Maize Transposable Elements Ac/Ds as Insertion Mutagenesis Tools in Candida albicans

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ABSTRACT In nonmodel systems, genetic research is often limited by the lack of techniques for the generation and identification of gene mutations. One approach to overcome this bottleneck is the application of transposons for gene tagging. We have established a two-element transposon tagging system, based on the transposable elements Activator (Ac)/Dissociation (Ds) from maize, for in vivo insertion mutagenesis in the fungal human pathogen Candida albicans. A nonautonomous Ds transposon carrying a selectable marker was constructed into the ADE2 promoter on chromosome 3 and a codon usage-adapted Ac transposase gene was inserted into the neutral NEUTSL locus on chromosome 5. In C. albicans cells expressing the transposase, the Ds element efficiently excised and reintegrated elsewhere in the genome, which makes the Ac/Ds transposons promising tools for saturating insertion mutagenesis in clinical strains of C. albicans.

The autonomous transposable element Activator (Ac) from Zea mays, originally discovered as the first transposon by McClintock (1951), is one of three prototypic elements of the hAT superfamily of "cut-and-paste" transposons. Soon after its discovery, the potential of Ac and its nonautonomous derivatives, termed Dissociation (Ds), for gene tagging and insertion mutagenesis in maize and heterologous plants was recognized (Walbot 1992). Ac/Ds elements are convenient to use in heterologous plants because the single Ac-encoded transposase protein (TPase) is sufficient for transposition, the transposon ends with the essential TPase-binding sites are only ~240 bp in length, and the nonautonomous Ds elements can carry > 15 kb of cargo DNA. Ac/Ds has been used in ~ 20 higher plants, among them model plants and agriculturally important species [reviewed in Kunze and Weil (2002)]. Ac/Ds elements tend to transpose into genetically linked sites [Dooner and Belachew 1989; Vollbrecht et al. 2010; reviewed in Lazarow et al. (2013)], but they do not have the target sequence preference exhibited by other transposons (Spradling et al. 2011). More recently, the focus of insertion mutagenesis approaches has shifted from single-gene tagging toward the generation of large insertion mutant populations. The use of transposons for this purpose is highly attractive for the study of plants because transposons enable the formation of a large number of insertion mutations in plant species where ballistic or Agrobacterium tumefaciens-based T-DNA (T-DNA) transformation is inefficient [reviewed in Lazarow et al. (2013)]. Ac/Ds transposons also function in vertebrate animals (Emelyanov et al. 2006; Quach et al. 2015; Vrljicak et al. 2016) as well as in the budding yeast Saccharomyces cerevisiae (Weil and Kunze 2000). Recently, Ac/Ds was employed for saturated transposon mutagenesis in S. cerevisiae, facilitating the identification of conditionally essential genes and identifying important functional protein domains (Michel et al. 2017). An important advantage is the facility of generating libraries of insertion mutations in mutant strain backgrounds. A similar approach, using the hAT element Hermes from the house fly (Musca domestica), achieved saturated transposon insertion mutagenesis in the fission yeast Schizosaccharomyces pombe (Guo et al. 2013).

Transposable elements in eukaryotic genomes are mostly inactive and transpose only rarely. This relative stability of transposon insertions...
is due to epigenetic silencing, the low activity of the TPase, as well as post-translational negative autoregulation, such that increased TPase levels result in reduced transposition frequency [reviewed by Bire et al. (2013)]. This phenomenon, termed TPase "overexpression inhibition," presumably underlies the "inverse dose effect" of Ac/Ds first discovered by McClintock (1951) and later also reported in heterologous plants (Scofield et al. 1993, Heinlein et al. 1994). The naturally low activity of transposons limits the efficiency of transposon mutagenesis experiments; hyperactive TPase variants that catalyze more frequent transposition reactions have increased the efficiency of transposon mutagenesis with several prokaryotic and eukaryotic transposons (Goryshin and Reznikoff 1998; Lampe et al. 1999; Beall et al. 2002; Zayed et al. 2004; Mátés et al. 2009; Yusa et al. 2011; Lazarow et al. 2012). For example, AcTPase4x, a hyperactive Ac TPase, catalyzes a higher Ds excision frequency compared to the wild-type AcTPase [100-fold higher in budding yeast and sixfold higher in Arabidopsis thaliana (Lazarow et al. 2012)].

