Original Article

Gradual Loss of DNA-PK Activity from the Cytoplasm Is Coincident with the Nuclear Translocation of Its Activator Ku during Early Development of *Xenopus laevis*

(Ku / DNA-PK / gastrulation / *Xenopus*)

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Abstract. DNA-dependent protein kinase is a serine/threonine kinase consisting of a catalytic subunit, p460, and a regulatory subunit called Ku (p80/p70). DNA-dependent protein kinase plays a role in transcription, non-homologous recombination, and DNA repair. Previous data have shown the presence of DNA-dependent protein kinase in *Xenopus* oocytes and changes in its activity during vitellogenesis. Metabolic labelling studies have shown that the increased enzyme activity in vitellogenic oocytes correlates with increased levels of Ku protein, and compared to the pre- and early-vitellogenic oocytes, vitellogenic and post-vitellogenic oocytes show an increased level of DNA-dependent protein kinase activity. Whether DNA-dependent protein kinase activity is altered during early embryogenesis in *Xenopus* is not known. The present study demonstrates that DNA-dependent protein kinase activity is gradually lost from the cytoplasm in the early embryonic cells of blastulae and gastrulae. The gradual loss of DNA-dependent protein kinase activity during post-fertilization early embryogenesis in *Xenopus* is consistent with the reports on *Arbacia punctulata*. Immunohistochemistry of the oocytes at various stages and early embryos (gastrulae) shows that Ku70, a regulatory subunit of DNA-dependent protein kinase, is present both in the cytoplasm and nucleus in the pre-vitellogenic oocytes, full-grown post-vitellogenic oocytes and in the gastrula cells. However, Ku70 appears to accumulate in higher concentrations in the nuclei of gastrula cells. These results suggest that gradual loss of DNA-dependent protein kinase activity from the cytoplasm of the early embryos could be the consequence of Ku translocating to the nuclei that may be necessary for post-zygotic transcription followed by cellular differentiation.

Introduction

DNA-dependent protein kinase (DNA-PK) is a multisubunit enzyme consisting of a catalytic subunit, p460, and a regulatory subunit, called Ku. The Ku protein is a heterodimer composed of 70 kDa (p70) and 80 kDa (p80) subunits, which has the capability of binding selectively to specific forms of DNA (Mimori and Hardin, 1986; de Vries et al., 1989). In this capacity it functions as the regulator of DNA-PK that is active in transcription, DNA recombination, and DNA repair (Carter et al., 1990; Jackson et al., 1990; Satoh and Lindahl, 1992). DNA-PK activity has been detected in rabbit reticulocyte lysate; in eggs and oocyte extracts obtained from *Xenopus*, clam (*Spisula*), sea urchin (*Arbacia*); and in cellular extracts of mouse, hamster, and *Drosophila* (Anderson and Lees-Miller, 1992; Gottlieb and Jackson, 1993; Kanungo et al., 1997). Earlier reports suggest a role of DNA-PK in apoptosis (Mimori et al., 1986) and early embryogenesis of sea urchin (Kanungo et al., 1996b, 1997; Kanungo, 2002). Additionally, DNA-dependent phosphorylation of histones during nucleosome assembly has been demonstrated in *Xenopus* oocytes (Finnie et al., 1995). DNA-PK and Ku80 have been reported to suppress RNA polymerase I transcription in extracts of embryonic kidney cells of *Xenopus* and in murine P19 embryonic carcinoma cells (Feldmann and Winnacker, 1993; Cao et al., 1994). Furthermore, experiments with extracts of *Xenopus* eggs have indicated that DNA-PK may be involved in the phosphorylation of P1 protein (Taccioli et al., 1994). In studies relating to other cellular functions, Ku70 as a tumour suppressor (Li et al., 1998) and Ku80 as a somatostatin receptor have been demonstrated (Le Romancer et al., 1994).

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Abbreviations: DNA-PK – DNA-dependent protein kinase, DTT – dithiothreitol, GV – germinal vesicle, OCT – optimal cutting temperature, PBST – phosphate-buffered saline Tween-20.

