Hormonal Dependence of DNA Synthesis in Mammary Carcinoma Cells in vitro

Abstract. *Explants of C3H-mouse and rat R3230AC mammary carcinomas were cultured on chemically defined medium for study of the effects of the hormonal environment on synthesis of DNA. Synthesis in the more slowly proliferating C3H carcinoma cells is stimulated by insulin and inhibited by estrogenic hormones as in normal mammary epithelial cells. Rat R3230AC mammary carcinoma cells can initiate synthesis of DNA independently of insulin or estrogenic hormones. Autonomous growth with respect to these hormonal controls correlates with rapid proliferation, but it is not an essential characteristic of the neoplastic mammary cell.*

A fundamental characteristic of neoplastic cells is unrestricted proliferation. In mammary carcinoma cells the rate of cellular proliferation may be modified by various hormonal factors. In order to define more completely the role of the hormonal environment in growth of mammary carcinoma cells we have compared the rates of synthesis of DNA in mammary epithelial cells and in mammary carcinoma cells cultured in chemically defined medium with various hormones added.

The tumors studied were the spontaneous, virus-associated mammary adenocarcinoma of the C3H/HeJ mouse, and the R3230AC mammary carcinoma, a spontaneous tumor that is transplantable in the Fischer rat (*1*). The latter tumor was maintained by trochar transplantation into 90-g female Fischer rats 3 weeks after ovariectomy. Small nonnecrotic tumors were removed aseptically and cut into explants weighing about 0.1 mg. Explants prepared from normal tissues weighed approximately 0.3 mg.

Twelve to 14 explants were placed on a siliconized lens paper floating on 3 ml of sterile Medium 199 (Microbiological Associates) and incubated at 37°C in an atmosphere of 95 percent oxygen and 5 percent carbon dioxide. The medium contained crystalline beef insulin (Eli Lilly; 23.6 international units per milligram) at 5 μg/ml and sodium penicillin at 35 μg/ml. Steroid hormones were dissolved in absolute alcohol before addition to the medium. Rates of synthesis of DNA were determined by allowing the explants to incorporate tritiated thymidine (1 μc/ml; specific activity, 8.0 c/mmole) during 4-hour pulse-labeling periods (*2*).

Tumor explants were maintained in organ culture for as long as 4 days without evidence of necrosis. However, central necrosis invariably occurred within 24 hours during incubation in an atmosphere of air and 5 percent carbon dioxide. The mouse tumors were typical adenocarcinomas of Dunn's type-A (*3*). The explants of both tumors each tended to become enveloped by a single layer of squamous epithelium during the first 2 days of culture (*4*).

Mouse mammary epithelial cells in organ culture are stimulated by insulin to initiate DNA synthesis and subsequently divide (*5*, *6*). After an initial lag of 12 to 24 hours, synthesis of DNA in virgin mammary epithelial cells in-

Table 1. Mitotic and labeling indexes of mammary epithelial and carcinoma cells in explants cultured in Medium 199 with or without insulin. Mammary explants from 3-months-old virgin C3H mice were cultured for 48 hours; all other explants, for 24 hours. During the final 4 hours of incubation all explants were exposed to Medium 199 containing tritiated thymidine at 0.5 μc/ml. Tissues were fixed in Bouin solution, sectioned at 5 μ, and stained with hematoxylin and eosin. Slides for autoradiography were dipped in Kodak NTB-3 emulsion and developed 2 weeks later. At least 1000 cells were counted for determination of each mitotic or labeling index. Average grain counts above the labeled nuclei were similar in all systems.

