Original Article

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

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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 NDPKs 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 NM23 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; Hsu *et al.*, 1994,1995). 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-myc* promoter and transactivates *c-myc* transcription both *in vitro* and *in vivo* (Postel *et al.*, 1993; Berberich and Postel, 1995; Hildebrandt *et al.*, 1995).

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

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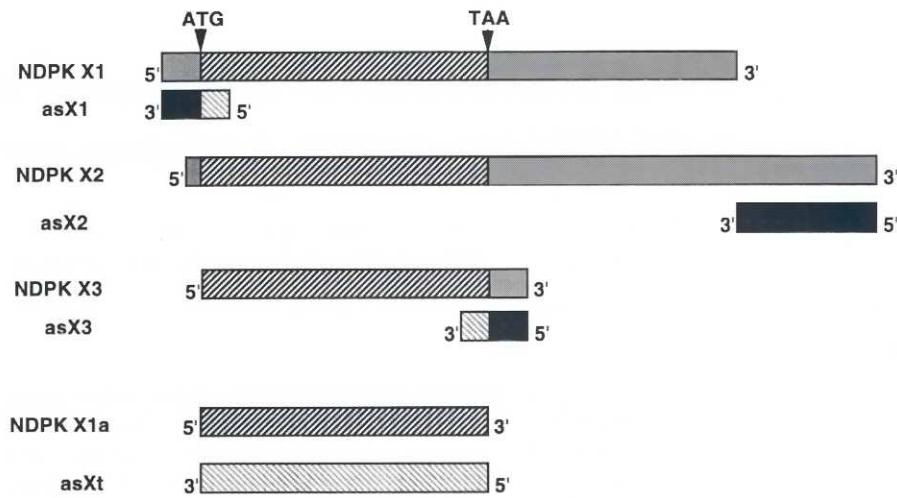

Fig. 1. Schematic representation of the different anti-sense probes asX specifically recognizing each of the *NDPK* mRNAs. *The open reading frame is hatched. The initiation codon (ATG) and the stop codon (TAA) are indicated. The differently shaded rectangles correspond to the 5' or 3' UTR regions. Construction of asX1, asX2, and asX3 which recognize NDPK X1, NDPK X2, and NDPK X3 mRNAs, respectively, and of the anti-sense probe asNDPK Xt recognizing every NDPK mRNA, is described in methods.*

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* (awd^{Kpn}) 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^{Kpn} allele also suppresses hematopoietic defects associated with the *Tum-l* oncogene. *Tum-l* lethality is also suppressed by *pn* mutations, indicating the existence of a hematopoietic regulatory pathway involving Tum-l, Awd^{Kpn} 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 pseudotetraploïd 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 latters 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 mRNAs 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.

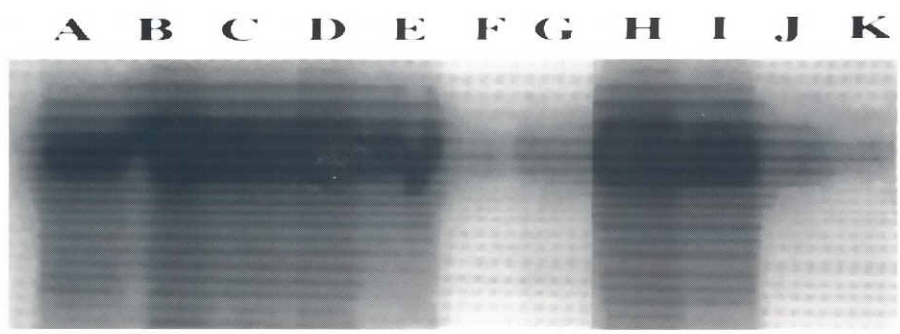

Fig. 2. N*DPK X1* mRNA stability in acellular extracts. *1ng mRNA was incubated alone (D, H), with a yeast extract (A, E, I), or with a S100 Xenopus oocyte extract (B, C, F, G, J, K) at O°C for 1h (A, B, C), at 20°C for 5 min (D, E, F, G) or 30 min (I, J, K).*

