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Reviews

DNA polymerases in prokaryotes and eukaryotes: Mode of action and biological implications

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Background

DNA transactions such as replication, repair and recombination require the concerted action of many proteins either alone or in a complex[1]. Nevertheless, in all these processes one protein, the DNA polymerase, plays a central role. Its function is to ensure the accurate incorporation of the 4 deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP) on a mother template according to the Watson-Crick base pairing rules (A to T and G to C).

Since the discovery of the first DNA polymerase about 25 years ago[2] such enzymes have been found in all prokaryotic and eukaryotic organisms tested[1,3-15,162,163]. Although they differ in chemical, physical or immunological properties they all share the following important similarities: a) they incorporate a complementary deoxyribonucleoside triphosphate as monophosphate on a template by hydrolyzing the α-β phosphodiester bond in the presence of cofactors (divalent cation and DNA), b) they all need a short complementary piece of nucleic acid (DNA or RNA), called primer, with a free hydroxyl group at the third carbon position of the (deoxy)-ribose to attach the first nucleotide and c) they all cause polymerisation in the 5′ to 3′ direction of the growing daughter strand. It is the aim of this review to compare DNA polymerases from prokaryotes and eukaryotes and especially to point out similarities and differences. In particular, attempts are made to compare *Escherichia coli* DNA polymerases I, II and III[1] with the different vertebrate DNA polymerases α, β and γ[16]. Basic rules of DNA synthesis have been described for prokaryotic and eukaryotic cells and organisms[1]. These include, e.g., semiconservative mode of replication, RNA priming, direction of replication, leading and lagging strand replication, excision repair processes. It seems that this extends to the DNA polymerase level since growing pro- and eukaryotic cells possess at least three different DNA polymerases[1,3-16,162,163].

Biological functions of the different DNA polymerases have been well studied in prokaryotes because of their genetics (conditional lethal mutants of certain DNA polymerase genes), and the ease of obtaining large cell quantities due to the short generation time of bacteria. On the other hand only indirect in vitro experimental evidence is available so far for the involvement of the mammalian DNA polymerases in replication and/or repair. Such descriptive results will always be tentative until conditional lethal DNA polymerase mutants are found. However, there have been reports of mutants in structural genes of DNA polymerases of lower eukaryotic cells, such as fungus and fruit-fly[17,18], indicating a direct involvement of these enzymes in chromosomal DNA replication. The DNA polymerase(s) involved in a particular DNA replication process is/are most likely to act in a complex with other proteins (factors) and enzymes, called the replisome[19]. Similar concerted actions can also be postulated for DNA repair[20] or DNA recombination[21]. In DNA replication the multipolypeptide complex responsible for the overall DNA polymerization is henceforth called the DNA elongation complex or DNA elongation apparatus. In recent years a number of laboratories have described complexes of

Abbreviations

DNA pol:	DNA polymerase (E.C. 2.7.7.7.)
dATP:	Deoxyadenosintriphosphate
dCTP:	Deoxycytidintriphosphate
dGTP:	Deoxyguanosintriphosphate
dTTP:	Deoxythymidintriphosphate
ss:	Single-stranded
ds:	Double-stranded
SDS:	Sodium dodecyl sulfate
ΦX174, G4, M13:	Bacteriophages ΦX174, G4 and M13
RF:	Replicative form of a single-stranded DNA

DNA polymerases and accessory proteins. In prokaryotes these proteins are called DNA polymerase III holoenzyme subunits[22], DNA elongation factors[23] and DNA polymerase auxiliary proteins[24].

In eukaryotes on the other hand, there are only fragmentary data about such complexes. Until recently few attempts have been made to use naturally occurring template/primer systems (e.g. singly RNA primed single-stranded DNAs, mitochondrial DNA containing a D-loop or single-stranded parvoviral DNA with a natural primer) to isolate an in vivo-like DNA elongation apparatus[25,26]. In vitro model replication systems such as papovavirus[27-29] or adenovirus[30] proved to be effective tools in identifying proteins that interact with DNA polymerase(s). The concerted action of several proteins in connection with polymerases helps to achieve the main goal in replication, namely to act promptly, quickly and accurately.

Although there are differences in DNA replication between prokaryotes and eukaryotes, and sometimes even within a single cell type, it is the objective of this review to find and present similarities of DNA polymerizing enzymes from prokaryotic and eukaryotic organisms. Replicative DNA polymerases and their auxiliary proteins are especially good candidates for comparison since their function in all cells is to produce an accurate copy of the genetic material. The high accurancy of these polymerases and their associated proteins ensures the overall consistency of a species but on the other hand their mistakes, although very limited in number (less than $1:10^9 - 1:10^{10}$), are one of the sources of evolutional variability[31].

After a comparative description of prokaryotic and eukaryotic DNA polymerases I will first focus on their respective biological functions. Then, proteins interacting with replicative DNA polymerases are presented. This is followed by the description of interactions of the DNA elongation apparatus with primers at the leading and lagging strand at the replication fork, the processive translocation and the accuracy of polymerisation. Finally some newer aspects will be summarized. These include proteolytic cleavage and the conservation of DNA polymerases in evolution.

1. Description of prokaryotic and eukaryotic DNA polymerases

a) Isolation: Problems and progress

Soon after the discovery in *Escherichia coli* of an enzymatic activity capable of polymerizing the 4 deoxyribonucleoside triphosphates on a DNA template[2,32] it became clear that a corresponding DNA polymerizing activity also existed in animal cells[33,34]. Thus it was assumed that such an enzymatic activity is universal. DNA polymerases were subsequently identified, isolated and characterized in all cells, tissues and organisms tested. About a dozen years later it became evident that prokaryotes and eukaryotes possess several types of DNA polymerases. In *E. coli*, in addition to the above-mentioned DNA polymerase (henceforth called DNA polymerase I), 2 new enzymes were found, named in order of their discovery DNA polymerase II[35-37] and DNA polymerase III[38,39]. Also, in animal cells it became clear, even without having conditional lethal mutants, that there are again at least 3 different DNA polymerizing enzymes[16]: First a high-molecular-weight DNA polymerase[40] (henceforth called DNA polymerase α), followed by the small-molecular-weight DNA polymerase β[41,42], and the DNA polymerase γ[43]. Finally data were presented suggesting the existence of a 4th polymerase, called δ[44].

It is now established that organisms ranging from bacteria to human cells contain several DNA polymerases. It is not the aim of this review to present all the laborious and often controversial work that has been carried out in this field. After describing some of the most highly purified enzymes, from bacteriophage to mammalian ones, I have chosen mainly the DNA polymerases from *E. coli* and vertebrate cells for a detailed comparison.

In *Escherichia coli* the DNA polymerases I[32,45,46], II[47-49] and III[50-54] were purified to apparent homogeneity (for discussion of multiple bands on SDS-polyacrylamide gels of pure DNA polymerase preparations, see below). Bacteriophages exist that code for and possess their own replicase. Examples are: T7[55,56], T4[57], T5[58] and SPO1[59]. Other prokaryotic cells from which homogeneous or near homogeneous preparations have been reported are: *Bacillus subtilis* DNA polymerase I[60], II[61] and III[62], *Micrococcus luteus*[63,64] and *Salmonella typhimurium*[65].

In eukaryotes the most extensive and successful efforts were made using a) unicellular and lower eukaryotes such as *Ustilago maydis*[66], *Saccharomyces cerevisiae*[67,68], *Euglena gracilis*[69], *Physarum polycephalum*[70], *Paramecium*[71], *Tetrahymena pyriformis*[72], *Dictiocoelium discoideum*[73]; b) insects such as *Drosophila melanogaster* (DNA polymerase α[74,75] or β[76]); echinodermata e.g. sea urchins[77-80]; d) amphibia e.g. *Xenopus laevis* (DNA polymerase α[81-83], β[84] and γ[83]); e) birds e.g. chicken embryo (DNA polymerase α[85], β[86] and γ[87,88]); and finally f) mammals e.g. rat, calf or human (DNA polymerase α[89-95], β[96,97], γ[98,100,360] and the possible δ[101]).

Some difficulties should be mentioned at this stage in order to see the often controversial reports in the right perspective. Among these are a) the low amounts of certain DNA polymerases per cell, and b) the small amount of defined material, especially from mammals, owing to limited cell sources and high costs. c) The existence of multiple DNA polymerase species in

all cells and tissues render isolations cumbersome. d) Further complications arise from the occurrence of multiple peaks of DNA polymerases upon ion exchange- and affinity-chromatography; these are not due to different DNA polymerases but rather to different forms of DNA polymerase-protein complexes, as is known today from extensive work with *Escherichia coli* DNA polymerase III holoenzyme[22,54,102-105]. e) The majority of replicative DNA polymerases, even when purified to homogeneity as judged by electrophoresis in non-denaturing polyacrylamide gels, are themselves found to be complexes of two and more polypeptides[75,89-95]. The quantitative and/or qualitative variations of DNA polymerases in different physiological stages of cells and organisms is another explanation for controversal findings. Finally f), in early developing and rapidly differentiating tissues, the DNA polymerase structure may be altered by generalized or genetically programmed proteolysis creating fragments still active in in vitro assays (see below).

In contrast to the situation of protein synthesis, where tens of thousands of ribosomes are present per cell, enzymology of DNA replication is hampered by the very small quantities of proteins and by inability to isolate the intact replisome. However, some of these obstacles have now been overcome and progress has been made: 1. Genetic engineering allows the overproduction of replication proteins[104], and in vitro construction of an intact and fully active prokaryotic replisome has recently been achieved[19,106-110]. Furthermore, 2. laborious collection of fast replicating tissues such as fly eggs gives reasonable quantities of DNA polymerases and other replication proteins[75], 3. antibodies[111-116], and inhibitors[117-120] can distinguish between multiple DNA polymerases. 4. Assays for DNA polymerase auxilary proteins[24] and DNA polymerase subunits[22,102-104,121] can solve the problem of multiplicity of identified DNA polymerizing enzymes. 5. The catalytic subunits of DNA polymerases can now be identified because a) the *dna* E gene of *Escherichia coli* DNA polymerase III can be cloned[122], b) all other proteins can be removed from prokaryotic or eukaryotic DNA polymerase complexes[54,123] and c) the catalytic DNA polymerase activity can be regained after SDS-gel electrophoresis[124,125]. 6. Defined eukaryotic cells and tissues can be used to estimate the quantitative variation pattern of DNA polymerases to provide information about their functional roles. Examples are: Synchronized cell cultures in various phases of their cell cycle[126,127], in vivo differentiating or proliferating cells such as neurons and spleen cells, respectively[128-130], regenerating liver after partial hepatectomy[131,132] and phytohemagglutin-stimulated lymphocytes[133-135]. 7. Last but not least, the availability of potent protease inhibitors has improved the reproducibility of isolation procedures and has made comparison between DNA polymerases more meaningful.

b) Physico-chemical and other properties of highly purified or homogeneous enzymes

Detailed properties of DNA polymerases from all kinds of biological sources have been summarized in books and review articles[1,3-16,162,163]. Especially in the case of eukaryotic DNA polymerases the authors were forced to use information about preparations which were far from being homogeneous. In table 1 an attempt is made to compare a variety of DNA polymerases from organisms ranging from bacteriophages to mammals with respect to four criteria, namely molecular weight, number of subunits, homogeneity (a DNA polymerase is defined as homogeneous if one stained band is seen on a native polyacrylamide gel and this band corresponds to the enzyme activity) and associated enzyme activities.