Although DNA-PK activity has been shown in a number of different species, its role in early development has not been fully explored. Whether its DNA-associated functions, such as DNA repair and transcription, are the exclusive cellular functions remains to be established. The presence of DNA-PK activity in the unfertilized eggs of *Xenopus* (Kanungo et al., 1997) and sea urchin (Kanungo et al., 1996a; Kanungo et al., 1997; Kanungo, 2002) suggests a critical role of the holoenzyme in early development. Metabolic labelling studies in oocytes at different stages of vitellogenesis have demonstrated that Ku (p80/p70) expression is higher in later stages of vitellogenesis and is implicated in being stored in abundance as a maternal protein to be used once post-fertilization transcription and differentiation begin (Kanungo et al., 1997). Furthermore, a role of Ku seems critical for early embryogenesis in sea urchin since microinjection of the purified anti-Ku antibody arrests development (Kanungo et al., 1999).

In the present study, results based on immunohistochemistry demonstrate that Ku70 is present in the nuclei of oocytes and gastrulae, but appears more abundant in the nuclei of the latter. DNA-PK activity assays suggest that in the blastula and gastrula stages, DNA-PK activities are significantly lower compared to those in the full-grown oocytes. A gradual loss of DNA-PK activity from the cytoplasm during early development may correlate with the nuclear translocation of its activator, Ku. These results suggest that DNA-PK activity is altered during developmental progression, which may be necessary for normal embryonic development of *Xenopus*.

Material and Methods

Xenopus oocytes

Frogs (*Xenopus laevis)* were purchased from Nasco (Nasco International, Fort Atkinson, WI). Oocytes were collected by surgically removing a piece of ovary. Oocytes were defolliculated using a pair of watchmaker's forceps under a dissecting microscope and all the oocytes including the pre-vitellogenic (smallest) and post-vitellogenic full-grown (largest) oocytes (Dumont, 1972) were collected in Barth's modified saline (Millipore, Billerica, MA) (Gurdon, 1976).

Cytoplasmic and nuclear extract preparation from oocytes and embryos

After washing the oocytes in the homogenization buffer (50 mM HEPES, pH 7.4; 10 mM EGTA, 40 mM NaCl, 100 mM potassium acetate, 8.5 mM $CaCl_2$, 2.29 mM $MgCl_2$, 277 mM glycerol), nuclei or germinal vesicles (GVs) of the post-vitellogenic full-grown oocytes were manually dissected and recovered. The enucleated oocytes were collected and dithiothreitol (DTT) was added to a final concentration of 1 mM. The oocytes were then spun in an airfuge for 30 min. The clear supernatant was collected and filtered through an eppendorf microfilter unit (0.45 μ). This extract was designated as the cytoplasmic extract. The recovered GVs were flushed several times using a heat-blunted Pasteur pipette to get rid of the attached residual cytoplasm. The clean, glistening GVs were resuspended in 10 μl homogenization buffer (with DTT) per GV. The GVs were spun in the airfuge for 30 min. The supernatant was filtered through an eppendorf microfilter unit. The extract thus prepared was designated as nuclear extract. Early embryo (16-cell stage blastula and gastrula) cytoplasmic extracts were prepared by gently disrupting the embryos in homogenization buffer (containing DTT) by pipetting a few times through a P1000 eppendorf pipette (Daigger, Vernon Hills, IL). The homogenate was spun for 5 min at 5000 rpm. The supernatant (cytoplasmic fraction) was spun for 30 min in an airfuge. The clear supernatant was collected and filtered through an eppendorf microfilter unit (0.45 μ).

Immunohistochemistry

Xenopus oocytes and early embryos (16-cell stage blastula and gastrula) were fixed in 4% paraformaldehyde overnight, embedded in OCT compound, quick-frozen on dry ice, sectioned (10 μm), placed on poly-L-lysine-coated slides, and stored at 80 °C. The sections were washed three times for 5 min each with PBS, blocked with 10% sheep serum in PBST (PBS + 0.05% Tween 20) for 1 h at room temperature, washed twice with PBST for 5 min each, incubated with the primary antibody (N3H10, a monoclonal mouse anti-human Ku70 (Neomarkers, Fremont, CA)) antibody at 1 to 50 dilution in blocking solution for 1 h at room temperature, washed thrice with PBST for 10 min each, incubated with horse-radish peroxidase-coupled secondary anti-mouse IgG antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at 1 to 100 dilution for 1 h at room temperature, washed thrice with PBST for 10 min each, mounted with anti-fade gel/mount (Biomeda Corp, Foster City, CA), and observed with a Zeiss Axioplan 2 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

