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Spinach thioredoxin m inhibits DNA synthesis in fertilized *Xenopus* eggs

(DNA initiation/gastrulation/cytokinesis)

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ABSTRACT **A role for thioredoxin in metazoan DNA synthesis has been assessed by injecting rapidly dividing *Xenopus* eggs with purified heterologous thioredoxins, which might act as inhibitors if they were to replace resident thioredoxins in some but not all reaction steps. Of 10 tested proteins, spinach chloroplast thioredoxin m is the most potent inhibitor. Eggs cleave and produce cells lacking nuclei. DNA synthesis is severely reduced. Development arrests before gastrulation. In egg extracts, thioredoxin m inhibits incorporation of radioactive dCTP into DNA of sperm nuclei and M13 phage. Inhibition exceeds 90% when thioredoxin m and M13 DNA are preincubated together. The data support the interpretation that thioredoxins normally participate in initiation of metazoan DNA synthesis.**

Thioredoxins are a widely distributed group of proteins that mediate both redox and nonredox reactions in prokaryotic and eukaryotic cells (1–4). In the latter capacity, thioredoxin functions as an essential subunit of phage T7 DNA polymerase (5, 6) and in the maturation of DNA filamentous phages F1 and M13 (7, 8). Aside from yeast, where inactivation of the two resident genes for thioredoxin (9–11) results in a 4-fold longer S phase (10), there is little information on the involvement of thioredoxins in metazoan DNA synthesis. We chose to test for such involvement by asking whether injected foreign thioredoxins can interrupt DNA synthesis in *Xenopus* eggs shortly after fertilization. It was expected that a foreign thioredoxin might sufficiently resemble metazoan homologs to act as a substitute in certain, but not all, cellular reactions and therefore act as a specific inhibitor. Of the representatives selected, thioredoxin m from spinach chloroplasts shows the most effective inhibition and blocks embryonic development. The fertilized eggs undergo cell cleavage, but DNA synthesis is severely reduced, and the resultant cells lack nuclei. Thioredoxin m also inhibits incorporation of labeled precursor into DNA of sperm nuclei and M13 phage. The data suggest a function for thioredoxin in initiation of DNA synthesis.

MATERIALS AND METHODS

Proteins and DNA. M13 phage DNA was obtained from R. Harland (University of California, Berkeley) as a stock sample [1 mg/ml in TE buffer (10 mM Tris/Tris·HCl/1 mM Na_2EDTA, pH 8.0)]. Spinach thioredoxin m was used as a stock solution of 0.38 mg per ml of TB (thioredoxin buffer; 30 mM Tris/Tris·HCl, pH 7.8).

Egg Injection and Scoring of Embryos. *Xenopus* eggs were obtained and fertilized *in vitro* by standard procedures (12), and each was injected with 30 nl of the specified thioredoxin in TB at the stock concentrations indicated in the figure legends and tables or with TB (buffer) alone 40–60 min postfertilization—that is, prior to first cleavage. Embryos were allowed to develop in MMR/5 medium at 21°C (see ref. 12 for composition of MMR medium). The progress of development was scored at various intervals. Arrested blastulae were identified as eggs that had cleaved to numerous cells but did not form a blastopore and did not initiate gastrulation. Arrested gastrulae were identified as those that initiated gastrulation but failed to close the blastopore (large yolk plug remaining) and did not initiate neurulation. Arrested neurulae closed the blastopore and raised neural folds but did not complete closure of the folds or differentiate specific tissues such as eyes or muscle.

DNA Staining and Microscopic Analysis of Nuclei. Eggs were injected and allowed to develop in MMR/5 medium at 21°C for 8 hr to reach the midblastula transition (13). They were fixed in 4% formaldehyde in MMR/5 for 1 hr. Animal caps were cut off and transferred to MMR/5 containing Hoechst dye 33258 (bisbenzimide), flattened under a coverslip, and observed with Zeiss fluorescence optics.

Preparation of *Xenopus* Sperm Nuclei and Egg Extracts. *Xenopus* sperm nuclei were prepared by the method of Lohka and Masui (14) and were demembranated by the inclusion of 400 μg of lysolecithin per ml of sperm heads (≈10^6 sperm heads per ml). Extracts from unfertilized *Xenopus* eggs were also prepared by published methods (14, 15), with a final centrifugation at 10,000 × *g* for 5 min in a microcentrifuge at a 90° angle.

