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Application of an In Vitro System in the Study of Chemotherapeutic Drug Effects on DNA Replication

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Abstract DNA replication machinery is an important target for chemotherapeutic drugs. We have used an in vitro system to study the effect of drugs on mammalian DNA replication, either by direct interaction with the DNA structure or with replication proteins and machinery. The anthracycline doxorubicin (Dox) showed a dose-dependent inhibitory effect on DNA replication, whether incubated with HeLa cell extracts or with DNA and nucleotides. Earliest-labeled fragment analysis revealed that inhibition of replication began within the origin-containing fragment in both control and Dox-containing reactions in vitro. AraC, a nucleoside analog, had no significant effect on DNA synthesis. In contrast, araCTP was able to inhibit DNA replication in vitro. Since metabolism is diminished in this in vitro system, the degree of phosphorylation of araC was apparently low. Progesterone showed an increase in nucleotide incorporation (sensitive to BuPdGTP inhibition of replication-specific polymerases α and δ) after preincubation with HeLa cell extracts, although progesterone receptors were not detectable in the HeLa cell extracts. In addition, we observed an inhibition in DNA replication when progesterone was preincubated with DNA and nucleotides. These results suggest that progesterone may have a mechanism of action that is different from any known to be mediated through progesterone receptors. In conclusion, these results indicate that this mammalian in vitro replication system will be useful for the study of mechanisms and design of therapeutic drugs that inhibit mammalian DNA replication.

Key words: in vitro DNA replication, mammalian, doxorubicin, araC, progesterone

DNA replication is one of the most fundamental biological processes in living cells. Alterations in DNA replication may result in uncontrolled cell proliferation which can contribute to cancer and other abnormal growth disorders, or block propagation and maintenance of cells. There is an intense search for new antitumor agents with more favorable therapeutical profiles; among these is an important group of drugs that target the machinery of DNA replication. However, in the evaluation of drug efficacy of new compounds, there are many biological factors, such as transport of the drug into the cell and cell nucleus, which make it difficult to determine the efficacy of these drugs as related to structure. In order to overcome these problems, investigators have used in vitro systems [Bachur et al., 1992; Ciarroechi et al., 1991; Frosina and Rossi, 1992; Bigioni et al., 1994]. There are several in vitro cell-free systems which seem to mimic closely eukaryotic nuclear DNA replication in vivo, where the efficacy of drugs can be studied in the absence of confounding factors [Li and Kelly, 1984; Pearson et al., 1991; Berberich et al., 1995]. One of the current models of eukaryotic DNA replication is based on SV40 [Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985], and although it is very well characterized, it uses a viral origin of replication requiring a specific viral initiator protein, the SV-40 large T-antigen (T-Ag). Specific protein–protein interactions, DNA–protein interactions, and drug interactions with complexes essential for DNA replication in the SV40 in vitro system only mimic certain eukaryotic DNA replication traits, which may not be entirely appropriate models for the evaluation of the sensitivity of mammalian cellular DNA replication systems to drugs. We and others have used an in vitro DNA replication system which uses a mammalian origin of replication and extracts of human cells, HeLa [Pearson et al., 1991, 1994] and 293S [Berberich et al., 1995].

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Plasmids containing monkey origin–enriched sequences (*ors*) which replicate in extracts from HeLa cells were used by Pearson et al. Berberich et al. reported that a plasmid containing the c-*myc* insert can be recognized by the mammalian replication machinery from 293S cells to initiate semiconservative DNA synthesis. Since proteins involved in DNA replication and the DNA molecule itself are potential targets for chemotherapy of cancer, we have designed the in vitro assay protocol to better discriminate between the effect of drugs on DNA and the effect on the replication proteins that are present in the HeLa cell extracts, as well as to assess the overall efficacy of drugs on DNA replication. In this initial study we have investigated compounds belonging to three different families. We have studied the effects of the anthracycline doxorubicin; the nucleoside analog araC; and the steroid progesterone. The results demonstrate that we can use this mammalian in vitro replication system to study the effect of drugs on DNA or on proteins involved in the process of DNA replication. In particular, we found that the steroid progesterone could have an effect or mechanism of action apparently unrelated to receptor–ligand nuclear interactions.

