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Evidence that a high molecular weight replicative DNA polymerase is conserved during evolution

(enzyme renaturation/assay after $NaDodSO_4$ gel electrophoresis/proteolysis/protease inhibitors)

ULRICH HÜBSCHER*†, AD SPANOS*, WALTRAUD ALBERT‡, FRIEDRICH GRUMMT‡, AND GEOFFREY R. BANKS*

*National Institute for Medical Research, Mill Hill, London, NW7 1AA; and ‡Institut für Biochemie, D-87 Würzburg, Federal Republic of Germany

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ABSTRACT **Using a technique developed recently to detect DNA polymerase activity *in situ* after $NaDodSO_4$ gel electrophoresis (Spanos, A., Sedgwick, S. G., Yarranton, G. T., Hübscher, U. & Banks, G. R. (1981) *Nucleic Acids Res.* 9, 1825–1839), we present evidence that a high M_r (≥125,000) polypeptide is responsible for chromosomal DNA replication in prokaryotes, lower eukaryotes and high eukaryotes. Not only extracts from *Escherichia coli*, *Ustilago maydis*, *Drosophila melanogaster*, rat neurones, calf thymus, human fibroblast, and HeLa cells possess such high M_r activities, but also highly purified *E. coli* DNA polymerase III core enzyme, *U. maydis* DNA polymerase, and *D. melanogaster* embryo and calf thymus DNA α polymerases. The evidence that these activities are responsible for chromosomal DNA replication is genetical (*E. coli*, *U. maydis*, and *D. melanogaster*); also, the high M_r activity disappears from rat neurones during differentiation from an actively dividing precursor cell to a postmitotically mature neurone. Furthermore, when limited proteolysis is allowed to occur, a defined and remarkably similar pattern of intermediate M_r activities is generated in lower eukaryotic and high eukaryotic extracts and, to some extent, in prokaryotic extracts. In higher eukaryotic extracts, a low M_r activity of ≈35,000 is also generated. Protease inhibitors can retard formation of these catalytically active proteolytic fragments. We propose that the replicative DNA polymerase complex of both prokaryotes and eukaryotes contains a high M_r polypeptide responsible for chain elongation which might be conserved during evolution and which is extremely sensitive to proteolytic cleavage.**

Although DNA replication requires a concerted action of many proteins in a complex, the DNA polymerase plays a central role (1–6). The function of the replicative polymerase, to catalyze DNA chain elongation, appears to be similar in all organisms investigated, and it might be expected to be highly conserved during evolution (1). Despite different susceptibilities to some inhibitors, these enzymes are similar in most biochemical properties. Antibodies generated against calf thymus DNA polymerase α crossreact with several mammalian α polymerases but not with *Drosophila melanogaster* DNA polymerase α and *Escherichia coli* DNA polymerase I (7). The majority of replicative polymerases, even when purified to homogeneity as judged by electrophoresis in nondenaturing polyacrylamide gels, are themselves found to be complexes of two or more polypeptides; in general, it has not been possible to determine in which polypeptide the polymerizing activity resides and what functions may be provided by the others (8–17). There is now genetical and biochemical evidence that some proteins that copurify with *E. coli* DNA polymerase III are necessary for and do contribute defined functions to DNA replication *in vivo* and *in vitro* (18–21). Biochemical evidence also indicates that in eukaryotes there are proteins associated with DNA α polymerases that increase the processivity of polymerization (22, 23), permit chain elongation on single-stranded DNA (23), and bind diadenosine tetraphosphate, an initiator signal for DNA replication (12, 24). Another possible source of heterogeneity in the polypeptide band patterns of these enzymes after electrophoresis in denaturing gels is proteolytic degradation. This can generate low M_r polypeptide fragments which, however, still possess catalytic activity (11, 25).

We have developed a technique (26) to assay DNA polymerase activity *in situ* after electrophoresis of crude cell extracts, highly purified, or homogeneous enzymes in a $NaDodSO_4$/polyacrylamide gel containing a DNA template. We now have used this technique to identify and characterize (*i*) polypeptides with DNA polymerase activity in cell extracts and (*ii*) highly purified replicative DNA polymerase enzymes from both prokaryotes and eukaryotes. We present evidence that a high M_r polypeptide is responsible for DNA chain elongation in all cases, but that proteolysis can generate catalytically active fragments, even in the presence of some protease inhibitors. The similarity in sizes and numbers of active fragments so generated leads us to propose that aspects of the primary or tertiary structures of replicative DNA polymerases may be conserved during evolution.

