Characterization and cloning of the African horsesickness virus genome

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The dsRNA profiles of all nine African horsesickness virus (AHSV) serotypes were compared by agarose gel electrophoresis and PAGE. The agarose profiles were identical, but a unique profile was obtained for each of the nine serotypes by PAGE. Nine of the 10 dsRNA genome segments of AHSV-3 were cloned and the clones were used in dot-spot and Northern blot hybridization experiments to determine intra- and inter-serogroup nucleic acid similarities. Segments 1, 3, 4, 5, 7 and 8 were highly conserved in the AHSV serogroup and no genetic relationship with any of the other orbiviruses was observed. Of these segments 3, 5 and 8 showed the largest degree of cross-hybridization to the cognate genes of all the serotypes. These clones did not cross-hybridize to other orbiviruses such as epizootic haemorrhagic disease virus, bluetongue virus or equine encephalosis virus and are therefore recommended for use as group-specific probes for the identification of the AHSV serogroup. Genome segments 6 and 10 showed an intermediate degree of conservation, whereas segment 2 is serotype-specific and therefore probably codes for the outer capsid protein VP2.

Introduction

African horsesickness is an economically important viral disease of solipeds, causing a high mortality in horses. Clinically it is characterized by hyperthermia, oedema of the lungs, pleura and subcutaneous tissues, as well as haemorrhages in the internal organs (McIntosch, 1958). The aetiological agent, African horsesickness virus (AHSV), is a member of the orbivirus genus of the Reoviridae family (Verwoerd et al., 1979). Nine different serotypes have been identified (McIntosch, 1958; Howell, 1962). The disease is enzootic in Africa and severe epizootics have occurred at infrequent intervals in Egypt, the Middle East and Yemen (McIntosch, 1958). In 1987, 1988 and 1989 outbreaks were reported in Spain, and recently AHSV has also spread to Portugal (B. J. Erasmus, personal communication).

The virus is transmitted by a Culicoides vector (Du Toit, 1944). The involvement of a reservoir host of the virus has also been suggested, but attempts to determine the presence of the virus in a variety of wild mammals, birds and amphibians were unsuccessful (McIntosch, 1958). At present routine detection of AHSV infection relies largely on virological procedures such as propagation by passaging in mouse brain (Howell, 1962), cell cultures or embryonated eggs, and serological typing is done by plaque reduction neutralization (personal communication, B. J. Erasmus). However, these methods are time-consuming and often present problems due to their requirement for viable virus.

In view of the serious economic implications of outbreaks of AHSV, particularly in the racing industry, it is essential to have available more rapid, sensitive and specific diagnostic tools. Of particular importance is the need to be able to distinguish between vaccinated and naturally infected animals. The development of genomic probes offers many of these advantages and would certainly supplement the existing serological tests. The identification of group- and serotype-specific genomic probes has been extensively investigated for other orbiviruses such as bluetongue virus (BTV) (Roy et al., 1985; Huismans & Cloete, 1987; Huismans et al., 1987; Gould, 1988a), equine encephalosis virus (EEV) (Viljoen & Huismans, 1989) and epizootic haemorrhagic disease virus (EHDV) (Nel & Huismans, 1990). Genomic probes have also been developed for members of the rotavirus genus of the Reoviridae family (Pedley & McCracke, 1984).

Like BTV, the prototype orbivirus, the AHSV virion consists of a double-layered protein capsid containing 10 dsRNA genome segments (Oellermann et al., 1970). The capsid is composed of four major and three minor structural proteins; two of the major proteins form the outer capsid layer (Oellermann et al., 1970; Bremer, 1976). In this paper we report on the cloning of fragments of nine of the 10 dsRNA genome segments of AHSV-3 and we have also investigated the genetic relatedness of a
number of cognate genes within the AHSV serogroup using both dot-spot and Northern blot hybridization. From these results the best candidates for a group-specific and serotype-specific probe were identified.