Here, we asked if the maize Ac/Ds transposon system could be applied to nonmodel micro-organisms more distantly related to the model budding and fission yeasts. The commensal ascomycete Candida albicans is the most common fungal pathogen of humans (Kim and Sudbery 2011) and differs in several genetic and morphological features from S. cerevisiae. In addition, C. albicans diverges from the universal genetic code at one codon: CTG encodes serine instead of leucine, which complicates the design of heterologous molecular genetic systems. Furthermore, C. albicans was long thought to be an obligate diploid with the ability to undergo a "parasexual cycle" between generations without conventional meiosis from diploid to tetraploid and back [reviewed by Noble and Johnson (2007)]. However, mating-competent haploid cells were discovered recently (Hickman et al. 2013). Haploids appear to arise via a concerted chromosome loss mechanism rather than meiosis. The initial isolates grew slower than diploids and were relatively unstable, reverting to "autodiploids" with high frequency (Hickman et al. 2013). Here, we describe a haploid strain selected for its faster growth rate and use it to test the design of an inducible two-component Ac/Ds transposon system for C. albicans, consisting of a codon-adapted hyperactive TPase gene and a nonautonomous Ds element carrying a selection marker. We demonstrate that the TPase mobilizes the Ds element and that the Ds excision footprint sequences exhibit subtle differences compared to those seen in S. cerevisiae and plants.

MATERIALS AND METHODS

Construction of Ac/Ds components

The codon-adapted AcTPase4xCa open reading frame (Supplemental Material, Figure S1 in File S1) was constructed by fusing three de novo-synthesized DNA fragments (Thermo Fisher Scientific GENEART GmbH, Regensburg, Germany) and cloned into pJET1.2 via the ClonJET kit (Thermo Fisher Scientific). The CaMAL2 promoter (Backen et al. 2000), amplified from pKB2019 with primers oMAL2-F and oMAL2-R, and the CaADH1 terminator, amplified from pMG2120 (Gerami-Nejad et al. 2012) with primers oADHT-F and oADHT-R, were also ligated into pJET1.2. The AcTPase4xCa expression cassette was assembled by fusing the promoter, AcTPase4xCa-coding region, and terminator in pJET1.2. From this plasmid, the AcTPase4xCa expression cassette was excised with EcoRI and ligated into EcoRI-linearized pDUP5 (Gerami-Nejad et al. 2013), yielding pKM300 (Figure 1A, top line).

To construct a nonautonomous miniDs element with a selectable marker inserted into the 5′-UTR of the C. albicans ADE2 gene, the miniDs from pBBdXhko (Laufs et al. 1990) was excised with SalI and cloned into pUC19. The resulting plasmid was pKR86. The nourseothricin acetyl N-transferase gene NAT1, under the control of the TEF promoter, was amplified from pDUP3 (Gerami-Nejad et al. 2013) with the primers oTEFmut-F and oTEFmut-R, and the PCR product was digested with SalI and ligated into Xhol-linearized, dephosphorylated pRK86 to form pRK86-NAT1, carrying the transposable element Ds-NAT1. A 528-bp segment of C. albicans chromosome 3, centered around an artificial SalI site 24-bp upstream of the ADE2 start codon, was de novo synthesized (Thermo Fisher Scientific) and ligated into pJET1.2. The resulting plasmid, pRK401, was linearized with SalI and dephosphorylated with FastAP (Thermo Fisher Scientific). Ds-NAT1 was excised with SalI from pRK86-NAT1 and ligated into SalI-linearized pRK401, followed by the exchange of two nucleotides flanking the Ds-NAT1 5′-end using primers oKM34 and oKM35, and the Q5 site-directed mutagenesis kit (New England Biolabs, Beverly, MA). The resulting plasmid pRK403a was used for chromosomal integration via homologous recombination of Ds-NAT1 into the 5′-UTR of the C. albicans ADE2 gene (Figure 1B, top line).

