DNA ligase I from *Xenopus laevis* eggs

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ABSTRACT

We have purified the major DNA ligase from *Xenopus laevis* eggs and raised antibodies against it. Estimates from SDS PAGE indicate that this DNA ligase is a 180 kDa protein. This enzyme is similar to the mammalian type I DNA ligase which is presumed to be involved in DNA replication. We have also analysed DNA ligase activity during *X. laevis* early development. Unfertilized eggs contain the highest level of activity reflecting the requirement for a large amount of DNA replicative enzymes for the period of intense replication following fertilization. In contrast with previous studies on the amphibians axolotl and *Pleurodeles*, the major DNA ligase activity detected during *X. laevis* early development is catalysed by a single enzyme: DNA ligase I. And the presence of this DNA ligase I in Xenopus egg before fertilization clearly demonstrates that the exclusion process of two forms of DNA ligase does not occur during *X. laevis* early development.

INTRODUCTION

DNA ligases catalyse the formation of a phosphodiester bond at single stranded breaks in double stranded DNA (1). This activity is required during replication, repair and recombination of DNA. A single NAD-dependent DNA ligase has been found in bacteria (2, 3) while two different ATP-dependent DNA ligase activities (type I and type II) have been described in mammalian cells (4–6). The respective roles of the type I and the type II eukaryotic DNA ligases, which possess different catalytic properties (7,8) and are not serologically related (9,10), are still under investigation. At present, type I DNA ligase seems to be implicated in DNA replication (11) whereas type II DNA ligase would be involved in DNA repair (12–14) and possibly in DNA replication (15). Eukaryotic DNA ligases have been purified from many different organisms including mammals (8,16,17,18), plants (19), and insects (20), but not from an amphibian. In many respects the amphibian egg provides a potentially valuable system for purifying the enzymes involved in DNA metabolism (21–26) because they are stored in mature oocyte waiting to be used during segmentation (27). The availability of purified enzymes combined with the recent development of systems using cell free extracts have made *X. laevis* an excellent model to study both DNA replication (28) and DNA repair (29,30). Having these considerations in mind, we purified *X. laevis* DNA ligase I and raised antibodies against it. We also studied DNA ligase activity during *X. laevis* early development. We found that DNA ligase I is present from the oocyte through the embryo. These findings are different from previous reports on other amphibians (axolotl and *Pleurodeles*) where the authors David *et al.* have assumed that the only DNA ligase activity detected in the unfertilized egg was a type II DNA ligase because of its sedimentation coefficient (31,32).

EXPERIMENTAL PROCEDURES

Reagents and enzymes

Oligo(dT)$_{16}$, and poly(dA)$_{400}$ were obtained from Pharmacia. Poly(rA)$_{400}$ and bacterial alkaline phosphatase were from Sigma. Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase were supplied either by Biolabs or Boehringer. Phosphocellulose (P11), and Blue Sepharose CL6B, G25 Sephadex and Mono Q were purchased from Whatman and Pharmacia respectively. [α-^{32}P]ATP (400 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. The rabbit polyclonal antibodies raised against bovine DNA ligase I were a generous gift from Tomas Lindahl.

Xenopus laevis eggs and embryos

Unfertilized eggs were obtained from laboratory reared *X. laevis* females by injection of 800 Units of human chorionic gonadotropin. For fertilization, the eggs were squeezed from an induced female directly into a Petri dish and fertilized with a fresh sperm suspension that has been prepared from testes crushed with forceps in F1 medium pH 7.8 (31 mM NaCl, 1.7 mM KCl, 1 mM $CaCl_2$, 2 mM NaOH, 10 mM Hepes). Eggs and embryos staged according to Nieuwkoop and Faber (33) were dejellied in 2% cystein in F1 medium and frozen in liquid nitrogen.

