Most short DNA molecules isolated from 3T3 cells are not nascent

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ABSTRACT

The population of short DNA molecules (less than $10^3$ nucleotides) in 3T3 cells has been studied using in vivo and in vitro pulse labeling techniques and in vitro end-labeling. There is a large number of molecules of less than 100 nucleotides present in equal numbers in both G0 and S phase cells. In S phase cells, most of these molecules are not replicating intermediates because they do not become density-labeled after a moderate period of substitution of BrdUMP, although they are detected by end-labeling in vitro. This population includes the nascent Okazaki pieces that can be labeled in a short pulse with $[^3H]$dThd or $[^3H]$dTTP, however, these represent less than 10% of the total population. Alkaline hydrolysis of the molecules that had been end-labeled with $^{32}$P using $[^32P]$ATP and polynucleotide kinase did not reveal significant release of $[^32P]$ 2'-(3'), 5' ribonucleoside diphosphates.

INTRODUCTION

A large part of our understanding of the nature of DNA replication comes from studies employing incorporation of radioactive precursors into DNA either in vivo or in a variety of in vitro systems. In eukaryotic replication systems, small DNA pieces of 50-300 nucleotides in length are believed to be involved in the discontinuous synthesis of at least one of the two strands at the replication fork, both in vivo and in vitro (1,2,3,4). Thus, short length in DNA is usually associated with replication. The synthesis of at least some of these molecules is believed to be initiated by the synthesis of a ribonucleotide primer. The evidence for this comes from experiments involving the phosphate transfer method of Josse et al., 1961, indicating the presence of RNA-DNA junctions in molecules newly synthesized in vitro (5,6, 7,8,9). Other experiments on the molecules synthesized in vitro indicate the presence of 7-10 ribonucleotides terminated in a triphosphate at the 5' end of the DNA (10,11,12). However, there is yet no evidence to indicate the involvement of ribonucleotides in the synthesis of DNA in vivo analogous.
to that for prokaryotes (13,14,15,16). It was with the aim of comparing the 
nature of the 5' ends of short DNA molecules synthesized in vitro and in 
vivo that we originally undertook this work.

The nature of the total population of both nascent and pre-existent DNA 
molecules from mammalian cells as determined by in vitro labeling with poly-
nucleotide kinase after isolation is not known. In E. coli, Jacobson and 
Lark (17) reported that nascent and non-nascent short DNA molecules were 
present in about equal numbers; however, Anderson (18) reported that the 
great majority was not nascent. A detailed study of the population of 
short molecules in E. coli pol Aex2 revealed 5' end-labeled molecules from 
the cell possessed at least one ribonucleotide at their 5' ends (16). A 
similar approach was used here in the analysis of DNA molecules isolated from 
mammalian cells.

We isolated short DNA molecules from cultured 3T3 cells directly and 
from an in vitro replication system prepared from these cells. Substitution 
with BrdUMP was employed to distinguish nascent from pre-existent DNA 
molecules. This DNA was extensively purified and labeled at the 5' ends 
with 32P using [γ32P]ATP and T4 polynucleotide kinase. It was then character-
ized with respect to size, density, and the presence of 5' terminal ribo-
nucleotides detected as 32P-labeled 2'(3'), 5'-ribonucleoside diphosphates 
released by alkaline hydrolysis.

We have demonstrated the existence in mammalian cells of a population 
of DNA molecules less than 100 nucleotides in length which do not possess 
ribonucleotides at their 5' ends. These molecules are not nascent and their 
presence in the cell does not correlate with DNA replication. We estimate 
that they are present in 10-100 fold excess over the number of nascent 
molecules expected. No clear evidence was found for the presence of ribo-
nucleotides at the 5' ends of any of the DNA molecules analyzed.

MATERIALS AND METHODS

Cells - The cells employed in this work were a line of 3T3 Balb/c mouse 
fibroblasts (obtained from Dr. B. Mukherjee, McGill University). Cell stocks 
were assayed for mycoplasma contamination by Dr. P. Gill (McGill University), 
and were stored frozen in liquid nitrogen. Cells were maintained in monolayer 
cultures seeded at 1-2 x 10^4 cells per 75 cm^2 passed every 4-5 days up to 
a maximum of 15 passages. Cultures were maintained in Dulbecco-modified 
Eagle medium containing 10% fetal calf serum, 50 mg/l Gentamycin (gift of 
Schering Corporation), 0.25 mg/l Amphotericin B (gift of Squibb and Sons Ltd)
Nucleic Acids Research

in a humidified atmosphere at 10% CO₂ in air at 37°C. One to two days after reaching confluence, cells were seeded at 1 x 10⁷ cells per 150 cm² plate in medium containing fresh fetal calf serum.

