REVIEW

A developmental biological study of aldolase gene expression in *Xenopus laevis*

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ABSTRACT

We cloned cDNAs for *Xenopus* aldolases A, B and C. These three aldolase genes are localized on different chromosomes as a single copy gene. In the adult, the aldolase A gene is expressed extensively in muscle tissues, whereas the aldolase B gene is expressed strongly in kidney, liver, stomach and intestine, while the aldolase C gene is expressed in brain, heart and ovary. In oocytes aldolase A and C mRNAs, but not aldolase B mRNA, are extensively transcribed. Thus, aldolase A and C mRNAs, but not B mRNA, occur abundantly in eggs as maternal mRNAs, and strong expression of aldolase B mRNA is seen only after the late neurula stage. We conclude that aldolase A and C mRNAs are major aldolase mRNAs in early stages of *Xenopus* embryogenesis which proceeds utilizing yolk as the only energy source. aldolase B mRNA, on the other hand, is expressed only later in development in tissues which are required for dietary fructose metabolism. We also isolated the *Xenopus* aldolase C genomic gene (ca. 12 kb) and found that its promoter (ca. 2 kb) contains regions necessary for tissue-specific expression and also a GC rich region which is essential for basal transcriptional activity.

Key words: *aldolase A, B and C mRNAs, embryo-type aldolase mRNA composition, tissue specific expression pattern.*

INTRODUCTION

Studies on the temporally- and spatially-regulated expression of multilocus-type isozyme genes are important for understanding control mechanisms

underlying growth and differentiation of cells in development of the animal embryo[1]. In *Xenopus*, L-lactate dehydrogenase (LDH) consists of two major [LDHA (muscle-type) and LDHB (liver-type)] and one minor [LDHC (oocyte-type)] subunits, and these subunits, coded for by different genes, are expressed in a tissue-specific manner during embryogenesis[2]. Creatine kinase consists of two (in tetrapods) or four (in amphibia and teleost fish) different subunits, and expression of these subunits also occurs in tissue-specific and developmentally-regulated manners[3]. Here, we review our recent studies on properties and spatial and temporal regulation of *Xenopus aldolase* genes in developing

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embryos and tissues.

aldolases as materials for studies on differential gene expression

aldolase, or fructose-1, 6-bisphosphate *aldolase* (EC 4, 1, 2, 13), provides a typical example of developmentally-controlled multilocus-type isozymes. Its functional form is either homo- or hetero-tetramer composed of two of the three different ca. 40,000-dalton proteins (A, B and C), each coded for by different genes[4],[5]. Holoenzymes catalyse different reactions depending on the nature of the constituent subunits. Basically, *aldolases* A and C catalyze the cleavage of fructose-1, 6-bisphosphate in the glycolytic pathway, giving rise to glyceraldehyde-3-phosphate and dihydroacetate phosphate[4],[6], whereas *aldolase* B catalyses cleavage of fructose-1-phosphate

derived from dietary carbohydrates to give rise to glyceraldehyde and dihydroacetate phosphate[6-8]. In addition, *aldolase* B catalyses gluconeogenesis or the hexose formation[7],[8].

Interestingly, expression properties of *aldolase* subunit genes (A, B and C) differ according to the specialized roles which they perform. Thus, *aldolase* A is expressed mainly in muscles and other tissues with a high rate of glycolysis[9], and *aldolase* C is expressed specifically in brain and nervous tissues[10]. By contrast, *aldolase* B, adapted to dietary fructose metabolism and gluconeogenesis, is expressed in liver, kidney and intestine which are active in these pathways[4], [7],[8]. As a result, mutation of this gene in humans causes the severe hereditary congenital disease fructose intolerance[11],[12].

We have been interested in control mechanisms of the expression of the three *aldolase* genes in *Xenopus* embryogenesis and we asked which of the three *aldolase* genes (so-called muscle-type A, liver-type B and brain-type C) are utilized in early embryonic development, during which an unicellular and totipotent egg cell cleaves rapidly and becomes

a multicellular embryo composed of many differentiated cells. We also cloned a genomic DNA of the *aldolase C* gene and characterized its promoter function.

