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APEX/Ref-1 (apurinic/apyrimidic endonuclease DNA-repair gene) expression in human and ascidian (*Ciona intestinalis*) gametes and embryos*

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In recent years, the impact of sperm DNA damage on fertility has become an important issue. The different technologies developed to check sperm DNA fragmentation lead to the same conclusion: DNA damage negatively impacts upon reproductive processes. Oocyte DNA repair capacity is one of the cues to understanding embryo developmental arrest. *APEX/Ref-1* (apurinic/apyrimidic endonuclease) is an enzyme involved in the DNA base excision repair pathway removing the abasic sites, the most common DNA decays. In humans, *APEX* has a multifunctional role, including the control of the redox status of transcription factors. RT-PCR allowed us to detect human *APEX* transcripts in oocytes, spermatozoa and preimplantation blocked embryos. In parallel, a comparative study on sea squirt *Ciona intestinalis* (ascidian) indicated that *APEX* transcripts are clearly detectable in oocytes and embryos until the larva stage, but not in spermatozoa, suggesting the appearance of the paternal contribution to DNA repair during development having arisen only late in Vertebrate evolution. Of additional phylogenetic significance is the observation that sea squirt *APEX* appears to lack redox transcriptional activity.

Q2 *Keywords:* DNA repair; *APEX/Ref-1*; human gametes; human embryos; sea squirt; *Ciona intestinalis*

Introduction

The impact of male infertility is still a matter of debate. For many years male fertility has been defined as the ability of sperm to fertilize oocytes and obtain early cleavage stage embryos. In human *in vitro* fertilization (IVF), the generation of two to four cell embryos was considered a test for sperm fertilizing ability, assuming that all resulting embryos had the same developmental potential, irrespective of sperm and oocyte quality (Menezo, 2006; Menezo *et al.*, 2006). Now, it is well established that differences in male fertility are not simply related to sperm penetration failure (Ron-el *et al.*, 1991). In some cases, ICSI allows a better embryo development; however, this technique presents potential harmful aspects (Morozumi and Yanagimachi, 2005). More recently, careful attention has been paid to the quality of sperm DNA, and new methods have been developed to evaluate paternal DNA integrity, which can be totally independent of all semen parameters, including sperm morphology, concentration and motility (Evenson *et al.*, 2002; Oger *et al.*, 2003). Different techniques reached the same conclusions: sperm DNA fragmentation and increased reactive oxygen species (ROS) concentration are responsible for reduced fertility (Henkel *et al.*, 2004). ROS have a heavily deleterious impact on sperm DNA (Lopes *et al.*, 1998a,b), since

they cause the formation of apurinic/apyrimidic (AP) sites (Xu *et al.*, 1998; Hsieh *et al.*, 2001), that, if left un-repaired, have profound effects on sperm physiology.

All mammalian cells possess DNA repair systems (Bessho *et al.*, 1993; Wood *et al.*, 2001). In oocytes and zygotes, DNA repair is probably one of the most important processes to be performed at the time of fertilization and immediately after, in order to allow complete embryonic development. The product of the *APEX/Ref-1* (AP excision/Redox factor-1) gene is an enzyme capable of initiating the repair of AP sites, the most common decay in damaged DNA (Demple *et al.*, 1991; Hsieh *et al.*, 2001; Kelley and Parsons, 2001). *APEX/Ref-1* is known by different names (e.g. APE; APX; APE1; APEN; HAP1; REF1; REF-1) and encodes the major AP endonuclease in human cells. Splice variants have been found for this gene: all encode the same protein. Variant-1 contains the full-length first exon and is the longest transcript (Fritz, 2000). In human cells, *APEX/Ref-1* not only initiates the removal of baseless sites in DNA through the catalytic scission of the phosphodiester bond 5' and adjacent to an AP site, but also possesses a Ref-1 activity, i.e. the ability to control the redox status of a number of transcription factors including Fos, Jun and p53 (Xanthoudakis and Curran, 1992; Jayaraman *et al.*, 1997).

The importance of metabolic pathways involving *APEX/Ref-1* activity, led us to check the presence of *APEX/Ref-1* mRNA in human oocytes and preimplantation embryos, in order to clarify the

*A part of this work was presented (oral presentation) at the 2004 ESHRE meeting in Berlin.

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115 role of the enzyme in these models in response to oxidative stress. Accordingly to recent estimations, spermatozoa can carry up to 5000 mRNAs (Ostermeier *et al.*, 2002; Miller *et al.*, 2005); therefore, we tested the expression of *APEX/Ref-1* also in spermatozoa.

To investigate how evolutionarily conserved was the expression of *APEX/Ref-1* mRNA during the early stages of development, we extended the study to oocytes and spermatozoa of the ascidian *Ciona intestinalis* (sea squirt), an organism intensively studied in developmental biology (Satoh, 2003) and, more recently, proposed as a model to study meiotic regulation (Russo *et al.*, 1996; Nixon *et al.*, 2000; Russo *et al.*, 2004). From an evolutionary point of view, Tunicates (appendicularians, salps and sea squirts) have very recently been re-evaluated as the closest relatives of vertebrates, more than cephalochordates, like amphioxus (Delsuc *et al.*, 2006). This important discovery has been made possible since the advent of genomics that actually provide the opportunity for phylogenetics to resolve a number of outstanding evolutionary questions (Delsuc *et al.*, 2005). In this respect, the draft copy of the *C. intestinalis* genome became publicly available, providing new insights into origin and evolution of chordates (Dehal *et al.*, 2002; Ciona Genome Group, 2003). Based on this evidence, we used *C. intestinalis* gametes and embryos as comparative models to study the conservation of *APEX/Ref-1* functions during evolution.

