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Mammalian DNA Polymerase <latex>α</latex>: A Replication-Competent Holoenzyme Form from Calf Thymus

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Mammalian DNA polymerase α: a replication-competent holoenzyme form from calf thymus

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Calf thymus DNA polymerase α, like the replication-specific DNA polymerase III holoenzyme of *Escherichia coli*, can be isolated as a distinct complex. A specific multiprotein form of the polymerase α, a form designated replication-competent (RC) holoenzyme, consists of a complex of a polymerase–primase core and at least six other polypeptides. The RC holoenzyme can efficiently replicate several naturally occurring templates, including the genomic DNA of the porcine circovirus (PCV). The DNA of this virion consists of a single-stranded circle with a defined replication origin, and its replication requires the cellular DNA replication machinery. It might therefore provide an invaluable opportunity to investigate chromosomal replication mechanisms, analogous to the way that studies on *E. coli* bacteriophage DNA replication elucidated host DNA replication mechanisms. Calf RC holoenzyme α selectively initiates PCV DNA replication *in vitro* at a site that possibly represents a consensus sequence of cellular DNA replication origins. The cell-free PCV replication system will be exploited for the *in vitro* dissection and reconstitution of the RC holoenzyme and the functional analysis of its component polypeptides.

Introduction

DNA replication requires the concerted action of many proteins and enzymes, acting either separately or in a complex (Kornberg 1980, 1982; Hübscher & Spadari 1984). The replication-specific proteins include DNA polymerases, DNA polymerase accessory proteins, DNA primase, DNA topoisomerase, DNA helicase, DNA binding proteins, RNase, DNA ligase and a variety of factors conferring specificity at the origins of DNA replication. Since the discovery of DNA polymerase in *Escherichia coli* 30 years ago (Kornberg *et al.* 1956), DNA polymerases have been found in all organisms containing DNA genomes. Three general classes of DNA polymerase have been identified in both prokaryotes (polymerases I, II and III) and eukaryotes (polymerases α, β and γ). Replication of chromosomal DNA requires DNA polymerase III in *E. coli* (Kornberg 1980) and DNA polymerase α or a DNA polymerase α-like enzyme in eukaryotes (Campbell 1986; Fry & Loeb 1986). The function of a replication-specific DNA polymerase, regardless of the system from which it is derived, is to produce an accurate copy of the genetic material which specifies it. A replication-competent (RC) form of a

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DNA polymerase must execute several functions other than that of the polymerization of deoxyribonucleoside triphosphates. Such functions include: (1) participation in the unwinding of the parental DNA strands; (2) extension of a continuous daughter strand along the 3′→5′ parental template; (3) cooperation with primases in the synthesis of oligomeric RNA required to prime the discontinuous synthesis of the lagging daughter strand along the 5′→3′ parental template; (4) the processive translocation of the polymerase and its associated proteins along the parental DNA strands; (5) the production of a faithful complementary copy of parental template; and (6) the capacity to cooperate with all the other enzymes and factors involved in the recognition and binding to the origins of genome replication (Kornberg 1980, 1982; Campbell 1986).

The extensive characterization of the DNA replication machinery of *E. coli* (Kornberg 1984) and several of its bacteriophages (Richardson 1983; Alberts 1984) has shown that the replication-specific DNA polymerases of these systems exert their multiple functions in the form of a multisubunit, holoenzyme structure in which the catalytic 'core' binds and functions in concert with non-core accessory proteins in the form of a 'replisome' (Kornberg 1980, 1982, 1984; McHenry 1985). The elucidation of the prokaryotic replisome has prompted the search in mammalian and other eukaryotic cells for similar forms organized about an holoenzyme form of DNA polymerase α (Campbell 1986; Fry & Loeb 1986). The results of the latter search have been generally successful. Mammalian DNA polymerase α in essentially all the systems examined can be isolated as a tight complex of DNA polymerase and DNA primase, and a more loosely affiliated assortment of 'accessory' proteins of unassigned, or at best, uncertain replicative functions. As a first step towards understanding what function(s) the polymerase α accessory proteins might serve in genome replication, we have adopted a strategy for calf thymus DNA polymerase α like that developed to elucidate the 'physiological' replicative functions of the accessory proteins present in the *E. coli* DNA polymerase III holoenzyme. Our specific approach has exploited the capacity of selected enzyme forms to catalyse site-specific DNA synthesis and to utilize origin-specific initiation on naturally occurring prokaryotic and eukaryotic viral templates. Our initial results, which form the basis of this paper, suggest specific tasks for at least six non-core, accessory proteins.