DNA-PK activity assay

A DNA-PK-specific peptide was used as the substrate for the kinase assay and a non-specific peptide phosphorylation assay was performed in parallel for comparison. The sequences of these peptides are as described earlier (Anderson and Lees-Miller, 1992; Kanungo et al., 1997). A crude extract kinase assay (15 μl final volume) constituted 10 μl extract, 75 ng of sonicated calf thymus DNA, 200 μM peptide substrate, 2 mM $MgCl_2$, 130 μM ATP and 10 μCi of (^{32}P)γ-ATP (6000 Ci/mmol) (NEN, Du Pont, Boston, MA). The reaction was performed at room temperature for 30 min. Acetic acid was added to a final concentration of 30% to stop the reaction. The reaction product was spotted onto P81 phosphocellulose discs (Whatman, Clifton, NJ). The discs were washed extensively, first with 30% acetic acid following three washes with 15% acetic acid. The discs were finally washed with acetone, air-dried and counted in a scintillation counter.

Results and Discussion

Well before the cloning and characterization of DNA-PK subunits in *Xenopus*, we reported that *Xenopus* oocytes begin to synthesize Ku protein during oogenesis especially at the vitellogenic Y4 stage (Kanungo et al., 1997). These results based on metabolic labelling by ^{35}S-methionine only showed Ku synthesized during the culture but not the maternal Ku protein that might have been present as a maternal protein. Increased synthesis of Ku was noticeable at the next stage, Y5, the late-vitellogenic stage. However, metabolic labelling of oocytes did not detect any newly synthesized Ku in the full-grown post-vitellogenic oocytes (Y6) (Kanungo et al., 1997) indicating that the full-grown post-vitellogenic oocytes may be translationally quiescent. Corresponding *in vitro* DNA-PK activity assays showed the maximal enzyme activity being present in Y4 through Y6 oocytes and during the same time the levels of its cofactor Ku (both p80 and p70) heterodimer also increased from early vitellogenic (Y1-Y2) to vitellogenic (Y3-Y5) oocytes. These results indicated that not only Ku is a maternal protein, but also the oocytes keep synthesizing Ku proteins during oogenesis. Based on these results, we concluded that the accumulation of the enzyme in the oocytes prior to fertilization might as well have an implication for use in later embryogenesis (Kanungo et al., 1997). The present study followed up on the localization of Ku in the pre-vitellogenic oocytes, post-vitellogenic full-grown oocytes, and the early embryonic cells of the blastula and gastrula. A monoclonal antibody that recognizes a subunit (Ku70) of the DNA-PK holoenzyme was used for immunolocalization of DNA-PK in frozen sections of the oocytes and embryos. A pre-immune mouse IgG was used as control to monitor background staining (Fig. 1 A, C). In the pre-vitellogenic oocytes, Ku protein was mostly present in the nucleus, although its presence in the cytoplasm appeared possible (Fig. 1 B). In the post-vitellogenic full-grown oocyte, there appeared an equal distribution of Ku in the nucleus (or germinal vesicle, marked by an arrow) as well as in the cytoplasm (Fig. 1D), since there was no differential staining between the nuclear and cytoplasmic regions. In the oocytes and early embryos, soluble yolk proteins in the prepared extracts hinder immunoblot analyses as the yolk proteins distort the SDS-PAGE gel lanes and also major yolk proteins like vitellogenin that are present in high abundance overshadow the specific immunosignals in immunoblots, especially when resolving polypeptides of 80 or 70 kD molecular weights. Although the immunohistochemical analyses did not reveal a quantitative presence of Ku as was observed in earlier studies based on ^{35}S-methionine incorporation during metabolic labelling of oocytes maintained in culture (Kanungo et al., 1997), the fact that Ku as a nuclear protein was present in the nucleus of both pre-vitellogenic and post-vitellogenic oocytes suggests that although oocytes are transcriptionally quiescent, the nuclear proteins still localize to the nucleus, possibly due to their inherent nuclear localization signals. While the metabolic labelling experiments revealed only the levels of nascent Ku, the present study based on immunohistochemical analyses shows the absolute amount of Ku70 present in the specific stages of oocytes and embryos.

