NORMAL MAMMALIAN MUSCLE DIFFERENTIATION AND GENE CONTROL OF ISOCITRATE DEHYDROGENASE SYNTHESIS*

BY BEATRICE MINTZ AND WILBER W. BAKER

INSTITUTE FOR CANCER RESEARCH, PHILADELPHIA, PENNSYLVANIA

A new kind of laboratory mammal, the *allophenic* mouse, has been described by Mintz.[1,2] These animals are artificial creations, since they are formed by aggregating cleavage-stage blastomeres *in vitro* from embryos of different genotypes, and the composites are then transferred to an "incubator" mother for further development. Despite their strange history, hundreds of such mosaic embryos have gone on to become healthy and long-lived adults. The mice are called *allophenic* because they display an orderly arrangement of two concurrent, allelically alternative cellular phenotypes, or *allophenes*.[2] Many biological problems are open to examination in these individuals, inasmuch as their component genotypes can be chosen without restriction. In the present report, a question is raised which could have many implications for development or disease: Do cells fuse in the organism?

The possibility that fusion between somatic cells might be made to occur experimentally was first suggested by Schultz[3] and by Lederberg.[4] It was soon taken up with methods chiefly applicable to cell cultures, in a series of imaginative *in vitro* experiments.[5-8] The allophenic mouse provides an ideal way to obtain an unambiguous answer to the question of spontaneous cell fusion *in vivo*, for any normal tissue or for malignant ones.

This study will be primarily concerned with skeletal muscle because, unlike most of the other tissues in the body, its cells are multinucleated rather than uninucleated. Definitive muscle arises from narrow tubules, or myotubes, in which many nuclei are arranged in approximately single file within a common cytoplasm. Myotubes in turn, are derived from uninucleated cells, the spindle-shaped myoblasts. The developmental transition from a uninucleated to a multinucleated state might, hypothetically, occur either by repeated nuclear division without cytoplasmic cleavage or by fusion of myoblasts. The debate as to which of these is in fact the normal mechanism has been sustained for well over half a century. There have been many observations on growth and regeneration of muscle *in vivo*, but the chief arena in which hypotheses of myogenesis have been tested experimentally has been the *in vitro* one. Here myoblasts of mammalian and of avian origin have been found to form myotubes by fusion.[9-13] The query which has persisted is whether this behavior reflects the situation in the organism, or is an *in vitro* peculiarity.

Within an allophenic mouse, if some cells were to fuse, multinucleated heterokaryons could be formed. In the event that the two strains of cells were each homozygous for a different allele at an appropriate enzyme locus, *hybrid enzyme* molecules might be synthesized in the heterokaryons. Thus occurrence of cell fusion could be detected by simple biochemical means. An example of an enzyme favorable for this purpose is the nicotinamide-adenine dinucleotide phosphate (NADP)-dependent isocitrate dehydrogenase found in the supernatant fraction of homogenates of many mouse tissues, including muscle. The autosomal *isocitrate*

dehydrogenase-1-locus, as first reported by Henderson,[14] governs synthesis of two allelic enzyme variants, or isozymes,[15] differing in electrophoretic mobility. The more slowly migrating cathodal (Id-1^a/Id-1^a) and the more rapid anodal (Id-1^b/Id-1^b) types have been identified in different inbred strains. Each yields only a single band in starch gel. The F_1 hybrid between the parental strains (Id-1^a/Id-1^b) generates the two original bands plus an intermediate, or hybrid, isozyme in a ratio of approximately 1:2:1. From this it was concluded that the enzyme is probably a dimer.[14] The pure-strain variants do not hybridize spontaneously outside the organism under the conditions of extraction and electrophoresis—a necessary prerequisite to a search for hybrid enzyme in allophenic muscle (see Fig. 2).

