Differentiation expression of nucleoside diphosphate kinases (NDPK/NM23) during *Xenopus* early development

TAOUFIK OUATAS1**, MYRIAM SÉLO1, ZAHIA SADJI1, JACQUES HOURDRY2, HERMAN DENIS1 and ANDRÉ MAZABRAUD1

1Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette Cedex and 2Laboratoire de Biologie du Développement, Université de Paris XI (URA CNRS 1134), Orsay Cedex, France

**ABSTRACT** In *Xenopus laevis*, three nucleoside diphosphate kinase (NDPK) monomers have been described (NDPK X1, X2 and X3) (Ouatas et al., 1997). In eucaryotes, this kinase is known as a hetero- or homohexamer. Here, we examine the distribution of the enzyme and its different subunit mRNAs during oogenesis and early embryogenesis of *Xenopus laevis*, respectively by immunohistofluorescence and whole-mount in situ hybridization. These analyses show that NDPKs and their mRNAs are differentially distributed throughout the oocyte and early embryos with a high level of transcription in somites and brain. We emphasize two points. First, each mRNA displays a distinct subcellular localization in somites, suggesting a complex regulation of NDPK genes both at the transcriptional and translational level and a possible involvement of NDPK X2 homohexamers in the dorsal muscle differentiation. Second, in oocytes and early embryos, the proteins are mainly localized in the nucleus, suggesting a new mechanism for their nuclear import, since they do not possess any known nuclear import sequences.

**KEY WORDS:** nucleoside diphosphate kinase, NM23, *Xenopus laevis*, nuclear localization, somitogenesis

**Introduction**

Nucleoside diphosphate kinases (NDPK; E.C.2.7.4.6) catalyze the γ-phosphate transfer from ATP to nucleoside diphosphates via a ping-pong mechanism involving a phospho-histidine intermediate (Edlund et al., 1969; Agarwal et al., 1978). X-ray crystallography data (Gilles et al., 1991; Dumas et al., 1992) confirmed the previous biochemical analyses showing that eukaryotic NDPKs associate into hexamers composed of 17–20 kDa monomers (Palmieri et al., 1973). In mammals, NDPK is encoded by two distinct but closely related genes (Steeg et al., 1988a; Kimura et al., 1990; Urano et al., 1992; Shimada et al., 1993). Human NDPK genes *nm23-H1* and *nm23-H2* encode respectively the acidic NDPK-A and the basic NDPK-B, that are 88% identical (Rosengard et al., 1989; Stahl et al., 1991) and can form homo- and heterohexamers (Presecan et al., 1989; Gilles et al., 1991). In human, two other additional genes have been recently discovered: *DR-nm23* and *nm23-H4*, encoding less related proteins, with 60% to 67% amino acid identity to Nm23-H1 and Nm23-H2 proteins (Venturelli et al., 1995; Milon et al., 1997).

In addition to their "house keeping" function as nucleoside triphosphates providers, it has been suggested that NDPKs could play an important role in cell proliferation and differentiation, and tumorigenesis. Different studies have shown a significant correlation of NDPK expression with some aggressive tumors (Hailat et al., 1991; Lacombe et al., 1991; Sastre-Gareau et al., 1992; Leone et al., 1993b; Luo et al., 1993; Walther et al., 1995; Lindmark, 1996; Myers et al., 1996). The human *nm23-H1* gene product has been characterized as a putative metastasis suppressor (*nm23*) in several tumors (Steeg et al., 1988a,b; Rosengard et al., 1989; Leone et al., 1991, 1993a). Low levels of *nm23-H1* mRNA and protein have been detected in highly invasive breast tumors and other metastatic cell lines. In addition, transfection of mouse and human tumoral cells with mouse, human or rat *nm23* cDNAs inhibits their metastatic potential. Moreover, mammalian ND23 proteins were found to inhibit differentiation of leukemia cells into the monocyte/macrophage or erythrocyte pathway (Okabe-Kado et al., 1992, 1995a,b). In terms of cell signaling, *nm23-H1* has been shown to be involved in the TGFβ1 pathway (Leone et al., 1991, 1993a). In terms of gene expression, the human *NDPK-B* was identified as the *PUF19* transcription factor that associates with a nuclease hypersensitive element on the human c-mycpromoter and transactivates c-myc transcription both in vitro and in vivo (Postel et al., 1993; Berberich and Postel, 1995; Hildebrand et al., 1995).

