Published in final edited form as:

DNA Repair (Amst). 2017 February ; 50: 71–76. doi:10.1016/j.dnarep.2016.12.004.

Mouse DNA polymerase ι lacking the forty-two amino acids encoded by exon-2 is catalytically inactive *in vitro*

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Abstract

In 2003, we reported that 129-derived strains of mice carry a naturally occurring nonsense mutation at codon 27 of the *Poli* gene that would produce a polι peptide of just 26 amino acids, rather then the full-length 717 amino acid wild-type polymerase. In support of the genomic analysis, no polι protein was detected in testes extracts from 129X1/SvJ mice, where wild-type polι is normally highly expressed. The early truncation in polι occurs before any structural domains of the polymerase are synthesized and as a consequence, we reasoned that 129-derived strains of mice should be considered as functionally defective in polι activity. However, it has recently been reported that during the maturation of the *Poli* mRNA in 129-derived strains, exon-2 is sometimes skipped and that an exon-2-less polι protein of 675 amino acids is synthesized that retains catalytic activity *in vitro* and *in vivo*. From a structural perspective, we found this idea untenable, given that the amino acids encoded by exon-2 include residues critical for the coordination of the metal ions required for catalysis, as well as the structural integrity of the DNA polymerase. To determine if the exon-2-less polι isoform possesses catalytic activity *in vitro*, we have purified a glutathione-tagged full-length exon-2-less (675 amino acid) polι protein from baculovirus infected insect cells and compared the activity of the isoform to full-length (717 amino acid) GST-tagged wild-type mouse polι *in vitro*. Reaction conditions were performed under a range of magnesium or manganese concentrations, as well as different template sequence contexts. Wild-type mouse polι exhibited robust characteristic properties previously associated with human polι's biochemical properties. However, we did not detect any polymerase activity associated with the exon-2-less polι enzyme under the same reaction conditions and conclude that exon-2-less polι is indeed rendered catalytically inactive *in vitro*.

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Conflict of Interest

The authors declare that they have no conflict of interest

Keywords

DNA polymerase ι; 129-derived strains of mice; Y-family DNA polymerase; Mutagenesis; Translesion DNA synthesis

1. Introduction

The genes encoding human and mouse DNA polymerase ι (*POLI* and *Poli*, respectively) were cloned in 1999 [1]. Both genes exhibit the same general genomic organization and are composed of 10 exons. Exons 1–9 are all relatively small and combine to produce the ~450 amino acid catalytic core of the DNA polymerase, while the largest exon, exon-10, encodes a C-terminal peptide of ~275 amino acids that participate in the protein-protein interactions that regulate polι's activities *in vivo*.

At the time the genes were cloned, the human *POLI* gene was believed to encode a 715 amino acid protein and the mouse *Poli* gene a 717 amino acid protein. Advances in whole genome sequencing subsequently revealed an in-frame methionine initiation codon that is conserved in eukaryotes that would lead to a human polι protein of 740 amino acids and a mouse polι protein of 737 amino acids, and Genbank files were updated to reflect the longer forms of the polι proteins in the database. However, as far as we are aware, there is no experimental evidence actually supporting the existence of the longer isoform *in vivo* and based upon our own unpublished observations, the shorter 715 amino acid human polι is significantly more prevalent than the 740 amino acid isoform (if it exists at all). As a consequence, we will use the amino acid numbering of the original shorter isoforms of polι throughout this manuscript to avoid confusion over amino acid residues that are the subject of investigation.

Shortly after the *POLI* and *Poli* genes were cloned, the respective encoded polι proteins were overexpressed, purified and their biochemical properties assayed [2–8]. Both enzymes exhibited near identical properties that are best characterized by the template dependent misincorporation pattern of the enzyme. When replicating template dT, polι prefers to misincoporate dG over the normal Watson-Crick base dA, by a factor of 3–10. In contrast, when replicating template dA, polι is quite accurate and only misincorporates an incorrect base with a frequency of 1×10^{-4}. Thus, the fidelity of polι can vary by a factor of 10^5, depending upon the template base replicated *in vitro* [2, 6, 7].

