Hormonal Induction of Mammary Tumor Viruses and Its Implications for Carcinogenesis

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Abstract

The purpose of this study was to evaluate the possibility that hormones and mouse mammary tumor virus (MuMTV) are cocarcinogenic for mammary epithelium. Results of our investigations in vivo and in vitro suggest: (a) Hormones promote mammary carcinogenesis in BALB/c females whether MuMTV is germinally (BALB/c) or horizontally (BALB/cfC3H) transmitted; the rate of carcinogenesis in BALB/cfC3H females is substantially faster than it is in BALB/c, but the final mammary carcinoma incidence is approximately the same. The rate-limiting step in malignant transformation in BALB/c, which infectious MuMTV overcomes, is in premalignant transformation from normal. (b) One characteristic of horizontal MuMTV transmission in BALB/c that is not observed in germinal transmission is integration of new MuMTV sequences in mammary cell DNA. Integration is mammary cell specific and constant at 2 to 3 copies/cell from tumor to tumor. (c) MuMTV expression is changed in mammary epithelial cells during hormonal carcinogenesis. The nature of the change is qualitatively similar in both BALB/c and BALB/cfC3H. Expression of envelope glycopeptides (glycoprotein with a molecular weight of 52,000) is induced, which correlates with amplification of MuMTV RNA sequence content. Quantitative differences exist in induced levels in BALB/c and BALB/cfC3H. (d) MuMTV RNA and a glycoprotein with a molecular weight of 52,000 were not inducible with dexamethasone in normal mammary epithelial cells in culture. These structural components were induced in both premalignant (BALB/cfC3H) and malignant (BALB/c and BALB/cfC3H) cells. MuMTV RNA was induced by dexamethasone in normal cells pretreated with 5-iodo-2'-deoxyuridine. (e) Both premalignant and malignant cells have altered (vis-a-vis normal) surfaces, discernible by differences in reactivity with concanavalin A in hemadsorption assays. Indirect evidence suggests that the alteration includes membrane incorporation of MuMTV-related determinants of a glycoprotein with a molecular weight of 52,000. (f) Malignant cells exhibit enhanced sensitivity to insulin for reinitiation of DNA synthesis and mitosis in contact-inhibited homotypical monolayers. These findings have been organized into a hormonal cocarcinogenesis hypothesis in which expression of germinally transmitted MuMTV genes is the proximal cause of neoplastic transformation.

Introduction

Genetic (2, 22, 25) and hormonal (33, 34) etiological components have been established in human breast carcinogenesis from epidemiological studies. In recent years a third etiological component, viruses, has been added; RNA tumor virus markers not observed in normal breast epithelium or in nonbreast cancers have been repeatedly identified in breast cancers (reviewed in Ref. 32). These markers include nucleic acids and proteins homologous to the genome and structural gene products of the MuMTV. These discoveries have made the mouse, in which genetics, hormones, and viruses play etiological roles, the most comprehensive experimental organism for definition of causal elements of breast cancer in humans.

The certain involvement of MuMTV, hormones, and genetics notwithstanding, the precise role of these factors in malignant transformation of mouse mammary epithelium is imperfectly understood. Thirty years after their initial description (7), they are understood only as "influences" in disease onset and progression. A viral etiology for murine mammary cancer is an attractive hypothesis because of the unique position that MuMTV occupies in ordering the other 2 levels of causality. MuMTV is genetically transmitted (3, 4); host genes regulate the life cycle of MuMTV, including infectivity patterns and intracellular expression (3); mammotropic hormones can activate MuMTV genes in mammary cells (13, 15, 29, 44, 47, 57); and high levels of MuMTV in mammary tissues, milk, and blood directly correlate with high mammary tumor incidence (40).

Alignment of virus, genetic, and hormone components in this way suggests a unifying hypothesis for disease etiology: mammotropic hormones act as cocarcinogens, by altering expression of genetically transmitted MuMTV in the normal mammary cell. In its simplest form the hypothesis predicts that mammotropic hormones would promote mammary tumorigenesis in mouse strains that germinally transmit MuMTV and that expression of endogenous virus information would be altered in the mammary epithelial cell en route to malignant transformation that occurred during abnormal hormonal stimulations.

Definition of roles for hormones and MuMTV in hormonal carcinogenesis was pursued both in vivo and in vitro. We present results of 2 lines of investigation in vivo: (a) efficacy of mammotropic hormones in promoting premalignant and malignant transformations; and (b) changes in MuMTV...
expression in mammary cells during these hormonal-modulated transformations. Studies in vivo were complemented by studies in vitro to determine whether mammotropic hormones directly alter transcription of MuMTV in mammary cells and whether cell transformation resulted. These studies were conducted in BALB/c mice that transmitted MuMTV either germinally or horizontally. Results obtained in this multivariate system have been organized into a 2-step hormonal cocarcinogenesis model in which the expression of genetically transmitted MuMTV information is the proximal cause of neoplastic transformation.

Materials and Methods

Mice and Tissues. BALB/c (MCF) mice were obtained from the Cancer Research Laboratory (Berkeley, Calif.) colony from K. DeOme and S. Nandi (University of California, Berkeley) in 1972. These mice have been maintained inbred at the Michigan Cancer Foundation. Mammary tumor incidence is 0.5% at 12 months in virgin females. BALB/cfC3H (MCF) was generated by fostering BALB/c infants on C3H mothers (40) and has been maintained inbred at the Michigan Cancer Foundation since 1972.

Hyperplastic alveolar nodules and tumors were generated in BALB/c and BALB/cfC3H as described in Chart 1 and "Results." Methods of pituitary implantation, visualization, and transplantation of hyperplastic nodules have been adequately described by Medina (35).

Molecular Hybridization. All methods and reagents used have been previously described (30). The MuMTV cDNA was prepared from MuMTV-P grown in primary tumor cell culture (28). The cDNA was prepared in endogenous polymerase reactions, was 4S to 6S in size, was specific for MuMTV RNA, and represented 76% of the 35S MuMTV genome at a 10:1 cDNA:RNA ratio (47% at 1:1). Hybridizations to DNA were time variable; hybridizations to RNA were RNA concentration variable. Hybridization was monitored by $S_i$ enzymatic methods (30). $C_{i}$ and $C_{I}$ values were calculated as described by Britten and Kohne (10) and corrected to a monovalent cation concentration of 0.18 M (11). DNA:cDNA hybridizations were conducted at $65^\circ$ in 0.5 M Na$^+$; RNA:cDNA hybridizations were conducted at $68^\circ$ for 66 hr in 0.5 M Na$^+$. The MuMTV sequence copy number in DNA was calculated relative to a mouse cell DNA reassociation standard (30). MuMTV sequence content in cell RNA was expressed either as the percentage of cell RNA MuMTV content:

$$\frac{C_{i,15} \text{MuMTV RNA}}{C_{i,15} \text{cell RNA}} \times 100$$

or as genome equivalents (55).

$$\frac{C_{i,15} \text{MuMTV RNA}}{C_{i,15} \text{cell RNA}} \times \frac{\text{Wl. RNA:cell}}{\text{Wl. 35S RNA}}$$

Immunofluorescence. Indirect immunofluorescence tests were performed in 4 steps as described by McGrath and Blair (29) and by Yang et al. (59). Monospecific MuMTV antisera to p28, gp52, and gp36 were obtained by Dr. R. D. Cardiff (University of California, Davis, Calif.). The method of preparation has been described (12, 52).