Materials and Methods

Collection of human sperm, oocytes and embryo

140 Human oocytes were collected from an IVF centre (IRH/Laboratoire Marcel
Q3a Mérioux). Hormonal stimulation was performed according to classical protocols involving a semi-long treatment with gonadotrophin-releasing hormone (GnRH) agonists (decapetyl or busserelin) followed by ovarian stimulation with urinary or recombinant follicle stimulating hormone (FSH). In the absence of fertilization, metaphase II (MII) oocytes were collected 48 h after insemination. All the fertilization and embryo culture procedures were performed under oil to allow better developmental potential and to avoid oxidative stress. Germinal vesicle (GV) oocytes were collected from intracytoplasmic sperm injection (ICSI) patients, when maturation was not completed. MII oocytes (one pool of eight, and two pools of five) were gathered when, in two cases, the husband was not able to produce sperm at the time of IVF/ICSI. As the access for GV was not restricted, most of our controls (β -actin) were performed on pools of 10 GV oocytes. Tests were also performed on individual GV oocytes. Care was taken to ensure the total absence of corona cells on the oocytes and to avoid any extra cellular contamination by observation under an inverted microscope at $\times 200$ magnification. Arrested or lysed embryos (after freezing and thawing) were used for the determination of *APEX* according to the French bioethical law. Analyses were performed on two pools of five and one pool of two 2–3 cell blocked embryos, two pools of eight, and one pool of four early blocked morula (eight to 16 cell stages), of two blocked late morulas and five blocked/lysed blastocysts. No four-cell blocked or lysed embryos were available. This protocol was performed in accordance with an agreement of the CNMBRDP (Commission Nationale de Medecine, Biologie de la Reproduction et Diagnostic Preimplantatoire). This agreement allows research only on embryos that were unsuitable for transfer. Six sperm samples, with characteristics largely over WHO parameters were treated twice with 45/90% Percoll (Suprasperm, Medicult France) gradient. We especially took care, in the crude samples, of the absence of round cells and blood cells. After treatment the samples were checked under an inverted microscope to be absolutely sure of the total absence of foreign cells.

RNA extraction, reverse transcription (RT) and polymerase chain reaction (PCR) from human samples

170 Total RNA was extracted from human sperm (5×10^5 highly selected spermatozoa) using Trizol protocol (Invitrogen, Milano, Italy). For human oocytes and blocked embryos, a direct thermolysis was performed. Briefly, samples were placed in PCR tubes in 2 μ l of sterile diethylpyrocarbonate (DEPC)-treated

water and covered with one drop of mineral oil. Samples underwent thermolysis for 1 min at 100°C in order to release nucleic acids.

The reverse transcription reaction was performed in a final volume of 20 μ l containing RT buffer 1 \times , 10 mM dithiothreitol (Sigma, Milan, Italy), 0.5 mM of each dNTP (Invitrogen), 0.5 μ g oligo(dT)_{12–18} (Invitrogen), 10 IU RNase inhibitor (Promega, Chrabonnières, France) and 200 IU SuperScript reverse transcriptase (Invitrogen). For each sample, 18 μ l of the RT mix was added. RT reaction was carried out at 42°C for 50 min followed by heating to 70°C for 15 min to inactivate the reaction.

180 PCR analyses were carried out in a final volume of 50 μ l containing the cDNA (half of the RT product) from 10 oocytes and a minimum of five blocked embryos, or from 5×10^5 spermatozoa, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.2 mM each of dNTP, 0.4 μ M of each primer (MWG biotech, France) and 2 IU of *Taq* DNA polymerase (Perkin Elmer Cetus, Courtaboeuf, France). To avoid any risk of genomic contamination during PCR, the forward APEX-F and the reverse APEX-R were designed on exons three and five of human *APEX* gene, respectively (Accession Number M80261, Table 1). Internal positive control of PCR was performed on the same samples using human β -actin specific primers (Table 1, Accession Number E00829). A positive control was also performed on 1 μ g of total RNA from human amniotic cells, which we knew to express human *APEX*, under the same conditions described above. After an initial denaturation step of 1 min at 94°C, 35 amplification cycles were performed. Each cycle included denaturation at 94°C for 45 s, annealing at 56°C for 1 min and extension at 72°C for 1 min. A final extension step of 10 min at 72°C was performed in order to complete the PCR reaction. The size of *APEX* external product was 499 base pairs (bp), while the couple of nested primers generated an internal amplicon of 292 bp. To confirm the identity of the human *APEX* transcripts, each RT–PCR product was cleaved with AluI restriction enzyme. PCR products and AluI reaction mixtures were separated by electrophoresis on 2% agarose gel, stained by ethidium bromide and visualized under UV.

DNA/RNA extraction from *C. intestinalis* gametes and embryos

200 *Ciona intestinalis* individuals were supplied from Stazione Zoologica of Naples ‘A. Dohrn’. Unfertilized oocytes and spermatozoa were collected as reported (Russo *et al.*, 1996). Genomic DNA was prepared from gametes and embryos using a commercially available kit (Wizard, Promega, Milano, Italy). Typically, each single sperm preparation started from 50–100 μ l of dry sperm ($4–6 \times 10^{10}$ spermatozoa/ml). Oocytes were collected from the ovary of three to five samples, depending on their size, and fertilized to obtain embryos at different stages. RNA extraction from sperm, unfertilized oocytes and selected embryos was performed using Trizol reagent (Invitrogen), quantitated by spectrophotometric reading and analysed on denaturing agarose gel as reported (Sambrook and Russell, 2001). The total RNA extracted was subjected to extensive treatment with DNase–RNase/free (BD-Clontech, Milano, Italy), in order to eliminate any trace of contaminating genomic DNA, before cDNA synthesis. The amount of unfertilized oocytes and embryos collected was enough to isolate 1–3 μ g of total RNA.

