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Prolactin effects on the dietary regulation of mouse mammary tumor virus proviral DNA expression

(dopaminomimetic agent/pituitary transplantation/chronic energy-intake restriction)

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ABSTRACT **Chronic energy-intake restriction inhibits mouse mammary tumor virus (MMTV)-induced mammary tumors in C3H/Ou mice by >90%. We have shown that associated with suppression of mammary tumorigenesis there is a reduction or inhibition of circulating prolactin, MMTV particles expressed, and MMTV mRNA transcription in mammary glands (and in most organs tested). To understand the concerted action of prolactin, energy-consumption level, and MMTV on inducing mammary tumors, experiments were designed to control prolactin and energy levels in order to evaluate their effects on MMTV mRNA expression. Mice on restricted diets were grafted with adenohypophyses, and mice fed ad libitum were treated with the dopaminomimetic agent octahydrobenzo[g]quinoline. Adenohypophyseal grafting significantly increased prolactin in dietary (energy)-restricted mice, and this effect was associated with an increase in MMTV mRNA expression within the mammary gland; a linear correlation between prolactin levels and MMTV mRNA expression in the mammary gland was found. Conversely, elimination of the nocturnal peak of circulating prolactin by i.p. injection of dopaminomimetic octahydrobenzo[g]quinoline to mice fed ad libitum delayed (by 8 weeks) and reduced (even as long as 25 weeks) mammary gland MMTV mRNA expression. These findings associate prolactin influences with MMTV mRNA production in mice and help explain the link between chronic energy-intake restriction and reduced MMTV gene expression.**

That chronic energy-intake restriction (CEIR) delays or inhibits the development of experimental mammary adenocarcinoma has become increasingly evident (1–3). Although diet, body weight, and breast tumorigenesis had been strongly associated from critical analysis of human epidemiological data, little was known of the underlying mechanisms that conferred the protective effect (4). The C3H/Ou mouse is a model strain for investigating the biology of spontaneous mammary tumorigenesis. Mammary adenocarcinomas occur in 50% of female C3H/Ou mice within 30–35 weeks of age (5). To explain the mechanisms of tumorigenesis, an insertional-mutagenesis model of the milk-transmitted type-B retrovirus mouse mammary tumor virus (MMTV) near the *Int-1* locus and subsequent clonal growth of the mutant cells have been reported (6). In addition, hormones, genetic make-up, and immunological status may be important in the initiation and/or development of mammary tumors.

Prior studies have shown that calorie restriction (40%) delays dramatically the development of mammary tumors in C3H mice (3). Fewer precancerous hyperplastic alveolar nodules (HAN) are found, MMTV expression is suppressed, and blood prolactin levels are lower (1–3) in mice subjected to CEIR. Because blood prolactin levels appear affected by calorie restriction and prolactin levels *in vitro* apparently regulate MMTV expression (7), alterations of prolactin levels with calorie restriction may be important in breast tumorigenesis for their mammotrophic effects, acute somatogenic effects, and for action on proviral expression (3, 7, 8).

Development of HAN is suppressed and tumor formation is reduced among C3H mice treated daily with dopamine analogs such as 2-bromo[α]ergocryptine (CB-154), which lower blood prolactin (9). Conversely, multiple adenohypophyseal grafts, which are principally prolactin-secreting, to adreno-ovariectomized C3H mice shorten the interval to median tumor incidence (10); expression of MMTV in the tissues, however, was not investigated in the latter studies.

To further our understanding of the mechanisms by which calorie restriction reduces risk for mammary cancer, virgin C3H/Ou mice were fed a semi-purified diet either ad libitum or restricted 40% in calorie consumption (CEIR), and basal serum prolactin levels were experimentally reduced or elevated. Serum prolactin was elevated by grafting two adenohypophyses under the renal capsule. Serum prolactin was reduced by i.p. injection of the dopamine analog octahydrobenzo[g]quinoline (CV205-502). Levels of proviral mRNA expression in mammary gland, serum prolactin levels, and prevalence of HAN lesions were then assessed under different conditions of dietary calorie level and prolactin levels. Our data indicate a crucial role for prolactin in the early expression of MMTV mRNA *in vivo*, regardless of calorie levels that otherwise inhibit or permit mammary adenocarcinoma development in C3H/Ou mice.