**Autoradiography** - Whole cell autoradiography was performed as previously described (19).

**Labeling and Extraction of DNA** - DNA was labeled either *in vitro* or *in vivo*. *In vitro* labeling was carried out as described by Hunter & Francke (20). Briefly, this involves permeabilization of the cells by hypotonic treatment and incubation of the concentrated lysate with added nucleoside triphosphates. [³H]dTTP, was incorporated into DNA linearly for 60 minutes. In a 30 sec pulse of [³H]dTTP, 60% of the radioactivity is found in molecules sedimenting at about 4S in alkaline sucrose gradients. These molecules were chased into higher molecular weight intermediates during incubation with an excess of unlabeled dTTP (data not shown) as previously observed in this system (21).

DNA replicating *in vivo* was labeled with [³H]dThd (100-200 µCi/ml; 40-60 Ci/mMol or with [³H]dCyd (50 µCi/ml; 20-40 Ci/mMole) and 20 µM BrdUrd. The density labeling medium also contained 2 µM FdUrd. Pretreatment of cells with FdUrd was not employed.

DNA synthesis was stopped by one of three different ways. When 5' terminal ribonucleotides were sought, synthesis was terminated by the addition of 1% sodium dodecyl sulfate (SDS) - 10 mM EDTA - 2% phenol at 100°C to either the *in vitro* incubation or directly to cells on the plate. The lysate was then heated at 100°C for 5 min to denature the DNA and chilled at 4°C. When 5' terminal ribonucleotides were not sought, cells were lysed with 0.2 M NaOH - 10 mM EDTA. The lysate was heated at 50°C for 30 min, neutralized, made 1% in SDS, and chilled to 4°C. When the intracellular location of short DNA molecules was being studied, cells were lysed with 1% SDS - 1 mM EDTA at 25°C and lysate was chilled to 4°C. In each case, an SDS precipitate formed about the high molecular weight DNA and was pelleted by centrifugation at 45 min at 45 K rpm in the 60 Ti rotor at 4°C. This is designated the Hirt pellet. DNA in the pellet was sheared by several passages through a syringe. The nucleic acids were deproteinized either by the addition of auto-digested Pronase B (200 µg/ml, 37°C, 16 hours) or by extraction twice with phenol-chloroform (1:1) saturated with 0.01 M Tris HCl pH 7.4 - 0.1 M NaCl - 10 mM EDTA. Nucleic acids were then precipitated with 2 volumes isopropanol at -20°C overnight in the presence of 0.3 M sodium acetate at pH 5.5.

**Nitrocellulose Chromatography** - Nucleic acids were redissolved in 10 mM
Tris-10 mM EDTA, heated to 100°C for 5 min, adjusted at 0.4 M KCl, applied to a 0.8 x 7 cm column of nitrocellulose. The column was washed with 10 sample volumes of 10 mM Tris HCl pH 7.4 - 0.5 mM EDTA - 0.4 M KCl and DNA was eluted with 10 mM Tris-HCl pH 7.4 - 0.5 mM EDTA (16,22).

Labeling of 5' Ends with Polynucleotide Kinase - DNA purified as described above through the first nitrocellulose chromatography step was redissolved in 100 μl 50mM Tris-HCl pH 8.1 and the amount of nucleic acid was determined by spectrophotometry. A typical preparation from 10⁶ cells contained 20-30 μg of nucleic acid from the Hirt supernatant fraction at this point. The sample was diluted, if necessary, to 300 nmoles nucleotide/ml. Bacterial alkaline phosphatase was added to a concentration of 1 unit/ml and the sample was incubated at 65°C for 30 min. EGTA was then added to 7 mM and incubation continued at 65°C for 30 min to inactivate the phosphatase. The sample was then adjusted to 2 mM potassium phosphate pH 6.8, 10 mM MgCl₂ and 20 mM 2-mercaptoethanol. [γ²³P]ATP (1-4 x 10⁶ cpm/nMole) prepared as described by Miyamoto and Denhardt (16) was added to a concentration of 10 μM, polynucleotide kinase (purified as described by Richardson (23) was added to a final concentration of 4-8 units/ml and incubation was carried out at room temperature for 90 min. The reaction was stopped by the addition of EDTA to 30 mM. We have previously determined that under these conditions, the extent of end-labeling agrees well with the number of 5' ends present (16). 50 μg of denatured salmon sperm DNA was added and nucleic acids were precipitated with isopropanol. End-labeled DNA was purified away from [γ²³P]ATP and residual RNA by a second chromatography on a 0.5 x 4 cm column of nitrocellulose.