Methods

Virus and cells. The AHSV, BTV and EEV serotypes used in the investigation were all South African isolates; EHDV-New Jersey (NJ) was an American isolate. The AHSV serotypes were passaged three times in suckling mice and thereafter propagated in baby hamster kidney monolayer cells using modified Eagle's medium (Verwoerd, 1969) supplemented with 5% bovine serum. The origin and cultivation of EEV-Cascara has been described by Viljoen & Huismans (1989) and that of BTV-10 by Huismans & Cloete (1987).

Purification of dsRNA. The dsRNA from AHSV-3 for cloning was isolated by SDS-phenol extraction from purified virus (Bremer, 1976). When required, the dsRNA was enriched for certain fragments using methylated albumin Kieselguhr columns (Huismans & Cloete, 1987). Individual dsRNA segments were obtained by cutting bands containing the different segments from preparative polyacrylamide gels. These were homogenized in 0.15 M-STE buffer (0.15 M-NaCl, 0.01 M-Tris and 0.001 M-EDTA, pH 7.4) using an Ultra Turrax homogenizer and the slurry was shaken at 37 °C for 4 h. Acrylamide was removed by centrifugation and, after phenol extraction of the supernatant, dsRNA segments were further purified by centrifugation on 10 to 40% sucrose gradients in 0.15 M-STE. dsRNA was also purified by SDS-phenol extraction from infected cells followed by CF-11 cellulose chromatography (Franklin, 1966), as described by Huismans & Bremer (1981).

Polyacrylamide and agarose gel electrophoresis. dsRNA was separated on 7% polyacrylamide gels using the buffer systems of either Loening (1967) or Laemmli (1970). The latter gel system did not yield the ‘trailing’ of segments that often occurs on Loening gels. Agarose (Seakem ME) gel electrophoresis of dsRNA was carried out in buffer containing 0.04 M-Tris-acetate and 0.002 M-EDTA, pH 8.

Cloning of dsRNA genome segments and characterization of recombinant plasmids. The dsRNA of AHSV-3 was cloned using a modification of the method of Casadilol et al. (1984). Preparations of dsRNA were denatured with 10 mM-methylmercuric hydroxide and polyadenylated (Sippel, 1973). Complementary DNA was synthesized and fractionated on alkaline sucrose gradients, as described by Huismans & Cloete (1987). DNA fractions were dC-tailed and cloned into dG-tailed PstI-cut pBR322, as described by Huismans et al. (1987). Plasmids were purified using the alkaline lysis method of Birnboim & Doly (1979). Recombinant plasmids were labelled with 32P by nick translation (Rigby et al., 1977; Maniatis et al., 1982) and their origin was assigned by hybridization to Northern blots of AHSV-3 dsRNA, as described by Huismans & Cloete (1987).

Dot-spot and Northern blot hybridization. Dot-spot hybridization was carried out as described by Huismans & Cloete (1987) using Hybond-N membranes (Amersham). For Northern blot hybridization dsRNA was separated on a 7% polyacrylamide gel (Laemmli, 1970). Before blotting the dsRNA was denatured by soaking the gel in 0.1 M-NaOH for 20 min, then twice for 10 min in 5 × TAE buffer (1 × TAE contained 40 mM-Tris, 20 mM-sodium acetate and 1 mM-EDTA, pH 7.8) and then 10 min in 0.5 × TAE. RNA was then transferred to a Zeta-Probe membrane in a Trans-Blot electrophoretic transfer cell (Bio-Rad) in 0.5 × TAE at 0.8 A for 1 to 2 h. The dsRNA was fixed on the membrane by exposure to u.v. light for 5 min on each side. Prehybridization and hybridization were carried out as described in the Zeta-Probe blotting membranes instruction manual, using the formamide protocol. Probes were labelled with 32P by a nick translation reaction (Rigby et al., 1977; Maniatis et al., 1982).