DNA ligase assays

The DNA ligase substrate, [^{32}P]oligo(dT)$_{16}$-poly(dA)$_{500}$ was prepared as described by Arrand *et al.* (7). 4 to 5×10^4 cpm of substrate were used per 20 μl assay which contained 60 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 50 μg/ml BSA. The reaction was started by the addition of 5 μl of cellular crude extract or partially purified enzyme fractions and incubated for 20 min at 37°C. The reaction was stopped by heating at 100°C for 2 min. Aliquots (10 μl) were removed and treated twice for 15 min at 80°C with 0.2 unit of bacterial alkaline

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phosphatase. The reaction prroducts were precipitated in 10% TCA, filtered through GF/C paper and radioactivity determined by in liquid scintillation counting. One unit of DNA ligase converts 1 nmol of [^{32}P]oligo(dT)$_{16}$ into an alkaline phosphatase resistant form in 20 min at 37°C. Specific activities are expressed per mg of protein determined according to Bradford (34). Aliquots (5 µl) from the standard assay were removed at the end of the incubation and the reaction products were analysed following electrophoresis through a 20% polyacrylamide/8M urea gel. Blunt ended DNA ligation was assayed in a 20 µl reaction containing 20 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$, 10 mM DTT, 20 µg/ml BSA, 1 mM ATP, 200 mM NaCl, 10% PEG 6000, 0.25 µg EcoRV digested Bluescript DNA and 5 µl of protein extract. After incubation at 37°C for 15 min the reaction was analyzed by electrophoresis on 0.8% agarose gel.

Purification

All operations were carried out at 2–4°C. Dejellied eggs were homogenized in a glass and teflon homogenizer in two volumes of extraction buffer (20 mM Tris/HCl pH 7.5, 2 mM DTT, 500 mM KCl, 0.2% NP40, 5% glycerol, 5% sucrose, 1 mM PMSF, 1 µg/ml leupeptin, 1 mg/ml pepstatin A, 20 mM $Na_2S_2O_5$). The homogenate was sonicated (3×15 s) and centrifuged for 60 min at 12,000 g. The supernatant was filtered through glass wool (crude extract) and then proteins were separated by ammonium sulphate fractionation. The 40–60% suspension containing ligase activity was chromatographed through a G75 Sephadex column (2.5×100 cm) pre-equilibrated in buffer A (50 mM Tris/HCl pH 7.5, 0.1 mM DTT, 1 mM EDTA, 10 mM KCl, 0.2% NP40, 5% glycerol). Active fractions were directly applied to a phosphocellulose column (5×15 cm) equilibrated with Buffer A and after washing with buffer A, proteins were eluted with the same buffer containing 300 mM NaCl. The DNA ligase activity, present in the 300 mM NaCl eluate was precipitated with ammonium sulphate (60%), dissolved in buffer A and desalted through a G25 Sephadex column (2.5×20 cm). The resulting fraction was applied to a blue Sepharose CL6B column (2.5×5 cm), pre-equilibrated with buffer B (buffer A + 10 mM $MgCl_2$) and proteins were eluted with the same buffer containing 2.5 M NH_4Cl. Proteins in the high salt eluate were precipitated by ammonium sulphate, desalted through a Sephadex G25 equilibrated with buffer A and then were applied to a ssDNA Ultrogel column (1×3 cm), pre-equilibrated with buffer A. Proteins were eluted with the same buffer containing 1M NaCl. Active fractions were pooled, dialysed against buffer C (30 mM NaCl, 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 0.5 mM DTT and 10% glycerol) and were then loaded onto a FPLC Mono Q column (Pharmacia) equilibrated with buffer C. Proteins were eluted with a 20 ml linear gradient of 0 to 1 M NaCl in buffer C. Fractions containing DNA ligase I activity were pooled, adjusted to 20% glycerol and stored at −80°C.

Formation of the DNA ligase adenylylated complex

The DNA ligase-AMP adduct reaction was performed as described by Banks and Barker (35) with slight modifications. Reactions were incubated at room temperature for 30 min in 50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 40 mM KCl, 0.03% NP40, 0.25 µM ATP containing 2 µCi of [α-^{32}P]ATP in a total volume of 20 µl. Reactions were terminated by addition of 10 µl sample buffer containing 185 mM Tris/HCl pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue, heat denatured and then electrophoresed through a polyacrylamide gradient (2–22.5%) SDS gel. Adenylylated polypeptides were detected by autoradiography on Amersham MP film.

Antibody preparation

Proteins in the active fraction eluted from the FPLC Mono Q column were separated by SDS PAGE, electrophoretically transfered onto a nitrocellulose membrane and stained with Indian Ink. The band corresponding to the DNA ligase polypeptide was cut out and the piece of nitrocellulose was dissolved in Me_2SO. After adding Freund's adjuvant the solution was injected every 15 days into New Zealand white rabbits. Serum was directely used for antigen-antibody reactions.