DNA Replication by Sperm Nuclei. Egg extract (20 μl) was added to 2 μl of sperm nuclei, 1 μl of [α-^{32}P]dCTP (0.1 mCi; 1 Ci = 37 GBq), and 1 μl of spinach thioredoxin m [in TB (pH 7.8) at the concentrations indicated in the figure legends and tables, which are the final concentrations of thioredoxin in the reaction mixtures] or TB alone. The samples were mixed and incubated at 21°C for 60 min. Thereafter, samples were frozen, and the next day they were thawed and incubated with an equal volume of 1% SDS/0.5 mg of proteinase K per ml (Sigma) at 37°C for 60 min. This preparation was then homogenized with 60 μl of water-saturated phenol and centrifuged at 10,000 × *g* for 2 min to separate the phases. The water-soluble portion was withdrawn and 50 μl was applied to the sample well of a 1% agarose gel and electrophoresed at 200 V for 30 min with electrode buffers of TAE (40 mM Tris/Tris acetate/1 mM Na_2EGTA, pH 8.0). The dried gel was autoradiographed to locate bands of radioactive macromolecular products (16-hr exposure). The major band was found 5 cm into the gel for each lane. The gel was cut at this position and radioactivity was quantified by scintillation spectrometry. These values are expressed as cpm incorporated in Table 2.

M13 DNA Synthesis. Egg extract (16 μl) was combined with 1 μl of M13 DNA, 1 μl of radioactive deoxynucleotides ([α-^{32}P]dCTP; 10^5 dpm per reaction), 1 μl of 5 mM dithio-

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Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

threitol (DTT), and 1 μl of TB or thioredoxin m in TB at 23°C. Aliquots of 2 μl were removed from the reaction mixture at 12.5-min intervals, and each was added to 1 ml of 5% trichloroacetic acid (TCA) in a vial at 0°C. The precipitate was collected by filtration on a Whatman GF/C filter and washed with 5% TCA before counting in a Beckman LS7000 scintillation spectrometer with ^{32}P settings (Cerenkov radiation). The supernatant was also counted, and the extent of synthesis was expressed as a percentage of total radioactivity incorporated into acid-insoluble material.

For preincubations of M13 DNA and thioredoxin, these were mixed (1 μl of each plus 1 μl of 5 mM DTT) and kept in a sealed 0.5-ml Eppendorf tube (to prevent evaporation) for various lengths of time as indicated in Fig. 2, and then egg extract and radioactive deoxynucleotides were added to the tube to initiate the reaction.

Electrophoretic Analysis of M13 DNA. M13 DNA (1 mg/ml in TE) was mixed with an equal volume of spinach thioredoxin m (0.38 mg/ml in TB) and an equal volume of 5 mM DTT and allowed to incubate at 23°C for 60 min. The sample (9 μl per lane) was then analyzed by electrophoresis in a 1.2% agarose gel with ethidium bromide (1 μg/ml) for 30 min at 200 V, with luminol blue as a marker dye. The gel was photographed with long UV illumination, as shown in Fig. 3. DNA species were assumed to be those identified by Mechali and Harland (16).

RESULTS

For quantification of effects on DNA synthesis, we chose to inject thioredoxins of significant sequence divergence from those of metazoa (17) into *Xenopus* eggs shortly after fertilization. This egg is a particularly favorable cell for such a test because (*i*) foreign proteins can be easily injected into the cytoplasm, owing to its large size, (*ii*) DNA synthesis is a major synthetic activity (S phase, 10–15 min; cell cycle, 25–30 min), (*iii*) many other products have been stockpiled before fertilization, and so inhibition of their synthesis should not affect development [e.g., deoxynucleotides are present in amounts for 12 cell cycles of DNA synthesis (18)], and (*iv*) damage to DNA synthesis should be seen at the late blastula/early gastrula stages when zygotic gene expression is first required for continued development (13).