Labeling of 3' ends with Terminal Transferase - The reaction mixture contained 1 μg of DNA, 0.1 mM [³H]dCTP (4 Ci/nmole) 1.2 mM CoCl₂, 250 mM potassium cacodylate, pH 7.6 and 120 Units of terminal deoxynucleotidyl transferase (Miles Laboratories). The reaction was carried out for 90 min at 37°C and was stopped by the addition of EDTA to 20 mM. 50 μg of denatured salmon sperm DNA was added as carrier and DNA was precipitated with isopropanol. [³H]dCTP was removed by chromatography on nitrocellulose.

CsCl and Cs₂SO₄, Isopycnic Centrifugation - The low-salt fractions from the second nitrocellulose column containing labeled DNA were heated to 100°C for 30 sec, made 56% (w/w) in CsCl (58.3% when bromodeoxyuridine substituted DNA was present) and centrifuged to equilibrium in a Beckman type 40 rotor at 40 Krpm for 40 hours at 20°C. Fractions were collected from the top with a Buchler Auto Densi Flow Apparatus. For Cs₂SO₄, centrifugation nucleic acid
dissolved in 10 mM Tris-HCl, pH 7.4 - 10 mM EDTA was heated to 100°C for 30 sec and the solution was made 41% (w/w) in Cs$_2$SO$_4$. Centrifugation was performed as described above. Alkaline gradients contained 0.3 M NaOH.

**Sucrose Gradients** - Neutral sucrose gradients were 3.6 ml of 5% to 20% sucrose in 1 M NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 8, over a 0.3 ml cushion of saturated CsCl. Centrifugation was performed in the Beckman SW56 rotor at 50 Krpm for 3 hours at 20°C. Fractions were collected from the bottom.

Alkaline sucrose gradients were 3.6 ml of 5% to 20% sucrose in 0.2 M NaOH, 0.8 M NaCl, 2 mM EDTA, and 0.05% Sarcosyl over a 0.3 ml cushion of saturated CsCl. Centrifugation was performed in the Beckman SW56 rotor for 4 hrs (Fig. 1) or 24 hrs (Fig. 5) at 48 Krpm at 20°C. Fractions were collected from the bottom.

**Alkaline Hydrolysis** - Alkaline hydrolysis of RNA was performed by the addition of NaOH to 0.4 M and incubation at 37°C for 8-16 hrs. Glacial acetic acid was added to neutralize the sample.

**Chromatography of 2'(3'), 5' - Ribonucleoside Diphosphates** - Chromatography of 2'(3'), 5' - ribonucleoside diphosphates resulting from alkaline hydrolysis of end-labeled DNA preparations was performed as described previously (16). Samples of 10-15 µl were applied to polyethyleneimine cellulose (PEI) thin layer plates and the plates were washed in water to remove excess salt. Ascending chromatography was carried out with 3 M sodium formate, pH 3.5. The resulting chromatogram was cut into 1.5 x 1 cm pieces for scintillation counting.

**Determination of Radioactivity** - Samples were prepared for liquid scintillation counting by different methods depending on the efficiency desired and the amount of acid-soluble radioactivity present. Fractions from nitrocellulose columns, sucrose gradients, and equilibrium centrifugations were either spotted directly onto 1 inch squares of Whatman No.5 paper, dried and counted in a toluene cocktail, or were diluted into 0.5 ml water and counted in a triton: toluene cocktail. Strips of PEI cellulose chromatograms were counted in the toluene cocktail.

Radioactivity was determined by counting in a Intertechnique SL36 scintillation counter and appropriate corrections were made for background and channel spillover.

**RESULTS**

**Pulse Labeling and Purification of Short DNA Molecules and Labeling of**
5' Ends - Part of this work was to compare the 5' ends of nascent DNA molecules synthesized in vitro to the 5' ends of molecules synthesized in vivo. In vitro DNA synthesis was performed essentially as described for 3T3 cells by Hunter and Francke (20). Hypotonically treated 3T3 cells were incubated with \[^{3}H\]dTP or with a combination of \[^{3}H\]dCTP and BrdUTP for 3 min. In vivo labeling was performed directly on cell monolayers either with \[^{3}H\]dThd for approximately 30 sec, or with a combination of \[^{3}H\]dCyd and BrdUrd for 10 min. Generally, the labeling intervals were kept as short as possible in order to monitor recovery of the most recently synthesized molecules through the purification. The longer labeling interval in vivo with BrdUrd was found necessary in order to obtain a significant amount of tritium labeled DNA substituted with BrdUMP in the absence of prolonged treatment with FdUrd to deplete the dTPP pool.

