Effects of Estrogen and Antiestrogen on DNA Polymerase in Human Breast Cancer

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ABSTRACT

We have investigated the effects of estrogens and antiestrogens on cellular DNA-dependent DNA polymerase activity in human breast cancer, using as a model the MCF-7 human breast cancer cell line which contains estrogen receptor. 17β-Estradiol had little if any effect on cytosol DNA polymerase activity or growth (total DNA per flask) of MCF-7 cells. Incubation of the cells for 4 to 6 days with the antiestrogen nafoxidine, however, resulted in a dose-dependent reduction in cytosol DNA polymerase activity to one-half that observed in untreated cells. Enzyme activity in antiestrogen-treated cells was restored to levels contained in untreated cells by removing antiestrogen from the growth medium and incubating the cells for an additional 4 days with 17β-estradiol. The restoration required estrogenic steroids specifically, and the time course, magnitude, and dose dependence of the response were similar to estrogen-stimulated increases in DNA polymerase activity described in other estrogen target tissues. Estrogen-mediated reversal of antiestrogen suppression of DNA polymerase activity was paralleled by increases in total DNA synthesis.

INTRODUCTION

In many eukaryotic systems, rapid cell proliferation is accompanied by increases in DNA-dependent DNA polymerase activity. At least 4 molecular species of DNA polymerase have been characterized in eukaryotic cells. DNA polymerase α and β are the principal molecular forms. The α enzyme is a high-molecular-weight protein found predominantly in the cytosol fraction, and the lower-molecular-weight β polymerase is located in the nuclear fraction (13, 23, 28, 34). Mitochondrial DNA polymerase and DNA polymerase γ, which are found both in cytoplasm and nucleus (6, 36), constitute a minor portion of the total cellular DNA polymerase activity.

The exact physiological role for each enzyme species in DNA synthesis is not certain. In rapidly proliferating cells (2, 25), including some human tumors (11), DNA polymerase α in general accounts for the majority of total cellular activity, whereas quiescent cells may contain predominantly the β enzyme (19). Cellular activity of DNA polymerase α has been correlated with the proliferative state in a number of eukaryotic systems (2, 3, 5, 9, 10, 18, 19, 25, 29, 32), including some steroid hormone target tissues such as the estrogen-stimulated uterus (14, 33), oviduct (27, 31), Leydig cells (30), and androgen-stimulated ventral prostate (26). Because DNA polymerase β does not fluctuate appreciably in these systems, the α en-

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(β-aminoethyl ether)N,N'-tetraacetic acid: 2 mM dithiothreitol buffer, and the supernatant was added to the crude cytosol. This was centrifuged at 105,000 × g in a Beckman fixed-angle rotor, and the supernatant (cytosol) was either used immediately or frozen at −70° until use. The crude nuclear pellet was resuspended in 1.0 ml of 10 mM Tris-HCl (pH 8.0) 2 mM dithiothreitol buffer at 4°, containing 0.4 M KCl and was incubated for 1 hr at 4°, during which time the pellet was intermittently agitated by vortexing. Extracted nuclear proteins were then separated from other nuclear material by centrifugation at 105,000 × g. The supernatant was either used immediately or frozen at −70° until use. Protein content in cytosol and nuclear extracts was measured by the method of Lowry et al. (22), and frozen at −70° until use. The crude nuclear pellet was centrifuged for no longer than 1 week before use.

Assay of DNA Polymerase Activity. DNA polymerase activity was measured as incorporation of [3H]TTP into an acid-insoluble product, using activated calf thymus DNA as the template primer (1, 4). Assays were carried out in a 300-μl volume containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, 2.5 mM dithiothreitol, activated calf thymus DNA (165 μg/ml or approximately 33 μg/assay), 0.1 mM dATP, dCTP, and dGTP each, and 15 μM [3H]TTP diluted to a specific activity of 1900 to 2000 cpm/pmol with unlabeled dTTP. The reaction was initiated by addition of 100 μl of either cytosol or crude nuclear extract (1 to 2 mg protein per ml). Assays of nuclear extracts (taken as a measure of DNA polymerase β) contained 0.1 mM KCl in addition to the above and were adjusted to pH 8.8 rather than pH 7.4. Incubations were for 30 min at 30°. Reactions were stopped by addition of 2 ml of cold 10% TCA containing 1% sodium pyrophosphate to each tube. The acid-insoluble product was collected on Millipore filters (type HAMK with 0.45 μm pore size) under vacuum and washed with 20 ml of cold 5% TCA containing 1% sodium pyrophosphate. Filters were then placed on the bottom of scintillation vials with 0.5 ml of 0.5 N HCl and heated in a water bath for 15 min at 100°. The hydrolysate was cooled, neutralized with 0.45 ml of 0.2 N NaOH, and counted in 10.0 ml of toluene-based liquid scintillation fluid (4.0 g of PPO, 0.05 g of POPOP, and 1 liter of toluene) containing 15% BBS-3 (Biosolv aqueous solubilizer, Beckman Instruments Inc.), in a Beckman LS-233 counter with a counting efficiency of 38% for tritium. Assays were in triplicate, and background incorporation was determined by adding cold TCA to assay tubes immediately after addition of enzyme samples. Background incorporation did not exceed 5% of the incorporation obtained by 30 min incubation at 30°, and has been subtracted. All values for enzyme activity are expressed as cpm of [3H]TTP incorporated in 30 min per mg of DNA.

