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Poxviruses and the Origin of the Eukaryotic Nucleus

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Abstract. A number of molecular forms of DNA polymerases have been reported to be involved in eukaryotic nuclear DNA replication, with contributions from α-, δ-, and ε-polymerases. It has been reported that δ-polymerase possessed a central role in DNA replication in archaea, whose ancestry are thought to be closely related to the ancestor of eukaryotes. Indeed, in vitro experiment shown here suggests that δ-polymerase has the potential ability to start DNA synthesis immediately after RNA primer synthesis. Therefore, the question arises, where did the α-polymerase come from? Phylogenetic analysis based on the nucleotide sequence of several conserved regions reveals that two poxviruses, vaccinia and variola viruses, have polymerases similar to eukaryotic α-polymerase rather than δ-polymerase, while adenovirus, herpes family viruses, and archaeotes have eukaryotic δ-like polymerases, suggesting that the eukaryotic α-polymerase gene is derived from a poxvirus-like organism, which had some eukaryote-like characteristics. Furthermore, the poxvirus's proliferation independent from the host-cell nucleus suggests the possibility that this virus could infect non-nucleated cells, such as ancestral eukaryotes. I wish to propose here a new hypothesis for the origin of the eukaryotic nucleus, posing symbiotic contact of an orthopoxvirus ancestor with an archaebacterium, whose genome already had a δ-like polymerase gene.

Key words: DNA polymerase α — DNA polymerase δ — DNA replication — Molecular evolution — Phylogenetic tree — Poxvirus — Eukaryotic nucleus

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Introduction

Symbiosis is a system of physical contact in which living things of different species provide benefits to each other, which facilitate their mutual existence. Eukarya are thought to have arisen by the permanent incorporation of bacteria-like organisms, which have no nuclei, inside other prokaryotic cells (Margulis 1970; Martin and Müller 1998). To date, phylogenetic evidence has accumulated suggesting that the ancestor may have been an archaea-like cell rather than an eubacteria host, and that eubacteria-like cells, such as α-proteobacteria, as symbionts evolved to form mitochondria (Margulis 1970; Margulis 1996; Doolittle and Brown 1994; Martin and Müller 1998). However, the first experiments of living things, which created the nucleus surrounded by a lipid membrane, have not been elucidated.

DNA polymerases can be classified into several families based on the amino acid sequences of their catalytic subunits (Kornberg and Baker 1992). Among them, family B includes viral DNA polymerases, eubacteriotic DNA polymerase II, archaeotic DNA polymerases, and eukaryotic nuclear DNA polymerases (α-, δ-, ε-, and ζ-polymerases). These polymerases are all thought to be replicases. Numerous experiments have indicated that DNA polymerases in family C play roles in replication of genomic DNA in eubacteriotes (Kornberg and Baker 1992) and mitochondria (Yoshida and Keshav 1998), while those in family B are active in archaeotes and eukaryotes (Wang 1991; Waga and Stillman 1998). A number of the archaeotes DNA replication proteins, such as proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and origin recognition complex (ORC), are similar to those of eukaryotes (Edgell and Doolittle

1997), suggesting that these, different from eubacteriotes, have evolved from archaeote-like organisms.

It remains unclear how family B polymerases arose during evolution of eukaryotes. In *Escherichia coli*, it has been elucidated that the main DNA chain elongation is performed by a DNA polymerase III holoenzyme that has dimeric subunit structures (Kornberg and Baker 1992). Discontinuous lagging strand synthesis is thus coordinated with continuous leading strand synthesis by dimeric core enzyme (Kornberg and Baker 1992). On the other hand, in eukaryotes coordinated synthesis of both strands is thought to be accomplished by asymmetric polymerases (Wang 1991; Waga and Stillman 1998). First, new short RNA-DNA primer is synthesized by α-polymerase-primase complex, and then processive polymerases, δ-polymerase or ε-polymerase, take over and synthesize long DNA to the end (Wang 1991; Waga and Stillman 1998). This two or three-polymerase system seems to be well conserved among eukaryotes of *protocista*, *fungi*, *animalia*, and probably *plantae*, while the homo-dimeric or mono-polymerase system prevails in prokaryotes, viruses, and mitochondria.

