Restriction Endonuclease Cleavage Site and Length Polymorphisms in Mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae)

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**ABSTRACT**

Restriction endonuclease cleavage maps of mitochondrial DNAs of Scandinavian *Apis mellifera mellifera* L., of German, Austrian, and Yugoslavian *A. m. carnica* Pollman, and of Austrian “Nigra” honey bees are compared with previously published maps of mitochondrial DNA from North American bees of European ancestry and Brazilian Africanized bees. *A. m. mellifera* mitochondrial DNA is characterized by a pattern of cleavage sites unique among the honey bee populations thus far investigated. Variation in size of the mitochondrial DNA molecule is common among families (hives) of *A. m. mellifera* and appears to involve several distinct regions that span a region at least 5.1 kilobase pairs in length. Some elements of size variation seem to be confined to the *A. m. mellifera* population, whereas others are shared with Africanized bees. *A. m. carnica* mitochondrial DNA is characterized by a pattern of cleavage sites, which differs from that of *A. m. mellifera* and the Africanized bees but is similar to that of the domestic North American bees of European ancestry.

**KEY WORDS**

Insecta, *Apis mellifera*, Africanized honey bee, mitochondrial DNA

*Apis mellifera* L. is subdivided into approximately 24 named geographic races or subspecies (Ruttner 1988). These subspecies are believed to be recent in origin; for example, the principal subspecies in Europe may have differentiated from one another during the Pleistocene glacial periods, when European honey bee populations were probably confined to refugia around the Mediterranean (Ruttner 1988). *A. mellifera* subspecies are defined and identified largely by morphometric criteria (e.g., Alpatov 1929, Cornuet et al. 1975, Daly & Balling 1978, Ruttner et al. 1978, Ruttner 1986), but they also differ in behavior, physiology, and ecology (Adam 1951, 1954, 1961, 1964, 1977; Ruttner 1988). Differences in allele frequencies have been found among subspecies at several polymorphic loci (e.g., Mestriner & Contel 1972, Contel et al. 1977; Martins et al. 1977; Sylvester 1982; Del Lama et al. 1985, 1988; Spivak et al. 1988). These polymorphisms are useful in the study of honey bee biogeography and population biology (e.g., Cornuet 1979; Sheppard & Berlocher 1984, 1985; Cornuet et al. 1986; Sheppard & McPherson 1986) and in the study of Africanized bees (Nunamaker & Wilson 1981, Sylvester 1982, Nunamaker et al. 1984).

Polymorphisms in mitochondrial DNA (mtDNA) complement and extend studies of allozyme variation in a number of ways. Because animal mtDNA is typically maternally inherited without recombination (e.g., Dawid & Blackler 1972, Reilly & Thomas 1980; Lansman et al. 1983; Brown 1985), genetic markers on mtDNA can be used to determine the maternal ancestry of hybrid individuals and, thus, the directionality of gene flow in hybrid zones or hybrid populations (Wright et al. 1983, Avise & Saunders 1984, Gyllensten et al. 1985). In addition, because animal mtDNA does not undergo recombination during sexual reproduction, it is passed intact along maternal lineages. This allows identification of the populations that have contributed to a hybrid population even after many generations of hybridization or backcrossing, or both (Ferris et al. 1982, Wright et al. 1983, Tegelström 1987).

Several practical considerations make the use of mtDNA particularly attractive in the study of honey bee population biology. First, although honey bees appear to have relatively low levels of allozyme variability (e.g., Sheppard & Berlocher 1984, 1985), the level of variation in their mtDNA is well within the range found in other species (Avise & Lansman 1983). Second, although no fixed differences in allozymes have been found among honey bee subspecies, preliminary studies of the mtDNAs of European and African subspecies (Smith 1988; D.R.S., unpublished data) indicate that at least some have unique cleavage site patterns. Finally, because all the offspring of a queen inherit the same
mtDNA, large quantities of mtDNA from a single source can be prepared by pooling tissue from hive mates.

