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Cytoplasmic Control of Nuclear DNA Synthesis During Early Development of *Xenopus laevis*: A Cell-Free Assay

(DNA replication/initiation factor/embryogenesis/electron microscopy/[^{3}H]dTTP)

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ABSTRACT **Nuclei isolated from nondividing cells were induced to synthesize DNA by incubation with cytoplasm from early embryos of *Xenopus laevis*. Numerous replication eyes were formed in the nuclear DNA molecules, and high levels of [^{3}H]dTTP were incorporated. With this assay a protein(s) which appears to initiate DNA synthesis was found at high levels in the cytoplasm of eggs, blastulae, or gastrulae, but only at low levels in the cytoplasm of oocytes, hatched embryos, or adult tissues.**

Nuclear activity during early development in *Xenopus laevis* is controlled by components from the cytoplasm (1). For example, nuclei from nondividing cells start to synthesize DNA after they are injected into the cytoplasm of intact eggs (2, 3). The cytoplasmic components which induce this nuclear DNA synthesis *in vivo* are not active in oocytes (3), and are not species specific (2).

To identify these cytoplasmic components we have developed an assay to measure the initiation of DNA replication in isolated nuclei. Although numerous measurements of DNA synthesis in isolated nuclei have been reported (4–20), our assay differs from these in several respects. First, the amount of incorporation of nucleotides into DNA is high, reaching a level equivalent to replication of 20% or more of the starting amount of nuclear DNA. Second, the appearance during incubation of small replication eyes (21–26) in the nuclear DNA molecules has been demonstrated by electron microscopy. Third, over 99% of the nuclei incorporate [^{3}H]dTTP as measured by autoradiography. Finally, the cytoplasmic components have been shown to retain their ability to induce nuclear DNA synthesis after fractionation by Sephadex G-200 or DE-52 column chromatography.

Using this assay, we have identified a component from rapidly dividing embryos which appears to be involved in the initiation of nuclear DNA synthesis.

MATERIALS AND METHODS

Isolation of Nuclei from Nondividing Cells. All operations were carried out at 0–4°. Livers from five 1-year-old *Xenopus laevis* females (older frogs have too much pigment) were removed into Ca- and Mg-free saline (27), cut free from the gall bladder, chopped with scissors in 10–15 volumes of 0.25 M sucrose–1 mM $MgCl_2$, and converted to a slurry with an MSE 7700 whirling blade homogenizer (1000 rpm for about 1 min). The slurry was subjected to five up-and-down strokes in a Teflon-glass homogenizer, and was sieved through eight layers of butter muslin.

To reduce the number of red blood cells the homogenate was centrifuged at maximum acceleration to 500 × *g*, at which time the centrifuge was quickly decelerated. The supernatant was immediately decanted, recentrifuged for 10 min at 2500 × *g*, and the resulting pellet was resuspended by gently pipetting with 18.0 ml of 2.0 M sucrose–1 mM $MgCl_2$.

Nuclei were purified on the basis of density (28). Five milliliters of 2.4 M sucrose–1 mM $MgCl_2$; 4.5 ml of 2.0 M sucrose–1 mM $MgCl_2$; and 3.0 ml of the above suspension of nuclei were layered successively in a two-step gradient. Centrifugation was for 75 min at 37,000 rpm (170,000 × *g*) in an MSE 6 × 14 swing out titanium rotor. Nuclear pellets were removed after cutting the tube with shears 2 cm above the pellet, and were resuspended by gently pipetting in 5.0 ml of 0.25 M sucrose–1 mM $MgCl_2$.

Nuclei were counted with a Petroff–Hauser counter, and were either prepared fresh or were stored at −20° after addition of glycerol to 20% (v/v). Nuclei stored for up to 3 months gave identical results except that [^{3}H]dTTP incorporation often increased up to a factor of 2 after *repeated* freezing and thawing.