Even in the eukaryotic family B DNA polymerases, enzymatic properties are diverse. In eukaryotic cells, except for the *Drosophila* case, δ- and ε-polymerases have intrinsic 3′-5′ exonuclease activity, but not α-polymerase, probably causing their differences in replication fidelity (Wang 1991). Their accessory proteins are also diverse, e.g., PCNA is a processive factor of δ- and probably ε-polymerases, but not of α-polymerase (Wang 1991; Waga and Stillman 1998), and some stimulators and inhibitors affect only α-polymerase (Takemura et al. 1997; Takemura et al. 1999; Mizushina et al. 2000). The δ- and ε-polymerases possess high processivity, but α-polymerase has low processivity (Wang 1991). These differences may reflect not only evolutionary changes in the role of each polymerase in DNA replication, but also the original characters of these enzymes.

Information about several aspects of DNA polymerases of archaea has been reported. Edgell et al. found that the crenarchaeote *Sulfolobus solfataricus* has some family B polymerases, which are related to the eukaryotic δ-polymerase catalytic subunit (1997). Klenk et al. reported that *Archaeoglobus fulgidus* has two genes encoding δ-type polymerases in its genome (1997). On the other hand, Uemori et al. and Cann et al. respectively, found that the euryarchaeote *Pyrococcus furiosus* has δ-like polymerases I and II (Uemori et al. 1993; Cann et al. 1998). The suggestion is that δ-polymerase possessed a central role in DNA replication in archaea and eukarya during their evolution. These reports indicate that the ancestor of δ-polymerase participated in initiation of DNA replication without any α-like polymerase. In the present study, I have found that the δ-polymerase could initiate chain elongation, and hypothesized that the α-polymerase was a product of symbiosis, according to the phylogenetic analysis of the nucleotide sequences of these polymerases.

Materials and Methods

Materials

Baculoviruses encoding subunits of mouse α-polymerase (p180, p58, and p48) were kind gifts of Dr. Heinz-Peter Nasheuer of Institut für Molekulare Biotechnologie of Germany. Catalytic subunit's gene of human δ-polymerase (p125) was cloned from placental cDNA library by Dr. Susumu Suzuki of MBL Co. Ltd. of Japan. Hybridoma SJK287-38, producing anti-α-polymerase monoclonal antibody, was purchased from the American Type Culture Collection. Single-stranded synthetic oligonucleotide, including calf thymus primase-preferred sequence (Suzuki et al. 1993), 5′-AAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAACCCTTTCCATCCACC-3′ (CTPPS-POLA), was synthesized by, and purchased from Amersham-Pharmacia.

Purification of DNA Polymerase Subunits

Mouse catalytic subunit of α-polymerase (p 180) was purified using baculovirus expression system (GIBCO BRL, Bac-to-Bac™ System) by a column chromatography on anti-α-polymerase antibody, (SJK287-38)-Sepharose, as previously described (Stadlbauer et al. 1994) with several modifications. The primase subunits (p58/p48) were purified using baculovirus expression system (GIBCO BRL, Bac-to-Bac System) by column chromatographies on DEAE-Sephacel (Amersham-Pharmacia) and phosphocellulose (Whatman, P11) as previously described (Stadlbauer et al. 1994). Human catalytic subunit of δ-polymerase (p 125) was purified by Dr. Aki Tosaka of my laboratory using baculovirus expression system (GIBCO BRL, Bac-to-Bac System) by column chromatographies on DEAE-cellulose-(Whatman, P11) and cobalt-affinity resin (Clontech, TALON™ Metal Affinity Resin).

DNA Polymerase Assay

De novo DNA synthesis were performed using CTPPS-POLA as a template. Standard reaction mixture (25 μl) contained 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 5 mM $MgCl_2$, 50 μM [^{3}H]dTTP, 2 mM ATP, 4 μg/ml of synthetic DNA CTPPS-POLA, and 0.2 units of α- or δ-polymerase, with or without 50 μM each of GTP and UTP, and primase subunits (p58/p48). After the incubation at 37°C for 1 hour, acid-insoluble radioactivity was measured as previously described (Yoshida et al. 1974).