METHODS

Plasmids and Cell Extracts

Plasmid pX24 of Dr. J. Hamlin (University of Virginia) was provided by Dr. M.L. DePamphilis (Roche Institute of Molecular Biology). pX24 is able to replicate autonomously in a cell-free system that uses HeLa cell extracts [Zannis-Hadjopoulos et al., 1994]. Plasmid pX24 contains a 4.8 kb *Xba* I fragment of the DHFR origin of replication region, from the *Xba* I site at 14.0 kb to the *Xba* I site at 18.8 kb, inserted into the *Xba* I site of pUC13 [Burhans et al., 1986]. Plasmid 30.4 carries a randomly selected sequence of 0.7 kb inserted into the pBluescript vector, obtained from human breast tumor DNA, and without autonomous replication activity.

HeLa S3 extracts (nuclear and cytoplasmic) were prepared as described previously by Pearson et al. [1991].

Replication Reactions

The cell-free replication assay was adapted from the method described previously by Pearson et al. [1991] with slight modifications. The system essentially consists of nuclear and cytoplasmic extracts from HeLa cells, a mixture of nucleotides (ATP, CTP, GTP, UTP, dATP, dGTP, dTTP and dCTP), 10 μCi of [α-^{32}P] dCTP and 10 μCi of [α-^{32}P] dTTP, an ATP regenerating system, and equimolar amounts of a plasmid containing either a specific mammalian origin of replication (pX24) or a random human breast tumor DNA sequence (30.4). The drugs have been tested using concentrations which include reference values within the pharmacological margin, i.e., IC_{50} in HeLa cells for doxorubicin (0.13 μM) and araC (4.5 μM), and C_{max} in serum for progesterone (200 nM). The IC_{50} for araC was taken as the reference value for araCTP. The experiments were performed by preincubation of the different drugs with either HeLa cell extracts or plasmid DNA and nucleotides at 30°C. After 15 min, the remaining components, plasmid DNA and nucleotides (preincubations with HeLa cell extracts) or HeLa cell extracts (preincubations with DNA and nucleotides), were added and the samples were incubated at 30°C for 1 hr. The reactions were terminated by the addition of a stop mix (30 mM EDTA, 1% SDS) and proteinase K, and the samples were incubated at 37°C for 1 hr. DNA was purified by standard procedures [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994], and the reaction products were divided in three portions. One of the three aliquots was digested with 1 U *Dpn* I (New England Biolabs) at 37°C for 1.5 hr. The undigested and *Dpn* I–digested products were resolved on a 1% agarose gel in 1× TAE (Tris-acetate, EDTA) buffer (16 hr, 2.5 V/cm). The gels were fixed, dried, and exposed to NEF-496 film (Dupont). Quantification was performed on *Dpn* I–digested products by densitometric measurements using a Phosphoimager analyzer (Fuji BAS 2000). Quantification involved the measurement of the density of bands corresponding to forms II and III of pX24 plasmid DNA, and normalized by subtracting the background and for the amount of DNA in ethidium bromide gels. The amount of [α-^{32}P] dCTP and [α-^{32}P] dTTP incorporated into DNA was expressed as a percent of a control pX24 containing no drug. The total incorporation into DNA was 0.033 pmols for pX24 and 0.009 pmols for the control plasmid 30.4; *Dpn* I–resistant incorporation was approximately one-third of the total incorporation for pX24; plasmid 30.4 did not show *Dpn* I resistance.

Stock solutions of drugs were: 0.1 M araC (SIGMA) in water, 1 mM araCTP (SIGMA) in water, 3.5 mM doxorubicin (Adria laboratories)

in DMSO, and 1 mM progesterone (SIGMA) in ethanol. Dilutions were made in water to the desired concentration. The final concentrations of DMSO and ethanol never exceeded 0.1%. Solutions were stored at −20°C.

Earliest-Labeled DNA Fragment

In vitro DNA replication of pX24 in the absence or presence of 0.33 μM concentration of doxorubicin was performed, and the reactions were stopped at 4, 8, or 12 min of incubation. The DNA products were digested overnight with 1 U of each of the restriction enzymes, *BamH* I, *Bgl* I, and *Xba* I. The digestion products were electrophoresed on a 1.5% agarose gel in 1× TAE buffer (16 hr, 2.5 V/cm). Incorporation of [α-^{32}P] dCTP and [α-^{32}P] dTTP into each fragment was quantitated by densitometry of a phosphoimager screen using the Fuji BAS 2000 analyzer, and expressed as incorporation/kb of DNA.

