Enolase isoenzymes in adult and developing *Xenopus laevis* and characterization of a cloned enolase sequence

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As part of a study of glycolysis during early development we have examined the pattern of expression of enolase isoenzymes in *Xenopus laevis*. In addition, the nucleotide sequence of a cDNA clone coding for the complete amino acid sequence of one enolase gene (ENO1) in *X. laevis* was determined. *X. laevis* ENO1 shows highest homology to mammalian non-neuronal enolase. Analysis of enolase isoenzymes in *X. laevis* by non-denaturing electrophoresis on cellulose acetate strips revealed five isoenzymes. One form was present in all tissues tested, two additional forms were expressed in oocytes, embryos, adult liver and adult brain, and two further forms were restricted to larval and adult muscle. Since enolase is a dimer, three different monomers (gene products) could account for the observed number of isoenzymes. This pattern of enolase isoenzyme expression in *X. laevis* differs from that of birds and mammals. In birds and mammals the most acidic form is neuron-specific and there is only one major isoenzyme expressed in the liver. RNAase protection experiments showed the presence of ENO1 mRNA in oocytes, liver and muscle, suggesting that it codes for a non-tissue-restricted isoenzyme. ENO1 mRNA concentrations are high in early oocytes, decrease during oogenesis and decrease further after fertilization. Enolase protein, however, is maintained at high concentrations throughout this period.

INTRODUCTION

Alterations in glycolytic flux have long been associated with changes in the growth properties of cells and with the acquisition of a transformed phenotype (Warburg, 1956). The glycolytic enzyme enolase has been identified in relation to transformation and growth regulation in several experimental systems: (1) a cDNA clone for enolase was identified in a screen for sequences specifically induced in quiescent rat fibroblasts by growth factors and serum (Matrisian *et al.*, 1985); (2) transcriptional induction of enolase has been shown upon mitogenic stimulation of human peripheral-blood lymphocytes (Giallongo *et al.*, 1986); (3) a specific isoenzyme of enolase in yeast is induced in the resting phase of the cell cycle (G_0), as well as upon heat shock (Iida & Yahara, 1985); (4) enolase is a major substrate for tyrosine phosphorylation in Rous-sarcoma-virus-transformed chick cells (Cooper *et al.*, 1983, 1984); (5) enolase mRNA is highly expressed during oogenesis in *X. laevis* and shows increased expression in a tumorigenic *Xenopus borealis* cell line compared with its non-transformed parent line (Picard *et al.*, 1986).

In avian and mammalian tissues three types of enolase isoenzymes (α , β and γ) have been described. The active form of the enzyme is a dimer composed of α -subunits in immature organs and in adult liver, of β -subunits in adult skeletal muscle and of γ -subunits in adult neurons (Rider & Taylor, 1974; Fletcher *et al.*, 1976; Zomzely-Neurath, 1983). In developing muscle and in heart muscle the $\alpha\beta$ -heterodimer (Rider & Taylor, 1974) and in developing nervous system the $\alpha\gamma$ -heterodimer (Fletcher *et al.*, 1976; Jørgensen & Centervall, 1982) are also detected. The two enolase genes coding for α - and γ -subunits have been

cloned (Sakimura *et al.*, 1985a,b; Giallongo *et al.*, 1986). Chicken muscle (β) enolase protein has been sequenced (Russell *et al.*, 1986) and is likely to be encoded by a separate gene. In the present paper we describe the tissue distribution of enolase isoenzymes in *X. laevis* and characterize the sequence of *Xenopus* enolase ENO1 derived from the cDNA clone ENO1 (formerly 207A2). We also present measurements of enolase protein concentrations during oogenesis and early development.

MATERIALS AND METHODS

DNA sequencing

All DNA sequencing was done by the method of Maxam & Gilbert (1980). Fragments used for sequencing were obtained by digestion with restriction endonucleases of cDNA clone ENO1 (clone 207A2 in Picard *et al.*, 1986) or of subclones containing *Bal*31-digested restriction fragments of clone ENO1. Labelling of 5' ends and 3' ends was done as described previously (Dworkin-Rastl *et al.*, 1984). All fragments were sequenced in both strands.

Preparation of cells and extraction of RNA

Embryos were staged in accordance with Nieuwkoop & Faber (1967), and oocytes in accordance with Dumont (1972). Oocytes were isolated from the follicle cells as described previously (Dworkin *et al.*, 1984), except that no Pronase was used and collagenase treatment was for up to 1 h. Eggs and embryos were dejellied in 2% (w/v) cysteine, pH 8.0, before use. Liver and leg muscle were extracted fresh from individual adult females, and brain was dissected, frozen in liquid N_2 and stored at $-80^\circ C$. Brain extracts and RNA from brain were prepared from

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the tissue of several animals. Chicken brain extracts were a gift from the 1985 Physiology Course at the Marine Biological Laboratory, Woods Hole, MA, U.S.A.

Oocytes and embryos were directly extracted in phenol/chloroform and polyadenylated RNA was isolated as previously described (Dworkin & Dawid, 1980). Brain from five animals was homogenized in a Sorvall Omnimix homogenizer in 10 vol. of 10 mM-sodium acetate buffer, pH 6.0, containing 0.1 M-NaCl, 5 mM-EDTA and 1% (w/v) SDS, and extracted as described previously (Dworkin & Dawid, 1980). Liver and muscle were minced and directly homogenized in 10 vol. of the above buffer plus 10 vol. of phenol/chloroform (1:1, v/v). Total RNA from liver and muscle was treated with DNAase I and proteinase K as described by Long & Dawid (1979) before polyadenylated RNA was isolated.

