INFLUENCE OF GENE DUPLICATION AND *X*-INACTIVATION ON MOUSE MITOCHONDRIAL MALIC ENZYME ACTIVITY AND ELECTROPHORETIC PATTERNS[1]

EVA M. EICHER AND DOUGLAS L. COLEMAN

The Jackson Laboratory, Bar Harbor, Maine 04609

Manuscript received December 1, 1975
Revised copy received December 10, 1976

ABSTRACT

We have investigated, with and without the influence of *X*-inactivation, the relationship between autosomal gene-dosage and gene-product in a mammalian system, the mouse. The gene was mitochondrial malic enzyme (*Mod-2*), shown to lie on Chromosome *7* between the albino (*c*) and shaker-1 (*sh-1*) loci, and the enzyme was its product, mitochondrial malic enzyme (MOD-2). Gene duplication, with and without the influence of *X*-inactivation, was achieved using a translocation that involves the insertion of a portion of Chr *7*, including *Mod-2*, into the *X*, *T(X;7)1Ct*. A 1:1 relationship for *Mod-2* dosage and MOD-2 activity was found in heart mitochondria. Evidence of *X*-inactivation of *Mod-2* was noted in heart and kidney preparations from females carrying a *Mod-2* duplication (one copy of *Mod-2* in the *X* and two copies of *Mod-2* on Chr *7*). We conclude that the expression of an autosomal locus attached to *X*-chromatin depends upon whether the translocation is in a balanced or unbalanced state.

IN mammals there appears to be a one-to-one relationship between gene-dosage and gene-product (protein) when one or two functional copies of an autosomal structural locus are present (reviewed by KIRKMAN 1972). It is unknown whether this relationship still persists when three or more copies of a locus are present as a result of duplication or chromosomal trisomy.

It has been hypothesized that, although a direct relationship between gene-dosage and gene-product may exist as long as the involved autosomal loci remain surrounded by autosomal chromatin, this relationship may change when the autosomal loci are attached instead to inactive *X* chromatin (see review by EICHER 1970). To date, no evidence has accumulated at the protein level to confirm the hypothesis that autosomal loci attached to inactive *X*-chromosomal material are, in fact, inactivated.

Recently, a mammalian system was found that allowed questions to be answered concerning *X*-inactivation of autosomal loci and the relationship

[1] This investigation was supported in part by research grants GM 20919 from the National Institute of General Medical Sciences and CA 05873 from the National Cancer Institute, and by an allocation from Public Health Service General Research Support grant RR-00545 from the Division of Research Resources to the Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

between gene-dosage and gene-product in a duplicated state, both with and without the influence of *X*-chromosomal inactivation. The mammalian organism is the mouse carrying the *T(X;7)1Ct* (*TCt*) translocation, the enzyme is mitochondrial malic enzyme (MOD-2), and the locus is mitochondrial malic enzyme (*Mod-2*). We present here evidence that (1) the *Mod-2* locus is located between the Chromosome 7 loci *c* (albino) and *sh-1* (shaker-1) and is, therefore, translocated to the *X*-chromosome in the *TCt* translocation; (2) the specific activity of mitochrondrial malic enzyme is increased in heart mitochondria in *TCt* animals carrying a duplication for the *Mod-2* locus; and (3) inactivation of the *Mod-2* locus occurs in unbalanced (those carrying the duplication), but not balanced female *TCt* mice.

MATERIALS AND METHODS

Linkage of Mod-2

SM/J is the only inbred strain known to contain an inherited electrophoretic variant at the *Mod-2* locus, the $Mod\text{-}2^a$ allele. It also carries the Hbb^s (single) allele at the *Hbb* locus (β-hemoglobin chain). The FS/Ei strain is homozygous for the Chr7 alleles *p* (pink-eyed diluiton), c^{ch} (chinchilla), $Mod\text{-}2^b$, *sh-1*, Hbb^d (diffuse), and *fr* (frizzy).

In order to determine the exact location of *Mod-2* in Chr 7, mice of the two inbred strains SM/J and FS/Ei were crossed to each other. The $p\ c^{ch}\ Mod\text{-}2^b\ sh\text{-}1\ Hbb^d\ fr/+\ +\ Mod\text{-}2^a\ +\ Hbb^s\ +$ F_1 offspring were crossed back to FS/Ei mice. Each offspring was classified for its phenotype at all six loci.

Production of MOD-2A stock

Several backcross mice from the previous cross that were $p\ +/p\ c^{ch}$, thus crossovers between *p* and c^{ch}, were mated together and their *p/p* offspring kept. Each *p/p* offspring was mated to an *SM/J* animal and 7 to 10 of their offspring were tested to determine whether they were homozygous $Mod\text{-}2^a$ and Hbb^s. The presence of only homozygous $Mod\text{-}2^a$ and Hbb^s offspring indicated that their *p/p* parent was also homozygous for the $Mod\text{-}2^a$ and Hbb^s alleles. A proven homozygous $p\ Mod\text{-}2^a\ Hbb^s$ female was crossed to an identical male to produce the homozygous $p\ Mod\text{-}2^a\ Hbb^s$ stock, hereafter designated MOD-2A, used in these studies.