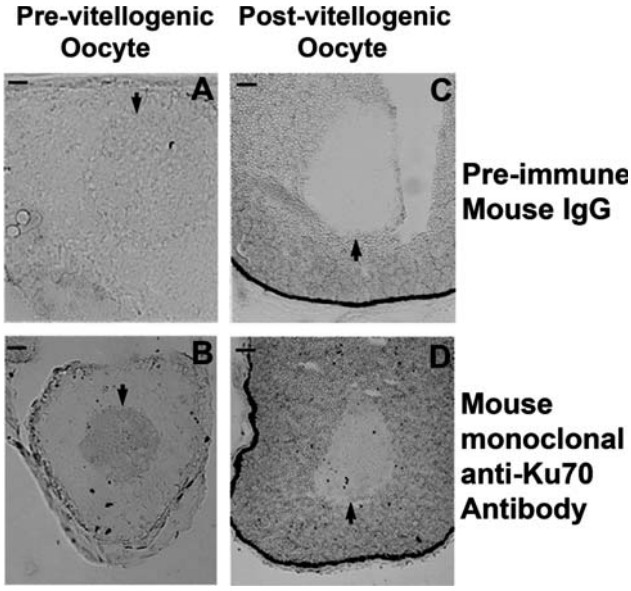

Fig. 1. Localization of Ku (p70) in *Xenopus* oocytes isolated from the ovaries. A piece of ovary was dissected out of the frog. Oocytes at different stages of development were isolated from the piece of ovarian tissue using a pair of watchmakers' forceps. The oocytes were then processed for cryostat sectioning as described in the Material and Methods section. Images of immunostained frozen sections of the oocytes with either the mouse pre-immune IgG or a mouse monoclonal antibody against human Ku 70 are shown. (A) Pre-vitellogenic oocyte immunostained with the preimmune IgG. (B) Pre-vitellogenic oocyte immunostained with the anti-Ku70 antibody. Nuclei are indicated by the arrows. (C) Post-vitellogenic full-grown oocyte immunostained with the preimmune IgG. (D) Pre-vitellogenic oocyte immunostained with the anti-Ku70 antibody. Nuclei (also called germinal vesicles) are indicated by the arrows. Scale bars, 10 μm.

Further analysis of Ku localization revealed that in the 16-cell stage (post-fertilization cleavage) blastula, Ku did not preferentially show nuclear localization (Fig. 2). The embryo sections immunostained with the pre-immune IgG were treated as controls (Fig. 1A). In the 16-cell early embryonic stage, the nucleus and cytoplasm were both positive for Ku (Fig. 2B). However, in the early gastrula stage, Ku seemed to have accumulated in the nuclei (Fig. 2D). In these embryos, the cytoplasm also stained positive for Ku although a preferential accumulation in the nuclei was obvious. The control gastrula stained with pre-immune IgG is shown for comparison of background staining (Fig. 1C). These results are analogous to what is observed in the sea urchin embryos (Kanungo et al., 1996a, b). The possibility that the nuclear translocation of Ku during early embryogenesis may be critical for post-zygotic transcription and further