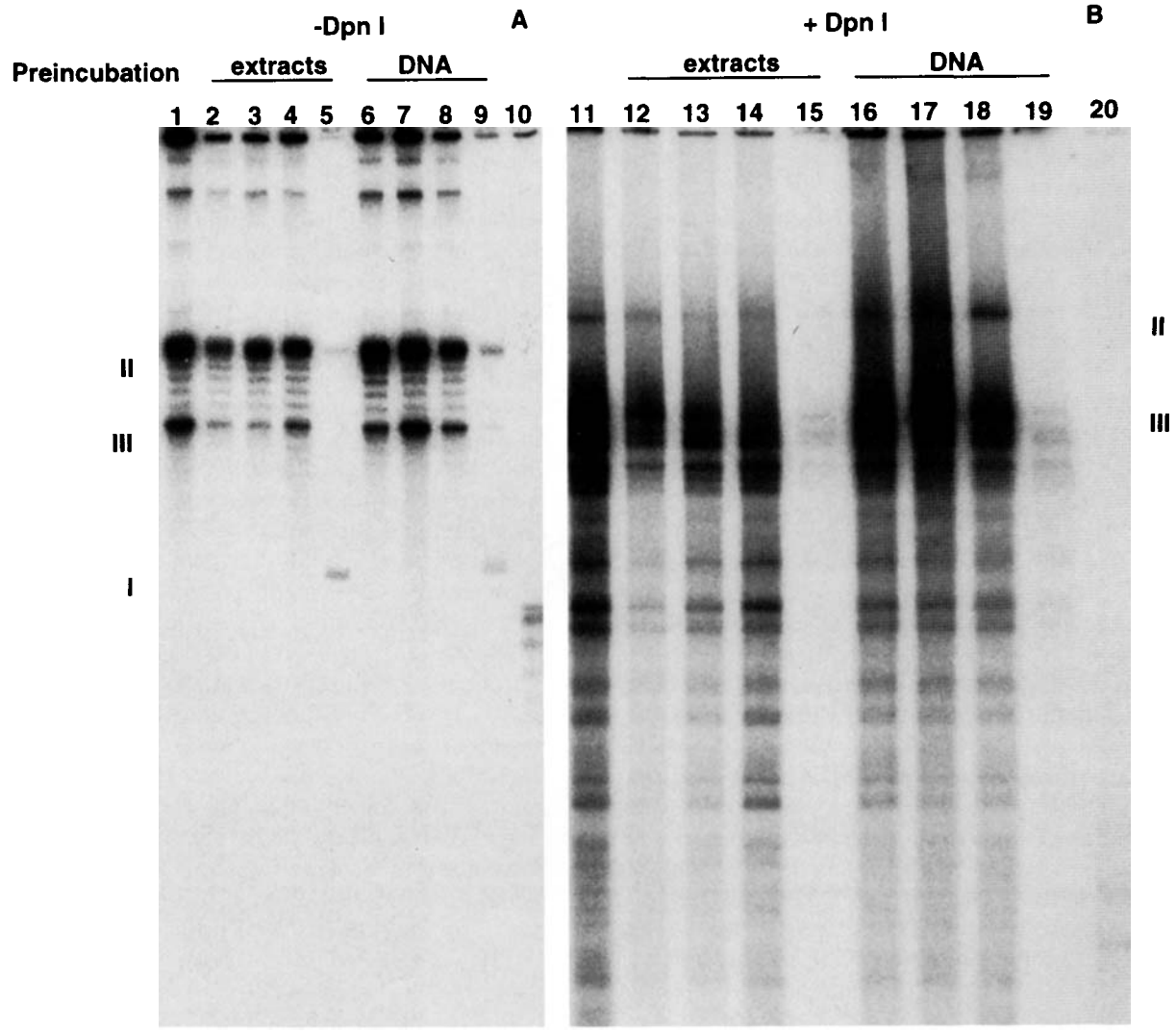

Fig. 1. pX24 replication in vitro in the presence of increasing concentrations of doxorubicin. Reactions (50 μl) contained 105 ng of pX24. Different drug concentrations were preincubated either with HeLa cell extracts or with DNA and nucleotides. Reaction mixtures were completed as described in the Methods. The products were divided in three aliquots; two of them (one untreated, **A**, and the other digested with 1 U *Dpn* I at 37°C for 1.5 hr, **B**) were electrophoresed on 1% agarose and analyzed by a phosphoimager analyzer. Electrophoresis of replication products without *Dpn* I digestion (A) and with *Dpn* I digestion (B) was performed in the same manner, excepting the comb size and the running time. *Lanes 1, 11:* positive control, no drug; *lanes 2, 6, 12, 16:* doxorubicin (0.13 μM); *lanes 3, 7, 13, 17:* doxorubicin (0.33 μM); *lanes 4, 8, 14, 18:* doxorubicin (1.3 μM); *lanes 5, 9, 15, 19:* doxorubicin (13 μM); *lanes 10, 20:* negative control (30.4). The mobilities of supercoiled (form I), relaxed (form II), and linear (form III) pX24 are indicated.

RESULTS AND DISCUSSION

The purpose of this study was to demonstrate the effect of different drugs, particularly chemotherapeutic drugs, on DNA replication. To address this, we chose a mammalian in vitro DNA replication system, dependent upon the presence of a mammalian origin of DNA replication, which is thought to closely mimic nuclear mammalian DNA replication [Pearson et al., 1991, 1994]. This system uses soluble extracts from cytoplasm and nuclei of HeLa cells, which provide the replication proteins to support replication of exogenous plasmid templates containing a mammalian origin of replication. The plasmid pX24 was chosen because it has been shown by several autonomous replication