# Replication occurs at a nucleoskeleton

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The site of S-phase DNA synthesis has been the subject of
recurring controversy. All recent evidence supporting a site
fixed to some nuclear sub-structure is derived from studies
in which cells or nuclei have been extracted in hypertonic salt
concentrations. The controversy centres on whether the re-
sulting nuclear matrices or cages have counterparts *in vivo*
or are simply artefacts. Using isotonic conditions throughout
the isolation and analytic procedures we have now reinvesti-
gated the site of replication. Cells are encapsulated in agarose
microbeads and lysed to leave encapsulated nuclei which are
nevertheless completely accessible to enzymes. After incuba-
tion with endonucleases, most chromatin can be electroeluted
from beads: however, nascent DNA and active DNA polymer-
ase remain entrapped. Since chromatin particles containing
DNA the size of 125 kbp can electroelute, we conclude that
the polymerizing complex is attached to a nucleoskeleton
which is too large to escape. We have also studied various
artefacts induced by departure from isotonic conditions.
Perhaps surprisingly, the hypotonic conditions used during
isolation of nuclei by conventional procedures are a signifi-
cant source of artefact.

*Key words:* chromatin/microbeads/nucleoskeleton/replication

## Introduction

Whether or not nuclear DNA is replicated at a fixed site—for
example, at the nuclear membrane or matrix—has been controver-
sial for many years (for review see Edenberg and Huberman,
1975). Recently it is becoming increasingly accepted that the
replication site is fixed and that DNA is polymerized as it moves
through a polymerization complex attached to part of a nuclear
sub-structure called variously the nuclear matrix or cage (Dijkwel
*et al.*, 1979; Pardoll *et al.*, 1980; McCready *et al.*, 1980; Smith
and Berezney, 1982, 1983). The evidence for such a view comes
entirely from studies on cells or nuclei that have been extracted
in hypertonic salt solutions: subsequent fractionation and auto-
radiography shows that much of the nascent DNA and some of
the relevant polymerase are closely associated with the sub-
structure. However, all this work is subject to one, as yet
unanswered, criticism: namely, that these associations involv-
ing nascent DNA and polymerase are simply artefacts induced
by exposure to hypertonic salt concentrations. An active
polymerase complex might be sticky, perhaps because it is rich
in DNA ends and single-strands, and so would precipitate when
disrupted on to any surrounding structure. Credence is lent to
such a trivial interpretation when minor alterations in the prepara-
tion of nuclear matrices profoundly alter their constitution (Kauf-
mann *et al.*, 1981; Kirov *et al.*, 1984) and there is controversy

as to whether nuclear DNA is ever specifically associated with
the matrix (Basler *et al.*, 1981; Kuo, 1982; Kirov *et al.*, 1984;
Mirkovitch *et al.*, 1984). Furthermore, the matrix itself may be
an artefact since no such structure is seen when nuclei are dispers-
ed under hypotonic conditions, for example in the preparation
of 'Miller' spreads (Miller, 1984). The view that the matrix plays
no part in replication is further reinforced by the isolation in
hypotonic solutions of soluble polymerases that work in the
absence of any skeletal elements (Ariga and Sugano, 1983; Li
and Kelly, 1984). We have now reinvestigated the site of DNA
synthesis using isotonic salt concentrations throughout the isola-
tion and analytic procedures. (Note that we necessarily use the
term isotonic rather loosely, for the precise ionic milieu of the
nucleus *in vivo* is unknown.)

Unphysiological salt concentrations have been used for the
studies described above because nuclei and chromatin general-
ly aggregate and jellify in and around 150 mM salt. Recently
we have developed a method for preparing chromatin contain-
ing intact DNA using isotonic salt concentrations (Jackson and
Cook, 1985a). First, cells are encapsulated in 0.5% agarose
microbeads using a simple procedure and then lysed with Triton
X-100 in a concentration of salt which lies roughly in the
physiological range. Most cytoplasmic proteins and RNA dif-
fuse rapidly out through pores in the beads to leave encapsulated
chromatin which is nevertheless completely accessible to
enzymes. This chromatin can be manipulated freely without
aggregation in a variety of conditions. It also contains intact
DNA since removal of the histones releases superhelical DNA.
Using this preparation we have investigated whether nascent
DNA and DNA polymerase α are associated with a nuclear sub-
structure. We have used the term nucleoskeleton to describe
the nuclear sub-structure found under isotonic conditions to
distinguish it from others isolated in hypertonic salt concentra-
tions (Jackson and Cook, 1985b). We would stress that the use
of our 'isotonic' conditions does not necessarily guarantee the
isolation of 'native' chromatin, only that artefacts are less like-
ly than if clearly hyper- or hypotonic conditions are used.

## Results

### Two models for replication

Two extreme views of how replication might occur are presented
in Figure 1. In A, the polymerase progresses along DNA un-
associated with any nucleoskeleton whereas in B, it is attached
to the nucleoskeleton and DNA moves through the polymeriza-
tion complex. The two can be distinguished using encapsulated
nuclei prepared under isotonic conditions, fragmenting the
chromatin with an endonuclease and finally electroeluting any
unattached material. If view B is correct then nascent DNA and
active polymerase will both remain associated with the nucleo-
skeleton which, being larger than bead pores, will remain en-
trapped: if view A is correct we might expect even low levels
of digestion to lead to their loss since replicating DNA is cut
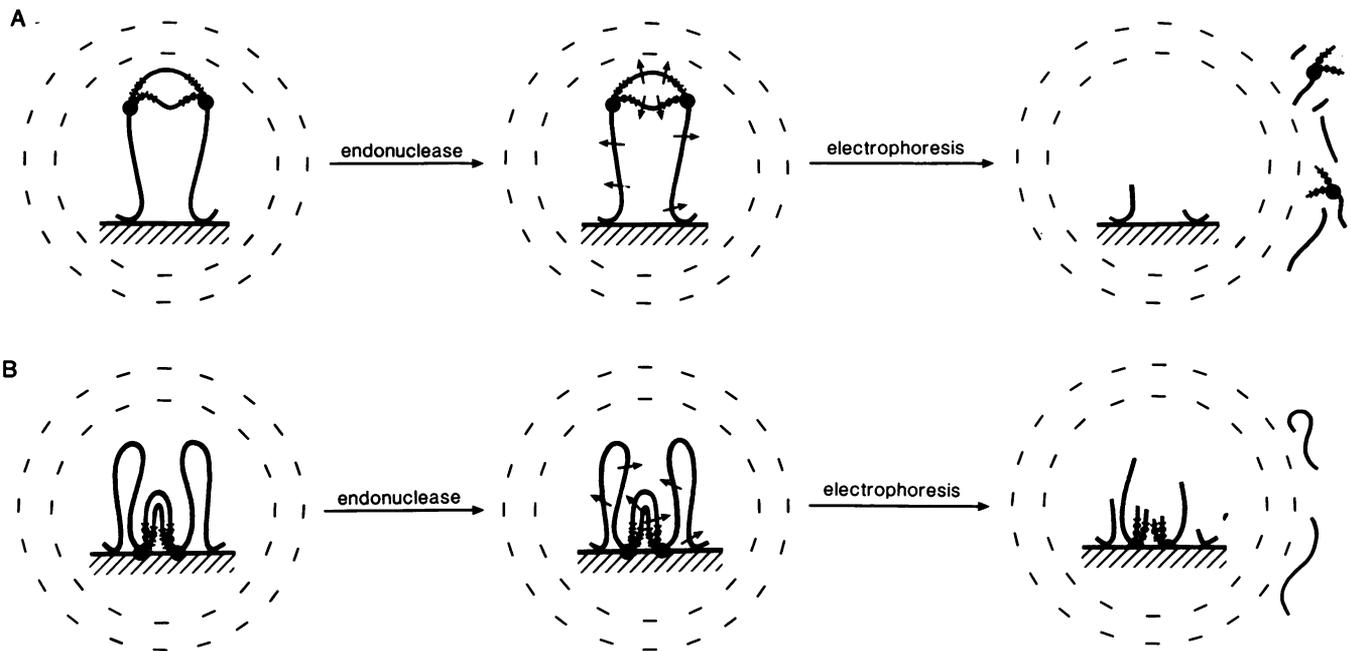preferentially by endonucleases (Seale, 1976).

