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Brief report

Near-full-length REV3L appears to be a scarce maternal factor in *Xenopus laevis* eggs that changes qualitatively in early embryonic development

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ABSTRACT

REV3 is the catalytic subunit of DNA polymerase ζ (pol ζ), which is responsible for the damage-induced mutagenesis that arises during error-prone translesion synthesis in eukaryotes. The related *REV3L* genes in human and mouse encode proteins of approximately 350 kDa, twice as large as yeast REV3, but full-length REV3L has not been identified in any vertebrate cell. We report that *Xenopus laevis REV3L* encodes a 352-kDa protein that has high overall amino acid sequence similarity to its mammalian counterparts, and, for the first time in a vertebrate species, we have detected putative REV3L polypeptides of 300 and 340 kDa in *X. laevis* oocytes. Only the 300-kDa form is stored in eggs, where its concentration of about 65 pM is much lower than those of other replication and repair proteins including the accessory pol ζ subunit REV7. In fertilized eggs, the levels of this polypeptide did not change until neurula; the larger 340-kDa form first appeared at stages after gastrula, suggesting a pattern of regulation during development. These observations indicate the existence of REV3L as a scarce protein, of approximately the full predicted size, whose level may impose severe constraints on the assembly of pol ζ in *X. laevis*.

1. Introduction

DNA lesions caused by endogenous and environmental agents are removed by repair systems to maintain genomic stability, but some of them nevertheless persist in the genome during replication. When a replicative DNA polymerase encounters such a lesion, DNA chain elongation is blocked and the replication fork stalls. To avoid cell death by fork arrest, cells deploy post-replication repair pathways [1], which are channeled by modifications of the sliding clamp molecule PCNA in eukaryotes [2]. One of these repair pathways is translesion synthesis (TLS), in which specialized translesion DNA polymerases directly copy the damaged DNA in either an error-free or an error-prone manner [3]. Error-prone TLS results in DNA damage-induced mutagenesis, which is implicated in carcinogenesis in higher eukaryotes [3].

DNA polymerase ζ (pol ζ) functions in error-prone TLS in eukaryotes [4]. Pol ζ is a B-family polymerase and has been extensively characterized in the budding yeast *Saccharomyces cerevisiae* [5,6]. The core complex of yeast pol ζ is at least a heterodimer of the catalytic subunit REV3 and the accessory subunit REV7 [3–7]; REV7 enhances the polymerase activity of REV3 [8]. Although yeast recombinant pol ζ can bypass certain lesions such as *cys-syn* cyclobutane pyrimidine dimers (CPDs) [8], its main function is to catalyze efficient extension of a misinserted nucleotide opposite a DNA lesion [9]. This extension activity constitutes the latter step in a two-polymerase model of the TLS process comprising insertion of the nucleotide opposite the lesion followed by extension reactions to elongate from the inserted nucleotide [10]. However, the native architecture of pol ζ is unknown because the endogenous pol ζ holoenzyme has not yet been isolated from any eukaryote.

Counterparts of the yeast *REV* genes have been identified in other eukaryotes [3,4]. Mouse and human REV3L (REV3-like; 350 and 353 kDa, respectively) orthologs have a large extra segment which is not conserved in yeast REV3, and are thus about twice the size of the 173-kDa yeast REV3 [11–14] (see Fig. 1). Knockdown of *REV3L* expression in cultured mammalian cells decreases damage-induced mutagenesis [15–18]. In contrast, disruption of mouse *REV3L* results in embryonic lethality, indicating a requirement in early development [19–23]. *REV3L*-deficient embryonic fibroblasts established from knockout mice are genetically unstable [24,25]. Some additional function of REV3L is also required for recombination and interstrand cross-link repair [3,4]. Despite the established participation of REV3L in many cellular processes, little

Abbreviations: BSA, bovine serum albumin; CPD, *cys-syn* cyclobutane pyrimidine dimer; IP, immunoprecipitation; LSS, low-speed supernatant; NP-40, Nonidet P-40; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; pol, DNA polymerase; RACE, rapid amplification of cDNA ends; TLS, translesion synthesis.

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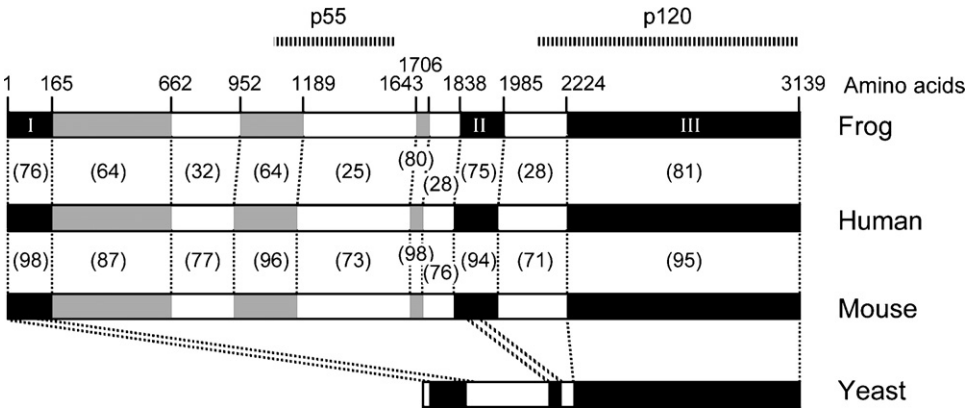

