Isolation of the messenger RNA for 8S DNA ligase in early developing axolotl egg and its cell free translation

Pierre Thiebaud1, J. Lefresne2, J. Signoret2, and Jean-Claude David1*

1Laboratoire de Biochimie du Développement, C.N.R.S. LA No 256, Université de Rennes I, Campus de Beaulieu, 35042 Rennes Cedex and 2Laboratoire de Biologie du Développement, Faculté des Sciences, 14000 Caen, France

Received 1 March 1983; Accepted 6 April 1983

ABSTRACT

A new DNA ligase activity is expressed when the Axolotl eggs enter cleavage. The messenger RNA can be labelled by [3H] uridine thereby indicating its de novo synthesis. This new genetic expression is occurring just before cleavage and is the earliest found during Amphibian development. The newly synthesized [3H] mRNA can be translated in vitro in the rabbit reticulocyte lysate system. The resulting product is a 160 K protein specifically immunoprecipitated with the antiserum directed against 8S DNA ligase. This in vitro translated polypeptide exhibits 8S DNA ligase activity specific of activated or fertilized eggs but does not display 6S DNA ligase activity of non activated eggs.

INTRODUCTION

DNA ligase can be considered as a key enzyme in DNA metabolism since it is involved in replication (1), repair (2) and recombination (3). In recent years it has been shown that this enzyme exists under two different forms in eukaryotic tissues (6-10). This molecular duality provides a system of limited complexity suitable for experimental study of gene control (10). In Amphibia, a heavy form of enzyme is observed in the embryo while a light form is present thereafter in differentiated tissues (11). In chicken retina and thymus the appearance of the light DNA ligase takes place respectively at 14 days of embryonic life (5) and at birth (6) and can be used as a marker of maturation for thymocytes (9). In ram spermiogenesis a light ligase is observed in round spermatids, whereas a heavy form is present in elongated ones (10).

An experimental analysis of the change in enzymatic form has been performed using Axolotl embryos which are of special interest for this purpose. The replacement of the 6S DNA ligase by the 8S form results from egg activation and is achieved before the egg enters cleavage. This biochemical event requires an intact maternal genome able to be expressed (12) and involves the activation of structural genes (13, 14). Therefore further investigations re-
quest an analysis at the messenger RNA level and this is the matter of the present report.

In the prospect of a possible isolation of mRNA coding for 8S DNA ligase, messenger supposed to be transcribed at the one cell stage, we first investigated the existence of a de novo synthesis of RNA in activated Axolotl eggs. The mRNA was obtained and translated in vitro in the reticulocyte lysate system.

**MATERIAL AND METHODS**

**Isolation of RNA**

Two sets of 200 non activated eggs were used. To ensure a reliable penetration of the precursor, the two sets were incubated separately for 24 hrs in 1 ml of Steinberg's solution containing 400 μCi of [3H] uridine (New England Nuclear 30 Ci/mmol) at 4°C and transferred to 20°C in the same solution. One set was artificially activated (15) when transferred and both were incubated for 6 hrs and RNA was extracted.

Unless otherwise indicated, extractions were carried out at 4°C in baked glassware with sterile solutions. Eggs were homogenized in 10 volumes of extraction buffer containing 20 mM Tris HCl pH 7.6, 0.1 M NaCl, 5 mM Mg acetate. Homogenization was performed in a motor driven teflon glass Potter homogenizer. The homogenate was centrifuged for 10 min at 10000 x g. Protease K and sodium dodecyl sulphate (final concentrations of respectively 500 μg/ml and 2 %) were added and incubation carried out for 10 min at 20°C (16). All further steps were performed at room temperature, NaCl added to a final concentration of 0.3 M and the solution was extracted twice with 2 ml of phenol-chloroform (1:1 v/v) followed by two extractions with 2 volumes of chloroform. 2.5 volumes of ethanol were added to the aqueous phase and RNA was allowed to precipitate overnight at -20°C. The RNA precipitate was washed twice with 3 M sodium acetate pH 6 (17), twice with 70 % ethanol containing 0.1 M potassium acetate and once with ethanol. The pellet was dried under vacuum and RNA was taken up in sterile distilled water and stored at -70°C.