Isolation of a DNA polymerase α form active in the replication of naturally occurring DNA templates

By including ATP and an extensive panel of inhibitors of proteolysis during purification we have isolated and defined two functionally different forms of DNA polymerase: one form, the so-called replication-competent (RC) holoenzyme, is able to replicate efficiently single-stranded parvoviral DNA and primed M13 DNA (Hübscher *et al.* 1982). The second, so-called replication-incompetent (RI) form was very active on gapped DNA, but could not copy the long single-stranded viral DNA templates (Hübscher *et al.* 1982; Albert *et al.* 1982). The RI form consisted of a high molecular mass catalytic subunit of 118–125 kilodaltons (kDa) and several smaller polypeptides displaying apparent molecular masses 54–64 kDa (Albert *et al.* 1982). Proteolytic peptide mapping of the high (118–125 kDa) and the low (54–64 kDa) molecular mass polypeptides revealed common peptides, suggesting that they may derive from a common precursor molecule of more than 140 kDa (Albert *et al.* 1982).

Two other research groups have exploited rapid immunoaffinity purification of calf thymus extracts to define a DNA polymerase α core polypeptide of greater than 160 kDa (Wahl *et al.*

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1984; Chang *et al.* 1984). The bulk of the DNA polymerase α in both cases was tightly bound to a DNA primase, in the form of a polymerase–primase complex (Campbell 1986) like that first described by Conaway & Lehman (1982) for *Drosophila melanogaster* DNA polymerase α. The DNA polymerase α–primase complex that we have purified with immunoaffinity chromatographic techniques has consistently been unable to copy long single-stranded DNA (data not shown). Apparently, preservation of the RC form of the holoenzyme, like the corresponding holoenzyme form of *E. coli* (McHenry & Kornberg 1981), is strongly dependent on the conditions of isolation. Indeed, care must be taken from the earliest steps of purification to probe RC activity with single-stranded templates, to include inhibitors of proteolysis, ATP, and to apply purification techniques that avoid the exposure of the enzyme to chaotropic agents (Ottiger *et al.* 1987).

Properties of the RC holoenzyme form

The RC holoenzyme had a specific activity (units per milligram of protein) of greater than 75000 on activated DNA and greater than 25000 on primed M13 DNA. Figure 1 shows the polypeptide pattern of the RC form on a Coomassie blue-stained SDS–polyacrylamide

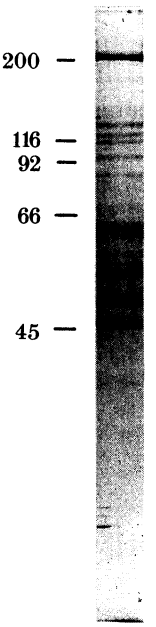

FIGURE 1. SDS–polyacrylamide gel of the replication-competent DNA polymerase α from calf thymus. 10 μg of the purified enzyme were electrophoresed in an 8% gel and subsequently stained with Coomassie brilliant blue.

electrophoresis gel. The enzyme complex yielded ten major protein bands with apparent molecular masses of approximately 200, 125, 118, 116, 98, 87, 63, 54, 49 and 47 kDa. An essentially identical pattern of bands was also found in gels stained with the ultrasensitive silver staining technique (data not shown).

The activity of the RC holoenzyme was compared with that of the replication-incompetent (RI) DNA polymerase α primase complex (Albert *et al.* 1982) on a variety of templates; the results, summarized in table 1, indicated that the RI form was deficient in the capacity to copy both primed and unprimed long, single-stranded viral DNA templates and poly(dT)-tailed pBR322 RFIII DNA.

Table 1. Template utilization by the replication-competent (RC) and replication-incompetent (RI) DNA polymerase α forms

template	DNA synthesis (% input DNA) RC-α	RI-α
parvoviral DNA	32	11
porcine circovirus s.s.DNA	9	1
primed M13 DNA	57	13
unprimed M13 DNA	51	5
poly(dT)tailed pBR322 DNA	4	0.5

Identification of polypeptides unique to RC holoenzyme

Table 2 summarizes the results of an extensive set of experiments designed to dissect the RC form (Ottiger *et al.* 1987). Treatment of the RC form with the core protein-specific neutralizing anti-DNA polymerase α monoclonal antibody SJK 132–20 (Tanaka *et al.* 1982) selected the complete set of ten polypeptides under non-denaturing conditions. Three of the ten bands (200, 118 and 63 kDa) displayed DNA polymerase activity in denaturing activity gel assays (Hübscher *et al.* 1981; Spanos & Hübscher 1983).