Results

dsRNA profiles

The previous work on AHSV was carried out with AHSV-3 and no comparison of the dsRNA profiles of the various serotypes has as yet been made. Although the
PAGE systems of Loening and Laemmli do not give an accurate reflection of the size of the genome segments (Clarke & McCrae, 1982; Pedley et al., 1988), the profiles do give an indication of genome heterogeneity based on both size and structure and can be useful in distinguishing between different virus serotypes and isolates.

dsRNA from the nine different AHSV serotypes was isolated from infected cells and, after purification, fractionated on a 7% Laemmli polyacrylamide gel together with dsRNA isolated from a number of other orbiviruses (Fig. 1a). Ten genome segments were resolved for AHSV serotypes 1, 2, 3, 6, 7 and 9, BTV-10 and EEV-Cascara. Bands 7, 8 and 9 of AHSV-4 (lane 4) coelectrophoresed and there was also no separation of segments 5 and 6 of serotypes 5 and 8 (lanes 5 and 8), but on 10% or 4% acrylamide many of these segments could be resolved (result not shown). Some AHSV serotypes could be clearly distinguished from one another (e.g. 1 and 4), whereas others, such as serotypes 1 and 2 and also serotypes 3 and 7, had very similar patterns. As has been reported for BTV (Mertens et al., 1987; Pedley et al., 1988) different isolates of the same AHSV serotype were found to vary in electropherotype (result not shown). On 1% agarose gels the dsRNA profiles of all the AHSV serotypes were identical and could be distinguished from that of BTV, EEV and EHDV (Fig. 1b). Genome segments 7, 8 and 9 of AHSV, EEV and EHDV did not separate under our gel conditions.

**Cloning of AHSV-3 dsRNA genome segments**

Cloning of AHSV-3 genome segments was undertaken to obtain genomic probes that could be used in investigating the degree of similarity amongst cognate genome segments within the AHSV serogroup and be further developed for diagnostic purposes. AHSV-3 dsRNA was converted to dsDNA and cloned into the PstI site of pBR322, as described in Methods. Hybridization of recombinants to a blot of electrophoretically separated dsRNA of AHSV-3 indicated that clones representing fragments of nine of the 10 AHSV dsRNA genome segments were obtained. The sizes of the determined cloned insertse were by agarose gel electrophoresis of PstI-digested recombinant plasmids (Table 1); none of the inserts had internal PstI sites. The size of the segment 7-, 8- and 10-specific clones indicated that they could be full-length clones. The order of the genome segments in Table 1 is that obtained on 1% agarose gels and 4% PAGE gels. The order of segments 5 and 6 is reversed on 7% polyacrylamide gels.

**Dot-spot hybridization**

In order to identify AHSV group-specific probes the genetic relatedness between cognate genome segments of the different AHSV serotypes needed to be established. Therefore, 32P-labelled segment 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- and 10-specific DNA probes of AHSV-3 were hybridized to dot-blots of dsRNA of the nine AHSV serotypes. Hybridization stringencies were varied by washing blots at different concentrations of SSC. The equation T_m (RNA-DNA) = 79.8 + 18.5 (log [Na+]) + 58.4 (GC) - 0.5 (formamide) was used to calculate the stringency conditions. The blots were washed using stringency conditions requiring more than 75% nucleic acid similarity (1 x SCC, 0-1% SDS, 65°C). Results obtained (Fig. 2) indicate that probes of segments 1, 3, 4, 5, 7 and 8 hybridized to dsRNA of all the different AHSV serotypes. These probes did not hybridize to the BTV dsRNA or to cellular RNA controls and are therefore possible candidates for a group-specific probe of the AHSV serogroup. The segment 2 probe was the only one that is serotype-specific. The weak cross-hybridization with serotype 4 could be eliminated using stringency conditions requiring more than 90% nucleic acid similarity (0-1 x SCC, 0-1% SDS, 65°C) (results not shown). The segment 6 probe of serotype 3 cross-hybridized strongly with cognate genes of both serotypes 6 and 9. The much weaker hybridization with serotypes 2, 4, 5 and 8 disappeared under high stringency washing conditions (result not shown). The segment 10 probe of AHSV-3 cross-hybridized only to serotype 7.