RT–PCR and real-time qPCR on *C. intestinalis* oocytes and embryos

215 The protocols for reverse-transcriptase PCR, opportunely optimized, were carried out on approximately 2 μ g of RNA total employing the One-Step and RT–Omniscrypt kits (Qiagen, Milano, Italy), following the manufacturer’s instructions. Synthesized cDNA was amplified using the following protocol: 5 μ l cDNA, 10 \times PCR buffer (100 mM Tris/HCl, pH 8.3; 500 mM KCl; 15 mM MgCl₂; Roche, Mannheim, Germany) 2.5 mM dNTP; 25 pmol of specific primer; 2.5 U *Taq* Polimerase enzyme (Biogem; supplied by Stazione Zoologica of Naples); water to a final volume of 50 μ l. PCR primers were generated using ‘Primer3’ software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by the Molecular Biology Core Facility at Stazione Zoologica of Naples. Their sequences are reported in Table 1. PCR reactions were placed in a Thermocycler Ptc-100 (M.J. Research, Inc.) using the following parameters: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 52–60°C for 1 min; extension at 72°C for 1 min; final extension at 72°C for 10 min. Only when genomic DNA was amplified, cycle numbers were lowered to 25. Amplified products were separated on 2% (w/v) agarose

Table 1: Sequences of primers employed in this study

Gene	Abbreviations	Primers (sequence 5'–3')
<i>HsAPEX</i> (intron-spanning – Exon 3–5)	APEX-F	GGCAAACCTGCCACACTCAAG
	APEX-R	GGGTTGCGAAGGTCAATTTCTTC
<i>HsAPEX</i> nested (intron-spanning – Exon 3–5)	APEX-N-F	TGGATGGGCTTCGAGCCTGG
	APEX-N-R	ACAAACGAGTCAAATTCAGCCAC
<i>Hs-β-actin</i>	β-actin-F	GTGGGGCGCCCCAGGCACCA
	β-actin-R	CTCGTTAATGTCACGCACGATTTTC
<i>CiAPEX</i> (mRNA)	AXM-1	GAATGTAGCTGGGGTTTCGAG
	AXM-2	AGCTCTGCAGTTTCCCATGT
<i>CiAPEX</i> (qPCR)	AP-RT-F	GAAATGGAGAAAAGGCCACA
	AP-RT-R	TTCACCCACTTTACCCCATC
<i>CiAPEX</i> (genomic)	AXG-F	GCAAATCCGAAAGGAAACAA
	AXG-R	GTTGCGTTTGAATGTCATGG
<i>C. intestinalis</i> Hexaminidase A	TS1-F	GTGCTGGAGACAAAAGCTC
	TS1-R	CCCCAACTGATTTTGCTGT
<i>C. intestinalis</i> β-Tubulin	CiTu-F	GACTCCTTTTGGACGTTGT
	CiTu-R	CCATTCACCGTCTTCACTT
<i>C. intestinalis</i> Ribosomal protein S-27	RP-S27-F	AATCCACCCTTCACCTTGTG
	RP-S27-R	GGGAGATCTTGCCATTTTCA

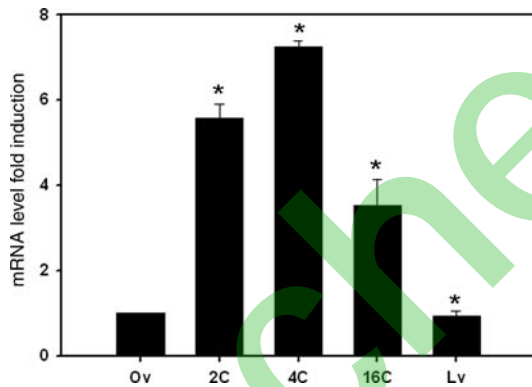


Figure 1: Temporal expression of *CiAPEX* in unfertilized oocytes and during early embryo developmental stages. mRNA levels were measured by real-time PCR (qPCR) from cDNA templates prepared from oocytes (Ov), or embryos at two (2C), four (4C), sixteen (16C) cells and larval stage (Lv). Data were normalized versus the ribosomal protein S-27 (RP-S27). Cts and results were reported as fold increase respect to the expression level in unfertilized oocytes, as described in Materials and Methods. Data represent the mean values \pm standard deviation of four independent experiments performed in triplicate. * $P < 0.01$ Student's *t*-test with Bonferroni adjustment for multiple-comparison

gel and visualized by ethidium bromide staining. The images were acquired and digitized using the Gel-Doc 2000 equipment (Bio-Rad).

Real-time PCR (qPCR) was performed using an ABI PRISM 7000 sequence detector system (Applied Biosystems) and SybrGreen (SYBR) chemistry as a double strand DNA-specific fluorescent (Invitrogen). Specific primer sets for each gene were designed on the basis of the recently sequenced *C. intestinalis* genome (<http://genome.jgi-psf.org>) (Dehal *et al.*, 2002) using the Primer3 software. Primers for housekeeping (ribosomal protein S-27; RP-S27) (Olinski *et al.*, 2006) and *CiAPEX* genes used in qPCR are reported in Table 1.