The TCt *translocation*

The nonreciprocal *X*-autosomal translocation *TCt* involves the insertion of a central region from Chr 7 into the distal medial region of the *X* chromosome (reviewed by EICHER 1970, and Figure 1). The *X* chromosome containing the inserted region of Chr 7 is commonly called the X^{Ct} or flecked *X* chromosome (X^{fd}). In relation to the autosomal genes of interest in this report, the X^{Ct} chromosome contains the wild-type allele at the *p* locus (*p*+) and the $Mod\text{-}2^b$ allele at the *Mod-2* locus. We have represented this *X* as X^{Ct} ($p+\ Mod\text{-}2^b$) to indicate the presence of the *p*+ and $Mod\text{-}2^b$. It should be noted in Figure 1 that the *Hbb* locus was not translocated to the *X* chromosome.

TCt animals are of two common chromosomal types, balanced and unbalanced (Figure 2). The balanced type contains an X^{Ct} chromosome, a normal *X* (or *Y*), a normal Chr 7, and a Chr 7 that has a deletion for the region contained in the X^{Ct} chromosome, hereafter called Chr 7^{Df}. In relation to specific genes carried on Chr 7 and Chr 7^{Df}, if we symbolize an individual as $p\ c^{ch}\ Mod\text{-}2^a\ Hbb^s/Df\ Hbb^d$, it indicates that this individual carries the *p*, c^{ch}, $Mod\text{-}2^a$, and Hbb^s alleles on Chr 7, and Hbb^d allele on Chr 7^{Df}, and a deficiency for the *p*, *c*, and *Mod-2* genes on Chr 7^{Df}. The unbalanced type contains an X^{Ct} chromosome, a normal *X* (or *Y*), and two normal Chr 7. Thus, unbalanced *TCt* individuals have a duplication for the Chr 7 region contained in the X^{Ct} chromosome.

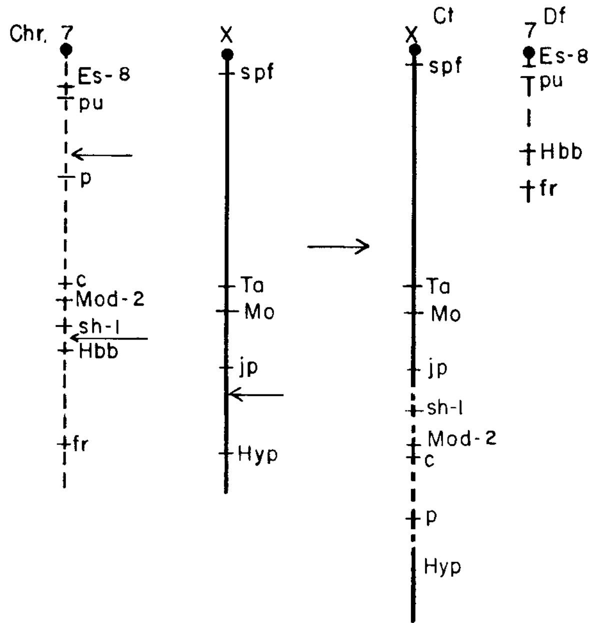

FIGURE 1.—Diagram of *T(X;7)1Ct* translocation. Two breaks occurred in Chr *7*, as indicated by arrows. This chromosomal region was inserted into the *X* chromosome in region of *X* designated by the arrow. Actually, it is unknown whether the piece was inserted distal (as shown), or proximal to *jp*. It should be noted that the piece of Chr *7* is inverted in the *X* chromosome relative to the centromere. The loci shown on the *X* and Chr *7* are *spf* (sparse fur), *Ta* (tabby), *Mo* (mottled), *jp* (jimpy), *Hyp* (hypophosphatemia), *Es-8* (esterase-8), *pu* (pudgy), *p* (pink-eyed dilution), *c* (albino), *Mod-2* (malic enzyme, mitochondrial), *sh-1* (shaker-1), *Hbb* (hemoglobin β chain), and *fr* (frizzy).

In order to produce the *TCt* mice and their normal sibs for analysis, females that were genetically X^{Ct} $(p+ Mod\text{-}2^b)/X\ p\ Mod\text{-}2^a\ Hbb^s/Df\ Hbb^d$ were crossed back to MOD-2A males. The six classes of expected offspring are listed in Table 1. The animals designated as Class 1 can be distinguished from those in the other two classes because of their nonpigmented eyes. The animals designated as Class 3 can be distinguished from those in Class 2 because of their HBB-S phenotype (see below).

In order to increase the number of Class 3 males for enzyme analysis, a few females of the genotype X^{Ct} $(p+ Mod\text{-}2^b)/X\ p\ Mod\text{-}2^a\ Hbb^s/p\ Mod\text{-}2^a\ Hbb^s$ (Class 3) were crossed to males of the MOD-2A stock. This cross produces offspring that are of the Classes 1 and 3.