MATERIAL AND METHODS

Animals. Six-week-old nulliparous C3H/Ou female mice (The Jackson Laboratory), maintained in accordance with the principles of the Animal Welfare Act as described in Public Health Service/National Institutes of Health publication 86-23, were separated into four experimental groups. Mice of group A (full-fed) consumed a semi-purified diet ad libitum at 16–18 kcal per day (1 cal = 4.184 J) in which calories were derived principally from carbohydrates. Group B (CEIR) mice consumed a proportionally similar complete semi-purified diet but were restricted 40% in calories consumed (10–11 kcal per day).

Mice of group AI were fed the same as mice in group A but were injected daily beginning at age 8 weeks with CV205-502. Group BG was fed the same as mice in group B but first were engrafted with two syngeneic adenohypophyses at age 7 weeks.

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Abbreviations: CEIR, chronic energy-intake restriction; HAN, hyperplastic alveolar nodules; MMTV, mouse mammary tumor virus; CV205-502, octahydrobenzo[g]quinoline.

Semipurified Diets. The composition of diets used has been described in detail (2, 3). All dietary constituents were obtained from ICN. Both diets A and B were low in dietary fat but differed by 40% in the level of calorie energy available; the diets were otherwise comparable. Equivalent amounts of vitamins, minerals, essential fatty acids, and 30% calories as protein were present in both diets. C3H mice fed either diet gain weight, exhibit normal estrous cycles, and have normal immunological capabilities. All mice were fed twice weekly and weighed weekly.

Manipulation of Serum Prolactin Levels *in Vivo*. *Lowering serum prolactin levels.* CV205-502 (Sandoz Pharmaceutical) is a highly potent nonergot dopaminomimetic, which by selective dopamine (D_2) receptor stimulation inhibits prolactin synthesis and secretion (11). To assess the effects of CV205-502 on serum prolactin levels of female C3H/Ou mice, in group AI, mice were injected i.p. with 100–500 μg of CV205-502 per kg in a 0.2-ml volume. The dose that ablated the nocturnal production peak of prolactin was 300 μg/kg. Daily administration of CV205-502 to group AI mice at 1600 hr was timed to counteract the nocturnal prolactin peak.

Elevating serum prolactin levels. Each mouse of group BG was engrafted with two syngeneic adenohypophyses under the left renal capsule. Donor anterior pituitary glands from male C3H/Ou mice were dissected free and placed in sterile physiologic saline on ice. The recipient was anesthetized, prepared, and incised along the dorsum. The left kidney was isolated, the capsule was incised, and the glands were placed beneath the capsule. A two-layer closure was made.

Enzyme Immunosorbent Assay of Prolactin. Standard mouse prolactin (AFP-6476-C) and rabbit anti-mouse prolactin antibody (AFP-131078) were provided by Albert F. Parlow (Pituitary Hormones and Antisera Center, Harbor–University of California, Los Angeles, Medical Center). A modification of methods described by Shrivastav *et al.* (12) was used (13). The enzyme immunosorbent assay for prolactin is as sensitive as the radioimmunoassay and has the advantages of being faster and requiring smaller amounts of both reagents and serum samples.

Purification and Analysis of Proviral mRNA. Mammary gland was excised and homogenized using a single-step method of RNA isolation by acid guanidium thiocyanate/phenol/chloroform extraction (14). Ethidium bromide-stained RNA was applied to a 1% agarose gel. The RNAs were transferred to nylon filter (Hybond N; Amersham) by using a blotting apparatus (LKB) and fixed by using UV irradiation (UV Stratalinker 1800; Stratagene). The RNAs were hybridized to ^{32}P-labeled pMTV-1, which includes all of the MMTV genome except for a 1-kilobase (kb) fragment within the *gag* gene (15); pMTV-1 was provided by M. R. Stallcup (University of Southern California). The murine β-tubulin probe, pSP65-MT49, was provided by S. Matsumoto (Tokyo Metropolitan Institute of Medical Science). The RNAs were labeled with ^{32}P by using a random priming kit (Boehringer Mannheim). The filters were incubated with 10^7 cpm of radioactive probe in a mixture of 50% (vol/vol) formamide/5× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/50 mM phosphate buffer, pH 7.0/salmon sperm DNA at 50 μg/ml (Sigma)/yeast tRNA at 50 μg/ml (Sigma)/0.04% Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.2% SDS at 42°C overnight. Filters were washed twice with 2× SSC (0.1% SDS) and 1× SSC (0.1% SDS) at 55°C, dried, and exposed to x-ray film (X-Omat; Kodak) with intensifying screens at −80°C overnight. The density of MMTV mRNA bands (8.9 and 3.8 kb) was semi-quantified by using laser densitometry (LKB).

