Nucleotide Sequence Variation in Mitochondrial Deoxyribonucleic Acid from Bovine Liver

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ABSTRACT
Mitochondrial deoxyribonucleic acid isolated from the liver of pedigreed cows was examined by restriction enzyme analysis. Differences in restriction enzyme patterns of deoxyribonucleic acid from different maternal lineages were observed. These nucleotide sequence differences were scattered over the mitochondrial genome, with some clustering near the origin of deoxyribonucleic acid replication. Most of them appeared to result from the simple loss or gain of a restriction enzyme site and did not appear to involve gross rearrangements of the mitochondrial genome.

Within one maternal lineage was a nucleotide sequence variation resulting in the loss of a Hae III restriction enzyme site mapping at 37.1 map units on the bovine mitochondrial genome. This polymorphism segregated among members of the lineage in a manner not easily understood by a simple mutagenic event. Amplification and segregation of mitochondrial deoxyribonucleic acid during maternal transmission to progeny most directly explains this extremely rapid variation in nucleotide sequence within a single maternal lineage.

These observations document the existence of a mitochondrial deoxyribonucleic acid polymorphism between and within Holstein cow lineages and should help in understanding molecular genetics of the dairy cow. They additionally demonstrate the utility of patterns of mitochondrial deoxyribonucleic acid restriction enzyme digestion in analyzing maternal lineages of dairy cows.

INTRODUCTION
A number of laboratories have reported that mitochondrial deoxyribonucleic acids (DNA) from closely related species and also from individuals within a species show variability of nucleotide sequence by restriction enzyme analysis (5, 6, 7, 9, 12, 13, 15, 16, 21, 22, 23, 25). Based on these data, it has been suggested that mitochondrial DNA may be evolving more rapidly than nuclear DNA sequences (5, 6). To test the potential of such rapid mitochondrial variation in dairy cow genetics, we examined mitochondrial DNA's from maternally related Holstein cows to find polymorphisms between Holstein cow lineages. Additionally, within one lineage was an extremely rapid and specific variation in the presence of a single Hae III restriction enzyme site, mapping at 37.1% on the bovine mitochondrial map (17, 18). In a more general sense, this type of sequence shift may have significant implications for the molecular mechanism of mitochondrial DNA inheritance, because the traditional view that mitochondrial genotypes undergo random segregation in progeny (2) does not explain this observation.

MATERIALS AND METHODS
Eighteen dairy animals from over 115 with known pedigrees from the Florida Agricultural Experiment Station Dairy Research Unit herd are examined in this report. Livers were obtained at slaughter or occasionally liver biopsies were of 20 to 60 g. For biopsies, a 15-cm vertical incision was made behind the animal's last rib on the right side after administration of
local anesthetic (Lidocaine, 150 to 200 ml). A 20 to 35 g sample was removed by tearing a segment from the margin of the liver (which causes less hemorrhage than cutting) and placed immediately on ice in isotonic buffer (18). Surgical procedures were standard. Mitochondrial isolation, DNA preparation, restriction enzyme digestion, and gel electrophoresis were as in (11, 18).

Briefly, fresh liver was chilled, ground, homogenized, and mitochondria were isolated by differential centrifugation. Mitochondrial DNA was extracted by an SDS-phenol procedure from the purified mitochondria, ethanol precipitated, and then centrifuged to equilibrium in a cesium chloride-ethidium bromide gradient. Further purification on preparative sucrose gradients was occasionally necessary. Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs, and DNA digests were under conditions suggested by the supplier. Gel electrophoresis was in short or long Hoeffer Scientific vertical electrophoresis apparatus, with either agarose or acrylamide gels, depending on the size range of fragments of interest. The cloning of bovine mitochondrial DNA followed standard practice.

Mitochondrial DNA purified from individual animals was digested with Pst I and ligated into the Pst I site of pBR 322 with T4 polynucleotide ligase (4, 11). Recombinant molecules then were transfected into E. coli HB 101 by the calcium-heat shock method (10, 19). Recombinant plasmid-containing colonies then were selected by their resistance to tetracycline and sensitivity to ampicillin, and recombinant-plasmid DNA was isolated from 1-liter cultures of the appropriate colonies (11).

RESULTS

Figure 1 shows a composite physical and genetic map of bovine liver mitochondrial DNA (10, 11, 18). This map was derived from mitochondrial DNA from a single animal. With it as a basis, comparisons can be to restriction enzyme digests of mitochondrial DNA from other animals, and nucleotide sequence differences can be mapped. A number of restriction site alterations are indicated in Figure 1, which have been seen between different maternal lineages.