Western blotting

The proteins to be analysed were separated by SDS PAGE and electrophoretically transfered onto a nitrocellulose membrane. The membrane was rinsed with 140 mM NaCl, 2.5 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5, then saturated with the same buffer containing 0.1% BSA and incubated for 3 h with the antibodies. The antigen-antibody complexes were detected by incubation with [I^{125}] protein A followed by autoradiography.

RESULTS

To determine whether the level of DNA ligase activity changes during early development, we carried out a quantitative assay on crude extracts obtained at various developmental stages. Neither phosphatase nor nuclease activity were detected (fig.1A) and the assay is linearly dependent on protein concentration (fig.1B). The fluctuation of DNA ligase activity during early development is shown in table I, Mature oocytes and unfertilized eggs have the highest level of ligase activity. From the egg onwards, the ligase activity drops through early development. Thus ligase activity is high in the rapidly dividing embryo and then drops when cell division slows down. Analysis of *X. laevis* DNA ligase by sedimentation through sucrose gradients detected a single peak of activity sedimenting at 4.2 S throughout the stages of early development (data not shown).

The DNA ligase from *X. laevis* egg is a type I DNA ligase

DNA ligase I has two specific catalytic properties that differentiate between it and DNA ligase II activity. DNA ligase I can join blunt ended DNA duplexes but can not ligate oligo(dT)$_{16}$ hybridized with a poly(rA)$_{500}$ polymer. We were unable to measure any ligation of the hybrid DNA/RNA substrate, but a partially purified extract from either oocyte, unfertilized egg or

Table 1. Quantification of the DNA ligase activity during *X. laevis* early development.

Developmental stage	Time after fertilization	Specific activity Units/mg×10^{-6}
Oocytes VI		12.0
Unfertilized eggs		12.5
Stage 5	2 h 45 min	12.3
8	5 h	11.5
14	16 h	11.7
20	22 h	10.7
35	50 h	8.1
Liver		1.3

embryo was able to ligate plasmid molecules with blunt ends (Fig.2). This demonstrates that the major DNA ligase activity found in *X. laevis* egg is a DNA ligase I activity, and that the enzyme is already present and active in oocyte before fertilization.

Identification of DNA ligase polypeptides in *X. laevis* egg extract

Since there is significant conservation of amino acid sequence between eukaryotic DNA ligases (36), we have used monospecific antibodies raised against bovine DNA ligase I (16) to detect cross-reacting polypeptides in extracts from *X. laevis* . As shown in Fig.3, partially purified extracts contain two major polypeptides of 180 and 76 kDa that cross-react with the antiserum. In some cases a minor 130 kDa polypeptide was also detected. Although there is some quantitative variation in the relative proportion of the different polypeptides, it is clear that they are all present in the different development stages.

During the first step of the reaction catalysed by the DNA ligase, called DNA ligase-AMP adduct reaction, the enzyme reacts with ATP to form a stable covalent intermediate between AMP and the protein. This property has been widely used to detect DNA ligase polypeptides. We therefore investigated the formation of enzyme adenylylated complexes using the different protein extracts. As shown in Fig.4 when the partially purified