Cloning of *Xenopus* *aldolases* A, B and C cDNAs

Fig 1. Structure of *Xenopus* aldolase A, B and C. (left, top): Amino acid sequences. {from Kajita et al[26]}. (left, bottom): Characteristic features of the three aldolases. The regions(1-7) highly conserved among all the three types of aldolases are indicated gray. Isozyme group-specific sequences (IGS) are indicated three colours; A-type specific as red, B-type as yellow, C-type as blue. Sequences which are not conserved are not coloured. Δ (right): Steric structure of aldolase A. Red regions are IGSs. The highly conserved regions among all types of aldolases are indicated by green. Gray-coloured regions are low conserved sequences. The orange portion is the active center. N-terminus and C-terminus are shown.

We first attempted cloning and expression studies of *aldolase* A, B and C cDNAs, and we obtained XALDA, XALDB and XALDC. Fig 1 shows amino acid sequences of XALDA, XALDB and XALDC (respectively 1.5, 1.8 and 1.8 kb in length). As for the number of amino acids deduced from cDNAs there was no difference in all the three *Xenopus aldolases* (A, B and C) (Fig 1, left, top). The overall identities at the amino acid sequence level between *Xenopus aldolases* A and B and between B and C were lower (67 and 68% respectively) than that between *aldolases* A and C (left, top) as in rats[10],[13] and humans[14]. In Fig 1 (left, bottom), we marked 4 isozyme group-specific regions (IGS I to IGS IV), which are conserved in each group of *aldolases* (A, B and C). We differently marked two of these (IGS I and IGS IV) (Fig 1, left, top), since these are particularly important for *aldolase* B-specific function (utilization of fructose-1-phosphate)[15]. Furthermore, we marked 7 regions which are highly or relatively highly conserved in all three types of *aldolases*[5]. Sequences in 4 IGSs were considerably different among the three *Xenopus aldolases* (A, B and C) (less than 50% identity) (Fig 1, left, top), yet those in 7 conserved regions were similar (between A and B, 85.0%; B and C, 85.0%; A and C, 93.8% in amino acid identity). *Xenopus aldolases* A, B and C, contained Lysine at amino acid number 230 (K; orange-colored in Fig 1, left, top) as an active center [4]. The three *aldolases* have a similar three dimensional structure (Fig 1, right).

The phylogenetic tree constructed by the neighbor-joining method showed that the ancestral gene of *aldolase* B genes segregated from the ancestral gene of *aldolase* A and C genes before segregation of *aldolase* A and C genes in *Xenopus* as in other animal species[10],[16]. We obtained a cell line from *Xenopus* adult kidney and Fluorescence in situ hybridization (FISH) studies showed that the three *aldolase* genes are located on different chromosomes[17].

Expression of three *aldolase* mRNAs in adult frogs

Northern blot analysis was done using RNAs extracted from various *Xenopus* adult tissues. We obtained a very strong *aldolase* A mRNA signal of

ca. 1.8 kb in muscle, a moderately strong signal in heart, brain and intestine, and low levels of expression in all the other tissues, probably due to the transcription from the minor "pan-expression promoter" [18]. *aldolase* B mRNA appeared as a signal of ca. 1.8 kb in kidney, liver, intestine, stomach and interestingly, in skin, as noted for humans [14], rat[19], sheep[20], sea bream (*Sparus aurata*) [21] and salmon [22]. The expression in skin is as in salmon[22]. We obtained a strong signal of *aldolase* C mRNA of ca. 2 kb in heart, brain and ovary, and a weak signal in intestine, testis, lung and other tissues.

