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Daunomycin Disrupts Nuclear Assembly and the Coordinate Initiation of DNA Replication in *Xenopus* Egg Extracts

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Abstract We have used *Xenopus* egg extracts to investigate the effects of the antitumor drug daunomycin on DNA replication in vitro. *Xenopus* sperm nuclei replicated nearly synchronously in our egg extracts, thereby allowing us to determine the effects of the drug on both replication initiation and elongation. Titration experiments demonstrated that daunomycin effectively inhibited replication in the extract, with 50% inhibition at a total drug concentration of 2.7 μM. However, a high concentration of daunomycin (50 μM) also inhibited nuclear envelope assembly, a prerequisite for the initiation of replication in this system. Therefore, to bypass the effects of daunomycin on nuclear envelope assembly, sperm nuclei were preassembled in extract prior to drug addition. Initiation of replication in preassembled nuclei was also inhibited by daunomycin, with 50% inhibition at a drug concentration of 3.6 μM. At low drug concentrations, where replication did occur, the synchrony of initiations within individual nuclei was lost. This drug-induced disruption of initiation events may provide important clues regarding the mechanism(s) by which these events are coordinated in eukaryotic cells. Daunomycin also inhibited replication elongation in preassembled, preinitiated nuclei. However, the concentration of drug required for 50% inhibition of elongation was nearly fourfold higher than that required for inhibition of initiation. Taken together, these data demonstrate that *Xenopus* egg extract can be used to investigate the effects of DNA-binding antitumor drugs on a number of interrelated cellular processes, many of which are less tractable in whole cell systems. J. Cell. Biochem. 64:476–491. © 1997 Wiley-Liss, Inc.

Key words: daunomycin; anthracycline; *Xenopus* egg extract; DNA replication; initiation; elongation; nuclear assembly; *Xenopus* sperm nuclei

Daunomycin (daunorubicin) is the parent drug of anthracycline antibiotics which are widely used in human cancer chemotherapy [Weiss, 1992]. Interaction of daunomycin with DNA is thought to be a crucial step in the molecular mechanism by which it inhibits DNA replication both in vitro [Zunino et al., 1975] and in vivo [Schellinx et al., 1979]. Several studies have shown that daunomycin is rapidly accumulated within the nuclei of sensitive cells, and intercalates into DNA [Gigli et al., 1988; Belloc et al., 1992]. It is arguably the best characterized DNA intercalator [Chaires, 1990]. Daunomycin also binds to nucleosomes [Chaires et al., 1983; Cera et al., 1991] and selectively displaces a unique set of nuclear proteins [Bartkowiak et al., 1989; Rabbani and Davoodi, 1994]. A direct relationship between DNA affinity and biological activity has been established for a series of 26 anthracycline derivatives [Valentini et al., 1985], indicating that DNA is an important target for these drugs. Although it has been proposed that topoisomerase II is a principal target of anthracycline antibiotics, DNA binding is also necessary for topoisomerase II inhibition by these drugs [Bodley et al., 1989; Capranico et al., 1990]. Recently, in vitro studies have shown that daunomycin inhibits RNA polymerase [Kriebardis et al., 1987], DNA ligase [David et al., 1985; Montecuccot et al., 1990], DNA helicase [Bachur et al., 1992], and DNA topoisomerase I [Crow and Crothers, 1994] efficiently. However, the precise mechanism by which daunomycin is cytotoxic remains unclear. The availability of a cell-free system that mimics many of the events that occur in whole

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cells would be of tremendous value in addressing this issue. Ideally, a cell-free system that is useful for this purpose should possess the following characteristics. First, DNA replication in the system should be governed by the regulatory mechanisms operating within eukaryotic cells. Second, the cell-free system should be capable of replicating a variety of DNA templates. Third, replication in the system should be highly efficient.

Cell-free extracts derived from the activated eggs of the frog *Xenopus laevis* meet all of these requirements. First, egg extracts initiate and complete semiconservative DNA replication under strict cell cycle control [Blow and Laskey, 1986; Newport, 1987; Hutchison et al., 1987]. Second, these extracts replicate purified plasmid or genomic DNA [Blow and Laskey, 1986; Newport, 1987; Blow and Sleeman, 1990] and nuclei isolated from *Xenopus* sperm cells [Blow and Laskey, 1986, 1988; Hutchison et al., 1987; Blow and Watson, 1987; Mills et al., 1989] or from a variety of somatic cells [Coppock et al., 1989; Leno and Laskey, 1991; Leno et al., 1992; Coverley et al., 1993; Leno and Munshi, 1994]. Third, replication is efficient in egg extracts. This is particularly evident when *Xenopus* sperm nuclei are used as the template. In this case, most nuclei are completely replicated within a 2 h incubation, with each individual nucleus requiring on average only 1 h to complete S phase [Blow and Watson, 1987]. Thus, all of these features make this system potentially very useful for studying the mechanism(s) of cytotoxicity of DNA binding drugs.

In the present study, we have used *Xenopus* sperm nuclei to investigate the effects of the anthracycline daunomycin on DNA replication in *Xenopus* egg extract. We found that individual sperm nuclei replicate rapidly and in near synchrony with each other within our extracts, thereby allowing us to determine the effects of daunomycin on both replication initiation and elongation. Daunomycin efficiently inhibited both the initiation and elongation phases of DNA replication; however, initiation was considerably more sensitive to the effects of this drug. Surprisingly, we found that while a low concentration of drug does not prevent the initiation of DNA replication, it does disrupt the coordination of initiation events within individual nuclei. This drug-induced loss of synchrony may be exploited to investigate the mechanism(s) by which initiation events are coordinated in eukaryotic cells. At higher drug concentrations, daunomycin also inhibited nuclear envelope assembly, suggesting that disruption of nuclear structure may also contribute to daunomycin's cytotoxicity in vivo.

MATERIALS AND METHODS

Materials

Daunomycin, propidium iodide, Hoechst 33258, pregnant mare's serum gonadotropin, human chorionic gonadotropin, calcium ionophore A23187, aprotinin, leupeptin, pepstatin, cytochalasin B, phenylenediamine, and lysophosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. A molar extinction coefficient of 11,500 $M^{-1}cm^{-1}$ at 480 nm was used to determine the concentration of daunomycin. [α-^{32}P]dATP (800 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and 5-biotin-16-deoxyuridine triphosphate and proteinase K were obtained from Boehringer Mannheim (Mannheim, Germany). RNase A, fluorescein streptavidin, and Texas Red streptavidin were purchased from Amersham Corp. (Arlington Heights, IL). Glass fiber filters (GF/C) were obtained from Whatman Inc. (Clifton, NJ).

Methods

Preparation of egg extracts. *Xenopus* egg extracts were prepared essentially according to Blow [1993] with the following modifications. Leupeptin, pepstatin A, and aprotinin were added to a final concentration of 1 μg/ml, cytochalasin B to a final concentration of 10 μg/ml, and EGTA to a final concentration of 0.4 mM. Extract was not diluted nor filtered. All centrifugation steps were carried out in a SW50.1 rotor (Beckman, Fullerton, CA).

Preparation of permeabilized *Xenopus* sperm nuclei. *Xenopus* sperm nuclei were prepared according to Philpott and Leno [1992] with the following modifications. Sperm cells from homogenized testes were sedimented through 70% percoll in modified Barth's saline (20 mM HEPES-KOH, pH 7.5, 110 mM NaCl, 1 mM KCl, 0.75 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$) at 2,800 rpm for 30 min at 4°C in a Juan CR4-22 centrifuge. The sperm sediment was washed one time in buffer NIM (10 mM HEPES, pH 7.5, 2.5 mM $MgCl_2$, and 0.2 M sucrose) and sedimented by centrifugation at

2,800 rpm for 15 min. The sperm sediment was resuspended in 10 ml buffer NIM containing 1.0 mg/ml lysophosphatidylcholine (LPC) with 100 μM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. LPC treatment of sperm cells disrupts both plasma and nuclear membranes, resulting in "demembranated" sperm chromatin. During incubation, the sample was mixed by inverting the tube every minute. Permeabilization was stopped by adding 5 ml ice-cold NIM with 3% bovine serum albumin. Permeabilized sperm nuclei (chromatin) were sedimented by centrifugation at 1,500 rpm for 15 min at 4°C, washed once in NIM, and finally resuspended in buffer XN/50% glycerol (buffer XN: 50 mM HEPES-KOH, pH 7.0, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine). DNA concentration was determined by counting nuclei with a hemacytometer and assuming a DNA mass of 3.15 pg per haploid genome [Dawid, 1965].