Given these unique properties, we were interested in generating a genetically modified mouse with defects in polι's catalytic activities, so as to identify any polι-associated phenotypes *in vivo*. During our attempts to do so, we discovered that 129-derived strains commonly used at that time to generate genetically modified embryonic stem cells actually carry a naturally occurring nonsense mutation that replaces Serine 27 in polι with a stop codon and would result in the synthesis of a polypeptide of just 26 amino acids [8]. The mutation was found in all three 129-derived strains assayed. This includes (129P3/J (Jax #000690); 129X1/SvJ (Jax #000691); and 129P1/ReJ (Jax #001137). Antibodies to mouse polι were used in western blots to probe testes extracts from C57BL/6 (Jax #000664) and 129X1/SvJ strains and confirmed the genomic analysis. A very strong signal to the wild-type

polι was observed in C57BL/6 testes extracts, but polι was undetectable in 129X1/SvJ testes extracts [8]. As a consequence, for the past 13 years, the 129X1/SvJ mouse, and its *Poli* derived gene have been widely used to study the effects of a polι-deficiency *in vivo* [8–14].

However, the assumption that 129-derived strains of mice are truly deficient in polι activity has recently been questioned by the fact that in 129-derived strains, exon-2 is skipped during mRNA maturation [15, 16]. The extent of the exon skipping appears to be dependent upon the precise 129-derived strain analyzed, with exon-2 skipping in 129X1/SvJ significantly lower than that observed in 129/Ola mice [16]. The extent of exon-2 skipping also appears to be tissue specific [17]. When translated, the full-length exon-2-less polι protein would contain exons 1 and 3–9 of the catalytic core, as well as exon 10, containing all of the motifs required for polι's protein interactions *in vivo*. Because of this fact, it has been hypothesized that the exon-2-less isoform may retain significant biological activity *in vitro* and *in vivo*. Indeed, a recent study by Aoufouchi *et al.*, appears to support this notion [16].

We were therefore interested in characterizing the biochemical properties of the exon-2-less mouse polι protein *in vitro*. Here, we present data on the structural considerations a deletion of the 42 amino acid exon-2 would have on the wild-type polι polymerase, as well as present our efforts to purify and characterize the exon-2-less mutant from *E.coli* and baculovirus infected insect cells *in vitro*.

2. Materials and Methods

2.1 Structural analysis

The structural model of mouse polι was made using PyMol (pymol.com) based on the homologous human polι structure (PDB: 3GV8) [18]. As with all Y-family DNA polymerases, polι consists of four structural domains; palm, finger, thumb and little finger. The three catalytic carboxylates of the polymerase active site reside in the palm domain. The region encoded by exon-2 in mouse polι (residue 14–55) spans from the palm to the finger domain.

2.2 Expression and purification of full-length wild-type polι and an exon-2-less derivative in E.coli and baculovirus infected insect cells Construction of E.coli expression vectors

The full-length (717 amino acid) mouse polι protein was first codon optimized for expression in *E.coli* and the gene encoding the codon optimized polι chemically synthesized (Genscript, Pistcataway, NJ). The DNA was cloned in to the low-copy expression vector, pJM871 [19], as an ~2.4 kb *Nde*I-*Xho*I fragment to generate pEC117, which expresses N-terminal 6-His tagged mouse polι. A derivative lacking exon-2 was generated by synthesizing an ~380 bp fragment (Genscript) encompassing the exon-2-less region of the codon optimized *Poli* gene and was sub-cloned into the unique *Nde*I-*Bsr*GI sites of pEC117. The resulting vector, pEC119, expresses His-tagged full-length exon-2-less pol iota (675 amino acids). Both *E.coli* vectors express wild-type mouse polι and exon-2-less polι at similar levels. The two His-tagged polι proteins were purified using the same protocols successfully employed to purify full-length human polι from *E.coli* [19]. This included Ni-

NTA, hydroxyapatite and HP-Q chromatography. Wild-type mouse polι behaved like its human counterpart and was readily purified. However, although initially expressed at the same level as wild-type polι, the exon-2-less derivative exhibited significant instability during the purification process. Based upon western blot analysis of the purified fractions with affinity purified antibodies generated to the C-terminus of mouse polι [8], at least 100-fold lower levels of the exon-2-less protein were obtained following the simple 3 step purification protocol.

