INSULIN-DEPENDENT DNA POLYMERASE AND DNA SYNTHESIS IN MAMMARY EPITHELIAL CELLS IN VITRO

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It has been established that mouse mammary gland tissue exposed to insulin, hydrocortisone, and prolactin in a chemically defined explant culture system responds with augmented DNA synthesis, subsequent epithelial cell proliferation, and biochemical and histologic differentiation.[1-3] This transformation into a secretory state is manifested by the production of the milk proteins casein, α-lactalbumin, and β-lactoglobulin by the newly formed cells.[2, 4]

Although differentiation requires cell division and the presence of all three hormones,[5, 6] augmentation of DNA synthesis and proliferation are dependent on insulin alone.[3] These phenomena are demonstrable in tissue from pregnant and virgin mice. It is presumed that the relative quiescence of the tissue from virgin animals as compared to the tissue from pregnant animals is responsible for the fact that mammary gland explants from virgin mice respond to insulin more dramatically. For this reason tissue from virgin mice was used as a convenient system for the study of the initiation of proliferation.

The present studies examine the relationship of DNA polymerase activity to insulin-dependent DNA synthesis in order to determine the factors that may play a role in the initiation and maintenance of insulin-dependent proliferation.

Materials and Methods.—Animals: Three-month-old virgin mice of either C3H/HeN or Balb/c strain were used.

Preparation of de-epithelialized mammary glands: The ventral segments of the abdominal mammary glands of 20-day-old Balb/c mice were surgically removed on one side according to the method of DeOme *et al.*[7] Removal of this segment of mammary gland prior to three weeks of age interrupts proliferation of mammary epithelial cells from the nipple bud into other areas of the mammary gland. Careful removal of the ventral segment does not interfere with the blood supply to the remainder of the gland, which is composed almost exclusively of fat cells and fibroblasts. After the animals had convalesced for nine weeks, the mammary glands were used for DNA synthesis and DNA polymerase studies, the contralateral unoperated gland serving as a control. Multiple histological preparations of de-epithelialized glands at three months of age revealed occasional sparsely scattered epithelial cells but these comprised only a few per cent of those present in the control side. Fibroblasts on the operated side were generally increased in an area around the incision line.

Culture method: Explants (0.5–1.0 mg) of abdominal mammary glands from groups of two to five animals were removed with aseptic technique and prepared and cultured in sterile medium 199 (Microbiological Associates) using a modified procedure of Chen as previously described.[2] Crystalline beef insulin was used at a concentration of 5 μg/ml and testosterone at a concentration of 10 μg/ml. In those

experiments in which insulin was omitted from the culture medium, medium 199 minus glucose was used and D-fructose was added to a final concentration of 1 mg/ml.

Assay for DNA synthesis: DNA synthesis was determined in explants exposed to 0.5 μc/ml of tritiated thymidine (Schwarz BioResearch, sp. act. 6.0 c/mM) for four hours.[5] Explants were weighed, defatted by acetone extraction, and then 2.5 mg of carrier protein, bovine casein, was added. The explants and the carrier were precipitated with an equal volume (2 ml) of cold 10 per cent TCA and then washed four times with cold 5 per cent TCA and three times with ether:ethanol (1:3). The precipitate was dissolved in Hyamine (1 ml) overnight and then counted in toluene-PPO-POPOP in a liquid scintillation spectrometer.

