ate and washed exhaustively with distilled water. The material eluting in the void volume was collected and stored overnight at 4°C. TFA was added dropwise to the stored eluate to a final concentration of 0.2% (v/v); the solution was then clarified by centrifugation at 20,000*g* for 5 min at room temperature. The supernatant was loaded onto a reversed-phase flash column (C18, 40 μm, 3 by 25 cm) previously equilibrated with aqueous 0.1% TFA. The column was eluted with the use of compressed nitrogen at 8 psi. After the sample was loaded, the column was washed with 200 ml 0.1% TFA; the retained material was then eluted with successive washes of 20, 40, and 60% methanol in 0.1% TFA. The methanol was removed with a rotary evaporator and the remaining liquid was frozen and lyophilized. Two kilograms of leaf material yielded about 1 g of crude material containing the active factor. The procedure was repeated 15 times. Samples (approximately 4 g) of crude material dissolved in 20 ml water and adjusted to pH 7.8 with 10 M ammonium hydroxide were loaded onto a G25 Sephadex column (4 by 44 cm) that was equilibrated with 50 mM ammonium bicarbonate, pH 7.8. The material eluting at and just after the void volume was recovered and lyophilized. Four identical runs through the entire procedure produced 1.25 g of partially purified factor. The 1.25 g was dissolved in 500 ml $H_2O$, the pH was adjusted to 6 with 1 M NaOH, and the sample was applied to a CM Sephadex column (2 by 17 cm) and washed with 0.01 M potassium phosphate, pH 6. The activity was retained by the CM Sephadex, eluted with 250 mM ammonium bicarbonate, and lyophilized. The yield was 190 mg.

33. The material was injected, one-fifth at a time, into a semi-preparative reversed-phase C18 column (Vydac, Hesperia, CA, Column 218 TP510, 10 by 250 mm, 5-μm beads, 300 Å pores). Solvent A consisted of 0.1% TFA in water. Solvent B consisted of 0.1% TFA in acetonitrile. Samples were injected in solvent A and, after 2 min, a 90 min gradient to 30% solvent B was begun for elution. The flow rate was 2 ml/min and eluted peaks were monitored at 225 nm. Several peaks of activity were found. The major peak of activity resided in tubes 43 to 46, which were pooled and lyophilized. Total protein content of the pooled fractions was estimated at 2.5 mg.
34. Chromatography was performed on a poly-SULFOETHYL Aspartamide column (4.6 by 200 mm, 5 μM, The Nest Group, Southborough, MA) with the use of the following solvent systems: Solvent A, 5 mM potassium phosphate, pH 3, in 25% acetronitrile; solvent B, 5 mM potassium phosphate, 500 mM potassium chloride in 25% acetonitrile, pH 3. The sample was dissolved in 2 ml of solvent A, filtered, and applied to the column. After a 5-min wash with solvent A, a 60-min gradient to 50% B was applied. The flow rate was 1 ml/min, and the elution profile was monitored by absorbance at 210 nm. The active fractions, 35 to 38, were pooled and reduced in volume to 1 ml by vacuum centrifugation.
35. Chromatography was performed on a Beckman Ultrasphere Ion pair column (4.6 by 250 mm, C18, 5 μm). Solvent A was 10 mM potassium phosphate, pH 6, and solvent B was 10 mM potassium phosphate, pH 6, containing 50% acetronitrile. The active fractions, 39 to 42, were pooled and vacuum centrifuged to a final volume of 1 ml. This fraction was applied to the same column as the previous run but under the solvent and gradient conditions of step 2. The sample was adjusted to pH 3 with TFA, filtered through a 0.45-μm syringe filter and chromatographed at a flow rate of 1 ml/min. The peaks were detected at 212 nm. The fractions containing the activity, eluting at 53.5 to 56.5 min, were pooled and vacuum centrifuged to a volume of 1 ml.
36. The column was run at 0% B for 5 min at which time a gradient to 30% B in 120 min was started. The profile was detected by absorbance at 210 nm. Fractions eluting at 76 to 78.5 min were pooled and vacuum centrifuged to reduce the volume to 1 ml.
37. A 60-min gradient to 30% solvent B was employed.
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# Evidence of Lactate Dehydrogenase-B Allozyme Effects in the Teleost, *Fundulus heteroclitus*