Two basic fixation regimens were followed. For determining the fraction of antigen-positive cells in mammary epithelial cells in vivo, mammary cells were collagenase dissociated, and the epithelial cells were recovered and fixed directly on glass microscope slides in acetone ($-20^\circ$). For determining the percentage of reactive cells after growth in culture, mammary cells dissociated by collagenase and grown in culture (56) were fixed in 10% aqueous formalin ($10^\circ$). Stained cells were examined under a Leitz fluorescence microscope with a Leitz 1 fluorescein isothiocyanate filter. For determining the percentage of reactive cells in a sample, every third field of 90 consecutive microscopic fields was counted at $\times 400$ magnification. The total cell number was then calculated with the aid of bright-field optics. An average of 2500 cells in 30 fields were counted to determine the percentage of reactive cells.

Cell Cultures. Cultures of normal mammary epithelial cells were obtained by collagenase dissociation of epithelial cells from minced glands of midterm, first-pregnancy BALB/c mice as previously described (56). Malignant and premalignant mammary epithelial cells were obtained from mammary adenocarcinomas or hyperplastic outgrowths as described in Chart 1. After collagenase dissociation the epithelial cells in the mixed mammary cell populations were selectively aggregated in dilute serum, gently sedimented, and then plated either in 35-mm Falcon dishes (transfor-
medium and refed with Dulbecco's medium, which contained 2 glass coverslips (9 x 22-mm), with insulin was added without a change of culture medium. Insulin was growth medium containing 15% serum and insulin was dissolved at 2.5 mg/ml in 0.005 M HCI and diluted in Dulbecco's medium without supplements immediately before use.

Tracer amounts (3 μCi/ml) of [3H]thymidine were added at various times after the addition of insulin. Control dishes, which received no insulin (0.1 ml volume of medium), were also labeled. After a 60-min label interval, the medium was removed and the monolayers were washed 3 times with ice-cold phosphate-buffered saline. One coverslip from each dish was removed and air dried without further treatment to determine the total amount of [3H]thymidine taken up during the labeling period. The remaining coverslip was fixed at 4° with 10% formalin in buffered 0.9% NaCl solution for 10 min, incubated with ice-cold 10% trichloroacetic acid 2 times for 15 min and 3 times for 5 min, washed twice with absolute ethanol, and air dried. The coverslips were placed directly into scintillation vials for radioactive counting. Total thymidine uptake and trichloroacetic acid-precipitable uptake were monitored by counting in a toluene-based PPO:POPOP cocktail.

 Autoradiography. Autoradiography was conducted on cells fixed in place on the surface of plastic culture dishes. Cells labeled with [3H]thymidine as described previously were fixed in formalin at 4° and washed with ice-cold 10% trichloroacetic acid and ethanol as described previously. The dishes were dip coated with Kodak NTB-2 nuclear emulsion diluted with an equal volume of distilled water and exposed for 7 days at 4°. The dishes were developed with Kodak D-19 and stained with hematoxylin and eosin. For determining each labeling index (the number of labeled cells:the total number of cells), 1400 cells were counted.

 Determination of Cells in Mitosis. Duplicated 35-mm dishes were fixed in 10% formalin, stained with hematoxylin, and examined for cells in mitosis at ×400 magnification by phase microscopy. Cells from late prophase through late telophase were counted. Each point in Chart 5 represents data derived from examination of at least 20,000 cells.

Results

Bisection of Hormonal Mammary Carcinogenesis in BALB/c into Premalignant and Malignant Transformation Steps

From the studies of Bern, DeOme, Muhlbock, Nandi, Medina et al. (reviewed in Refs. 5 and 35), it is well accepted that there are multiple stages in malignant transformation of mammary epithelium. The 2 major events are premalignant transformation from normal and malignant transformation from premalignant. Prolonged hormonal stimulation of mammary epithelium achieved by pituitary isografts increases incidence and decreases time of onset for mammary carcinomas in BALB/c (5, 35, 36). Freed from hypothalamic control, pituitary isografts secrete primarily luteotropic hormone (35, 39). The effect of the implant is to induce a rapid proliferation of mammary epithelium and recurrent pseudopregnancy (24, 39). Endocrine reconstruction experiments suggest that the primary hormones active in tumor promotion in BALB/c are estrogens, progesterone, and prolactin (5).

The effect of hormones on rates and incidences of premalignant and malignant transformations in BALB/c and the effect of horizontally transmitted MuMTV on these parameters were measured in the "hormonal carcinogenesis system" shown in Chart 1. In its essential features the system was adopted from that developed by DeOme et al. (14). Either BALB/c animals are infected at birth with MuMTV-S from C3H mothers, or they are not. At 3 to 4 weeks of age, mammary epithelium is stimulated to proliferate with hormones provided by pituitary implants in perirenal fat. Hyperplastic lesions of mammary alveolar buds, which contain premalignant cells in addition to normal cells, develop during hormonal stimulation. These hyperplastic nodules (HAN) are isolated by microsurgery, expanded in virgin BALB/c hosts, and transplanted into secondary virgin BALB/c hosts in which rates and frequencies of tumor formation are determined as a function of continued hormonal stimulation.

The effect of pituitary isografts on premalignant transformation in BALB/c (MCF) and BALB/cfC3H (MCF) is shown in Table 1. Only 2.0% (1 of 50) of uninfected BALB/c females contained HAN after 12 months of continuous hormonal stimulation, and that animal contained only 1 HAN. This low incidence of HAN correlates with a low tumor incidence in primary hosts (11% at 12 months; data not shown). In contrast, hormonal stimulation increased the incidence of HAN in BALB/cfC3H to 100% at 6 months; these animals contained multiple HAN. Thus, hormonal stimulation promotes premalignant transformations in noninfected as well as infected females, but the efficiency of premalignant transformation in infected females is substantially greater than that in noninfected females. These data are in agreement with those reported by Medina and DeOme (36). Our own studies (data not shown) and those of Medina and DeOme (36) have shown that the effect of hormones in premalignant transformation is to accelerate the rate of HAN formation and is not to generate premalignant cells with higher tumor-forming capability than those induced in...
the absence of hormonal stimulation.