1. Molecular weight: The replicative DNA polymerases possess high molecular weight ($> 110{,}000$–$120{,}000$) subunits in their purest fractions (see later sections for functional roles of DNA polymerases, for heterogeneity and also for the problem of generalized proteolysis). From Echinodermata to mammals a small molecular weight ($< 50{,}000$) β polymerase can be found. The β-like polymerase from lower eukaryotes has a molecular weight of more than 70,000 daltons. The subunit structure of DNA polymerase γ is controversial but the purest preparations from birds[87,88] and from mammals[360] are suggestive of a small molecular weight ($< 50{,}000$) catalytic subunit. All bacteriophage DNA polymerases have a molecular weight of more than 87,000 and bacteria and mycoplasms all possess DNA polymerases with a molecular weight of more than 105,000 daltons.

2. Number of subunits: It is evident that well-characterized replicative DNA polymerases from bacteriophage T4 and T7, DNA polymerase III holoenzyme from *Escherichia coli,* and eukaryotic DNA polymerases α exist as multipolypeptide complexes. DNA polymerase γ, the mitochondrial replicase (see next section), appears to exist as a tetramer complex.

3. Homogeneity: Due to considerable efforts in the last few years, DNA polymerases from a variety of lower and higher eukaryotes have been obtained in apparently homogeneous form. Work with prokaryotic DNA polymerases has taught us that this is an absolute prerequisite for performing meaningful in vitro experiments.

4. Associated enzyme activities are discussed in the following section.

c) Other enzyme activities associated with the DNA polymerase polypeptide chain

Certain DNA polymerases such as DNA polymerase I from *Escherichia coli* are able to carry out several

Table 1. Properties of prokaryotic and eukaryotic DNA polymerases

Organism		Molecular weight ($\times 10^{-3}$)[a]	Number of subunits[b]	Homogeneity[c]	Associated 3′→5′ exonuclease	Other associated activities[d]
Bacteriophages	T7	87 (56)[e]	2 (56, 136)	+(56)	+(137)	+(137)
	T4	114 (138)	1 (138) 5 (24)[f]	+(138)	+(138)	+(138)
	T5	96 (139)	1 (58)	+(58)	+(58)	+(58)
Mycoplasms		106–140 (140, 141)	1 (140, 141)	+(141)	−(141)	−(141)
Bacteria	*E. coli* DNA pol I[h]	109 (46)	1 (46)	+(45, 46)	+(142)	+(143)
	E. coli DNA pol II	120 (47–49)	1 (47–49)	+(47–49)	+(47–49)	−(47–49)
	E. coli DNA pol III	140 (52)	3 (54),	+(52, 54)	+(53, 54)	+(53, 54)
	B. subtilis DNA pol I	115 (60)	8 (105, 144)[f]			
	B. subtilis DNA pol II	? (61)[g]	1 (60)	+(60)	?	?
	B. subtilis DNA pol III	166 (62)	?	−(61)	−(61)	−(61)
			1 (62)	+(62)	+(62)	+(62)
Lower eukaryotes	*S. cerevisiae* DNA pol I (A)	70 (68)	2 (68)	−(68)	−(68)	−(68)
	S. cerevisiae DNA pol II (B)	70 (68)	2 (68)	−(68)	+(67, 68)	+(68)
	U. maydis DNA pol A	50–55 (66), >125 (124)	2–4 (66)	+(66)	+(145, 146)	+(145, 146)
	U. maydis DNA pol B	? (66)	?	−(66)	?	?
	E. gracilis DNA pol A	142–150 (69), 185 (69)	1–2 (69)	−(69)	−(69)	−(69)
	E. gracilis DNA pol B	38 (69)				
	P. polycephalum DNA pol α-like	98–136 (71, 147), 106 (148)	2–4 (69), 6 (69) 2 (148)	−(69) +(147)	+(69) ?	−(69) ?
	P. polycephalum DNA pol β-like	81 (71, 148)	?	−(148)	?	?
	T. pyriformis DNA pol α-like (I)	80 (72), 130 (149)	?	+(72)	?	+(72)
	T. pyriformis DNA pol β-like (II)	70 (149)	?	−(149)	?	?
	D. discoideum DNA pol α-like (A)	127 (73)	?	−(73)	?	?
	D. discoideum DNA pol β-like (B)	70 (150)	?	−(150)	?	?
Insects	*D. melanogaster* DNA pol α	148 (75, 123)	4 (75, 123)	+(75)	−(75)	−(75)
	D. melanogaster DNA pol β	? (76)	?	−(76)	?	?
Echinodermata	Sea urchin DNA pol α	150 (151)	2 (151)	−(151)	?	?
	Sea urchin DNA pol β	34 (152)	2 (152)	−(152)	?	?
	Sea urchin DNA pol γ	? (78)	?	−(78)	?	?
Amphibia	*X. laevis* DNA pol α	76 (83)	?	(83)	?	?
	X. laevis DNA pol β	45 (84)	1 (84)	−(84)	?	?
	X. laevis DNA pol γ	? (83)	?	−(83)	?	?
Birds	Chicken embryo DNA pol α	148 (85)	1 (85)	+(85)	−(85)	−(85)
	Chicken embryo DNA pol β	40 (86)	1 (86)	+(86)	?	?
	Chicken embryo DNA pol γ	47 (87, 88)	4 (88)	+(88)	?	?
Mammals	DNA pol α	47–156 (89–95, 125)	2–8 (89–95, 125)	+(89–95)	−(95)+(91)	−(95)+(91, 153)
	DNA pol δ	49, 60 (101, 154)	2–4 (101, 154)	+(101, 154)	+(101, 154)	+(44)
	DNA pol β	39–45 (96, 97, 155), 32 (157)	1 (96, 97, 155–157)	+(96, 97, 155–157)	−(97, 153, 156, 158, 159)	−(153, 156)+(97)
	DNA pol γ	140–180 (99, 100, 124), 47 (360), 60 (160)	4 (360)	+360	?	?

[a] Values taken from SDS-polyacrylamide gels, where possible, or from other molecular weight analyzing techniques, if they were performed under high salt conditions to prevent aggreagation. This should give a tentative idea about the molecular weights of the catalyzing subunits. [b] Number of stained bands after SDS-polyacrylamide gel electrophoresis, or oligomeric form of the putative catalytic subunits. [c] Homogeneity means 1. one stained band after SDS-polyacrylamide gel electrophoresis, 2. a single band on a native polyacrylamide gel which subsequently may produce several bands in a SDS-polyacrylamide gel on re-electrophoresis or 3. several bands copurified after extensive purification over several affinity chromatography of gel filtration columns. [d] Examples are: pyrophosphate exchange, pyrophosphorolysis, 5′→3′ exonuclease activity, RNase H activity. [e] Reference number in brackets, see literature. [f] Including all DNA auxiliary proteins or DNA polymerase III holoenzyme subunits. [g] ? means uncertain, not measured or not determined. [h] *E. coli: Escherichia coli; B. subtilis: Bacillus subtilis; S. cerevisiae: Saccharomyces cerevisae; U. maydis: Ustilago maydis; E. gracilis: Euglena gracilis; P. polycephalum: Physarum polycephalum; T. pyriformis: Tetrahymena pyriformis; D. discoideum: Dictiocoelium discoideum; D. melanogaster: Drosophila melanogaster; X. laevis; Xenopus laevis.*

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DNA transactions with a single polypeptide chain (109,000 daltons in the case of DNA polymerase I)[1,138,161]. The enzyme has different binding sites for DNA, for nucleoside monophosphates, and for nucleoside triphosphates. Furthermore, some of these enzymes combine various enzymatic functions in one polypeptide, namely polymerization in the 5′-3′ direction[45,46], exonucleolytic activity in the 3′-5′ direction (proofreading[142]) exonucleolytic activity in the 5′-3′ direction (excision, nick translation[139]), pyrophosphorolysis[143], and pyrophosphate exchange[143]. Some of these associated activities appear to contribute important functions in replication and repair[161]. Various associated activities were found in many bacteriophage and bacterial DNA polymerases (table 1 and references therein). There exists convincing evidence today that DNA polymerases from lower eukaryotes[67-69,145,146] and even from higher eukaryotes such as mammals[44,91,97,153,154] may possess at least one additional enzymatic activity beside the polymerization capacity (table 1).

Exonuclease working in the opposite direction of polymerization (socalled proofreading activity) have been reported in the lower eukaryotes *Ustilago maydis*[146], *Saccharomyces cerevisiae*[67,68] and *Euglena gracilis*[69] as well as in higher eukaryotic mammalian cells[91,101]. In the last case this enzyme is called DNA polymerase δ[44]. This activity might either be a distinct enzyme species or a special form (precursor?) of DNA polymerase α. DNA polymerase α, the putative vertebrate replicase (see next section), is reported to carry out pyrophosphate exchange and pyrophosphorolysis[153] and to possess exonucleolytic activity in the 5′-3′ direction[91], but one should bear in mind that this enzyme, even when purified to apparent homogeneity, has always 2 or more bands under the denaturing conditions of SDS-polyacrylamide gel electrophoresis. It appears that in higher eukaryotes the associated nuclease activities are in distinct polypeptides. Indeed, a variety of enzymes exonucleolytically active in the 5′-3′[212,213] or 3′-5′[214-216] direction have been isolated and characterized.

d) Escherichia coli polymerase compared with vertebrate DNA polymerase

Simple, single cell prokaryotes and mammalian cells alike possess at least 3 DNA polymerases. It is therefore tempting to speculate about analogous functions. From all the comparisons in table 2 a certain number of similarities between DNA polymerase III of *E. coli* and DNA polymerase α is evident. Inhibitor and functional studies further substantiate this (see next section). Properties such as molecular weight, K_m for deoxynucleoside triphosphates, pH optimum, inhibition by salts and sulfhydryl (−SH) blocking agents, template utilisation, biological functions, occurrence of a holoenzyme form and stimulation by homologous singlestranded DNA binding proteins are similar. One should bear in mind that many of these results were obtained in a variety of laboratories under different assay conditions.

Mitochondria have similarities to prokaryotic organisms[217]. These organelles harbor a small circular DNA[218,219] and their own DNA polymerase γ[100,160,260]. A comparison of the γ polymerase with either DNA polymerase I or II suggests many similarities. Whether this coincidence is fortuitous or reflects true similarity cannot be decided on the basis of such a simple comparison.

2. Discovery of biological functions

DNA polymerases play a central role in 3 important biological events, namely DNA replication, DNA repair and DNA recombination. With the discovery of several distinct DNA polymerizing enzymes in a single cell authors began to ascribe one or the other of these events to certain DNA polymerases. In this section I try to compare the progress in this field in *Escherichia coli* and higher eukaryotes, mainly mammals.

a) Genetic approaches mainly in prokaryotes

After the discovery of the *Escherichia coli* DNA polymerase (later called DNA polymerase I) it was assumed that this enzyme was the replicase and would also function in processes like repair and recombination[2]. However with the discovery of a viable, temperature sensitive mutant able to grow at low but not a higher temperatures, and which was severely deficient in this DNA polymerase[220] this view changed completely. Despite extremely low levels of DNA polymerase, this mutant (*pol*A) synthesized its DNA irrespective of temperature at the same rate as the wild type. Owing to improvements in assaying crude extracts other DNA polymerases were soon discovered in *pol*A mutants. They were isolated and called in order of their discovery DNA polymerase II[35-37] and DNA polymerase III[38,39].

After screening all the known DNA replication mutants for in vitro complementation of a receptor extract from a defective mutant with isolated wild-type DNA polymerases II and III it was found that *dna*E (*pol*C) was the genetic locus for DNA polymerase III[221]. Thus it was established that DNA polymerase III was one of the essential enzymes in bacterial DNA replication. What is the function of the other 2 enzymes?