Fig. 1. Primary structures of REV3 orthologs. Protein sequences of frog (*X. laevis*), mouse and human REV3L and yeast (*S. cerevisiae*) REV3 are shown schematically. *X. laevis* REV3L amino acids are numbered from the N to the C terminus; the human, mouse and yeast orthologs contain 3130, 3122 and 1504 amino acids, respectively. Black boxes indicate regions that are conserved among all four sequences, and gray boxes indicate REV3L-specific conserved regions. Numbers in parentheses show the percent amino acid sequence identity between collinear regions of adjacent sequences, as determined with GeneWorks software. The locations of polypeptides p55 and p120, which were used to raise anti-*X. laevis* REV3L antibodies, are shown by broken lines.

is known about the REV3L protein itself. The endogenous REV3 in higher eukaryotes has been purified only from *Drosophila* embryos, as an active 240-kDa enzyme [26], and full-length REV3L has not hitherto been identified in vertebrates, although REV3L protein in human cells was recently detected by immunofluorescence *in situ* [27]. Furthermore, no enzymatic functions, including polymerase activity, have been reported even for recombinant REV3L, since ectopic expression of REV3L is technically difficult using standard recombinant DNA techniques because of its high molecular mass.

The failure to detect full-length REV3L could reflect regulation of its expression so as to keep pol ζ levels low [3]. Fully grown oocytes or unfertilized egg cells of *Xenopus laevis* should contain a higher concentration of DNA-metabolizing enzymes as a maternal stockpile than somatic cells, to support the much smaller replicons that occur during the first 12 embryonic cell divisions [28,29]. Reasoning that these stockpiled proteins of *X. laevis* oocytes might include unusually high levels of REV3L, we report here, for the first time, the identification of two near-full-length, endogenous forms of REV3L. From an analysis of eggs and embryos, we find that these proteins are clearly limited quantitatively, but are altered qualitatively, during early embryonic development. These features may constrict the formation of pol ζ and thus help to minimize error-prone TLS in vertebrates.

2. Materials and methods

2.1. X. laevis cDNAs

General methods used for DNA manipulation and transformation followed standard procedures [30]. RNA was extracted from de-jellied *X. laevis* eggs with TRIzol (Invitrogen, USA), and poly(A)-RNA was isolated from the total RNA with Oligotex-dT30 beads (Takara, Japan) and used to synthesize cDNA with a Marathon cDNA amplification kit (Clontech, USA). A 360-bp DNA fragment was amplified from the cDNA by PCR using the primers dXL29 (5′-GCCCTTGAACTAACAAGGAAAATG-3′) and dXL30 (5′-GTCATCACACACAGGACAGTG-3′), corresponding to sequences near the 3′ end of the human *REV3L* gene. Sequential 5′- and 3′-RACE reactions on this fragment using the Marathon cDNA amplification kit resulted in the identification of a *REV3L* cDNA. To ensure that the determined nucleotide sequence of *REV3L* would be free of PCR-related errors, five overlapping DNA fragments spanning the open reading frame were amplified from the double-stranded cDNA mixture by proofreading-proficient Advantage cDNA polymerase mix (Clontech), and both strands of each fragment were sequenced directly without intermediate cloning procedures. The assembled *REV3L* sequence was deposited in GenBank (accession number GU072594). The 9420-bp ORF was reconstructed from the overlapping DNA fragments in the plasmid pTWV228 (Takara). The *POL3* gene, which encodes the catalytic p125 subunit of pol δ, was identified and sequenced in the same way (GenBank accession number GU072593). Using reported sequence information for *X. laevis*, cDNAs encoding REV7/MAD2L2 [31], pol β [32] and PCNA [33] were isolated from the *X. laevis* egg cDNA mixture by PCR and cloned into appropriate plasmids to express recombinant proteins; all plasmids were sequenced to verify that no mutations had been introduced during PCR amplification. Part of the *REV3L* coding sequence was cloned into pET22-b(+) (Novagen, USA) to yield pKEY3, which produces a 55-kDa polypeptide (p55) containing amino acid residues 1109–1597 of REV3L with a C-terminal His tag. Likewise, plasmid pTMN28 expresses the 120-kDa polypeptide (p120) encompassing residues 2121–3139 of REV3L, with a short PelB leader and a His tag. Plasmid pNR116 was constructed by cloning the *REV7* gene into pMAL-c2X (New England Biolabs, USA) to express MBP-tagged REV7.

2.2. X. laevis proteins

Protein preparations were carried out at 0–4 °C. Low-speed supernatants (LSSs) from *X. laevis* eggs and S150 extracts from oocytes were prepared as described [34,35]. Total proteins of *X. laevis* embryos were extracted by boiling the embryos in SDS-sample buffer [30]. *X. laevis* recombinant proteins were produced in bacteria and purified by column chromatography, and their concentrations were determined by SDS-PAGE using BSA as standard. *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, USA) was the host strain for plasmids pKEY3 or pTMN28, and the p55 and p120 polypeptides of REV3L expressed in these cells were purified successively on Ni^{2+}-charged chelating columns and HiTrap S columns (GE Healthcare, USA). MBP-REV7 expressed in *E. coli* was purified on an amylose affinity column (New England Biolabs) followed by a HiTrap Q column (GE Healthcare).

2.3. Antibodies

Rabbit polyclonal antibodies were raised against the following purified *X. laevis* polypeptides: p55 and p120 of REV3L, pep6 (15-SSQTKKLRGDWDDD-28 of the pol δ-p125 subunit), PCNA and pol β. REV3L-specific rabbit antibodies were affinity-purified from rabbit sera using antigen fixed on nitrocellulose filters [36]. Mouse