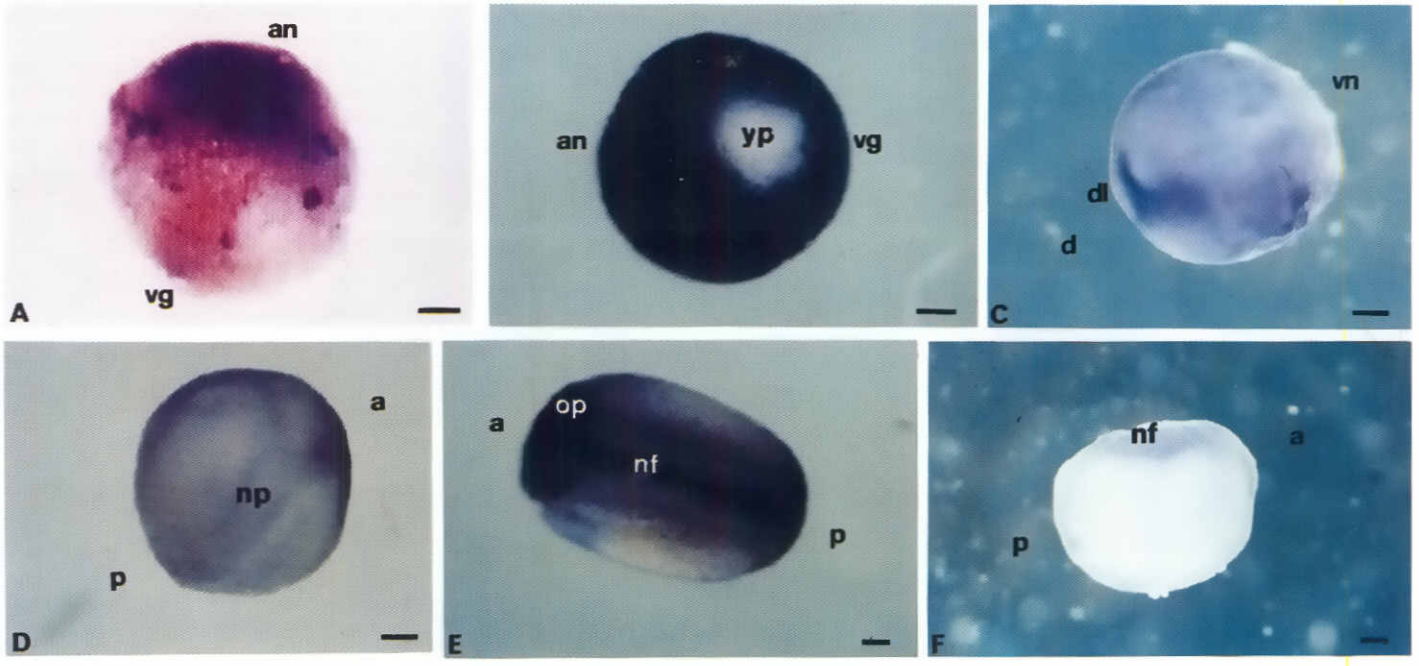

Fig. 3. Specific detection of NDPKs transcripts by whole-mount *in situ* hybridization on gastrula (A-C) and neurula embryos (D-F). (A and D) NDPK X1 mRNA. **(B and E)** *NDPK X2 mRNA.* **(C and F)** *NDPK X3 mRNA. a, anterior; an, animal; d, dorsal; dl, dorsal lip of the blastopore; nf, neural fold; 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 otic 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 metamerization. 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 metamerizing somites. At the tail bud stage, this transcript can also be detected as a single band in the center of each metamerizing 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