**Fig. 1.** Two models for replication. (**A**) Two replication complexes (●) incorporate pulse-label (X) as they travel around a DNA loop (—) attached to the nucleoskeleton (hatched area). After cutting with an endonuclease (arrow) and electrophoresis, both pulse-label and polymerase are lost from the bead (concentric broken circles). (**B**) The replication complex is associated with the nucleoskeleton so that after cutting and electrophoresis, pulse-label and polymerase remain in the bead. Pulse-label and polymerase are lost in variants of **A** in which replication is initiated at the nucleoskeleton by a mobile polymerase that then moves around the loops or in which the DNA is unattached to the nucleoskeleton. Both are retained in a variant of **B** in which the origin of replication remains attached.
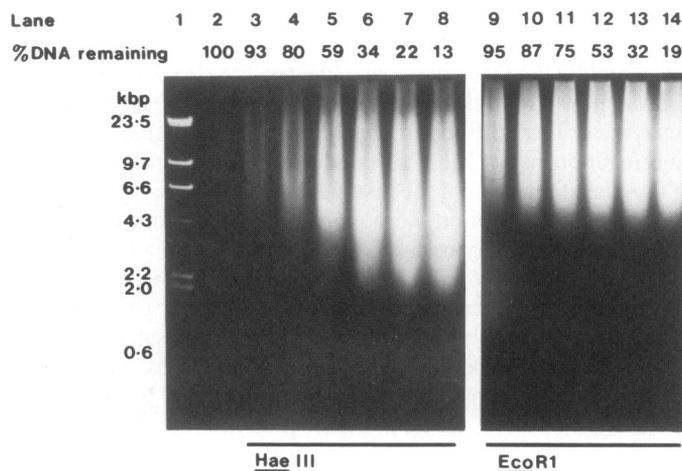


**Fig. 2.** Electroelution of chromatin from agarose beads. HeLa cells were labelled for 24 h with [$^3$H]thymidine (0.02 $\mu$Ci/ml), encapsulated ($10^7$ cells/ml), lysed and washed. MgCl$_2$ was added to 2.5 mM and the encapsulated chromatin treated with 500 units/ml HaeIII (**lanes 3–8**) or 5000 units/ml EcoRI (**lanes 9–14**) for 0 (**lane 2**), 2.5 (**lanes 3, 9**), 5 (**lanes 4, 10**), 10 (**lanes 5, 11**), 20 (**lanes 6, 12**), 40 (**lanes 7, 13**) and 80 mins (**lanes 8, 14**). EDTA was added to 2.5 mM and the samples subjected to electrophoresis in isotonic buffer supplemented with Tris. The gel was treated as described in Materials and methods, stained with ethidium and photographed. Beads were recovered and their radioactive content expressed as a percentage of that in the undigested control (**lane 2**). The sizes of marker λ DNA cut with HindIII (**lane 1**) are indicated in kbp on the left.

## Electroelution of chromatin particles from beads

Very large particles are able to pass through pores in agarose beads. For example, 1% beads are used routinely (e.g. as Biogel A-150m from Biorad) in chromatographic fractionation of par-

ticles up to 150 × 10$^6$ daltons and we have shown that chromatin containing DNA fragments of 125 kb can electroelute from 0.5% agarose beads (Jackson and Cook, 1985b). Since such particles have a weight at least thirty times greater than the weight of one mammalian replication complex (Noguchi *et al.*, 1983), we would expect the much smaller chromatin particles generated in our experiments to escape readily. Figure 2 illustrates a typical experiment in which encapsulated nuclei were incubated with HaeIII or EcoRI and the digestion mixture loaded on to an agarose gel under isotonic conditions and subjected to electrophoresis. Negligible amounts of chromatin escape from control beads untreated with enzyme (lane 2): as the amount of digestion increases, progressively more chromatin enters the gel. DNA fragments in chromatin migrate at almost half the rate that they do as free DNA (Jackson and Cook, 1985b) so that when >80% of the chromatin has been detached by EcoRI, most DNA has been cut to pieces of <30 kbp. The fragments are even smaller after cutting with HaeIII.