Using in vitro transcribed *aldolase* A, B and C mRNAs as references, northern blot analyses were done and calibration curves for determinations of absolute amounts of *aldolase* A, B and C mRNAs in each adult tissue (we estimated the amounts permg of total RNA). We then calculated the percent composition of the three *aldolase* mRNAs (A, B and C) for each tissue and expressed the results as circle graphs (Fig 2). The total amount of three *aldolase* mRNAs (A plus B plus C) per unit amount (1mg) of the total RNA is largest in muscle (270 pg/mg total RNA), and next largest in heart (120 pg/mg total RNA), due to the occurrence of an extremely large amount of *aldolase* A mRNA in muscle and *aldolase* C mRNA in heart. This result is reasonable in view of the fact that these two tissues are most active in energy consumption. It is interesting here that skeletal muscles express almost exclusively *aldolase* A but the heart (cardiac muscles) expresses much more *aldolase* C mRNA than *aldolase* A mRNA. *aldolase* A mRNA constituted as much as 99% of the total *aldolase* mRNAs in muscle, whereas *aldolase* C mRNA constituted 70% and 60%, respectively, of the total *aldolase* mRNAs in heart and brain. Thus, the percent composition of three *aldolase* mRNAs in brain, a typical ectodermal tissue, is similar to that in heart, a typical mesodermal tissue. *aldolase* B mRNA, on the other hand, constitutes the major *aldolase* mRNA in liver and kidney, and also in stomach and intestine, again reflecting the fact that *aldolase* B is actively expressed in tissues which are necessary for metabolism of dietary fructose[7].

It is worth pointing out that the kidney, which is mesodermal in origin, has an *aldolase* mRNA composition almost identical to that of the liver, which is

endodermal in origin: In both of these tissues *aldolase* B mRNA constituted ca. 85% of the total *aldolase* mRNAs, the rest being *aldolase* A mRNA with only a trace amount of *aldolase* C mRNA. In intestine and stomach, which are both endodermal, the percent composition of *aldolase* B mRNA exceeded 50%, and skin which is ectodermal also strongly expressed *aldolase* B mRNA albeit the amount being slightly lower than 50% of the total *aldolase* mRNAs. Thus, each tissue has its characteristic *aldolase* mRNA composition, thereby reflecting functional differences between the three *aldolases*, rather than differences in developmental origin of each tissue.

Expression of three *aldolase* mRNAs in oocytes and embryos

Northern blot analyses were made using RNAs from oocytes. *aldolase* A mRNA occurred as a major

aldolase mRNA and the level increased greatly as the oocyte grew, and the increased level was maintained during maturation. We detected no signal of *aldolase* B mRNA over the background level throughout oogenesis and oocyte maturation. We obtained a substantial amount of *aldolase* C mRNA, the level increased throughout oogenesis and remained constant during maturation.

Northern blot analysis were made using RNAs from embryos at different stages and in vitro transcribed three *aldolase* mRNAs. Using the calibration curves to correlate strengths of northern blot signals to the amounts of *aldolase* RNAs, we here again estimated the absolute amount of each of the three *aldolase* mRNAs in embryos. A *Xenopus* fertilized egg contained approximately 60 pg of *aldolase* A mRNA, 45 pg of *aldolase* C mRNA but only 1.5 pg or less *aldolase* B mRNA. Thus, *aldolase* A and C

Fig 2. *aldolase* mRNA expression in various adult organs {from Kajita et al[17]. Sizes of circles indicate the relative amount of *aldolase* mRNAs which is comparable among tissues.

Fig 3. Expression of three aldolase mRNAs in developing embryos from Kijita et al [17]. Size of the circle is comparable among different stages. The total amount of aldolase mRNAs in an egg is 106.5 pg.

mRNAs together constitute 99% of the total egg *aldolase* mRNAs (A plus B plus C), and *aldolase* mRNAs constitutes altogether ca. 0.0025% of the total egg RNA, since a *Xenopus* egg contains ca. 4 g of RNA a major portion of which is rRNA[23], [24]. During cleavage the amount of *aldolase* A mRNA per embryo decreases to a level as low as 15 pg/embryo (at the late gastrula stage). From the early neurula stage, however, the level increased sharply to a level as high as 600 pg/embryo at the tadpole stage (stage 35). The period of this great increase in the amount of *aldolase* A mRNA corresponds to the period of somitogenesis. The amount of *aldolase* C mRNA per embryo also decreased sharply until the gastrula stage (ca. 3 pg/ μ mbryo). Unlike that of *aldolase* A mRNA, however, the post-gastrular increase in the amount of *aldolase* C mRNA was not large and the level remained at 50 pg/embryo even at stage 35 (tadpole stage). The very low level of *aldolase* B mRNA per embryo was maintained until the midgastrula stage (stage 11). At the late gastrula stage (stage 13), however, a slight increase was detected, and from the late neurula stage (stage 22) on, the amount continuously increased and eventually reached the level as high as 140 pg/embryo at the tadpole stage (stage 35). Based on the quantitative data, we calculated the percent composition of three *aldolase* mRNAs for embryos at different stages (Fig 3). It is apparent that the A plus C-type *aldolase* composition is “egg-specific” or “early embryo-specific”. Probably, this *aldolase* composition is suitable for fructose metabolism during the yolk-dependent developmental stages, during which *aldolase* B is probably not necessary. *aldolase* B mRNA, on the other hand, is expressed only in later stages so that embryos get ready to metabolize dietary fructose at the feeding stages.