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**The evolutionary significance of protein polymorphisms has long been debated. Exponents of the balanced theory advocate that selection operates to maintain polymorphisms, whereas the neoclassical school argues that most genetic variation is neutral. Some studies have suggested that protein polymorphisms are not neutral, but their significance has been questioned because one cannot eliminate the possibility that linked loci were responsible for the observed differences. Evidence is presented that an enzymatic phenotype can affect carbon flow through a metabolic pathway. Glucose flux differences between lactate dehydrogenase-B phenotypes of *Fundulus heteroclitus* were reversed by substituting the *Ldh-B* gene product of one homozygous genotype with that of another.**

THE DEBATE OVER THE EVOLUTIONARY significance of protein polymorphisms has continued for over 20 years without resolution (*1, 2*). Yet, many of the recent advances in evolutionary biology depend on the implicit assumption that the majority of this genetic variation is selectively neutral. Arguments for the neutral theory are largely derived from the theoretical considerations of population genetics (*2*). They are bolstered by the view, advanced by Newsholme and Start (*3*), that metabolism is controlled by a few key regulatory or rate-limiting enzymes in a pathway, whereas other enzymes such as lactate dehydrogenase (LDH) participate in reactions that are near equilibrium and therefore of little or no importance in flux generation or metabolic control (*3*). Kascer and Burns (*4*) suggested that the control of metabolic rate is more generally shared among enzymes, including the so-called "equilibrium" enzymes like LDH. Because the neutralist hypothesis implies that polymorphic enzymes (allozymes) are functionally equivalent, some investigators have sought to resolve the controversy by examining the biochemistry of allozymes, while others have concentrated on life history correlates (*5–9*). Although these exciting studies present convincing evidence that one or more polymorphic loci are correlated with physiological or other differences between genotypes, they cannot eliminate the possibility that some unknown locus is actually responsible for the differences observed. Thus, the debate remains an important evolutionary question. We addressed this question by examining the metabolism of mummichog, *Fundulus heteroclitus* (Teleostei, Cyprinodontidae), embryos in which the native LDH-B isozyme was directly replaced with heterologous enzymes.

In the mummichog, LDH is a multilocus system that produces three gene products (LDH-$A_4$, LDH-$B_4$, and LDH-$C_4$): the *Ldh-A* locus is expressed in white muscle, *Ldh-B* in a variety of tissues including oocytes, and *Ldh-C* in eye and some nerve tissue (*10*). The *Ldh-B* locus has two major alleles, *Ldh-B*$^a$ and *Ldh-B*$^b$, that vary clinally over the geographical range of the species (*11*). The allelic isozymes (allozymes) encoded by this locus have been purified and kinetically characterized (*12–14*). Differences have been observed in product inhibition and reaction velocities at low substrate concentrations (*14*). Within a local population, *Ldh-B*$^a$ homozygotes develop faster than *Ldh-B*$^b$ homozygotes (*15*). This developmental variation has been correlated with differences in oxygen consumption (*16*), lactate and glucose utilization (*17*), and survival, that is, Darwinian fitness (*6*).

Recently, we have shown that mummichog eggs contain 40 to 50 mM lactate at the time of fertilization and that a major function of LDH-B in early development is oxidation of the lactate pool (*17*). Specific activity of LDH-B is similar between genotypes ($1.2 \times 10^{-3}$ IU per egg), but lactate concentrations found in newly fertilized eggs are correlated with LDH-B phenotype and are essentially equal to the inhibition constant of the purified allozymes of LDH-B: 51.8 ± 5.9 mM for LDH-$B_4^a$ and 36.6 ± 3.5 mM for LDH-$B_4^b$ (*14*). Thus, in vivo, the *Ldh-B*$^a$ homozygote accumulates more lactate and uses it at a higher rate. The *Ldh-B*$^a$ homozygote also uses glucose at a higher rate even though the glucose pool size is the same for both phenotypes. Since the LDH reaction and glycolysis are both functioning to produce pyruvate in mummichog embryos during the first 24 hours, it is difficult to explain how the two LDH-B allozymes could differentially affect glucose utilization.

