DNA intercalators differentially affect chromatin structure and DNA replication in *Xenopus* egg extract

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In this paper, we describe a scheme utilizing the *Xenopus* egg extract system to simultaneously evaluate DNA-interacting drugs as potential anti-cancer agents and gain insights into the mechanisms of drug action. We studied two DNA intercalators, daunomycin (DM), a cancer chemotherapeutic, and ethidium bromide (EtBr), a compound with no reported therapeutic value. Consistent with our earlier report, we find that DM inhibits DNA replication in a concentration-dependent manner. In contrast, EtBr does not inhibit replication over the same concentration range. The environment in which drug–DNA interactions take place is an important determinant of the effect of the drug on DNA replication. While neither intercalator inhibits nuclear membrane assembly nor nuclear protein import, DM does disrupt chromatin structure at very low concentrations, whereas EtBr does not. This system may prove useful for large scale screening of DNA-interacting chemotherapeutic compounds in a cellular milieu. *Anti-Cancer Drugs* 15:633–639 © 2004 Lippincott Williams & Wilkins.

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Introduction

Cytoplasmic extracts from *Xenopus laevis* eggs have been used extensively to study the mechanics of eukaryotic DNA replication [1]. This is a powerful *in vitro* system with *in vivo* merits. Any DNA template added to the *Xenopus* egg extract is first assembled into chromatin and surrounded by a nuclear envelope, following which it undergoes a single round of semi-conservative DNA replication under complete cell cycle control [2]. Initiation of replication within individual replicon clusters appears to be nearly synchronous in this system, although these clusters appear to fire at different times during S phase, similar to somatic cells [3]. Furthermore, the regular spacing of origins without respect to DNA sequence and the relatively small inter-origin distances reported (i.e. 5–15 kb), allow the DNA to be replicated completely in a very short period of time [3]. Finally, *Xenopus* extracts contain most, if not all, of the key replication proteins that are found *in vivo* and these proteins display similar, if not identical, function *in vitro* [4].

Drug–DNA interactions are typically studied using *in vitro* systems derived from purified components. The results from such studies provide valuable insights into many physical parameters, but may have questionable relevance in the biological setting. Drugs rarely have a single molecular target and, therefore, it is critical that more complex systems be employed to address these interactions and to better predict drug efficacy. Cytotoxic effects of drugs are studied in cultured cells or whole animal models. However, the results from such studies are often difficult to interpret as the effects observed are generally the result of many molecular interactions that may mask, or even prevent, interactions of the drug with its major targets. Manipulation of system components, to identify and to regulate specific interactions, for example, would be of great value in identifying efficacious drugs.

We have devised a simple scheme utilizing *Xenopus* egg extract to compare the inhibition of DNA replication by a chemotherapeutic DNA intercalator, the anthracycline daunomycin (DM), and a non-therapeutic intercalator, ethidium bromide (EtBr). DM intercalates into DNA specifically [5] and has therapeutic value towards the treatments of leukemias [6]. On the other hand, EtBr intercalates into both RNA and DNA [7], and is a powerful mutagen with no known therapeutic value. Inhibition of replication is a known mechanism of cytotoxicity by the anthracycline antibiotics. A benefit of our scheme is that we can manipulate system components to separate drug–DNA interactions from those interactions that may also impact drug efficacy *in vivo*. Consistent with this, we find that simultaneous exposure of DM to DNA and extract results in the inhibition of DNA replication, and the disruption of

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chromatin structure, at low drug concentrations. In contrast, EtBr does not inhibit DNA replication, or disrupt chromatin structure, at similar concentrations under these experimental conditions. However, if EtBr is pre-incubated with DNA before extract addition, inhibition of replication does occur. Neither intercalator inhibits DNA replication at low drug concentrations when pre-incubated with extract before the addition of DNA. Our data indicate that *Xenopus* egg extracts can be used to study the effects of DNA binding drugs on numerous cellular processes and may be a useful approach to screen DNA interacting drugs for chemotherapeutic value.

Materials and methods

Interphase extracts from *X. laevis* eggs and demembranated sperm nuclei were prepared as described [8]. *X. laevis* were obtained from NASCO (Fort Atkinson, WI). All animal protocols were approved by the IACUC. DM and EtBr were purchased from Sigma (St Louis, MO). Stock solutions (10 mM) were prepared in DMSO and aliquots were stored at −80°C. Dilutions were made from the stocks directly and used.