Table 2 shows first that HAN in both infected and uninfected females contain premalignant cells, as defined by a higher than normal rate of malignant transformation in secondary hosts. Second, continued hormone stimulation increases the frequency (approximately 2-fold) and decreases the time of onset (approximately 30%) of malignant transformations in BALB/c and BALB/cfC3H HAN in secondary BALB/c hosts; and, in the 2 outgrowth populations shown, the malignant potential of BALB/c pre malignant cells is as great as BALB/cfC3H premalignant cells. These data suggest that the rate-limiting step in hormonal carcinogenesis in non-MuMTV-infected females, which virus infection overcomes, is in conversion of normal cells to premalignant cells, i.e., in early transformation events. Further, our data suggest that, if the rate-limiting step is experimentally normalized, the final incidence of mammary tumors is the same in uninfected BALB/c animals as in MuMTV-S-infected BALB/cfC3H animals. Although there is considerable variation in malignant potentials of different isolated HAN outgrowth lines (35), our data to date suggest that the variation occurs independent of the fact of MuMTV-S infection.

Changes in MuMTV Proviral Sequence Content in BALB/c

Diploid normal BALB/c mammary epithelial cells contain multiple (5-7) copies of MuMTV sequences in DNA (Refs. 30 and 38; Table 3). MuMTV sequences present in mammary cells are present at the same frequency in nonmammary cells of adult animals, such as liver, spleen, or brain, and in embryonic cells (Table 3; Ref. 30). These sequences are present in nuclear DNA (30) with a molecular weight greater than 30 x 10^6 (unpublished observation). The uniform segregation of these sequences in chromosomal DNA of multiple somatic BALB/c cell types is consistent with the germinal transmission pattern of virus particles observed by Bentvelzen et al. (4) and suggest that the MuMTV variant transmitted by BALB/c is transmitted via the germ line. Based on the degree of relatedness between MuMTV sequences in DNA of divergent mouse species, MuMTV sequences in BALB/c appear to have been in the germ line of Mus for at least 3 x 10^6 to 4 x 10^6 years and to be as well conserved as cellular unique sequence DNA (38).

Germically transmitted MuMTV sequences in BALB/c are closely related to the genomes of highly oncogenic variants (MuMTV-S and MuMTV-P) indigenous to high mammary cancer incidence mouse strains (C3H and GR). We detected no difference in thermal stabilities (t_m) of hybrids formed between MuMTV-P cDNA and either normal BALB/c or malignant C3H cell DNA (30). However, because of the insensitivity of thermal denaturation measurements [Δt_m 1° = 1.5% base change (Ref. 9)], finding even identical (the inherent error in these measurements is ±1°) hybrid t_m's does not imply that MuMTV-S, MuMTV-O, and MuMTV-P are identical. Tryptic peptide mapping data from our own laboratory (unpublished observation) suggest that the major envelope polypeptides of these 3 variants are indeed different. Teramoto et al. (53) have clearly shown antigenic differences between MuMTV-S and MuMTV-P, which we were unable to distinguish as proviruses by differences in hybrid t_m's (30).

Malignant cells in BALB/cfC3H tumors contain a 2- to 3-fold increment in MuMTV sequence copy number in DNA (30, 38). The increment in proviral sequences is specific for mammary epithelium; neither normal cells of tumor-bearing females (Table 3), nor malignant nonmammary cells (e.g., lymphoid leukemias) contain extra MuMTV sequences in DNA. The extra MuMTV sequences in BALB/c DNA have not been distinguishable from MuMTV-O sequences by differences in t_m of hybrids [formed between BALB/cfC3H or BALB/c DNA and MuMTV-P cDNA (30)]. However, since MuMTV-O cannot be readily distinguished from MuMTV-S in mouse cell DNA by these methods (30), the data do not directly bear upon the origin of the extra sequences. Since experimental infection of mouse cells in vitro results in a similar 2- to 3-fold amplification of proviral sequences in cell DNA (54), extra sequences acquired by mammary cells during hormonal carcinogenesis in vivo are probably derived from infecting MuMTV-S. Peptide mapping data on BALB/cfC3H and C3H MuMTV variants will probably be necessary to unequivocally establish the origin of the extra sequences observed in BALB/cfC3H DNA.

Table 1

Effect of hormones on premaligRAFT development in BALB/c and BALB/cfC3H

<table>
<thead>
<tr>
<th>Animal age (mos.)</th>
<th>Pituitary</th>
<th>No. of HAN/ mouse</th>
<th>No. of HAN/ mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>+</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>0/50</td>
<td>10/10</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>0/10</td>
<td>2/10</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>1/50</td>
<td>3/3</td>
</tr>
</tbody>
</table>

a Two pituitaries were implanted in perirenal fat in animals 4 weeks of age (+); −, virgin females. At 6 and 12 months, pituitaries were removed, and HAN were counted as described in Ref. 35. Low sample number due to a high frequency of mammary tumors; HAN were not counted in tumor-bearing animals. NT, not tested.

Table 2

Effect of hormones on malignant transformation in 2 hyperplastic outgrowths

<table>
<thead>
<tr>
<th>Outgrowth</th>
<th>Pituitary</th>
<th>Tumor incidence</th>
<th>Mean latent period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>+</td>
<td>0.5</td>
<td>345</td>
</tr>
<tr>
<td>BALB/c</td>
<td>−</td>
<td>31</td>
<td>265</td>
</tr>
<tr>
<td>BALB/cfC3H</td>
<td>+</td>
<td>80</td>
<td>200</td>
</tr>
<tr>
<td>BALB/cfC3H</td>
<td>−</td>
<td>41</td>
<td>240</td>
</tr>
<tr>
<td>BALB/cfC3H</td>
<td>+</td>
<td>82</td>
<td>210</td>
</tr>
</tbody>
</table>

Hormonal-Viral Mammary Cocarcinogenesis

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Table 3

<table>
<thead>
<tr>
<th>DNA</th>
<th>Normal or neo-plastic</th>
<th>Maximum % of cDNA Annealed</th>
<th>C_d,f&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>Copies/ diploid cell</th>
<th>Hybrid t&lt;sub&gt;m&lt;/sub&gt; (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Mammary</td>
<td>Normal</td>
<td>72–80 700–800</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Premalignant</td>
<td>71–80 720–770</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant</td>
<td>72–77 760–800</td>
<td>5</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70–75 740–830</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Normal</td>
<td>73–79 780</td>
<td>5</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Normal</td>
<td>70–80 710</td>
<td>6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td>Normal</td>
<td>70–77 730</td>
<td>6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td>BALB/cfC3H Mammary</td>
<td>Normal</td>
<td>70–80 730–770</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Premalignant</td>
<td>71–80 720–780</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignant</td>
<td>70–76 270–400</td>
<td>10–15</td>
<td>82–83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70–75 700–800</td>
<td>5–6</td>
<td>82–83</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Normal liver from mammary tumor-bearing animals.
<sup>b</sup> Normal cells were from first-midpregnant animals 2 to 3 months of age. Spleen, brain, and whole embryo were from first-midpregnant animals. BALB/cfC3H tumors were from multiparous females; BALB/c tumors and premalignant outgrowths were from virgin females 8 to 12 months of age described in Tables 1 and 2.