Assay for DNA polymerase: The DNA polymerase assay was a modification of the procedure described by Bollum.[8] Tissue was homogenized (1:15, wt/vol) in a Teflon-glass homogenizer at 4°C in a solution containing 6 mM KCl, 0.25 M sucrose, and 12 mM Tris-HCl buffer (pH 7.8). The homogenate was centrifuged at 600 $\times$ g for 10 minutes. The supernatant was centrifuged at 100,000 $\times$ g for 60 minutes and the resulting clear supernatant was used for assay of DNA polymerase. The reaction mixture contained: 20 μM Tris-HCl buffer (pH 7.4); 2 μM $MgCl_2$; 0.25 μM 2-mercaptoethanol; 0.25 μM EDTA; 30 mμM each of dCTP, dGTP, and TTP; 15 mμM of dATP; 0.3 mμM dATP-H^3 (Schwarz BioResearch, sp. act. 3.8 c/mM); and 100 μg of heat-denatured salmon sperm DNA. Enzyme preparation (50 or 100 μl) was added to give a final volume of 0.250 ml. The reaction was carried out for either 30 or 60 minutes at 37°C in a Dubnoff shaker. Isotope incorporation was arrested by the addition of 1 ml of a 1 per cent casein solution containing 300 mμM ATP and an equal volume of cold 10 per cent TCA. The precipitate was washed four times with cold 5 per cent TCA, twice with ether:ethanol (1:3), and then dissolved in Hyamine overnight and counted in toluene-PPO-POPOP in a liquid scintillation spectrometer.

Results.—Some characteristics of the assay system for mouse mammary gland DNA polymerase are recorded in Table 1.

The results show that the incorporation of dATP-H^3 into acid-insoluble material was proportional to the concentration of extract and the duration of incubation up to 60 minutes. Omission of TTP reduced the incorporation of isotope over 50 per cent, while omission of TTP, dGTP, and dCTP reduced it 95 per cent. Although

TABLE 1

ASSAY FOR DNA POLYMERASE

Assay system	Radioactivity incorporated into DNA (cpm)
Complete (100 μl enzyme; 60 min)	165
50 μl enzyme; 60 min	75
100 μl enzyme; 30 min	95
No TTP	80
No TTP, dGTP, dCTP	8
No primer	0
60 min; 50 μg DNase added at 30 min	0
Boiled* enzyme	15
Enzyme from tissue cultured in the presence of insulin, F, and P	145

The assay system is described in the *Materials and Methods* section. The enzyme was prepared from tissue which had been cultured for 48 hr in insulin medium, except where otherwise noted. The final concentration of each hormone in the culture medium was 5 μg/ml. F = hydrocortisone; P = prolactin.

* For 5 min at 100°C.

not shown, when TTP-H^3 was used in place of dATP-H^3, similar incorporation occurred. The reaction was completely dependent on exogenous DNA. Double-stranded DNA was only half as effective as heat-denatured DNA (not shown). The radioactive acid-precipitable material was rendered acid-soluble by treatment with DNase. Addition of hydrocortisone and prolactin to the insulin culture medium did not enhance the activity of the polymerase. This is consistent with observations that whereas all three hormones are required for the overt differentiative responses,[2] insulin alone is necessary and sufficient for the initiation of DNA synthesis and subsequent cell division.[3]

It was previously reported[3] that insulin effects an increase in the rate of synthesis of DNA by mammary gland epithelial cells *in vitro*. Figure 1 illustrates the time course of this rate and also shows the activity of DNA polymerase as a function of duration of explant culture in insulin medium.

The initial DNA polymerase activity is unaltered for 48 hours when the explants are cultured in medium containing no insulin. Insulin stimulates DNA polymerase activity and the rate of DNA synthesis, the effects being of similar magnitude and having a similar time course. The greatest augmentations consistently occurred during the culture period between the 24th and 48th hours. In three such experiments parallel increases ranging from 5- to 20-fold have been observed.

Mammary gland tissue is comprised primarily of three cell types, namely, fat cells, fibroblasts, and epithelial cells. It was previously reported[3] that the fat cells do not synthesize DNA. In order to determine whether the initial DNA-polymerase activity and DNA synthesis observed in uncultured mammary gland tissue (cf.