DNA replication assays were carried out as follows. Demembranated *Xenopus* sperm nuclei were incubated in *Xenopus* egg extracts containing an energy regenerating system (150 μg/ml creatine phosphokinase, 60 mM phosphocreatine), 100 μg/ml cycloheximide, 2 mM ATP, 100 μCi/ml [α-^{32}P]dATP and 50 μM deoxynucleotides. Samples were either incubated with drugs or with buffer I (50 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM β-mercaptoethanol). Incubations were carried out at 25°C for 90 min. Reactions were stopped with stop mix C (0.5% SDS, 20 mM EDTA and 20 mM Tris, pH 8.0) supplemented with 500 μg/ml proteinase K. DNA was extracted with phenol chloroform and spotted on Glass microfiber filters. The filters were dried, washed with trichloroacetic acid and DNA was precipitated with ethanol. Incorporated radioactivity was counted in a scintillation counter to determine the amount of DNA synthesized.

Micrococcal nuclease digestion of *Xenopus* sperm chromatin was carried out as described [9] with modifications. First, 3 μg *Xenopus* sperm DNA was incubated with the drugs at the appropriate concentrations or Buffer I for 30 min. Unbound drug was removed by low speed centrifugation. Sperm DNA was assembled into chromatin using *Xenopus* egg extracts at a concentration of 25 ng DNA/μl of extract for 25 min. The reaction was diluted with cold buffer A (60 mM KCl, 15 mM Tris, pH 7.4, 15 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM spermidine and 0.15 spermine). Chromatin was purified and resuspended in MDB (60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 15 mM β-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine, 2 mM $CaCl_2$ and 15 mM Tris, pH 8.5) supplemented with 0.03 IU micrococcal nuclease. The digestion was carried out for 5 min at 25°C and stopped by adding MTB (20 mM EDTA, 0.5% SDS and 20 mM Tris, pH 8.0) supplemented with 0.5 mg/ml proteinase K. DNA was extracted with phenol chloroform and precipitated with ethanol. The precipitated material was digested with RNase and the products were resolved on a 1.8% Metaphor agarose gel. DNA was visualized by EtBr staining.

Nuclear envelopes were stained with Nile Red and visualized as described previously [10]. Nuclear import assays were carried out as described [11]. Briefly, *Xenopus* sperm nuclei were incubated in egg extract supplemented with either T7 RNA polymerase containing the NLS (T7 + NLS) or without the NLS (T7 – NLS). Reactions were carried out for 60 min in the same fashion as DNA replication assays except that the extracts were supplemented with 500 ng T7 RNA polymerase and no radiolabel was added. Nuclei were sedimented upon poly-L-lysine-coated coverslips through a 30% sucrose cushion. T7 RNA Polymerase was probed with a polyclonal anti-T7 RNA polymerase serum and detected with a FITC-conjugated donkey anti-rabbit secondary antibody. Nuclei were visualized and photographed on a Nikon Labophot-2 epifluorescence microscope equipped with a Sensys digital camera.

