

Nuclear Assembly with λ DNA in Fractionated *Xenopus* Egg Extracts: An Unexpected Role for Glycogen in Formation of a Higher Order Chromatin Intermediate

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Abstract. Crude extracts of *Xenopus* eggs are capable of nuclear assembly around chromatin templates or even around protein-free, naked DNA templates. Here the requirements for nuclear assembly around a naked DNA template were investigated. Extracts were separated by ultracentrifugation into cytosol, membrane, and gelatinous pellet fractions. It was found that, in addition to the cytosolic and membrane fractions, a component of the gelatinous pellet fraction was required for the assembly of functional nuclei around a naked DNA template. In the absence of this component, membrane-bound but functionally inert spheres of λ DNA were formed. Purification of the active pellet factor unexpectedly demonstrated the component to be glycogen. The assembly of functionally active nuclei, as assayed by DNA replication and nuclear transport, required that glycogen be pre-incubated with the λ DNA and cytosol during the period of chromatin and higher order intermediate formation, before the addition of membranes. Hydrolysis of glycogen with α -amylase in the extract blocked nuclear formation. Upon analysis, chromatin formed in the presence of cytosol and glycogen alone appeared highly con-

densed, reminiscent of the nuclear assembly intermediate described by Newport in crude extracts (Newport, J. 1987. *Cell*. 48:205–217). In contrast, chromatin formed from phage λ DNA in cytosol lacking glycogen formed “fluffy chromatin-like” structures. Using sucrose gradient centrifugation, the highly condensed intermediates formed in the presence of glycogen could be isolated and were now able to serve as nuclear assembly templates in extracts lacking glycogen, arguing that the requirement for glycogen is temporally restricted to the time of intermediate formation and function. Glycogen does not act simply by inducing condensation of the chromatin, since similarly isolated mitotically condensed chromatin intermediates do not form functional nuclei. However, both mitotic and fluffy interphase chromatin intermediates formed in the absence of glycogen can be rescued to form functional nuclei when added to a second extract which contains glycogen. This study presents a novel role for a carbohydrate in nuclear assembly, a role which involves the formation of a particular chromatin intermediate. Potential models for the role of glycogen are discussed.

WITH the advent of cell-free extracts capable of forming nuclei from chromatin or naked DNA templates, it is now possible to study discrete steps in nuclear assembly. Such extracts permit the analysis of the intermediate chromatin structures involved, as well as the components required for the assembly of functional nuclei *in vitro*. Several nuclear reconstitution extracts are available, among them one prepared from cultured mammalian cells (Burke and Gerace, 1986) and one from the eggs of *Xenopus laevis*. The latter is especially amenable to analysis since the *Xenopus* egg contains large pools of the components required for nuclear assembly stored in a soluble form for later embryonic development (for review see Laskey, 1985; Laskey and Leno, 1990). Harland and Laskey (1980) found that DNA microinjected into *Xenopus* eggs becomes assembled into nucleosome filaments and is replicated semi-conservatively. It was later found that bacteriophage λ DNA injected

into *Xenopus* eggs, in actuality, becomes assembled into “synthetic” nuclei, whose chromatin is composed entirely of λ DNA. These nuclei contain double nuclear membranes, a nuclear lamina, and nuclear pores, as determined by electron microscopy. The reconstituted “synthetic” nuclei formed *in vivo* respond normally to mitosis promoting factors (CSF and MPF)¹ by dissolution of their nuclear envelopes and DNA condensation (Forbes et al., 1983).

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1. *Abbreviations used in this paper:* HSA, human serum albumin; MPF, mitosis promoting factors; PEG, polyethylene glycol; PP-1, phosphatase 1; PVA, polyvinylalcohol; PVP, polyvinylpyrrolidone.

Subsequent work with lysates of *Xenopus* eggs ("crude extracts") demonstrated that nuclear assembly with a naked DNA template also occurs in vitro (Newport and Forbes, 1985; Newmeyer et al., 1986a; Blow and Laskey, 1987; for review see Laskey and Leno, 1990). A crude *Xenopus* extract can be fractionated by ultracentrifugation into soluble cytosol, a vesicular fraction, and a gelatinous pellet containing glycogen and ribosomes (Lohka and Masui, 1983; Newport, 1987; Sheehan et al., 1988). The cytosol and vesicular fractions have been shown to be sufficient to form functional nuclei with the complex chromatin template derived from demembrated *Xenopus* sperm (Lohka and Masui, 1983, 1984). Nuclei formed from such sperm chromatin have greatly facilitated the in vitro study of chromatin decondensation, vesicle targeting and fusion, lamin assembly, nuclear pore formation, nuclear transport, and DNA replication (Lohka and Masui, 1984; Sheehan et al., 1988; Wilson and Newport, 1988; Dabauvalle et al., 1990; Newport et al., 1990; Finlay and Forbes, 1990; Newmeyer and Forbes, 1990; Vigers and Lohka, 1991, 1992; Dasso et al., 1992; Boman et al., 1992; Newport and Dunphy, 1992; Philpott and Leno, 1992).

Much less is known of the early steps in chromatin and nuclear envelope assembly that must occur when, instead of sperm chromatin, naked DNA is used as the template. What is known is that using an unfractionated or crude assembly extract, exogenously added long linear DNA acquires nucleosomes and forms a highly condensed intermediate (Newport, 1987). This condensed spherical intermediate then binds nuclear membrane vesicles which fuse and form the double membrane of the nuclear envelope. Nuclear pores are also formed at this time. Once assembled, the synthetic nuclei decondense their DNA, carry out efficient nuclear transport (Newport and Forbes, 1985; Newmeyer et al., 1986a,b) and replicate their DNA semi-conservatively at discrete foci just as occurs in nuclei in vivo (Blow and Laskey, 1986; Newport, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990; Cox and Laskey, 1991). Many of these steps are much less efficient in a fractionated system. If DNA is incubated alone with cytosol, the intermediates appear visually different. For example, Hirano and Mitchison (1991) found that in cytosol alone, λ DNA does not form the highly condensed intermediate described above, but instead forms a "fluffy" non-condensed chromatin intermediate. Even so, the authors were able to show that the fluffy chromatin responded to mitotic inducing factors by undergoing a distinct mitotic chromatin condensation.

In all the experiments designed to ask what cellular components are necessary for nuclear assembly around a naked DNA template, it has been found that the process of nuclear assembly becomes less efficient with each step of fractionation. Microinjection of long linear DNA into eggs results in more efficient assembly than nuclear formation with a crude egg extract, which in turn is much more efficient than assembly with the cytosol and vesicular fractions of an ultracentrifuged extract. This is despite the fact that all of the above readily assemble nuclei around a complex sperm chromatin template. We have analyzed this major difference in requirements for the assembly of nuclei from λ DNA, attempting to identify the missing factor or factors lost upon fractionation of the cytoplasm. We find that in addition to the cytosol and membrane vesicle fractions, a component of

the gelatinous pellet is essential for nuclear assembly with λ DNA. Surprisingly, the component required is glycogen, an abundant component of the *Xenopus* egg. In the presence of glycogen, large synthetic nuclei form that are capable of high levels of DNA replication and nuclear transport. In the absence of glycogen, λ DNA forms small inert DNA spheres which bind membrane vesicles, but neither replicate efficiently nor carry out nuclear transport. Glycogen thus dramatically promotes the formation of functional nuclei. Upon analysis, glycogen appears to be required during the formation of a chromatin intermediate in nuclear assembly. Conditions for the formation and isolation of this higher order chromatin intermediate are described.

Materials and Methods

Reagents

Porcine pancreatic α -amylase was obtained from Worthington Biochem. Corp. (Freehold, NJ) (26 mg/ml protein; 709 U/mg in NaCl suspension). The *Streptomyces tendae* α -amylase inhibitor tendamistat, Hoechst 33258, and 3,3'-dihexyloxycarbocyanine (DHCC) were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Methyl-adenine-free λ DNA was obtained from New England Biolabs (Boston, MA), prepared in the absence of ethanol precipitation. Polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), dextran, mannose, maltose, amylopectin, and *N*-acetylglycosamine were purchased from Sigma (St. Louis, MO). Polyethylene glycol (PEG), dextrose, and galactose were purchased from Fisher Scientific (Pittsburg, PA). Sucrose was purchased from GIBCO-BRL (Gaithersburg, MD).

Xenopus Egg Extract Preparation

Xenopus eggs were collected, dejellied, and lysed to prepare an interphase extract essentially as described (Smythe and Newport, 1991). Egg lysis buffer for the preparation of interphase extracts consisted of 250 mM sucrose, 50 mM KCl, 3 mM MgCl₂, 10 mM Hepes/NaOH, pH 7.5, supplemented with 1 mM DTT, 100 μ g/ml cycloheximide, and 10 μ g/ml aprotinin and leupeptin immediately before use. Eggs were packed by spinning for 15 s at setting No. 3 in a clinical table top centrifuge (International Equipment Co., Needham Heights, MA). Excess buffer above the packed eggs was removed and a crude extract prepared by centrifugation at 10,000 rpm in Sorvall HB-4 rotor (DuPont Co., Newton, CT). This low-speed centrifugation resulted in a small lipid layer, a crude extract fraction containing cytosol and membranes, and large pellet of yolk and pigment granules. The crude extract was supplemented with 5 μ g/ml cytochalasin, and further separated into cytosol, membrane-rich, and gelatinous pellet fractions by ultracentrifugation at 200,000 g for 60 to 75 min in a Beckman TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The cytosol generated was then re-centrifuged at 200,000 g for 30 min to remove residual membranes and glycogen, divided into 25- μ l aliquots, frozen in liquid nitrogen, and stored at -70°C until used. The membrane fraction was resuspended in 5 to 10 vol of cold lysis buffer (without ATP) and centrifuged (TLS-55 rotor, 2°C, 20 min, 26,000 g) into a cushion consisting of lysis buffer + 0.5 M sucrose. It was essential that ATP not be present during this step to consistently observe nuclear formation in frozen extracts using a naked DNA template. Membranes were resuspended in cushion buffer (egg lysis buffer with 0.5 M sucrose) to a final volume that was ~10% of the original volume of crude extract (10 \times membranes), divided into 10- μ l aliquots, frozen in liquid nitrogen, and stored at -70°C until used. It should be noted that in our study the initial centrifugation of the crude extract at 200,000 g occasionally generated a biphasic cytosolic fraction which was not completely clear but composed of a lighter clarified layer and a denser yellowish phase (Vigers and Lohka, 1991). We found that it was important to continue centrifugation of the extract at 200,000 g for an additional 10 to 15 min to completely clarify the supernatant and reduce the background of nuclei in incubations which did not contain exogenously added glycogen.