**Abbreviations used in this paper:** NDPK: nucleoside diphosphate kinase, NM23: non metastatic 23rd clone (Steeg et al., 1988a).

*Present and corresponding address for reprints:* Laboratoire de Physiologie, Museum National d’Histoire Naturelle, 7 Rue Cuvier, 75005 Paris, France. FAX: 33 1 40 79 36 18. e-mail:ouatas@mnhn.fr

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Studies on Drosophila have shown that NDPKs play a crucial role in development. In Drosophila, the abnormal wing discs (awd) gene product exhibits NDPK activity that accounts for up to 95% of the whole NDP kinase activity that can be recovered from larvae (Biggs et al., 1990). A null mutation (awd^b3) results in development arrest at the third larval instar (Dearolf et al., 1988a,b). The Killer of prune (Kpn) mutation of awd(awdKpo) has no phenotype of its own but causes dominant lethality in individuals that cannot make a functional prune (pn) gene product (Sturtevant, 1956; Biggs et al., 1988). This indicates an interaction between the NDPK, the product of the awd gene, and the product of the pn gene (Timmons and Shearn, 1996). The awd^Kpo allele also suppresses hematopoietic defects associated with the Tum-I oncogene. Tum-I lethality is also suppressed by pn mutations, indicating the existence of a hematopoietic regulatory pathway involving Tum-I, Awd^Kpo and Pn proteins (Zinyk et al., 1993). During normal mouse development, nm23 genes are weakly but widely expressed in proliferating tissues (Lakso et al., 1992). At the onset of organogenesis, NDPKs accumulate mainly in the heart and nervous system, which are the first structures to differentiate. During Dictyostelium discoideum development, it has been reported that the NDPK encoding gene gip17 is down-regulated at the transcriptional level during the first hours of differentiation (Wallet et al., 1990).

We used the Xenopus laevis developmental model to analyze the function of NDPK genes during oogenesis and early development. Xenopus laevis is a pseudotetraploid species with 38 chromosomes, resulting from an ancient duplication of the 20 chromosomes still existing in the related diploid species Xenopus tropicalis. Since two genes encode NDPK in mammals, it was not surprising to isolate four NDPK cDNAs (X1, X1a, X2 and X3) in Xenopus laevis (Ouatas et al., 1997). These latter encode three different NDPKs (X1, X2 and X3), since NDPK X1 and X1a cDNAs encode the same protein (NDPK X1). As in mammals, NDPK X1 (but not NDPK X3) displays DNA binding properties with pyrimidine-rich sequences. We also reported the existence of 3' processing variants of NDPK mRNA in X. laevis oocytes (Ouatas et al., 1997).

To gain further insight into the biological significance of these variants, we performed whole-mount in situ hybridization using RNA probes that specifically recognize three NDPK mRNAs. The protein pattern at the subcellular level was analyzed by immunofluorescence histology, using polyclonal antibodies recognizing the different gene products.

**Results**

**NDPK mRNA localization during early development**

After fertilization and until the mid-blastula transition (MBT), no NDPK mRNAs were detected by non-radioactive in situ hybridization (methods). Moreover, experiments with in situ radioactive hybridization showed that fertilized eggs contain only small amounts of NDPKs mRNAs concentrated in the animal hemisphere (not shown). Also, an in vitro stability assay revealed NDPK mRNA to be unstable in an S100 oocyte extract, while remaining stable in a yeast extract (Fig. 2). Therefore, the results presented here concern newly (zygotic) synthesized mRNAs.