Preparation of "Cytoplasmic" Extracts. Eggs were laid into saline and were dejellied by swirling in 2% cysteine (pH 8.0) for 5 min, followed by washing in four changes of cold tap water. Undiluted cytoplasm was prepared by homogenizing dejellied eggs at 1000 rpm for 3 min in the MSE whirling blade homogenizer, followed by centrifugation at 2500 × *g* for 15 min. The supernatant (undiluted cytoplasm) and some of the lipid was removed by pipetting; most of the lipid pellicle was not collected. Cytoplasm could be stored at −20° for at least 4 months after the addition of glycerol to 25% (v/v).

Extracts of oocytes, eggs, and embryos were prepared in 0.025 M basic homogenization buffer (pH 9.5) at a concentration of 50 embryos per ml as described previously (29). Basic homogenization buffer contained 0.025 M Tris (pH 9.5 at 25°), 0.005 M $MgCl_2$, 0.002 M 2-mercaptoethanol, 0.001 M EDTA, 0.005 M KCl, and 25% glycerol.

Assay for Nuclear DNA Synthesis: [^{3}H]dTTP Incorporation. Reaction components were selected to optimize conditions for swelling of the nuclei, and for incorporation of [^{3}H]dTTP by the known DNA polymerase enzymes (29, 30) (unpublished observations). One milliliter of the reaction medium contained 10 mM Tris (pH 7.5 at 37°), 1.5 mM $MgCl_2$, 100 mM KCl, 2 mM ATP, 2 mM 2-mercaptoethanol, 50 μM of dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim GmbH), 20 μCi of [^{3}H]dTTP (Amersham, 15.1 Ci/mmol), 200 μg of bovine serum albumin (fraction V), 1 to 10 × 10^6 nuclei (in 0.2 ml of 0.25 M sucrose–1 mM $MgCl_2$), and "cytoplasmic" extract (usually 1

Abbreviations: CAPS, 3-cyclohexylaminopropanesulphonic acid.

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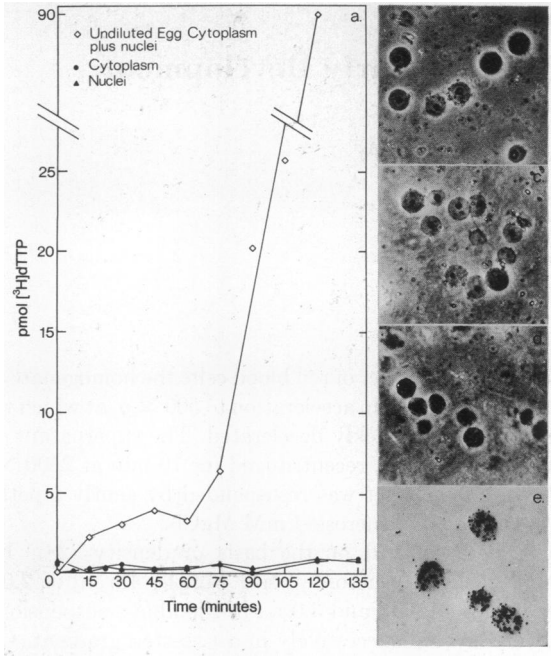

FIG. 1. Incorporation of [³H]dTTP by isolated nuclei. (a) Liver nuclei (1.6×10^7) were incubated with 3.6×10^3 egg equivalents of undiluted egg cytoplasm. Aliquots containing 8×10^5 nuclei were removed at various times, precipitated, filtered, and counted as described above. Control assays contained homogenization buffer in place of the missing component. The shape of the incorporation curve is characteristic: the abrupt rise in incorporation at 75–90 min in this experiment is observed from 60 to 240 min after the start of incubation, depending on the particular preparation of nuclei and cytoplasm. This later incorporation increased for up to 15 hr (i.e., assuming no bacterial incorporation, 20–40% of the input DNA is "replicated" in 15 hr). Identical results were obtained with nuclei isolated (2) from brains of adult *Xenopus laevis*, or with undiluted cytoplasm prepared from stage 11–12 gastrulae. (b) Isolated liver nuclei prior to incubation with cytoplasm. (c) Isolated liver nuclei from the same preparation incubated with egg cytoplasm for 90 min. (d) Incubated for 15 hr. We chose to study periods of less than 2 hr because it is difficult to exclude bacterial contamination during extended incubations. (e) Autoradiographs of liver nuclei after incubation with undiluted egg cytoplasm for 90 min in the standard assay. Nuclei were pelleted, resuspended in one drop of 0.2% bovine serum albumin–2 mM $MgCl_2$–10 mM NaCl–10 mM Tris·HCl at pH 7.5, smeared, dipped in Ilford K-2 emulsion, and processed according to the manufacturers' instructions after 12 days at 0–4°. Over 99% of the liver nuclei incubated for 90 min were labeled; in contrast, roughly 1% of control nuclei incubated with homogenization buffer were labeled.