<sup>c</sup> Hybridizations were conducted at a DNA:cDNA ratio of 9 x 10<sup>6</sup>:1 and to a cellular C<sub>d,f</sub> of 5 x 10<sup>6</sup> mol x sec/liter. Unique sequence reassociation was 93 to 96% at the same C<sub>d,f</sub> value. Background hybridization (9 to 10%) to salmon sperm DNA is not subtracted. Data are maximum-minimum and average (maximum variation, 12%) values from duplicated experiments on DNA from pooled organs or individual tumors. Ranges in C<sub>d,f,1/2</sub> for hybridization to normal BALB/c DNA and BALB/c liver DNA were obtained from 3 groups of mice, 10 mice pooled/group. Ranges for hybridization to malignant BALB/c and BALB/cfC3H cells were obtained from 5 and 7 individual tumors, respectively.

<sup>d</sup> Copy number was derived from C<sub>d,f,1/2</sub> values, with a C<sub>d,f</sub> of 2000 mol x sec/liter for unique sequence reassociation (see Ref. 30).

In contrast to infected heterologous (non-mouse) cells, in which only a fraction of sequences are integrated (46), all extra sequences detected in malignant BALB/cfC3H cells are integrated into chromosomal DNA (Footnote 5 and Ref. 46). We therefore refer to these sequences as somatic proviral sequences. At appropriate high cell DNA driver excess, MuMTV cDNA hybridized to normal BALB/c DNA to the same final extent as did malignant BALB/cfC3H DNA (Table 3; Ref. 30), indicating that somatic proviral sequences that we detected are not novel to the malignant BALB/cfC3H cell.

Each of 10 BALB/cfC3H tumors that we have tested to date have contained extra sequences in DNA (see Ref. 30; Table 3). The level of sequence integration (2 to 3 copies/cell) has been the same for each tumor. Apparently, some mechanism operates to limit the extent of MuMTV integration. The constant level of integration is consistent with a hypothesis that mouse cells contain a fixed (limiting) number of integration sites or that integration establishes a form of immunity to superinfection by the same MuMTV variant.

We have not detected extra MuMTV sequences either in normal mammary cells of MuMTV-S-infected females 2 to 3 months of age or in premalignant BALB/cfC3H outgrowth populations maintained in virgin BALB/c (Table 3; Ref. 30).

In contrast to malignant BALB/cfC3H cells, malignant cells in BALB/c mammary tumors derived from transplanted BALB/c HAN in virgin BALB/c secondary hosts (Table 2) contain the same number of sequence copies as do normal BALB/c mammary cells (Table 3; Ref. 30). These data suggest that malignant BALB/c cells contain only germinal MuMTV proviral sequences.

Changes in MuMTV Expression in Mammary Cells during Hormonal Mammary Carcinogenesis

Normal Cells. MuMTV-specific RNA and structural poly-peptides are synthesized in normal BALB/c mammary epithelial cells. Concentrations of MuMTV RNA contained in normal mammary cells have been recently described in detail (30) and will therefore be treated only briefly here. The concentration of MuMTV-specific RNA in normal mammary cells in vivo varies as a function of the physiological state of the gland and animal age. We detected the highest concentrations in midpregnant and lactating glands of animals 1 to 3 months of age (30). Mammary cells from animals 8 to 12 months of age (matched in age with tumor-bearing animals), whether lactating, midpregnant, or resting, contained less than 1 full equivalent of the MuMTV genome per epithelial cell (30). Even in normal cells of young animals, the MuMTV RNA appears to be an incomplete or highly disproportionate transcript of the MuMTV genome. RNA from normal cells never completely hybridized MuMTV cDNA even at excesses of RNA 50-fold beyond
that required to saturate the cDNA at submaximal levels (30). Some sequences were detected in multiple copies, some less than uniquely in mammary cells (30).

Antigenic determinants of the major MuMTV core polypeptide, p28 (18, 48, 53), are also synthesized in normal mammary epithelial cells. Earlier studies in our laboratory to detect p28 relied on radioimmunoassay of detergent-solubilized (16) gland extracts. The amount of p28 detectable by radioimmunoassay was extremely variable in normal cells taken from different individual animals such that positive results were difficult to reproduce (unpublished observation). We therefore turned to fixed-cell immunofluorescence assays in an attempt to characterize the variability in terms of cellular heterogeneity in expression. Results of indirect immunofluorescence assays with monospecific p28 antisera in serum dilution experiments indicate that the variability in p28 expression in normal cells is primarily in the number of cells that are active in p28 expression (Table 4).

Antigenic determinants of the major envelope glycopeptides [gp52 and gp36 (18, 48, 52)] have not been detected in normal mammary epithelial cells by using monospecific sera in immunofluorescence assays. gp52 has not been detected in cells that contained the highest concentrations of MuMTV RNA (midpregnant and lactating cells of 2-month-old animals) or cells that contained the highest concentrations of p28 (Table 4). Results of radioimmunoassays for gp52 in our own (unpublished observation) and other (41) laboratories have also been negative. Yang et al. (58) were unable to show gp52 on the surface membranes of normal cells. The sensitivity of our own competitive radioimmunoassay for gp52 fixes an upper limit of 20 ng gp52-related protein per mg cell protein in normal mammary epithelial cells.

**Malignant Cells.** Malignant cells contain an average of 30 to 40 times higher MuMTV RNA concentration than do those normal cells that express highest levels of MuMTV RNA [midpregnant cells of animals 1 to 3 months of age (Table 4)]. High MuMTV RNA concentrations (vis-à-vis normal) are maintained in malignant cells grown in virgin BALB/c hosts, indicating that maintenance of abnormally high MuMTV RNA concentrations in malignant cells does not depend on that hormonal regimen that initially promoted tumor development.

MuMTV sequences are contained in multiple copies (30 × normal to 40 × normal) in RNA of malignant BALB/c as well as BALB/cfC3H cells (30). Thus, the increase occurs whether cells contain only germinally transmitted MuMTV or additional somatic proviral sequences, although malignant BALB/cfC3H cells in general contained 2- to 4-fold higher MuMTV RNA concentrations than did malignant BALB/c cells (30). Sequences in malignant BALB/c RNA were indistinguishable from sequences in malignant BALB/cfC3H RNA by differences in thermal stabilities of hybrids formed with MuMTV-P cDNA (30).