Nuclear Assembly Assays

For an assembly experiment, aliquots of cytosol were rapidly thawed in room temperature water, pooled, and supplemented with an ATP-regenerating system consisting of 2 mM ATP, 20 mM creatine phosphate, and

0.1 mg/ml creatine kinase final concentration. A typical assembly reaction contained 25 μ l of cytosol to which either glycogen or buffer was added such that the cytosol was diluted by no more than 20%. In a given experiment the extent of dilution by buffer, resuspended gelatinous pellet, or glycogen was always kept constant. 1 μ l of λ DNA at 250 μ g/ml was added to the above incubation with a 1–10- μ l Eppendorf pipette (Brinkman Instruments Inc., Westbury, NY) to ensure accuracy (final concentration, 10 μ g/ml). The sample was immediately (within 15 s) pipetted up and down 10 times with a yellow Micropipette tip and a P-200 Pipette (set at 25–30 μ l), without extensive foaming to thoroughly mix the DNA into the extract. This was a critical step in producing nuclei. Inadequate mixing resulted in large masses of DNA which eventually were packaged at the edges into nuclei, with the bulk of DNA remaining largely unincorporated. In nuclear assembly experiments, after incubation at room temperature for 60 min 3 μ l of membrane or cushion buffer was then added to samples. As described in the text, preincubation of the DNA and cytosol with the pellet fraction of the egg extract greatly enhanced nuclear formation and function. The protocol for these preincubations involved mixture of the λ DNA and cytosol with such fractions as described above, incubation for 0–60 min depending on the experiment, followed by the addition of membranes.

Nuclear formation at various times after membrane addition was monitored by placing 4- μ l aliquots of the above incubations on a microscope slide and mixing with 1 μ l of sample buffer containing 200 mM sucrose, 30% formaldehyde, and 10 μ g/ml Hoechst 33258, and DHCC dyes (to visualize DNA and membrane components, respectively). An 18 mm² coverslip was gently placed on the sample and the specimen examined by fluorescence microscopy using a Zeiss Photomicroscope III (Wilson and Newport, 1988). Samples were generally examined at 730 \times , resulting in fields of view \sim 200 μ m in diameter. Where the average number of nuclei/field is reported, a minimum of 10 fields were observed over a wide area of the microscope slide. Successful nuclear formation was judged to be the appearance of multiple (5–20) nuclei per 200- μ m field as visualized with the fluorescence microscope. (A smaller plasmid of 3 kb, pBsKII+, did not efficiently form transporting nuclei even when preincubated with added glycogen; data not shown. Long linear DNA is by far the most efficient template for synthetic nuclear assembly; see also Blow and Sleeman, 1990).

Nuclear Transport Assay

Nuclear transport substrate consisted of rhodamine-labeled human serum albumin (HSA) coupled to multiple copies of the SV-40 large T antigen nuclear localization sequence, as described previously (Newmeyer and Forbes, 1988; Finlay and Forbes, 1990). To assay nuclear transport, transport substrate (RITC-ss-HSA; 1.2 mg/ml) was diluted 30–150-fold in nuclear formation reactions 90 min after the addition of membranes. The level of accumulation of transport substrate was visualized 30 to 60 min later by fluorescence microscopy (Newmeyer and Forbes, 1988, 1990).

DNA Replication Assay

Nuclei or chromatin assembly reactions using λ DNA were prepared as described above. Competence for DNA replication was assayed by addition of 0.5 mCi of ³²P-labeled α -dCTP (New England Nuclear) to reactions immediately after the addition of membrane or cushion buffer. Reactions were terminated 120-min later by the addition of an equal volume of stop mix containing 1% SDS, 20 mM EDTA, 20% glycerol, and 50 mM Tris-Cl, pH 8.0. Proteinase K was added to 1 mg/ml, and the mixture incubated at 37°C for 1 h. Aliquots were electrophoresed in 1% agarose-TBE gels. After electrophoresis gels were dried onto Whatman paper (Whatman Laboratory Products Inc., Clifton, NJ) and exposed to Kodak X-OMAT AR X-ray film.

Supercoiling Analysis of DNA

Plasmid DNA (pBsIIKS+, from Promega Biotec, Madison, WI) was added to egg cytosol that contained either 15 mg/ml glycogen or buffer alone, such that the DNA was at a final concentration of 15 μ g/ml. At different times aliquots were removed and mixed with an equal volume of 1% SDS, 20 mM EDTA, 50 mM Tris-Cl, pH 8.0, and 2 mg/ml Proteinase K. Samples were incubated at 37°C for 1 h and then electrophoresed in 1% agarose-TBE gels. Gels were stained with ethidium bromide in TBE and destained in water. DNA was visualized with ultraviolet illumination.

Glycogen Purification and Quantitation

Glycogen was purified from the gelatinous pellet of ultracentrifuged *Xenopus* egg extracts according to the protocol of Kennedy et al. (1985). For

large scale purification, pellet fractions from numerous egg extracts were collected and stored at -70°C until used. The fractions (\sim 4 ml) were then resuspended in an equal volume of cold egg lysis buffer and centrifuged at 10,000 rpm in an HB-4 rotor at 4°C to remove contaminating pigment granules. The supernatant was made 10% with respect to trichloroacetic acid (on ice) using a 100% stock solution of TCA, then centrifuged at 10,000 rpm for 5 min. The supernatant which contained glycogen was immediately precipitated with 2 vol of 100% ethanol, centrifuged, and resuspended in G buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 3 mM MgCl₂). The glycogen-containing sample was extracted with chloroform:octanol (3:1) and precipitated again with ethanol. The pellet was resuspended in 5 ml of G buffer, and the chloroform/octanol extraction and ethanol precipitation steps repeated. The resulting pellet was resuspended in 50 mM ammonium bicarbonate, dialyzed briefly, and passed through a DEAE-Sepharose column equilibrated in ammonium bicarbonate. The flowthrough was then lyophilized and stored at -20°C . For nuclear formation experiments, a stock solution of glycogen was prepared by resuspension of a carefully weighed sample of purified glycogen in G buffer to 150–300 mg/ml; this stock could be stored at -70°C indefinitely, provided the glycogen was purified as above.

To determine the glycogen concentration in the crude and fractionated extracts, a sample of each fraction was extracted once with TCA to remove protein, ethanol precipitated to pellet larger molecular weight carbohydrates, and resuspended in buffer G. The glycogen concentration was determined by hydrolysis to glucose and spectrophotometric quantitation, according to the procedure of Dubois et al. (1956). Glycogen from rat liver (freshly isolated) and rabbit skeletal muscle (Sigma) was subjected to the same purification procedure and tested for activity in the same manner as the *Xenopus* egg glycogen. Oyster glycogen (mol. biol. grade; Calbiochem-Behring Corp.) was also tested.

α -Amylase Digestion of Glycogen in Reconstituted Extracts

For the experiment shown in Fig. 5, 30 μ l of α -amylase, supplied as a suspension in 50% NaCl, was centrifuged briefly and resuspended in 120 μ l of G buffer + 0.5 mM CaCl₂ + 0.1 mM PMSF such that the final concentration of enzyme was 1 U/ μ l. The α -amylase inhibitor tendamistat (a naturally occurring inhibitor protein from *Streptomyces tendae*; Calbiochem-Behring Corp.; Vertesy et al., 1984; Pflugrath et al., 1986) was resuspended in G buffer + 0.5 mM CaCl₂ to a final concentration of 10 mg/ml. Where indicated, 0.5 U of α -amylase or 10 μ g of the inhibitor were added to nuclear assembly reactions at the initiation of the experiment. In reactions containing both enzyme and inhibitor, the reagents were mixed and then simultaneously added to the nuclear assembly reaction. Digestion proceeded for 10 min before addition of the DNA template for nuclear assembly.

It should be noted that digestion of isolated or swollen sperm chromatin with α -amylase had no effect on nuclear formation: good nuclear assembly was still observed in cytosol plus membranes. However, assembled sperm nuclei did show some enhancement of nuclear transport when glycogen was added if the extracts were of marginal quality (data not shown).

Sucrose Gradient Isolation of Chromatin Intermediates

Chromatin was assembled by incubation of λ DNA (10 μ g/ml) for 2 h in cytosol-containing buffer G or 20 mg/ml glycogen. The mixture containing chromatin was diluted 5.6-fold with buffer D (200 mM sucrose, 15 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM DTT, 10 mg/ml aprotinin and leupeptin, 250 μ M spermidine). The diluted sample (1.4 ml final volume) was layered on a sucrose step gradient composed of 1 ml of buffer D + 1 M sucrose and 0.2 ml of buffer D + 2 M sucrose in a TLS-55 centrifuge tube (Fig. 8A). The gradient was centrifuged in a TL-100 ultracentrifuge at 7,000 rpm for 15 min at 4°C . Fractions (0.3 ml) were collected as indicated in Fig. 8A, divided into 25- μ l aliquots, frozen in liquid nitrogen, and stored at -70°C until use.

