EVIDENCE FOR RNA LINKED TO NASCENT DNA
IN HELA CELLS

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ABSTRACT

Rapidly labeled, i.e., nascent, DNA from HeLa cells was separated from the bulk DNA by ultracentrifugation. Further characterization of the rapidly labeled component revealed that its sedimentation coefficient is in the range of 4S and that it exists in a single- and double-stranded conformation. Moreover, analysis by nitrocellulose chromatography and CsSO₄ density sedimentation of the nascent DNA labeled with ³H-uridine revealed that it is covalently linked to short chains of RNA, indicating that in HeLa cells an RNA primer is involved in DNA replication.

Studies of DNA replication indicate clearly that synthesis occurs in a discontinuous manner. This has been observed in viruses such as adenovirus type 2 (28), polyoma (13), Simian virus (SV)₂₅ (6), in bacteria (14, 15) and in eukaryotic cells (9, 23, 26, 27). At the present time a major question concerns participation of an RNA primer in the replication of DNA. Since its original discovery in M 13 phage DNA replication (3), the existence of such an RNA primer in mammalian cells has remained a controversial issue. Evidence for RNA-DNA hybrids in Physarum polycephalum (27), Ehrlich ascites tumor cells (22), human lymphocytes (7), and mitochondrial circular DNA (29) has been presented. In contrast, Berger et al. (1) using mouse myeloma cells and Gautsch and Clarkson (8) working with mouse P 815 cells failed to find any RNA primer.

In the context of our studies on the mechanism of vaccinia virus-mediated inhibition of host DNA replication (16, 18, 19), we undertook to demonstrate the presence of RNA linked to nascent DNA in HeLa cells. The experimental system utilized has these advantages: one can (a) slow down the rate of DNA synthesis at 4°C coupled with enhanced [³H]thymidine (TdR) uptake pretreatment with 5-Fluoro-deoxyuridine (F UdR), (b) obtain rapidly labeled, i.e., nascent, DNA separable from the bulk DNA by ultracentrifugation, and (c) accomplish DNA purification obviating the use of phenol. The results obtained provide evidence for the existence of an RNA primer.

MATERIALS AND METHODS

Cell Culture

HeLa cells free of detectable pleuropneumonia-like organism (PPLO) contamination were employed in all experiments and were cultured in monolayers in Auto-Pow MEM (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (FCS) and maintained at 37°C in a gas mixture of air and 5% CO₂. All experiments were performed with cells in the stationary phase of growth.

Chemicals

TdR and deoxycytidine (dCyt) were purchased from Calbiochem Corp., San Diego, Calif. and F UdR from Hoffman-LaRoche, Inc., Nutley, N. J. Calf thymus
DNA was obtained from Worthington Biochemical Corp., Freehold, N. J.; \([^{3}H]TdR\) 13 Ci/mmol from INC Pharmaceuticals Inc., Plainview, N. Y.; \([^{3}H]Tdr\) 21 Ci/mmol and \([^{3}H]UdR\) 40 Ci/mmol from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; \([^{14}C]tRNA\) (1.25 \(\mu\)Ci/OD260) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind., and CaSO4 from Garrard and Schlesinger. Samples of \(^{3}H\)-labeled yeast RNA (1,000 cpm/\(\mu\)g), \(^{3}H\)-labeled Bacillus subtilis DNA (1.13 \(\times\) 10³ cpm/\(\mu\)g), and \[^{3}H\]UdR 40 Ci/mmol from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J., \([^{14}C]tRNA\) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind., and CaSO4 from Garrard and Schlesinger. Samples of \(^{3}H\)-labeled yeast RNA (1,000 cpm/\(\mu\)g), \(^{3}H\)-labeled Bacillus subtilis DNA (1.13 \(\times\) 10³ cpm/\(\mu\)g), and \[^{3}H\]UdR 40 Ci/mmol from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J., \([^{14}C]tRNA\) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind., and CaSO4 from Garrard and Schlesinger. Samples of \(^{3}H\)-labeled yeast RNA (1,000 cpm/\(\mu\)g), \(^{3}H\)-labeled Bacillus subtilis DNA (1.13 \(\times\) 10³ cpm/\(\mu\)g), and \[^{3}H\]UdR 40 Ci/mmol from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J., \([^{14}C]tRNA\) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind., and CaSO4 from Garrard and Schlesinger.