Antigenic determinants of the major MuMTV core polypeptide (p28) are expressed in malignant cells. The fraction of positive cells in malignant populations is higher and the variation is less than is in normal cells (Table 4), suggesting that there is a selection for cells in which MuMTV p28 is expressed during malignant cell growth. However, expression of p28 or linked functions does not itself confer malignant growth properties on cells since the same fraction of normal cells can express p28 determinants as malignant cells at concentrations no more than 2-fold less than in the average malignant cell (Table 4).

In contrast to their inexpression in normal cells, gp52 and gp36 determinants were readily detectable in malignant cells in immunofluorescence assays. The range in percentage of gp52-positive cells varied between 60 and 89% for BALB/cfC3H and between 37 and 70% for BALB/c. Thus, expression of MuMTV envelope glycopeptides occurs in malignant cells whether cells contain germinally transmitted or somatic proviral sequences, although the mean fraction of gp52-positive cells in BALB/cfC3H tumors was 10% higher than it was in BALB/c tumors (Table 4). **Hyperplastic Alveolar Cells.** Hyperplastic alveolar cells

<table>
<thead>
<tr>
<th>Mammary cell</th>
<th>% of cell RNA MuMTV specific (av.)</th>
<th>Mean % of cells expressing structural antigens at the following reciprocals of antiserum dilution</th>
<th>gp52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premalignant</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Malignant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premalignant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Two normal preparations represent epithelial cells pooled from the glands of 2 individual first-midpregnant females. Malignant and premalignant are from tissues described in Table 2, from virgin secondary hosts.

$^b$ Averages are derived from hybridizations to RNA from: normal glands of pooled first-midpregnant mice, 10 mice/pool, 3 pools; hyperplastic glands from virgin BALB/c secondary hosts (Table 2), 3 pools of 4 pooled outgrowths; 5 individual tumors (Table 2) in virgin BALB/c.

$^c$ NT, not tested.

$^d$ Numbers in parentheses, ranges of positive cells in pooled glands of individual animals.

$^e$ MuLV+, murine leukemia virus.
also contain higher concentrations of MuMTV-specific RNA than do normal cells. The average MuMTV RNA concentration was approximately 10-fold higher in hyperplastic alveolar cells than it was in normal cells and 10- to 30-fold lower in hyperplastic alveolar cells than it was in malignant cells (Table 4). Multiple copies of MuMTV sequences were detected in RNA of premalignant BALB/c as well as premalignant BALB/cfC3H cells; however, as in the case with malignant cells, BALB/cfC3H cells contained an approximately 2- to 4-fold higher MuMTV RNA concentration than did the BALB/c cells.

Thus, during hormonal carcinogenesis, i.e., in premalignant outgrowths, MuMTV genomic RNA is induced, and the increase in RNA content occurs to approximately to the same extent in premalignant outgrowths, independent of the large difference in their rates of formation (Table 1). Further, induced MuMTV RNA levels are maintained in premalignant cells in virgin hosts, indicating that increased levels are maintained in the absence of the hormonal stimulus that resulted in cell transformation. MuMTV envelope glycopeptides are also induced during hormonal carcinogenesis in BALB/cfC3H. Experiments are in progress to determine whether premalignant BALB/c mammary epithelial cells express MuMTV envelope glycopeptides.

Hormonal Induction of MuMTV RNA and Glycopeptides in Mammary Epithelial Cells in Cell Culture

The results of steady-state measurements of MuMTV RNA and polypeptides in normal, premalignant, and malignant cells during hormonal carcinogenesis in vivo are consistent with the hypothesis that hormones promote mammary carcinogenesis by altering expression of MuMTV in mammary epithelial cells. Although the results support the hypothesis, steady-state in vivo measurements cannot distinguish between cause and effect, in this case whether enhanced expression of MuMTV results in malignant properties or whether acquisition of malignant properties results in enhanced MuMTV expression. Thus, we have attempted hormonally to induce those changes in MuMTV expression, which we observed in malignant cells in vivo, in normal and premalignant cells in vitro to determine whether cell transformation results. These experiments only recently became possible with the development of methods to cultivate normal and premalignant epithelial cells in dispersed homotypical cultures (56).

Prior to development of these culture methods, several studies were conducted defining hormonal control of intracellular MuMTV expression in malignant cells. Of the several groups of mammotropic hormones tested, glucocorticoids have been shown to have the most dramatic inductive effect (13, 15, 28, 43, 44, 47, 57). The glucocorticoid-inductive effect is mediated by increasing rates of MuMTV RNA transcription from proviral sequences in DNA (reviewed in Ref. 57).

All of the published studies on glucocorticoid-controlled synthesis have been conducted on MuMTV-infected cells (reviewed in Ref. 57), cells containing somatic proviral sequences in DNA. Studies of glucocorticoid-controlled MuMTV expression in these cells have shown 2 distinct levels. The first level of control is represented by a cis-dominant expression of glucocorticoid-independent, constitutive MuMTV levels (42). The second level of control is represented by a glucocorticoid stimulation of MuMTV synthesis via high-affinity cytoplasmic receptor proteins (50, 60). Constitutive levels of MuMTV are responsive to insulin (28) and to a minor extent to sex steroids (13, 15).

Constitutive levels of MuMTV RNA in normal and malignant cells in BALB/c and BALB/cfC3H are shown in Chart 2. In our experiments, constitutive levels of MuMTV RNA were defined as those levels maintained in cells cultivated in the presence of 5% calf serum and insulin (10 µg/ml) and were measured 7 days after plating (3 days after reaching a stationary growth phase). Approximately 0.05% of the malignant BALB/cfC3H cell RNA, 0.0006% of malignant BALB/c cell RNA, and 0.00004% of normal BALB/c cell RNA were MuMTV specific after 7 days in culture, corresponding to an order of magnitude difference in constitutive MuMTV RNA levels maintained in the 3 cell types. These differences were maintained in the absence of significant differences in rates of cell RNA synthesis (Chart 3). Thus, normal cells are characterized by a highly repressed constitutive level of MuMTV genomic RNA vis-à-vis malignant cells, but malignant cells in which somatic proviral sequences were detected contained 10 times more MuMTV RNA than did those
MuMTV RNA was not induced with dexamethasone in normal mammary epithelial cells (Charts 2 and 4), with the hormone concentration and assay intervals optimal for malignant cells (Charts 2 and 4). Concentrations of 10^{-5} to 10^{-7} M dexamethasone were equally noninductive (data not shown). Thus, under a defined set of conditions of cell growth and hormonal milieu, normal cells are refractory to glucocorticoid, lacking the glucocorticoid-modulated mechanism of MuMTV induction characteristics of malignant cells. Normal cells therefore maintained MuMTV RNA levels 1,000- to 10,000-fold lower than those of malignant cells, at concentrations less than 1 35S genome equivalent/cell.