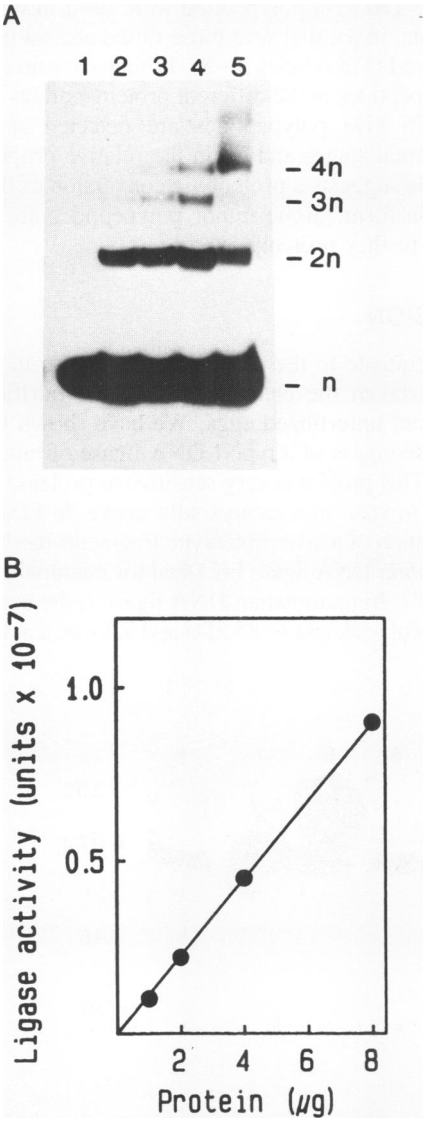

Figure 1. Quantification assay for DNA ligase activity. Linear relationship between DNA ligase activity and protein content. DNA ligase assay was performed as described in Experimental Procedure using oligo$(dT)_{16}$-poly$(dA)_{500}$ as a substrate and crude protein extract from a stage 14 *X. laevis* embryo. **A**, analysis of DNA ligation products on denaturing gel. **lane 1**, control with 0 µg of protein extract; **lanes 2 to 5**, respectively 1 µg, 2 µg, 4 µg, 8 µg of protein. **B**, quantification plot curve: DNA ligase as a function of protein is expressed in units as defined in experimental procedures.

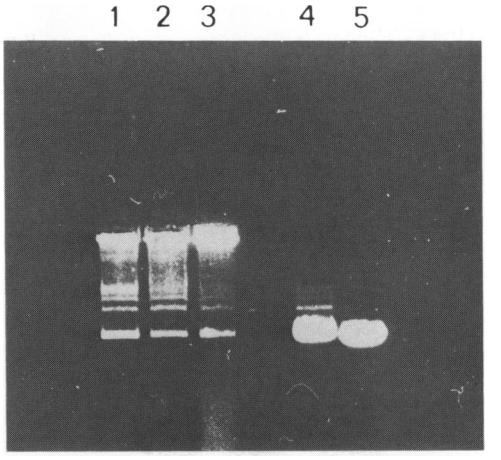

Figure 2. DNA ligase I activity in *Xenopus laevis*. DNA ligase activity partially purified by ammonium sulfate precipitation and gel filtration was assayed on EcoRV digested Bluescript DNA as described in Experimental Procedures. Protein extracts were as follows: **1**, oocyte; **2**, unfertilized egg; **3**, stage 8 embryo; **4**, control with T4 DNA ligase; **5**, control with no protein extract.

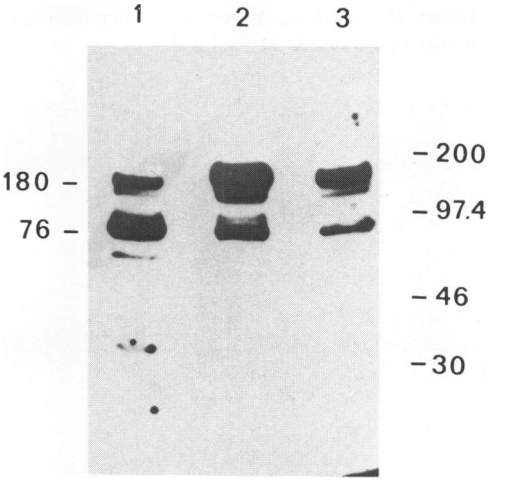

Figure 3. Immunological cross reaction between calf thymus DNA ligase I antibodies and *X. laevis* DNA ligase I. *X. laevis* DNA ligase I partially purified by ammonium sulfate precipitation and gel filtration was electrophoresed through a 7.5% SDS polyacrylamide gel and transferred onto nitrocellulose. Polypeptides recognized by 1:500 dilution of the antiserum were detected as described in Experimental Procedures. The molecular mass markers are: myosin, 200 kDa; phosphorylase b, 97.4 kDa; ovalbumin, 46 kDa and carbonic anhydrase, 30 kDa. The protein extracts are as follows: **1**, oocyte; **2**, unfertilized egg; **3**, stage 8 embryo.

extracts are incubated with [α-^{32}P] ATP two polypeptides of 180 and 76 kDa are labelled. This strongly suggests that these polypeptides are related to the DNA ligase enzyme. As already observed in immunoblotting experiments the relative abundance of the two adenylylated complexes is subject to variation that depends on the preparation and delay in the purification procedure. In initial purification experiments we obtained a single 76 kDa adenylylated polypeptide that retained ligation activity. When a 180 kDa adenylylated polypeptide was digested with a non-specific protease, it was rapidly converted to a 76 kDa polypeptide, indicating that the 76 kDa polypeptide is a proteolytic fragment of the 180 kDa enzyme (data not shown).