Northern blots

Polyadenylated RNA (0.1–0.5 µg) or total RNA (≤ 10 µg) was denatured with glyoxal (McMaster & Carmichael, 1977), separated on 1.2% agarose gels, transferred to Nytran membrane (Schleicher and Schuell) and hybridized with radiolabelled RNA synthesized *in vitro* (see below) or nick-translated plasmid DNA (2×10^8 c.p.m./µg; Rigby *et al.*, 1977) in accordance with Church & Gilbert (1984).

Transcription of RNA probes *in vitro* and RNAase protection experiments

Template DNA was subcloned from cDNA clone ENO1 into plasmid pSP65 or plasmid pGEM4 (Promega Biotec, Madison, WI, U.S.A.). Subclone 1 contained the fragment between the *Pst*I site at nucleotide 402 and the *Eco*RI site at nucleotide 533 (see Fig. 1), cloned into the respective sites of the polylinker of plasmid pSP65. The plasmid was linearized at the *Pvu*II site at nucleotide 235 of plasmid pSP65, and anti-sense RNA was transcribed with SP6 RNA polymerase (New England Biolaboratories or Boehringer Mannheim) in accordance with Melton *et al.* (1984), yielding a transcript of 331 nucleotide residues (probe 1). Subclone 2 contained the fragment between the *Bgl*II site at nucleotide 1232 and the *Hind*III site at nucleotide 1514 (see Fig. 1), replacing the *Bam*HI–*Hind*III fragment in plasmid pGEM4. The plasmid was linearized at the *Nhe*I site at nucleotide 2628 of plasmid pGEM4, and anti-sense RNA was transcribed with T7 RNA polymerase (New England Biolaboratories) in accordance with the manufacturer's recommendations, yielding a transcript of 571 nucleotide residues (probe 2). The specific activity of the RNAs synthesized *in vitro* was approx. 2×10^8 c.p.m./µg. After removal of the template DNA (Melton *et al.*, 1984), the RNA probes were precipitated twice and then dissolved in hybridization buffer.

RNAase protection assays and analysis of hybrids on denaturing gels were carried out as described by Melton *et al.* (1984). Per assay, 2×10^6 c.p.m. of probe was added; the amounts of RNAase used in the digestion step (at 30 °C for 1 h) are indicated in the legend to Fig. 4.

Preparation of a β-galactosidase–*X. laevis* ENO1 fusion protein

The fragment between the *Pst*I site at nucleotide 402 and the *Hind*III site at nucleotide 1514 was cloned into

the respective sites in the polylinker of the β-galactosidase fusion vector pUR292 (Rüther & Müller-Hill, 1983), resulting in an in-frame fusion of the ENO1 fragment to the C-terminal end of β-galactosidase. The resulting plasmid was used to transform *Escherichia coli* K-12 71-18 (Messing *et al.*, 1977). The detailed procedure for production of fusion protein and immunization are not described in detail in the present paper. Briefly, an exponentially growing bacterial culture was induced with 1 mM-isopropyl β-D-thiogalactoside for 2 h. The fusion protein was found in the insoluble fraction of the lysed cells and was purified from preparative SDS/polyacrylamide gels after solubilization of this fraction in 8 M-urea. The gel-eluted fusion protein was precipitated with 40%-satd. (NH₄)₂SO₄, dissolved in 8 M-urea and dialysed against 130 mM-NaCl/16 mM-sodium phosphate buffer, pH 7.4. This material was used for immunization of rabbits.

Cellulose acetate electrophoresis

Extracts for cellulose acetate electrophoresis were prepared by Dounce homogenization of oocytes or disruption of adult tissues in a Sorvall Omnimix homogenizer in 2–3 vol. (10 vol. for brain) of 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgSO₄, 1 mM-EDTA, 10 mM-2-mercaptoethanol, 100 mM-KCl and 0.5 mM-phenylmethanesulphonyl fluoride. Extracts were centrifuged at 2000 rev./min for 5 min and then at 10000 rev./min for 5 min in a Sorvall SS34 rotor, and small portions of the supernatant were frozen and stored at –80 °C. The following procedure for electrophoresis was modified from published protocols (Susor *et al.*, 1975; Oskam *et al.*, 1985). Cell lysates (0.5 µl) were applied to cellulose acetate strips (Sepraphore III; Gelman Sciences, Ann Arbor, MI, U.S.A.), and strips were electrophoresed at room temperature in 20 mM-sodium phosphate buffer, pH 7.0, for 1 h at 100 V (for separation of *X. laevis* isoenzymes). Enolase activity was localized by incubating strips against a substrate transfer membrane (Gelman Sciences) that had been soaked in the following solution: 100 mM-Tris/HCl buffer, pH 7.0, containing 10 mM-MgCl₂, 100 mM-KCl, 0.5 mM-EDTA, 1.5 mM-ADP, 20 mM-AMP, 10 mM-NADP⁺, 1 mM-glucose, 0.5 mM-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.17 mM-phenazine methosulphate, 10 mM-2-phosphoglycerate, 4 units of pyruvate kinase/ml, 6.5 units of hexokinase/ml and 2.5 units of glucose-6-phosphate dehydrogenase/ml. Incubation was at 37 °C for approx. 10 min under conditions of high humidity and low light. Incubation was stopped by washing in 5% (w/v) trichloroacetic acid for 5 min, followed by a wash with electrophoresis buffer.