Generation of the MTLa haploid Candida albicans strain GZY896

To isolate a MTLa haploid strain from YJB12881 (Hickman et al. 2013), single colonies were subjected to repeated rounds of subcultivation in glucose minimal medium supplemented with 40 μg ml−1 histidine (GMM + His) medium (Zeng et al. 2014). Haploid colonies were identified by flow cytometry analysis and then stored at −80°C. Strains were revived on GMM + His plates and several more subcultivation cycles were performed, yielding a haploid strain, GZY892, that appeared stable by flow cytometry. A ura3Δ derivative of the strain was constructed by excising the ura3Δ::His4 cassette from plasmid pYG1023 (Hickman et al. 2013) and transforming it into GZY892, according to the method of Zeng et al. (2014), where it inserted by homologous recombination at the URA3 locus. The resulting strain was termed GZY896 (MTLa ura3Δ::imm434 his4 gal1Δ:: ura3Δ::His4).

Generation of Ac/Ds transposable element-carrying Candida albicans strains KMY100 and KMY103G1

GZY896 transformation was performed according to the method of Zeng et al. (2014). First, linearized pKM300 was used to integrate the AcTPase4xCa expression cassette via homologous recombination into the NEUTS1 locus (Gerami-Nejad et al. 2013) (Figure 1A). Transgenic cells were selected on complete synthetic defined minimal (SDC) plates lacking uridine. In a second step, these transformants and, in parallel, GZY896 cells were transformed with linearized pRK403a to integrate the Ds-NAT1 into the 5′-UTR of ADE2 on chromosome 3 (Figure 1B). Transformants were selected by plating the cells on yeast extract peptone (YP) medium + adenine + glucose with 0.4 mg/ml nourseothricin. The resulting strains carrying the ade2::Ds-NAT1 construct alone, or in combination with the AcTPase4xCa expression construct, were termed KMY100 and KMY103G1, respectively.

Isolation of Ds transposants

KMY103G1 cells were grown in SDC + 3% maltose liquid medium at 30°C for 24 hr, and then plated on SDC − adenine + glucose plates to select for Ade2 revertants. To select for reintegration of Ds-NAT1 elements, Ade2 revertant colonies were replica-plated on YP + adenine + glucose plates with 0.4 mg/ml nourseothricin and grown for 2 days at 30°C.

Flow cytometry

Flow cytometry was performed as described previously (Hickman et al. 2013) using a MACSQuant FLOW cytometer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).
Ds excision and reintegration site analysis

ADE2 revertant colonies were picked, resuspended in 10 μl H2O containing 2.5 U Zymolyase (Zymo Research, Irvine, CA) and incubated for 15 min at 30°C. Next, 2 μl of a 1:10 dilution of these suspensions was used as template for PCR amplification of Ds excision sites with CaADE2 primers F2 and R2 (Table S1 in File S1) and Q5 DNA Polymerase (New England Biolabs). PCR products were purified on NucleoSpin Gel and PCR Clean-up columns (MACHEREY-NAGEL GmbH, Düren, Germany) and sequenced.

Reintegration sites of Ds-NAT1 elements were mapped by modified FPNI-PCR (Fusion Primer and Nested Integrated PCR; Wang et al. 2011) on ADE2+::NatR cells to amplify genomic DNA flanking the Ds.
3’-end. Target site duplications at three Ds reinsertion sites were identified by amplification of novel Ds-NAT1 insertion sites with pairs of Ds-flanking primers (Table S1 in File S1) and sequencing of the PCR products.

**DNA gel blot analysis**

Genomic DNAs were isolated from 2 ml cultures of ADE2 revertants grown in SDC + 2% glucose liquid medium at 30°C for 24 hr with the Yeast DNA Preparation Kit (Jena Bioscience GmbH, Jena, Germany). The quality of the isolated DNAs was analyzed on a 1% agarose gel. Next, 200 ng DNA of each sample were digested with 10 U of EcoRI (Thermo Fisher Scientific) for 4 hr at 37°C and size fractionated on a 0.9% agarose gel overnight at 2 V/cm. The gel was successively soaked for 20 min in 0.2 M HCl, 20 min in 0.5 M NaOH/1.5 M NaCl, and 30 min in 1 M Tris Cl/1.5 M NaCl pH 7.4. Following overnight capillary transfer of the DNA to a Hybond NX membrane (GE Healthcare, Little Chalfont, UK) the DNA was UV cross-linked (120 J at 254 nm). The membrane was successively prehybridized, hybridized with a Digoxigenin (DIG)-labeled NAT1 probe, washed, and detected with anti-DIG-AP, Fab fragments, and CDP-Star (Roche Applied Science, Penzberg, Germany) following the manufacturer’s protocols. The DIG-labeled NAT1 probe was amplified with primers oKM222 and oKM223 using pRK403a as template.