Tissue-specific expression of *aldolase* genes in embryos

According to whole mount in situ hybridization, the earliest stage at which we detected localized signals for *aldolase* A, B and C mRNA was stage 16 (early neurula stage), 25 (late-neurula stage), and 21 (neurula stage), respectively[25], [26]. Throughout later stages, the *aldolase* A mRNA signal was noted in somites. At the tailbud stage the *aldolase* B

mRNA signal appeared in regions where liver rudiments and proctodeum are formed. After hatching, *aldolase* B mRNA was strongly detected in pronephros, liver rudiments and hindgut, and at this stage, the tail fin (epidermis) was also stained. At stage 23, the *aldolase* C mRNA signal was in the neural tube, and at stage 33, it was in brain, spinal cord, heart anlage, and pronephros. Interestingly, *aldolase* A mRNA was detected in pronephros at stage 36.

In the dorsal view of hatched embryos, the regionally-different expression of three *aldolase* mRNAs was clearly seen (Fig 4). Thus, the *aldolase* A mRNA signal was detected exclusively in somites. *aldolase* B mRNA signals occurred in pronephros and in epidermis. *aldolase* C mRNA signals occurred in retina, mesencephalic optic tectum, and rhombencephalon. *aldolase* C signals occurred also in the spinal cord which is elongated toward the tail

Fig 4. Differential expression of three *aldolase* mRNAs detected by whole mount in situ hybridization. A, B and C indicate expression pattern of *aldolase* A, B and C mRNA, respectively {from Kajita et al[26]}.

tip. We obtained essentially the same results in sectioned materials of tailbud embryos.

Genomic DNA structure of *Xenopus aldolase C*

Genomic DNA cloning of *aldolase C* was also done[27]. We obtained a clone named XACl3-1, containing a 12.2 kb DNA insert. XACl3-1 contains the entire sequence for 5'-non-coding, coding, and 3' -non-coding regions of *Xenopus aldolase C* gene (Fig 5A). From comparison of this genomic gene with *Xenopus aldolase C* cDNA[10], the *Xenopus* gene was found to consist of nine exons and spanned a total 9.6 kb from the beginning of exon-1 to the end of exon-9 (Fig 5A). Fig 5A shows a comparison of this *Xenopus* gene with rat and human counterparts. *Xenopus aldolase C* gene closely resembled human and rats *aldolase* genes[28-30]. Thus, the number of exons was the same, and furthermore, lengths of all the exons, except for those including 5' -UTR and 3' -UTR, were quite similar between *Xenopus* and mammalian genes. However, size of the gene as a whole was quite different between *Xenopus* and mammals, due to the fact that all the introns in the *Xenopus* gene are several-fold larger than human and rat counterparts.

Primer extension analyses was done using oocyte poly(A)+ RNA as a template, and we determined the transcription initiation site (+1) (G). We made a sequence analysis of the 5'-flanking region (in total 1596 bp), conventionally dividing it into six different regions (A-F) (Fig 5B). The proximal part of the 5'-flanking region (regions D, E and F) contained a number of potentially cis-acting elements, as compared with mammalian genes. Like rat and human genes[31], the *Xenopus aldolase C* gene contained a GC box (-47 bp in region E) and an *aldolase C*-specific element (ACSE) (from -14 to +2). Furthermore, in the *Xenopus* gene a CCAAT box (at -62 bp in region E) and a TATA-like element (at -28 bp in region F) were found, and these not found in rat and human genes[31] (Fig 5B).