Western blots

Protein polyacrylamide-gel electrophoresis and immunoblotting were done by standard procedures (Laemmli, 1970; Towbin *et al.*, 1979; Burnette, 1981). Oocytes, eggs or embryos, adult liver, adult leg muscle or adult brain were homogenized in 2–3 vol. of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 M-urea, 2% (v/v) Nonidet P-40, 1 mM-phenylmethanesulphonyl fluoride and 5% (v/v) 2-mercaptoethanol, centrifuged at 2000 rev./min for 10 min and then at 10000 rev./min for 10 min in a Sorvall SS34 rotor, and small portions of the supernatant were frozen and stored at –80 °C. Lysates equivalent to 3–60 µg of protein were added to 1 vol. of

M S I K N I R A R E I F

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1      GGAAGAGAACGGTTGCACCCACAAGAAAGTGAAATGTCTATCAAGAACATCCGTGCACGTGAAATATTT
71     D S R G N P T V E V D L Y T C K G L F R A A V P
      GATTCTCGTGGGAACCCCTACAGTGGAGGTTGACCTATACACTTGCAAAGGACTGTTCCGTGCTGCTGTTCCA
143    S G A S T G I Y E A L E L R D N D K T R Y L G K
      AGTGGAGCTTCCACTGGTATTTATGAAGCACTAGAACTCCGGGACAATGACAAGACTCGCTACCTGGGAAAG
215    G V G R A V K Y V N E F L G P A L C T Q N L N V
      GGTGTCGGCAGAGCTGTCAAATATGTAATGAGTTTCTGGGACCTGCATTATGCACCCAGAATCTAAATGTT
287    V E Q E K I D K L M I E M D G T E N K S K F G A
      GTTGAGCAGGAAAAGATTGACAAGCTCATGATTGAAATGGATGGAAGTGAACAAATCCAAATTTGGTGCT
359    N A L L G V S L A V C K A G A A E K G V P L Y R
      AATGCACCTCGTGGAGTGTCCCTGGCAGTGTTGTAAGCTGGAGCTGCAGAGAAGGGAGTCCCAGCTGTACCGT
431    H I A D L A G N P E V I L P V P A F N V I N G G
      CACATTGCTGACCTGGCTGGGAACCCCTGAGGTCATACTTCTGTACCGCATTTAATGTTATCAATGGTGCC
503    S H A G N K L A M Q E F M I L P V G A D S F K E
      TCTCATGCTGGCAACAAGCTGGCTATGCAAGAATTCATGATCTTGCTGTGGGTGCAGACAGCTTTAAGGAG
575    A M R I G A E V Y H N L K N V I K E K Y G K D A
      GCCATGCGCATAGGGGCGAGAGGTGTATCACAATCTGAAGAATGTATCAGGAGAAATATGGAAGGATGCC
647    T N V G D E G G F A P N I L E N K E A L E L L K
      ACAAATGTTGGTGATGAAGGGGTTTCGCTCCCAATATCTTGAGAGAACAAGAAGCCTTGGAGCTTCTGAAA
719    T A I N K A G Y P D K I V I G M D V A A S E F Y
      ACTGCCATTAACAAGGCAGGATACCCAGATAAGATTGTCTATGGGATGGACGTGGCAGCATCTGAATTCTAC
791    R D G K Y D L D F K S P D D P S R Y I S P D K L
      CGTGATGGAAATATGATCTGGACTTCAAGTCCCGGATGACCCGAGGAGTACATCTCACCTGACAGAGCTG
863    A E L Y M S F V K N Y P V V S I E D P F D Q D H
      GCTGAAGTGTACATGAGCTTTGTCAAAAATTATCCAGTGGTATCCATTGAAGATCCCTTTGACCAGGATCAT
935    W E A W T K F T A A S G I Q V V G D D L T V T N
      TGGGAAGCCTGGACAAAGTTCACAGCAGCTTCTGGTATCCAGGTAGTTGGTGATGACTTGACTGTGACCAAC
1007   P K R I A K A V E E K A C N C L L L K V N Q I G
      CCTAAGCGTATTGCCAAGGCTGTGGAGGAGAAGGCTTGTAAGTGTCTGCTTCTGAAAGTCAATCAGATTGGC
1079   T V T E S L E A C K L A Q S N G W G V M V S H R
      ACAGTGACAGAATCCCTGGAGGCGTGAAGCTGGCTCAGTCAAACGGCTGGGGTGTGATGGTCAGCCATCGT
1151   S G E T E D T F I A D L V V G L C T G Q I K T G
      TCTGGGGAGACAGAAGATACTTTTCATTGCTGACCTAGTGTTGGTCTTTGCACTGGACAGATAAAGACTGGG
1223   A P C R S E R L A K Y N Q L L R I E E E L G S K
      GCTCCATGCAGATCTGAGCGACTTGCAAAAGTACAACCAATTGCTCAGGATTGAAGAAGAAGTGGCAGCAAG
1295   A R F A G K N F R K P V F N
      GCCCGCTTTGCTGGAAAAAAGTTCAGAAAACAGTTTTCAACTAATTGGCGACTTCCCTAGGCAATCACCCA
1367   ACTGATGTAGTAGCTGTGCACCCAGGAGAGACTACAAGAGGTGGCTTACTTGTCACTGCTGAGCCCTCGCTT
1439   AATTTCAAGCACTTCCAGCGATGTTAGATGTTACGGAGCCTCTCCAGCATAACCCTGCCATCTTTCCAATT
1511   CAAAAGCTTTGCGCTGCTGCTGTTTAGGATTACTCCTAATTCAGTGTCACCTCTGGTGCTGCATGTCGTA
1583   TGTCTTTTCTATTGTGGCTGTGGTCTGTCACGTCTTCTTTGTAGTCCTGTGTTCTCATCCTGTTGCAGTT
1655   TCACAATAGATAATGGTTTGTGTTACACTGTAGAGATTAAGAGGCAATAAACTCTTTTGAAGGTG

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Fig. 1. Nucleotide sequence of *X. laevis* ENO1 cDNA and predicted amino acid sequence