assay techniques, in vivo and in vitro, to contain a mammalian origin of replication from the DHFR locus [Zannis-Hadjopoulos et al., 1994]. Several mapping techniques, such as nascent DNA PCR mapping [Vassilev et al., 1990] and Okazaki fragment distribution mapping [Burhans et al., 1990], have also shown that pX24 contains a bidirectional origin of replication. In this initial study, we have investigated the effects of three compounds belonging to different families of drugs, i.e., having different mechanisms of action. Specifically, we describe results for β-cytosine arabinoside (araC), doxorubicin, and progesterone. The first two agents are thought to interact with the DNA replication machinery, such as DNA polymerase, topoisomerase II, DNA ligase, and helicase [Kuchta et al., 1992; Bodley et al., 1989; Ciarroacchi et al., 1991; Bachur et al., 1992], and the third one is thought to exert an indirect effect on DNA replication by binding to the progesterone receptor [Spelsberg and Toft,

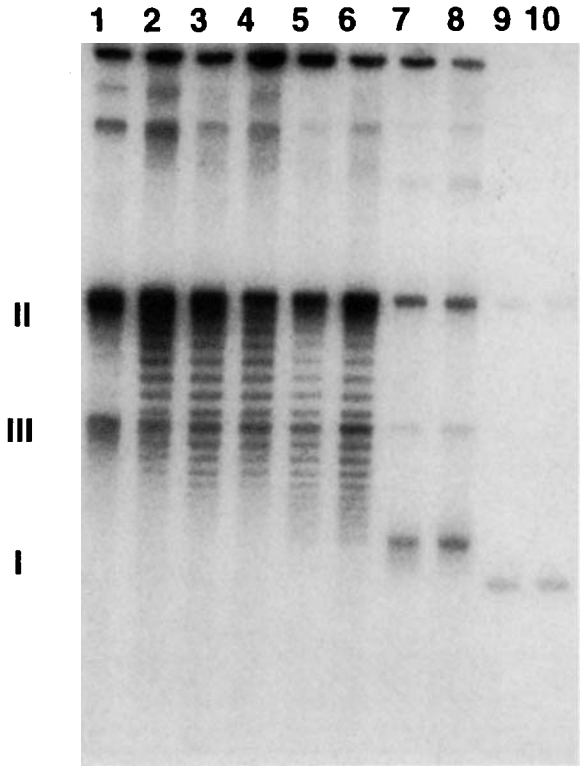

Fig. 2. Reproducibility of the in vitro DNA replication assay, and effect of doxorubicin on DNA structure and DNA replication. Replication was carried out as previously indicated in the Methods. This gel represents the undigested replication products, after template DNA and nucleotides were preincubated with different concentrations of doxorubicin. The samples are in duplicate. *Lanes 1, 2:* positive control, no drug; *lanes 3, 4:* doxorubicin (0.13 μM); *lanes 5, 6:* doxorubicin (0.33 μM); *lanes 7, 8:* doxorubicin (1.3 μM); *lanes 9, 10:* doxorubicin (13 μM). The positions of pX24 form I, II, and III DNA are indicated. The ladder of bands migrating between forms II, III, and I indicate the presence of a series of topoisomeric molecules with supercoils.