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**Table 1. The sizes of full-length dsRNA genome segments of AHSV-3 and of the cloned DNA fragments**

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Size of dsRNA genome segment (bp)</th>
<th>Cloned DNA fragment</th>
<th>Size of the cloned DNA fragment as a percentage of the size of the total corresponding dsRNA genome segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3314</td>
<td>2139 (pCW1)</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>3038</td>
<td>1619 (pCW2)</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>2663</td>
<td>1118 (pCW3)</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>2033</td>
<td>671 (pCW4)</td>
<td>33</td>
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<tr>
<td>5</td>
<td>1804</td>
<td>1476 (pCW5)</td>
<td>65</td>
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<tr>
<td>6</td>
<td>1639</td>
<td>1067 (pCW6)</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>1137§</td>
<td>1284 (pCW7)</td>
<td>113</td>
</tr>
<tr>
<td>8</td>
<td>1137§</td>
<td>1345 (pCW8)</td>
<td>118</td>
</tr>
<tr>
<td>9</td>
<td>1137§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>693</td>
<td>771 (pCW10)</td>
<td>111</td>
</tr>
</tbody>
</table>

* The order of the genes are those obtained on a 4% PAGE gel and on 1% agarose gels. The order of segments 5 and 6 is reversed on polyacrylamide gels of 6% and higher.
† The sizes of the dsRNA genome segments were obtained by electrophoresis on agarose gels using known size values for BTV-10 (Roy, 1989) as standards.
‡ Calculated from the size of the clones obtained from recombinant plasmids after digestion with PstI.
§ These segments coelectrophoresed under our gel conditions.
¶ The more than full-length size of cloned segments is probably due to dGC-homopolymer tails as a result of the cloning strategy.
AHSV serotype

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>BTV-10</th>
<th>C2</th>
<th>C1</th>
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<tbody>
<tr>
<td>pCW1</td>
<td>pCW2</td>
<td>pCW3</td>
<td>pCW4</td>
<td>pCW5</td>
<td>pCW6</td>
<td>pCW7</td>
<td>pCW8</td>
<td>pCW10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Autoradiograph of the hybridization of $^{32}$P-labelled AHSV-specific DNA probes to the dsRNA of different AHSV serotypes and BTV-10, as well as RNA of uninfected cellular controls, immobilized on Zeta-Probe membrane. The probes listed on the left-hand side of the figure were labelled with $^{32}$P in a nick translation reaction and hybridized to a strip containing the different dsRNAs mentioned above. The different dsRNA isolates are listed on top. Uninfected cellular controls from the ssRNA and dsRNA fraction of a CF-11 cellulose column are indicated by C1 and C2, respectively.

Northern blot hybridization

Reports in the literature suggest that Northern blot hybridization results are more consistent for comparing RNA of different virus serotypes or isolates than dot-spot hybridization (Unger et al., 1988a). Therefore we investigated to what extent we could confirm our dot-spot hybridization data on the genomic relatedness of cognate genome segments amongst AHSV serotypes by Northern blot hybridization.

Double-stranded RNA of the nine AHSV serotypes and of BTV-10, EEV-Cascara and EHDV-NJ was separated on 7% polyacrylamide gels (Laemmli, 1970) and blotted onto Zeta-Probe membranes. Since genome segments 5 and 6, as well as 7 and 8, coelectrophoresed in some serotypes, two separate gels were blotted. $^{32}$P-labelled DNA probes of the various segments were hybridized to the blots and washed in 1 x SSC and 0.1% SDS at 65 °C (six times for 15 min), which required 75% nucleic acid similarity. All the probes hybridized well to serotype 3 (the homologous serotype). Results obtained (Fig. 3) were essentially similar to those of the dot-spot hybridization.