The underlined sequences are complementary to the probes used in the RNAase protection experiments (Fig. 4), nucleotides 401–538 to probe 1 and nucleotides 1233–1519 to probe 2. Numbers on the left margin refer to the first nucleotide residue of each line.

a 2 × sample gel buffer and separated by electrophoresis on 12.5% polyacrylamide gels (Laemmli, 1970). Gels were transferred to nitrocellulose filters by using the Hoefer TE42 electrophoresis cell, and filters (two to ten lanes) were incubated in 20 ml of buffer with a 1:400 dilution of anti-(*X. laevis* ENO1) serum or 1:1000 dilutions of anti-(rat α -enolase) serum or anti-(rat γ -enolase) serum washed, incubated with 2×10^7 – 2.5×10^7 d.p.m. of 125 I-Protein A (New England Nuclear) in a volume of 20 ml for 1 h, washed, dried and placed under X-ray film for autoradiography for 24–48 h as described by Burnette (1981).

RESULTS

Identification of clone ENO1 as coding for enolase

Clone ENO1 (formerly 207A2 or 101F12) was derived from a cDNA library of stage 41 *X. laevis* tadpoles (Dworkin & Dawid, 1980). The complete nucleotide sequence of its cDNA insert was determined and is shown in Fig. 1. The cloned sequence spans 1720 nucleotide residues and contains an open reading frame coding for 434 amino acid residues, preceded by a 5' non-translated region of 34 nucleotide residues and followed by a 3' non-translated region of 384 nucleotide residues.

	1
<i>X. laevis</i> ENO1	MS I K N I R A R E I F D S R G N P T V E V D L Y T C K G L F R A A V P S G A S T G I Y E A L E L R D N D K T
Human α	-- L K - H - - - - - F - S - - - - -
Rat α	-- L K - H - - - - - A - - - - -
Rat γ	-- Q K - W - - - L - - - - - H - A - - - - - G - Q
Chicken β	-- Q K - H - - - L - - D - - - - H - A - - - - H - - - - G - K
Yeast ENO1	AVSKVY - SVY - - - - - E - T - E - V - S I - - - - - V H - - - M - G - S
Yeast ENO2	AVSKVY - SVY - - - - - E - T - E - V - S I - - - - - V H - - - M - E - S
	56
<i>X. laevis</i> ENO1	R Y L G K G V G R A V K Y V N E F L G P A L C T Q N L N V V E Q E K I D K L M I E M D G T E N K S K F G A N A
Human α	-- M - - - S K - - E H I - K T I A - - V S K K - - - T - - - - Q - - - -
Rat α	- F M - - - S K - - E H I - K T I A - - V S K K - - - - - Q - - - - -
Rat γ	- - - - - L K - - D H I - S T I A - - I S S G - S - - - L - N - - L - L - - -
Chicken β	- F - - - L K - - E H I - K T I - - - I E K K I S - - - - - V V - - - -
Yeast ENO1	K W M - - - L H - - - N - D V I A - - F V K A - I D - K D - K A V - D F L - S L - - - A - - - L - - -
Yeast ENO2	K W M - - - M N - - N N - - N V I A A - F V K A - - D - K D - K A V - D F L L S L - - - A - - - L - - -
	111
<i>X. laevis</i> ENO1	L L G V S L A V C K A G A A E K G V P L Y R H I A D L A G N * * P E V I L P V P A F N V I N G G S H A G N K L
Human α	I - - - - - V - - - - - * * S - - - - -
Rat α	I - - - - - V G - - - - - * * - - - T - - - - -
Rat γ	I - - - - - - - - - - D L - - - - - Q - - - - - * * S D L - - - - -
Chicken β	I - - - - - S H - - - - - - - - - - * * T - L - - - - -
Yeast ENO1	I - - - - - A S R - A - - - N - - - K - L - - - S K S K T S P Y V - - - F L - - L - - - - G A -
Yeast ENO2	I - - - - - M - A A R - A - - - N - - - Q - L - - - S K S K T S P Y V - - - F L - - L - - - - G A -
	166
<i>X. laevis</i> ENO1	A M Q E F M I L P V G A D S F K E A M R I G A E V Y H N L K N V I K E K Y G K D A T N V G D E G G F A P N I L
Human α	- - - - - A N - R - - - - -
Rat α	- - - - - S - R - - - - -
Rat γ	- - - - - E - R D - - L - - - - T - G - - D - - - -
Chicken β	- - - - - V - - - A - H D - - V - - - - S - G - - A - - - -
Yeast ENO1	- L - - - A - T - K T - A - L - - - S - - - - S L T - K R - - A S - G - - - V - - - Q
Yeast ENO2	- L - - - A - T - K T - A - - - - S - - - - S L T - K R - - A S - G - - - V - - - Q
	221
<i>X. laevis</i> ENO1	E N K E A L E L L K T A I N K A G Y P D K I V I G M D V A A S E F Y R D G K Y D L D F K S P D * D P S R Y I S
Human α	- - - G - - - - - G - - - - - T - - V - - - - - F - S - - - - -
Rat α	- - - - - S - - A - - - T - Q V - - - - - A - - - - - * - A - - - T
Rat γ	- S - - - - V - E - D - - - T E - M - - - - - A * - - - C - T
Chicken β	D - H - - - - A - A Q - - T - V - - - - - C - - R - - - - P * - K - L - T
Yeast ENO1	T A E - - - D - I V D - - K A - - H D G - V K - - L - C - S - - - F K - - - - - N - N S - K - K W L T
Yeast ENO2	T A E - - - D - I V D - - K A - - H D G - V K - - L - C - S - - - F K - - - - - N - N S - K - K W L T
	276
<i>X. laevis</i> ENO1	P D K L A E L Y M S F V K N Y P V V S I E D P F D Q D H W E A W T K F T A A S G I Q V V G D D L T V T N P K R
Human α	- - Q - - D - - K - - I - D - - - - - D - G - - Q - - S A - - - - -
Rat α	- - Q - - D - - K - - I - D - - - - - D - D - - Q - - T A - - - - -
Rat γ	G - Q - G A - - Q D - - R - - - - - D - A - - S - - N V - - I - - - -
Chicken β	G E Q - G - I - R G - I - D - - - - - D - - - K R - V S H V D - - - - A - - - -
Yeast ENO1	G P Q - - D - - H - L M - R - - I - - - - - A E - D - - - S H - F K T A - - - I - A - - - - -
Yeast ENO2	G V E - - D M - H - L M - R - - I - - - - - A E - D - - - S H - F K T A - - - I - A - - - - - A - - - -
	331
<i>X. laevis</i> ENO1	I A K A V E E K A C N C L L L K V N Q I G T V T E S L E A C K L A Q S N G W G V M V S H R S G E T E D T F I A
Human α	- - - N - - S - - - - - S - - - - - Q - - - - - A - - - - -
Rat α	- - - A G - - S - - - - - S - - - - - Q - - - - - E - - - - -
Rat γ	- E R - - - - - S - - - - - S - - A I Q - - - - - E - - - - -
Chicken β	- - H - A - Q H - - - - - G - - - - - S - - - - - I Q - - - - - H - - - - -
Yeast ENO1	- - T - I - K - - A D A - - - - - L S - - I K - A Q D S F A A - - - - -
Yeast ENO2	- - T - I - K - - A D A - - - - - L S - - I K - A Q D S F A A N - - - - -
	386
<i>X. laevis</i> ENO1	D L V V G L C T G Q I K T G A P C R S E R L A K Y N Q L L R I E E E L G S K A R F A G K N F R K P V F N
Human α	- - - - - - - - - - - - - - - - - K - - - R - - - N - L A K
Rat α	- - - - - - - - - - - - - - - - - K - - - R S - - - N - L A K
Rat γ	- - - - - - - - - - - - - - - - - M - - - - E E - - - H - - - N - S V L
Chicken β	- - - - - E Q - - - - - - - - - - M - - - - A - D - - K - - R K - - N - K A K
Yeast ENO1	- - - - - R - - - - - A - - - - - L - - - - - D N - V - - - E - - H H G D K L
Yeast ENO2	- - - - - R - - - - - A - - - - - L - - - - - D - - V Y - - - E - - H H G D K L