**NDPK X1 mRNA can be detected in the animal hemisphere of the gastrula embryo but is undetectable in the vegetal hemisphere** (Fig. 2).
Fig. 3. Specific detection of NDPKs transcripts by whole-mount in situ hybridization on gastrula (A-C) and neurula embryos (D-F). (A and D) NDPK X1 mRNA. (B and E) NDPK X2 mRNA. (C and F) NDPK X3 mRNA. a, anterior; an, animal; d, dorsal; dl, dorsal lip of the blastopore; nf, neural fold; np, neural plate; op, optic vesicle; p, posterior; v, vegetative; vn, ventral; ym, yolk mass; yp, yolk plug. Bars, 200 μm.

3A). During neurulation, levels of this mRNA drop down transiently and become undetectable in the anterior part of the neural plate (Fig. 3D; Table 1) until the late neurula. At this stage, it is present in the neural tube and the developing brain but less abundant in the pharynx and endoderm (Fig. 4A; Table 1). By the tail bud stage, its expression is the highest in the brain, the optic vesicle, and the branchial arches (Figs. 4D and 5A; Table 1).

NDPK X2 mRNA is uniformly distributed in the whole gastrula embryo, except the invaginating endoderm (Fig. 3B). In the non involuting endodermal cells, this mRNA seems to be associated to the vegetal cortex (not shown). During neurulation, it is present in the neural folds, and is highly abundant in the anterior and posterior parts of the neurula (Fig. 3E). In the late neurula, this mRNA drops down and has a particular localization in the rotating somites (Fig. 4B). By the tail bud stage, NDPKX2 expression is mainly detected in the brain, branchial arches and optic vesicle (Figs. 4E and 5B-E). The other tissues expressing this mRNA are summarized in Table 1.

NDPK X3 mRNA appears at the MBT and is highly concentrated in the dorsal marginal zone (DMZ) migrating cells (Fig. 3C). During neurulation, this mRNA is only detectable in the neural plate (Fig. 3F) and at the late neurula stage, it is mainly detected in the brain and the spinal cord (Fig. 4C). Also, its expression is the highest in the brain and spinal cord of the tail bud embryos (Figs. 4F and 5F-G; Table 1).

Further analysis of NDPK mRNA distribution from tissue sections of the in toto hybridized embryos is summarized in Table 1.

Specific subcellular localization of the different NDPK mRNAs within the somites
A particular distribution pattern of the three NDPK mRNAs was observed during somite maturation. In Xenopus, somite segmentation starts during neurulation (stage 17), and goes on until the late tail bud stage (Hamilton, 1969). During this process, aggregates of unsegmented mesoderm elongating cells undergo a 90° rotation and elongate according to the anterior-posterior axis while remaining mononucleated. Each resulting metamer forms a somite and the mechanism proceeds from the anterior post-otic region to the posterior end of the tail bud, generating about 47 somites. The somites consist of three cell types: the dermatome which contributes to the connective tissue layer of the skin, the myotome which gives rise to the axial musculature, and the sclerotome which generates the axial skeleton (Hamilton, 1969). The myotome cells consist of superposed elongated cells with their nuclei in the center (Fig. 6C).

In the late neurula, only NDPK X2 mRNA was detected as an early marker of rotating somites. The staining appears as single bands restricted to the myotome of the most anterior metamersizing somites. At the tail bud stage, this transcript can also be detected as a single band in the center of each metamersizing somite. This location coincides with that of the aligned nuclei in the somites as confirmed by Hoechst nuclear staining performed on sections of the in toto hybridized embryos (Fig. 6D-F). All three somitic components show high levels of NDPK X2 transcript in the nuclei.

NDPK X1 and X3 mRNAs staining appears, on the contrary, as band doublets in the anterior and posterior parts of each somite. Hoechst nuclear staining clearly shows that NDPK X1 staining is present in the cytoplasm (Fig. 6A). One band of the doublet is localized in the posterior part of a post-otic somite (n) and the other band of the same doublet squares with the anterior part of the adjacent somite (n+1, Fig. 6C). The same results were obtained for NDPK X3 (not shown).