Purified thioredoxins from 10 bacterial, plant, and animal sources were tested. These include representatives from chloroplasts (m type from spinach), heterotrophic bacteria (*Escherichia coli*), and photosynthetic bacteria (*Chromatium vinosum*). As detailed in Table 1 and the following text, most of the thioredoxins did not affect development, but thioredoxin from spinach chloroplasts (m type) had a strong inhibitory effect when injected into eggs from stock solutions of 0.1 mg of protein per ml. Although injected embryos appeared to cleave normally for at least 10 division cycles, none was able to gastrulate at the normal time (10 hr postfertilization) as reflected by the absence of a blastopore. Shortly thereafter, the embryos disaggregated into single cells—an effect well known for *Xenopus* embryos in which zygotic transcription has been inhibited [e.g., by α-amanitin (19)]. Spinach thioredoxin m was the most inhibitory of the proteins tested; when the stock was diluted to 25 μg of thioredoxin per ml, and 30 nl was injected per egg, 50% of the embryos still failed to gastrulate. With this amount (0.75 ng per egg), the internal concentration of thioredoxin m would be ≈1 μM, approximately the level at which thioredoxins are thought to occur in cells (1). At lower concentrations (0.37 ng of protein per egg), most embryos initiated gastrulation, but all arrested in the midst of this process or at neurulation (data not shown). No tadpole hatched from the injected eggs at this or higher concentrations. Inhibition was observed with four different spinach thioredoxin m samples purified in different ways, suggesting that this thioredoxin, and not a trace contaminant, is the inhibitor.

Table 1. Inhibition of *Xenopus* development by injected thioredoxins

Thioredoxin injected	% injected eggs arresting at stage			% not arresting
	Blastula	Gastrula	Neurula	
Spinach m (0.1 mg/ml; n = 80)	90	10	0	0
Spinach m NEM (0.1 mg/ml; n = 33)	27	73	0	0
E. coli (0.2 mg/ml; n = 22)	22	78	0	0
Chromatium (1.6 mg/ml; n = 39)	0	3	3	94
TB buffer (n = 40)	0	0	0	100
Chromatium + *E. coli* (0.5 + 0.1 mg/ml; n = 16)	0	80	20	0

Fertilized *Xenopus* eggs were each injected with 30 nl of the specified thioredoxin in TB at the stock concentration shown or with TB alone prior to first cleavage. Progress of development was scored at various intervals. Arrested blastulae were identified as eggs that had cleaved to numerous cells but did not form a blastopore and did not initiate gastrulation. Arrested gastrulae were identified as those that initiated gastrulation but failed to close the blastopore (large yolk plug remaining) and did not initiate neurulation. Arrested neurulae closed the blastopore and raised neural folds but did not complete closure of the folds or differentiate specific tissues such as eyes or muscle.

When spinach thioredoxin m was reduced with 10 mM DTT to eliminate its hydrogen-accepting disulfide group prior to injection, its inhibitory activity was not altered. When this reduced material was subsequently derivatized with *N*-ethylmaleimide (NEM) to prevent disulfide bond formation and to eliminate redox cycling, and then injected, it was still inhibitory, although requiring 4 times more material for an effect equal to that of the underivatized form. Thus, the catalytic redox cycle is not needed for the inhibitory effect. (The effectiveness of the NEM derivatization of thioredoxin was measured in the NADP-malate dehydrogenase activation assay, and thioredoxin was found to be inactivated by at least 98%.)

As shown in Table 1, *E. coli* thioredoxin is also inhibitory, but at least 10-fold more protein is required than in the case of the spinach thioredoxin m protein. The *E. coli* protein blocked gastrulation in 50% of the cases when injected at ≈9 ng per egg; the blocked embryos resembled those injected with the spinach thioredoxin m material. Other thioredoxins were not effective at the highest doses injected per egg—namely, those from *Chromatium* (48 ng), other photosynthetic bacteria (*Chlorobium limicola* forma thiosulfatophilum, 15 ng; *Rhodospirilum rubrum*, 12 ng), a fermentative bacterium (*Clostridium pasteurianum*, 15 ng), from plants (the spinach f type, 5 ng), and from animals (calf thymus, 15 ng; human reticulocyte, 15 ng). Similarly, *E. coli* glutaredoxin—a disulfide protein that can replace thioredoxin in ribonucleotide reduction and certain other reactions (1, 2)—was also ineffective (30 ng per egg). Thus, inhibition was specific with regard to the thioredoxin source.