Normal mammary epithelial cells from young first-mid-pregnant females contained approximately 100 times less MuMTV-specific RNA in primary 7-day homotypical cell culture than did cells in the corresponding physiological state in vivo (30). It thus appears that levels attained in normal cells of young animals in vivo are actually induced levels, and conditions attained in vitro are insufficient to maintain the in vivo concentrations. Experiments are in progress to define conditions (e.g., hormones other than glucocorticoid) that maintain in vivo levels in cultivated normal cells. It should be noted, however, that the noncoordinate expression of gp52 and p28 in normal cells (Table 4) was observed under conditions of induced MuMTV RNA content; i.e., in normal cells freshly isolated from midpregnant glands.

Treatment of normal cells with IdUrd converted dexamethasone-resistant normal cells to dexamethasone-responsive cells. Table 5 shows that, after normal cells had been exposed to 20 μg IdUrd per ml for 48 hr during log phase growth, dexamethasone increased the MuMTV RNA concentration 7 to 8 times. IdUrd treatment alone did not activate MuMTV RNA expression (Table 5). Treatment of nondividing normal cells with IdUrd did not sensitize normal cells to the MuMTV-inductive effect of dexamethasone (Table 5), and the sensitizing effect required exposure to visible light (Table 5). Both observations suggest that incorporation of IdUrd into DNA is required for its effect to enhance sensitivity of the cell to dexamethasone (51).

Additional experiments (51) are needed to confirm that suggestion.

MuMTV RNA and gp52 are inducible with dexamethasone in premalignant BALB/cfC3H cells without prior IdUrd treatment (Table 6). MuMTV RNA concentrations were increased 9 to 10 times, and MuMTV gp52 levels were increased approximately 2- to 4-fold 24 hr after the addition of 10^{-8} M dexamethasone (Table 6).

**Transformed Mammary Epithelial Cell Phenotype in Culture**

Does glucocorticoid induction of MuMTV RNA and glycopeptide expression in normal or premalignant cells result in conversion to a malignant phenotype in culture? It has been impossible to answer this question because of the paucity of markers for transformed mammary epithelial cells in culture. The several most common markers (phenotypic changes) that have been identified to distinguish malignant from normal fibroblasts [a change in morphology (26), a change in the intercellular junctional complex (8),
Chart 4. Basal and dexamethasone-induced levels of MuMTV-specific RNA in BALB/c mammary epithelial cells. 
MuMTV-P cDNA was hybridized to RNA from primary 7-, 7.5-, 8-, and 9-day cultures of normal (midpregnant), malignant BALB/c (hormone-induced), and malignant BALB/cfC3H (exogenous MuMTV-induced) cells as described in "Materials and Methods." The number of MuMTV 35S genome equivalents in cells was calculated from the equation described in "Materials and Methods," with 5 x 10^-13 g as the average RNA content of mouse cells, 5 x 10^-12 g as the weight of 1 molecule of 35S RNA (55), and Ci/Co of cDNA x 35S RNA hybridization of 4 x 10^-3, and Ci/Co of cDNA hybridization to cell RNA determined in hybridizations as described in the legend to Chart 2. •, minus hormone; O, plus hormone.

Table 5
Effect of IdUrd in sensitizing normal cells to dexamethasone

IdUrd was first added to dividing (D) or stationary (S) cultures of normal cells from first-midpregnant glands. In each case, cells were exposed to IdUrd for 48 hr. All cultures were confluent at IdUrd termination. At termination, cells were exposed to visible light [with one exception, maintained under conditions of dark exposure (DE)]. Dexamethasone was added for 24 hr after washing the IdUrd from cultures. MuMTV RNA content was measured 24 hr after dexamethasone addition by hybridization to MuMTV cDNA (see "Materials and Methods.").

<table>
<thead>
<tr>
<th>IdUrd treatment (µg/ml)</th>
<th>Growth state</th>
<th>Dexamethasone (M)</th>
<th>% of cell RNA MuMTV specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>D</td>
<td>10^-4</td>
<td>0.0005</td>
</tr>
<tr>
<td>20</td>
<td>D</td>
<td>None</td>
<td>0.0007</td>
</tr>
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<td>20</td>
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<tr>
<td>20</td>
<td>S</td>
<td>10^-4</td>
<td>0.0007</td>
</tr>
<tr>
<td>20 (DE)</td>
<td>D</td>
<td>10^-4</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

increased longevity (26), and decreased response to contact (1)- or density (20)-mediated signals that inhibit growth] do not distinguish normal mammary epithelial cells from BALB/c or BALB/cfC3H mammary tumor cells in primary culture. Morphologically, these cells are not readily distinguishable from normal mammary epithelial cells (31, 40, 56; Fig. 1). They retain a polygonal epithelioid shape with microvilli and organelles polarized to the medium-bathed surface (49). Nuclear:cytoplasm ratios are the same (H. D. Soule, personal communication). Malignant cells are interconnected by a junctional complex characteristic of normal transporting mammary epithelium (49). Ocluding junctions are functional between interconnected malignant cells, acting as low-resistance barriers to passage of ions and small molecules (49).

The longevity of BALB/c and BALB/cfC3H mammary tumor cells is the same as the longevity of normal mammary cells from midpregnant 1- to 2-month-old females. Both tumor and normal cells stop dividing after 3 to 4 divisions (T_d = 24 hr) in primary culture.²

We have identified 2 alterations in malignant mammary epithelial cells that serve to distinguish them from normal cells. One is a surface membrane alteration detectable by an abnormal interaction with Con A-coated human RBC in hemadsorption assays (56).

Malignant BALB/c or BALB/cfC3H mammary epithelial cells in primary culture adsorbed human type O erythrocytes coated with low concentrations of Con A, whereas normal cells were completely unreactive at 5- to 6-fold higher concentrations (56). Premalignant BALB/c and BALB/cfC3H mammary epithelial cells were as reactive in Con A-mediated hemadsorption assays as were malignant cells (56).

The Con A receptor on the surface of malignant cells has not been identified; however, several observations suggest
that the receptor is a determinant of MuMTV gp52: (a) gp52 readily binds Con A (unpublished observation); (b) gp52 is detectable at the surface of malignant and premalignant cells (this study); and (c) brief treatment of malignant cells with anti-gp52 sera diminishes reactivity with Con A-coated RBC (unpublished result). However, normal cells do not react with gp52 antisera either before or after a hyaluronidase treatment that results in "exposure" (realignment) of Con A receptors (56). It is likely that multiple glycoproteins with appropriate sugar hapten specificity and steric arrangement for Con A reactivity are present on mammary cell surfaces, only one of which is gp52.