Under these conditions, the reaction was linear for at least 60 min, and incorporation was a linear function of cytosol protein concentration (0.3 to 4.5 mg of protein per ml). Freezing the cytosol at −70° resulted in minimal loss of DNA polymerase activity (less than 10%); samples were routinely stored at −70° for no longer than 1 week before use.

To confirm that the product of in vitro incubation was thymidine incorporation into DNA (20), incorporation was found to require the presence of all 4 deoxyribonucleotide triphosphates, was absolutely dependent on magnesium, and was prevented by simultaneous digestion with deoxyribonuclease I but not by the action of ribonuclease. The insoluble product was stable to alkali and hydrolyzed at 100° by 0.5 N HCl.

RESULTS

MCF-7 DNA Polymerase. The cytosol of MCF-7 cells contains the majority of cellular DNA polymerase activity. Approximately 95% of total enzyme activity was contained in the cytosol, and the remainder was present in nuclei. Addition of 10 mM of the sulfhydryl reagent N-ethyl-maleimide to the assay inhibited approximately 91% of the enzyme activity in cytosols compared with controls and about 40% of the activity extracted from crude nuclei. On 5 to 20% sucrose density gradients prepared in 0.01 M Tris-HCl (pH 7.8), 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.45 mM NaCl, cytosol enzyme activity sedimented as a heterogeneous peak at about 6 to 8 S. The nuclear enzyme sedimented as a single peak in the 4S region of the gradient (data not shown). Additionally, cytosol enzyme displayed optimal activity at 5 mM MgCl₂. These properties are similar to those described for DNA polymerases in other tissues (23, 28, 34, 36) and are compatible with the cytosol containing predominantly DNA polymerase α and the crude nuclear fraction DNA polymerase β. Since DNA polymerase α activity has been correlated with the proliferative state in a number of other tissues, and because the major portion of enzyme activity is contained in the cytosol, we have measured only cytosol DNA polymerase in this study.

Effects of Estradiol and Nafodixone on Cell Growth and DNA Polymerase Activity. Cytosol DNA polymerase activity and cell growth (total DNA content/flask) were measured for 8 days in untreated cells and in cells treated continuously with either the antiestrogen nafodixone (1 μM) or a physiological concentration of 17β-estradiol (0.1 nM) (Chart 1). At the dose used, 17β-estradiol had no effect on either the growth of the cells (Chart 1, inset) or DNA polymerase activity relative to

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† The abbreviation used is: TCA, trichloracetic acid.
controls, whereas both were markedly reduced by nafoxidine. To define further the role of 17ß-estradiol in regulation of growth and DNA polymerase activity, cells were grown for either 4 or 8 days with medium containing a wide range of 17ß-estradiol concentrations (0.0001 to 1000 nM). Repeated experiments failed to show a significant increase in either growth or DNA polymerase activity in response to any 17ß-estradiol dose tested.

Despite the failure to observe an estradiol response, the antiestrogen nafoxidine is undoubtedly effective in reducing cytosol DNA polymerase activity and inhibiting cell growth. Maximal inhibition of DNA polymerase activity in repeated experiments occurs anywhere between 4 and 6 days of incubation with nafoxidine. Chart 2 shows the effect of increasing concentrations of nafoxidine on cytosol enzyme activity after 6 days. Although not shown, decreases in DNA polymerase activity in response to increasing doses of nafoxidine were paralleled by decreases in total DNA content per flask. Because enzyme activity is expressed in units per mg of DNA, the effect of nafoxidine is not a result of there being fewer cells; rather nafoxidine has caused a reduction in enzyme activity per cell.

Reversal of Antiestrogen Inhibition of DNA Polymerase Activity By 17ß-Estradiol. Antiestrogen suppression of cytosol DNA polymerase activity was reversed by 17ß-estradiol in a dose-dependent manner (Chart 3). Nafoxidine appears to be more effective than tamoxifen in suppressing cytosol DNA polymerase activity. Growth and enzyme inhibition caused by tamoxifen was gradually reversed by 17ß-estradiol doses between 0.01 nM and 100 nM, whereas slightly more 17ß-estradiol was required to begin reversal in nafoxidine-inhibited cells. The difference in sensitivity of DNA polymerase to nafoxidine compared to tamoxifen is probably due to their differing affinities for the estrogen receptor. Nafoxidine has a 10-fold lower affinity for the MCF-7 estrogen receptor than 17ß-estradiol, whereas tamoxifen has a 20-fold lower affinity (17). Reversal of antiestrogen inhibition of DNA polymerase activity by 17ß-estradiol was coincident with restimulation of cell growth (DNA/flask).