NDPK localization during oogenesis
The specificity of the anti-NDPK X1 and NDPK X3 sera was tested on Western blots (not shown). The antibodies recognize
almost equally the two homohexameric *Xenopus* NDPKs expressed from *E. Coli*. This is not surprising because these enzymes are very similar in sequence (Ouatas et al., 1997). Therefore, the immunodetection results presented here will refer to all types of homo- or heteromeric *Xenopus* NDPKs.

During oogenesis, the NDPKs are mainly localized within the germlinal vesicle (Fig. 7A). In previtellogenic oocytes, they are also associated with the Balbiani body (also called mitochondrial cloud), a rounded structure consisting of an aggregate of mitochondria associated with several cement proteins and stored RNAs (Raven, 1961; Guraya, 1979; Denis, 1996). In vitellogenic oocytes, the NDPKs are located at the periphery of the nucleoli (Fig. 7B). At the end of vitellogenesis, the stained material moves to the cytoplasm and becomes concentrated in the animal pole (Fig. 7C). In the present study, we could not detect any staining of cytoskeletal structures with either the anti-Xenopus NDPK polyclonal antibodies we generated, nor with anti-human NDPK A or B affinity-purified antibodies kindly provided by M-L Lacombe (data not shown).

**NDPK localization during early embryogenesis**

Immunohistofluorescence experiments show that during the first cell cycles, the NDPKs remain abundant in the animal pole of the egg and become localized mainly in the cytoplasm of the morula embryos (Fig. 7D). After the MBT, high levels of NDPKs are detected in dorsal ectoderm and later in the cell membranes and nuclei of the involuting cells of the DMZ. By contrast, the endodermal cells have uniformly low levels of NDPK mainly localized within nuclei (Fig. 7E).

At the tail bud stage, NDPKs accumulate in the neural tube and concentrate in the brain and somites. The rapidly dividing notochordal sheath cells contain large amounts of NDPKs (data not shown). In stage 44 larvae, the NDPKs are abundant in the eye cups (Fig. 7F), brain, dorsal muscle cells and midgut epithelium (not shown). In the retinal cells and myocytes, the NDPKs are mainly localized in the cytoplasm. In other tissues of the larvae, NDPK are equally distributed in the cytoplasm and the nuclei of the cells.

**Discussion**

**NDPK during oogenesis**

Timmons et al. have shown that the AWD protein is required during *Drosophila* oogenesis for normal oocyte differentiation and female fertility (Timmons et al., 1993; Xu et al., 1996). We report here that *Xenopus* NDPKs are mainly localized in the germlinal vesicle of previtellogenic oocytes. This is consistent with an involvement of the enzyme in transcription, but could also reflect a function in nuclear import (see below). We also show that NDPKs are present in the Balbiani body. Such a localization could be explained by the association of NDPK with the GTP-binding and GTP-hydrolyzing proteins of the translation apparatus. NDPK may bind to the GTP-hydrolyzing/elongation factor EF1α (Djé et al., 1990) which is concentrated in the Balbiani body during previtellogenesis (Viel et al., 1990). Interactions between NDPK and GTPases have been reported in other species (Kikkawa et al., 1990; Kimura and Shimada, 1990; Bominaar et al., 1993; Orlov et al., 1996). Furthermore, membrane-associated NDPK was also purified from mouse cultured cells as a complex with the α subunit of membrane associated G-protein which also binds GTP (Kimura and Shimada, 1990).

The present immunofluorescence (IIF) study has revealed that at the end of vitellogenesis, NDPKs move to the cytoplasm and become mainly located in the animal pole. During gastrulation, most of the NDPKs reenter the nuclei of the embryo. This is similar to the situation prevailing in dividing cells (Kraeft et al., 1996). During mitosis, human NDPK-B moves to the cytoplasm but returns to the nucleus after cytokinesis.