Sucrose density gradient centrifugation of RNA was performed in 15-30 % sucrose (18), 10 mM Tris HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA for 210 min at 45000 rpm in a SW28 rotor at 23°C. Fractions (0.4 ml) were collected and tested for RNA content.

**In vitro translation of the messenger RNA**

The mRNA dependant rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (19) from rabbits injected with acetylphenylhydrazine
with minor modifications: creatine kinase, haemin and dithiotreitol were added to the rabbit lysate at final concentrations of 50 μg/ml, 25 μM and 5.4 mM respectively. The lysate was subsequently treated with micrococcal nuclease (20 μg/ml) in the presence of 2 mM CaCl₂ for 15 min at 20°C. EGTA was added at a final concentration of 4 mM.

Translation incubations contained in a final volume of 60 μl: 30 μl nuclease treated lysate, 100 mM KCL, 10 mM creatine phosphate, 10 μg/ml calf liver tRNA, 30 μM of the 19 unlabelled aminoacids (minus methionine), 40 μCi of [³⁵S] methionine (Radiochemical Centre Amersham 1000 Ci/mmol) and 20 μg of total RNA or 2 μg of Poly (A⁺) RNA as further indicated. After incubation for 1 h at 30°C, hot trichloroacetic acid precipitable radioactivity incorporation was measured on 2 μl aliquots (controls of translation).

DNA ligase activity of the translation products

Ten pooled reticulocyte translation fractions were analysed for ligase activity on sucrose gradients as previously described (5, 6, 11, 12). DNA ligase assay is based upon a modification (6) of the method described by Olivera (20). This assay involves the sealing of [³²P] 3'-5' phosphodiester bonds between oligo dT (12-18) (PL. Biochemicals) hybridized by adequate amounts of Poly dA (PL. Biochemicals). One unit of DNA ligase activity is defined as the activity which renders 1 nmole of [³²P] 5' oligo dT resistant to alkaline phosphatase in 30 min under standard conditions.

Partial purification of RNA from activated Axolotl eggs

After checking that the translation ability for 8S DNA ligase was displayed in the single radioactive peak, the fractions containing ^3H]-uridine radioactivity were pooled and submitted to oligo dT cellulose (Collaborative Research Grade T3). Chromatography was carried out according to Aviv and Leder (21) as modified by Bantle et al. (22).

Polyacrylamide Gel electrophoresis

Aliquots (5 or 10 μl of translation products) were removed and analysed by gel electrophoresis according to Laemmli (23). 12.5 % polyacrylamide, 7M urea gels were used at pH 7. In some experiments radioactive protein markers (the Radiochemical Center Amersham) were used. After electrophoresis, gels were impregnated with PPO, dried and exposed for about one week at -70°C using flash activated (24) Kodak films.

Immunoprecipitation of synthesized proteins

Prior to specific immunoprecipitation, 0.5 million cpm of TCA precipitable material were incubated with normal rabbit serum and a suspension of Staphylococcus aureus (to reduced non specific binding (24)) then immunopre-
Nucleic Acids Research

cipitated specifically with anti-DNA ligase 8S (25) serum, according to the method described by Wolf et al. (26). The supernatant was analysed by SDS electrophoresis (16) and fluorography performed according to Laskey and Mills (24).

RESULTS

Extraction of RNA from [3H] uridine incubated Axolotl eggs

As described under Methods, total RNA from activated or non activated eggs was extracted. With activated eggs (Fig. 1A) a peak of radioactivity was observed with a sedimentation coefficient of 24S representing the totality of the [3H] radioactivity. When non activated eggs were incubated in the same conditions with [3H] uridine, no radioactivity peak was detected on the RNA separation sucrose gradient (Fig. 1B).