Whole-Mount Preparations. The inguinal mammary fat pad from some of the mice was dissected at euthanasia, fixed in 10% (vol/vol) buffered formalin cleared of fat in acetone and serial alcohol baths, stained with hematoxylin, and scored for the degree of mammary development and prevalence of HAN.

Experimental Plan. Group A and AI mice were fed identically, 16–18 kcal per day, but AI mice were injected with 300 μg of CV205-502 per kg each day as described to suppress the nocturnal prolactin peak. Group B and BG mice were fed identically but consumed 40% fewer calories than mice of the A and AI groups. BG mice were engrafted with two adenohypophyses at age 7 weeks. At 8 weeks, feeding of the semi-purified diets was initiated. Gradual restriction of energy consumption by B and BG group mice was such that total calorie intake was restricted to 40% by 14 weeks of age. Proviral mRNA expression and serum prolactin were evaluated at 17, 21, and 25 weeks of age, and whole mounts of mammary tissue were prepared when the mice were 21 weeks old. Mice were euthanized between 0900 and 1000 hr at each interval of evaluation, so as to minimize variations attributable to circadian rhythms.

RESULTS

Effect of CV205-502 on Serum Prolactin. Basal serum prolactin levels, the prolactin circadian rhythm of C3H/Ou mice, and the effects of CV205-502 administration were assessed in preliminary evaluations. Three groups of 28 mice each were injected i.p. at 1600 hr with either sterile phosphate-buffered saline, or CV205-502 at 100 μg/kg or 300 μg/kg in a 0.2-ml volume. Trunk blood was collected, and prolactin levels were determined by enzyme immunosorbent assay for four mice from each of the three experimental groups before injection and 2, 4, 6, 8, 16, and 24 hr after injection.

Administration of the dopaminomimetic CV205-502 at 300 μg/kg effectively ablated the nocturnal production peak of prolactin (Fig. 1). Saline-injected control mice were found to have a regular circadian pattern of prolactin. Administration of CV205-502 at 100 μg/kg lowered serum prolactin levels for ≈8 hr, but a peak of production reappeared at the 16-hr assessment, indicating that the nocturnal peak had been transiently suppressed and was consequently shifted to the right. A daily dose of 300 μg of CV205-502 per kg administered at 1600 hr was thus selected for our studies. Note that at 16 hr after injection (or 0800 hr the next morning) little difference in serum prolactin was found between the saline and CV205-502 (300 μg/kg)-injected mice and that the principal difference in serum prolactin between these groups was the significant loss of the nocturnal production peak in the CV205-502-treated mice.

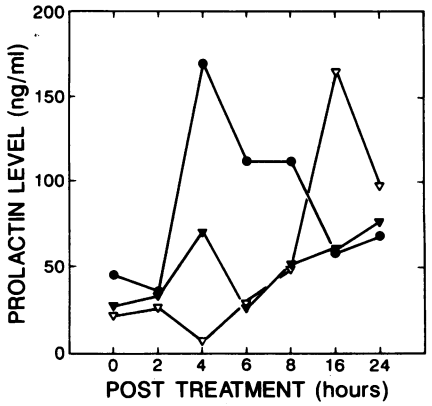

FIG. 1. Effect of CV205-502 injection on serum prolactin. ●, Saline injected i.p.; ▽, 100 μg/kg of CV205-502; ▼, 300 μg/kg of CV205-502. Prolactin level was measured periodically for 24 hr after treatment.

Expression of MMTV mRNA in Mammary Glands of Mice at 17, 21, and 25 Weeks of Age. Fig. 2 *Upper* demonstrates that mammary glands of four of four 17-week-old mice engrafted with adenohypophyses (group BG) one of four full-fed (group A) mice expressed MMTV mRNA strongly. By contrast, weak expression of provirus was found in mammary glands of one of four CEIR (group B) mice and only one of four of the full-fed mice injected with CV205-502 (group AI).