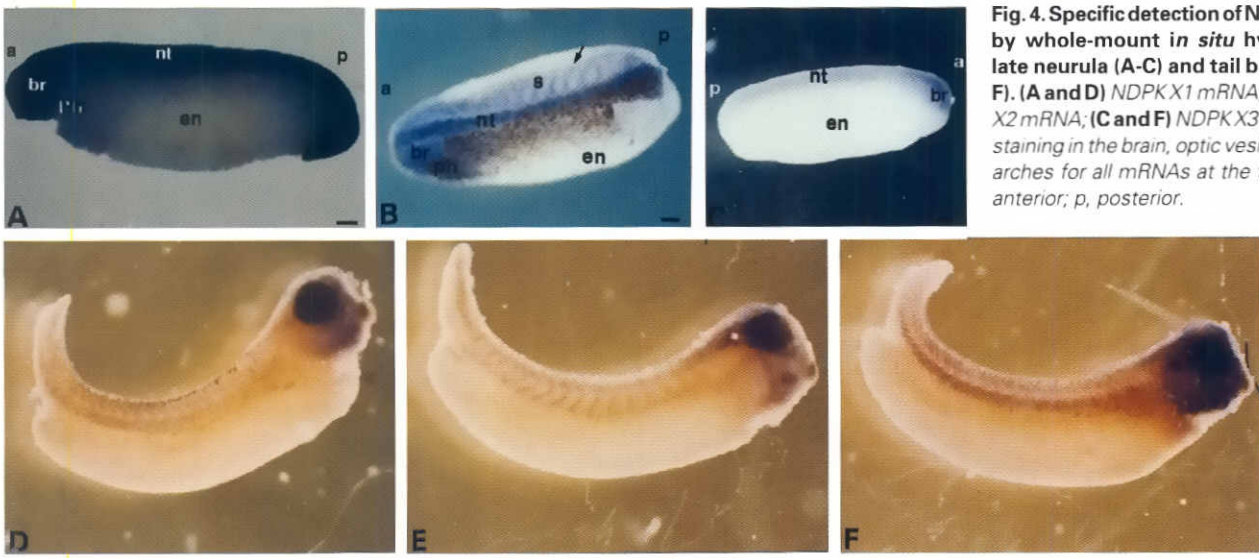

Fig. 4. Specific detection of NDPK transcripts by whole-mount *in situ* hybridization on late neurula (A-C) and tail bud embryos (D-F). (A and D) *NDPK X1 mRNA;* **(B and E)** *NDPK X2 mRNA;* **(C and F)** *NDPK X3 mRNA. Note the staining in the brain, optic vesicle and branchial arches for all mRNAs at the tail bud stage. a, anterior; p, posterior.*

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 germinal 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 germinal 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 EF1aO (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 a 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 Xlgv7 (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

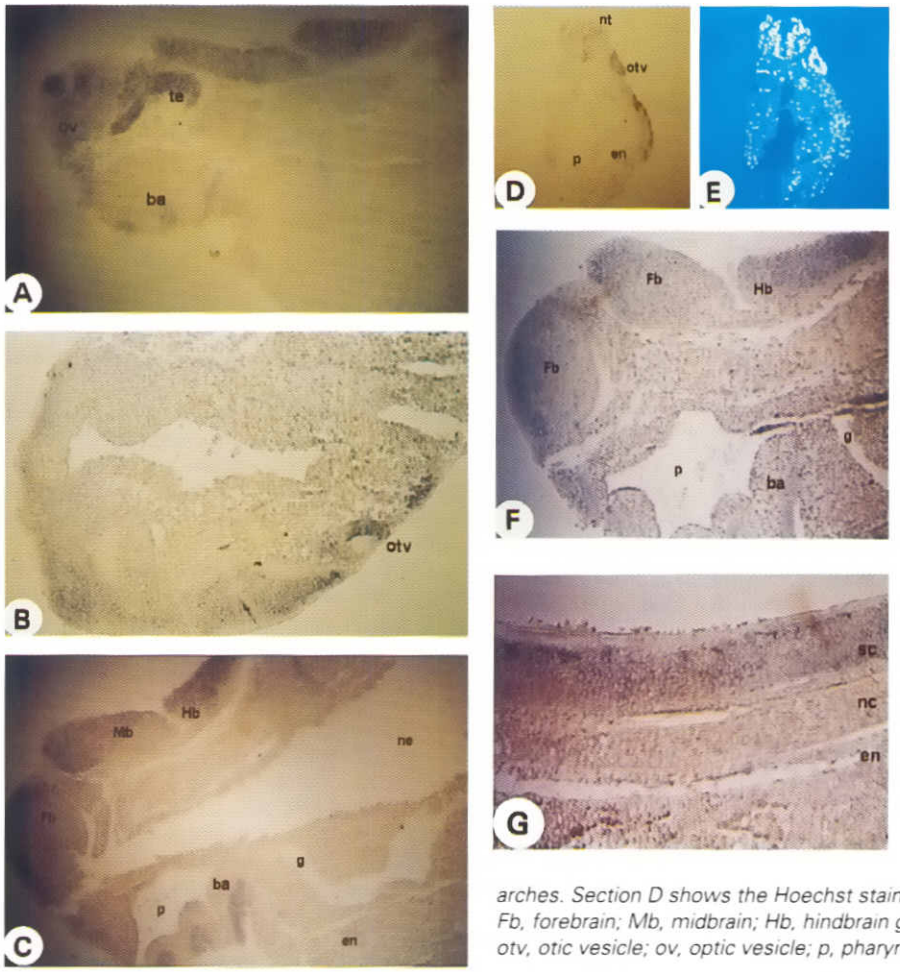

Fig. 5. Tissue section visualization of the *in toto* hybridized tail bud embryos. (A) *NDPK X1 mRNA localization.* **(B-E)** *NDPK X2 mRNA localization.* **(F and G)** *NDPK X3 mRNA. Sections show the presence of NDPK X1 mRNA in the optic vesicle, telencephalon and brachial arches. NDPK X2 mRNA is present in the otic 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, otic vesicle; ov, optic vesicle; p, pharynx; sc, spinal cord; te, telencephalon.*

any other NDPK known sequences. Only mitochondrial NDPKs such as that from *Dictyostelium discoïdeum* (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; Siomi 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, germinal 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* NDPKs 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 metamerization

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. CCCACCC 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 *Xhox1A* or *Xtwi* that have polypyrimidine rich sequences or CCCACCC 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 metamerizing 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.