MATERIALS AND METHODS

Chemicals. Acrylamide and *N,N'*-methylenebisacrylamide were from Bio-Rad, $NaDodSO_4$ (specially pure, product no. 30176), glycine, and *N,N,N',N'*-tetramethylethylenediamine were from British Drug House, calf thymus DNA and pancreatic DNase I were from Worthington, and deoxyribonucleoside triphosphates were from PL-Biochemicals. [^{32}P]TTP (2000–3000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from the Radiochemical Centre (Amersham, England).

Protease Inhibitors. Phenylmethylsulfonyl fluoride, purchased from Calbiochem, was dissolved in isopropanol to make a stock solution of 0.1 M. Analytical grade British Drug House sodium bisulfite was dissolved in water, the pH was adjusted to 7.5 with solid NaOH, and the solution was diluted to a concentration of 1 M $NaHSO_3$. Pepstatin (Sigma) was dissolved in water to give a final concentration of 1 mM.

Tissues. The following cells and tissues were used: *E. coli* HMS83 *pol* A1, *pol* B1, *thy*, *lys*, *lac*, *rha*, *str*^{r} from C. C. Richardson (27) was grown as described (18); the basidiomycete *Ustilago maydis*, *D. melanogaster* early embryos, and neurones from SIV-50 rats of different developmental stages were isolated by the method of Kuenzle *et al.* (28); calf thymus tissue (frozen

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† Present address: Institut für Pharmakologie and Biochemie, Medizinisch und Veterinär Fakultät de Universität Zürich, CH-8057 Zürich, Switzerland.

immediately in liquid nitrogen after slaughtering), MRC-5 human fibroblasts, and HeLa cells were grown as described (29).

Preparation of Crude Extracts. Crude enzyme fractions were prepared as described: *E. coli* HMS83, ammonium sulfate-backwashed fraction 2 (18); *U. maydis* fraction 1 (10); and *D. melanogaster* embryo fraction 1 (11). Crude extracts of rat neurones, calf thymus, MRC-5 human fibroblasts, and HeLa cells were as described (12). All extractions, unless otherwise stated, were performed at or near 0°C in the presence of phenylmethylsulfonyl fluoride (1 mM) in sterile buffers and were prepared for electrophoresis within 60 min of thawing the cells (4 hr in the case of *E. coli* ammonium sulfate-backwashed fraction 2). Alternatively, the extracts were immediately frozen and stored in liquid nitrogen until further use.

Enzymes. Homogeneous DNA polymerase I with or without the Klenow fragment was as described (26). DNA polymerase fractions were purified by established methods: homogeneous *E. coli* DNA polymerase III core (fraction VI) (9), *U. maydis* DNA polymerase (fraction IV) (10), *D. melanogaster* embryo DNA polymerase α (fraction VIII) (11), and calf thymus DNA polymerase α (fraction E) (12). PL-Biochemicals supplied the commercial DNA polymerase α from calf thymus (lot no. 0896). One unit of activity is defined as the incorporation of 10 nmol of dNTP into acid-insoluble DNA in 30 min at 37°C in the corresponding *in vitro* assay (9–12).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Detection of DNA Polymerase Activities. Details of this procedure have been described (26). Briefly, the steps were as follows. Extracts or enzymes in 65 mM Tris·HCl, pH 6.8/2 mM EDTA/125 mM 2-mercaptoethanol/10% (vol/vol) glycerol/1% (wt/vol) $NaDodSO_4$ were heated at 37°C for 3 min and electrophoresed in a 7.5% (wt/vol) polyacrylamide gel containing $NaDodSO_4$ and gapped calf thymus DNA. $NaDodSO_4$ was removed (60 min in 50 mM Tris·HCl pH 7.5/10 mM 2-mercaptoethanol at 37°C); proteins were renatured (30 hr in 50 mM Tris·HCl, pH 7.5/ 5 mM 2-mercaptoethanol/ 1 mM EDTA at 4°C); the gel was incubated in 70 mM Tris·HCl, pH 7.5/7 mM $MgCl_2$/10 mM 2-mercaptoethanol/45 μCi [α^{32}-P]dTTP (2000–3000 Ci/mmol)/14 μM each of dATP, dGTP, and dCTP for 18 hr at 37°C (the reaction is linear for up to 24 hr); and unincorporated [α-^{32}]dTTP was washed out with 5% (wt/vol) trichloroacetic acid/1% sodium pyrophosphate. The gel was dried and autoradiographed.

