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Characterization of *Choristoneura fumiferana* Genes of the Sixth Subunit of the Origin Recognition Complex: CfORC6

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A new protein was cloned and identified as the sixth subunit of *Choristoneura fumiferana* origin recognition complex (CfORC6). The newly identified 43 kDa protein CfORC6 is much bigger than DmORC6 (25.7 kDa) and HsORC6 (28.1 kDa), though it's 23.85% identical to DmORC6 and 23.81% identical to HsORC6. Although the molecular weight of CfORC6 is close to ScORc6 (50 kDa), CfORC6 is only 14.03% identical to ScORC6. By alignment, it was found that the N-terminal of CfORC6 has about 30% identities with other ORC6s, but about 100aa of C-terminal of CfORC6 has no identity with other ORC6s. Like ScORC6, CfORC6 has many potential phosphorylation sites, (S/T)PXK. Like DmORC6, CfORC6 has leucine-rich region in the relevant site. Northern Blot showed that CfORC6 mRNA is about 2,000nt. Southern Blot confirmed that there is one copy of CfORC6 gene in spruce budworm genome. Western blot showed that infection of Cf124T cells with CfMNPV didn't affect the expression levels of CfORC6, at least up to 26 hr post infection.

Keywords: *Choristoneura fumiferana* ORC6, Expression characteristics, Sequence characteristics, Transcription characteristics

Introduction

The replication of DNA in eukaryotic cells is tightly controlled and coordinated with other events in cell division cycle. This control is thought to be exerted primarily at the initiation of DNA replication. Eukaryotic chromosomal replication initiates at multiple sites in the genome and proceeds bidirectionally. The position of these sites are believed to be specified by DNA elements called origins of replication. Initiation of DNA replication is mediated by a conserved set of proteins, including origin recognition complex (ORC), a heteromeric six subunit protein, which is bound to origins of DNA replication and is highly conserved, serving as a landing pad for the assembly of a multiprotein prereplicative complex (Kelly *et al.,* 2000; Bell, 2002; Archambault *et al.,* 2005). The orc6 (the smallest subunit of ORC) gene is the least conserved of the ORC subunits, and amino acid alignments with the budding yeast ORC6 and the metazoan smallest subunit show no statistically significant homologies (Kelly *et al.,* 2000). The *Drosophila melanogaster* ORC6 (DmORC6) (Chesnokov *et al.,* 2001) and *Homo sapiens* ORC6 (HsORC6) (Dhar *et al.,* 2000) are homologues and similar in size to the *Schizosaccharomyces pombe* counterpart (SpORC6) (Moon *et al.,* 1999), all of which are considerably smaller than the *Saccharomyces cerevisiae* ORC6 (ScORC6).

In *Saccharomyces cerevisiae*, orc6 is an essential gene, but *in vitro* it is not required for ORC binding to the origins of DNA replication (Moon *et al.,* 1999). Yeast ORC6, however, undergoes cell cycle-regulated phosphorylation by cyclin-dependent protein kinase and is dephosphorylated as cells exit mitosis (Lee and Bell, 1997). DmORC6 is required for ORC DNA binding (Weinreich, 2001) but is not required for replication licensing in *Xenopus* (Gillespie *et al.,* 2001). In human and *Drosophila* cells, there is a major fraction of ORC6 that is not part of the ORC complex (Chesnokov *et al.,* 1999, 2001); furthermore, ORC6 localized to cell membrane and cleavage furrow and the midbody during cell division as well as in the nucleus. Thus, ORC6 may be involved in functions other than initiation of DNA replication (Chesnokov *et al.,* 2003; Prasanth *et al.,* 2003). Silencing of orc6 expression resulted in cells with multipolar spindles, aberrant mitosis, formation of multinucleated cells, and decreased DNA replication. Prolonged periods of ORC6 depletion caused a

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decrease in cell proliferation and increased cell death. These results implicate orc6 as an essential gene that coordinates chromosome replication and segregation with cytokinesis (Chesnokov *et al.,* 2003; Prasanth *et al.,* 2003). Further research demonstrated that C-terminal of DmORC6 has a domain critical for cytokinesis, and N-terminal part of DmORC6 has a core replication domain (Prasanth *et al.,* 2003).