Since thioredoxins participate in the reduction of ribonucleotides to deoxyribonucleotides, we tested the capacity of deoxynucleotides to reverse the spinach thioredoxin m inhibition of *Xenopus* development, but we found no rescuing effect with injected mixtures of the four deoxynucleoside triphosphates in amounts to allow at least one extra cell cycle (0.6 mM each in the stock solution; 30 nl delivered per egg). Higher levels of injected deoxynucleotides cannot be tested since, as shown by others (20), they themselves block cleav-

age after the 12th cycle. Thus, the primary inhibitory effect of spinach thioredoxin m is probably not on ribonucleotide reduction, a suggestion fitting the fact that *Xenopus* eggs store sufficient deoxynucleotides for 12 rounds of DNA synthesis (18).

To analyze further the dramatic inhibition of *Xenopus* development by spinach thioredoxin m, we observed the early cleavages of injected eggs by time-lapse videomicroscopy. Cytokinesis was complete and normally timed for the first 9 or 10 divisions, but then it became increasingly irregular. The midblastula transition (at the 12th cycle) from rapid, synchronous to slower, asynchronous cell cycles was not sharp, as it is in normal embryos (13) or in ones in which transcription has been blocked by α-amanitin (13, 19). As pointed out to us by John Newport, the thioredoxin-affected cleavage pattern resembles that seen for *Xenopus* eggs injected with aphidicolin, an inhibitor of DNA synthesis (15, 21). Dasso and Newport (15, 21) discovered that when DNA synthesis is retarded in *Xenopus* eggs at early cleavages, nuclei fail to form; there is, nonetheless, a cyclic formation of centrosomes, spindles, and cleavage furrows. [These early cell cycles are under cytoplasmic control and do not require zygotic transcription (12).] In aphidicolin-treated eggs, several thousand cells are formed without nuclei (15).

The effect of spinach thioredoxin m on DNA synthesis was examined in two ways. First, eggs at the one-cell stage were doubly injected with the protein (3.8 ng per egg) and with [α-^{32}P]dCTP (≈0.03 μCi per egg) and then sampled at 1-hr intervals until 8 hr postfertilization to assess the incorporation of radioactivity into DNA (acid-insoluble radioactivity; see *Materials and Methods*). By 8 hr, incorporation in the thioredoxin-containing eggs was <4% that of control eggs injected with the same amount of [α-^{32}P]dCTP in conjunction with thioredoxin buffer. Second, eggs injected at the one-cell stage with spinach thioredoxin m were examined 8 hr after fertilization, when several thousand cells were present. Indeed, nuclei were absent, as scored by Hoechst staining (Fig. 1*A*). Not even small nuclei or DNA fragments were observable. Eggs injected with the less effective *E. coli* thioredoxin, by contrast, contained nuclei, but these were smaller and fewer in number than in normal embryos (data not shown). Thus, we conclude that spinach thioredoxin m-injected embryos fail to gastrulate simply because they lack DNA and are therefore unable to synthesize the zygotic mRNAs needed for gastrulation and for the continued adhesion of cells. RNA synthesis in thioredoxin m-injected embryos was indeed depressed to <10% of normal at the scheduled time of gastrulation (assessed as the incorporation of [α-^{32}P]GTP radioactivity, injected in the one-cell stage, into acid-insoluble material; data not shown).

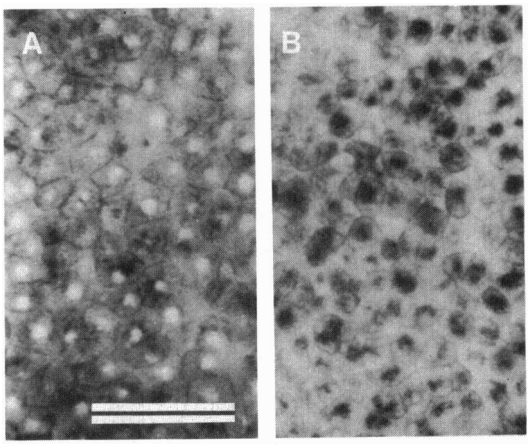

FIG. 1. Reduced DNA in cells of *Xenopus* embryos cleaved from eggs injected with spinach thioredoxin m. (*A*) DNA in nuclei stained with Hoechst dye appears as white spots (control embryo). Low-level white light epiillumination (from a fiber optic lamp) was used to reveal cell outlines of the embryo's surface. (*B*) Surface of a thioredoxin-injected embryo. Notice the cell outlines, documenting the occurrence of many cleavages, but the reduction or absence of Hoechst-staining nuclei. (Bar = 50 μm.)