Hemoglobin determinations

The Hbb^s/Hbb^s or Hbb^s/Hbb^d genotype (HBB-S and HBB-SD phenotypes, respectively) of most of the mice was determined using the method of HUTTON (1969) for the preparation of mouse hemoglobin for starch gel electrophoresis, as modified in our laboratory for cellulose acetate electrophoresis. The modified method consists of collecting approximately 0.2 ml of whole blood in 2 ml 0.8% NaCl containing 1 drop of 5000 U/ml Heparin solution. The cell suspension is centrifuged and the supernatant removed. Lysis is accomplished using 1 part distilled H_2O and 1 part toluene per 1 part of packed RBC's. The lysate is centrifuged at 15,000 rpm for 30 minutes. Three parts of the supernatant are mixed with 2 parts of a 15.38 mg/ml solution of iodoacetate in 0.2 M phosphate buffer, pH 7.0, and left at room temperature for 2 hrs. Thirty minutes before electrophoresis, 1 drop of a 250 mg percent solution of potassium

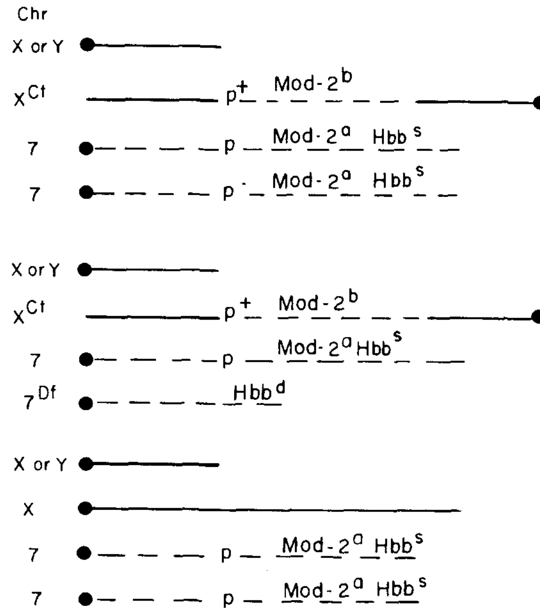

FIGURE 2.—Diagram of three types of mice used in experiments. At top, Class 3 females and males; at middle, Class 2 females and males; and, at bottom, Class 1 females and males. Dark knob at end of each chromosome represents the centromere position.

ferricyanide is added to each tube. This final solution is applied directly to cellulose acetate strips (Cellogel, Kalex Scientific Company; or Titan III, Helena Laboratories).

Electrophoresis is conducted at 300 volts, cathode to anode, for approximately 30 minutes in a buffer containing 25 parts distilled H_2O and 3 parts of EBT (109 g Tris base; 7.45 g EDTA, disodium salt; 30.9 g boric acid per liter of solution, pH 8.6). No staining is necessary to distinguish the two hemoglobin phenotypes.

Some individuals were classified using a much easier procedure to distinguish the HBB-S from the HBB-SD phenotype. The packed RBCs in a 1.6 cm length of a hematocrit tube are lysed in 3 drops of lysis solution (1 g EDTA, tetrasodium salt, per liter). The lysate is applied directly to cellulose acetate gels. Electrophoresis is conducted at 300 volts, cathode to anode, for 15 minutes in a buffer containing 25.2 g Tris base; 2.5 g EDTA, free acid; and 1.9 g boric acid per liter. No staining is necessary to distinguish the two hemoglobin phenotypes. The EBT buffer previously described does not allow HBB-D and HBB-SD to be distinguished with this shortened procedure.

Electrophoresis of MOD-2

Electrophoresis of heart or kidney extracts was performed on starch gels or cellulose acetate gels. Most starch gels were run according to a modified method of SHOWS, CHAPMAN and RUDDLE (1970). Single hearts or both kidneys were homogenized in 0.4 or 0.3 ml cold distilled H_2O, respectively. Two drops of a 10% Triton-X 100 solution was added to each tube and the contents mixed. Supernatants were collected after centrifugation in the cold at 15,000 rpm and used directly for electrophoresis or after a maximum of one week, if frozen. Electrophoresis was performed at 150 volts, cathode to anode, at 4° C for 18 to 20 hrs. The bridge buffer consisted of a one-to-one dilution of Shows' buffer (54 g Tris base: 36.14 g citric acid, monohydrate; per liter, pH 6.2). Gels consisted of 58 g Electrostarch, 9 ml SHOWS' buffer, and 491 ml distilled H_2O. The staining method was identical to that used by SHOWS, CHAPMAN and RUDDLE.

During the course of these studies, we were able to improve the above buffer system so as to avoid the heating problems encountered by the high ionic strength of SHOWS' buffer. The bridge buffer (TC buffer) consisted of a 0.1 M Tris citrate solution, pH 7.6, (12.1 g Tris base titrated to pH 7.6 with citric acid and then brought to one liter.) The gel buffer consists of 1:9 dilution of TC buffer. Electrophoresis was conducted at 180 volts, cathode to anode, 4° C, 18–22 hours. Identical MOD-2 patterns were observed with TC buffer as with SHOWS' buffer.