Database

The nucleotide sequences (Wang et al. 1989) of the catalytic polypeptides of each of the DNA polymerases were obtained from GenBank:

(Eukarya) *Homo sapiens* α- and δ-polymerases, X06745 and M80397; *Mus musculus* α- and δ-polymerases, D13543 and Z21848; *Bos tarus* δ-polymerase, M80395; *Drosophila melanogaster* α- and δ-polymerases, D90310 and X88928; *Leishmania donovani* α-poly-

merase, U78172; *Caenorhabditis elegans* δ-polymerase, Z81497; *Plasmodium falciparum* α- and δ-polymerases, L18785 and X62423; *Trypanosoma bruceii* α-polymerase, S71823; *Oxytricha nova* α-polymerase, U02001; *Saccharomyces cerevisiae* α- and δ-polymerases, J03268 and X15477; and *Shizosaccharomyces pombe* δ-polymerase, X59278.

(Archaea) *Pyrococcus furiosus*, D12983; *Methanococcus jannaschii*, U67532; *Sulfolobus solfataricus* P2 B1, U92875; *Sulfolobus solfataricus* P2 B3, Y08257; and *Archaeoglobus fulgidus*, AE000782.

(Eubacteria) *Escherichia coli* polymerase II, X54847.

(Viruses) vaccinia virus, M13213; variola virus, L22579; adenovirus 2, J01917; herpes simplex virus type I, X04771; human cytomegalovirus, M14709; bacteriophage PRD1, J03018; and T4 bacteriophage, M10160.

Phylogenetic Analysis

The phylogenetic trees were constructed using both the fast DNA maximum likelihood program supplied by VPP500 super computer of Nagoya University Computation Centre, and the neighbor-joining algorithm supplied with the GENETYX package, based on the nucleotide sequences (Wang et al. 1989) of conserved regions and their neighbors of DNA polymerases from various species.

Results and Discussion

Substitution of δ-polymerase for the Function of α-polymerase In Vitro

I obtained results that δ-polymerase could substitute for the function of α-polymerase in vitro. I highly purified the catalytic subunits of α-polymerase (p 180) and δ-polymerase (p125), and two DNA primase subunits (p58 and p48) using baculovirus expression system respectively. I tested the ability of δ-polymerase to perform α-polymerase-like activities, which can elongate DNA immediately after the synthesis of RNA primer. As a result, the p125 subunit of δ-polymerase has a potential function to start DNA synthesis directly after DNA primase (Fig. 1). The δ-polymerase could not elongate short DNA without primase subunits, but adding primase subunits did result in elongation (Fig. 1). Further, the dependence of δ-polymerase elongation activity upon the presence of ribonucleotides suggests that DNA synthesis by δ-polymerase starts immediately after RNA synthesis (Fig. 1).

From this, we can estimate how this kind of work sharing in DNA replication has arisen during the evolutionary history. If δ-polymerase has α-polymerase-like characteristics, the ancestral δ-polymerase may work on both initiation and elongation steps.