The second alteration in malignant mammary epithelial cells, one which distinguishes them from normal cells, is an altered response to insulin as a mitogen. Malignant BALB/c and BALB/cfC3H mammary epithelial cells exhibit an enhanced response to insulin as an "overgrowth stimulating factor." Both malignant and normal mammary epithelial cells exhibit a high degree of contact (1)- or density (20)-mediated inhibition of division in homotypical culture (30). Contact-inhibited normal and malignant cells are shown in Fig. 1. Each cell type stops dividing at the identical density (30). When proliferation of malignant cells is inhibited by intercellular contact, insulin reinitiates DNA synthesis and mitosis. 4 The optimum insulin concentration is 10 \( \mu \text{g/ml} \). Chart 5 shows that the maximum DNA-synthetic response to insulin occurs 24 hr after addition of the hormone. Approximately 6% of malignant cells are stimulated to reinitiate DNA synthesis and mitosis by insulin. Physiological concentrations of insulin do not stimulate malignant cells to enter DNA synthesis. A wave of mitosis follows approximately 14 hr after the wave of DNA synthesis, suggesting that malignant cells are arrested in G\(_1\) of the cell cycle by intracellular contact. The length of G\(_1\) + S can be estimated to be approximately 19 hr, from the ascending slopes of DNA-synthetic and mitotic curves.

The mechanism of insulin action in promoting DNA synthesis in contact-inhibited malignant cells is under study. Insulin stimulates uptake of DNA precursors into acid-soluble cell pools, but pool sizes achieved by normal cells, which do not initiate DNA synthesis, are the same as those achieved by malignant cells (Table 7). Thus, alterations in the DNA-synthetic machinery rather than transport-dependent functions appear particularly relevant to altered responsiveness to insulin. Studies are in progress to determine whether malignant mammary cells respond to other normally tropic hormones in a similar way, as they responded to insulin. Additionally, by using combined techniques of immunofluorescence and autoradiography, it is now possible to determine whether hormonal induction of MuMTV glycopeptides in normal cell populations results in altered response to insulin as a mitogen.

### Discussion

The problem addressed by these studies is the mechanisms by which hormones promote mammary tumorigenesis in mice. At issue is the likelihood that hormones act as cocarcinogens by promoting expression of endogenous mammary tumor virus genes in mammary cells. Our approach has been to compare rates of premalignant and malignant transformation in cells carrying genetically transmitted and somatic MuMTV proviral sequences and to identify specific alterations in MuMTV expression during hormonal carcinogenesis in vivo. These measurements in vivo are complemented by an in vitro study to determine whether the altered expression of MuMTV observed during hormonal carcinogenesis in vivo could be reproduced under defined conditions of hormonal stimulation in vitro.

The results of our investigations in the multivariate BALB/c system have been organized into a model for continued investigation into the roles of hormones and MuMTV in malignant transformation of mammary epithelium. The hormonal-MuMTV cocarcinogenesis model is shown in Chart 6.

In the model we suggest that hormones play 2 roles in

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Table 7

<table>
<thead>
<tr>
<th>Cell</th>
<th>Insulin</th>
<th>Total</th>
<th>Soluble</th>
<th>Precipitable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10 \mu \text{g/ml} )</td>
<td>(18.1)</td>
<td>(15.9)</td>
<td>(2.2)</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>(4.3)</td>
<td>(2.9)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>Malignant</td>
<td>None</td>
<td>(11.3)</td>
<td>(8.5)</td>
<td>(2.8)</td>
</tr>
</tbody>
</table>

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Note: Normal, cells from first-midpregnant females 2 months of age; malignant, primary BALB/cfC3H tumor cells. Cells were labeled with \(^3\)H-thymidine as described in "Materials and Methods." Soluble cpm = total – precipitable cpm; the percentage of total \(^3\)H-thymidine incorporated is (precipitable + (total)) \times 100. Measurements were made 24 hr after insulin addition. A 2000 cpm "0-time" background is subtracted from total and precipitable values. Numbers are the average of 1 duplicated experiment. Maximum variation in cpm was 19%.

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Hormonal-Viral Mammary Cocarcinogenesis

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mammary cell transformation: the first role is a permissive one, promoting a genetic change in the cell, which relaxes repressive controls over specific MuMTV genes. This step results in the generation of a "high-risk cell," which is defined as a cell with an enhanced responsiveness to the MuMTV gene-inductive effects of mammmotropic hormones. The second role of hormones is direct, inducing expression of viral-coded products in mammary epithelial cells, which are essential for events leading to expression of a transformed phenotype. The transformed phenotype is defined functionally as an enhanced responsiveness to trophic hormones for overgrowth.

In the model, we suggest that the proximal cause of malignant transformation is expression of MuMTV genes in the 3'-region of the genome in normal mammary epithelial cells. The evidence for this is circumstantial, based on finding markers (env gene products) for the 3'-region of the MuMTV genome expressed in malignant cells but not in normal cells and on finding these markers expressed en route to malignant transformation, i.e., in premalignant cells. Although MuMTV gene order has not been established, the similarities between MuMTV and RNA tumor viruses in which gene order has been established (6) in terms of nucleotide length (17), size of primary gene products (18), and molecular details of replication (6, 18) suggest that the env gene is in the 3'-third and the gag gene in the 5'-third of the MuMTV genome. Results of radioimmunoassays for MuMTV gp52 not described here indicate that normal cells contain at least 50 times less gp52 than do malignant cells and fix an upper limit of 20 ng gp52 per mg normal cell protein (C. M. McGrath, unpublished result). In conjunction with data on p28 expression, in which we demonstrated that normal cells can express nearly identical levels as malignant cells, the difference in gp52 contents in normal and malignant cells suggests noncoordinate repres-

sion of 3'-region MuMTV gene products in normal cells. The noncoordinate regulation appears to be at the level of RNA processing from proviral DNA, as controlled rates of either transcription or turnover. Experiments are in progress with cDNA tailored to the 3'- and 5'-regions of the MuMTV genome to determine directly the extent to which message transcripts of these genetic regions are present in normal and malignant cells. If the cocarcinogenesis model is accurate, we would predict that malignant cells would contain a messenger RNA species with 3'-marker gene sequences that is not present (or present at much reduced concentration) in normal cells.