The Carniolan honey bee, Apis mellifera carnica Pollmann, is native to Yugoslavia, Austria south of the Alps, and parts of Hungary, Romania, and Bulgaria (Ruttner 1975, 1988). Because of its gentle disposition, good overwintering abilities, and good honey production, this subspecies is popular among commercial and amateur beekeepers and has been imported to other parts of Europe and to the New World. It is now one of the most widely distributed of the honey bee subspecies (Ruttner 1975). Apis mellifera carnica L. originally occupied Britain and north and central Europe (Ruttner 1988). Today its range, at least in domesticated populations, as been dramatically altered by human preferences. A. m. ligustica Spinola and A. m. carnica have been introduced into many parts of A. m. mellifera’s range, and in some domestic populations (e.g., Germany, Switzerland) they have largely replaced native A. m. mellifera (Ruttner 1975). Thus care was exercised in the selection of the A. m. mellifera populations we sampled. The Swiss and Austrian “Nigra” honey bees are hybrids of A. m. mellifera, originally native to these areas, and imported A. m. carnica.

Our study presents cleavage site maps and an analysis of length variation in A. m. mellifera, A. m. carnica, and “Nigra” mtDNA as well as comparisons between these mtDNAs and those of North American bees of European ancestry and Brazilian Africanized bees. This is part of a continuing survey of mtDNA in A. mellifera subspecies.

Materials and Methods

Collections. Fifty to 200 adult worker bees were collected from each hive. Heads and thoraces were frozen in liquid nitrogen and transported to the laboratory, where they were stored at −80°C until they were used in the preparation of mtDNA.

Samples of A. m. carnica were collected from Graz, Villach, and the Institut für Bienenkunde, Lunz-am-See, Austria. The research apiary at Lunz-am-See maintains imported colonies as well as local honey bees. Samples collected at the Institut für Bienenkunde included hives from Medvode (Slovenia) and Split (Dalmatia), Yugoslavia; Hamburg, West Germany; and Vienna and Lunz-am-See, Austria. Graz, Villach, Vienna, Medvode, and Split lie within the original range of A. m. carnica. Lunz-am-See lies outside the original range of A. m. carnica, but A. m. carnica is well established in the area now and most domestic hives are this subspecies (Ruttner 1988). The Hamburg sample is an example of the A. m. carnica that have been imported extensively into northern and western Europe and that have largely replaced domesticated A. m. mellifera over much of that subspecies’ original range.

Because A. m. mellifera has been replaced or hybridized with other subspecies throughout much of its range, bees for this study were collected from apiaries whose hives were known to consist mainly of A. m. mellifera and for which the history of importation and breeding was relatively well known. Samples of A. m. mellifera were collected from Laesø, Denmark (the island of Laesø is now a preserve for A. m. mellifera, and importation of other subspecies is forbidden; S. Toft, personal communication); Billingstad, near Asker, Norway; and Uppsala, Sweden. Samples of the honey bee strain “Nigra” were collected from Otzalbahnhof Austria. Subspecies identifications have been corroborated by morphological measurements and allozyme analysis (D.R.S. & B. Crespi, unpublished data).

MtDNA was prepared from three hives each from Graz (Graz1–3), Villach (Vill1–3), and Split (Dalm1–3); two hives each from Vienna (Wien1–2) and Medvode (Slov1–2); one hive each from Lunz-am-See (Lunzl) and Hambach (Ham1); seven hives from Laesø (Laesol–7); three hives each from Asker (Asker1–3) and Uppsala (Uppsl–3); and from nine hives from Otztalbahnhof (Nigrfl–9).

Preparation and Analysis of mtDNA. Mitochondrial DNA was prepared from frozen thoraces of about 25 adult worker bees from each hive by the method described by Wright et al. (1983) with the following modifications of the tissue homogenization procedure. Batches of 15–25 thoraces were ground to a powder in liquid nitrogen with a ceramic mortar and pestle. The powdered tissue (mostly flight muscle) was resuspended either in 10 ml of 10 mM NaCl, 10 mM Tris, 200 mM EDTA, pH 7.5, or in 10 ml of 10 mM Tris, 10 mM EDTA (TE), pH 7.5. 1.2 ml of 20% sodium dodecyl sulfate (SDS) was added, and the mixture was incubated for 10–20 min at room temperature to lyse cell and organelle membranes. 3.5 ml of CsCl-saturated TE was then added, and the mixture was incubated on ice for 15–30 min to precipitate SDS and proteins. The mixture was centrifuged at 17,000 × g (12,000 rpm in a Beckman JA-17 rotor) at 4°C for 10 min to pellet cellular debris; 1–2 ml of propidium iodide (2 mg/ml in TE) was added to the supernatant, and the density of the solution was adjusted to 1.56–1.57 g/ml with solid CsCl. CsCl density centrifugation and sample recovery followed the methods described in Wright et al. (1988).