Electrophoresis of MOD-2 was also performed on cellulose acetate gels (Titan III strips, Helena Laboratories) using a 1:9 dilution of the TC buffer. We obtained as detailed an MOD-2 pattern with the cellulose acetate system as with the starch gel system.

We determined the MOD-2 electrophoretic pattern from lysates of heart, kidney, or both, of more than 50 females each from Class 2 and 3, and 20 males each from Classes 2 and 3.

Enzyme determinations

Mice were killed by cervical dislocation and the hearts removed. Each heart was washed in isotonic saline, minced, and homogenized in 1 ml 0.44 M sucrose. After 10 min centrifugation at 800 × g, the supernatants were removed and centrifuged at 10,000 × g in a Spinco ultracentrifuge for 10 min. The supernatant was discarded. The resulting mitochondrial pellet was washed in 1 ml 0.44 M sucrose and then centrifuged as before. Again the supernatant was discarded. The mitochondrial pellet was suspended in 0.30 ml of mixture of 5 parts of 0.25 M sucrose to 1 part 10% Triton-X 100. The pellet material and suspension solution were thoroughly mixed on a vortex mixer, and then set aside for 10 min at room temperature. The mixture was then centrifuged at 10,000 × g for 10 min to separate the soluble fraction from the membrane fraction. The soluble fraction was removed and 0.1 ml taken for a protein determination by the biuret method and the rest used to determine malic enzyme activity (MOD-2: EC 1.1.1.40 L-malate : NADP oxidoreductase, decarboxylating). Malic enzyme was determined essentially as described by HSU and LARDY (1969) except that 0.1 ml of a 0.03 M malic acid solution, rather than 0.05 ml, was used in the reaction mixture. All assays were conducted at room temperature using a Beckman DB spectrophotometer. The conduct of these assays at ambient temperature in different seasons of the year may have contributed to the different values obtained for male and female mice studied at different times.

Like-sexed groups of 6 to 8 individuals of comparable ages were always analyzed together. The investigator (DLC) conducting the enzyme determinations did not know how many or which individuals were Class 2 or Class 3 within any individual group. Each individual group contained at least one mouse of each of the three classes. All determinations were conducted on freshly prepared soluble material obtained from mice killed the same day. The analysis of MOD-2 levels of heart mitochondria isolated from individual females was conducted during the first part of 1974 and that from individual males conducted during the second part of 1974. A separate group of Class 1, 2, and 3 females and males were used for analysis of heart and kidney MOD-2 electrophoretic patterns.

RESULTS AND DISCUSSION

Location of Mod-2 *in Chr 7 and the* TCt *translocation*

It became clear in the early part of these studies that *Mod-2* was located either between c^{ch} and *sh-1* or between *sh-1* and *Hbb*. Therefore, we can focus our attention only on the c^{ch} to *Hbb* interval of Chr 7. Of the 478 mice produced in the cross *p* c^{ch} *Mod-2*^{b} *sh-1 Hbb*^{d} *fr/p* c^{ch} *Mod-2*^{b} *sh-1 Hbb*^{d} *fr* × + + *Mod-2*^{a} + *Hbb*^{s} +/*p* c^{ch} *Mod-2*^{b} *sh-1 Hbb*^{d} *fr*, 25 were recombinants between the *c* and *sh-1* loci, indicating that the percent recombination between *c* and *sh-1* was 5.23 ± 1.02. Nineteen of these mice were successfully classified for MOD-2.

TABLE 1

Expected offspring of the cross: ♀ X^{Ct} (p+ Mod-2^b)/X p Mod-2^a Hbb^s/Df Hbb^d × ♂ +/Y p Mod-2^a Hbb^s/p Mod-2^a Hbb^s

Class	Sex	Genotype	
1	♀	+/+	*p Mod-2^a Hbb^s/p Mod-2^a Hbb^s*
1	♂	+/Y	*p Mod-2^a Hbb^s/p Mod-2^a Hbb^s*
2	♀	X^{Ct} (*p+ Mod-2^b*)/X	*p Mod-2^a Hbb^s/Df Hbb^d*
2	♂	X^{Ct} (*p+ Mod-2^b*)/Y	*p Mod-2^a Hbb^s/Df Hbb^d*
3	♀	X^{Ct} (*p+ Mod-2^b*)/X	*p Mod-2^a Hbb^s/p Mod-2^a Hbb^s*
3	♂	X^{Ct} (*p+ Mod-2^b*)/Y	*p Mod-2^a Hbb^s/p Mod-2^a Hbb^s*

Fifteen were recombinants between the *Mod-2* and *sh-1* loci but not recombinants between the *c* and *Mod-2* loci, and four were recombinants between both the *c* and *Mod-2* loci and the *Mod-2* and *sh-1* loci. Thus, *Mod-2* is located closer to the *c* locus than to the *sh-1* locus. The relative distance from *c* to *Mod-2* and from *Mod-2* to *sh-1* can be determined by dividing the recombination fraction from *c* to *sh-1* (5.2%) into 4/19 and 15/19, respectively. The order of loci in Chr 7 with percentage recombination is *c*–1.1–*Mod-2*–4.1–*sh-1*.