**Data availability**

Plasmids and strains are available upon request.

**RESULTS**

An Ac/Ds two-component transposon system in *C. albicans*

*C. albicans* belongs to the CUG clade of pathogenic yeasts, in which the trinucleotide CTG specifies serine instead of the commonly encoded leucine (Santos and Tuite 1995). Furthermore, *C. albicans* does not have naturally occurring or engineered plasmids that are maintained autonomously. Therefore, the Ac/Ds transposon components previously constructed for *S. cerevisiae* (Lazarow et al. 2012) were not suitable for application in *C. albicans* and a new Ac/Ds two-component system for *C. albicans* was required. The system includes a nonautonomous Ds element carrying the nourseothricin N-acetyl transferase (NAT1) gene as a selectable marker gene, Ds-NAT1, and a codon-adapted (“candidized”) hyperactive Tpase-coding sequence (AcTPass4xCa) under control of the inducible CaMAL2 promoter. The original AcTPass4xCa-coding region with an N-terminally-fused SV40 nuclear localization sequence (NLS) was candized by replacing two CTG codons with TTG to ensure translation into leucine and by exchanging all codons that are rarely (≈11%) used in *C. albicans* with more frequently used codons to ensure efficient translation (Figure S1 in File S1). The AcTPass4xCa expression cassette was inserted into an intergenic region on chromosome 5 (NEUT5L; Gerami-Nejad et al. 2013) of the haploid *C. albicans* strain GZY896 (Figure 1A). The Ds-NAT1 element was cloned into the 5’-UTR of ADE2, 26-bp upstream of the ADE2 start codon on chromosome 3 (Figure 1B), and recombined into GZY896 with or without the AcTPass4xCa gene, respectively. The resulting strains KMY100 (ade2::Ds-NAT1) and KMY103G1 (AcTPass4xCa::NEUT5L ade2::Ds-NAT1) grow a little slower than ADE2 cells and form red colonies on SDC medium.

**AcTPass4xCa expression induces Ds-NAT1 excision**

The stability of the Ds-NAT1 insertion in the ADE2 5’-UTR was examined by propagating KMY100 cells for four passages onYP + adenine + glucose medium. After each passage, we plated three colonies and observed only red colonies, suggesting that the ade2::Ds-NAT1 locus is stable. Accordingly, amplification of the Ds-NAT1 from cells of each passage with flanking ADE2-primers yielded a single 2.6-kb band that was indistinguishable from that obtained from cells after the first passage (Figure 1C), indicating that the Ds-NAT1 insertion remained stably inserted in the ADE2 promoter.

Similarly, KMY103G1 cells grew as red colonies on SDC plates after propagation for 24 hr in noninducing SDC + adenine + glucose liquid medium. In contrast, when cells were precultured in glucose-free SDC + adenine + maltose medium to induce AcTPass4xCa expression, white ADE2 revertant colonies appeared with a frequency ranging from 0.0001 to 0.01%.

From 128 independent ADE2 revertant KMY103G1 colonies, the Ds-NAT1 excision sites were amplified. The PCR bands from all revertants were indistinguishable in size from the 510-bp band in the progenitor strain GZY896, suggesting that the Ds-NAT1 had been excised from the ADE2 promoter locus (Figure 1, D and E). Sequencing of the PCR bands revealed 28 different Ds excision footprints (Figure 2A). Two predominant footprints, which differed in a single nucleotide at the fusion site, were recovered in 35 and 18 clones, respectively. In addition to these two major footprints, 26 other, different footprints were observed less frequently, with half of them appearing only once. Despite the limited number of footprints analyzed, the bias in excision site repair products was obvious. All footprints exhibited short deletions or palindromic insertions (also termed P-nucleotide insertions; Huefner et al. 2011) at either side of the breakpoint, which closely resembled the Ac/Ds excision footprints in plants and in budding yeast.