Preparation of \(^{3}H\) Rapidly Labeled and Bulk DNA Fractions

Cells were plated at a density of 6 \(\times\) 10⁶ cells per 100-mm petri dish and allowed to grow to confluence in Eagle’s MEM (6) supplemented with 10% FCS dialyzed according to Dulbecco and Vogt (5). After the cells reached a density of 10⁶ per plate, \(\mu\)-FUDR was added to the above medium for 1 h at 4°C. Upon removal of the FUDR medium, the cultures were incubated for only 1 min at 25°C with 50-100 \(\mu\)Ci/ml of \([^{3}H]Tdr\) (sp act 13 Ci/mmol) or \([^{3}H]UdR\) (21 Ci/mmol) in a solution containing 60 mM Tris-HCl pH 7.4, 60 mM NaCl, 11 mM glucose, and 2 mM MgCl₂. Incorporation of \([^{3}H]Tdr\) was terminated by lysing the cells and sedimenting the bulk DNA by means of hydroxyapatite column chromatography. Both DNA species were first sheared by extrusion several times before and after the column chromatography, further purified on nitrocellulose columns. Rapidly labeled ss or ds DNA containing incorporated \([^{3}H]UdR\) was, after isolation by hydroxyapatite chromatography, further purified on nitrocellulose columns as described above. As described by Sato et al. (22), the \(^{3}H\)-UdR-labeled DNA was eluted with SDS-TSE buffer and dialyzed for 2 days at 4°C against 3 liters of distilled water for further analysis in CsSO₄ equilibrium density gradients.

Characterization of the Rapidly Labeled DNA Fractions

Determinations of the amount of \([^{3}H]Tdr\) and \([^{3}H]\)-UdR incorporated into rapidly labeled and bulk DNA were made on 100-\(\mu\)l duplicate aliquots of cell lysate.
Samples to which 50 μg calf thymus DNA was added as carrier were precipitated in ice-cold 10% TCA. The acid-insoluble cpm contained in the precipitates were retained by Millipore filtration and determined by scintillation counting. Estimates of the isotope present in the bulk DNA fraction before hydroxyapatite chromatography were made by subtracting radioactivity incorporated into the rapidly labeled DNA from that present in the whole lysate. The [3H]TdR incorporation was expressed as a percentage of the total.

Velocity sedimentation analyses of the DNA were performed by centrifugation for 6–7 h at 56,000 rpm in a SW 65 rotor in gradients consisting of 5–20% wt/vol sucrose, 1 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. [14C]tRNA was added as a marker. Fractions collected from the bottom were mixed with 10% TCA, and the precipitates formed were collected by Millipore filtration and prepared for scintillation counting.

Equilibrium density analyses were made in CsSO₄. All samples were heat-denatured at 100°C for 5 min followed by rapid cooling on ice, and to each was added 1 OD₅₀₀ of bacteriophage Fd ss DNA as carrier. The sample was mixed with saturated CsSO₄ in a final volume of 10.8 ml of 5 mM EDTA at a p 1.506 g/cm³, and centrifugation was performed at 20°C for 60 h at 38,000 rpm in a 50 Ti rotor (Beckman Instruments). When [3H-]UdR rapidly labeled DNA was analyzed, 1% of formaldehyde was added to the gradient mixture as recommended by Sugino et al. (25). Fractions were prepared for scintillation counting as described above.

Electrophoresis in a mixture of 0.5% agarose and 1.7% acrylamide was performed according to Dingman et al. (4), using 15-cm long gels and applying 8.8 V/cm of gel for either 16–17 h at 4°C or 5–6 h at 25°C. The buffer contained 0.04 M Tris, pH 7.8, 1 mM EDTA, 5 mM

### TABLE 1

|        | % of total label | % of total label* |
|--------|------------------|-------------------|
| Supernatant | 77.5            | 22.5              |
| rapidly  | 0.5             | 99.5              |
| labeled DNA |                |                   |
| Pellet DNA | (Bulk DNA)      |                   |