To test mitotic chromatin for nuclear assembly competence, mitotic chromatin was formed as follows: λ DNA or *Xenopus* DNA (10 μ g/ml) was incubated with clarified interphase cytosol and added recombinant GST-cyclin- Δ 13 fusion protein (Solomon et al., 1990; Newport and Smythe, 1992). After 2 h the mixture which now contained mitotic chromatin was diluted fivefold with modified egg lysis buffer (200 mM sucrose, 50 mM KCl, 3 mM MgCl₂, 10 mM Hepes/NaOH, pH 7.4, 1 mM DTT, 10 μ g/ml aprotinin and leupeptin, 5 μ g/ml cytochalasin B, 250 μ M spermidine). The diluted sample was underlaid with 0.2 ml of 0.5 M sucrose modified egg

lysis buffer in a TLS-55 centrifuge tube. The sample was centrifuged in a TL-100 ultracentrifuge at 7,000 rpm for 10 min at 4°C. The diluted supernatant was aspirated off, leaving 50–100 μ l remaining on top of the cushion. Four fractions (50- μ l each) were collected by using a yellow pipette tip starting from the bottom of the cushion. 3 μ l from each of the four fractions were checked for the presence of chromatin. Mitotic chromatin intermediates formed in the absence of glycogen were usually found near the interface. For use as a control, interphase chromatin was assembled by incubation of λ DNA or *Xenopus* DNA (10 μ g/ml) for 2 h in clarified interphase cytosol containing buffer G (to form "fluffy chromatin") or 20 mg/ml glycogen (to form highly condensed intermediates), and centrifuged through an identical 0.5 M sucrose cushion. The interphase chromatin intermediates from the sample containing glycogen were usually found in the bottommost fraction, while those formed in the absence of glycogen remained near the interface. These three types of chromatin intermediates were tested for nuclear assembly by adding 5 μ l intermediates to 25 μ l cytosol, with or without 3 μ l glycogen. 45 min later, 3 μ l of membranes were added for a 60 min incubation. After 60 min, transport substrate was added and nuclear assembly and transport assayed visually 60 min later.

Electron Microscopy

Nuclei were assembled from λ DNA as described above. 2 h after membrane addition, 25- μ l samples were fixed by mixing with an equal volume of cold fixative, consisting of 100 mM sucrose, 200 mM cacodylate, pH 7.4, 8% glutaraldehyde, 1% paraformaldehyde, and 1% tannic acid. Samples were stored at 4°C overnight, washed in 0.1 M phosphate buffer, pH 7.4, and pelleted in an Eppendorf centrifuge for 10 s. Samples were then postfixed with 2% OsO₄ for 3 h, dehydrated through a graded ethanol series, and embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate and visualized with a Philips EM 300 electron microscope.

Quantitation of Transport Substrate Accumulation and Nuclear Size

The data shown in Figs. 3 and 5 were generated using a fluorescence video image analysis system described by Newmeyer and Forbes (1990). Microscope slides of transport reactions, prepared as described above, were visualized on a Nikon Optiphot fluorescence microscope. Images were captured with an Imaging Technologies PC Vision Plus 512 frame grabber board installed in an IBM AT-compatible computer. Data was collected with Optimas software as described (Newmeyer and Forbes, 1990).

Results

Nuclear Assembly on a Naked DNA Template

To examine nuclear assembly using a naked DNA template, crude cytosol capable of nuclear assembly was prepared from *Xenopus* eggs and separated by ultracentrifugation (200,000 *g* for 60–75 min) into three fractions: cytosol, a membrane vesicular fraction, and a golden gelatinous pellet. The cytosol fraction was further clarified by a second centrifugation at 200,000 *g* for 30 min to remove residual membranes and a second small golden pellet. The membrane fraction was washed free of any cytosolic contamination by dilution, recentrifugation, and resuspension. Upon addition of demembrated sperm chromatin, as expected, the cytosol and membrane fractions were fully sufficient to form nuclei. By fluorescence microscopy, the nuclei appeared as large, spherical, membrane-enclosed, DNA-containing structures, which were capable of efficient DNA replication and accumulation of a nuclear transport substrate (data not shown; Sheehan et al., 1988; Wilson and Newport, 1988; Forbes and Finlay, 1990). In contrast, when protein-free phage λ DNA was added to the cytosol and membrane fractions, the DNA formed only very small (\sim 1 μ m) spherical structures which bound membrane vesicles (Fig. 1 C). For

the most part, these spheres were too small to judge whether complete membrane enclosure had occurred, but rare larger spheres were sometimes completely enclosed (data not shown). These structures did not generate a significant number of nuclei capable of nuclear transport (Fig. 1 D), the criteria we subsequently used to define functional nuclear assembly.

The data suggested that fractionation resulted in the loss of a component that is needed for the formation of large, functional nuclei from a naked DNA template. The data also suggested that sperm chromatin contains this component (or a functionally equivalent homolog), since demembrated sperm chromatin routinely formed large transporting nuclei with only the clarified cytosol and membrane fractions. To attempt to reconstitute nuclear assembly on a λ DNA template, a portion of the gelatinous pellet fraction was added back to the clarified cytosol and membrane fractions. When this pellet was resuspended in an equal volume of buffer and added at its original concentration to the clarified cytosol, membranes, and λ DNA, the formation of nuclei was indeed rescued. DNA became enclosed in a nuclear envelope and nuclear transport was now observed. Highly efficient rescue, however, was observed only when the pellet material was preincubated with cytosol and DNA for 30–60 min, before the membrane addition (data not shown; see below). This result suggested that the pellet material was influencing the formation of a chromatin intermediate, before the membrane binding and enclosure steps of nuclear assembly.

The pellet fraction is thought to contain primarily ribosomes and all of the detectable glycogen present in the crude extract (Laskey et al., 1985). We therefore asked whether either of these macromolecular components was responsible for the rescue observed. Ribosomes, purified as described by Wolffe and Brown (1987), did not rescue nuclear formation (not shown). Glycogen, however, purified from the pellet as described in Materials and Methods, surprisingly rescued nuclear formation quite effectively when preincubated with cytosol and λ DNA before membrane addition (Fig. 1, A and B). Rescue of nuclear formation in incubations to which glycogen was added was dramatic (Fig. 1, compare A and B to C and D). The nuclei that formed in the presence of glycogen ranged in size from 5–15 μ m or more, rather than the 1- μ m structures formed in its absence, and rapidly accumulated a nuclear transport substrate (Fig. 1 B). At a final concentration of 10 μ g/ml λ DNA, the number of transporting nuclei formed in the presence of glycogen ranged from 10–20 per 200 μ m field, as compared to virtually no transporting nuclei/field in the absence of glycogen. Rescue with purified glycogen, as with the crude gelatinous pellet, was optimal when the glycogen was pre-incubated with λ DNA and cytosol for 30 to 60 min before the addition of membranes. A simultaneous combination of cytosol, membrane, DNA, and glycogen fractions most often generated membrane-enclosed, spherical aggregates of DNA which transported poorly (data not shown). Similar results showing dependence on glycogen were also obtained using a 50-kb circular COS plasmid as template, although the nuclei formed tended to be smaller (3–5 μ m in diameter; data not shown). Further evidence that glycogen itself was the key agent in this large enhancement of nuclear formation was provided by the finding that purified glycogen from *Xenopus* eggs, rat liver, rabbit skeletal muscle, and oyster all greatly