Fig. 2. Comparison of the predicted amino acid sequence of *X. laevis* ENO1 with other enolase sequences

Only the sequence of *X. laevis* ENO1 is given completely; in other enolases an amino acid is only indicated if it differs from the corresponding amino acid in *X. laevis* ENO1. The numbers above the first amino acid in each line refer to the position of the respective amino acid residue in the sequence. Amino acids 141, 142, and 268 are only present in yeast enolases; their absence from vertebrate enolases is indicated by an asterisk.

A putative polyadenylation signal AATAAA is located 14 nucleotide residues upstream of the 3' end of the cloned insert, suggesting that the clone contains the complete 3' non-coding sequence. Translation of the open reading frame sequence (Fig. 1) and computer comparison with the sequences in the Dayhoff protein data bank identified it as coding for the glycolytic enzyme enolase. Fig. 2 shows a comparison of *X. laevis* enolase (which we refer to in the present paper as

X. laevis ENO1) with other eukaryotic enolase sequences known to date. These are rat non-neuronal enolase (α -enolase; Sakimura *et al.*, 1985a), rat neuron-specific enolase (γ -enolase; Sakimura *et al.*, 1985b), human non-neuronal enolase (Giallongo *et al.*, 1986), chicken muscle enolase (β -enolase; Russell *et al.*, 1986) and two yeast enolases (yeast ENO1 and ENO2; Holland *et al.*, 1981). *X. laevis* ENO1 exhibits homology to all these enolase sequences (Table 1), ranging from 88% identity with

Table 1. Homology of amino acid sequences between *X. laevis* ENO1 and other enolases

Enolase	Percentage identity
Human α -enolase	88
Rat α -enolase	87
Rat γ -enolase	82
Chicken β -enolase	79
Yeast ENO1	62
Yeast ENO2	61

human α -enolase to 61 % identity with yeast ENO2. In a comparison with the other vertebrate enolases, *X. laevis* ENO1 shows least similarity to chicken β -enolase, and it is more similar to rat α -enolase than to rat γ -enolase. When only the 77 amino acid positions that differ between the two rat enolases are compared with the respective positions in *X. laevis* ENO1, it is found that ENO1 matches with rat α -enolase in 37 cases (48 %) and with rat γ -enolase in only 17 cases (22 %). Therefore *X. laevis* ENO1 may represent a homologue of the α subtype rather than of the neuron-restricted γ subtype.

Enolase isoenzymes in *X. laevis*

In mammalian and avian tissues three homodimeric enolase isoenzymes have been described (see the Introduction). Chicken and rat $\alpha\alpha$ - and $\gamma\gamma$ -enolase can be separated by DEAE-cellulose chromatography (Clark-Rosenberg & Marangos, 1980) as a result of the difference in the acidity of the two proteins (the pI of $\gamma\gamma$ -enolase is 4.7, and the pI of $\alpha\alpha$ -enolase is 5.9; Marangos *et al.*, 1978b). However, when adult frog (*Rana catesbeiana*) was analysed under these conditions, no peak as acidic as rat $\gamma\gamma$ -enolase was detected (Clark-Rosenberg & Marangos, 1980). Nevertheless, frog brain did give rise to several less acidic peaks with enolase activity.

