

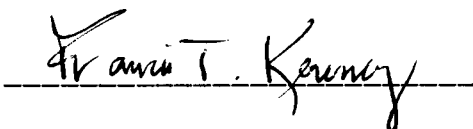
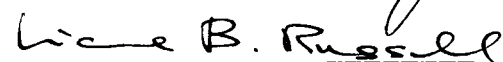
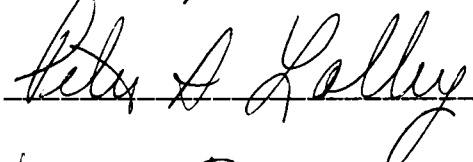
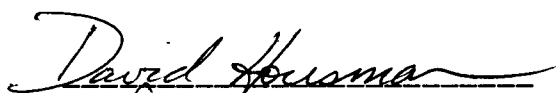
To the Graduate Council:

I am submitting herewith a thesis written by Jane E. Mendel entitled "Molecular and Genetic Analysis of the Gene Encoding the A Subunit of Lactate Dehydrogenase in the Mouse." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.



Edward G. Bernstine,
Major Professor

We have read this thesis and
recommend its acceptance:



Accepted for the Council:



Vice Provost and Dean of
the Graduate School

MOLECULAR AND GENETIC ANALYSIS OF THE GENE ENCODING
THE A SUBUNIT OF LACTATE DEHYDROGENASE
IN THE MOUSE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jane E. Mendel
August 1987

DEDICATION

This thesis is dedicated to my mother, Montrue Worthington Mendel. Through her example, I learned the value of education, and also the courage to pursue a long-term goal.

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ABSTRACT

In higher vertebrates, the genes encoding the A and B subunits of lactate dehydrogenase (E.C.1.1.1.27, LDH) show complex patterns of developmental and tissue-specific regulation.

In order to determine whether the genes encoding LDH in mammals are controlled by feedback regulation, a mouse stock, 46DFiOD, having a recessive lethal deletion of *Ldh-1*, the mouse gene encoding the LDH-A subunit, was identified in a screen of several presumptive deletion carriers affected at a closely linked marker, *p*, on mouse chromosome 7. Analysis of LDH activity in tissues of this stock provided no evidence for feedback regulation of mouse LDH genes.

A molecular analysis of *Ldh-1* was initiated by using a partial LDH-A cDNA, obtained from Dr. Kenneth Fong, to isolate full-length LDH-A cDNA and genomic clones.

Cloning of expressed LDH-A genomic sequences was complicated by the presence of several LDH-A pseudogenes. A 14 kilobase pair (Kb) EcoR1 restriction fragment having homology to mouse LDH-A cDNA was localized to mouse chromosome 7 using Southern analysis of DNA from a somatic-cell hybrid mapping panel. Southern analysis of DNA from offspring of a cross between 46DFiOD, the *Ldh-1* deletion stock, and *Mus spretus*, a species having a polymorphism for the 14 Kb restriction fragment was used to further localize this fragment to the chromosomal region containing *Ldh-1*. Genomic clones containing that fragment were

shown to direct the synthesis of mouse LDH-A following transfection into Chinese hamster cells.

Sequence analysis of a portion of the LDH-A genomic clone showed that *Ldh-1* has a promoter region that is G-C rich, lacks TATAA and CAAT boxes, and contains consensus sequences for Sp1 binding and cAMP induction.

The 46DFiOD deletion was also characterized for the involvement of nearby markers using the 46DFiOD x *Mus spretus* offspring. *Xld-1* and *LH β* are not included in the chromosomal segment missing in 46DFiOD, while the *Saa* gene complex, and 24.2, a probe from a syntenic region of human chromosome 11, are included in that segment.

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LIST OF SYMBOLS

Symbol

LDH-A	The A subunit (polypeptide) of lactate dehydrogenase.
LDH-B	The B subunit of lactate dehydrogenase.
LDH-C	The C subunit of lactate dehydrogenase.
LDH-5	The LDH isozyme composed of four A subunits.
LDH-4	The A ₃ B isozyme of LDH.
LDH-3	The A ₂ B ₂ isozyme of LDH.
LDH-2	The AB ₃ isozyme of LDH.
LDH-1	The LDH isozyme composed of four B subunits.
LDH-X	The LDH isozyme composed of four C subunits.
<i>Ldh-a</i>	A gene encoding the A subunit of LDH.
<i>Ldh-b</i>	A gene encoding the B subunit of LDH.
<i>Ldh-c</i>	A gene encoding the C subunit of LDH.
<i>Ldh-1</i>	The gene encoding the A subunit of LDH in the mouse.
<i>Ldh-2</i>	The gene encoding the B subunit of LDH in the mouse.
<i>LDHA</i>	The gene encoding the A subunit of LDH in humans.
<i>LDHB</i>	The gene encoding the B subunit of LDH in humans.
<i>Ldr-1</i>	A mouse gene controlling the expression of LDH isozymes in erythrocytes.
<i>Ldr-2</i>	A mouse gene controlling the expression of LDH isozymes in fetal liver.

Chapter I

INTRODUCTION

Gene duplication has played a prominent role in the evolution of vertebrates. The advantages of gene duplication for diploid organisms are obvious: one copy of a gene can continue to fulfill its original function while the other is free to diverge to form a new gene. In fact, it has been suggested that new genes can arise only by gene duplication (Ohno, 1970), and that all known vertebrate genes arose through the duplication and subsequent divergence of a small number of ancestral genes. The number of ancestral genes may be as small as 500-1000 (Dayhoff, 1975; Zuckerkandl, 1975). The evolutionary relationships of many genes have been determined by protein and deoxyribonucleic acid (DNA) sequencing, and many genes are now known to be members of multigene families. The genes encoding vertebrate globins, immunoglobins, ribosomal ribonucleic acids (RNAs), histones, serine proteases and several dehydrogenases are members of multigene families, each arising through duplications of a single ancestral gene (Markert et al., 1975; Li, 1982).

Divergence of duplicated genes has led to differences in the structure and function of encoded proteins, and also, in some multigene families, to differences in gene regulation. The molecular and genetic analysis of duplicated genes, therefore, may

provide important information concerning the evolutionary changes that affect not only protein structure and function but also gene regulation. Multigene families such as multi-locus isozyme systems, in which the structure and function of the encoded proteins remains similar but the level of expression of the those proteins varies in specific and measurable ways, may be particularly informative in terms of gene regulation. Not only can the end result of gene regulation, the phenotypic realization of a protein, be readily quantitated, but the homology of the encoded proteins can be used to determine the degree of gene relatedness, providing a framework for the assessment of conservation or divergence of putative regulatory regions (Whitt, 1983).

The multi-locus isozyme system of lactate dehydrogenase (E.C.1.1.1.27; L-lactate: NAD oxidoreductase [LDH]) provides a particularly attractive system for both molecular and genetic analysis. LDH is a very stable enzyme, easily assayed and purified. Since the enzyme is so easy to analyze, a great deal of detailed information has been gathered concerning its genetic determination and phenotypic expression. In vertebrates, LDH occurs as several different molecular forms or isozymes that arise from the combination of different types of subunits into a tetramer. The subunits are encoded by separate genes which are regulated differently with respect to time of activation in development and degree of expression in various adult tissues, resulting in characteristic stage- and tissue-specific LDH isozyme profiles (Markert et al., 1975). The developmental and tissue-

specific isozyme profiles have been determined for several vertebrate species (Whitt, 1983), and the amino acid sequences of the murine LDH subunits have been determined and the degree of divergence of each gene has been estimated (Pan et al., 1983; Whitt, 1983). In addition to a wealth of biochemical data concerning the structure and expression of the LDH subunits, there is some knowledge about the genetic system controlling the production of LDH isozymes. The genes encoding some of the LDH subunits have been mapped in man and in the mouse (Boone et al., 1972; Chen et al., 1973; Lalley et al., 1978; Soares, 1978; Peters and Andrews, 1985) and genetic variants are known in mice and other mammals that affect the distribution of LDH isozymes (Shows and Ruddle, 1968; Khlebodarova and Serov, 1980). Additionally, mouse strains carrying presumptive chromosomal aberrations near the structural gene for one of the LDH subunits have been isolated (L. B. Russell, personal communication), providing the possibility of examining the effect of gene dosage on LDH expression. There are also some *in vitro* systems in which the levels of LDH isozymes can be altered (Maker et al., 1972; deVillis and Brooker, 1973; Schapira, 1981).

This introduction describes what is known about the phenotypic realization of LDH and about the genes that control the expression of LDH isozymes. It is primarily limited to the LDH isozyme systems of mammals, particularly that of mice which has been well characterized. Some of the systems in which LDH expression can be manipulated are also described, and what is

known about the level of regulation of LDH expression in various systems is discussed.

PROTEIN STRUCTURE:

LDH catalyzes the interconversion of lactate and pyruvate using nicotinamide adenine dinucleotide (NAD^+) as coenzyme (Everse and Kaplan, 1973). Although LDH was purified as early as 1940 (Straub, 1940), crystalline preparations of the enzyme were later shown to be heterogeneous (Meister, 1950). This heterogeneity, appearing as distinct peaks separable by electrophoresis, was at first attributed to copurification of inactive contaminants (Meister, 1950). Each peak, however, was shown to have LDH activity, demonstrating that LDH exists in multiple molecular forms termed isozymes (Markert and Moller, 1959).

In somatic tissues of mammals and birds there are five major isozymes of LDH (Markert, 1968). The isozymes are readily separable by electrophoresis and can be visualized by a specific histochemical stain (Markert and Moller, 1959). Using a variety of agents known to disrupt the tertiary structure of proteins, Appella and Markert (1961) demonstrated that the active form of LDH is a tetramer that, in somatic tissues, can be composed of two types of subunits, A and B. The five isozymes result from the combination of the two types of subunits to form both homo- and heterotetramers: $\text{LDH-1} = \text{B}_4$, $\text{LDH-2} = \text{A}_1\text{B}_3$, $\text{LDH-3} = \text{A}_2\text{B}_2$, $\text{LDH-4} = \text{A}_3\text{B}_1$ and $\text{LDH-5} = \text{A}_4$ (Appella and Markert, 1961; Markert, 1962). Microheterogeneity of LDH-5, the A_4 isozyme, has been

demonstrated by isoelectric focusing, but appears to be due to post-transcriptional modification of A subunits rather than the presence of additional subunits (Susor et al., 1969). A sixth isozyme, LDH-X, composed of homotetramers of a third type of subunit, LDH-C, is present in adult testis and spermatozoa of mammals and some birds (Blanco and Zinkham, 1963).

The LDH isozymes differ in physicochemical properties such as pH optima, response to cofactor analogs, kinetic properties, affinity for substrates and inhibition by excess substrate concentrations (Holbrook et al., 1975). They also differ in immunological properties, and, as mentioned above, in electrophoretic mobility. This difference in mobility is due to a difference in net electrical charge (at neutral or alkaline pH, LDH-1 has the highest migration rate toward the anode, LDH-5 the lowest) and indicates a difference in amino acid composition. The primary structures of LDH subunits from various vertebrate species have been determined and reveal that the A, B and C subunits are distinctly different but closely related proteins each having a molecular weight of approximately 35,000 (Klitz et al., 1977; Li et al., 1983; Pan et al., 1983; Li et al., 1985).

GENETICS:

In several organisms, genetically determined variation in the structure of each of the subunits has been described, indicating that each subunit is encoded by a distinct structural gene. In the mouse, three variant alleles of *Ldh-1*, the gene encoding the A

subunit, have been described. *Ldh-1^l*, found in a wild-living population of *Mus musculus* in Hungary, and *Ldh-1^b*, which arose from a spontaneous mutation in the inbred strain DBA/2J, affect the electrophoretic mobility of isozymes containing LDH-A subunits (Britton-Davidian et al., 1978; Soares, 1979). *Ldh-1^a*, induced by procarbazine, affects both the electrophoretic mobility and the staining intensity of LDH-A containing isozymes (Charles and Pretsch, 1981). There are also three variant alleles of *Ldh-2*, the mouse gene encoding the LDH-B subunit. All affect the electrophoretic mobility of LDH-B containing isozymes. Two of these, *Ldh-2^m* and *Ldh-2^k*, occur in the feral mouse species *Mus spretus*, the third, *Ldh-2^b*, was detected in two of the founding mice of the Peru-Coppock stock (Britton-Davidian et al., 1978; Berry and Peters, 1981). Although no murine variants of LDH-C have been discovered, mutant varieties of this subunit have been described in pigeons, providing evidence that the C subunit is encoded by a gene different from either the *Ldh-a* or *Ldh-b* genes (Blanco et al., 1964; Zinkham et al., 1966; Zinkham, 1968).

In mice, *Ldh-1* has been mapped to chromosome 7, and *Ldh-2* to chromosome 6 (Lalley et al., 1978; Soares, 1978; Peters and Andrews, 1985). The human genes encoding the A and B subunits, *LDHA* and *LDHB*, have been mapped to chromosomes 11 and 12, respectively (Boone et al., 1972; Chen et al., 1973). The gene encoding the C subunit has not been mapped in mice or humans, but it has been shown to be tightly linked, perhaps even contiguous, to the *Ldh-b* gene in pigeons (Zinkham et al., 1969).

In addition to the LDH structural genes, two regulatory genes affecting LDH expression have been described in the mouse: *Ldr-1*, which controls the expression of B subunits in erythrocytes, and *Ldr-2*, which affects the expression of B, and possibly A, subunits in the liver (Shows and Ruddle, 1968; Khlebodarova and Serov, 1980). Both *Ldr-1* and *Ldr-2* are linked to *Ldh-2* on mouse chromosome 6 (Hutton and Roderick, 1970; Khlebodarova and Serov, 1980).

In some inbred mouse strains, LDH-B subunits are not expressed in erythrocytes: the absence of B subunits in erythrocytes is controlled by the *Ldr-1^a* allele, while the *Ldr-1^b* allele controls the presence of these subunits (Shows and Ruddle, 1968). The action of *Ldr-1* is determined by the presence of a low-molecular-weight molecule that prevents hybridization of A and B subunits, resulting in the formation of only LDH-A homotetramers in the erythrocyte (Glass and Doyle, 1972). An *Ldr-1*-like gene is also known in foxes, sheep and rabbits. This gene, however, has a phenotypic effect opposite to that produced by *Ldr-1* in mice: isozymes containing LDH-A are absent from erythrocytes, those containing LDH-B are present (Serov and Khlebodarova, 1973; Coggan et al., 1974; Serov et al., 1975). In the fox, the action of *Ldr-1* is associated with a decrease in the number of LDH-A subunits (Khlebodarova et al., 1978).

Ldr-2 controls the expression of LDH subunits, primarily the B subunit, in murine liver. In mice of the CBA/Lac and AKR/J inbred lines, all five LDH isozymes are seen in the liver. This

phenotype is controlled by the *Ldr-2^b* allele. A second phenotype, controlled by the *Ldr-2^a* allele, is seen in most other inbred strains including DBA/2J, C57Bl/6J, and C3H/J. In these strains, a decrease in the activity of LDH-1, LDH-2, and LDH-3 isozymes is observed in the liver beginning on the sixth to eighth day of development. By the middle of the first month of development, activity of the LDH-1 and LDH-2 isozymes is undetectable. This decrease in activity has been shown to be due to a decrease in the concentration of LDH-B subunits. The concentration of LDH-A subunits also shows some decrease (Khlebodarova and Serov, 1980).

EVOLUTION:

Based on protein homology, it is believed that the three genes encoding LDH subunits arose from a single ancestor by gene duplication followed by divergence (Markert et al., 1975; Chang, 1979; Li, 1983; Li et al., 1983). The ancestral gene is believed to have resembled the contemporary *Ldh-a* gene since only one LDH gene, encoding an A-like subunit, is found in the lamprey (Markert et al., 1975). The gene encoding the C subunit was formerly believed to have been derived from the *Ldh-b* gene since the physical, kinetic, and immunological properties of the C subunit resemble those of the B subunit more closely than those of the A subunit (Markert et al., 1975). Additionally, the close linkage of the genes encoding the B and C subunits in pigeons

suggests that the *Ldh-c* gene arose by a tandem duplication of the *Ldh-b* gene (Zinkham, 1969).

A comparison of the amino acid sequence of the LDH-A, B, and C subunits from several species, as well as tryptic peptides maps of the murine A, B, and C subunits, however, failed to reveal closer homology between C and B subunits than between C and A subunits. In these studies, A and B subunits were shown to be more closely related to each other than either is to the C subunit. Amino-acid-sequence data, therefore, indicate that the gene duplication leading to the *Ldh-c* gene antedates that leading to the *Ldh-a* and *Ldh-b* genes. Based on sequence data, this duplication is estimated to have occurred more than 750 million years ago in the late Precambrian age. The gene duplication leading to the *Ldh-a* and *Ldh-b* genes is estimated to have occurred in the early Cambrian, 500-600 million years ago. (Markert et al., 1975; Li et al., 1983).

Estimates of the time of duplication of genes by means of sequence data are based on the assumption that all genes are evolving at the same rate. There is some evidence that the *Ldh-c* gene is evolving more rapidly than the *Ldh-a* and *Ldh-b* genes. A comparison of the amino acid sequence of rat and mouse LDH-C revealed 32 differences (approximately 10%) accumulating over an organismic divergence of about 35 million years (Pan et al., 1983). The A and B subunits exhibit only a 25% difference in sequence after 500-600 million years of divergence (Whitt, 1983). If this relatively rapid rate of change of the LDH-C subunit has been

constant over time, the amino acid sequence data would indicate that the *Ldh-c* gene arose more recently than the *Ldh-a* or *Ldh-b* genes (Whitt, 1983).

Other lines of evidence also suggest the the *Ldh-c* gene is younger than the *Ldh-a* or *Ldh-b* genes. Testis-specific LDH-C is found only in mammals and in one bird species (Wheat and Goldberg, 1983), arguing for a more recent origin of the *Ldh-c* gene than of the *Ldh-a* and *Ldh-b* genes which arose in the earliest vertebrates (Markert et al., 1975). Additionally, in other multi-locus isozyme systems and in the LDH isozyme system of some fish, the subunits most restricted in developmental or tissue specificity are encoded by the most recently derived genes (Markert, et al., 1975; Fisher and Whitt, 1978; Whitt, 1981). The expression of LDH-C is restricted to the testis and to a specific time in spermatocyte maturation as described below.

A phylogenetic comparison of developmental and tissue-specific LDH isozyme patterns can lead to inferences concerning not only the relative age of the genes encoding the LDH subunits, but also the relative age of different regulatory patterns. The tissue-specific distribution of LDH isozymes is generally conserved throughout the mammalian kingdom, while the developmental isozyme patterns are more species specific (Markert et al., 1975). This suggests that the mechanisms responsible for tissue-specific regulation arose prior to those responsible for developmental regulation. The tissue-specific regulatory mechanisms, therefore, should be conserved between mammalian species, while the

developmental mechanisms should be conserved only between closely related species (Whitt, 1983).

EXPRESSION:

The duplication and subsequent divergence of the LDH genes produced changes not only in the amino acid sequence of each subunit, but also in the regulation of expression of each gene. Vertebrate species show characteristic LDH isozyme patterns in embryonic and adult tissues. The amount of each isozyme present in a tissue appears to follow a binomial distribution reflecting the relative amount of each subunit in the cells of that tissue. The ontogenic and tissue specificity of isozyme patterns, therefore, appears to be due to a difference in levels of expression of each of the LDH genes at different times and in different tissues of an animal (Markert et al., 1975).

In most vertebrates, LDH-B subunits predominate in the unfertilized egg and through early cleavage. Later in development, an abrupt increase in A subunits occurs followed by a tissue-specific resurgence of B subunits (Markert et al., 1975).

In the mouse, where the ontogeny of isozyme patterns has been well characterized, newly ovulated ova and zygotes contain very high levels of LDH activity, at least six times that of any adult tissue (Brinster, 1979). This activity, which does not require protein synthesis and is apparently accumulated during oogenesis (Auerbach, 1969; Epstein et al, 1969; Brinster, 1973a), remains high for approximately 60 hours after ovulation, then falls over

the next 48 hours to about 10% of that found at ovulation (Brinster, 1965). The LDH present in oocytes and zygotes is composed almost exclusively of B subunits (Auerbach and Brinster, 1967). At or around implantation, from day 4 to day 5 after ovulation, the isozyme pattern shifts to predominantly A subunits (Markert and Ursprung, 1962), while the maternal B subunits are apparently rapidly and selectively degraded (Sellens and Sherman, 1980). Embryonic LDH-A subunits first appear in inner cell mass-derived tissue and then in trophoblast-derived tissues (Monk and Ansell, 1976).

Throughout early development, LDH-A subunits predominate in all tissues except both male and female germ cells where high levels of LDH-B subunits are seen (Brinster, 1979). With increasing age up to adulthood, B subunits gradually appear in somatic tissues until the characteristic adult isozyme profiles are reached. The shift from the isozyme patterns of the embryo to those of the adult does not occur synchronously throughout the animal: each tissue matures in terms of LDH expression at its own characteristic rate (Brinster, 1979). Once adult isozyme proportions are reached, they remain generally invariant throughout the life of the animal (Markert and Ursprung, 1962).

The expression of the C subunit is also regulated temporally. By following changes in LDH-X activity and biosynthesis in gamma irradiated mouse testes, Meistrich et al. (1977) have shown that LDH-X synthesis is initiated in midpachytene stage spermatocytes and continues throughout spermatid differentiation. A and B

subunits are apparently not synthesized in spermatocytes since heterotetramers involving the C subunit are not seen *in vivo* although they are readily formed *in vitro*. In addition, in mature sperm, LDH activity appears as a single band attributable to the C₄ isozyme following electrophoresis (Markert et al., 1975). Although LDH-X is present in mature sperm, there is no evidence of this isozyme in the zygote (Brinster, 1979).

It should be stressed that the ontogenic profiles described above are true only for mice. Preimplantation human embryos, like those of mice, express primarily LDH-B subunits. Following implantation, however, both A and B subunits are present: LDH-3 (A₂B₂) is the predominant isozyme (Pfleiderer and Wachsmuth, 1961). In rabbits, the ontogenic pattern of LDH isozymes is the reverse of that seen in mice: preimplantation embryos express predominantly A subunits, while B subunits are expressed predominantly following implantation (Brinster, 1973b).

In adult mammals, although most tissues contain both A and B subunits, each tissue has a characteristic pattern of isozymes in terms of relative abundance (Markert et al., 1975). Generally, A subunits predominate in skeletal muscle while B subunits predominate in the heart (Markert et al., 1975). In fact, in much of the literature, the A subunit is termed the "M" or muscle subunit, while the B subunit is termed the "H" or heart subunit. In some mouse tissues, the number of LDH-A and B subunits per cell has been determined and is presented in Table I (Nadal-Ginard, 1978). From these data, it can be calculated that in the mouse,

TABLE I*

**SUBUNITS OF LACTATE DEHYDROGENASE PER CELL
IN ADULT MOUSE TISSUES**

Tissue	Number of lactate dehydrogenase subunits per cell		
	Total	A subunits	B subunits
Heart	1.13×10^8	2.56×10^7	8.71×10^7
Muscle	3.60×10^8	3.34×10^8	2.56×10^7
Liver	3.94×10^7	3.78×10^7	1.60×10^6
Kidney	3.91×10^7	1.11×10^7	2.80×10^7

*From Nadal-Ginard, 1978.

there are thirty times more A subunits per skeletal muscle cell than per liver cell, and fifty-four times more B subunits in a heart-muscle cell than in a liver cell (Nadal-Ginard, 1978).

The tissue specificity of the A and B subunits is a matter of degree of expression: the *Ldh-a* and *Ldh-b* genes are expressed in all somatic tissues, but at different levels. By contrast, the expression of the *Ldh-c* gene is primarily restricted to adult testes in mammals and birds although C subunits have been seen in neural tissues in some species (Schapira, 1981). In mice, the C subunit is seen only in spermatids and mature sperm (Meistrich et al., 1977).

The distribution of LDH isozymes in adult mammalian tissues is also species-specific, although the differences between species are not as striking as the ontogenic differences described above. Throughout the course of primate evolution, for example, there has been a general increase in the relative expression of LDH-B subunits in all tissues (Markert et al., 1975).