DNA polymerase I was shown to be involved in a variety of biological functions (see Kornberg[1] for details). 1. Involvement in chromosomal DNA replication[161,222-224]: DNA polymerase I removes the RNA primer from the nascent DNA fragments (Okazaki

fragments[225]) with its 5′-3′ exonuclease function and then fills the gaps by polymerization; the pieces are finally joined together by an enzyme called DNA ligase[226]. In addition it has been discovered that DNA polymerase I or a DNA polymerase I-like enzyme can abolish the temperature-sensitivity of a DNA polymerase III (*pol*C) mutant[232,233] suggesting a surrogate function of DNA polymerase I in replication. 2. Involvement in extrachromosomal DNA replication[234]: DNA polymerase is the replicase of certain plasmid DNAs (e.g. Col El). 3. Involvement in excision repair of damaged bases[227-230]: mutants in DNA polymerase I are more sensitive to UV and X-ray irradiation[227-229] and to treatment with the base altering agent methylmethane sulfonate[230] suggesting an important role in restoring functionally damaged DNA. 4. Involvement in repair of misincorporated bases[231]: it was found that DNA polymerases I and III are both necessary for this process and 5. involvement in recombination[315]: DNA polymerase I has been found to be necessary for transposition.

The function of DNA polymerase II is obscure. *Pol*B mutants defective in this enzyme were obtained by heavily mutagenising *pol*A1 cells[235]. These mutagenized cells grew normally at 25 and 42 °C and supported the growth of all known phages. Reports concerning DNA polymerase II mutants in DNA repair and recombination are controversial[235-237]. The single-stranded DNA-binding protein (SSB) of *E. coli*, which is essential for chromosomal replication[238-240] stimulates DNA polymerase II[180] giving circumstantial evidence of a role in DNA replication.

DNA polymerase III is essential for chromosomal[221] and bacteriophage[241] replication. Furthermore, participation in excision[3,236,242,243] and postreplication repair[231] mechanisms have been reported.

In summary, *Escherichia coli* polymerases seem to have more than one in vivo function. DNA polymerase III is the major replicase but also assists in DNA repair. DNA polymerase I is the major repair enzyme but also functions during replication as a gap-filling enzyme; apart from that it fulfills other essential replication tasks. Much more work is necessary to establish the role(s) of DNA polymerase II.

b) Genetic 'surrogate' systems mainly in eukaryotes

The rapid rise of interest in the DNA polymerases of lower and higher eukaryotes has led to a good knowledge of the physical, chemical and catalytic properties of the least 3 DNA polymerases, named α, β and γ[5-16,163]. In contrast, identification of their respective functions has lagged behind. This was mainly due to the lack of eukaryotic mutants defective in DNA replication. Indirect evidence is available which suggests the involvement of one or the other enzyme in a particular DNA transaction. A few years ago, a replication mutant in the smut fungus *Ustilago maydis* was found to have a defective DNA polymerase[17]; this was followed recently by reports of DNA polymerase mutants in higher eukaryotes such as the fly[18] and possibly in mammalian cells[244]. Each of the 3 DNA polymerases α, β and γ and their putative involvement in different DNA events will now be discussed in more detail.

DNA polymerase α is believed to play a major role in DNA replication. For a long time its localization provoked a lot of controversy, but by using refined extraction methods several authors showed the nuclear localization of this putative replicase[245-248]. Recent immunocytochemical methods, however, seem to indicate that the α polymerase might be localized in the perinuclear region of the cytoplasm[249]. First evidence for an involvement of this enzyme in DNA replication came from studies with cells that were stimulated to divide. Examples include liver regeneration after partial hepatectomy and stimulation of lymphocytes with phytohemagglutinin. Such cells had an increase of DNA polymerase α which paralleled the rate of DNA synthesis[131,133,250,251]. These studies were followed by using synchronized cells where the α-polymerase was enhanced in the late G1 and early S phases of the cell cycle[126,127]. DNA polymerase levels in physiologically differentiating and growing cells, such as neurons, spleen and muscle cells, showed an excellent correlation of stopping and onset of DNA replication with the decrease and increase of DNA polymerase α[128,129,252,253]. Isolation of replicating DNA further illuminated the function of α-polymerase. Replicating Simian virus 40 DNA[118,254-256] (the small genome of Simian virus is replicated in nuclei of mammalian cells and, with the exception of the virus coded T antigen[257], relies entirely on the cellular DNA replication machinery), replicating adenovirus DNA[258-262] (adenovirus DNA replication is dependent on the cellular DNA polymerases, but the viral DNA codes for several proteins that are involved in its own replication[263]), and isolated replicating chromatin from mammalian cells[264] all have an associated DNA polymerase α. In vitro replication of parvovirus DNA is also dependent on the α-enzyme[265,266], and gene amplification during *Xenopus laevis* development appears to rely on DNA polymerase α[267].

DNA polymerases are unable to initiate de novo DNA synthesis in the absence of a 3′OH primer terminus. Short fragments of RNA serve as primers for DNA synthesis in vivo[268]. They are generated by RNA polymerases or by special primases which thus fulfil this function in a putative replication complex[1]. DNA polymerase α is the only mammalian enzyme that is able to mimic in vivo DNA replication by elongating such RNA primers[186]. DNA polymerase α is furthermore able, probably as a DNA polymerase

holoenzyme, to elongate in vitro long single-stranded primed DNAs[25,269-272]. Stimulation of DNA polymerase α by single-stranded DNA-binding proteins, and other factors including the interaction between structural proteins and the replicase at the replication fork will be discussed later. Adenovirus replication is the best characterized eukaryotic DNA replication system functioning in vitro; with this system, adenovirus DNA is fully replicated with partially purified proteins, and among them is DNA polymerase α[273] or a specially modified DNA polymerase α form[378]. Finally, there are a few reports claiming that the α-polymerase acts not only in replication but in addition has functional responsibilities in excision repair mechanisms[274,275].

DNA polymerase δ displays similarity to DNA polymerase α[101,154]. The novel and exciting finding is that this enzyme has a putative associated 3'-5' exonuclease proofreading activity. Whether this enzyme is a precursor form of DNA polymerase α or whether it is a distinct enzyme which serves a special function cannot be decided yet.

The function of DNA polymerase β was obscure for a long time. It was considered to be a repair enzyme (for a review of DNA repair and enzymes involved see Hanawalt et al.[20]), more because of a need for a repair DNA polymerase, in analogy to bacteria, than on a basis of fact. No fluctuations can be measured during the cell cycle[126,127,131,133,250,251] or in physiological development[128,129,252,253]. In phytohemagglutinin-stimulated lymphocytes the major increase in DNA polymerase β was found to coincide with the peak of DNA repair activity and minimal DNA replication[135]. Later it became evident that the β-polymerase can participate in excision repair of UV-damaged DNA of non-dividing neurons[158,207] that do not contain DNA polymerase α[128]. These results were confirmed in a variety of other systems[199,276-279]. Finally, with an elegant in vitro system where UV-treated DNA which had been incised with *Micrococcus luteus* correndonuclease[200] was used, evidence was provided that DNA polymerase β is indeed able to use such an incision as a target to fill gaps and to repair the DNA in the 'patch and cut mechanism'[199]. DNA polymerase α, on the contrary, is unable to do this. All these facts, together with many catalytic properties of DNA polymerase β[184,185,197,198], indicate that DNA polymerase β is involved in DNA repair.

On the other hand, it seems that at least in vitro DNA polymerase β is able to participate in DNA elongation. Elongation of DNA primed ΦX174 DNA[270,271] and in vitro replication of adenovirus DNA with isolated proteins need, in addition to DNA polymerase α, the β-enzyme as well[273]. These properties are reminiscent of DNA polymerase I, which is also a multifunctional enzyme in DNA synthesis[161].

Following the discovery of DNA polymerase γ[43] it took more than 5 years to realize that the mitochondrial DNA polymerase is a γ-type enzyme[100,160,206]. An in vitro DNA replication system was developed using synaptic end knobs from neurons[280]. They contain mitochondria completely unassociated with nuclei, and these particles (called synaptosomes) can be isolated surrounded by a sealed membrane. Thus mitochondria of high integrity and purity could be obtained. These mitochondria carried out replicative DNA synthesis in vitro[207,253], probably catalyzed by γ-polymerase which was the only DNA polymerizing enzyme present. The claim that aphidicolin, a potent inhibitor of DNA polymerase α (see later), has an effect on mitochondrial DNA replication[281] has recently been ruled out[282], which suggests that γ-polymerase is the only replicase in mitochondrial DNA.

DNA polymerase γ does not only occur in mitochondria, it is also present in the nucleus. The nuclear γ-polymerase is involved in adenovirus[293] and parvovirus single-strand to double-strand replication[26]. In both systems it seems, however, that DNA polymerase α[258-262], and in adeno-replication even DNA polymerase-β[273], have certain roles in viral replication. Both mitochondrial[283] and adenovirus[263] DNA replication proceed in a continuous fashion, called strand displacement synthesis. The participation of γ-polymerase in strand displacement synthesis has been proposed[14]. Strand displacement synthesis needs a DNA polymerase that translocated in a non-distributive (processive) manner along the mother template. This is indeed the case for γ-polymerase[87].

What is the function of nuclear γ-polymerase besides replication of 'parasites'? Speculations could be a) a so far unknown function in nuclear DNA replication, b) involvement in amplification of genes or c) participation in recombinational events. In synchronized HeLa cells DNA polymerase γ in nuclei and cytoplasm increases more than twofold in early S-phase[126].

Summarizing all the evidence available for the functions of these 3 polymerases, I conclude that DNA polymerase α is the replicase for the chromosomal DNA; DNA polymerase δ, if it is indeed a distinct species, may help the α-enzyme by proofreading during replication. DNA polymerase γ is responsible for strand displacement synthesis in mitochondria and in certain viruses (e.g. adenovirus and parvovirus) and may have some specialized functions in chromosomal DNA synthesis like gene amplification or recombination. Finally, strong evidence is available that DNA polymerase β appears to be the excision-type repair enzyme.

c) Inhibitors and effectors

Inhibitors of DNA polymerases will be discussed exclusively in connection with selective inhibition of different enzymes and not with respect to their

Table 2. Comparison between *Escherichia coli* and vertebrate DNA polymerases[a]