embryo equivalent per 10^4 to 10^5 nuclei). The effect of varying each of these components on the incorporation of [³H]dTTP will be described elsewhere (C. C. Ford and R. M. Benbow, to be published). After incubation at 37° for various periods, 100 μl aliquots were taken into 1.0 ml of 0.1 M $Na_4P_2O_7$–0.001 M EDTA that contained 500 μg/ml of salmon sperm DNA (Sigma type III) at 0°. Samples were heated to 100° for 2 min, chilled, and 1.0 ml of cold 10% trichloroacetic acid was added. Samples were filtered on *wet* Whatman GF/C glass fiber filters (29) (presoaked in 0.2 M $Na_4P_2O_7$, 0.1 M EDTA, 100 μg/ml of dATP, dGTP, dCTP, and dTTP), and washed with three 15 ml aliquots of cold 5% trichloroacetic acid.

Samples were dried with an infrared lamp and counted in 10 ml of NCS-liquifluor-toluene (31) in a Wallac liquid scintillation counter. NCS-liquifluor-toluene contains 90 ml of NCS (Amersham), 42 ml of liquifluor (New England Nuclear), 858 ml of toluene, and 10 ml of water.

Preparation of Nuclear DNA Molecules for Electron Microscopy. Nuclei were collected from a 5 or 10 ml incubation mixture by centrifuging for 25 min at $2500 \times g$, were resuspended in 0.75 ml of 200 mM EDTA, 10 mM 3 cyclohexylaminopropane sulfonic acid (CAPS), pH 10.4 at 25°, followed by 0.75 ml of the same buffer containing 2% Sarkosyl (25). After 5 min of gentle agitation at 60°, the lysate was gently layered over a preformed CsCl gradient. The linear CsCl gradient was made from 4.5 ml of $\rho = 1.69$ g/cm³ of CsCl solution and 4.5 ml of $\rho = 1.76$ g/cm³ of CsCl solution with an MSE gradient maker, and was layered over a 2.0 ml pad of $\rho = 1.81$ g/cm³ of CsCl solution. All CsCl solutions contained 200 mM EDTA, 10 mM CAPS (pH 10.4 at 25°). After centrifugation for 12–15 hr at 17,000 rpm ($35,000 \times g$) in the MSE 6×14 swing out titanium rotor at 0°, fractions were collected slowly (with back pressure) through a 14 gauge needle (to minimize breakage of DNA molecules). Fractions around $\rho = 1.64$–1.67 g/cm³ usually contained the longest molecules; all fractions were dialyzed against 0.05 M Tris·HCl (pH 7.5 at 25°)–0.005 M EDTA–0.1 M NaCl, and were spread and viewed by the procedures of Davis, Simon, and Davidson (32).

RESULTS

(1) *Isolated Nuclei Incorporated [³H]dTTP During Incubation with Undiluted Egg Cytoplasm.* DNA synthesis was induced when isolated liver nuclei were incubated with undiluted egg cytoplasm (Fig. 1a). In contrast, nuclei alone or cytoplasm alone did not incorporate [³H]dTTP above background levels. When nuclei and cytoplasm were incubated together, incorporation rose sharply during the first 45 min. After a characteristic lag (from 45 to 75 min in this experiment), incorporation again rose rapidly, reaching a level equivalent, to synthesis of 1–2% of the added nuclear DNA in the first 2 hr (5% after 3 hr; up to 40% after 15 hr).