DNA synthesis was rapidly terminated by raising the temperature to 100°C in the presence of 1% sodium dodecyl sulphate (SDS) and 2% phenol. This ensures prompt interruption of replication and at the same time the combination of phenol, dodecyl sulphate, and high temperature promotes the immediate inactivation of cellular nucleases and minimizes further alteration of newly synthesized DNA by cellular enzymes in the lysate. In most experiments the lysate was heated at 100°C for 5 min at this point to denature the DNA and to release small newly formed DNA molecules from the template. The majority of high molecular weight DNA was removed by performing a modified Hirt extraction of the lysate. This procedure routinely released between 75% and 95% of the radioactivity incorporated into DNA in a short pulse into the supernatant while 99.9% of the total cellular DNA (pre-labeled with \[^{14}C\]dThd) was pelleted with the dodecyl sulphate. Figure 1 compares the size of pulse-labeled DNA recovered from the pellet to the total pulse-labeled DNA. About half of the total radioactivity incorporated is found in molecules sedimenting at less than 10s. The pellet usually contained 5-25% of the pulse-labeled DNA comprising molecules sedimenting predominantly greater than 10s.

Other methods were also used to terminate replication. These included, lysis with SDS at 25°C (i.e. the DNA was not denatured before centrifugation) and lysis by alkali at 4°C. In both cases, SDS precipitation and centrifugation were used to remove the majority of high molecular weight cellular DNA. These methods revealed similar population of short molecules, and provided evidence that the technique of lysing cells at 100°C is not introducing artifacts. We have demonstrated elsewhere (16,24) that DNA molecules are not degraded to short pieces by this step.
Figure 1: Velocity sedimentation in alkaline sucrose gradients of DNA pulse labeled for 20-30 seconds with \([^{3}H]dThd\) in vivo. Cells were lysed at 100°C, the DNA was heat-denatured, and high molecular-weight DNA was separated from the low molecular weight DNA by centrifugation of the SDS precipitate. DNA from part of the unfractionated lysate and from the pellet of the centrifugation, was sheared with a syringe, digested with pronase, precipitated with isopropanol, and centrifuged in parallel alkaline sucrose gradients. 4X174 DNA was used as a sedimentation marker. Total DNA in the unfractionated lysate, (O——O); DNA recovered from the SDS pellet, (■——■). The DNA in the pellet represented 5-25% of the total of 10⁶cpm incorporated by 4 x 10⁷ cells.

Nucleic acids in the supernatant were then extracted twice with a 1:1 mixture of phenol and chloroform. Recovery of pulse labeled acid-insoluble radioactivity from this step was usually about 85%, however, the quantitation is not accurate because of the presence of large amounts of acid-soluble radioactivity. There is evidence (25) to indicate that short DNA chains are preferentially lost from phenol or chloroform extractions of mammalian cell lysates. We have found that when de-proteinization is performed by pronase treatment in the absence of phenol-chloroform extraction, the yield of small DNA molecules is improved 3-4 fold. However, incubation of crude cell lysate at 37° for pronase treatment could give rise to degradation of RNA by endogenous nucleases. Thus, both procedures of proteinization were used and compared with respect to presence of 5' ribonucleotides on DNA.

The purpose of the remaining steps in the purification was to remove free RNA from the preparation prior to analysis of DNA for alkali labile 5' terminal ribonucleotides. When the presence of terminal ribonucleotides was not being measured, alkaline hydrolysis was performed at this point to
remove free RNA. Single-stranded DNA was adsorbed to nitrocellulose in a high-salt buffer and eluted with a low-salt buffer. The efficiency of separation of DNA from RNA on nitrocellulose columns has been described previously (16). In addition to two nitrocellulose columns, the purification usually included one separation based on density and one based on size. Before each step, the preparation was briefly heated to 100°C to minimize non-covalent association of DNA with RNA.

Labeling of 5’ ends of nucleic acid molecules in the preparation was performed after the first nitrocellulose chromatography. This permitted the addition of carrier DNA to improve recovery in the subsequent steps of the purification. Overall recovery of 3H-labeled DNA relative to the acid insoluble radioactivity incorporated into DNA during the pulse labeling was 5%-10%.

Characterization of End Labeled DNA Molecules - The size distribution of pulse-labeled DNA molecules and of 32P labeled 5’ ends is shown in Figure 2. 3H pulse-labeled DNA sediments as a broad distribution between 10S and 3S with a peak at approximately 5S. The ratio of 32P and 3H counts increases toward the top of the gradient because each molecule, regardless of its size, has only one 32P atom; the amount of 3H, in contrast, will be proportional to the size of the molecule if the molecules are uniformly labeled and have uniform base composition. These data are consistent with the assumption that all of the 32P-labeled molecules are newly-synthesized. However, data described below will show that this is not so.