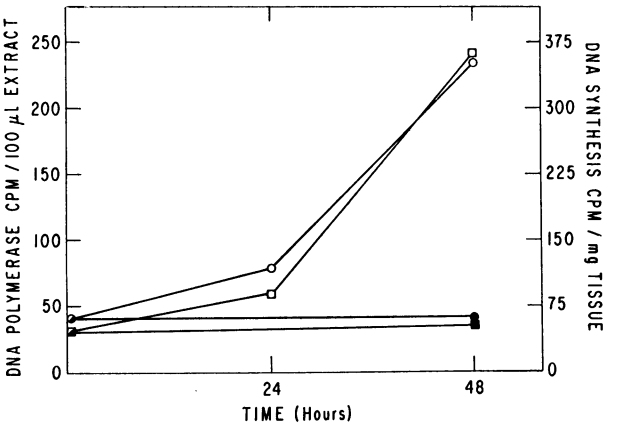

FIG. 1.—Time course of DNA synthesis within explants, and of DNA polymerase activity. Explants were cultured in insulin medium except where otherwise noted. In studies on DNA synthesis explants were exposed to tritiated thymidine (cf. *Materials and Methods*) for 4 hr prior to precipitation of DNA. Determinations of the two parameters were performed on explants derived from the same group of virgin animals, cultured in parallel. ○, DNA polymerase activities in extracts of explants cultured in insulin medium; ●, DNA polymerase activities in extracts of explants cultured in the absence of insulin; □, rates of DNA synthesis in explants cultured in the presence of insulin; ■, rates of DNA synthesis in explants cultured in the absence of insulin.

TABLE 2

COMPARISON OF DNA POLYMERASE ACTIVITY AND DNA SYNTHESIS IN DE-EPITHELIALIZED GLANDS AND CONTRALATERAL CONTROL GLANDS

System	DNA polymerase (cpm)	DNA synthesis (cpm/mg tissue)
Uncultured control tissue	22	20
Uncultured de-epithelialized tissue	20	20
Cultured control tissue	250	320
Cultured de-epithelialized tissue	45	81

The surgical procedure used for removal of epithelial cells is described in *Materials and Methods*. The methods employed were the same as those recorded in Fig. 1.

Fig. 1) were related to epithelial cells, de-epithelialized glands were compared with contralateral control glands (cf. *Materials and Methods*). Table 2 gives the results of such experiments. The initial values for the DNA polymerase and DNA synthesis are the same in de-epithelialized tissues as in the control tissue. However, the effect of culture in insulin medium is much greater on the control glands than on the operated glands. These results suggest that the initial values for both parameters relate to fibroblasts, and that DNA synthesis and DNA polymerase activity in the epithelial cells of mammary gland of mature virgin animals approach zero. The augmentations of both parameters which are effected by insulin appear, then, to approach *de novo* appearance of these activities in the epithelial cells. This suggestion is supported by autoradiographic evidence. Exposure of mammary explants to thymidine-H^3 (0.5 μc/ml) for four hours during the initial culture period, and after 44 hours of culture in the absence and presence of insulin, resulted in labeling of only about 1 per cent of the epithelial cells in the first two instances, and labeling of 20–25 per cent of these cells in the third instance. Moreover, 24 hours after the intraperitoneal injection of thymidine-H^3 (1 μc/gm body weight) into three-month-old virgin mice, only 1.5 per cent of the mammary epithelial cells had synthesized isotopic DNA. The small stimulations observed with the operated tissue are probably a consequence of the few epithelial cells remaining in this tissue after surgery (cf. *Materials and Methods*).

In order to determine whether the initial very low level of DNA polymerase activity in extracts of uncultured tissue is due to the presence of inhibitors or hydrolases capable of destroying the substrates or DNA, mixing experiments were performed. Activities in extracts of uncultured tissue, cultured tissue, and in mixtures of the two extracts were determined. It was found that the DNA polymerase activity in the mixture corresponded to the sum of the individual activities. This indicates that the very low activity in the extract of uncultured tissue is not due to the presence of inhibitors or hydrolases. The results also indicate that the enhanced activity in cultured tissue extracts is not due to the presence of activators which function under these conditions.