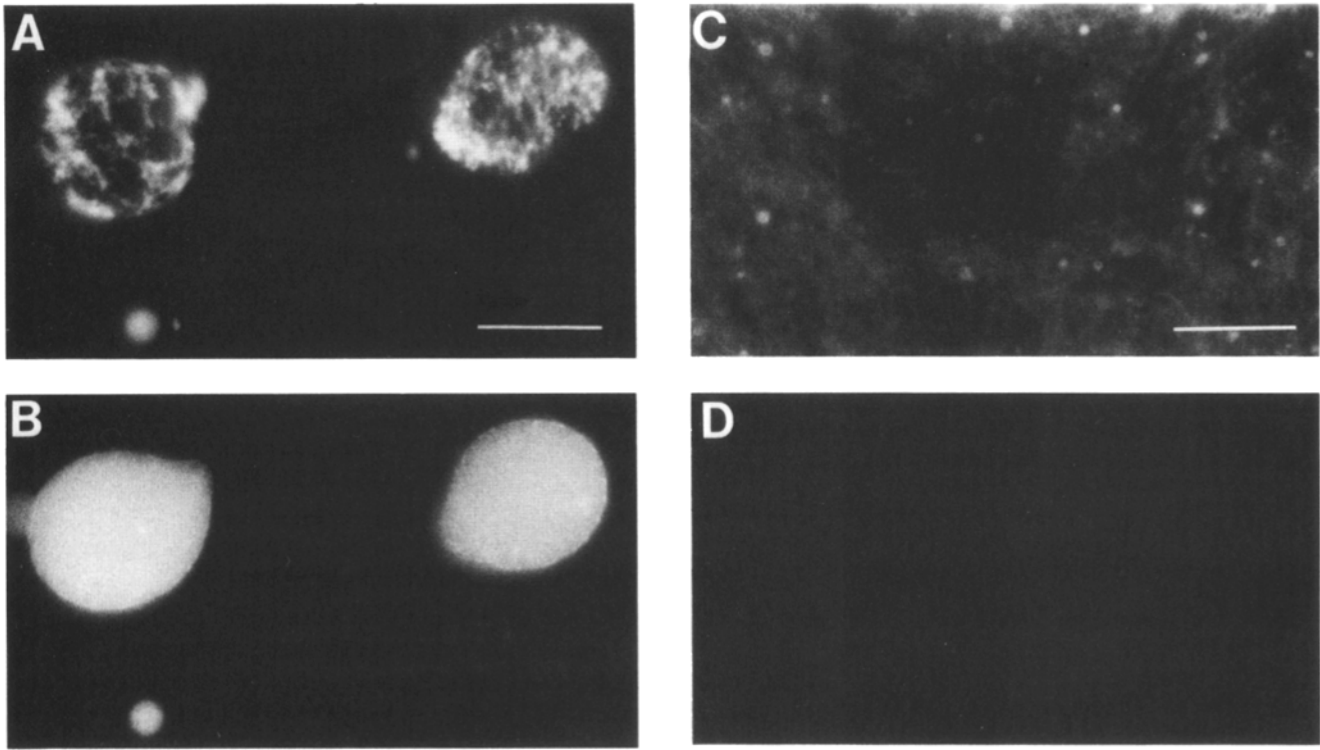


Figure 1. Formation of nuclear structures using naked λ DNA in reconstituted *Xenopus* egg extracts. Extracts were fractionated as described in Materials and Methods. λ DNA ($10 \mu\text{g/ml}$) was incubated for 45 min in clarified cytosol containing either glycogen purified from the gelatinous pellet (*A* and *B*), or buffer (*C* and *D*). Washed membranes were then added, followed by rhodamine-labeled nuclear transport substrate 90 min later as described in Materials and Methods. The resulting structures formed were examined by fluorescence microscopy 30 min after addition of transport substrate. DNA was stained with Hoechst (*A* and *C*) and transport substrate accumulation was visualized by fluorescence of the rhodamine-labeled substrate (*B* and *D*). Because of their small size, the DNA-containing structures in *C* (no glycogen) were difficult to photograph. They appeared in the microscope as small membrane-bound spheres (membrane binding not shown). The DNA inside was more condensed than linear λ DNA at $t = 0'$ of addition. Very rare large spheres formed in the absence of glycogen (not shown) excluded large cytoplasmic proteins, indicating full membrane enclosure, at least in these large spheres. Bar, $10 \mu\text{m}$.

enhanced nuclear assembly, as long as the glycogen was purified (Kennedy et al., 1985; data not shown). Highly purified *Xenopus* egg glycogen was used for the experiments that follow.

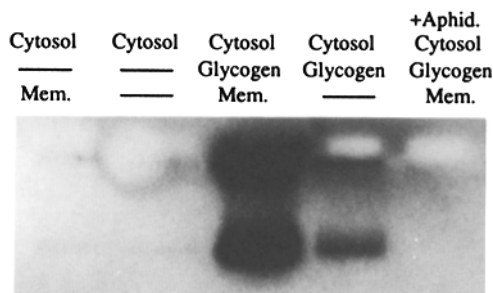


Figure 2. λ DNA replication in reconstituted egg extracts is largely glycogen dependent. DNA ($10 \mu\text{g/ml}$) was incubated for 1 h in cytosol containing glycogen (15 mg/ml) or G buffer. Membranes or membrane cushion buffer was then added to the incubations, followed within 5 min by ^{32}P -labeled α -dCTP. Reactions were terminated 2 h later, processed, electrophoresed, and the gel exposed to X-ray film as described in the Materials and Methods. The upper band in the figure contains concatamerized DNA which did not enter the gel. The rightmost lane indicates the extent of replication in the presence of aphidicolin (*Aphid.*) which inhibits DNA polymerase α .

In most cases, when glycogen was not added, few if any transporting nuclei were found. A low background of transporting nuclei was sometimes observed, however, if the clarified cytosol was not re-centrifuged to remove the residual golden pellet. This second pellet also had the rescue activity found with the primary pellet (data not shown). The level of removal of glycogen may account for the disparity among extracts and between investigators (see Discussion).

The enclosure of double-stranded DNA by nuclear membranes is essential for semi-conservative DNA replication to occur. This is true for both sperm chromatin and naked DNA templates (Newport, 1987; Sheehan et al., 1988; Blow and Sleeman, 1990; Newport et al., 1990; Cox and Laskey, 1991). We thus tested the effect of glycogen on DNA replication in the fractionated extract. In control assays, when replication was assayed in cytosol alone, little λ DNA replication was observed (Fig. 2, lane 2). In incubations containing λ DNA, cytosol, and membranes, but lacking glycogen (where small inert DNA spheres formed as in Fig. 1 *C*), replication was also negligible (Fig. 2, lane 1). However, in nuclei formed in the presence of cytosol, membranes, and glycogen, highly efficient DNA replication was observed (Fig. 2, lane 3). This replication was sensitive to aphidicolin, indicating that DNA polymerase α was being utilized for semi-conservative replication (Fig. 2, lane 5). We conclude that with a λ DNA template not only the assembly of nuclei, but also the function of those nuclei, defined as efficient DNA

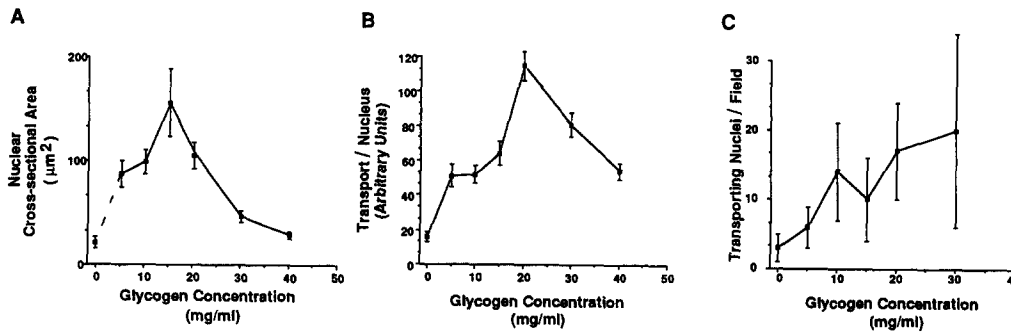


Figure 3. Effect of glycogen concentration on formation and function of nuclei using λ DNA as template. Cytosol was preincubated with λ DNA (10 μ g/ml final) and different final concentrations of glycogen, as indicated in the figure, for 60 min. Membranes and transport substrate were then added as described in Materials and Methods. The average size of nuclei (nuclear

cross-sectional area) (A), transport substrate accumulation by nuclei (transport/nucleus) (B), and average number of transporting nuclei found in a 200- μ m diam field (transporting nuclei/field) (C) are shown. The data was accumulated and processed as described in the Materials and Methods. Error bars represent standard error of the mean. Note: The structures formed in 0 mg/ml glycogen in Fig. 1 C were quite small and difficult to measure. This uncertainty in size is indicated by the dotted line. Also, the appearance of a field of nuclei at the highest concentration of glycogen (40 mg/ml) was quite distinct; small nuclei of irregular shape tended to be found in distinctive large clusters rather than as individual round nuclei evenly distributed across the coverslip. This aggregation of small nuclei is reflected in the large standard error of the mean at the highest glycogen concentration in C.

replication and nuclear transport, are greatly enhanced by the presence of glycogen.

Analysis of the Glycogen Dependence of Nuclear Formation

The optimal concentration of glycogen required for formation of functional nuclei from λ DNA was assessed by determining the number of transporting nuclei formed per field, the level of transport substrate accumulated by these nuclei, and their size at varying glycogen concentrations (Fig. 3). (The presence of transporting nuclei was used as a criteria

for successful nuclear assembly since the structures in Fig. 1 C were too small to define as "nuclei.") When glycogen was pre-incubated with λ DNA and cytosol for 1 h before membrane addition, the formation of nuclei was found to be optimal at glycogen concentrations of 15–20 mg/ml (Fig. 3, A and B). The concentration of glycogen in the crude extract was $\sim 43 \pm 7$ mg/ml (Table I). After fractionation, no detectable glycogen was found in the clarified cytosol and only a minute amount was found in the membrane fraction. The gelatinous pellet contained the vast majority of glycogen (326 ± 68 μ g/ml; Table I). The nuclei assembled from λ DNA at optimal glycogen concentrations were found to be morphologically normal when viewed by electron microscopy in that they contained a double nuclear membrane and nuclear pores (Fig. 4).

It was a formal possibility that glycogen might facilitate the formation of nuclei for a relatively non-specific reason, i.e., by increasing the viscosity of the incubation medium, or by functioning as a macromolecular crowding reagent to increase the effective concentration of components involved in assembling nuclei (Minton, 1983). Alternatively, glycogen might change the osmolarity of the reaction if it were degraded upon addition to the cytosol. Another non-specific option might be that glycogen degradation could provide additional ATP to the system which then stimulates nuclear assembly and transport. To test for such non-specific roles, we first determined whether compounds known to affect viscosity or reactant concentrations in *in vitro* reactions would rescue nuclear formation (Zimmerman and Harrison, 1987). The effect of PVP, PVA, and PEG were tested in our assay. PEG was found to strongly inhibit all nuclear formation even with sperm chromatin. When PVA and PVP (15 mg/ml) were added to the extract instead of glycogen, DNA showed a certain amount of clumping, but never became enclosed in a nuclear envelope, decondensed, or subsequently accumulated a transport substrate (Table II). Neither PVP nor PVA adversely affected the formation of transporting nuclei using a sperm chromatin template, but as stated showed no positive effect on nuclear assembly with a naked DNA template. Similarly, increasing the osmolarity of the cytosol and membranes with sucrose or the sugar *N*-acetylglucosamine did

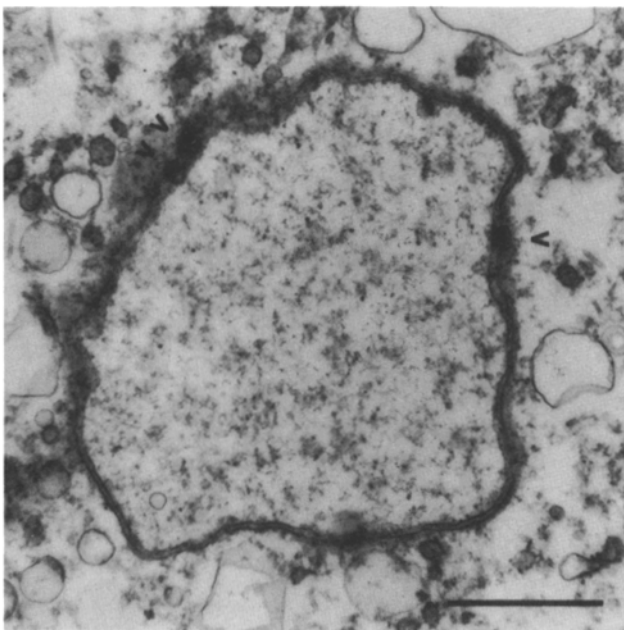


Figure 4. Electron micrograph of nuclei formed from λ DNA in reconstituted extracts. Nuclei were formed from λ DNA using 15 mg/ml glycogen. Samples were fixed and prepared for electron microscopy as described in Materials and Methods. A typical nucleus is shown. Nuclear pores are marked with arrows. Bar, 1 μ m.