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The ideal way to determine whether genetic variation at a particular locus directly affects a biochemical or metabolic function would be to study organisms that only vary at that locus. The construction of such strains can take years or decades and is not always feasible. An alternate experiment would be to change an organism's enzyme phenotype by exchanging its allozyme with that of a different genotype.

Klyachko *et al.* (*18*) successfully inserted exogenous LDH in loach blastomeres by microinjection into the yolk shortly after fertilization. These experiments showed that LDH was disproportionately accumulated in the blastomeres through binding of the enzyme to some cellular component and that the foreign protein competes with the native enzyme for binding sites. We hypothesized that it is possible to quantitatively replace an embryo's native LDH-$B_4$ (the only LDH isozyme present in eggs) with exogenous enzyme by injecting an excess of foreign protein and then, after an incubation time to allow the enzyme to distribute itself, wash out the yolk and excess LDH. We tested this hypothesis by attempting to create blastulas whose native LDH content was replaced by either the LDH-$B_4$ allozyme of a different genotype or one of two porcine LDH isozymes. Our results generally confirmed those of Klyachko *et al.* (*18*). The blastulas from injected eggs contain about 62% of the LDH originally present in oocytes; the same amount as in uninjected or saline injected blastulas (Table 1). Electrophoretic analysis showed that the LDH in the blastulas was of the type injected, whereas no detectable amounts of the native enzyme remained. Because it is possible to quantitatively replace an embryo's native LDH-B with foreign enzymes of different kinetic or structural properties, we used these preparations to test whether it is the physical LDH type present in the blastula that influences glucose utilization or some other factor.

Blastulas were prepared as above, but $^{14}$C-6-glucose (specific activity, 0.2 μCi/μmol) was added to the washing buffer. Ten embryos were sealed in 1.2-ml tubes without additional buffer. A KOH trap was affixed to the tube cap to collect $CO_2$ given off by the eggs. These were incubated at 20°C for an additional 12 hours. The tubes were then acidified with trichloroacetic acid and equilibrated for 1 hour. The traps were then removed and counted. The data in Table 2 show that the actual LDH-$B_4$ homotetramer incorporated into the blastula affected glucose utilization rates. Genotypes injected with their own enzyme used glucose in a pattern similar to whole eggs; that is, the *Ldh-B*$^a$ homozygote used glucose at a higher rate (*17*). However, when a genotype's allozyme was replaced by the alternate allozyme, glucose was used at a rate determined by the injected allozyme rather than by the genotype.

These results indicate that the genetic differences between LDH-B allozymes can alter glycolytic flux by approximately 30% (Table 2). We hypothesized that the substitution of other, genetically more divergent LDH isozymes, might have an even greater influence. Therefore, we replaced the native enzyme of *Ldh-B*$^a$ homozygotes with two porcine LDH isozymes. Porcine heart type LDH is physiologically optimized to oxidize lactate to pyruvate. In this regard it is more like mummichog LDH-$B_4^b$ than mummichog LDH-$B_4^a$ (*12*), and its effect on the *Ldh-B*$^a$ homozygote was in the same direction and of a greater magnitude than mummichog LDH-$B_4^b$ (Table 3). Conversely, the physiological function of the porcine muscle type LDH is the reduction of pyruvate to lactate. Its effect on the *Ldh-B*$^a$ homozygote was to increase glucose utilization. Thus, the results of the porcine LDH replacement studies are consistent with and reinforce the native allozyme replacement studies.

The mechanism by which LDH influences glucose metabolism in mummichog embryos is not clear. Since both the LDH reaction and glycolysis are functioning to produce pyruvate and the phenotype that is using glucose faster is also using lactate faster (*17*), it is unlikely that feedback inhibition, competition for nicotinamide adenine dinucleotide (NAD), or some similar mechanism is responsible for the observed differences. Srivastra and Bernhard and their colleagues (*19*) have shown that enzyme-enzyme interactions, including those between LDH and other glycolytic enzymes, have significant effects on the kinetics of