Property	*Escherichia coli* DNA polymerase I	II	III[b]	Vertebrate DNA polymerase α[c]	β	γ
Physico-chemical						
Molecular weight, native	109,000	120,000	185,000;500,000	190,000-450,000	40,000	180,000-330,000
Molecular weight, denatured[d] (dl)	109,000	120,000	140,000	120,000-180,000	40,000	47,000
Catalytic subunit (d2)	109,000	120,000	140,000	120,000-180,000	40,000	47,000;160,000
Homogeneity	+	+	+	+	+	+
Isoelectric point	Acidic	Acidic	Acidic	Acidic	Acidic	Acidic
Order of elution from phosphocellulose	2	3	1	2	1	3
Catalytic						
Specific activity of the purest preparations[e]	360,000	5,300	192,000	800,000	200,000	600,000
K_m for deoxynucleosidetriphosphates	Low	Low	High	High	High	Low
pH optimum	7.0	7.5	7.2	7.2	8-9	8-9
Effect of ionic strength (100 mM KCl)[f]	Stimulation	Slight inhibition	Strong inhibition	Strong inhibition	Stimulation	Slight stimulation
Effect of sulfhydryl (−SH) blocking agents	No effect	Inhibition	Inhibition	Inhibition	No effect	Inhibition
Divalent kation optimum (mM)						
$MgCl_2$	2	10	6-15	2-7	5-25	5-12
$MnCl_2$	0.1	0.5	1	0.5	0.3-0.8	0.1-0.6
Functional and biological[g]						
Template utilization						
ss DNA	−	−	−	−;(+)	−	−
ds intact DNA	−	−	−	−	−	−
ds extensively nicked[h] DNA	+	+	+	+	+	+
ds gapped DNA:						
gaps of < 10	+	−	−;−	−	+	?
gaps of 10-100	+	(+)	+;(+)	+	(+)	+
gaps of > 100	+	−	−;+	−;+	−	+
DNA primed ssDNA[i]	+	(+)	-;+	−;+	(+)	+
RNA primed ssDNA[i]	+	(+)	-;+	−;+	−	−
Synthetic homopolymer DNA[j]:						
d template · d primer	+	+	+	+	+	+
d template · r primer	(+)	?	+;+	+	−	(+)
r template · d primer	+	?	−;−	−	(+)	+
Main biological function:						
DNA replication	(+)	?	+	+	?	+
DNA repair	+	(+)	(+)	?	+	?
Polymerization: 5′→3′	+	+	+	+	+	+
Exonuclease: 5′→3′	+	−	+	−;(+)[k]	−	?
Exonuclease: 3′→5′	+	+	+	−;(+)	−	?
Pyrophosphorolysis	+	+	+	+	−	?
Pyrophosphatexchange	+	+	+	+	−	?
Relative amount in growing cell %	> 90	4-5	2-3	> 80	10-15	2-5
Number of molecules per cell	400	17	10	6×10^4	2.5×10^4	4×10^4
Number of molecules per *Escherichia coli* genome size[l]	400	17	10	600	250	400
Polymerization rate (nucleotides × sec^{-1} × $enzyme^{-1}$)	10-20	10-15	100-150; > 300	30	2.5	32
Processivity (nucleotides × $enzyme^{-1}$)	188	?	10−15; > 5000	9−17	1	4000
Accuracy	1.6×10^{-6}	?	5×10^{-7}	8×10^{-5}	3×10^{-5}	$>10^{-4}$
Strand displacement synthesis	+	−	−	−	+	+
Occurence of holoenzyme form	−	?	+	+	−	−
Effect of homologeous single-stranded DNA binding protein	No effect	Stimulation	Inhibition; Stimulation	Stimulation	Stimulation	?
Effect of histones:						
core histones	Inhibition[m]	?	Inhibition[m]	Inhibition	Inhibition	Inhibition
H1 histones	Inhibition[m]	?	Inhibition[m]	Inhibition	Inhibition	Stimulation

[a] Data were collected from literature references 1, 45, 46, 142, 143, 164-174 for DNA pol I; 1, 35, 38, 47-49, 175-180 for DNA pol II; 1, 22, 50-54, 102, 104, 122, 125, 144, 176, 181-183 for DNA pol III; 11, 89, 91, 93-95, 125, 184-196, 210, 211 for DNA pol α; 11, 86, 96, 97, 155-158, 167, 184, 195-202 for DNA pol β; and 10, 11, 87, 88, 98-100, 160, 203-207 for DNA pol γ. [b] If there are 2 numbers or statements, the first stands for the DNA polymerase III core enzyme and the second for the DNA polymerase III holoenzyme. The parameters were determined with specific assays. [c] If there are 2 numbers or statements, the first stands for DNA polymerase α and the second for a DNA polymerase α multipolypeptide complex ('holenzyme or holoenzyme fragment'). [d] Determined after SDS-polyacrylamide gel electrophoresis either by a conventional staining method (dl) or by activity measurements after renaturation (d2) of the enzyme in the gel (see ref. 124 for explanation). [e] Specific activity is expressed as units × mg^{-1}. One unit is defined as the incorporation of 1 nmol total deoxynucleoside triphosphate into acid insoluble material in 60 min at 37 °C. [f] Compared to an assay in the absence of any salt. [g] The minus sign means absolutely negative; the plus sign positive and the plus sign in parentheses either poorly positive or moderate evidence. A question mark

Table 3. Inhibitors of DNA polymerases

	DNA polymerase Prokaryote			Eukaryote		
	I	II	III	α	β	γ
Arabinosyl NTP's	−(117)[a]	++(117)	+ (177)	++(288)	− (289)	− (288)
Dideoxy NTP's	−(1)	++(1)	++(1)	− (118)	++(118)	++(118)
N'ethylmaleimide	−(1)	++(1)	++(1)	++(7)	− (7)	++(7)
Aphidicolin	−(286)	− (286)	− (286)	++(119)	− (119)	− (119)
Phosphonoacetate	−(287)[b]			++(120)	− (120)	− (120)

++, Strong inhibition; +, slight inhibition; −, no inhibition. [a]References numbers in brackets. [b]*M. luteus* DNA polymerase I.

mechanism of action on the enzyme itself (for detailed mechanisms for inhibition of DNA polymerases see Brown and Wright[284] and Cozzarelli[285]. Inhibitors have been extremely useful in elucidating DNA polymerase functions, especially in vertebrates and mammalian cells.

Table 3 outlines the effects of known DNA polymerase inhibitors of the following 5 types: arabinofuranosyl-nucleoside triphosphates, 2′,3′-dideoxynucleoside triphosphates, the sulfhydryl blocking reagent N-ethylmaleimide, the tetracyclic diterpenoid aphidicolin[290-292] and phosphonoacetic acid. DNA polymerase α is inactivated by all inhibitors except 2′,3′-dideoxynucleoside triphosphates. In contrast, 2′,3′-dideoxynucleoside triphosphates inhibit DNA polymerase β and γ. These two enzymes can then be distinguished by the resistance of the β-polymerase to high concentrations (10 mM) of N-ethylmaleimide. By working with these substances it has been demonstrated that the α-polymerase functions in chromosomal[119,294-305] and viral (papova[118,256,298] or adenovirus[262,306-308]) DNA replication. It has been similarly shown that the γ-enzyme is solely responsible for mitochondrial DNA replication[207,282] and, together with α-polymerase, is involved in adenovirus[261,293,301,307] but not in papovavirus replication[298]. The data obtained with these inhibitors were all in agreement with the results of the approaches mentioned in the previous section.

The uncommon purine nucleotide diadenosine 5′,5‴-p^1,p^4-tetraphosphate (Ap_4A) has been found to be a trigger of DNA replication in mammalian cells[309,310]. The target of this molecule seems to be the DNA polymerase α holoenzyme[311,312]. This target is lost in postmitotic cells again suggesting a role for DNA polymerase α as the replicase[92]. The effect of Ap_4A seems to be ubiquitous since it also binds to *Escherichia coli* DNA polymerase III holoenzyme[313] and to *Physarum polycephalum* DNA polymerase[314].

d) Comparative summary

Comparison of prokaryotic and eukaryotic DNA polymerase functions manifests that each enzyme seems to have more than one particular role in DNA transactions, probably in collaboration with subunits or auxiliary proteins. Taking into account all the physico-chemical, biochemical and biological comparisons the following statements are permissible (see tables 1-3): DNA polymerase α is analogous to DNA polymerase III, DNA polymerase β can be likened to DNA polymerase I, and DNA polymerase γ resembles to some extent DNA polymerase II.

3. Proteins interacting with DNA polymerases

With the discovery and description of multienzyme systems involved in *Escherichia coli* replication, using the small bacteriophages ΦX174, G4 and M13[316,317] as model replicons it became evident that the in vitro DNA elongation step needs DNA polymerase III too. Soon it was realized that it must be a complex form of this polymerase. Such a multipolypeptide aggregate was isolated and called DNA polymerase III holoenzyme[241]. This DNA elongation complex is required for elongation of primed single-stranded DNA[316] and for specifically nicked double-stranded ΦX174 DNA[318], while on nicked or gapped DNA's the DNA polymerase core alone is active[51-54]. Such artificial templates are DNAs of any source that have been partially digested with a deoxyribonuclease in order to create gaps containing a free 3′OH group as primer for the enzyme.

What functions do these additional factors, called holoenzyme subunits or DNA polymerase accessory proteins, provide during the elongation step? It is essential for the DNA elongation machinery to synthesize the daughter strands efficiently and accurately. Interaction at the primer terminus with the primosome[106] or recognition of the termination site(s) is

indicates that a property is not known or was not measured with homogeneous enzymes. [h] Optimal template activity is reached after extensive treatement with DNase I, when 5-20% of the DNA was rendered acid soluble. Such digested DNA contains structures that are similar to gapped DNA with random gap sizes. [i] A primed single-stranded DNA is a long single strand that contains a short complementary nucleic acid strand annealed to it. Examples are: Multi- or singly-primed ss bacteriophage DNA's such as φX174, G4 or M13 or, as an in vivo example, parvovirus DNA. [j] d: deoxy; r: ribo [k] See Chen et al.[91]. [l] The vertebrate genome is for this purpose assumed to be 1000 times larger than that of *Escherichia coli*. [m] Tested either with the eukaryotic histones or with a prokaryotic histone-like protein[208,209].

possible. In this chapter proteins interacting with DNA polymerases are introduced and compared. Table 4 gives an overview of the structure and function of replicative DNA polymerases from *Escherichia coli, Drosophila melanogaster* and calf thymus. It is evident that much more functional information is available for the prokaryotic than for the eukaryotic systems, because of the advantages mentioned previously. In what follows I would like to present four replicases and their subunits in more detail.

These are: T4 DNA polymerase, *Escherichia coli* DNA polymerase III, *Drosophila melanogaster* DNA polymerase α and mammalian DNA polymerases α. Bacteriophage T4 DNA polymerase (gene 43 protein)[326] needs 4 additional proteins in order to elongate a primed T4 DNA[24]. One is the single-stranded DNA binding protein encoded by gene 32[327] and the others are the three accessory proteins specified by genes 45, 44 and 62[24]. This elongation machinery proceeds at an extremely fast rate[328] and is highly accurate[329]. The gene 44/62 complex possesses a DNA-dependent ATPase (or dATPase) activity[330] and binds directly to DNA[331], while 45 protein serves as a linker between the polymerase and the 44/62 replication complex[331]. These proteins help the DNA polymerase to pass hairpin loops efficiently and also act as a 'sliding clamp' to enhance the binding of the polymerase to the 3′OH primer terminus, thus making the polymerase more processive and accurate[331] (see also next section).

DNA polymerase III holoenzyme of *E. coli* has an even more complex structure (table 4) than the T4 elongation apparatus. While the latter cannot be isolated as a entity from bacteriophage T4 infected cells[332], it is possible to purify a DNA polymerase III holoenzyme several thousand-fold as a stable multipolypeptide complex[22]. The DNA polymerase III core enzyme contains three polypeptides called α, ε and θ of 140,000, 25,000 and 10,000 daltons, respectively[54]. In addition there are at least 5 polypeptides associated with highly purified enzyme preparations. These are: the β-subunit of 37,000 daltons[103], the γ-subunit of 52,000 daltons[104], the δ-subunit of 32,000 daltons[102], the ζ-subunit of 48,000 daltons[324] and finally the τ-polypeptide, a 83,000 daltons DNA-dependent ATPase[325]. As far as the genetic loci are concerned, the *dna*E gene codes for the α-subunit[322], the *dna*N for the β-subunit[105], the *dna*Z for the γ-subunit[104], and *dna*X for the δ-subunit[102]. The *mut*D gene might specify one of the small core subunits ε or θ[323]. The α-subunit is the catalytic DNA polymerizing activity[122,125], the β-subunit transfers the holoenzyme to the primers or to the primosome[102,105,333], the γ- and δ-subunits might be involved in processivity and fidelity events[182,183] as well as in the interaction with the 3′OH primer[333]. The DNA-dependent ATPase activity of the τ polypeptide is not required for in vitro replication of small bacteriophages but might have analogous function to the 44/62 proteins in T4 replication[331], namely to make the DNA polymerase more processive[334] (see also next section). The energy derived from ATP hydrolysis by the τ protein may be used to activate the holoenzyme complex[335]. The *E. coli* DNA elongation factors I, II and III[23,336] could have the following relationship to the holoenzyme subunits: factor I might be identical to the β-subunit, factor II to a mixture of γ- and δ-subunits and factor III to the δ-subunit alone[102,104]. Finally it should be mentioned that the single-stranded DNA binding protein is required for in vitro[337] and in vivo[238] elongation of *E. coli* DNA.