Choristoneura fumiferana multicapsid nucleopolyhedrovirus (CfMNPV) is a baculovirus that specifically infects a major forestry pest, the Spruce budworm (*Choristoneura fumiferana*). Viral gene products including LEF-1, LEF-2, LEF-3, IE-1, P143, DNApol and P35 are essential for baculovirus DNA replication in transient replication assays (Li, *et al.*,, 1993; Kool *et al.*,, 1994b; Wu *et al.,* 1996), but additional proteins, both viral and cellular, may be required for viral DNA replication in infected host insects. It's reported that human DNA replication factors, ORC and MCM, can bind *oriP* of Epstein-Barr virus (Biswendu *et al.*, 2001). We hypothesize that identifying cellular factors that control the ability of baculoviruses replication in host cells will identify important determinants of viral virulence and host specificity. To identify Cf homologues, ORC sequences from *Schizosaccharomyces cerevisiae, Drosophila melanogaster, Anopheles gambiae* and *Homo sapiens* were obtained from NCBI GenBank and compared by BLAST searches against sequences from the Spruce budworm EST database. Two EST-containing plasmids were identified as carrying possible ORC6 homologues. Here we report the cloning, expression and characteristics of the sixth member of spruce budworm ORC, the CfORC6 homologous to other ORC6s. Identifying CfORC6 will lead us to our ultimate goal to study whether CfORC6 functions in CfMNPV replication.

Materials and methods

Cloning of C*forc6*. One clone, JH39B6, similar to the cellular DNA replication gene ORC6 was identified when screened the Cf EST databank with consensus sequences of genes in the MCM and ORC family of replication proteins. JH39B6 is a cDNA cloned into pBlueScriptSK as pBSC*forc6*, which has been confirmed by sequencing. JH39B6 carried a full length cDNA clone of a gene homologous with ORC6. The original clone JH39B6 was PCR amplified with primers N-CfORC6 (5'CTGGATCCGCTTCGTAC AATAAAACTCTTC 3') and C-CfORC6 (5'CTCTCGAGTTAGG ATATTTCCAAACATATCTTCC 3'). To express ORC6 protein fused to a His epitope tag, the PCR product was cut with BamHI and XhoI, then cloned into pRSET-A cut with *Bam*HI and *Xho*I to produce pRSETA-CfORC6, then transformed into DH5α. All clones were confirmed by restriction enzyme and nucleotide sequence analysis.

Preparation of polyclonal antibodies. The recombined expression vector pRSETA-CfORC6 was transformed into BL21(DE3) pLysS cells. When cells grew up (in LB medium, at 37°C, 220 rpm) to an OD600 = 0.4-0.6, IPTG was added to a final concentration of 2 mM and cultured cell at 26°C, 200 rpm for half hour. The cells were collected followed by purification on a nickel resin column under native conditions (QIAGEN), then the purified protein was used to raise antibodies in rabbits (Invitrogen). New Zealand White rabbits were injected intramuscularly with 100 μg of CfORC6. The rabbits were boosted three times every 3 weeks.

Cells, viruses infection and Western blot. The *Choristoneura fumiferana* continuous cell line Cf124T was maintained at 28°C in TC100 medium supplemented with 10%fetal calf serum. CfMNPV was prepared and titrated as previously described (Lu and Carstens, 1991).

Cf124T cells, seeded into 35-mm dishes were incubated at 28°C for 4 hr, then Cells were infected with CfMNPV, incubated at 28°C again. Harvest cells at different time points postinfection. Cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond-C). The membranes were blocked with 5% skimmed milk powder overnight at 4°C and then incubated with primary polyclonal antibody against CfORC6 (1 : 2,500), for 1 hr at room temperature. Following three washes with PBS-T (phosphate-buffered salineplus 0.05% Tween 20), the membranes were incubated for 30 min with the appropriate goat secondary antibodies (Molecular Probes) conjugated with horseradish peroxidase (1 : 50,000 dilution). The immunoreactive proteins were detected with a chemiluminescent detection system (New England Nuclear).

Probed with monoclonal antibody against the six-His tag (Sigma) (1 : 10,000), immunoblotting of cells (BL21(DE3) pLysS lysates were carried out using the same protocols as above.