**Table 1.** Activity of LDH in isolated blastulas after microinjection of exogenous enzyme. Ripe adults were captured near Shady Side, Maryland, and genotyped for LDH-B. Pooled gametes were crossed to produce either *Ldh-B*$^a$ or *Ldh-B*$^b$ homozygotes with a common genetic background (*15, 16, 21*), except the controls, which were based on crosses of mixed genotype. The eggs were then microinjected with $1.2 \times 10^{-2}$ IU of LDH (approximately 10× the native enzyme concentration) purified from either mummichog liver or from porcine heart or muscle (Sigma). The eggs were incubated at 20°C for 6 hours. The yolk sac was then pierced with two needles. Yolk sac contents were withdrawn from one needle and 10 to 15 egg volumes (40 to 60 μl) of a buffer (tris-HCl, pH 7.0; 40 mM lactate; 5 mM glucose) was injected through the other. This procedure effectively washed out the entire contents of the yolk sac while leaving it inflated. The buffer lactate and glucose concentrations mimicked whole egg concentrations (*15*). Thus, the experimental preparation consisted of a developing blastula resting on a nutrient-enriched buffer-filled sac. These preparations were viable for at least 24 hours and appeared to blastulate normally. However, they did not gastrulate. The experiments were conducted on four occasions to obtain three replicates of each treatment (a sufficient number of preparations for all eight treatments could not be prepared each time). The SAS general linear models procedure (*22*) was used to correct small differences in enzyme concentration between replications (significant at $P > 0.001$). There were no differences between treatment ($F7,13) = 1.46$; $P = 0.26$). LDH activity was determined by the decrease in absorbance of NADH at 25°C under saturating conditions of pyruvate and NADH (*14*). Specific activities of the enzymes injected were: 425 IU/mg protein for both *Fundulus* allozymes; 386 IU/mg protein for the porcine heart LDH; and 450 IU/mg protein for the porcine muscle LDH. At the activity levels accumulated in the blastulas, differences in apparent enzyme concentration due to the small activity differences would be below detection limits.

| Injected enzyme | Phenotype injected | LDH activity* (IU × $10^{-3}$/egg) |
|---|---|---|
| None | | 0.72 ± 0.03 |
| Saline | | 0.81 ± 0.03 |
| Porcine (heart) | LDH-$B^aB^a$ | 0.76 ± 0.03 |
| Porcine (muscle) | LDH-$B^aB^a$ | 0.79 ± 0.03 |
| LDH-$B_4^a$ | LDH-$B^aB^a$ | 0.83 ± 0.03 |
| LDH-$B_4^b$ | LDH-$B^aB^a$ | 0.78 ± 0.03 |
| LDH-$B_4^a$ | LDH-$B^bB^b$ | 0.72 ± 0.03 |
| LDH-$B_4^b$ | LDH-$B^bB^b$ | 0.78 ± 0.03 |

*Data are the means (± SEM) of three pools of ten eggs each.

**Table 2.** Glucose utilization rates (μmol × $10^{-8}$ $s^{-1}$ per egg) of injected eggs. Data are means (± SD) of three pools of ten eggs each. Saline-injected controls were not significantly different from phenotypes injected with their own allozyme; *Ldh-B*$^a$ and *Ldh-B*$^b$ homozygotes injected with the same allozyme were not different from each other; phenotypes injected with LDH-$B_4^a$ were significantly different from those injected with LDH-$B_4^b$ (ANOVA, $P < 0.005$).

| Phenotype | Injected enzyme | | |
|---|---|---|---|
| | Saline | LDH-$B_4^a$ | LDH-$B_4^b$ |
| LDH-$B^aB^a$ | 6.6 ± 0.5 | 7.1 ± 0.9 | 5.1 ± 0.4 |
| LDH-$B^bB^b$ | 4.3 ± 0.8 | 6.3 ± 0.8 | 4.4 ± 0.5 |

**Table 3.** Glucose utilization rates (μmol × $10^{-8}$ $s^{-1}$ per egg) of injected eggs. Data are means (± SD) of three pools of ten eggs each. All means are significantly different from each other (ANOVA, $P < 0.001$).

| Original phenotype | Injected enzyme | | |
|---|---|---|---|
| | Porcine (heart) | LDH-$B_4^a$ | Porcine (muscle) |
| LDH-$B^aB^a$ | 3.2 ± 0.4 | 7.1 ± 0.9 | 14.7 ± 0.5 |

enzyme reactions and consequently metabolic flux. They suggested that an important aspect of enzyme evolution may be related to changes in protein structure that alter metabolite transfer within a pathway. Perhaps genetic variation in the LDH-B isozyme of the mummichog affects its association with other glycolytic enzymes and thereby affects the rate of glucose use.

