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Calorie restriction suppresses subgenomic mink cytopathic focus-forming murine leukemia virus transcription and frequency of genomic expression while impairing lymphoma formation

(diet/retroviral expression/proviral transcription)

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ABSTRACT **Calorie restriction suppresses mammary proviral mRNA expression and protooncogene activation in breast tumor-prone C3H/Ou mice while inhibiting tumor formation. To determine whether the beneficial effects of chronic energy-intake restriction (CEIR) can be extended to an organ site of retrovirus-induced tumorigenesis where the dynamics of growth and sexual maturity are not paramount as they are in breast tissue, calorie restriction of 40% was imposed on thymic lymphoma-prone AKR mice when 4 weeks old. Recombination between various murine leukemia virus (MuLV) mRNAs, resulting in the generation of an 8.4-kilobase genomic-length transcript with mink cytopathic focus-forming (MCF) characteristics, is considered the proximal retroviral event in AKR lymphomagenesis. Thymic expression of subgenomic MCF MuLV mRNA was uniformly suppressed among 6- and 8-week-old CEIR mice ($P < 0.02$). This suppression of MuLV transcription preceded a 25% reduction in the appearance of genomic-length MCF transcripts among CEIR mice and a 28% reduction in cumulative lymphoma mortality. The latency to median tumor incidence was extended >3 months by calorie restriction, and median lifespan was extended ≈50%. Survival curves for the full-fed and CEIR dietary cohorts were found to be significantly different ($P < 0.0001$), with full-fed mice experiencing a 3 times greater risk of lymphoma mortality. These findings extend the known range of pathologic states influenced by CEIR in inbred mice and show that retroviral mechanisms involved in generation of lymphoid malignancy can be significantly impaired by calorie restriction.**

Chronic energy-intake restriction (CEIR) without essential nutrient deficiency extends the latency to onset and reduces the frequency of neoplastic and non-neoplastic disease in inbred mice. Diseases delayed or prevented by calorie restriction include the systemic amyloidosis of senescence-accelerated mice (1), the systemic lupus erythematosus-like syndrome of B/W F_1 mice (2, 3), the breast cancer of C3H mice (4–6), the nephrophthisis of *kd/kd* mice (7), and the lymphoproliferative syndrome of autoimmune-prone MRL mpj *lpr/lpr* mice (8, 9).

The influences of calorie restriction on leukemogenesis and formation of lymphoma have been inferred from disease pattern surveys of large populations of outbred rats (10) and long-lived inbred (11) and hybrid (12) mice fed semipurified diets. In these surveys, cumulative frequencies of mortality due to lymphoma among calorie-restricted cohorts were lower and maximum longevities were extended. Further evidence that CEIR can regulate leukemogenesis is implied by an early report (13) of the stringent underfeeding of a nonpurified diet to inbred, lymphoma-prone AK mice. In that study, food restriction was severe enough to produce adult mean body weights of <17 g, organ underdevelopment, and high mortality attributable to causes other than lymphoma, but this restriction also lowered the incidence and delayed the onset of lymphoma (13).

That calorie restriction can influence mechanisms of retrovirus-induced tumorigenesis has been demonstrated by our prior investigations using breast cancer-prone C3H/Ou mice. Our findings showed that CEIR can modulate mouse mammary tumor virus (MMTV) proviral expression at the mRNA level, delay protooncogene activation, and abrogate the development of breast tumors (6, 14, 15). In the current study, we show that the protective influences of CEIR can be imposed in an organ site of retrovirus-induced tumorigenesis other than the breast by demonstrating that calorie restriction can significantly impair retrovirus-induced lymphomagenesis in AKR mice.

The AKR mouse develops thymic lymphoma with high frequency after 6 months of age and harbors multiple endogenous murine leukemia virus (MuLV) proviruses as well as numerous replication-defective, subgenomic MuLV-related sequences in their genomes. MuLVs are classified on the basis of host range in culture, which is determined by envelope glycoprotein gp70, encoded by the envelope gene (*env*), and are referred to as either ecotropic, infecting only murine cells, xenotropic, replicating primarily in heterologous cells, or dualotropic (16).