To test more directly the effect of spinach thioredoxin m on DNA synthesis, we prepared lysolecithin-permeabilized *Xenopus* sperm heads as a source of nuclei and DNA, combined them with extracts of activated *Xenopus* eggs [Lohka–Masui extracts (14)], and followed the *in vitro* incorporation of [α-^{32}P]dATP into acid-precipitable material (DNA). Compared to controls, sperm nuclei underwent normal swelling (related to the uptake of nuclear proteins and formation of the nuclear envelope) in the presence of spinach thioredoxin m. However, as shown in Table 2, DNA synthesis was inhibited 70–80% in the first hour, with thioredoxin concentrations equivalent to those used *in vivo* to produce anucleate cells and to block gastrulation. The NEM derivative of thioredoxin m was also inhibitory in this assay for DNA synthesis. In the presence of the native protein or the NEM derivative, the reaction had an increased lag period, as if initiation might be particularly sensitive to inhibition.

Finally, we attempted to determine under more defined conditions whether inhibition of DNA synthesis concerned the initiation or elongation phase of replication (16). *Xenopus* egg extracts were supplemented with phage M13 single-stranded circular DNA as a template suitable to detect both initiation and elongation. DNA synthesis was quantified by the incorporation of [α-^{32}P]dCTP into acid-insoluble material. As shown in Fig. 2, spinach thioredoxin m strongly inhibits DNA synthesis under preincubation conditions—i.e., when thioredoxin was preincubated with DNA for 15 min or more at room temperature (23°C in TB), after which time *Xenopus* extract and radioactive dCTP were added in rapid succession. Under these conditions, DNA synthesis was inhibited 50% with 20 min of preincubation and >90% when preincubation lasted 60 min. In contrast, when spinach thioredoxin m was added to the extract at the same time as DNA and nucleotides, little or no inhibition was observed in the amount of incorporation into DNA after a 60-min period (<15%; Fig. 2, see 0 min PI entry). In other assays with some preparations of extract (data not shown), thioredoxin m inhibited incorporation by 30–50% at the early times of the reaction (15–30 min after initiation), when added simultaneously with DNA and radioactive dCTP, although incorporation levels eventually reached those of controls by 60 min. This transient inhibition shows itself as an increased lag

Table 2. Inhibition of DNA synthesis in *Xenopus* sperm nuclei by spinach thioredoxin m

Material added to reaction mixture	cpm incorporated	% of control
None (TB buffer control)	11,321	100
Thioredoxin m (16 μg/ml)	3,641	32
Thioredoxin m (32 μg/ml)	1,667	14
Thioredoxin m NEM (14 μg/ml)	4,543	40

Xenopus sperm nuclei and unfertilized egg extracts were prepared as described. For assays of DNA replication, extract, sperm nuclei, [α-^{32}P]dCTP, and spinach thioredoxin m or the NEM derivative in TB buffer, or TB buffer alone, were mixed and incubated at 21°C for 60 min. Thereafter, samples were frozen and the next day were thawed and treated with SDS, proteinase K, and phenol as described. The water phase was withdrawn and 50 μl was applied to the sample well of a 1% agarose gel and electrophoresed at 200 V for 30 min with TAE electrode buffers. Dried gel was autoradiographed to locate bands of radioactive macromolecular products, and the major band was found 5 cm into the gel for each lane. The gel was cut at this position and radioactivity was quantified by scintillation spectrometry. These values are entered as cpm incorporated.

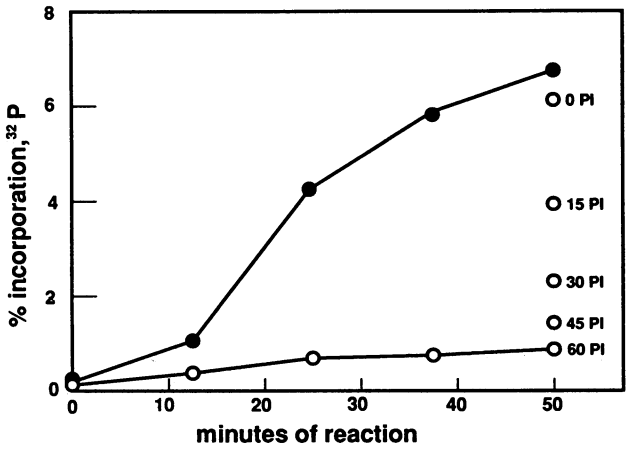

FIG. 2. Inhibition of DNA synthesis from M13 single-stranded circular DNA by spinach thioredoxin m. Vertical axis indicates percentage of total radioactivity incorporated into acid-insoluble material (DNA); horizontal axis indicates time (min) elapsed from addition of [α-^{32}P]dCTP to the mixture of M13 DNA, egg extract, and thioredoxin or thioredoxin buffer. ●, Reactions run in the absence of thioredoxin; ○, reactions run in the presence of thioredoxin. Numbers to the right of the open circles indicate time (min) of preincubation (PI) of DNA and thioredoxin before extract and radioactive dCTP were added.