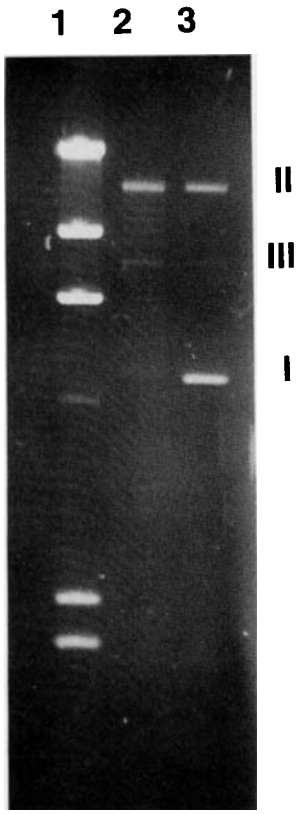

Fig. 3. Increase in the amount of supercoiled DNA in the presence of 13 μM doxorubicin. Electrophoretic mobility of DNA in the absence (*lane 2*) and presence (*lane 3*) of 13 μM doxorubicin in the ethidium bromide–stained gel. Marker in *lane 1*. The mobilities of form I, II, and III DNA are indicated.

1976]. The drugs have been tested using concentrations which include reference values within the pharmacological margin. Different drug concentrations were preincubated with either DNA or cell extracts, in an attempt to differentiate direct effects of drugs on the DNA molecule or on the proteins involved in the process of DNA synthesis.

Preincubation of doxorubicin with both DNA and cell extracts, which contain the proteins involved in DNA replication, resulted in a concentration-dependent inhibition of DNA synthesis (0.13–13 μM) (Fig. 1, and see Fig. 5A). However, at low concentrations of doxorubicin (0–0.33 μM), the inhibitory effect was higher when the drug was preincubated with the HeLa cell extracts (Fig. 1, lanes 2, 3, 12, 13) than with the DNA and nucleotides (Fig. 1, lanes 6, 7, 16, 17). The results obtained with this in vitro assay showed that the method is reproducible, and an example is shown in Figure 2. Moreover, we observed that the increase of doxorubicin concentration produced an alteration in topoisomeric forms of DNA, increasing the amount of form I (supercoiled) plasmid (Fig. 2, lanes 7–10). The maximal amount of form I DNA was reached with 1.3 μM doxorubicin (Fig. 2, lanes 7, 8) (100%); the relative amounts of supercoiled plasmid with respect to form I DNA were 9.2% (Fig. 2, lanes 1, 2), 11.6% (0.13 μM, lanes 3, 4), 28% (0.33 μM, lanes 5, 6), and 32.4% (13 μM, lanes 9, 10). There was similar electrophoretic mobility of DNA in the 13 μM doxorubicin sample for both the autoradiogram of the gel and in the ethidium bromide–stained gel (Fig. 3, lane 3). This effect is probably due to the intercalation of doxorubicin in the duplex of DNA, as has been reported previously [Bodley et al., 1989]. To determine whether replication of pX24 initiated within the DHFR fragment, pX24 was incubated in the in vitro replication system for 4, 8, or 12 min and subsequently digested with *BamH* I, *Bgl* I, and *Xba* I, and fractionated on a 1.5% agarose gel. The digestion yielded eight fragments ranging from 6 to 1,750 bp. The incorporation of [α-^{32}P] dCTP and [α-^{32}P] dTTP into each restriction fragment was quantitated by densitometry using a phosphoimager analyzer, and normalized as indicated in the Methods and for the size of the fragment. The results showed that the DHFR-containing fragment 1.6 kb has the highest incorporation/kb, and is the earliest-labeled fragment. Moreover, the same experiment in the presence of 0.33 μM doxorubicin showed that the inhibitory effect of the drug did not change the apparent initiation site on overall profile of incorporation (Fig. 4).