TABLE1

***NDPKS* MRNAS DISTRIBUTION DURING EARLY DEVELOPMENT. (A): ANTERIOR PART OF THE EMBRYO; (C): CYTOPLASMIC LOCALIZATION; (N): NUCLEAR LOCALIZATION**

	NDPK mRNAs			
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	+	+	+	++
Epihysis	+	+	-	++

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 (3-3.5 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 4500g, 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 NDPKX1 and NDPKX3 do not discriminate between the two proteins. Whole ovaries 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 or fertilized eggs were recovered after classical gonadotrophin treatment (Kay, 1991). Embryos were staged according to Nieuwkoop and Faber (1967) (Nieuwkoop and Faber, 1967) and fixed in Bouin's fixative for immunofluorescence assays or in MEMPFA 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 pBluescript KSII+ (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'-GCTG**GGATCC**ATCAGGCTTGATGGC-3') primer designed to hybridize to the 5'-coding region of *NDPK X1* (position 64 to 88) in combination with the reverse primer of pBSIIKS+. 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

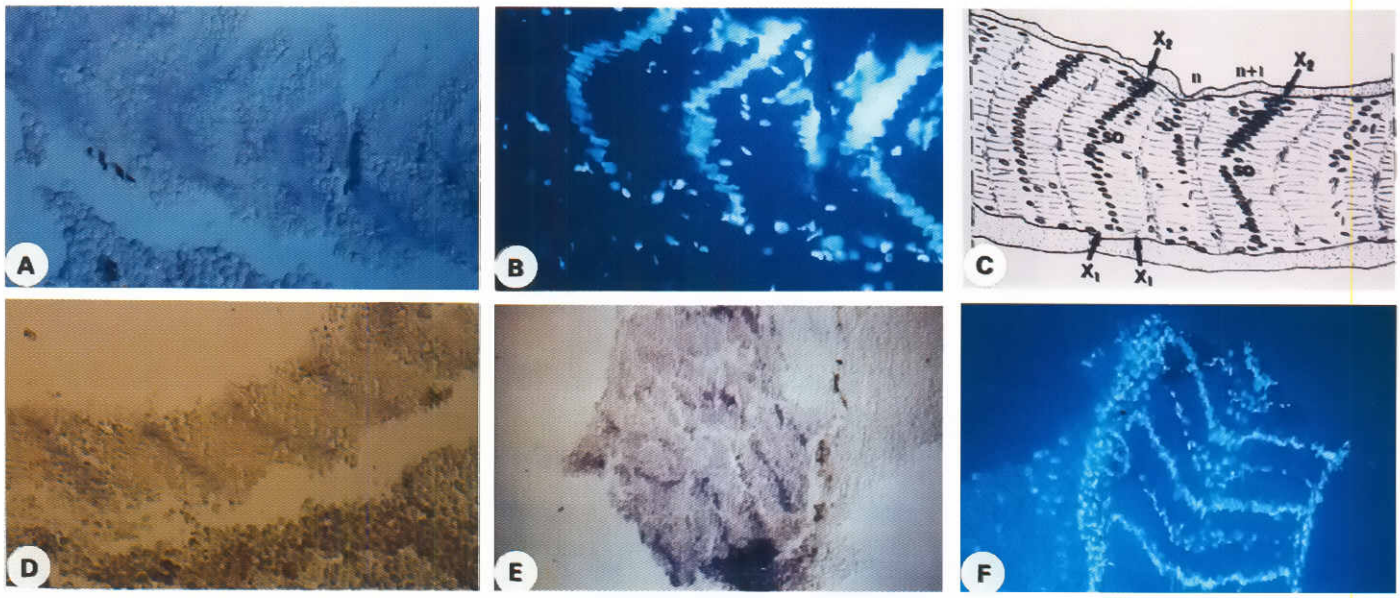

Fig. 6. N*DPK X1* and *NDPK X2* mRNA localization in the somites of tail bud embryos. (A-B) *NDPK X1 mRNA localization.* **(D-F)** *NDPK X2 mRNA localization.* **(B, F)** *Hoechst staining of sections in A and in E, respectively.* **(C)** *Scheme of the tail bud somites (so) showing the alignement of nuclei in the myotome (n and n+1 represent adjacent somites). Arrows point on the nuclear localization of X2 mRNA and cytoplasmic localization of X1 mRNA. NDPK X1 mRNA is present in the two anterior and posterior cytoplasmic parts of the somitic cells (A). NDPK X2 mRNA co-localize with the nuclei in the center of the somitic cells (D, E, F). NDPK X3 mRNA has the same localization as of NDPK X1 mRNA (data not shown). Section in D was revealed by the NBT-BCIP procedure, as opposed to sections in A and E, revealed with the BM purple procedure (cf methods).*