Phylogenetic Analysis of DNA Polymerases

To elucidate the origin of α-polymerase, I have analyzed the sequence data for numerous polymerases, from viruses to eukarya, and constructed phylogenetic trees of DNA polymerases based on their conserved regions I, II, and IV (Wang et al. 1989). As shown in Fig. 2, DNA polymerases can be clearly divided into two major groups on this basis (Fig. 2, α-type and δ-type), using neighbour-joining algorithm. Focusing on viruses, the phylogenetic trees presented here reveal that two types are distinguishable, one with α-like polymerase, and the other with δ-like polymerase. Adenovirus and herpes family viruses, including herpes simplex virus and human cytomegalovirus (HCMV), have δ-like polymerases, while the two orthopoxvirus families, vaccinia and variola viruses, and T4 phage have α-like polymerases. This clarification of viral polymerases could also be obtained using fast DNA maximum likelihood program (data not shown). The similarity of vaccinia virus and T4 phage according to the present analysis corresponds to the previous report, which described these two as derived from a common ancestor (Bernad et al. 1987). Phylogenetic analysis suggests eukaryotic α-polymerase and orthopoxviruses to have a closely related ancestry, with links to the δ-polymerase, adeno, and herpes families, as well. Although almost no eukaryotic α-polymerases have any intrinsic 3′-5′ exonuclease activity, this is not the case for vaccinia polymerase (Challberg and Englund 1979). The 3′-5′ exonuclease activity was probably lost during evolution due to a mutation involving aspartic acid residue in the active centre of α-polymerase (Derbyshire et al. 1991). On the other hand, my phylogenetic trees indicate that some archaeotes (*Archaeoglobus fulgidus*, *Pyrococcus furiosus*, *Methanococcus jannaschii*, and *Sulfolobus solfataricus* P2) have δ-like polymerases, in line with previous reports (Edgell et al. 1997; Uemori et al. 1993; Klenk et al. 1997; Cann et al. 1998). In this analysis, *Homo sapiens* and *Saccharomyces cerevisiae* ε-polymerases were classified in δ-type (data not shown).

Eukaryotic mitochondria contain their own small ge-

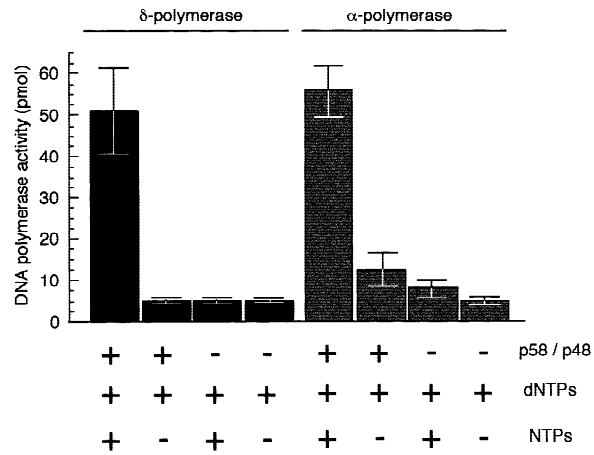

Fig. 1. DNA synthesis by δ-polymerase immediately after RNA synthesis. Purified catalytic subunits of α- or δ-polymerases were assayed in the presence or absence of purified primase subunits (p58/p48) or ribonucleotides (NTPs) as described in "Materials and Methods". The data represent the means of three determinations ± S.D.