Several nuclear proteins localized in the animal pole of the *Xenopus* oocyte at the end of vitellogenesis and during the first cell cycles, reenter the nuclei during gastrulation or neurulation. Most of those, such as A33 or Xig7 (Miller et al., 1989; Bellini et al., 1993), are nucleic acids binding proteins possessing nuclear localization signal sequences (NLS) characterized by clusters of basic amino acids (Dingwall and Laskey, 1992). Although Kraeft et al. (1996) have suggested that the nuclear localization of NDPK was DNA dependent, such NLS are not present in *Xenopus* nor in
Fig. 5. Tissue section visualization of the in toto hybridized tail bud embryos. (A) NDPK X1 mRNA localization. (B-E) NDPK X2 mRNA localization. (F and G) NDPK X3 mRNA. Sections show the presence of NDPK X1 mRNA in the optic vesicle, telencephalon and brachial arches. NDPK X2 mRNA is present in the optic vesicle, neural tube, brain and branchial arches. NDPK X3 mRNA is mainly detected in the brain, spinal cord and branchial arches. Section D shows the Hoechst staining of section E. ba, branchial arches; en, endoderm; Fb, forebrain; Mb, midbrain; Hb, hindbrain g, gut; nc, notochord; ne, neurocoele; nt, neural tube; otv, optic vesicle; ov, optic vesicle; p, pharynx; sc, spinal cord; te, telencephalon.

NDP kinases (NM23) during Xenopus development

any other NDPK known sequences. Only mitochondrial NDPKs such as that from Dictyostelium discoideum (Wallet et al., 1990; Troll et al., 1993) are presently known to possess a localizing sequence permitting mitochondrial import. Recently, other receptors mediating nuclear protein import by other routes have been discovered (Gerace, 1995; Aitchison et al., 1996; Pollard et al., 1996). However, neither the M9 sequence present on hnRNP proteins A1 and A2/B1 from Xenopus and humans (Michael et al., 1995; Sioni and Dreyfuss, 1995) nor nuclear export signals (Gerace, 1995) are present on Xenopus NDPK sequences. Thus, the NDPK nuclear import mechanism remains to be determined. It would also be worth finding out if NDPK nuclear localization indeed is due to its DNA binding properties or, alternatively, if nucleus import of NDPK could involve an association, either direct or indirect, with a GTPase such as RAN (Ras-related nuclear protein)/TC4 (Melchior et al., 1993; Moore and Blobel, 1993). This latter possibility is strengthened by the following recent findings. A new member of the Nm23/nucleoside diphosphate kinase family, isolated from a fish hepatocyte cell line, has been shown to interact with the cytoplasmic heat shock cognate protein hsc70, which is a multifunctional molecular chaperone, crucial in importing cytoplasmic proteins into the nucleus (Leung and Hightower, 1997). It is known that hsc70 recycles across the nuclear envelope (Mandell and Feldherr, 1990). Moreover, the guanine nucleotide exchange factor RCC1 has been shown to interact with the proteins RAN and hsc70 as well as two other proteins (RanBP1 and a 340-kDa protein) in Xenopus extracts, probably as part of a large complex containing multiple proteins (Saitoh and Dasso, 1995).

NDP Kinase during mesoderm and neural inductions

In Xenopus, NDPK expression starts earlier than in Drosophila and mouse. In Drosophila, a slight transcription of zygotic awd gene is detected during the second larval instar, after mesoderm induction, germinall band retraction and imaginal disc determination (Timmons et al., 1993). In mouse, nm23 genes were reported to be actively transcribed after the onset of organogenesis (day 10.5), when cell fate was determined in almost all the presumptive territories of the embryo (Lakso et al., 1992). Xenopus NDPK transcripts become detectable at MBT. Specific in toto hybridization showed that among the three Xenopus NDPK genes, NDPK X3 is the first gene transcribed in the involuting dorsal blastoporal lip, during the response to the induction by the Nieuwkoop center. This may probably reflect two phenomena. The first one is that the NDPK activity from the maternal stock is not sufficient for the gastrula embryo which requires new NDPK synthesis. The second one is that NDPKs may play an important role in fate determination and their synthesis is therefore required during mesoderm and neural inductions.