### Figure 1

Monolayers containing 8 × 10⁶ cells were incubated as described in Materials and Methods. Supernate (rapidly labeled) and bulk DNA isolated from lysates were separated on hydroxyapatite columns after adsorption. The absorbance at 260 nm was measured after alkaline hydrolysis. (●—●) rapidly labeled; and (O—O) bulk DNA.
TABLE II
Distribution of ss or ds DNA in the Rapidly Labeled and Bulk Fraction as a Percent of the Total CPM Released by Hydroxyapatite Chromatography

| Molarity of PO₄ | Percent Specific of radioactivity | cpm | cpm/µg |
|----------------|----------------------------------|-----|-------|
|                | ssDNA                             | 86  | 8    |
|                | dsDNA                             | 13  | 1    |
| Bulk DNA       | ssDNA                             | 0   | 0    |
|                | dsDNA                             | 100 | 10   |

Monolayers containing 8 × 10⁶ cells were incubated with 50 µCi/ml of [³²P]Tdr (sp act 21 Ci/mmol) for one min. Rapidly labeled and bulk DNA were isolated and separated as in Fig. 1. The results are expressed as percent of the total cpm or cpm/µg DNA released from the columns.

sodium acetate, and 0.5 µg/ml ethidium bromide. The rapidly labeled ss DNA, purified by phenol-m-cresol, was added to gels along with marker molecules, including [³⁵S]RNA, ds calf thymus, and Fd DNA. The position to which the unlabeled DNA had migrated in gels could be observed under a UV lamp due to the fluorescence at 366 nm of the DNA ethidium bromide complex. Whenever appropriate, RNA was removed by alkaline hydrolysis at 100°C in 0.3 M NaOH for 5 min. The hydrolysate was neutralized with HCl before cooling on ice.

Enzymatic Assays

The Neurospora crassa ss endonuclease (Miles Laboratories) was assayed according to Rabin et al. (21). Termination of the reaction was achieved by adding as carrier, to each sample, 25 µg of calf thymus and 0.5 M perchloric acid (PCA), and acid-soluble cpm were measured. Pancreatic DNase (2,130 U/mg, Worthington), free from RNase, was assayed under the same conditions as described above. [³²P]UdR rapidly labeled DNA was incubated with 20 µg of enzyme in a final volume of 100 µl of 0.05 M Tris pH 7.0 and 1 mM MgCl₂ for 1 h at 37°C. To each sample were added 25 µg of yeast RNA as carrier and the reaction was terminated by mixing with 100 µl of 1 M ice-cold PCA. Acid-soluble material which was formed in the precipitate was sedimented by centrifugation at 2,000 rpm for 10 min and hydrolyzed DNA in 50-µl duplicate aliquots were assayed in a scintillation counter.

Susceptibility of [³²P]UdR rapidly labeled DNA was also tested with a combination of two RNases, one from the bovine pancreas and the other from Aspergillus oryzae (Worthington), termed the T1 enzyme. Before hydrolysis the RNases were placed for 5 min at 100°C to destroy any contaminating DNase activity. After incubation with the DNA samples for 1 h at 37°C the reaction was terminated as described above in the DNase assay, and acid-insoluble material present in the precipitate was collected by Millipore filtration for scintillation counting.

RESULTS

Isolation of Labeled Products after Short-Term Synthesis of DNA

After labeling with [³²P]Tdr during pulses of 1 min and chasing for 10 min, the DNA was separated from cell lysates by ultracentrifugation. The data summarized in Table I showed that most of the label incorporated during the pulse was present in rapidly labeled DNA whereas that acquired during the pulse-chase period occurred predominantly in the bulk DNA fraction. This suggests a transfer into the bulk fraction during the chase and also indicated that the ultracentrifugation procedure employed effectively separated rapidly labeled from bulk DNA. Therefore, during the chase, elongation of the DNA must have occurred.

Purification of Rapidly Labeled and Bulk DNA

After its isolation from the crude lysates, the rapidly labeled DNA was separated and purified by hydroxyapatite column chromatography into double-stranded (ds) and single-stranded (ss) components. As a check of the procedure used for HeLa cell DNA, denatured and native calf thymus DNA were shown to be eluted from the columns.