## Detaching chromatin detaches little nascent DNA

We first determined whether nascent DNA was retained within beads after removing most chromatin. HeLa cell DNA was uniformly labelled with $^{14}$C, the cells encapsulated and grown in [$^3$H]thymidine for 30 s to label nascent DNA (Figure 3a). After lysis, encapsulated nuclei were treated with HaeIII for different times and then subjected to electrophoresis. In untreated controls, all the DNA—whether labelled with $^{14}$C or $^3$H—remains associated with beads, confirming the remarkable stability of encapsulated chromatin. In treated samples, variable amounts of chromatin were electroeluted so that less $^{14}$C (i.e. bulk DNA) remained. If replication sites in loops were scattered at random relative to the nucleoskeleton as in Figure 1A, fragmentation by HaeIII should lead to subsequent loss of $^{14}$C and $^3$H in equal proportions. As a result the normalized ratio
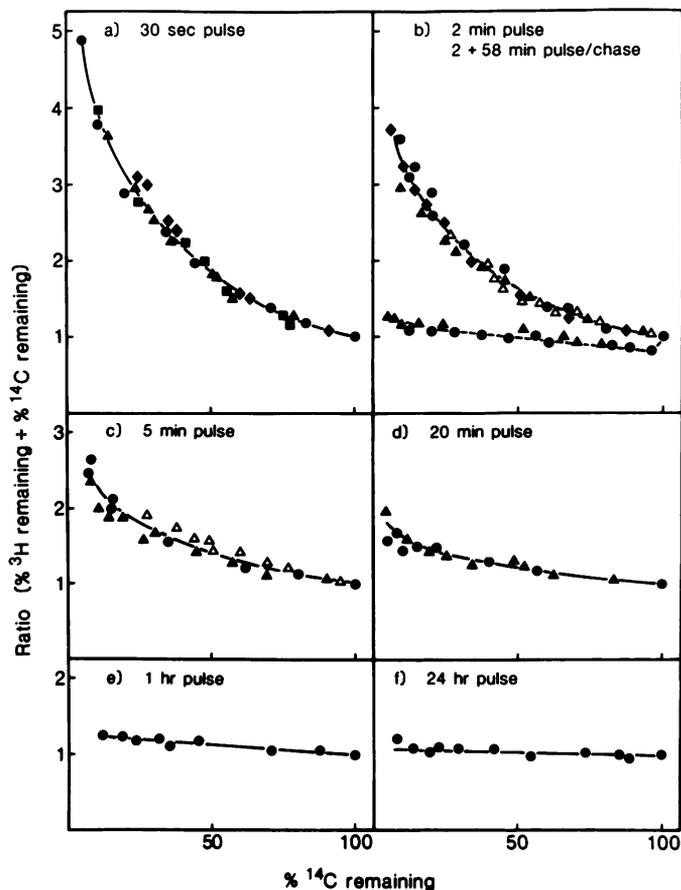
Fig. 3. Nascent DNA is closely associated with the nucleoskeleton. HeLa cells were labelled for 24 h with [$^{14}$C]thymidine, encapsulated and then pulse-labelled with [$^3$H]thymidine for various times (0.5 mins−24 h). An additional experiment involving a 2 min pulse then a 58 min chase is included in panel b (lower line). After lysis, encapsulated nuclei were incubated with HaeIII for various times, any detached chromatin removed by electrophoresis and the amount of radioactivity remaining in beads determined and expressed as a percentage of that obtained with undigested control samples. The ratio is (% [$^3$H] remaining in beads) ÷ (% [$^{14}$C] remaining in beads). Ratios greater than unity indicate that the sample contains more nascent DNA than total DNA relative to undigested controls. Different symbols in each panel refer to different experiments, closed symbols to electroelution in TEA and open symbols to electroelution in isotonic buffer supplemented with Tris.

(%[$^3$H] remaining in beads) ÷ (%[$^{14}$C] remaining) should remain at unity irrespective of the length of digestion or pulse. Indeed, since nascent DNA is more accessible than bulk DNA to endonucleases (Seale, 1976), we would expect more pulse-label to be lost than bulk DNA so that this ratio should fall from unity with increasing digestion.

In contrast, loss of $^{14}$C was accompanied by little loss of $^3$H so that the ratio increased above unity (Figure 3a). For example when all but 20% of the $^{14}$C has been removed, 63% of the $^3$H remains (i.e. the ratio is 63% ÷ 20% = 3.15; Figure 3a). This clearly shows that bulk chromatin can be removed without much nascent DNA, consistent with Figure 1B. As the pulse-length is increased the enrichment of $^3$H falls, i.e. increasing amounts of nascent DNA are lost with bulk chromatin so that the ratio declines towards unity (Figure 3). Presumably this is because nascent DNA synthesized at the nucleoskeleton early on in the pulse has time to move away and so be detached subsequently. After a 60 min pulse the distribution of pulse-labelled DNA almost mirrors that of bulk DNA and the ratio approaches

unity. When pulsed for 24 h both labels become detached equally and the ratio is unity irrespective of the degree of detachment (Figure 3f).

The association of pulse-label with the nucleoskeleton is not due to a labelling artefact since the pulse-label can be chased to give a ratio approaching unity at all degrees of detachment (Figure 3b). (The slight fall in ratio below unity on detaching very little $^{14}$C is reproducible and probably results from a persistent preferential sensitivity of some nascent DNA.) Furthermore, although it remains formally possible that the branched replication complex cannot escape because of its extended shape, this seems unlikely since pulse-label can be electroeluted if the encapsulated and digested nuclei are treated with SDS (results not shown), a procedure which we might expect to further extend it. We conclude that all the results in Figure 3 are consistent with the model in Figure 1B, but not 1A.

In similar experiments involving nucleoids isolated in 2 M NaCl we calculated the time taken to replicate a loop to be 11 min by analyzing a set of curves like those in Figure 3 (McCready et al., 1980). Assuming bidirectional replication and a synthetic rate of about 1 μm/min, this gave a size for the replicating loop of 22 μm or 60 kbp, consistent with other estimates of replicon size. A similar analysis of Figure 3 yields a time of 2 min [results not shown of J.Godwin of an analysis with α and λ both zero: see Appendix to McCready et al., (1980)]. This low value probably results from the preferential sensitivity of replicating chromatin to nucleases (Seale, 1976) which is not taken into account by the model.

Detachment of nascent DNA with DNase and micrococcal nuclease

The experiments described above used HaeIII to fragment DNA. Since deoxyribonuclease I (DNase I) was often used in analogous experiments with matrices, we have investigated the effect of this enzyme, as well as micrococcal nuclease, on detachment of nascent DNA from encapsulated nuclei (Figure 4). The way DNase I cleaves DNA depends upon the ionic composition of the medium (Campbell and Jackson, 1980). In Mg$^{2+}$ few double-strand cuts occur, with single-stranded breaks predominating whereas in Ca$^{2+}$ or Mn$^{2+}$ double-strand cutting becomes significant. Micrococcal nuclease mainly cuts double-strands in chromatin (Noll and Kornberg, 1977).

