A Cell-cycle-dependent DNA Polymerase Activity that Replicates Intact DNA in Chromatin

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An insoluble DNA polymerase activity that replicates the intact chromatin template at 85% of the rate found in vivo has been partially characterized. HeLa cells, encapsulated in agarose microbeads, are lysed using an isotonic salt concentration: the resulting encapsulated nuclei contain polymerase associated with a nucleoskeleton and the unbroken template. This preparation can be manipulated freely without aggregation or breaking the DNA and yet is accessible to enzymes and other probes. The major activity, which is sensitive to aphidicolin, is found only in S-phase nuclei and replicates DNA semi-conservatively, forming intermediates that are ligated efficiently into larger products.

1. Introduction

Nuclei and chromatin aggregate and jellify in an isotonic salt concentration (see, e.g., Ohlenbusch et al., 1967). As a result, they are generally studied under hypo- or hypertonic conditions in the presence of "stabilizing" cations. Such conditions significantly affect both the template and replicating complex. For example, the stabilizing cations activate degradative nucleases so that the template is inevitably broken. As we know that template integrity and supercoiling are essential prerequisites for efficient replication of plasmids in bacteria (Gellert, 1981; Mukherjee et al., 1985), and that breaks and nicks activate certain polymerase activities (for a review, see Kornberg, 1974), broken templates are far from ideal. Furthermore, non-physiological concentrations of salt may not only alter the properties of the chromatin template but also those of the polymerase, again generating aberrant activities (Jackson & Cook, accompanying paper). Thus nuclei prepared from non-replicating cells (i.e. those in G₀ or G₁) using conventional procedures synthesize significant amounts of DNA in vitro so that S-phase synthesis can only be seen above this background. For example, S-phase nuclei replicate their DNA in vitro at a rate only two to seven times that of their G₁-phase counterparts (Friedman, 1970; Seki & Mueller, 1976; Brun & Weissbach, 1978) and crude activities assayed using broken DNA also vary less than fivefold when extracted from cells or nuclei at different phases of the cell cycle (Spadari & Weissbach, 1974; Chu & Baril, 1975; Delfini et al., 1985; Foster & Collins, 1985; for a recent review, see Thömmes et al., 1986).

Recently we have isolated chromatin containing intact DNA using an isotonic concentration of salt (Jackson & Cook, 1985a). Living cells are encapsulated in 0.5% agarose microbeads, which are about 50 μm in diameter. The bead pores permit free exchange of protein <1.5 x 10⁻⁵ M, but not chromosomal DNA or chromatin. Therefore, when encapsulated cells are immersed in Triton X-100 and EDTA at an isotonic salt concentration, most cytoplasmic proteins and RNA diffuse out through the pores to leave encapsulated nuclei containing unbroken DNA and surrounded by a residual cytoskeleton. This chromatin, whilst protected from aggregation and shearing, is nevertheless completely accessible to enzymes and other probes used in modern molecular biology. Our nuclei differ from their unencapsulated counterparts prepared by conventional procedures in that they contain unbroken DNA and have never been exposed to non-isotonic salt-concentrations. (Note that since the ionic conditions within living nuclei are unknown, we necessarily use the term "isotonic" loosely and cannot guarantee that our procedure yields "native" nuclei or chromatin. Our buffers contain 150 mM monovalent cations.)

Previously we have used this preparation to investigate the site of transcription and replication, and have suggested that both occur at the nucleoskeleton (Jackson & Cook, 1985b, 1986a). We now report on some of the properties of the major
DNA polymerase activity within these encapsulated nuclei. We would stress that this insoluble activity is easily obscured by two other soluble activities that prefer broken templates. One arises by dissociation of the insoluble activity and the other is released from cells whatever their stage in the cell cycle (Jackson & Cook, 1986b, accompanying paper).

2. Materials and Methods

(a) Cells

Unsynchronized HeLa were used unless stated otherwise and were grown as suspension cultures in minimal essential medium (Eagle, 1959) plus 10% foetal calf serum. CV1 and Cos7 cells (Gluzman, 1981) were grown as monolayers in Dulbecco's modification of minimal essential medium (Smith et al., 1980) supplemented with 10% newborn calf serum.

HeLa cells were synchronized in mitosis (mitotic index >95%) using a single thymidine block, followed by nitrous oxide under high pressure (Rao, 1968; Warren & Cook, 1978) and at the G1/S boundary by a double thymidine block (thymidine block for 24 h, reverse the block and grow for a further 10 h in 5 mM-deoxyctydine, before blocking again with thymidine for 14 h).

In most experiments cells were grown for 18 to 24 h in [3H]thymidine (0.05 μCi/ml; ~60 Ci/mmol) to label their DNA uniformly. This enabled corrections to be made subsequently for any slight variations in cell or nuclear numbers.

(b) Cell encapsulation and lysis

Cells were encapsulated in 0-5% agarose, lysed at 4°C for 30 min by addition of 3 vol. isotonic lysis mixture and encapsulated nuclei washed 3 times in an isotonic buffer (Jackson & Cook, 1985a). The isotonic lysis mixture contained: 0-5%, Triton X-100, 100 mM-KCl, 10 mM-Tris-HCl (pH 8.0), 25 mM-Na3EDTA, 1 mM-dithiothreitol; and the isotonic buffer: 100 mM-KCl, 25 mM-(NH4)4SO4, 10 mM-Tris-HCl (pH 8.0), 1 mM-EDTA, 1 mM-dithiothreitol and 0.5% glycerol.