Four different sample preparations were carried out for each time point analysed: unfertilized oocytes, two-, four-, 16-cell stages, larvae (legend of Fig. 1). Each reaction was performed at least in triplicates in optical strip tubes in two different runs, one for each sample preparation. The qPCR mixture, in the final volume of 25 μ l, contained 2 μ l of cDNA (dilution 1 : 2 of starting material); 12.5 μ l of Platinum SYBR Green qPCR super mix-UDG with Rox (Invitrogen) and 10.5 μ l of specific primer pairs (7.5 pmol). The following experimental run protocol was used: denaturation program (95°C for 10 min), 40 cycles of

amplification (15 s at 95°C; 1 min at 60°C and 1 min at 72°C). Specificity of every amplification reaction was verified by melting curve analysis. For all experiments, raw data output were internally normalized against RP-S27 mRNA (the endogenous control), whose expression levels remain constant during all the developmental stages examined (data not shown). Analyses were performed using the standard curve method. The relative standard curve method uses a set of relative standards from which unknown samples are quantitated. The quantity is expressed relative to some base sample, such as the calibrator (e.g. unfertilized oocytes). A calibrator is a sample used as the basis for comparing results. For all experimental samples, target quantity is determined by interpolating from the standard curve and then dividing by the target quantity of the calibrator. The calibrator becomes the 1RELSP \times fold and all other quantities are expressed as an *n*-fold difference relative to the calibrator. qPCR results have been reported as fold increase/decrease compared to the expression level of *CiAPEX* determined in unfertilized oocytes (Fig. 1).

Results

Expression of APEX in human gametes and early embryos

In order to verify the expression of *APEX* (*HsAPEX*) in human oocytes and preimplantation embryos, a RT-PCR was performed employing the primers listed in Table 1. Fig. 2a shows that the expected 499 bp product was clearly detectable in GV oocytes, two cell embryos, morula (Mo) and blastocyst (BL). No clear signal was evidenced in MII oocytes (Fig. 2a). An internal positive control was represented by the amplification of human β -actin transcripts starting from all cDNA preparation showed in Fig. 2 (data not shown). A subsequent nested-PCR led to the amplification of a 292 bp fragment, reinforcing the presence of *HsAPEX* transcripts in GV and preimplantation embryos. In addition, the increased sensitivity due to the nested-PCR allowed us to detect a clear transcript in MII oocytes (Fig. 2b), suggesting that the negative result shown in Fig. 2a was probably due to a limited amount of *HsAPEX* mRNA available for the RT-PCR reaction. The careful design of primers employed in the primary PCR and in the following nested-PCR (Table 1) excluded any possible contaminating amplification due to the presence of genomic DNA in the cDNA preparation.

The same 499 bp amplicon, corresponding to *HsAPEX* transcript, was also observed in human sperm cells (Fig. 2c). No nested-PCR was necessary on these samples, confirming the high expression of *HsAPEX* in human sperm. As a further characterization of *HsAPEX* transcript in

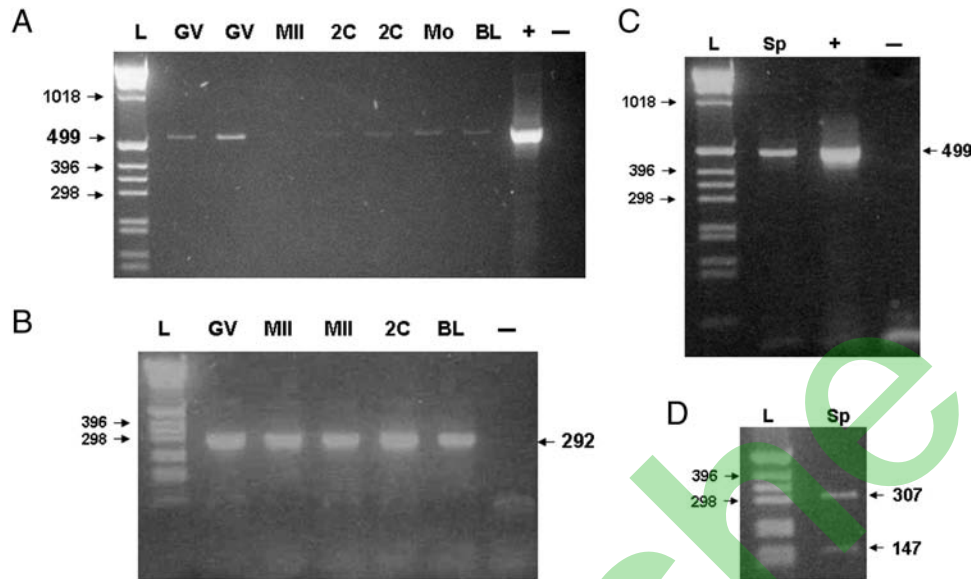


Figure 2: mRNA expression analysis of *HsAPEX* in human gametes and embryos. Panel A: the amplicons of 499 bp, detected following RT-PCR in oocytes and embryos, represent the transcript encoding for *HsAPEX*. Panel B: 292 bp amplicons were detected following nested-PCR using *HsAPEX* specific primers (Table 1). GV, MII, 2 cells (2C), Mo and BL stages are indicated. Panel C: presence of *HsAPEX* transcripts in human sperm (Sp). The amplicon of 499 bp, detected following RT-PCR, represents the transcript encoding for *HsAPEX*. Panel D: restriction profile of *HsAPEX* PCR amplicons reported in Panel a. The expected fragments obtained after AluI digestion are of 307 and 147 bp, respectively. Lanes L, + and - represent 1 kb DNA ladder (Invitrogen cat n. 15615-016), positive (1 μ g of total RNA from human amniotic cells reverse-transcribed to cDNA and PCR-amplified with *HsAPEX* primers) and negative (1 μ g of total RNA from mouse liver tissue reverse-transcribed to cDNA and PCR-amplified with *HsAPEX* primers) controls, respectively. Numbers on both sides of the images indicate the size of DNA ladder and PCR products

the sperm, an AluI restriction profile was performed. The enzyme generated the two expected fragments of 307 and 147 bp (Fig. 2d), confirming the identification of *HsAPEX* transcript in human sperm.