In vitro replication. Egg extract was thawed and supplemented with an energy-regenerating system [Blow and Laskey, 1986], cycloheximide to a final concentration of 100 μg/ml, ATP to a final concentration of 2 mM, permeabilized *Xenopus* sperm nuclei at ~1 ng DNA/μl, 100 μCi/ml [α-^{32}P]dATP or 20 μM 5-biotin-16-deoxyuridine triphosphate and daunomycin or an equivalent volume of water. The extent of extract dilution was kept constant at 20% for all incubations. dNTPs were added to a final concentration of 50 μM to readjust pool sizes following dilution. The final volume of each incubation was 12 μl.

Determination of [α-^{32}P]dATP incorporation. Incubations containing [α-^{32}P]dATP were stopped by adding stop mix C (0.5% SDS, 20 mM EDTA, 20 mM Tris-HCl, pH 8.0). Proteinase K (10 mg/ml in 10 mM HEPES, 50% glycerol) was added, and the mixture was incubated for 1 h at 37°C. The DNA was extracted with phenol chloroform. Incorporation into acid-insoluble material was determined by spotting samples onto glass fiber filters followed by TCA precipitation, washing with ethanol, drying, and counting in a Beckman LS6500 Scintillation Counter. Quantitation of DNA replication was based on a dATP pool size of 50 μM [Blow and Laskey, 1986], and the nanograms of DNA synthesized per microliter of extract was determined.

Detection of incorporated biotinylated dUTP. To detect incorporation biotinylated dUTP, nuclei were spun onto polylysine-coated coverslips, rinsed with Dulbecco's phosphate-buffered saline (Gibco BRL, Gaithersburg, MD), treated in ice-cold methanol for 15 min, and rinsed in buffer A (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1 mM β-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine). Each coverslip was then incubated for 10 min in 0.5 ml buffer A supplemented with either fluorescein streptavidin (5 μl) and propidium iodide (0.5 μl of a 1 mg/ml solution) or Texas red streptavidin (5 μl) and Hoechst 33258 (1 μl of a 100 μg/ml solution). Coverslips were then rinsed in buffer A, containing RNase A (0.5 μl of a 10 mg/ml solution), and water and then mounted in phenylenediamine mounting medium [Leno and Munshi, 1994]. Nuclei were viewed with either a Labophot-2 epifluorescence microscope (Nikon Inc., Metairie, LA) or a Odyssey laser confocal microscope (Noran Instruments, Middleton, WI).

Nuclear membrane formation. Nuclear membranes were identified by staining unfixed *Xenopus* sperm nuclei assembled in the egg extract with Hoechst 33258 (100 μg/ml) and the lipid dye, Nile red (0.1 μg/ml). Specifically, permeabilized sperm nuclei were incubated in egg extract for the time indicated in each experiment. Nile red and Hoechst 33258 were then added to an aliquot of each sample, and this mixture was placed on a glass slide. Nile red fluorescence was viewed with a Odyssey laser confocal microscope (Noran Instruments, Middleton, WI).

RESULTS

Dose-Dependent Inhibition of DNA Replication by Daunomycin

Following addition to *Xenopus* egg extract, each sperm nucleus undergoes nuclear envelope breakdown followed by decondensation and remodeling of its highly condensed chromatin. A new envelope is then formed around the decondensed chromatin, leading to pronuclear assembly. Once nuclear assembly is complete, the initiation of DNA replication can occur. As a first step in our studies, we sought to determine the kinetics of replication in our extracts. Time-course experiments were conducted in which nascent DNA was labeled with [α-^{32}P]dATP. A representative time-course experiment is shown in Figure 1. Following a 30 min delay, during

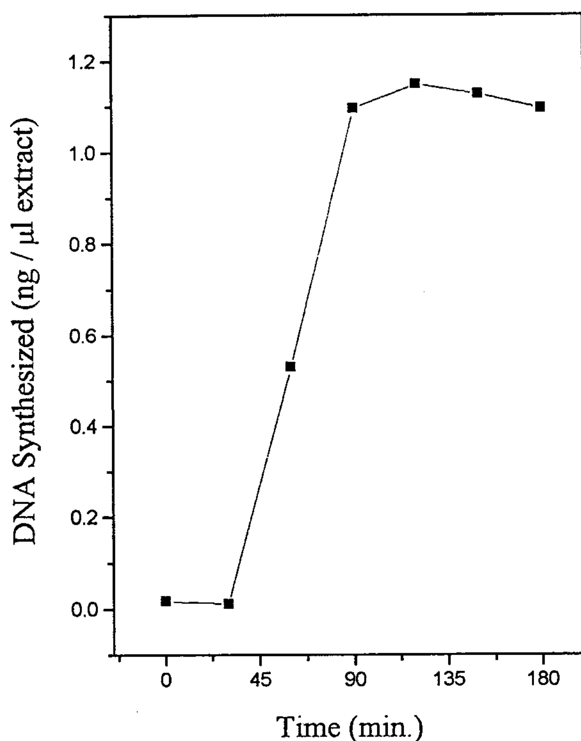

Fig. 1. Replication of *Xenopus* sperm nuclei in homologous egg extract. Permeable sperm nuclei at ~1 ng DNA/µl were incubated in egg extract containing [α-^{32}P] dATP for up to 180 min. The reactions were stopped at the times indicated and processed as described in Materials and Methods. DNA replication is expressed as nanograms of DNA synthesized per microliter of extract.

which nuclear assembly occurs, incorporation of label increases very rapidly, reaching a plateau by 90–120 min. The mass of DNA synthesized during this period is approximately equal to the mass of input DNA (i.e., ~1.0 ng/µl extract), demonstrating that virtually all nuclei underwent complete replication in the extract. Thus, sperm nuclei replicate rapidly and completely in our egg extracts.

Titration studies were conducted next to determine the effect of daunomycin on DNA replication in the extract. Sperm nuclei were incubated in extract without drug (control) or with various concentrations of daunomycin ranging from 0.1–50 µM. Daunomycin was added to the samples at time 0, and the reactions were incubated for 75 min. Figure 2A shows the mean values along with the standard error of the mean from three separate experiments in which two different extracts were used. DNA replication is expressed as a percentage of the control sample. The actual mass of DNA synthesized in the three control samples ranged from 0.67–0.86 ng/µl extract. These results indicate that daunomycin inhibits DNA replication completely at concentrations ≥10 µM, while concentrations of ≤1 µM had little or no effect. At a concentration of 5 µM, daunomycin inhibited replication approximately 80% relative to the control. Thus, daunomycin effectively inhibits DNA replication in egg extract, with 50% inhibition at a total drug concentration of 2.7 µM.