Table I. Glycogen Concentration in Crude and Fractionated *Xenopus* Egg Extracts

Extract	Concentration
Whole Crude (2 ml)	43 ± 7 mg/ml
Clarified Cytosol (1 ml)	<0.1
Washed Membranes (0.2 ml)	0.6
Gelatinous Pellet (0.25 ml)	326 ± 68

Extracts were fractionated as described in the Materials and Methods. The glycogen concentration of each fraction was determined as described in the Materials and Methods. The mean and standard error of at least four measurements for each sample are shown.

not facilitate the formation of nuclei (Table II). Increasing the ATP concentration of the reaction mix to 10 mM had no effect. In combination then, these data suggest that a simple change in the effective concentration of reactants, viscosity, osmolarity, or ATP concentration is not the explanation for the effect of glycogen on the formation of nuclei.

Several components, however, did somewhat facilitate nuclear assembly. Amylopectin, a polymer of glucose that differs from glycogen only in being less branched in structure, was able to promote some nuclear assembly (Table II). Glucose, a precursor to glycogen, which is known to be readily polymerized into glycogen when injected into *Xenopus* eggs (Dworkin and Dworkin-Rastl, 1989), rescued nuclear formation about 50–60% as well as glycogen in 2/3 of the extracts (data not shown; see Discussion). Maltose, a dimer of glucose, also had this effect, as did mannose, an epimer of glucose, although the latter had to be preincubated

for at least 3 h in cytosol before DNA addition (data not shown). We predict that these small sugars, like glucose, are being converted to glycogen in the extract by the highly active glycogen conversion system (Dworkin and Dworkin-Rastl, 1989) and are therefore active. Significantly though, none of these compounds approached glycogen in efficiency.

Next, we determined whether glycogen, if hydrolyzed in the extract, could rescue formation. To test this, α -amylase which hydrolyzes glycogen at α -D-(1-4) glycosidic linkages (Bernfeld, 1951) was added to incubations containing glycogen. Formation of nuclei from λ DNA was substantially inhibited (Fig. 5, A–C; also see below). Nuclear formation activity was recovered, however, when a specific inhibitor of α -amylase (Vertesy et al., 1984) was simultaneously incubated with the enzyme in egg cytosol (Fig. 5, A–C). These data indicate that it is the polymeric nature of glycogen that is required to facilitate nuclear formation, rather than the breakdown products of glycogen hydrolysis. Indeed, neither glucose, maltose, or mannose had any stimulatory effect on nuclear assembly if α -amylase was included in the extract (data not shown), arguing that these compounds must be converted to a polymeric form to have rescue activity.

A Higher Order Chromatin Intermediate Is Assembled in Extracts Containing Glycogen

The finding that preincubation of glycogen with cytosol and DNA was required before the addition of membranes suggested that a higher order chromatin intermediate might be generated during the preincubation step. A highly condensed DNA intermediate, lacking membranes, had been observed previously as a transient intermediate in nuclear assembly when naked λ DNA was added to a crude extract (Newport, 1987). These observations prompted us to examine the chromatin formed in the presence and absence of glycogen.

Table II. Compounds Affecting Nuclear Formation and Function

	Nuclear formation	Average diameter (size range)	Transport	Number of transporting nuclei/field	DNA replication
Buffer	±*	1 μ m (1–5 μ m)	–	–	–
Glycogen	+++++	8–10 μ m (5–15 μ m)	+++++	10–20	+++++
Polyvinylpyrrolidone (40K)	±	–	–	–	±
Polyvinylalcohol (8,20K)	±	–	–	–	±
Amylopectin	+++†	2–5 μ m	+++	5–10	ND
Dextran	+	(1–10 μ m)§	±	0.3	ND
ATP (10 mM)	±	–	–	–	ND
Sucrose	±	–	–	–	–
Galactose	++	3–4 μ m (2–5 μ m)	+	0.5¶	ND
N-Acetylglucosamine	+	–	–	–	ND

The compounds indicated (15 mg/ml, except where noted) were incubated in egg cytosol with 10 μ g/ml λ DNA for 60 min. The membrane fraction was then added, followed 90 min later by transport substrate. Nuclear transport, nuclear formation, and DNA replication were assayed as described in the Materials and Methods. Samples were quantitated relative to incubations containing glycogen, which were assigned a relative value of +++++. Nuclear formation is defined as the formation of DNA-containing structures which are membrane-enclosed, as well as functionally active in transport and replication assays. ND indicates not determined.

* With buffer, PVP, PVA, ATP, or sucrose, most structures were membrane bound, but not necessarily membrane-enclosed, and of very small size (1 μ m).

† Amylopectin was difficult to solubilize and solubility varied with the source. Potato amylopectin gave the results shown. Corn amylopectin was poorly soluble and therefore ineffective.

§ Occasional rare structures up to 5 μ m were seen, which sometimes showed transport. These rare nuclei varied greatly in size such that no obvious average size was seen.

|| One transporting nucleus was seen per every three 200 μ m fields.

¶ One transporting nucleus was seen every two 200 μ m fields.

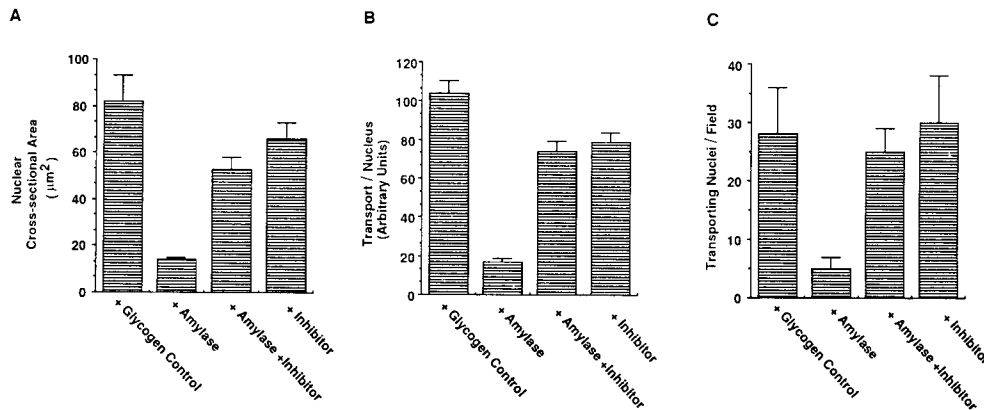


Figure 5. Effect of α -amylase digestion on nuclear formation. Glycogen, λ DNA, and cytosol were incubated as described for optimal nuclear formation in the previous figures. Samples containing glycogen were treated with α -amylase, α -amylase inhibitor, or both as described in Materials and Methods. The effect of digesting glycogen on nuclear formation was assayed and quantitated as described in Fig. 3. Nuclear size, transport substrate accumulation, and number of transporting nuclei formed are shown in A, B, and C, respectively, and described in Fig. 3.

Nucleosomes are known to be readily assembled onto linear or circular DNA in *Xenopus* egg extracts (Laskey et al., 1977; Dilworth et al., 1987; Hirano and Mitchison, 1991). This nucleosome formation can be assayed by observing the state of supercoiling of a closed circular plasmid DNA template added to the extract. Upon plasmid addition, relaxation of the supercoiled plasmid occurs quickly through topoisomerase I-induced nicking. The addition of each nucleosome to the relaxed DNA circle changes the linking number by -1 and this can be visualized as an increasingly supercoiled plasmid DNA ladder on gels (Laskey, 1977; Simpson et al., 1985; Dilworth et al., 1987; Hirano and Mitchison, 1991). When supercoiled plasmid DNA (Fig. 6, form *I*) was added to clarified cytosol either in the presence or absence of glycogen, the DNA was rapidly relaxed (Fig. 6, form *Ir*), and then progressively supercoiled as nucleosomes became assembled over the 150-min incubation (Fig. 6, ladder bands). Significantly, glycogen had no visible effect on the rate or ex-

tent of supercoiling in this assay, suggesting that glycogen does not affect the process of nucleosome formation in these extracts. In addition, subjecting the supercoiled chromatin generated in the presence or absence of glycogen to electrophoresis in the presence of chloroquine, a process which can reveal more subtle differences in nucleosome distribution, also did not reveal any differences (data not shown; Dilworth et al., 1987).

After λ DNA becomes packaged into nucleosomes in egg cytosol, it then undergoes a series of visibly distinct condensation events (Newport, 1987; Hirano and Mitchison, 1991). In our clarified cytosol, the DNA was initially observed as long thin filaments which became progressively more compact during the course of chromatin formation, but by 90 min still appeared as diffuse fluffy structures (Fig. 7 B). Using deproteinated *Xenopus* genomic DNA or phage λ DNA, Hirano and Mitchison (1991) describe the formation of similar "fluffy chromatin-like structures" in a clarified egg cytosol and did not observe further compaction. When, however, we incubated λ DNA in cytosol containing glycogen, the chromatin formed by 90 min consisted of highly compact structures (Fig. 7 A). These structures closely resemble the intermediates in nuclear assembly described by Newport (1987) in crude extracts.