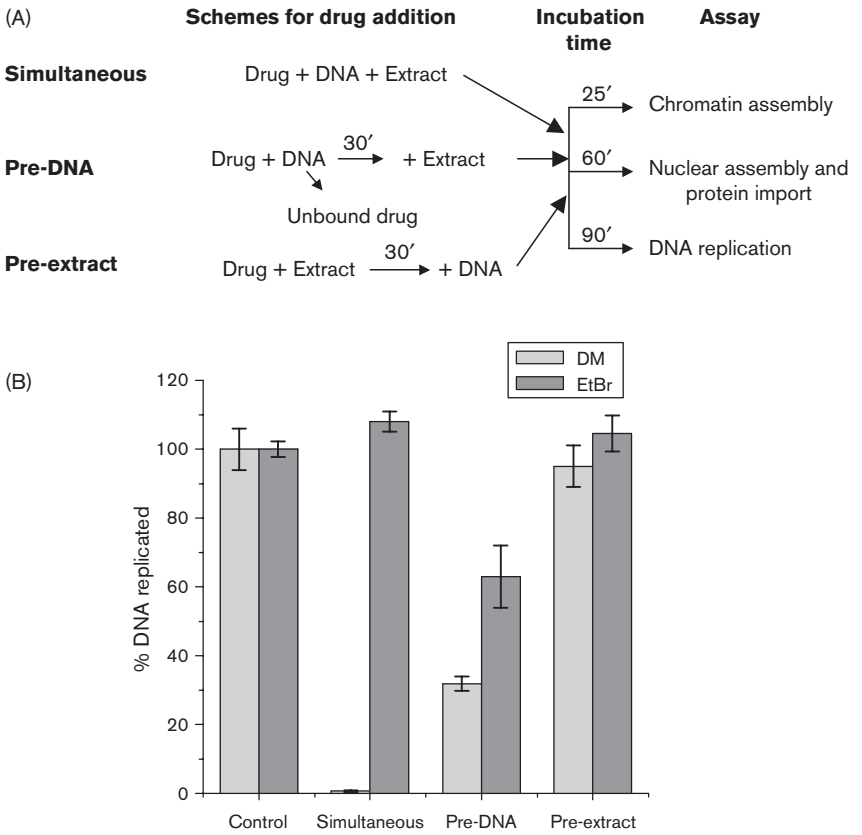
Results and discussion

We have previously shown that *Xenopus* egg extract can be used to study the effects of the DNA intercalator, DM, on nuclear assembly and DNA replication [10]. Here we extend this study in two important ways. First, by ordering the exposure of drug to DNA and extract, we have developed a simple scheme to study the impact of drug–DNA interactions on DNA replication in this system (Fig. 1). Second, we have used this scheme to compare the effects of two DNA intercalators, DM and EtBr, on DNA replication and chromatin structure. DM is a clinically relevant drug while EtBr is not.

Effects of DM and EtBr on DNA replication in *Xenopus* egg extracts

Simultaneous addition of DM, DNA and extract resulted in a complete inhibition of DNA replication at a drug concentration of 5 μM (Fig. 1B, simultaneous). In contrast, we observed no inhibition of replication by EtBr following the simultaneous addition of system components (Fig. 1B, simultaneous). However, when EtBr was pre-incubated with DNA before extract addition (Fig. 1B, pre-DNA), replication was inhibited by approximately 30–40%. DM, on the other hand, was less inhibitory when pre-incubated with DNA before extract addition than when it was added simultaneously with DNA and extract (Fig. 1B, pre-DNA versus Fig. 1B, simultaneous). Finally,

Fig. 1



Effect of DM and EtBr on DNA replication in *Xenopus* egg extract. (A) Experimental schemes employed for drug assessment. (B) *Xenopus* sperm nuclei were incubated in *Xenopus* egg extracts supplemented with [α-^{32}P]dATP, ATP, cycloheximide and an energy regenerating system either with or without 5 μM drug in accordance with the basic strategies outlined in (A). Each bar represents the mean value of at least four independent experiments using different egg extracts. The error bars represent the SEM.

pre-incubating drug with extract before the addition of DNA resulted in nearly complete replication of all DNA in both samples (Fig. 1B, pre-extract). Taken together, these data illustrate three important points. First, DM and EtBr differ in their ability to inhibit DNA replication in egg extract. Second, this differential inhibition is at least partly dependent upon the order in which drug is exposed to the DNA and to the extract. Third, most of the observed inhibition of replication appears to be due to the direct interaction of the drugs with DNA. Minimal inhibition following pre-incubation of drug with extract supports this view (Fig. 1B, pre-extract). However, we cannot rule out the possibility that interaction of the drugs with non-DNA components of sperm chromatin interferes with replication.

The nearly complete inhibition of DNA replication by DM following simultaneous addition of all system components (Fig. 1B, simultaneous) agrees well with our earlier study [10], with *in vivo* studies using mammalian cells [12,13] and with *in vitro* studies using purified components [14]. The comparative loss of inhibitory activity when the drug was mixed with DNA before extract addition (Fig. 1B, pre-DNA) may be due to the loss of weakly bound DM molecules from the DNA when the nuclei are removed from the drug milieu and placed in drug-free extract (Fig. 1A, pre-DNA). DM has been shown to exhibit different affinities for different DNA sequences and DNase footprinting studies have revealed that it preferentially binds to the triplet sequences 5 (A/T) GC and 5 (A/T) CG [15]. The difference in inhibition may also relate to the nature of sperm chromatin prior to extract exposure. Sperm chromatin is highly compact until it undergoes rapid decondensation in egg extract. The decondensing activity in the extract may facilitate the binding of drug molecules to all possible binding sites resulting in a greater inhibition of replication. On the other hand, when DM is pre-incubated with highly condensed sperm nuclei, drug binds only to accessible regions of the sperm DNA limiting its impact on replication in extract. Finally, it is possible that the inhibition of DNA replication in

this system is the cumulative effect of drug interactions with DNA and extract components. This possibility seems less likely, however, in that pre-incubation of DM with extract results in very little inhibition of replication following the addition of DNA (Fig. 1B, pre-extract).