Construction of baculovirus expression vectors—We have previously constructed a baculovirus expression plasmid, pJM306, which expresses an N-terminal GST-tagged mouse polι protein [8]. An exon-2-less derivative was generated by synthesizing an ~65bp gene fragment (Genscript) encompassing the exon-2 boundaries and was subsequently sub-cloned into the unique *Nco*I-*Bst*EII sites of pJM306 to generate pJM844. Exon-2-less polι was subsequently expressed in Sf9 insect cells and purified by Glutathione affinity chromatography followed by size exclusion chromatography as a custom service by scientists at Eurofins (Dundee, UK). Based upon western blots, the yield of exon-2-less polι obtained is only slightly lower than that previously obtained with the wild-type protein. Expression of GST-tagged polι in baculovirus infected insect cells therefore appears to have circumvented most of the instability observed when the His-tagged exon-2-less polι mutant was expressed in *E.coli*.

2.3 In vitro primer extension replication reactions

Primers and Templates—All oligonucleotides were synthesized at Lofstrand Laboratories (Gaithersburg, MD) and gel purified prior to use. The 17mer primer, "17ssp1" (5′-ATG GTA CGG ACG TGC TT-3′) was labeled at its 5′ end with ^{32}P by polynucleotide kinase and annealed to either the 48-mer template "UTTA" (5′-TCG ATA CTG GTA CTA ATG ATT AAC GAA TTA AGC ACG TCC GTA CCA TCG-3′), or the 29-mer template "T10AGC" (5′–CGA AAA AAA AAA AAG CAC GTC CGT ACC AT-3′) (Primer binding sites are underlined).

Standard replication reactions (10 μl) contained 40 mM Tris•HCl (pH 8.0), 2.5% glycerol, 0.1 mg/ml bovine serum albumin, 10 mM dithiothreitol, 100 μM aphidicolin, 10 nM of 5′-^{32}P-labeled primer-template DNA and 2 nM glutathione S-transferase- polι. After 20 minutes incubation at 37°C, reactions were terminated by addition of 10 μl of 95% formamide, 10 mM EDTA, and samples were heated to 100°C for 5 min and then immediately transferred on ice. The reaction products were separated by 18% polyacrylamide, 8M urea gel electrophoresis and analyzed using a Fuji FLA-5100 PhosphorImager and ImageGauge software.

3. Results

3.1 Structural considerations of the effects of deleting the 42 amino acids encoded by exon-2 on the integrity of wild-type polι

While the crystallographic structure of mouse polι has yet to be solved, many crystal structures of human polι now exist [18, 20–23]. Of particular relevance, is that the N-

terminus of mouse and human polι is highly conserved and from residue 26 to 52 of exon-2 they are identical (Fig. 1A). Like all Y-family DNA polymerases [24], polι possesses four structurally distinct domains: The fingers domain that encompasses the so-called “substrate lid” of the enzyme; the thumb domain which binds DNA; the palm domain that includes the catalytic active site of the polymerase; and the little finger domain that together with the thumb domain helps grasp DNA and hold the polymerase to the template [25]; As seen in Fig. 1B, the amino acids encoded by exon-2 (shown in red) encompasses residue D34, which is one of the three carboxylates required to coordinate the metal ions necessary for catalysis as well as F38 and Y39 residues necessary for the binding of incoming nucleotides (Fig. 1C). In addition, deletion of exon-2 also destroys the structural integrity of the polymerase by removing the central strand of the five-stranded β sheet in the palm domain and the first α helix in the finger domain. The tertiary structure of polι without the 42 residues encoded by exon 2 is therefore highly likely to be partially unfolded. Given this scenario, it is hardly surprising that the mutant protein is incredibly unstable *in vivo* and is rapidly degraded by the proteasome in human cells [16].