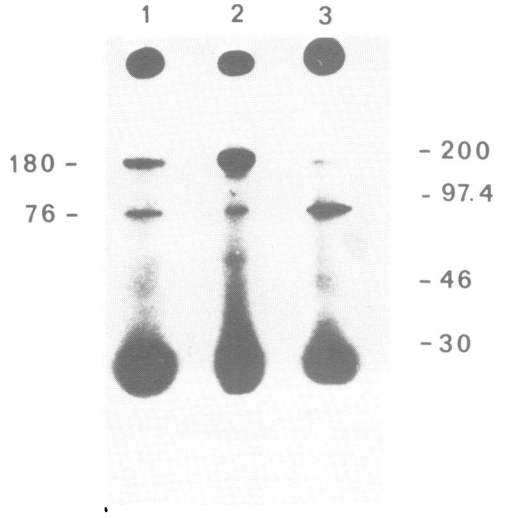

Figure 4. DNA ligase AMP adducts. DNA ligase [^{32}P] AMP adducts, formed as described under experimental procedures, were electrophoresed through a 7,5% SDS polyacrylamide gel which was fixed, dried down and exposed to X-ray film. The protein extracts are as follows: **1**, oocyte; **2**, unfertilized egg; **3**, stage 8 embryo. Molecular weight marker as for Fig.3.

Purification of the 180 kDa polypeptide

The previous results suggested that the DNA ligase I from *X. laevis* is a 180 kDa polypeptide that is sensitive to proteolysis. We therefore purified that polypeptide from unfertilized eggs and raised antibodies against it. To minimize proteolysis, the purified extract was resolved on SDS PAGE and blotted onto nitrocellulose membrane. The portion of the membrane containing the 180 kDa polypeptide was used directely to raise antibodies. As shown in Fig.5 the nitrocellulose membrane stained with Inda ink after transfer shows a single polypeptide of 180 kDa molecular weight (lane 1). An aliquot of the purified extract labelled with [α-^{32}P] ATP and resolved on the same SDS gel shows that the major adenylylated complex co-migrated with the 180 kDa polypeptide (lane 2). An over exposure of the autoradiograph shows lower molecular weight adenylylated complexes resulting from proteolysis. The antibodies raised against the 180 kDa polypeptide were used in western blotting experiments, in parallel with those raised against the mammalian DNA ligase I (Fig.5 lanes 3–8). Both antiserum recognized the same polypeptides in the different protein extracts. The 180 kDa and the 76 kDa polypeptides are detected at the different developmental stages and again the relative proportion of each polypeptide suggests a proteolytic conversion of the 180 kDa to the 76 kDa form. Some minor polypeptides are also detected reflecting further proteolysis steps.

DISCUSSION

As a prerequisite to the study of DNA ligase in *X. laevis*, we have undertaken the characterization and purification of this activity from unfertilized eggs. We have shown that the major ligase in the egg is of a type I DNA ligase of molecular weight 180 kDa. This protein is very sensitive to protease and is rapidly converted *in vitro* to a catalytically active 76 kDa polypeptide. The generation of active proteolytic fragments has been described for *Drosophila* DNA ligase I (21)and for mammalian DNA ligase I (16,37,38). In mammalian DNA ligase I, the catalytic domain has a molecular weight of 85 kDa and is located at the C-terminal

