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Isolated HeLa cell nuclei synthesize meaningful DNA

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ABSTRACT

DNA replicated at the beginning of S phase was labelled by incubating nuclei isolated from cells arrested at the G /S border with radioactive deoxyribonucleoside triphosphate in a reaction mixture sustaining DNA synthesis. By hybridization against ribosomal RNA bound to nitrocellulose, the fraction of the labelled DNA which was complementary to rRNA could be quantified, and the stability of the RNA-DNA hybrids could be estimated by sequential elution of DNA at increasing temperatures. The results obtained indicate that the isolated nuclei make "meaningful" DNA, as judged by the melting characteristics of the hybrids between rRNA and the in vitro replicated DNA. Hybridization of the labelled DNA against rRNA fractionated by electrophoresis and blotted onto nitrocellulose verified the presence of sequences complementary to 18 S and 28 S rRNA.

INTRODUCTION

Isolated nuclei have frequently been used in mechanistic studies of the DNA replication process. They are a convenient source of the basic DNA replication apparatus of the cell, can be isolated in a state of high purity (1) and allow the use of deoxyribonucleoside triphosphates as precursors, thus avoiding pool considerations which may complicate interpretation of experiments with intact cells.

Earlier work has demonstrated that isolated nuclei continue ongoing replication (1-5), initiate new Okazaki pieces, and ligate full length Okazaki pieces into high molecular weight DNA (5). It has also been reported that isolated nuclei can initiate replication of new replicons in vitro (6,7). Nuclear replication systems are, however, rapidly exhausted. This can to some extent be prevented or delayed by adding back cytosol or cytosol subfractions to the nuclei (3,7-9).

Clearly, one fundamental question about the suitability of

the nuclear system as recently developed remains to be answered: Do nuclei in vitro produce "meaningful" DNA? Is their DNA product limited to for instance highly repetitive sequences, or is the process of low fidelity? In the present work we have tried to answer these questions. DNA synthesized in isolated S phase nuclei has been hybridized against rRNA bound to nitrocellulose. The thermal stability of the hybrids, which consist of rRNA and ribosomal genes synthesized in vitro, has been investigated. Our results suggest that the replication of DNA in isolated nuclei produces meaningful DNA with a high fidelity, and that the degree of replication of a given DNA sequence varies with the temporal position of the nuclei in the S phase.

MATERIALS AND METHODS

The chemicals and other materials were obtained as follows: Eagle's MEM for suspension cultures (MEMS), newborn bovine serum and Eagle's nonessential amino acids from Flow, Irvine, Scotland; Pluronic F 68 from Maries-Kuhlmann-Wyandotte, Pas de Calais, France, Methocel MC 4000 CP from Fluka, Buchs, Switzerland; proteinase K from Boehringer, Mannheim, Germany; polyvinylpyrrolidone from Serva, Heidelberg, Germany; formamide from E. Merck, Darmstadt, Germany, [^{32}P]dCTP from The Radiochemical Centre, Amersham, England; sodium dodecyl sulphate (SDS) from Bio-Rad, Richmond, Ca., USA; and RNase A, bovine serum albumin, salmon sperm DNA, Ficoll and nucleoside- and deoxynucleoside triphosphates from Sigma, St. Louis, USA. Ribonucleoside-vanadylate complex was prepared as described by Berger and Birkenmeier (10).

Cell culture and synchronization

HeLa cells were cultured in suspension in a gyratory shaker in MEMS supplemented with 10% newborn bovine serum, 25 mM Hepes, 2 mM glutamine, non-essential amino acids, 0.1% Pluronic and 0.03% Methocel, and transferred to fresh medium three times a week. Synchronization of the cells was achieved by blocking with amethopterin and adenosine (final concentration 10^{-6} M and $5x10^{-5}$ M, respectively) and release by addition of thymidine (3µg/10^6 cells) (1,2). Based on the observed increase in

DNA content within 8-10 hrs after the addition of thymidine and the subsequent increase in cell number a synchrony of 75-80% was obtained.

Isolation of cell nuclei

Unless otherwise stated, the cells were harvested 10 min after reversal of the amethopterin block with thymidine. Nuclei were isolated as previously described (1), by washing the cells with buffer A (10 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 2 mM EGTA), resuspension in the same buffer at $8x10^7$ cells/ml, swelling on ice for 10 min, and homogenization in a Dounce homogenizer. Buffer B (340 mM Tris-HCl pH 8.1, 150 mM glucose, 3 mM $MgCl_2$, 2 mM EGTA) (0.5 vol) containing 0.15% Brij 58 was added, and the nuclei pelleted at 200xg for 4 min. After one wash with a 2:1 mixture of buffer A and buffer B, the pelleted nuclei were suspended in replication buffer (see below) for labelling of replicating DNA.

