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DNA Bending Near the Replication Origin of IncFII Plasmid NR1

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The DNA replication origin of plasmid NR1 is located approximately 190 base pairs downstream from the 3′ end of the *repA1* gene, which encodes the essential initiation protein for replication of the plasmid. Restriction endonuclease fragments that contain the NR1 replication origin and its flanking sequences at circularly permuted positions were obtained by digesting oligomers of *ori*-containing DNA fragments with sets of enzymes that each cut only once in every *ori* fragment. Polyacrylamide gel electrophoresis of these permuted restriction fragments showed anomalous mobilities, indicating the presence of a DNA bending locus. Through analysis of the relative mobility plots of these permuted fragments, we found one or two possible DNA bending sites located in the intervening region between the *repA1* gene and the replication origin of NR1. It seems possible that DNA bending in this region might help to orient the replication origin alongside the *repA1* gene, which could contribute to the *cis*-acting character of the RepA1 initiation protein.

The DNA double helix is usually a rod-shaped molecule. However, DNA can undergo bending owing to the binding of specific proteins (19, 27, 28) or to the presence of specific nucleotide sequences in the molecule (1, 3, 22, 25, 27, 29). Sequence-specific static DNA bending was first found in trypanosome kinetoplast minicircle DNA, which contains a series of $CA_{5-6}T$ sequences separated from each other by two to three nucleotides (i.e., a set of A tracts at 10- to 11-base-pair [bp] intervals) (2, 13, 27). A characteristic of a bent DNA fragment is its anomalously slow electrophoretic mobility in a polyacrylamide gel. A set of circularly permuted restriction endonuclease fragments having a variable position of the same DNA sequence relative to the fragment ends has been used to locate bending sites (7–9, 12, 13, 27). When a bending site is in the middle of a DNA fragment, it causes the most pronounced retardation, whereas when it is at the end, it causes the least retardation.

Static DNA bending sites have been found associated with many DNA replication systems, such as the phage λ replication origin (29), the simian virus 40 origin (22), the origins of plasmids R6K and pT181 (11, 19), and in yeast autonomously replicating sequences (24). Most of these bending sites contain similar adenine tracts with 10- to 11-bp intervals. It has been suggested that DNA bending (static or protein induced) may play a role in initiation of DNA replication (11, 19, 22, 23, 29), in regulation of gene expression (10, 27), in DNA packaging (16), and in recognition of target sites in DNA molecules by specific proteins (22, 30). The A tracts in the bacteriophage λ replication origin were shown to be essential for the binding of λ initiator protein O, even though they are not in contact with the protein (30). Those bending loci are probably also important for the formation of the λ O′-some structure, which is formed by wrapping and folding of the λ origin sequence around the O-protein aggregate (17). O′-some formation may play a role in the storage of free energy, which may be used in subsequent stages of the initiation reaction such as strand separation (17).

In light of the discovery of DNA bending sites in several other DNA replication systems, we undertook studies on the IncFII plasmid NR1 replication region. The replication initiator protein of plasmid NR1 is the *cis*-acting RepA1 protein (4, 18, 20), which can form large aggregates (Dong et al., unpublished data). The replication origin of NR1 is located 187 bp downstream from the end of the *repA1* gene (20). RepA1 protein binds to the DNA sequences within the origin (15), and it may form an O′-some-like structure with the NR1 origin. Through gel retardation experiments, we show that a