Thymic leukemogenesis in AKR mice requires the early expression of ecotropic MuLV and the subsequent emergence of recombinant, dualotropic MuLVs which are distinguished by their ability to form cytopathic foci in cultured mink lung cells (17). Mink cytopathic focus-forming (MCF) MuLVs have been isolated primarily from tumors and preleukemic thymuses, are moderately leukemogenic in mouse strains with low leukemia incidence, and accelerate lymphoma development in weanling AKR mice (16–18).

The independent temporal and tissue-specific expression of proviral and subgenomic ecotropic, xenotropic, and MCF mRNAs has been described (19, 20). Ecotropic MuLV mRNAs, including a genomic-length ecotropic transcript, are expressed during late embryogenesis and peak in intensity around 2 months of age, with continued expression in all tissues examined throughout life. Subgenomic MCF MuLV mRNAs are expressed perinatally in many tissues, peak in expression around 2 months of age, and regress in expression level after 4 months of age. Genomic-length MCF MuLV mRNA first appears around 3–4 months of age and is found only in the thymus. Whereas ecotropic MuLV can be isolated from many tissues during embryogenesis and throughout life, MCF MuLV isolation is possible only when AKR mice are at

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Abbreviations: MMTV, mouse mammary tumor virus; MuLV, murine leukemia virus; MCF, mink cytopathic focus-forming; CEIR, chronic energy-intake restriction.

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least 5 months old, and then only from thymic or lymphoma tissue. Ecotropic, xenotropic, and MCF-related transcripts are concurrently expressed exclusively in thymus tissue of AKR mice prior to detection of genomic-length MCF transcripts and isolation of MCF MuLV (19). It has been proposed that interaction and recombination among the genomic-length ecotropic MuLV transcript, subgenomic MCF transcripts, and xenotropic mRNA within thymocytes generates the potentially leukemogenic genomic-length MCF MuLV mRNA (19, 20). Nucleotide sequence analyses suggest that substitutions within the genomic-length ecotropic transcript have occurred, with MCF sequences substituting at the 5′ gp70 and 3′ p15E regions, and xenotropic sequences replacing the U3 region of the long terminal repeat (16, 19–21).

In the current study, we show that by restriction of calories consumed by AKR mice, MCF subgenomic mRNA expression is suppressed, the frequency of appearance of genomic-length MCF MuLV transcripts is reduced, the latency to median tumor incidence is extended by >3 months, the cumulative tumor incidence is reduced by 28%, and the median lifespan of CEIR mice is increased by ≈50%. These findings lend credence to the proposed mechanism of retroviral action in AKR leukemogenesis that involves generation of recombinant MCF MuLV genomes. The findings support the premise that calorie restriction without other essential nutrient deficiency can directly influence retrovirus-induced tumorigenesis, delay or prevent altogether tumor formation, and extend healthful lifespan.

MATERIALS AND METHODS

Animals. One hundred fifty-eight 4-week-old nulliparous AKR female mice (The Jackson Laboratory) maintained in accordance with the principles of the Animal Welfare Act and as described in Public Health Service/National Institutes of Health Publ. 86-23 were separated into two experimental groups. Mice of group A (full-fed) were housed in pairs and fed a semipurified diet ad libitum at 16–18 kcal/day (1 cal = 4.184 J) in which calories were derived principally from carbohydrates. Mice of group B (CEIR) were housed singly, were fed a proportionally similar complete semipurified diet, but were restricted 40% in calories consumed (10–11 kcal/day).