Since one of the *TCt* breakpoints in Chr 7 is between *Hbb* and *sh-1*, the *Mod-2* locus is carried on the X^{Ct} chromosome. As expected from the linkage study, Class 3 males had an MOD-2 isozyme pattern different from that of Class 1 and Class 2 males (see Table 1).

TABLE 2

Enzymatic activity of MOD-2 *in heart mitochondria*

Class	Dosage of *Mod-2*	Enzymatic activity, Heart: N	*Mod-2**
Males			
1	2 Copies-Normal	12	15.92 ± 0.47**
2	2 Copies-Balanced *TCt*	19	15.13 ± 0.28**
3	3 Copies-Unbalanced *TCt*	20	22.84 ± 0.44
Per cent increase, Class 3			47.9
Females			
1	2 Copies-Normal	13	21.68 ± 1.13††
2	2 Copies-Balanced *TCt*	12	20.21 ± 0.96††
3	3 Copies-Unbalanced *TCt*	15	29.13 ± 1.27
Per cent increase, Class 3			38.9

* Nanomoles NADP/mg protein/min room temperature ± S.E.

** Values not significantly different from each other according to the Student-Newman-Keuls multiple range test.

†† Values not significantly different from each other according to the Student-Newman-Keuls multiple range test.

Analysis of enzyme levels

The results of the enzyme studies are given in Table 2. It is appropriate to consider first the enzyme levels in males because of complication of inactivation of the *Mod-2* locus by the X^{Ct} chromosome in Class 2 and 3 females.

Class 1 and 2 males differ in two ways in relation to the *Mod-2* locus (see Figure 2 and Table 1): (1) Class 1 males are homozygous *Mod-2^a*, whereas Class 2 males are heterozygous, and (2) Class 1 males have both their alleles on Chr 7, whereas Class 2 males have one of their alleles on the X chromosome and one on Chr 7. We had determined early in our investigation that mice having the MOD-2A and MOD-2AB forms of the enzyme did not show significant differences in activity. Therefore, if differences were noted between Class 1 and 2 males, the cause must be due to the influence of the X chromosome on the *Mod-2* locus in Class 2 males (see difference number 2 above). Because Class 1 and 2 males contain the same MOD-2 activity in their heart mitochondria, we conclude that the presence of the *Mod-2* autosomal locus in the X^{Ct} chromosome does not in itself influence its expression. In addition, we conclude that any increase in the level of MOD-2 enzyme in Class 3 males will be a direct reflection of their duplicated *Mod-2* state.

As seen in Table 2, Class 3 males contain approximately a 50 per cent increase in MOD-2 activity in heart mitochondria, as compared to Class 1 and 2 males. This is strong evidence that in the duplicated state, the *Mod-2* locus maintains a 1:1 relationship with its product in heart tissue.

It is pertinent to note that similar results on the effect of dosage of *Mod-2* on MOD-2 levels were also noted by DIAMOND and ERICKSON (1974). They measured the MOD-2 levels in whole heart extracts of mice heterozygous for one of the radiation-induced albino mutations (c^{25H}) that has been shown to be a deletion covering the *Mod-2* locus (ERICKSON, EICHER and GLUECKSOHN-WAELSCH 1974). Controls consisted of homozygous normal littermates. In the heart they found a 1:1 relationship between the number of *Mod-2* genes (one *versus* two) and the MOD-2 enzyme level. That is, individuals heterozygous for the deletion had half the MOD-2 level compared to normal individuals. We conclude from their studies and our studies that the level of MOD-2 in heart mitochondria in relation to a *Mod-2* gene dosage of 1:2:3 is 0.5:1:1.5, respectively.

Analysis of MOD-2 levels in the females is more complicated than in the males because of the influence of X-inactivation on the attached *Mod-2* gene. Class 3 females showed a significant increase in heart mitochondrial MOD-2 levels compared to Class 1 and 2 females. This increase indicates that the extra *Mod-2* copy carried on their X^{Ct} chromosome is increasing the level of MOD-2. If the *Mod-2^b* allele carried on the X^{Ct} chromosome was always inactivated when the X^{Ct} chromosome was the inactive X *and* the X^{Ct} chromosome was inactive in 50 per cent of the heart cells, we would expect that Class 3 females would have half of the Class 3 male MOD-2 increase. The observed increase was 39 percent. This increase is greater than the 25 percent increase expected (half of that observed

in Class 3 males) and may indicate that in heart cells these females have either (1) the normal *X* preferentially inactivated, or (2) the *Mod-2*^{b} allele in the X^{fd} chromosome is active when X^{fd} is the inactive *X* (in some cells *Mod-2*^{b} is never inactivated or it can be reactivated). Further research is needed to clarify this point.