The presence of ribonucleotides at the 5’ termini of DNA molecules was assessed as described previously (16). 2′(3′)5′-ribonucleoside diphosphates (pNp) released by alkaline hydrolysis from the 32P-labeled 5’ ends of nucleic acid molecules in the preparation were detected by chromatography on PEI cellulose thin layer plates. The distribution of 32P on a representative chromatogram is shown in Figure 3. The average proportion of 32P-labeled ends released as pNp in three different preparations was 2-3% (Table 1).

The proportion of 32P-labeled molecules that had the density of free RNA was determined by equilibrium centrifugation in Cs2SO4, as shown in Fig. 4. This analysis shows that the degree of contamination of the preparation by free RNA was indeed very low. However, it was not possible to exclude the possibility that the [32P]pNp released by alkali resulted from the hydrolysis of a very small amount of free 32P end-labeled RNA molecules, since the proportion of 32P released as pNp moieties was so low. These results are in agreement with the experiment performed by Gautschi and Clarkson (2).
Figure 2: Neutral sucrose gradient velocity sedimentation of DNA. DNA was labeled in vitro with [3H]dCTP and BrUdUTP for 3 min, purified as described in the text, and labeled with 32P at the 5' end. An aliquot was centrifuged in a neutral sucrose gradient. (• •), 3H radioactivity (0 - 0), 32P radioactivity. The vertical arrow denotes the position of \( \phi X 174 \) DNA centrifuged in a parallel gradient.

Figure 3: Thin layer chromatography of 2'(3'), 5'-ribonucleoside diphosphates released by alkaline hydrolysis of DNA. DNA was labeled with \([3H]dTTP\) in vitro for 3 min, purified as described in the text and 32P-end-labeled with polynucleotide kinase. Half the sample was subjected to alkaline hydrolysis and both samples were chromatographed in PEI cellulose thin layer plates. (0 - 0), control without alkali; (0 - 0), DNA treated with alkali.

Quantitation of these data is presented in Table I, Expt. III. The approximate positions of pNps were determined from the RF values reported previously (16).
Table I  Summary of pIP Release Experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Size Class</th>
<th>$^{32}$P cts/min analyzed ($\times 10^3$)</th>
<th>% of total $^{32}$P found as pIP after exposure to alkali</th>
<th>% of total $^{32}$P found as RNA in Cs$_2$SO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Total</td>
<td>6.7</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>&quot;</td>
<td>21</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>&quot;</td>
<td>17</td>
<td>0.9</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

DNA of low molecular weight purified from hypotonically treated cells replicating their DNA in vitro was subjected to alkaline hydrolysis and chromatographed on PEI-cellulose as described in Figure 3. Experiments I, II, and III are identical. In experiment III, Cs$_2$SO$_4$ centrifugation was carried out on DNA not subjected to alkaline hydrolysis to assess the proportion of $^{32}$P-labeled molecules having the density of free RNA. This Cs$_2$SO$_4$ gradient is shown in Figure 4.

Figure 4: Cs$_2$SO$_4$ isopycnic centrifugation of DNA. An aliquot of the DNA analyzed for 5' terminal ribonucleotides (Table I, Expt. III) was heated at 100°C for 3 min, made 41% (w/w) in Cs$_2$SO$_4$, and centrifuged to equilibrium. A parallel gradient contained a mixture of DNA and RNA labeled with $[^{32}$P]-orthophosphate in vivo and purified for use as a density marker. (0 - 0), end-labeled sample; (O - O) internally labeled marker.

Miyamoto and Denhardt (16) observed similarly that the proportion of ribonucleotide termini in the total population of end-labeled molecules from E. coli polAex was of the order of 2%. Fractionation according to size revealed that a large proportion of very small molecules did not have ribo-
nucleotide termini whereas, larger molecules, which constituted only a small proportion of the total population, released 20-30% of their $^{32}$P termini as pNps on exposure to alkali. A similar experiment on low molecular weight DNA fractionated according to size as in figure 2, revealed that there was no substantially different proportion of $[^{32}P]$pNp released by alkali from molecules in three different size classes in the gradient. It is a well-established observation that Okazaki pieces in eukaryotic replication systems are an order of magnitude smaller than in prokaryotes. This could explain why size fractionation does not detectably separate them from the class of pre-existent DNA molecules described below.

Thus, we conclude that the DNA molecules detected by polynucleotide kinase labeling have the characteristics of replication intermediates with respect to their size but do not show substantial evidence for the presence of ribonucleotides at their 5' ends.