TABLE III
Hydrolysis of Rapidly Labeled and Bulk DNA by Neurospora Crassa Endonuclease

| DNA fraction | Percent hydrolyzed* |
|--------------|---------------------|
| Rapidly labeled ss | 100 |
| Rapidly labeled ds | 40.7 |
| Bulk ds | 17 |

Rapidly labeled ss and ds DNA isolated on hydroxyapatite columns was incubated at 37°C for 60 min with 3.3 U/ml of N. crassa endonuclease. The reaction was terminated by adding to each sample 25 µg of calf thymus carrier DNA and 0.5 M perchloric acid. The precipitate was centrifuged into pellets in 10 min at 12,000 rpm. Cpm measured in the acid-soluble component was calculated as the percentage of the total cpm added initially. Each point in the table represents the mean value of duplicate samples.

* Enzymatic hydrolysis was tested after alkaline degradation because this Neurospora endonuclease is also active with RNA as a substrate.
FIGURE 2 Monolayers containing $8 \times 10^6$ cells were incubated with 50 $\mu$Ci/ml of $[^3H]$TdR (sp act 21 Ci/mmol) for 1 min. Rapidly labeled ss DNA fractions were purified on hydroxyapatite according to Fig. 1 and centrifuged at 20°C for 6 h in (a) or 7 h in (b) at 56,000 rpm in neutral gradients containing 5-20% wt/vol sucrose, 1 M NaCl, 10 mM Tris-Cl pH 7.5, and 1 mM EDTA. $[^4C]$tRNA from *E. coli* was added as an external 4S marker. After collecting dropwise 0.2-ml fractions, the DNA was precipitated by adding 10% TCA, collected on Millipore filters, and prepared for scintillation counting.

FIGURE 3 Rapidly labeled DNA fractions, isolated as described in Materials and Methods, were extracted with phenol and electrophoresed in a mixture of 0.5% agarose and 1.7% acrylamide according to procedures described in Materials and Methods. The external markers added were: ds calf thymus DNA, Fd bacteriophage DNA, and *E. coli* $[^{14}C]$tRNA. Each gel was divided into 2-mm thick slices which were incubated at 45°C for 2 h and dissolved in 0.5 ml of N,N-dimethyl formamide, then mixed with 10 ml of the toluene-Triton (2:1) scintillation liquid mixture in preparation for counting. (●●●) $[^3H]$TdR pulse-labeled for 1 min; (○○○) $[^3H]$TdR pulse-labeled for 1 min, chased for 10 min; and (×××) $[^3H]$TdR pulse-labeled 1 min, chased for 30 min.
with 0.15 M and 0.25 M phosphate buffer pH 7.0, respectively. An elution profile of supernatant DNA equivalent to rapidly labeled and bulk DNA are shown in Fig. 1. Since RNA became eluted at the same molarity (0.15 M) as ss DNA, alkaline hydrolysis was performed before measuring the absorbancy at 260 nm. The results indicated that the supernate (rapidly labeled DNA) existed in the ss and ds conformations and that more ss than ds DNA was present. With bulk DNA the reverse situation was found whereby most of the material at 260 nm occurred as ds DNA (Fig. 1). The nature of the ss component, observed reproducibly in the bulk DNA fraction, remains to be elucidated. The distribution of rapidly labeled and bulk DNA between the ss and ds fractions, after 1 min pulse with \[^{3}H\]Tdr, as determined by hydroxyapatite chromatography is given in Table II. The data indicated that rapidly labeled DNA component contained approximately equal amounts of ds- and ss-labeled material but the specific radioactivity of ss rapidly labeled DNA was less than that of ds rapidly labeled DNA. In the bulk fraction, only labeled ds DNA was detected, possessing a low specific radioactivity.

**Characterization of Rapidly Labeled DNA**

**Susceptibility to Neurospora Crassa Nuclease:** The rapidly labeled and bulk DNA isolated by hydroxyapatite columns was incubated with *Neurospora crassa* endonuclease. The results summarized in Table III indicated that rapidly labeled ss DNA was more susceptible to hydrolysis by this enzyme than rapidly labeled ds DNA. By contrast, bulk DNA was relatively insoluble. As a control of the specificity of this enzyme, heat-denatured \[^{3}H\]-labeled *B. subtilis* DNA was found to be totally hydrolyzed, but the same undenatured DNA was hydrolyzed only 15% and yeast \[^{3}H\]-labeled RNA, 56%.