Is the [³H]dTTP incorporation within *undamaged* intact nuclei? We cannot determine this with certainty. The morphology of the nuclei does not alter dramatically during incubation: the nuclei swell slightly (3- to 4-fold volume increase), nuclear boundaries become diffuse, and a few pigment granules may adhere to each nucleus (Fig. 1b–d). The number of intact nuclei does not decrease during incubation. Most of the incorporated [³H]dTTP counts were recovered in nuclear pellets during preparation of incubated nuclei for electron microscopy. Furthermore, the incorporated radioactivity was localized over nuclei in autoradiographs (Fig. 1e), and over 99% of all nuclei incubated for 90 min were labeled. Therefore, most of the observed [³H]dTTP incorporation occurred in nuclei† which were at least superficially undamaged. These *in vitro* results are consistent with *in vivo* results (2, 3), which show that cytoplasmic proteins can enter injected nuclei and induce normal replication of nuclear DNA molecules.

(2) *Replication Eyes Appear in the Nuclear DNA Molecules After Incubation of the Isolated Nuclei with Cytoplasm from Eggs or Early Embryos.* Characteristic structures called replication eyes (21–23) can be observed by electron microscopy

† Preliminary experiments suggest that chromatin or even certain kinds of native DNA molecules (polyoma, erythrocyte) can serve as templates in our assay system.

in nuclear DNA molecules isolated from rapidly replicating embryonic cells [in *Drosophila* (24–26); in *Xenopus* (C. C. Ford, J. Heath, and R. M. Benbow, to be published)]. These structures were not observed in DNA molecules prepared from isolated liver or brain nuclei: less than 0.02% of the control molecules examined contained replication eyes (1 in 5000; 0 in 2000). After incubation of the liver nuclei for 90 min in assay mixture plus homogenization buffer (*no* cytoplasm), less than 0.05%‡ (3 in 7000) of the subsequently purified molecules contained replication loops. However, after incubation of the liver nuclei for 90 min or 15 hr with undiluted egg cytoplasm, or for 60 or 120 min with an extract of cytoplasm of stage 11–12 gastrulae, over 1%‡ of the subsequently purified nuclear DNA molecules contained one or more *small* replication eyes, i.e., 32 in 1800, 18 in 2000, 54 in 5000, 9 in 1000.

About 20% of the replication eyes seen in DNA molecules isolated after incubations of less than 2 hr were identical in size and configuration to those observed previously in DNA molecules isolated from rapidly replicating embryonic cells (refs. 24–26, C. C. Ford, J. Heath, and R. M. Benbow, to be published). After 15 hr incubation with undiluted egg cytoplasm, over 70% of replication eyes were of this previously described type. However, *most* replication eyes formed during the 1–2 hr incubations were *considerably smaller* (Fig. 2a–2d) and were either of the type postulated (23) to be "initiation" eyes (Fig. 2b), or were of a type we have not seen described previously in nuclear DNA molecules (Fig. 2a and c). The latter, which apparently consist of one single and one double-strand arm, are consistent with current theories of initiation of DNA replication in eukaryotes (33) and superficially resemble mitochondrial D loops (36).

To the extent that the observed structures are diagnostic of DNA replication, these data suggest that the observed [^{3}H]-dTTP incorporation is associated with the initiation (and continuation?) of chromosomal DNA replication in the isolated nuclei. Control experiments without nuclei established that we were not looking at DNA molecules carried in with the cytoplasm, and that we did not count mitochondrial DNA replicative intermediates.