To determine the isoenzyme distribution of enolase in *X. laevis*, tissue extracts were analysed by cellulose acetate electrophoresis and enolase isoenzymes were localized by their activity in a colorimetric assay (see the Materials and methods section). Fig. 3 shows the enolase isoenzyme profile in *X. laevis* oocytes, adult brain, adult liver and adult muscle in comparison with rat muscle, liver, and brain. The distribution of enolase isoenzymes in the rat tissues was as expected (Rider & Taylor, 1974; Oskam *et al.*, 1985) and consistent with the presence of an $\alpha\alpha$ form in liver, a mixture of $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$ forms in brain, and a $\beta\beta$ form in muscle. However, *X. laevis* oocytes, liver and brain unexpectedly all showed a pattern of three equidistant bands (bands 1–3 in Fig. 3). All three bands migrated close to the position of the rat $\alpha\alpha$ -enolase (compare lanes 3 and 4 in Fig. 3). No enolase isoenzyme as acidic as the rat $\gamma\gamma$ form is present in *X. laevis*, and no isoenzyme is restricted to brain.

The enolase isoenzyme distribution in adult *X. laevis* muscle displayed a pattern similar to that of rat muscle (Fig. 3, lanes 1 and 8) consisting of a major, relatively basic, species (band 5), a minor species (band 4) migrating between the main muscle isoenzyme and the isoenzymes from non-muscle tissues, and another very minor band migrating at the position of the most basic isoenzyme present in non-muscle tissues (band 3). Isoenzyme forms 4 and 5 are absent from oocytes (Fig. 3, lane 5) and from early embryos (blastulae, gastrulae and neurulae; results not shown). To determine whether the muscle-specific enolase isotype was already present in developing larvae, the enolase pattern was analysed in extracts of 3-day-old (stage 41) tadpoles (Fig. 3, lane 9). Five equidistant bands were observed, the upper three corresponding to the oocyte/liver/brain forms (bands 1–3), and the lower two corresponding to the muscle forms (bands 4 and 5). The muscle forms are presumably derived from the developing larval muscle tissue. Tail muscle of prometamorphic tadpoles (stage 55) yielded bands 4 and 5 (Fig. 3, lane 10) and, to a lesser extent, band 3 (hardly

Fig. 3. Enolase isoenzymes in *X. laevis* and in the rat

Tissue extracts from rat (lanes 1–3) and *X. laevis* (lanes 4–10) were separated by non-denaturing electrophoresis on cellulose acetate strips, and enolase activity was localized on the strips. Stage 41, total stage-41 (3-day old) tadpoles; stage 55 muscle, tail muscle of stage-55 (prometamorphic) tadpoles. For *X. laevis* extracts the amount of protein per lane was 0.75 μ g (oocyte VI), 0.65 μ g (brain), 12.5 μ g (liver) or 0.65 μ g (muscle). For other extracts protein amounts were not determined. Note that enolase concentrations are considerably lower in liver than in oocytes or in muscle. $\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, and $\alpha\gamma$ refer to rat isoenzymes; bands 1–5 refer to *X. laevis* isoenzymes. Electrophoresis was towards the anode; electrophoresis time for lanes 5–10 was longer than for lanes 1–4.

Fig. 4. Analysis of enolase mRNA from different tissues in RNAase protection experiments

RNAase protection experiments were carried out as described in the Materials and methods section, with RNA from the indicated tissues. The amounts of polyadenylated RNA per assay (lane) were approx. 0.3 μ g (oocyte I–VI), 0.5 μ g (oocyte I–II), 0.7 μ g (muscle) and 1.3 μ g (liver). Two different probes were used for protection: probe 1 spanning a coding region fragment and probe 2 overlapping the 3' portion of the coding region and the 3' non-coding region (see Fig. 1 for the exact positions of the probes). The amounts of RNAase used in the protection assays are indicated on top of each lane (1 $\times$, 2 $\times$ and 10 $\times$; 1 $\times$ = 2 μ g of RNAase A + 0.05 unit of RNAase T₁ per reaction volume of 330 μ l). Note that for probe 1 larger amounts of RNAase were required to digest unhybridized probe completely, and intermediate digestion products (e.g. the doublet of bands just above the protected fragment) can be seen. The calculated lengths of the protected fragments [nucleotide residues (nt), in parentheses] agreed with the actual migration of the fragments with respect to a pBR322/*Hpa*II marker (not shown). The results of two experiments are presented, one with oocyte I–VI RNA (an RNA preparation from mixed-stage oocytes), the other one with oocyte I–II, muscle and liver RNA. The exposure time shown for the oocyte I–II lanes is one-third of that for the muscle and liver lanes.

visible in Fig. 3). Thus the enolase isoenzyme characteristic of adult muscle (band 5) is already expressed early in larval development and increases in intensity relative to band 4 during development from the larval to the adult stage.

***X. laevis* ENO1 corresponds to non-muscle enolase**

The clone ENO1 had been derived from a cDNA library made from stage-41 tadpoles (Dworkin & Dawid, 1980). Since at that stage the major muscle-specific isoenzyme is already expressed (Fig. 3, lane 9), *X. laevis* ENO1 could correspond to any of the embryonic or muscle isoenzymes. To determine the expression of ENO1 mRNA in different adult tissues, RNAase protection experiments were carried out. Two RNA probes complementary to the coding region and to the 3' flanking region of *X. laevis* ENO1 mRNA were prepared by subcloning of the respective fragments of plasmid ENO1 in pSP 65 and pGEM 4 and synthesis of ³²P-labelled RNA *in vitro* (see the Materials and methods section). The exact positions of the two probes are

indicated in Fig. 1. Probe 1 covers a 138-nucleotide-residue fragment located in the first half of the enolase coding sequence, and probe 2 spans the junction between coding region and 3' non-coding region, containing 104 nucleotide residues of coding region and 183 nucleotide residues of non-coding region. Both RNA probes were synthesized such that they contained several hundred bases of plasmid sequence adjacent to the enolase sequence in order to permit clear identification of full-length protected fragments separate from undigested probe. RNA probes were hybridized to RNA preparations from muscle, liver and oocytes, then digested with RNAases A and T₁, and the protected fragments were separated by electrophoresis under denaturing conditions on a polyacrylamide gel. The results are shown in Fig. 4. Both probes yielded full-length protection with muscle, liver and oocyte RNA. No tissue-specific sub-bands indicative of a second cross-hybridizing enolase mRNA species forming imperfect hybrids with the probe were seen. In particular, no sub-band formed at a position around 104 nucleotide residues with probe 2, thus