Northern Blot and Southern Blot. Taking JH39B6 as template, and T3 promoter (5'CAATTAACCCTCACT3') and CfORC6 B1 (5'CCTCTTTTTGTGGGCTACC3') as primers, the CfORC6 gene was amplified by PCR method. Then the product was purified with PCR purification kit (Invitrogen) and labelled with digoxigenin-dUTP (Roche) according to the manufacture's introduction. This probe was used in Northern and Southern Blot. The hybridization was performed as described by Koetsier (1993) and Thompson (1994) by using downward alkaline blotting of RNA or DNA.

Phylogenetic Tree Construction. Database searches were performed with BLASTP programs on the NCBI non-redundant database. Determination of the modular structure of the proteins was performed using the Conserved Domain Database at NCBI. Multiple alignments were performed using CLUSTAL W and phylogenetic analysis was conducted using the neighbor-joining method as implemented in the CLUSTAL W program. The origin recognition complex subunit 6 (ORC6) sequences and accession numbers used in this paper are as follows: Homo sapiens (HsORC6, NP055136), *Drosophila melanogaster* (DmORC6, AAD39474), *Saccharomyces cerevisiae* (ScORC6, AAB68869) and *Anopheles gambiae str.* (AgORC6, XP309400).

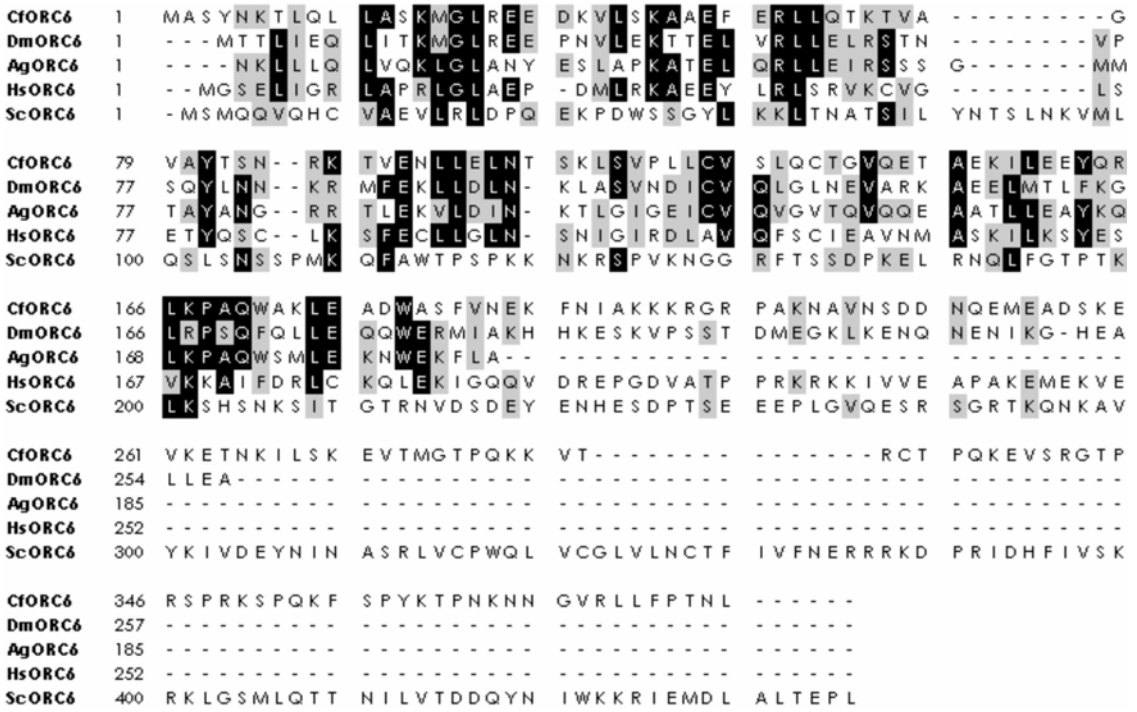

Fig. 1. Comparison of protein sequences of *Choristoneura fumiferana* (CfORC6), *Homo sapiens* ORC6 (HsORC6), *D. melanogaster* ORC6 (DmORC6), *Anopheles gambiae* ORC6 (AgORC6) and *S. cerevisiae* ORC6 (ScORC6). *A*lignment of protein sequences of CfORC6, HsORC6, DmORC6, AgORC6 and ScORC6. The alignment and shading was performed using Bioedit program. Identical residues are marked by *dark shading*, and similar residues are marked by *light shading*. The *numbers* on the *right* indicate the amino acid residues of ORC6 in each species.