Analysis of *C. intestinalis* APEX gene and protein

To verify if the presence of APEX transcripts in gametes and preimplantation embryos was an event that is evolutionarily conserved, we measured its expression in the ascidian *C. intestinalis*. The rationale of this choice is based on the observation that sea squirts are closest relatives of vertebrates, more than cephalochordates (Delsuc *et al.*, 2006). Therefore, using the Smith-Waterman algorithm into the Joint Genome Institute (JGI) browser, we found clone fgenes3_pm.C_chr_12p000020 (*CiAPEX*), protein ID: 203216, located on chromosome 12p:962878–963822 of *C. intestinalis* and homolog to *HsAPEX* (*CiAPEX*). Fig. 3a reports the gene sequence downloaded from the JGI website (<http://genome.jgi-psf.org/ciona4/ciona4.home.html>). Different from *HuAPEX*, *CiAPEX* does not present introns. In accordance with the evolutionary position of *C. intestinalis*, at the diverging point between invertebrates and chordates, similarity, for both mRNAs and proteins, between *CiAPEX* and its vertebrate homologs (human, mouse, frog and fish) was generally higher compared to invertebrate species, such as insects and protozoa (Table 2). Clustal alignment method (Higgins *et al.*, 1996) for both DNA and amino acid sequences between *HsAPEX* and *CiAPEX* showed a 33.5 and 44.3% identity, respectively, with several functional regions perfectly conserved between them (Fig. 3b). The four acidic residues, namely Asp-90, Glu-96, Asp-219, Asp-308 of human protein, essential for DNA repair activity are conserved in *CiAPEX*, including Asp-219 (Asp-215 in *C. intestinalis*), whose mutation causes loss of both enzyme functions: DNA binding and AP-endonuclease activity (Fig. 3b). Conversely, residues Cys-65 and Cys-93, forming a disulphide bond in *HsAPEX*, are not conserved in *CiAPEX*. The absence of these two cysteine residues in *CiAPEX* suggests that the ascidian enzyme may lack redox transcriptional activity (Fig. 3b). This

conclusion is also supported by the absence of PKC phosphorylation sites in *CiAPEX*, which are thought to be an important regulatory element for the human enzyme increasing its binding activity to transcription factors such as Fos, Jun and AP-1 (Hsieh *et al.*, 2001). It is worthwhile to note that among the alignments shown in Table 2, acidic residues Asp-90, Glu-96, Asp-219, Asp-308 of the human protein are perfectly conserved in all species reported, from mouse to protozoa, in accordance with the primary role of APEX as a DNA repair gene. However, APEX Cys-65 and Cys-93 (numbers refer to human residues) are not conserved among invertebrates, from *C. intestinalis* to *D. discoideum*, suggesting the later evolution of Ref-1 activity (Table 2).

Expression of APEX in ascidian gametes and early embryos

Before determining if *CiAPEX* was expressed in unfertilized oocytes, a β -tubulin control was routinely performed to exclude the presence of contaminating genomic DNA in RNA preparations. Fig. 4a shows amplification products obtained from genomic DNA (lane 1), or cDNA synthesized from total RNA isolated from *C. intestinalis* oocytes (lane 2) using β -tubulin specific primers (CiTu-F/CiTu-R; Table 1). Similar to humans, *C. intestinalis* oocytes presented a clearly detectable expression of *CiAPEX* mRNA (Fig. 4b, Ov), that persisted during the early phase of embryo development (two to 16 cells stage; Fig. 4b) and remained detectable during larva stages (Fig. 4c). Nested PCR and sequence analyses confirmed the identity of the 618 bp band shown in Fig. 4b–c as *CiAPEX* transcript (data not shown), while PCR amplifications using primers specific for genomic *CiAPEX* (AXG-F/AXG-R in Table 1 and Fig. 5), or *C. intestinalis* β -tubulin (Fig. 4c, + lane) excluded the possible amplification of genomic DNA contaminating sequences.

