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 100 aa of C-terminal of CfORC6 has no identity with other ORC6s. Like ScORC6, CfORC6 has many potential phosphorylation sites, (ST)PXK. Like DmORC6, CfORC6 has leucine-rich region in the relevant site. Northern Blot showed that CfORC6 mRNA is about 2,000 nt. 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 *Drosophila melanogaster* orc6 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
Characterization of \textit{Choristoneura fumiferana} Genes of the Sixth Subunit of the Origin Recognition Complex: CiORC6

...take cDNA clones into pBlueScriptSK as pBS-CiORC6, then transformed into DH5α, and XhoI, then cloned into pRSET-A cut with BamHI. Cloning of \textbf{of} was fused to a His epitope tag, the PCR product was cut with BamHI and XhoI, then cloned into pRSET-CiCORC6, then transformed into DH5α. All clones were confirmed by restriction enzyme and nucleotide sequence analysis.

Preparation of polyclonal antibodies. The recombinant expression vector pRSETA-CiORC6 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 (Innovogen). New Zealand White rabbits were injected intramuscularly with 100 μg of CiORC6. The rabbits were boosted three times every 3 weeks.

\textbf{Cells, viruses infection and Western blot.} The \textit{Choristoneura fumiferana} continuous cell line CJT24T was maintained at 28°C in TC100 medium supplemented with 10% fetal calf serum. CiMNPV was prepared and titrated as previously described (Lu and Carstens, 1991).

CJT24T cells, seeded into 35-mm dishes were incubated at 28°C for 4 hr, then Cells were infected with CiMNPV, 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 CiORC6 (1 : 2,500), for 1 hr at room temperature. Following three washes with PBS-T (phosphate-buffered saline) plus 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.

\textbf{Northern Blot and Southern Blot.} Taking JH39B6 as template, and T3 promoter (5'CAATTAACCCCTACT3') and CiORC6 B1 (5'CTCTTTTTTGGGCTACCC) as primers, the CiORC6 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 manufacturer'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.

\textbf{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, AAB68889) and Anopheles gambiae str. (AgORC6, XP308400).
Results

Cloning and Sequence Analysis of CfORC6. While searching the database to get possible candidates for putative CfORC6 subunit, we found expressed sequence tags with considerable homology with Dm ORC6 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 Dm ORC6 (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 DmORC6, HsORC6, and AgORC6. About 100 amino acids in C-terminal of CfORC6 have no homology to any other ORC6s, which is extra part compared with DmORC6 and HsORC6. 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 (14.03% identical) and S. cerevisiae (10.03% identical). The alignment 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 HsORC6 (32.84% identity) while DmORC6 and HsORC6 only share 26.97% identity.

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 interacting with the other ORC subunits.

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.

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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 CMNPV LEF3, whose mRNA is close to 2000 nt and whose molecular weight is 43 kDa. But in contrast to the mRNA of CMNPV LEF3, the mRNA of CfORC6 signal is very weak. We speculated that it may be 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 recombinant CfORC6, pRSET-ACforc6 was transformed into BL21(DE3) plyS 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...
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 has the same role in cell cycle. Furthermore, from alignmen t (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 amphithetic helix. By communo-precipitation, this region mediate DmORC6 interaction with Drosophila peanut, a member of septin family of proteins important for cell division; therefore, CfORC6 may also has a role in cytokinesis and chromosome 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-immunoprecipitating Cf124T cells (infected with CfMNPV) lysate supemant 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 viral 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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References