Some additional observations relating to the DNA polymerase activity present in uncultured tissue were made. When homogenization was performed in the presence of all four deoxynucleoside triphosphates (300 mμM each of dCTP, dGTP, and TTP per ml of homogenizing medium; 150 mμM dATP), no enhancement of the enzyme activity was noted. This result demonstrates that the higher enzyme activity observed in extracts of cultured tissue is not due to stabilization of the enzyme, during tissue homogenization, by the potentially higher substrate concentrations which may be present in such tissue. Insulin added to the extract (5 μg/ml) does

TABLE 3

EFFECT OF INSULIN ON DNA POLYMERASE ACTIVITY OF EXPLANTS PREVIOUSLY CULTURED IN THE ABSENCE OF INSULIN

Culture conditions	DNA polymerase activity (cpm/100 μl extract)
4 Hr without insulin	8
48 Hr without insulin	8
96 Hr without insulin	15
48 Hr without insulin and then 48 hr with insulin	250
96 Hr with insulin	265

DNA polymerase was assayed as described in *Materials and Methods*. In each case D-fructose had been substituted for D-glucose in the medium.

not activate the enzyme. Of the total enzyme activity present in the 600 × *g* pellet and the high speed supernatant almost 80 per cent resides in the latter when homogenization is carried out as described in *Materials and Methods*. This implies that the greater activity observed in extracts of insulin-cultured explants is not a consequence of augmented release of enzyme from nuclei into supernatant during homogenization.

It was desirable to determine whether insulin promoted the augmentation of DNA polymerase (cf. Fig. 1) by simply permitting the explants to survive during 48 hours of culture, or whether insulin elicited this effect in a more specific way. Accordingly, explants were cultured in the absence of insulin for 48 hours and were then transferred to an insulin medium for an additional 48 hours. The results of DNA polymerase determinations on such explants are shown in Table 3. It is clear that the augmentation induced by insulin is not merely a consequence of increased explant survival promoted by the hormone. In fact, the extract derived from explants which had been cultured for the first 48 hours in the absence of insulin and for the second 48 hours in the presence of insulin had essentially the same activity as that obtained from explants cultured for 96 hours with insulin. The results also demonstrate that the effect of insulin on DNA polymerase is not dependent on insulin-stimulated glucose transport; this is consistent with a previous observation[9] to the effect that stimulation of DNA synthesis by insulin is also independent of glucose.

It was previously reported that androgens inhibit the differentiation of mammary gland explants by reducing the number of epithelial cells which syntheisze DNA.[6] The experiments recorded in Table 4 were performed in order to determine whether testosterone exerted a corresponding effect on the activity of DNA-polymerase. It is apparent that the magnitude of inhibition of the enzyme is essentially the same as the diminution of DNA synthesis.

Figure 1 showed that during the first 48 hours of culture, insulin effects a parallel rise in the rate of DNA synthesis and DNA polymerase activity. It was previously reported[5] that DNA synthesis by mammary gland explants from virgin mice drops

TABLE 4

EFFECT OF TESTOSTERONE ON DNA POLYMERASE

	I medium	I and T medium	Inhibition by T (%)
DNA synthesis (cpm/mg tissue)	325	195	40
DNA polymerase (cpm)	675	415	38

Explants were cultured for 48 hr in the presence of insulin, or in the presence of insulin and testosterone. Determination of DNA synthesis and DNA polymerase activities were then performed (cf. *Materials and Methods*). I = insulin; T = testosterone.

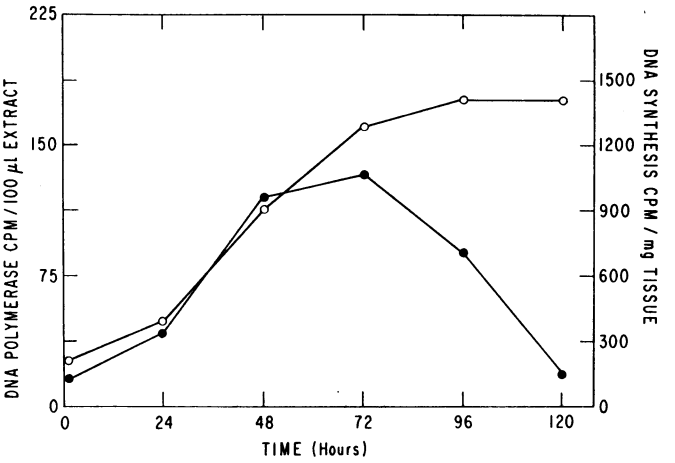

FIG. 2.—Extended insulin culture time study of DNA synthesis and DNA polymerase. Refer to legend of Fig. 1 for details. ○, DNA polymerase; ●, rates of DNA synthesis.

precipitously after five days in culture. It was of interest to determine whether DNA polymerase activity paralleled this fall in DNA synthesis. The results are shown in Figure 2. Parallelism during the initial phases of culture is again observed, but in the later phases DNA polymerase activity is maintained at an elevated level while DNA synthesis markedly diminishes.