Figure 2 shows 11 Hae III restriction enzyme digest patterns of mitochondrial DNA from animals of three breeds. Each individual represented a single maternal lineage that was unrelated to other animals examined within that breed for at least 100 yr. Although the overall patterns of restriction fragments were similar, a number of differences were observable on the gels. Mapping studies (10, 11, 17, 18) suggest that each of the observed differences represents a single base change leading to a loss or creation of a restriction enzyme recognition sequence, rather than deletions, rearrangements, or substitutions of new DNA. In addition, mutations showed clustering in certain regions of the mitochondrial genome, particularly near the origin of replication (D-loop) (17). These differences, plus others, are summarized in Figure 1. We also confirmed that, as in insects, amphibians, and three other species of mammals, maternal inheritance of mitochondrial DNA occurs (unpublished data). All of these results, although interesting, are overshadowed by an unexpectedly rapid varia-
tion from detailed study of animals within a single Holstein cow maternal lineage.

Maternal descendents of a registered Hol-

stein cow (barn number H15, registration number 3797669) are in Figure 3. When mitochondri-
drial DNA's from livers of nine animals of this maternal lineage were compared by Hae III
restriction enzyme analysis, size of a single restriction fragment varied. As in Figure 4, frag-
ment L (about 520 base pairs) was in four ani-
mals whereas it was missing and a larger frag-
ment, L* (about 550 base pairs), was in three
animals. Because all other Hae III fragments
visible on the gel were identical for all animals,
it is probable that fragments L and L* arose
from the same genome location.

Hae III fragment L maps between 37.1 and
40.6 map units (Figure 5), because it contains
the Eco RI site at 39.3 and the Bgl II site at
37.9 (10). To confirm the relationship between
L and L*, H493 and H512 mitochondrial DNA
(data not shown) or cloned mitochondrial DNA
from these same two animals (Figure 6) was
digested with Hae III and then either with Bgl
II or Eco RI. All resulting fragments longer
than 200 base pairs were identical in size, ex-
cept that the Hae III + Eco RI digest of H493
DNA contained a fragment of about 390 base
pairs whereas the same digest of H512 DNA
was missing this fragment and contained a new
fragment of 420 base pairs, the same length
difference as between L and L*. Hence, L and
L* map to the same genome location, and the
additional 30 base pairs of L* must map to the
right of 37.9 map units (Figure 5).

Two general possibilities remain for the

Figure 2. Hae III restriction enzyme digests of
mitochondrial DNA. Liver mitochondrial DNA puri-
fied from 11 animals of 10 maternal lineages was
digested with Hae III and compared by electrophoresis
on 6% polyacrylamide gels. These gels separate DNA
molecules of different lengths (i.e., molecular weight),
with the largest molecules migrating more slowly.
Deoxyribonucleic acid is visualized by staining with
the dye ethidium bromide. Gels contained standard
fragments of either SV40 or pBR322 DNA to allow
size determination of mitochondrial fragments; these
lanes have been removed in this figure and Figure 4 to
allow easy comparison of the different digests. Ani-
mals are indicated by barn number. H493 and H737B
belong to the same maternal lineage (see Figure 3).
Letters on the right side of the gel refer to “standard”
Hae III digest products, arranged in order of size (see
Figure 1). Letters (') on the left side indicate new frag-
ments visible in different digests; the letters in paren-
theses refer to missing standard fragments, if they can
be determined. These differences are mapped in Figure
1.

Figure 3. Maternal descendents of registered Hol-
stein cow, barn number H15. Solid underlines of de-
sendants indicate mitochondrial DNA samples hav-
ing the Hae III-L* fragment; dashed underlines indicate
those having the Hae III-L fragment; * denotes animal
is still alive. For clarity, only the relevant relationships
are shown; 19 descendents that have not yet been
analyzed are still alive.
structure of \( L^* \) relative to \( L \); either the extra DNA segment in \( L^* \) arises from an insertion of DNA into an original fragment \( L \) between 37.1 and 37.9 map units, or the extra length of \( L^* \) resulted from the loss of a Hae III site at 37.1 map units and the subsequent joining of a small, previously unmapped Hae III fragment to fragment \( L \). If an insertion of about 30 base pairs occurred between 37.1 and 37.9, this size difference would be apparent in other restriction fragments mapping to this region. Sau 3A digestion produces a fragment (Sau 3A G) of 685 base pairs mapping between 33.7 and 37.9 (J. Alger and P. J. Laipis, unpublished). Comparison of Sau 3A digests of H493 and H512 mitochondrial DNA shows no differences in Sau 3A G or any other Sau 3A fragment (Figure 6). Additionally, a Hinf I digest comparison between H495, H496, and H949B shows no differences in Hinf I J, which maps between 4.8 and 7.9 map units (10). These results are inconsistent with the insertion model and suggest that \( L^* \) has lost a Hae III restriction site at 37.1 and is about 30 base pairs longer than \( L \) for that reason.