**Ds-NAT1 efficiently reinserts into the genome**

Reintegation of the Ds-NAT1 transposon was investigated by cultivating 10 individual red KMY103G1 colonies in SDC + adenine + maltose liquid medium and spreading aliquots on SDC — adenine + glucose plates, yielding 1722 ADE2 revertant colonies. The white ADE2 colonies were replica-plated onto nourseothricin-containing SDC — adenine + glucose plates and grown for 2 days at 30°C. In total, 1528 colonies (~90%) grew on nourseothricin. This unexpectedly high frequency (in plants and *S. cerevisiae* reinsertion frequencies of ~60% were observed) prompted us to scrutinize the ploidy of the cells. All five KM103G1 colonies that we tested by flow cytometry were diploid. A DNA gel blot analysis of genomic DNA from white ADE2 revertant colonies revealed that, with only one exception, all clones still contained the ade2::Ds-NAT1 allele (Figure 1F). Fourteen clones exhibit an additional, different-sized band, indicating reinsertion of the Ds-NAT1 transposon elsewhere in the genome. One exceptional clone showed no ade2::Ds-NAT1 band, with one smaller and one larger band. These data demonstrate that: (i) the KMY103G1 progenitor cells had undergone autodiploidization prior to transposon mobilization, (ii) the Ds-NAT1 transposon had excised from only one of the two alleles with the exception of one clone, and (iii) in 15 of the 28 ADE2 revertant clones, the transposon reintegrated in different positions in the *Candida* genome. In the one exceptional clone (marked with a red asterisk), the Ds-NAT1 transposons apparently transposed from both alleles and reintegrated in two novel chromosomal sites.

For three ADE2 revertant clones, the genomic reinsertion sites were identified by FPNI-PCR of the 3’-end of Ds-NAT1 (Figure 2B and Table S2 in File S1). Two of the Ds-NAT1 elements reintegrated in chromosome 3, 174- and 266-kb distal and proximal to the ADE2 locus, respectively. The third element transposed to chromosome 5. Amplification and sequencing of both Ds-NAT1 flanking sequences revealed the canonical 8-bp target site duplications generated upon integration.
of the Ds-NAT1 transposable elements (Figure 2B). Thus, transposition occurred, both close to the original insertion site as well as on a different chromosome.

**DISCUSSION**

Approaches for genome-wide, untargeted insertion mutagenesis of eukaryotic genomes are frequently based on chemical or ballistic transformation with selectable DNA marker molecules [in fungi, sometimes boosted by restriction enzyme-mediated integration (REMI) reviewed in Jiang et al. (2013)], on A. tumefaciens infection and T-DNA insertion in plants, or by transposon tagging. In micro-organisms that can be efficiently transformed, large mutant collections can be generated by transformation with plasmid-based mutant libraries produced by in vitro transposition of bacterial transposons (see for example Kumar et al. 2004). However, transformation is inefficient in the majority of eukaryotes. This limitation can be avoided by using in vivo transposon tagging that exploits endogenous or heterologous transposons. The advantage of using a heterologous transposon is that it will be unique in the genome and is easily detected. The diploid C. albicans WO-1 genome is composed of 2.1% retroelements and contains only two copies each of a Tc1/mariner-like DNA transposon and a (defective) Mutator-like element (Butler et al. 2009). However, endogenous transposition activity has not been reported for any of these elements, despite a report that C. albicans transposons are not modified by DNA methylation (Mishra et al. 2011). In this study, we explored the suitability of the maize Ac/Ds transposons for in vivo transposon insertion mutagenesis in C. albicans.
Like in all heterologous host organisms tested, the nonautonomous Ds element in *C. albicans* is genetically stable and its mobilization strictly depends on expression of the Ac TPase. The Ds excision footprints display sequence features similar to those that have been reported for Ac/Ds in other plants and in *S. cerevisiae* (Lazarow et al. 2012): deletions and palindromic P-nucleotide insertions at the repaired DNA joint are centered around the complement of the base adjacent to the transposon ends. This indicates that in haploid *C. albicans*, the DNA double-strand break (DSB) repair pathway forms a DNA hairpin followed by hairpin resolution and nonhomologous end joining repair of the empty donor sites, essentially as has been suggested for plants and *S. cerevisiae* (Coen et al. 1986; Lazarow et al. 2012). However, an interesting distinction is that the structural heterogeneity of the footprints in *C. albicans* is lower than in Arabidopsis and budding yeast. For example, the longest P-nucleotide insertions were three nucleotides and occurred only in a single footprint type three times among the 128 footprints (2.3%) (Figure 2A, line 11), whereas in *S. cerevisiae*, the maximal palindromic arm length was 20 nucleotides and 66 of 140 footprints (47%) contained palindromes with $\geq$3 nucleotide-long arms (Lazarow et al. 2012). Also in Arabidopsis, the frequency of palindromes at least three nucleotides in length was much higher than in *C. albicans* (20–30%) (Huefner et al. 2011; Lazarow et al. 2012). The shorter palindromic lengths in *C. albicans* indicate that the (unknown) hairpin-opening enzymes introduce nicks closer to the apex of the hairpin than in *S. cerevisiae* and plants. In *S. cerevisiae*, mutations in the Artemis-related Pso2 protein, which cleaves DNA hairpins (Tiefenbach and Junop 2012), reduced Ds excisions 10-fold and increased atypical Ds excision site repair products (Yu et al. 2004). It will be interesting to determine if the *C. albicans* and *S. cerevisiae* Pso2 orthologs have different activities, or if other enzymes are responsible for the distinct footprints and excision frequencies.