Aliquots of each mtDNA sample were digested with each of 15 restriction enzymes (AccI, Aval, BglII, EcoO109, EcoRI, EcoRV, HindII, HindIII, NdeI, PstI, PvuII, SpeI, XbaI, and XhoI), using the buffer conditions recommended by the suppliers (Bethesda Research Laboratories, Gaithersburg, Md.; International Biotechnologies, New Haven, Conn.; New England BioLabs, Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis). Each of these enzymes recognizes and cleaves a particular sequence or sequences of six basepairs. The resulting DNA fragments were ra-
Table 1. Percentage sequence divergence among the mitochondrial genomes of A. m. carnica, A. m. mellifera, a North American hive of European ancestry (USA1), and a Brazilian Africanized hive (Brzl)

<table>
<thead>
<tr>
<th></th>
<th>Car1</th>
<th>Car2</th>
<th>Car3</th>
<th>USA1</th>
<th>Brz1</th>
<th>Mel1</th>
<th>Mel2</th>
<th>Mel3</th>
<th>Mel4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Car1</td>
<td>—</td>
<td>0.0097</td>
<td>0.0033</td>
<td>0.0033</td>
<td>0.0269</td>
<td>0.0372</td>
<td>0.0340</td>
<td>0.0291</td>
<td>0.0324</td>
</tr>
<tr>
<td>Car2</td>
<td>0.0057</td>
<td>—</td>
<td>0.0063</td>
<td>0.0063</td>
<td>0.0291</td>
<td>0.0388</td>
<td>0.0356</td>
<td>0.0310</td>
<td>0.0341</td>
</tr>
<tr>
<td>Car3</td>
<td>0.0033</td>
<td>0.0045</td>
<td>—</td>
<td>0.0065</td>
<td>0.0304</td>
<td>0.0405</td>
<td>0.0372</td>
<td>0.0324</td>
<td>0.0356</td>
</tr>
<tr>
<td>USA1</td>
<td>0.0033</td>
<td>0.0045</td>
<td>0.0047</td>
<td>—</td>
<td>0.0225</td>
<td>0.0324</td>
<td>0.0291</td>
<td>0.0246</td>
<td>0.0278</td>
</tr>
<tr>
<td>Brz1</td>
<td>0.0108</td>
<td>0.0111</td>
<td>0.0116</td>
<td>0.0106</td>
<td>—</td>
<td>0.0109</td>
<td>—</td>
<td>0.0054</td>
<td>0.0068</td>
</tr>
<tr>
<td>Mel1</td>
<td>0.0129</td>
<td>0.0129</td>
<td>0.0135</td>
<td>0.0117</td>
<td>0.0101</td>
<td>0.0055</td>
<td>—</td>
<td>0.0105</td>
<td>0.0068</td>
</tr>
<tr>
<td>Mel2</td>
<td>0.0123</td>
<td>0.0123</td>
<td>0.0129</td>
<td>0.0111</td>
<td>0.0109</td>
<td>0.0049</td>
<td>0.0063</td>
<td>—</td>
<td>0.0033</td>
</tr>
<tr>
<td>Mel3</td>
<td>0.0111</td>
<td>0.0112</td>
<td>0.0117</td>
<td>0.0099</td>
<td>0.0109</td>
<td>0.0049</td>
<td>0.0063</td>
<td>—</td>
<td>0.0033</td>
</tr>
<tr>
<td>Mel4</td>
<td>0.0117</td>
<td>0.0117</td>
<td>0.0123</td>
<td>0.0106</td>
<td>0.0056</td>
<td>0.0033</td>
<td>0.0049</td>
<td>—</td>
<td>0.0033</td>
</tr>
</tbody>
</table>

The method of Nei & Tajima (1983) was used to estimate the mean number of nucleotide substitutions per site (above the diagonal) and standard deviation of the mean (below the diagonal) using comparisons of the mapped cleavage sites produced by nondegenerate six base pair restriction enzymes (BglII, BglIII, EcoO109, EcoRI, EcoRV, HindIII, NdeI, PvuII, PstI, PvuII, Spel, and Xhol). A. m. mellifera restriction morphs (Mel1-Mel4) as in Fig. 2. A. m. carnica restriction morphs (Car1-Car3) as in Fig. 3.