Loss of the Hae III site at 37.1 could occur either by a limited alteration in base sequence or perhaps by base modification of an unaltered sequence. To test whether H493 and H512 mitochondrial DNA differ in their modification, the Pst I (19.2/62.2) fragments of H493 and H512 were each cloned via the plasmid pBR 322 into \( E. coli \). Hae III digests of the cloned DNA from these two animals are compared in Figure 6. Because the \( L, L^* \) difference is retained when both DNA’s are grown in the same host (and in presumably the identical modification background), it appears that the difference is from a limited base sequence difference affecting the Hae III restriction site rather than a difference in base modification. Direct nucleotide sequencing of this region of the bovine mitochondrial genome by ourselves (10) shows that the difference in Hae III restric-

Figure 4. Hae III digestions of mitochondrial DNA from seven maternal descendants of Holstein H15 compared by electrophoresis on a 6% polyacrylamide gel. Size calibration is based on Hae III digestion products of SV40 DNA run on the same gels.

Figure 5. Physical map of restriction sites between 31.8 and 41.4 map units for Holsteins H493, H624, H455, H393, and H949B. The dashed line indicates the position of Hae III-L* found in H512, H634, H496, H737B.
tion enzyme patterns between H493 and H512 is from the change of a cytosine to a thymine in the Hae III site. Their single base change alters the restriction site and leads to the observed difference in restriction pattern between animals.

DISCUSSION

Comparison of restriction enzyme digestion patterns of mitochondrial DNA from the livers of pedigreed cows shows that variations in the nucleotide sequence of mitochondrial DNA exist among individuals of the same breed as well as between breeds. These patterns serve as markers for mapping descendents of a single female and, as such, may be useful to breeders in the future.

Physical mapping studies to locate these differences, as well as biochemical studies that examine the molecular details of each sequence change, are beginning to yield important information about the rates and mechanisms of normal mutational events in mammals. In addition, work in our laboratory that compares restriction enzyme patterns of a variety of other bovid species (water buffalo, yak, American bison) is yielding estimates of mutational rates over long times that are of evolutionary significance, as well as answering questions of interest in bovid taxonomy.

Of more fundamental importance is our observation that analysis of maternally related animals suggests that rapid sequence variations are occurring by a presently unknown mechanism. We saw a rapid shift in restriction patterns of mitochondrial DNA isolated from direct maternal descendents. The shift in pattern involved a change in one base of a Hae III site mapping at 37.1 map units (18% clockwise from the 5' end of the D-loop). It is not possible to conclude, with certainty, in which direction the variation.

Figure 6. Polyacrylamide gels of restriction enzyme digests of H493 and H512 mitochondrial DNA and Pst I (19.2/62.2) cloned fragments from those DNAs. Lanes a and b: Sau 3A digests of H493 and H512 DNA, respectively. "G" of H493 and H512 DNA, respectively. "L" and "L*" indicate the positions of Hae III L (37.1/40.5) and Hae III L* (36.9/40.5). Lanes c and f: Bgl II digest of clones of the Pst I (19.2/62.2) fragments of H493 (lane e) and H512 (lane f) in pBR 322. "B" denotes the Hae III/Bgl II (37.9/40.5) fragment. Lanes g and h: Eco RI digests of the same clones in lanes e and f. "E" and "E*" indicate the positions of Hae III/Eco RI (37.1/39.3) and (36.9/39.3), respectively. "E-9" indicates the 176 bp fragment resulting from Eco RI digestions of Hae III 9 (4344-174) of pBR. Lanes i and j: Hae III digests of the same clones in lanes e and f. "L" and "L*" denote the positions of Hae III L (37.1/40.5) and Hae III L* (36.9/40.5). Lane k: Hae III digest of pBR 322 DNA.
is occurring. We note, however, that the Hae III-L fragment is in the mitochondrial DNA of all other cows we have examined, including 24 Holstein and 9 Jersey cows. It is, therefore, reasonable to conclude that the Hae III site at 37.1 map units represents the predominant sequence in cows. The alternatives are then that either loss of Hae III-L*, as evidenced by presence of fragment Hae III-L, is the result of a relatively recent mutational event that is now beginning to spread through the population, or that loss of Hae III-L reflects an ancient vestigial or perhaps less "fit" sequence that is disappearing from dairy cow mitochondria. We cannot conclude which view is correct without knowledge of the functions of the mitochondrial protein coded by this region. Both genotypes are clearly viable, but small differences in "fitness" may not be obvious in the animal.