Results

Cloning and Sequence Analysis of CfORC6. While searching the data base to get possible candidates for putative CfORC6 subunit, we found expressed sequence tags with considerable homology with DmORC6 and HsORC6 subunits. Ours is the first published report on this new ORC6 subunit. The cDNA encoded an open reading frame corresponding to 374 amino acids with a predicted molecular mass of 42.3 kDa. The predicted molecular mass is close to that of ScORC6 (50 kDa), but much bigger than that of DmORC6 (25.7 kDa) and HsORC6 (28.1 kDa). An alignment of the ORC6 subunit in *Choristoneura fumiferana*, *Drosophila melanogaster*, *Homo sapiens*, *Anopheles gambiae,* and *S. cerevisiae* is showed in Fig. 1. The result shows that about two thirds of CfORC6 from the N-terminal is homologous to the whole ORC6 subunit of *Drosophila melanogaster*, *Homo sapiens* and *Anopheles gambiae*. About 100 amino acids in C-terminal of CfORC6 have no homology to any other ORC6s, which is extra part compared with DmORC6, HsORC6 and AgORC6. By pairwise alignment, it was found that CfORC6 is significantly homologous to the ORC6 subunit of *Drosophila* (23.85% identical) and *Homo sapiens* (23.81% identical) over the entire coding region, but less homologous to the ORC6 subunit of *Anopheles gambiae* (19.63% identical) and *S. cerevisiae* (14.03% identical). The homology of the *Drosophila* and human and spruce budworm ORC6 subunits with *S. cerevisiae* ORC6 is low. The *S. pombe* ORC6 is also significantly different from *S. cerevisiae* ORC6 (Joachim and Ira 1993). The 260aa from N-terminal of CfORC6 is significantly homologous to the whole DmORC6 (32.73% identity) and HmORC6 (32.84% identity) while DmORC6 and HsORC6 only share 26.97% identity.These results suggest that ORC6 has evolved faster than the other ORC subunits in eukaryotes.

CfORC6, DmORC6, AgORC6, HsORC6 and ScORC6 were used in a phylogenetic analysis. The neighbor-joining tree method was used and the results were shown in Fig. 2. CfORC6 was most closely related to insect ORC6s, followed by HsORC6. These four ORC6s seemed to belong to one cluster, metazoan; while another cluster, containing ScORC6 (monadic eukaryotic living), appeared to be more distantly related. The ORC6s of metazoan were quite distinct from yeast.

CfORC6 contains 10 potential phosphorylation sites, (S/T)PXK, for cyclin-dependent protein kinase (Joachim and Ira 1993) clustered in the last one third of the molecular (aa 255 to 364, Fig. 3). In contrast, ScORC6 (435aa) just contains four potential phosphorylation sites and clustered in the half of the molecular (aa 105 to 150). As to HsORC6 and DmORC6, there is no (S/T)PXK; furthermore, immunoprecipitation experiments showed that HsORC6 was very weakly

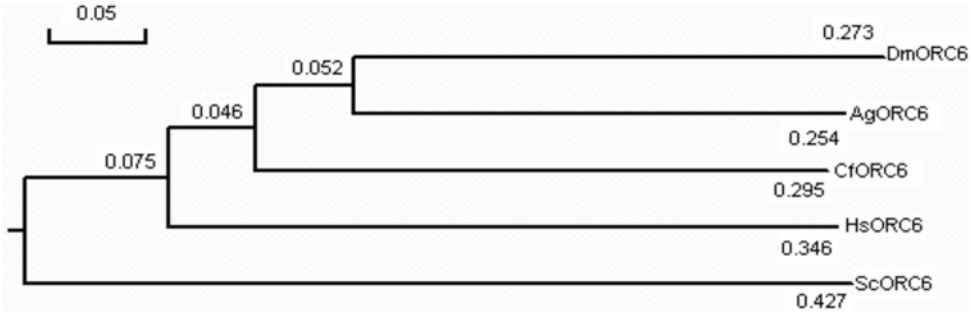

Fig. 2. Phylogenetic analysis of CfORC6, DmORC6, AgORC6, HsORC6 and ScORC6 based on the whole amino acid sequence.