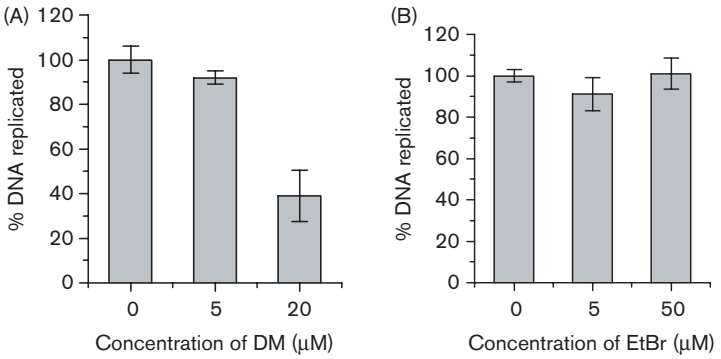
Incubation of DM with extract, prior to addition of sperm DNA, did not impact DNA replication at low drug concentrations (Fig. 2A, 5 μM). This suggests that extract components sequester the drug and reduce its binding to DNA. If so, increasing DM concentration should saturate the extract with drug and result in greater DNA binding and an inhibition of replication. A 4-fold higher concentration of drug inhibits replication by around 60% (Fig. 2A, 20 μM), suggesting that DM may be sequestered by components of the extract that are not critical for DNA replication. However, we cannot rule out subtle drug-extract effects that, along with DNA binding, are important regulators of inhibition. DM is known to bind lipids [16,17] and the extract is a membrane-rich environment.

EtBr did not inhibit DNA replication at all in our assay when mixed with DNA and extract simultaneously (Fig. 1B, simultaneous). Indeed, even when the concentration of drug was raised 10-fold, to 50 μM, no inhibition of replication was observed (Fig. 2B). This was somewhat surprising given that EtBr is a known inhibitor of nucleic acid synthesis *in vivo* [18] and *in vitro* [19]. EtBr binds more avidly to RNA than DNA [5] and given that egg extracts contain large quantities of RNA, it is possible that RNA sequesters the drug thereby preventing inhibition. RNase treatment of the extract was unsuccessful due the presence of ribonucleoprotein complexes (data not shown). Unlike DM, pre-incubating EtBr with DNA resulted in more inhibition than did simultaneous addition of EtBr to DNA and extract (compare Fig. 1B, pre-DNA with simultaneous). This observation reinforces the idea that EtBr is sequestered by extract components before it can bind DNA. As compared to drug-free samples (Fig. 1B, control), pre-incubation of DM with DNA results in around 70% inhibition of replication in extract compared to around 40% inhibition with EtBr, around 1.75-fold difference in efficiency (Fig. 1B, pre-DNA). DM has a 1.8-fold higher binding affinity for DNA than EtBr [20 and references therein] consistent with our results. However, when allowed to bind DNA in the cellular milieu, DM inhibits replication completely, whereas the same concentration of EtBr has little to no effect. These results reinforce the importance of the environment in determining the efficacy of molecular interactions.

The impact of DM and EtBr on nuclear membrane assembly and nuclear protein import

Sperm chromatin must decondense, undergo remodeling and assemble into a functional nucleus prior to the initiation of DNA replication in egg extract. Thus, we can assess drug-induced changes in these processes through a number of simple assay methods. In this way, we may gain valuable mechanistic insights in to the mode of drug action. One of the prerequisites for replication is the formation of an intact nuclear membrane surrounding the DNA [21]. The extract is membrane-rich and given that DM is known to interact with membranes [16,17] we wanted to determine if DM inhibits DNA replication by preventing the formation of an intact nuclear membrane. The fluorescent lipid dye Nile Red was employed to visualize membrane integrity directly. Following simultaneous addition of DM, DNA and extract, no membrane disruption was observed at the concentration of drug that completely abolishes replication (i.e. 5 μM), suggesting