Figure 1 presents diagrammatically the enzyme results which the two alternative hypotheses of myogenesis would predict. The diagram refers entirely to allophenic mice constituted from two homozygous embryos, genotypically Id-1^a/Id-1^a
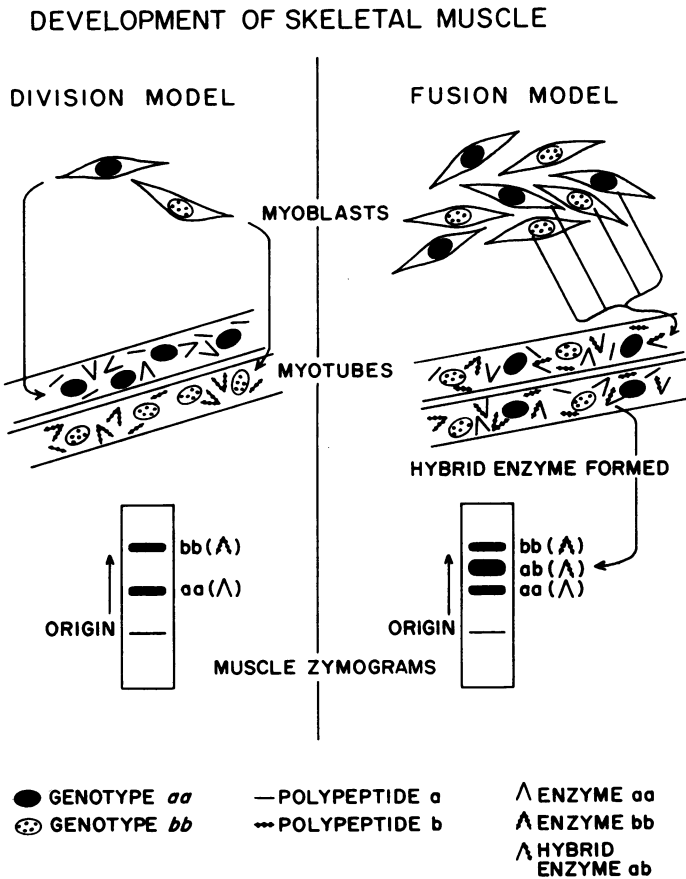

Fig. 1.—Diagram of expected isozyme results in *allophenic* mice, on the "division" (*left*) vs. "fusion" (*right*) models of skeletal muscle development. When homozygous cells of different NADP-isocitrate dehydrogenase genotypes coexist, heterokaryons would result in the event of myoblast fusion, and *hybrid enzyme* could be formed. Enzyme molecules are represented as dimers formed in the cytoplasm from polypeptide subunits (see text).

and Id-$1^b/Id$-1^b. On the "division" model (*left*), each myotube would contain nuclei of only one genotype, and only the two pure-strain isozymes (shown as aa and bb) would be recovered from muscle. On the "fusion" model (*right*), genetically different nuclei would be found in single myotubes and this could be expected to account for formation of hybrid enzyme (ab), in addition to the two pure types.

Two different pairs of inbred strain combinations were examined, and many samples of separate muscles and of other tissues were taken from one adult animal of each of the two combinations. The allophenics are C3Hf ↔ DBA/2 and C57BL/6 ↔ CBA, respectively; in each, the Id-$1^a/Id$-1^a member is indicated first and the Id-$1^b/Id$-1^b second. The coat colors of both component strains were visible. These particular individuals were designed to serve not only this investigation but also several others, and some of their other features are therefore analyzed elsewhere.[2, 16]

Materials and Methods.—The mice were killed by cervical dislocation and their tissues cut into pieces and frozen. Tissues were stored at −57°C and thawed for analyses. Preparation of extracts and identification of isozymes generally followed the procedures used by Henderson,[14] with some modifications. Muscle was homogenized in one volume of cold glass-distilled water at high speed in a Virtis tissue disintegrator for 20–30 seconds, and the homogenate washed out with another half volume of water. All other tissues were less tough and were therefore homogenized in a Ten Broeck homogenizer, in four volumes of water. Homogenates were centrifuged in a Sorvall model RC2-B refrigerated centrifuge at 0–3°C for 20 minutes at 600 × g, and the supernatant fraction was recentrifuged at 40,000 × g for 20 more minutes. The final supernate was used for electrophoresis.