200 VNSDDNQEME ADSKEEGPTE IEIEPYETWK QRMLESAYEE LKQLKMKENE
251 TRLK**SPRK**AT VKETNKILSK EVTMG**TPQK**K VTRC**TPQK**EV SRG**TPQK**VTS
301 G**TPSK**ENTRG **SPQK**EVSRG**T PHK**EVTRYTP PEDMFGNILN NMSPRR**SPRK**
351 **SPQK**F**SPYKT PNK**NNGVRLL FPTNL

Fig. 3. C-terminal predicted amino acid sequence of CfORC6. Matches to the consensus phosphorylation site (S/T)PXK of cyclin-dependent protein kinase in bold italics.

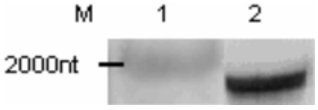

Fig. 4. Northern blotting showed the size of the mRNA of CfORC6. Lane 1: total RNA isolated from Cf124T cells 24 hr after passage, hybridized with CfORC6 probe; lane 2: total RNA isolated from Cf123T cells 24 hr post infected by CfMNPV, hybridized with CfMNPV LEF3 probe.

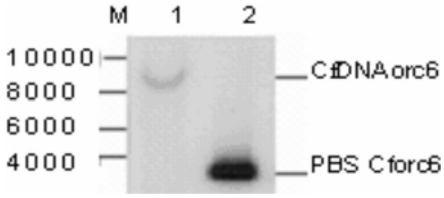

Fig. 5. Southern blotting showed C*forc6* is single copy gene. Lane 1 showed the fragment contains C*forc6* gene; lane 2 showed the control, the plasmid PBSCfORC6. Both DNA were hybridized with same probe.

phosphorylated, but its phosphorylation level did not change with the cell cycle (Dhar and Dutta 2000).

Characteristics of CfORC6 gene transcription. To confirm whether the molecular weight of CfORC6 meets the putative size (42.5 kDa), the CfORC6 gene from JH39B6 was amplified by PCR, and labelled with digoxigenin-dUTP. The cellular total RNA was extracted with one-step method (Chomczynski, 1987, 1993). After 20 μg total RNA was separated on denatured gel and transferred to nylon membrane, Northern blot was performed. The result showed that the mRNA of CfORC6 is about 2000 nt (Fig. 4.), similar to the size of the mRNA of CfMNPV LEF3, whose mRNA is close to 2000 nt and whose molecular weight is 43 kDa. But in contrast to the mRNA of CfMNPV LEF3, the mRNA of CfORC6 signal is very weak. We speculated that it may because CfORC6 is single copy gene, and transcription level is low; or CfORC6 is cell cycle-dependent protein, and there was less transcription in asynchronization cell.

To know whether the CfORC6 is single copy gene, we purified Cf123T cellular DNA, and digested it with EcoRI (for there is no EcoRI site in CfORC6 gene). The digestion mixture was separated on 0.7% agarose gel. After being transferred to nylon filter, it was hybridized with digoxigenin labelled DNA probe. The Southern blotting analysis showed that there was only one weak band which contains ORC6 gene on the membrane (Fig. 5); therefore, CfORC6 gene is single copy gene. It maybe the main reason to explain why CfORC6 mRNA level is low.

Characteristics of CfORC6 expression. The recombine CfORC6, pRSET-ACforc6 was transformed into BL21(DE3) plysS cells. Pilot experiment demonstrated that the fusion protein was expressed as a soluble but unstable product (Fig. 6), especially at high temperature. The protein was purified and used as antigen to immunize rabbit. Polyclonal antibody was successfully generated. This antibody lights up a polypeptide of about 43 kDa protein in Cf124T cells (FIg. 7). The results show that CfORC6 increased with time after infection. This increase also occurred in uninfected cells, suggesting that the amount of CfORC6 protein increased over 26 hr of the experiment, and this increase was not affected by

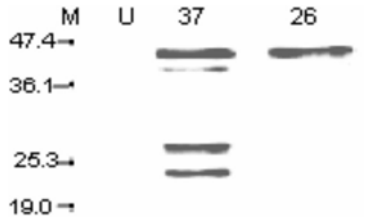

Fig. 6. Recombinant CfORC6 expressed in *Ecoli* and probed with Anti-Hisx6 tag. Lane1, M, Marker; Lane2, U, uninduced cells ascontrol; lane3, expressed at 37°C in1 hr after induction with 2 mM IPTG; lane3, expressed at 26°C in 0.5 hr after induction with 2 mM IPTG.