Tissue	Mitotic figures per 1000 cells			Cells labeled (%)		
	Initial	Insulin		Hours ≤ 4	Insulin	
		Minus	Plus		Minus	Plus
C3H Mouse						
Virgin mammary gland	1	1	10	2	2	29
Midpregnancy mammary gland	9	7	22	6	5	36
Mammary carcinoma	12	9	34	18	14	61
Fischer rat						
Host mammary gland	1	1	4	4	5	16
R3230AC carcinoma	24	70	70	25	80	85

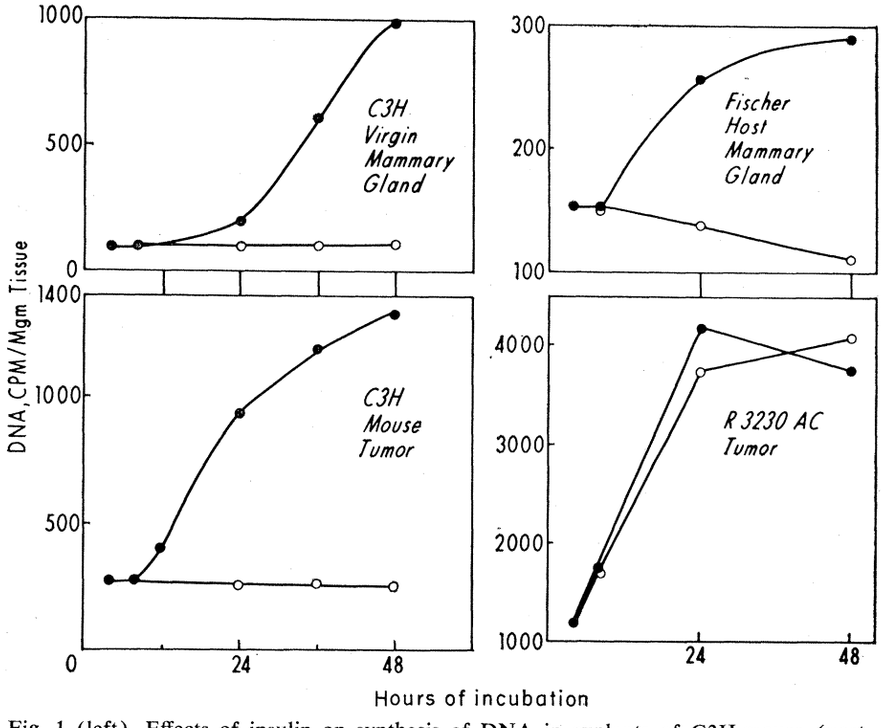

Fig. 1 (left). Effects of insulin on synthesis of DNA in explants of C3H-mouse (postadolescent virgin) mammary gland and C3H-mouse mammary tumor. Each point represents the incorporation of tritiated thymidine into DNA during the preceding 4-hour period. Solid circles, insulin present; open circles, insulin absent. These results are representative of 18 such tumors studied. Fig. 2 (right). Rates of synthesis of DNA in the presence (solid circles) and absence (open circles) of insulin in explants from the R3230AC carcinoma and from a normal mammary gland of the host Fischer rat. Each point represents incorporation of tritiated thymidine into DNA during the preceding 4-hour period. These results are representative of 20 such experiments.

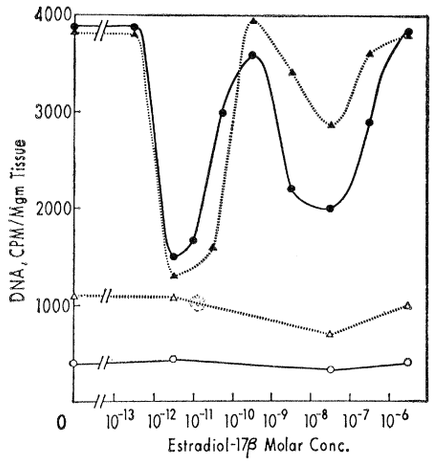

Fig. 3. Effects of estradiol-17β on synthesis of DNA in explants, from a C3H-mouse midpregnancy mammary gland (circles, solid line) and from C3H-mouse carcinoma (triangles, dotted line), incubated for 20 hours in the presence (solid symbols) or absence (open symbols) of insulin and pulse-labeled with tritiated thymidine during the period between 20 and 24 hours of incubation. These results are representative of four such experiments.