The results in Figure 2A indicate that high concentrations of daunomycin inhibit completely the replication of sperm nuclei in the extract. However, it is conceivable that under these conditions nuclei are able to initiate but are unable to complete the replication process. Initiation alone may not be detected by incorporation of ^{32}PdATP. Therefore, to determine if nuclei initiate replication in the presence of high concentrations of daunomycin, we used the highly sensitive technique in which nascent DNA is visualized by fluorescence microscopy. Nuclei were incubated in egg extract supplemented with biotinylated dUTP for 75 min, both in the absence of daunomycin (control = C) and in the presence of 5 µM or 50 µM of the drug. Biotin dUTP, a thymidine analog, is readily incorporated into nascent DNA [Langer et al., 1981] and can be visualized by staining with fluorochrome-conjugated streptavidin. The extent of biotin incorporation and thus streptavidin fluorescence is proportional to DNA content and therefore illustrates the extent of replication of each sperm nucleus in the extract [Blow and Watson, 1987; Leno and Laskey, 1991]. Incubated nuclei were spun onto coverslips, fixed, and stained with propidium iodide to show total DNA and with fluorescein streptavidin to detect incorporated biotin-dUTP. Two hundred nuclei from each sample were examined for fluorescein fluorescence, and the percentages of biotin-labeled nuclei in each sample are shown in Figure 2B. In the absence of daunomycin (C), >99% of the sperm nuclei showed uniformly bright streptavidin fluorescence, demonstrating that virtually all nuclei were replicating in the extract. Surprisingly, in the presence of 5 µM daunomycin, which reduces the overall levels of replication by 80% (Fig. 2A), >94% of the nuclei incorporated biotin-dUTP, demonstrating that initiation of replication occurs within most nuclei under these conditions. By contrast, streptavidin fluorescence was absent from all nuclei in the presence of 50 µM of the drug, confirming that initiation

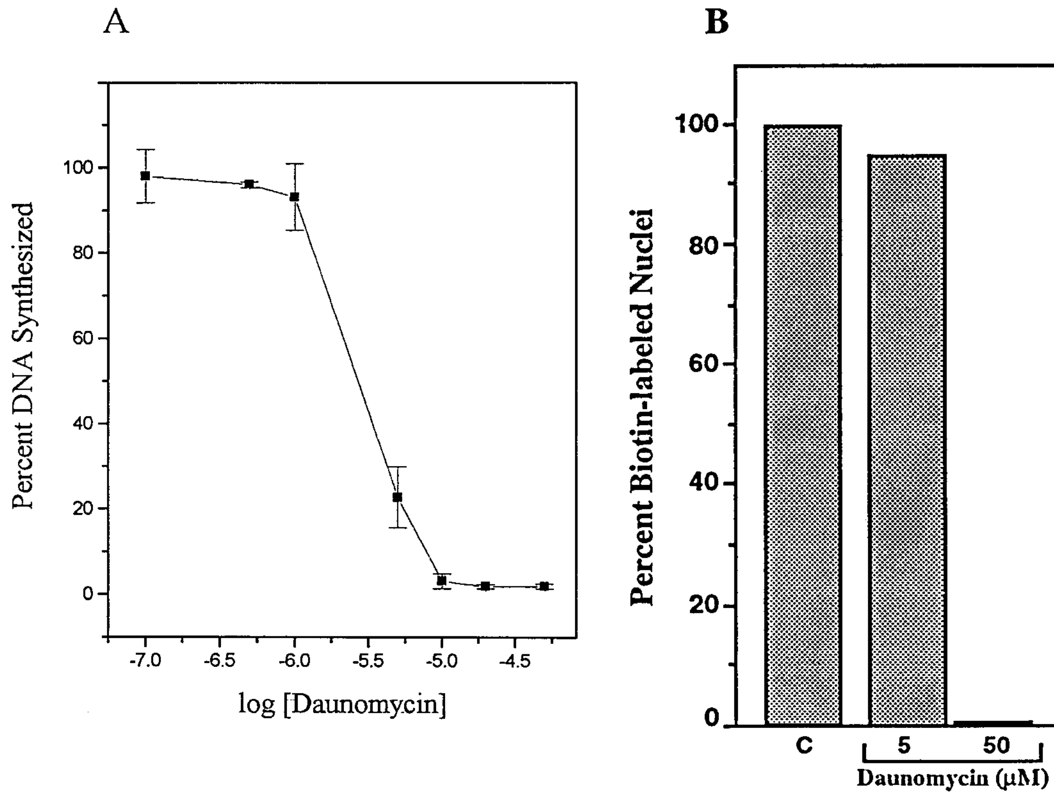

Fig. 2. Daunomycin inhibits replication of sperm nuclei in egg extract. **A:** Permeable sperm nuclei were added to egg extract supplemented with [α-^{32}P] dATP and daunomycin to concentrations of 0.1, 0.5, 1, 5, 10, 20, and 50 μM. The reactions were incubated for 75 min, stopped, and processed as described in Materials and Methods. Shown are the mean values along with the standard error of the mean from three separate experiments in which two different extracts were used. DNA replication is expressed as a percentage of the control sample which contained no drug. **B:** Permeable sperm nuclei were incubated in extract supplemented with 20 μM biotinylated-dUTP in the absence of drug (control = C) or in the presence of 5 μM or 50 μM daunomycin. Nuclei were processed as described in Materials and Methods and stained with propidium iodide to show total DNA and with fluorescein streptavidin to detect biotin-dUTP incorporated into nascent DNA. Two hundred nuclei from each sample were examined for fluorescein fluorescence, and the percent of biotin-labeled nuclei in each sample is shown.

of DNA replication was completely inhibited under these conditions.

High Concentrations of Daunomycin Inhibit Nuclear Envelope Assembly

The data presented so far demonstrate that high concentrations of daunomycin (i.e., 50 μM) completely inhibit DNA replication in the extract. In theory, daunomycin could inhibit initiation indirectly by preventing nuclear envelope assembly. To investigate this possibility, we incubated sperm nuclei in egg extract for 90 min without the drug (control) or with 5 μM or 50 μM daunomycin and subsequently stained unfixed nuclei from each sample with the lipid dye, Nile red (Fig. 3). A continuous, peripheral Nile-red fluorescence indicates complete nuclear membrane formation around sperm chromatin in the extract [Cox and Leno, 1990]. As expected, a continuous, distinct fluorescence was observed around nearly all sperm nuclei in the absence of daunomycin (Fig. 3A). Likewise, complete nuclear membrane formation was observed around most nuclei in the presence of 5 μM of the drug (Fig. 3B). This result is not surprising considering that initiation of replication is dependent upon the formation of an intact nuclear envelope [Blow and Sleeman, 1990] and that most nuclei initiate replication in the extract under these conditions (Fig. 2B). In a small percentage of nuclei (<5%), discontinuous or punctate fluorescence was observed around sperm chromatin in the presence of 5 μM of the drug (Fig. 3C), indicating vesicle binding without fusion. By contrast, nuclear membrane formation was completely inhibited by 50 μM dau-

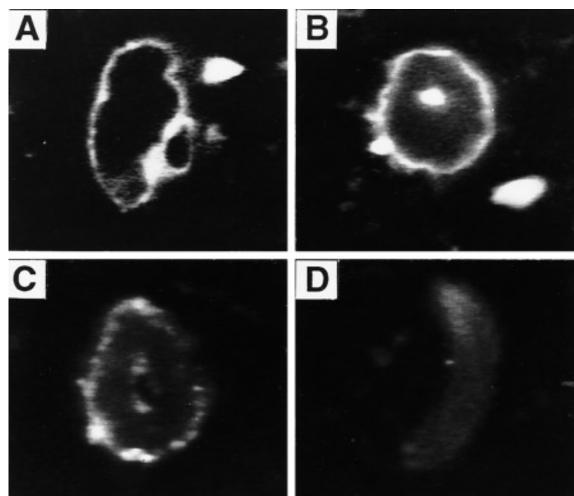

Fig. 3. A high concentration of daunomycin inhibits nuclear assembly. Permeable sperm nuclei were incubated in extract for 90 min in the absence of drug (**A**) or in the presence of 5 μM (**B**,**C**) or 50 μM (**D**) daunomycin. Nile red was added to each unfixed sample to label nuclear membranes. Membrane fluorescence was viewed by confocal microscopy. In the absence of drug (A) and in the presence of 5 μM daunomycin (B), most nuclei showed a continuous Nile-red fluorescence around the perimeter of the decondensed chromatin, indicating the presence of complete nuclear membranes. Only a small percentage (<5%) of nuclei in the 5 μM sample showed a punctate fluorescence at the chromatin periphery, indicating the attachment of discrete membrane vesicles to the chromatin (C). In the presence of 50 μM daunomycin, however, little or no nuclear membrane fluorescence was observed (D), indicating that high concentrations of the drug prevent the association of membrane vesicles with chromatin and thus the formation of a complete nuclear membrane. The general fluorescence associated with the chromatin in B–D is due to the autofluorescence of daunomycin in these samples.