For protein staining, the gel was hydrated after autoradiography, stained in 0.25% (wt/vol) Coomassie blue/50% (vol/vol) methanol/10% (vol/vol) acetic acid for 90 min, destained first in 50% methanol/10% acetic acid for 60 min and then in 5% methanol/7% acetic acid for 24 hr.

Other Methods. Protein determination was by the Coomassie blue method of Bradford (30).

RESULTS

High M_r DNA Polymerases Are Found in Crude Extracts and Highly Purified Enzyme Preparations. Autoradiograms of crude extracts from *E. coli* and *U. maydis* cells, *D. melanogaster* embryos, rat neurones, calf thymus tissue, human fibroblasts, and HeLa cells showed multiple bands of activity of $M_r \geq 125,000$ in each case (Fig. 1) when assayed under conditions optimal for polymerase α activity (26). The activities in excess of M_r 125,000 undoubtably comprise a small but significant fraction of the total cellular activity because they were detected only with high protein concentrations and after deliberate overexposure of autoradiograms as in Fig. 1 (which, therefore, cannot be interpreted quantitatively). After a short exposure, only the M_r 125,000 fraction of the high M_r activities in mammalian extracts was detected, and it must represent the major activity

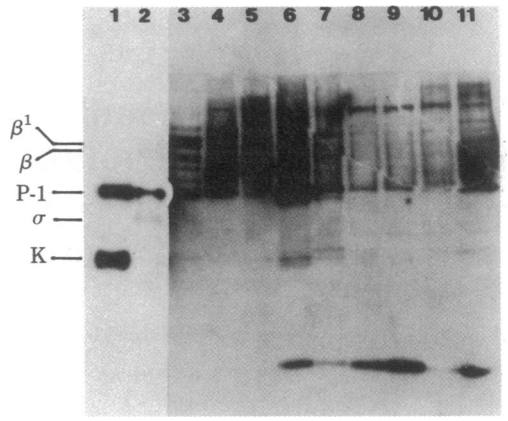

FIG. 1. Autoradiogram of DNA polymerase activities after $NaDodSO_4$/polyacrylamide gel electrophoresis of different prokaryotic and eukaryotic cell extracts. Lanes: 1, homogeneous *E. coli* DNA polymerase 1 containing a Klenow fragment (1.5 units); 2, DNA polymerase I lacking the Klenow fragment (1 unit); 3, *E. coli* HMS83 ammonium sulfate-backwashed fraction 2 (200 μg of protein); 4, *U. maydis* fraction 1 (100 μg); 5, *D. melanogaster* embryo fraction 1 (200 μg); 6, rat neuronal extract, 4 days before birth (100 μg); 7, same as lane 6, but 7 days after birth; 8, calf thymus extract (100 μg); 9, same as lane 8, but phenylmethylsulfonyl fluoride was omitted; 10, MRC-5 human fibroblasts extract (100 μg); 11, HeLa cell extract (100 μg). M_r markers were *E. coli* RNA polymerase β^1, β and σ subunits (165,000, 155,000, and 95,000, respectively) and *E. coli* DNA polymerase 1 (P-1; 109,000) and its Klenow fragment (K; 74,000) from the same gel but with a short autoradiography exposure time to calculate their exact positions (lanes 1 and 2).

(compare Fig. 1, lanes 6 and 7 with Figs. 4 and 5). The mammalian extracts also exhibited a low M_r activity of $\approx$35,000. The extensively purified *E. coli* DNA polymerase III core enzyme, *U. maydis* DNA polymerase, and *D. melanogaster* and calf thymus DNA α polymerases again all possessed a high M_r activity (Fig. 2). In addition, *U. maydis* DNA polymerase and calf thymus DNA polymerase α show three intermediate M_r activity bands and a low M_r activity band, respectively, which appear to be proteolytic degradation products.