creases about tenfold (Fig. 1). An increase in the rate of DNA synthesis in explants of mouse carcinoma similarly depends on the presence of insulin in the medium; this increase is detectable after 12 hours of incubation, and the rate of synthesis continues to rise during at least 96 hours of culture. The stimulatory effect of insulin is independent of its role in augmenting glucose transport, since it was observed in both neoplastic and normal cells when glucose was replaced by fructose in the incubation medium.

Stimulation of synthesis of DNA in mammary epithelial cells derived from the Fischer rat also depends on the presence of insulin (Fig. 2). However, explants of the R3230AC tumor synthesized DNA at a markedly accelerated rate when placed in vitro, and this stimulation was independent of insulin; in contrast to the time course of synthesis of DNA in the normal tissue and the C3H mouse tumor, no initial lag was observed. During the first 24 hours of culture, 100 percent of the carcinoma cells became labeled with tritiated thymidine.

The rates of synthesis of DNA observed in these experiments primarily reflect the numbers of cells engaged in synthesis (Table 1). Parallel changes in mitotic indexes indicate that the thymidine labeling reflects cell proliferation rather than changes in uptake of the radioactive precursor.

Observations of mammary cancers in several experimental animal lines and in man have indicated that estrogenic hormones play a major role in modification of growth of mammary tumors. However, we know of no previous experimental demonstration that estrogens can interact directly with neoplastic mammary cells to alter cell proliferation.

Estradiol-17β has a markedly inhibitory effect on the rate of insulin-stimulated synthesis of DNA in mammary epithelial cells, and this effect is highly dependent on the concentration of the hormone (Fig. 3). At $4 \times 10^{-12}M$, estradiol-17β markedly inhibits synthesis of DNA in insulin-stimulated cells, although synthesis in the absence of insulin is not altered. As the concentration of estradiol increases over a "physiological" range, synthesis of DNA again increases to a maximum rate at approximately $4 \times 10^{-10}M$. At an estradiol concentration of $4 \times 10^{-8}M$, synthesis of DNA is markedly inhibited in the insulin-containing system and slightly inhibited in the insulin-free system. Since the final concentration of ethanol was the same in all systems shown in Fig. 3, these effects cannot be due to the ethanol used as the initial solvent of estradiol.

Very similar patterns of inhibition were observed with other estrogenic hormones in the following order of relative potencies: estradiol-17β, 1.0; estriol, 1.0; diethylstilbestrol, 0.5; estrone 0.4. Autoradiographic data indicated that these responses to estrogenic hormones reflected primarily changes in the number of cells engaged in synthesis of DNA rather than in the rate of synthesis per cell. The response of the C3H-mouse carcinoma cells was very similar to that of the normal tissue (Figs. 3 and 4), whereas synthesis of DNA was little altered in the explants of rat R3230AC carcinoma exposed to estradiol-17β.

Insulin induces normal mammary epithelial cells to enter the *S*-phase (DNA-synthesis phase) of the cell cycle, although it does not appear to alter the rate of replication of the DNA chromosomes (duration of *S*-phase) (*5*). Since cell division must subsequently occur, the initiation of synthesis of DNA may be an important step in the regulation of cell proliferation (*7*).