nomycin (Fig. 3D). Not only was a continuous peripheral fluorescence absent from all these nuclei, but very few vesicles were found associated with the chromatin, indicating that daunomycin may affect both binding and fusion of nuclear membrane vesicles around sperm chromatin in the extract. Taken together, these data indicate that the inhibition of replication observed in the presence of a high concentration of drug is due, at least in part, to its effect on nuclear assembly. This does not seem to be the only mechanism by which replication is inhibited, however, as low drug concentrations, which have little or no effect on nuclear envelope assembly (Fig. 3B) or on the number of replicating nuclei (Fig. 2B) dramatically reduce the overall levels of replication in the extract (Fig. 2A).

Time Course of Daunomycin Inhibition of DNA Replication

In the presence of 5 μM daunomycin, initiation of DNA replication occurs within >94% of the nuclei by 75 min (Fig. 2B), yet the mass of DNA synthesized at this time is nearly fourfold lower than in the control sample (Fig. 2A). There are at least two possible explanations for these results. First, daunomycin induces a delay in the timing of initiation of replication, or, second, the rate of replication elongation is reduced by this drug. Clearly, these two possibilities need not be mutually exclusive, and thus both could contribute to the observed effects. Therefore, to determine which of these or other possibilities are indeed correct, we carried out time-course studies to investigate the kinetics of replication in the presence of two concentrations of the drug. The results of a typical experiment are shown in Figure 4. Clear differences in the kinetics of replication were observed between the 5 μM daunomycin sample and the no-drug control. The presence of daunomycin in the extract consistently nearly doubled the length of the lag phase (i.e. from 30 min

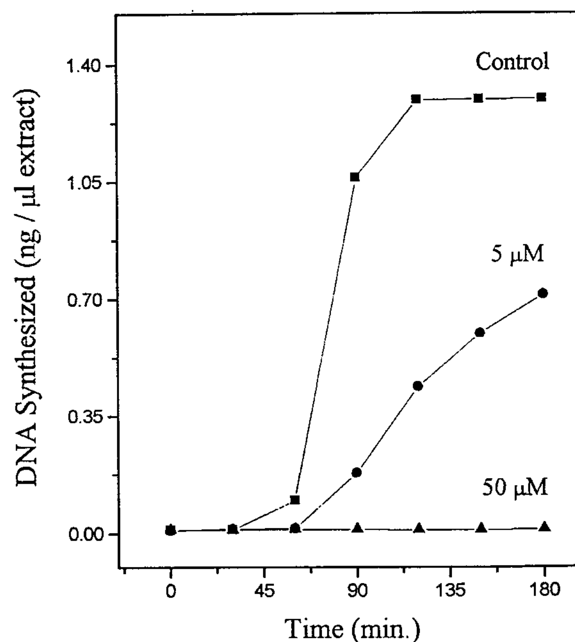

Fig. 4. Time course of replication inhibition by daunomycin. Permeable sperm nuclei were incubated in egg extract containing [α-^{32}P]dATP in the absence (Control) or presence of 5 μM or 50 μM daunomycin. Reactions were stopped at the times indicated. DNA replication is expressed as nanograms of DNA synthesized per microliter of extract.

(control) to 60 min) and also significantly reduced the rate of replication. By 120 min, the time required to replicate all the DNA in the control sample, <40% of the input DNA was replicated in the presence of 5 μM of the drug. Even after 180 min, only ~50% of the DNA was replicated in the presence of the drug. Consistent with our previous results, replication was completely inhibited in the presence of 50 μM daunomycin.

The extended lag phase observed in the presence of 5 μM daunomycin could be due to a delay in the timing of replication initiation. Therefore, to determine whether or not initiation occurs during this extended lag, we carried out time-course studies labeling nascent DNA with biotinylated dUTP. Specifically, permeabilized sperm nuclei were incubated for up to 90 min in egg extract in the presence of 5 μM daunomycin (Fig. 5 Daunomycin) or in the absence of drug (Fig. 5 Control). Reactions were stopped at 30 min intervals, and the nuclei were pelleted onto coverslips, fixed, and stained with Hoechst 33258 to label total DNA and with Texas red streptavidin to detect incorporated biotin-dUTP. The results from a representative experiment are shown in Figure 5. As expected, no streptavidin fluorescence was detected in any nuclei from the control or the daunomycin sample during the first 30 min in the extract, confirming that initiation of replication does not occur during this assembly phase (Fig. 5a). However, by 60 min, >95% of all nuclei in the control sample showed bright streptavidin fluorescence, demonstrating that DNA replication was initiated within most nuclei during the period between 30 and 60 minutes in the extract. Furthermore, streptavidin fluorescence was distributed uniformly throughout each sperm nucleus (Fig. 5a, Uniform Fluorescence, and Fig. 5b, A,B), consistent with previous results showing that replication occurs at discrete foci spaced uniformly throughout sperm nuclei [Mills et al., 1989]. Greater than 75% of the nuclei in the daunomycin sample also showed streptavidin fluorescence after 60 min in the extract (Fig. 5a) even though virtually no incorporated radiolabel was detected during this period. This result was not too surprising, however, considering our previous observation that >94% of all nuclei were labeled with biotin after a 75 min incubation in the presence of 5 μM of the drug (Fig. 2B). However, we were very surprised to find that, in >60% of these

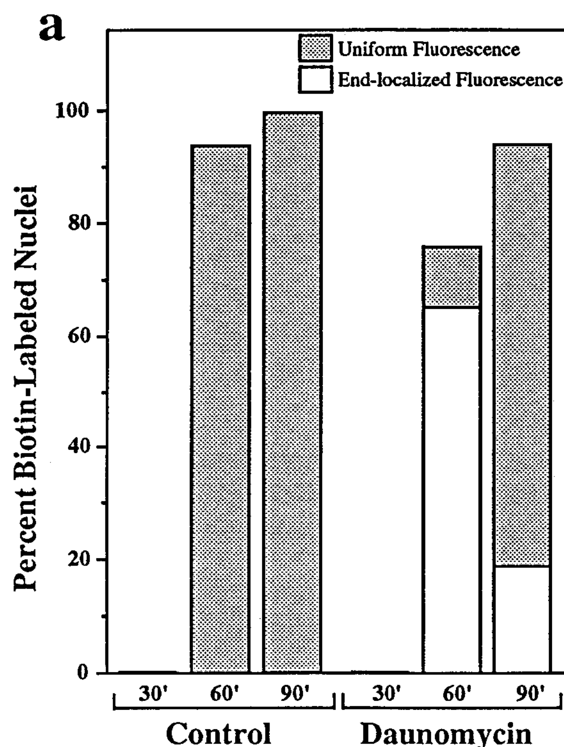

Figure 5

replicating nuclei, fluorescence was limited to one end of the nucleus (Fig. 5a, End-localized Fluorescence, and Fig. 5b, C–F). This is in striking contrast to the uniform fluorescence observed in control samples. By 90 min, however, most of the nuclei in the daunomycin sample resemble control nuclei, showing uniform fluorescence throughout each nucleus (Fig. 5a, Fig. 5b, G,H). Taken together, these data demonstrate that while a low concentration of daunomycin does not prevent the initiation of replication in sperm nuclei, it does disrupt the coordination of initiation events that normally occurs within an individual nucleus [Blow and Watson, 1987; Mills et al., 1989]. Eventually, however, nuclei become uniformly labeled even in the presence of drug.