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-PstI 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 PstI and HindIII, respectively.

The *NDPK X3* specific probe was generated by treating pBS X3 with DdeI. 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 Xl*), 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 Transprobe T 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 BM purple (Boehringer Mannheim) following the manufacturer's instructions. The detection reactions were allowed to proceed for 2h (*NDPKXt* and *NDPK X2*) or 6h (*NDPK X1* and *NDPK X3*). *In toto* hybridized embryos were refixed in MEMPFA, 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 $MgCl_2$; 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 10.000g for 20 min, the supernatant was centrifuged for 1h at 100.000g. 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 $\alpha(^{32}P)$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% polyvinylalcool; 10 µg E. coli tRNA 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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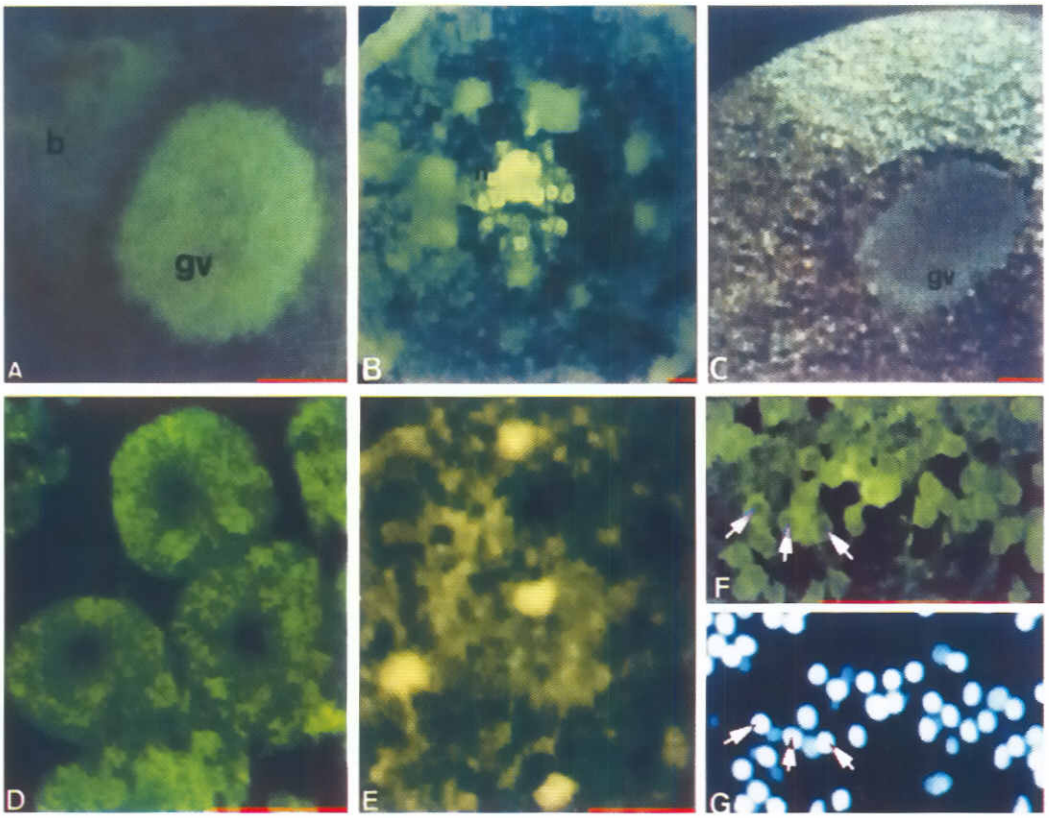

Fig. 7. Indirect immunohistofluorescence detection of NDPKs in X*enopus* oocytes and early stage embryos. (A-D) *Oocytes. A, previtellogenic oocyte; B, mature oocyte; C, vitellogenic oocyte; D, morula macromeres; note the weak staining (faint green) of nuclei compared to cytoplasm.* **(F)** *Gastrula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

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