Table 2. Polypeptides in the replication-competent DNA polymerase α

polypeptide (kDa)	required for *in vitro* replication	possible function
200	+	polymerase
125	?	?
118	+	polymerase
116	?	calmodulin binding
98	+	?
87	+	?
63	+	polymerase
54	+	primase ?
49	+	primase ?
47	+	nuclear factor I?

To determine which polypeptides were required for replication activity, the RC holoenzyme was exposed to more than 30 chromatographic systems. The approach included: (1) equilibration of the holoenzyme in loading buffer; (2) adsorption to the selected chromatographic support; (3) washing of the column with adsorption buffer, and (4) salt-induced elution. The flow through and eluate of each system were collected, dialysed, and subjected to: (*a*) assay of DNA polymerase α on activated DNA versus primed M13 DNA and (*b*) SDS–PAGE electrophoresis. A loss of more than 90% of replication activity on primed M13 DNA was scored as inactivation of the replication activity. The following chromatographic media removed polypeptides (the relative molecular mass of the polypeptide removed is given in parentheses): blue dextran Sepharose (98), Biorex (87), DEAE–Sephadex (63), valyl–Sepharose (54 and 49) and heparin–Sepharose (47). In summary, the results of the chromatographic experiments suggest that efficient replication activity of RC holoenzyme requires the function of a non-proteolysed form of the 200 kDa catalytic DNA polymerase α core and at least six other auxiliary polypeptides of 98, 87, 54, 49 and 47 kDa. We do not yet

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know whether the 118 kDa and 63 kDa polypeptides are artefacts of proteolysis of a larger, peptide or 'physiologically' relevant RC holoenzyme proteins. However, we hope to resolve this question by applying a newly devised protocol for purifying the RC polymerase α at −20 °C, a temperature that greatly reduces proteolysis (S. Spadari & U. Hübscher, unpublished results).

Finally, it may be significant that the 116 kDa polypeptide, which was not apparently required in the M13 replication system, can bind calmodulin in a calcium-dependent reaction (M. Berchtold, M. Gassmann, N. C. Brown and U. Hübscher, unpublished results).

The RC holoenzyme can specifically initiate DNA replication on porcine circovirus DNA

Porcine circovirus (PCV) is the only mammalian virus known to contain a single-stranded (s.s.) closed circular DNA (1.76 kilobases) as its genome (Tischer *et al.* 1982). Structurally, the PCV genome resembles those of the small *E. coli* bacteriophages φX174, G4, fd and M13, all of which have been used so successfully to unravel the mechanisms of bacterial DNA replication (Kornberg 1980, 1982). The PCV genome is only one third as large as these bacteriophage DNAs. Therefore its replication is likely to be even more dependent on host cell functions than that of the corresponding prokaryotic systems. The latter possibility has prompted us to exploit the PCV genomes as a naturally occurring s.s.DNA template to study the sequence requirements for initiation of DNA replication by the RC holoenzyme.

To define the region of the PCV genome where priming occurs, newly synthesized, radioactively labelled DNA was hybridized to PCV restriction fragments. First, DNA polymerase α was provided with ribonucleoside triphosphates and allowed to prime for 30 min. Subsequently, the product was exposed for another 10 min to DNA polymerase I and deoxyribonucleoside triphosphates to allow chain elongation. The newly synthesized chains were limited to an average length of 200–300 bases by addition of the chain terminator, dideoxy-TTP. The reaction was stopped by heating, and the unreplicated s.s. regions of the template DNA were digested with S1 nuclease. The nuclease-resistant, radioactively labelled DNA was extracted with phenol, precipitated with ethanol, and hybridized to Southern-blotted PCV restriction fragments originating from the viral DNA cloned into a plasmid. The results suggest (H.-J. Buhk & U. Hübscher, unpublished results) that RC holoenzyme selectively primes DNA replication *in vitro* at a specific site on the PCV genome, a site that possibly represents a consensus sequence of mammalian origins of DNA replication.