In a few cases, higher enzyme concentrations were desired in skeletal muscle extracts and these were obtained by centrifuging the thick homogenate at 80,000 × g in a Spinco model L ultracentrifuge at 0–3°C for 30 minutes, recentrifuging the supernatant fraction under the same conditions, and lyophilizing the second supernatant in a Virtis freeze-dryer. The dried preparation was then redissolved in cold distilled water so as to yield a two- to fourfold concentrate of enzyme. The isozyme patterns obtained after such treatment were identical with those from the nonlyophilized muscle preparations described above.

Vertical electrophoresis of samples was conducted in starch gel (Buchler Instruments apparatus and Heathkit constant voltage power supply), with phosphate-citrate buffer. The stock buffer, containing 0.32 M Na_2HPO_4 adjusted to pH 7.0 with citric acid, was diluted 1:80 in the gel and 1:10 in the electrode chambers.[17] Gels contained 14–15 per cent hydrolyzed starch (Connaught Medical Research Labs.). Following 16 hours of electrophoresis at 3°C and 225 volts (7.5 v/cm), the block was cut into horizontal slices 2 mm thick. Enzyme was visualized by incubation of slices at 37°C in buffered substrate with NADP, phenazine methosulfate, and nitro blue tetrazolium.

Results and Discussion.—*Skeletal* muscle from both kinds of allophenic mice contained appreciable quantities of hybrid enzyme (Fig. 2a). We can rule out the possibility that the molecular hybrid was assembled from subunits diffusing across cell boundaries; if enzyme subunits were indeed passing between cells and then forming the total enzyme macromolecule, the simultaneous presence of cells of both genotypes in the other tissues analyzed (cardiac muscle, liver, spleen, etc.) should
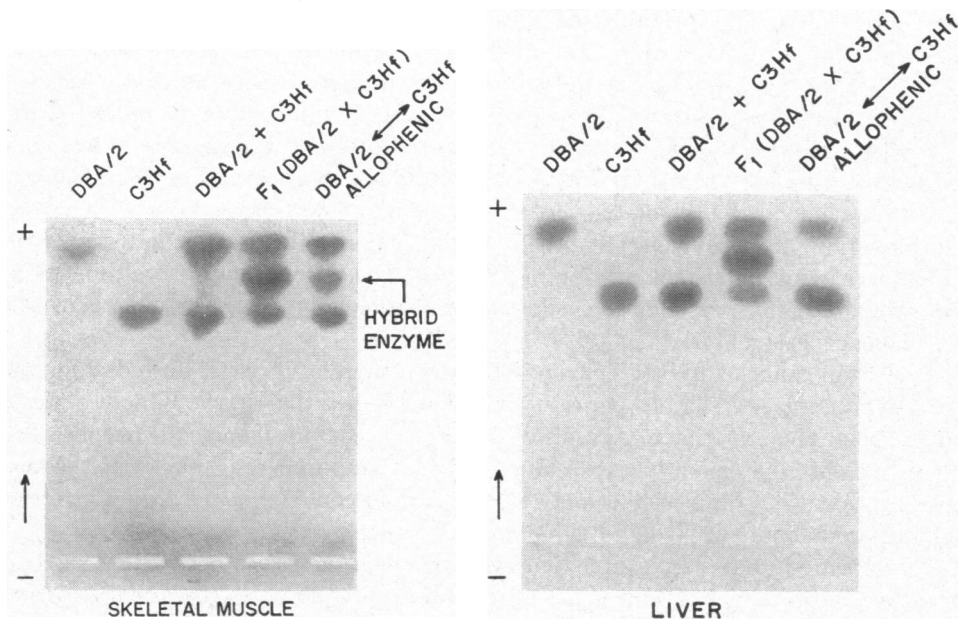
FIG. 2.—Starch gel electrophoretic patterns of NADP-isocitrate dehydrogenase in homogenates (supernatant fraction) of mouse tissues. (*a*) Skeletal muscle; (*b*) liver. From left to right in each gel: the single anodal isozyme in *Id-1b/Id-1b* (DBA/2 strain) homozygotes; the single more cathodal isozyme in *Id-1a/Id-1a* (C3Hf strain) homozygotes; both homozygous isozymic types, in a homogenate of pooled DBA/2 and C3Hf tissues; the F₁ heterozygote (*Id-1a/Id-1b*) between the parental strains, showing the original variants plus an intermediate hybrid isozyme; tissue from an *allophenic* mouse, with both DBA/2 and C3Hf cells combined *in vivo*. In allophenic skeletal muscle (*a*), *hybrid enzyme is formed*, demonstrating muscle origin by myoblast cell fusion. In allophenic liver (*b*) no hybrid enzyme is detectable.