Discussion.—Differentiation of mouse mammary gland requires three hormones: insulin, a glucorticoid,[9] and prolactin. Overt differentiation is elicited by prolactin only after proliferation has occurred in the presence of insulin and hydrocortisone.[10] The requirement for proliferation reflects the fact that the *in vitro* action of hydrocortisone must occur before mitosis of the mammary epithelial cells is completed. In terms of hormonal requirements, insulin is necessary and sufficient for the initiation of DNA synthesis by mammary epithelial cells present in explants of tissue derived from midpregnancy and postpubertal mice.[5] The DNA synthesizing system includes multiple components, namely, the four deoxynucleoside triphosphates, DNA template, and DNA polymerase. This report is concerned only with the effect of insulin on DNA polymerase; the other components are added in excess to the assay system.

The results presented suggest that not only is the rate of DNA synthesis by epithelial cells in explants of mammary glands derived from postpubertal mice limited by the activity of DNA polymerase, but that the level of this activity approaches zero in fresh tissue. Results shown indicate that during 48 hours of culture in insulin medium (Fig. 1) and in insulin-testosterone medium (Table 4) the rate of DNA synthesis is closely paralleled by activity of DNA polymerase. Augmentations of DNA polymerase and DNA synthesis have been observed in liver after the administration of insulin to severely diabetic rats.[11] Experiments with de-epithelialized tissues (Table 2) suggest that the enzyme activity observed in tissue prior to culture resides almost exclusively in fibroblasts, i.e., that the activity present in the epithelial cells of such tissue approaches zero. This implies that the large augmentation consequent to the action of insulin may represent *de novo* appearance of

enzyme activity in the epithelial cells. The virtual absence of isotope incorporation when dGTP, dCTP, and TTP are absent from the assay system (Table 1) indicates that the enzyme is the replicative rather than the terminal addition type.[12]

These observations do not, of course, exclude the possibility that insulin may also augment the activities of other enzymes related to the DNA synthesizing system. A cluster of other enzyme activities, such as those involved in the synthesis of the deoxynucleoside triphosphates, may concomitantly make their appearance under the influence of the hormone.

When puromycin, 10^{-4} M, or actinomycin D, 5 μg/ml, are present in the insulin medium during the second day of culture, the activity of DNA polymerase is equivalent to that usually found after only one day of culture. This suggests that the hormone effect may relate to *de novo* synthesis of enzyme molecules rather than to activation of pre-existing inactive molecules.

The parallelism which exists during the first two days of culture between the rate of DNA synthesis and DNA polymerase activity is not manifest during three additional days of culture (Fig. 2). Diminution of DNA synthesis has been shown to precede diminution of DNA polymerase in salivary gland after adminsitration of isoproterenol[13] and in regenerating liver.[14] The implication of this report is that whereas insulin initiates mammary epithelial cell DNA synthesis in part, at least, by eliciting DNA polymerase activity, the lack of some other factor precludes synthesis after 120 hours of culture.

Summary.—*In vitro* differentiation of mouse mammary gland requires insulin, hydrocortisone, and prolactin. Cell division is a prerequisite for this differentiation. Insulin initiates DNA synthesis in the epithelial cells of mammary explants derived from three-month-old virgin mice. This effect is elicited, at least in part, by the insulin-dependent emergence of DNA polymerase activity in these cells.

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Abbreviations: TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetate; d-, deoxy form of compound; CTP, GTP, TTP, and ATP, 5′-triphosphates of cytidine, guanosine, thymidine, and adenosine, respectively.

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