In Fig. 1, nuclei were prepared using a conventional procedure. Cells were washed 3 times in a hypotonic buffer containing 5 mM-Tris-HCl (pH 8.0), 1 mM-EDTA, 0.2 mM-EGTA, 1 mM-dithiothreitol, 10 mM-KCl, 2.5 mM-MgCl2, swollen for 30 min on ice, broken by 15 strokes of a Dounce homogenizer and Triton X-100 was added to 0.5%. Nuclei were pelleted (100 g, 10 min at 4°C) and washed successively with 20 vol. of ice-cold hypotonic buffer twice, a hypotonic/isotonic buffer mixture (1:1, v/v) twice, and finally in isotonic buffer.

(c) Replication assays

Assays (500 μl) were generally conducted in isotonic buffer supplemented with 10 mM-potassium phosphate (pH 7-4), 5 mM-MgCl2, 1-25 mM-ATP, 0-1 mM-CTP, GTP and UTP, 0-25 mM-dATP, dCTP and dGTP, 2-5 mM-dTTP and various amounts of [32P]dTTTP (~3000 Ci/mmol; typically 20 μCi/ml). Encapsulated nuclei in isotonic buffer and a freshly made 10 times concentrated solution of supplements were incubated separately on ice, then at 37°C for 5 min before mixing. Samples (100 μl) were removed at various times subsequently and mixed with 250 μl 2%, sodium dodecyl sulphate. More than 2 h later 3 100-μl samples were spotted onto GF/C discs and the discs were extracted successively with 5% trichloroacetic acid, ethanol and ether, then dried and their radioactivity was estimated using a Packard 300 CD scintillation counter.

Individual DNA polymerase activities were distinguished by preincubation (15 to 30 min at 4°C) with aphidicolin (20 μg/ml), dideoxymidine triphosphate (5 μM) or N-ethylmaleimide (10 mM).

(d) Autoradiography of cells and nuclei

Cells were encapsulated, the beads washed in medium, grown at 37°C in [3H]thymidine (10 μCi/ml; 59 μCi/mmol) for 30 min to label S-phase cells and lysed. Encapsulated nuclei within washed beads were incubated for 15 min in the replication mixture containing 0-01 mM-UTP, [3H]dTTP (50 μCi/ml; 97 Ci/mmol) and no unlabelled dTTP. Labelled samples were washed 3 times with isotonic buffer and eosin added to 0-01% to mark the nuclei. A 250-μl sample of these beads, with a packed volume of 80% (Jackson & Cook, 1986a), was introduced as a droplet into the centre of 2-5 ml Tissue-Tek II O.C.T. compound (R. A. Lamb) and frozen in liquid nitrogen. Frozen sections (10 μm) were air-dried, fixed by rinsing in methanol (2 x 15 s) and washed successively in 5% trichloroacetic acid (3 x, 1 h) and distilled water (5 x, 1 h; 2 x, 12 h). Slides were dipped in K-5 emulsion (Ilford Scientific Products), exposed for 3 to 10 days at 4°C and silver grains developed with D19 developer (Kodak).

(e) Preparation of DNA

Plasmid DNA was purified in gradients of CsCl containing ethidium (Maniatis et al., 1982), and genomic DNA by the proteinase K/SDS method (Gross-Bellard et al., 1973). DNA in encapsulated nuclei was purified as follows. After replication, 0-25 mM-dTTP was added to prevent further incorporation of [32P]TP, chromatin was fragmented with HaeIII (1000 units/ml, 30 min at 37°C) to reduce the size of the DNA, the volume increased 3-fold, and RNase A (25 μg/ml) and Sarkosyl (0-5%) added for 2 h at 45°C, followed by SDS (0-5%) and proteinase K (1 mg/ml) for 1 h at 37°C. Agarose was removed by centrifugation (30,000 g for 20 min) and DNA purified from the supernatant by extraction with phenol. More than 90% DNA prepared in this way is between 1 and 5 kb long (Jackson & Cook, 1980b, 1986).

(f) Gel electrophoresis and blotting

DNA was cut with restriction enzymes (Boehringer), fragments were separated by electrophoresis in 0-8% agarose gels containing 40 mM-Tris, 2 mM-EDTA and 20 mM-sodium acetate (pH 8-3), electro-eluted from gels and precipitated with ethanol as described by Maniatis et al. (1982).

Prior to hybridization, DNA in gels was visualized by staining with ethidium bromide (0-1 μg/ml; 30 min) and transferred to nitrocellulose (Southern, 1975). Nitrocellulose filters were prehybridized (4 to 16 h; 42°C) in 0-1 ml/cm² of: 50% formamide, 0-45 mM-NaCl, 45 mM-trisodium citrate, 0-6%; Ficoll, bovine serum albumin and polyvinylpyrrolidone, and 500 μg yeast tRNA/ml and then hybridized (16 to 48 h; 42°C) in 0-05 ml/cm² of the above solution containing heat-denatured [32P]DNA.

