Gene 485 (2011) 91–101

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Characterization of a CENP-B homolog in the holocentric Lepidoptera *Spodoptera frugiperda*

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ARTICLE INFO

Article history:
Accepted 7 June 2011
Available online 25 June 2011

Keywords:
CENP-B
Centromere
Holocentric
Transposable element
Transposon domestication

ABSTRACT

The discovery of an homolog of the human centromeric protein B, CENP-B, in an EST database of the holocentric insect species *Spodoptera frugiperda* prompted us to further characterize that gene because i) CENP-B has not been described in invertebrates yet ii) it should be a milestone in the molecular characterization of the holocentric centromere of Lepidoptera.
Like its human counterpart, the *Sf* CENP-B protein is related to the transposase of the pogo transposable element (TE) of *D. melanogaster*. In this paper, we show evidences that the lepidopteran *cenp*B gene has evolved from domestication of a transposase. Furthermore, the *Sf* CENP-B nuclear location and its ability to bind to a retrotransposon derived sequence *in vivo* argue in favor of a functional homology to CENP-B proteins.

1. Introduction

Holocentric chromosomes differ from the monocentric ones in centromere structure. In *C. elegans*, during mitosis, kinetochore spans most of the chromosome length (Albertson and Thomson, 1982). At meiosis, conventional electronic microscopy failed to show a trilaminar kinetochore structure (Albertson and Thomson, 1993; Wicky and Rose, 1996). However confocal microscopy allowed visualization of kinetochore proteins (KNL-1, HIM-10) forming a cup-like structure that encloses the ends of the chromosomes (Howe et al., 2001; Monen et al., 2005). Although reverse genetics *via* RNA interference in *C. elegans* led to the discovery of more than 30 kinetochore proteins among which, HCP-3 and HCP-1, homolog to the human centromeric chromatin binding proteins CENP-A and CENP-C (see (Maddox et al., 2004) for review), DNA sequences interacting with them have not been identified yet (Buchwitz et al., 1999; Desai et al., 2003; Moore and Roth, 2001; Oegema et al., 2001).

The chromosomes of several insect orders separated by at least 300 million years have been described as holocentric (Hughes-Schrader and Rise, 1941; Schrader, 1947; Suomalainen, 1953). Some chromosomal fragments obtained after irradiation (inducing chromosome breakage) can be maintained over several generations (Fujiwara et al., 1991; Hughes-Schrader and Rise, 1941; Murakami and Imai, 1974). Several authors have described holocentric chromosomes in meiotic cells on testis or ovarioles of Vth instar insect larvae (see review by (White, 1973)). The existence of kinetochore plaques extending over 50% to 70% of the chromosome pole ward surface of the chromosome was confirmed by electronic microscopy (Wolf, 1994). New insights on holocentric chromosome structure and segregation should come from the study of lepidopteran species, with the recent availability of the *B. mori* genome (Mita et al., 2004; Xia et al., 2008; The International Silkworm genome Consortium, 2008) which seems to be somehow differently organized than the genome of the holocentric model *C. elegans* (45% of repeated sequences in *B. mori* (Berry, 1985) compared to 17% in *C. elegans* (Sulston and Brenner, 1974)).

The discovery of an homolog of CENP-B in an EST database of the holocentric insect species *Spodoptera frugiperda* prompted us to further characterize that gene because i) CENP-B has not been described yet in invertebrates and ii) it could be a milestone in characterization of the holocentric centromere of Lepidoptera.

