with relative indifference to calorie source.

MMTV-specific transcripts were dramatically suppressed in both mammary and hepatic tissues by CEIR, suggesting a direct association between calorie intake and expression of MMTV proviral DNA at the mRNA level. However, the spleen seems less sensitive to the influence of CEIR. The reason for such tissue-specific differences in MMTV expression must be addressed in future studies. Nonetheless, suppression of MMTV-specific transcription attributable to CEIR in so many tissues might decrease availability of virus for integration and thus decrease the consequent activation of cellular protooncogenes thought to represent a crucial step in initiation of mammary tumorigenesis during the chronic phase of virus infection.

Transformation of mammary epithelial cells by MMTV appears to be a multistep process whereby tumorigenesis is fostered by persistent activation of a set of cellular genes over a long period of latency in tumor development. The cellular genes (putative protooncogenes) are considered to affect growth and development processes (25). Host genomic sequences that flank proviral integration sites—namely, the *int-1*, *-2*, and *-3* genes—appear important in early steps of mammary tumorigenesis, since their active transcription is frequently detected subsequent to proviral integration. Furthermore, *int-1* also is tumorigenic in transgenic mice and can stimulate growth of mammary epithelial cells (26).

In the present study, either *int-1* or *int-1* plus *int-2* gene messages, but not *int-3*, were found in MTs of C3H/Ou full-fed mice. These findings confirm reports by others (19, 20) and suggest that once a MT has been initiated, *int* genes and MMTV provirus transcripts might work together to influence MT growth. Relatively high levels of MMTV provirus and *int-2* but not *int-1* or *int-3* gene expression were found in liver tissue of MT-bearing mice, and hepatic *int-2* transcription was shown to be suppressed by CEIR.

It has also been proposed that protooncogenes *myc* and *ras* play a functional role in malignant transformation (26) and may contribute to induction of mammary tumors in transgenic mice (3, 6). In our studies both *myc* and *ras* gene transcripts were present in all mammary tumors tested irrespective of diet. Hepatic *ras* gene transcription, like hepatic *int-2* expression, was impressively reduced by CEIR. Hepatic protooncogene expression by *int-2* and *ras* appeared also to be reduced by CEIR. It can be speculated that a CEIR-induced suppressive effect on MMTV and certain protooncogene expressions in mammary gland may contribute to prevention of MTs during the initiation and promotion of mammary adenocarcinoma in C3H/Ou mice.

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