Although the developmental and adult LDH isozyme patterns are generally invariant within a particular species, several systems have been described in which the relative proportions of isozymes can be induced to change. The expression of LDH-A isozymes in cultured chick embryos can be affected by oxygen pressure: high oxygen pressure inhibits the production of A and stimulates the production of B subunits (Chan, 1964; Goodfriend et al., 1966; Maker et al., 1972). The isozyme profile in the liver of rats has been shown to be affected by diet: LDH-B subunit levels are

increased in the livers of rats fed a high starch diet (Ogihara, 1975). In rabbits, estradiol has been shown to increase the synthesis of A subunits in the uterus (Goodfriend and Kaplan, 1964). In rats and humans, there is an increase in LDH-A synthesis in malignant, but not in benign tumors. Since LDH-A is the predominant LDH isozyme in the mammalian fetus, and since fetal isozymes of other enzymes, such as aldolase and pyruvate kinase, are also expressed in malignant tissues, the increase in LDH-A synthesis in malignant tumors is believed to be the result of a general shift toward a fetal pattern of gene expression in cancerous tissues (Schapira, 1981). LDH-A synthesis has also been shown to increase in a rat brain glial tumor cell line, RGC6, following treatment with catecholamines (deVillis and Brooker, 1973). Catecholamines cause a large increase in the intracellular concentration of cyclic adenosine monophosphate (cAMP) and the increase in LDH-A subunits is also observed following treatment with N⁶,O^{2'}-dibutyryl adenosine 3':5'-monophosphate (dibutyryl cAMP). Although RGC6 cells synthesize some LDH-B, no increase in its synthesis is observed (deVillis and Brooker, 1973).

PHYSIOLOGICAL SIGNIFICANCE:

Throughout evolution, the relative abundance of LDH isozymes in a given mammalian tissue has been generally conserved, providing evidence that the isozyme profile in a particular tissue has physiological significance. The physiological significance of the LDH isozymes is generally attributed to the

difference in inhibition by high concentrations of substrate, either pyruvate or lactate, exhibited by the A₄ and B₄ homotetramers: the B₄ isozyme is more strongly inhibited by high substrate concentrations than the A₄. In the heart, where B subunits predominate, the accumulation of pyruvate inhibits LDH so that pyruvate metabolism is shifted toward oxidation through the citric acid cycle. In skeletal muscle, where A subunits predominate, glycolysis can continue even in the presence of high concentrations of lactate (Markert, 1968). Generally, LDH-B subunits predominate in tissues that receive a relatively constant supply of oxygen such as the heart, brain, and kidney cortex, while LDH-A subunits predominate in tissues that are exposed to transient periods of anaerobiosis such as skeletal muscle (Markert, 1968).

This physiological role for the LDH isozymes is not universally accepted. Several studies have indicated that substrate concentrations do not reach inhibitory levels *in vivo* (Vesell and Pool, 1966; Everse et al., 1970; Wuntch et al., 1970). Additionally, LDH-A subunits predominate in human and rodent liver, tissues that have a very active aerobic metabolism, while human erythrocytes, which have no citric acid cycle, contain predominantly LDH-B subunits (Schapira, 1981).

While the physiological significance of LDH isozymes is far from established, the preferential localization of LDH-B₄ with mitochondria, and the association of LDH-A₄, but not LDH-B₄ with particulate matter in skeletal muscle, argues that the tissue

specific distribution of LDH isozymes does have some significance for cell metabolism (Holmes and Masters, 1979).

LEVEL OF REGULATION:

Although a great deal is known about the level of expression of LDH isozymes in various systems, very little is known about the mechanisms that regulate those levels. In most of the systems described above, the level of expression of the *Ldh-a*, *b*, and *c* genes was inferred from the level of active enzyme in a particular tissue. Except for the determination of the mode of action of *Ldr-1* (Glass and Doyle, 1972), no attempt has been made to quantitate LDH subunit monomers directly. The quantitation of LDH subunits in mouse tissues in Table I, for example, is calculated from enzyme activity data (Nadal-Ginard, 1978). It is generally assumed that the amount of LDH activity in a tissue is a direct reflection of the numbers of LDH-A and B subunits present in each cell of that tissue since the abundance of each isozyme can be explained by a binomial distribution of the subunits (Appella and Markert, 1961; Markert and Ursprung, 1962; Nadal-Ginard, 1978). The correlation of LDH gene activity with isozyme abundance, however, has not been proved directly.

The regulation of LDH gene activity can occur at all possible levels: at the level of the rate of transcription, at the level of messenger RNA (mRNA) processing, transport or stability, at the level of the rate of translation initiation or chain elongation, or at

the level of post-translational modification of the subunits, assembly of the tetramer, or the rate of protein degradation.

Almost nothing is known about the level of regulation for LDH genes during development. Transcription of the LDH genes must be initiated in the embryo, but this could occur at any time, possibly long before the appearance of the active isozymes. The appearance of active LDH-5, the A₄ isozyme, in the embryo requires some positive signal: embryonic LDH-A is not seen in the inner cell mass in the absence of implantation or implantation-like events such as trophoblast outgrowth, aggregation of blasocysts, or collapse of the blastocoele which allows contact of the blastomeres with each other (Sellens and Sherman, 1980). Synthesis of embryonic LDH-A in the trophoblast requires the presence of the inner cell mass, suggesting the possibility of an inductive interaction between the two tissues (Monk and Ansell, 1976). Neither the level at which implantation induces LDH production in the inner cell mass nor the level at which the inner cell mass induces LDH production in the trophoblast is known.

Nothing is known about the level of regulation of either the developmental timing or the tissue-specificity of LDH-C expression, however, the regulation of the tissue-specific expression of LDH-A and LDH-B is understood in part. Nadal-Ginard (1978) has calculated the rate of synthesis and the rate of degradation for each isozyme in several mouse tissues and has shown that each LDH isozyme has the same half-life in a given tissue. Although each LDH isozyme is degraded at the same rate within a particular

tissue, LDH isozymes are degraded at different rates in different tissues as shown in Table II. The rate of degradation of the LDH isozymes in a particular tissue is similar to the the rate of degradation of the bulk of protein in that tissue (Nadal-Ginard, 1978). The difference in degradative rates for LDH seen in different tissues accounts for the tissue-specific differences in overall LDH activity. For example, the specific activity of LDH in skeletal muscle is six-fold higher than that in heart muscle. This difference is due to the greater stability of LDH in skeletal muscle (Nadal-Ginard, 1978). Differences in LDH gene expression between tissues are, therefore, due to post-translational regulation. The difference in the amount of LDH-A and LDH-B subunits within a particular tissue is due to a difference in the rate of synthesis (shown in Table II) of each subunit in that tissue (Nadal-Ginard, 1978). It is not yet known whether the rate of synthesis of either subunit is controlled transcriptionally or post-transcriptionally.

The regulation of LDH gene expression in mouse erythrocytes occurs post-translationally in some strains. As described above, the regulatory gene *Ldr-1* controls the formation of a low-molecular-weight inhibitor that prevents the formation of LDH-B containing tetramers, resulting in the predominance of LDH-5 in the erythrocytes of some mouse strains (Glass and Doyle, 1972).

The level at which LDH gene expression is regulated has been determined in detail for catecholamine or cAMP stimulated rat glioma cells (Jungmann et al., 1983a). When cells of the rat

TABLE II*

HALF-LIVES, DEGRADATION COEFFICIENTS, AND RATES
OF SYNTHESIS OF LACTATE DEHYDROGENASE
IN DIFFERENT MOUSE TISSUES

Tissue	T _{1/2} days	K _d (rate constant of degradation)	K _s (rate of synthesis)		
			Total	A	B
			molecules/cell ⁻¹		
1/s ⁻¹					
Heart	8.18	0.084	110	25	85
Muscle	43.0	0.016	67	62	5
Liver	4.3	0.161	74	71	3
Kidney	6.1	0.113	52	15	37

*From Nadal-Ginard, 1978.

glioma line RGC6 are treated with catecholamines, isoproterenol, or dibutyryl cAMP, the level of LDH-A activity increases eight to ten fold over that seen in untreated controls (deVillis and Brooker, 1973). The increase in LDH activity is due to an increase in the rate of synthesis of LDH-A (Derda et al., 1980). This increase in the rate of synthesis is due in part to a two to three fold increase in the rate of transcription of LDH-A mRNA, and in part to a four to five fold increase in the half-life of the LDH-A message (Miles et al., 1981; Jungmann et al., 1983b). Regulation of the gene expression of the *Ldh-a* gene in this system, therefore, occurs both transcriptionally and post-transcriptionally.

The level of LDH-A mRNA also increases following serum or growth-hormone stimulation of quiescent rat fibroblasts, providing another example of pre-translational regulation of *Ldh-a* gene expression (Matrissian et al., 1985).

The genes encoding LDH-A and LDH-B have thus been shown to be regulated transcriptionally, post-transcriptionally, and post-translationally. Since the *Ldh-a*, *b*, and *c* genes arose through duplication and divergence of a single ancestral gene, a molecular analysis of each gene may provide insights into the sequences that are involved in the various levels of gene regulation. Sequence comparisons of structural genes can be informative for differential gene regulation only if it occurs within a particular cell, since differences in regulation that occur between species, or between different tissues of the same species can be due to species- or tissue-specific expression of a variety of processing, regulatory,

and temporal genes (Paigen, 1979). The differences in LDH activity seen between different mouse tissues, for example, are controlled by tissue-specific factors that regulate the overall rate of protein degradation (Nadal-Ginard, 1978). Within a single cell, however, the three LDH genes exhibit unique patterns of regulation. For example, in mouse liver and muscle, the *Ldh-a* gene is expressed at a higher level than the *Ldh-b* gene, and the *Ldh-c* gene is not expressed (Markert et al., 1975). In rat glial cells, the A gene responds to cAMP, the B and C genes do not (deVillis and Brooker, 1973).

Differential regulation of the LDH genes that occurs post-translationally must be due to variation in the amino-acid sequence of the three subunits and therefore to changes in the coding region of each gene. For example, the ability of the *Ldr-1*-controlled inhibitor molecule to discriminate between LDH-A and B subunits in mouse erythrocytes must be due to structural differences between the two subunits (Glass and Doyle, 1972).

Regulation that occurs at the level of mRNA transport or stability, or of the rate of translation initiation can be attributed to variation in the untranslated or translated portions of the message. Regulation that occurs at the level of mRNA processing can be due to variation in the intervening sequences as well.

Differential regulation that occurs at the level of transcription can arise in duplicated, diverged genes in a variety of ways. In eukaryotes, regulation of transcription initiation requires two DNA sequence elements, promoters and enhancers (see

Maniatis, 1987, for review). When a gene is duplicated, the promoter may or may not be duplicated as well. Duplication of promoter sequences along with a gene seems likely since promoters are immediately upstream from the start site of transcription and are typically only about 100 base pairs in length (Dyran and Tjian, 1985; McKnight and Tjian, 1986). Insertions, deletions, or other rearrangements, as well as "point" mutations can affect the promoter, either in sequence or in distance from the start site of transcription, and so affect regulation. Unlike promoters, enhancers can influence the expression of *cis*-linked genes at great distances in an orientation-independent manner and can also function downstream from the transcription start (Serfling et al., 1985). During duplication, an enhancer may simply be left behind, a more likely occurrence than in the case of promoters since enhancer elements are not necessarily tightly linked to the genes whose expression they control. Insertions and deletions as well as point mutations can cause loss, gain or divergence of enhancer elements resulting in differential transcriptional regulation of duplicated genes.

The determination of the level at which gene expression is regulated as well as a comparison of the sequence of duplicated genes can therefore be useful in identifying possible regulatory elements. Such analysis of the *Ldh-a*, *b*, and *c* genes should be particularly informative since the expression of these genes is known to be regulated at several different levels.

Additionally, since A and B subunits interact to form tetramers, and since the resulting isozymes show specific distributions that may be related to physiological function, it is possible that the A and B subunits are themselves regulatory. One may thus ask: do the levels of A or B subunits influence the expression of either the *Ldh-a* or *Ldh-b* genes? A possible regulatory role for LDH-A is suggested by the observation that rat liver LDH-A₄ possesses DNA-binding activity (Williams et al., 1984), and that chicken LDH-A is phosphorylated by tyrosine kinase (Cooper et al, 1984). The influence of LDH levels on LDH expression can be examined genetically using null mutations for either the A or B subunits.

THESIS GOALS:

One goal of this thesis was to identify a true null mutation for one of the LDH subunits. A null mutation is one that results in the absence of gene product. For the purpose of determining whether LDH is autoregulatory, a null mutation in which neither the protein product of the *Ldh* gene nor its mRNA is produced would be the most informative. A deletion of one of the *Ldh* genes would, therefore, be ideal.

As a result of specific-locus mutation-rate experiments, conducted at the Oak Ridge National Laboratory, there are a number of mouse mutants that carry presumptive deletions affecting the pink-eyed-dilute (*p*) locus, a coat color marker that maps genetically very close to *Ldh-1*, the structural gene for

mouse LDH-A. In the specific-locus test, mice of a uniform genetic background are treated with a mutagen, and then mated to a test stock homozygous for seven recessive visible markers (Russell, 1951). In the experiments conducted at Oak Ridge, mutant strains affected at one of the visible markers present in the test stock were further characterized for pleiotropic effects of the mutation, particularly pre- and post-natal lethality. Involvement in a mutation of two or more functional units such as lethality and a coat color marker is usually taken to indicate a chromosomal aberration (Russell, 1971). Lethal deficiencies involving the *p* locus, however, cannot be assumed to be deletions since *p* has not yet been shown to be a non-vital gene. Some of the *p*-locus lethal deficiencies may be deletion, and since the *p* locus maps genetically only two centimorgans (cM) distal to *Ldh-1* (Davisson and Roderick, 1986), it is possible that some of these presumptive *p*-locus deletions include *Ldh-1*.

Mutants affected at the *p* locus that were also recessive lethals were screened for an alteration in LDH levels overall and LDH-A levels in particular, using both spectrophotometric and electrophoretic assays [Chapter II]. Of thirty-four *p*-locus lethals and sublethals screened, one strain, 46DFiOD, was identified as being heterozygous for an LDH-A deficiency. Quantitation of LDH activity and isozyme distribution in several tissues of this strain provided no evidence for dosage compensation by either the LDH-A or LDH-B subunit, demonstrating that, at least in terms of

tissue-specificity, LDH-A levels do not regulate the expression of either the *Ldh-1* or *Ldh-2* genes in mice.

Quantitation of the LDH isozymes in heterozygous deletion carriers and normal littermates of the 46DFiOD strain also provided a formal demonstration that the isozyme distribution in mammalian tissues is a direct reflection of the amount of each LDH subunit produced in that tissue.

Another goal of this thesis was to obtain and characterize full length cDNA and genomic clones for mouse LDH-A.

Northern analysis of RNA from several mouse tissues (Maniatis et al., 1982), using a partial mouse LDH-A complimentary DNA (cDNA) probe having an insert size of 900 base pairs (bp) provided by Dr. Kenneth Fong (Akai et al, 1985), showed that full length LDH-A cDNA is approximately 1.6 kilobase pairs (Kb) in length. The 900 bp partial cDNA probe was used to screen a mouse cDNA library prepared from uninduced mouse erythroleukemia (MEL) cell mRNA [Chapter III]. A clone containing an insert of 1.6 Kb was obtained, and partially sequenced. This clone contains 80 bp of five prime (5') untranslated (UT) sequence, the AUG start site of translation, the full coding region of mouse LDH-A (Li et al., 1985), and 488 bp of three prime (3') UT sequence.

As a first step in obtaining genomic LDH-A clones, the full-length mouse LDH-A cDNA was used as a probe in Southern analysis of mouse DNA (Southern, 1975). This analysis showed that there are a large number of sequences (20-30 hybridizing

restriction fragments when DNA was digested with the restriction endonuclease EcoR1) in the mouse genome having homology to LDH-A. These sequences have a high degree of homology to mouse LDH-A cDNA (hybridization to LDH-A cDNA is seen under conditions of high stringency), and are dispersed throughout the genome (the number of hybridizing restriction fragments remains similar when mouse DNA is digested with a variety of restriction enzymes). Some of these hybridizing sequences have since been shown to be LDH-A pseudogenes (Fukawasa et al., 1986; Fukasawa and Li, 1986; Akai et al., 1987) and will be referred to as such throughout this thesis.

To determine which of the restriction fragments contained the expressed LDH-A genomic sequences, the full-length LDH-A cDNA was hybridized to DNAs from 16 well-characterized mouse-Chinese hamster somatic cell hybrids obtained from Dr. Peter Lalley [Chapter IV]. An EcoR1 restriction fragment of 14 Kb was identified that showed 100% concordance with the presence or absence of mouse chromosome 7 in the somatic cell hybrids indicating that this fragment contains either the expressed LDH-A gene or a linked pseudogene.

In order more rigorously to test the identity of the 14 Kb EcoR1 restriction fragment as containing the expressed LDH-A gene, experiments were performed to correlate the absence of this fragment with the LDH-A deficiency carried by 46DFiOD. Animals of the stock 46DFiOD are deficient for at least four adjacent markers; the *p* locus, a functional unit required for viability, *Ldh*-

1, and the ruby-eyed-2 locus *ru-2* (Clyde Montgomery, personal communication). The mutation carried in 46DFiOD is therefore considered to be a deletion of a portion of chromosome 7. Similar deficiencies at the albino (*c*) locus (Disteche and Adler, 1984) and at the dilute (*d*) locus (Rinchik et al., 1986) have been directly shown to be deletions. Since the deletion carried by 46DFiOD is lethal when homozygous, 46DFiOD animals will have at least one normal chromosome 7. Thus, when DNA from 46DFiOD deletion carriers is probed with pML1.6, the absence of the 14 Kb EcoR1 fragment from the deleted chromosome will be obscured by its presence on the normal chromosome. To separate the normal from the deleted chromosome 7 and thus to test for the absence of the 14 Kb EcoR1 restriction-fragment-believed to contain the expressed LDH-A gene, heterozygous 46DFiOD deletion carriers were mated to *Mus spretus*, a feral mouse species shown to have a restriction fragment length polymorphism (RFLP) for the restriction fragment in question. Progeny from this mating were tested for LDH activity. Of thirteen progeny, seven had reduced LDH activity in liver, kidney, and muscle indicative of the LDH-A deficiency. Southern analysis of DNA from the 46DFiOD x *Mus spretus* progeny clearly showed that the 14 Kb EcoR1 restriction fragment cosegregates with normal LDH activity [Chapter IV].

EcoR1-digested DNA from a mouse-Chinese hamster somatic-cell hybrid containing mouse chromosome 7 was used to make a recombinant library in a bacteriophage lambda vector. The library was screened with LDH-A cDNA [Chapter V]. Two different clones

having 14 Kb EcoRI inserts were isolated. A single-copy probe from one of the cloned sequences was shown to hybridize to a single restriction fragment in Southern analysis of mouse DNA, and to be absent from the deleted chromosome 7 carried by 46DFiOD. The restriction map of the cloned genomic LDH-A sequences was determined, and the approximate positions of the start sites of translation and transcription were identified by hybridizing portions of the full length LDH-A cDNA to restriction fragments of the genomic clone [Chapter V].

The 14 Kb genomic DNA insert, which contains about 3 Kb of flanking sequence 5' to the start site of transcription, was transfected into Chinese hamster cells where it directed the synthesis of mouse LDH-A. A 12 Kb truncated construct, containing about 1000 bp of 5' flanking DNA, was also shown to be capable of directing the synthesis of mouse LDH-A following transfection into Chinese hamster cells [Chapter V].

The nucleotide sequence of approximately three Kb of the LDH-A gene was determined. This segment extends from a Hind3 restriction site about 1000 bp 5' to the start site of transcription to the AUG start site of translation [Chapter VI]. Analysis of the sequence immediately upstream of the first exon revealed that LDH-A, like many "housekeeping" genes, lacks the consensus sequences TATAA and CAAT associated with the promoter region of many eukaryotic genes (Dyanan, 1986; Maniatis et al., 1987). The promoter region of *Ldh-1* does, however, contain putative regulatory elements: the hexamer CCGCCC associated with the

SV40 enhancer and the transcription factor SP1 occurs three times immediately upstream from the presumed site of transcription initiation, and a 12 base pair sequence (CTGACGTCAGAG) shown to be necessary for cAMP stimulation of several other eukaryotic genes (Montminy et al., 1984; Tsukada et al., 1985; Short et al., 1985; Comb et al., 1986) occurs just upstream from the presumed mRNA cap site.

The mutant 46DFiOD was further characterized with respect to inclusion or exclusion of nearby markers on mouse chromosome 7 [Chapter VII]. The nearby markers *Xld-1*, the structural gene for xylose dehydrogenase (XLD) (Newton et al., 1982), and *LH β* the beta subunit of luteinizing hormone (Chin et al., 1983), were excluded from the region missing in 46DFiOD. Included in this region are *Saa*, the serum amyloid gene complex linked to the transaminase locus *Tam-1* (Taylor and Rowe, 1984), and a human probe 24.2, which maps to a region of the short arm of human chromosome 11 (Tom Glaser, personal communication), that shares homology with mouse chromosome 7 at several loci including *Ldh-1* (Glaser et al., 1985).

In this thesis, a strain of mice carrying a deficiency for one of the LDH subunits, LDH-A, is identified, and the effect of this deficiency on LDH regulation is determined [Chapter II]. It is demonstrated that the deficiency is due to a chromosomal deletion, and the extent of this deletion is roughly determined by the inclusion or exclusion of adjacent genetic markers [Chapters V and VII]. Full length cDNA and genomic clones encoding mouse LDH-A

are isolated and characterized [Chapters III, IV, V, and VI]; and, finally, possible upstream regulatory elements in the mouse *Ldh-a* gene are described [Chapter VI].

Chapter II

SCREENING OF *P*-LOCUS LETHALS FOR LDH-A DEFICIENCY

SUMMARY:

In order to determine whether the expression of the *Ldh-a* gene is linearly related to gene dosage, a number of presumed mouse deletion stocks affected at the *p*-locus, a coat color marker mapping genetically very close (2 cM) to *Ldh-1*, the mouse structural gene for LDH-A, were screened for LDH activity and LDH isozyme profiles in various tissues. One stock, 46DFiOD, was identified as carrying a deletion for *Ldh-1*. Analysis of the LDH activity in various tissues, and the isozyme profile in the kidney, of 46DFiOD heterozygous deletion carriers and their normal littermates showed that expression of *Ldh-1* is linearly related to gene dosage.

INTRODUCTION:

The tissue specificity of LDH isozymes in a given mammalian tissue has been generally conserved throughout evolution, providing convincing evidence that the isozyme profile in a particular tissue has physiological significance (Markert, 1975). As described in Chapter I, the physiological significance of the LDH isozymes is generally attributed to the difference in inhibition by high concentrations of substrate, either pyruvate or lactate, exhibited by the A₄ and B₄ homotetramers (Holbrook, 1975). If

the isozyme profile of a particular tissue is physiologically important, it is possible that mechanisms to maintain that profile have evolved.

One type of mechanism that could control the relative proportion of LDH isozymes in a particular tissue is feedback regulation of the expression of the *Ldh* genes by their gene products. This regulation could be autogenous, with the level of LDH-A mRNA or LDH-A subunits regulating expression of the *Ldh-a* gene, for example. Regulation could also be heterogenous, with the level of the LDH-A message or protein regulating the expression of the *Ldh-b* gene, for example. Feedback regulation could occur directly since LDH binds to nucleic acids (Keck and Choules, 1962; Williams et al., 1984), or indirectly via a 'sensor' mechanism that detects the level of LDH mRNA or protein in the cell.

One way to determine whether LDH is autoregulatory, is to alter the abundance of one of the two LDH subunits. This can be accomplished mechanically by injecting LDH subunits into a cell, or genetically through alterations in gene dosage by either duplications or deletions. Alterations in gene dosage might be more informative, since LDH mRNA levels would probably also be changed.

In humans, chromosomal aberrations resulting in monosomy and trisomy of portions of chromosome 12 exist, providing alterations in gene dosage for *LDHB*, the human gene encoding the B subunit of LDH. LDH-B activity is proportional to *LDHB* gene

dosage while LDH-A activity remains unaffected by changes in *LDHB* dosage, indicating that LDH-B levels do not regulate the expression of *Ldh* genes in humans (Krone and Wolf, 1977).

Nadal-Ginard (1976) has injected both frog and rat LDH-A₄ into frog oocytes. No alteration in the gene expression of frog LDH isozymes was seen. Feedback regulation of the *Ldh* genes by LDH-A is not ruled out by this experiment, however, since this regulation could depend on the levels of LDH-A mRNA or free LDH-A monomer rather than on those of the mature homotetramer. LDH tetramers, once formed, do not dissociate *in vivo* (Nadal-Ginard, 1976). The possible feedback regulation of the *Ldh* genes by LDH-A can be more informatively tested by examining the effect of altered gene dosage of *Ldh-a* genes.