Fig. 2 *Lower* presents data concerning mRNA expression in mammary glands of all four groups of mice at 21 weeks of age. Four of four mammary glands of group BG-engrafted mice and three of four full-fed group A mice expressed MMTV mRNA strongly. Strikingly, mammary glands of the CV205-502-injected mice (group AI) and the CEIR mice (group B) showed faint-to-undetectable viral mRNA expression in four separate experiments. By 25 weeks, expression of provirus was evident in mammary glands of mice from all four groups. However, much stronger expression was evident among mice of groups A and BG mice (Fig. 3) than among CEIR mice or CV205-502-treated full-fed mice.

It is of interest that the expression of β-tubulin was greater in mammary glands of groups A and AI mice fed ad libitum than in the 40% energy-restricted group or the pituitary-engrafted (BG) groups.

Serum Prolactin Levels. Enzyme immunosorbent assay levels of serum prolactin for mice of each of the four experimental groups were determined at 17, 21, and 25 weeks of age. Serum was collected between 0900 and 1000 hr to minimize the effects of circadian rhythms. Basal prolactin levels of laboratory chow-fed 17- and 25-week-old mice were 46 ± 11 and 40 ± 7 ng/ml, respectively. Prolactin levels of ad libitum-fed group A, CV205-502-injected group AI, and CEIR group B mice did not differ significantly from these levels at 21 and 25 weeks of age, but prolactin levels of group AI mice