Semipurified Diets. The preparation and composition of the two semipurified diets used have been described in detail (6, 14) (Table 1). All dietary constituents were obtained from ICN. Both diets were low in dietary fat (≈6% of total calories) but differed by 40% in the level of total caloric energy available; the diets were otherwise comparable. The amounts of essential dietary constituents added to each diet were determined with regard to the gram and calorie consumption of mice fed ad libitum. As shown in Table 1, the diet prepared for CEIR mice (group B) was further enriched so that although the CEIR diet contained 40% fewer calories, equivalent amounts of vitamins, minerals, essential fatty acids, and 30% of calories as protein were consumed by all mice. Approximately 60% of total calories in both diets were derived from carbohydrates. All mice were fed twice weekly and weighed weekly.

Table 1. Composition of diets

Constituent	Group A diet		Group B diet	
	g	kcal	g	kcal
Sucrose	47.25	189.0	26.51	106.05
Glycerol	16.0	64.0	8.98	35.91
Casein	29.4	117.6	17.64	70.56
Methionine	0.6	2.4	0.36	1.44
Safflower oil	2.0	18.0	2.0	18.0
AIN vitamin mix	1.0	3.95	1.0	3.95
AIN mineral mix	3.5	1.65	3.5	1.65
Inositol	0.05	0.2	0.05	0.2
Choline bitartrate	0.2	0.8	0.2	0.8
Total	100.0	397.6	60.24	238.56
Energy, kcal/g	3.98		3.96	
kcal ratio				
Protein/total	0.302		0.302	
Carbohydrate/total	0.636		0.595	
Fat/total	0.045		0.075	

Purification and Analysis of RNA. Total RNA was isolated from excised thymus by an acid guanidinium isothiocyanate method (22), with tissues mechanically disrupted in a tissue homogenizer. RNA purity and quantity were ascertained from scanning spectrophotometric analysis.

RNA was denatured in a loading premixture containing 8.0% formaldehyde, 60% deionized formamide, 0.12% ethidium bromide, and 1.2× Mops buffer (1× Mops buffer is 20 mM 4-morpholinepropanesulfonic acid/50 mM sodium acetate/1 mM EDTA). Samples were heated for 15 min at 55°C and chilled on ice prior to electrophoresis in formaldehyde denaturing gels (2.2 M formaldehyde/1% agarose/1× Mops buffer). RNA sizes were estimated by semilogarithmic graph comparison with ribosomal RNA bands and with an RNA ladder on the same gel (GIBCO/BRL). RNA was transferred from gels to Hybond-N nylon hybridization membranes (Amersham) with a vacuum blotting apparatus (2016 Vacugene, Pharmacia LKB) and then was bound to the membranes by exposure to UV light (UV Stratalinker 1800, Stratagene).

Prehybridization (duration, 8–24 hr) and hybridization (8–16 hr) were at 42°C in buffer containing 5× SSC (1× SSC is 0.15 M NaCl/15 mM trisodium citrate), 50% formamide (deionized), denatured salmon sperm DNA (60 μg/ml), yeast tRNA (37 μg/ml), 0.4% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate (pH 7; prepared from 1 M stocks of mono- and dibasic sodium phosphate), 0.1% sodium pyrophosphate and 2× Denhardt's solution (1× is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin).

Probes were radiolabeled with [α-^{32}P]dCTP by use of a random-priming kit (Boehringer Mannheim). Each 11 × 14-cm filter was incubated with 10^7 cpm of radiolabeled probe in 10 ml of hybridization buffer in a small (14-cm) hybridization chamber tube (Robbins Scientific, Sunnyvale, CA).

Probes were prepared from recombinant plasmids grown in *Escherichia coli*. A. S. Khan (Laboratory of Molecular Microbiology, National Institutes of Health) kindly provided plasmid pMCF-1, which contains an MCF envelope-specific fragment (23). S. K. Chattopadhyay (Pediatric Oncology, National Cancer Institute) kindly provided a plasmid containing a 400-base-pair fragment from the envelope region of AKR ecotropic MuLV (24). Probe identity and specificity were confirmed from Northern and Southern blots of cell lines singly infected with MCF (MCF247-infected mink lung cells), ecotropic (pAKR623-infected NIH 3T3 mouse fibroblasts), and xenotropic (NFS-Th-1 mink lung cells) leukemia viruses, all kindly provided by A. S. Khan (20).