Our results suggest that Xenopus NDPKs expression displays two distinct phases during early development. First, NDPKs are
expressed early and transiently during mesoderm and neural inductions and may be required in cell fate establishment or totipotency maintenance. Second, Xenopus NDPKs are abundantly expressed in the differentiated structures, especially in neural derivatives. The expression in neural tissue is particularly interesting in the light of the recent findings showing that in the presence of nerve growth factor, overexpression of nm23 in PC12 neuronal precursor cells delays cell cycle transition and rapidly induces neurite outgrowth (Gervasi et al., 1996). This indicates that neural cell proliferation and/or differentiation can be modulated by NDPK expression levels.

**NDPK X2 mRNA as an early marker of somite metamaterization**

NDPK X2 mRNA is abundant in the axial and lateral mesoderm during gastrulation and is later an early marker of somite rotation. Therefore, it probably plays an important role during muscle differentiation. NDPK X2 presumably has nucleic acids-binding properties since it differs by only one aminoacid from NDPK X1 which has been shown to have such properties. CCCACC motifs and polypyrimidine-rich sequences have been shown to interact with human and Xenopus NDPK in the promoter of the c-myc gene and in **in vitro** assays (Postel et al., 1993, 1996; Hildebrandt et al., 1995; Ouatas et al., 1997). Therefore, one can imagine that NDPK X2 could influence either the transcription or the translation of homeobox genes implicated in muscle differentiation such as Xho1X or Xtwi that have polypyrimidine rich sequences or CCCACC motifs in their 5'UTR, respectively (Harvey and Melton, 1988; Hopwood et al., 1989). During somitogenesis, early expressed NDPK X2 mRNA may encode a protein that folds in a homo-oligomeric state in the metabolizing somites since neither NDPK X1 nor NDPK X3 are yet transcribed. Thus, NDPK X2 may act as a key factor in cell fate establishment in the dorsal muscle, by modulating the expression of target genes such as Xtwi, implicated in somite differentiation. By the tail bud stage, all three mRNAs are present in the somites and their protein products may form hetero-hexameric NDPKs that would no longer influence the cell fate.

**Materials and Methods**

**NDPK and anti-NDPK immune serum purification and western blotting**

NDPK X1 and X3 purification was performed as described in (Ouatas et al., 1997). New Zealand male rabbits (3.5 kg) were immunized by injecting 200 μg NDPK in 500 μl 0.9% NaCl containing 1.5 (V/V) Freund adjuvant. After four weekly injections, 50 ml of blood was recovered and left overnight at 37°C. The supernatant was centrifuged three times at 4500 g, then sodium azide was added (0.02%, W/V).

The immune serum was tested for NDPK specificity at a dilution of 1/1000 (V/V) on Western blot membranes. Antibodies prepared against NDPK X1 and NDPK X3 do not discriminate between the two proteins. Whole oocytes were dissected from immature or adult X. laevis females and homogenized. The resulting supernatant was sonicated and clarified by centrifugation for 15 min at 17000 g. 50-100 μg of whole oocyte soluble proteins were analyzed by standard SDS-PAGE (12% acrylamide separating gels and 3% stacking gels). Transfer was performed by the semi-dry technique (Biometra). Nitrocellulose membranes were treated with phosphate-buffered saline (PBS)-Tween 0.05% (V/V) ph 7.4 containing 5% (W/V) skim milk. All washing and incubation steps were performed using this buffer. Finally, the bound antibodies were revealed by means of mouse anti-rabbit IgG alkaline phosphatase conjugate (Promega).

**Oocytes and embryos collection**

Oocytes of fertilized eggs were recovered after classical gonadotrophin treatment (Kay, 1991). Embryos were staged according to Nieuwkoop and Faber (1967) and fixed in Bouin's fixative for immunofluorescence assays or in MEMFA fixative for the whole-mount **in situ** hybridization (Harland, 1991).