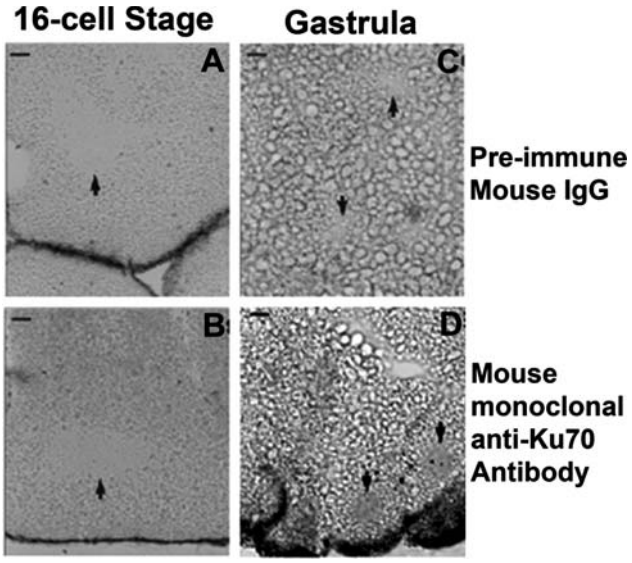

Fig. 2. Localization of Ku (p70) in *Xenopus* embryos during early development. Fertilized eggs from *Xenopus* were allowed to develop for a desired time period. Embryos at various times of development were collected and processed for cryostat sectioning as described in the Material and Methods section. Images of immunostained frozen sections of the blastula and gastrula with either the mouse preimmune IgG or a mouse monoclonal antibody against human Ku 70 are shown. (A) Blastulae (16-cell stage) were immunostained with the preimmune IgG, or (B) with the anti-Ku70 antibody, (C) gastrula immunostained with the preimmune IgG, or (D) with the anti-Ku70 antibody. Nuclei are indicated by the arrows. Scale bars, 10 μm.

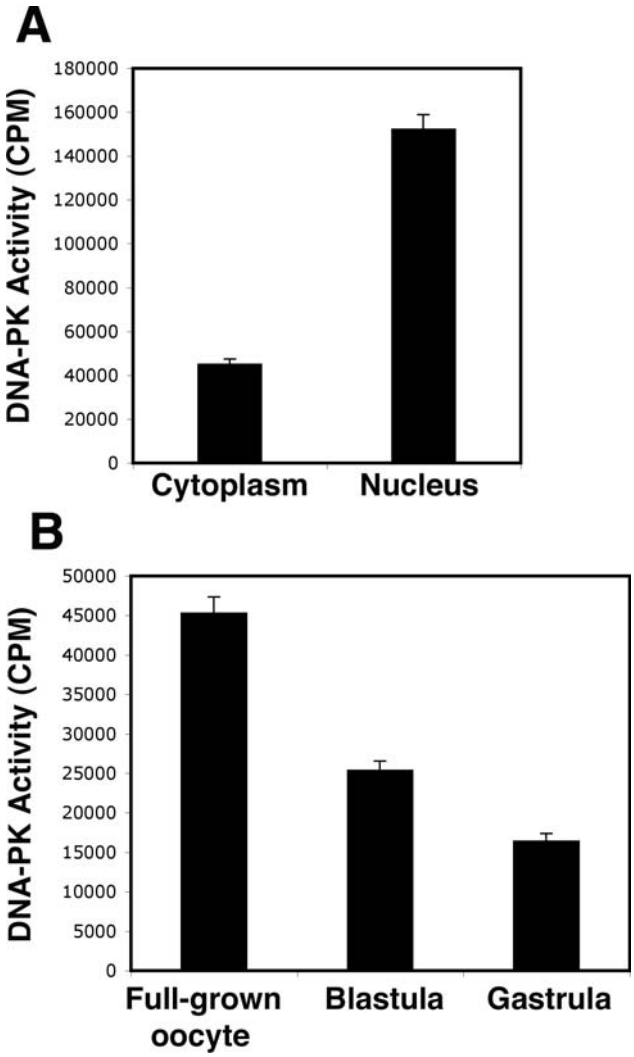

Fig. 3. DNA-PK activity in the oocytes, blastulae and gastrulae of *Xenopus*. Nuclear and cytoplasmic extracts of post-vitellogenic full-grown oocytes were prepared as described in the Material and Methods. Cytoplasmic extracts of the early developmental stages (16-cell stage blastulae and gastrulae) were prepared by gently disrupting the embryos in homogenization buffer by passing through a pipette tip followed by low-speed centrifugation. All the extracts were then spun in an airfuge to obtain clear, debris-free extracts for DNA-PK assay. Each assay contained the cytoplasmic or nuclear extracts, 200 μM of the specific peptide substrate, sonicated double-stranded calf thymus DNA (dsDNA). The control reactions contained all the constituents except either the dsDNA or the specific substrate, which was replaced with a non-specific peptide. The assay method was essentially similar to the previously reported procedure (Kanungo, 2008). (A) DNA-PK activity in the nuclear and cytoplasmic extracts of the post-vitellogenic full-grown oocytes, (B) DNA-PK activity in the cytoplasmic extracts of the post-vitellogenic full-grown oocytes, 16-cell stage blastulae, and gastrulae. The enzyme activities are presented in CPM representing the ^{32}P-γATP incorporated into the peptide substrate. The data represent the averages of three different experiments with corresponding standard errors.