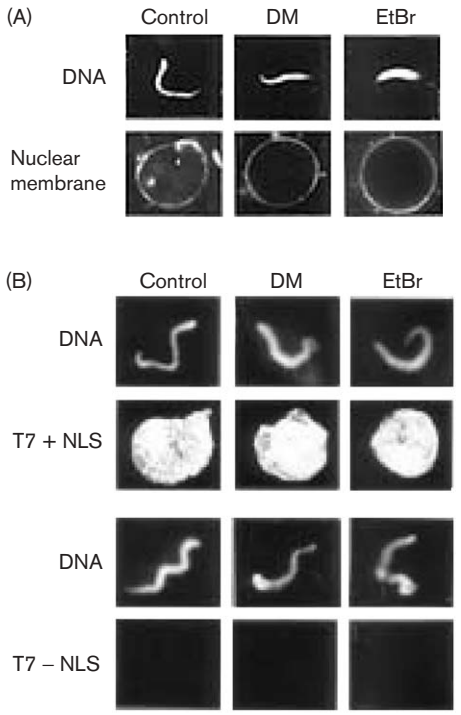
Fig. 2



High drug concentrations impact DNA replication differentially. (A) DM was incubated, at the concentrations indicated, in *Xenopus* egg extracts supplemented with [α-^{32}P]dATP, ATP, cycloheximide and an energy regenerating system for 30 min according to the 'pre-extract' scheme described in Figure 1(A). Sperm nuclei were subsequently added and the extent of DNA replication determined after an additional 90-min incubation (Fig. 1A, pre-extract). (B) EtBr was incubated, at the indicated concentrations, in egg extracts supplemented with [α-^{32}P]dATP, ATP, cycloheximide, an energy regenerating system and sperm nuclei for 90 min according to the 'simultaneous' strategy described in Fig. 1(A) (simultaneous). Each bar represents the mean value of two independent experiments using different egg extracts. The error bars represent the SEM.

that inhibition is not due to a failure to assemble a nuclear membrane. This conclusion is also supported by our observation that DNA replication occurs in extract even when DM is pre-incubated with the membrane vesicles that are required for nuclear membrane assembly (Fig. 1B, pre-extract). However, if the concentration of DM is increased 4-fold (20 μM), individual membrane vesicles fail to fuse and do not give rise to a complete nuclear membrane in some nuclei [data not shown; 10]. This could account for the reduction in replication observed in Figure 2(A). As expected from the replication data, EtBr did not inhibit nuclear membrane assembly at the same concentrations (Fig. 3A). DNA replication also requires import of nuclear proteins from the extract through a structurally intact, and functional, nuclear membrane. To determine if nuclei assembled in the presence of drug retain this function, we monitored the import of a transport probe, the bacteriophage T7 RNA polymerase constructed with a NLS, following simultaneous addition of system components. Import of the probe occurred in most nuclei (Fig. 3B, T7 + NLS). By contrast, the T7 RNA polymerase lacking the NLS (negative control) was not imported into nuclei (Fig. 3B, T7 – NLS). These results confirm the presence of intact and functional nuclear membranes surrounding sperm DNA and indicate that DM does not inhibit replication at low drug concentrations by disrupting nuclear structure and/or function.

Fig. 3



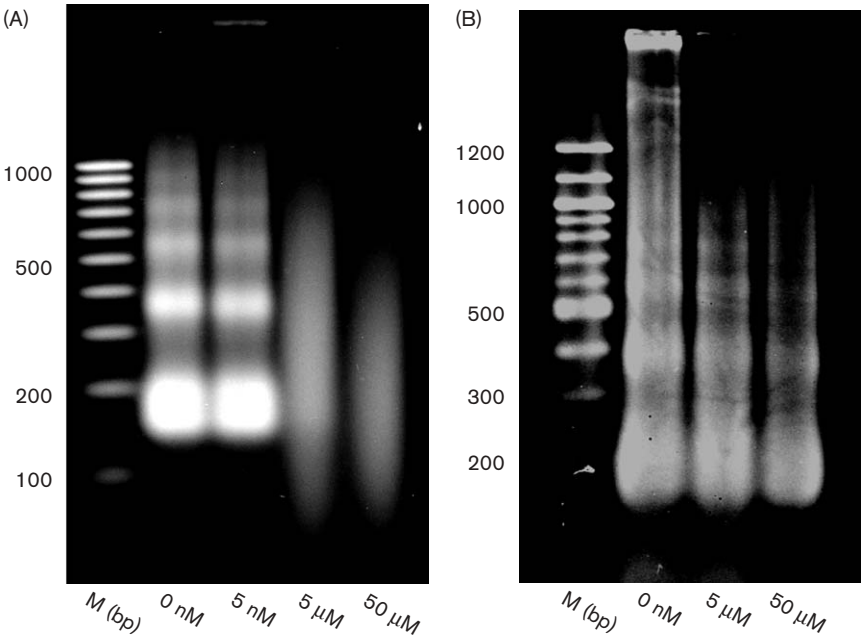
Drug treatment does not inhibit nuclear membrane assembly or nuclear protein import. (A) DM or EtBr were incubated with 5 μM drug in egg extracts supplemented with ATP, an energy regenerating system, cycloheximide and *Xenopus* sperm nuclei for 90 min (Fig. 1A, simultaneous). Nuclear membranes were stained with the lipid dye Nile Red. DNA was visualized by staining with Hoechst 33258. (B) DM or EtBr were incubated with 5 μM drug in egg extracts supplemented with ATP, an energy regenerating system, cycloheximide, T7 RNA polymerase containing the NLS (T7 + NLS) and T7 RNA polymerase without the NLS (T7 – NLS) and with *Xenopus* sperm nuclei for 90 min (Fig. 1A, simultaneous). Nuclei were fixed and probed with a polyclonal anti-T7 RNA polymerase anti-serum that was detected by a FITC-conjugated donkey anti-rabbit secondary antibody.