3.2 Purification of wild-type polι and an exon-2-less derivative from E.coli and insect cells

We were interested in purifying and characterizing the biochemical properties of the mouse polι-exon-2-less mutants and initially attempted to purify the enzyme from *E.coli*. To do so, the mouse *Poli* gene was codon-optimized for optimal expression in *E.coli* (Genscript, Piscataway, NJ). The synthesized gene was then cloned into pJM871, a low copy expression vector that adds a 6x-his tag to the N-terminus of any gene cloned into the unique *Nde*I site of the vector [19]. A plasmid expressing the exon-2-less mutant was constructed by replacing the 5′ end of the wild-type *Poli* gene with a chemically synthesized gene fragment that deleted exon-2. We then attempted to purify both wild-type and mutant polι proteins using the same protocol previously used to purify full-length human polι from *E.coli* [19]. While the wild-type mouse polι behaved in a similar manner to its human counterpart and was readily purified to a high degree of homogeneity, we had very limited success with the purification of the exon-2-less mutant. The protein was very unstable and while western blot analysis confirmed the presence of small quantities of the mutant protein (Fig 2A), Coomassie blue staining of these fractions revealed that they contained many impurities (unpublished observations) and were unsuitable for further biochemical characterization.

We have previously purified mouse polι as a GST-tagged fusion protein from baculovirus infected insect cells [8]. We therefore attempted to purify the exon-2-less mutant using the same expression and purification system. To do so, an exon-2-less mutant was generated in the wild-type BV expression plasmid, pJM306 [8], by sub-cloning a short 65 bp chemically synthesized fragment encoding the exon-2-less region into the wild-type vector. The mutant protein was expressed in baculovirus infected insect cells and purified in a two-step process of Glutathione-S-transferase affinity chromatography and size exclusion chromatography, as a custom service by scientists at Eurofins (Dundee, UK). The yield of the exon-2-less mutant was slightly lower than that previously obtained for the wild-type GST-tagged mouse polι enzyme, but much higher than that purified in the *E.coli* system (Fig. 2B) and was deemed suitable for further biochemical characterization.

3.3 In vitro characterization of exon-2-less polι

The wild-type GST-tagged polι and exon-2-less mutant were then assayed for their ability to extend a radiolabeled primer-template *in vitro*. Initial experiments indicated that the exon-2-less protein preparation exhibited very weak polymerase activity, but the pattern of misincorporated bases promoted by this enzyme did not match the characteristic misincorporation profile of polι and we suspected that the preparation was contaminated by small quantities of an insect-encoded DNA polymerase (unpublished observations). Indeed, that appears to be the case, as the addition of the B-family DNA polymerase inhibitor, aphidicolin [26], to the reactions completely eliminated the weak polymerase activity in the exon-2-less preparation (unpublished observations). Similar to human polι [3], aphidicolin had no effect on the polymerase activity of wild-type polι (unpublished observations).

Human polι has previously been shown to exhibit greatest activity when low levels of magnesium, or manganese ions are provided for catalysis [27]. We therefore assayed the activity of wild-type mouse polι and the exon-2-less mutant over a wide range of manganese and magnesium concentrations using a radiolabeled primer annealed to the 10AGC template (Fig. 3A). This template was chosen since the wild-type human enzyme readily incorporates dT opposite the run of 10 template dAs [27]. Similar to human polι, wild-type mouse polι exhibited best primer extension in the presence of manganese (peak activity was 0.5 mM) (Fig. 3B). However, in contrast to human polι, which is also active in low levels of magnesium [27], the mouse polι required higher levels of magnesium for optimal catalysis (peak activity was 5 mM).

Significantly, we observed no exon-2-less polι-dependent primer extension under any manganese or magnesium concentrations assayed on this template (Fig. 3C).

Next, we assayed the misincorporation pattern of the two enzymes on the UTTA template, which has two template Ts as the first two bases to be encountered during primer extension (Fig. 4A). As previously reported [8], in the presence of magnesium, wild-type mouse polι prefers to misincorporate dG opposite template T [8]. In these experiments misincorporation of dT opposite template T appears better than dA. This is in contrast to the human polι enzyme where the correct incorporation of dA is slightly favored over the misincorporation of dT [8].