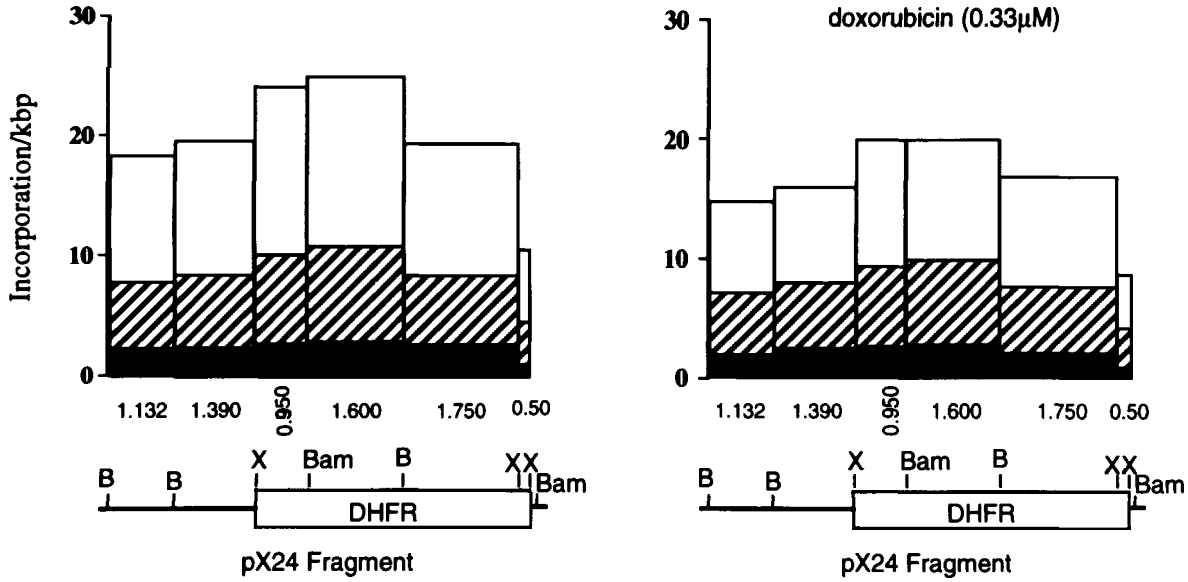

Fig. 4. Preferential labeling in the DHFR insert of pX24 at early times of in vitro replication. pX24 was assayed in an in vitro replication reaction in the absence (left) or presence (right) of 0.33 μM doxorubicin, and the reactions were stopped at 4 (black bars), 8 (striped bars), or 12 (white bars) min. After extraction and purification of DNA, the products were digested with *BamH* I (Bam), *Bgl* I (B), and *Xba* I (X) before analysis by agarose gel electrophoresis and quantitative densitometry. The results were expressed as incorporation per kb for each fragment. At the bottom of the graphic is shown the linearized map of pX24, where the box represents the DHFR insert.

The nucleoside analog araC showed only a slight inhibition of nucleotide incorporation ($\leq 20\%$, at concentrations up to 13 μM) when it was preincubated with DNA or cell extracts, which contain the proteins involved in the mechanism of DNA replication (Fig. 5B). Since araC needs to be phosphorylated to araCTP to interact with DNA or DNA replication proteins, the suboptimal metabolism afforded by these HeLa cell extracts probably failed to adequately duplicate the phosphorylation of araC that can be obtained in intact cells. This was confirmed by using araCTP, which showed a concentration-dependent inhibition of DNA synthesis after preincubation with DNA or HeLa cell extracts (Fig. 5C).

In addition, we observed new activities for the steroid progesterone. Cells are generally considered to be positive for progesterone receptor if they contain greater than 20 fmol/mg total protein [Leclercq et al., 1977]; however, measurements of receptor levels in the HeLa nuclear and cytoplasmic extracts that were used in the in vitro replication system did not reach this threshold (data not shown). The progesterone receptors were measured in the cytoplasmic and nuclear extracts by the standard dextran-coated charcoal adsorption method using frozen cell extract pellets (RIANEN [^{3}H] Progestin Receptor Assay Kit, Dupont, Billerica, MA) [King et al., 1979; Bloom et al., 1980]. Preincubation of progesterone with the HeLa cell extracts resulted in an enhancement of DNA synthesis (Fig. 5D). The maximal stimulation was reached at a concentration of 8 nM, and the same level of DNA replication was maintained up to 200 nM progesterone. At concentrations between 200 nM and 500 nM the stimulatory effect decreased toward no drug control levels. To determine whether this effect of progesterone was due to DNA replication or repair, the ability of progesterone to increase the incorporation of precursor nucleotides into DNA was tested in the presence of the DNA synthesis inhibitor butylphenyl