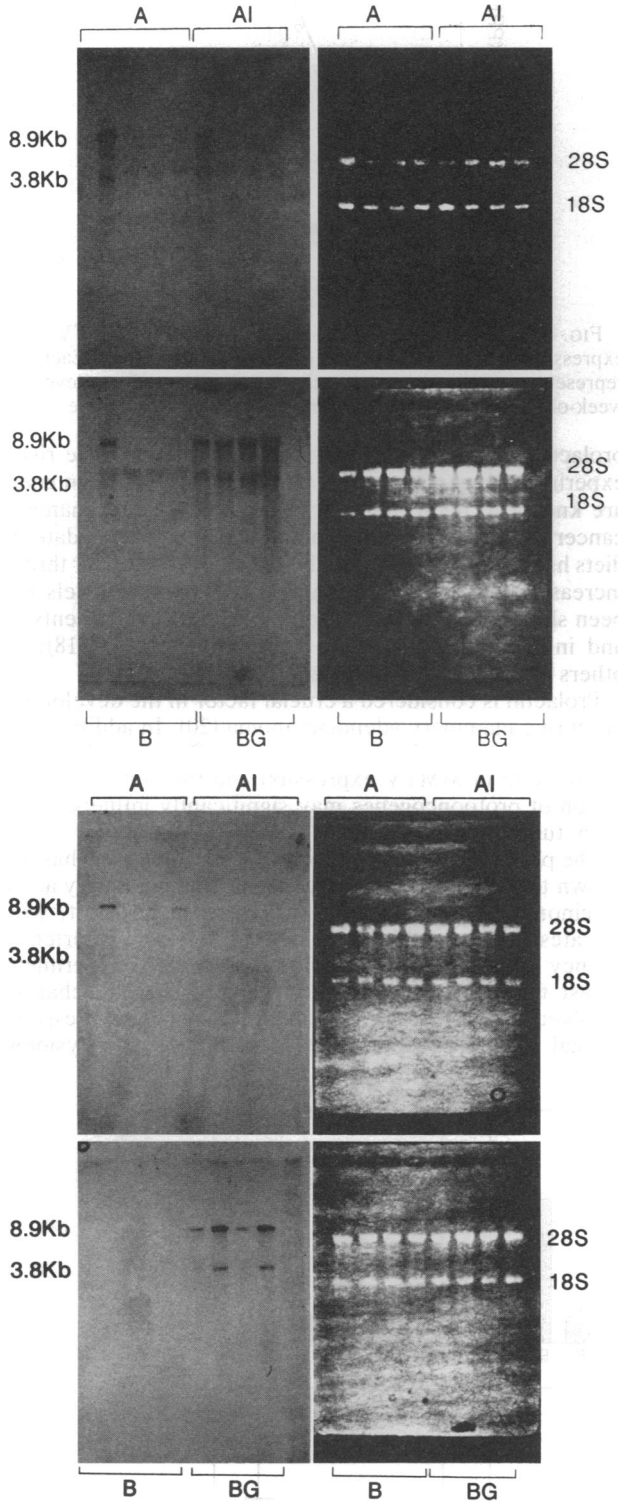

FIG. 2. Northern (RNA) blot analysis of RNA (10 μg was loaded on each lane) from mouse mammary gland from 17-week-old (*Upper*) and 21-week-old (*Lower*) mice. (*Right*) Ethidium bromide-stained filters. Major RNA bands are designated 28S and 18S. Each lane represents a different mouse and corresponds to a *Left* lane for that mouse. (*Left*) Autoradiograms using ^{32}P-labeled pMTV-1 probe. Bands of major MMTV transcripts are designated 8.9 and 3.8 kb. A, full-fed group; AI, full-fed CV205-502-injected group; B, CEIR group; BG, CEIR and pituitary-grafted group. The data from each experimental group thus represent four separate analyses.

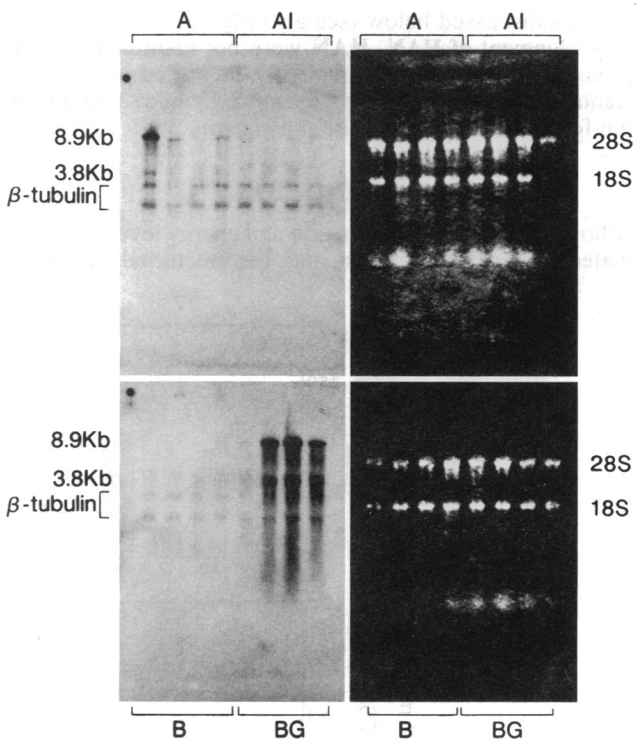

FIG. 3. Northern blot analysis of mammary gland from 25-week-old mice. RNA was hybridized to pMTV-1 (containing MMTV genome) and pSP65-MT49 (containing β-tubulin gene). On right-side lane in AI group only 5 μg of RNA was loaded; otherwise 10 μg was used. Major RNA bands are designated 28S and 18S; bands of major MMTV transcripts are designated 8.9 and 3.8 kb. Each lane in a group represents a different mouse, except that *Left* and *Right* lanes correspond. A, full-fed group; AI, full-fed CV205-502-injected group; B, CEIR group; BG, CEIR and pituitary-grafted group.

Table 1. Serum prolactin variation in experimental groups

Age, weeks	Serum prolactin, ng/ml				
	A	AI	B	BG	C
17	32 ± 13	65 ± 23	23 ± 7	101 ± 20	46 ± 11
21	20 ± 4	33 ± 6	21 ± 6	122 ± 37	ND
25	32 ± 5	50 ± 16	21 ± 10	93 ± 13	40 ± 7

A, full-fed group; AI, full-fed CV205-502-injected group; B, CEIR group; BG, CEIR and pituitary-grafted group; C, chow-fed control group.

were somewhat greater at 17 weeks (Table 1). Note that CV205-502-treated AI mice exhibit no nocturnal production peak, as described above. Whether the circadian pattern of prolactin production was altered by CEIR in the group B mice was not determined. Prolactin levels of group BG mice were significantly greater than those of all other mice evaluated and showed a 2- to 4-fold elevation of prolactin depending on the time of comparison.