† Abbreviations used: SDS, sodium dodecyl sulphate; kb, 10³ base-pairs; bp, base-pairs.
Filters were rinsed 3 times in 0.3 M-NaCl, 30 mM-trisodium citrate, 0.1% SDS (20 min; 20°C) then twice in 15 mM-NaCl, 1.3 mM-trisodium citrate, 0.1% SDS (20 min; 60°C). Autoradiographs were prepared using pre-flashed (Laskey & Mills, 1977) Fuji RX X ray film and Ilford intensifying screens at -70°C.

(g) Analysis of the size of nascent DNA
Following replication in vitro encapsulated nuclei were washed 5 times in 30 vol. of ice-cold isotonic buffer and incubated (36°C; 2 h) in 1% SDS and proteinase K (1 mg/ml). NaOH was added to 0.2 M and after 5 min at room temperature an equal volume of 2 x electrophoresis buffer supplemented with 5% Ficoll was added and the sample subjected to electrophoresis in a 2% agarose gel. Gel slices were heated to 90°C in 200 µl 1 M-HCl, 1% SDS and their radioactivity was determined.

(h) Fluorometry
The ethidium-binding capacity of encapsulated nuclei was determined on ice-cold samples as described (Cook & Brazell, 1978; Jackson & Cook, 1985a). Every experiment involved a comparison of the fluorescence of γ-irradiated (9-6 J/kg) and unirradiated samples. Encapsulated nuclei were incubated in replication mixture at 37°C and after various times EDTA and NaCl were added to final concentrations of 10 mM and 2 M, respectively. Half were γ-irradiated, ethidium was added to both samples (8 µg/ml final concentration) and their fluorescence was measured. After subtraction of appropriate blanks, the fluorescence of dye bound to unirradiated nuclei was subtracted from that of irradiated nuclei and expressed as a percentage of the difference between the fluorescence of dye bound to unirradiated and irradiated controls incubated on ice.

3. Results

(a) DNA replication in vitro
Figure 1 illustrates the rate of incorporation of [32P]dTTP into acid-insoluble material by HeLa nuclei prepared conventionally by homogenization in a hypotonic buffer. Encapsulated HeLa nuclei (prepared using an isotonic salt concentration) incorporate label initially at about twice the rate and this incorporation continues for longer (Fig. 1). It is absolutely dependent on unlabelled triphosphates and MgCl₂ (Table 1). However, omission of CTP, GTP or UTP from the replication mixture.

![Figure 1. Polymerase activity in free and encapsulated nuclei. Cells were labelled for 24 h with [3H]thymidine, free nuclei isolated by a conventional procedure () or encapsulated nuclei prepared by isotonic lysis (■) and the incorporation of [32P]dTMP into acid-insoluble material measured using our standard conditions. The third curve (□), with the scale on the right, illustrates the incorporation by encapsulated nuclei under optimum conditions, i.e. standard replication mixture adjusted to pH 7.75, 7 mM-MgCl₂ and 250 µM-[32P]dTTP (1.6 Ci/mmol). Samples contained roughly 5 x 10⁶ nuclei/ml; the incorporation of ³²P by exactly equal numbers of nuclei was determined by normalization using the radioactivity due to ³H.

Table 1
<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>Treatment with nuclease following polymerization</th>
<th>Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>- CTP or - GTP or - UTP</td>
<td>90–95</td>
<td></td>
</tr>
<tr>
<td>- dCTP or - dGTP</td>
<td>92–97</td>
<td></td>
</tr>
<tr>
<td>- dGTP and GTP</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>- ATP</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>- All ribonucleoside triphosphates</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>- All unlabelled deoxyribonucleoside triphosphates</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>- All unlabelled triphosphates</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>- MgCl₂</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>DNase</td>
<td>0–5</td>
</tr>
<tr>
<td>Complete</td>
<td>RNase</td>
<td>99</td>
</tr>
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</table>

Cells were labelled overnight with [3H]thymidine, encapsulated (10⁷/ml agarose) and lysed in isotonic buffer. Reactions were initiated by mixing encapsulated nuclei with complete or deficient mixtures containing 2.5 µM-dTTP and 20 µCi [32P]dTTP/ml (~3000 Ci/mmol). The incorporation of ³²P into acid-insoluble material was determined 15 min later. In 2 samples, further incorporation of ³²P was reduced by adding dTTP to 0.25 mM and nucleases added (25 µg/ml, 30 min, 37°C) before counting.
which usually contains all four ribo- and deoxyribo-
nucleotides, has little effect, while omission of ATP
roughly halves incorporation (Table 1). Incorpora-
tion is reduced significantly when a particular base
is omitted (i.e. no GTP and no dGTP), but omission
of all four unlabelled deoxyribonucleoside triphos-
phates leaves 40% of the incorporation, implying
an interconversion of ribose moieties to their deoxy
counterparts. In spite of this we have been unable
to show (unpublished results) any metabolic
channelling of ribonucleoside diphosphates into
DNA, using the conditions described by Reddy &
Pardee (1980). As expected, label is incorporated
into material that is sensitive to deoxyribo-
nuclease I but not ribonuclease A (Table 1).