Fig. 5. Northern-blot analysis (a) and Western-blot analysis (b and c) of enolase expression

(a) Total RNA from the indicated stages was denatured with glyoxal, separated on a 1.2% agarose gel, transferred to a Nytran membrane and hybridized with nick-translated plasmid DNA from cDNA clones ENO1 and 207H4 (Dworkin & Dworkin-Rastl, 1985). In oocyte VI and egg lanes 10 μ g of total RNA/lane was used; the amount of oocyte I-II RNA was adjusted as described in the Results section. (b) Approx. 20 μ g of protein from oocytes, eggs and embryos ranging from stage 2 (two-cell stage) to stage 23 (neurula) were analysed by immunoblotting with an anti-(*X. laevis* ENO1) polyclonal serum as described in the Materials and methods section. (c) Approx. 20 μ g of protein from early oocytes (oocyte I-II), 60 μ g of protein from adult liver, 3 μ g of protein from adult muscle and undetermined amounts of protein from *X. laevis* and chicken brain were analysed as in (b). The primary antibody used is indicated below the respective lanes.

excluding the presence of an enolase mRNA that matched well with ENO1 mRNA in the coding region but diverged in the 3' flanking region. RNA prepared from embryos at several stages of development up to the swimming tadpole stage was also tested with probe 1 and yielded complete protection (results not shown). Consistent with the results of the RNAase protection experiments, Northern-blot analysis of RNA from oocytes, liver, brain and muscle revealed a single hybridizing band of about 1900 nucleotide residues in each of these tissues (results not shown). Since only one enolase isoenzyme is expressed in common among oocyte, liver, brain and muscle, as assayed by cellulose acetate electrophoresis, namely the isoenzyme corresponding to band 3 (Fig. 3), we tentatively conclude that the cloned sequence ENO1 codes for the enolase isoenzyme represented by band 3.

Enolase mRNA and protein concentrations during oogenesis and early development

X. laevis ENO1 mRNA is one of the most abundant mRNAs in early (stage-I-II) oocytes (N. Segil, A. Shrutkowski, M. B. Dworkin & E. Dworkin-Rastl, unpublished work). Most maternal mRNAs accumulate during the early stages of oogenesis and then do not change in titre during the growth phase of oogenesis (Golden *et al.*, 1980). ENO1 mRNA is among a small group of oogenic messages that decrease in titre during oogenesis (Dworkin & Dworkin-Rastl, 1985; see also Picard *et al.*, 1986). A Northern blot comparing the concentrations of ENO1 mRNA in stage-I-II oocytes, full-grown (stage-VI) oocytes and eggs (i.e. after oocyte maturation) is shown in Fig. 5a). Since a substantial

amount of mRNA in early oocytes is not polyadenylated, total RNA was used in this experiment. Because the amount of rRNA increases significantly during oocyte growth while mRNA concentrations stay relatively constant, the amount of RNA used to assay ENO1 mRNA concentrations in stage-I-II oocytes and in stage-VI oocytes was adjusted to yield equal hybridization signals for a different sequence, 207H4 (Dworkin & Dworkin-Rastl, 1985). This sequence is typical of the majority of moderately abundant sequences whose titres remain relatively constant during this period, as revealed by DNA dot-blot hybridization, with the use of radiolabelled cDNA as a probe (a method independent of rRNA concentrations). Equal amounts of total RNA were used in comparing stage-VI oocytes and eggs (matured oocytes). The result shows a severalfold decrease in *X. laevis* ENO1 mRNA concentration during oogenesis and no change during oocyte maturation. Further decreases in the concentration of ENO1 mRNA occur after fertilization (Dworkin *et al.*, 1985; M. B. Dworkin, A. Shrutkowski & E. Dworkin-Rastl, unpublished work).

Although ENO1 mRNA concentrations decrease during oogenesis and early embryonic development, enolase enzyme activity does not decrease. Rather, it increases 2–3-fold during oogenesis and then stays constant up to at least the blastula stage (Dworkin *et al.*, 1987). Since the enolase activity measured is a composite of the activities of all isoenzymes expressed in a tissue, this result could indicate a decrease in the amount of ENO1 that is not paralleled by the other isoenzymes. This possibility, however, is unlikely on the basis of the cellulose acetate electrophoresis; all three isoenzymes expressed in early oocytes continue to be expressed in the

same proportion in full-grown oocytes and early embryos (Fig. 3, lanes 4 and 5, and results not shown).

To measure enolase protein concentrations directly, polyclonal antibodies were prepared in rabbits against a β -galactosidase-*X. laevis* ENO1 fusion protein (see the Materials and methods section). The antiserum obtained did not exhibit tissue specificity, and in protein blots of *X. laevis* oocytes, liver, muscle and brain it reacted with a single band of the size expected for enolase (Fig. 5c). The antiserum also reacted with both chicken α -enolase and γ -enolase, which migrate slightly differently on electrophoresis on SDS/polyacrylamide gels. For comparison, an anti-(rat α -enolase) serum recognized only chicken α -enolase, whereas an anti-(rat γ -enolase) serum recognized both chicken α -enolase and γ -enolase but reacted more strongly to the latter isoenzyme (Fig. 5c). Both these antisera, however, reacted with all *X. laevis* tissues (results not shown). Immunologically reactive *X. laevis* enolases, in contrast with chicken enolases, did not exhibit size differences on electrophoresis on SDS/polyacrylamide gels.