developmental progression can be rationalized based on the earlier reports that injection of Ku antibody halts development in sea urchin embryos (Kanungo et al., 1999). In the sea urchin, a gradual shift of Ku from the cytoplasm to the nucleus was simultaneous with early developmental progression, which entailed a gradual loss of DNA-PK activity from the cytoplasm during early embryogenesis (Kanungo et al., 1996a).

DNA-PK plays a role in transcription, DNA repair and recombination (Carter et al., 1990; Jackson et al., 1990; Satoh and Lindahl, 1992). Cytoplasmic localization of Ku, the activator of DNA-PK, is observed in the oocytes, where these nuclear processes are dormant, and therefore, the nuclei might not have a requirement for active DNA-PK. After the mid-blastula transition, activity around nuclear DNA increases and so does the requirement for the molecules that participate in the machinery. Although this notion would support an already identified role of DNA-PK in transcription and DNA repair, the accompanying cellular differentiation during gastrulation certainly claims attention as to the use of DNA-PK in the process. DNA-PK activity, especially influenced by the presence of its cofactor, Ku, is required for the early cleavages of sea urchin embryos (Kanungo et al., 1999). Additionally, a critical role of Ku 80 in endodermal differentiation of the murine embryonic

pluripotent P19 cells (Kanungo et al., 2004) and retinoic acid-induced differentiation of F9 teratocarcinoma cells (Datta et al., 1997) has also been documented along with a possible role of DNA-PK in adipogenesis (Meulle et al., 2008).

In this study, whether the physical localization of Ku in the embryonic cells correlates with DNA-PK activity in that particular sub-cellular fraction was explored. Cytoplasmic and nuclear extracts were prepared from the full-grown post-vitellogenic oocytes as a manual enucleation process provides pure cytoplasmic and nuclear extracts (Kanungo, 2008). The manual enucleation process to isolate the nuclei or the germinal vesicles is possible since the nuclei (germinal vesicles) of the oocytes are large enough for manual isolation. However, this process cannot be used to isolate the nuclei of the multicellular early embryos. In these embryos, nuclei are smaller and no more look like the sac-like germinal vescicles. Moreover, routine nuclear extract preparation from these embryos may not rule out possible contamination of the cytoplasmic fraction. Therefore, DNA-PK activity in the cytoplasmic extracts of the oocytes and early embryos was sought for comparative DNA-PK activity assays. First, the results show that the full-grown oocyte cytoplasm contained a modest level of DNA-PK activity compared to the nucleus (Fig. 3A). However, in the 16-cell stage there was a slight shift in the trend (Fig. 3B). The cytoplasmic fraction had lower DNA-PK activity as compared to the oocyte cytoplasm. In the gastrula stage, there was further decrease in the DNA-PK activity level in the cytoplasm (Fig. 3B). These results demonstrate that during early embryogenesis, there is a gradual decline in DNA-PK activity from the cytoplasm. Ku70, the activator of DNA-PK, also shows preferential accumulation in the nuclei of the early embryos, suggesting that the loss of cytoplasmic enzyme activity may be the consequence of the translocation of Ku from the cytoplasm to the nucleus. A similar observation in the sea urchin embryos further indicates that the nuclear translocation of Ku may be critical for developmental progression (Kanungo et al., 1996a) and the process may be evolutionarily conserved. Interference with Ku translocation to the nuclei by microinjecting specific monoclonal antibodies is under investigation, since antisense morpholino technology or siRNA cannot be used for an already accumulated maternal protein in the oocytes. Whether such interference would compromise early development in *Xenopus* remains to be determined.

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