(b) Damage to the template during replication

The isotonic replication conditions described in
detail in Materials and Methods have been
developed, bearing in mind a number of constraints.
The MgCl₂ concentration used routinely is sub-
optimal (Fig. 2) and reflects a compromise between
having sufficient to support replication without too
much to activate endogenous nucleases and so
damage the DNA. (Note that the 1 mM-EDTA and
2.3 mM-triphosphates will chelate most of the 5 mM-
Mg²⁺ present in the mixture.) The damage induced
in encapsulated DNA was investigated using the
intercalating dye, ethidium (Fig. 3). Supercoils can
only be maintained in intact circles of pure DNA;
nicking anywhere in the circle releases all super-
coiling. At high concentrations of ethidium less dye
binds to intact DNA than to its nicked and relaxed
counterpart. (This difference enables supercoiled
plasmid DNA to be freed of relaxed plasmid or
chromosomal DNA in caesium chloride gradients.)
As the fluorescence of ethidium is enhanced when
it binds, binding (and hence integrity) can be
monitored conveniently by fluorometry (Le Pecq,
1971). Adding 2 mM-NaCl to encapsulated nuclei
extracts the histones, leaving naked DNA loops of
about 200 kbp attached to a nuclear cage (Cook &
Brazell, 1978; Jackson & Cook, 1985a). If each loop
is nicked at least once by γ-irradiation, the
ethidium-binding increases from a normalized value
of 1.0 to about 1.25, indicating that nearly all the
DNA is supercoiled initially and can be relaxed by
irradiation (Jackson & Cook, 1985a).

When encapsulated nuclei were incubated at 37°C
for 15 minutes in the replication mixture, the
difference in dye-binding of unirradiated and
irradiated samples (determined after raising the salt
concentration to 2 M) was halved (Fig. 3). Since this
assay is so sensitive (one nick per loop giving the
maximum effect) this means that there is very little
net nucleolytic activity during the first five minutes
when the initial rate is measured. Integrity can be
maintained for longer in lower concentrations of
Mg²⁺ (Jackson & Cook, 1985a) but this reduces the
replication rate (Fig. 2).

(c) The efficiency of replication

Another important constraint on optimization of
the composition of the replication mixture was an
economical use of label. The concentration of dTTP
used (2.5 μM) is sub-optimal and supports an initial
rate of incorporation of 0.66 pmol dTMP/10⁶ cells
The effect of the nucleotide triphosphate concentration on the rate of dTTP incorporation. Cells were labelled for 24 h with [3H]thymidine, encapsulated (10^7/ml agarose) and lysed. A 50-μl sample of beads was incubated for 5 min in reaction mixtures containing equal amounts of each dNTP (2.5 to 100 μM; dTTP at 10 μCi/pmol) in a final volume of 100 μl and incorporation into acid-insoluble material was determined. V, amount of dTMP incorporated in pmol/10^6 cells per min; S, concentration of each dNTP (M).

This is about 8% of the rate found in vivo. (We assume that an average nucleus contains 12 pg DNA (Colman & Cook, 1977) and that one cell cycle takes 22 hours (Warren & Cook, 1978).) Increasing the dTTP concentration has a dramatic effect, which is reflected by the double-reciprocal plot illustrated in Figure 4. This gives a K_m of 85 μM for dTTP and a V_max of 5.6 pmol/10^6 cells per minute. As we shall see, reducing the pH from 8.0 to 7.75 also increases the rate. Therefore, the initial rate in vitro becomes 85% of the rate found in vivo at pH 7.75 in 250 μM-dTTP and 7 mM-MgCl_2 (Fig. 1). Under these optimal conditions about 0.06% of the template in the unsynchronized culture is replicated in 1 min.

(d) Replication throughout the cell cycle

The activity in encapsulated nuclei is not only very efficient, it is restricted to cells in S-phase (Fig. 5). Cells were synchronized at mitosis or the G1/S boundary, encapsulated and regrown: at hourly intervals samples were removed, encapsulated nuclei prepared and their polymerizing activity assayed. Nuclei isolated from a population in which >95% of the cells were in mitosis polymerized little dTTP, but what activity there was showed linear kinetics (Fig. 5(a) left-hand panel). The activity was due to polymerase β (see below, and unpublished results) and remained at a low level in nuclei derived from cells throughout G2. Cells in S yielded a much greater activity, with different kinetics, due to polymerase α (see below). It declined in G2 nuclei (Fig. 5(b)). These initial rates are replotted in Figure 6: the activity found in vitro reflects the polymerizing activity in vivo, the maximal rate in S-phase nuclei being 50 times greater than the rate in mitotic nuclei. (We show in the accompanying paper that M and G2 cells contain inactive polymerase α that can be activated on provision of a broken template.)

This correlation between activities found in vitro and in vivo in populations of cells was extended to single cells by autoradiography. Unsynchronized HeLa cells were encapsulated and split three ways...
Figure 6. Polymerase activity through the cell cycle. The initial rates of DNA synthesis at different stages of the cell cycle are taken from Fig. 5. Maximal rates obtained with each set of synchronized cells were aligned. Rough times of the different phases of the cell cycle are indicated and these agree with values obtained by conventional methods (Warren & Cook, 1977). The length of $S$ and $G_1$ were determined as follows. In experiment b pairs of daughter cells in beads (visualized by fluorescence microscopy of ethidium-stained beads (10 µg/ml)) first became evident 13 h after release of the thymidine block (i.e. 13 h after $G_1(S)$ and by 14 h 60% of the cell population had divided. The overall cell cycle time is about 22 h.