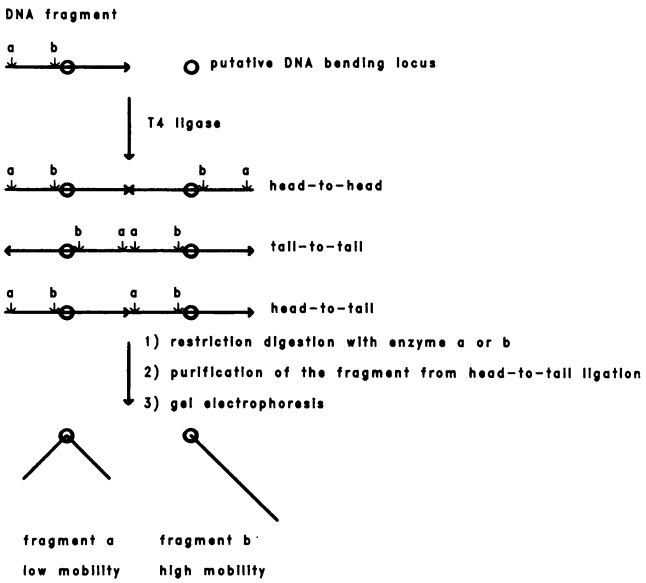

FIG. 1. Strategy for generating a series of permuted DNA restriction endonuclease fragments. DNA fragments containing a putative DNA bending locus (○) are ligated by T4 ligase in three possible orientations, which are represented here by three dimers. Restriction digestion with enzyme a or b, each of which has a single recognition site in the fragment distant from or close to the putative DNA bending site, yields fragments either larger or smaller than the input fragment when the ligation products are in the head-to-head or tail-to-tail orientation. Digestion of the tandem head-to-tail dimers with either of these enzymes generates fragments with the same size as the input fragment, with the putative DNA bending site either in the middle or at the end of the fragment. Fragment a is strongly bent and therefore of low gel mobility, whereas fragment b is nearly linear and therefore of high mobility.

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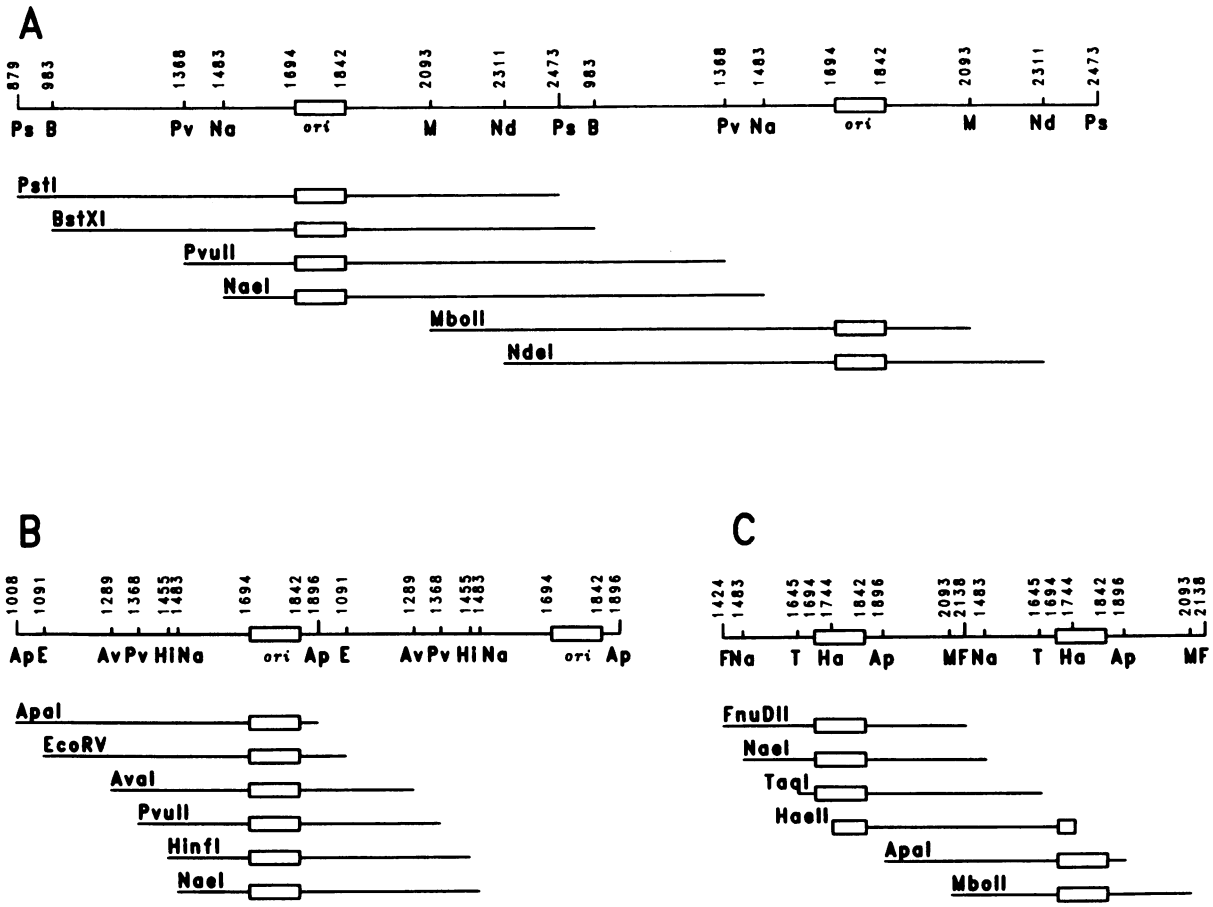