Results and Discussion

Both cleavage site and length polymorphisms occur in honey bee mtDNA. Fig. 1 shows examples of restriction-site polymorphisms among the mtDNAs of A. m. carnica, A. m. mellifera, and a hive of Brazilian Africanized bees. Two distinct restriction enzyme cleavage maps were found in this study, one typical of A. m. mellifera (Fig. 2) and a second typical of A. m. carnica (Fig. 3). Seven of the nine “Nigra” samples had A. m. mellifera mtDNA and two, Nigra 5 and 7, had A. m. carnica mtDNA.

The mtDNAs of A. m. mellifera and A. m. carnica show very little intrapopulation variation in cleavage sites. Only the presence of two Spel sites and one BglI site varied among Scandinavian A. m. mellifera and mellifera-like “Nigra” hives (Fig. 2). A. m. carnica and carnica-like “Nigra” hives differed in the presence or absence of two XbaI sites and one NdeI site (Fig. 3). The similarity of mtDNA cleavage maps within each subspecies is reflected in the low estimate of sequence divergence among the A. m. mellifera mtDNAs (0.33-
Fig. 2. Restriction enzyme cleavage site map of mtDNAs of Scandinavian *A. m. mellifera* and *mellifera*-like Austrian "Nigra" bees. The cleavage site for *PvuII* was arbitrarily chosen as the starting point for the map. Cleavage sites above the horizontal were common to all *A. m. mellifera* and *mellifera*-like "Nigra" mapped. The presence of sites below the horizontal (*SpeI* at 0.5 kb, *SpeI* at 15.16 kb, and *BclI* at 6.89 kb) varied among restriction morphs (*Mel*-1-Mel4) as follows: *Mel1*, both *SpeI* sites present, *BclI* site absent, *Laesel*-1, -3, *Askel* and 5, *Nigra6* and 8; *Mel2*, *SpeI* at 0.05 present, *BclI* and *SpeI* at 15.16 absent, *Uppel* and 2; *Mel3*, *SpeI* at 15.16 and *BclI* present, *SpeI* at 0.05 present, *Uppel* and 2; *Mel4*, all 3 sites present, *Laesel*-4, -7, *Ask2*, *Nigra2,4,5,9*. Restriction-site mapping indicated that the *xcl* and *PstI* sites at 2.4 kb were approximately 10bp apart, the *HinClI* site occurring before the *PstI* site subsequent sequencing of this region by Vlasak et al. (1987) shows these sites to be 9bp apart; the region sequenced (0-3.0 kb on our map) encompasses most of the large subunit ribosomal DNA (Vlasak et al. 1987). The *Ndel* and *MII* sites at approximately 6.4 kb were indistinguishable by restriction-site mapping. Subsequent sequencing of this region (Grozier et al. 1989) shows that these two restriction sites are overlapping. The *HindIII* and *XbaI* sites at 7.2 kb are approximately 50bp apart, the *HindIII* site occurring before the *XbaI* site. The *HindIII* and *XhoI* sites at approximately 7.8 kb are approximately 15bp apart; their relative positions have not been determined. The letters below the horizontal refer to regions of size variation presented in Table 2.

1.00%) and among the *A. m. carnica* mtDNAs (0.33–0.97%; Table 1).