Nuclear factor I appears to copurify with the RC holoenzyme

The PCV sequence at which DNA replication was selectively initiated by the RC holoenzyme contains part of a consensus sequence for nuclear factor I (Gronostaiski *et al.* 1985; H.-J. Buhk and U. Hübscher, unpublished results). The latter observation and the observation that the RC holoenzyme contained polypeptides in the relative molecular mass range of nuclear factor I (Nagata *et al.* 1982) prompted us to examine the RC holoenzyme for the presence of nuclear factor I. Specifically, we incubated the enzyme with a ^{32}P-labelled nuclear-factor-I-specific oligonucleotide sequence from adenovirus type 5 DNA and examined its binding in a gel retention assay (Schneider *et al.* 1986). The results summarized by figure 2 indicated that one

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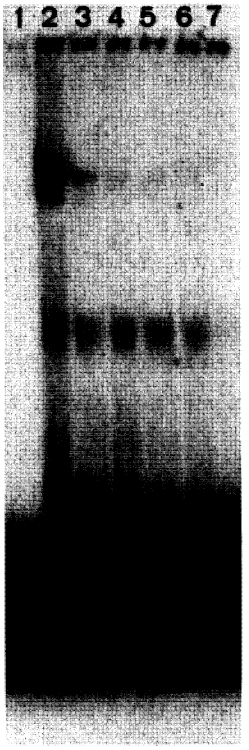

FIGURE 2. Gel retention assay for nuclear factor I. Binding and electrophoresis were done as outlined by Schneider *et al.* (1986). The nuclear factor I consensus oligonucleotide $A_1 B_1$ from adenovirus type 5 was 5′ labelled with [^{32}P] ATP by T4 polynucleotide kinase (Schneider *et al.* 1986). A final volume of 50 µl contained: 25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, glycerol (10% by volume), 150 mM NaCl, 10 fmol [^{32}P]oligonucleotide (specific activity 10^3 counts per minute fmol^{-1}) and 14 units of DNA polymerase α (units determined on activated DNA). Lane 1: control, no enzyme; lanes 2–7 contained in addition to radioactively labelled oligonucleotide unlabelled oligonucleotide in amounts of, respectively, 0, 95, 190, 475, 950 and 4750 fmol.

polypeptide from the RC holoenzyme specifically bound the oligonucleotide and the binding was suppressed by increasing amounts of unlabelled nuclear factor consensus oligonucleotide sequence (figure 2, compare lanes 2 to 7). If the RC holoenzyme was subjected to heparin–sepharose chromatography, the resultant multiprotein complex lost both a 47 kDa polypeptide and the capacity to bind the nuclear factor I-specific DNA sequence (data not shown).

We plan now to purify *bona fide* nuclear factor I according to established protocols. The rationale for this approach is to attempt: (1) to determine if the nuclear factor I 'candidate' is in fact the *bona fide* protein, and (2) to understand how it is associated functionally and physically with the RC holoenzyme.

CONCLUSIONS AND PERSPECTIVE

We have isolated a form of calf thymus DNA polymerase α active in the replication of naturally occurring genomes. This enzyme form, which we have named RC holoenzyme, appears to be considerably more complex than the DNA polymerase–primase aggregate described for the calf thymus polymerase α complex by other research groups (Campbell 1986). The RC holoenzyme can specifically initiate replication of the single-stranded circular

genome of the porcine circovirus DNA, a mammalian virus that probably relies entirely on host cell functions in replication. By applying the general approaches that used single-stranded bacteriophage DNAs to dissect the replication of *E. coli*, we now hope to exploit the PCV DNA to delineate the functional roles of individual polypeptides of the RC holoenzyme. Considering that genes for the catalytic subunits of several eukaryotic replicative DNA polymerases have now been cloned (Johnson *et al.* 1985; Foster *et al.* 1986; Sen Gupta *et al.* 1986) we also hope to gain a more thorough understanding of the DNA polymerase α core itself through the study of genetically engineered forms.

We thank Dr E. L. Winnacker for a gift of nuclear factor I consensus sequence oligonucleotide and B. Ruf and P. Frei for excellent technical assistance. This work was supported by the Swiss National Science Foundation, grant number 3.604-0.84 to E. F. and U. H, by a grant from the Stipendienfonds der Basler Chemishen Industrie to M. G. and by the CNR Progetti Finalizzati 'Controllo delle Malattie da Infezione' and 'Oncologia' to S. S.; N. C. B. was supported by a John Simon Guggenheim Memorial Foundation Fellowship and a grant-in-aid from the Roche Research Foundation.