Contrary to what we expected, Class 2 females showed no decrease in heart mitochondrial MOD-2 levels, indicating they did not inactivate the *Mod-2*^{b} locus in the X^{Ct} chromosome (see results from electrophoretic gels below.) There is evidence that autosomal loci attached to inactive *X* chromatin are not always inactivated. For example, evidence exists that the further removed an autosomal locus is from inactive *X* chromatin, the greater is the probability that it will escape inactivation (reviewed by EICHER 1970). This possibility is highly unlikely for the *Mod-2* locus in the X^{Ct} chromosome. The order of Chr 7 loci in the X^{Ct} chromosome is centromere—*X* chromosome—*sh-1*$^+$—*Mod-2*^{b}—c^+—p^+—*X* chromosome such that *Mod-2*^{b} is located between the *sh-1*$^+$ and c^+ loci, both of which are inactivated in both Class 2 and 3 females (EICHER and WASHBURN unpublished data; EICHER 1967; DEOL and GREEN 1969; CATTANACH 1961; EICHER 1970). Further, we have presented evidence here that the *Mod-2*^{b} locus in Class 3 females can be inactivated (see results from electrophoretic gels below). Thus, in most heart cells of Class 2 females, either (1) the Chr 7 region in the X^{Ct} chromosome is not inactivated when the X^{Ct} is the inactive *X*, or (2) the normal *X* is always the inactive *X*, or (3) the *Mod-2*^{b} allele is reactivated in the inactive X^{Ct} chromosome. Unfortunately, there are presently no *X*-linked isozyme variants in the mouse that can be used for an efficient test of possibilities 1 and 2. Regarding possibility 3, CATTANACH (1974) has presented evidence that there is reactivation of the inactive c^+ locus during the lifetime of Class 3 females. However, no such finding has been reported for Class 2 females. Until we have information on the MOD-2 levels of embryonic and very young Class 2 females to indicate whether they have an inactive *Mod-2*^{b} allele in some of their cells during development that is reactivated by adulthood (or those cells are selected against), possibility 3 cannot be eliminated from consideration.

It is appropriate to note here that we obtained different levels of MOD-2 in heart mitochondria of Class 1 males and females (Table 2). As pointed out previously (see MATERIALS AND METHODS), the heart determinations for males and females were done at room temperature at separate times in different seasons. At the end of these experiments (end of 1974), we analyzed the heart mitochondria MOD-2 levels for two groups of Class 1 individuals, each group consisting of four females and four males. No differences were noted between males and females. Therefore, the previous difference between sexes in some way reflects either changes in our efficiency of measuring MOD-2, or unknown environmental changes that affected the MOD-2 level, or both. Thus, although the absolute values of MOD-2 present in heart mitochondria are in question, the differences noted between Class 1, 2 and 3 females or Class 1, 2 and 3 males are not in question.

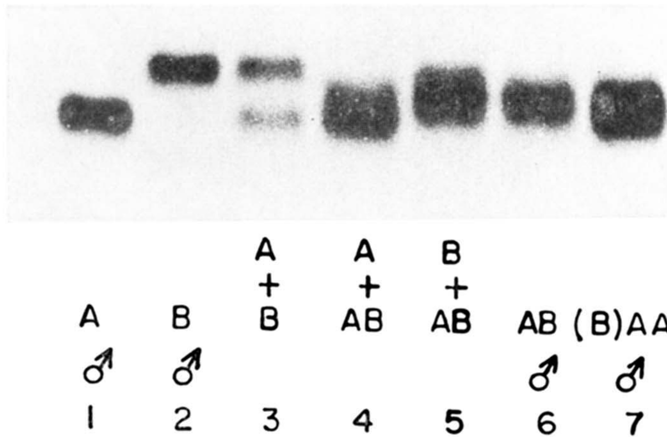

FIGURE 3.—Electrophoresis of heart MOD-2 cellulose acetate. The slot number, sex of animal, and phenotype are given. "A" represents the phenotype of a $Mod\text{-}2^a/Mod2^a$ mouse, "B" a $Mod\text{-}2^b/Mod\text{-}2^b$ mouse, and "AB" an $Mod\text{-}2^a/Mod\text{-}2^b$ mouse. When the symbol is surrounded by parentheses, it indicates that the allele responsible for that part of the phenotype is in the X chromosome (X^{Ct}). For example, "(B)AA" is the phenotype observed in the X^{Ct} ($Mod\text{-}2^b$)/X $Mod\text{-}2^a/Mod\text{-}2^a$ female (or male, if indicated). Slot 3 is a mixture of slots 1 and 2; slot 4 of 1 and 6; slot 5 of 2 and 6. Note similarity of slots 4 and 7.