In order to examine the effects of *Ldh-a* gene dosage on LDH expression, a number of mouse stocks carrying presumptive deletions at the pink-eyed-dilute (*p*) locus, were screened for LDH activity. The *p*-locus maps genetically very close (2 cM distal) to *Ldh-1*, the structural gene for mouse LDH-A (Davisson and Roderick, 1986). The presumptive *p*-locus deletions were isolated at the Oak Ridge National Laboratory in specific locus mutation rate experiments (Russell, 1951). In the specific locus experiments, wild-type mice of a uniform genetic background were treated with a mutagen, then crossed to a tester stock homozygous for seven recessive alleles including *p* (Russell, 1951). In the experiments performed at Oak Ridge, mutants visibly affected at *p*, which is a coat-color marker, were isolated and

tested for other phenotypes such as pre- and post-natal lethality of homozygotes. *p*-locus lethals, particularly those induced by ionizing radiation, are good candidates for *Ldh-1* deletions since mutations at the *d*, *se*, and *c* loci obtained in the same experiments have been shown to be deletions (Disteche and Adler, 1984; Rinchik et al., 1986), some of which extend as much as 6-11 cM on either side of the marker locus (Russell et al., 1982).

Thirty-four *p*-locus lethals and sublethals were screened for overall LDH activity in the liver and for the proportion of each LDH isozyme in the kidney. One stock, 46DFiOD, had an alteration of LDH-A activity indicating involvement of *Ldh-1* in the deletion carried by this stock.

In order to examine the effects of reduced *Ldh-1* gene dosage, the specific activity of LDH was determined in several tissues of 46DFiOD heterozygous deletion carriers and their normal littermates. Additionally, the abundance of each of the five LDH isozymes in kidneys of deletion carriers and their normal sibs was quantitated.

MATERIALS AND METHODS:

Mice: *p*-locus deficiency stocks were provided by Dr. Liane B. Russell, and were maintained as closed-colony, noninbred stocks at the Biology Division of the Oak Ridge National Laboratory. *p* region mutants were identified by the specific-locus method among offspring of irradiated or control parents (Russell, 1951). The symbol *p** is used to designate the induced allele isolated in

the specific-locus experiments. *p*-locus lethal stocks were maintained by outcrossing to a stock, 2A, homozygous for the chinchilla allele, *c^{ch}*, at the *c*-locus which is linked to *p* on mouse chromosome 7. Non-mutant offspring are $+ c^{ch}/+ c^{ch}$ and mutant offspring are $p^* +/+ c^{ch}$. Stocks were periodically tested for recombination between *c^{ch}* and *p*. All mice used in the LDH screen had been tested for recombination. At least two mutant and two non-mutant animals of each *p*-locus stock were tested for LDH activity.

Preparation of tissue extracts: Animals were sacrificed by cervical dislocation. 100 milligrams (mg) of tissue, either kidney or liver was homogenized in [150 millimolar (mM) NaCl; 0.5% Triton X-100; 50 mM Tris-HCl, pH 8.6] using ten strokes of a motor driven teflon pestle in a Potter-Elvehjem homogenizer at 4° C. Homogenates were centrifuged at 100,000 x g for 45 minutes. Supernatants were analyzed immediately or stored overnight at 4° C. No loss of LDH activity was observed after short term storage under these conditions.

LDH assays: Assays of LDH activity in tissue homogenates were made in a recording spectrophotometer (Beckman Acta C III) using pyruvate as a substrate at pH 7.5 as described by Markert and Ursprung (1962). The reaction mixture contained [0.1 molar (M) potassium phosphate, pH 7.0; 20 mM pyruvate; 0.13 mM NADH; 1-10 microliters (μl) of tissue homogenate] (Markert and Ursprung, 1962). One international unit of LDH is defined as the amount of enzyme that catalyzes the formation of one micromole

(μ mole) of NAD⁺ and lactate per minute under the described conditions (Lee et al., 1982). The specific activity of LDH is given as international units of LDH per mg total protein. The total amount of protein in each tissue extract was determined by the method of Lowry (1951).

Gel electrophoresis: Kidney homogenates were diluted 1:1 with [65mM Tris-borate, pH 9.0; 5% beta-mercaptoethanol; 10% glycerol] and 50 μ l were loaded on a 100 millimeter (mm) x 140 mm x 1.5 mm discontinuous polyacrylamide slab gel having a 4.5% acrylamide stacking gel and a 7.5% acrylamide separating gel. All gel solutions were thirty parts acrylamide:one part bis-acrylamide (Biorad). Gel buffer was 0.3 M Tris-citrate, pH 9.0; running buffer was 0.065 M Tris-borate, pH 9.0. Pre-electrophoresis was carried out at 35 milliamps (mA) for 30 minutes at 4° C. Sample was applied, focused for 10 minutes at 15 mA, and the gel was run for 3-3.5 hours at 25 mA. Bromophenol blue at 0.001% was used as a tracking dye. Following electrophoresis, gels were stained in [100 milliliters (ml) 50 mM Tris-HCl, pH 8.3; 40 mg NAD⁺; 1 gram L-lactic acid, lithium salt; 40 mg nitroblue tetrazolium, 3 mg phenazine methosulfate] (Markert and Moller, 1959). When staining had reached the desired intensity, gels were fixed in [10% acetic acid; 10% methanol]. For quantitation of isozymes, kidney homogenates were applied to similar polyacrylamide gels cast in 0.6 mm x 12 mm glass tubes. Electrophoresis and staining of tube gels was as described for slab gels. Tube gels were scanned in a

Beckman Acta C III spectrophotometer at a wavelength of 560 nanometers.

RESULTS AND DISCUSSION:

Thirty lethal, four sub-lethal, and two viable *p*-locus mutant stocks were tested for LDH activity. At least two heterozygous mutants and two normal littermates from each stock were tested. The specific activity of LDH in liver and kidney homogenates of each animal was determined using a spectrophotometric assay, as described above. In addition, the relative proportion of each of the five LDH isozymes was determined following electrophoresis of kidney extracts from each animal. Soluble proteins in the kidney extracts were separated by electrophoresis through polyacrylamide slab gels, and the LDH isozymes were visualized by a specific histochemical stain. The intensity of the staining is proportional to the amount of activity of each isozyme (Markert and Moller, 1959).

In the absence of dosage compensation, an animal heterozygous for a deletion involving *Ldh-1* would have reduced LDH activity in all tissues, with the degree of reduction depending on the proportion of LDH-A containing isozymes produced in that tissue. For example, in liver and skeletal muscle, where most of the LDH is LDH-5, the A₄ isozyme, there would be close to a 50% reduction in LDH activity in a heterozygous deletion carrier. In kidney, where the proportion of LDH-A subunits is estimated to be 42% (Nadal-Ginard), there would be a 21% reduction in activity.

This expected degree of reduction is based on the assumption that all LDH subunits produced in a cell combine to form active tetramers. In addition to the reduction in LDH activity, the proportion of each isozyme in a heterozygous deletion carrier should change such that the number of LDH-A containing isozymes is reduced, and the number of LDH-B containing isozymes is increased, since more B subunits are free to form LDH-1 and LDH-2. The amount by which each isozyme is changed should follow a binomial distribution within a particular cell, provided that all LDH subunits form active tetramers. This binomial shift in distribution can be visualized in whole tissue homogenates only if the majority of cells in that tissue have the same LDH isozyme pattern. Nadal-Ginard (1978) has shown that the isozyme profiles of heart, muscle, and liver, can be explained by a binomial distribution of LDH subunits. The isozyme profile of kidney, however, does not follow a binomial distribution because the three major types of cells in this tissue, the cortex, papilla, and medulla, have different isozyme distributions (Nadal-Ginard, 1978).

If cells are capable of compensating for a reduction in the number of LDH-A subunits produced, a variety of changes in LDH activity or isozyme distribution might be produced. Compensation for a heterozygous deletion of *Ldh-1* could result in an increase in the expression of the remaining copy of that gene. If such compensation were perfect, there would be no effect on either LDH activity or isozyme distribution, and it would not be possible to detect the inclusion of *Ldh-1* in a deletion. If this type of dosage

compensation were not perfect, however, there would be some reduction of LDH activity and of LDH-A-containing subunits in the deletion carriers compared to normal sibs, but the amount of reduction would be less than expected. Dosage compensation that resulted in a change in the activity of the *Ldh-b* gene would be manifested by either a change in LDH activity, or a change in the LDH isozyme profile. If dosage compensation evolved to maintain the total amount of LDH activity, or the total number of subunits, then the activity of the *Ldh-b* gene would be expected to increase, resulting in a maintenance of LDH activity, but a change in the isozyme profile toward more LDH-B containing isozymes. If, on the other hand, dosage compensation evolved to maintain the relative proportion of each isozyme, there would be a decrease in the number of functional LDH-B subunits so that the isozyme profile would remain the same, but the LDH activity would be reduced.

In the absence of perfect dosage compensation, therefore, a mouse heterozygous for a deletion involving *Ldh-1* would show a change in LDH activity, isozyme distribution, or both.

Out of thirty-six lethal, sublethal, or viable *p*-locus deficiency stocks screened, one, 46DFiOD, showed an alteration in LDH activity. The specific activity of LDH in several tissues of 46DFiOD deletion carriers and normal littermates is shown in Table III. LDH activity in 46DFiOD deletion carriers is reduced in proportion to the percentage of LDH-A subunits relative to total LDH produced by each tissue. The percentage of LDH-A subunits was

TABLE III

LACTATE DEHYDROGENASE ACTIVITY IN TISSUES OF 46DFiOD

Tissue	Number tested	% LDH-A subunits [#]	LDH Activity		Reduction in Activity	Expected reduction
			+ / +	p [*] / +		
			(Units/mg protein)			(no compensation)
Heart	8	35	1.11 ± 0.2	0.92 ± 0.4	17.1%	17.5%
Muscle	8	96	6.00 ± 1.1	2.94 ± 0.5	49.0%	48.0%
Liver	10	98	1.62 ± 0.4	0.83 ± 0.1	48.7%	49.0%
Kidney	10	42	2.13 ± 0.1	1.67 ± 0.3	21.5%	21.0%

#from Nadal-Ginard, 1978

estimated by Nadal-Ginard (1978) based on the relative abundance of each isozyme in the tissues listed in Table III. In each tissue examined, LDH activity was reduced in the deletion carriers by approximately one half of the estimated percentage of LDH-A subunits (Table III).

The distribution of LDH isozymes in kidney extracts of 46DFiOD deletion carriers and their normal littermates is shown in Figure I. A spectrophotometric scan of similar tube gels of kidney extracts of normal and heterozygous deletion carriers is shown in Figure II. It is evident from the spectrophotometric scan that the proportion of LDH-A containing isozymes is reduced in the mutant, while the proportion of LDH-B containing isozymes is increased. Quantitation of each isozyme by weighing the area under each peak of the spectrophotometric scan showed that the change in proportion of each isozyme results from a reduction of the number of LDH-A subunits by approximately one half as shown in Table IV.

The reduction in LDH activity and the change in isozyme profile in 46DFiOD deletion carriers is in very good agreement with the variation expected in the absence of dosage compensation. There is no evidence, therefore, for feedback regulation of the *Ldh* genes by their products. This is not a particularly surprising result since in mammals, gene expression generally follows gene dosage (Krone and Wolf, 1977). For example, expression of the structural gene for mitochondrial malic enzyme, *Mod-2*, which is also on

Figure I.

**POLYACRYLAMIDE GEL OF 46DFiOD KIDNEY
EXTRACTS STAINED FOR LDH ACTIVITY**

The subunit composition of each band is indicated at the left of the gel. Lanes 1 and 2: kidney extracts of $+/+$ animals. Lanes 3 and 4: kidney extracts of $p^{*}/+$ animals. A marked reduction in the staining intensity of LDH-A containing isozymes is seen in $p^{*}/+$ extracts as compared to $+/+$ extracts.

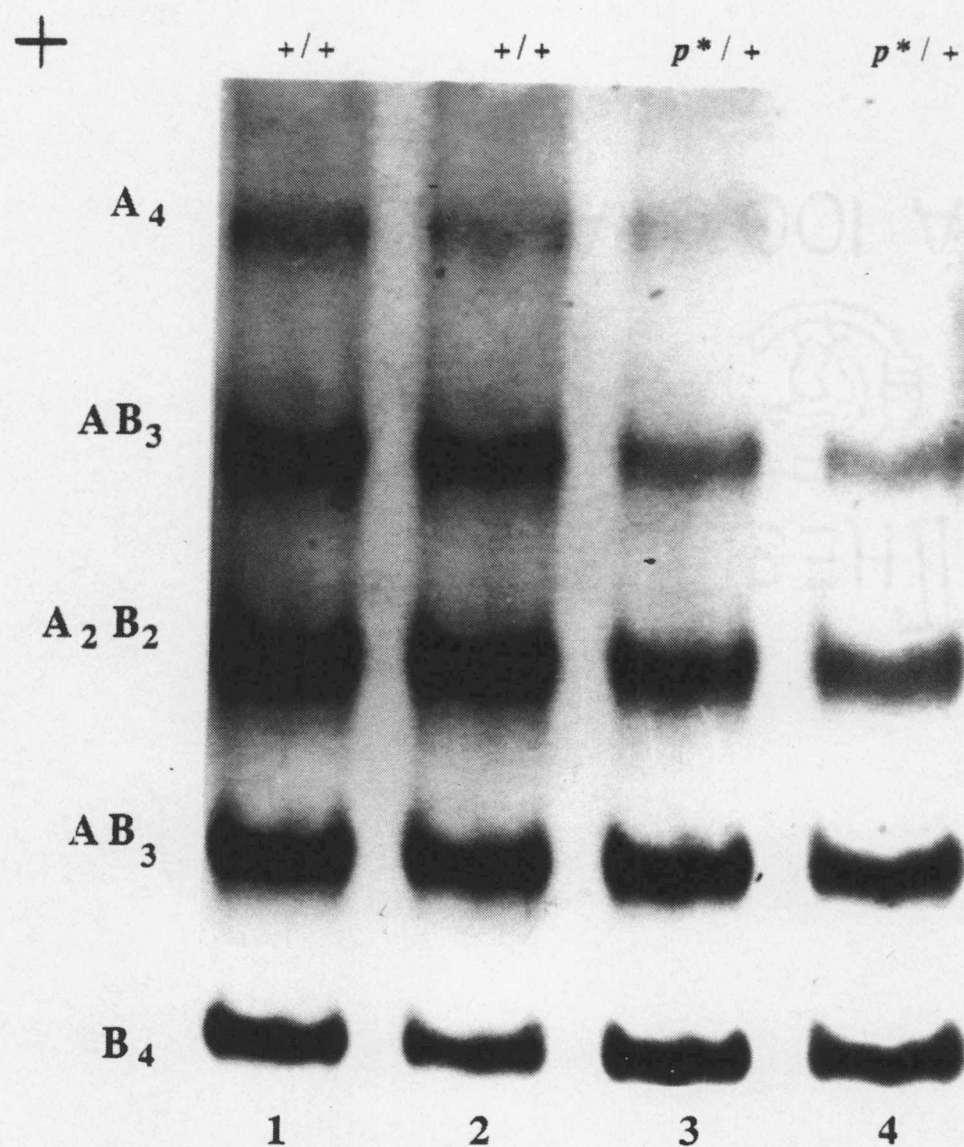


Figure I.

Figure II.

**SPECTROPHOTOMETRIC SCAN OF POLYACRYLAMIDE
GELS OF 46DFiOD KIDNEY EXTRACTS
STAINED FOR LDH ACTIVITY**

Polyacrylamide tube gels were stained as described and scanned in a Beckman Acta III spectrophotometer at a wavelength of 560 nanometers. Staining intensity is proportional to LDH activity. The upper panel, A, shows a drawing derived from the tracing of a gel of kidney extracts of $+/+$ 46DFiOD mice. The lower panel, B, shows a drawing derived from the tracing of a gel of kidney extracts of $p+/+$ 46DFiOD mice. The subunit composition of each peak is indicated.

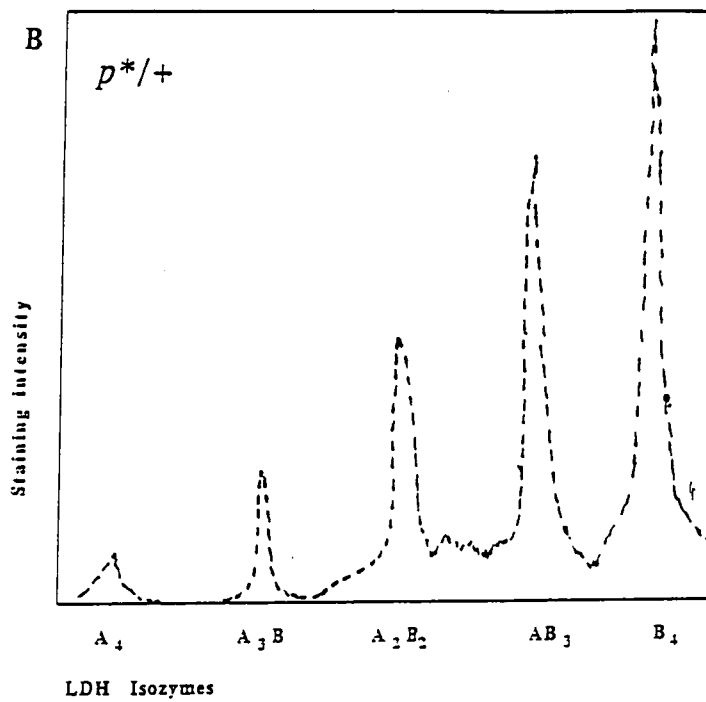
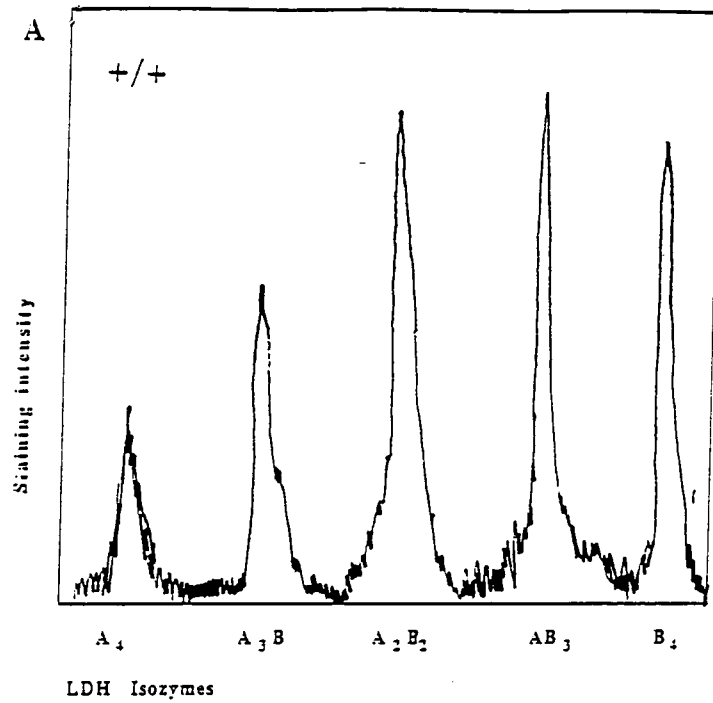


Figure II.

TABLE IV

**QUANTITATION OF STAINING INTENSITY IN
POLYACRYLAMIDE GELS OF 46DFiOD
KIDNEY EXTRACTS**

Isozyme	Proportion +/+	Staining Intensity $p^*/+$
A ₄	4.0%	2.0%
A ₃ B	15.0%	7.0%
A ₂ B ₂	31.0%	17.0%
AB ₃	27.0%	29.0%
B ₄	23.0%	45.0%
Total		
A	37.5%	23.0%
B	62.5%	77.0%
Ratio		
A/B	0.6	0.3

mouse chromosome 7, is linearly related to gene dosage (Eicher and Coleman, 1976; Bernstine et al., 1978).

The proportionate dosage-dependent expression of the LDH loci confirms the conclusion of Nadal-Ginard (1978) that tissue-specific isozyme profiles are regulated at the level of synthesis of each LDH subunit. Analysis of LDH isozyme levels in heterozygous mutants of this stock also demonstrates formally that the proportion of LDH isozymes in a particular cell reflects the number of subunits produced in that cell. Although analysis of whole kidney homogenates does not reveal a binomial distribution of LDH subunits (Nadal-Ginard, 1978), quantitation of the isozymes in kidneys of mutant and normal 46DFiOD sibs shows the variation expected by a 50% reduction in the number of LDH-A subunits.

Chapter III

ISOLATION AND CHARACTERIZATION OF A FULL LENGTH LDH-A cDNA CLONE

SUMMARY:

Northern analysis of RNA from several mouse tissues using a partial mouse LDH-A cDNA probe showed that the mRNA for LDH-A in the mouse is approximately 1.6 Kb in length. The partial LDH-A cDNA was used to screen a mouse erythroleukemia cell cDNA library, and recombinant clones having insert sizes of 1.6 Kb were obtained. Sequence analysis showed that these clones contain 80 bp of 5' untranslated sequence, the full coding region for mouse LDH-A, and 488 bp of 3' untranslated sequence.

INTRODUCTION:

Since the three genes encoding LDH subunits arose from a single ancestor and are differentially regulated as described in Chapter I, a molecular analysis of each gene from a single species can provide information concerning both evolution and gene regulation. Full understanding of the evolutionary changes that led to differences in regulation of the LDH genes requires detailed knowledge about the exon/intron structure of each of the three LDH genes, the start site of transcription for each gene, and the sequence of flanking, intervening, and coding regions of each gene. As a first step in this molecular analysis, full length cDNA and

genomic clones of mouse LDH-A were isolated and characterized. This chapter describes the isolation and characterization of a full length, or nearly full length, murine LDH-A cDNA clone.

MATERIALS AND METHODS:

Probe: A partial mouse LDH-A cDNA clone, pML2, was obtained from Dr. Kenneth Fong. This clone, which appears to be identical to pML73 described by Akai et al. (1984), has an insert size of approximately 900 bp, 488 bp of which is the 3' untranslated region. The 393 bp of coding region are in agreement with the COOH-terminal sequence of residues 201-331 of mouse LDH-A (Akai et al., 1984; Li et al., 1975).

Northern blot analysis: RNA from several mouse tissues and uninduced mouse erythroleukemia (MEL) cells was prepared by the following method: Cells or tissues were homogenized in 5 volumes of [4 M guanidinium isothiocyanate; 1 M β -mercaptoethanol; 50 mM sodium acetate, pH 5.0; 1 mM (Ethylenedinitrolo)-tetraacetic acid, disodium salt (EDTA)] using a two speed biohomogenizer (Biospec products). One gram of CsCl was added per ml of homogenate and the resulting solution was loaded into centrifuge tubes, underlayered with 0.3 volumes [5.7 M CsCl; 50 mM sodium acetate, pH5.0; 1 mM EDTA] and centrifuged in a Type 50 rotor in a Beckman ultracentrifuge at 45,000 rpm for 48 hours. Bands of RNA were pulled from each gradient, diluted with water and repeatedly ethanol precipitated in 0.3 M sodium acetate, pH 5.0 to remove excess CsCl. Purified RNA was

resuspended in water at 5 mg/ml and stored at -70° C. RNA was separated by electrophoresis through a 1% agarose gel containing formaldehyde and transferred to nitrocellulose as described by Maniatis et al. (1982). Northern blots prepared in this way were hybridized to a labelled fragment of pML2 and washed as described (Maniatis, 1982). Blots were exposed to Kodak XAR X-ray film with an intensifying screen (DuPont) at -70° C for one to several days.

Labelling of probes: Probes were labelled using the random-primer method modified from Feinberg and Vogelstein (1983) as follows: pML2 DNA was digested with Pst1 and applied to a 1.5% low gelling temperature agarose (Seakem) gel in Tris-borate buffer. Following electrophoresis, a 400 bp insert fragment containing the coding region of pML2 was cut out and trimmed of excess agarose. Three ml of water was added per gram of gel slice, and the slice was boiled for 10 minutes. An appropriate amount of boiled gel slice containing 50 nanograms (ng) DNA was added to [50 micro-Curies of alpha-³²P dCTP (2000-3000 Curies/millimole from New England Nuclear); 20 micrograms (μg) of bovine serum albumin (nuclease-free from Bethesda Research Laboratory); 10 μl of OLB {0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 1.0 M Hepes pH 6.6, and 15 μg/ml random undecanucleotides (P. L. Biochemicals)}} in a reaction volume of 50 μl. Four units of the large fragment of *E. coli* DNA polymerase I (New England Biolabs) were added, and the reaction was incubated overnight at 25° C. Labelled probe was separated from free nucleotides by spin

dialysis through Sepharose CL6B (Pharmacia) as described by Maniatis et al. (1982).