FIG. 2. Permuted DNA fragments containing the replication origin of plasmid NR1 used in bending analyses. The permuted DNA fragments were generated from a 1.6-kb *Pst*I fragment (A), an 888-bp *Apa*I fragment (B), and a 714-bp *Fnu*DII fragment (C). The minimal origin of replication of NR1 (positions 1694 through 1842) is shown as a box. The sites of restriction enzyme cleavage are indicated on the top of the maps for enzymes *Apa*I (Ap), *Ava*I (Av), *Bst*XI (B), *Eco*RV (E), *Fnu*DII (F), *Hae*II (Ha), *Hin*fI (Hi), *Mbo*II (M), *Nae*I (Na), *Nde*I (Nd), *Pst*I (Ps), *Pvu*II (Pv), and *Taq*I (T).

DNA sequence located in the region between the *repA1* gene and the replication origin, but probably not within the origin itself, can cause static DNA bending.

MATERIALS AND METHODS

Plasmids. Plasmid pDXRR50, in which the 1.6-kilobase (kb) *Pst*I restriction fragment that contains the NR1 plasmid replication origin (18) is inserted into vector plasmid pUC8 (26), was used as the source of NR1 origin DNA. pDXRR50 plasmid DNA was isolated from *Escherichia coli* KP245 (pDXRR50) as previously described (6, 18).

Isolation of permuted DNA fragments. Purified plasmid pDXRR50 DNA was digested with the restriction enzyme *Pst*I, *Apa*I, or *Fnu*DII (all from New England Biolabs). The individual restriction fragments obtained that contain the NR1 origin were recovered from agarose gels by the method of Dretzen et al. (5) with DEAE-cellulose paper. The DNA fragments were each ligated with T4 DNA ligase (New England BioLabs). Individual portions of the DNA ligation mixtures were digested with a set of restriction enzymes (New England BioLabs), each of which had a single recognition site in the original fragment. The resulting permuted fragments were isolated from agarose gels as described above (5).

Gel retardation. The gel retardation assay of each set of the permuted fragments was performed as described by Koepsel and Khan (11). The fragments were electrophoresed on 6 or 8% polyacrylamide gels (acrylamide/bisacrylamide ratio of 70:1) in TBE buffer (89 mM Tris borate, 89 mM boric acid, and 2 mM disodium EDTA [pH 8.0]) at 10 V/cm for 20 h with recirculation of the buffer and the cooling water to maintain a relatively stable temperature. DNA molecular weight standards (1 Kilobase Ladder; Bethesda Research Laboratories) were electrophoresed on both sides of the sample fragments. The resulting gels were stained with ethidium bromide to visualize the DNA bands.

RESULTS AND DISCUSSION

Permuted restriction fragments can be obtained by linearizing the circular form of a DNA molecule with different restriction enzymes that have only one recognition site on the molecule or by digesting a tandem head-to-tail dimer or higher oligomer clone of the fragment with these single-site enzymes (Fig. 1). The NR1 replication origin and the 3′ end of the *repA1* initiation protein gene are located within a 1.6-kb *Pst*I restriction fragment (18). Unfortunately, attempts to clone two tandem 1.6-kb *Pst*I origin fragments into a high-copy-number vector plasmid, pUC8, were not successful. Deletions of one of the NR1 origins were obtained instead. This is consistent with previous observations made in this laboratory that cointegrates of two NR1 replicons were not stable. The resulting stable plasmids obtained from