We are also suggesting in the model that the information contained in the endogenous MuMTV is sufficient MuMTV information for mammary cell transformation. This is based on 2 observations: (a) altered expression of MuMTV was observed during hormonal carcinogenesis in cells in which only germinally transmitted sequences were detected, an altered expression that was qualitatively the same as in cells in which somatic proviral sequences were detected; and (b) the incidence of carcinomas in BALB/c was the same as the incidence in BALB/cfC3H; integration of somatic proviruses only served to accelerate the rate of malignant transformation (by accelerating the rate of pre-malignant transformation). However, we cannot claim unequivocally that somatic proviral sequences are not present in malignant BALB/c cells (or that the endogenous MuMTV variant is not defective in genes related to MuMTV-S or MuMTV-P). The MuMTV cDNA used in the experiments described, which detected only germinal levels of MuMTV sequences in malignant BALB/c cells, was prepared by using endogenous primers and was therefore probably not uniformly representative of the entire MuMTV genome (30). Thus, MuMTV sequences not detected by this cDNA could have been present at amplified levels in malignant BALB/c

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DNA (or absent in normal cell DNA). Hybridizations with well-characterized cDNA, which represent 3'-, 5'-, and middle thirds of the MuMTV genome, probes that have only recently become available, are necessary to determine whether extra and novel MuMTV sequences are integrated in mammary cell DNA during hormonal carcinogenesis in BALB/c. Likewise, whereas we have shown that 1 BALB/c premalignant outgrowth produced malignant carcinomas at the same frequency as 1 BALB/cfC3H premalignant outgrowth, Medina (35) reported considerable variability in malignant potential of different premalignant BALB/c outgrowths. Malignant potential of several BALB/c and BALB/cfC3H outgrowths must be compared before concluding that only rates of malignant transformation and not incidences are increased by infection of BALB/c mammary epithelium by MuMTV-S.

In the hormonal-MuMTV cocarcinogenesis model shown in Chart 6, we suggest that hormones play a more fundamental role in malignant transformation than do activating endogenous MuMTV genes. This role is to promote a change in cell DNA, a change that makes the 3'-genetic region of endogenous MuMTV inducible with either the same or different mammotrophic hormones that promoted the change in DNA.

The evidence for this indirect role of hormones in malignant transformation is also circumstantial: (a) when applied during periods of hormone-induced DNA synthesis, carcinogens, such as X-irradiation or mutagenic chemicals, increase the incidence of premalignant and malignant transformations in BALB/c to more than that observed during prolonged hormonal stimulation alone (35); (b) a major genetic change occurs during transformation in the presence of infectious MuMTV, integration of somatic provirus sequences (30, 38); MuMTV genes are inducible in those cells; (c) endogenous MuMTV RNA and 3'-region gene products, induced during hormonal carcinogenesis in vivo, are not inducible in normal cells by glucocorticoids at concentrations optimal for induction in malignant cells; (d) MuMTV RNA is inducible with glucocorticoid hormone in normal cells after IdUrd treatment, but only under conditions that suggest that the conditioning effect of IdUrd is mediated by a perturbation of chromosomal DNA (51); and (e) MuMTV RNA and 3'-region gene products are inducible in premalignant cells, cells at higher than normal risk for malignant transformation.

In the model, we propose that mammotrophic hormones promote malignant transformation in BALB/c by promoting extensive cell division, which maximizes activity of low level environmental carcinogens and allows accumulation of random mutations; this results in entry of MuMTV genes into a hormonal domain (23). In BALB/cfC3H, in which infectious MuMTV is the primary carcinogen (40), hormones promote infection and thereby the genetic change, proviral sequence integration. Indeed, it is tempting to speculate that the difference in rate of premalignant transformation in BALB/c and BALB/cfC3H is due precisely to the nature of the change in chromosomal DNA: the tropism of infectious virus in BALB/c for mammary cells (40) and the specificity of sequence integration for sites that abrogate control of endogenous sequence replication versus the accumulation of random base changes in the presence of low-level environmental carcinogen exposure. If this speculation is valid, we would anticipate that premalignant BALB/cfC3H cells would contain somatic proviral sequences. These were not detected in the present study but, in considering the cellular constituency of hyperplastic outgrowths used as a source of premalignant cells, we do not consider our data as evidence that premalignant cells do not contain extra sequences. Hyperplastic outgrowths are mixtures of normal and premalignant cells (14, 35, 36). Moderate excesses of normal cells with germinal levels of MuMTV sequences in DNA would easily dilute 2-fold extra somatic proviral sequences detected in malignant cells to non-detectable levels in our DNA hybridization. Cloned premalignant cells are needed for hybridization experiments to determine whether extra sequences are integrated.

A priori, the genetic change in cells promoted by hormones, which results in glucocorticoid activation of endogenous MuMTV, could occur by several mechanisms. A provirus may be activated by a proximal DNA change (e.g., creating an initiation or readthrough site for RNA polymerase (27)) or a distal change, moderated by a change in activity of difusible controlling compounds. Preexisting template-active MuMTV sequences may be modified to bring the active genes into the glucocorticoid domain.

The hormonal-viral cocarcinogenesis model should be considered at present a fabric in which to conduct finer investigations into its nature. The major predictions of the model are accessible by experimentation both in vivo and in vitro. The MuMTV structural and nonstructural genes activated in vivo during hormonal carcinogenesis need to be identified; activated germinal and somatic proviral genes need to be distinguished. If pleiotropic genetic alterations of the type described previously are fundamentally responsible for premalignant and malignant transformation, acting by sensitizing the normal cell to the 3'-MuMTV gene-inductive effects of mammotrophic hormones, treatment of normal cells in vitro that are hormonally primed to divide, such as to reproduce the proposed changes in DNA (transfection of proviral sequences and application of physical and chemical mutagens), would accelerate malignant transformation, and the acceleration would necessarily follow acquired sensitivity to mammotrophic hormones for activation of 3'-region MuMTV genes. Further, properly tailored chemotherapy of intervention of MuMTV gene activation, after the high-risk cell is generated, would prevent transformation mediated by hormonal induction of MuMTV genes.

Acknowledgments

Much of the molecular hybridization data was the result of excellent technical work of E. J. Marineau. Dr. B. A. Voyles and Dr. H. D. Soule contributed significantly to the cell culture aspects of this study.

References

Fig. 1. Normal (midpregnant) and malignant BALB/c mammary epithelial cells in primary culture. A and B, cells stained at confluence with hematoxylin. x 440; A, normal; B, malignant (BALB/cIC3H). C and D, unstained autoradiographs (x 440) of [3H]thymidine-labeled cells (see "Materials and Methods"); C, normal cells at the edge of an artificially created wound to demonstrate that normal cells not responsive to insulin for initiation of DNA synthesis are capable of DNA synthesis. Cells were labeled 24 hr after wounding culture. D, malignant (BALB/cIC3H) cells 24 hr after the addition of insulin (10 μg/ml).
Hormonal Induction of Mammary Tumor Viruses and Its Implications for Carcinogenesis

Charles M. McGrath and Richard F. Jones


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