Library screening: A cDNA library prepared from uninduced MEL cell mRNA in the bacteriophage vector lambda-gt11 (Young and Davis, 1983), was obtained from Dr. Vikram Patel. The library had been amplified, and contained inserts of an average size of 2 Kb (Vikram Patel, personal communication). Clones were propagated in the E. coli strain Y1090 (Young and Davis, 1983) as described by Maniatis et al. (1982). One-hundred twenty thousand plaques were screened as described by Benton and Davis (1977) using a 400 bp Pst 1 fragment of pML2 (containing 393 bp of coding region) as probe. Clones hybridizing to the pML2 probe were plaque purified as described by Benton and Davis (1977).

Restriction mapping: DNA was prepared from plaque purified positive clones as described by Maniatis et al. (1982). Restriction mapping was performed by standard methods (Maniatis et al., 1982) with restriction enzymes purchased from New England Biolabs.

Sequencing: Relevant portions of positive clones were subcloned into BLUESCRIPTTM (Stratagene) plasmid vectors. Isolation of insert DNA was performed as follows: DNA from plaque purified recombinant clones was digested with appropriate restriction enzymes, and the resulting restriction fragments were separated by electrophoresis through agarose as described by Maniatis et al. (1982). GTG grade agarose (Seakem) was used for

all DNA purifications. The desired bands were excised from the gel, finely minced, mixed with an equal volume of Tris-HCl equilibrated phenol, pH 8.0, vortexed for 30 seconds, frozen at -70° C, and centrifuged in an Eppendorf microcentrifuge for 15 minutes at 4° C. The freezing and centrifugation steps were repeated three times. The supernatant was then extracted with phenol and chloroform, ethanol precipitated and resuspended in [10 mM Tris-HCl, pH 8.0; 1 mM EDTA]. Insert DNA was ligated to plasmid vector as described by Maniatis et al. (1982) using T₄ DNA ligase (New England Biolabs). Bacteria of the strain, JM109 [recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lambda⁻, del(lac-proAB)] (Stratagene) were made competent for transformation and transformed with recombinant plasmids as described by Hanahan and Meselson (1980). Transformed bacteria were plated on Luria broth (LB) (Maniatis et al., 1982) containing 13% agar, 100 µg/ml ampicillin, 40 µg/ml (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). White colonies were selected, and DNA from each colony was isolated by the alkaline lysis mini-prep procedure described by Ish-Horowicz and Burke (1981). Mini-prep DNA was analyzed by restriction enzyme digestion and electrophoresis through agarose or polyacrylamide gels as described by Maniatis et al. (1982). DNA from desired recombinant plasmids was prepared as described by Maniatis et al. (1982) with the following modifications: Bacteria were grown overnight in LB at 37° C with constant agitation. Bacterial pellets from one liter of growth were resuspended in 12 ml [25% sucrose;

50 mM Tris-HCl, pH 8.0]. Lysozyme (Sigma) was added to 1 mg/ml. Following a 5 minute incubation at 0° C, EDTA was added to 50 mM. Bacteria were lysed by the addition of 18 ml 0.1% Triton X-100 in [50 mM Tris-HCl pH 8.0, 50 mM EDTA]. Bacterial debris was removed by centrifugation for 30 minutes at 30,000 rpm in an SW27 type rotor (Beckman). Plasmid DNA was purified from the supernatant by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982). Sequencing was performed by the chain termination sequencing method (Sanger et al., 1977) using the GemSeqTM double stranded (ds) DNA sequencing method as described by Promega Biotec. Primers were purchased from Stratagene, and nucleotide mixes were purchased from Promega Biotec.

RESULTS AND DISCUSSION:

LDH-A cDNA sequences were first cloned by Miles et al. (1981) using poly-A⁺ RNA from cAMP stimulated rat glioma cells. The cDNA clone isolated by these researchers hybridized to a single band of approximately 1.6 Kb in Northern blot analysis of rat RNA (Miles et al., 1981). Using this clone to screen a mouse liver cDNA library, Akai et al.(1984) obtained a partial mouse LDH-A cDNA, pMLA 73, having an insert size of approximately 900 bp. A mouse liver LDH-A cDNA clone, pML2, also having an insert size of approximately 900 bp and apparently identical to pMLA 73 was obtained from Dr. Kenneth Fong, one of the co-authors of Akai et al. (1984). When used as a probe in Northern blot analysis of

RNA from several mouse tissues, this clone, pML2, also hybridized to a single band of approximately 1.6 Kb as shown in Figure III.

In order to obtain a full-length mouse LDH-A cDNA, pML2 was used to screen a cDNA library made from uninduced mouse erythroleukemia (MEL) cell mRNA. An MEL-cell cDNA library was used because RNA from these cells contains a relatively large amount of LDH-A message as shown in Figure III. The MEL-cell cDNA library screened was obtained from Dr. Vikram Patel and contained an average insert size of approximately 2 Kb.

Using as probe a 400 bp Pst1 restriction fragment containing the most 5' region of pML2 as shown in Figure IV, 120,000 recombinant clones of the MEL cDNA library were screened. Based on an estimate of the abundance of LDH-A subunits relative to total protein in MEL cells, the number of recombinant clones screened should be more than adequate to isolate a full length LDH-A cDNA clone. The specific activity of LDH in MEL cells, which is almost exclusively LDH-5 the A₄ isozyme, is 1.3 units per milligram of total protein, while that of purified LDH-5 is 200 units per milligram protein (data not shown). Therefore, LDH-A makes up about 0.5% of the total protein in MEL cells. LDH-A is therefore an abundant protein in these cells and, with the usual caveats, it can be estimated that one in every two-hundred clones in an MEL cell cDNA library will contain LDH-A sequences.

Fourteen positive recombinant clones were obtained and plaque purified. The longest of these, ML4A and ML14C, had

Figure III.

NORTHERN ANALYSIS OF RNA FROM MOUSE TISSUES AND MOUSE ERYTHROLEUKEMIA CELLS

RNA from several mouse tissues and mouse erythroleukemia cells was probed with a partial mouse cDNA, pML2, obtained from Dr. Kenneth Fong. Fifteen micrograms of total RNA were loaded per lane. Lane 1: MEL cell RNA. Lane 2: mouse kidney RNA. Lane 3: mouse liver RNA. Lane 4: Mouse heart RNA. Lane 5: mouse muscle RNA. The band hybridizing to pML2 is slightly smaller than 18S ribosomal RNA, and is approximately 1.6 kbp in length.

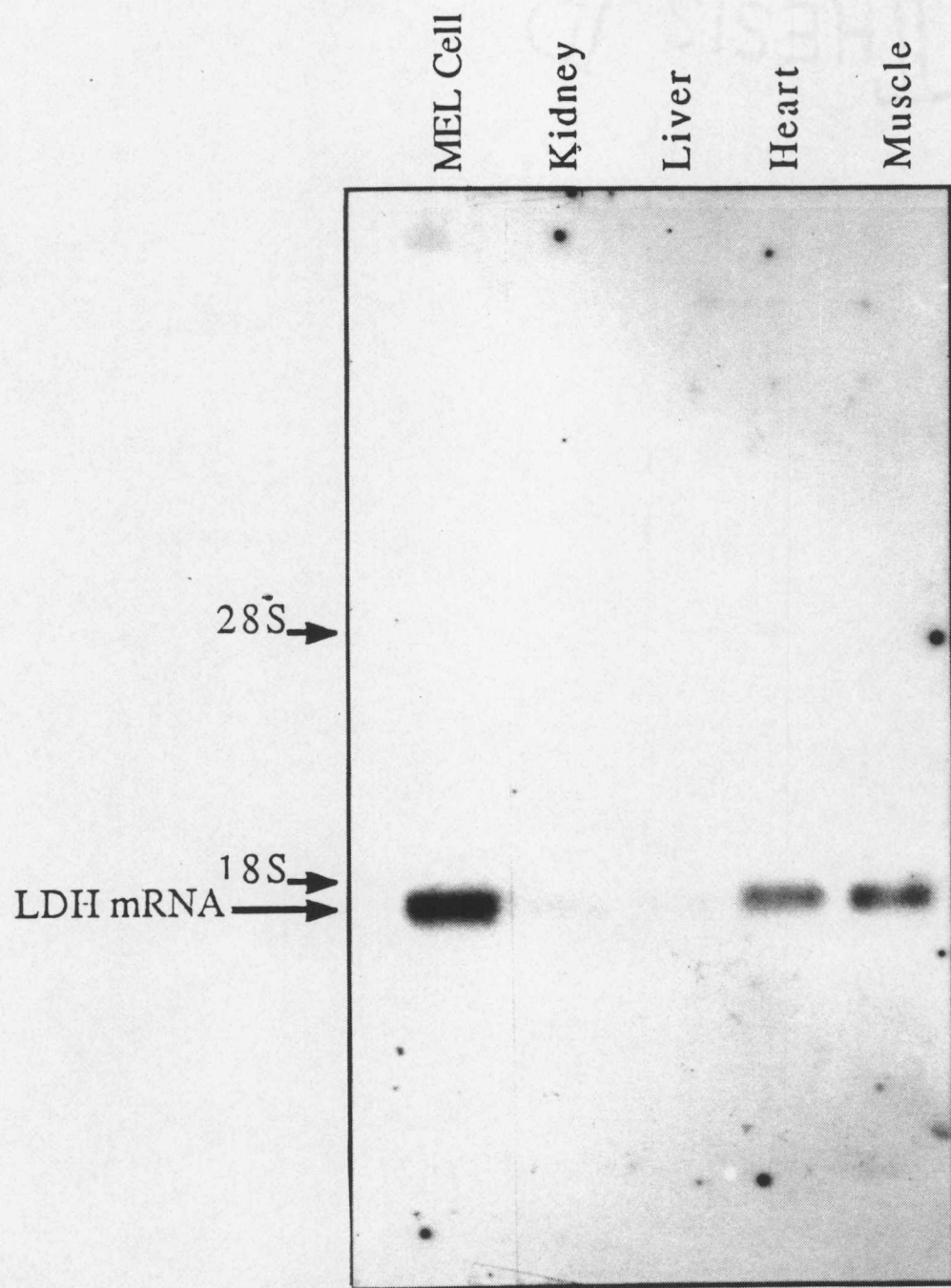


Figure III.

Figure IV.

**RESTRICTION MAPS OF PML2 AND PML1.6,
MOUSE LDH-A CDNA CLONES**

The complete restriction map for EcoR1, EcoRV, and Pst1 are shown. The maps for Hinf1 and HincII are partial. pML2 is cloned into the Pst1 site of pBR322; pML1.6 is cloned into the EcoR1 site of pUC. Arrows show the regions sequenced. The translated portion of pML1.6 is indicated by a dotted line.

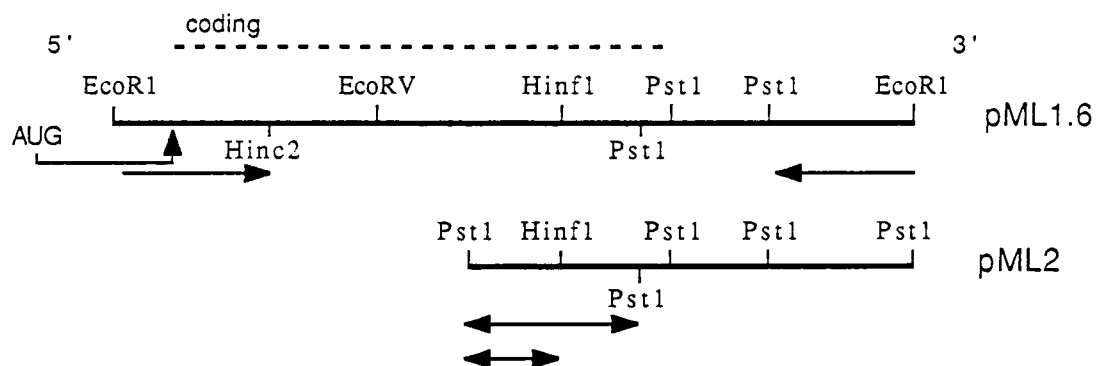


Figure IV.

insert sizes of approximately 1.6 Kb. Sequence analysis, as described below, showed that ML4A and ML14C are identical, so only ML4A will be described. The 1.6 Kb insert of ML4A, which is an Eco R1 restriction fragment, was subcloned into a plasmid vector and renamed pML1.6. The restriction map of pML1.6 was determined and compared to that of pML2, the mouse LDH-A cDNA clone obtained from Dr. Fong. Both maps are shown in **Figure IV**.

An EcoR1-Hinc II restriction fragment from the 5' end, and a Pst1-EcoR1 restriction fragment from the 3' end of pML1.6 (**Figure IV**) were subcloned into the plasmid vector BLUESCRIPT™ (Stratagene). The BLUESCRIPT™ vectors are pUC derivatives having T7 and T4 promoter/primer sequences flanking the polylinker cloning sites. Single-stranded T7 or T4 promoter sequences were hybridized on either side of the pML1.6 subcloned inserts and used as primers in DNA sequencing by the chain-termination method (Sanger et al., 1977). The 5' and 3' terminal DNA sequences of pML1.6 are presented in **Figure V**. As shown in the figure, pML1.6 is identical to pML2 at the 3' terminus. The 5' region of pML1.6 contains 80 bp of untranslated sequence 5' to the AUG start site of translation. The amino acid sequence of mouse LDH-A is from Li et al. (1985).

It is not yet known whether pML1.6 contains the entire transcribed mRNA of mouse LDH-A. Full length rat LDH-A cDNA has been obtained from growth hormone-stimulated fibroblasts, and the 5' end of the rat LDH-A message was confirmed by primer

Figure V.

**PARTIAL SEQUENCE ANALYSIS OF PML1.6,
A MOUSE LDH-A CDNA CLONE**

This figure shows the nucleotide sequence of a 5' EcoR1-HincII subclone containing the first 241 base pairs of pML1.6 as shown in Figure IV. The sequence of this fragment is compared to the amino acid sequence of mouse LDH-A (Li et al., 1985). The nucleotide sequence of a 3' Pst1-EcoR1 subclone containing the terminal 266 bp of pML1.6 is also shown. This sequence is compared to the nucleotide sequence of the partial cDNA clone pML2 obtained from Dr. Kenneth Fong. The sequence of pML1.6 fragments are shown in capital letters. Small letters denote the sequence of pML2. pML1.6 and pML2 have the same 3' terminus.

CTGTGCGCCGAGCTCGGCCACGCTGCTTCTCTCTCGCCAGTCGCCCCCCCCATCGTGCCATC

TAGCGGTCTAAAGATTCAAAGTCCAAG ATG GCA ACC CTC AAG GAC CAG CTG
Met Ala Thr Leu Lys Asp Gln Leu

ATT GTG AAT CTT CTT AAG GAA GAG CAG GCT CCC CAG AAC AAG ATT
Ile Val Asn Leu Leu Lys Glu Glu Gln Ala Pro Gln Asn Lys Ile

ACA GTT GTT GGG GTT GGT GCT GTT GGC ATG GCT TGT GCC ATC AGT
Thr Val Val Gly Val Gly Ala Val Gly Met Ala Cys Ala Ile Ser

ATC TTA ATG AAG GAC TTG GCG GAT GAG CTT GCC CTT GTT GAC
Ile Leu Met Lys Asp Leu Ala Asp Glu Leu Ala Leu Val Asp

.....

..... CTGCAGGCTTCGAT
ctgcaggcttcgat

TACCCCTGTGAGCCTGCTGCATTGCTGCCCTGCACCAAACAGCCTAGGCCGACGAGTTC
taccctgtgagcctgctgcattgctgccctgcaccaaacagcctaggccgacgagttc

CCAGTTAAGTCGTATAACCTGGCTCCAGTGTGTACGTCCATGATGCATATCTTGTGCAT
ccagttaagtcgtataacctggctccagtggtgtacgtccatgatgcatatcttgtgcat

AAATGTTGTACAGGATATTTTATATATTATATGTGTCTGTAGTGTGCATTGCAATATTA
aaatgttgtacaggatattttatatattatatgtgtctgtagtgtgcattgcaatatta

TGTGAGATGTAAGATCTGCATATGGATGATGGAACCAACCACCCAAGTGTTCATGCCAAA
tgtgagatgtaagatctgcatatggatgatggaaccaaccacccaagtgatgccaaa

TAAACCTTGAACAGTGAAAAAAAAA
taaaccttgaacagtgaaaaaaaaaa

Figure V.

extension (Matrisian et al., 1985). Rat LDH-A mRNA has a 5' untranslated region of 103 bp. A human LDH-A cDNA having a 5' untranslated region of 97 bp has also been obtained (Tsujibo et al., 1985). It is possible therefore that the mouse LDH-A message is slightly longer than pML1.6. In addition, it is possible that more than one mRNA is transcribed from *Ldh-1*. Other genes that are regulated in a tissue-specific manner, notably mouse alpha-amylase, have been shown to encode messages differing in the 5' untranslated portion (Hagenbüchle et al., 1981; Schibler et al., 1982). Confirmation of the 5' terminus or termini of mouse LDH-A mRNA requires primer extension of RNA from several mouse tissues

Chapter IV

IDENTIFICATION OF A RESTRICTION FRAGMENT CONTAINING MOUSE LDH-A GENOMIC SEQUENCES

SUMMARY:

When the mouse LDH-A cDNA pML1.6 is used as a probe in Southern analysis of mouse DNA, several hybridizing bands are seen. In order to determine which of these bands contains *Ldh-1*, pML1.6 was used as a probe in Southern analysis of DNA's from a well-characterized mouse-Chinese hamster somatic cell hybrid mapping panel informative for mouse chromosome 7, the chromosome containing *Ldh-1*. A 14 Kb EcoR1 restriction fragment hybridizing to pML1.6 segregated with mouse chromosome 7. This band was shown to be absent from the 46DFiOD deletion and, therefore, was identified as containing the expressed copy of the mouse gene for LDH-A.

INTRODUCTION:

As a first step in isolating genomic clones containing mouse LDH-A sequences, the mouse LDH-A cDNA clone pML1.6 was used as a probe in Southern analysis (Southern, 1975) of mouse genomic DNA. Surprisingly, although pML1.6 hybridizes to a single band in Northern analysis of mouse RNA from MEL cells, liver, kidney, heart, and muscle (data not shown), this clone hybridizes

to multiple restriction fragments in mouse genomic DNA as shown below. These restriction fragments have a high degree of homology to LDH-A cDNA since hybridization was seen even under conditions of very high stringency. Attempts were made to reduce the number of hybridizing restriction fragments by restricting the genomic DNA with various enzymes, and by using various portions of pML1.6 as probe. Several bands (20-30 with most restriction enzymes) were seen under all conditions tried.

In order to determine which of the genomic restriction fragments hybridizing to pML1.6 contains the expressed mouse gene for LDH-A, pML1.6 was used as a probe in Southern analysis of DNA from several well-characterized mouse-Chinese somatic cell hybrids containing varying complements of mouse chromosomes (Minna et al., 1975; Lalley et al., 1978). Through this analysis, a 14 Kb EcoR1 restriction fragment was identified that hybridized to pML1.6 and segregated with mouse chromosome 7, the chromosome containing *Ldh-1*, the mouse gene for LDH-A. Further identification of this restriction fragment with *Ldh-1* was accomplished using Southern analysis of DNA from offspring of a cross between 46DFiOD deletion carriers and *Mus spretus*, a feral mouse species having a restriction fragment length polymorphism (RFLP) for the 14 Kb EcoR1 fragment in question.

MATERIALS AND METHODS:

Cell lines: The somatic cell hybrids, resulting from a fusion of a BALB/c mouse spleen to E36 Chinese hamster cells deficient

for hypoxanthine phosphoribosyl transferase (HPRT) as described by Minna et al. (1975), were obtained from Dr. Peter Lalley. The presence or absence of mouse chromosome 7 in each hybrid was determined by isozyme and cytogenetic analysis performed by Dr. Lalley.

Mice: Female 46DFiOD deletion carriers described in Chapter II were mated to males of the feral mouse species *Mus spretus* obtained from Dr. Verne Chapman. All matings were done at the Oak Ridge National Laboratory Biology Division under the supervision of Dr. Liane Russell and Clyde Montgomery.

LDH assays: The specific activity of LDH in *Mus spretus* and 46DFiOD x *Mus spretus* progeny was determined as described in Chapter II.

Preparation of DNAs: Somatic cell hybrid DNAs were obtained from Dr. Lorraine Albritton. Mouse genomic DNAs were prepared by homogenization of livers in five volumes [0.25 M sucrose; 50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 10 mM KCl] in a Potter-Elvehjem homogenizer with a motor driven pestle. Nuclei were pelleted by centrifugation at 1000 rpm for 12 minutes in a Sorvall RCB5 centrifuge. Nuclei were rinsed in 5 ml TNE [10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA] and resuspended in 5 ml TNE containing [300 µg/ml proteinase K (Sigma); 0.5% SDS]. After incubation at 37° C. for two to eight hours, DNA was extracted with phenol and chloroform, ethanol precipitated and resuspended at 1 gram/ml in TE [10 mM Tris-HCl, pH 8.0; 1 mM EDTA].

Southern blots: Hybridization of probes to restriction enzyme digests of DNAs from hybrid cells or mouse tissues was as described by Southern (1975), except that DNA was transferred to nylon membranes (zetabind, American Lab Supplies). After baking, filters were washed for one hour in [0.5% sodium dodecyl sulfate (SDS), 0.1X standard saline citrate (SSC)] at 65° C. Prehybridization and hybridization were at 42° C. in [50% formamide, 5X SSC, 1X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA]. Probes were labelled as described in Chapter III, using the appropriate restriction enzymes to generate the desired fragment of pML1.6. Prehybridization was 8-16 hours, hybridization was 42-48 hours. Following hybridization, filters were washed to 0.1X SSC at 65° C. and exposed for one to several days to Kodak XAR X-ray film with an intensifying screen (DuPont) at -70° C.

RESULTS AND DISCUSSION:

When pML1.6 is used as a probe in Southern analysis of mouse DNA, a relatively large number of hybridizing restriction fragments or bands are seen as shown in Figure VI. These bands are seen even under conditions of high stringency indicating a high degree of homology with pML1.6 (Figure VI). Three of these bands have been cloned, sequenced, and identified as processed pseudogenes of *Ldh-1* (Akai et al., 1984; Fukasawa and Li, 1986; Fukasawa et al., 1986). Although the identity of the other hybridizing sequences is not yet known, for convenience all bands

Figure VI.

**SOUTHERN ANALYSIS OF MOUSE-CHINESE HAMSTER
MAPPING PANEL DNAs**

DNAs from several well-characterized mouse-Chinese hamster somatic cell hybrids were digested with the restriction enzyme EcoR1 and probed with pML1.6. The + and - symbols indicate the presence or absence of mouse chromosome 7 in each somatic cell hybrid. The position of a 14 kbp band that segregates with mouse chromosome 7 is indicated.

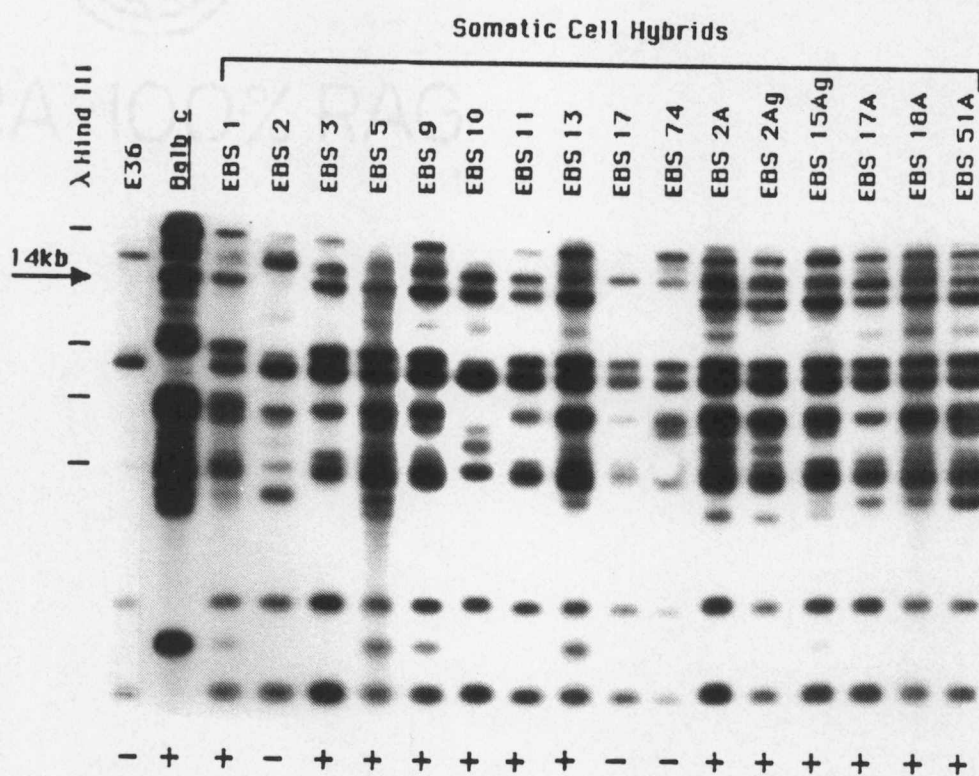


Figure VI.

hybridizing to pML1.6, except for the expressed LDH-A gene, will be referred to as LDH-A pseudogenes.