After hybridization, filters were washed at 55°C three times for 15 min each in 2× SSC/0.1% SDS and then once for 15 min in 1× SSC/0.1% SDS. Filters were sealed in plastic bags and then exposed to Kodak X-Omat RP x-ray film at −70°C with Spectroline L Plus intensifying screens (Kodak; Sigma).

Banding patterns of MuLV RNA on Northern blots were as previously reported (20). The density of MuLV genomic and subgenomic transcript bands was semiquantified by laser densitometry (Ultrascan XL, Pharmacia LKB).

Experimental Plan. Four mice each of group A or B were euthanized when 6, 8, 12, 16, 17, 21, and 25 weeks old to

assess genomic and subgenomic ecotropic and MCF MuLV transcription levels and to determine the age of onset and frequency of appearance of genomic, full-length MCF MuLV transcripts, considered the MuLV genome required for leukemogenesis in the AKR mouse (19, 20). An additional eight mice each of group A or B were euthanized when 21 weeks old for histologic examinations (sections were fixed in buffered 10% formalin and stained with hematoxylin and eosin) and for calculating organ/body weight ratios so as to address the prior observation by others that underfed AK mice experience organ underdevelopment (13). The balance of 86 group A and B mice were followed to assess the age of onset, manifestations, and frequency of lymphoma formation. Mice were examined three times weekly for signs of illness, including anorexia, dyspnea, and peripheral lymphadenopathy. Each mouse was necropsied at the time of natural death.

Statistics. The mean densities of ecotropic and MCF-related MuLV bands on Northern blots from 6- and 8-week-old mice were compared using a one-way analysis of variance. Development of thymic lymphoma in group A and B mice were compared by Kaplan–Meier product limit analysis of individual groups (25).

RESULTS

Physical Parameters. Nulliparous AKR female mice fed semipurified diets either ad libitum (group A, full-fed mice) or restricted 40% in calories (group B, CEIR mice) gained weight, with mean body weights of adult CEIR mice 25–30% less than that of full-fed controls (Fig. 1). Ratios of mean uterine weight to body weight, mean hepatic weight to body weight, and mean renal weight to body weight for CEIR mice were higher than similar ratios for full-fed mice (data not shown). Mammary pads from full-fed mice weighed 4 times more than those of CEIR mice, a reflection of reduced body fat among CEIR mice. Significant differences in the histologic appearance of organs from either dietary cohort were not apparent.

Expression of Ecotropic and Subgenomic MCF MuLV Transcripts. Fig. 2 is a Northern blot of thymic RNA, probed for subgenomic MCF MuLV expression in eight individual 6-week-old group A or B mice. In every case, thymic expression of subgenomic MCF MuLV transcripts is lower among individual group B (CEIR) mice when compared with group A (full-fed) controls. In 8-week-old mice thymic subgenomic MCF MuLV expression was again lower among three of four CEIR mice. The fourth CEIR mouse exhibited thymic expression at a level equivalent to the lowest individual expression among full-fed controls.

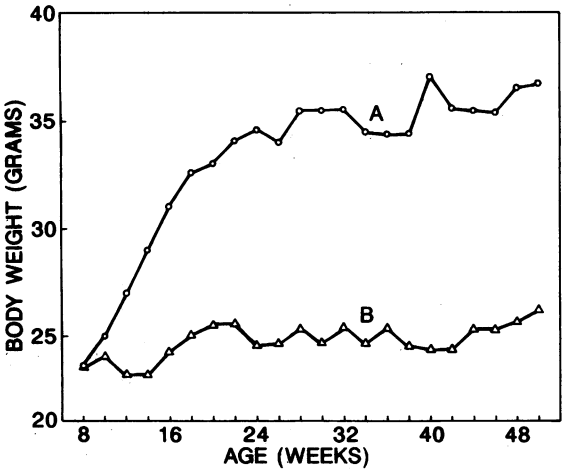

FIG. 1. Mean body weights of full-fed (group A) (○) and 40% calorie-restricted (group B) (△) mice.