To investigate if a variable level of expression of *CiAPEX* was present during development, a qPCR was performed on the same

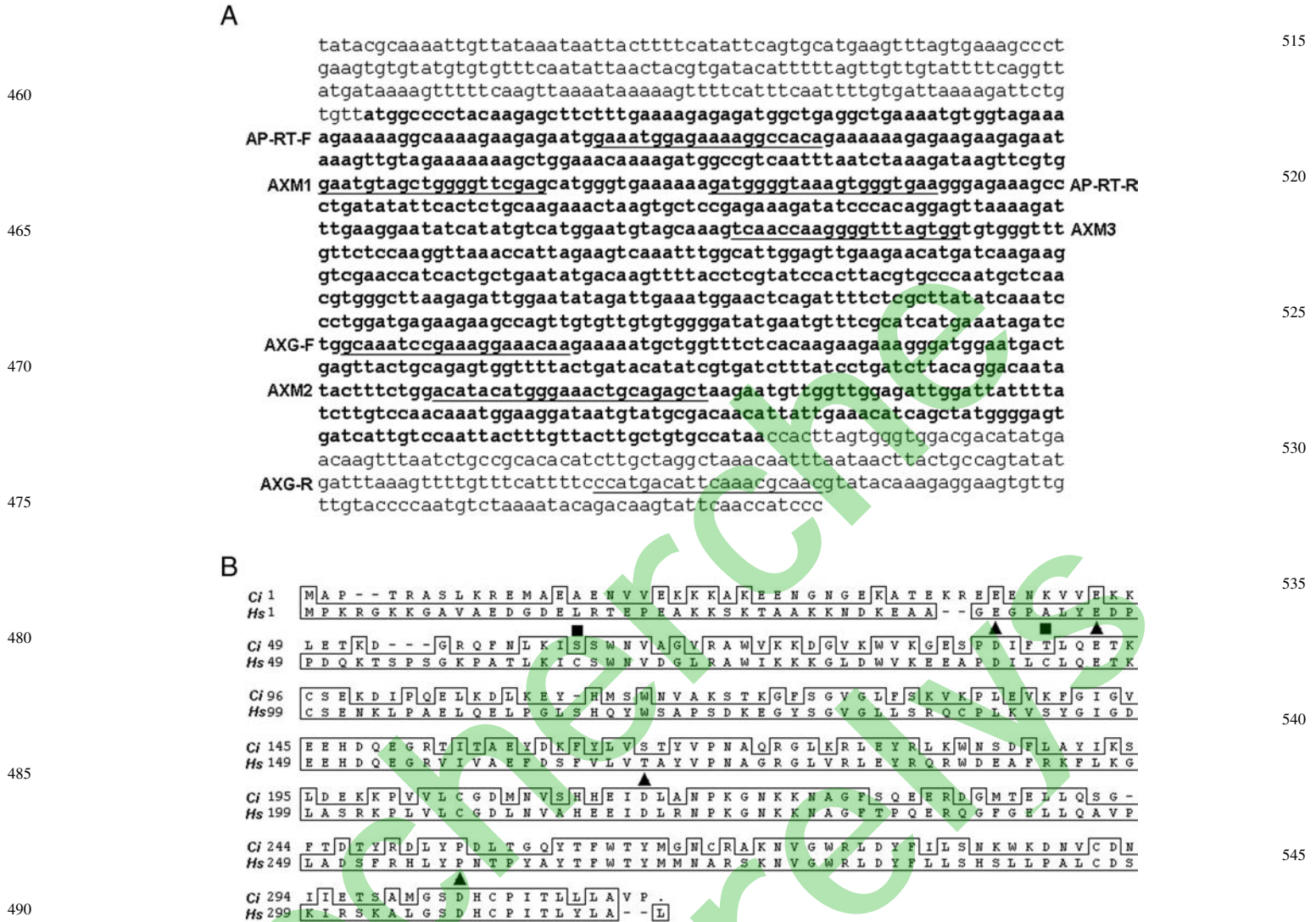


Figure 3: Gene sequence and the Clustal alignment method for both DNA and amino acid sequences between *HsAPEX* and *CiAPEX*. Panel A: DNA sequence of *CiAPEX* gene as downloaded from the Ciona Genome website (http://genome.jgi-psf.org). Bold letters indicate open reading frame, while underlined sequences are referred to PCR primers employed in this study (Table 1). Panel B: alignment of protein sequences corresponding to *HsAPEX* (M80261; top) and *CiAPEX* (bottom). Sequence alignment was obtained using ClustalW (multiple sequence alignment) and MegAlign 4.0 software (DNASTAR Inc.). Symbols indicated functionally conserved (filled triangles), or not conserved (filled squares) residues between the human and ascidian protein (see text for description)

samples reported in Fig. 4. Data were normalized using as endogenous reference the gene encoding for the ribosomal protein S-27 (Table 1), whose expression remains constant in all the developmental stages examined (data not shown). *CiAPEX* was present as maternal transcript at relatively low levels. Its expression significantly changed during early divisions of the embryos, reaching its maximal expression at four-cells stage (about eight-fold compared to the expression in oocytes). At larva stage, *CiAPEX* mRNA expression decreased reaching a value lower compared to that observed in unfertilized oocytes (Fig. 1).

Overall, these results suggest that the expression of the *APEX* gene in oocytes and early embryos might have an important functional role, since it is conserved during evolution from Urochordata to humans.

To verify if also in the male gametes the expression of *APEX* was conserved during evolution, total RNA was isolated from *C. intestinalis* spermatozoa and employed to synthesize the corresponding cDNA. The procedure was carefully carried out in order to exclude the presence of genomic DNA in the preparation, a problem often encountered when cDNA is synthesized from sperm. Fig. 5a shows that

AXG-F/AXG-R primers, specific for genomic *CiAPEX* (lane G), did not amplify any transcript among the population of cDNA synthesized from *C. intestinalis* sperm (lane 1). However, any combination of primers specific for *CiAPEX* mRNA used in both, direct or nested-PCR, failed to detect any amplification product (data not shown). The good quality of *C. intestinalis* sperm cDNA preparation was confirmed by the positive control showed in Fig. 5b. A search in the Ciona Genome website led us to select the *C. intestinalis* homolog of human hexosaminidase A (Cioin2/chr_04q:1503589–1509804, protein ID 201985), a gene specifically expressed in testis (http://hoya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/ci). We designed the TS1-F/TF1-R PCR primers that were able to selectively amplify a 175 bp fragment (Fig. 5b, lane 2). The size of the fragment was exactly that expected based on the sequence of hexosaminidase A transcript reported on the JGI website, and its identity was also confirmed by sequence analysis (data not shown).