The great abundance of LDH-A pseudogenes seen in mouse DNA seems to be restricted to the Muridae. Twenty to thirty hybridizing bands are seen in Southern analysis of DNA from mice and rats (data not shown), but only five to seven bands are seen in Southern analysis of DNA from hamsters (see Lane 1, Figure VI) and humans (Chung et al., 1985). It is interesting to note that the number of pseudogenes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), another enzyme in the glycolytic pathway, also shows an increase specific to the Muridae (Piechaczyk et al., 1984; Tso et al., 1985). The significance of this increase is not yet known.

In this study, a mouse-Chinese hamster somatic cell hybrid mapping panel was used to correlate the presence or absence of restriction fragments hybridizing to pML1.6 with mouse chromosome 7 which contains *Ldh-1*, the mouse gene encoding the A subunit of LDH. The use of somatic cell hybrid panels for mapping loci in the mouse has been well established (Minna et al., 1975; Lalley et al., 1978; D'Eustachio et al., 1981; Albritton, 1986).

The somatic cell hybrid mapping panel used in this experiment was made by fusing clonal Chinese hamster cells to fresh, non-cultured mouse spleen cells (Minna et al., 1975; Lalley et al., 1978). Hybrid cells made in this manner segregate mouse chromosomes and enzymes (Minna et al., 1975). The mouse chromosome composition of each cell line was determined using

chromosome banding techniques and electrophoretic separation of mouse and Chinese hamster enzymes (Minna et al., 1975; Lalley et al., 1978). Assignment of a genetic locus to a particular mouse chromosome is made by comparing the segregation of a phenotypic marker for that locus with the segregation of mouse chromosomes in several somatic cell hybrids (Minna et al., 1975). For most loci mapped in this way, concordance of the tested marker with the presence or absence of a particular chromosome is not always one-hundred percent since the segregating chromosomes are often fragmented. For LDH-A, however, concordance should be exact because each cell line is typed for the presence or absence of mouse LDH-A activity as a marker for mouse chromosome 7.

Southern analysis of EcoR1 restricted mapping panel DNAs using pML1.6 as a probe showed at least one band, of approximately 14 Kb, segregating with mouse LDH-A activity (Figure VI). As expected, this band shows exact correlation with the presence or absence of mouse chromosome 7, providing strong evidence that the expressed gene for mouse LDH-A is contained, at least in part, in a 14 Kb EcoR1 restriction fragment. It is possible, however, that the 14 Kb EcoR1 band contains a pseudogene linked to *Ldh-1* on chromosome 7.

Loss of the 14 Kb EcoR1 hybridizing fragment in the deletion carried by 46DFiOD would more closely correlate this band with *Ldh-1*. Since the deletion carried by 46DFiOD is a recessive lethal, however, it is not possible to perform Southern analysis on

homozygous deletion DNA. In order to separate the deleted chromosome 7 from its normal homolog in heterozygotes, 46DFiOD females heterozygous for the p^* deletion characterized in Chapter II were mated to males of the feral mouse species *Mus spretus*. *Mus spretus* mice are polymorphic for the 14 Kb EcoR1 restriction fragment in question as shown in Figure VII. Progeny of the 46DFiOD x *Mus spretus* mating were tested for LDH activity in several tissues. Out of thirteen progeny tested, seven had reduced LDH activity in liver and skeletal muscle characteristic of the *Ldh-1* deletion (data not shown). Southern analysis of DNA from the thirteen 46DFiOD x *Mus spretus* progeny is shown in Figure VII. When pML1.6 is used as a probe, the complete absence of the 14 Kb EcoR1 band in deletion carrying progeny is obscured by a comigrating band. There is a marked difference in the intensity of hybridization in the deletion carriers, however (Figure VII). When a portion of the coding region of pML1.6 [a Pst1-Hinf-1 fragment of pML2 (Figure III, page 57)] is used as probe, the comigrating band is not seen and, as is evident in Figure VIII, deletion carriers in progeny of the 46DFiOD x *Mus spretus* cross lack the 14 Kb EcoR1 hybridizing fragment, providing convincing evidence that this fragment contains the expressed sequences for mouse LDH-A. It is possible, however, that the 14 Kb EcoR1 fragment contains an LDH-A pseudogene that is very tightly linked to *Ldh-1*, and so included in the 46DFiOD deletion. There do not appear to be any other hybridizing bands associated with both mouse chromosome 7 and the 46DFiOD deletion. Figures VII and

Figure VII.

**SOUTHERN ANALYSIS OF DNAs FROM 46DFiOD X
MUS SPRETUS PROGENY PROBED WITH pML1.6**

DNAs from 46DFiOD, *Mus spretus*, and F1 progeny of a mating between 46DFiOD deletion carriers and *Mus spretus* were digested with EcoR1 and probed with pML1.6. Lane 1: DNA from a +/+ 46DFiOD mouse. Lane 2: DNA from a *p*/+* 46DFiOD mouse, Lane 3: DNA from *Mus spretus*. Lanes 4-9: DNA from F1 progeny having normal LDH activity. Lanes 10-16: DNA from F1 progeny having reduced LDH activity. The 14 kbp EcoR1 fragment that segregates with mouse chromosome 7 (Figure VI) is indicated. The total absence of this band in F1 progeny carrying the 46DFiOD deletion of *Ldh-1* is obscured by a comigrating, but much less intense, band of hybridization.

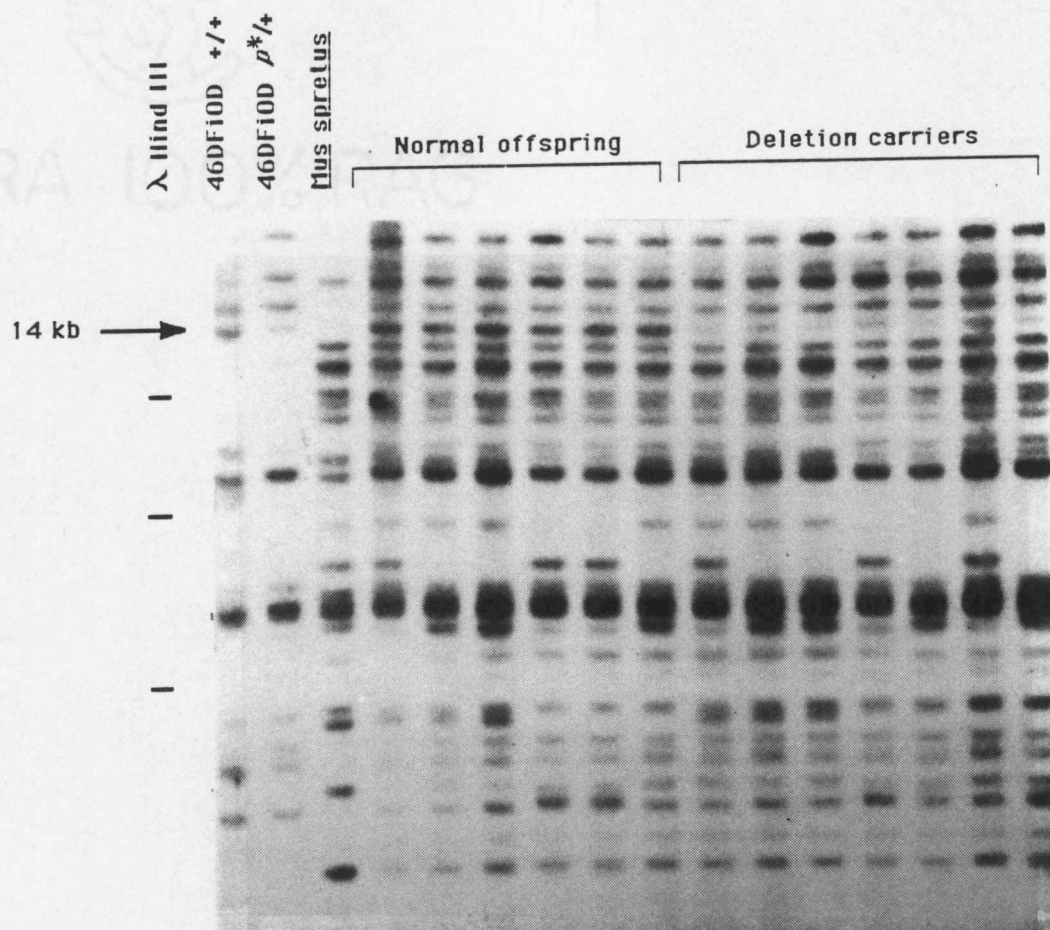


Figure VII.

Figure VIII.

**SOUTHERN ANALYSIS OF DNAs FROM 46DFiOD X
MUS SPRETUS PROGENY PROBED WITH
A FRAGMENT OF pML1.6**

DNA from 46DFiOD, *Mus spretus*, and F1 progeny of a mating between 46DFiOD deletion carriers and *Mus spretus* was digested with EcoR1 and probed with a restriction fragment from the coding region of pML1.6 (see Figure IV). Lane 1, DNA from a +/+ 46DFiOD mouse. Lane 2, DNA from *Mus spretus*. Lanes 3 and 4, DNA from F1 progeny having normal LDH activity. Lanes 5 and 6, DNA from F1 progeny having reduced LDH activity. The band comigrating with the 14 kbp EcoR1 restriction fragment that segregates with mouse chromosome 7 is not hybridized by this probe. F1 progeny having reduced LDH activity show a complete absence of the 14 kbp EcoR1 restriction fragment.

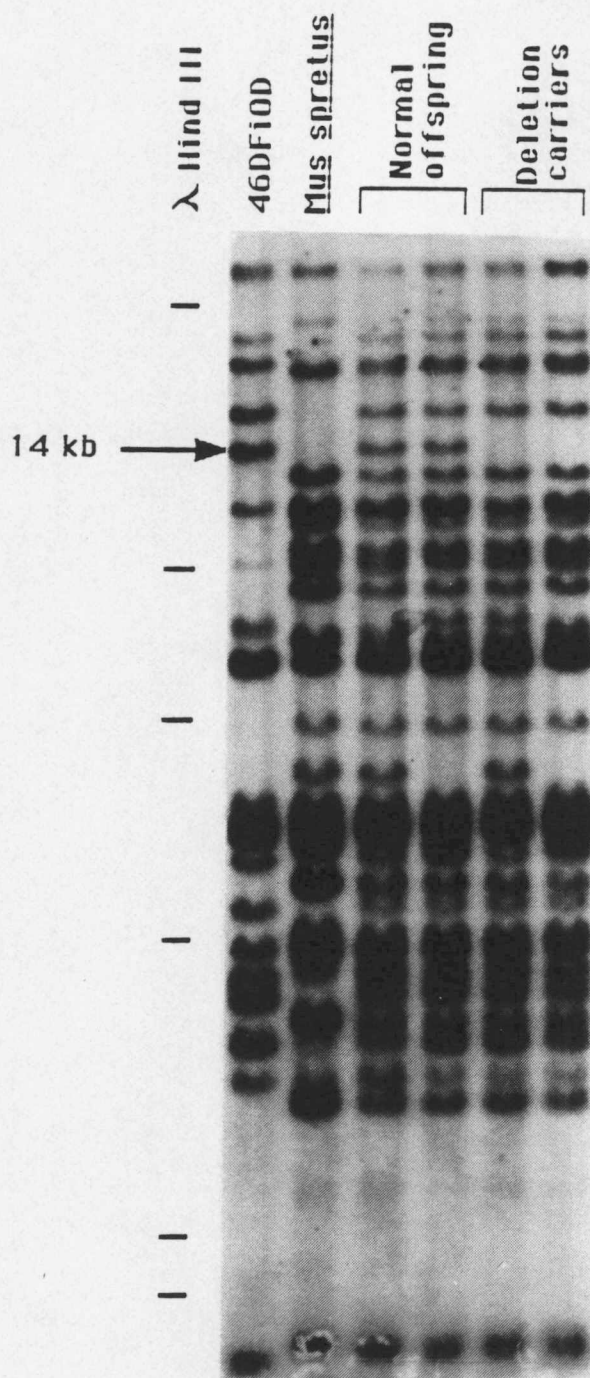


Figure VIII.

VIII also show many other polymorphisms between the hybrid offspring, but only the 14 Kb EcoR1 fragment segregates with LDH-A activity.

Chapter V

CLONING OF A MOUSE LDH-A GENOMIC SEQUENCE

SUMMARY:

DNA from a mouse-Chinese hamster somatic cell hybrid containing mouse chromosome 7 was digested to completion with EcoR1, size selected for 14 Kb fragments, and cloned into a bacteriophage lambda vector. This recombinant library was screened with pML1.6 and positive clones having EcoR1 inserts of 14 Kb were obtained. A single copy fragment from one of these clones, lambda LD14, did not hybridize to DNA from 46DFiOD x *Mus spretus* hybrid progeny carrying the *Ldh-1* deletion of mouse chromosome 7, and also segregated with mouse chromosome 7 in Southern analysis of DNA from the somatic cell hybrid mapping panel described in Chapter IV. The restriction map, orientation, and exon/intron structure of the DNA from this clone were determined, and it was shown that all of the exons represented in pML1.6 are present in this genomic clone. This clone was transfected into Chinese hamster cells where it directed the synthesis of large amounts of mouse LDH-A, demonstrating that this clone contains the expressed mouse gene for the A subunit of LDH, *Ldh-1*, and includes the promoter sequences necessary for transcription of that gene.

INTRODUCTION:

As described in **Chapter IV**, a somatic cell hybrid mapping panel and a mouse stock carrying a deletion of *Ldh-1* were used to identify a 14 Kb EcoR1 restriction fragment that probably contains the expressed sequences encoding mouse LDH-A. This chapter describes the isolation and characterization of a recombinant clone containing that 14 Kb restriction fragment. This clone was shown to direct the synthesis of mouse LDH-A following transfection into Chinese hamster cells.

MATERIALS AND METHODS:

Preparation of insert DNA: DNA from EBS11B6, a somatic cell hybrid containing mouse chromosome 7 (Albritton, 1986), was prepared by the following procedure. Monolayer cultures of EBS11B6 were grown in 15 centimeter (cm) dishes to confluence. Cells were rinsed in phosphate buffered normal saline, and ten ml lysis buffer [10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 10 mM NaCl; 0.5% SDS; 5.0 µg/ml proteinase K (Promega Biotec)] was added to each plate. After four hours of incubation at 37° C., another aliquot of 50 µg/ml proteinase K was added, and the incubation was continued for an additional four hours. After eight hours at 37° C., 10 ml water-saturated phenol, pH 8.0 was added to each plate, and plates were incubated at room temperature for three hours. The aqueous layer was then decanted and the phenol extraction was repeated. The aqueous layer was then similarly extracted with chloroform two times. Following the second

chloroform extraction, the aqueous layer, containing purified DNA, was dialyzed against TE [10 mM Tris-HCl pH 8.0; 1 mM EDTA], and stored at 4° C.

EBS11B6 DNA was digested to completion with the restriction enzyme EcoR1 (New England Biolabs), and separated by electrophoresis through agarose in a Tris-acetate buffer (Maniatis et al., 1982). A slice containing restriction fragments from 9 to 18 Kb was cut out of the gel, and DNA was extracted from the slice as described in Chapter III.

Library construction and screening: Insert DNA was ligated in a standard ligation reaction (Maniatis et al., 1982) to EcoR1 digested DNA from the bacteriophage lambda replacement vector, EMBL4 (Frischauf et al., 1984). Recombinant DNA molecules were packaged with Gigapak™ (Stratagene) extracts following manufacturers recommendations. Packaged bacteriophage were infected into the bacterial strain DB1161 (recA, recB21, recC2, sbcB15, rk-mk⁺) obtained from Dr. Arlene Wyman, and were plated and screened, as described by Benton and Davis (1977), with the LDH-A cDNA, pML1.6 which was labelled as described in Chapter III. Positive clones were plaque purified (Benton and Davis, 1977) and DNA was prepared from purified bacteriophage as described by Maniatis et al. (1982). DNA from recombinant bacteriophage was digested with Hinf1 and EcoR1 (New England Biolabs). Hinf1 digests were analyzed by electrophoresis through polyacrylamide gels in a Tris-borate buffer as described by Maniatis et al. (1982). EcoR1 digests were

analyzed by electrophoresis through agarose gels in a Tris-acetate buffer (Maniatis et al., 1982).

Isolation of single-copy fragments: Positive clones having inserts of 14 Kb were digested with various restriction enzymes and analyzed by the technique of Southern (1975) using pML1.6 and sheared mouse DNA as probes. Restriction fragments that did not hybridize to either pML1.6 or the repetitive sequences in mouse DNA were isolated and labelled as described in Chapter III, and used as probes in Southern analysis of EcoR1 digested mouse DNA. Restriction fragments of positive clones that hybridized to a single band in Southern analysis of mouse DNA were identified as single-copy sequences and used as probes in Southern analysis of DNA from the somatic cell hybrid mapping panel and the 46DFiOD x *Mus spretus* F1 progeny described in Chapter IV.

Restriction mapping: Cloned inserts of 14 Kb were digested with several restriction enzymes and the resulting fragments were resolved by electrophoresis through agarose or polyacrylamide gels by standard methods (Maniatis et al., 1982). All restriction enzymes were purchased from New England Biolabs. Insert DNA digested with various restriction enzymes was analyzed by the technique of Southern (1975) using various restriction fragments of pML1.6 as probes.

Transfections: DNA from a recombinant clone containing presumptive LDH-A sequences (pUCLD14) was digested to completion with EcoR1 and transfected into the Chinese hamster

fibroblast line CHTG49 by the method of Parker and Stark (1979). CHTG49 cells were grown as monolayer cultures in 100 mm dishes in Dulbecco's modification of Eagle's medium (DME) (Gibco) supplemented with 10% fetal calf serum. Transformations were done when cells were 50-60% confluent. For each 100 mm plate of cells transfected, 12 μ g of EcoR1 digested pUCLD14 were mixed with one μ g pSV2neo plasmid DNA (Southern and Berg, 1982) and precipitated with calcium chloride in the presence of sodium phosphate. The precipitated DNA was added to the cells, and cells were incubated at room temperature for twenty minutes, then at 37° C. for four hours. As controls, cells were transfected with pSV2neo DNA alone, and were also mock transfected with no DNA. Following the four hour incubation at 37° C., cells were washed in fresh medium and refed. After two days of growth, cells were split into DME containing 10% fetal calf serum plus 0.5 mg/ml G418 (Gibco). After one week to ten days in selective media, G418 resistant colonies of cells were isolated, grown to the desired density and analyzed for LDH activity.

LDH assays: Clonally isolated transformed cell lines were grown to confluence in 100 mm dishes. Cells from each 100 mm dish were scraped into 3 ml phosphate buffered normal saline and pelleted by centrifugation at 1000 x g for 15 minutes. Cell pellets were rinsed in 3 ml lysis buffer [10 mM sodium phosphate, pH 7.5; 1 mM EDTA; 5 mM β -mercaptoethanol], repelleted, and suspended in 100 μ l lysis buffer. Cells were lysed by repeated freezing at minus 70° C. and thawing at 37° C. Following three rounds of

freezing and thawing, lysed cells were centrifuged in an air driven ultracentrifuge (Airfuge™, Beckman) at 100,000 x g for one hour. The LDH specific activity was determined in supernatants from each cell line as described in Chapter II. Cell lines having elevated levels of LDH were analyzed by polyacrylamide gel electrophoresis as described in Chapter II, except that gels were loaded for equal LDH activity rather than equal protein, and electrophoresis was continued for 12 hours rather than 3.5 hours. The increased time of electrophoresis was necessary to separate Chinese hamster LDH-5 from mouse LDH-5.

RESULTS AND DISCUSSION:

In order to determine the exon/intron structure of *Ldh-1* and to analyze possible regulatory elements in that gene, it is necessary to obtain recombinant clones containing *Ldh-1* sequences. In the previous chapter, Southern analysis of DNA from a somatic cell hybrid mapping panel and similar analysis of DNA from the F1 offspring of an interspecific cross between a mouse stock heterozygous for a deletion of *Ldh-1* and *Mus spretus* was used to identify a 14 Kb EcoR1 restriction fragment of *Mus musculus* DNA that probably contains sequences of *Ldh-1*, the expressed gene for mouse LDH-A.

Recombinant clones containing that 14 Kb restriction fragment could be obtained by using pML1.6 to screen a recombinant library prepared from total mouse genomic DNA. Such a screen, however, would yield a large number of

recombinant clones containing LDH-A pseudogenes as well as clones containing the expressed gene for LDH-A since there are so many sequences in the mouse genome having homology to pML1.6. In order to reduce the number of pseudogene sequences obtained in a library screen, a size-selected recombinant library was prepared from genomic DNA of a mouse-Chinese hamster somatic cell hybrid cell line, EBS11B6.

Size selection was accomplished by digesting genomic DNA to completion with EcoR1 and gel purifying restriction fragments of approximately 14 Kb. Complete digestion of genomic DNA with a restriction enzyme such as EcoR1, whose target sites are not distributed randomly throughout the mouse genome, markedly reduces the complexity of a genomic library constructed from that DNA. It has been calculated that over 65% of mouse DNA digested to completion with EcoR1 is less than five Kb in length (Botchan et al., 1973).

A mouse-Chinese hamster somatic cell hybrid was used as a source of insert DNA because, as shown in Figure VI, page 69, mouse LDH-A cDNA hybridizes to fewer sequences in Chinese hamster DNA than in mouse DNA. The somatic cell hybrid used, EBS11B6, is a thioguanine-resistant derivative of EBS11, one of the cell lines in the somatic cell hybrid mapping panel described in Chapter IV. As shown in Figure VI, page 69, EBS11, which contains a small complement of mouse chromosomes including mouse chromosome 7, shows a relatively simple pattern of hybridization to pML1.6. The cell line EBS11B6, due to thioguanine

selection, has fewer mouse chromosomes than EBS11 (Albritton, 1986), and has a correspondingly simpler pattern of hybridization to pML1.6 (data not shown). Prior to DNA extraction, the EBS11B6 cells used to construct the recombinant library were re-tested for the production of mouse LDH-A and found to be positive for that marker.

Size-selected, EcoR1-digested, EBS11B6 DNA was cloned into a bacteriophage lambda replacement vector (Frischauf et al., 1984), and the recombinant molecules were packaged *in vitro* into infectious virions (Benton and Davis, 1977). The recombinant bacteriophage were propagated in a recombination-negative (recABC) bacterial host since previous attempts to obtain the desired 14 Kb EcoR1 restriction fragment insert from recombinant libraries propagated in recombination-competent hosts had proved unsuccessful. A total of 300,000 plaque-forming units were screened with pML1.6 (Benton and Davis, 1977). Since the complexity of the recombinant library screened was reduced by EcoR1 digestion and size selection, the number of plaques screened should represent several genomes of EBS11B6 DNA (Botchan et al., 1973).

Thirty-five positive clones hybridizing to pML1.6 were obtained from this screen. Digestion of purified DNA from each clone with a restriction enzyme that cuts mammalian DNA frequently (Hinf1) showed that the thirty-five positive clones represented five unique insert sequences. Digestion of DNA from

each clone with EcoR1 showed that two of the five unique unique insert DNA sequences were 14 Kb in length.