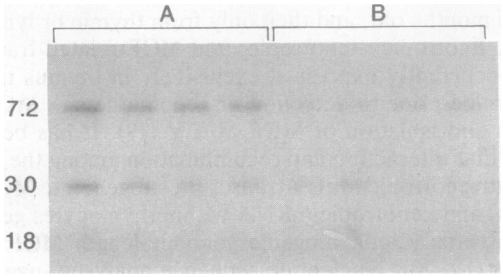

FIG. 2. Northern blot analysis of thymic RNA from individual 6-week-old AKR mice. Thymic RNA (10 μg) from an individual mouse was loaded on each lane and, after electrophoresis and blot transfer, hybridized to ^{32}P-labeled pMCF-1 probe. Bands of subgenomic MCF MuLV transcripts are designated 7.2, 3.0 and 1.8 (length in kilobases). Lanes A, full-fed mice; lanes B, CEIR mice.

Fig. 3 depicts the mean thymic expression levels of both ecotropic MuLV and subgenomic MCF MuLV among 16 individual group A or B mice when 6 or 8 weeks old. Ecotropic and subgenomic MCF MuLV expression increased with age in both dietary cohorts (note increase in units of MuLV expression for mice 8 weeks of age in Fig. 3). Mean subgenomic MCF MuLV expression among 6-week-old CEIR mice was suppressed by 69% when compared with the expression of full-fed mice. When mice were 8 weeks old, thymic expression of subgenomic MCF MuLV mRNA was again significantly suppressed ($P < 0.02$) in CEIR mice. Mean thymic expression of ecotropic MuLV was one-third lower among calorie-restricted mice when 6 weeks old, but strong and equivalent among all mice when 8 weeks old (Fig. 3). As can be seen in Figs. 2 and 3, persistent suppression of subgenomic MCF MuLV mRNA expression was imposed by the 40% restriction of calories.

Appearance of Genomic-Length MCF MuLV Transcripts. Appearance of genomic-length MCF MuLV transcripts solely in thymic tissue is considered the proximal retrovirologic event of AKR leukemogenesis. Northern blots of thymic RNA from 40 individual group A or B mice, 12–25 weeks old, showed that 12 of 20 (60%) full-fed (group A) mice and only 9 of 20 (45%) CEIR (group B) mice expressed the 8.4-kilobase full-length MCF MuLV transcript prior to 25 weeks of age (Table 2). The rather uniform suppression of subgenomic MCF MuLV mRNA expression among CEIR mice early in life (Figs. 2 and 3) may partially explain the 25% reduction by CEIR in the frequency of generating the recombinant, genomic-length MCF MuLV transcript.

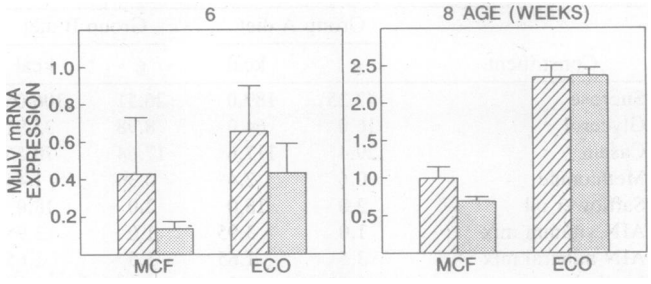

FIG. 3. Mean thymic ecotropic and subgenomic MCF MuLV transcription of 16 group A or B AKR mice at 6 (*Left*) or 8 (*Right*) weeks of age. Each bar represents mean ± SD of four individual mice. Ordinates show arbitrary units of MuLV mRNA expression determined by quantifying bands of either MCF MuLV or ecotropic (ECO) MuLV transcripts with a laser densitometer. Note increase in the arbitrary units of MuLV mRNA expression indicated on the ordinate for 8-week-old mice. Hatched bars, full-fed (group A) mice; stippled bars, CEIR (group B) mice.