Cells were labelled for 24 h with [$^{14}$C]thymidine, encapsulated and then pulse-labelled for 2 min with [$^3$H]thymidine. After lysis, the encapsulated nuclei were treated with DNase or micrococcal nuclease, detached chromatin removed by electrophoresis and the relative proportions of $^{14}$C and $^3$H determined. As expected, the curve obtained with micrococcal nuclease is very similar to its counterpart obtained with HaeIII (Figure 4d): both enzymes make double-stranded cuts in chromatin. In contrast, DNase treatment yields different curves. In the presence of Mg$^{2+}$, the ratio initially rises and then falls below unity (Figure 4a). The ratio is very sensitive to enzyme concentration, being greater at any one degree of detachment of $^{14}$C if less enzyme is used for longer (Figure 4a, different symbols). If label incorporated during a 1 min pulse is chased for 1 min, the ratio falls below unity without an initial increase (Figure 4b), presumably because no label is now close to the nucleoskeleton. In Mn$^{2+}$, DNase yields a curve intermediate between those obtained with Mg$^{2+}$ and micrococcal nuclease (Figure 4c). These curves with DNase must reflect the complex interplay between single- and double-stranded cutting, the relative accessibility of nascent and bulk DNA, and the precise proportions and positions of pre-existing nicks and single-
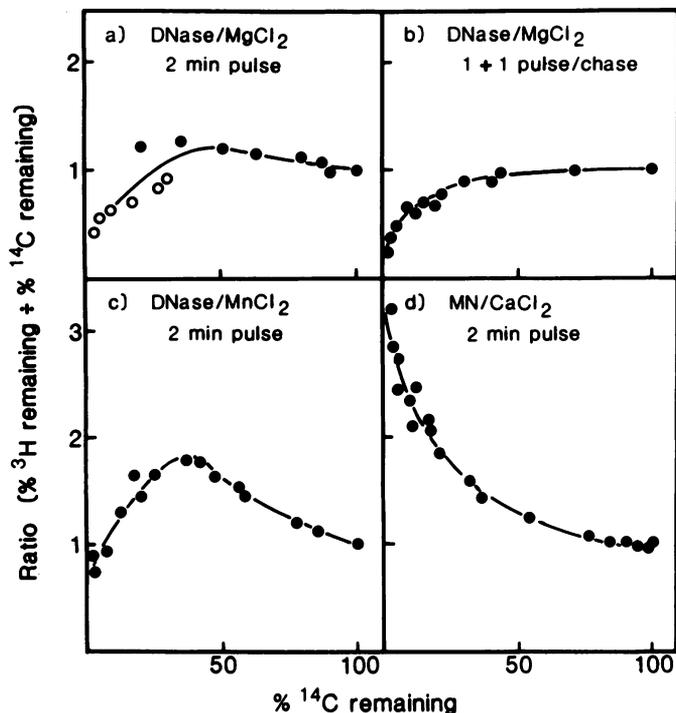
**Fig. 4.** The effect of DNase and micrococcal nuclease (MN) on the attachment of nascent DNA. HeLa cells were labelled for 24 h with [$^{14}$C]thymidine, encapsulated and then pulse-labelled with [$^3$H]thymidine for 2 min. In (b), a 1 min pulse was followed by a 1 min chase. After lysis, encapsulated nuclei were incubated for various times in the isotonic buffer supplemented as indicated, then any detached chromatin was removed by electrophoresis and the amount of radioactivity remaining in beads determined and expressed as a percentage of that obtained with undigested controls. The ratio is (% [$^3$H] remaining in beads) $\div$ (% [$^{14}$C] remaining in beads). The open and closed circles in (a) refer to incubations for various times (2.5−80 min) in the presence of two different concentrations of DNase (10 and 20 $\mu$g/ml): though this gives rise to two different curves, only one is drawn for ease of comparison with curves in b−d.



**Fig. 5.** The effect of S1 nuclease treatment on attachment of nascent DNA. Cells were labelled for 24 h with [$^{14}$C]thymidine, encapsulated and then pulse-labelled with [$^3$H]thymidine for 1 min. After lysis, encapsulated nuclei were incubated with S1 (0, 1000 or 10 000 units/ml; 37°C; 1 h). (a) After adding an equal volume 2× TEA, samples were subjected to electrophoresis and the radioactivity in gel slices measured. No $^3$H or $^{14}$C was electroeluted from untreated controls and no $^{14}$C from treated beads: some $^3$H electroeluted as chromatin fragments. The single-headed arrows indicate the position of marker $\lambda$ DNA cut with HindIII and double-headed arrows that of chromatin fragments containing 200 and 400 bp DNA. (b) Part of the samples that had been incubated with S1 were washed in isotonic buffer and treated with HaeIII, detached chromatin removed by electrophoresis and the radioactivity remaining in beads determined and expressed as a ratio (relative to undigested control) as described in Figure 3.

stranded regions relative to the nucleoskeleton. Nevertheless, one trend is clear: the enrichment of pulse-label seen with the various enzymes declines in the series: HaeIII or micrococcal nuclease > DNase in $Mn^{2+}$ > DNase in $Mg^{2+}$. This mirrors the decline in double-stranded cutting.

*The effect of pretreatment with S1 nuclease on subsequent detachment*

Whether pre-existing nicks influence subsequent detachment was investigated using the single-strand specific nuclease, S1. Sequences in and around replication complexes are rich in single-stranded regions (Dijkwel *et al.*, 1979) so S1 treatment should detach nascent DNA unless it is attached or too large to escape. (Unfortunately, the acidic conditions required for single-stranded cutting might well alter attachments, so that any conclusions drawn from such experiments must be treated with caution.) To investigate the effect of S1 treatment, cells were labelled for 24 h with [$^{14}$C]thymidine, encapsulated, pulse-labelled for 1 min with [$^3$H]thymidine, lysed, and the encapsulated nuclei incubated with S1 nuclease, layered on a gel and subjected to electrophoresis. All the $^{14}$C remained in beads. The $^3$H could be divided into three fractions. 13% migrated into the gel as a twin peak at the chromatin front and contained DNA fragments of 200 and 400 bp (Figure 5a, shaded area). One-tenth the concentration of nuclease released almost as much of this fraction, showing that it was very accessible. Since its total
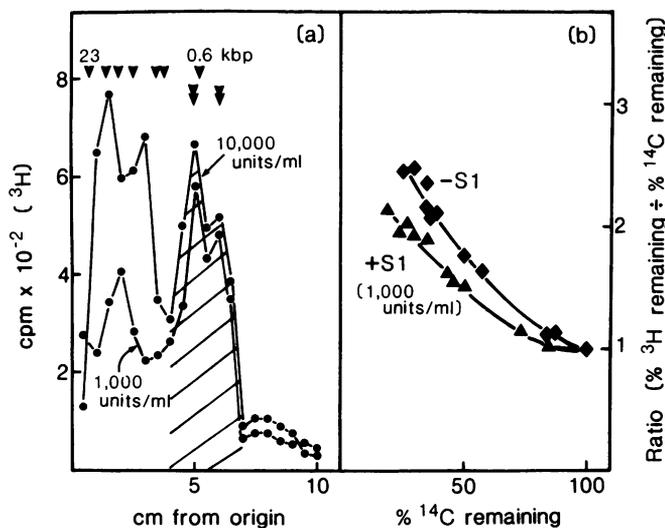
radioactive content was also equal in amount to that of single-strands of 200 and 400 nucleotides released on treatment of encapsulated nuclei with SDS and NaOH (unpublished data), we conclude that this fraction is released by cuts made in single-stranded regions between 'Okazaki' fragments. The second, more resistant, fraction of 20% migrated behind the first and so contained larger particles, presumably ligation intermediates. After pulse-labelling for 2 min these two fractions remained constant in absolute amount, but declined to 5 and 8% respectively of the total pulse label (unpublished data). This is consistent with their being kinetic intermediates. The third and largest fraction of 67% remained in beads and increased to 87% after a 2-min pulse (unpublished data). Presumably it contains accumulating and fully ligated end products.