What is the source of the observed variations in restriction patterns of maternally related animals? In the most general sense, these differences could arise through either a mutational mechanism or through a pattern of mitochondrial inheritance in which only subsets of the total mitochondrial DNA molecules of an animal are distributed to her progeny. If heterozygosity exists within the animal, then variant progeny could arise from this source without requiring a temporally-linked mutational event. Toward deciding which mechanism is more plausible it is possible to estimate the mutation rate necessary to account for the observed variation. To date we have compared a total of 95 mapped restriction sites between H493 and H496 (17) and only the one Hae III site at 37.1 map units is altered. If we assume the change results from a mutational process and that the alteration is a point mutation, the apparent mutation rate is \(1.4 \times 10^{-4}\) changes/base pair per yr by either the method of Upholt (24) or Brown (6). This rate is 10,000 times more rapid than that calculated from evolutionary divergence of mitochondrial DNA between primate species (6). Even if this single Hae III site difference is the only difference in the entire sequence between the mitochondrial DNAs of these two animals, our calculated mutation rate is still 200 times more rapid than interspecies rates. Additionally, inspection of the maternal lineage (Figure 3) shows that this event occurred independently at least four times, at the same apparent site, to give the observed distribution of descendents. Hence, in addition to its rapid rate, this change appears to be site specific. Both of these properties would require an unprecedented mutational process which is both uniquely rapid and sequence specific. It, therefore, seems reasonable to consider non-mutational mechanisms to explain this sequence variation.

One alternative, as suggested, is process of mitochondrial inheritance, which effectively amplifies a small subset of parental mitochondrial DNA molecules. If the mitochondrial DNA molecules of a single cell are heterogeneous either because of a recent mutation or through evolutionary accumulation of divergent mitochondrial DNA molecules, then the animal will be heterozygous for different mitochondrial DNA molecules. Subsequent amplification and inheritance of such a divergent molecule from a heterozygous population could account for a rapid shift between restriction pattern types. The shift then could occur in a few (possibly only one) generations without invoking an unprecedented mutational mechanism. Unfortunately, little is known about the molecular details of mitochondrial inheritance in higher eukaryotes. Within present limits of detection, mitochondria appear to be inherited strictly maternally (8, 15, 16) (detection techniques would have missed paternally inherited mitochondrial DNA if the ratio of maternal/paternal were greater than 500). Therefore, the frequency of variation seems to rule out paternal inheritance. Additionally, breeding records within the H15 lineage yield no obvious paternal pattern to account for the variation. Whereas mammalian somatic cells have \(1 \times 10^3\) mitochondrial DNA molecules per cell (3), mammalian ova have 100 to 1000 times more copies per cell (20). Clearly, then, mitochondrial DNA amplification has occurred during development of the mature ovum. There are, however, no data bearing on whether all mitochondrial DNA molecules are amplified equally or only a subset is amplified. However, it is not unreasonable to speculate that the special replication apparatus necessary to amplify mitochondrial DNA during ovum development may use only one or a few original template molecules. Without further information as to the exact fate of individual mitochondria DNA molecules during ovum development, it is difficult to make specific predictions based on a
mitochondrial inheritance model except to say that it cannot be ruled out.

Such rapid shifts in mitochondrial DNA populations between maternally related animals may provide a mechanism for selecting and testing the fitness of mitochondrial DNA variants. In the present case, both mitochondrial genotypes are approximately equally viable. The general procedures and techniques in this work would be applicable to any study of material inheritance of phenotype in mammals.

In particular, the presence of basic mapping data (10, 18) and the complete nucleotide sequence of bovine mitochondrial DNA (1) make this an ideal system to investigate the genetic basis of maternally inherited phenotype. Examples of such inheritance would be of great interest to us. Finally, we wish to emphasize that this observation could only have been made in a system where careful, long records of parentage are kept. In this regard, the bovine system offers an unexcelled data base that should be employed profitably in many other studies.

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