However, the restriction site maps show substantial variation among subspecies. *A. m. mellifera* mtDNAs differ from those of *A. m. carnica* and the American and Brazilian bees examined (Fig. 4), and from those of other subspecies currently under investigation (*A. m. ligustica*, *A. m. scutellata* Lepeletier, *A. m. capensis* Escholtz, and *A. m. Iberica* Goetze; Smith [1988] and unpublished data). In particular, the *HinClI* site at 7.7 kb, the *EcoRI* site at 14.7 kb, and the *SpeI* site at 16.16 kb (Fig. 2) have been found so far only in *A. m. mellifera* samples. Likewise, the absence of the *HindIII* site at approximately 6.3 kb and the *AccI* site at 11.03 kb appear to be characteristic of *A. m. mellifera*. The mtDNAs of *A. m. carnica* are not substantially different from that of *USA1*, a domestic North American hive (0.33–0.47% sequence divergence; Table 1). This is not surprising, because domestic North American bees are primarily descendants of *A. m. ligustica* and *A. m. carnica*, and these two subspecies are themselves thought to be very closely related (Ruttner 1988). The differences between the cleavage maps of *A. m. mellifera*, *A. m. carnica*, *USA1*, and a Brazilian Africanized hive

Fig. 3. Restriction enzyme cleavage site map of mtDNAs of *A. m. carnica* Cleavage sites above the horizontal were common to all *A. m. carnica* mapped. The presence of the sites below the horizontal (*XbaI* at 5.25 and 12.88 *Ndel* at 15.74) varied among restriction morphs (*Carl*-1–*Carl*3) as follows: *Carl1*, both *XbaI* sites and *Ndel* site absent, *Graz1* and 3, *Slov1*; *Carl2*, both *XbaI* sites and *Ndel* site present, *Vill3*, *Dalm1* and 3; *Carl3*, both *XbaI* sites absent, *Ndel* site present, *Hambl*, *Wienl* and 2, *Lunzl*, *Vill1* and 2, *Graz2*, *Dalm2*, and *Slov2*. The letters below the horizontal refer to regions of size variation discussed in Table 2.
(BRZ1) are reflected in their higher estimated sequence divergences (2.23–4.05%; Table 1).

There is substantial variation among families (hives) in the size of the mtDNA molecule as well as size variation in mtDNA among subspecies (Table 2). Two regions of size variation are of particular interest. The region between the XbaI site at 5.23 kb (Fig. 2, b) and the AccI site at approximately 5.8 kb (Fig. 2, c) is longer in $A. m. mellifera$ and the $mellifera$-like "Nigra" than the corresponding region in the American and Brazilian mtDNAs; it also is variable among $A. m. mellifera$ families (Fig. 5). This variation is most easily explained by postulating a sequence 80–100 bp in length which is present once (in Laesø1-7, Asker1-3, and Upps3), twice (in Upps1-2), or three times (Nigra1-2,4-6,8-9) in $A. m. mellifera$ and $mellifera$-like "Nigra" mtDNA, and which is absent from the mtDNAs of $A. m. carnica$ and the North American and Brazilian bees sampled.

The region of $A. m. mellifera$ mtDNA the BclI sites at 7.9 kb (Fig. 2 and 3, k) is 270 bp large corresponding region in the mtDNA of this respect $A. m. mellifera$ and the Brazilian mtDNAs are similar. The corresponding region in the mtDNA of hive Upps3 is 540 bp larger than in USA1; it is explained by assuming that the 270-bp site is present twice in the mtDNA of Upp.

The region between the NdeI sites (Fig. 2, g) and 12.26 kb (Fig. 2, h) is approximately 80 bp larger in $A. m. mellifera$ than in $A. m. carnica$ Africanized hive than in $A. m. carnica$

Size variation in mtDNA has been reported in individuals, among conspecific individuals (e.g., Faustenholme 1976, 1980a,b; Densmore et al. 1985; Moritz & Brown 1986; Moritz et al. 1987) and among closely related species (e.g., Faustenholme 1976, 1980a,b; Densmore et al. 1985; Moritz & Brown 1986; Moritz et al. 1987).