To test whether the condensed chromatin intermediates formed in the presence of glycogen could be isolated and then prove functional for subsequent nuclear assembly in an extract lacking glycogen, the following was done: λ DNA was incubated in clarified cytosol for 2 h, either in the presence or absence of glycogen, and chromatin was allowed to form. The resulting assembly reactions were diluted, applied to sucrose step gradients, and centrifuged as shown in Fig. 8 A. The condensed structures that formed in the presence of glycogen (Fig. 7 A) were able to sediment through a 1 M sucrose layer and were recovered in the 2 M sucrose cushion (Fig. 8 B, + Glycogen, fraction 4). The appearance of the chromatin in this fraction was indistinguishable by fluorescence microscopy from the same intermediates prior to gradient centrifugation (data not shown; see Fig. 7 A). In contrast, the fluffy chromatin which forms in the absence of glycogen (Fig. 7 B) sedimented to the diluted extract/1 M su-

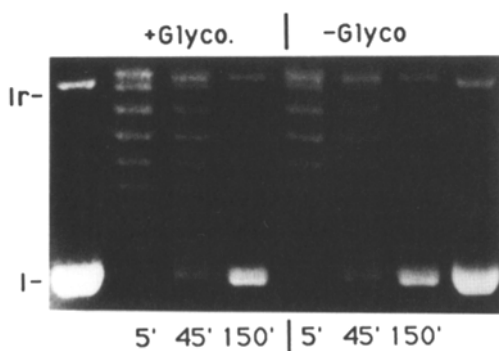
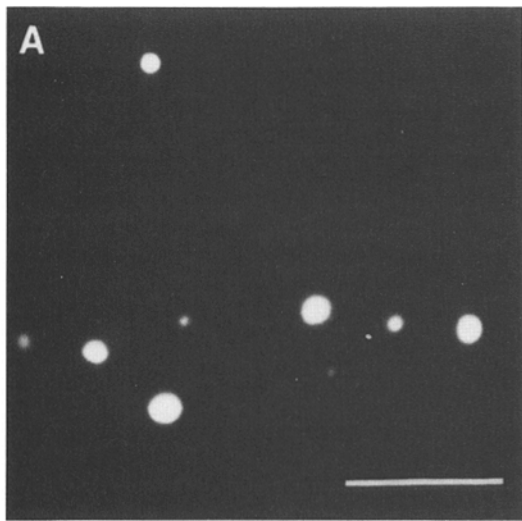
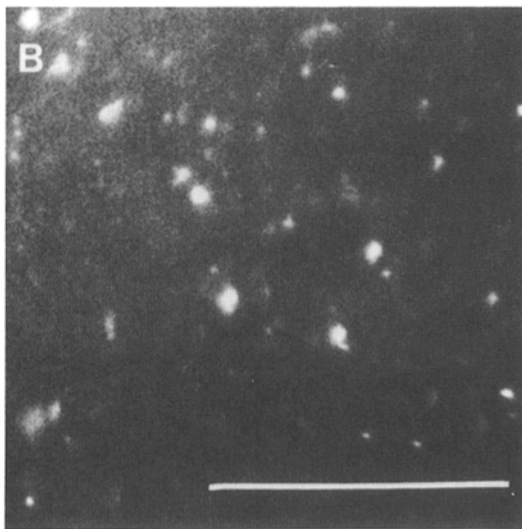


Figure 6. Glycogen has no effect on nucleosome assembly in *Xenopus* cytosol. Plasmid DNA (pBsIIKS+; 10 mg/ml) was added to clarified cytosol, containing either 15 mg/ml glycogen (+Glyco) or buffer (-Glyco), to a final concentration of 15 μ g/ml DNA. At the times indicated in the figure, samples were removed from the incubations, terminated, processed, and analyzed by electrophoresis in 1% TBE agarose gels, as described in the Materials and Methods. The first and last lanes of the figure contain input DNA. Fully supercoiled (*I*) and relaxed circular (*Ir*) topoisomers are indicated.



+Glycogen



-Glycogen

Figure 7. Higher order chromatin structures form in clarified cytosol in the presence of glycogen. λ DNA ($10 \mu\text{g/ml}$ final) was incubated in cytosol plus or minus added glycogen for 2 h. The chromatin formed in an incubation containing 20 mg/ml glycogen is shown in *A*; the chromatin formed in an incubation containing buffer is shown in *B*. The intermediates formed in the presence of glycogen appeared in the microscope much more condensed and spherical than those formed in the absence of glycogen, the latter appearing fluffy with non-defined edges. DNA was visualized with Hoechst 33258. Bar, $10 \mu\text{m}$.

crose interface (Fig. 8 *B*, + Buffer, fraction 2). Chromatin from each of the two fractions (+ Glycogen, fraction 4, and + Buffer, fraction 2) was tested for its ability to undergo nuclear assembly in a reaction lacking glycogen. The fluffy chromatin from the +Buffer gradient did not form nuclei (data not shown). In contrast, the rapidly sedimenting chromatin intermediates formed in the presence of glycogen (+ Glycogen, fraction 4) acquired nuclear envelopes, decondensed, and readily accumulated transport substrate. A rep-

A Sucrose Step-Gradient Isolation Of Chromatin

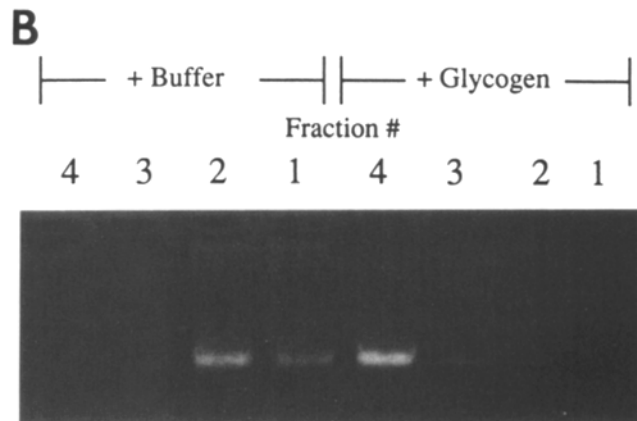
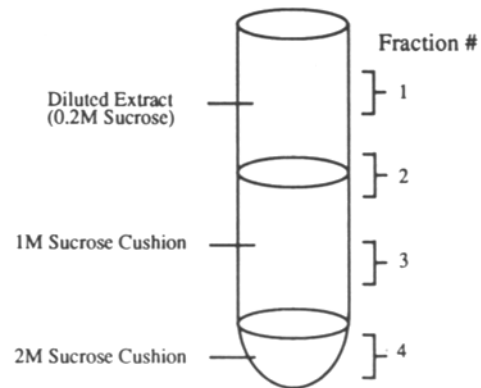


Figure 8. Sucrose step-gradient isolation of chromatin formed in clarified cytosol. Chromatin was formed by incubation of λ DNA in clarified cytosol either in the presence of buffer or glycogen for 2 h, as described in Fig. 7 and Materials and Methods. Extracts were then diluted 5.6-fold and centrifuged on a sucrose step gradient as depicted in *A*, and described in detail in Materials and Methods. Fractions (0.3 ml) were collected from the sections of the gradient shown in *A*. Aliquots of these fractions were de-proteinized and analyzed for DNA content by electrophoresis on a 1% agarose-TBE gel, followed by ethidium bromide staining (*B*) as described for plasmid DNA in Fig. 6.

resentative nucleus is shown in Fig. 9 (*A* and *B*). The efficiency of forming transporting nuclei from such rapidly sedimenting chromatin structures was high, ranging from 50 to 90%. (In a separate experiment, we showed that fortuitous sedimentation of glycogen into Fraction 4 did not occur and thus was not responsible for the formation of nuclei from chromatin in this fraction; data not shown.) These data indicate that two forms of chromatin with similar kinetics of nucleosome assembly, but completely different sedimentation and nuclear formation properties, are being generated in the presence and absence of glycogen. With glycogen, naked DNA is assembled by egg cytosol into a condensed intermediate which can then serve as an effective precursor for functional nuclear assembly without further requirement for free glycogen. In the absence of glycogen, fluffy chromatin with different sedimentation properties is formed which does not form nuclei in a second extract depleted of glycogen. Interestingly, fluffy chromatin recovered from the gradient,

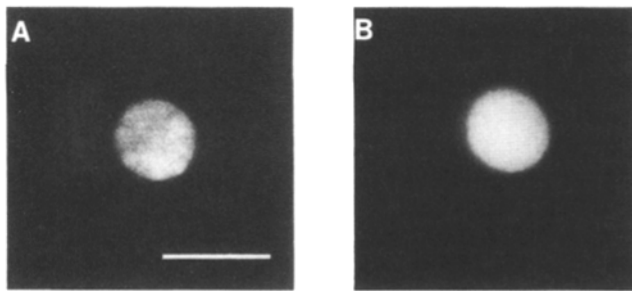


Figure 9. Nuclear assembly from chromatin isolated by sucrose step-gradient fractionation. An aliquot of chromatin, formed in cytosol containing glycogen and recovered in Fraction 4 of the sucrose step gradient of the previous figure, was added to a reconstituted extract containing both clarified cytosol and washed membranes, but no added glycogen. The incubation was assayed for the DNA decondensation coincident with nuclear assembly (A) and for transport substrate accumulation (B) after 2 h. A typical structure is shown. Bar, 10 μm .

however, was competent for nucleus formation when added to a second extract now containing glycogen (Table III). This indicates that formation of the condensed nuclear assembly intermediate can be temporally separated from nucleosome assembly.

Hirano and Mitchison (1991) have shown that the fluffy chromatin formed in interphase cytosol alone is converted to compact chromatin in mitotic extracts. This mitotic chromatin is somewhat similar in appearance to the glycogen-induced intermediates generated here. One possibility was that it is the highly compact nature of the glycogen intermediate that promotes nuclear assembly. We therefore tested the capacity of compact mitotic chromatin structures to form nuclei when added to our interphase cytosol lacking glycogen. We found that isolated mitotic chromatin when added to clarified interphase cytosol and membranes was not competent to form nuclei (Table III), unless glycogen was also present. Similarly, the fluffy interphase chromatin isolated

in parallel could form nuclei only when added to extracts containing glycogen (Table III). Thus, the formation of condensed chromatin in a mitotic extract did not mimic the glycogen-induced condensed chromatin. We conclude that the "activation" to nuclear assembly competence that glycogen induces is not simply the result of chromatin condensation, but must be the result of some other altered aspect of the chromatin. The data in Table III also demonstrate that this activation can occur after chromatin formation, although glycogen added later has a smaller effect on nuclear size than glycogen added at $t = 0'$ (Table III). In all cases, however, nuclei capable of DNA replication and transport form when glycogen is present.