The best-characterized eukaryotic replicative DNA polymerase holoenzyme is the α polymerase from *D. melanogaster* (table 4). It has been purified to homogeneity[75] and it consists of at least 4 subunits

Table 4. Functional roles of prokaryotic and eukaryotic DNA polymerase subunits

E. coli DNA polymerase III holoenzyme (144, 319)				*Drosophila melanogaster* DNA polymerase α (75, 123)			Calf thymus DNA polymerase α (92, 94, 95, 124, 125, 320)	
Subunit	kdaltons	Genetic locus	Function	Subunit	kdaltons	Function	kdaltons	Function
α	140	*dna* E (122)	Catalytic subunit (125, 322)	α	148	Catalytic subunit (123, 125)	210–230 158, 155, 148	Precursor? (125)
β	37	*dna* N (105)	Transfer of holoenzyme to primosome (105, 322)	β	58	?	134, 125, 110 105, 100 90, 74	Catalytic subunit ? ?
γ	52	*dna* Z (104)	Interaction with primosome, processivity, fidelity (104, 182, 183)	γ	46	?	70–50[a] 57	? $A_{p4}A$ binding protein (92, 321)
δ	32	*dna* X (102)		σ	43	?		
ε	25	one	Part of DNA polymerase III core (54)				50–40[a]	?
θ	10	*mut* D? (323)					25[b]	?
ξ	48	?	? (324)					
τ	83	?	DNA dependent ATPase (325)					

[a]Several bands each; [b]from HeLa cells 272. References numbres in brackets.

called α, β, γ and δ of 148,000, 58,000, 46,000 and 43,000 daltons, respectively[76,123]. The term holoenzyme should be taken with some caution, since the enzyme has not been isolated with an elongation assay similar to *E. coli* and it might therefore be a fragmentary complex. The same statement is also valid for mammalian DNA polymerases α (discussed below). Indeed, if a single RNA primed single-stranded M13 template is elongated with the *Drosophila melanogaster* α-enzyme the polymerisation rate is an order of magnitude lower than that of *Escherichia coli* DNA polymerase III holoenzyme[25]. The α subunit is the catalytic polypeptide[25,125], while the functions of the other 3 proteins are unknown. It remains to be seen whether the *Drosophila* replicase is more complex when it is isolated with an elongation assay.

The mammalian replicase, DNA polymerase α, seems to be an extremely complex entity. It is impossible to give a conclusive picture about the structure of this enzyme (complex) at this time. In table 5 some of the purest mammalian DNA polymerases α are compared to homogeneous replicases from other organisms. All these complexes are pure in the sense that one stained band on a native polyacrylamide gel coincides with the polymerase activity. However, after denaturation and SDS-polyacrylamide gel electrophoresis, they show 2 or more polypeptides. What does this imply? Isolation of holoenzyme forms or fragments might be a likely explanation. Depending on the tissues and isolation assay more or less complex structures might have been isolated. One has to perform the isolation with specific elongation assays in order to solve this question. Indeed, if a ssΦX174 DNA is used as a template with a complementary restriction fragment as a primer and a partially purified DNA polymerase α as the enzyme, an α polymerase stimulatory protein can be isolated[270]. It is known from work with *Escherichia coli* DNA polymerase III holoenzyme that multiple peaking on affinity or ion exchange columns are due to different forms or fragments of a holoenzyme, e.g. the presence or absence of an additional protein in the DNA polymerase complex[319,322]. Different holoenzyme forms could be one of the reasons for different findings and for the postulation of different DNA polymerase α forms[339,340]. Proteolysis and different physiological complexes are other possible explanations for conflicting reports (see e.g. Albert et al.[320] and later).

From table 5 it seems that high-molecular-weight (200,000 dalton) complexes of DNA polymerases α are found in all cases. On the other hand, on denaturing gels, a high-molecular-weight subunit ($\geq$ 125,000 dalton) often appears when protease inhibitors are used during the isolation procedures. Applying a recently described technique to detect DNA polymerase activities after SDS gel electrophoresis[124] it could be demonstrated that a high-molecular-weight polypeptide ($\leq$ 125,000 dalton) is responsible for chromosomal DNA replication both in lower and higher eukaryotes[125]. Furthermore it is evident that intermediate (75,000–110,000 dalton) and low-molecular-weight (35,000–75,000) active fragments are detected if extraction and isolation are performed under conditions where proteolysis is allowed to occur[125]. Comparing the *Escherichia coli* DNA polymerase III holoenzyme subunits with the different polypeptides reported to occur in calf thymus DNA polymerase α it is striking that almost all reported DNA polymerase III holoenzyme subunit molecular weights can be identified in DNA polymerase α preparations of different authors (tables 4 and 5).

Another DNA polymerase α subunit which has been characterized is the 57,000 dalton protein, the binding target for the small-molecular-weight DNA replication trigger Ap_4A[92,321]. Association of DNA polymerase α with tryptophanyl-tRNA synthetase[311]

Table 5. Subunit structure of apparently homogeneous replicases

Source	Molecular weight $\times 10^{-3}$ Native Electrophoresis	Filtration	Sedimentation	Denatured	Protease inhibitor	Reference
Human[a]	265–280	140	140	(150–170), 76, 66	–	89
Calf thymus[a]			250	230–210, 155, 140, 103, (70–50)	–	90, 339
	410	400		(125)[b], 64, 63, 62, 60, 57, 55, 52	–	92
				125[b], 105, 100, 95, 64, 63, 62, 60, 57, 55, 52,	+	125
			250	(150), 134, 123, (70–40)	+	94
				158, 148, 140, 134, 123, 110, 90, 74, 59, 55, 48, 30, 10	+	95, 272
Rat[a]	1300			156, 64, 61, 58, 54	+	93
Mouse[a]	450–525		190	54, 47	–	91
Drosophila melanogaster[a]	550		280	148, 58, 46, 43	+	75
Ustilago maydis			180	55[b], 50	–	66
				200[b], 115, 68, 62	+	125
Escherichia coli	528–580[c]		240–250[d]	140, 83, 52, 48, 37, 32, 25, 10	–	22, 54, 324

[a]DNA polymerase α.
[b]Upper row stained bands only, lower row stained bands and activity bands, not seen upon staining, after renaturation (see ref. 124, 125 for explanation).
[c]DNA polymerase III holoenzyme.
[d]U. Hübscher, unpublished results.

might be of significance in this connection, since this enzyme, at least in vitro, is involved in the synthesis of Ap_4A in the backreaction of the amino-acid activation process[341].

A fair number of proteins that interact with DNA polymerase α have been described in recent years. Among these are homologous single-stranded DNA binding proteins[269,342-349], protein kinase[350], DNA dependent ATPase[351], proteins that can convert the α-polymerase into another structural form[352], a basic non-enzymatic protein factor of 30,000 daltons[353], and α-polymerase accessory proteins from HeLa[272] and rat giant trophoblast cells[354]. Similar proteins have been identified and isolated from *Tetrahymena pyriformis*[355] and the sea urchin[356]. There exist also proteins which inhibit various DNA polymerase forms in different ways, e.g. ricin, hemin and non-histone chromosomal proteins[357].

Finally it should be mentioned that stimulatory proteins have been described for DNA polymerase β, e.g. a heat stable single-stranded DNA binding protein[358] or factor IV from hepatoma cells[359], which is most probably a 5′-3′ and 3′-5′ exonuclease[215].

In summary, it may be stated that the proteins working with replicases during the elongation step are well characterized in prokaryotic systems, while in eukaryotes the knowledge is still fragmentary. It is therefore impossible to compare proteins with putatively similar roles. In conclusion, the DNA polymerase polypeptide alone is not able to elongate long stretches of DNA. Additional auxiliary subunits are needed to support the DNA polymerase in prokaryotes as well as in eukaryotes.

4. Action of replicative DNA polymerases and their associated proteins at the replication fork

In the previous section the DNA polymerase and its accessory subuntis were discussed by themselves. It is, however, obvious that other proteins and cofactors have to be present at the replication fork in order to enable the replicase to perform its job.

DNA replication is controlled at the point(s) of initiation[361]. After initiation events, e.g. recognition of specific DNA sequences by proteins such as *Escherichia coli* protein n′[362] or gene A protein from ΦX174[363,364], formation of a prepriming complex[365] and the synthesis of oligoribonucleotides by primases[366], it is the task of the DNA elongation machinery to replicate the primed or specifically nicked DNA. Several events have to be considered: a) the unwinding of the double-stranded DNA in advance of replication, b) the continuous synthesis in the overall 5′-3′ direction, c) the discontinuous synthesis in the 3′-5′ direction, d) the translocation along the mother DNA and e) the degree of accuracy during polymerization.

It is beyond the purpose of this review to give detailed information on mechanisms of initiation. This has been reviewed[361] and detailed data on formation, movement and conservation of primosomes have been published[19,106,107]. The functional interaction of the DNA elongation apparatus with primers and priming proteins during DNA synthesis will be described. That there are several possibilities for a replicase to start and proceed will come clear from the following discussion.

a) The unwinding reaction ahead of leading strand polymerization

Unwinding ('unzippering') of double-stranded DNA is necessary since most of the DNA polymerases are incapable of strand displacement. Exceptions are *Escherichia coli* DNA polymerase I[1], T5 DNA polymerase[58] and DNA polymerase γ[87]. The 2 prokaryotic model systems of bacteriophage T4 and bacteriophage ΦX174 again give us the best information in this field.

The T4 DNA polymerase gains limited displacement ability in the presence of the 'helix destabilizing protein' (gene 32 protein) even in the absence of ATP[367]. This capability is greatly enhanced by the three auxiliary proteins 45 and 44/62[328,330,331,368]. It seems, but is not proven yet, that ATP is the energy source required for this process[330,331,368]. The displacement mechanism seems to be coupled with DNA synthesis[368]. The same proteins seem to enhance the rate at which T4 DNA polymerase traverses helical regions in single-stranded templates[331]. Whether T4 topoisomerase is involved in unwinding is not clear at this time[369].

In vitro studies with *E. coli* DNA polymerase III holoenzyme are performed with circular viral DNA. Bacteriophage ΦX174 requires by far the largest number of proteins and is therefore considered to be the model system for *E. coli* chromosomal DNA replication[121,317]. The conversion of single-stranded DNA to the double-stranded form (RF I) is an analogue for discontinuous replication on the lagging strand[370], while synthesis of positive single-stranded viral DNA from RF I is the model for the continuous mode of replication on the leading strand[325]. The *rep* protein, a helicase, is necessary for the unwinding of this ΦX174 DNA[318] after a specific nick has been introduced by the ΦX174 encoded gene A protein[363]. This unwinding process can be uncoupled from replication[371,372]. At the same time as unwinding takes place, the single-stranded regions have to be stabilized by the single-stranded DNA binding protein (SSB)[372]. Finally the DNA polymerase III holoenzyme copies the genome concurrently with unwinding[373]. In addition, *E. coli* has several other helicases of which the in vivo functions are so far unknown[374]. Unwinding possibly

needs the participation of DNA topoisomerases of type II[369,375]. One of these enzymes (*E. coli* DNA gyrase) can, among other reactions, introduce negative superhelical twists into closed circular DNA molecules with ATP as a cofactor[375] and such turns may give the motive force for the unwinding of the double helix[376]. Nicking closing is another possible mechanism by which topoisomerases of type I can facilitate unwinding[376].