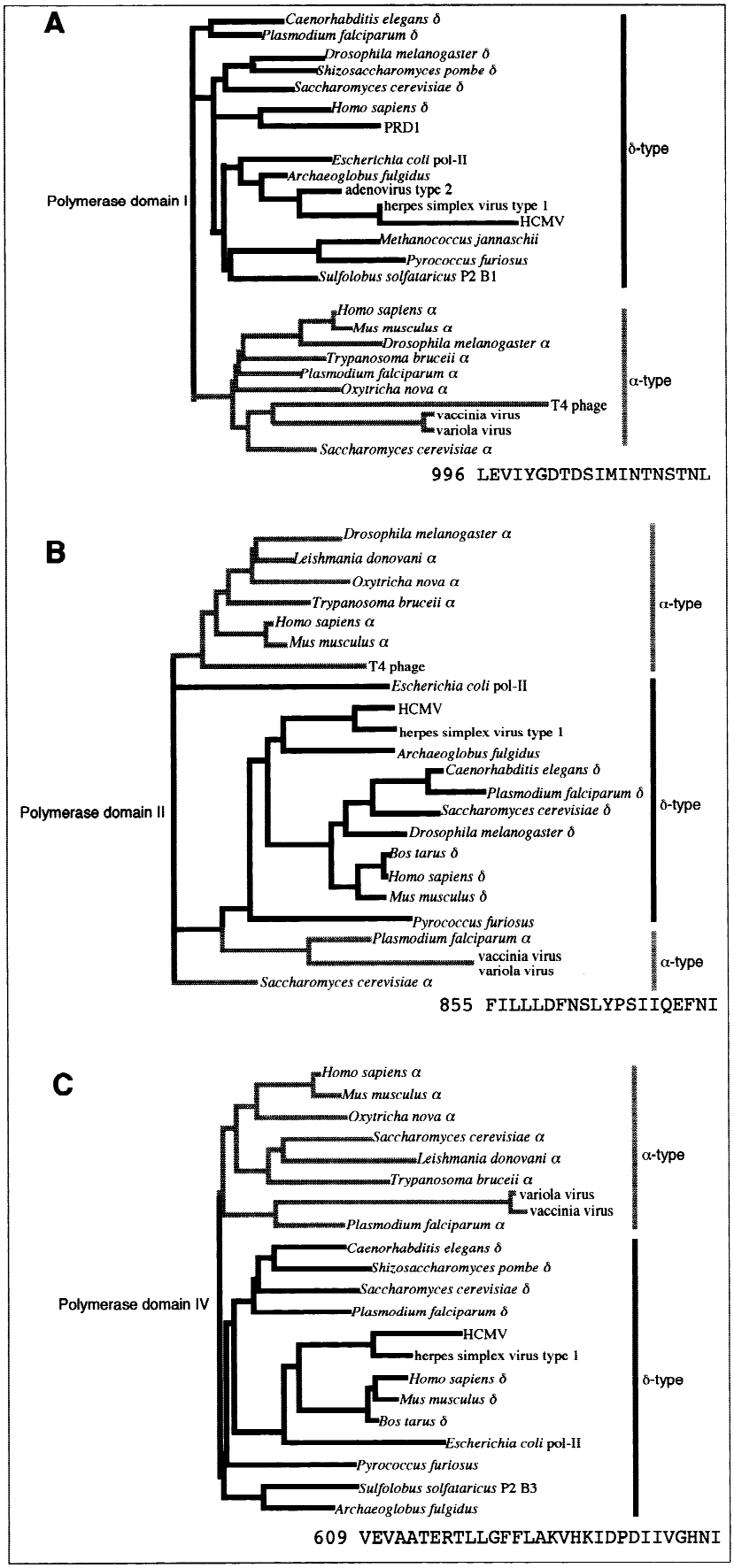

Fig. 2. Phylogenetic trees constructed using neighbour-joining algorithm based on the nucleotide sequences (Wang et al. 1989) of well-conserved DNA polymerase domain I (A), II (B), and IV (C), including their neighbour sequences. Insets indicate the corresponding amino acid sequences of each domain of *Homo sapiens* α-polymerase to nucleotide sequences used for phylogenetic analysis.

nome (16.5 kb) which is separate and distinct from the nuclear DNA (Yoshida and Keshav 1998) and lack an associated DNA polymerase gene for replication. In other words, mitochondrial DNA replication is performed by γ-polymerase, which is encoded by a nuclear gene (Yoshida and Keshav 1998). Genome analysis of mitochondria has revealed that in evolutionary history many genes may have vanished by several possible mechanisms. One of pathways by which mitochondrial genes could have disappeared is migration from mitochondria to the nucleus (Lang et al. 1999). According to this hypothesis, the DNA polymerase gene of the symbiont ancestor of mitochondria might have integrated into the genome of the host-cell nucleus and evolved into γ-polymerase. It is quite possible that the same phenomenon occurred for other polymerases in ancestral cells. I hypothesize that the α-polymerase of eukaryotes was a product of ancestral orthopoxviral gene integration into an archaeotic host genome, which already had a δ-polymerase ancestor. On the other hand, polymerases of herpes or adenoviruses might come from eukaryotic δ-like polymerase via the transposable acquisition of host genes, according to the known characteristics of these viruses, such as the prerequisite host nucleus for their infection and proliferation.

The Poxviruses

Poxviruses (Moss 1996) are exceedingly complicated. The largest are around 350 by 270 nm (vaccinia virus). The vaccinia virus, one orthopoxvirus, has a 192 kb linear duplex genome, which has covalently closed hairpin termini. This encodes a large number of proteins responsible for interaction with the host, and for viral DNA replication, transcription, and protein phosphorylation, reminiscent of eukaryotes. One notable characteristic of poxviruses is the ability to replicate its genome in the cytoplasm of host cells (Traktman 1990). Why does this not occur in the nucleus?