(3) *The Ability of Embryonic Cytoplasm to Induce DNA Synthesis in Isolated Nuclei Varies with the Developmental Stage of the Embryo.* Nuclei injected into the cytoplasm of intact large oocytes are not induced to synthesize DNA, whereas those injected into the cytoplasm of intact eggs are (2, 3). Similarly, nuclei incubated with extracts of oocytes did not incorporate *high* levels of [^{3}H]dTTP, whereas those incubated with egg extracts did (Fig. 3). It should be noted, however, that there was a significant level of incorporation with extracts of oocytes; preliminary experiments suggest that oocyte *cytoplasm* is unable to induce DNA synthesis in isolated nuclei, whereas the contents of the oocyte germinal vesicle (nucleus) are somewhat effective.

"Cytoplasmic" extracts of blastulae or gastrulae induce high levels of [^{3}H]dTTP incorporation in isolated liver nuclei, whereas extracts of adult liver cells do not (Fig. 3). Experiments with extracts of neurulae and of hatched embryos are complicated by a high background of incorporation in the cytoplasmic extract, i.e., without added nuclei. Most of this can be removed by high-speed centrifugation (35,000 × *g* for 15 hr in an MSE 18 ultracentrifuge), and appears to arise from efficient utilization of endogenous chromatin (unpublished observations). The high-speed supernatants of neurulae or of hatched embryos did not induce high levels of [^{3}H]dTTP incorporation in isolated nuclei (Fig. 3). In contrast, high-speed supernatants of extracts of eggs or gastrulae were as effective as crude extracts or low-speed supernatants. A simple interpretation of these results is that the cytoplasm of eggs, blastulae, and gastrulae contains high levels of a factor inducing DNA synthesis, whereas the *cytoplasm* of later stage embryos, and of adult tissues contains low levels of this factor.

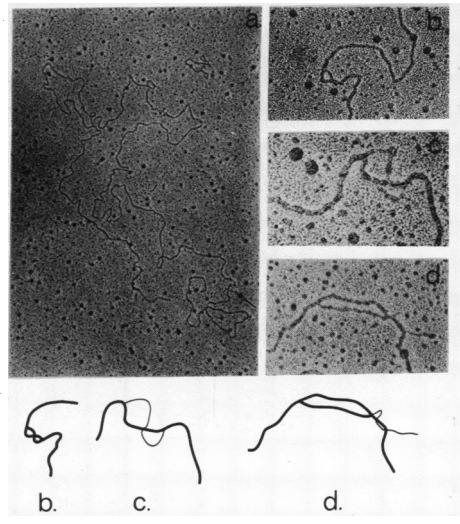

FIG. 2. Replication eyes appear in nuclear DNA molecules during incubation with egg cytoplasm. DNA molecules were isolated from liver (or brain) nuclei after incubation with homogenization buffer (control), undiluted egg cytoplasm, or an extract of gastrulae in homogenization buffer. After incubation the average contour length of the subsequently extracted DNA molecules was 2- to 3-fold shorter, paralleling the results obtained *in vivo* (ref. 34 and C. C. Ford, J. Heath, and R. M. Benbow, unpublished observations). (a) A molecule 19 μm long isolated from liver nuclei incubated for 90 min in undiluted egg cytoplasm. The arrow points to a small (0.23 μm long) replication eye which appears to have one single-strand and one double-strand arm. A "twist" or crossover is observed in about 50% of all small eyes. (b) A highly magnified portion of a DNA molecule isolated from liver nuclei incubated for 90 min in undiluted egg cytoplasm. This type of structure, 0.13 μm long (440 base pairs) with two apparently double-strand arms and a twist, was previously postulated to be an initiation eye (23) in DNA molecules isolated from *Saccharomyces cerevisiae*. (c) A highly magnified portion of a DNA molecule isolated from liver nuclei incubated for 90 min in undiluted egg cytoplasm. This eye, 0.23 μm long (740 base pairs), apparently consists of one single-strand and one double-strand arm. This is the most common eye found after incubations of less than 90 min with undiluted cytoplasm, and after all incubations with extracts of eggs or gastrulae. (d) A highly magnified portion of a DNA molecule isolated from liver nuclei incubated for 90 min in undiluted egg cytoplasm. This appears to be a small, traditional replication eye, 0.32 μm long (1070 base pairs), with a putative branch migrated single-strand "whisker" 0.27 μm long (885 base pairs) (25, 26). Larger but similar structures are the most common eye forms seen after long incubations. All contour lengths are relative to a value of 1.695 μm for φX174 replicative form DNA (35). Some of the early isolations of DNA molecules were done at 37°C in 10 mM EDTA.