(Table 2). One sample was regrown in [14C]thymidine for 30 minutes. Another was used to prepare encapsulated nuclei, which were then labelled in vitro using [3H]dTTP. The third was labelled first with [14C]thymidine in vivo and then with [3H]dTTP in vitro. Beads from each sample were sectioned and autoradiographs prepared. A similar fraction of the population was labelled in vitro as in vivo (cf. samples 1 and 2). Differences in grain density and distribution (i.e. intense local label due to 3H and light diffuse label due to 14C) enable cells with dual labelling to be identified (Fig. 7); most labelled cells in sample 3 are doubly labelled. The small number of diffusely labelled nuclei in sample 3, which were also very lightly labelled, are probably from the 5% of cells that passed from S into $G_2$ during the 30-minute labelling in vivo. Remarkably, only two nuclei were locally labelled without any surrounding diffuse label, in these the grains due to 14C were probably below the detection level. Clearly, $S$-phase nuclei incorporate label in vitro, and conversely, non-$S$-phase nuclei do not.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Labelling regime</th>
<th>% Cells labelled</th>
<th>Diffuse label</th>
<th>Localized label</th>
<th>Diffuse + localized label</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>[14C]thymidine, in vivo</td>
<td>32</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>[3H]dTTP, in vitro</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[14C]thymidine, in vivo then [3H]dTTP, in vitro</td>
<td>5</td>
<td>&lt;1</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

Unsynchronized cells were encapsulated and labelled: sample 1, in vivo for 30 min in [14C]thymidine; 2, in vitro for 15 min with [3H]dTTP; 3, in vivo for 30 min in [14C]thymidine, then in vitro for 15 min with [3H]dTTP. After cutting sections, autoradiographs were prepared, stained and the labelled cells were expressed as a percentage of the total. Grain patterns were categorized as localized (due to 3H) or diffuse (due to 14C). About 500 cells were counted for each sample.

Figure 7. Autoradiography of cells and nuclei labelled in vivo and in vitro. An illustration of typical autoradiographs used for the analysis described in Table 2. After a 10-day exposure, samples 1 (in (a)) and 2 (in (b)) showed about 75 and 500 silver grains/nucleus by autoradiography, compared with 250 and 6000 disints/cell per 10 days by scintillation counting, respectively. Background levels were < 1 grain/nuclear area. Note that in sample 1 the grains are much more widely scattered than the highly localized and denser pattern in 2. As a result dual labelling in sample 3 (in (c)) can be detected. An unlabelled nucleus from sample 3 is also shown in (d). The bar represents 5 µm.
Figure 8. DNA polymerase activities at different pH values. Cells were labelled for 24 h with $[^{3}H]$thymidine, encapsulated and lysed. Identical samples were washed 3 times with 20 vol. of isotonic buffer with different pH values and their polymerizing activity was determined (●). In some cases inhibitors were present for 30 min before (preincubation on ice) and during synthesis. α (●) and β + γ (■) DNA polymerase activities were distinguished using aphidicolin or dideoxythymidine triphosphate, respectively.

(e) Polymerase α is the major activity

Different DNA polymerase activities have different pH optima and sensitivities to inhibitors; aphidicolin and high pH inhibiting polymerase α, and dideoxythymidine triphosphate inhibiting polymerases β and γ (Krokan et al., 1979). Between pH 7.5 and 8.0, >90% of the activity in unsynchronized cells is sensitive to aphidicolin (Fig. 8). The minor activity sensitive to dideoxythymidine triphosphate becomes relatively more active above pH 8.0 but the total activity declines as polymerase α is inhibited.

These results show that polymerase α is the major activity. We have not yet characterized the minor activity further but these inhibitor studies indicate that most of it is polymerase β. Furthermore, the following experiment (unpublished results) suggests that polymerase γ (the enzyme involved in replicating mitochondrial DNA) constitutes only a minute fraction of the total activity.

(f) Properties of replication in vitro

The polymerizing activity is clearly efficient and restricted to S-phase nuclei. Can it initiate synthesis of new chains and can it do so specifically? The origin of replication in simian virus 40 (SV40) is one of the few sequences at which specific initiation has been demonstrated: initiation depends on functional T antigen (De Pamphilis & Wassarman, 1980; Ariga & Sugano, 1983; Li & Kelly, 1984; Wobbe et al., 1985). Therefore, we added plasmid DNA containing the SV40 origin (i.e. pSVtkneo; Townsend et al., 1984) to encapsulated nuclei prepared from a monkey cell-line that expressed T antigen (i.e. Cos7; Gluzman, 1981). Monkey cells lacking T antigen (i.e. CV1) and plasmid DNA lacking the origin (i.e. pBR322) provided suitable controls. After replication in $[^{32}P]$dTTP, DNA was purified, hybridized with fragments of plasmid DNA bound to filters and any hybridization of radiolabelled DNA was detected by autoradiography (Fig. 9). Replication proceeds in the suboptimal concentration of dTTP used in Figure 9 at a fraction of 1% of the rate in vivo, so we would expect only the origin to be replicated in 30 minutes if specific initiation was occurring. When mixed with encapsulated nuclei from Cos7 cells, pSVtkneo DNA became labelled uniformly (Fig. 9, panel b). This labelling depended on added DNA (Fig. 9, panel c), an active polymerase α (Fig. 9, panel d) and (only slightly) on the superhelical state of the template (Fig. 9, panels g and h). However, neither an origin or T antigen (Fig. 9, panels e and f) was required so that although synthesis was initiated, little (if any) appeared to be specific. Perhaps this is not too surprising as the nucleoskeleton-associated activity detaches on incubation, generating a soluble activity (Jackson & Cook, accompanying paper), which is largely responsible for the observed synthesis of plasmid DNA (D. A. Jackson & P. R. Cook, unpublished results). We are currently investigating whether the remaining synthesis is correctly initiated by a nucleoskeleton-associated activity.