Single-copy probes were obtained from each of the unique positive clones having EcoR1 insert sizes of 14 Kb and used as probes in Southern analysis of DNA from the 46DFiOD x *Mus spretus* progeny described in Chapter IV. Although a single-copy probe from one of the 14 Kb EcoR1 inserts hybridized to *Mus musculus* DNA in 46DFiOD deletion carriers, a single-copy probe from the other recombinant clone having a 14 Kb insert produced the hybridization pattern shown in Figure IX. This probe hybridizes to a single band of fourteen Kb in EcoR1-digested 46DFiOD DNA, and to a single band of approximately twelve Kb in *Mus spretus* DNA. Hybrid offspring of the 46DFiOD x *Mus spretus* mating having normal LDH activity have both the fourteen and twelve Kb bands hybridizing to this single copy probe while hybrid offspring having reduced LDH activity have only the twelve Kb *Mus spretus* hybridizing band. Hybridization of this probe also segregated with mouse chromosome 7 in Southern analysis of the mouse-Chinese hamster somatic cell hybrid mapping panel described in Chapter IV. The blot shown in Figure VI, page 69, was used for this analysis. This clone therefore contains the presumptive sequence encoding mouse LDH-A, and was named lambdaLD14. The EcoR1 insert from this clone was subcloned into a plasmid vector, pUC, and the subclone was named pUCLD14.

The restriction map of pUCLD14 was determined and is shown in Figure X. DNA from pUCLD14 was digested with various

Figure IX.

**SOUTHERN ANALYSIS OF DNAS FROM 46DFiOD
X *MUS SPRETUS* PROGENY PROBED WITH
A FRAGMENT OF λ LD14**

DNAS from 46DFiOD, *Mus spretus*, and F1 progeny of a mating between 46DFiOD deletion carriers and *Mus spretus* were digested with EcoR1 and probed with an EcoR1--Nco1 single copy restriction fragment of pUCLD14. Lane 1: DNA from a +/+ 46DFiOD mouse. Lane 2: DNA from a $p^{*}/+$ 46DFiOD mouse, Lane 3: DNA from *Mus spretus*. Lanes 4-9: DNA from F1 progeny having normal LDH activity. Lanes 10-16: DNA from F1 progeny having reduced LDH activity. The 14 Kb EcoR1 fragment that segregates with mouse chromosome 7 (Figure VI) is indicated.

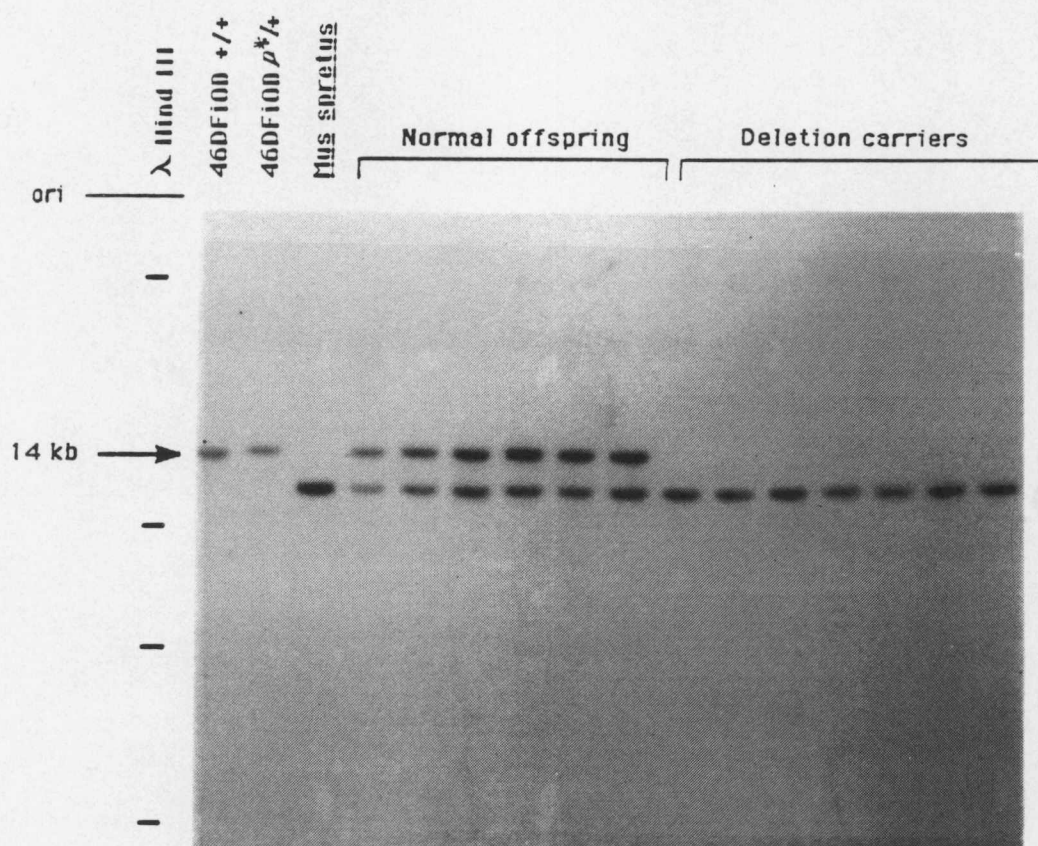
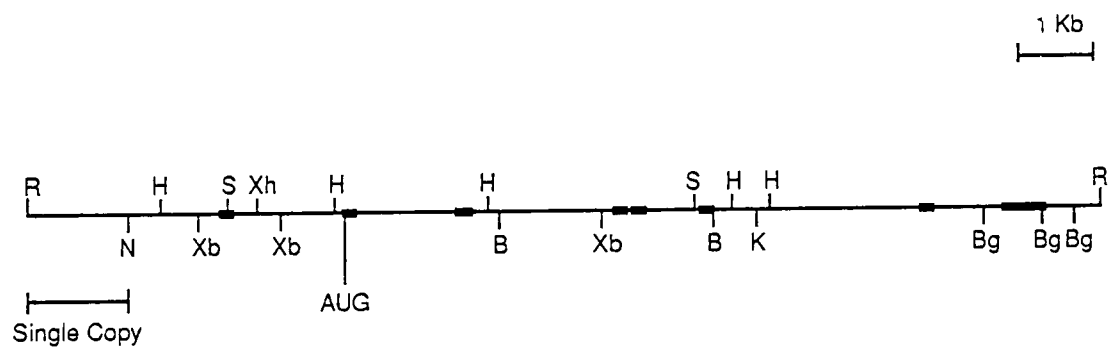


Figure IX.

Figure X.

**RESTRICTION MAP OF pUCLD14, A
MOUSE LDH-A GENOMIC CLONE**

The restriction sites in pUCLD14 are shown for several enzymes. The exons represented in pML1.6 are indicated by dark boxes and the location of the AUG start site of translation is shown. Exons are not drawn exactly to scale. The positions of exons 2-8 are from Li et al. (1985). The position of exon 1 is approximate. The position of the single copy probe used in **Figure IX** is shown.



R --- EcoR1
 N --- Nco1
 Xb --- Xba1
 S --- Sac1
 Xh --- Xho1
 H --- Hind3
 B --- BamH1
 K --- Kpn1
 Bg --- Bgl2

Figure X.

restriction enzymes and probed in Southern analysis (Southern, 1975) with fragments from the 5' and 3' termini of pML1.6 to determine the orientation of pUCLD14 with respect to transcription. The approximate position of the exons and introns of *Ldh-1* were determined by probing digests of pUCLD14 with small fragments from all regions of pML1.6. pUCLD14 appears to be identical to a mouse genomic LDH-A clone obtained by Li et al. (1985). These researchers have determined the exact position of exons two through eight using DNA and amino acid sequence data. The exact positions of exons two through eight from Li et al. (1985), and the approximate position of exon one, as determined by hybridization to the 5' region of pML1.6, are shown in **Figure X**.

Since pUCLD14 contains both exons and introns, it almost certainly contains the expressed gene for mouse LDH-A. It is possible, although unlikely, that pUCLD14 contains an unprocessed pseudogene or a heretofore undiscovered second *Ldh-a* gene tightly linked to *Ldh-1* rather than the genetically identified *Ldh-a* gene expressed in adult mouse tissues and somatic cell hybrids. Additionally, although pUCLD14 contains all of the exons represented in pML1.6, it is possible that pML1.6 is not actually full-length since the 5' terminus of mouse LDH-A RNA has not been confirmed by primer extension. Therefore, there may be another LDH-A exon upstream from the first exon shown in

Figure X. Additionally, full expression of LDH-A may require enhancer elements that are not included in pUCLD14.

In order to obtain critical evidence on whether or not pUCLD14 contains the expressed mouse gene for LDH-A, and to determine whether this clone contains all of the sequences necessary for expression of the LDH-A protein, DNA from pUCLD14 was transfected into a Chinese hamster cell line, CHTG49, along with DNA from the plasmid pSV2neo (Southern and Berg, 1982), which confers resistance to the antibiotic G418. The DNA used to transfect CHTG49 cells was a co-precipitate of pSV2neo and pUCLD14, containing a ten-fold excess of pUCLD14 DNA. Under these conditions, nearly all of the cells that take up pSV2neo DNA will also take up pUCLD14 DNA. As controls, cells were mock transfected with no DNA, and also transfected with pSV2neo alone.

Following transfection, cells were cultured in the presence of G418 for a period of ten days. After ten days of growth in selective media, mock transfected plates of cells showed no growth, while plates transfected with pSV2neo and pSV2neo plus pUCLD14 had several clonal colonies. Clones resistant to G418 from the transfection of pSV2neo and pSV2neo plus pUCLD14 were isolated and grown to confluence. Extracts from each cell line were made as described above, and analyzed for LDH specific activity. Out of five independently isolated clones transfected with pUCLD14, four had LDH-specific activities approximately ten-fold higher than those of CHTG49 cells or of CHTG49 cells transfected

with pSV2neo alone. The remaining pUCLD14 transfectant had slightly elevated levels of LDH activity compared to controls.

Extracts from the five transfected cell lines having elevated LDH activity were assayed for mouse LDH-A by electrophoresis through a polyacrylamide gel followed by staining for LDH activity. The results of this assay are shown in Figure XI. Figure XI shows the LDH isozyme pattern for Chinese hamster extracts from CHTG49 cells, mouse extracts from NIH 3T3 cells, extracts of EBS11B6, the somatic cell hybrid line from which pUCLD14 was isolated, and extracts from the five pUCLD14/pSV2neo transfectants. The bands shown in lanes one and two of Figure XI correspond to Chinese hamster and mouse LDH-5, the A₄ isozyme, since rodent cells in tissue culture synthesize primarily LDH-A subunits (Lalley et al., 1978).

The bands shown in lane three, which contains extracts from EBS11B6, represent hybrid tetramers between mouse and hamster LDH-A subunits. As shown in lanes four through eight, CHTG49 cells transfected with pUCLD14 synthesize large amounts of mouse LDH-A. In some cell lines, the amount of mouse LDH-A is so great that most of the LDH activity is in mouse LDH-A homotetramers. Hybrid tetramers of mouse and hamster LDH-A subunits are seen at very low levels. The amount of mouse LDH-A produced by the cell lines shown in lanes four, five, seven, and eight, is even greater than this gel indicates since each lane was loaded with approximately equal amounts of LDH activity rather than equal amounts of protein to avoid over-development of the

Figure XI.

**POLYACRYLAMIDE GEL OF CHINESE HAMSTER CELLS
TRANSFECTED WITH pUCLD14 AND STAINED
FOR LDH ACTIVITY**

Extracts of Chinese hamster, mouse, EBS11B6, and pUCLD14 transfectants were separated by electrophoresis through polyacrylamide gels and stained for LDH activity. Lane 1: Extract from CHTG49, a Chinese hamster fibroblast cell line. The position of Chinese hamster LDH-5 is indicated. Lane 2: Extract from mouse NIH 3T3 cells. The position of mouse LDH-5 is indicated. Lane 3: Extract from EBS11B6. Bands shown represent Chinese hamster LDH-5, and hybrid tetramers composed of Chinese hamster and mouse LDH-A subunits. Lanes 4-8: extracts from CHTG49 cells transfected with pUCLD14. Bands in lanes 4, 5, 7, and 8 represent mouse LDH-5 and hybrid tetramers containing three mouse LDH-A subunits and one Chinese hamster LDH-A subunit. Bands in lane 6 are Chinese hamster LDH-A₄ and Chinese hamster LDH-A₃:mouse LDH-A₁.

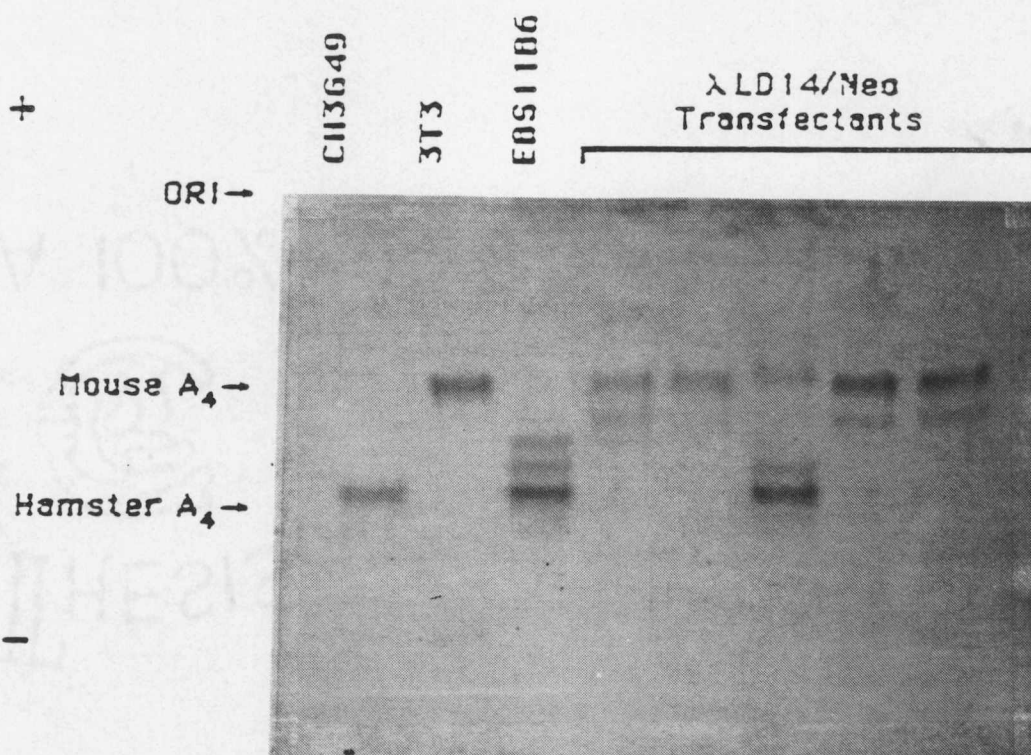


Figure XI.

histochemical stain in the lanes containing extracts from the transfected cell lines. The large amount of mouse LDH-A produced in most of the transfectants is probably due to the integration of multiple copies of pUCLD14 into the CHTG49 genome, since cells that are competent to take up DNA by the transfection protocol used usually take up large amounts of DNA (Parker and Stark, 1979).

The result presented in **Figure XI** demonstrates conclusively that pUCLD14 contains the gene encoding mouse LDH-A, and further demonstrates that this clone, which contains approximately three Kb of sequence flanking the 5' terminus of pML1.6, contains the promoter sequences necessary for transcription of LDH-A mRNA.

Chapter VI

SEQUENCING OF A PORTION OF THE MOUSE LDH-A GENE

SUMMARY:

A portion of pUCLD14 from a Hind3 site one Kb upstream from the 5' terminus of the full-length LDH-A cDNA, pML1.6, to the second exon containing the start site of translation, was sequenced. This analysis showed that the promoter region of *Ldh-1* is G-C rich, lacks the consensus sequences TATAA and CAAT, and contains several possible regulatory elements. The hexamer CCGCCC associated with the transcription factor Sp1 occurs three times upstream from the putative start site of transcription. The sequence CTGACGTCAG, a consensus sequence necessary for cyclic AMP induction of a number of eukaryotic genes is also found upstream from the transcriptional start site.

INTRODUCTION:

In the previous chapter a cloned sequence, pUCLD14, was shown to direct the synthesis of mouse LDH-A in a heterologous rodent cell, the Chinese hamster fibroblast line, CHTG49. A comparison of pUCLD14 with pML1.6, a mouse full-length LDH-A cDNA, showed that pUCLD14 contains approximately three Kb of flanking sequence upstream from the 5' terminus of pML1.6. Since pUCLD14 is expressed in cells, the promoter sequences must be contained within three Kb of the 5' terminus of pML1.6. In

order to identify putative regulatory regions and to determine the structure of the promoter of *Ldh-1*, the nucleotide sequence of a portion of the 5' region of pUCLD14 was determined. Sequence analysis extended from a Hind3 restriction site approximately one Kb upstream from the region hybridizing to the 5' terminus of pML1.6 to the AUG start site of translation of the LDH-A message, about three Kb downstream.

MATERIALS AND METHODS:

Subcloning: Several subclones of the 5' region of pUCLD14 were constructed by standard methods (Maniatis et al., 1982). Insert DNAs were prepared from the appropriate restriction enzyme digests of pUCLD12 as described in **Chapter III**, and cloned into the plasmid vector BLUESCRIPT™ (Stratagene). Recombinant subclones were propagated in the *E. coli* strain JM109 as described in **Chapter III**. DNA isolations were as described in **Chapter III**.

Sequencing: DNA was sequenced by base-specific chemical cleavages as described by Maxam and Gilbert (1980). The desired restriction digest of the appropriate pUCLD12 subclone was end labelled as described by Maniatis et al. (1982). Following end labelling, DNAs were extracted with phenol and chloroform, ethanol precipitated, and redigested overnight with the appropriate second restriction enzyme. DNA fragments labelled on one strand were isolated following electrophoresis through polyacrylamide gels in a Tris-borate buffer (Maniatis et al., 1982).

Polyacrylamide gel slices containing labelled DNA fragments were crushed with a glass pestle and soaked overnight at 37° C. in [0.5 M ammonium acetate, pH 5.5; 1 mM EDTA]. DNA was recovered from the crushed gel slices by filtration through a 0.4 micron filter (American Lab Supply) followed by repeated ethanol precipitation. Chemical cleavages were as described by Maxam and Gilbert (1980). Cleaved fragments were resolved by electrophoresis through 8% polyacrylamide gels containing [7.6% acrylamide, 0.4% bisacrylamide; 8.3 M Urea, 100 mM Tris-borate, pH 8.3; 2 mM EDTA]. Gels were cast between 30 x 50 centimeter glass plates and were mounted in a Biorad DNA sequencing apparatus. Electrophoresis was at 2000 V in Tris-borate, pH 8.3 (Maniatis et al., 1982). Each sample was loaded three times at three hour intervals for a total of nine hours of electrophoresis. Following electrophoresis, gels were dried under vacuum at 80° C. (LKB gel dryer) and exposed to Kodak XAR film. Approximately three-hundred base pairs of DNA could be resolved per restriction fragment using this system.

Sequence analysis: Sequence data were analyzed on a Macintosh Plus™ personal computer using *The DNA Inspector II+* (Textco) software.

RESULTS AND DISCUSSION:

In order to facilitate the detailed restriction mapping and preparation of labelled restriction fragments required by DNA sequencing using the chemical cleavage method of Maxam and

Gilbert (1980), a number of subclones containing overlapping fragments of pUCLD14 were generated. Figure XII shows a diagram of those subclones. The sequencing scheme used is also shown in Figure XII. DNA sequence was generated by standard methods (Maxam and Gilbert, 1980), and the sequence analysis extended from a Hind3 site approximately one Kb upstream from the 5' terminus of pML1.6 to the AUG start site of LDH-A translation. The nucleotide sequence of approximately three Kb of pUCLD14 is shown in Figure XIII.

In order to determine whether pML1.6 contains all the exons present in full-length mouse LDH-A mRNA, the sequence of pUCLD14 flanking the 5' terminus of pML1.6 was examined for homology to the full-length rat LDH-A mRNA described by Matrisian et al. (1985). The 5' terminus of the rat LDH-A mRNA has been confirmed by primer extension (Matrisian et al., 1985).

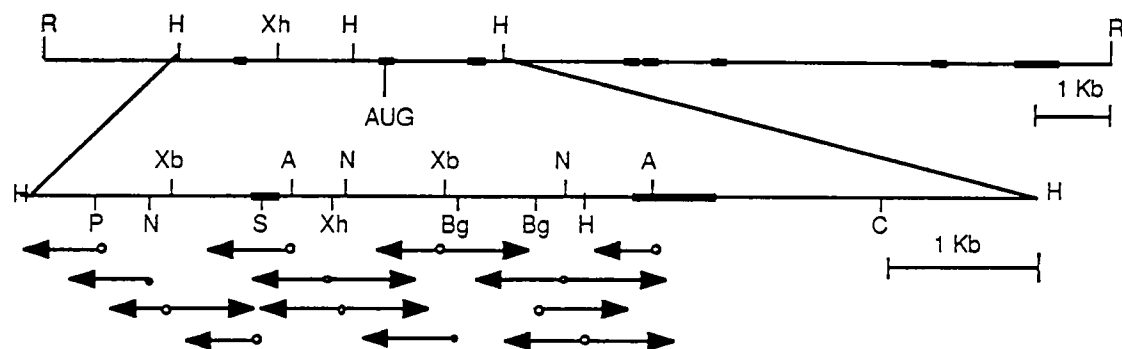
The position of the first exon represented in pML1.6 is shown in Figure XIII. Figure XIV shows an expanded version of the region immediately flanking this exon. The sequence immediately adjacent to the 5' terminus of pML1.6 has homology to the additional seventeen nucleotides present in full-length rat LDH-A mRNA indicating that, at least in cultured cells such as the MEL cells that were the source of pML1.6, transcription of *Ldh-1* probably begins about seventeen base pairs 5' to the terminus of pML1.6.

Analysis of the sequence upstream from the presumptive start site of transcription of *Ldh-1* showed that this gene lacks

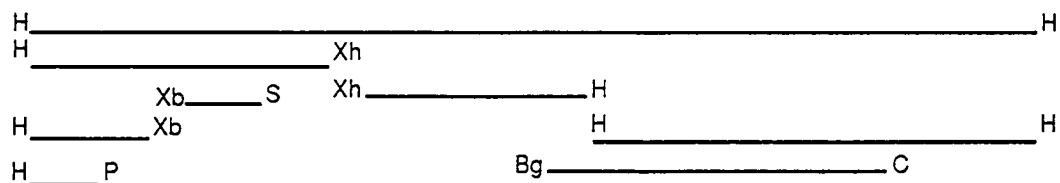
Figure XII.

**DIAGRAM OF SUBCLONES AND SEQUENCING
SCHEME OF pUCLD14**

A restriction map of pUCLD14 is shown. Subclones used for sequencing are shown by lines below the restriction map. The scheme used to determine the sequence of the 5' region of pUCLD14 is shown by directional arrows. Bold dots below a restriction site indicate the position at which a DNA fragment was labelled. Arrows indicate the direction of sequencing.



SUBCLONES



R --- EcoR1
 N --- Nco1
 Xb --- Xba1
 S --- Sac1
 Xh --- Xho1
 H --- Hind3
 A --- Ava2
 P --- Pst1
 Bg --- Bgl2
 C --- Cla1

Figure XII.

Figure XIII.

SEQUENCE ANALYSIS OF A PORTION OF pUCLD14

The nucleotide sequence of approximately three kbp of the 5' region of pUCLD14 is shown. The positions of Hind3, Xba1, Sac1, and Xho1 restriction sites are shown for orientation with the restriction map shown in Figure X. Double underlines indicate transcribed regions. Exons one and two of *Ldh-1* are indicated. Sp1 binding sites are underlined and G-C rich regions are indicated by a wavy underline. The consensus sequence for induction by cAMP is underlined and in italics. The first six codons and amino acids of LDH-A are indicated.