DNA ligase activity of the translation products

DNA ligase activity was determined using RNA from pooled fractions of RNA separation on sucrose (Fig. 2). RNA was precipitated in ethanol from 5 pooled fractions (1-5, 6-10, 11-15, 16-20, 21-25) and each fraction redissolved in 20 μl of sterile water. The RNA of these fractions was translated in the reticulocytes system as described under Methods. 60 μl of the translation mixture was layered on 5-20 % sucrose gradients as described under Methods. No translation activity for the 8S DNA ligase was found in any of RNA fractions from the non activated eggs. However in the pooled fractions 11 to 15, a significant peak of DNA ligase activity was observed with a sedimentation coefficient of 6S (Fig. 1A). In the RNA extracted from activated eggs no DNA ligase activity was found in the different fractions except in the pooled fractions 6-10 (corresponding closely to the [3H] radioactivity peak). When these pooled fractions were translated in the reticulocytes system and the sucrose gradient analysed for DNA ligase activity, a single peak was observed with a sedimentation coefficient of 8S. No activity was found corresponding to the 6S DNA ligase observed in the non activated eggs extract, though several peaks of [35S] methionine were observed. Neither DNA ligase activity nor intense peaks of [35S] methionine were observed when the sucrose gradient

Fig. 1. Total RNA from activated (A) and non activated Axolotl eggs (B) incubated in [3H] uridine and analysed on 15-30 % sucrose gradient. The extracts were centrifuged 210 min at 45000 rpm in a SW50-1 rotor at 17°C. RNA markers 18S and 28S are from mouse testes and 4S is from beef liver tARN. Absorbance at 260 nm (-) and [3H] radioactivity of 100 μl aliquots in cpm per μg RNA (--).

2567
Fig. 2. DNA ligase activity in translation products of total RNA of pooled fractions 11-15 from the non activated eggs (A) or 6-10 from the activated eggs (B). 60 μl of the translation incubation lysate were layered and analysed on 5-20% sucrose gradients. Centrifugation was for 15 h at 45000 rpm in a SW50L-1 rotor at 4°C. Fractions of 200 μl were collected and ligase activity determined on 50 μl aliquots (-) [35S] radioactivity was counted directly (in cpm) on 100 μl aliquots (-).

was made with extracts from reticulocytes in the absence of RNA (Fig. 3B).

Since this result appears original the mRNA was further studied and partially purified as Poly (A⁺) on oligo dT cellulose as described under Methods. The Poly (A⁺) mRNA from the peak of [3H] uridine radioactivity (fraction 6-10, see Fig. 1A) was translated in the reticulocytes system and the resulting incubation mixture layered on a 5-20% sucrose gradient. As shown in Fig. 3A, a single 8S peak of DNA ligase activity was observed under these conditions. The peak of [35S] methionine incorporated was found to comigrate with DNA ligase activity.
In vitro translation of the polyadenylated mRNA

Polyadenylated $[^3H]$ labelled mRNA was translated in the mRNA dependant rabbit reticulocyte system. In vitro translation products were analysed by gel electrophoresis. When 5 μl of the translation mixture was used a major band was noticed, migrating with the same velocity as pure 8S DNA ligase from activated Axolotl eggs. When 10 μl of the translation mixture was tested for electrophoresis this 160 k protein increased in intensity and is assumed to be the newly synthesized 8S DNA ligase (Fig. 4), since it comigrates with the pure 8S DNA ligase. Other proteins appear as minor bands and this may in-
Nucleic Acids Research