How may these results be interpreted in the light of different models of replication? If replication occurs bidirectionally from any origin, and is continuous in one stand but discontinuous in the other no attachments other than those through polymerase are required. Alternatively, if synthesis is discontinuous in both stands there must be additional attachments, perhaps at the origin. If these additional attachments were not present mild S1 digestion would release all the chromatin between the two forks of a replication bubble.

We next determined whether S1 pretreatment sensitized pulse-label to subsequent detachment by HaeIII. As bulk chromatin is detached (i.e. as %[$^{14}$C] remaining falls) more $^3$H is lost from the S1-treated sample than from the control, so that the ratio is less (Figure 5b). As expected introducing double-stranded breaks within the nascent DNA enhances its detachment by subsequent restriction endonuclease digestion.
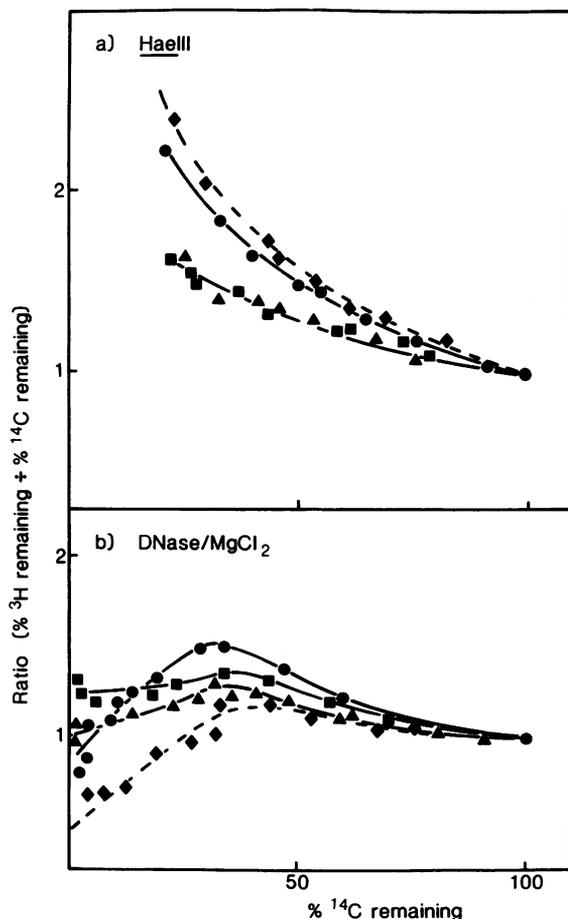
**Fig. 6.** The effect of various salt concentrations on attachments studied using (a) *Hae*III and (b) DNase. Cells were prelabelled with $^{14}$C, encapsulated, pulse-labelled with $^3$H for 2 min, lysed and washed in isotonic buffer (see Figure 3). Beads were incubated in various buffers for 15 min on ice, washed in isotonic buffer, treated with (a) *Hae*III or (b) DNase in 2.5 mM MgCl$_2$ and the ratios determined (see Figure 3). The points from matched controls (◆) lay on the broken lines which are taken from Figures 3b and 4a. Buffers (▲) hypertonic (2 M NaCl), (■) hypotonic (1.5 mM NaCl), followed by hypertonic (2 M NaCl) for 15 min on ice; (●) hypotonic (1.5 mM NaCl).

## The effect of various salt concentrations on attachments

Since salt-induced artefacts are central to the problem under consideration, we have investigated how salt affects attachments of nascent DNA (Figure 6). Cells, prelabelled with [$^{14}$C]thymidine, were pulse-labelled for 2 min with [$^3$H]-thymidine, lysed and washed in isotonic buffer as in Figure 3b. Beads were then treated with different salt concentrations before rewashing in isotonic buffer, digestion with *Hae*III or DNase, electroelution, and calculation of the ratio. With *Hae*III, treatment in hypotonic conditions used in the preparation of 'Miller' spreads yielded a curve in which the ratio was slightly lower than that obtained with untreated controls (Figure 6a). This could mean that some nascent DNA had been detached or that the treatment altered the relative accessibilities of nascent and bulk DNA. 2 M NaCl—whether alone or following a hypotonic treatment—reduced the ratio even further (Figure 6a). 2 M NaCl strips histones from DNA, eliminating the preferential sensitivity of nascent DNA to subsequent nucleolytic attack, and so might be expected to increase the ratio. The fall in ratio implies that some nascent DNA has been detached artefactually by the treatment. With DNase, lowering and/or raising the salt concentra-
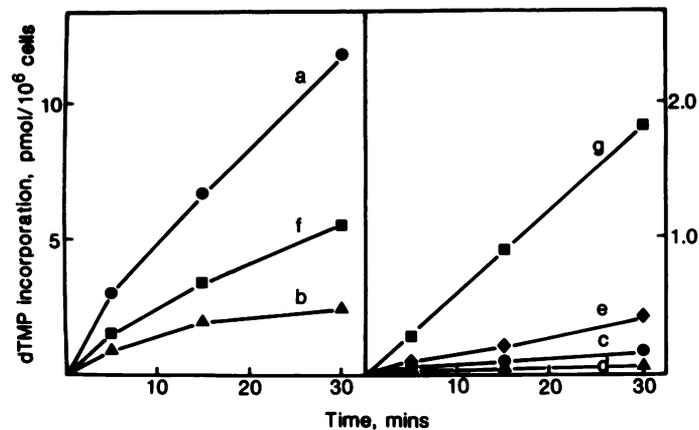


**Fig. 7.** The effect of various salt concentrations on replication. Cells were labelled for 24 h with [$^3$H]thymidine (0.02 $\mu$Ci/ml) and encapsulated (20 × 10$^6$/ml beads). (a–e) Cells were lysed and beads washed 3 times in isotonic buffer and then treated for 30 min on ice variously. (a) Control, (b) hypotonic (1.5 mM), (c) 0.5 M KCl, (d) 1 M KCl, (e) 2 M KCl, (f) encapsulated nuclei prepared by a conventional (hypotonic) procedure, (g) encapsulated 'matrices'. After the various treatments samples were washed 3 times in 10 vols isotonic buffer and the rate of incorporation of [$^{32}$P]dTTP into DNA determined.

tion changes the curve, with each procedure yielding a curve of characteristic shape (Figure 6b). We have no complete explanation for these differences but we would stress that departure from isotonicity alters subsequent detachment by DNase.