such experiments always had deleted one of the NR1 origin regions (21). To overcome this problem, all of the manipulations to obtain the permuted DNA fragments were done in vitro (Fig. 1). Restriction fragments containing the NR1 origin were first isolated from plasmid pDXRR50, which is a pUC8 clone of a single copy of the *Pst*I 1.6-kb *ori* fragment. The fragments used in our studies were the 1.6-kb *Pst*I fragment, an 888-bp *Apa*I fragment, and a 714-bp *Fnu*DII fragment (Fig. 2). Each of these fragments was ligated separately into oligomers with T4 DNA ligase. The ligation products were digested separately by a set of enzymes that cut only once in every fragment. The nonligated fragments and ligation products in the head-to-head or tail-to-tail fashion yielded fragments either larger or smaller than the input fragment in these digestions, whereas restriction fragments obtained from the self-ligated circular molecules or head-to-tail ligated products had the same size as the input fragment (Fig. 1). Fragments that contained the same DNA sequences but at permuted positions (Fig. 2) were separated by size from the other fragments by agarose gel electrophoresis and extracted from the gel.

Each set of permuted fragments was examined by electrophoresis on 6% or 8% polyacrylamide-TBE gels. The test fragments were run side by side with DNA molecular weight standards, from which the expected mobility of a fragment with a given molecular weight was calculated. The mobilities within each set of permuted fragments were different from each other even though they had identical molecular weights (Fig. 3). This indicated the presence of DNA bending sites within the fragments. The relative mobility of each fragment was calculated by the ratio of the expected mobility according to the molecular weight of the fragment to the apparent mobility observed on the gel. The larger the relative mobility value is compared with 1.0, the slower the fragment runs on the gel. The closer the relative mobility value is to 1.0, the faster the fragment runs, and therefore the closer the putative DNA bending site is to the end of the fragment. The relative mobility of each permuted fragment, averaged from three experiments, was plotted against the position of the restriction site in the DNA fragment (Fig. 3). The relative mobility curves were extrapolated to the position of the cut that would yield maximum gel mobility (relative mobility of 1.0) to locate the center of the DNA bending. For the experiment with permuted *Pst*I fragments (1.6 kb), the DNA bending center was located approximately at base 1585 (Fig. 3A); for the *Apa*I fragments (888 bp), the center was located at approximately base 1605 (Fig. 3B); and for the *Fnu*DII fragments (714 bp), the center was located at approximately base 1645 (Fig. 3C). Since the permuted *Fnu*DII fragments were the smallest, and the fragment cut at the *Taq*I site had a relative mobility of almost 1.0, the results obtained from this experiment (Fig. 3C) might be considered the most accurate of the three. Cutting at the *Hae*II site, which is located in the middle of the replication origin (base 1744), did not yield the fragment with the lowest relative mobility (Fig. 3C). This implies that the DNA bending site is probably not in the origin but rather is upstream from it.

Polyacrylamide gel electrophoresis of all three sets of permuted restriction fragments (*Pst*I, *Apa*I, *Fnu*DII) that contain the NR1 origin region and the flanking sequences showed similar patterns of anomalous mobilities, which is a hallmark of DNA bending. Through plots of the relative mobility curves, the putative DNA bending site was located to the region between the *repA1* gene and the replication origin, from base numbers 1585 to 1645. Examination of the DNA sequence in this region (Fig. 4) revealed several A (or