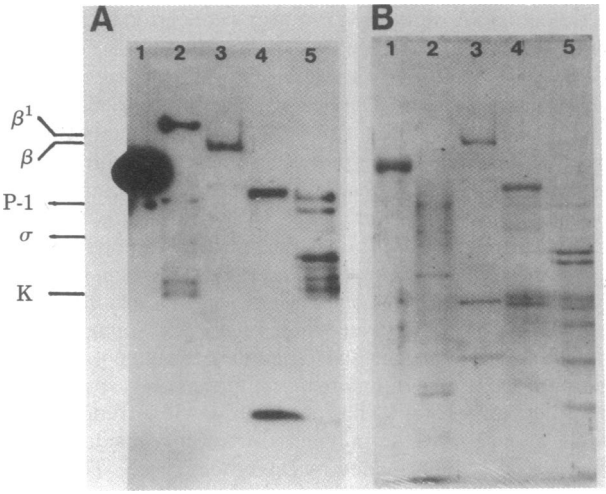

FIG. 2. Autoradiogram (*A*) and protein staining (*B*) of a $NaDodSO_4$/7.5% polyacrylamide gel of highly purified or homogeneous DNA polymerases. Lanes: 1, *E. coli* DNA polymerase III core enzyme (5 units); 2, *U. maydis* DNA polymerase (13 units); 3, *D. melanogaster* embryo DNA polymerase α (14 units); 4, calf thymus DNA polymerase α (20 units); 5, calf thymus DNA polymerase α from PL-Biochemicals (18 units). M_r markers are as in Fig. 1.

Storage of Crude Extracts Triggers the Generation of Active Proteolytic Fragments That Are Similar Throughout Evolution. That proteolysis might be responsible for generating the low M_r activities and, perhaps, the multiplicity of high M_r activities was investigated further by storing extracts in ice before electrophoresis to encourage limited proteolysis. Except for *U. maydis*, no extract then contained detectable polymerase activity of M_r >125,000 (Fig. 3), even after overexposure of the autoradiogram (not shown). In fact, there was a corresponding increase in activities in the intermediate (74,000–110,000) and low (35,000) M_r ranges (compare Figs. 1 and 3). The other striking aspect of Fig. 3 is the remarkable similarity in size and number of the major intermediate and low M_r activities generated by storage of the three mammalian extracts (calf thymus, human fibroblasts and HeLa cells). Storage of lower eukaryotic (*U. maydis* and *D. melanogaster*), and even to some extent prokaryotic (*E. coli*), extracts also generated activities with size similarities, but degradation to a M_r 35,000 activity did not occur. Furthermore, it is evident (Fig. 2, lanes 4 and 5) that a highly purified polymerase α from calf thymus (12) had only the genuine high M_r 125,000 band and the low M_r proteolytic fragment, whereas the commercially obtained, partially pure enzyme from the same tissue had at least six intermediate M_r activity bands, very reminiscent of those found after storage of the crude extract on ice (Fig. 3, lane 4). Although some proteolytic activity fragments showed DNA polymerase activity, they did not have a corresponding stained band, suggesting a very active proteolytic fragment (see, e.g., Fig. 2, lane 4).

Protease Inhibitors Diminish the Formation of Some Intermediate M_r and Mainly the Low M_r Catalytic Fragments. Fig. 4 shows the effect of different protease inhibitors on the activity pattern of calf thymus extracts. Pepstatin and sodium bisulfite reduced one or two of the intermediate M_r activities and more clearly the low M_r one. Thus, it appears that, depending on the isolation conditions, different molecular weight DNA α polymerases may be isolated.

The High M_r DNA Polymerases Are Involved in DNA Replication. Genetic evidence has established that the *E. coli* DNA polymerase III (5), the *U. maydis* DNA polymerase (6), and the *D. melanogaster* DNA polymerase α (31) all function in chromosomal DNA replication. Fig. 5 shows that the M_r 125,000 activity from rat neurones is the DNA polymerase α and also is involved in chromosomal DNA replication because (*i*) it has the same M_r as the isolated mammalian α-polymerase (Fig. 2) and, more significantly, (*ii*) this activity band decreased during late embryonic and early postnatal neuronal development with the arrest of cell division; this is quantitated and compared with the *in vitro* results (32) in Table 1.