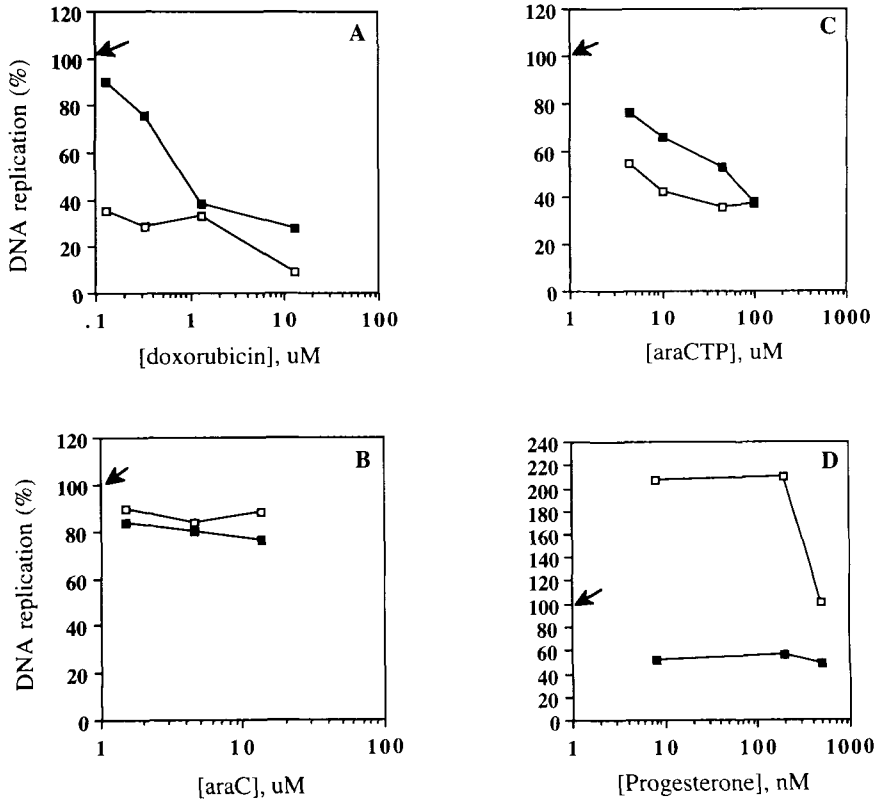

Fig. 5. Effect of different drugs on DNA replication. pX24 replication was assayed in HeLa cell extracts in the presence of increasing concentrations of doxorubicin (A), araC (B), araCTP (C), or progesterone (D). Incorporation of radioactive nucleotide precursor was determined for each sample by autoradiography and quantitative densitometry scanning. DNA replication (%) was plotted against the drug concentration. Each point represents the mean of duplicate samples, with a maximal variation of 15%. Open squares, preincubation with HeLa cell extracts; solid squares, preincubation with DNA and nucleotides. Arrows denote control values.

deoxyguanosine triphosphate (BuPdGTP) (Fig. 6). BuPdGTP inhibits polymerases α and δ [Byrnes, 1985], both of which participate in the process of nuclear DNA replication. Preincubation of HeLa cell extracts with two different concentrations of BuPdGTP (100 and 200 μM) in the presence and absence of 150 nM progesterone showed an inhibition of incorporation of approximately 65% with 100 μM, and 75% with 200 μM BuPdGTP (Fig. 6, lanes 3–6, 3′–6′). These data indicate that the enhancement of nucleotide incorporation observed in the presence of progesterone is due to DNA replication. The stimulatory effect of progesterone on in vitro DNA replication may be due to the interaction of progesterone with one or more proteins involved in the process of DNA replication, a mechanism likely different from any known to be mediated through progesterone receptors. Furthermore, preincubation of progesterone with DNA and nucleotides indicated an inhibition of DNA synthesis (Fig. 5D), which was maximal at 8 nM concentration, and was maintained between 8 nM and 500 nM progesterone. The observed effects of progesterone are specific since prednisolone (C_{max} approximately 2.8 μM) [Hill et al., 1990], a structural analog of progesterone, showed no effects in this in vitro DNA replication system (data not shown). These studies indicate that this in vitro DNA replication assay will be useful for determining the multiple effects of compounds on mammalian DNA repli-