have led to production of hybrid enzyme in those tissues also, and it apparently did not (Fig. 2*b*). Consequently, hybrid enzyme seems to have been formed within the boundaries of the multinucleated muscle cells. The only logical explanation for intracellular formation of hybrid in this case is the "fusion" model of muscle development (Fig. 1). The results can therefore be taken as critical proof that skeletal muscle development in the organism proceeds via fusion of myoblasts rather than by repeated nuclear divisons within one cell body.

Resolution of the problem of myogenesis *in vivo* provides some of the necessary foundation for future investigation of a number of problems, such as gene control of normal myogenesis and etiology of genetically determined diseases of muscle in mammals. A basis also exists for examining how genetic mechanisms can lead to formation in the embryo of such widely different tissues as muscle, bone, and dermis from a single pool of ostensibly similar cells within each somite.

The preceding evidence for normal myogenesis by cell fusion rests upon a clear-cut phenotype (hybrid enzyme). There have been some other attempts to investigate muscle development (in man) by genetic analyses, but the phenotype employed was muscular degeneration, which creates inevitable ambiguities of interpretation. Since the degeneration itself is progressive, the ostensible "phenotype" at any one time does not necessarily reflect the genetic constitution of the

cell.   Thus, females heterozygous for a sex-linked type of dystrophy were described in one study as having only two populations of fibers, normal or dystrophic,[18] while in a conflicting report a wide range from normal to severely abnormal was found.[19]   Intergrades, in the second instance, were interpreted in terms of the Lyon hypothesis,[20] and were taken to signify cell fusion.   A similar view was suggested in a third study of a dystrophic XO/XX/XXX mosaic.[21]   Further complications here included lack of knowledge of any hereditary basis for the muscular degeneration, and occurrence of rubella in the subject's mother during pregnancy, possibly leaving persistent mitotic anomalies.   These investigations, although interesting, therefore remain inconclusive because of the intrinsic nature of the available genetic material.

The proportion of hybrid enzyme in a given muscle, of those tested from the allophenics, was less than 50 per cent, and therefore less than in the F_1 heterozygote (Fig. 2a).   This experimental result could be accounted for if some fibers contained nuclei of only one genotype.   Possibly the relative numbers of nuclei of the two genotypes within a mixed fiber may also vary, and could lead to varying levels of hybrid product among such individual fibers.

As indicated above, hybrid enzyme was not seen in tissues other than skeletal muscle from the allophenic mice, though each tissue had the two bands characteristic of the homozygous strains and therefore contained both genotypes of cells.   Analyses were conducted on cardiac muscle, liver, kidney, lung, and spleen.   The absence of visible hybrid enzyme does not exclude the possibility that small amounts might be present, below the limits of detection by this method, as might be the case if small numbers of cells had fused.   More suitable markers, detectable within individual cells, are being employed to ascertain whether or not occasional fusions have taken place.   In addition, the isozymic studies are being extended to many more allophenic animals and also to other kinds of tissues in them.   The results obtained here nevertheless suggest that cell fusion is not a frequent phenomenon, if it occurs at all, in any of the tissues which yielded no hybrid enzyme.

The most interesting of these tissues is perhaps cardiac muscle, which superficially resembles a syncytium.   The syncytial nature of heart muscle has, however, been seriously questioned.   The present preliminary results on cardiac tissue contrast markedly with the skeletal muscle data and appear to support a nonsyncytial view of the former.   Hearts from other allophenic mice are being analyzed in order to learn whether this is upheld in a larger sample.   The conclusions, together with a more detailed discussion of the problem, will be presented elsewhere.