Work in eukaryotes using model replication forks includes the previously mentioned papova-, parvo- and adenovirus systems. The former two have not been sufficiently fractionated and characterized to allow the attribution of functional roles to proteins other than the DNA polymerase core enzyme itself[15]. The adenovirus in vitro system, however, although it relies on a few adenovirus-encoded replication proteins[30] is being worked out in some detail. A protein fraction from adeno-infected HeLa cells is able to replicate in vitro adenovirus DNA in the presence of an adenovirus DNA binding protein[273] and the 55,000 dalton protein which is covalently linked to the 5′-prime end of the DNA[273,377]. This protein fraction was been purified, and found to contains DNA polymerase α, a DNA dependent and an independent ATPase activity[273] and a functional precursor 80,000 dalton form of the covalently bound protein[378]. No nicking closing activity, DNA ligase, exonuclease, DNA gyrase or single-stranded DNA binding protein could be detected in these preparations. These viral systems are promising tools to elucidate DNA elongation mechanisms, because they rely completely on the host's enzyme machinery.

Apart from these viral studies, enzymes and proteins which probably help the replicase to function at the fork have been isolated and characterized. Among those are single-stranded DNA binding proteins[379-382] single-stranded DNA dependent ATPase[382,383]- and topoisomerases of type I (nicking closing enzyme) or type II[384].

b) Interaction with multiple primers in advance of and during lagging strand polymerization

Because DNA polymerases synthesize exclusively in the 5′-3′ direction, one strand, the lagging strand, has to be synthesized discontinuously in small pieces, called Okazaki fragments[225]. Each piece has to be initiated individually, so that the replicase can elongate. The DNA elongation machinery has to interact frequently with the primers or the primosome[106]. Again I would like to discuss, first, the more highly elaborated T4 and the *Escherichia coli* bacteriophage systems, and then at the end, to give the fragmentary data available for eukaryotes.

In T4 the seven proteins 32, 41, 44, 45, 61, 62 and 43 (DNA polymerase) can synthesize DNA in vitro discontinuously on the lagging side giving rise to Okazaki fragments of 10,000 nucleotides in length[328] (see Liu et al.[24] for more details). This system, however, is incapable of removing the RNA primers, of sealing the Okazaki pieces together and of reinitiating replication forks at defined origins[328].

The most elegant system by far for lagging strand synthesis is the conversion of single-stranded ΦX174 DNA to its double-stranded form[325]. The primosome[106], containing all proteins needed to form a primer at a defined place in the ΦX174 chromosome, moves after complex formation at the replication origin in the antielongation direction[107]. This processive movement with the advance of the replication fork is an attractive proposal for repeated initiation of nascent (Okazaki) fragments[107]. Thus, the DNA polymerase III holoenzyme always finds primers 'backwards' and starts to elongate as soon as it reaches the previous synthesized RNA primer. The *dnaN* gene product[105], which is the β-subunit of the holoenzyme, is responsible for the interaction with the primer synthesized by the *dnaG* primase[322] and probably also for the interaction with the primosome[324]. Thus it appears that this protein enables the replicase to find the primosome efficiently in order to carry out the concerted actions of frequent priming and elongation. In contrast to T4, in this ΦX174 lagging strand model small gaps can be filled in vitro, the primers removed by DNA polymerase I, and the remaining nick sealed by the action of DNA ligase[317].

In eukaryotes the systems are either not defined or not fractionated enough to permit comparisons. The DNA polymerase α of *Drosophila melanogaster* was tested on RNA primed M13 DNA[25]. Using a multiprimed template the *Escherichia coli* single-stranded DNA binding protein enhanced the rate and extent of replication, while on singly-primed DNA, the same protein had no effect. The product size was 200–600 nucleotides in the absence and 2300–2400 in the presence of the binding protein. The catalytic α-subunit alone is unable to use such a template[25], which is very reminiscent of the DNA polymerase III core[22]. Thus it seems that the 3 additional DNA polymerase subunits may have functional responsibilities in the elongation process.

The primase described so far in mammalian cells[385] was not pure enough for elongation studies. Artificially primed systems such as DNA or RNA primed single-stranded ΦX174 DNA[270,271] give insight into some aspects of the elongation process. It seems, as in *E. coli,* where the concerted action of DNA polymerase III holoenzyme and DNA polymerase I is needed for a complete synthesis of an Okazaki fragment, that in eukaryotes as well 2 distinct DNA polymerases are required. RNA primers are elongated by DNA polymerase α to a certain extent[186,270,271] and the β-enzyme is necessary for continuation[270,271]. In

vitro elongation of initiator RNA in isolated nucleoprotein complexes from polyoma infected cells can be performed by DNA polymerase α and these in vitro synthesized Okazaki fragments can be joined by extracts of early *D. melanogaster* embryos[388]. All these results have to be interpreted with caution, since the DNA polymerases used were either not homogeneous or, most probably, were fragments of a holoenzyme.

Even though it is speculative at this time, an attractive hypothesis is that 2 DNA polymerases share the work on the lagging strand: DNA polymerase III or -α does the major work of replication, while DNA polymerase I or -β, together with an associated or attached 5′-3′ exonuclease[215], fills in the gaps and remove the primers.

c) Mode of translocation

Generally, a DNA polymerase can work in 2 different ways during elongation. In the 1st case the enzyme at the actual primer terminus binds a deoxyribonucleoside triphosphate, catalyses polymerization on a mother template and finally translocates to the next base without leaving the DNA. A 2nd, alternative mode is the release of the enzyme after each polymerization step. The former mechanism is called processive and the latter distributive. A 3rd mechanism has been proposed and called 'quasi' processive; it is somewhere between the two extremes[194]. For reasons of economy, speed and fidelity (see below) one might postulate that a replicase should work in the processive mode of translocation.

Recently 2 methods to determine the degree of processivity have been worked out. The 1st[167] measures the rate of DNA synthesis in the presence of all four deoxyribonucleoside triphosphates versus the rate in the absence of one, two, or three of the four. If the reaction is carried out with excess template:primer, the number of nucleotides polymerized for each binding event involving the DNA polymerase and the template can be calculated. The 2nd method[194] uses the artificial template $(dA)_{300} \cdot (dT)_{10}$ as template/primer. The reaction is carried out under conditions, where most probably the enzyme uses the template/primer only once during a polymerization step. The product is then purified over an oligo(dT)-cellulose column, digested by micrococcal nuclease and spleen phosphodiesterease and the nucleosides and nucleotides separated. The degree of processivity can finally be calculated from the nucleoside/nucleoside × nucleotide ratio.

Before discussing the data it should be stated that reaction conditions such as DNA structure, ionic strength and temperature can affect processivity. In addition, as will become clear later, the source and particularly the purity of an enzyme can influence the results due either to 'intrinsic' nucleases or to factors and proteins that make the enzyme more processive.

The T4 DNA polymerase alone has a processivity of 3–12 nucleotides[194] and is alone only slightly or 'quasi' processive. . If the accessory proteins 45 and 44/62 are added the DNA polymerase enhances its rate and becomes fully processive, that is several hundred nucleotides are added per binding event[386]. These proteins together with the DNA polymerase could build the DNA 'walking machine'[331]. The ATPase of the 44/62 complex serves as the 'sliding clamp' and the 45 protein is essential for the formation and maintenance of this 'walking machine'[331].

In Escherichia coli most studies have been carried out with DNA polymerase I. Depending on the reaction conditions and on the assay the enzyme is highly processive (188 nucleotides at low salt and high temperature) or only 'quasi processive' (3.3 nucleotides at high salt and low temperature)[167]. A 'quasi processive' value was obtained when an artificial template was used in the assay[387]. A mutant of DNA polymerase I (*pol* A5) has a 5-fold decreased processivity[387]. DNA polymerase III core enzyme has a processivity of 10–15 nucleotides[183]. On the other hand, DNA polymerase III holoenzyme is extremely processive[183]. On an artificial homopolymer or on a randomly primed template the processivity is more than 100 nucleotides. Using a single-stranded G4 DNA template that has been primed with the *dna*G primase in the presence of SSB the holoenzyme can replicate the full genome with one binding event. Thus the processivity is greater than 5000 nucleotides. This experiment shows that in vivo-like replication systems are necessary to work out the true processive translocation. The DNA polymerase III holoenzyme subunits γ, δ and τ are the most likely candidates for functions analogous to those of the above-mentioned T4 proteins 45 and 44/62.

In lower eukaryotes, recent studies with the *Drosophila melanogaster* α-polymerase[25], using a multiprimed M13 DNA, demonstrate that the α-polymerase (α, β, γ and δ subunits) has a processivity of 14. The catalytic α-subunit alone, on the other hand, has one of only 5. Heterologous single-stranded binding proteins enhance the processivity more than 20 times to 285 for the 4 subunit α-polymerase, while the α-subunit alone in the presence of binding protein becomes fully distributive. It seems, as for T4 and *Escherichia coli*, that subunits other than the catalytic one serve as clamp holders for DNA polymerase at the replication fork.

In higher eukaryotes pure DNA polymerase α preparations are either distributive[193] or 'quasi' processive (11 nucleotides)[188,192–194]. Other forms of DNA polymerases α (more holoenzyme-like?) are more processive[192,406]. DNA polymerase β is found to be fully distributive[167,202] but a 'quasi' processiveness of 9 nucleotides has also been found[194]. The γ-polymerase, as one expects for an enzyme that is capable

of strand displacement, is a highly processive enzyme[87]. In conclusion, it seems likely that replicative DNA polymerases need additional proteins to become fully processive. This high processivity may be responsible for the fast rate at which replication proceeds in vivo.

d) Accuracy of polymerization

Accurate duplication of the genetic material in advance of cell division is a prerequisite for any living organism. DNA polymerases play a central role in the correct incorporation of a precursor nucleotide on a mother template. However, there are other factors, enzymes and structural proteins that influence the accuracy of replication. Several mechanisms support the high degree of fidelity which is estimated to be on the order of 10^8–10^{11} in vivo[31,195,389]. These are: 1. the correct selection of the nucleotide by the enzyme at the 3′OH terminus of specific primers, 2. an editing mechanism to throw out incorrect bases as soon as they are incorporated (proofreading) and 3. an elimination process that recognizes wrong bases after replication.

1. How is the DNA polymerase able to select a correct base? Two hypotheses are proposed. The 1st states[390] that the strength of the hydrogen bonding between the complementary bases (A–T and G–C) is stronger than other pairing possibilities[222,392–396] and this so-called passive DNA polymerase model predicts that the most stable base pairs are favored for incorporation while the more weakly ones are rejected before incorporation or, if they are by any chance incorporated, immediately removed by the 3′-5′ exonuclease[142]. The 2nd[5,397,398], the so-called active DNA polymerase model, proposes that hydrogen bonding alone cannot fully explain the accuracy, and postulates allosteric sites on the DNA polymerase that react to the nucleotides in connection with base pairing.