‡ To simplify counting, we calculated all frequencies as a fraction of all molecules over 10 μm in contour length; all replication eyes, even if found on molecules of less than 10 μm, were included. About 15% of all eyes were in molecules of less than 10 μm.

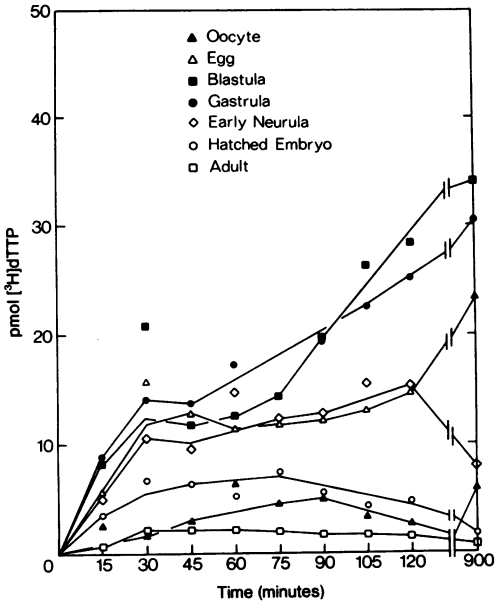

FIG. 3. Incorporation of [³H]dTTP by isolated liver nuclei incubated with extracts of early embryos of *Xenopus laevis*. Extracts of embryos of *Xenopus laevis* were used in place of undiluted cytoplasm in the standard assay. Isolated liver nuclei were induced to incorporate [³H]dTTP at a level dependent on the stage of the embryo from which the "cytoplasm" was prepared. The [³H]dTTP incorporation induced by an extract at 50 embryos per ml was comparable to that induced by undiluted cytoplasm during the first 2 hr; however, during long incubations the extracts did not continue to incorporate [³H]dTTP, whereas those with undiluted cytoplasm continued for 15–24 hr. The data for high-speed supernatants of each embryonic stage are given; crude extracts and low-speed supernatants gave the same results for stages prior to neurulation. "Cytoplasm" isolated from liver cells of adult *Xenopus laevis* was also included as a control.

It is of interest to note that the cytoplasmic extracts of embryos taken at various stages of development did not induce the second sharp rise (Fig. 1a) in [³H]dTTP incorporation, even after incubation for 24 hr. This is in contrast to the results obtained with *undiluted* cytoplasm from either eggs or gastrulae (Fig. 1a). However, extracts of gastrulae were able to induce the formation of small replication eyes within 2 hr (Section 2).

(4) *The Cytoplasmic Components Inducing DNA Synthesis in Isolated Nuclei Retain Their Activity After Column Chromatography.* In order to show that the observed [³H]dTTP incorporation was not a trivial stimulation of endogenous incorporation by small molecules (cations, for example) and to establish that the cytoplasmic components could be fractionated by column chromatography, we assayed fractions from a Sephadex G-200 column (29) for their ability to induce [³H]dTTP incorporation in isolated liver nuclei. Components of less than 60,000 molecular weight were ineffective as shown in Table 1. Similar experiments (data not shown) using DEAE-cellulose column chromatography established that the factor(s) inducing [³H]dTTP incorporation in isolated liver nuclei could be distinguished from *Xenopus laevis* DNA polymerases X-I, X-II, and X-III (29, 30). Control experiments showed that most activity in unfractionated extracts or in undiluted cytoplasm of eggs or gastrulae was abolished by heating to 100° for 5 min, to 65° for 15 min, or by pretreatment with trypsin (Table 1). Furthermore, chloroquine diphosphate (intercalative inhibitor), daunomycin hydrochloride (intercalative antibiotic), and *p*-chloromercuribenzoate (blocks free S-H groups), which are known to inhibit *Xenopus* DNA polymerases X-I and X-II, also eliminate [³H]dTTP incorporation in our nuclear assay (Table 1). Similarly, nalidixic acid [which specifically inhibits *semi-conservative* DNA replication in bacteria (47)], and *N*-hydroxyurea both strongly inhibit [³H]dTTP incorporation in our assay (Table 1).