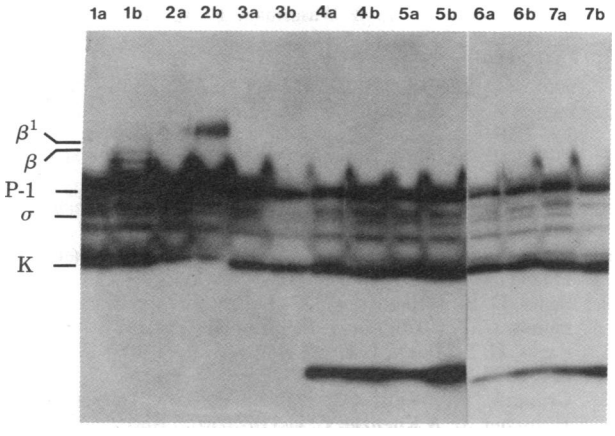

FIG. 3. Autoradiogram of DNA polymerase activities after a NaDodSO$_4$/7.5% polyacrylamide gel electrophoresis of cell extracts after storage on ice. The extracts were kept on ice for 18 hr before electrophoresis of 100 μg (*a*) and 500 μg (*b*) of the extracts. Lanes: 1, *E. coli* HMS83; 2, *U. maydis*; 3, *D. melanogaster* embryos; 4, calf thymus; 5, calf thymus with phenylmethylsulfonyl fluoride omitted; 6, MRC-5 human fibroblasts; 7, HeLa cells. (Note that in this autoradiogram the total activity in a pair of lanes is not always proportional to the amount of protein applied because of saturation effects). M_r markers are as in Fig. 1.

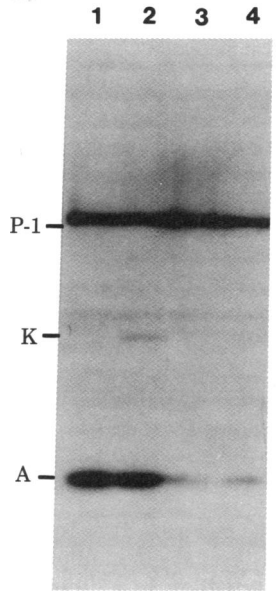

FIG. 4. Autoradiogram of DNA polymerase activities after a NaDodSO$_4$/7.5% polyacrylamide gel electrophoresis with calf thymus extracts isolated with different protease inhibitors. Freshly prepared calf thymus extract (50 μg) was electrophoresed immediately after preparation in the presence of different protease inhibitors. Lanes: 1, no protease inhibitor; 2, phenylmethylsulfonyl fluoride (1 mM); 3, pepstatin (1 μM); 4, sodium bisulfite (10 mM). M_r markers are as in Fig. 1, except for A (35,000), which was a M_r extrapolated from the other markers.

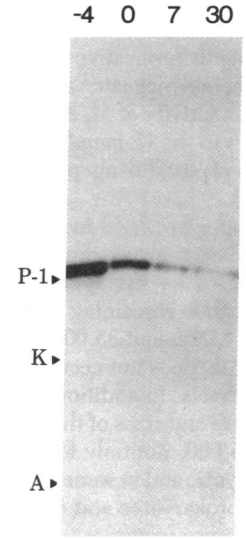

FIG. 5. Autoradiogram of DNA polymerase activities after a NaDodSO$_4$/7.5% polyacrylamide gel electrophoresis of neuronal extracts from different developmental stages. The lane numbers are days in relation to birth, the mean gestation period of the rats being 22 days. Fifty micrograms of protein of each extract was electrophoresed immediately after preparation. The assay incubation time was reduced to 10 hr. M_r markers are as in Fig. 1 and Fig. 4.

Table 1. DNA polymerase α activity is lost during terminal differentiation of rat neurones

Developmental stage, days*	Relative DNA polymerase α activity, %	
	In vitro†	Autoradiogram‡
−4	100	100
0	21	19
7	10	8
30	2	4

* See legend to Fig. 5.
† Data from reference (32).
‡ The autoradiogram of Fig. 5 was scanned with a Joyce Loebl Autodensidater type MK.3.