**Indirect Immunofluorescence (IIF)**

Esterwax-embedded sections of Bouin’s fixed embryos were dewaxed in toluene, rehydrated, and pretreated with 1% bovine serum albumin (BSA) in PBS. The sections were incubated with anti-NDPK immune serum (1/2000 V/V) overnight at 4°C, washed 3x15 min in PBS and incubated with mouse anti-rabbit IgG biotin conjugate (Amersham) for 1 h at 37°C. After three washes in PBS at room temperature, sections were incubated for 30 min with streptavidin-fluorescein conjugate at room temperature and washed 3x15 min in PBS. Sections were air dried and mounted in mowiol 40-88 (Aldrich) for histological examination.

**Generation of specific probes for NDPK mRNAs**

All Xenopus NDPK cDNAs have been previously cloned in the EcoRI site of pBlueScrip KS+ (Stratagene) (Ouatas et al., 1997) and are referred here as pBS X1, pBS X1a, pBS X2 and pBS X3 cDNA, pBS X1 and pBS X1a encoding the same protein NDPK X1. The lengths and positions of the antisense RNA probes used in this study are described in Figure 1. For NDPK X1 mRNA, a polymerase chain reaction (PCR) was performed on pBS X1 cDNA, using a (5'-GCTGGATCC|CATCAAGGTTGATGCG-3') primer designed to hybridize to the 3'-coding region of NDPK X1 (position 64 to 88) in combination with the reverse primer of pBSIKS+. The primer also contains a BamHI restriction site (in bold). The amplified DNA contains the 5'UTR and the 50 nucleotides downstream of the initiation codon of

| TABLE 1 | NDPKS MRNAS DISTRIBUTION DURING EARLY DEVELOPMENT. (A): ANTERIOR PART OF THE EMBRYO; (C): CYTOPLASMIC LOCALIZATION; (N): NUCLEAR LOCALIZATION |
|---|---|---|---|---|---|
| Tissues/Organs | NDPK X1 | NDPK X2 | NDPK X3 | Whole NDPKs |
| Gastrula | + | - | - | + |
| Ectoderm | - | + | + | + |
| Mesoderm | - | + | + | + |
| Endoderm | + | - | + | + |
| Neurula | + | - | + | + |
| Neural plate | +(a) | + | + | + |
| Axial/lateral mes. | -/- | +/+ | +/- | ++ |
| Neural fold | + | + | + | +++ |
| Tail bud | + | + | + | + |
| Spinal chord | + | + | + | ++ |
| Brain | + | + | + | ++++ |
| Notochord | + | - | + | + |
| Branchial arches | + | + | + | +++ |
| Optic vesicle | + | + | + | + |
| Otic vesicle | + | + | + | + |
| Somites | +/(c) | +(n) | +/(c) | ++ |
| Sclerotome | - | + | - | + |
| Pronephros | + | - | - | + |
| Larvae | + | + | - | + |
| Heart | + | - | + | + |
| Midgut | + | + | + | + |
| Pharynx | + | + | + | + |
| Epiphysis | + | + | - | +++ |
NDPK X1 mRNA. This DNA was purified by GeneClean II (Bio 101, La Jolla, CA), digested with EcoRI and BamHI to generate a 88bp fragment which was subcloned in pBluescriptIIKS+. The resulting plasmid was digested by EcoRI to generate the antisense probe, or BamHI to generate the sense probe.

For the specific detection of NDPK X2 mRNA, the HindIII-Pstl 3' UTR DNA fragment of NDPK X2 cDNA (position 1244 to 1494) was cloned in pBluescriptIIKS+. The sense and antisense probes were generated after linearization of the resulting plasmid with Pstl and HindIII, respectively.

The NDPK X3 specific probe was generated by treating pBS X3 with Ddel. This digestion generates a DNA fragment containing the T3 promoter and the 3'UTR of NDPK X3. Therefore, the antisense probe that hybridizes to the 75 nucleotides of the 3'UTR of NDPK X3 mRNA (position 471 to 546) was synthesized by T3 RNA polymerase.

To synthesize the RNA probe recognizing all the NDPK mRNAs (NDPK X1, X2, and X3), pBS X1a (Ouatas et al., 1997) was digested by BamHI to generate a sense probe or by HindIII to generate an antisense probe.