As with the 10AGC template, no primer extension activity was observed on the UTTA template with the exon-2-less polι enzyme (Fig. 4B).

4. DISCUSSION

We have expressed, purified and characterized a derivative of mouse polι that lacks the 42 amino acid residues encoded by exon-2. As reported by others, the exon-2-less derivative is unstable [16, 17], presumably because its structural integrity is severely compromised (Fig 1.). While we were unable to purify sufficient quantities of the mutant protein from *E.coli* extracts, we were able to purify the exon-2-less polι mutant as a GST-fusion protein from baculovirus infected insect cells (Fig. 2) that allowed us to assay the activity of the mutant polymerase *in vitro*. Using the purified protein, we were unable to detect any activity of the

exon-2-less polι derivative *in vitro* under a variety of metal ion concentrations and template sequence contexts, despite observing vigorous polymerase activity of the wild-type mouse polι under the same assay conditions (Figs. 3 & 4), These observations are consistent with the structural ramifications of the loss of exon-2 containing amino acid residues on polι and we conclude that the exon-2-less mouse polι polymerase is catalytically inactive *in vitro*.

Our observations are in agreement with those of Kazachenko *et al.*, who have also recently expressed and purified the mouse exon-2-less polι protein from *S. cerevisiae* and found no polymerase activity associated with the mutant protein [17]. It is also consistent with conceptually similar studies with a human variant lacking exon-2, that was also shown to be catalytically inactive *in vitro* [28], as well as a single D34A amino acid substitution in human and mouse polι that inactivates the polymerase [16, 28]. However, they are not in agreement with that of Aoufouchi *et al.*, [16] who reported weak polymerase activity attributed to the polι-exon-2-less mutant in a highly sensitive radioactive gel assay after extended assay times (16 hours). The polι preparation used in those assays was apparently obtained after immunoprecipitation of the mutant polι protein following the protocol of Karawya *et al.*, [29]. Since the mutant protein contains all of the residues required for interactions between polι's protein partners and it is known that polι physically interacts with polη [30, 31], we hypothesize that the weak activity attributed to the exon-2-less polι may actually be due to a contamination of the immunopreparation sample with native polη, which is roughly the same size as exon-2-less polι.

Aoufouchi *et al.*, have also reported the construction of a genetically modified mouse with a complete "knock-out" (KO) of *Poli* [16]. Mouse embryonic fibroblasts (Mefs) with the *Poli* KO apparently exhibit moderate sensitivity to ultraviolet light compared to the wild-type *Poli* Mefs at a UV dose of 3 Jm^{-2} and this UV-sensitivity is abrogated when the exon-2-less polι is expressed in the *Poli* KO cell line, thereby implying the exon-2-less polι is functionally active *in vivo* [16]. However, in another report using the same KO allele, no UV sensitivity was observed for the *Poli* KO Mef cell line in UV dose response curves ranging from 0 – 5 Jm^{-2} [32]. As a consequence, it remains unclear whether the exon-2-less polι retains any capacity to restore UV resistance to the KO *Poli* cell line, since there is conflicting evidence in the literature as to whether the KO *Poli* cell line is actually sensitive to UV-light, or not.

As previously mentioned, the 129X1/SvJ mouse and its encoded mutant *Poli* gene have been used by a variety of researchers around the world as an accepted *Poli* null allele since 2003 because these studies have revealed clear phenotypes that are inconsistent with the notion that the exon-2-less polι mutant protein retains functionality [9–14].

Based upon the observations reported here and elsewhere, we believe that 129-derived strains of mice remain a good source of a naturally occurring genetic mutation conferring a polι-deficiency. However, care should be taken with the derivative used for analysis, since the extent of exon-2 skipping appears to be strain dependent. Indeed, 129X1/SvJ appears to give the least exon 2 skipping [16]. For researchers looking for alternate sources of a murine polι-deficiency, we have recently reported the generation of C57BL/6-derived mice that express a catalytically inactive polι protein (D126A and E127A substitutions) [14], and

these mice are openly available from the Mutant Mouse Regional Resource Center (MMRRC) as MMRRC:042060. Two “knock-out” strains of polι mice have also been reported in the literature [16, 33] that will also hopefully be available to the scientific community upon request.