Detection of Small End-labeled DNA in $G_0$ Cells - One possible explanation for the above result is that the end-labeled molecules are not replication intermediates. We thus proceeded to determine if cells that are not replicating DNA will give rise to molecules with similar properties. Mouse fibroblasts can be efficiently arrested in the $G_0$ phase of cell growth by depletion of serum growth factors. Restoration of serum growth factors results in a partially synchronized entry of cells into $S$ phase (19, 26). The proportion of cells in the culture that were synthesizing DNA was determined by autoradiography. Since the pNp release experiments described in the previous section revealed few ribonucleotide termini, alkaline hydrolysis of the preparation was performed to remove RNA prior to $^{32}$P-end-labeling with polynucleotide kinase. The end-labeled material was DNA as judged by its complete resistance to alkaline hydrolysis measured by PEI chromatography, its digestion to mononucleotides by DNase I and snake venom phosphodiesterase, and by its buoyant density in CsCl (not shown).

Figure 5 compares the size distribution of $^{32}$P end-labeled DNA molecules from $G_0$ and $S$ phase cells. Clearly, there is no difference in the size of molecules recovered from cells replicating DNA and cells in $G_0$. Quantitation of the number of molecules recovered from cells in each of the two phases was done and the results are presented in Table 2. Experiment 1 and 2 are identical. Experiment 3 employed lysis of the cells by SDS at 25°C and Experiment 4 employed lysis at 4°C by alkali. There is some variation in recovery from experiment to experiment, but the ratio of small DNA molecules on $G_0$ to $S$ phase cells was always near unity. These numbers represent a
Figure 5: Velocity sedimentation in alkaline sucrose gradients of $^{32}$P-end-labeled DNA from Go and S phase cells. Go or S phase cells were lysed with SDS at 25°C and the lysate was fractionated into a Hirt supernatant and pellet. DNA in the pellet was sheared and both fractions were purified in parallel as described in the text and labeled with $^{32}$P using polynucleotide kinase. Three separate gradients run in parallel are shown (O - O), Go Hirt supernatant; (I - I) S Hirt supernatant; (□ - □), Go Hirt pellet.

The intracellular location of these DNA molecules was investigated in the following experiment. Cells arrested in Go were lysed with SDS at 25°C and the high molecular weight DNA was precipitated by the Hirt procedure, without denaturation. DNA from both the supernatant and pellet was purified and labeled at the 5' ends with $^{32}$P and centrifuged in alkaline sucrose gradients (Figure 5). Molecules of about 0-3s, in size predominate in the Hirt supernatant. However, about 3/4 of the molecules of this size in the cell are derived from the Hirt pellet under these conditions of lysis. This suggests that the majority of them are stably associated with the chromosomal DNA, presumably in the double stranded form.
Table II  Quantitation of the number of small DNA molecules recovered from G0 and S Phase cells

<table>
<thead>
<tr>
<th>Percent Cells in S</th>
<th>Number of DNA molecules recovered per cell</th>
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<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>0.1</td>
<td>$10^6$</td>
</tr>
<tr>
<td>40-60</td>
<td>$6 \times 10^3$</td>
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</table>

Cells were accumulated in G0 by serum starvation and then half the cultures were re-seeded in fresh serum for 15 hours. The proportion of cells synthesizing DNA was determined by autoradiography. Cells were lysed with 1% SDS-10 mM EDTA at 100°C (expt. 1 and 2) or at 25°C (expt. 3). In experiment 4, cells were lysed with 0.2 M NaOH-10 mM EDTA at 4°C and alkaline denaturation replaced heat denaturation throughout purification. Quantitation is based on knowledge of the specific activity of $\gamma$-ATP in the polynucleotide kinase reaction (16).

Purification of Density-labeled Nascent DNA - One of the original aims of this work was to label the 5' ends of nascent Okazaki pieces. The above results show that the presence of DNA of this size in the cell does not correlate with replication. We thus attempted to determine what proportion of the small DNA in S phase cells was nascent. DNA was synthesized in vitro or in vivo under conditions where BrdUMP substituted for dTMP. It was purified as described above, labeled at the 5' end with $^{32}$P and separated according to buoyant density in CsCl. Figure 6a shows that the majority of small DNA molecules in the cell are not nascent. $^{32}$P-labeled molecules banding at densities greater than that of unsubstituted DNA were in the minority. These molecules were analyzed for the presence of 5' terminal ribonucleotides as described above. The level of release of terminal label as pNP upon alkaline hydrolysis increased from 2% in the region of light DNA to about 5% in the region of heavy DNA. The proportion of $^{32}$P termini due to the presence of RNA not covalently joined to DNA was determined by re-banding of the nucleic acid in Cs$_2$SO$_4$ after brief denaturation as described in Figure 4. It was found that the level of $^{32}$P-pNP release correlated reasonably well with the proportion of free RNA contaminating the heavy and intermediate regions of the CsCl gradient. Furthermore, many of the molecules from these regions of the gradient reband in the light position in Cs$_2$SO$_4$. Thus most of the molecules of intermediate density in CsCl (figure 6a) appear to result from non-covalent association of the small non-nascent molecules described above and residual free RNA.
Figure 6: CsCl Equilibrium Centrifugation of BrdUMP Substituted DNA.