The size of DNA synthesized in vitro was analysed by gel electrophoresis after denaturation (Fig. 10). After labelling for only ten seconds, 70% of the incorporated label migrates to the same position as markers <600 nucleotides long and so was in “Okazaki fragments”. Almost all the remaining 30%, is in fragments too long to be electroeluted from the beads. As the labelling time is increased, the label in Okazaki fragments increases until it reaches a constant level after 2-5 minutes (Fig. 11), even though the overall rate of incorporation is linear for ten minutes. This must mean that Okazaki fragments are efficiently ligated into larger molecules.

We next used the classic density-labelling procedure to see whether DNA was replicated semi-
Figure 9. Replication of plasmid DNA in vitro. Cos7 or CV1 cells were labelled with [3H]thymidine for 1 day, encapsulated (1.5 × 10⁷ cells/ml agarose), lysed and incubated with pSVtkneo or pBR322 DNA (10 µg/ml in panels b to f or 2.5 µg/ml in panels g and h) for 90 min at 4°C. The mixtures containing: b, Cos7 nuclei and pSVtkneo DNA; c, Cos7 nuclei alone; d, Cos7 nuclei and 10 µg aphidicolin/ml; e, Cos7 nuclei and pBR322 DNA; f, CV1 nuclei and pSVtkneo DNA; g, Cos7 nuclei and supercoiled pSVtkneo DNA; and h, Cos7 nuclei and relaxed pSVtkneo DNA, were allowed to synthesize DNA at 37°C with 50 µCi [32P]dTTP/ml (≈3000 Ci/mmol). After 30 min, unlabelled dTTP was added to 0.25 mM to dilute the label; 100 ± 5% DNA was prepared from each sample of 5 × 10⁶ labelled cells; heat denatured, hybridized with DNA bound to filters (3 × 10⁶ to 5 × 10⁶ cts/min, or 0.5 × 10⁶ cts/min in d, for each 2 × 10 cm filter for 48 h) and autoradiographs were prepared. Seven filters were prepared by Southern blotting gels like that illustrated in panel a, which shows a gel stained with ethidium and photographed under ultraviolet light. The left-hand channel of each gel contained 250 ng λ DNA cut with HindIII, the middle channel 50 ng each of pSVtkneo cut with HindIII and the following pSVtkneo fragments: 2.3 kb PvuII–EcoRI fragment; 0.9 kb EcoRI–HindIII fragment and 0.3 kb HindIII–PvuII fragment, which contains the origin; and the right-hand channel 5 µg genomic DNA from Cos7 cells cut with EcoRI plus 50 ng pSVtkneo DNA. In panels a to c the pSVtkneo DNA in the right-hand channel was linearized with HindIII and in panels d to h it was also cut with EcoRI. The genomic Cos7 DNA, with a doublet due to repeated sequences at approximately 7 kb, provides a suitable internal control. Exposures (panels b to h) were for 2 days.

Figure 10. The size of products of replication in vitro. Cells were labelled for 24 h with [3H]thymidine, encapsulated (10⁷/ml agarose), lysed and incubated in the replication mixture for 0, 10 s, 30 s, 1 min, 2.5 min, 3.0 min, 10 min, 30 min or 60 min. Next, DNA in beads was freed of protein and RNA, denatured and 50 µl containing DNA from approximately 0.25 × 10⁶ cells was applied to a 2% agarose gel and subjected to electrophoresis. Finally, the gel was silvered (0.5 cm) and the radioactivity in each slice was counted. Sample 1 contains beads recovered from the gel following electrophoresis and sample 2 is the first slice of the gel. In every case 3H was recovered only in the beads and the first gel slice. There was some variation (<10%) in the 3H recovered and so the cts/min due to 32P have been normalized to 3H present in all samples for each experiment. Arrowheads indicate the position of marker λ DNA cut with HindIII and denatured in NaOH (sizes in kb).
and the gradient fractioned. Finally the radioactive content and size of DNA in each fraction were determined, bulk DNA being visualized and sized by ethidium staining after electrophoresis and nascent DNA by autoradiography of the gel. Fraction 9 contained the most \(^3\)H, little \(^32\)P and fragments of 50 to 10,000 bp (with a centre of intensity of ethidium staining at 900 bp). This DNA had the buoyant density of unsubstituted molecules (i.e. bulk L/L DNA; see legend to Fig. 12). Unifilarly substituted DNA (i.e. L/H nascent DNA) banded around fraction 15. Autoradiography showed that it contained \(^32\)P-labelled fragments of 50 to 2500 bp (with a centre of labelling intensity at 500 bp). Ethidium staining gave a pattern similar to the \(^32\)P profile. The DNA fragments in the substituted (L/H) position clearly result from semi-conservative replication and have the expected size.