These data indicated the putative absence of *CiAPEX* transcript in ascidian spermatozoa, different from the observation reported for human sperm (Fig. 2).

Table 2: Comparison of *APEX* transcripts and proteins from different species

Species ^a	Accession	Alignment data ^b					
		CDS Protein	Sequence length	Identity	Similarity	Difference	% change
<i>Homo sapiens</i> ^c	AY892060	957	957			0	0
	AAX28977	318	318	0		0	0
<i>Mus musculus</i>	NM_009687	954	828			126	20.76
	NP_033817	317	294	9		14	8.97
<i>Xenopus laevis</i>	BC072056	951	622			329	40.47
	AAH72056	316	207	36		73	36.11
<i>Danio rerio</i>	NM_213421	933	607			326	41.91
	NP_998586	310	204	37		69	35.44
<i>C. intestinalis</i> ^d		945	528			417	49.47
		314	127	47		140	59.81
<i>Strongylocentrotus purpuratus</i>	XM_784422	909	532			377	49.09
	XP_789515	302	126	50		126	58.68
	XM_623548	1011	471			540	54.92
<i>Apis mellifera</i>	XP_623551	336	117	51		168	65.58
<i>Drosophila melanogaster</i>	AF073994	2040	525			1515	75.27
	AAC27621	679	116	45		518	83.06
<i>Dictyostelium discoideum</i>	XM_637426	1086	451			635	60.26
	XP_642518	361	139	57		165	62.02

^aSpecies considered in this comparison are as follows: human (*H. sapiens*); mouse (*M. musculus*); frog (*X. laevis*); fish (*D. rerio*); ascidian (*C. intestinalis*); sea urchin (*S. purpuratus*); insecta (*A. mellifera* and *D. melanogaster*); protozoa (*D. discoideum*).

^bData obtained using *emma* and *infoalign* program enclosed in the EMBOSS software package available on the Web (<http://emboss.ch.embnet.org/wEMBOSS/>).

^cSequences from *H. sapiens* has been used as references.

^dClone fgenes3_pm.C_chr_12p000020; protein ID: 203216, located on chromosome 12p:962878-963822 of *C. intestinalis* assembled genome. Downloaded from the JGI web site (<http://genome.jgi-psf.org/ciona4/ciona4.home.html>).

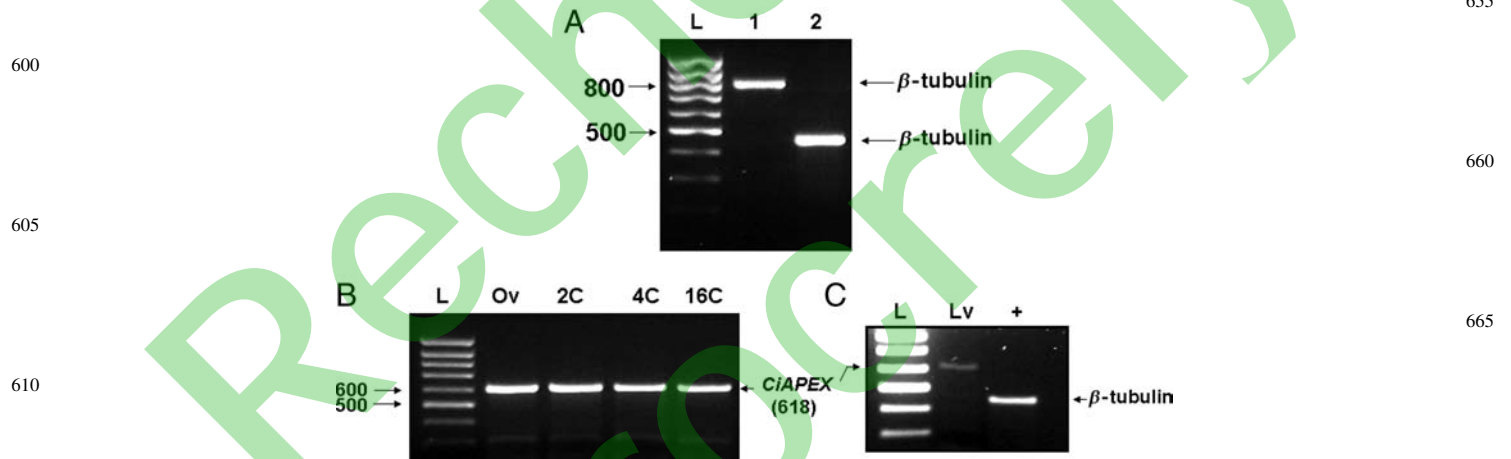


Figure 4: Amplification products obtained from genomic DNA and detection of *CiAPEX* mRNA in *C. intestinalis* oocyte persisted during the early phase of embryo development. Panel **A**: amplification products of genomic DNA (lane 1) and cDNA (lane 2) prepared from *C. intestinalis* oocyte total RNA using (β -tubulin specific primer, CiTu-F/CiTu-R (Table 1). Panel **B** and **C**: detection of *CiAPEX* mRNA in *C. intestinalis* oocyte persisted during the early phase of embryo development (two-, four-, 16-cell stages and larva). PCR amplification of *CiAPEX* cDNA prepared from unfertilized oocytes (Ov), or embryos at two (2C), four (4C), sixteen (16C) cells and larval stage (Lv) was performed using primers AMX1/AMX2 (Table 1) able to generate a 618 bp PCR product. L and + represent 1 kb DNA ladder and positive control (Panel c), respectively. Numbers on both sides of the images indicate the size of DNA ladder and PCR products