CENP-B is a centromere associated protein originally identified in human cells as a 65 kDa autoantigen (apparent molecular weight of 80 kDa) recognized by sera from patients with anti-centromere antibodies (ACA) (Earnshaw and Rothfield, 1985). CENP-B interacts with centromeric heterochromatin in human chromosomes (Earnshaw et al., 1987) and binds specifically to a 17 bp sequence embedded in the alphoid repeats, the CENP-B box (Masumoto et al., 1989; Yoda et al., 1992). CENP-B is thought to form a higher-order chromatin structure required for kinetochore formation by virtue of its ability to dimerize (Masumoto et al., 1989; Muro et al., 1992; Yoda et al., 1992). Supporting

Abbreviations: LTR, Long Terminal Repeat; ACA, anti-centromere antibodies; TBP, TATA box Binding Protein; ChIP, Chromatin Immunoprecipitation; TE, transposable element.

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doi:10.1016/j.gene.2011.06.007

this idea, CENP-B box is required for *de novo* centromere assembly on human alphoid DNA (Ohzeki et al., 2002). CENP-B homologs have been described in mammals (Sullivan and Glass, 1991; Yoda et al., 1996), fission yeast (Halverson et al., 1997) and plants (Barbosa-Cisneros and Herrera-Esparza, 2002), however its biological role has remained elusive in some species because of functional redundancy or maybe because of a dual role of this protein. CENP-B protein is able to target alphoid DNA by the N-terminal DNA-binding domain and to initiate nucleation of CENP-A chromatin on naked DNA in mammalian cell lines. By its C-terminal domain, it may be able to interact directly or indirectly with chromatin modifying enzymes like histone methyl tranferases and to enhance heterochromatin formation (Okada et al., 2007).

Here we describe the *Sf cenpB* gene structure, its syntenic genomic environment in related lepidopteran species, putative properties of the encoded polypeptide, its cellular expression and localization, its *in vivo* DNA targets, the first attempts to assess its biological function and we discuss the possibility that, like fission yeast and human CENP-B homologs, it may result from convergent domestication of a type II transposase (Casola et al., 2008).

2. Material and methods

2.1. Isolation of the Sf cenpB gene and sequence analysis

An EST database from *Sf*9 cells (Landais et al., 2001) was searched for homology with the human CENP-B protein sequence (accession number P07199) using TBLASTN (Altschul et al., 1997). One EST, *Sf*9L08080 matches to the query (30% identities over 121 amino acids, E Value 1e-06). Sequencing of the 1.7 kb insert carried by the *Sf*9L08080 cDNA clone was completed using primers p120 = CAAAACCTTCTT-GATTGGTTTGAA, p123 = CTTGGTACAACAGAACTAAGC. To obtain the corresponding genomic sequence, high density filters spotted with an *S. frugiperda* BAC library (d'Alencon et al., 2004) were screened by hybridization with the 1.7 kb cDNA insert as probe. DNA extracted from one of the 5 positive clones, BAC 72 F01, was used for direct sequencing with primer p137 = CATTTTTAAGTCCAGGATTCC to recover the promoter region, and a PCR fragment encompassing the gene obtained with pEA137 = CCGGAATTCATGTCAATTTGGAATCCTGGAC and pEA140 = CGCGGATCCGGAATCATTTGTACTAGC was also sequenced with the same primers. The whole BAC sequence, accession number FP340415, was obtained at Genoscope, France. Repeats and genes annotation as well as synteny with *H. armigera* and *B. mori* genomes can be visualized in the genomic database Lepidodb, at www.inra.fr/lepidodb (d'Alençon et al., 2010). *Sf* CENP-B gene corresponds to gene 31 on the BAC called 72F01_SFBAC (see the menu "Targets" in LepidoDB database and choose "tbp").

2.2. Codon-based test of neutrality for lepidopteran cenp-B evolution

Coding sequences for 3 CENP-B orthologs in 3 lepidopteran species were aligned by ClustalW (Thompson et al., 1994). *Sf* CENP-B ortholog in *Helicoverpa armigera* corresponds to gene 25 on the BAC 94B11_HaBAC and its ortholog in *Bombyx mori* corresponds to prot 20 on nscaf2998, manually annotated as CENP-B like in LepidoDB (www.inra.fr/lepidodb).

