Gene 519 (2013) 41–49

Contents lists available at SciVerse ScienceDirect

Gene

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

Identification, quantification, and evolutionary analysis of a novel isoform of MCM9

Elizabeth P. Jeffries, William I. Denq [1], Jonathan C. Bartko, Michael A. Trakselis *

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, 15260, USA

ARTICLE INFO

Article history:
Accepted 28 January 2013
Available online 9 February 2013

Keywords:
Alternative splicing
DNA replication
DNA repair
MCM9
qRT-PCR
Cell cycle

ABSTRACT

The minichromosome maintenance (MCM) family of proteins is conserved from archaea to humans and is required for assembly of pre-replication complexes (pre-RCs) to initiate DNA replication. MCM9 is an uncharacterized member of the eukaryotic MCM protein family that contains conserved ATP binding and hydrolysis motifs. We have identified a novel alternatively spliced isoform of *Hs*MCM9 that results in a medium length protein product ($MCM9^{M}$) that eliminates a long C-terminal extension of the fully spliced product ($MCM9^{L}$). Quantitative real-time reverse transcriptase PCR (qRT-PCR) separated and measured the relative mRNA isoform expression levels across a variety of cell lines. Although there is some variability in expression levels, the full length $MCM9^{L}$ transcript is more abundant than the $MCM9^{M}$ variant in all cell lines tested. The expression of both MCM9 isoforms is cell cycle regulated: induced in S-phase, decreases through G2/M, and becomes constant through G1. Consistent with recent reports suggesting MCM9 participates in repair or prevention of double strand breaks, mitomycin C significantly induces the specific expression of $MCM9^{L}$, while the replication fork inhibitor, hydroxyurea, has no effect. Evolutionary analysis indicates that the $MCM9^{M}$ isoform is a conserved variant, whereas the addition of the terminal exon producing $MCM9^{L}$ appears to be a more recent event present only in the highest order of eukaryotes.

1. Introduction

DNA replication is an essential yet complex process in all living cells. It is vital to cellular survival and genomic stability that faithful genome duplication occurs exactly once per cell cycle during S-phase (Machida et al., 2005; Nishitani and Nishimoto, 2000; Ritzi and Knippers, 2000; Sivaprasad et al., 2007). Aberrant DNA replication has been linked to several cancer types, and certain replication factors, when overabundant, have been identified as potential clinical biomarkers in cancerous tissues (Coleman and Laskey, 2009; Coleman et al., 2006; Freeman et al., 1999; Gonzalez et al., 2004; Williams et al., 1998). Both timing and spatial location of DNA replication initiation are used as mechanisms of cell cycle control. Eukaryotic genomes are replicated from multiple origins of replication, located throughout the genome (Diffley, 2011; Douglas and Diffley, 2012). The controlled recruitment and function of a diverse group of catalytically active proteins to multiple origins of replication are necessary to initiate faithful genome replication within S-phase (Bielinsky, 2003). The assembly of these proteins, termed the pre-replication complex (pre-RC), is complete upon recruitment of the minichromosome maintenance proteins (MCM2–7) or replicative helicase (Bochman and Schwacha, 2008; Boos et al., 2012) to chromatin; a process defined as the licensing of replication origins (Remus and Diffley, 2009; Tsakraklides and Bell, 2010). Activation of the MCM2–7 helicase is thought to occur through the concerted action of GINS and Cdc45 to encircle single-stranded DNA to begin unwinding (Bruck and Kaplan, 2011; Ilves et al., 2010). Following recruitment of the remaining members of the replisome, replication forks progress along double-stranded DNA until meeting head-on, terminating replication (Davey and O'Donnell, 2000).

MCM8 and MCM9 are recently identified members of the MCM family of AAA^{+} ATPases (*A*TPases *a*ssociated with a variety of cellular *a*ctivities) (Lutzmann et al., 2005). The initial discovery of MCM9 from homology to expressed sequence tags (ESTs) identified a short isoform ($MCM9^{S}$) of only 391 amino acids that lacks complete conservation of the AAA^{+} MCM core motifs (Yoshida, 2005). Full-length MCM9 protein was subsequently identified in *Xenopus*, and a homology search revealed the fully spliced human isoform ($MCM9^{L}$), 1143 amino acids in length (Lutzmann et al., 2005). Immunodepletion of *Xl*MCM9 from egg extracts causes interruption of pre-RC formation resulting in inhibition of DNA replication (Lutzmann and Méchali, 2008). *Xl*MCM9 binds chromatin in a manner dependent on ORC recruitment and forms a stable complex with Cdt1 presumably within the pre-RC (Lutzmann and Méchali, 2008).