Fig. 4. In vitro translation of polyadenylated RNA from activated Axolotl eggs. Standard in vitro translations were performed as described under Material and Methods using the mRNA dependant rabbit reticulocyte lysate. Track b, 5 μl of reticulocyte mRNA incubation mixture. Track c, 10 μl of reticulocyte mRNA incubation mixture. Track d, reticulocyte lysate without mRNA. Standard [14C] protein markers (Amersham) were electrophoresed in track a of the SDS gel with: Pure 8S DNA ligase (160 K): phosphorylase b (92.5 K), bovine serum albumine (68 K) ovalbumine (43 K) α chymotrypsinogen (57 K) β lactoglobulin (18.4 K/not shown) and cytochrome c (12.3 K).

cicate that the major protein synthesized by the reticulocytes lysate is a 160 k enzyme presumably DNA ligase 8S.

Immunoprecipitation of the synthesized proteins

The synthesized proteins were immunoprecipitated as described under Methods using a rabbit antiserum directed against 8S DNA ligase purified from activated Axolotl eggs. The immunoglobulin was purified on sepharose 4B(25). This antiserum specifically immunoprecipitated the 160 k protein (Fig. 5, track c). This protein appears to comigrate with pure 8S DNA ligase. The anti 8S DNA ligase antibody does not reduce the activity or precipitate the 6S DNA ligase found in the eggs before activation.

DISCUSSION AND CONCLUSIONS

A discrete synthesis of mRNA has been reported in Amphibia blastula(27). A minor fraction of newly synthesized RNA has been detected at this stage in Xenopus polysomes (28). In Axolotl, newly synthesized RNA has been found in the nucleus of the 64 cells cleavage stage (29). Never any transcription has
been claimed to occur before cleavage in any non mammalian zygote. The present report establishes unequivocally a da novo RNA synthesis in activated eggs in the first 6 hrs after activation, before the eggs undergo fragmentation. This result agrees with previous conclusions, based upon experimental arguments reporting an early genetic activity as a consequence of activation (11, 12). Subsequently the presence of mRNA for 8S DNA ligase in this newly synthesized RNA has been tested. The first observation of this report is that total RNA extracted from either non activated or activated Axolotl eggs can be translated in vitro in the reticulocyte lysate system to synthesize respectively a 6S or a 8S DNA ligase.

Moreover, mRNA coding for the 8S DNA ligase has a sedimentation coefficient of 24S while mRNA coding for 6S DNA ligase has a smaller sedimentation coefficient between 18 and 22S. The mRNA coding for the heavy enzyme comigrates with the radioactivity of uridine [\(^{3}H\)] origin and may indicate that the messenger is newly synthesized while no equivalent peak of radioactivity is found in the RNA sucrose gradient from non activated eggs. After purification of the Poly (A\(^{+}\)) messenger RNA from activated eggs on oligo dT cellulose
it can be observed that the major protein synthesized is a 160 k protein still catalytically active while no 80 k is observed. When the translation products are incubated in the presence of specific anti 8S DNA ligase antibody the major protein precipitated has a molecular weight of 160 k, corresponding to DNA ligase 8S. Moreover, no ligase activity can be detected if the reticulocyte translation products are precipitated by the 8S DNA ligase antibody. This observations is in favor of the identity between the 8S DNA ligase found in activated Axolotl eggs and the enzyme resulting of the translation.

The major conclusions of this report are:

1) Structural gene(s) for DNA ligase are transcribed into mRNA in activated eggs.

2) Unexpectedly this mRNA can be translated in vitro to give an active enzyme at an exceptionally high rate. Since DNA ligases display important physiological functions in DNA replication (5) and repair (8, 10) investigations are in progress to obtain the cDNA for 8S DNA ligase mRNA. Besides a practical interest in the prospect of genetic engineering, this probe could provide a unique tool for the study of gene activity control for the DNA ligase in Axolotl.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. J. Elsevier and to Dr. A.L. Haenni for their help in translational experiments. This work was supported by a grant from Ministère de la Recherche et de la Technologie N° 82.E.1149.

*To whom correspondence should be addressed

REFERENCES