Acknowledgments

We thank Alena Makarova for kindly sharing unpublished data on her own group’s studies on the exon-2-less polι mutant and for helpful and stimulating discussions. This work was supported by funds from the National Institutes of Health/National Institute of Child Health and Human Development Intramural Research Program to RW and National Institutes of Health/National Institute Diabetes and Digestive and Kidney Diseases Intramural Research Program to WY.

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Highlights

- 129-derived strains of mice carry a C→A mutation in codon 27 of polι
- The naturally occurring mutation changes Ser27 (TCG) to an amber (TAG) stop codon
- Exon-2 is sometimes skipped and results in a 675 amino acid exon-2-less polι protein
- The exon-2-less 675 amino acid polι protein is catalytically inactive *in vitro*
- The 129X1/SvJ strain of mice remains a good source of a *Poli* null allele

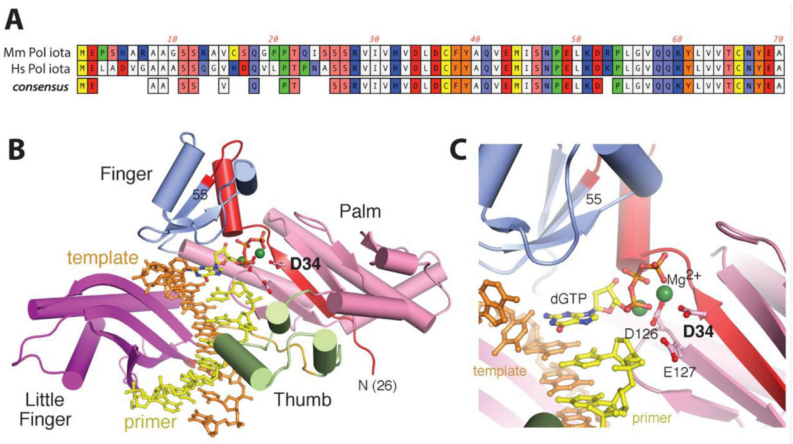

Fig. 1. Ramifications of an exon-2 deletion on the tertiary structure of polι

A: Alignment of the first 70 primary amino acids at the N-terminus of mouse and human polι. The region is highly conserved, and is invariant in exon-2 residues 26 through 52 (exon 2 spans residues 14–55). **B**: Structure of the catalytic domain of human DNA polymerase ι (PDB: 3GV8). The structure starts at residue 26 of polι. The four structural domains found in all Y-family polymerases are shown in pink (palm), light blue (finger), light green (thumb) and magenta (LF). The region deleted when exon 2 is skipped, is shown in red. To emphasize the critical importance of exon-2 on the structure of polι, a helix in the thumb domain (residues 202–212) has been omitted for clarity. : The three carboxylates in the polymerase active site of polι (D34/D126/E127) are shown as sticks and the two Mg^{2+} ions are shown as green spheres.

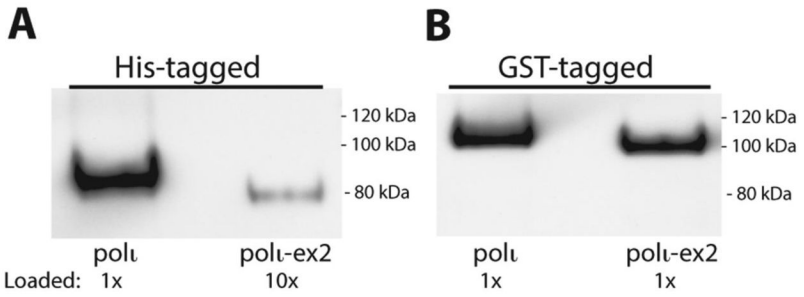

Fig. 2. Western blot analysis of purified wild-type polι and exon-2-less polι

A: Levels of His-tagged proteins purified from *E.coli*. **B**: Levels of GST-tagged proteins purified from baculovirus infected insect cells. Recombinant protein fractions were probed with polyclonal affinity purified antibodies to the C-terminus of mouse polι. Levels of the polι exon-2-less protein purified from *E.coli* were significantly lower than the wild-type protein, but were similar to the wild-type protein in baculovirus expressed insect cells.