2. An editing mechanism has been detected in DNA polymerase I[142]. The polymerase polypeptide possesses and additional enzyme activity, the 3′-5′ exonuclease. This activity is able to remove incorrect bases as soon as they are incorporated. Such a 'proofreading' exonuclease was later found in T4[138], T5[399] and T7[137], as well as in the *Escherichia coli* replicase, DNA polymerase III[53]. It was detected in lower eukaryotes[145] and even in mammalian DNA polymerase[44,91]. This enzyme, DNA polymerase δ, has been purified to apparent homogenity[101] and appears to have this associated exonuclease in the DNA polymerase polypeptide[91,101]. As mentioned earlier, it cannot be ruled out that the δ polymerase is a form of DNA polymerase α and this has indeed been proposed[91].

Genetic studies have given insight into this editing mechanism. Mutants of T4 with mutator and antimutator phenotype, mutated in gene 43, the genetic locus for the DNA polymerase, seem to have either an altered base-selection mode[400,401] or an altered ratio of DNA polymerase to 3′-5′ exonuclease activity[392,395].

3. The postulation of the third step, editing after replication, has led to the discovery of enzymes that can recognise and eliminate incorrect bases. Among these are DNA glycosylases and apurinic endonucleases[402] and also an 'insertase', an enzyme capable of purine insertion at apurinic sites in doubel-stranded DNA[403]. Newly replicated DNA might be undermethylated[404], allowing mother and daughter strands to be discriminated and a mechanism has been described that scans the newly replicated DNA for mismatched base pairs and excises the incorrectly incorporated nucleotide on the daughter strand[405].

Several in vitro fidelity assays have been developed, and give some insight into the correct copying ability of different DNA polymerases. The first used artificial polynucleotide template such as homopolymers and heteropolymers[172,195,407]. The incorporation of a correct versus an incorrect nucleotide gives an estimate for misincorporation. Results strongly depend on the extremely high purity of the template and the nucleotides. Using such artificial templates, the following estimated misincorporations were obtained: DNA polymerase I, 1.3×10^{-5}[172,173], T4 DNA polymerase, 10^{-5}[407], vertebrate DNA polymerases α[195,196] from sea urchin, 8×10^{-5} and calf thymus, 1.1×10^{-4}, vertebrate DNA polymerases β[195,196] from human placenta, 2.5×10^{-5} and calf thymus, 3.3×10^{-5}. The accuracy of a variety of other DNA polymerases have been determined[5,196,408–410] and sometimes conflicting results have been reported. These results have to be seen in the context of differences in the degree of purity of templates and substrates and the various forms of enzymes that were used. Additional proteins, or different divalent cations and other factors are sometimes used by different authors. Using these accuracy measurement methods it seems that the β enzyme (repair) is more faithful than the α-enzyme (replication)[195,196,411].

Better in vivo systems were developed by using ΦX174 DNA with an amber mutation (*am*3)[412]. A restriction fragment is hybridized just ahead of the mutation. After the DNA polymerase reaction has taken place, the DNA is isolated, *Escherichia coli* spheroblasts are infected and on indicator bacteria the reversion frequencies are determined. With this system the accuracy of DNA polymerase I was 1.5×10^{-6}, an order of magnitude higher than previously determined[174]. On the other hand, highly purified α-polymerases had an accuracy of 3×10^{-5} and the β and γ enzymes of 2×10^{-4} and 1.4×10^{-4}, respectively[413]. This suggests that the highly purified enzymes lack factors that are involved in accurate replication. Using randomly nicked double-stranded ΦX174

DNA again with an amber mutation the T4 replication apparatus can replicate such a molecule in the 'rolling circle' mode[329]. After isolation and specific restriction and ligation of the DNA, a transfection is performed as mentioned above. Error rates of 2×10^{-6}–2×10^{-7} were determined with this intact replication apparatus[329,414]. Finally, the system closest to the situation in vivo is the double-stranded to single-stranded ΦX174 DNA replication[370]. Using *gene*A protein, *rep* protein, SSB and DNA polymerase III holoenzyme, 10–20 intact viral single-stranded DNA copies can be generated[370]. Applying the mentioned tranfection protocol and extrapolation to physiological precursor nucleotide concentrations in *E. coli* an error rate of less than 5×10^{-7} was calculated[182], very similar to that predicted from genetic studies with ΦX174[184]Using this type of in vivo system measurements of mismatches of certain base pair combinations have validated theoretical in vitro models[415].

Both the *E. coli* and the T4 systems give an error rate close to the in vivo rate, in phages. It can therefore be inferred that base selection and proof-reading alone contribute to a high degree of fidelity. However, there is still a factor of 1000 missing compared to the in vivo rate in *E. coli.* A recently described post-replication repair system increases the accuracy by a factor of 10^3–10^4[405]. Therefore the in vitro determined fidelity of 10^{-5}–10^{-6} is sufficient to account for a DNA elongation machinery like that in vivo[182,329].

From this discussion it seems that the additional proteins in the elongation machinery significantly enhance accuracy. This is evident from comparisons of values between the in vivo-like systems of T4 and *E. coli* versus isolated pure or paritally pure enzymes. Such proteins may help to transfer and to complex the replicase to the right primer terminus[322], proof-read the DNA, or make the DNA polymerase more processive[183,331]. *E. coli* single-stranded DNA binding protein increased the fidelity of prokaryotic and eukaryotic DNA polymerases, probably by enhancing the base selection by the enzyme[416].

A new protocol for testing the error rate of chromatin-associated DNA polymerase β has been published[417]. With this method the accuracy is different from previously determined values[195,413], probably due to factors associated with the chromatin-DNA polymerase complex. Finally, age-dependent changes in fidelity have been detected in some cases[408,418], but not in others[419–421].

This could reflect another case of the presence or absence of fidelity factors in different DNA polymerase preparations (see e.g. Murray[422]). Therefore increased errors may be due to alteration in factors rather than to the DNA polymerase itself.

In conclusion, the DNA polymerase peptide alone seems to contribute substantially to the correctness of replication. Studies with in vivo-like prokaryotic systems predict that additional 'accuracy helper proteins' are present.

e) Comparative summary

The action of DNA polymerases and their associated proteins at the replication fork is extremely complex. In prokaryotes, systems elongating in vitro primed DNAs with in vivo speed and accuracy have been designed. In comparison, knowledge of DNA elongation in eukaryotes is still very fragmentary. The use of well-defined prokaryotic template systems and improved knowledge of the genetics of eukaryotes may help to overcome this problem.

5. Proteolysis: an undesirable artefact or an important physiological event?

Many proteins and enzymes have to be cleaved proteolytically before they are able to perform in vivo functions[391]. The molecules may thus be activated (e.g. hormones or other secretory proteins)[423] or fit into a higher order structure (functional complex)[424]. Proteolytic inactivation of a protein is another example of regulation (e.g. inactivation of *E. coli* bacteriophage lambda repressor by proteolytic cleavage mediated by *rec*A protein)[425].

Proteolysis can either be genuine (genetically controlled) or artificial (uncontrolled during fractionation and isolation). Uncontrolled proteolysis is probably responsible for a lot of the controversy in the field of eukaryotic DNA polymerases. The use of efficient protease inhibitors should soon give more consistent results. It has been demonstrated that the popular phenylmethylsulfonyl fluoride is inefficient in preventing the formation of low-molecular-weight DNA polymerases[125]. Indeed, the DNA polymerase α from *Drosophila melanogaster* with a high-molecular-weight catalytic subunit can only be isolated in the presence of more suitable inhibitors[25,75]. In their absence a great variety of different forms is found[74]. Generally it can be stated that the molecular weights of DNA polymerases are found to be higher in the presence of protease inhibitors (table 5).

These facts do not, however, exclude the possibility that genetically programmed conversion of a DNA polymerase precursor by proteolytic cleavage may regulate DNA replication in advance of initiation and elongation. Precursor and product may have different affinities for the primers[426,427] or for the primosome[324].

In a proteolysis model, as presented in figure 1, a precursor DNA polymerase is cleaved so that the product DNA polymerase can form a holoenzyme complex with auxiliary proteins (subunits). This inactive holoenzyme is further cleaved, so that in-

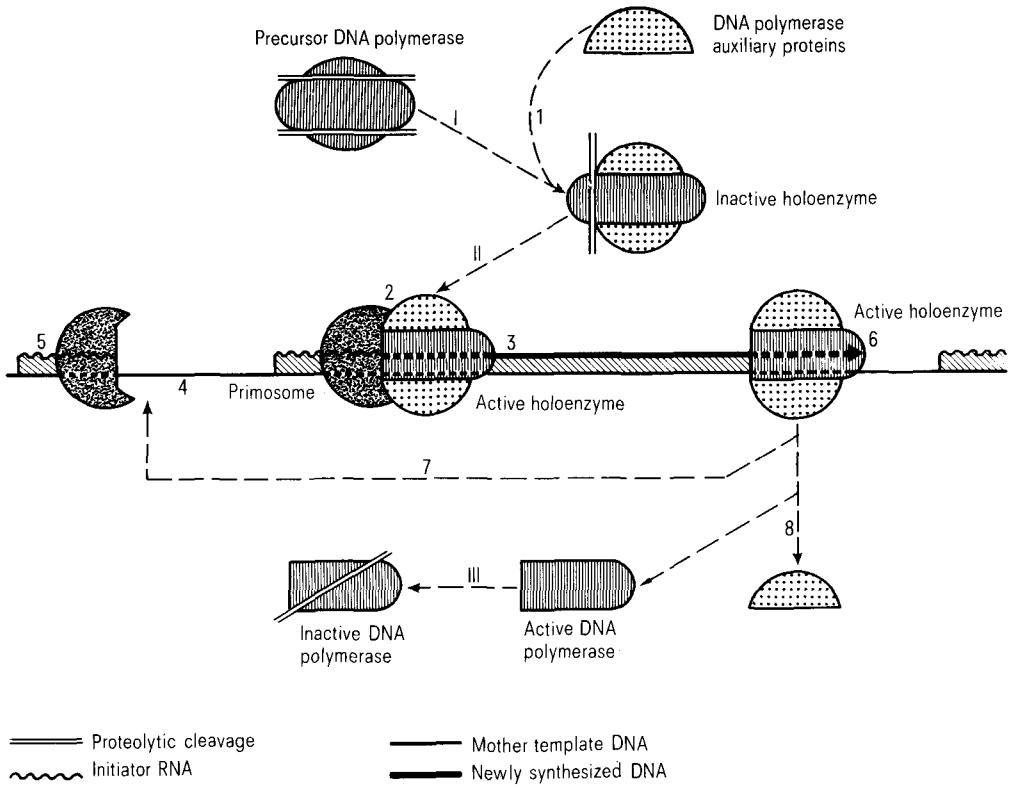

Figure 1. Model postulating proteolytic regulation of a replicative DNA polymerase. Three proteolytic steps (I-III) are proposed for the activation and inactivation of a DNA elongation mechanism. I A precursor DNA polymerase is cleaved so that the DNA polymerase can form a holoenzyme-complex with DNA polymerase auxiliary proteins (1). II This inactive holoenzyme is further cleaved so that interaction with the primosome at the primer terminus is possible (2). After complex formation the DNA polymerase starts DNA synthesis (3) and the primosome moves in anti-elongation direction (4) to form another primer (5) and to wait for another active holoenzyme. When replication is completed (6) (e.g. the previous primer is reached), the active holoenzyme either moves or jumps in the anti-elongation direction to the next primosome (7) or dissociates (8) into the core enzyme and the auxiliary proteins, III The core DNA polymerase is eventually cleaved and inactivated.

teraction with the primosome at the primer/terminus is possible. After complex formation the DNA polymerase starts to synthesize DNA and the primosome moves in the antielongation direction to form another primer and to wait for another holoenzyme. When replication is completed (e.g. the previous primer is reached, the active holoenzyme either moves or jumps in the antielongation direction to the next primosome or dissociates into the core enzyme and the auxiliary proteins. The core DNA polymerase is envetually cleaved and inactivated.