Drug treatment and chromatin structure

The DNA in all eukaryotes exists as a nucleoprotein filament called chromatin [22]. The nucleosome is the basic repeating unit of chromatin, and consists of an octamer of two molecules each of the core histones H2A, H2B, H3 and H4, around which DNA is wrapped. Histones not only help to package the DNA, but also serve to regulate access to the information contained within the primary sequence. Changes in chromatin structure can affect essential processes such as replication and, therefore, we sought to determine if DM or EtBr disrupts the regular spacing of nucleosomes assembled on DNA by the extract [1,8]. DM or EtBr were incubated with sperm nuclei prior to extract addition (Fig. 1A, pre-DNA). Chromatin structure was assayed by digestion with micrococcal nuclease that cleaves the DNA in between nucleosomes giving rise to a ladder of bands corresponding to mono-, di-, tri- nucleosomes and higher oligomers. Addition of DM at 5 nM results in no change in the nucleosome repeat pattern relative to the untreated control (0 nM); however, a concentration of 5 μM drug severely disrupts chromatin structure as judged by the loss of the nucleosomal ladder (Fig. 4A). This property of DM may contribute to its potency as a chemotherapeutic by inhibiting the chromatin-mediated regulation of replication, transcription, etc. These results are consistent with previous studies using assembled chromatin isolated from HeLa S3 cells [23]. EtBr did not disrupt chromatin structure even at a concentration of 50 μM (Fig. 4B) suggesting that the inhibition of replication observed in Figure 1(B) (pre-DNA) is mediated through other mechanisms. The appearance of highly condensed (nuclease insensitive) chromatin in the non-treated control sample (Fig. 4B, 0 nM) may be attributed to incomplete decondensation and/or aggregation that occasionally occurs during the recovery of chromatin from the extract. DM has been previously reported to disrupt chromatin structure more severely than EtBr [23]. Our results demonstrate that a similar difference exists when chromatin assembly occurs on drug-bound DNA.

In summary, we have utilized the *Xenopus* egg extract system for comparative analyses of DNA interacting drugs, aiming to explore its potential for screening DNA interacting compounds that may have therapeutic value against cancer. By comparing the effects of two known

Fig. 4



Effect of DM and EtBr on chromatin structure. *Xenopus* sperm nuclei were incubated with the indicated concentrations of DM (A) or EtBr (B) for 30 min. Unbound drug was removed and the nuclei were incubated in egg extract for 25 min (Fig. 1A, pre-DNA). Chromatin structure was assayed by digesting the DNA with micrococcal nuclease. The digestion products were resolved on a 1.8% metaphor agarose gel and stained with EtBr for visualization.

DNA intercalators in this system, we have clearly demonstrated that DM, a clinically useful chemotherapeutic, is a more potent inhibitor of DNA replication than EtBr, a compound with no known therapeutic value. Furthermore, we were able to study the impact of these drugs on DNA replication independent of any ongoing transcription or translation. Our data also suggest that the inhibition we observe at low drug concentration is predominantly due to the association of DM with DNA and the resultant disruption of chromatin structure, and not due to the inactivation of essential replication factors from the cytoplasm or due to the disruption of the nuclear membrane or nuclear protein import. This experimental system should allow for the dissection of multiple interactions that these drugs can have in complex biological systems.

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