(g) Replication under modified ionic conditions

Figure 13(a) illustrates how increasing concentrations of KCl affect replication: the initial rate of synthesis falls progressively. To assess whether the salt disrupted the replication complex and template, or whether it directly affected the polymerization rate, encapsulated nuclei were pre-incubated in buffer containing up to 1.0 \(\times\) KCl, before replication was monitored in isotonic buffer (Fig. 13(b)). Pretreatment with >200 \(\text{mM}\) KCl dramatically reduces activity even though some persists after treatment with 500 \(\text{mM}\) KCl. Activity is maximal after pretreatment with 200 \(\text{mM}\) KCl, but this concentration, if present during synthesis, reduces the initial rate to 12%. Presumably the template is decompacted irreversibly whilst the activity is inhibited reversibly.

Figure 14 illustrates the kinetics of incorporation after various hypertonic or hypotonic pretreatments. Omission of KCl during pretreatment (leaving 50 \(\text{mM}\) monovalent cation) increases the initial rate by 10 to 15% though this higher rate is not sustained (cf. Fig. 14, a and b; these slight differences are quite reproducible). Omission of both KCl and (NH\(_4\))\(_2\)SO\(_4\) reduces dramatically both the initial rate and the overall incorporation after 30 minutes (Fig. 14(c)). It is probably this reduction in activity by hypotonic pretreatment that accounts for the reduced replicative activity of nuclei prepared by conventional procedures, seen in Figure 1. Hypertonic pretreatments further reduce incorporation, giving curves with profiles that are not characteristic of those seen in isotonic conditions (Fig. 14(d) to (f)). This aberrant activity is probably related to the activity seen in nuclear matrices (Smith & Berezney, 1982, 1983). Pretreatment with 2 \(\text{mM}\) KCl gives a higher subsequent
Figure 13. The effect of ionic conditions on replication. Cells were labelled for 24 h with [3H]thymidine, encapsulated (107/ml agarose) and lysed. Replication assays were performed using the standard mixture with 0 M to 1000 mM-KCl. For each salt concentration, samples were taken at 0, 5, 15 and 30 min and the initial rate of incorporation was determined. The initial rate at different KCl concentrations is shown in (a). In (b) encapsulated nuclei were pretreated for 30 min on ice in isotonic buffer supplemented with KCl (50 to 1000 mM) as shown on the abscissa before restoration of isotonicity and determination of the initial rate under isotonic conditions.

Figure 14. The effect of hypotonic and hypertonic pretreatment on the kinetics of replication. Encapsulated nuclei were prepared from [3H]-labelled cells and identical samples pretreated for 30 min on ice before washing in isotonic buffer and assay polymerase activity. Pretreatments were: curve a, isotonic control; b, isotonic buffer containing no KCl; c, isotonic buffer containing no KCl or (NH4)2SO4; d, isotonic buffer with 0.5 M-KCl; e, isotonic buffer with 1 M-KCl; f, isotonic buffer with 2 M-KCl.

Figure 15. The size of nascent DNA following various pretreatments. After 30 min replication, samples (0.5 x 10^6 cells) from the experiment illustrated in Fig. 14 were freed of protein and RNA, denatured and subjected to electrophoresis as described in the legend to Fig. 10. (a) to (f) illustrate the size profiles of single-stranded nascent DNA after pretreatments a to f in Fig. 14. The radioactivity in each gel slice is expressed as a percentage of the total. The incorporation after 30 min relative to that in the control (a) is indicated in the top right-hand corner of each panel.

4. Discussion

(a) Replication in a physiological salt concentration using an intact template

Replication has been studied in a wide variety of experimental systems, ranging from whole cells and their permeabilized derivatives, through nuclei and crude chromatin preparations to reconstituted soluble systems made from purified components (De Pamphilis & Wassarman, 1980; Ariga & Sugano, 1983; Li & Kelly, 1984; Wobbe et al., 1985). Perhaps not surprisingly, activities from each differ in their efficiency, template preference and other characteristics. We have developed yet another system with two main aims in mind; namely, that both the ionic environment of the nucleus and the integrity of the template should be preserved. We have only partly achieved these aims. As the ionic milieu of the nucleus is unknown, we can only guess at what buffers to use and have chosen to maintain the monovalent cations at roughly the physiological activity than 1 M-KCl (cf. Fig. 14(e) and (f)), perhaps because all the histones have been stripped from the template.