Figs. 4 and 5 summarize observations of MMTV mRNA expression and serum prolactin levels. A linear correlation between serum prolactin and MMTV mRNA expression was clearly evident for mammary glands of group BG mice studied at 25 and 21 weeks of age (Fig. 4), $r = 0.98$ and 0.85, respectively. Among full-fed (group A) mice (Fig. 5) and CEIR (group B) mice, the level of MMTV mRNA expression (Fig. 5 *Lower*) paralleled the serum prolactin level (Fig. 5 *Upper*); that is, both were greater among full-fed mice and lower among CEIR mice. The relationship, however, lacked a precise linear correlation. A paradoxical observation was that group AI mice seemed to have elevated 0900 levels of prolactin at 17 weeks. This observation should be interpreted in light of the ablation of the nocturnal prolactin production peak, as discussed below (see also Fig. 1).

Development of HAN. HAN were not identified in CEIR (group B) mice but were regularly identified and large in adenohypophyseal-engrafted (group BG) mice and ad libitum-fed (group A) mice (data not shown).

DISCUSSION

Although (*i*) dietary composition and energy level have been related to serum prolactin and the nocturnal release of prolactin (16), (*ii*) diets high in energy increase the risk of experimental mammary cancer, and (*iii*) diets low in energy are known to decrease the risk of experimental mammary cancer (1–3), there is no commanding evidence to date that diets high in energy increase the risk of breast cancer through increased prolactin secretion. Serum prolactin levels have been shown to be higher in some breast cancer patients (17) and in daughters of women with breast cancer (18); yet, others found no such elevation (19).

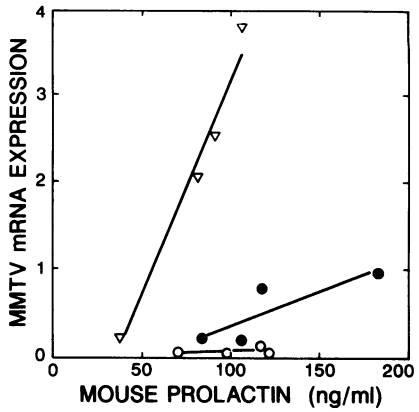

FIG. 4. Correlation between serum prolactin and MMTV mRNA expression *in vivo* of mice in the pituitary-grafted group. Each point represents a single mouse at each period evaluated. ○, Seventeen-week-old mice; ●, 21-week-old mice; ▽, 25-week-old mice.

Prolactin is considered a crucial factor in the development of murine mammary adenocarcinoma (20). In addition to its mammotrophic and acute somatogenic effects (8, 21), the role of prolactin in MMTV expression and the consequent activation of protooncogenes may significantly influence mammary tumorigenesis (3, 7).

The pharmacological lowering of serum prolactin has been shown to reduce the risk for experimental mammary adenocarcinoma (9), and adenohypophyseal engraftment, which elevates serum prolactin, has been shown to shorten the latency period and increase the incidence of experimental breast tumors (10). However, the molecular mechanisms involved have not yet been elucidated. Proviral expression in normal mammary epithelium is regulated by physiologic