In the experiments described in Figure 6, nuclei were pre-treated with various salt concentrations before restoration of isotonicity and digestion with endonucleases. In an influential series of experiments, Pardoll *et al.* (1980) treated nuclear matrices with DNase in 2 M NaCl: they found that nascent DNA resisted detachment and concluded that the matrix was the site of replication. We were able to confirm their general conclusion, namely that pulse-labelled DNA was relatively resistant to detachment [i.e. results generally similar to the squares in Figure 6a were obtained (unpublished data)]. However, when 'encapsulated matrices' were prepared using their methods (see Materials and methods) and then digested with DNase in 2 M NaCl, unusually variable results were obtained. This is perhaps a consequence of nicking when nuclei are incubated overnight in hypotonic buffers containing Mg$^{2+}$ ions. Such nicking might also account for the aberrantly high levels of bulk DNA found attached to 'matrices' after complete digestion. Thus, removal of as much DNA as possible from isotonically prepared nuclei (by electrophoresis and high concentrations of DNase), leaves 0.2% DNA in beads. The corresponding figure is 0.1% for isotonically prepared nuclei which have subsequently been extracted with 2 M NaCl but 1–2% for 'encapsulated matrices'.

## The effect of salt concentration on replication in encapsulated nuclei

Encapsulated nuclei contain a polymerase activity sensitive to 10 $\mu$g/ml aphidicolin and that incorporates radiolabelled dTTP into DNA (D.A.Jackson and P.R.Cook, in preparation). This activity is only found in S-phase cells, it is not stimulated by added 'activated' templates, preferring the endogenous chromatin and, most importantly, it is extremely efficient. For example, under the sub-optimal concentration of dTTP used in Figure 7a, the initial rate of incorporation is 9% of that *in vivo*: under more optimal concentrations it exceeds 75% (D.A.Jack-
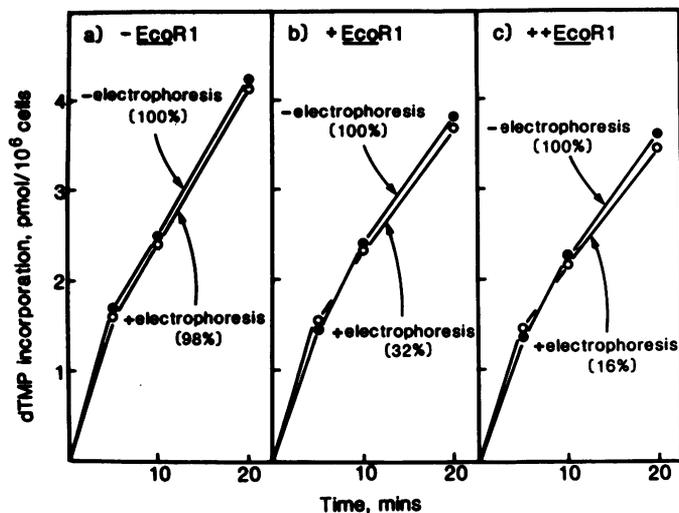
**Fig. 8.** The replication complex is closely associated with the nucleoskeleton. Cells, labelled for 24 h with [³H]thymidine (0.05 μCi/ml) were encapsulated (5 × 10⁶/ml beads), lysed and beads washed. MgCl₂ was added to 2.5 mM and samples incubated (37°C; 20 mins) with (a) 0, (b) 1000 and (c) 5000 units/ml EcoRI. EDTA was added to 2 mM and half each set of beads subjected to electrophoresis for 6 h in isotonic buffer supplemented with Tris. After recovering beads, the rate of incorporation of [³²P]dTTP (0.5 μM; 40 μCi/ml) into DNA was determined. The amount of ³H in equal volumes of each sample was determined and expressed as a percentage (brackets) of the sample in (a) that had not been treated with EcoRI or subjected to electrophoresis.
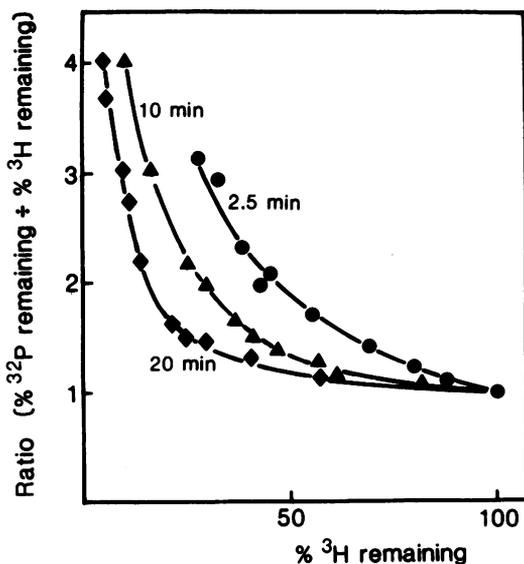


**Fig. 9.** Cells were prelabelled with [³H]thymidine (0.02 μCi/ml; 24 h), encapsulated, lysed, washed in isotonic buffer and allowed to incorporate [³²P]dTTP into DNA for 2.5, 10 and 20 min. Beads were washed 3 times in isotonic buffer, incubated with different concentrations of HaeIII subjected to electrophoresis and the ratio determined as described for Figure 3.

son and P.R.Cook, in preparation). As we shall show, this activity is relatively stable at 4°C and resists electroelution, about 90% of the activity being recovered in beads after electrophoresis for 5 h in isotonic buffer. The important point in the present context is that we are not dealing with a residual activity but most of the activity in the cell. However, this activity is relatively unstable at 37°C, becoming soluble, able to escape from beads and more like the activities studied by others (e.g.

it is now stimulated by added activated templates or by nicking or cutting the endogenous template). These aberrant activities easily obscure the authentic activity if broken templates are available.