Starch gel and cellulose acetate electrophoretic patterns

We obtained the same gel patterns for MOD-2 as those reported by SHOWS, CHAPMAN and RUDDLE (1970): The MOD-2A form migrated faster than the MOD-2B form, with the MOD-2AB form migrating in an intermediate position (Figure 3). The same patterns have been observed for mitochondrial malic

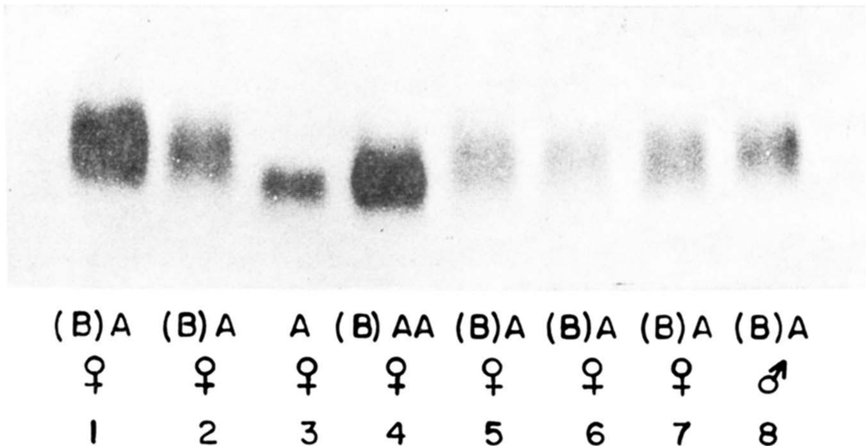

FIGURE 4.—Electrophoresis of heart MOD-2 on cellulose acetate. Note that phenotypes of females in slots 1, 2, 5, 6, and 7 are identical to that of the male in slot 8 (interpretation: $Mod\text{-}2^b$ in X^{Ct} chromosome is active).

enzyme in human brain and *Macaca nemestrina* brain (COHEN and OMENN 1972a,b). Mitochondrial malic enzyme is probably a tetramer (POVEY *et al.* 1975).

The MOD-2 starch gel and cellulose acetate electrophoretic patterns observed from kidney and heart preparations of Class 1, 2, and 3 individuals were consistent with our findings of MOD-2 enzyme levels. As can be seen in Figures 3 and 4, the Class 2 males and females showed the MOD-2AB pattern typical of the *Mod-2ᵃ/Mod-2ᵇ* mouse indicating no influence of *X*-chromosomal material on the *Mod-2ᵇ* allele in either sex. Furthermore, as seen in Figure 3, the Class 3 males had a MOD-2 pattern similar to that obtained if supernatants from *Mod-2ᵃ/Mod-2ᵃ* and *Mod-2ᵃ/Mod-2ᵇ* individuals were mixed before electrophoresis.

It was very striking that each Class 3 female displayed a unique MOD-2 pattern, indicating that each contained different proportions of cells with the *Mod-2ᵇ* allele active (or inactive). As can be seen in Figure 5, some females had a pattern similar to Class 3 males (*Mod-2ᵇ* allele was active in all cells), some had a pattern showing increased activity in the MOD-2A region of the gel (*Mod-2ᵇ* allele was active in some cells and inactive in others), and some displayed a pattern similar to Class 1 females (*Mod-2ᵇ* allele was inactive in most cells).

A number of hypotheses related to the mechanism of *X*-inactivation have been postulated that rely on observations in females heterozygous for *X-A* translocations (RUSSELL and MONTGOMERY 1970; RUSSELL 1963, 1964; CATTANACH 1961, 1974; LYON 1971, 1972, 1974; GRUENBERG 1967a,b; EICHER 1970; BROWN and CHANDRA 1973; CHANDRA and BROWN 1975; COOPER 1971). Several of these

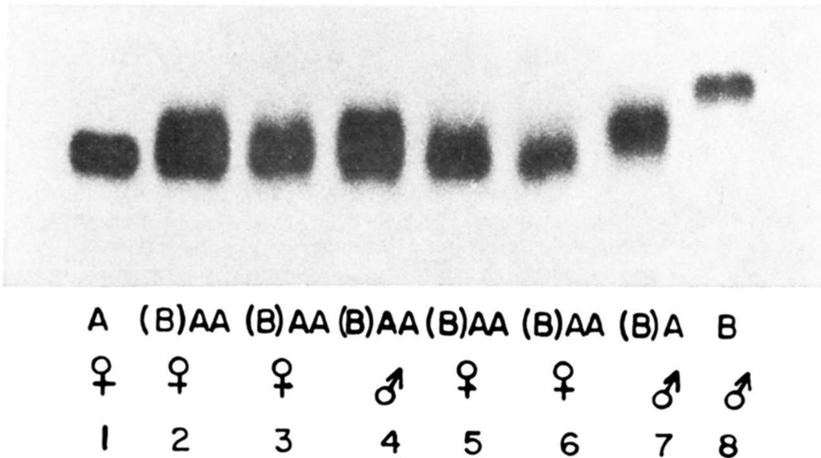

FIGURE 5.—Electrophoresis of heart MOD-2 on cellulose acetate. Note variation in phenotype of X^{Ct} (*Mod-2ᵇ*)/*X Mod-2ᵃ/Mod-2ᵃ* females. For example, phenotype of female in slot 2 is like that of male in slot 4 (Interpretation: *Mod-2ᵇ* in X^{Ct} chromosome is active in most cells), whereas that of female in slot 6 is like that of individual in slot 1, Figure 3 (Interpretation: *Mod-2ᵇ* in X^{Ct} chromosome is inactive in most cells).

hypotheses were based on the fact that lack of inactivation for X-linked or variegation-position effect for autosomal loci occurred in females heterozygous for one of several translocations. As previously pointed out, the lack of variegation in one tissue does not rule out the presence of variegation in other tissues (EICHER 1970). A case in point is the Class 2 *TCt*/+ females; they have variegation for *c* and *p* in melanocytes but not for *Mod-2* in kidney or heart cells.