### Table 2. Comparison of size variation in kilobase pairs (kb) in the mitochondrial genomes of $A. m. ca$ mellifera, and a Brazilian Africanized bee (BRZ1)

<table>
<thead>
<tr>
<th>Location of fragment</th>
<th>$A. m. carnica$</th>
<th>$A. m. mellifera$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PstI &quot;a&quot; to XbaI &quot;b&quot;</td>
<td>2.78</td>
<td>2.8–2.97</td>
</tr>
<tr>
<td>2. XbaI &quot;b&quot; to Accl &quot;c&quot;</td>
<td>0.53</td>
<td>0.62 in Laesø1-7, Asker1-3, Upps3</td>
</tr>
<tr>
<td>3. BclI &quot;d&quot; to Bell &quot;e&quot;</td>
<td>0.43</td>
<td>0.70 in Upps1 and 2</td>
</tr>
<tr>
<td>4. Bell &quot;e&quot; to BclI &quot;f&quot;</td>
<td>0.54</td>
<td>0.79 in Nigra 1-2, 4-6, 8-9</td>
</tr>
<tr>
<td>5. Bell &quot;f&quot; to Bell &quot;g&quot;</td>
<td>1.19</td>
<td>Site &quot;d&quot; absent</td>
</tr>
<tr>
<td>6. NdeI &quot;g&quot; to NdeI &quot;h&quot;</td>
<td>2.87</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>USA1 = 2.85</td>
<td>1.73 in Upps3, 1.46 in all others</td>
</tr>
</tbody>
</table>

$A. m. carnica$ fragment sizes are like those of a North American honey bee of European ancestry (USA1) except when lowercase letters in quotes refer to restriction sites in Fig. 2 and 3.
in Brown 1983, 1985). In many cases, this variation is located in or adjacent to the noncoding control region. However, the regions to which size variation in A. mellifera mtDNA have been mapped span a minimum of 5.1 kb, or approximately one-third of the 16.8-kb genome, making it unlikely that all of these regions of size variation are in or adjacent to the control region.

In addition, Crozier et al. (1989) have sequenced a part of an A. m. ligustica mtDNA corresponding to the region from 6.5 to 9.5 kb on our maps. This region was found to include (reading from 6.5 to 9.5 kb) aspartate tRNA, cytochrome oxidase I, leucine tRNA, a small unidentified reading frame, cytochrome oxidase II, and lymphocyte tRNAs. The position of the (as yet) unidentified reading frame corresponds to one of the regions of size variation, namely the site which is 270 bp larger in A. m. mellifera and Brazilian Africanized bees. This element of size variation clearly does not lie in the AT-rich control region.

Maternally inherited mtDNA polymorphisms can be used in conjunction with biparentally inherited characters such as allozyme or morphological phenotypes to detect the direction of gene flow in hybrid populations. This has practical application in the study of honey bee populations in the New World. For example, A. m. mellifera was the subspecies imported to North America by European colonists in the 17th century and was probably the most commonly imported bee until the middle of the 19th century (Oertel 1976, Pellet 1938). Today, A. m. mellifera has been largely replaced in North American apiaries by other subspecies, particularly A. m. ligustica, A. m. carnica, and their hybrid. It is possible that the descendants of the early A. m. mellifera imports are present in North American feral populations, as has been suggested by Sheppard (1988) on the basis of allozyme studies.

Maternally inherited mtDNA polymorphisms also are useful in analyzing the pattern of gene flow between European and Africanized bees in the New World. Earlier work has shown differences in mtDNA cleavage patterns between North American bees of European ancestry and some Brazilian Africanized bees (Smith & Brown 1988) and between European and African subspecies (Smith 1988). One critical question for the management of Africanized bees is the extent to which gene flow takes place from managed European apiary populations into feral Africanized populations. If swarms headed by European queens or hybrid queens (from European queen–African drone matings) routinely enter and survive in the feral population, the feral populations should gradually acquire more European characteristics. On the other hand, if swarms headed by European queens or European–African hybrid queens are selected against in feral populations, the Africanized population advancing north might show relatively little European influence.

The success of queens of European maternal ancestry in feral Africanized populations can be assessed by the relative frequencies of European and Africanized mtDNAs in these populations.

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Fig. 5. Autoradiograph of 1% agarose gel showing A. m. mellifera and “Nigra” mtDNA digested with the enzyme XbaI. Lane 1, Laesø1; 2, Laesø2; 3, Laesø3; 4, Asker1; 5, Asker2; 6, Asker3; 7, Upps1; 8, Upps2; 9, Upps3; 10, Nigra1; 11, Nigra2; 12, A. m. carnica (Graz)). Size standard is HindIII-digested lambda phage DNA. Note size variation in smallest XbaI fragment.
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