Ac/Ds excision footprints in both *S. cerevisiae* and *A. thaliana* are accompanied by putative microhomologies (MHs) of the repair intermediates after DNA hairpin opening (Yu et al. 2004; Huefner et al. 2011; Lazarow et al. 2012). The frequency of footprints with MHs ranged from 85 to almost 100% in wild-type *A. thaliana* (Huefner et al. 2011; Lazarow et al. 2012) and from 88 to 89% in *S. cerevisiae* (Yu et al. 2004; Lazarow et al. 2012). In contrast, only 51% of the footprints in *C. albicans* had putative MHs. This suggests that DNA hairpin opening and DSB repair proceeds somewhat differently in *C. albicans* than in the model yeast *S. cerevisiae* and plants. Shorter P-nucleotide insertions in *C. albicans* may indicate that the nuclease that binds to and opens DNA hairpins is more stringently locked to the hairpin apex in *C. albicans* than the functional homologs in plants and budding yeast. While MHs are not essential for the fusion of open ends, terminal MH in double-stranded DNA with short 3’ overhanging single strands stabilizes DSB repair intermediates and enhances the efficiency of repair (Gu et al. 2007). Since DNA ligase IV (encoded by *LIG4*) is critically involved in the repair of DSBs in all eukaryotes and also can ligate DNA ends lacking MHs (Gu et al. 2007), the lower frequency of MHs at Ds excision footprints suggests that *LIG4*-dependent DSB repair is likely to be more active in *C. albicans* than in the other organisms tested.

The high efficiency of the Ac/Ds transposon system demonstrated in this study makes it a potentially superior molecular tool for genomewide in vivo insertion mutagenesis in *C. albicans* compared to alternative approaches. Several in vitro insertion mutant libraries were constructed in diploid *C. albicans* using bacterial Tn7 or Tn5 derivatives that were transposed into isolated genomic DNA fragments, cloned, amplified in *Escherichia coli*, and then transformed into *C. albicans*, where the transposon together with flanking genomic DNA integrated by homologous recombination into the chromosomes (Uhli et al. 2003; Oh et al. 2010; Bharucha et al. 2011). However, this approach was labor-intensive and yielded 18,000, 3633, and 6528 heterozygous transformants, respectively, in the three studies. Thus, the in vitro approach is much less practical for repeated application in multiple different strains, for example clinical isolates. Another advantage of the transposon system described here is that, in addition to generating total loss-of-function alleles, it should be useful to identify haploinsufficient genes in diploid cells and other conditionally essential genes, as was recently demonstrated for Ac/Ds insertions in *S. cerevisiae* (Michel et al. 2017). Furthermore, it can easily be modified for activation-tagging applications, as has been demonstrated in plants [reviewed in Lazarow et al. (2013)], as well as for the generation of overexpression and epitope tagging libraries. The beauty of the system is the ease in generating new libraries with thousands, to hundreds of thousands, of new mutants in a single strain background. When coupled with deep sequencing technologies, this provides the potential for facile, rapid generation of large libraries of random insertion mutants without the need for efficient transformation frequencies.

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