Yha1
 AAGCTTTGTGATATTAAATGGCAGACTGCTCTCCAGGTCACACTGTTTACTTTTAAATTTTTAAATTTTC
 GCTGTGCTGAGGGACTCAGGCTTGGTGTATGCAAGACAAAGTGTCCCTGTCTGGCCACACTGCTAGGCTG
 TTGTTTGTATAAAGAGATCACAAGGATACAGAGCCAGCCAAATAGGGGGGCTGTGAGCTTCATTCC
 CTCTCACATGATCCCTGCATATCCAGCACCTGCAACCAAGCTTCTTCAAACTCTTGTCAAGACTGTATAGA
 CTTAATCTGCAAGTGAACACATCCTTCCGGGGAGATGGGTGAGCAGGGTTGAAGTCAAGTTCTTCCCATC
 ACTAAGGAATCAACAGCTTGCACACTCCATTTTCACTCCTATCAGTGGTGGAGTCAAGATTAGCATC
Yha1
 CCGTTGATCGGGAGCTGGGAGAGGACAGGAACTGGCCTCACAGCTTAAAGAGCTCTAGAAAGACCTTTA
 AAGGCAGAGGGGTGTGTGAACACAGCAGGGGCTGATACCTGTTGTAAAGCTAAACCAATGCTGCTC
 GGGATGGCATGGGAGAGGGCAGATATGGATGTAAAGCTGGCAAGCCATCAGAGCTGAGCCGACCCGCTCC
 CCCCATGCTTTGGGAGATGGAAGTGGGGCAGGAGAAAGGCCATCTCATAGGCTGCTATGCCATAGACCG
 GCACGCTGCTGGCATGTGCTTTCAACCAATATTTACTGAAGGCTGTGCTTGGCAGGAGTTGTTCCGGGG
 CCGGAACAGCAATGAAGAAAGTGAACAGGGTTTTTTCTGAGTCTCACAACTTTTCCGGTGAAGGAGGGGAG
 GATCGATGCAATTTCCGGCTCCTGCTTCTGAGGCTGAGGAGCATGTCCGGTTGGCCTTTCTTTGGGTGTCCG
 AGCACACCTGGAGCCCACTCTTGCAGGGACATCTGCTGGGGGGGGGGGGGGTCTCGGTGGCCCTAGCC
 CGGCTGGAGCGGGGGGGGGGGGGGGGGGGTACACGTGGGTCCGACCTCCGCTGGGCTCCCACTCTGAGCT
Sac1
AGCGGAGCTTCATTTAAGGGCGGGGGGGGGTCTGCTGCTGCTGCTGGAGCCACTGTCCCGGAGCTCGGG
 Exon 1
CCACGCTGCTTCTGCTGGCAGTCCGCGCCCATCTGATCTAGCGTTGGGCGCCACCTGCGCGCACAG
 GGGGTCCCTATCCGGGTGGAGGTGCACGGGTGTTCAAGTTTGGGCACCGTGGGCTACTGTGCTTTGGGA
 ACTGAGCAGGCGCGCCACCCAGCTTGGGGCGCGAGACCTGTAAAGAGGACTAAGGGTGTGGCTGAGC
Yha1
 AAGGTTGGGAGCCACCAACCGGGGGCGAGGGAGCGGTGCACAACTTGAAGTCCGCTAACTCAGCCATGGGG
 GCGCAAAAGCCTTGACCTCCGGGGCGGGTGTCTCGAGCAGCCGTGCGGTTTGCATTTCTTTGCGTGG
 GCTCGGTGAGTGGTTGTTCTGTCAGATTATAGGGCGCTCTTGGCCTAAACCGCGCTGCTGGCGAGAGGA
 AGCGGGAGAGTCCAGTCCCTTCTGTCACCTAGCGGAGGAGGAACTGTGCTGAGAGGCTGCAGCAC
 CCATCGGTCTAGGTGGGAAGTAGAGGCACCTGTCTTAATAGAGCACAGACTTGAGGTTGCTGAGTA
Yha1
 GATTCTGTAGGGAGAGGCCCCACGCCCTGCCCCAGTGAGCAGAGACCTCACAGGCTGTCTAGAAATAGC
 AGCGTTGTAAAGAGACCGATCCCTGCTTGAATGGCCCTAGCGTCTTGGCAGAGATCTTGTCCAGTCTCT
 TTGCACCTTGAACGATTTCAAAATAGACATGGTGTCTGCTGGGAAGGTGGCCATCCCGGGGTGGAGT
 GGGCTCCAGGCTCAGGCTCCGCATATTGTATCCCATTTTGAAGTGAAGAAATTTCCCTTAGCGGCCACA
 TCCTGGGTAAAGAGCCCGCTGTGGTGAAGGCCCTAATCCTGTTAGTGGCATTTTGAATCTCACTCTG
 GCACCAGGCTAAGGTGGCTGCCAGCTCCACTTACCGCGCTTGTGCTTTGGGCTCTAGTAAGGGCCAACTT
 TGACCCCAACTGCTGCCAGAGGTCTCATCCATGCCAGCAGTCCGTAGACCTATATATATGCACCTGATT
 CTGAATATACGCGCACTGCTTCCCGCGCTGCCAGTCTTCTGATCTGGGCTGGTCATACACTTGGGTT
Yha1
 TGCGGGGTGGGGGGTTAGAAGAGCTTGCSCGTGAGGCTTAAGCACCTTGCTATGCTTGGGGTCCGAC
 CTTGTGGCGTTATTGGCGCCCTGTGCTTGTGATTTTGGTACTTCTGGAGCACTGGCGCTACTTGGT
 GTAGGGCTCTGGGTGATGGGAGAGAGCGGGAGCGGGAGGGCAGCTTCTAACCATATAAGAGGAGATACCA
 TCCGCTTTTGGTTTATCAGATGAGTAACTCCTCAGGCGCTACACGTACACGGAGACCTCGGTATTATTTT
 TCCATTTCAAGGTAGAGCTTCTGTAGAGCCAGAACCATCTGCCGCTGCTATTCTTGGTTTTCCACT
 TCTGTTCTTTGTACATTTGCTACTTAATGAAGAGGAGTCCAGGTAGCAAGTCAAGCTTTTTTTTTTTTTT
 TTAATACAGGGACTTGGTGAATATACCTTGGGAGGTTACAAATGACACACGGTGTATACCCCTTAGGCTC
 Met Ala Thr Leu Lys Asp
 AAAAGATTCAAGTCCAG ATG GCA ACC CTC AAG GAC
 Exon 2

Figure XIII.

Figure XIV.

SEQUENCE ANALYSIS OF THE PUTATIVE
REGULATORY REGION OF *Ldh-1*

The nucleotide sequence immediately flanking the first exon of *Ldh-1* is shown. The portion represented in pML1.6 is shown by a railroad underline. The additional sequence homologous to rat LDH-A mRNA is shown by a dashed underline. Consensus sequences for Sp1 binding (CCGCCC) are surrounded by boxes. G-C rich regions are shown by a dotted underline. The consensus sequence for induction by cAMP is shown by a bold dotted underline.

5' T G T T G C T T G C C A G G A G T T G T T C C C G G C G C C G G A A C A G C A A T G A A
 G A A A G T G A C C A G G G T T T T T T C T G A G T C T C A C A G T T T T C C G G T G
 A A G G A G G C G G A G G A T C G A T G C A T T T C G G G C T C C T G C T T C T G A G G
 C T G A G G A G C A T G T C G G G T T G G C T T T C T T T G G G G T G T C G C A G C A
 C A C G T G G A G C C C A C T C T T G C A G G G A C A T C G T G C T G C G C G C G C C G
C C C C G G T C T C G G T G G C G C C T A G C C C G G C T G G A C G C C C G C C C C G
G C C C A G C C T A C A C G T G G G T T C C G C A C G T C C G C T G G G C T C C C A C T
C T G A C G T C A G G C G G A G C T T C C A T T T A A G G C C C G C C C G C G T G C T
 G C T C T G C G T G C T G G A G C C A C T G T C G C C G A G C T C G G G C C A C G C T G
C T T C T C C T C G C C A G T C G C C C C C C A T C G T G C A T C T A G C G G T T G G
 G C C C C A C G C T G C C G G C A C A G G G G T C C C T A T C C G G G G T G G A G G T
 G C A C G G G T G T T C A G A T T T G G G C A C G C G T G G G C T A C T G T G C T T T G 3'

===== Transcribed region from pML 1.6
 ----- homology to rat LDH-A mRNA
 ===== cAMP responsive site
 [] Sp 1 binding site
 G-C rich region

Figure XIV.

both the TATAA and CAAT consensus sequences that are characteristic of many eukaryotic promoters (Maniatis et al., 1987).

Recently, a number of promoter regions of eukaryotic genes have been described that also lack TATAA and CAAT boxes (see Dynan, 1986, for review). Such promoter regions belong to housekeeping genes, genes whose expression is necessary for general cell metabolism and that are therefore expressed in all cells of an organism. Instead of TATAA and CAAT boxes, many housekeeping genes, such as the mouse genes for hypoxanthine phosphoribosyl transferase (HPRT) and dihydrofolate reductase (DHFR), contain G-C rich regions immediately upstream from the transcriptional initiation site. Some of the G-C rich regions of the HPRT and DHFR promoters contain the sequence CCGCCC, the the consensus sequence for binding of the cellular transcription factor Sp1 (Melton et al., 1986; Dynan et al., 1986). In both genes, the Sp1 binding sites occur within fifty base pairs of the start site of transcription, and deletion analysis has shown that sequences within fifty base pairs of the start site are sufficient for transcription of these genes (Dynan, 1986). The G-C rich regions of the DHFR promoter have been shown by direct *in vitro* experiments to bind Sp1 (Dynan et al., 1986). In addition to Sp1 binding sites, the promoter of mouse DHFR contains direct repeats of twelve to fifteen base pairs (McGrogan et al., 1985).

Since *Ldh-1* is expressed in most cells of the mouse, it is possible that this gene has a promoter region similar to that of

housekeeping genes. In order to determine whether *Ldh-1* has such a promoter, the DNA sequence upstream from the first exon was searched for direct repeats, G-C rich regions, and the Sp1 binding consensus sequence CCGCCC.

No obvious pattern of direct repeats was seen, but there are four G-C rich regions within three-hundred and fifty base pairs of the presumptive start site of transcription as shown in **Figure XIV**. Three of these, which lie within one-hundred and fifty base pairs of the transcriptional start, contain potential Sp1 binding sites (**Figure XIV**). *Ldh-1*, therefore, has a promoter region that is characteristic of housekeeping genes.

Since the *Ldh-a* gene in rats is inducible by cAMP in the glioma cell line RGC6, the sequence of *Ldh-1* was examined for the presence of consensus sequences shown to be involved in the cAMP responsiveness of other eukaryotic genes such as the rat somatostatin gene (Montminy et al., 1986), the rat phosphoenolpyruvate carboxykinase gene (Short et al., 1986), and the human proenkephalin gene (Comb et al., 1986). The sequence CTGACGTCA, which is identical to the responsive region of the rat somatostatin gene (Montminy et al., 1986), and similar to the responsive region of several eukaryotic genes including those mentioned above (see Montminy et al., 1986), occurs once in *Ldh-1*, about forty base pairs upstream from the transcriptional start site, as shown in **Figure XIV**. In the rat somatostatin gene, the sequence CTGACGTCA occurs at a similar distance (thirty to

seventy base pairs) from the transcriptional initiation site (Montminy et al., 1986).

The promoter region of *Ldh-1*, therefore, contains several presumptive regulatory regions: three possible binding sites for the cellular transcription factor Sp1, and a site necessary for induction by cAMP. Deletion mapping of the *Ldh-1* promoter has been initiated in order to determine whether these consensus sequences actually do play a role in LDH-A expression.

Additionally, since Sp1 binding appears to direct synthesis of transcripts from both strands of DNA in some promoters (Saffer and Singer, 1984; Farnham et al., 1985; Dynan, 1986), it will be interesting to determine whether transcription occurs from both strands of *Ldh-1*.

Chapter VII

FURTHER CHARACTERIZATION OF THE 46DFiOD DELETION OF MOUSE CHROMOSOME 7

SUMMARY:

The 46DFiOD deletion was originally identified by deletion of the *p* locus on mouse chromosome 7. The chromosomal segment missing in 46DFiOD also includes *Ldh-1* as described in Chapter II. Genetic studies at the Oak Ridge National Laboratory have shown that the coat color marker, *ru-2*, is also included in that segment. Several additional markers mapping to mouse chromosome 7 were tested for inclusion in or exclusion from the 46DFiOD deletion. The markers *Hras-1*, *Xld-1*, *Lh β* , and *Atpa-2* are not included in the chromosomal segment missing in 46DFiOD, while the markers *Saa* and 24.2 are included in that segment. The deletion carried by the mutant mouse stock, 46DFiOD, thus extends approximately five cM and includes five known genetic markers.

INTRODUCTION:

In this thesis, a mouse stock heterozygous for a deletion of a portion of chromosome 7 was used to provide information concerning the regulation of expression of a mouse structural gene. This deletion stock was also used to distinguish the expressed copy of a gene from numerous pseudogenes. Deletion stocks can also be used to provide information about the linkage map of the mouse.

By inclusion or exclusion in a deletion, the relative order of genetic markers on a chromosome can be determined. In particular, knowledge of the markers included in a deletion such as 46DFiOD, which is one of a number of overlapping deletions involving the *p* locus on mouse chromosome 7, can provide a framework for complementation analysis of other *p*-locus deletions. Such complementation analysis has been done for similar deletions at the *c*, *d*, and *se* loci and has yielded fine-structure maps of functional units in the chromosomal regions surrounding those markers (Russell, 1971; Russell et al., 1982).

Several markers mapping to mouse chromosome 7 were tested for inclusion in the 46DFiOD deletion using the 46DFiOD x *Mus spretus* progeny described in Chapter IV. This testing takes advantage of the high degree of natural polymorphism, at both the protein and the DNA levels, between the two mouse species *Mus musculus* and *Mus spretus* (Bonhomme et al., 1984; Albritton, 1986). The markers tested include: *Hras-1*, the Harvey murine sarcoma provirus (Ellis et al., 1980), *Atpa-2*, the structural gene for one isoform of the alpha subunit of the sodium-potassium ATPase (Kent et al., 1987), *LH β* , the structural gene for the β -subunit of lutropin (Chin et al., 1983), *Xld-1*, the structural gene for xylose dehydrogenase (XLD) (Newton et al., 1982), *Saa*, the serum amyloid gene complex, (Taylor and Rowe, 1984), and 24.2, a random probe from a region of the short arm of human chromosome 11 (Tom Glaser, unpublished data). The coat color marker, *ru-2*, had previously been tested for inclusion in the

46DFiOD deletion by Clyde Montgomery at the Oak Ridge National Laboratory Biology Division using standard genetic crosses.

MATERIALS AND METHODS:

Mice: 46DFiOD x *Mus spretus* mice were those described and characterized for LDH activity in Chapter IV.

Southern analysis: DNA from 46DFiOD x *Mus spretus* was extracted as described in Chapter IV. Southern blots were prepared as described in Chapter IV, except that DNAs were digested with various restriction enzymes as described below and probed with a variety of probes. The *Hras-1* probe was provided by Lorraine Albritton. The *Atpa-2* probe was provided by Rachel Kent. The *LH β* , *Saa*, and 24.2 probes were provided by Tom Glaser. When 24.2 was used as a probe, hybridization was in [30% formamide; 1 M NaCl; 50mM Tris-HCl, pH 7.4; 10% Dextran sulfate; 0.5% SDS; 1X Denhardt's solution; 1 mg/ml denatured salmon testes DNA]. When blots were reprobed, filters were washed for 15-20 minutes at 70° C. in [50% formamide, 5X SSC, 1X Denhardt's solution, 0.1% SDS, 100 μ g/ml denatured salmon sperm DNA]. Probes were labelled as described in Chapter III. Prehybridization was at 42° C. for 8-16 hours, hybridization was at 42° C. for 42-48 hours. Following hybridization, filters were washed to the stringency appropriate for each probe: Filters probed with *Hras-1*, *Saa*, or *Atpa-2* were washed to 0.1X SSC, 55° C; filters probed with 24.2 or *Lh β* were washed to 0.5X SSC, 55°.

Filters were exposed for one to several days to Kodak XAR X-ray film with an intensifying screen (DuPont) at -70° C.

XLD assays: Hybrid offspring were typed for XLD by the procedure of Newton et al. (1982). Liver extracts were prepared from each animal as described in Chapter II. XLD assays were done within two hours of sacrificing the animal. Extracts were applied twice with a zipzone loader onto Titan III cellulose acetate plates (Helena Laboratories, Inc.) which had been soaked in Tris-glycine pH 8.5. Electrophoresis was carried out for thirty minutes at 200 V in Tris-glycine pH 8.5. Xylose dehydrogenase was stained by an agar overlay technique in [0.26 mM NADP; 50 mM L-xylose; 65 mM Tris-HCl, pH 7.4; 0.3 mM PMS; 0.9 mM MTT]. Chemicals used in the stain were purchased from Sigma. Stain was allowed to develop to the desired intensity, and plates were fixed in [10% acetic acid; 10% methanol].

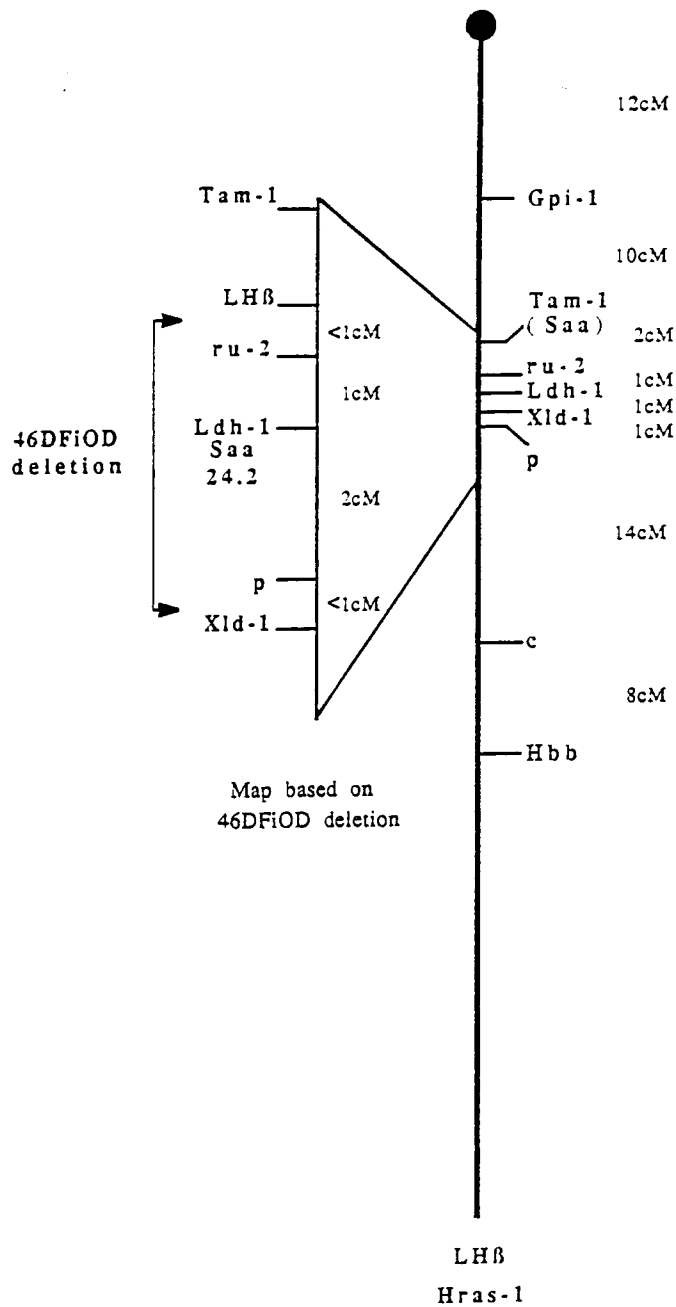
RESULTS AND DISCUSSION:

Figure XV shows a partial linkage map of mouse chromosome 7 (Davisson and Roderick, 1986; Tom Glaser personal communication). The markers tested for inclusion in the 46DFiOD deletion are surrounded by boxes. In general, the markers tested were those that have been mapped to chromosomal positions near the *p* locus on mouse chromosome 7. The markers *Hras-1* and *Atpa-2* were tested for their inclusion in the 46DFiOD deletion prior to their assignment to a particular locus on mouse chromosome 7.

Figure XV.

PARTIAL LINKAGE MAP OF MOUSE CHROMOSOME 7

A diagram of a portion of mouse chromosome 7 is shown. The region from below *p* to above *Tam-1* is shown at the left of the figure. The gene order in the expanded version is based on the order indicated by mapping of markers to the region of chromosome 7 missing in 46DFiOD (see text). The approximate boundaries of the 46DFiOD deletion are shown by arrows.



Linkage Map by
Standard genetics

Figure XV.

The tested markers had been positioned on mouse chromosome 7 in a variety of ways. The markers *ru-2*, *p*, *Ldh-1*, and *Xld-1*, the structural gene for xylose dehydrogenase (XLD), were assigned to chromosomal loci through standard genetic three-point crosses (see references in Peters, 1987). *Saa*, the serum amyloid gene complex, was shown to be linked to *Tam-1*, a marker five cM proximal to *p* (Davisson and Roderick, 1986), by recombinant inbred analysis and congenic strains (Taylor and Rowe, 1984). *Hras-1*, the Harvey murine sarcoma provirus (Ellis et al., 1980), *Atpa-2*, the structural gene for one isoform of the alpha subunit of the sodium-potassium ATPase (Kent et al., 1987), *LH β* , the structural gene for the β -subunit of lutropin (Chin et al., 1983), and 24.2, a random probe from a region of the short arm of human chromosome 11 syntenic to the *Ldh-1* region of mouse chromosome 7 (Lalley et al., 1978; Glaser et al., 1985), were mapped to mouse chromosome 7 using a mouse-Chinese hamster somatic cell hybrid mapping panel (Kent et al., 1987; Tom Glaser, unpublished data). These markers were assigned to loci on chromosome 7 by analyzing the frequency of meiotic recombination in offspring of interspecific matings between *Mus musculus* and *Mus spretus*. This method of genetic mapping was developed by Benoit et al. (1985), and exploited by Albritton (1986) and Tom Glaser (unpublished data) to map several markers to various mouse chromosomes, particularly mouse chromosome 7.

In order to test markers previously assigned to mouse chromosome 7, advantage was taken of the high degree of

polymorphism at the DNA and protein level between the two mouse species, *Mus musculus* and *Mus spretus* (Bonhomme, 1984). Results of testing each of the markers mentioned above for inclusion of the 46DFiOD deletion are shown in Table V.

With the exception of *Xld-1*, all markers tested exist as DNA probes, and show restriction fragment length polymorphisms (RFLPs) between *Mus musculus* and *Mus spretus*. Typing for the DNA markers was done by Southern analysis of 46DFiOD x *Mus spretus* F1 progeny, similar to the analysis for *Ldh-1* described in Chapter IV. Typing for *Xld-1* was done by an assay for the XLD protein. Hybrid progeny were identified as deletion carriers based on LDH activity in muscle and liver extracts as described in Chapter IV. These animals were conclusively shown to be deletion carriers by Southern analysis using a single copy probe from pUCLD14 as described in Chapter V.

When used as probes in Southern analysis of DNA from 46DFiOD x *Mus spretus* F1 progeny, DNA markers that are included in the chromosomal segment missing in 46DFiOD should show two bands of hybridization to DNA from F1 progeny having normal LDH activity, but only one band, that of the *Mus spretus* parent, in F1 progeny having reduced LDH activity. Markers that are not included in the 46DFiOD deletion should show the same pattern of hybridization to DNA from all F1 progeny. Figures XVI and XVII show examples of each type of result.

TABLE V
RESULTS OF TESTING MOUSE CHROMOSOME 7 MARKERS
FOR INCLUSION IN THE 46DFiOD DELETION

Marker	Polymorphism	Distance from <i>Ldh-1</i>	Inclusion in the 46DFiOD deletion*
Centromere	-----	25 cM	-----
<i>Atpa-2</i>	Taq1	12.9 cM	+
<i>LH-β</i>	Taq1	1.1 cM	+
<i>Saa</i>	Msp1	?	-
24.2	Taq1	?	-
<i>ru-2</i>	genetic	1 cM	-
<i>Ldh-1</i>	EcoR1	0	-
<i>p</i>	genetic	2 cM	-
<i>Xld-1</i>	protein	1 cM (?) see text	+
<i>Hras-1</i>	BamH1	49.1 cM	+

*Minus (-) means included in the 46DFiOD deletion, i. e. missing from the deleted chromosome 7 carried by 46DFiOD. Plus (+) means present on the deleted chromosome 7.

Figure XVI shows a Southern blot of DNA from 46DFiOD x *Mus spretus* F1 progeny probed with *Saa*, a marker that is included in the 46DFiOD deletion. As shown, a hybridizing band of approximately ten Kb present in the 46DFiOD lane but missing in the *Mus spretus* lane segregates with normal LDH activity.