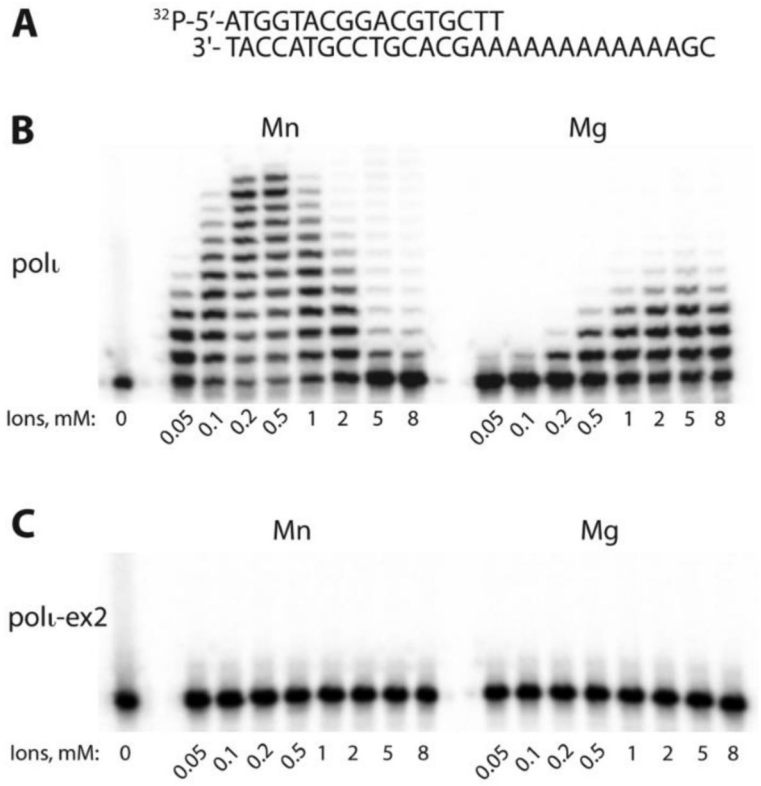

Fig. 3. ***In vitro*** **characterization of wild-type polι and exon-2-less polι on the "10AGC" template**
A: Nucleotide sequence of the primer-template used in the primer extension assays. **B**: Determining the optimal metal ion concentration for wild-type polι when it replicates the 10AGC template, **C**: Activity of exon-2-less polι under the same assay conditions.

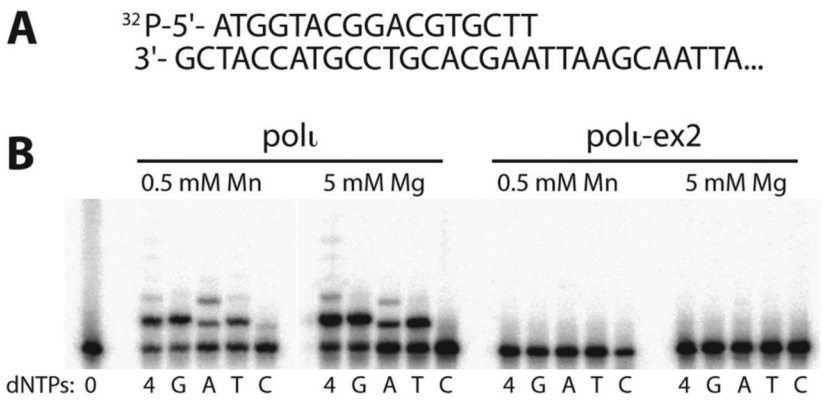

Fig. 4. ***In vitro*** **characterization of wild-type polι and exon-2-less polι on the "UTTA" template**
A: Nucleotide sequence of the primer-template used in these assays. **B**: Reactions were performed at optimal metal ion concentrations (0.5mM Mn^{2+} or 5mM Mg^{2+}) in the presence of all four dNTPs (4) or individual dNTPs (G, A, T, C).