Although glycogen is clearly essential for formation of the chromatin intermediate, we asked whether glycogen becomes dispensable once the intermediate is formed. To test this, we allowed chromatin intermediates to form for 2 h in cytosol containing glycogen. We then added α -amylase to the reaction for 30 min followed by membrane addition and a further 90-min incubation. The reaction was assayed for transport-competent nuclei (Fig. 10). As expected, addition of α -amylase to cytosol and λ DNA at $t = 0'$ virtually abolished the formation of transporting nuclei (Fig. 10 A; see also Fig. 5). In contrast, addition of α -amylase to extracts after the assembly of the condensed intermediate ($t = 90'$) did not significantly reduce the formation of transporting nuclei (Fig. 10 C). These data suggest that glycogen is required for the assembly of a chromatin intermediate competent to form nuclei, but that once this structure is assembled it can either be isolated from the initial glycogen-containing assembly extract (Fig. 9) or exposed to α -amylase (Fig. 10) without affecting subsequent nuclear assembly.

Discussion

In this report we have fractionated *Xenopus* egg extracts to analyze the requirements for assembly of nuclei around a protein-free DNA template. We find that when λ DNA is added to a nuclear reconstitution system consisting of highly clarified cytosol and membrane fractions, the DNA becomes

Table III. Nuclear Assembly Using Isolated Chromatin Intermediates

Intermediate	Glycogen		Number of structures/slide		Percent of structures transporting	Average Volume
	Pre	Post	Number intermediates*	Number nuclei‡		
Mitotic	—	+	20	20	50	8
	—	—	40	0	0	0.5§
Interphase	—	+	15	25	63	8
	—	—	36	4	10	0.5
	+	+	10	40	80	264
	+	—	10	30	75	113

The isolation of chromatin intermediates and testing for nuclear formation is presented in detail in the Material and Methods. Briefly, DNA was added to interphase or mitotic cytosol plus or minus glycogen. After 2 h, the mixture was diluted and applied over a sucrose cushion. After centrifugation, fractions were collected and analyzed for chromatin content. The fraction(s) containing the majority of the chromatin were used to test for nuclear assembly by addition to a second reaction mix containing interphase cytosol plus or minus glycogen. At 45 min membranes were added and nuclei allowed to form for 60 min. Transport substrate was then added and transport assayed 60 min later. Similar results were obtained in five separate experiments. The results of a representative experiment are shown. The nuclei formed in reactions where glycogen was added late were significantly smaller than when glycogen was present at $t = 0'$. However, the small nuclei were much larger than structures formed in the absence of any glycogen and, in addition, were functional.

* Chromatin intermediates are defined as 1–2 μm DNA-containing structures. In general, they were too small to determine if they were membrane enclosed.

‡ Nuclei were defined as DNA-containing structures which had become enclosed in membrane and were capable of transport.

§ No nuclei formed; the average volume of the chromatin intermediate is given.

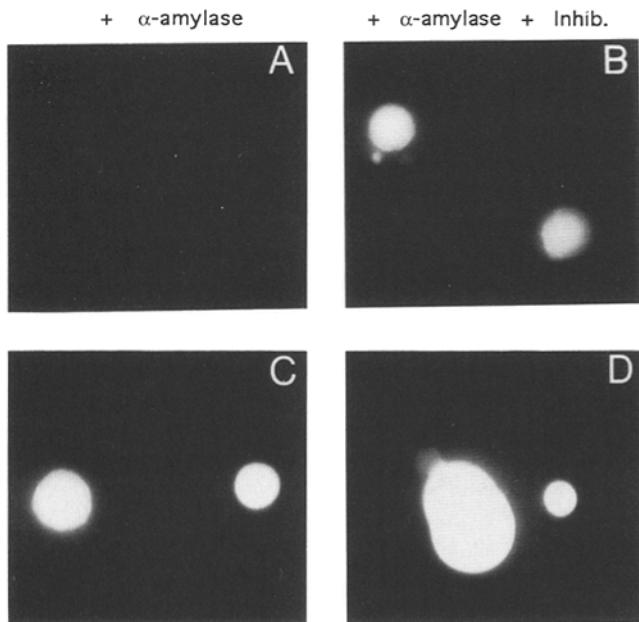


Figure 10. α -Amylase treatment of extracts containing chromatin intermediates: effect on nucleus formation. Condensed chromatin was formed by incubation of λ DNA (10 $\mu\text{g}/\text{ml}$ final concentration) in cytosol containing glycogen (15 mg/ml final) for 90 min. 1 μl of α -amylase (C) or α -amylase + α -amylase Inhibitor (D) was then added to extracts containing the intermediates and digestion permitted to proceed for 30 min. Inhibitor + membranes or membranes alone were then added to the respective incubation at $t = 120'$ and the capacity to form transport-competent nuclei determined by the addition of transport substrate 90 min later, followed by observation in the fluorescence microscope. Accumulation of a TRITC-labeled nuclear transport substrate is shown in all panels. A and B show control nuclear assembly reactions where amylase was added to cytosol containing glycogen at $t = 0'$ before the addition of λ DNA, as in Fig. 5.

packaged into nucleosomes and binds membrane vesicles. However, these structures are quite small and are functionally inert, capable neither of DNA replication nor nuclear transport. In consequence, we refer to them as small, inert DNA spheres, rather than "nuclei". Surprisingly, we find that a component of the gelatinous pellet of fractionated extracts dramatically restores the formation of large functional nuclei, when preincubated with the clarified cytosol before membrane addition. This pellet component is glycogen. Nuclei formed in the presence of glycogen are capable of very efficient nuclear growth, DNA replication, and nuclear transport. Prior treatment of the cytosol and glycogen with α -amylase negates the effect of glycogen and results once again in the formation of small, inert DNA spheres, indicating that the polymeric nature of glycogen is necessary for its assembly promoting activity. Precursors to glycogen which are thought to be quickly polymerized into glycogen can substitute for glycogen, albeit less efficiently, but no non-specific agents were found which could substitute for glycogen. The necessity for preincubation of glycogen with cytosol and λ DNA implied a role for glycogen in the assembly of some type of chromatin intermediate. Upon testing this hypothesis using cytosol alone, nucleosomes were found to assemble onto naked DNA at the same rate, both in the pres-

ence and absence of glycogen. However, with further incubation glycogen promoted the formation of highly condensed, presumably higher order chromatin intermediates. These could be isolated on a sucrose step gradient and differed considerably in their sedimentation properties from the fluffy, non-glycogen intermediate. When isolated, they were capable of efficient nuclear formation in extracts lacking glycogen, arguing that the role of glycogen in assembly is fulfilled in the initial preincubation. Consistent with this, post-treatment with α -amylase did not prevent these intermediates from becoming transport-competent nuclei. In addition, mitotically condensed chromatin intermediates formed in the absence of glycogen could not form nuclei unless glycogen was later added. This argues that it is not simply and condensed state of chromatin that is induced by glycogen, but another essential modification of the chromatin.

Chromatin and Nuclear Formation Using Glycogen

The requirement for glycogen in the formation of nuclei from naked DNA is in sharp contrast to nuclear formation with a complex frog sperm chromatin template, which requires only clarified cytosol and membrane fractions (Lohka and Masui, 1984; Sheehan et al., 1988; Wilson and Newport, 1988; Finlay and Forbes, 1990). Workers using cytosolic and membrane fractions of the extract (Newport, 1987; Blow and Sleeman, 1990) have also described nuclear formation using a naked λ DNA template. These investigators, however, did not recentrifuge the initial cytosolic fraction, which we now know removes residual glycogen, nor did they report on the efficiency of formation, as assayed by the number of nuclei formed or by transport substrate accumulation. Presumably those studies contained sufficient glycogen in the cytosol for nuclear assembly to take place, although relative to the *in vivo* assembly of synthetic nuclei, the earlier *in vitro* assembly reactions in fractionated egg extracts were often inefficient and variable (J. Newport, personal communication; Blow and Sleeman, 1990). We found that clarification of the cytosolic fraction by a second centrifugation step virtually eliminate the ability of cytosol and membranes to assemble condensed intermediates and nuclei. Moreover, such centrifugation generated a second small gelatinous pellet which could also rescue nuclear formation. We think this accounts for the disparity in different investigators' work. The finding that glycogen plays a role in nuclear assembly in egg extracts now allows one to reproducibly and very efficiently form λ nuclei using frozen fractionated extracts.

Optimal nuclear formation required that glycogen be preincubated with naked DNA and cytosol before the addition of membranes. This suggested that glycogen facilitates the formation of a nuclear assembly intermediate before membrane binding, a suggestion supported by our subsequent biochemical isolation of the intermediate. Although nuclear formation does proceed in unfractionated assembly extracts where all components are present simultaneously (Newmeyer et al., 1986a; Blow and Laskey, 1986; Newport, 1987), the number of nuclei formed, as well as the level of nuclear transport, was 2-5 fold lower (P. Hartl and D. Forbes, unpublished data).