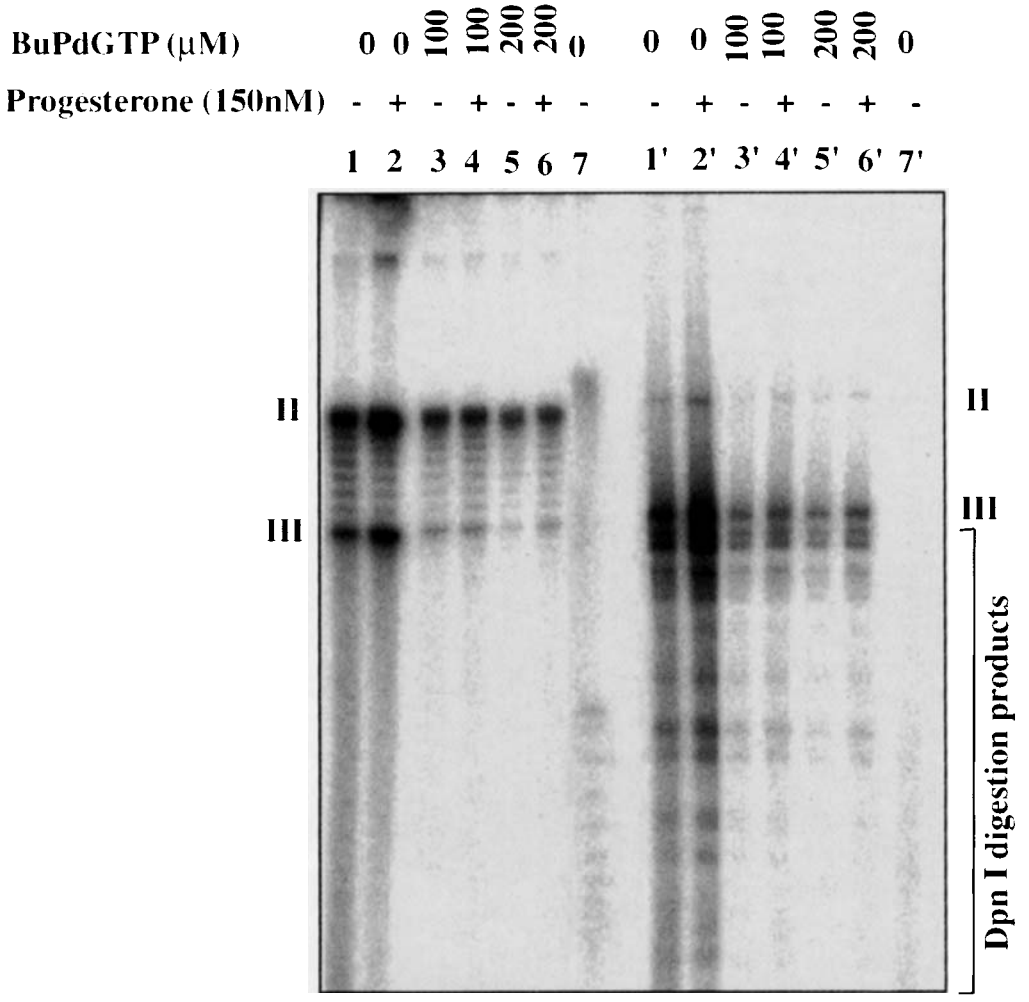

Fig. 6. Effect of BuPdGTP on in vitro reactions. pX24 was incubated with HeLa cell extracts and different concentrations of BuPdGTP (100 and 200 μM), in the absence (−) or presence (+) of 150 nM progesterone, as described in Methods. *Lanes 1–7* represent total incorporation, and *lanes 1′–7′*, *Dpn* I–digested samples. Lanes 7 and 7′ contain the reactions done with the negative control, p30.4. Relaxed circular (II) and linear (III) forms of plasmid DNA are indicated, as well as the area corresponding to *Dpn* I digestion products.

cation, whether interacting directly with DNA or replication proteins and machinery. The greater flexibility of this in vitro mammalian system offers unique opportunities to uncover new mechanisms of drug function, optimize drug efficacy, and provide for greater selectivity in design and development of new anticancer agents.

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