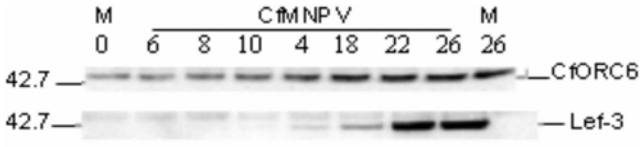

Fig. 7. Time course of CfORC6 level in Cf124T cells infected with CfMNPV. The cells were harvested at various time points after infection and analyzed by Western blotting using polyclonal, anti-CfORC6 antibody. Mock infected cells, harvested at 0 and 26 hr after passage served as controls (row1). The Western blot was stripped and then reprobed with polyclonal anti-CfMNPV LEF-3 to confirm the time course of virus infection (row2).

virus infection. In contrast, Lef3 increased with time course after infection. Infection of Cf124T cells with CfMNPV didn't affect the expression levels of CfORC6, at least up to 26hr post infection, as determined by Western blot analysis.

Discussion

We report here the identification of a sixth member of the *Choristoneura fumiferana* origin recognition complex, CfORC6. This protein is highly homologous to HsORC6 and DmORC6. DmORC6 is part of the sixprotein ORC that is required for DNA replication in the *Drosophila* and *Xenopus* egg extract, and so is HsORC6 in human cells, which suggests that this CfORC6 have the same role in cell cycle. Furthermore, from alignment (Fig. 1), we also can see that in the last conservation domain CfORC6 contains a leucine-rich region, just like the C-terminal 25 aa of DmORC6, which may mediate protein-protein interactions through an amphipathic helix. By coimmuno-precipitation, this region mediate DmORC6 interaction with Drosphila peanut, a member of septin family of proteins important for cell division; therefore, CfORC6 may also has a role in cytokinase and chromatin segments.

CfORC6 is much bigger than DmORC6, HsORC6 and AgORC6. In contrast, the size of CfORC6 is close to ScORC6, though they share fewer identities. Just like ScORC6, CfORC6 contains a 'RXL' (aa232-234) domain which can be recognized by Clb5. Clb5 binding to ORC provides an origin-localized replication control switch that specifically prevents reinitiation at replicated origins. Furthermore, like ScORC6, CfORC6 has many potential phosphorylation sites, though they have different distribution. The analysis above illustrated again that ORC6s are diversity. The results so far indicate significant differences in CfORC6 and other ORC6s might predict differences in how replication is regulated in different species.

CfORC6 expressed as unstable soluble protein in BL21(DE3)plysS cells, which indicated that CfORC6 did harm to the cells; therefore it was degraded by cellular enzymes. Considered that ORC6 takes part in DNA replication, CfORC6 may bind to bacterial DNA and interfere its replication and other activities, so it's degraded by cells when over expressed in bacterial cells. During the purification, CfORC6 used to be coeluted with a 63 kDa protein in 50 mM imidazole buffer; therefore, it interacted with other bacterial protein. But whether CfORC6 interacts with Cf124T cellular proteins and whether CfORC6 interacts with viral proteins remains to make sure by immunoprecipitate experiments. By co-immunoprecipating Cf124T cells (infected with CfMNPV) lysate supernatant with anti-CfORC6 and other cellular and viral antibodies, we will know whether CfORC6 interacts with other cellular proteins and with viral proteins. Homologous repeat regions in CfMNPV genome were speculated to be origins for vial replication. By yeast one-hybridizing, we will know whether CfORC6 recognize the viral replication origins. In a word, we have a long way to go to make sure what roles does CfORC6 take in host DNA replication and viral DNA replication.

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