Whole-mount in situ hybridization and nuclear staining

In toto hybridization was performed as described by Harland 1991 (Harland, 1991), except that all washes and hybridization steps were performed at 65 °C and RNase treatment was omitted. Sense and antisense probes were generated by T3 or T7 RNA polymerase using the Pharmacia Transscript kit. Digoxigenin-labeled RNA probes were prepared with the T7 digoxigenin labeling kit (Boehringer Mannheim). The RNA probes were used at a concentration of 0.5 μg/ml.

Hybridized probes were revealed using anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim), and detected with either NBT/BCIP (Sigma) or EM purple (Boehringer Mannheim) following the manufacturer's instructions. The detection reactions were allowed to proceed for 2h (NDPK X1 and NDPK X2) or 6h (NDPK X1 and NDPK X3). In toto hybridized embryos were refixed in MEMFA, photographed, dehydrated in butanol, and embedded in paraplast. Histological sections (10 μm) were dewaxed in toluene, rehydrated by successive washes in ethanol (100-30%), then washed twice in PBS for 15 min. Rehydrated sections were incubated for 5 min in Hoechst dye (10 mg/ml, Sigma) to stain the nuclei, washed successively in PBS and in water for 5 min. Sections were finally air-dried and mounted in Mowiol.

S100 Xenopus oocyte and yeast extracts preparations

All the preparation steps are performed at 4 °C. Yeast extracts were prepared as described in Bonneaud et al. (1994). Xenopus extracts were prepared by homogenization of stage VI oocytes in an equivalent volume of extraction buffer containing: 50 mM Tris-HCl pH 7.5; 5 mM MgCl₂; 1 mM EDTA; 10% glycerol; 2 mM DTT; 1 mM PMSF; 10 mM sodium fluoride; 1 mM benzamidine; 2 mg/ml pepstatin; 2 mg/ml antipain; 2 mg/ml leupeptin; 2 mg/ml chymostatin. After centrifugation at 10.000 g for 20 min, the supernatant was centrifuged for 1h at 100.000 g. The resulting supernatant was aliquoted, frozen in liquid nitrogen then stored at -80 °C.

RNA stability assay

pBluescriptIIKS+ containing NDPK X1 cDNA at the EcoRI site was digested with HindIII. The linearized plasmid was transcribed according to the T7 labeling kit from Pharmacia, using α32P)-CTP (Sigma) to generate the NDPK X1 sense cRNA. 1ng labeled RNA (48.000 cpm) was incubated in 25 μl final volume containing: 0.1 M KCl; 20 mM HEPES pH 7.9; 10% glycerol; 0.2 mM EDTA; 0.1 mM PMSF; 0.25 mM DTT; 2% polyvinylalcohol; 10 μg/ml RNase and 0.5 ml RNAguard (Pharmacia). The RNA was incubated either with a yeast extract (2.5 μl) or a stage VI oocyte extract (0.4 vol). The mixtures were incubated for 1h at 0 °C, or 5 min or 30 min at 20 °C, treated with proteinase K (20 μg/ml, 42 °C, 30 min), phenol extracted and ethanol precipitated. The RNA stability was assayed by electrophoresis on a denaturing 6% polyacrylamide gel (8,3M urea).

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Fig. 7. Indirect immunohisto-fluorescence detection of NDPKs in Xenopus oocytes and early stage embryos. (A-D) Oocytes. A, previtellogenic oocyte; B, mature oocyte; C, vitellogenic oocyte; D, morula macromeres; note the weak staining (faint green) of nuclei compared to cytoplasm. (E) Gastrula endodermal cells; note the strong staining (yellow) of nuclei. (F-H) stage 44 larvae. G, optic vesicle cells; note the weak staining of nuclei (arrows). H, Hoechst staining of section. B, Balbiani body; G, germinal vesicle of the oocyte; e, endoderm; m, mesoderm; n, nuclei. Arrows point to nuclei. Bars, 50 μm.

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