Before investigating whether this activity was attached we studied how salt affected it (Figure 7). (As unsynchronized cells are used, activity results from polymerase α, halted by lysis, which recontinues the synthesis of the nascent chain using the endogenous chromatin template.) Hypotonic treatment of the kind used in the generation of 'Miller' spreads, followed by assay under isotonic conditions, reduces the initial rate of incorporation of dTTP into DNA to 30% (Figure 7b) and the total incorporation even more. Hypotonic buffers used conventionally in the preparation of nuclei also extract activity (Figure 7f). Hypertonic treatment reduces the rate dramatically, the residual activities having linear kinetics (Figures 7c−e: note the difference in ordinate scales). Perhaps surprisingly, the activity following treatment in 2 M KCl (Figure 7e) is greater than that found after 1 M treatment (Figure 7d). Encapsulated 'nuclear matrices' have an even higher activity (Figure 7g); this is stimulated by added 'activated' template (unpublished data). Since more than 70% of the DNA can be released from encapsulated 'matrices' following alkali treatment (unpublished data), perhaps this aberrant activity is generated from polymerase stabilized at nicks. Whatever the reason, it is clear that this residual activity in matrices cannot provide strong evidence for replication at some nuclear sub-structure.

*The polymerase is closely associated with the nucleoskeleton*

We next determined whether the polymerase was also retained within beads after removing most chromatin (Figure 8). Since these experiments involve assaying polymerase following nuclease treatment when the ends generated stimulate an aberrant activity (D.A.Jackson and P.R.Cook, in preparation), we use EcoRI to fragment chromatin, as this enzyme cuts less frequently than HaeIII. EcoRI treatment without electrophoresis reduced the polymerizing activity slightly, presumably due to template truncation. In contrast, electroelution removed up to 84% of the chromatin but no activity: the active polymerizing complex resists electroelution. [We know that on incubation at 37°C a polymerase complex of about 3 × 10⁶ daltons detaches from the nucleoskeleton and that this complex is able to escape from beads (D.A.Jackson and P.R.Cook, in preparation).]

*Nascent DNA synthesized in vitro is associated with the nucleoskeleton*

The polymerase activity in encapsulated nuclei enabled us to label nascent DNA *in vitro* and then to see if it resisted detachment by HaeIII (Figure 9). Cells were labelled with [³H]thymidine for 24 h, encapsulated, lysed and DNA synthesized *in vitro* using [³²P]dTTP. Next, beads were incubated with HaeIII and any detached chromatin electroeluted. Finally, the relative proportion of the two labels that remained in beads was determined and expressed as a ratio. Like nascent DNA labelled *in vivo*, the DNA synthesized *in vitro* proved more resistant to detachment than the majority (i.e. relatively more ³²P than ³H was retained in beads so that the ratio was >1.0). As the period of labelling *in vitro* was increased, the relative enrichment of ³²P fell. The curves obtained by labelling *in vivo* and *in vitro* (cf. curves in Figures 3 with 9) are generally similar. The slight differences we attribute to differences in rate of polymerization and accessibility of the two kinds of nascent DNA due to differential packaging into chromatin.

## Conclusion

### Association of the replication complex with the nucleoskeleton

When encapsulated chromatin is incubated with endonucleases and subjected to electrophoresis, most chromatin fragments can be electroeluted from the beads but not most nascent DNA—whether synthesized *in vivo* or *in vitro*—or active DNA polymerase. There are two explanations of these results that involve a replication complex unattached to any nucleoskeleton. First, the complex might have no net charge and so be unable to migrate electrophoretically. If so, it must fail to do so when associated with each of the wide range of randomly sized chromatin fragments that are generated by endonuclease digestion and the charge must remain at zero in the different electrophoretic buffers used (see Figure 3 and Materials and methods). This seems improbable. Second, an unattached complex might be too large to escape through the bead pores or densely packed material in the nucleus. Then it must remain too large when 84% of the chromatin has been fragmented into pieces containing <20 kbp DNA and then removed (Figures 2 and 8c). We know chromatin fragments containing at least 125 kbp DNA can escape (Jackson and Cook, 1985b) as can a detached polymerase complex of $3 \times 10^6$ daltons (D.A.Jackson an P.R.Cook, in preparation). Therefore the polymerase complex must either electroelute very slowly, for example because it is assymetrically shaped, or be at least 30 times larger than known soluble replication complexes (Noguchi *et al.*, 1983). Whichever is so, it would be unlikely to diffuse around the nucleus so that this alternative becomes equivalent to one involving attachment to the nucleoskeleton.

As we have pointed out in a different context (Jackson and Cook, 1985b), we have not directly addressed the question: to what is the complex attached? Our results only suggest that the structure is large because it resists electroelution. Likely derivatives of such a structure—isolated in 2 M NaCl—are the nuclear cage or matrix.

### Artefacts in hyper- and hypotonic salt solutions

Our results partly explain the variable results obtained using nuclear matrices (see Introduction). These are prepared by first isolating nuclei under hypotonic conditions in the presence of $Mg^{2+}$ ions, followed by extraction with 2 M NaCl. Subsequently, attachments are probed by digestion with endonucleases, e.g. DNase. In the past, variability has been attributed to artefacts induced by high salt concentrations (Kaufmann *et al.*, 1981; Kirov *et al.*, 1984; Mirkovitch *et al.*, 1984). Although hypertonic treatment without prior hypotonic treatment does induce an artefact, it is not that of a salt-induced association of nascent DNA imagined by most critics; rather 2 M NaCl leads to dissociation of nascent DNA (Figure 6a). Our results show that the low salt concentrations used during the initial isolation of nuclei are a significant source of artefact and that sequential extraction with low and high concentrations generates additional ones not found with either procedure alone (e.g. Figures 6, 7b, g). Thus, during the hypotonic treatment, DNA is variably damaged by endogenous nucleases and polymerase activity extracted. DNase is a particularly poor choice of enzyme to use to detach DNA subsequently, since its type of cutting—whether single or double-stranded—is so sensitive to ionic conditions (Campbell and Jackson, 1980) and pre-existing nicks. We have shown that by judicious choice of DNase concentration, digestion conditions and pre-treatment we can increase or decrease the relative proportion of nascent DNA associated with the nucleoskeleton

(Figures 4 and 5) and so provide apparent support both for, or against, a fixed replication site.

### A model for replication

We envisage the nucleoskeleton as much more than a passive framework to which DNA is attached: it is seen as one part of the active site of the replication complex, organizing the template in three-dimensional space into close proximity to the polymerization site and so dictating the sequence of replicon firing. Replication then proceeds by passage of DNA through the complex which might be dedicated to replicating a few—perhaps only one—adjacent replicon(s).