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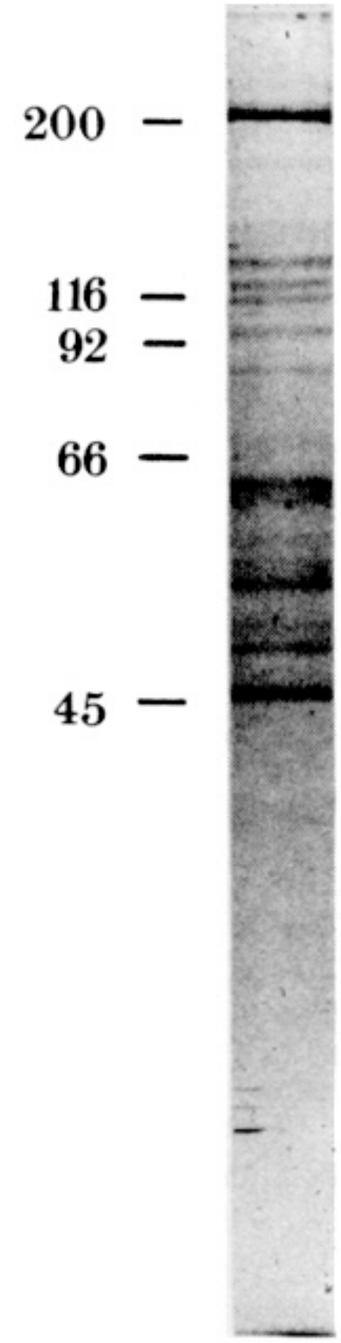

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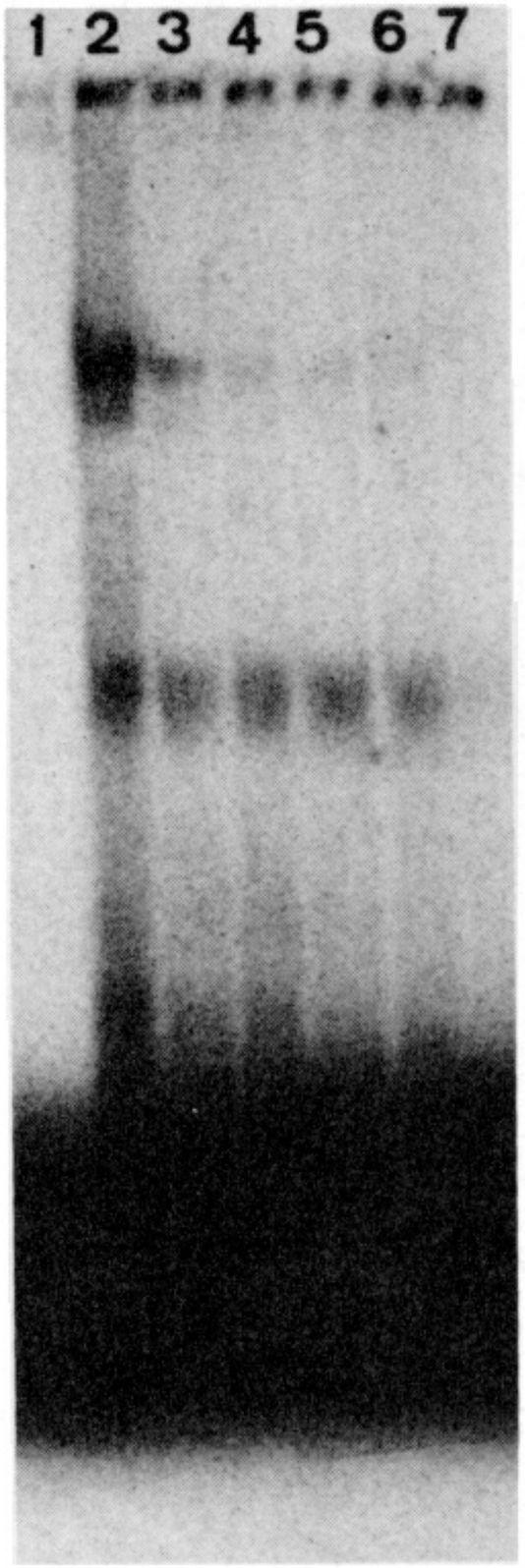

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