Daunomycin Inhibits the Initiation of DNA Replication in Preassembled Nuclei

Within an individual sperm nucleus, entry into S phase is characterized by synchronous or near synchronous initiation events; however, individual nuclei enter S phase at different times during a single incubation in the extract [Blow and Watson, 1987]. In our egg extracts, however, individual nuclei appear to be entering S phase in near synchrony with each other.

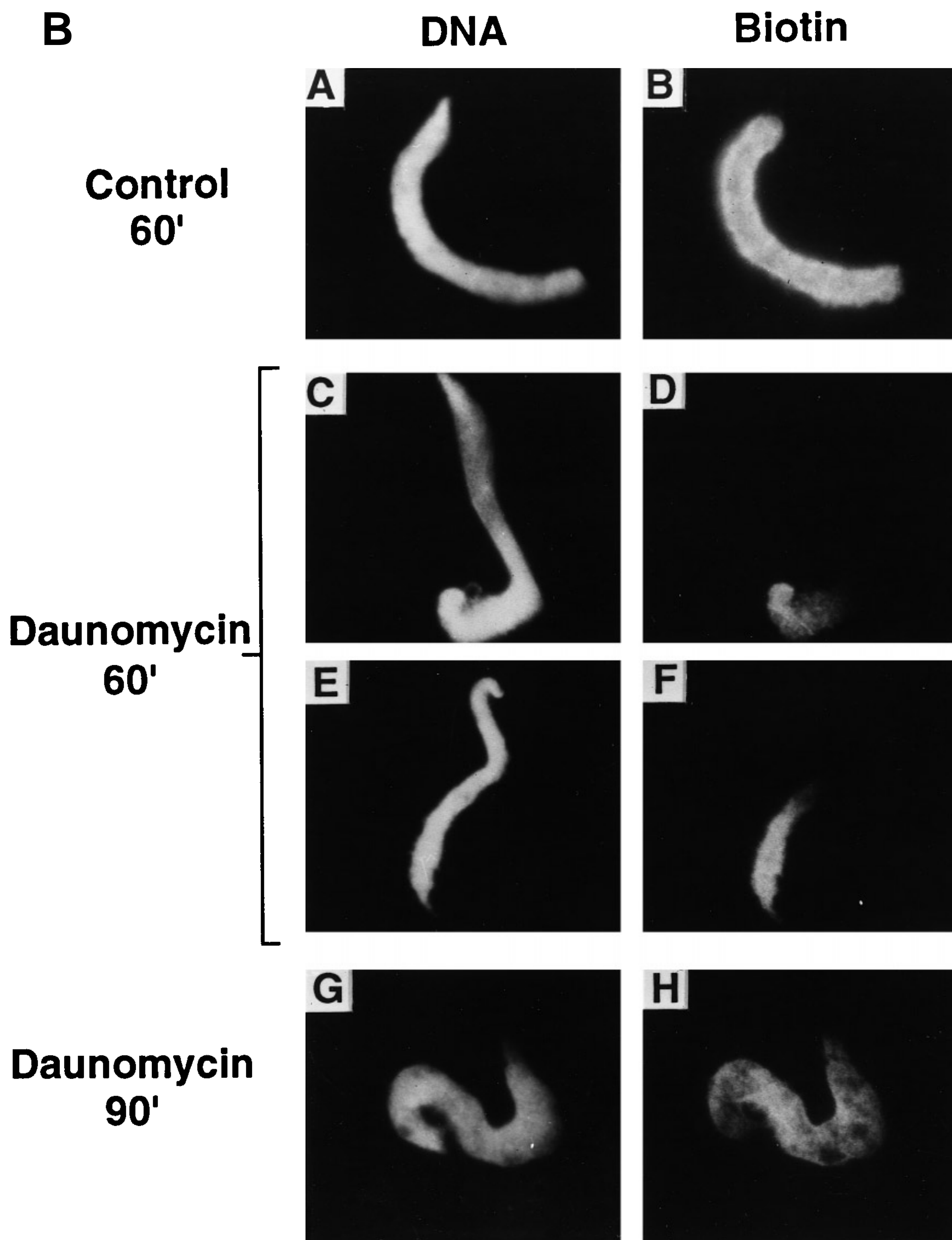

Fig. 5. Daunomycin disrupts the coordination of initiation events within individual sperm nuclei. Permeable sperm nuclei were incubated for 30, 60, or 90 min in egg extract supplemented with 20 μM biotinylated-dUTP in the absence (Control) or presence of 5 μM daunomycin (Daunomycin). Nuclei were spun onto coverslips, fixed, and stained with Hoechst 33258 to label total DNA (DNA) and with Texas Red streptavidin to detect biotin-dUTP incorporated into nascent DNA (Biotin). Two hundred nuclei from each sample were selected at random and classified according to the presence or absence of streptavidin fluorescence and the distribution of fluorescence within each biotin-labeled nucleus. Three classes were observed: negative (no fluorescence), end-localized fluorescence (bright fluorescence limited to a discrete region at one end of the nucleus), and uniform fluorescence (bright fluorescence uniformly distributed throughout the entire nucleus). **a:** The percent of biotin-labeled nuclei and the distribution of labeled nuclei displaying end-localized vs. uniform fluorescence is shown for each time point. **B:** Representative nuclei from the 60 min control sample (A & B), from the 60 min daunomycin sample (C–F), and from the 90 min daunomycin sample (G & H) are shown.

This conclusion is based upon the observation that most sperm nuclei are labeled with biotin-dUTP within the 30 min period following nuclear assembly (Fig. 5a, Control, 30′ vs. 60′ time points). Individual nuclei require approximately 60 min to completely replicate their DNA [Blow and Watson, 1987], and therefore the observation that nearly all input DNA is completely replicated by 90–120 min in our extracts (Figs. 1, 4) is consistent with the view that most nuclei do indeed initiate replication within the 30 min period following assembly. The fact that initiations are synchronous within individual sperm nuclei and that individual nuclei replicate in synchrony with each other in our extracts indicates that this in vitro system can be used to determine the effect(s) of daunomycin on both replication initiation and elongation provided the drug's effect on nuclear assembly can be circumvented.

The simplest way to bypass the effects of daunomycin on nuclear assembly is to "preassemble" nuclei in egg extract prior to the addition of drug. Therefore, permeable nuclei were incubated in egg extract supplemented with [^{32}P]dATP or biotinylated dUTP for 30 min prior to the addition of drug. During this time, nuclear assembly occurs, but the initiation of replication does not (Fig. 5a). After 30 min, various concentrations of daunomycin were added to the extract, and the samples were incubated an additional 45 min for a total time in extract of 75 min. Samples were processed for scintillation counting or fluorescence microscopy as described in Materials and Methods. The results of our studies with [^{32}P]dATP appear in Figure 6A. Shown are the mean values along with the standard error of the mean from four separate experiments in which three different extracts were used. DNA replication is expressed as a percentage of the control sample which contained no drug. Under these conditions, initiation of replication was almost completely inhibited by concentrations of daunomycin greater than 10 μM, while low concentrations of drug had little or no effect. At a concentration of 5 μM of the drug, replication was reduced ~60% relative to the control. Fifty percent inhibition of replication occurred at a total drug concentration of 3.6 μM. This concentration is very similar to the concentration of drug required for 50% inhibition of replication in the absence of nuclear preassembly (i.e., 2.7 μM) (Fig. 2A).