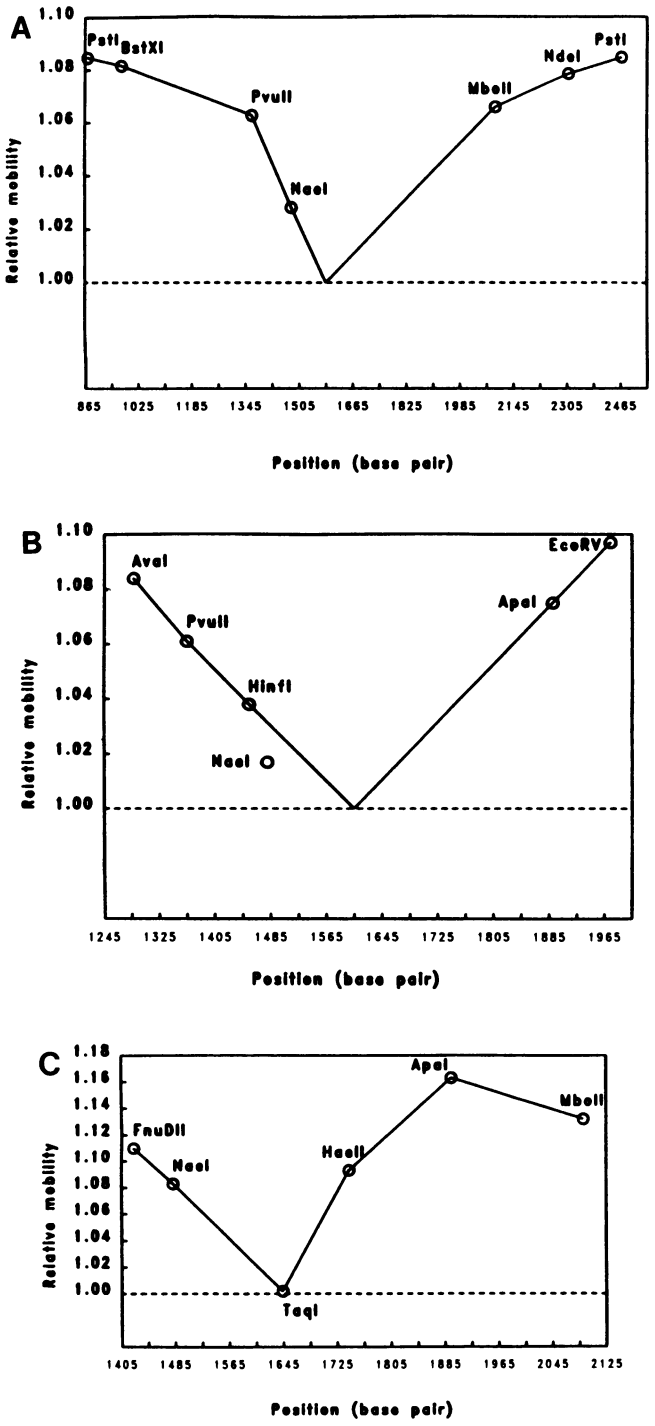

FIG. 3. (A) Mapping of the bending site on the permuted 1.6-kb fragments (Fig. 2A). The relative mobilities of these permuted fragments in 6% polyacrylamide gels were plotted against the position of the restriction cleavage sites in the DNA sequence. (B) Mapping of the bending site on the permuted 888-bp fragments (Fig. 2B), determined by electrophoresis in 8% polyacrylamide gels. (C) Mapping of the bending site on the permuted 714-bp fragments (Fig. 2C), determined by electrophoresis in 8% polyacrylamide gels. For each plot, the relative mobilities were averaged from three independent experiments.

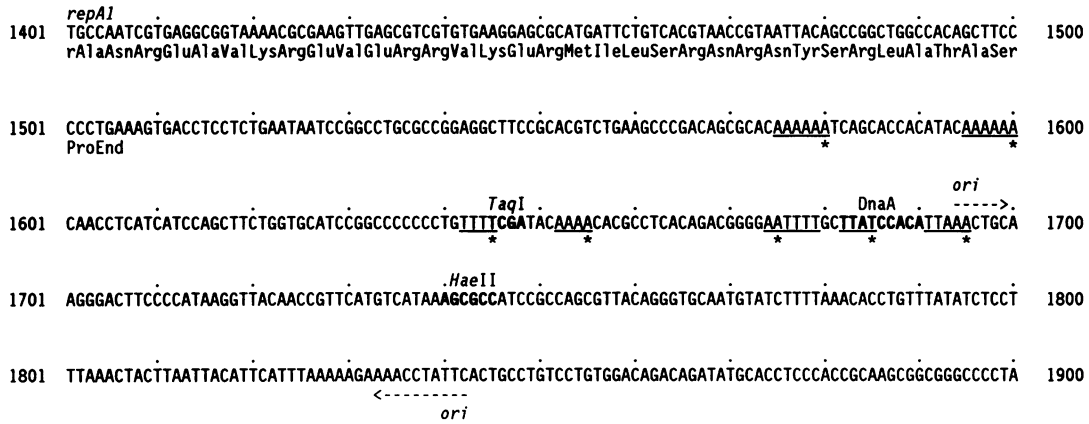

FIG. 4. DNA sequence of the NR1 replication origin and its flanking regions, including the 3′ end of *repA1*. Recognition sequences for restriction enzymes *Taq*I and *Hae*II and the DnaA binding site are in bold type. The 149-bp minimal origin (*ori*) is indicated by arrows between positions 1694 and 1842 (20). The RepA1 binding sites are located in the 5′ half of the minimal origin (15). The A or T tracts in the intervening region, which are possibly involved in DNA bending, are underlined. The A or T residues in these tracts that are in phase with each other with respect to the DNA helix are marked with an asterisk (*).