Labelling and purification of DNA

DNA synthesis in isolated nuclei was achieved as described previously (1), by suspending $5x10^6$ nuclei in 100 µl of a replication buffer consisting of 65 mM Tris-HCl pH 8.0, 65 mM NH_4Cl, 50 mM glucose, 10% glycerol, 10.7 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 2 mM EGTA, 10 mM ATP, 50 µM each of UTP, CTP, and GTP, 100 µM of each of three deoxyribonucleotide triphosphates (dGTP, dTTP and either dATP or dCTP), and 0.5 µM of either [^{32}P]dCTP or [^{32}P]dATP (approx. 1300 Ci/mmol). After 10 min at 37°C the nuclei were pelleted by a 15 s centrifugation in an Eppendorf centrifuge and suspended in 300 µl of 50 mM Tris-HCl pH 7.5 containing 0.15 M NaCl, 12.5 mM EDTA, 1% sodium dodecyl sulphate and proteinase K (0.2 mg/ml). After at least 2 h at 37°C the lysate was sequentially extracted with 1 vol of buffer-saturated phenol, buffer-saturated phenol:chloroform:isoamylalcohol 24:24:1 and chloroform:isoamylalcohol 24:1, and nucleic acids were precipitated with 2 vol of ethanol after adjustment of the water phase to 2 M ammonium acetate. After at least 1 h at -70°C or 6 h at -20°C, the nucleic acids were pelleted by centrifugation for 10 min in an Eppendorf centrifuge. The pellet was washed with ethanol, dried briefly in a vacuum desiccator, and dissolved in 300 µl of 10 mM Tris-HCl pH

7.4, 1 mM EDTA containing 50 µg/ml of heat-treated RNase A. After at least 1 h at 37°C the extraction and precipitation steps described above were repeated. The redissolved DNA was sonicated for 2x20s on ice with a Branson Sonifier B12 equipped with a micro tip. This treatment produced DNA fragments of 200-500 bp, as judged by agarose and polyacrylamide gel electrophoresis (not shown). Aliquots of this solution was hybridized to nitrocellulose-bound rRNA as described below.

Isolation, fractionation and nitrocellulose fixation of ribosomal RNA

Ribosomes were isolated from HeLa cells by ultracentrifugation of a post-mitochondrial fraction and washed with 50 mM Tris-HCl pH 7.6 containing 0.5 M NH_4Cl, 10 mM $MgCl_2$ and 6 mM 2-mercaptoethanol. RNA was purified by protease treatment and extraction and precipitation as described above for DNA, except that 10 mM nucleoside-vanadylate complex was included during the protease treatment.

RNA was denatured by treatment with formamide and transferred to nitrocellulose (BA85, Schleicher & Schüll, Dassel, Germany) pretreated with 20xSSC. The transfer was either by dot-blotting directly as described by Thomas (11,12), or after fractionation by electrophoresis in 0.8% agarose gels. Denaturation and electrophoresis was performed as described by Lehrach et al. (13). RNA was transferred from the agarose gels to nitrocellulose using 20xSSC. Dot-blotted RNA was cut into 4 mm circles prior to their baking in vacuum for 2 h at 80°C.

pBR322, which was used as a heterologous hybridization probe, was denatured and dot-blotted in the same manner.

Hybridization

The hybridization buffer consisted of 50% (v/v) deionized formamide, 50 mM sodium phosphate pH 7.4, 0.9 M NaCl, 5 mM EDTA, 0.1% each of Ficoll, polyvinyl-pyrrolidone, bovine serum albumin and SDS, 0.1 mg/ml of denatured salmon sperm DNA, and heat-denatured DNA labelled with[^{32}P]dCTP in isolated nuclei (see above).

Nitrocellulose filters containing dot-blotted rRNA or heterologous DNA (pBR322) were prehybridized, and incubated for 40 h at 42°C in 100 µl[^{32}P] DNA-containing hybridization

buffer. The filters were then washed with 6x40 ml of 2xSSC, 0.1% SDS at room temperature, and with 6x40 ml 0.1xSSC, 0.1% SDS at 50°C. The radioactivity remaining on the filters was quantitated by scintillation counting after solubilization of the filters in Filtercount (Packard Co). For melting curves the filters were washed at room temperature as above, and then washed individually in 2x100 l 0.1xSSC, 0.1% SDS at increasing temperatures. Radioactivity in the washing fluids and the activity remaining on the filters after the final wash were measured by scintillation counting.