Table 2. Thymic expression of MCF MuLV mRNA

Mouse age, weeks	No. of full-fed mice		No. of CEIR mice	
	Subgenomic only	Genomic length	Subgenomic only	Genomic length
12	4	0	4	0
16	1	3	1	3
17	2	2	3	1
21	1	3	3	1
25	0	4	0	4
Total	8	12 (60%)	11	9 (45%)

Effect of CEIR on Lymphoma Formation. Of the 86 group A and B mice at risk, 93% of those that died during the 60 weeks of study died with overt manifestations of thymic lymphoma. Among calorie-restricted (group B) mice the first death from lymphoma occurred later (27 weeks versus 25 weeks old for group A), mean survival time after first recognition of illness was greater (21 weeks versus 12 weeks for group A), and lymphoma was less grossly disseminated. Although some mice exhibited only thymic lymphoma, the majority of mice had thymic and other organ involvement; the order of frequency being nodal, splenic, and alimentary lymphoma. Whereas 50% of the full-fed mice dying with lymphoma had four or more lymphoma sites, 46% of the calorie-restricted mice showed involvement only of thymus and one other site.

During the 60-week study, all but one of the full-fed mice at risk had died from lymphoma (97%), while only 69% of the CEIR mice had developed tumors. Age at median tumor incidence was delayed 13 weeks for group B mice (50 weeks versus 37 weeks old for group A).

Fig. 4 shows the Kaplan–Meier survival curves for the full-fed and CEIR cohorts. The two survival curves were found to be quite different ($\chi^2 = 15.67$ on 1 degree of freedom, $P < 0.0001$). Full-fed mice experienced 3 times the risk of lymphoma mortality compared with CEIR mice; a 95% confidence interval for the relative hazards was 1.6–4.7. Median longevity for CEIR mice was ≈50% longer than that of full-fed mice and would have been even longer had the continued longevity of the remaining lymphoma-free CEIR mice been determined.

DISCUSSION

Prior studies in our laboratory using the model of MMTV-induced breast malignancy in C3H/Ou mice have shown that delayed and reduced proviral expression accompanies calorie restriction and is associated with impaired tumorigenesis

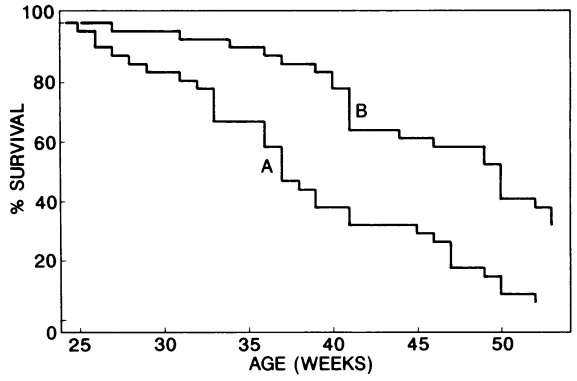

FIG. 4. Influence of calorie restriction on survival among 86 AKR mice. Ninety-three percent of all mortalities were attributable to lymphoma. Full-fed mice (group A) experienced 3 times greater risk of lymphoma mortality compared with 40% calorie-restricted mice (group B) ($P < 0.0001$).

(14). We have also shown that this suppression of proviral transcription can be imposed by CEIR rather acutely, even in parous females following a period of ad libitum feeding and elevated MMTV expression during lactation (26).

In the current study, we show that the beneficial influences of calorie restriction can be imposed in an organ site of tumorigenesis where the dynamics of growth and sexual maturity are not paramount, as they are in the breast. Calorie restriction of lymphoma-prone AKR mice resulted in the lowering of proviral and subgenomic provirus-related transcription in the thymus, reduction in the frequency of generating a recombinant, potentially leukemogenic transcript, and abrogation of tumorigenesis, thus yielding lowered lymphoma mortality and extended longevity. The beneficial influences of CEIR were imposed without altering normal AKR organ development or resulting in high mortality attributable to causes other than lymphoma. Further, we present evidence supporting the premise that MuLV mRNAs interact to form a genomic-length MuLV transcript that contributes to AKR leukemogenesis.