Expression studies with the variously-modified promoters were done using *Xenopus* embryos by

DNA microinjection and a kidney cell line by DNA transfection. The GC box is the crucial element in the basal promoter, although the TATA-like element appeared to have a slightly stimulative effect on GC box functioning. We showed by gel retardation and foot-printing assays the occurrence in A6 cells of a nuclear factor(s) that binds specifically to the GC box[27].

Expression characteristics of genomic *Xenopus aldolase C* gene

To test if the 5'-upstream region obtained here directs temporally and regionally regulated expression of the *aldolase C* gene in developing *Xenopus* embryos, we first injected an *aldolase C* promoter connected to bacterial CAT gene (pXAC-CAT) into *Xenopus* embryos and tested the CAT gene expression at different stages of development. pXAC-CAT was not expressed during cleavage to the blastula stage, expressed only weakly at gastrula and early neurula (neural plate) stages, but was strongly expressed at the late neurula (neural tube) stage (22 h). This time course was reproducible and roughly coincided with that of mRNA expressed from the endogenous *aldolase C* gene[10]. Strong expression of the *aldolase C* gene in the neurula stage is reasonable, since this gene is expressed extensively in neural tissues (Figs 2 and 4).

We then injected pXAC-lacZ, consisting of the *Xenopus aldolase C* gene promoter (1596 bp) and lacZ gene, at the animal side of both of the blastomeres at the two-cell stage. We examined X-gal expression at late neurula and tail-bud stages (Fig 6). Most of the injected embryos showed a blue colour which reflects lacZ gene expression mainly at the dorsal and central portion of the embryo axis, where the neural tube is formed. Fig 6A shows an example of an embryo with such an expression pattern. At the tail-bud and swimming tadpole stages, lacZ gene expression was found mainly in the head part. Fig 6B shows an example of the late tail-bud stage embryo which showed the signal at the hind brain. Fig 6C shows a swimming tadpole with the signal apparently seen in the midbrain region. From these observations, we concluded that the 1596 bp of the

<Fig 5. Structure of the *Xenopus aldolase C* genomic gene. A: The overall structure. B: various elements found in the promoter region. {from Yatsuki et al[27]}.

Fig 6. Expression in brain and spinal cord regions of injected aldolase C genomic gene promoter connected to Lac Z gene [Yatsuki et al, [27]].

5'-flanking sequence supported the temporally and spacially regulated expression pattern characteristic of the *aldolase C* gene in developing embryos.

Conclusion

In amphibian embryogenesis, it is a general phenomenon that a large amount of maternal stockpiles support early development and these are replaced by materials which are newly formed, based on zygotic gene expression during development[32],[33]. Such a switch from the maternal to zygotic mRNA was also observed for *aldolases A* and *C*. The maternal *aldolase B* mRNA occurs only at a residual level and extensive transcription takes place only after the late neurula stage. This agrees with the zymogram analysis: Predominant enzymes present in early embryos were *aldolase A* and *C*[25]. We confirmed that the tissue-specific expression pattern in embryos was mostly as in adults. It should be emphasized here that *aldolase A* and *C* genes are quite

actively expressed in oocytes and are preserved abundantly in early embryos which develop as a closed system depending on the yolk as the only energy source. By contrast, active *aldolase B* gene expression begins only late during embryogenesis, but before embryos reach the feeding tadpole stage. It follows then that the zygotic expression of *aldolase B* gene is to prepare for food intake. In mammals, there are data which parallel our present results.

Finally, one may ask why the *aldolase B* gene which is needed for metabolism of dietary fructose is expressed so early (at the gastrula stage, albeit at a low level) during embryogenesis. In embryos, however, such a precocious activation also occurs in other genes. For instance, transcription of rRNA genes begins as early as at the late blastula stage [34], despite the fact that embryos contain ribosomes of an amount sufficient support development up to stage 41 (feeding tadpole stage)[35].

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