Fig. 3. Autoradiograms depicting hybridization of $^{32}$P-labelled cloned fragments of AHSV-3 to Northern blots of dsRNA of the nine different AHSV serotypes, BTV-10, EEV-Cascara, EHDV-NJ and RNA obtained from uninfected cellular controls, separated by 7% PAGE (Laemmli, 1970). Lanes are AHSV-1 (lane 1), AHSV-2 (lane 2), AHSV-3 (lane 3), AHSV-4 (lane 4), AHSV-5 (lane 5), AHSV-6 (lane 6), AHSV-7 (lane 7), AHSV-8 (lane 8), AHSV-9 (lane 9), BTV-10 (lane 10), EEV-Cascara (lane 11) and EHDV-NJ (lane 12). Uninfected cellular controls from the dsRNA fraction (lane 13) and the ssRNA fraction (lane 14) of a CF-11 cellulose column are included. Probes of the different genome segments are listed on the left.
experiments. Probes of segments 1, 3, 4, 5, 7 and 8 cross-hybridized to various degrees with the cognate genes of all the heterologous AHSV serotypes. However, the segment 1 probe cross-hybridized poorly to serotypes 5, 6, 8 and 9, which is due to poor transfer of the large segment to the blot. A longer blotting period and denaturation of the dsRNA after blotting significantly improved hybridization of segment 1 to all the serotypes (result not shown). The only heterologous cross-hybridization of the segment 10 probe was again with dsRNA of serotype 7. The AHSV-3 segment 2 probe hybridized only to the serotype 3, confirming its serotype specificity.

Discussion

Until now the only AHSV serotype that had been biochemically characterized to a limited extent was AHSV-3 (Bremer, 1976). This investigation was therefore initiated to characterize the genomes of all the AHSV serotypes, as well as to identify and prepare group- and serotype-specific genomic probes of AHSV.

In this paper we report on the electropherotypes of the dsRNA genome segments of isolates of all nine serotypes on 7% polyacrylamide gels using the Laemmli buffer system and on 1% agarose gels. Similar to what has been reported for other orbiviruses, including BTV (Squire et al., 1983; Kowalik & Li, 1987; Mertens et al., 1987; Pedley et al., 1988), EEV (Viljoen & Huismans, 1989), EHDV (Brown et al., 1988), Palyam (Bodkin & Knudson, 1985) and members of the Wallal, Warrego and Eubenangee serogroups (Gonzalez & Knudson, 1988) the PAGE dsRNA profiles of the various AHSV serotypes differed from one another (Fig. 1a), whereas the agarose gel profiles were identical (Fig. 1b). Profiles of AHSV serotypes were distinct from those of other orbiviruses on both agarose and Laemmli PAGE systems, although they showed some similarity to that of EEV virus (Fig. 1b). Electrophoretic analysis in agarose gels is therefore not suitable for distinguishing between different AHSV serotypes. This is in agreement with results obtained for rotavirus (Pedley & McCrane, 1984) and other members of the Reoviridae family, with the exception of viruses belonging to the cytoplasmic polyhedrosis virus genus, which can be classified on the basis of distinctive agarose gel profiles (Mertens et al., 1989). Much of the variation in genomic profiles on polyacrylamide gels is due to differences in secondary structure of cognate genome segments rather than size differences. By using a Tris–borate–urea buffer system, which eliminates most of this secondary structure, much less variation should be observed.

Both dot-spot and Northern blot hybridization studies were used to investigate the intra- and inter-serogroup relationships of members of the AHSV serogroups using cloned genome segments as nucleic probes. These probes consisted of cloned fragments of nine different genome segments of AHSV-3. Of these only segments 7, 8 and 10 have sizes that indicate that they could be full-length clones. Nucleotide sequencing of segment 10 has confirmed that the complete gene has indeed been cloned (V. van Staden, unpublished results) and other than the results obtained by Unger et al. (1988a), a good correlation between the two hybridization methods was observed.