T) tracts separated with multiples of 10-bp intervals, which could cause DNA curvature. The first group started at position 1575, whereas the second group was near the DnaA box, starting from position 1642. According to the asymmetric shapes of the relative mobility curves (Fig. 3), it is likely that there is more than one DNA bending site in this region (28, 29). The NR1 origin apparently does not contain a significant static DNA bending site, because when the origin was placed at the end of a fragment by digestion with *Hae*II, the fragment did not have an electrophoretic mobility that was closest to the expected mobility (Fig. 2 and 3C). However, it is still possible that the binding of RepA1 protein to the origin might induce the bending of this region.

The biological significance of the DNA bending site (or sites) near the NR1 replication origin has not yet been determined. By deleting various sequences between the *repA1* gene and its binding site in the origin and replacing them with *Eco*RI or *Bam*HI linkers, Ohtsubo et al. (20) showed that the intervening sequences were not absolutely essential. However, those deletions had some effects on the transformation frequency and copy number of the resulting plasmids (20). Whether those effects were directly caused by removing the DNA bending site is not known. Since the plasmid used in those experiments (pSM1) was derived from a high-copy-number mutant of NR1 (20), the abilities of the plasmids with deletions to replicate were determined relative to that of the high-copy-number parental plasmid. A deletion that caused a severalfold reduction in copy number might be scored as replication proficient (Rep^+) for a plasmid that has a high copy number. However, such deletions might result in a Rep^- phenotype for a plasmid with a low copy number such as wild-type NR1. Therefore, it is not clear whether the DNA sequence between the *repA1* gene and the replication origin is essential for the replication of wild-type NR1, even though it was not essential for the high-copy-number mutant pSM1 (20).

The function of the RepA1 protein in the process of initiation of NR1 replication has not yet been determined. However, purified RepA1 protein binds in vitro specifically to the left half (as shown in Fig. 4) of the minimal replication origin (15). Interestingly, RepA1 protein is primarily *cis* acting both in vivo (4, 18) and in vitro (14). The *repA1* gene of NR1 has been cloned into high-expression vector plasmids under control of the λ p_L promoter (4). When the left half of the origin region that contains the RepA1 binding sites is included along with the *repA1* gene in these plasmid clones, very little, if any, RepA1 protein product is detectable even under inducing conditions (4). When the origin sequences are subsequently deleted from such a clone, large quantities of RepA1 protein are detectable after induction (4). It is also possible to detect large yields of RepA1 protein from clones that still contain the origin sequences if the cloned *repA1* gene contains a mutation that inactivates the RepA1 protein, such as a temperature-sensitive or small in-frame deletion mutation (4). It is possible that the binding of RepA1 protein to its target sites in the origin causes it to be rapidly degraded, which would reduce its detectable yield. Alternatively, the binding of some RepA1 protein to the origin sequences downstream from the *repA1* gene may somehow impede the synthesis of additional RepA1 protein. It is possible that the RepA1 protein produced from the temperature-sensitive or deletion mutants cannot bind to the origin, and therefore synthesis or degradation of the mutant proteins is not affected by the presence of the origin in those clones.

DNA bending in the intervening region may bring the NR1 replication origin alongside the *repA1* gene and enable the origin to interact with the gene product immediately after or even before completion of its synthesis. If so, this could form a complex that might impede the further synthesis of RepA1 protein, as mentioned above, or, alternatively, might induce its rapid degradation. Such phenomena could also account for the *cis*-acting character of the RepA1 protein. If RepA1 protein is synthesized in only small amounts from individual plasmids, these kinds of processes could prevent it from diffusing to an origin in *trans*. Therefore, DNA bending of the sequences between *repA1* and the origin, even if not absolutely essential, might enhance the efficiency of initiation of replication of plasmid NR1.

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