The populations of chromatin intermediates formed in the absence or presence of glycogen in our fractionated extract differ not only in their capacity to form nuclei, but in their sedimentation properties. Chromatin intermediates formed

in the presence of glycogen are of sufficient density to sediment through a 1 M sucrose cushion and, when isolated, are fully competent for nuclear formation in a reconstituted extract not containing glycogen. On the other hand, fluffy chromatin formed in cytosol lacking glycogen is not able to enter a 1 M sucrose cushion and does not form nuclei. The addition of glycogen promotes the formation of a chromatin intermediate reminiscent in appearance and biochemical extraction behavior to the assembly intermediate described by Newport (1987) using both crude and extracts less fractionated than ours. The intermediate described by Newport (1987) showed a proteinaceous central scaffold with a peripheral halo of DNA upon treatment with detergent and high salt extraction, a scaffold also seen with somatic nuclei (for a review see Gasser et al., 1989). Our readily sedimenting chromatin intermediates formed in the presence of glycogen showed an identical behavior upon detergent and salt extraction (P. Hartl and D. Forbes, unpublished data), suggesting that the two sets of highly condensed intermediates are similar. The chromatin formed in the absence of glycogen is of a dispersed or "fluffy" appearance. Hirano and Mitchison (1991) describe similar "fluffy chromatin-like structures" when working only with the clarified cytosol, which they produce with a 50,000 g, 4-h spin and which can be frozen; these authors specifically comment on their inability to form the intermediates described by Newport (1987). We do not know whether the failure to form condensed intermediates in their system was due to the fact that their clarified cytosol lacked sufficient glycogen, or to such a lack combined with freezing of the extract. It should now be possible, however, with the fractionation procedure used here to form complete condensed intermediates or the less condensed fluffy chromatin, as desired for given experiments.

The involvement of glycogen in the formation of nuclei was unexpected. Compounds such as polyvinylalcohol, polyvinylpyrrolidone, dextran, sucrose, and *N*-acetylglucosamine, which change effective reactant concentrations, viscosity, or osmolarity could not substitute for glycogen in the nuclear reconstitution assay (Table II), arguing against these possibilities as the explanation for the large enhancement of nuclear assembly by glycogen. In addition, the digestion of glycogen by α -amylase prevented nuclear formation, indicating that glycogen is required as a polymer rather than a product of hydrolysis.

In considering the mechanism by which glycogen promotes nuclear formation, it is interesting to note that glycogen makes up $\sim 5\%$ of the dry weight of the *Xenopus* egg. Surprisingly, this embryonic glycogen is not degraded for use as an energy source until the early gastrula stage of development (for review see Dworkin and Dworkin-Rastl, 1991). Thus, large stores of glycogen are continuously present in the *Xenopus* embryo during the rapid cell cycles of the cleavage stage, which precedes the mid-blastula transition. Indeed, it has been observed that if intermediates in glycolysis, such as glucose 6-phosphate and phosphoenolpyruvate, are microinjected into *Xenopus* eggs or early embryos, they are quantitatively converted to glycogen rather than used as an energy source (Dworkin and Dworkin-Rastl, 1989). Consistent with these data, we find that glucose, the monomeric sugar from which glycogen is synthesized (BeMiller, 1989; Smythe and Cohen, 1991), maltose, a disaccharide com-

posed of two glucose monomers, and mannose, an epimer of glucose, will rescue nuclear formation 50 to 60% as well as glycogen in approximately two-thirds of the fractionated extracts tested, as judged by the number of nuclei formed and by their size (P. Hartl and D. Forbes, unpublished data). If, however, α -amylase is present, no such rescue is seen. Melibiose, *N*-acetylglucosamine, glycerol, and sucrose have no effect. We hypothesize that the extract is converting added glucose (and related sugars) to larger polymers in a manner identical to that observed in an injected egg, or through a back reaction of glycogen's degradative enzymes (Dworkin and Dworkin-Rastl, 1989) and that the glycogen formed then promotes nuclear assembly. The early embryo of *Xenopus* undergoes extremely rapid cell division, alternating between the S and M phases of the cell cycle. Multiple mechanism to speed the assembly of nuclei may have evolved that are unique to the embryo. One such mechanism could be predicted to use glycogen in the manner observed here. Such mechanisms may also occur in somatic cells but this has not yet been examined.

Models for Glycogen Activity

Glycogen could play a number of roles in nuclear assembly, both structural and catalytic. Two types of structural models are possible. In one, glycogen could form a structural component of the chromatin intermediate. In this model, glycogen could facilitate the assembly of the intermediate itself, but not mediate nuclear membrane assembly. The observation that α -amylase does not abolish nuclear formation when added after to intermediate formation is consistent with such a model, but we cannot exclude the possibility that the glycogen assembled into the intermediate is resistant to α -amylase digestion. Alternatively, glycogen could be a structural component of the chromatin intermediate, but be primarily involved in the subsequent binding of nuclear membrane vesicles (Wilson and Newport, 1988). One possible precedent for such a model is the finding in skeletal muscle that a 161-kD protein, the "G-targeting unit" of protein phosphatase 1, binds specifically both to glycogen and sarcoplasmic reticulum membranes, thus potentially linking these components together (Hubbard and Cohen, 1989; Hubbard et al., 1990; for reviews see Cohen, 1989; Hubbard and Cohen, 1993). Those authors conclude that formation of a trimeric complex of (phosphatase 1)-(glycogen)-(SR membrane) is a likely possibility. The small inert DNA spheres observed here in the absence of glycogen contain bound membrane; we cannot judge whether membrane completely encloses all such spheres, but one possibility is that added glycogen helps form a more productive surface for vesicle fusion or a later step in nuclear envelope assembly.

A number of studies have reported glycogen in association with the nuclear envelope, the endoplasmic reticulum, and with annulate lamellae of embryonic and transformed cells (Coimbra and Lebland, 1966; Flaks, 1968; Eyal-Giladi et al., 1985; Kessel, 1989; Hubbard et al., 1990; Kessel and Beams, 1990). Glycogen has also been reported inside the nucleus (Himes and Pollister, 1962; Karasaki, 1971; Mori et al., 1970; Granzow et al., 1981; Kopun et al., 1989; for a review of intranuclear glycogen see Ferrans et al., 1975). These reports of glycogen in close apposition with nuclear envelopes and annulate lamellae may represent a structural association, or merely a circumstantial association. Further

work will be required to determine whether glycogen is an actual component of the chromatin intermediates we observe. It should be noted that carbohydrate modifications have been found in increasing numbers on nuclear proteins, including the *N*-acetylglucosamine residues found on many nuclear pore proteins and transcription factors. It has been hypothesized that lectin-protein interactions may well contribute to the structure of nucleus or to the function of its components (Hart et al., 1989). Glycogen may prove to directly mediate the binding of two protein moieties and in doing so promote nuclear formation.

Alternatively, glycogen could play several types of catalytic roles in nuclear assembly. Specifically, glycogen could bring into apposition DNA and chromatin proteins to form the chromatin intermediate, or could mediate the interaction between the chromatin intermediate and nuclear membrane vesicles, but after catalyzing the association or stabilization, glycogen would dissociate.

One possibility was that glycogen acts by somehow promoting high levels of chromatin condensation and it is this condensed state of the chromatin that facilitates nuclear assembly. If this were so, one would argue that condensed mitotic chromatin, when added to a mixture of interphase cytosol and membranes lacking glycogen, would form functional nuclei. We found this not to be true. Mitotic chromatin, upon isolation and addition to cytosol and membranes, formed small inert, membrane-bound structures. However, if glycogen was added to the isolated chromatin in the latter mix, functional nuclei were formed. The parallel reaction with isolated interphase ("fluffy") chromatin showed the same rescue by glycogen (Table III). Thus, we conclude that the effect of glycogen is not simply the induction of a highly condensed state, but the induction of some other alteration in the chromatin state. We also conclude that a defective intermediate can be "cured", i.e., converted to an assembly-competent intermediate, by the later addition of glycogen.

In consideration of possible models for glycogen action, it is intriguing that the highly conserved protein phosphatase 1 (PP-1) is found associated with the particulate fraction of most cells and is effectively sequestered by glycogen itself in muscle cells (for review see Hubbard and Cohen, 1993). In muscle cells when PP-1 is bound to glycogen, the phosphatase is not only compartmentalized but greatly activated (Hubbard et al., 1990). This has prompted Hubbard and Cohen (1993) to propose a model whereby phosphatases and kinases with generalized specificity are converted to more selective enzymes, by virtue of their binding to a "targeting" subunit which localizes the phosphatase or kinase to a particular cellular structure, such as microtubules, chromosomes, organelle membranes, etc. Many nuclear events during the cell cycle are strongly repressed or activated by changes in the protein phosphatase/kinase balance, including cell cycle progression, chromatin condensation, nuclear membrane binding, and RNA transcription (Hirano and Mitchison, 1991; Pfaller et al., 1991; Boseman-Roberts et al., 1991; Hartl et al., 1993). In our experiments, glycogen could well be affecting the localization and activity of PP-1, shifting the balance of protein kinase and phosphatase activities such that it favors the assembly of higher order chromatin intermediates and thus subsequent nuclear formation. Relevant to this model, Walker et al. (1992) have demonstrated that PP-1 activity is required for maintaining the in-

terphase state and for DNA replication in cycling *Xenopus* egg extracts. Significantly, PP-1 is tightly associated with mitotic chromosomes in somatic cells and, moreover, is required for nuclear reformation at the end of mitosis (Fernandez et al., 1992). Studies in non-mammalian systems also implicate PP-1 as being an essential element for regulating the events at the end of mitosis, including chromatin decondensation and nuclear reassembly (Doonan et al., 1989; Boehr and Beach, 1989; Stone et al., 1993). One possibility is that it is glycogen that sequesters PP-1 to mitotic chromosomes in vivo and to the condensed chromatin intermediate we observe, which both then become functional for nuclear assembly.

Clearly, the experiments described here do not yet distinguish between the above possible models. However, the different models are presented so that one can see the range of possibilities that must be considered to explain the large experimental effect of glycogen. The studies presented here should permit the future manipulation of *Xenopus* egg extracts for the formation of distinct populations of chromatin and nuclear structures, which can then be used to study the macromolecular components of these structures, as well as their role in replication, transcription, and nuclear transport. The intriguing role of glycogen in nuclear assembly can then be approached in such intermediates. A similar nuclear assembly extract derived from *Drosophila* eggs has been described; a role for glycogen in nuclear assembly in this organism can also now be tested (Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990).

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