The allophenic mice also afford an opportunity to elucidate some of the steps in normal biosynthesis of isocitrate dehydrogenase, and their genetic control.   From the fact that the F_1 heterozygote contains hybrid enzyme, it is reasonable to hypothesize[14] that the two different subunits are synthesized and released to dimerize at random within the cytoplasm of each cell.   Two interrelated difficulties remain, however, which cannot be easily clarified in the F_1 heterozygote: one is the lack of information, prior to the present study, on whether subunits can diffuse between cells before assembling into complete enzyme; the other is the uncertainty as to whether both alleles, or only one, are active per nucleus at the *Id-1*-locus.   It is, for example, admissible that one allele acts per nucleus and that hybrid enzyme forms in a uninucleated F_1 cell after monomers have been exchanged between cells.

Certain unique features of the allophenic mouse are especially favorable for resolving the issue. Genetically different, homozygous, uninucleated cells coexist, to test the matter of diffusion of monomers across cell membranes; and, in muscle heterokaryons, a common cytoplasm is shared by different homozygous nuclei which are individually incapable of producing the hybrid product.

As pointed out earlier in the discussion, intercellular diffusion of polypeptides has indeed been excluded, owing to absence of detectable hybrid enzyme in the nonmuscle allophenic tissues examined, despite presence of both genotypes of cells. The hybrid enzyme found in skeletal muscle must therefore be produced within the multinucleated cell itself. All available *in vivo* evidence points to retention of nuclear individuality in muscle, unlike the frequent nuclear fusion observed in cell cultures of other types.[5-8] In regenerating adult mouse muscle, mitotic replication is visible only in mononucleated myoblasts and the nuclei of multinucleated myotubes consistently possess the diploid DNA value.[22] Without nuclear fusion, only two homozygous strains of nuclei would be found in a heterokaryon, and the manufacture of an intracellular hybrid enzyme would require genetic information from both kinds of nuclei. We can conclude that hybrid enzyme in allophenic muscle is being made in a two-step sequence: first, monomeric subunits or polypeptides of each of the pure types are separately synthesized on templates received from each nucleus; then the two kinds of subunits are assembled in the cytoplasm into the final macromolecule (Fig. 1). In other words, the genetic material codes independently for each polypeptide, not for the completed enzyme as a single unit. A specific messenger RNA from each nucleus presumably mediates polypeptide formation on ribosomes in the cytoplasm. The two-step mode of NADP-isocitrate dehydrogenase synthesis must also apply to ordinary mice, and to cells with single nuclei, whether homozygous or heterozygous. A comparable series of events may take place with some other mammalian enzymes, such as lactate dehydrogenase.[23, 24, 7] Various questions remain. Is there, for example, any difference in ribosomes in the two strains of an allophenic mouse, and is there any specificity of association between them and the different messages? And are the polypeptide chains, in cells of normal or of experimental animals, put together into protein on the surface of any cytoplasmic organelle, or essentially in the cytoplasmic fluid?

The remaining uncertainty concerning the F_1 heterozygote can now be removed. Since enzyme appears to be made without intercellular diffusion, and with templates coding only for subunits, both kinds of templates would have to be formed in the nucleus of an F_1 uninucleated cell. Therefore, in a cell in which the isocitrate dehydrogenase-1-locus is active, both genes are functioning. This conclusion should also be valid for homozygous cells.

Summary.—Tissues from *allophenic* mice, which contain two separate genetic types of cells,[1, 2] were electrophoretically analyzed for isocitrate dehydrogenase isozymes in order to learn whether or not cell fusions are a normal concomitant of development. The pure-strain allelic variants at the autosomal *isocitrate dehydrogenase-1*-locus are known to produce only a single band each upon electrophoresis; the heterozygote produces the two parental enzymes and a third or hybrid enzyme.[14] The hybrid enzyme was present in skeletal muscle of allophenic animals, thus conclusively demonstrating the *in vivo* origin of this syncytium by myoblast fusion, rather than by repeated nuclear division in a nondividing cell body. Analyses of a

variety of nonmuscle tissues from two allophenics with different pairs of pure-strain variants disclosed no hybrid enzyme. Cardiac muscle from these animals also lacked hybrid product. The skeletal muscle heterokaryons provide evidence that in the intact organism the monomeric polypeptides are synthesized separately and then are apparently assembled into the complete protein as a second step in the cytoplasm. In addition, we can conclude that the genes on both chromosomes at this locus are functioning in each nucleus when the locus is active.

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