Prehybridized single lanes from filters containing fractionated RNA were incubated with 1 ml hybridization buffer containing labelled, denatured DNA in a heat-sealed plastic bag for 40 h at 42°C. The filters were then washed as above, except that 3x150 ml washing solution was used at each temperature. Radioactivity on the moist filters was detected with X-ray film and intensifying screens. The filters were then washed at 60 and 70°C with subsequent exposure to X-ray films for equivalent lengths of time.

RESULTS

In four separate experiments DNA labelled with [^{32}P]dATP in nuclei from the beginning of S phase was hybridized against purified rRNA fractionated by electrophoresis and blotted onto nitrocellulose. Results similar to those shown in Figure 1 were obtained in every case, showing the presence in the labelled DNA of sequences complementary to 28S and 18S rRNA and thus indicating that ribosomal genes are labelled in these nuclei.

The fraction of the labelled DNA which was complementary to rRNA was determined by hybridization against dot-blotted rRNA and counting of hybrid-bound material (Table 1). The amount of hybrid-bound material was proportional to the input of radioactivity (data not shown). About 0.007% of the input DNA remained bound after the wash when DNA labelled in early S phase nuclei was used, whereas no DNA labelled in mid-S phase nuclei was retained on the filters.

The stability of the DNA/RNA hybrids was investigated by sequential elution of hybrid-bound radioactivity at increasing

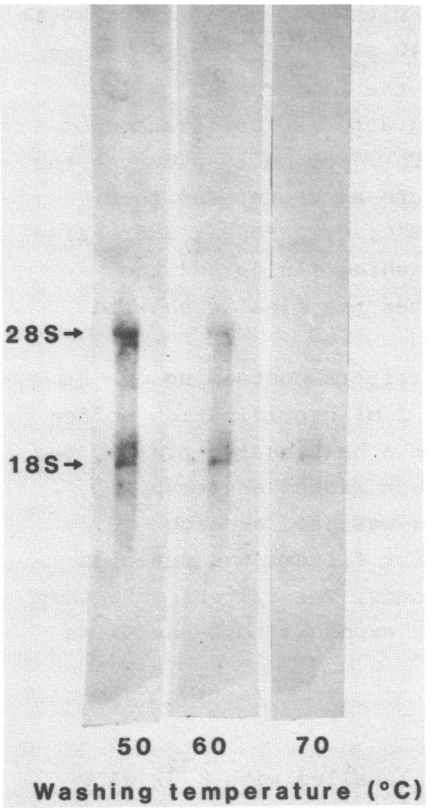

Figure 1. Sequences complementary to rRNA in DNA synthesized in isolated nuclei. RNA was denatured with formaldehyde, fractionated by electrophoresis, transferred to nitrocellulose and hybridized against [32P]DNA synthesized in nuclei harvested from synchronized cells at the beginning of S phase (see Methods). The positions of 18 S and 28 S rRNA are marked with arrows. The blot was exposed to X-ray films for equivalent lengths of time after washing at 50, 60 and 70°C.

Table 1. Hybridization of in vitro synthesized DNA against rRNA and heterologous DNA.

Labelling periods in S phase (min)	Filter bound probe (per cent) rRNA	pBR322	Blank
10-20	0.007 (range 0.006-0.008)	<0.001	<0.001
240-250	<0.001	<0.001	<0.001

DNA labelling in isolated nuclei was started at two different time points in S phase (10 min and 4 h after thymidine addition). Isolated labelled DNA was hybridized against dot-blotted rRNA, pBR322, or blank filters. The percentage of input radioactivity (approx. 2×10^6 dpm) which remained hybrid-bound after washing at 50°C is given as the mean of 3 experiments, each tested in duplicate.