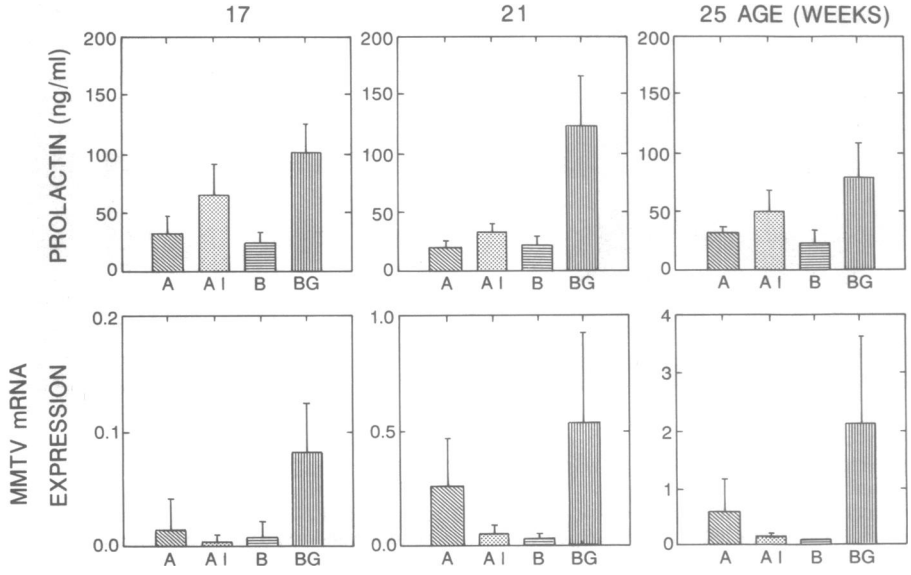

FIG. 5. Association of serum prolactin and MMTV mRNA expression *in vivo*. (*Upper*) Serum prolactin level of mice of full-fed (A), full-fed CV205-502-injected (AI), CEIR (B), and pituitary-grafted CEIR (BG) mice at 17, 21, and 25 weeks. (*Lower*) MMTV mRNA expression at each period evaluated. Each value represents mean ± SD. Ordinate shows the arbitrary units of MMTV mRNA expression by measuring bands (8.9, 3.8 kb) with a laser densitometer.

concentrations of prolactin *in vitro* (7), whereas other hormones, such as glucocorticoids, which enhance MMTV expression in mammary cell lines (7, 22), play only a permissive role. The current investigations show that prolactin influences MMTV mRNA expression *in vivo*. Latency to full proviral expression is dramatically shortened by adenohypophyseal engraftment, as shown in evaluations of group BG mice, and the level of proviral expression in these mice, as measured using densitometric scanning, correlates linearly with their serum prolactin levels.

Somewhat less clear, but also noteworthy, is the effect of ablating the nocturnal prolactin production peak using CV205-502 in the group AI mice. Proviral expression of group AI mice was significantly reduced when compared with expression levels of group A mice. Although 0900-hr prolactin levels of group AI mice appeared unchanged or slightly greater than those of group A mice, blood levels of both groups remained within the basilar range, and group AI mice treated with CV205-502 lacked the very high nocturnal production peak seen in group A mice. Consequently, MMTV mRNA expression levels and 0900-hr serum prolactin levels of AI mice do not correlate precisely, but the circadian rhythm of prolactin production of AI mice may have been sufficiently suppressed to account for the delay and reduction in proviral expression.

The same concept could be proposed for group B mice. Because diet restricted in calories may affect the nocturnal release of prolactin, CEIR may modify prolactin levels and rhythms not revealed in a single 0900-hr assessment. The great number of animals required to evaluate prolactin circadian rhythms prevented our evaluating this possibility thoroughly in the present experiment. Certainly, relatively lower serum prolactin in CEIR (group B) mice corresponded to a delayed and reduced MMTV mRNA expression, as suggested by prior experiments and shown here.

A reduction in cellular transcriptional rate may be one reason for the difference in β-tubulin expression in the mammary gland between ad libitum and CEIR mice, reflecting a difference in the kinetics of the predominant cell type present in mammary glands from mice of these dietary groups. Terminal end buds are known to have a profuse mitotic and DNA synthetic activity, whereas ductal and stromal cells are only occasionally labeled in microautoradiographic preparations (23). From our evaluation of whole mounts, ductal arborization and budding were decreased in CEIR inguinal fat pads compared with ad libitum-fed animals. HAN were not identified in CEIR mice but were regularly identified and large in adenohypophyseal-engrafted mice. Conversely, HAN were present in significant numbers in ad libitum-fed group A mice but were expressed to a lesser degree in CV205-502-injected mice. (R.W.E., N.K.D., Y.T., H.I., N.H., and R.A.G., unpublished work).

These studies indicate that diet and prolactin both play important roles in mouse tumorigenesis. The investigations also show that while these complex mechanism(s) appear related, they can be manipulated separately. Administration of the dopaminomimetic agent CV205-502 to full-fed mice prevents expression of MMTV and development of the precancerous hyperplastic lesions. By contrast, in mice subjected to CEIR, increasing the prolactin level by pituitary grafting increases expression of HAN and MMTV.

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