Figure XVII shows a Southern blot of DNA from F1 progeny probed with *LH β* , a marker that is not included in the 46DFiOD deletion. All F1 progeny show both parental hybridizing bands.

Xld-1, the structural gene for xylose dehydrogenase (XLD), has not been cloned, so it is not possible to test for inclusion of this marker in the 46DFiOD deletion by Southern analysis. XLD is, however, polymorphic at the protein level between *Mus musculus* and *Mus spretus*. The XLD produced by both *Mus spretus* and 46DFiOD is in the form of homo-dimers that are electrophoretically separable and can be visualized by a specific histochemical stain. Hybrid offspring between the two mouse stocks should have three staining bands of XLD activity, two representing the parental homo-dimers, and one representing the *Mus musculus*/*Mus spretus* hetero-dimer. If *Xld-1* is included in the 46DFiOD deletion, hybrid progeny having reduced LDH activity should have only one staining band, that representing the *Mus spretus* homodimer. Figure XVIII shows a cellulose acetate plate of liver extracts of (46DFiOD x *Mus spretus*) F1 progeny stained for XLD activity. All progeny, regardless of LDH activity levels, produce both *Mus musculus* and *Mus spretus* XLD subunits, indicating that *Xld-1* is not in the 46DFiOD deletion.

Figure XVI.

**SOUTHERN ANALYSIS OF DNAS FROM 46DFiOD X
MUS SPRETUS PROGENY PROBED WITH *Saa***

DNAS from 46DFiOD, *Mus spretus*, and F1 progeny of a mating between 46DFiOD deletion carriers and *Mus spretus* were digested with MspI and probed with *Saa*. Lane 1: DNA from a +/- 46DFiOD mouse. Lane 2: DNA from *Mus spretus*. Lanes 3-8: DNA from F1 progeny having normal LDH activity. Lanes 9-15: DNA from F1 progeny having reduced LDH activity. A 10 Kb MspI fragment detected by this probe is missing in F1 progeny carrying the 46DFiOD deletion of *Ldh-1*. Faint bands of approximately 10 Kb are seen in lanes 12-15 but the hybridization intensity of these bands relative to those seen in lanes 3-8 is greatly reduced. Equality of the amount of DNA loaded per lane can be inferred from the hybridization intensity of a 6 Kb band present in lanes 2 and 6-15. Results of a Southern blot in which the same hybrid DNAs were digested with BamHI and probed with *Saa* confirm that assignment of this marker to the portion of chromosome 7 missing in 467DFiOD (data not shown).

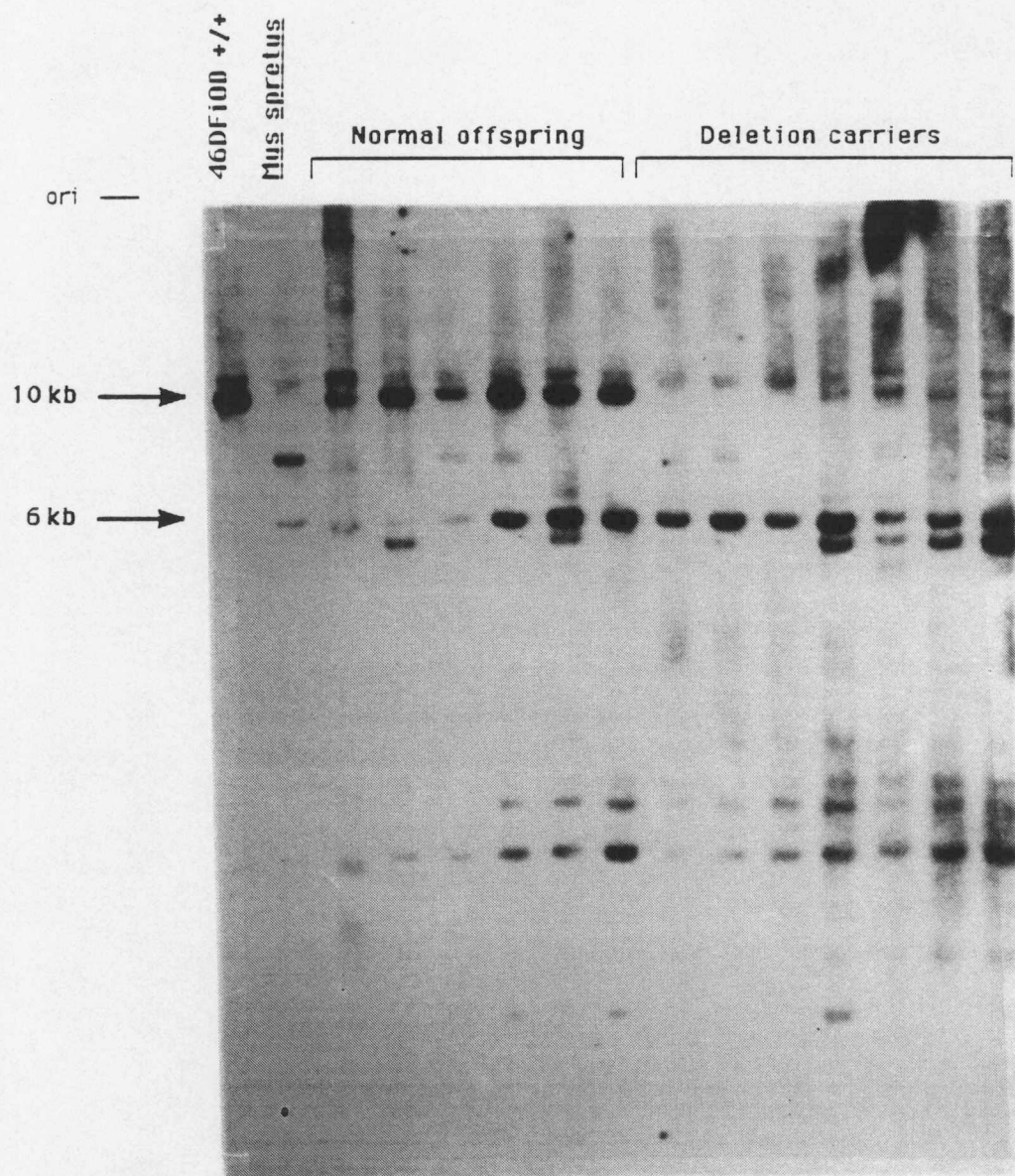


Figure XVI.

Figure XVII.

**SOUTHERN ANALYSIS OF DNAS FROM 46DFiOD X
MUS SPRETUS PROGENY PROBED WITH *LH β***

DNAS from 46DFiOD, *Mus spretus*, and F1 progeny of a mating between 46DFiOD deletion carriers and *Mus spretus* were digested with *TaqI* and probed with *LH β* . Lane 1: DNA from a +/+ 46DFiOD mouse. Lane 2: DNA from *Mus spretus*. Lanes 3-7: DNA from F1 progeny having normal LDH activity. Lanes 8-12: DNA from F1 progeny having reduced LDH activity. All progeny have the same pattern of hybridization to the *LH β* probe.

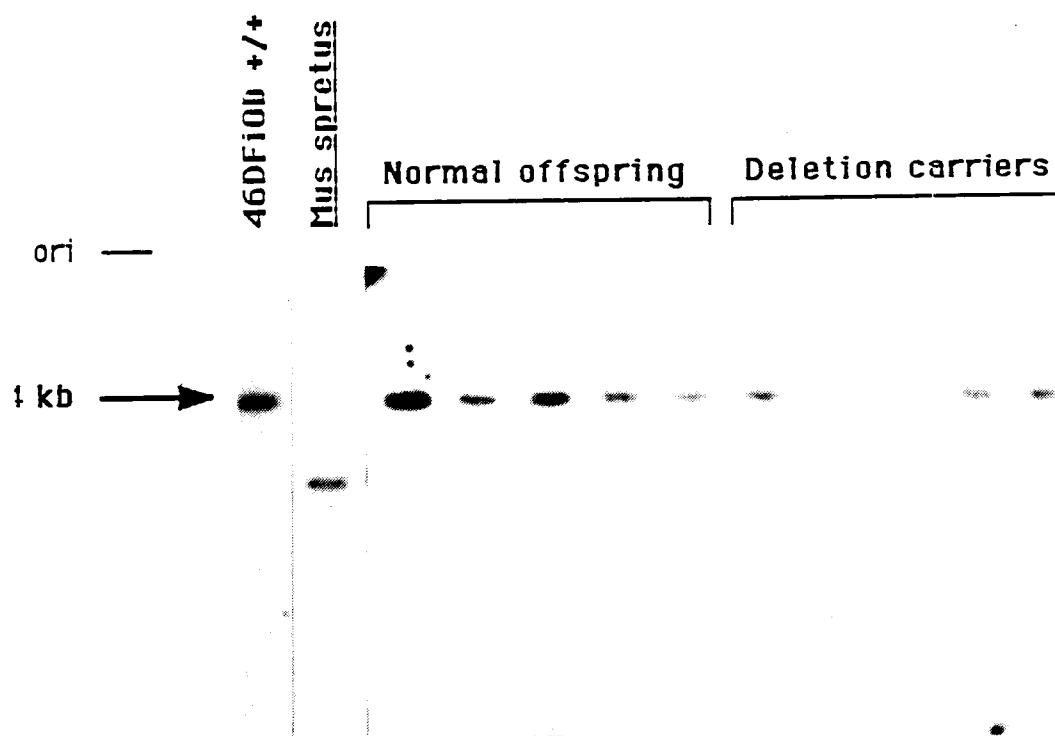


Figure XVII.

Figure XVIII.

CELLULOSE ACETATE PLATE OF LIVER EXTRACTS
OF 46DFiOD X *MUS SPRETUS* PROGENY
STAINED FOR XLD ACTIVITY

Cellulose acetate plate showing XLD polymorphism between *Mus musculus* and *Mus spretus*. All lanes were loaded with equal amounts of protein. Lane 1, 46DFiOD (+/+). Lane 2, extracts of 46DFiOD and *Mus spretus* mixed together. Lane 3, *Mus spretus*. Lanes 4 and 5, hybrid offspring having normal LDH activities. Lanes 6 and 7, hybrid offspring having reduced LDH activities. All offspring show the *Mus musculus* XLD band indicating that XLD is not included in the 46DFiOD deletion. Two bands of staining activity are seen in hybrid offspring corresponding to the *Mus musculus* Xld homodimer and a *Mus musculus/Mus spretus* heterodimer. The *Mus spretus* homodimer is not seen due to the reduced activity of Xld in *Mus spretus* liver relative to that of *Mus musculus* liver (compare lanes 1 and 3).

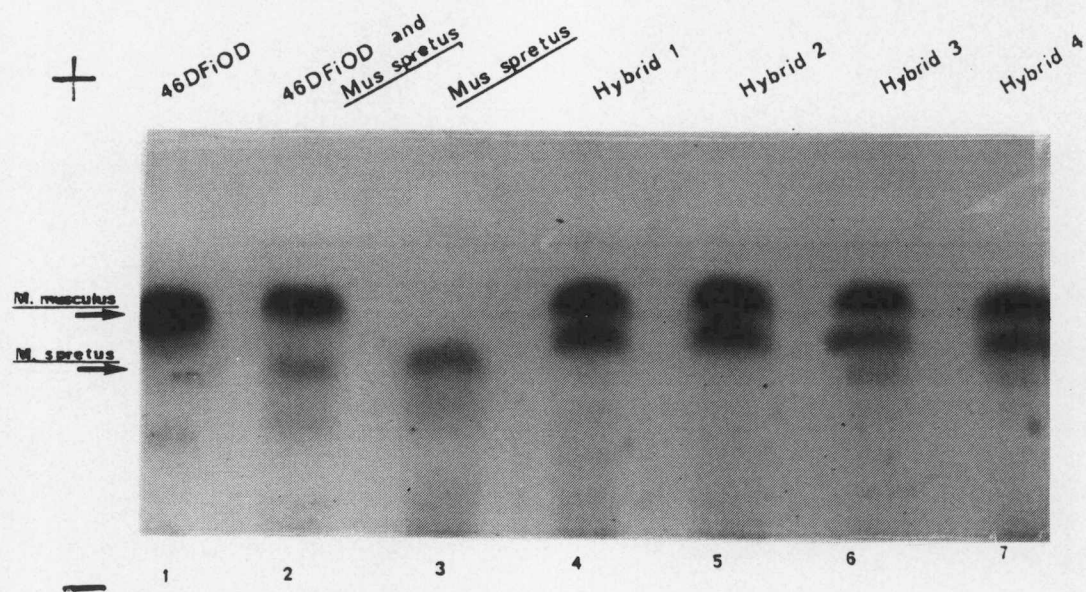


Figure XVIII.

As shown in Table V, page 119, the 46DFiOD deletion includes the markers *p*, *Ldh-1*, *ru-2*, *Saa* and 24.2, and excludes the nearby markers *LH β* and *Xld-1*. The proximal boundary of this deletion is, therefore, between *LH β* and the nearest included marker (see Figure XV, page 115). Although the gene order of *Saa* and 24.2 with respect to each other and to *Ldh-1* has not been determined (there were no meiotic recombinations observed between these markers in ninety-six *Mus musculus* x *Mus spretus* N1 progeny tested; Albritton, 1986, and Tom Glaser, personal communication) the recombination distance between these three markers and *LH β* is approximately one cM (Tom Glaser, unpublished data). The proximal boundary of the 46DFiOD deletion is, therefore, very close to *ru-2*.

The distal boundary of the 46DFiOD is more difficult to ascertain. *Xld-1* has been mapped genetically using two three-point crosses, one involving *Gpi-1* and *Hbb*, the other involving *Tam-1* and *Hbb* (Newton et al., 1982), to a locus between *p* and *Ldh-1*, as shown in Figure XV, page 115. There are two possible explanations for the exclusion of *Xld-1* from the 46DFiOD deletion. The map position of *Xld-1* could be inaccurate since it is difficult to assign map positions accurately within one and two cM distances using a three point cross involving a marker as distant as *Hbb*, which is twenty-two cM distal to *p* (Davisson and Roderick, 1986). The mapping data for *Xld-1*, taken together, allow for an error of 1.6 to 1.8 cM in the distal direction. Therefore, it is possible that

the physical location of *Xld-1* is actually distal to *p*. The assignment of *Xld-1* to the least conservative chromosomal position based on the mapping data of Newton et al. (1982) would indicate that the distal boundary of the 46DFiOD deletion is within one cM of *p*. The other possible explanation for the exclusion of *Xld-1* from the 46DFiOD deletion is that this deletion is not a simple aberration, but is actually the result of at least two events: one affecting the chromosomal region around *p*, the other affecting the region around *Ldh-1--ru-2*.

It is more probable that the aberration carried by 46DFiOD is a single deletion. The mutation-rate experiments of Russell (1951) indicated that the specific-locus method measures mostly single-hit events. Mutation rate curves produced by the specific-locus method are either linearly related to dose, or, when not linear, the curve is convex upward. The typical curve for two-hit aberrations is concave upward (Russell, 1965). In particular, analysis of the mutation rate for induction of deficiencies that simultaneously involve *d* and *se*, closely linked markers (recombination frequency 0.16%; Davisson and Roderick, 1986) on chromosome 9, indicates the single-hit nature of mutations recovered in the specific-locus mutation-rate experiments. The frequency of induced deficiencies involving both *d* and *se* relative to the total of all mutations does not decline at lower dose rates as is expected for two-hit events (Russell, 1965).

In addition to the kinetics of specific-locus mutations, there are other observations that support the hypothesis that the

deletion carried by 46DFiOD is the result of a one-hit event. The mutagenized mouse that produced 46DFiOD was a male, X-irradiated and mated so that mutations induced at the spermatogonial cell stage were recovered (Liane Russell, personal communication). At this stage, aberrations that are definitely due to two-hit events, such as translocations, are only rarely induced (Russell, 1965). Additionally, analysis of mutants at the *d*, *se*, and *c* loci produced by the specific-locus mutation-rate experiments, indicate that the majority of these deficiencies can be explained as the result of single deletions (Russell, 1971; Russell et al., 1982). Complimentation analysis of 102 deficiencies at *d* and *se* showed that, with the exception of two mutations, all could be fitted onto a linear map (Russell, 1971). Similar analysis of thirty-four lethal *c*-locus deficiencies indicated that all could be alligned in a linear pattern (Russell et al., 1982).

Although it is more likely that the aberration carried by 46DFiOD is a single deletion, more data is needed to determine whether this is indeed the case. Mapping of *Xld-1* by a more rigorous three-point cross would be one way to determine whether this locus lies proximal or distal to *p*. The physical gene order of *p*, *Xld-1*, and *Ldh-1* may also be determined by complementation mapping of the other *p*-locus mutations recovered in the specific-locus mutation-rate experiments conducted at Oak Ridge.

Complementation mapping of the other *p*-locus mutants at Oak Ridge may also be used to order the markers *Saa* and 24.2,

included in the 46DFiOD deletion. Another possible way to determine the relative gene order of those markers for which DNA probes exist, *Ldh-1*, *Saa*, and 24.2, would be to use them as probes in Southern analysis of DNA from 46DFiOD deletion carriers and their normal littermates using pulse-field gels (Schwartz and Cantor, 1984; Carle et al., 1986; Smith et al., 1987). Pulse-field gels separate DNA molecules as large as two hundred Kb, and have been shown to be useful in ordering markers in and around deletions of human chromosome 11 involving the Wilm's tumor-aniridia complex (Elise Rose, unpublished results).

The determination of the boundaries of the 46DFiOD deletion, as well as the ordering of markers in that deletion, can provide information about the linkage map of the mouse, and can also provide additional phenotypes for complementation mapping of the series of *p*-locus deficiencies isolated at the Oak Ridge National Laboratory. Analysis of the 46DFiOD deletion has also revealed a possible discrepancy between the genetic and the physical maps of mouse chromosome 7 involving the position of *Xld-1* relative to the *p*-locus.

Chapter VIII

CONCLUSIONS

The lactate dehydrogenase genes of mammals provide a useful and interesting system for studying gene regulation. The LDH protein is easily purified and, as a result, the amino acid sequences of the three mouse LDH subunits have been determined (Pan et al., 1983). The structural analysis has made it possible to estimate the evolutionary relationships between the subunits and, by extension, the evolutionary relationships between the genes that encode those subunits.

Another strength of the LDH system in mammals is the ease with which the LDH isozymes can be assayed. The facility with which changes in the levels of each subunit can be detected has led to detailed knowledge of the phenotypic expression of the genes encoding each LDH subunit. The LDH genes are regulated in a variety of ways. The gene encoding the C subunit in mammals is expressed in a very restricted cell type, the maturing spermatocyte, and at a restricted time in development, the mid-pachytene stage of spermatid development. The gene encoding the B subunit is expressed constitutively in all cells, except for the maturing spermatocyte, but the level of expression of the *Ldh-b* gene varies from tissue to tissue. This gene is activated in each tissue at a specific stage in the development of the animal as well. In addition to being expressed constitutively in almost all cells, the

Ldh-b gene shows a tissue-specific surge of expression in the developing oocyte. The gene encoding the A subunit of LDH is also expressed at constitutive levels in all cells but, in addition to some modulation in levels of expression in different tissues, this gene is inducible by agents that elevate the intracellular levels of cAMP. A comparison of the conservation or divergence of putative regulatory elements from each gene, therefore, should provide information about constitutive, tissue-specific, developmental, and inducible gene regulation.

The molecular analysis of *Ldh-1* described in this thesis showed that at least one mammalian *Ldh-a* gene has a promoter-regulatory region similar to those of housekeeping genes. This type of promoter is associated with genes that are expressed at relatively low levels, with little temporal regulation, and little tissue specificity. One can hypothesize that the putative regulatory elements seen in the promoter region of *Ldh-1* direct the expression of LDH-A at a relatively low but constant level in all tissues, and additional regulatory sequences are responsible for developmental activation of the gene, for the differences in levels of expression in various tissues, and for the response to induction by cAMP. One such "regulatory cassette" is the presumptive cAMP responsive sequence, CTGACGTCA, found in the promoter region of *Ldh-1*. Such sequences have been shown to be necessary, but not sufficient, for cAMP induction of the rat somatostatin gene (Montminy et al., 1986). Deletion mapping of the promoter region of *Ldh-1*, which has been initiated, should help to determine

whether the presumptive regulatory elements found in the *Ldh-1* promoter are actually involved in the constitutive or inducible expression of mouse LDH-A.

Recent analyses of the promoter regions of housekeeping genes have indicated that the promoters containing the consensus sequences TATAA and CAAT direct the synthesis of large amounts of RNA during restricted phases of development or restricted phases of the cell cycle. Since both LDH-B and LDH-C are synthesized at high levels in restricted cell types, it would be of considerable interest to examine the molecular structures of the promoter regions of the genes encoding those subunits. In particular, the molecular structure of the *Ldh-b* gene would be interesting since this gene is expressed both at constitutive levels in most mouse tissues, and at high levels in developing oocytes, suggesting the possibility that the promoter region of this gene might contain both G-C rich regions and TATAA and CAAT boxes. There is one example of a housekeeping gene with such a 'hybrid' promoter. The human gene for triosephosphate isomerase, which is also a glycolytic enzyme, has TATAA and CAAT sequences, but is considerably G-C rich (Brown et al., 1985). The significance of the presence of both housekeeping- and facultative-type promoter regions in this gene is not yet known.

In addition to the molecular analysis of some of the nucleotide sequences possibly involved in regulation of *Ldh-1*, the possibility of feedback regulation of the *Ldh* genes was examined in a mouse stock, 46DFiOD, heterozygous for a deletion of mouse

chromosome 7 including *Ldh-1*. Analysis of LDH activity levels and levels of each LDH isozyme in tissues of 46DFiOD deletion carriers and their normal littermates showed that expression of *Ldh-1* is linearly related to gene dosage. Lack of dosage compensation for *Ldh-1*, combined with the previously observed lack of dosage compensation for the *LDHB* gene in humans (Krone and Wolf, 1977), demonstrates that there is no feedback regulation of the *Ldh* genes by their products. This result also confirms the assumption, based on biochemical data, that the LDH isozyme profile in a tissue results from a binomial distribution of the A and B subunits produced in that tissue. Lack of compensation for a reduction in the number of genes encoding LDH-A also confirms the conclusion of Nadal-Ginard (1978) that differences in the number of A and B subunits in a particular tissue are due to differences in the rate of synthesis of each subunit in that tissue.

The deletion stock, 46DFiOD, used to examine the effects of gene dosage on LDH expression, also proved instrumental in distinguishing a restriction fragment containing the expressed copy of the mouse *Ldh-a* gene from a large number of restriction fragments containing presumptive mouse LDH-A pseudogenes. This analysis made use of a restriction fragment length polymorphism between 46DFiOD, a *Mus musculus* stock, and the feral mouse species, *Mus spretus*. Interspecific hybrid animals were produced by crossing 46DFiOD mice heterozygous for the recessive lethal deletion of mouse chromosome 7 with *Mus spretus*, in order to uncover the deleted copy of chromosome 7.

Southern analysis of DNA from hybrid animals using a mouse LDH-A cDNA probe showed that *Ldh-1* is contained in a 14 Kb EcoRI restriction fragment. This analysis also demonstrated directly that the deficiency carried by 46DFiOD is a physical deletion of a portion of mouse chromosome 7.

The production of interspecific hybrids using a deletion carrier as one parent has also provided a powerful tool for mapping genetic loci in the mouse. Since there is such a high degree of natural polymorphism between *Mus musculus* and *Mus spretus* (Albritton, 1986), it was possible, using a total of thirteen offspring from three matings, to determine the position of seven genetic markers relative to the region of mouse chromosome 7 missing in 46DFiOD. Using restriction fragment length polymorphisms between 46DFiOD and *Mus spretus*, two additional markers, *Saa* and 24.2, were shown to be included in the chromosomal segment missing in 46DFiOD. A third marker *Xld-1*, that maps genetically between two markers contained in that segment, was shown, by a protein polymorphism, to be excluded from the deletion carried by 46DFiOD. This exclusion of *Xld-1* indicates either that the published genetic map of the *p--Ldh-1* region of mouse chromosome 7 is incorrect, or that the deficiency carried by 46DFiOD is a complex chromosomal aberration. The chromosome-7 markers *Saa*, 24.2, and *Xld-1* can now be used in complementation mapping of a large number of *p*-locus deficiency animals generated in specific-locus mutation rate experiments at the Oak Ridge National Laboratory. Since 46DFiOD

is the only *p*-locus deficiency stock that involves *Ldh-1*, that marker constitutes the proximal end point for the complementation map of the *p*-locus deficiency mutants.

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