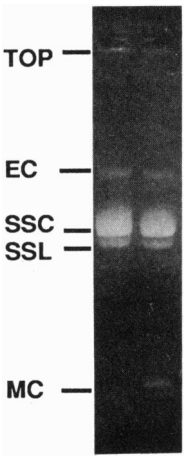

FIG. 3. Electrophoretic analysis of M13 DNA after incubation with thioredoxin m. M13 DNA was incubated with thioredoxin m for 60 min as described. The sample was then analyzed by gel electrophoresis. The gel was photographed with long UV illumination. Fluorescent bands include the following: TOP, top of gel; EC, *E. coli* DNA, minor contamination; SSC, single-stranded circular M13 DNA; SSL, single-stranded linear M13 DNA; MC, minicircle single-stranded M13 DNA. See ref. 16 for identification of DNA species. Left lane, M13 DNA plus thioredoxin; right lane, DNA alone. Note the absence of the minicircle form of M13 DNA in the left lane.

period before maximum rates of incorporation are reached, as if initiation had been slowed. Similar inhibitory effects, with a dependence on the time of preincubation with DNA, are also found for NEM thioredoxin m, indicating the dispensability of free thiol groups for the effect.

The results presented so far suggest that the initiation phase of DNA synthesis, rather than elongation, is preferentially inhibited. To test this possibility further, we added DNA to the extract alone and let the mixture incubate at room temperature for 10 min to allow the initiation to proceed before adding thioredoxin m and radioactive deoxynucleotide. In this case, we observed no inhibition even at the early times of incorporation. Thus, the incorporation pattern is consistent with the proposal that initiation of DNA synthesis is preferentially affected by thioredoxin m.

Alternatively, thioredoxin m could of course inhibit by degrading DNA during the long preincubation period. That this was not the case is shown by the near-normal electrophoretic profile of M13 DNA (Fig. 3) after 60 min of incubation with thioredoxin m. The only differences seen for DNA exposed to this protein are (*i*) the absence of a minor species, the small circular form of M13 still containing the origin sequence for DNA synthesis in *E. coli* (see ref. 16); (*ii*) a slight smearing of the full-sized circular DNA; and (*iii*) the possible trapping of a small amount of DNA at the top of the gel. Particularly intriguing is the removal or displacement of the small circular form, presumably as protein–DNA complexes. This may indicate a sequence-specific binding of thioredoxin.

As a summary comparison of the effects of various thioredoxins, the spinach thioredoxin m material strongly inhibits M13 DNA synthesis *in vitro*, inhibits DNA synthesis and eliminates nuclei in cleaving *Xenopus* eggs, and blocks development at low concentrations. The *E. coli* thioredoxin did not inhibit M13 synthesis *in vitro* and was 10-fold less effective at eliminating nuclei and blocking development in *Xenopus* eggs. The *Chromatium* protein inhibited M13 DNA synthesis slightly (<20% after 60 min of preincubation) *in vitro*, but it had no effect *in vivo*. Thus, the effects of the proteins in the egg and in extracts are not fully parallel, and DNA synthesis in the *Xenopus* egg is probably not equivalent to M13 DNA synthesis in extracts with regard to inhibition.