DISCUSSION

We have shown that cell extracts of both lower and higher eukaryotic organisms contained multiple high M_r ($\geq$125,000) DNA polymerase activities. In addition, higher eukaryotic extracts contained a low M_r activity of $\approx$35,000. Several lines of evidence indicate that this is derived from a higher M_r activity and is not DNA polymerase β or deoxyribonucleotidyl transferase activity because (*i*) the assay conditions were optimal for polymerase α activity; (*ii*) it decreased concomitantly with polymerase α activity during the development of rat neurones; (*iii*) rat neurones, human fibroblasts, and HeLa cells contain no transferase; (*iv*) no transferase activity was detected in the purified calf thymus enzyme, which also possessed the M_r 35,000 activity; and (*v*) it decreased when protease inhibitors were included during extract preparation.

Of the inhibitors tested, the popular phenylmethylsulfonyl fluoride was very inefficient in preventing the appearance of the low M_r activity in calf thymus tissue extracts, but the effects of this inhibitor are limited to the serine proteases. Pepstatin and sodium bisulfite were much more (but not completely) effective. In fact, sodium bisulfite was found to prevent the proteolytic degradation of the *D. melanogaster* DNA polymerase α during purfication to homogeneity (11, 25).

Highly purified preparations of *E. coli* DNA polymerase III core enzyme, *U. maydis* replicative DNA polymerase, and the α-polymerases of *D. melanogaster* and calf thymus tissue also all possessed a major activity of $M_r \geq$125,000. The three intermediate M_r activities in the *U. maydis* preparation appear to be generated by proteolysis during prolonged storage (unpublished observations).

When cell extracts were held in ice before electrophoresis to encourage limited proteolysis, the multiple high M_r activities disappeared, paralleled by the appearance of lower M_r ones. The major activities then remaining in the mammalian extracts were M_r 125,000, 74,000, and 35,000. Conversion to an active M_r 35,000 polypeptide does not occur in *E. coli*, *U. maydis*, or *D. melanogaster* extracts. In addition, there was a striking similarity in the numbers and sizes of the minor activities between M_rs 74,000 and 125,000, not only for mammalian extracts but also for lower eukaryotic and to some extent *E. coli* extracts. We propose that both prokaryotes and eukaryotes possess a replicative DNA polymerase (complex) containing a high M_r polypeptide responsible for DNA chain elongation. For higher eukaryotes we favor the activity of M_r 125,000 because it is the major one, especially in dividing rat neurones (Fig. 1, lane 6, and more clearly in Fig. 5). Furthermore, the remarkable similarity in the sizes and numbers of the minor activities generated by storage of extracts may result from the conservation, especially in higher eukaryotic organisms, of localized amino acid sequences or polypeptide conformations that are particularly susceptible to proteolytic cleavage, generating active fragments of defined sizes, a situation clearly illustrated by *E. coli* DNA polymerase I (1, 26). Such limited proteolysis would not necessarily lead to the dissociation of the resulting components of the polypeptide chain under native conditions, but would do so after reduction and denaturation by mercaptoethanol and $NaDodSO_4$, respectively. This proteolysis in conjunction with the complex structure of replicative DNA polymerases and their associated auxiliary proteins (11–13, 17–21) are possible reasons why the identity of the catalytic units of these enzymes is so controversial.

The origin of the multiple high M_r (>125,000) activities in cell extracts remains unknown. They comprise only a minor fraction of the total cellular DNA polymerase activity, and a meaningful estimate cannot be made at this time because of our present inability to inhibit proteolysis completely and because of possible differential specific activities and variations during the cell cycle under different conditions of growth. Thus, we cannot evaluate their significance or role in chromosomal DNA replication. They may represent cellular DNA polymerase γ or δ (33) or perhaps intermediates in the conversion of a precursor protein molecule to a form active in DNA replication *in vivo*. A serious problem lies in our lack of information on whether or not proteolysis occurs within an intact cell. Genetically programmed conversion of a DNA polymerase precursor by proteolytic cleavages is an attractive hypothesis to regulate DNA replication in advance of initiation and/or elongation. Precursor and products may then have different affinities for the type of replication complexes found for ϕX174 and T4 bacteriophages (34, 35).

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