Our studies show that cells of C3H-mouse mammary carcinoma are hormonally responsive in vitro in that they are induced by insulin to initiate synthesis of DNA, and that the rate at

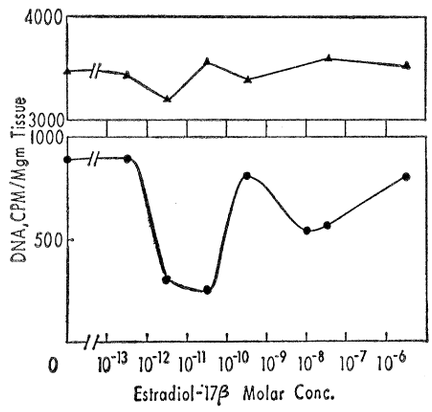

Fig. 4. Synthesis of DNA in explants from a mammary gland of a midpregnant Fischer rat (bottom) and from an R3230AC carcinoma (top) incubated in Medium 199 containing insulin and various concentrations of estradiol-17β. The explants were allowed to incorporate tritiated thymidine into DNA during the period between 20 and 24 hours of incubation.

which insulin-stimulated cells enter the *S*-phase can be further modified by estrogenic hormones. When explants of the R3230AC tumor are placed in vitro they are induced to initiate synthesis of DNA independently of insulin. It appears that the mechanisms that are induced by insulin in the normal cell may have become constitutive in the cell of the R3230AC carcinoma. However, this loss of hormonal regulation of synthesis of DNA is not an essential characteristic of the neoplastic mammary cell, as demonstrated by the cell of the C3H-mouse carcinoma.

No exceptions to the above patterns of response to hormonal stimuli were encountered among the tumors tested. Recently it was shown (*8*) that carcinogen-induced mammary carcinomas in Sprague-Dawley rats are heterogeneous with respect to their dependence on insulin for stimulation of cell proliferation. In our experiments hormonal dependence correlated with restriction of growth. The mitotic and labeling indexes noted in the C3H-mouse mammary carcinomas indicate that these small tumors proliferate relatively slowly. Autonomous growth with respect to hormonal controls may represent a factor in the rapid proliferation of the R3230AC cells.

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9. Aided by PHS grant CA-10268. We thank Elaine Nyberg and O. T. Ward for technical assistance.

22 April 1968

Comparison of RNA Terminal Sequences of Phages f2 and Qβ: Chemical and Sedimentation Equilibrium Studies

Abstract. *The terminal fragment obtained by hydrolysis with ribonuclease T_1 of the ribonucleic acid from the bacteriophage Qβ has been isolated and purified. The results of chemical and enzymatic hydrolysis of this fragment and of the intact RNA itself indicate that the fragment has the composition (10 Cp, 4 Up), and that the RNA has the terminal sequence -GP(9 Cp, 4 Up) CpA. These conclusions are supported by the results of an application of the sedimentation-equilibrium method in which the molecular weight of the Qβ fragment was compared with that of the corresponding fragment from f2 phage RNA for which the terminal sequence, -GpUpUpApCpCpApCpCpCpA had previously been determined.*

In studies on the relation between function and primary structure of large RNA molecules we have concentrated initially on the development of new methods for the determination of nucleotide sequences near the terminals of these molecules (*1–3*). In the case of the RNA from bacteriophages, examination of the sequences at the 3′ terminals has been of particular interest since, with the assumption that the replicative mechanism in such organisms involves the synthesis of complementary strands of RNA in the usual 5′ → 3′ direction, these sequences would be expected to contain the initiation sites for this mechanism. Some of the enzymes involved in replication exhibit a unique specificity in that, for synthetic activity, they require intact homologous viral RNA as template (*4*); the basis for this specificity may reside in the nucleotide sequences at the 3′ terminals. We have reported (*3*) the terminal undecanucleotide sequence of f2 RNA as -GpUpUpApCpCpApCpCpCpA (5). The same terminal sequence has also been reported by De Wachter and Fiers (*6*) for the serologically related phage, MS2. We now report the characterization of the terminal sequence of the RNA obtained from the serologically unrelated phage Qβ and show that this virus has a terminal nucleotide sequence that differs from those of phages f2 and MS2.