DISCUSSION

Ten heterologous thioredoxins, many differing significantly in sequence from vertebrate forms, have been tested for their effects on DNA synthesis in *Xenopus* eggs, and the spinach chloroplast thioredoxin m-type protein is found to inhibit strongly. By the midblastula stage of thioredoxin m-injected embryos, nuclei are absent, and the incorporation of radioactive deoxynucleotides into DNA is reduced by 95%, even though cytokinesis continues. Ribonucleotide reductase is not a likely target of inhibition since *Xenopus* eggs contain an abundance of deoxynucleotides (18). Anucleate cells are also produced in *Xenopus* eggs by aphidicolin, a well known inhibitor of DNA synthesis (15, 21). We cannot estimate thioredoxin's inhibition of DNA replication in eggs since chromosomal damage at mitosis probably also contributes to the loss of DNA. Before the midblastula stage, cleavages occur on schedule (driven by oscillations of M-phase promoting factor; ref. 12) even when replication is unfinished. Any slight inhibition of DNA synthesis by thioredoxin m would lead to incompletely replicated chromosomes, and these would be fragmented at mitosis (22) with the loss of non-centromere-containing pieces. After a few rounds of slightly incomplete replication followed by fragmentation, chromosomal DNA may be largely eliminated.

We therefore turned to a study of DNA synthesis *in vitro* in extracts of *Xenopus* eggs to learn more about the inhibition. When *Xenopus* sperm nuclei are used as a DNA source, thioredoxin m inhibits the incorporation of radioactive deoxynucleotides into DNA by 70–80% over short intervals (60 min) under circumstances where chromosome fragmentation is not a consideration. The inhibition involves a lengthening of a lag period before synthesis reaches its maximum rate, as if initiation is particularly sensitive. Thus, our *in vitro* results complement our *in vivo* results.

We then switched to the use of M13 DNA as a defined template for DNA synthesis in *Xenopus* egg extracts and found that thioredoxin m does not inhibit synthesis (dCTP incorporation) if added after the reaction has started, inhibits weakly if added at the same time as the extract, and inhibits strongly if preincubated with M13 DNA before extract is added. With increasing preincubation periods, the subse-

quent synthetic reaction has an even longer lag period, as if initiation has been inhibited. Gel electrophoresis of the preincubation mixture shows that DNA is still intact, although a quantitatively minor, minicircle form of DNA is not detected. These results indicate that spinach thioredoxin m can bind to M13 DNA, at least under the preincubation conditions in which DNA and protein are both 10-fold more concentrated than later during the synthetic reaction, and suggest that this binding inhibits initiation. We cannot conclude that thioredoxin m inhibits initiation in *Xenopus* extracts by binding to a specific M13 phage origin because in *Xenopus* eggs, and presumably extracts as well, replication is thought to be initiated at virtually any sequence (16). Thus, if thioredoxin m inhibits initiation, it may do so by binding at many sites on M13 DNA. Still, thioredoxin binding may be favored by the origin sequence, as reflected by the selective loss from electropherograms of M13 minicircles, which are enriched for the M13 origin of replication (as used in *E. coli*).

Finally, we have begun to study the effects of thioredoxin m on M13 DNA and purified polymerases. In preliminary results, this thioredoxin does not inhibit synthesis of a polymerase α-mediated elongation assay but, surprisingly, leads to a 10-fold activation of deoxynucleotide incorporation when the Klenow fragment of *E. coli* DNA polymerase I (23) is used with M13 DNA. While this is activation rather than inhibition, the result further suggests the capacity of thioredoxin to interact with replication components and perhaps with the M13 origin of initiation. Whether these interactions of thioredoxin, M13 DNA, and purified *E. coli* proteins are relevant to what happens in the *Xenopus* extract or egg remains a question for future investigation, but clearly thioredoxin m affects DNA synthesis in all the different assays we have applied.

An attractive explanation for the *in vivo* and *in vitro* results with *Xenopus* eggs and extracts is that an endogenous thioredoxin is a normal component of the initiation complex of *Xenopus* DNA synthesis and that spinach thioredoxin m serves as an imperfect substitute and upsets the normal function of the *Xenopus* thioredoxin. If thioredoxin is involved in metazoan DNA replication, it may act as a proteinaceous second messenger analogous to calmodulin, serving in its reduced form (or experimentally in its reduced alkylated form) in initiation of DNA synthesis, while in its oxidized form it might be inactive. Mechanisms for controlling the redox state of thioredoxin in metazoan cells remain to be elucidated, but the known association of thioredoxin with the cell membrane (1) suggests that this could be the site of such redox changes. In the case of the inhibition of M13 DNA synthesis by reduced thioredoxin m, the affected polymerase is likely to be DNA polymerase α (16), which requires an RNA primase to initiate lagging strand-type DNA synthesis. It is a plausible conjecture that thioredoxin in its reduced form is normally involved in primase activity and that a heterologous protein such as spinach thioredoxin m interferes with this function in *Xenopus* eggs.

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