The results of our experiments in which preassembled nuclei were labeled with biotin-dUTP are shown in Figure 6B. A pronounced drop in the percent of labeled nuclei is observed at concentrations of daunomycin >5 μM (Fig. 2B), with only 14% of the nuclei showing biotin incorporation in the presence of 50 μM of the drug. While this is a considerable increase over the number of nuclei that incorporated label when nuclear assembly was required (Fig. 2B), it is still far below the levels observed in control samples. Control levels should be reached under these conditions if inhibition of nuclear assembly were the sole mechanism by which daunomycin inhibits initiation. Clearly, this is not the case. Thus, these data indicate that daunomycin effectively inhibits the initiation of DNA replication in egg extract through mechanisms separate from those that prevent nuclear assembly.

Daunomycin Inhibits Replication Elongation in Preassembled, Preinitiated Nuclei

Over 95% of sperm nuclei initiate DNA replication within the first 60 min of extract incubation (Fig. 5a); however, less than 50% of the total input DNA is replicated by this time (Fig. 1). An additional 30–60 min is required for complete replication of all DNA. Thus, the vast majority of the label that is incorporated during this final 30–60 min incubation is due to replication elongation. To determine what effect, if any, daunomycin has on replication elongation in this system, we carried out the following experiments. Preassembled, preinitiated nuclei were prepared by incubating permeable sperm nuclei in egg extract for 60 min in the presence of [^{32}P]dATP. The 60 min control reactions were stopped, and various concentrations of daunomycin were then added to the remaining non-control samples. These samples were incubated an additional 60 min for a total incubation time in extract of 120 min.

The results of these experiments appear in Figure 7. Shown are the mean values along with the standard error of the mean from three separate experiments in which two different extracts were used. DNA replication is expressed as a percentage of the 120 min control sample which contained no drug. From 27–35% of the total input DNA was replicated during the initial drug-free 60 min incubation period. Therefore, complete inhibition of elongation by daunomycin would still result in replication of

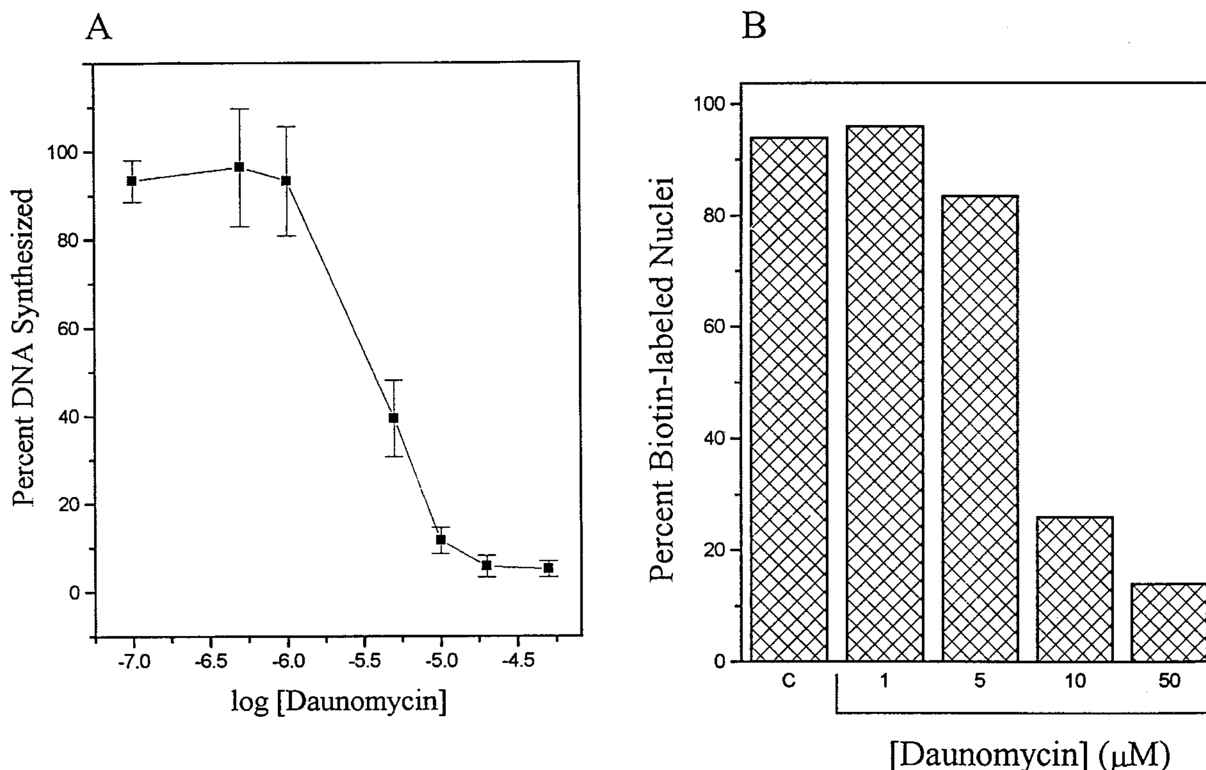

Fig. 6. Daunomycin inhibits the initiation of DNA replication in preassembled nuclei. Permeable sperm nuclei were incubated in egg extracts containing [α-^{32}P]dATP (**A**) or 20 μM biotinylated-dUTP (**B**) for 30 min. Various concentrations of daunomycin (0.1, 0.5, 1, 5, 10, 20, 50 μM) were then added to the extract which was incubated for an additional 45 min. The reactions were then processed as described in Materials and Methods. A: Shown are the mean values along with the standard error of the mean from four separate experiments in which three different extracts were used. DNA replication is expressed as a percentage of the control sample which contained no drug. B: Two hundred nuclei from each sample were selected at random and examined for streptavidin fluorescence. The percentage of biotin-labeled nuclei from a single, representative experiment are shown.

~30% of the input DNA after 120 min in the extract. In general, replication elongation is considerably less susceptible to the effects of daunomycin than is initiation (compare Fig. 7 with Fig. 6A). In fact, the concentration of daunomycin required to inhibit elongation by 50% in preinitiated nuclei is 11.7 μM, nearly four times higher than the concentration required for 50% inhibition of initiation. Taken together, these data demonstrate that daunomycin inhibits both initiation and elongation of replication in egg extract. However, initiation is more susceptible to the effects of this drug.

Daunomycin Does Not Promote Disassembly of Preassembled Nuclei

High concentrations of daunomycin (50 μM) inhibit nuclear envelope assembly in the extract (Fig. 3). This observation, coupled with the fact that an intact, functional nucleus is required for both initiation and elongation of replication in this system [Blow and Sleeman, 1990; Cox, 1992], prompted us to investigate the possibility that daunomycin inhibits both of these processes by promoting disassembly of preformed nuclei. To test this hypothesis, we incubated permeable sperm nuclei in egg extract for 90 min in the absence of daunomycin. Immediately thereafter, 50 μM of the drug was added to the extract, and the sample was incubated an additional 120 min. Unfixed nuclei were stained with Nile red and viewed by confocal microscopy. A continuous Nile-red fluorescence around the perimeter of nearly all nuclei was observed (Fig. 8), demonstrating that, even after extended exposure of preformed nuclei to a high concentration of daunomycin, nuclear disassembly does not occur.