Anti-(*X. laevis* ENO1) serum was used to quantify enolase protein concentrations during oogenesis and early embryonic development. We do not know the relative affinities of the anti-(*X. laevis* ENO1) serum to enolase isoenzyme bands 1, 2 and 3 (Fig. 3). However, since the relative proportions of these isoenzymes do not change during early development (results not shown), concentrations of immunoreactive enolase represent a constant proportion of total enolase concentrations. Equal amounts of total protein from early, intermediate-stage and full-grown oocytes, as well as from unfertilized eggs, cleavage, blastula, gastrula and neurula stages, were separated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose and treated with the antiserum (Fig. 5b). Enolase protein concentrations increased throughout oocyte growth and by the end of oogenesis reached a plateau level that was maintained at least until neurulation. The protein concentrations thus correlate with enolase enzyme activity. The high mRNA titre in early oocytes presumably is required to establish the necessary protein concentration, but is not needed for its maintenance.

DISCUSSION

In the present paper we have reported the isolation of a cDNA clone for *Xenopus laevis* enolase. Enolase isoenzymes in *X. laevis* showed a pattern distinct from that found in birds and mammals. At least five isoenzymes were distinguished, three of which (bands 1–3) are co-expressed in oocytes, embryos, adult liver and adult brain. No brain-specific form, and no isoenzyme as acidic as avian or mammalian $\gamma\gamma$ -enolase, was observed. A single enolase isoenzyme (band 5), comparable with the muscle-specific isoenzyme in rat, is the predominant form expressed in *X. laevis* muscle. The muscle-specific enolase isoenzyme is not inherited maternally. The non-tissue-specific enolase band 3 also persists in adult muscle, but only at a concentration much below that of the main muscle-specific band-5 isoenzyme. The muscle-specific band-4 isoenzyme may be a heterodimer between band-3 and band-5 monomers (see below).

Since enolase is a dimer, three isoenzyme forms (two homodimers and one heterodimer) can potentially be

formed from two different monomers. The three enolase bands seen in *X. laevis* non-muscle tissues are equally spaced after electrophoresis on cellulose acetate and could represent the homodimer and heterodimer forms derived from two different gene products. Likewise, the three isoenzymes expressed in *X. laevis* muscle could be the result of co-expression of two genes. In rat brain extracts the enolase isoenzyme of intermediate pI (Fig. 3, lane 3) is a heterodimer consisting of both an α - and a γ -subunit (Fletcher *et al.*, 1976). It has been argued that in adult brain α - and γ -subunits are not co-expressed in the same cell and that the $\alpha\gamma$ -heterodimer is an artifact formed during the preparation of tissue extracts (Jørgensen & Centervall, 1982; Marangos *et al.*, 1978a). Since *X. laevis* stage-VI oocytes are a 'single-cell type' (after removal of follicle cells), a heterodimer might, in this case, exist *in vivo*. Two alternative explanations for the multiple enzyme forms detected by cellulose acetate electrophoresis are either the expression of more than three enolase genes or post-translational modification of some enolase molecules. Preliminary experiments show that phosphatase treatment of *X. laevis* tissue extracts before electrophoresis does not change the migration pattern of the enolase isoenzymes. Therefore the isoenzyme pattern is not likely to be generated by phosphorylation. By analogy to the avian and mammalian systems the expression of different enolase isoenzyme forms can most probably be attributed to the existence of multiple genes.

The cloned enolase sequence *X. laevis* ENO1 has tentatively been identified as corresponding to the gene encoding the subunit of the cellulose acetate electrophoretic band 3 (Fig. 3). This is one of the isoenzymes expressed in stage-41 tadpoles, the source of the mRNA used to construct the cDNA library from which the enolase clone was isolated. The band-3 isoenzyme form of enolase is the only form present in all tissues, within the limits of sensitivity of the assay. RNAse protection experiments (Fig. 4) demonstrated that ENO1 mRNA is present in all tissues tested, although it is much more abundant in oocytes than in muscle and liver. There is little or no cross-hybridization of the ENO1 gene to other *X. laevis* enolase genes. Sequence analysis of *X. laevis* ENO1 shows that it is considerably more similar to rat α -enolase than to rat γ -enolase or to chicken β -enolase, and it probably represents the *X. laevis* homologue of α -enolase.

The likely co-expression of at least two enolase genes, giving rise to bands 1, 2 and 3, in *X. laevis* liver, is different from the expression of a single gene (α -enolase) in the liver of birds and mammals. At least two scenarios are possible for the origin of two co-expressed *X. laevis* genes. The first possibility is that a duplication of a primordial α -like enolase gene occurred in a common ancestor of amphibians, birds and mammals, and that in birds and mammals one of the two genes evolved neuron-specific expression subsequent to the divergence of these phylogenetic classes from amphibians. Alternatively, the second gene could have been generated by a genome duplication that occurred during the evolution of the genus *Xenopus* (Thiébaud & Fischberg, 1977) and could thus be restricted to *X. laevis* and its closest relatives. Curiously, the ENO1 gene is among the most polymorphic genes in *X. laevis* (described as clone F12 in Okada *et al.*, 1985). Analysis of the enolase isoenzyme pattern in other amphibians and in reptiles should reveal

the time in evolution when the enolase gene duplication occurred.

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