The probability of rejecting the null hypothesis of strict neutrality (dN = dS) was calculated and considered significant at the level of 0.05. dN and dS are the numbers of synonymous and nonsynonymous substitutions per site, respectively. The variance of the difference dN–dS was computed using the bootstrap method (500 replicates). Analyses were conducted using the Nei–Gojobori method (Nei and Gojobori, 1986) in MEGA4 (Tamura et al., 2007). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 422 positions in the final dataset.

2.3. Plasmids

In order to purify the N-terminal domain of *Sf* CENP-B (177 first amino-acids), the 5′ end of the *Sf*9L08080 cDNA insert was PCR-amplified with primers pEA136 = GAATTCCATATGTCAATTTGGAATC-CTGGA and pEA133 = GGTGGTTGCTCTTCCGCATCCCAAATAAACAT-CATTTGGCTG, containing *Nde*I and *Sap*I restriction sites, respectively (underlined sequence). The PCR fragment cut with *Nde*I and *Sap*I was ligated to plasmid pTYBI, linearized by the same enzymes, resulting in plasmid pTYB1Nt-*Sf*CENP-B.

2.4. RT PCR

Total RNA was isolated from different developmental stages (2.5 days old eggs, L1-L6 larval stages, 12 days old pupae and adults) of *S. frugiperda* with Trizol. cDNA preparation was done with SuperScript® III Reverse Transcriptase kit (Invitrogen) according to manufacturer instructions. Semi quantitative PCR was done with the primer pairs pEA139 = CCGGAATTCGAAGATAGTGATAATGATC-ATAGT and pEA140 (see above) for *cenp*B and for TBP, primers TBP1 = CAGCCACAAACACCACAAAACTTAATGTCA and TBPR = CACACTTATTGCTTTTTAAAGCTCTTCAATAATGGG.

2.5. Proteins and antibodies

The N-terminal part of *Sf* CENP-B was purified from *E. coli* cells harboring plasmid pTYB1Nt-*Sf*CENP-B as an Intein-Chitin Binding Domain protein fusion on a chitin beads column and eluted in presence of DTT to induce self-cleavage of the fusion protein (IMPACT-CN System, Biolabs). 1 mg of purified protein was run on SDS-PAGE electrophoresis, the *Sf* CENP-B band was excised and sent to Eurogentec for rabbit immunization.

2.6. Protein determination

Protein concentration was measured by using the Bradford assay (Bradford, 1976).

2.7. Western blotting

Samples were separated by 10% SDS/PAGE (Laemmli, 1970), and the proteins were transferred to nitrocellulose membranes by using a semidry blotting apparatus (CBS) according to the manufacturer instruction. After blocking the membrane with TBS-Tween 20 0.05%-dry milk 5%, they were incubated with primary antibody (dilutions 1/5000) in TBS for 1 h at room temperature. After three rinses with TBS-Tween 0.05% and incubation with alkaline phosphatase-conjugated anti-rabbit IgG in TBS, the membranes were rinsed twice with TBS-Tween 20 0.05%, once with TBS. Bound antibodies were detected by using the NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) color reaction.

2.8. Cytological observations

*Sf*9 cells were grown on glass coverslides placed at the bottom of culture plates, rinsed once in PBS. Localization of *Sf* CENP-B was performed on fixed cells according to (Li et al., 1996) modified as follow: A 15 min blocking step in IF buffer containing 1% BSA at room temperature was added after cells and nuclei permeabilization with 3% Triton X100. Anti *Sf* CENP-B antiserum was diluted to 1:500 and 1:500 Alexafluor 488 conjugated anti rabbit antibodies (Molecular probes) were used as secondary antibodies. Anti-β tubulin (Sigma) antibodies were used at a dilution of 1:500 and revealed by use of 1:500 Alexafluor 494 (Molecular probes) conjugated anti mouse antibodies. Preparations were observed using a Leica Widefield /Zeiss SV11 microscope. Black-and-white images were recorded with a