Evidence from a variety of other animals and enzyme systems has also indicated that allozyme variation is associated with metabolic and organismal level differences that may affect fitness (*5–17, 20*). However, our experiments suggest that the organismal differences correlated with the *Ldh-B* polymorphism in the mummichog are causally linked. If this is not a unique observation, allozymic variation may be important in the evolution of species.


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# Related RNA Polymerase–Binding Regions in Human RAP30/74 and *Escherichia coli* σ70


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**RAP30/74 is a heteromeric general transcription initiation factor that binds to mammalian RNA polymerase II. The RAP30 subunit contains a region that is similar in amino acid sequence to the RNA polymerase–binding domain of the *Escherichia coli* transcription initiation factor sigma 70 (σ70). Mammalian RNA polymerase II specifically protected a serine residue in the σ70-related region of RAP30 from phosphorylation in vitro. In addition, human RAP30/74 bound to *Escherichia coli* RNA polymerase and was displaced by σ70. These results suggest that RAP30 and σ70 have functionally related RNA polymerase–binding regions.**


RNA POLYMERASE II REQUIRES several general factors for initiation of transcription at the promoters of protein-encoding genes (*1*). Although many of these factors have been purified extensively, the functions of most of them in the initiation of transcription are not well characterized. RAP30/74 (RNA polymerase–associated protein), also known as TFIIF (*2*), is a heterodimeric general transcription initiation factor that binds to eukaryotic RNA polymerase II (*3, 4*). An adenosine triphosphate (ATP)–dependent DNA helicase activity that associates with RAP30/74 may be related to its function (*5*). The amino acid sequence of RAP30 deduced from its cDNA (*5*) shows that RAP30 contains a region with sequence similarity to a domain of the bacterial transcription initiation factor σ70. This domain of σ70 is conserved throughout the σ factor family and is required for the binding of σ70 to RNA polymerase (*6–8*). The structural similarity between RAP30 and σ70 suggests that RAP30 could contain an RNA polymerase–binding region that is functionally related to that of σ70. The demonstration of functional similarity between RAP30/74 and σ70 would suggest the use of the well-characterized prokaryotic transcription initiation system as a paradigm for initiation factor function in higher organisms.

In order to identify a region of RAP30/74 that is associated with RNA polymerase II, we used the catalytic subunit of cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (pKA) as a reagent to modify RAP30/74 and assessed the ability of RNA polymerase II to protect RAP30/74 from phosphorylation by pKA (*9*) (Fig. 1). RAP30/74 that had been purified by RNA polymerase II affinity chromatography (*3*) was phosphorylated in vitro with pKA. The RAP74 subunit was phosphorylated ten times as efficiently as RAP30. RAP30 was then immunoprecipitated after denaturation of the RAP30/74 heterodimer (Fig. 1A, lane 5). Excess RNA polymerase II in the reaction blocked phosphorylation of RAP30 (lane 6). RNA polymerase II did not affect phosphorylation of

**Table 1.** Approximate molar dissociation constants for RAP30 and σ70 binding to RNA polymerase.

| Protein | *E. coli* RNA polymerase | Mammalian RNA polymerase II |
|---|---|---|
| σ70 | $<3 \times 10^{-10}$* | $>10^{-6}$ † |
| RAP30 | $<3 \times 10^{-8}$ ‡ | $2 \times 10^{-8}$ § |

*In (*28*). †Estimated from the partial cosedimentation of σ70 with $4 \times 10^{-7}$ M RNA polymerase II (Fig. 2F). ‡Estimated from the complete cosedimentation of RAP30/74 with $4 \times 10^{-7}$ M RNA polymerase (Fig. 2C) and the poor displacement of RAP30/74 from RNA polymerase by NusA. §In (*3*).


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