We can not directly visualize the events leading to the first eukaryote, but can make educated speculations. The evidence of nucleotide sequence similarity and eukaryote-like characteristics of poxviruses, e.g., hairpin termini, also observed in some eukaryotes, and the existence of regulatory proteins which are responsible for protein phosphorylation/dephosphorylation like an eukaryotic cell cycle regulator (Blackburn and Gall 1978; Forte and Fangman 1979; Traktman et al. 1989; Guan et al. 1991) are interesting in this context. Blackburn and Gall have reported that the ribosomal RNA gene of the ciliated protozoan *Tetrahymena thermophila* has a palindromic region in its terminus because of tandemly repeated nucleotide sequences (Blackburn and Gall 1978), and Forte and Fangman have also observed the cross-linked termini in *Saccharomyces cerevisiae* (Forte and Fangman 1979). Traktman et al. and Guan et al., respectively, have found that the vaccinia virus genome encodes essential protein kinases and phosphatases, which are homologous to eukaryotic species (Traktman et al. 1989; Guan et al. 1991). These findings point to close relationships between orthopoxviruses and eukaryotes. The orthopoxvirus genome encodes all proteins necessary for viral DNA replication and transcription, unlike other viruses, essentially performing DNA transactions in the cytoplasm of host cells (Traktman 1990; Moss 1996). Although the possibility that poxviruses could acquire some genes from their hosts during the evolution has not been ruled out, the orthopoxvirus's independence from the host-cell nucleus strongly suggests that the ancestor of this virus could have infected non-nucleated organisms, such as the ancestors of eukaryotes, and evolved into a nucleus.

Indispensability of α-Polymerase to the Nuclear Structure

The α-polymerase is essential for cells to proliferate their genome. Cells can not survive without α-polymerase gene because other polymerases such as δ- or ε-polymerases cannot start DNA chain elongation immediately after the synthesis of primer RNA (Wang 1991; Waga and Stillman 1998). Indeed *Saccharomyces cerevisiae* temperature-sensitive (ts) mutants of α-polymerase can not proliferate at the restriction temperature (Budd and Campbell 1987).

Previously, my colleagues have reported that α-polymerase can expand mammalian telomere repeats other than telomerase (Nozawa et al. 2000). Telomere maintenance is thought to be the significant matter for normalizing nuclear structure, which seems to be one of the factors to prevent senescence (Greider 1996). Therefore, telomerase activation represents an immortalizing step in carcinogenesis. On the other hand, my colleagues and I have observed that one of the cell cycle regulators, retinoblastoma protein, could stimulate the activity of α-polymerase, but not δ- and ε-polymerases (Takemura et al., unpublished data). Retinoblastoma protein distributes throughout the whole of cell nucleus and seems to bind various proteins, including nuclear structural proteins, suggesting that the interaction of α-polymerase with retinoblastoma protein can be responsible for the regulation of nuclear structure. Present and previous findings suggest that α-polymerase can contribute to the maintenance of nuclear structure, and that the cell nucleus has always had α-polymerase throughout its evolution, since symbiotic contact.

Conclusions

My conclusion and hypothesis are summarized in Fig. 3: (1) It is surmised that orthopoxvirus-like and archaeote-