At first glance, the isolation of working polymerase complexes free of any structural elements might suggest that the nucleoskeleton plays no role in replication. However, these complexes do not generally initiate specifically or efficiently. Specific, but inefficient, initiation of viral replication has been achieved using unphysiological concentrations of T antigen (Ariga and Sugano, 1983; Li and Kelly, 1984), a protein we might predict would subvert the normal operation of the host cell's machinery. We would expect that some constituents of an attached polymerase complex might become soluble—perhaps even as quite large multi-enzyme particles—and be able to catalyze partial reactions both *in vivo* and *in vitro*. Partial reactions of one such complex have been found *in vitro* (Noguchi *et al.*, 1983).

## Materials and methods

### Radiochemicals

Labels (Amersham International) included [methyl-$^{14}$C]thymidine ($\sim 60$ mCi/mmol), [methyl-$^3$H]thymidine ($\sim 50$ Ci/mmol), thymidine 5'- [$\alpha$-$^{32}$P]-triphosphate ($\sim 3000$ Ci/mmol).

### Cell growth, encapsulation and lysis

HeLa cells were grown, encapsulated in 0.5% agarose, lysed at 4°C in an isotonic lysis mixture for 30 min and encapsulated nuclei washed 3 times in an isotonic buffer as described by Jackson and Cook (1985a). The isotonic lysis mixture contained 0.5% Triton X-100, 100 mM KCl, 10 mM Tris (pH 8.0), 25 mM Na$_2$ EDTA, 1 mM dithiothreitol and the isotonic buffer 100 mM KCl, 25 mM (NH$_4$)$_2$SO$_4$, 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol.

### Hypo- and hypertonic treatments

Encapsulated nuclei in isotonic buffer were washed 2 times in 10 vol 100 $\mu$M borate buffer (pH 8.5) ('hypotonic treatment') or in the appropriate salt concentration in 10 mM Tris (pH 8.0), 1 mM EDTA ('hypertonic treatment'). In Figure 7f, encapsulated nuclei were prepared using a conventional procedure by washing encapsulated cells 3 times in 5 mM Tris (pH 8.0), 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10 mM KCl, 2.5 mM MgCl$_2$, swelling them for 30 min on ice, before adding Triton to 0.5%. In Figure 7g and in the discussion of Figure 6, 'encapsulated matrices' were prepared by allowing the 'nuclei' prepared as in Figure 7f to stand overnight, then washing 2 times in the same buffer before adding NaCl to 2 M.

### Nuclease digestions

The typical procedures used in Figure 3 are as follows: $10^8$ HeLa cells, prelabelled for 24 h with [$^{14}$C]thymidine (0.01 $\mu$Ci/ml) were encapsulated (10 $\times$ 10$^6$/ml beads), washed, resuspended in an equal volume of warm medium and pulse-labelled for various times with [$^3$H]thymidine (250, 100, 50, 20, and 5 $\mu$Ci/ml for a – e respectively). For sample f, [$^3$H]thymidine (0.05 $\mu$Ci/ml) was included for 24 h with [$^{14}$C]thymidine. Two samples were included in b, one a pulse alone, the other a pulse followed by a chase. The chase involved pelleting the pulse-labelled cells, and washing them twice in 10 vols of medium before growing them in the absence of [$^3$H]thymidine. Incorporation was stopped by adding 20 vols ice-cold phosphate buffered saline and cells pelleted and lysed immediately in the isotonic lysis mixture. In some cases beads were added directly to the lysis mixture with no difference in results. Washed beads were suspended in isotonic buffer supplemented with 2.5 mM MgCl$_2$, incubated with *Hae*III (200 – 1000 u/ml) and at various times later (2.5 – 80 mins) 150 $\mu$l samples were removed, added to 100 $\mu$l ice-cold 2 $\times$ TEA and subjected to electrophoresis. After electrophoresis, beads were recovered, suspended in an equal volume of TEA, the haematocrit measured and SDS added to 1%. 1 hr later triplicate

samples were spotted on to GF/C discs (Whatman), the discs extracted successively with 5% trichloroacetic acid, ethanol and ether, then dried, their radioactivity counted and expressed as a percentage of that obtained with undigested (or in some cases, digested but not electrophoresed) control samples. The labelling conditions described above give $10 \pm 2 \times 10^3$ d.p.m. $^{14}C$ and $40-120 \times 10^3$ d.p.m. $^3H$ in samples of $10^5$ cells, except for the 30 s pulse which gave $2-7.5 \times 10^3$ d.p.m. $^3H$. In a typical pulse−chase experiment (Figure 3b) the incorporation increased from 50 000 d.p.m. $^3H$ per sample following the pulse to 60 000 following the chase.

In the experiments described in Figure 4, DNase I (5−20 μg/ml) or micrococcal nuclease (0.1−2.0 u/ml) replaced the *Hae*III and the isotonic buffer was supplemented with 2.5 mM MgCl$_2$, 2.5 mM MnCl$_2$ or 5.0 mM CaCl$_2$ as shown. In those described in Figure 5, encapsulated nuclei were washed in 20 mM sodium acetate (pH 4.7), 130 mM KCl, 1 mM EDTA, 2.5 mM ZnSO$_4$ and 5% glycerol prior to incubation with S1 nuclease.

*Electrophoresis*

Beads were washed in electrophoresis buffer prior to loading on 0.8% agarose gels. Electrophoresis buffers were TEA [40 mM Tris (pH 8.3), 2 mM EDTA, 20 mM sodium acetate], equal volumes of TEA and isotonic buffer or isotonic buffer containing 20 mM Tris (pH 8.0). Buffers other than TEA were recirculated during electrophoresis. (Caution! The recirculating buffer is 'live'). Essentially similar proportions of chromatin electroeluted from beads in the different buffers (e.g. Figure 3 and unpublished data). After electrophoresis (2 v/cm for 4−6 h or 0.7 v/cm for 16 h at 4°C) beads were recovered and washed in isotonic buffer.

Gels were treated successively by washing in 50 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA with RNase (30°C; 1 h; 0.5 μg/ml), 0.5% SDS and proteinase K (5 μg/ml; 1 h; 20°C) and then SDS was removed by washing in the buffer and the gel stained with ethidium (0.1 μg/ml; 30 min), destained and photographed under u.v. light. Removal of the protein in this way increased fluorescence 5−10-fold.

*Replication assays*

Assays (500 μl) were generally conducted in isotonic buffer supplemented with 10 mM potassium phosphate (pH 7.4), 5 mM MgCl$_2$, 0.25 mM CaCl$_2$, 1.25 mM ATP, 0.1 mM CTP, GTP and UTP, 0.25 mM dATP, dCTP and dGTP, 2.5 μm dTTP and various amounts of [$^{32}P$]dTTP (~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. 100 μl samples were removed at various times subsequently and mixed with 250 μl 2% SDS: >2 h later 3 × 100 μl samples were spotted on to GF/C discs and the discs extracted with trichloracetic acid and counted as described above.

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