TABLE 1. *Incorporation of [³H]dTTP (pmol/hr)*

Complete assay*	10.6
Sephadex G-200 fractions (molecular weight)	
125–250,000	8.0
100–125,000	9.1
90–100,000	10.6
75–90,000	9.3
60–75,000	9.01
20–60,000	2.3
0–20,000	3.7
Trypsin-treated cytoplasm†	>0.1
Cytoplasm heated at 65° for 15 min	2.8
Cytoplasm heated at 100° for 5 min	1.9
+ Nalidixic acid† (Boehringer)	3.3
+ Chloroquine diphosphate† (Boehringer)	>0.1
+ Tubercidin† (Boehringer)	10.5
+ *N*-Hydroxyurea† (Boehringer)	5.6
+ Daunomycinhydrochloride‡ (Boehringer)	>0.1
+ *p*-Chloromercuribenzoate† (BDH)	>0.1

* Liver nuclei (5×10^6) were incubated with 2×10^2 gastrula equivalents of an extract of stage 11–12 gastrulae. Data are given in pmol of [³H]dTTP incorporated in duplicate assays in 2 hr.

† 200 μg/assay.

‡ 2 μg/assay

DISCUSSION

The purpose of this paper is to describe a cell-free assay for the cytoplasmic components responsible for the initiation of DNA synthesis during early embryogenesis in *Xenopus laevis*. The existence of these components was established *in vivo* by microinjection assays (2, 3); our cell-free system permits purification of the pertinent proteins.

(1) *Components from the Cytoplasm of Early Embryos Can Initiate DNA Replication in Isolated Nuclei.* The observed [³H]dTTP incorporation in more than 99% of incubated nuclei (Fig. 1), the sensitivity of this incorporation to various inhibitors of DNA synthesis (Table 1), and the appearance of DNA replication eyes in nuclear DNA molecules (Fig. 2) during incubation, suggests that extensive initiation (and continuation?) of DNA synthesis occurs in our system. Components from the cytoplasm of early embryos are essential, although our data do not exclude that some of these could have been associated with embryonic nuclei prior to preparation of the cytoplasm. Control experiments (Table 1 and unpublished observations) have established that endogenous DNA replication in the isolated nuclei is not simply stimulated by cations or small molecules present in the cytoplasm nor does the cytoplasm simply provide DNA polymerases X-I, X-II, and X-III.

(2) *I Factor.* One simple way to interpret our data is to postulate the existence of an initiation protein (or proteins),

which we shall call *I factor*, and to suppose that the level of putative I factor in the cytoplasm varies throughout development.

We postulate the appearance of an I factor during oocyte maturation because most if not all of the other components necessary for extensive DNA replication (DNA and RNA polymerases, ligase, nucleases, precursors) are found at high levels in oocytes as well as early embryos (29, 30).

On the basis of indirect evidence (30) and (R. M. Benbow and C. C. Ford, to be published), we suggest that I factor is a site-specific endonuclease which recognizes either arm of the cruciform hair-pin structures formed by long palindromic sequences (37) known to be present in *Xenopus laevis* nuclear DNA molecules, ultimately giving rise to clusters of replication eyes whose spacing roughly coincides with the spacing between replicons (38) in *Xenopus laevis*. Initiation of DNA synthesis in early embryos of *Xenopus laevis* is controlled, therefore, (*1*) by varying the level of I factor in the cytoplasm, i.e., frequency of initiation per site, and (*2*) by varying the configuration of the DNA molecules within the nucleus, i.e., number of accessible palindromic initiation sites.

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