DISCUSSION

The major conclusions of this report are fourfold. First, daunomycin effectively inhibits the

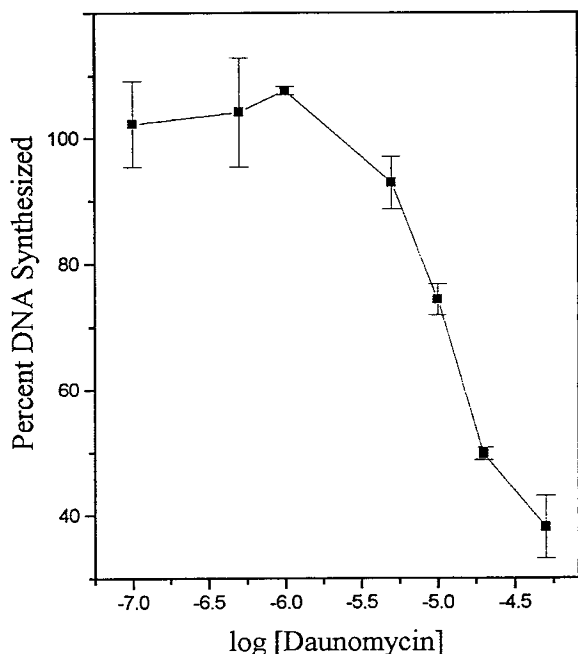

Fig. 7. Daunomycin inhibits replication elongation in preassembled, preinitiated nuclei. Permeable sperm nuclei were incubated in egg extracts containing [α-^{32}P]dATP for 60 min. Various concentrations of daunomycin (0.1, 0.5, 1, 5, 10, 20, 50 μM) were then added to the extract which was incubated for an additional 60 min. The reactions were then processed as described in Materials and Methods. Shown are the mean values along with the standard error of the mean from three separate experiments using two different egg extracts. DNA replication is expressed as a percentage of the control sample which contained no drug. Approximately 30% of the DNA was replicated during the first 60 min (drug-free) in the extract.

initiation of replication in preassembled, uninitiated nuclei (Fig. 6), with 50% inhibition occurring at a drug concentration of 3.6 μM. Second, replication elongation in preassembled, preinitiated nuclei is also inhibited by this drug (Fig. 7); however, initiation is nearly fourfold more sensitive to its effects than is elongation (compare Fig. 7 with Fig. 6). Third, low concentrations of daunomycin (5 μM), which do not prevent initiation, do disrupt the coordination of initiation events within individual nuclei (Fig. 5). Fourth, daunomycin in high concentrations (50 μM) completely inhibits nuclear assembly, a prerequisite for initiation of DNA replication in *Xenopus* egg extract (Fig. 3). Taken together, these data illustrate a number of important points. First, the daunomycin-induced disruption of initiation events may provide important clues regarding the mechanism(s) by which these events are coordinated in eukaryotic cells. Furthermore, if the inhibition of DNA replication is indeed a reliable indicator of antitumor activity by these drugs [Oki, 1984], then understanding the mechanism by which initiation events are perturbed may ultimately form the basis for the design of more effective anticancer drugs. Second, the daunomycin-induced disruption of nuclear envelope formation in egg extract raises the interesting possibility that this drug inhibits nuclear assembly *in vivo,* and that this inhibition may contribute to cytotoxicity. Third, the data presented here describe a novel cell-free system which can be used to investigate the effects of DNA-binding antitumor drugs on a number of interrelated cellular processes, many of which are less tractable in whole cell systems.

Inhibition of DNA Replication

A number of mechanisms have been proposed by which anthracyclines exert their cytotoxic effects. These include DNA binding, membrane binding, topoisomerase inhibition, or free radical production [Lown, 1988; Priebe, 1995]. To date, however, a consensus has not emerged. Anthracyclines including daunomycin have been shown to inhibit DNA replication both in vivo [Mizuno et al., 1975; Schellinx et al., 1979; Schott and Robert, 1989] and in vitro [Mizuno et al., 1975], and, indeed, the inhibition of DNA synthesis in leukemia cells may be the best

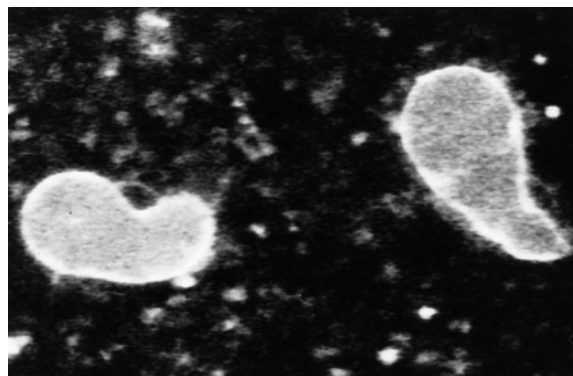

Fig. 8. Daunomycin does not promote disassembly of preassembled nuclei. Permeable sperm nuclei were incubated in egg extract for 90 min in the absence of daunomycin. Drug was then added to a final concentration of 50 μM, and the samples were incubated for an additional 120 min. Unfixed nuclei were stained with Nile red and viewed by confocal microscopy. Two nuclei are shown in this field. A bright, continuous fluorescence was observed around the perimeter of virtually all sperm nuclei, demonstrating the presence of complete nuclear membranes even after extended exposure of nuclei to a high concentration of drug.

indicator of antitumor activity by these drugs [Oki, 1984].

The inhibition of nuclear envelope assembly prevents the initiation of DNA replication in egg extract [Sheehan et al., 1988]. Given that high concentrations of daunomycin (50 μM) inhibit the assembly process (Fig. 3), it is therefore not surprising that initiation is also inhibited under these conditions (Fig. 2). However, daunomycin's effect on nuclear assembly can be circumvented by preassembling nuclei in egg extract in the absence of drug. Once assembled, these uninitiated nuclei can then be used to determine the effects of daunomycin on the process of initiation alone. Using this approach, we have demonstrated that daunomycin effectively inhibits initiation of replication in egg extracts (Fig. 6A,B), consistent with in vivo studies with cultured mammalian cells [Schellinx et al., 1979]. However, the inhibition of DNA replication in egg extract requires higher drug concentrations than does inhibition in whole cells. These concentration-dependent differences may be more apparent than real, however, given that whole cells are able to accumulate drug to intracellular concentrations that are several thousand fold greater than extracellular levels [Gieseler et al., 1994]. We do not know to what extent daunomycin accumulates within the nuclei formed in egg extract; however, it may be inappropriate to assume that concentrations similar to those in whole cells are reached. This is because it is not clear to what extent the plasma membrane and/or cytoplasmic components contribute to the final concentration of drug in whole cells. Clearly, if high nuclear concentrations of daunomycin are not reached in egg extract, it could be concluded that DNA replication in this system is actually more sensitive to the effects of the drug than it is in whole cells. A final interpretation awaits further analysis.

These data also support the view that inhibition of initiation occurs through mechanisms which are separate from those that inhibit nuclear assembly. Indeed, even in the presence of low concentrations of daunomycin where inhibition of nuclear envelope assembly does not occur, the normal patterns of initiation are perturbed (Fig. 5). Instead of synchronous initiations occurring uniformly throughout individual nuclei [Blow and Watson, 1987; Mills et al., 1989], initiations within these drug-treated nuclei occur asynchronously in localized regions, indicating loss of the coordination of initiation events within individual nuclei. This is clearly visible as end-localized streptavidin fluorescence which is observed within most nuclei following 60 min in the drug-treated extract (Fig. 5a,b). By 90 min, however, fluorescence is uniformly distributed within most of these nuclei similar to the control, demonstrating that initiation of replication does occur along the remaining DNA at later times.