a) Neutral CsCl equilibrium centrifugation of DNA labeled in vivo with [3H]dCyd and BrdUrd for 10 min. Cells were lysed with SDS at 100°C and low molecular weight DNA was purified and labeled with polynucleotide kinase. (0 - 0), 3HdCyd incorporated in vivo; (O - O) 32P-labeled 5' ends.

b) Alkaline CsCl equilibrium centrifugation of DNA labeled and purified as above but lacking in vivo 3H label. Half the preparation was labeled with [γ32P]ATP and polynucleotide kinase in the usual way while the other half was labeled with [3H]dCTP using terminal transferase. The two halves were recombined and centrifuged to equilibrium in alkaline CsCl. (O - 0), 32P-labeled DNA (28,000 cpm); (△ - △), 3H-labeled DNA (55,000 cpm).

A possible reason for failure to detect nascent molecules by this technique could be that the 5' ends of nascent molecules were not accessible to labeling with polynucleotide kinase or were resistant to the action of phosphatase. We tested this possibility by preparing DNA as described for Figure 6a and labeling half the preparation at the 5' end in the usual way. The other half was labeled at the 3' end using terminal transferase and [3H]dCTP. The two end-labeled populations were then analyzed together in a CsCl gradient as shown in Fig. 6b. In contrast to Fig. 6a, alkaline hydrolysis was used in the purification of the DNA. This probably accounts for the decreased amount of 32P-5' end-labeled nucleic acid molecules banding.
DISCUSSION

These studies have demonstrated the existence in mammalian cells of a population of DNA molecules of less than 100 nucleotides in length which do not possess ribonucleotides at their 5' ends and whose presence in the cell does not correlate with DNA replication.

Molecules with these properties have been previously observed in *E. coli*. Jacobson and Lark (17) reported the existence of DNA molecules labeled with polynucleotide kinase which had a size distribution identical to that of pulse-labeled molecules, but which did not behave as intermediates in DNA replication. Miyamoto and Denhardt (16) and Denhardt et al. (27) also observed a class of DNA molecules in *E. coli* which did not possess ribonucleotides at the 5' terminus and which were present in the cells under a variety of conditions, including chromosome initiation mutants held at the non-permissive temperature. Anderson (18) has presented evidence indicating that about 90% of the small DNA chains found in *E. coli* cannot be chased into higher molecular weight DNA.

In the present study, we have detected in mammalian cells a class of DNA molecules less than 100 nucleotides in length (3S and less in Figure 5). There are approximately 10^5 such molecules per cell, representing about 0.2% of the total DNA, and their number in cells replicating DNA does not differ from that in resting G0 phase cells. The ^32P-labeled material is DNA as judged by its buoyant density, its resistance to hydrolysis by alkali, and its digestion to mononucleotides by DNase I and snake venom phosphodiesterase.

One intriguing feature of these molecules is their small size. Oligonucleotides shorter than Okazaki pieces, have been previously observed in mammalian cells among the nascent DNA molecules (28,29,30). Thus, small size in DNA is usually associated with replication. However, polynucleotide kinase labels all of the molecules extracted from the cell. The ^32P-labeled molecules we detect by this method are not nascent because their density does not increase after moderate periods of labeling with BrdUUMP; also they are detected in resting G0 cells.