Fragmentary data suggest such a model. *Escherichia coli* DNA polymerase I can be proteolytically split into a lower molecular weight form[428,429]. The larger fragment (Klenow fragment) is the DNA polymerase with the associated 3′-5′ exonuclease[430], while the smaller has the 5′-3′ exonuclease activity[431]. In eukaryotes it was reported that high-molecular-weight DNA polymerase forms (DNA polymerases α?) can be converted via intermediate forms into small-molecular-weight ones[432-434]. In calf thymus extensive studies with DNA polymerase α suggested that proteolysis might be responsible for some of the 5 different forms (A_1, A_2, B, C and D) described[90,339,435]. It must be pointed out again, that the different types of DNA polymerase α reported are possibly fragmentary forms of a holoenzyme. 1 or 2 of these calf thymus forms were believed to be triggered by proteolysis[339]; this was based on the observation that the activity levels of the different forms have been found to vary in an unpredictable way from preparation to preparation[436]. Finally a protease from calf thymus was detected that can convert a larger DNA polymerase α (7.4S) to a smaller form (5.4S)[437].

Work with DNA polymerase β is much less controversial and the reported properties and sizes are very similar. Since the enzyme is a repair DNA polymerase[158,207] and constantly needed in the cell, without great fluctuations, it is not astonishing that it is relatively insensitive to proteolytic regulation.

In *Drosophila melanogaster* an active intermediate molecular weight 5.5S DNA polymerase α form was created from a 7.3S precursor by in vitro trypsin treatment[74,438]. Exposure of HeLa cells to the protein synthesis inhibitor cycloheximide creates a new form of DNA polymerase α[439] and this could be due to the action of a protease released in the cell during such treatment[439]. On SDS-polyacrylamide gels, after

renaturation of DNA polymerase α[124], at least 7 DNA polymerase activities can be detected when limited proteolysis is allowed to occur[125]. The formation of the lower molecular weight fragments can be prevented by more than 90% in the presence of potent protease inhibitors[125].

In conclusion, these results suggest that not all the proteolytic events detected or triggered in vitro are artefacts. Genetically programmed proteolysis may be an important step in controlling the active participation of a replicase in a replication complex.

6. *Evolutionary aspects*

Vital functions such as DNA replication and DNA repair are though to be conserved throughout biological evolution[1]. The role of a DNA polymerase, i.e. catalysis of DNA elongation, appears to be similar in all organisms investigated and this enzyme might be expected to be highly conserved. Indeed, all DNA polymerases so far described need a 3′hydroxyl group of a small RNA or DNA fragment hybridized to a DNA template in order to start polymerization. The direction of polymerization is always 5′-3′ in the newly synthesized strand.

Phylogenetic studies have given insight into the presence of different DNA polymerases in species ranging from bacteria to mammals[162,440-442] and immunological approaches have shown relatedness between different organisms[116]. These studies can be summarized as follows: A DNA polymerase α-like enzyme can be found in mammals, birds, reptiles, amphibians, echinoids, cephalopods, hydrozoas, sponges, ciliates, flagellates and even in plants and fungi[441]. A β-like enzyme seems to be present in all multicellular organisms other than plants and fungi[441]. The structural homology between DNA polymerases β from several mammals (mouse, rat, rabbit, pig, calf) and birds has been established[86,155,443]. Finally the γ-like polymerase is present in all mitochondria tested[442] and also occurs in chloroplasts from plants[444].

Since the major replicase, the DNA polymerase α appears even in plants and fungi its degree of conservation is higher than that of the β and γ enzymes. Limited proteolysis of crude enzyme fractions from *Escherichia coli* mutants (*pol*A, *pol*B), *Ustilago maydis, Drosophila melanogaster,* rat, calf and human followed by analysis of the activity patterns after SDS-gel electrophoresis and renaturation of the enzymes[124,338] gives fragments which show a remarkable similarity in size and enzymatic activity[125]. Even the major *Escherichia coli* replicase, the DNA polymerase III, has at least 3-4 proteolytic fragments in common with the vertebrate and mammalian DNA polymerases[125] (fig. 2). These similarities may result from the conservation of polypeptide conformations which are particularly susceptible to proteolytic cleavage. Whether only the three-dimensional structure of the replicases or even the primary one is conserved cannot be answered at this stage. It seems, however, that the basic reaction mechanism of incorporating a complementary nucleoside monophosphate has exerted an immense constraint on the overall three-dimensional shape of this biologically most important enzyme.

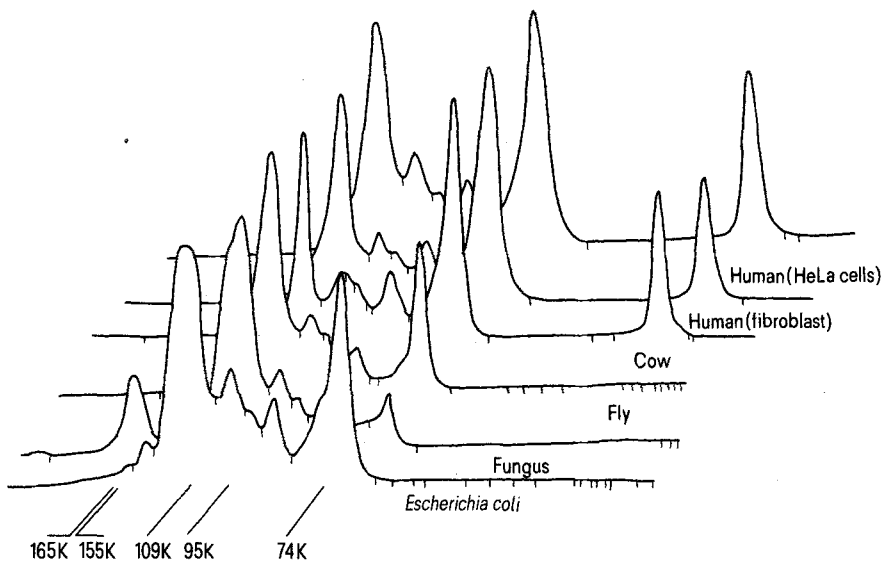

Figure 2. Densitograms of autoradiograms of DNA polymerase activities after SDS/7.5% polyacrylamide gel electrophoresis of different prokaryotic and eukaryotic cell extracts. Storage of crude enzyme fractions to trigger the generation of active proteolytic fragments, electrophoresis of 200 µg crude enzyme fraction of each tissue, renaturation of DNA polymerase activities in the gel and activity measurement were performed as described[125]. The cathode was on the left and the anode on the right, respectively. Molecular weight markers were *Escherichia coli* RNA polymerase β', β and σ subunits (165, 155 and 95 kdaltons (K), respectively) and *Escherichia coli* DNA polymerase I (109 K) and its Klenow fragment (74 K). Note that a defined and remarkably similar pattern of intermediate molecular weight activities is generated in extracts from prokaryotic, lower and higher eukaryotic cells.

In the same context it is worth mentioning that aphidicolin, a potent inhibitor of DNA replication and DNA polymerase α (see earlier), inhibits α-like polymerases from the whole range of eukaryotes[291,292]. Since aphidicolin is a competitive inhibitor of dCTP[445,446] one can argue that even a functional binding site of eukaryotic replicases has been conserved over several hundred million years.

To conclude, it appears that the DNA polymerase which is responsible for chromosomal DNA replication has resisted pressure of muation so efficiently that biochemical, physical and, to some lesser extent, immunological properties have been conserved for more than a billion years.

7. Conclusions and perspectives

Although DNA replication requires the concerted action of many proteins in a complex, the DNA polymerase plays a central role. The function of a DNA polymerase in a particular DNA synthesis process (e.g. DNA replication or DNA repair) is similar across the whole evolutionary spectrum and the basic reaction mechanism is the same for all DNA polymerases investigated.

The most detailed information available stems from DNA polymerases involved in DNA replication. Replicases not only from prokaryotes but recently also from eukaryotes can be obtained in pure form, and more accurate experiments may now be performed. Prokaryotes, due to the obvious advantages of their genetics, fast generation time and quantity, can teach us in astonishing detail how the mechanism of DNA elongation works. These processes include the interaction of the replicase (DNA polymerase holoenzyme) at the primer/terminus, the mode of translocation after condensation of a nucleoside triphosphate, the degree of accuracy and the concerted action of DNA synthesis and unwinding of the double helix. Even though information from eukaryotes is still fragmentary, comparisons between prokaryotes and eukaryotes may be permitted at this stage:

a) Several DNA polymerases have been identified in one cell and these enzymes have different in vivo functions.

b) One DNA transaction (e.g. DNA replication or DNA repair) seems to use more than one DNA polymerase.

c) The major replicase of prokaryotes and eukaryotes works at the replication fork as a multipolypeptide complex, also called DNA polymerase holoenzyme. The additional subunits, also called accessory proteins, may be important for processivity, accuracy and interaction with other proteins (e.g. primosome, unwinding proteins, single-stranded DNA binding proteins).

d) Proteolysis may be important as a regulatory step in prokaryotes as well as in eukaryotes.

e) Replicases are the most conserved DNA polymerases. Base selection as an absolute requirement for a DNA polymerase could have provided a high degree of conservation. This conservation is retained during evolution in the following order: base selection > editing > mode of translocation > accuracy > interaction with proteins at the replication fork. This means that the catalytic DNA polymerase polypeptide is more conserved than the polymerase holoenzyme and the latter more than the entire primosome or replisome.

Future studies with prokaryotes will lead to an understanding of the detailed mechanism of the concerted action between the core DNA polymerase and the associated subunits or auxiliary proteins. In eukaryotes DNA polymerase subunits will be identified and their interaction in the polymerase worked out. In 10 years time most details of the prokaryotic DNA elongation machinery will probably have been elaborated, while for eukaryotes we will have reached roughly the stage we have now reached in our understanding of prokaryotes.

New techniques such as genetic engineering, the use of monoclonal antibodies, and the study of eukaryotic genetics in cell cultures not depending on fetal calf serum, will hopefully help to narrow the gap that exists in the knowledge of prokaryotic and eukaryotic DNA enzymology.

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Full Papers

Time-energy budgets and optimization*

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Summary. Since the process of natural selection entails a comparison of phenotypes and choosing of the best, optimality theory appears appropriate to identify selection pressures. Optimality theory does *not* test whether an organism is designed optimally - it assumes it. The ingredients of a complete optimization model are outlined and two approaches are exemplified. Both time-energy-budgeting and Pontryagin's maximum principle lead to semi-quantitative predictions about, e.g., an animal's behavior; they merely entail an inequality formalism. A discrepancy between prediction and test would not yet show a behavior to be maladaptive since several other explanations are possible. Animals optimize their behavior over intervals ranging from less than a second to months or years. It is unknown whether, with a long interval, the animal makes use of the opportunity to revise its decision(s). Present optimal foraging models predicting, e.g., diet breadth are too simple in that foragers a) may not always maximize energy intake, as postulated, b) have to allow for nutrient, toxin and remedial content of food items, and/or c) have to allow for interaction of items, annihilating their ranking along a unidimensional scale of profitability.

Time-energy budgets and optimization

Natural selection is a process which maximizes 'inclusive fitness' or some other suitable quantity. In maximizing its inclusive fitness an animal cannot do better than survive; 'trying harder' does not yield greater rewards. Even the best genotype cannot live forever since there are certain constraints. From this it becomes clear that by 'optimal' one does not mean the commonplace sense of 'the best design'. The process

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