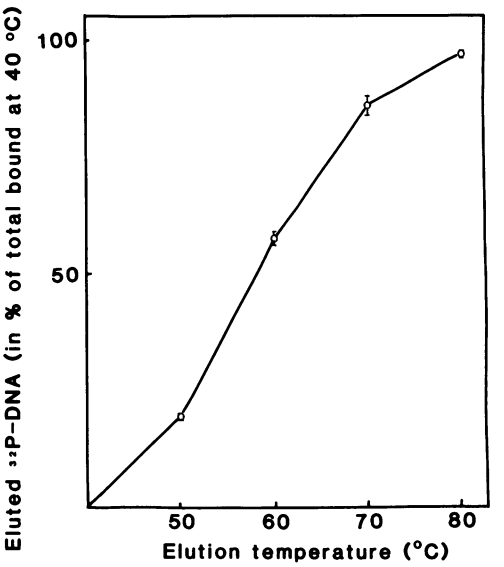

Figure 2. Thermal stability in 0.1xSSC, 0.1% SDS of the hybrid between ribosomal RNA bound to nitrocellulose and DNA synthesized in isolated nuclei. The radioactivity eluted at each temperature was corrected for the radioactivity eluted from an identically treated blank filter.

temperatures. A melting curve was constructed from the results of three independent experiments (Fig. 2). Of the radioactivity remaining on the filters after the 40^0 wash, more than 40% still remained after washing at 60^oC. Under the conditions used, a 50^oC wash will suffice to discriminate between embryonic and adult chick β-globin sequences (13). The results presented here thus indicate a high degree of sequence complementarity between the bound fraction of the in vitro synthesized DNA and rRNA.

We have previously (1,5,8,14) shown that the nuclear system utilized in the present work incorporates dNTP into DNA by a replication-type, rather than a repair-type mechanism. These observations are confirmed by the results (Table 2) showing that the incorporation of dNTPs was almost completely inhibited in the presence of aphidicolin, a DNA polymerase α-specific inhibitor. N-ethylmaleimide, which inhibits DNA polymerases α and γ, but not polymerase β, had the same effect, while ddTTP,

Table 2. Effect of DNA polymerase inhibitors on DNA synthesis in isolated nuclei.

Inhibitor	Incorporation (% of control)
Aphidicolin	6.8 ± 1.7
NEM	1.7 ± 0.2
ddTTP	76.0 ± 10.0

Aphidicolin (10 µg/ml), 5 mM N-ethylmaleimide (NEM), or 50 µM dideoxythymidine triphosphate (ddTTP) was included in replication mixtures with nuclei isolated from cells uniformly prelabelled with [3H]thymidine. DNA synthesis in the isolated nuclei was monitored by [32P]dCTP incorporation. 3H/32P ratios, which then relate the amount of DNA synthesized to cell number, were determined after purification of the DNA as described in Methods. The results are the means ± SD from 5 experiments.

which inhibits polymerases β and γ, had only little effect in our system.

DISCUSSION

The methods employed in the present work do not include the most stringent evaluation of the fidelity of DNA replication in isolated nuclei as compared to in vivo replication (i.e. sequence analysis). However, our results clearly demonstrate that the base sequence of rDNA synthesized in isolated HeLa nuclei is sufficiently correct to allow the in vitro replication product to form stable hybrids having the expected T_m with in vivo transcribed gene products. Previous reports have shown that the rate of overall DNA synthesis in isolated nuclei, although lower, is of a reasonable magnitude compared to that in intact cells. The results presented here show that about 0.007% of the DNA synthesized in isolated nuclei from the beginning of S phase is complementary to rRNA and thus probably represents ribosomal gene sequences. In contrast to this, DNA labelled in nuclei from middle S phase did not form hybrids with rRNA, suggesting no replication of the ribosomal genes of HeLa cells at this time in S. This difference in labelling of ribosomal genes observed in nuclei from early (10 min) and middle (240 min) S phase shows that the incorporation of dNTPs into these genes depends on the temporal position of the nuclei in S phase.

The results confirm and extend the conclusions drawn from

earlier work (1-5), namely that replication in isolated nuclei is a continuation of the on-going replication in the cells from which the nuclei were derived. The temporality of the incorporation of rDNA discussed above is also consistent with these conclusions. It has previously been shown that viral templates (e.g. SV 40 or polyoma DNA) are replicated in isolated nuclei in the same way as in intact cells (15,16). Our results indicate that replication of an endogenous template in isolated nuclei also proceeds with high fidelity.

The techniques described here may therefore also be of value in studies of the temporal distribution of the replication of particular genes throughout the S phase. The ribosomal genes, which number about 160 copies distributed over five chromosomes in the normal human haploid genome (17,18), are abundant genes, and the in vitro replicated genes are consequently fairly easy to detect. The high sensitivity offered by the use of [^{32}P] labelled DNA precursors should, however, allow the replication of less abundant genes to be studied in the same manner. Such a study is underway in our laboratory.

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