Hormone specificity of the reversal of antiestrogen suppression of DNA polymerase was determined by incubating nafoxidine-pretreated cells for 4 days with various hormones. Less biologically active estrogens such as estriol, 17a-estradiol at 1.0 μM, and estrone at 0.1 μM, were as effective, although at higher doses, as 17ß-estradiol in reversing the nafoxidine suppression of cytosol DNA polymerase activity. Testosterone, progesterone, cortisol, and insulin at 1.0 μM concentrations had no effect on DNA polymerase activity. The rate of 17ß-estradiol reversal of antiestrogen inhibition of DNA polymerase activity is shown in Chart 4. No significant increase in either growth or DNA polymerase activity was observed for the first 24 hr. A doubling in enzyme activity occurred by Day 2, and a 4-fold maximal stimulation (over zero...
time) was observed after 4 days of exposure to 17ß-estradiol. This increase in enzyme activity was paralleled by increases in total DNA/flask. Enzyme activity in cells maintained on nafoxidine for the entire incubation period were unchanged.

**DISCUSSION**

Although MCF-7 cells contain estrogen receptor, we have not observed significant stimulation of either cell growth or cytosol DNA polymerase activity. Antiestrogens, however, inhibit the growth of the cells, which is paralleled by a decrease in levels of cytosol DNA polymerase activity. Estrogens are able to reverse the antiestrogen suppression of growth and DNA polymerase. The time course, magnitude, dose dependency, and hormone specificity of the estrogen response in antiestrogen-inhibited cells is similar to estrogen-stimulated changes in cell proliferaton and DNA polymerase activity described in other estrogen target tissues (14, 27, 31, 33). The antiestrogens, tamoxifen and nafoxidine, bind to MCF-7 cytosol estrogen receptors and promote translocation of the receptor to the nucleus (17). Since estrogenic steroids are specifically required for the reversal of antiestrogen inhibition, fluctuations in DNA polymerase activity appear to be estrogen receptor-mediated. The apparent insensitivity of the cells to estrogens alone may be explained by 2 factors. (a) The cells may be replicating at a maximal rate due to the presence of serum growth factors in the medium or endogenous estrogen which has not been removed by charcoal stripping of the serum. In either case, the responsiveness of the cells to added estrogen may be masked by the stimulatory effects of these endogenous factors. By an estradiol exchange assay, however, we are not able to detect significant amounts of nuclear estrogen receptor bound with estradiol in cells grown on 5% charcoal-stripped serum (17, 38), and 17ß-estradiol failed to affect cell growth or cytosol DNA polymerase activity when lower amounts of charcoal-stripped serum (0.2 to 1.0%) were used in incubations to reduce the concentrations of putative serum growth factors (data not shown). (b) Alternatively transcribed sequences directed toward the synthesis of estrogen-regulated proteins, which are associated with DNA replication, may be copied at maximal rates in the absence of any estrogen. If this is true, the nafoxidine-receptor complex may sensitize the cells to estrogen by blocking transcription of these sequences. Estrogen receptors apparently do have the potential for regulating growth of MCF-7 cells, since inhibition and subsequent reversal of growth and DNA polymerase activity are highly specific events associated with the estrogen receptor. We do not know, however, whether antiestrogens specifically are required or whether any agent that slows cell growth will sensitize the cells to estrogen. Nor do we know whether estrogen action in breast cancer requires the permissive effect of other hormones or unknown factor(s) which may not be present in the growth medium.

We have seen that growth and at least one enzyme (DNA polymerase a) associated with DNA replication in MCF-7 cells are unaffected by estradiol in spite of the presence of estrogen receptor. However, the synthesis of certain MCF-7 proteins, including progesterone receptor (16) and lactate dehydrogenase (7), do appear to be regulated by estradiol. It is possible therefore, that estrogen control of cell replication and of the synthesis of some specialized protein(s) may involve separate mechanisms. There are studies in animal models to indicate this may be true in some cases. We found that growth of at least 2 rat dimethylbenz(a)anthracene-induced tumors was autonomous, while progesterone receptor remained estrogen-dependent (15), and progesterone receptor has been shown to be regulated by estrogens in the estrogen-independent MTW-9B transplantable mammary tumor (24).

Significant stimulation of MCF-7 cell growth by physiological doses of 17ß-estradiol has been observed by other investigators (21, 35). In the present study as well as in previous studies, however, we have not been able to stimulate reproducibly MCF-7 cell growth with estrogens (37). We have now examined, in addition to cell growth estrogen effects on DNA polymerase activity, an enzyme described in numerous other growing tissues to be associated with replicative DNA synthesis. Due to adaptation processes to tissue culture under slightly different conditions, growth of our MCF-7 cells may have lost its sensitivity to estrogens, yet the cells may have retained sensitivity to estrogen stimulation of progesterone receptor. Considering that about 40% of estrogen receptor-positive breast cancer patients fail to respond to endocrine therapies designed to reduce circulating estrogens (12), it is not surprising to find that growth of MCF-7 cells, which are derived from human breast cancer, may be insensitive to estrogens. It may be important, in fact, to our understanding of these classifications of estrogen receptor-positive breast tumors to study the mechanisms by which growth of MCF-7 cells has acquired resistance to estrogens.

**REFERENCES**


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