We thank RICHARD COPP, SALLIE C. REYNOLDS and JANICE L. SOUTHARD for technical assistance.

LITERATURE CITED

BROWN, S. W. and H. S. CHANDRA, 1973 Inactivation system of the mammalian X chromosome. Proc. Natl. Acad. Sci. USA **70**: 195–199.

CATTANACH, B. M., 1961 A chemically-induced variegation-type position effect in the mouse. Z. Vierbungslehre **92**: 165–182. ——, 1974 Position effect variegation in the mouse. Genet. Res. **23**: 291–306.

CHANDRA, H. S. and S. W. BROWN, 1975 Chromosome imprinting and the mammalian X chromosome. Nature **253**: 165–168.

COHEN, P. T. W. and G. S. OMEN, 1972a Genetic variation of the cytoplasmic and mitochondrial malic enzyme in the monkey *Macaca nemestrina*. Biochem. Genet. **7**: 289–301. ——, 1972b Human malic enzyme: High-frequency polymorphism of the mitochondrial form. Biochem. Genet. **7**: 303–311.

COOPER, D. W., 1971 Directed genetic change model for X chromosome inactivation in eutherian mammals. Nature **230**: 292–294.

DEOL, M. S. and M. C. GREEN, 1969 Cattanach's translocation as a tool for studying the action of the shaker-1 gene in the mouse. J. Exptl. Zool. **170**: 301–310.

DIAMOND, R. P. and R. P. ERICKSON, 1974 Gene dosage in a deletion for a nuclear-coded mitochondrial enzyme. Nature **248**: 418–419.

EICHER, E. M., 1967 The genetic extent of the insertion involved in the flecked translocation of the mouse. Genetics **55**: 203–212. ——, 1970 X-Autosome translocations in the mouse: Total inactivation versus partial inactivation of the X chromosome. Adv. Genet. **15**: 175–259.

ERICKSON, R. P., E. M. EICHER and S. GLUECKSOHN-WAELSCH, 1974 Demonstration in mouse of X-ray induced deletions for a known enzyme structural locus. Nature **48**: 416–418.

GRÜNEBERG, H., 1967a Gene action in the mammalian X-chromosome. Genet. Res. **9**: 343–357. ——, 1967b Sex-linked genes in man and the Lyon hypothesis. Ann. Human Genet **30**: 239–257.

HSU, R. Y. and H. A. LARDY, 1969 Malic enzyme. pp. 230–235. In: *Methods in Enzymology*. XIII. *Citric Acid Cycle*. Edited by J. M. LOWENSTEIN. Academic Press, New York.

HUTTON, J. J., 1969 Linkage analysis using biochemical variants in mice. I. Linkage of the hemoglobin beta-chain and glucosephosphate isomerase loci. Biochem. Genet. **3**: 507–515.

KIRKMAN, H. N., 1972 Enzyme defects, pp. 125–168. In: *Progress in Medical Genetics*. VIII. Edited by A. G. STEINBERG and A. G. BEARN. Grune and Stratton, New York.

LYON, M. F., 1971 Possible mechanisms of X chromosome inactivation. Nature New Biol. **232**: 229–232. ——, 1972 X-chromosome inactivation and developmental patterns in mammals. Biol. Rev. **47**: 1–35. ——, 1974 Mechanisms and evolutionary origins of variable X-chromosome activity in mammals. Proc. Roy. Soc. Lond. B. **187**: 243–268.

POVEY, S., D. E. WILSON, JR., H. HARRIS, I. P. GORMLEY, P. PERRY, and K. E. BUCKTON, 1975 Sub-unit structure of soluble mitochondrial malic enzyme: demonstration of human mitochondrial enzyme in human-mouse hybrids. Ann. Hum. Genet. Lond. **39**: 203–212.

RUSSELL, L. B., 1963 Mammalian X-chromosome action: Inactivation limited in spread and in region of origin. Science **140**: 976–978. ——, 1964 Another look at the single-active-X-hypothesis. Trans. N.Y. Acad. Sci. [2] **26**: 726–736.

RUSSELL, L. B. and C. S. MONTGOMERY, 1970 Comparative studies on X-autosome translocations in the mouse. II. Inactivation of autosomal loci, segregation, and mapping of autosomal breakpoints in five (T(X;1))'s. Genetics **64**: 281–312.

SHOWS, T. B., V. M. CHAPMAN and F. H. RUDDLE, 1970 Mitochondrial malic dehydrogenase and malic enzyme: Mendelian inherited electrophoretic variants in the mouse. Biochem. Genet. **4**: 707–718.

Corresponding editor: D. BENNETT