For the determination of the terminal nucleoside of phage Qβ (*7*) the RNA [1110 O.D.U. at 260 nm, 50 nmole based on a chain length of 3050] was hydrolyzed with alkali as described (*1*) except that the separation of the terminal nucleoside from the salt and the nucleotides obtained from the reaction mixture was carried out on a DEAE-Sephadex A25 column (HCO_3^- form, 1.0 × 50 cm) in place of the DEAE-cellulose column. The nucleoside was identified by chromatography on a column of DEAE-Sephadex A25 (HCO_3^- form; *p*H 9.0; 0.4 by 100 cm). On such a column, control experiments have shown that the four nucleosides can be separated and quantitatively eluted with water. With this procedure, the Qβ RNA gave adenosine (38 nmole, 76 percent yield) as the only nucleoside.

Additional information on the terminal sequence was obtained by isolation of a larger fragment as described for f2 RNA (*2*). In this method the RNA is specifically cleaved with ribonuclease T_1, the product is oxidized with periodate, and the oxidized terminal oligonucleotide is selectively adsorbed on an aminoethylcellulose column. Subsequently, the absorbed material is released from the column by a β-elimination reaction, and thus the terminal fragment is recovered in a form which lacks the original terminal nucleoside. Application of this method to Qβ RNA gave an oligonucleotide which, on subsequent chromatography on DEAE-Sephadex at *p*H 8, was eluted at a position corresponding to a chain length considerably greater than that of the f2 fragment. In a typical experiment the ribonuclease digestion of 5350 O.D.U. (260 nm) of the RNA gave 12 O.D.U. at 260 nm (a yield of 50 percent) of the oligonucleotide.

The distribution of pyrimidine nucleotides in this product was determined by pancreatic ribonuclease hydrolysis. The fragment (2.53 O.D.U. at 260 nm) was treated with the enzyme (0.05 mg) for 15 hours at 25°C, and the products were analyzed as described (*3*). The only products obtained were cytidine 3′-phosphate (244 nmole) and uridine 3′-phosphate (99 nmole), yielding the ratio Cp : UP = 5.0 : 2.03. The size of the oligonucleotide and the identity of its terminal nucleotide were determined by a procedure (*2*) which consists of enzymatic dephosphorylation of the polynucleotide and subsequent alkaline hydrolysis, yielding the terminal group as a nucleoside. The Qβ oligonucleotide (2.9 O.D.U. at 260 nm) was treated with alkaline phosphatase (0.1 mg) in 0.03*M* tris chloride buffer, *p*H 8.2, for 3 hours at 37°C, and the product was hydrolyzed with 0.25*N* sodium

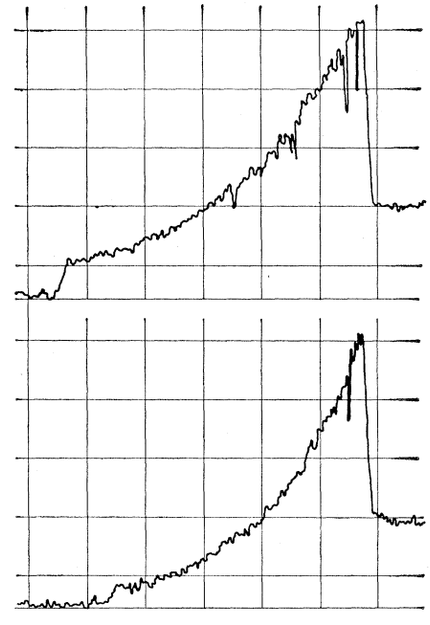

Fig. 1. Sedimentation equilibrium scanner traces for the two terminal oligonucleotides. Each was analyzed at 20°C in 0.2*M* potassium chloride–0.02*M* potassium phosphate buffer, *p*H 7, and at an initial concentration of 0.3 O.D.U. (260 nm) per milliliter. The scans were made at 260 nm after centrifugation for 20 hours. The Qβ fragment (upper trace) was run at 35,600 rev/min, and the f2 fragment at 50,740 rev/min.