The necessity of MCM9 for pre-RC formation cannot be generalized to all vertebrates. Knockouts of MCM8 and MCM9 in mice have shown

Abbreviations: qRT-PCR, quantitative real time reverse transcriptase polymerase chain reaction; FACS, fluorescence-activated cell sorting; MCM, minichromosome maintenance; pre-RC, pre-replication complex; EST, expressed sequence tag; C_T, cycle threshold.

* Corresponding author at: 219 Parkman Ave, 801 Chevron, Pittsburgh, PA, 15260, USA. Tel.: +1 412 624 1204; fax: +1 412 624 8611.
E-mail address: mtraksel@pitt.edu (M.A. Trakselis).
[1] Current address: University of Pittsburgh School of Medicine, 3550 Terrace Street, Pittsburgh, PA, 15261, USA.

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.01.054

that neither MCM8 nor MCM9 are essential for DNA replication or organism survival but are needed for germ-line stem-cell maintenance and efficient homologous recombination (HR) (Hartford et al., 2011; Lutzmann et al., 2012). Removal of MCM8 or MCM9 results in genomic instabilities, sex-specific tumor development, and sterility. MCM8 and MCM9 are thought to interact directly in a complex that participates in either preventing or repairing double strand breaks (Lutzmann et al., 2012; Nishimura et al., 2012). Both MCM8 and MCM9 are localized in cells as foci at DNA damage sites and are essential for the recruitment of other HR genes. It is proposed that a MCM8-MCM9 complex acts in some capacity as an ATP-dependent helicase to resolve complex DNA structures during HR. The contradiction regarding the necessity of MCM9 in eukaryotes could be explained by differences in *Xenopous* cell-free extract replication systems versus assays with whole cells or organisms. Alternatively, specific differences in alternative isoforms of MCM9 may provide differential or synergistic roles.

Although it was initially thought that MCM9 was a vertebrate-specific protein due to its absence in *Drosophila*, nematodes, and yeast (Lutzmann et al., 2005), it has now been identified in the genome of the invertebrate sea squirt (Dehal et al., 2002) as well as many plants, parasites, and even fungi (Iyer and Aravind, 2006; Liu et al., 2009) suggesting wider eukaryotic evolution (Aves et al., 2012). The collective findings suggest that MCM9 is a more recent evolutionary addition to the MCM family that is required for preventing or repairing DNA damage during replication that may lead to chromosomal instabilities and tumor development.

In this report, we have identified a novel mRNA isoform of *Hs*MCM9 resulting from an alternative splicing event in precursor mRNA (pre-mRNA) producing a medium isoform (MCM9^{M}) more consistent with the amino acid size of the rest of the MCM family members. Full length MCM9^{L} contains 12 contiguous exons, while in MCM9^{M}, exon 11 is excluded. This exclusion results in a frame shift from exon 10 to 12, causing a premature stop codon and truncation of the unconserved C-terminal extension. For the first time, we have separately quantified the individual mRNA expression levels of the two functional AAA^{+} MCM9 isoforms (MCM9^{L} and MCM9^{M}) in a panel of human cell lines. The expression profiles of both MCM9 isoforms are cell cycle regulated, peaking during S-phase and returning to basal levels as the cell cycle progresses through G2/M and into G1. The cell cycle profile of MCM9 differs slightly between the two isoforms examined and significant differences are noted in early S-phase and G2/M. Induction of DNA crosslinks using mitomycin C leading to double strand breaks causes MCM9^{L} specifically to be transcriptionally activated, whereas stalled replication forks from hydroxyurea treatment had no effect on MCM9 transcription. These results identify, characterize, and validate a novel MCM9^{M} isoform that lacks the long C-terminal extension placing it more consistent in length with the rest of the MCM family as well as medium length MCM9 variants in a variety of other eukaryotes. The cumulative expression data suggests that MCM9^{M} and MCM9^{L} may have different regulated roles for DNA maintenance.