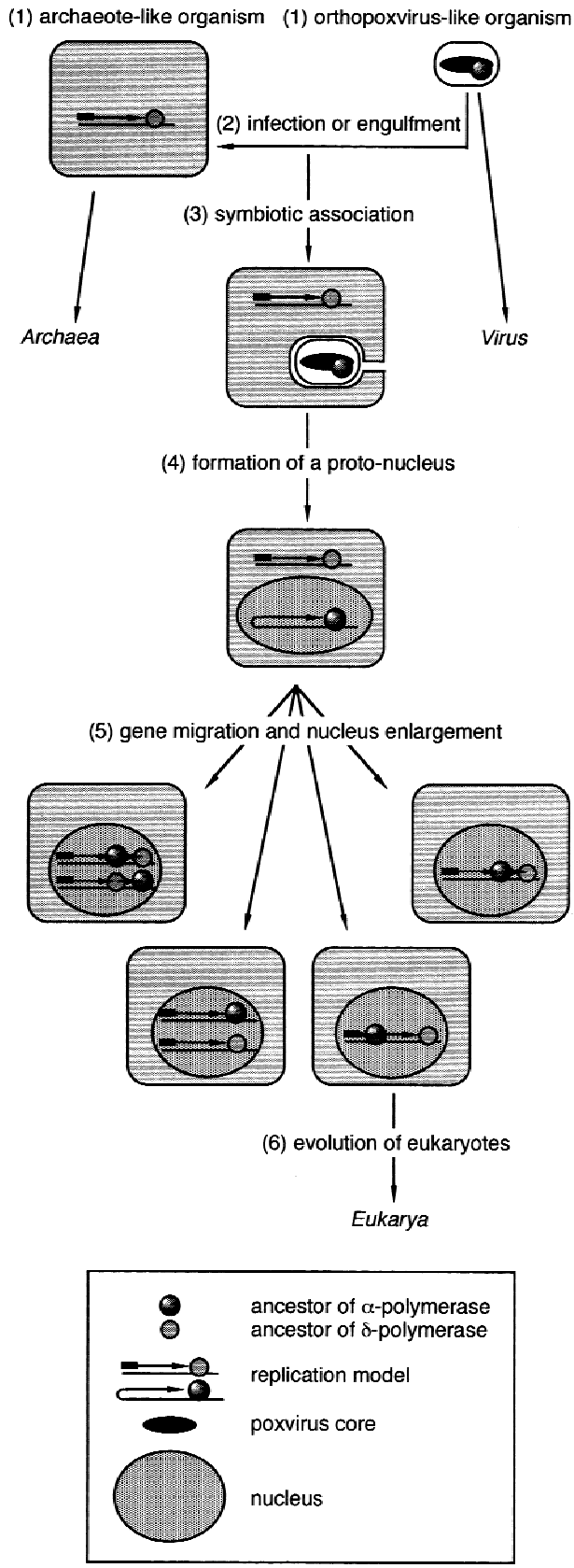

Fig. 3. Schematic summary of the origin of nucleus and asymmetric DNA polymerase system in eukaryotes. Italic words indicate today's taxonomic organisms.

like organisms existed which possessed ancestors of the α-polymerase and δ-polymerase genes; (2) The orthopoxvirus-like organism infected or was engulfed by the archaeote-like counterpart, but in some cells, was neither digested nor caused death; (3) These two organisms came to form a symbiotic association; (4) Undigested inner virion membranes fused with outer coated vesicle-like host membranes at numerous sites (future nuclear pores) resulting in the formation of a prototype nucleus dramatically smaller than today's; (5) The archaeote-like organism-derived genes migrated into the nucleus enlarging it, resulting in cooperation between the two kinds of DNA polymerases in DNA replication. As shown in Fig. 3 (6) in the early eukarya world, there might have been various types of DNA replication styles with different roles for polymerases of differing origins. From these, an asymmetric DNA polymerase system, in which α-polymerase performs initiation followed by chain elongation by δ-polymerase, is hypothesized to have been naturally selected during evolution. Beyond gene duplication (Edgell et al. 1997; Edgell et al. 1998), one of the factors leading to molecular superfamilies must be symbiotic associations of ancestral organisms, for example, in eukaryotic RNA polymerases (Pühler et al. 1989; Iwabe et al. 1991); and, as argued here, eukaryotic DNA polymerases could have a mixed archaeote and virus ancestry. However, the origin of ε-polymerase remains unclear according to symbiosis theory. As described previously (Edgell et al. 1998), it is possible that ε-polymerase was ancestral polymerase of eukaryotes, and δ-polymerase had arisen by the gene duplication.

Poxviruses are widely distributed all over the world and cause infectious diseases such as variola, cowpox, and molluscum contagiosum (Moss 1996). After the development of vaccines derived from studies of vaccinia viruses, their significance as diseases has diminished. However, these viruses may become a focus of public attention from the viewpoint of evolution. The hypothesis presented here provides clues for their evaluation in relation to the larger world of living things.

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