The concurrent expression of ecotropic, xenotropic, and subgenomic MCF mRNAs exclusively in the thymus prior to the appearance of the genomic-length MCF MuLV message, as well as the sequence similarities within regions of these mRNAs and the full-length MCF transcript, suggests that interaction and recombination between MuLV mRNAs generates the genomic-length MCF MuLV message (16, 19–21). We show that with restricted calorie intake, lowered subgenomic MCF transcription, and to a lesser extent lowered ecotropic MuLV transcription among 6- to 8-week-old CEIR mice, precedes a reduced frequency of generating the genomic-length MCF transcript among 12- to 25-week-old CEIR mice.

Thymic expression of subgenomic MCF mRNA was suppressed among all but one of the 6- to 8-week-old CEIR mice (seven of eight, 88%), compared with levels expressed by individual age-matched, full-fed controls. Mean thymic expression of subgenomic MCF mRNA was reduced by calorie restriction up to 69%, and expression of ecotropic mRNA was also transiently suppressed. This suppression of MuLV-related transcription preceded a reduced appearance of genomic-length MCF transcripts among CEIR mice. Of mice 12–25 weeks old, 25% fewer CEIR mice expressed the genomic-length MCF MuLV transcript.

Evidence that MCF MuLVs are potentially leukemogenic includes isolation of this virus only from tumors and preleukemic thymus, its moderate leukemogenicity in C3H/Bi mice, and its ability to accelerate lymphoma formation in weanling AKR mice (16–18). We show evidence that with CEIR, the reduced frequency of generation of genomic-length MCF transcript precedes the delayed and reduced frequency of lymphoma formation. Among calorie-restricted mice, a 25% reduction in the frequency of generating genomic-length MCF mRNA preceded a 28% reduction in cumulative lymphoma mortality. Median tumor incidence occurred more than 3 months later in calorie-restricted mice, and median lifespan was extended.

Calorie restriction prevents up to 90% of C3H/Ou breast tumors (6, 14). Differential impact by calorie restriction on retrovirus-induced tumorigenesis of the breast and thymus may partially reflect the distinct temporal patterns of MMTV and MuLV expression and the differences in the influences of CEIR on their expression. Transmission of MMTV in breast milk during nursing places the provirus in breast epithelium weeks after birth, with weak to undetectable MMTV mRNA expression among mice <16 weeks old (14). With calorie restriction, MMTV mRNA remains undetectable in most mice prior to 25 weeks of age (15). In contrast, MuLV proviral sequences are harbored within the AKR genome and expressed as message and as ecotropic virus *in utero* and

throughout life (16). In the present report, we show that with CEIR, MuLV mRNA expression is suppressed up to 69%, but all mice expressed detectable levels of MuLV mRNA.

Calorie restriction has been shown to decrease the DNA labeling indexes of many tissues, including the thymus and breast (27, 28). Since replication of retrovirus proceeds more efficiently when cells are cycling and not stationary (29), calorie restriction may limit proviral expression by lowering the cellular proliferative index. Further, since the fate of the breast is growth and maturation, and that of the thymus is one of gradual involution, calorie restriction may impact the proliferative index of breast and thymus differently and thus influence retroviral expression differently, particularly in degree.

In addition, calorie restriction has been shown to evoke a selective upregulation in repair and protective responses, including free-radical scavenging and DNA-repair processes (30, 31). Breast tumor-prone C3H/Ou mice might benefit from over 3 months of protective adaptations in response to calorie restriction before detectable MMTV expression, whereas lymphoma-prone AKR mice may benefit from only the acute influences of calorie restriction, since proviral mRNA expression and virus production occur even before birth.

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