2. Materials and methods

2.1. Materials

Fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM) supplemented with D-glucose, L-glutamine and sodium pyruvate were from Atlanta Biologicals (Atlanta, GA). Penicillin-streptomycin and trypsin were from Invitrogen (Carlsbad, CA). Adult human cervix RNA was from Agilent Technologies (Santa Clara, CA). Nocodazole and hydroxyurea were from Fisher Scientific (Waltham, MA) and Mitomycin C was from Roche (Indianapolis, IN). Taq DNA polymerase was from Bioline (Tauton, MA). Propidium iodide was from Sigma-Aldrich (St. Louis, MO). Ultraspec RNA isolation reagent was from Biotecx (Houston, TX). iScript One-Step RT-PCR Kit with SYBR Green was from Bio-Rad (Hercules, CA, USA). M-MuLV reverse transcriptase was from New England Biolabs. All other reagents were analytical grade or better.

2.2. Human Cell Culture and RNA Isolation

Cervical cancer (HeLa), lung cancer (A549), head and neck cancer (PCI-13), breast cancer (MDA-MB-231 and MCF-7) and normal skin keratinocyte (HaCaT) cell lines were maintained in a humidified 5% CO_2 incubator at 37 °C. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 4.5 g/L D-glucose, 4.5 g/L L-glutamine and 4.5 g/L sodium pyruvate, 10% fetal bovine serum and 5% penicillin-streptomycin. Mitomycin C at 2 μg/mL was added to the media for 6 h, before washing and harvesting. Hydroxyurea at 150 μM was added to the media for 48 h before washing and harvesting. Cells were grown, harvested by trypsinization, and total RNA was extracted using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's instructions. Total RNA concentrations were calculated from the absorbance at 260 nm using a DU730 UV-visible spectrophotometer (Beckman Coulter Inc., Brea, CA).

2.3. Semi-quantitative RT-PCR Detection of MCM9 Splice Variants

cDNA was generated from total RNA using reverse transcriptase according to the manufacturer's instructions. Four regions of MCM9 (E1-E4, E3-E6, E4-E10, and E9-E12) were specifically amplified from cDNA using Taq. The forward and reverse primers (sequences in Supplemental Table S1) were designed to overlap exons in the amplified regions. The A-tailed products of MCM9^{M} and MCM9^{L} were cloned into pGEM-T (Promega, Fitchburg, WI) and sequenced using the DNA sequencing facility at the University of Pittsburgh. For HeLa, A549, PCI-13, MDA-MB-231, MCF-7, and HaCaT cDNA, two sets of primers were used to specifically amplify each isoform. A single forward primer complementary to exon 9 (E9Fa) was used for amplification of both isoforms. A reverse primer specific to exon 11 (E11R) was used to amplify MCM9^{L} and a reverse primer complementary to the region bridging exons 10 and 12 (E11SkipR) was used to amplify MCM9^{M}.

2.4. Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Human cells were grown and harvested by trypsinization. DNaseI-treated total RNA (8 μg) was subjected to qRT-PCR analysis using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA, USA) and the real-time thermocycler iQ5 (Bio-Rad, Hercules, CA, USA). GAPDH was used as a reference gene. For qRT-PCR analysis of MCM9^{L} and MCM9^{M}, oligos E11R and E11SkipR were used, respectively with E9Fa (Supplementary Table S1). For amplification of reference housekeeping gene GAPDH, GAPDHF and GAPDHR were used and for simultaneous amplification of MCM9^{S}, MCM9^{M} and MCM9^{L}, oligos E3F and E5R were used (Supplementary Table S1). The cycle threshold or C_T value was determined for each amplified RNA sample and is the number of PCR cycles completed when the amplification curve intersects a specified threshold value. qRT-PCR was performed in triplicate on each plate and relative expression of mRNAs was evaluated by the difference in cycle threshold values from GAPDH (ΔC_T) or between isoforms ($\Delta\Delta C_T$) from at least three independent experiments (Livak, 2001). For all sets of data, p-values were calculated comparing the ΔC_T value for MCM9^{M} with that for MCM9^{L} or between different cell samples for the same isoform using a Student's t-test in Excel. A p-value of <0.05 was considered statistically significant.

2.5. HeLa S-Phase and G2/M Synchronization

HeLa cells were synchronized at the beginning of S-phase using a double thymidine block. Adherent cells were grown to 40% confluency in 150 cm^2 flasks with 30 mL growth medium. A final concentration