Preparation of nuclei under conditions where ribonucleoprotein particles leak out of the nucleus (31), did not result in the liberation of any DNA.
from the nucleus that could be detected by polynucleotide kinase labeling (data not shown). Thus, these DNA molecules are likely to be nuclear in origin. We routinely extracted these DNA molecules from cells under denaturing conditions employing either high temperature or high pH. During extraction under non-denaturing conditions 75% of them are stably associated with double stranded chromosomal DNA while 25% are either free or become liberated from chromosomal DNA during extraction. The liberation of some single-stranded DNA during extraction under non-denaturing conditions has been previously reported (28,32). A variable proportion of pulse labeled nascent DNA is usually found in this class together with some pre-existent DNA molecules. Presumably, these arise by a process of branch migration (33) during extraction or because of incomplete hydrogen-bonding to the template. Thus a chromosomal origin for the molecules we observe is compatible with the data. However, branch migration at replication forks, cannot be the sole explanation for our observation of these molecules in the Hirt supernatant, because we routinely extract molecules with identical properties from G0 cells in which all of the DNA is presumed to be in high-molecular weight unbranched duplex form. One could speculate that these short oligodeoxy-nucleotides are present in G0 DNA in the form of displacement loops similar to those observed in mitochondria (34). The approximate similarity of the number of molecules detected to the number of replication units in mammalian cells (35) is compatible with a role of these molecules in the priming of initiation of DNA synthesis at replicon origins. Of course, other functions for these molecules could also be postulated.

Where then are the nascent Okazaki pieces? Jacobson and Lark (17) detected nascent molecules in E. coli by labeling with BrdU and with polynucleotide kinase. The nascent molecules represented 50% of the short terminally labeled oligonucleotides extracted. Anderson (18) found, in contrast, that only some 10% of the molecules in this size class could be chased into high molecular weight DNA. We have been unable to detect nascent, density-labeled Okazaki fragments in either mammalian cells or in E. coli (27).

A very rough estimate of the expected number of nascent DNA pieces per S phase cell can be made. Mammalian DNA is comprised of replication units whose number is of the order of 10^6 per cell (35). Taking into consideration the average rate of fork movement (about 1 μm/min), and the average size of the portion of a replication unit replicated by one fork (about 30 μm) (35), and the length of S phase (8 hours), we can estimate that there are 10^3
replication forks in operation at any given time in S phase, assuming an even
distribution of replicon involvement throughout S phase. Since each
replication fork should contain at least one Okazaki piece, there should be
a minimum of $10^3$ nascent Okazaki pieces per cell at any time. Presumably,
these molecules can be labeled at the 5' end with polynucleotide kinase after
phosphatase treatment. Certainly failure to detect them cannot be explained
by a block at the 5' end since labeling of the 3' end by terminal transferase
produces the same picture.

We attempted to avoid selective loss of nascent molecules. Since
association with protein is thought to be a possible reason for loss of small
DNA molecules at the interphase in two phase solvent extractions (25), we
used pronase to deproteinize DNA preparations to maximize recovery. Although
we did not observe any difference in recovery of pulse-labeled DNA and pre-
labeled DNA, selective loss of a small portion of pulse-labeled DNA cannot
be ruled out.

Another reason for failure to detect nascent heavy DNA could be in-
efficient labeling with BrdUMP. We have observed that very short pulses of
BrdUMP labeling both in vivo (30 sec) and in vitro (3 min), produce fewer
fully substituted DNA molecules than expected. The results in vivo can be
explained by slow pool equilibration. However, it is more difficult to
explain in the permeabilized in vitro system. Nevertheless, longer pulses
(10 min) of BrdUrd in vivo produce detectable $^3\text{H}$-labeled substituted DNA
molecules (fig. 6a). A 10 min pulse in mammalian cells labels predominantly
molecules much larger than Okazaki pieces but the shortest, most recently
synthesized fragment should be substituted to the maximum extent with BrdUMP.

Failure to detect very many of these molecules with polynucleotide kinase
labeling is probably the result of the fact that they represent a minority
of the 5' ends relative to the light non-nascent molecules described above.
Jacobson and Lark (17) observed about 40 molecules per cell labeled with
polynucleotide kinase, 50% of which were nascent. This represents 10
molecules per fork. There would have to be in excess of 100 Okazaki pieces
per fork in mammalian cells for us to see 50% nascent and 50% non-nascent 5'
termini. Anderson (18) similarly observed 20-40 molecules of 700-9000
nucleotides per E. coli cell, however, only about 10% of these behave as
nascent molecules. The data presented here suggests a similar situation exists
in mammalian cells. Because of the high ratio of light pre-existent to heavy
nascent oligodeoxynucleotides residual free RNA molecules renature with pre-
existant DNA molecules and band in the intermediate density region in CsCl.
This is a likely reason for failure to detect terminal ribonucleotides as $[^{32}\text{P}]{\text{pNp}}$ molecules released by alkaline hydrolysis from density-labeled DNA fragments. Nevertheless, we do observe 2-3% of the total end-label released as $[^{32}\text{P}]{\text{pNp}}$ by alkali. This could be due to residual contamination with free RNA or it could be the result of release of terminal ribonucleotides from some or all of the nascent DNA molecules in the preparation. At present, we are unable to discriminate among these three possibilities.

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