Discussion

Even under physiological conditions, the genome is continuously subjected to aggressions. In human IVF/ICSI, *in vitro* manipulations are supposed to increase these assaults by generating ROS (Pabon *et al.*, 1989; Nasr-Esfahani *et al.*, 1990). Abasic sites (AP) are the most common DNA decays, especially those induced by ROS, which can form oxidized AP (Sung and Demple, 2006). In human cells, *APEX/Ref-1* is the major enzyme involved in the removal of baseless sites.

Since persistence of AP sites results in a DNA replication block, cytotoxic mutations and genetic instability, we hypothesized a functional role for *APEX/Ref-1* in human oocytes, supported by the presence of the corresponding mRNA. In sheep oocytes, completion of DNA repair involves upregulation of polymerase beta by estrogens and of DNA ligase (Murdoch and Van Kirk, 2001). In addition to its excision activity, *APEX/Ref-1* is a multifunctional protein, controlling the redox status of transcription factors such as Fos, Jun, hypoxia

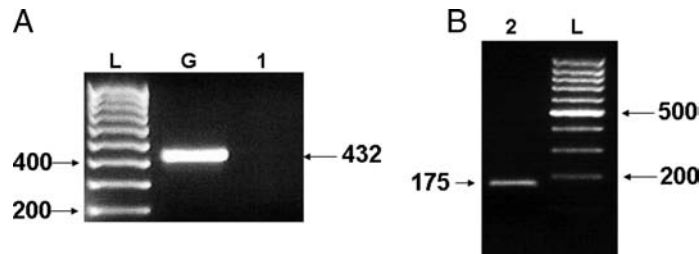


Figure 5: Absence of *CiAPEX* mRNA in *C. intestinalis* sperm. Panel A: AXG-F/AXG-R primers amplified genomic (G) DNA, but not cDNA prepared from total RNA isolated from spermatozoa (lane 1). Panel B: cDNA synthesized from *C. intestinalis* sperm (lane 2) was amplified by primers specific for the human homolog of hexosaminidase A gene (TS1-F/TF1-R) expressed in *C. intestinalis* testis. L represents 1 kb DNA ladder. Numbers on both sides of the images indicate the size of DNA ladder and PCR products. Data shown in figure are representative of one out of five experiments performed starting from independent sperm preparations. No significant differences were observed in band intensity (Panel b, lane 2)

inducible factor-1-alpha, and p53 maintaining them in an active reduced state (Kelley and Parsons, 2001). The *in vitro* redox activity of *APEX/Ref-1* is activated by PKC phosphorylation (Flaherty *et al.*, 2001; Pines *et al.*, 2005). In addition, *APEX/Ref-1* is associated with thioredoxin (Trx) in the up-regulation of the redox potential (Hedley *et al.*, 2004). Elevated levels of both *APEX/Ref-1* and Trx increases cell growth and resistance to programmed cell death (Powis *et al.*, 2000). Using Affimetrix chips, we are characterizing the presence of more than 10 000 human transcripts in different samples of pooled oocytes. Preliminarily, we identified mRNAs coding for Trx, Trx reductase and *APEX/Ref-1*. The transcripts for six members of the peroxiredoxin family, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase mRNAs were also detected. The pentose pathway generating NADPH necessary for Trx reductase is actively used in the human embryo (Menezes *et al.*, manuscript in preparation).

Many studies reported the presence of mRNA species in ejaculate spermatozoa, suggesting that these transcripts are delivered to the oocyte during the fertilization process (Ostermeier *et al.*, 2004). The function and utility of sperm mRNAs remains essentially unexplored, although circumstantial evidence suggests an involvement of sperm mRNAs in early embryonic development (Ostermeier *et al.*, 2004, 2005). In addition, it has been hypothesized that some of these mRNA species might represent a good marker for sperm quality (Ostermeier *et al.*, 2005). In this context, *HsAPEX* expression might be considered a valuable indicator of DNA repair capacity in pre-implantation embryos.

Our comparative study suggests that the male contribution to DNA repair functions, at fertilization and during the early stage of development, may have been established only later in the Chordate evolution. In fact, *CiAPEX* does not appear to be expressed in sea squirt spermatozoa (Fig. 5; Table 2). This observation is particularly interesting considering the structural differences existing between the human and ascidian enzymes: different from *CiAPEX*, *HsAPEX* transcript is present in sperm and *HsAPEX* enzyme is potentially capable of Ref-1 activity since the residues essential for this function (i.e. Cys-65 and Cys-93) are conserved. This might suggest that the presence of *HsAPEX* in the male gametes could not only be associated to a DNA-repair function, but also to a transcriptional activity in fertilized oocytes. An interesting exception to this is *D. melanogaster* where both Cys-65 and Cys-93 are conserved (Table 2), although the presence of a redox activity has not been demonstrated. Future studies in low vertebrates (Fishes, Amphibians, Reptiles) will be devoted to clarify when the Ref-1 function of *APEX/Ref-1* appeared during evolution. The constant publication of new genomes, including those of low vertebrates, will certainly facilitate this study.

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