The clones of AHSV segments 1, 3, 4, 5, 7 and 8 hybridized to all the different AHSV serotypes. No cross-hybridization occurred to BTV, EEV or EHDV using stringency conditions requiring more than 90% similarity in base sequence. The segment 3, 5 and 8 clones appear to be the most highly conserved and would therefore be suitable group-specific probes. Sequence data obtained for segment 3 of BTV serotypes 10 and 17 indicated 95-5% similarity (Roy, 1989) and although the segment 4 probe detected all the serotypes, it was distinctly less sensitive than the segment 3, 5 and 8 clones. The reason for this could be that the cloned fragment consisted of a largely non-conserved part of the genome segment. On Northern blot hybridization the segment 1 probe did not hybridize as well to the cognate segments of serotypes 5, 6, 8 and 9 as to those of serotypes 1 and 3. This result differs from that of the dot-spot experiment of segment 1 and the difference can probably be explained by weak transfer of large dsRNA segments during blotting. Similar to what has been reported for BTV (Huismans & Cloete, 1987; Kowalik & Li, 1987; Unger et al., 1988b), EHDV (Brown et al., 1988; Nel & Huismans, 1990) and EEV (Viljoen & Huismans, 1989) the segment 2 clone hybridized only to the homologous serotype 3 under high stringency conditions. Very slight cross-hybridization to serotype 4 occurred under low stringency conditions. Therefore segment 2 probably codes for the serotype-specific antigen, which would be the equivalent of the BTV outer capsid protein VP2. On a phosphate–urea gel system Bremer (1976) identified AHSV-3 virus protein VP3 as the BTV VP2 equivalent. When using the Laemmli PAGE system it appears that AHSV-3 VP2 and VP3 switch positions (C. W. Bremer, unpublished observation). Since genome segment 6 shows a considerable degree of variation amongst the different AHSV serotypes it most probably codes for VP3, the other outer capsid protein (Bremer, 1976). Similar variations in the genes coding for the outer capsid polypeptides of BTV have been reported (Huismans & Cloete, 1987; Huismans et al., 1987). These results are also in agreement with what has been described for cognate genome segments coding for the second outer capsid protein of Palyam virus (Bodkin & Knudson, 1985), viruses of the Eubenangee, Wallal and Warrego serogroups of orbivir-
uses (Gonzalez & Knudson, 1988), EHDV (Brown et al., 1988; Nel & Huismans, 1990) and EEV (Viljoen & Huismans, 1989).

The only heterologous serotype to which the cloned fragment of AHSV genome segment 10 cross-hybridized was serotype 7, even under conditions requiring only 75% base similarity. However, we probed only with AHSV-3 and this does not preclude the possibility that segment 10 of the non-reacting serotypes may be conserved. To investigate the full extent of the variation of AHSV segment 10 extensive sequencing and DNA-RNA or RNA–RNA cross-hybridization will have to be carried out. In the case of the EHDV serogroup segment 10 was also not highly conserved (Brown et al., 1988), whereas with BTV (Huismans et al., 1987), EEV (Viljoen & Huismans, 1989) and Palyam virus (Bodkin & Knudson, 1985) a high degree of conservation of segment 10 was observed. Nucleotide similarity of the segment 10 genes of BTV-1 (Australia) and BTV-10 (U.S.A.) is approximately 82% (Gould et al., 1988b). This is surprisingly high in view of the fact that the Australian BTV isolates have been shown to represent a distinct geographic group.

The use of nucleic acid probes offers distinct advantages for the detection of AHSV infection above time-consuming classical biological and serological diagnostic procedures. In view of the rapid pathogenesis and high mortality rate resulting of AHSV infection it is of the utmost importance to confirm the presence of AHSV as soon as possible. Infected animals also have to be identified during the very early stages of infection in order to initiate preventive measures and to control the spread of the disease. Provided the required level of sensitivity can be achieved then genomic diagnostic procedures could give results within a day. Epidemiological surveys of Culicoides populations would also benefit greatly by a more rapid, sensitive diagnostic procedure.

Our results indicate that genome segments 3, 5 and 8 are highly conserved within the AHSV serogroup and, under the conditions used, do not hybridize with other members of the orbivirus group, and are therefore good candidates for a group-specific diagnostic probe. Segment 2, on the other hand, is the obvious choice for an AHSV-3 serotype-specific probe. Serotype-specific recombinant DNA probes have already been used for the detection of BTV (Roy et al., 1985; Squire et al., 1985, 1986; de Mattos et al., 1989) and rotavirus (Pedley & McCrae, 1984) in cell cultures or field isolates.

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References


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