There are a number of possible ways in which daunomycin could uncouple or prevent initiations within individual sperm nuclei. First, daunomycin could bind DNA at the sites where initiation occurs, and it could displace or inhibit replication proteins. Indeed, Bartkowiak et al. [1989] showed that doxorubicin, a 14-hydroxyl derivative of daunomycin, selectively displaced a particular set of nuclear proteins from isolated rat liver nuclei. Thus, in the case of daunomycin, all initiation sites would be bound at high drug concentration (50 μM), and initiation would not occur. However, at low concentrations of drug (5 μM), only preferred sites would be bound, allowing initiation to occur at the remaining sites. Eventually, most or all initiation sites would be used in the presence of low concentrations of drug (Fig. 5a,b, 90 min time point). It has previously been shown that daunomycin preferentially binds specific DNA sequences in vitro [Chaires et al., 1987, 1990; Roche et al., 1994]; however, the relationship between this sequence specificity and initiation of replication in egg extract is not clear. We are currently investigating whether or not daunomycin disrupts the assembly or activation of prereplication centers on sperm chromatin [Adachi and Laemmli, 1994]. Second, the loss of coordinate initiation in the presence of daunomycin could be related to the extent of chromatin decondensation within sperm nuclei. Daunomycin binds more tightly to free or decondensed DNA than to condensed DNA [Chaires et al., 1983], and thus the end-localized replication we observe (Fig. 5) may reflect differential decondensation and daunomycin binding within these nuclei. However, this explanation seems unlikely for two reasons. First, uniformly decondensed nuclei still show end-localized fluorescence (data not shown), and, second, even when differential decondensation is observed, the more decondensed regions are often those that replicate (Fig. 5b, E,F). Third, daunomycin could also prevent or uncouple initiations within indi-

vidual nuclei by binding directly to proteins required for the initiation process. This possibility seems unlikely, however, since drug-DNA interactions appear to be required for the inhibition of a number of replication proteins in vitro (e.g., topoisomerase I [Crow and Crothers, 1994], topoisomerase II [Bodley et al., 1989; Capranico et al., 1990], DNA helicase [Bachur et al., 1992], and DNA ligase [David et al., 1985]. However, our data do not formally exclude the possibility that daunomycin binds directly to proteins required for initiation. Fourth, anthracyclines affect a number of membrane properties, including fluidity, ion transport, lipid organization, and morphology [Myers et al., 1988]. Conceivably, such daunomycin-induced changes in nuclear membrane structure and/or function could cause inhibition of initiation in the extract. Indeed, both initiation and elongation of replication are dependent upon the transport of nuclear proteins across the nuclear membrane [Cox, 1992]. The effects of daunomycin on nuclear transport are under investigation.

Nearly all sperm nuclei (96%) initiate DNA replication in the absence of daunomycin after 60 min in egg extract (Fig. 5); however, only 30–40% of the input DNA is replicated by this time (Figs. 1, 7). These observations, coupled with the fact that initiations occur synchronously at the beginning of S phase within individual sperm nuclei [Blow and Watson, 1987], demonstrates that most nuclei are undergoing replication elongation after a 60 min incubation in the extract. Therefore, to investigate the effects of daunomycin on replication elongation, preassembled, preinitiated nuclei were prepared by incubating permeable sperm nuclei in egg extract for 60 min before the addition of the drug. We found that daunomycin effectively inhibits replication elongation in the extract (Fig. 7); however, this inhibition is not due to disruption of preassembled nuclei (Fig. 8). Elongation is also less sensitive to the effects of daunomycin than is initiation (Fig. 6), consistent with the results of Schellinx et al. [1979]. Daunomycin may inhibit initiation and elongation processes through similar mechanisms. This would not be surprising if replication occurs at fixed sites within sperm nuclei, as has been proposed [Mills et al., 1989]. However, the fact that initiation and elongation display different sensitivities to daunomycin suggests that different mechanisms may exist.

Inhibition of Nuclear Assembly

DNA added to the egg extract is assembled into nuclei that resemble normal interphase nuclei both structurally and functionally [Lohka and Masui, 1983 a,b; Blow and Laskey, 1986; Newport, 1987]. Initiation of DNA replication occurs only on DNA that is present within these nuclei [Newport, 1987; Blow and Sleeman, 1990; Leno and Laskey, 1991]. If nuclear assembly is prevented [Sheehan et al., 1988] or if normal nuclear function is disturbed [Cox, 1992], initiation or elongation of replication will not occur. Even when the DNA template is in the form of a highly condensed cell nucleus, as in the case of *Xenopus* sperm nuclei [Lohka and Masui, 1983 a,b] or chicken erythrocyte nuclei [Leno and Laskey, 1991], these structures are reassembled into replication competent nuclei by the extract. In the case of sperm nuclei, this process initially involves extensive chromatin decondensation [Lohka and Masui, 1983a,b, 1984] and remodeling [for review see Poccia, 1986]. These processes are mediated by nucleoplasmin, which functions as both an assembly factor and as a disassembly factor in egg extract [Philpott et al., 1991; Ohsumi and Katagiri, 1991; Philpott and Leno, 1992; Leno et al., 1996]. Interestingly, daunomycin does not prevent stage I decondensation of sperm chromatin (Fig. 3D), suggesting that its effects are exerted at a later stage of the assembly process. The formation of a complete nuclear membrane by the extract constitutes a late stage in this assembly process. Thus, the presence of a continuous nuclear membrane surrounding decondensed sperm chromatin (Fig. 3A) is a reliable indicator of nuclear reassembly, while the lack of such a membrane, due to the presence of daunomycin for example (Fig. 3D), indicates at least one of the late stages of the assembly process is inhibited.

The mechanism(s) by which daunomycin inhibits nuclear envelope assembly in egg extract is not clear. However, given that DNA appears to be the prime target for daunomycin within the cell, it seems likely that the effect of this drug on nuclear assembly is mediated through these interactions. Belloc et al. [1992] used fluorescence resonance energy transfer (FRET) to show that daunomycin intercalates into the DNA of living cells. We also observed daunomycin binding to sperm DNA (Fig. 3B–D) and found FRET between this drug and Hoechst 33258 (data not shown), supporting the view

that drug-DNA interactions may mediate inhibition of assembly. However, other mechanisms, such as drug-membrane interactions, could theoretically inhibit this process.

Topoisomerase II activity has been implicated in the process of nuclear assembly in *Xenopus* egg extract. VM-26, a specific topoisomerase II inhibitor, was found to block nuclear membrane formation around sperm chromatin in the extract [Newport, 1987]. Interestingly, the anthracyclines also inhibit topoisomerase activity, a process that is mediated by drug-DNA interactions [Tewey et al., 1984; Bodley et al., 1989; Crow and Crothers, 1994]. Taken together, these data raise the interesting possibility that daunomycin inhibits nuclear assembly through its inhibition of topoisomerase II. Indeed, our results are similar to those described by Newport [1987], with the exception that membrane vesicles rarely bind to sperm chromatin in the presence of a high concentration of daunomycin (Fig. 3D), while vesicles bind but do not fuse in the presence of a high concentration of VM-26. Recently, however, Takasuga et al. [1995] have shown that ICRF-193, another inhibitor of topoisomerase II, does not inhibit nuclear assembly, nor does it inhibit the initiation of DNA replication in egg extract even when >98% of topoisomerase II activity is lost, calling into question the role of topoisomerase II in the assembly process. Taken together, these data indicate that even if daunomycin inhibits topoisomerase II activity in egg extract, this does not appear to be the primary mechanism by which this drug inhibits nuclear assembly or DNA replication in this system.

The extent to which daunomycin affects nuclear envelope assembly in egg extract may provide clues to its mechanism of inhibition. It has been proposed that binding of soluble and/or membrane-associated lamins to the chromosome surface is an early event in the assembly process. Targeting of additional vesicles to this site presumably involves binding of integral membrane proteins associated with these vesicles to the soluble lamins present on the chromatin surface [Lourim and Krohne, 1994]. Our observations that a high concentration of daunomycin prevents virtually all vesicles from binding to decondensed chromatin (Fig. 3D) suggests that this drug may be preventing the association of lamins with the chromatin surface. Alternatively, daunomycin could bind directly to the membrane vesicles and in this way prevent their association with chromatin. We are currently investigating these and other possibilities.

In summary, we have used *Xenopus* egg extract to investigate the effects of the antitumor drug daunomycin on DNA replication in vitro. Our data confirm and extend the results from previous in vivo and in vitro studies and establish that this cell-free system can be used to investigate the effects of DNA-binding antitumor drugs on normal cellular processes in vitro. It will be interesting to determine if the clinical efficacy of any of the anthracyclines can be correlated with their effects on nuclear assembly, DNA replication, or other processes in this cell-free system.

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