The size of the nascent DNA synthesized after these various pretreatments are illustrated in Figure 15. Pretreatment without KCl yields a profile like the control, with about 20% of nascent DNA as Okazaki fragments and the remainder as very large molecules retained within beads, i.e. in sample 1 (Fig. 15(a) and (b)). Pretreatment without both KCl and (NH4)2SO4 inhibits overall incorporation, especially into large fragments, and so probably inhibits ligation. After hypertonic pretreatment with 500 mM-KCl, the residual activity synthesizes nascent DNA with sizes roughly like the control (cf. Fig. 15(a) and (d)). However, after pretreatment with 1 M or 2 M-KCl, only short products are seen; ligation has been completely inhibited. The increased incorporation seen after pretreatment with 2 M-KCl (Fig. 14(f)), is due to the synthesis of a heterogeneous collection of fragments (Fig. 15(f)).
concentration. In contrast, divalent cations are removed during isolation and kept at a low level during replication to minimize nicking of the DNA. The hypotonic concentrations of salt usually used in the preparation of nuclei extract a quarter of the protein from encapsulated nuclei (Jackson & Cook, 1985a), and half their RNA and DNA polymerase activities (Jackson & Cook, 1985b; and Fig. 1). They also inhibit ligation of Okazaki fragments (Fig. 15). Similarly, hypertonic concentrations extract both polymerases and what remain have aberrant properties (Jackson & Cook, 1985b; and Figs 13 to 15).

We have been more successful in achieving our second aim. Initially the DNA in encapsulated nuclei is largely intact, as judged by the persistence of supercoiling after removing the histones. On replication, the DNA becomes progressively nicked so that little supercoiling can be seen after 30 minutes (Fig. 3). However, this represents very little damage since only one single-strand break in a loop of about 200 kb releases all supercoiling in that loop. It is clear that in the first few minutes an essentially intact template is replicated. The subsequent decline in synthetic rate cannot result from nicking since prior γ-irradiation does not decline is probably due to the dissociation of the polymerizing complex from its template or the nucleoskeleton (see accompanying paper).

The major activity in the S-phase nucleus is inhibited by aphidicolin and so is polymerase α (Fig. 8). Using our standard conditions the synthetic rate is about 80% of the rate in vivo and can be increased to 85%, of that in vivo by increasing the dTTP concentration and reducing the pH (Fig. 1). The activity is not found in G1-phase nuclei and, as the cells pass into S, it increases about 50-fold, declining slightly in the middle of S before increasing again at the end (see also Delfini et al., 1985; Foster & Collins, 1985).

(b) Comparison with other activities

The activity in S-phase nuclei, which is insoluble and attached to a nucleoskeleton (Jackson & Cook, 1986), is to be distinguished from at least two other soluble activities that are described in the accompanying paper. One can be extracted from cells at any stage of the cell cycle and the other is probably generated from the insoluble activity on incubation at 37°C. Both are active on broken templates and if these are provided they easily obscure the authentic activity described here. The activities studied by many others (Friedman, 1970; Spadari & Weissbach, 1974; Chiu & Baril, 1975; Seki & Mueller, 1976; Brun & Weissbach, 1978; Delfini et al., 1985; Foster & Collins, 1985) are probably mixtures of these three activities and so it is not surprising that the insoluble activity we have found has some similarity with them. It has similar K_m, pH optimum, and ATP requirement; in addition, ribonucleotide triphosphates will partly support DNA synthesis in the absence of their deoxy counterparts (for reviews, see Kornberg, 1974; De Pamphilis & Wassarman, 1980; Thiimmes et al., 1986). It differs in its (1) high efficiency, (2) absolute dependence on the phase of the cell cycle, (3) preference for an intact chromatin template (see also the accompanying paper), (4) salt optimum, which probably reflects its template preference, and in its (5) attachment to the nucleoskeleton (Jackson & Cook, 1986).

(c) Ligation

How efficient at ligation is our preparation? The following calculation shows that, under our standard conditions, ligation cannot limit the overall rate of synthesis. When living cells are labelled for one minute with [3H]thymidine, about 30% of the acid-insoluble label is found in Okazaki fragments and ligation intermediates (unpublished results). As the rate of synthesis in vivo is 8% of the rate in vitro, the same amount of DNA will be synthesized in (1 x 100)/8 – 12.5 min. After this time in vitro, the same percentage (i.e. 33%) is in Okazaki fragments (Fig. 11), so the efficiency of ligation must match the high overall efficiency. However, this may not remain true at the higher dTTP concentrations that are required to approach the rate in vivo.

Whether replication is discontinuous or semi-discontinuous is controversial (De Pamphilis & Wassarman, 1980). In principle, if synthesis is semi-discontinuous, no more than 50% of label can initially be in Okazaki fragments. We find about 70% of the label in Okazaki fragments in the first minute and, if we neglect the first two points, sampled after 30 seconds or less, our curve in Figure 11 extrapolates at zero time to 100%, consistent with discontinuous synthesis in both strands. However, it remains formally possible that the synthetic rate on one strand is lower than on the other under these conditions, so that we cannot yet clarify this issue.

(d) Initiation

How replication is initiated in eukaryotic nuclei remains poorly understood, the best-characterized system being that involving the SV40 T antigen and origin of replication (De Pamphilis & Wassarman, 1980; Ariga & Sugano, 1983; Li & Kelly, 1984; Wobbe et al., 1985). Although our preparation initiates replication of added DNA, it does not do so only at the origin (Fig. 9). However, it may be that the higher-order structure of the chromatin template and its relationship with the nucleoskeleton-associated polymerase determine the timing and specificity of initiation, so that it may prove difficult to achieve authentic and efficient replication using pure DNA. We hope that our preparation, in which the integrity of the template and the nucleoskeleton are preserved, will prove useful for such studies.
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References


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