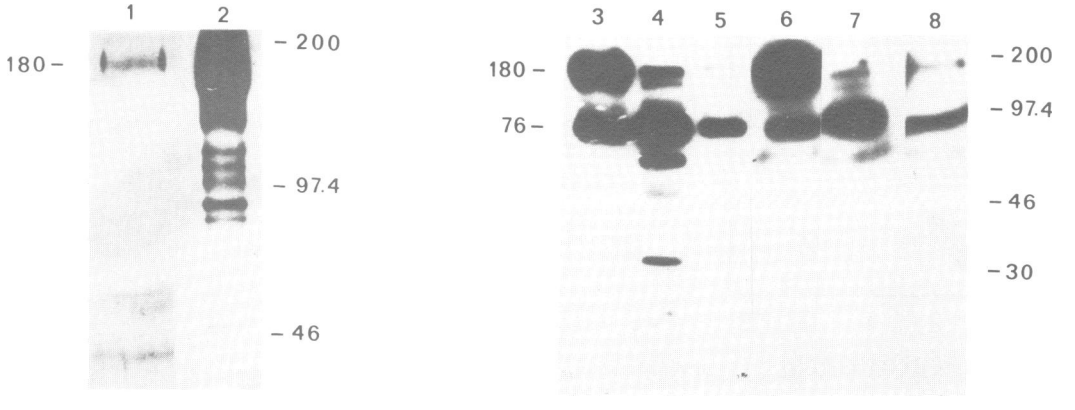

Figure 5. Immunospecificity of the antibodies raised against the *Xenopus laevis* 180 kDa DNA ligase. The *X. laevis* DNA ligase I purified fraction was electrophoresed through a 7.5% SDS polyacrylamide gel then transferred onto nitrocellulose and stained with India ink as described under experimental procedures (**lane 1**). An aliquot of the purified fraction was incubated with [α-^{32}P] ATP and run in the parallel lane then the gel was dried down and exposed to X-ray film (**lane 2**). *X. laevis* DNA ligase I partially purified by ammonium sulfate precipitation and gel filtration was electrophoresed through a 7.5% SDS polyacrylamide gel and transferred onto nitrocellulose. The polypeptides were detected by 1:500 dilution of the antiserum directed against calf thymus DNA ligase I (**lanes 3–5**) or 1:100 dilution of the antiserum directed against the *X. laevis* 180 kDa DNA ligase I (**lanes 6–8**). The protein extracts are as follows: oocyte (**lane 3, 6**); unfertilized egg (**lanes 4, 7**) and stage 8 embryo (**lanes 5, 8**). Molecular weight markers as for Fig.3.

region of the protein (16). This suggests that *X. laevis* DNA ligase I consists of a 76 kDa catalytic C-terminal domain with a N-terminal extension to produced the 180 kDa enzyme. The DNA ligase I activity associated with the 130 kDa protein in mammal cells has a half life of 7 h *in vivo* (39) indicating that the observed proteolysis occurs during extraction and purification. This type of proteolysis has also been observed during purification of other *X. laevis* DNA dependent proteins. For instance, DNA polymerase γ from ovarian mitochondria corresponds to a 140 kDa protein that is degraded to a 100 kDa and 55 kDa catalytically active polypeptides (24). Similarly, a 165 kDa topoisomerase I has been shown to be readily degraded to two polypeptides of 125 kDa and 88 kDa that are still able to relax DNA (27).

We also studied the DNA ligase activity during *X. laevis* early development. The highest level of activity was found in oocytes and unfertilized eggs. This high amount of DNA ligase I in the egg reflects the requirement for the egg to supply a large number of DNA replicating enzymes to be used during the six first hours of development, within which the embryo undergoes twelve rapid divisions without significant transcription (40). A similar situation has been observed in *Drosophila* (21) where DNA ligase I activity is maximal in the egg and drops during development. Both observations must be correlated with the high overall replication rate during the early development of these organisms, this reinforces the relation between DNA ligase I and replication.

We show that DNA ligase I is present in oocytes, unfertilized eggs as well as early embryos. We found no differences in DNA ligase I related polypeptides between the different development stages when using either antibodies directed against the type I bovine enzyme or the ligase AMP-adduct reaction. So in *X. laevis* the same protein clearly supporting DNA ligase I activity was present from the oocyte through to the embryo, this finding is totally inconsistent with studies on axolotl and *Pleurodeles* where the only DNA ligase activity detected in the egg before fertilization is a type II DNA ligase (32). Our results clearly demonstrate that in *X. laevis* DNA ligase I activity is already present in oocyte and unfertilized egg thus implying that the exclusion process of two forms of DNA ligase after fertilization described in axolotl and *Pleurodeles* early development does not occur in *X. laevis*. We are currently investigating the DNA ligase activity in the other amphibians in order to resolve these conflicting results.

In the light of our study, it is clear that the DNA ligase I accounts for almost all the ligase activity in the egg. But it has been shown that extract from *X. laevis* oocytes can repair DNA *in vitro* as well as *in vivo* (29,30) , if DNA ligase II is the true DNA repair ligase, which remains to be seen, the enzyme must be present at least in the oocyte. We failed to detect this activity, suggesting that DNA ligase I may also be involved in DNA repair. In the case of *Drosophila*, DNA ligase II has been found throughout the development (41). Now, if both DNA ligase I and II are present in the *X. laevis* egg this system offers a good situation for studying their respective roles.

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