**Velocity Sedimentation Analysis:** Characterization of ss and ds rapidly labeled DNA by velocity sedimentation was conducted in neutral 5-20% wt/vol sucrose gradients. The results of a representative experiment shown in Fig. 2a and b indicated that ss rapidly labeled DNA was heterogenous in size, having a skewed distribution with a peak in the vicinity of the 4S tRNA marker, but there was considerable material in the molecular range less than 4S (Fig. 2a). The amount of radioactive material occurring in peak fractions of 4S was calculated to be 85.8% of the total present in the gradient. By contrast, rapidly labeled ds DNA had a symmetrical sedimentation profile, with a peak in the zone of somewhat higher molecular weight than that to which the 4S marker had been sedimented (Fig. 2b).

**Gel Electrophoresis:** Phenol-extracted ss rapidly labeled DNA was also characterized by electrophoresis in agarose-acrylamide gels. Several molecular weight marker molecules were added to the labeled DNA samples to obtain a size estimate. They could be pinpointed under UV light at 366 nm after interacting with ethidium bromide added to the buffer solution used for electrophoresis. Another reference marker was 4S \[^{14}C\]tRNA. The data presented in Fig. 3 revealed that most of the ss rapidly labeled DNA synthesized in a 1-min pulse was slightly greater than 4S. However, labeled DNA isolated after the pulse and 10- or 30-min chase was no longer part of the 4S material but, instead, was co-electrophoresed with higher molecular weight ds DNA marker (Fig. 3).

**Equilibrium Density Sedimentation Analysis:** To ascertain whether rapidly
labeled nascent ss DNA was covalently linked to primer RNA. [3H]TdR pulse-labeled DNA was sedimented to equilibrium in CsSO₄ before and after alkali hydrolysis. It was found that alkali-untreated ss rapidly labeled DNA possessed a buoyant density greater than that of alkali-treated DNA (Fig. 4). The density of the latter was approximately coincident with that of the marker DNA. The presence of covalently linked RNA is, therefore, suggested by these data. By use of the formula of Hirose et al. (11), relating the shift of density with content of RNA in hybrid nucleic acid, it was calculated that RNA in the rapidly labeled ss DNA fraction was approximately 30% of the mass of the hybrid. On this basis, the RNA primer should contain about 30 nucleotide residues, presuming that on the average there were 100 residues in each rapidly labeled RNA-DNA molecule.

Further evidence for the presence of an RNA primer was obtained by isolating rapidly labeled DNA synthesized during a pulse in the presence of [3H]UdR. Cell nuclei were isolated, then lysed, and bulk DNA was separated from rapidly labeled ss and ds DNA on hydroxyapatite columns. Further purification, in order to remove the free RNA from the DNA-linked RNA, was carried out using nitrocellulose column chromatography according to Probst et al. (20). It is known from the work of Sato et al. (22) that the free RNA becomes eluted by TSE-buffer and RNA complexed with rapidly labeled DNA by SDS-TSE buffer. The data on the characteristics of the [3H]UdR rapidly labeled ss and ds DNA are presented in Figs. 5 and 6. In Fig. 5 a, labeled ss DNA in the eluate from nitrocellulose columns was distributed among a major fraction of free RNA and a smaller peak of hybrid material. The latter, after sedimentation to equilibrium in CsSO₄, became distributed among two peak fractions, one with a density somewhat greater than that of ss \textit{B. subtilis} DNA marker, and the other at a density coincident with that of the yeast RNA marker (Fig. 6 a). In Fig. 5 b, labeled ds DNA in the eluate from nitrocellulose
columns also became separated into two peak fractions. This material also was resolved by CsSO₄ equilibrium centrifugation into two peaks, one with a buoyant density greater than that of marker yeast RNA (Fig. 6 b). These results provided further evidence for the possible presence of a covalently linked RNA primer molecule in the rapidly labeled DNA fragments. In addition, the $^{3}H$-UdR-labeled material sedimenting closer to the buoyant density of DNA was not hydrolyzed upon incubation with pancreatic DNase, indicating that the label was not in DNA. Exposure of the $^{3}H$-UdR-labeled DNA fraction to combined T1 and pancreatic RNases caused the hydrolysis of only 10% of the total counts present in this fraction, but treatment with alkali released 75% of the total counts. This result implies that the label was in an alkali-labile (RNA) component which, because of its association with the DNA, remained mostly refractory to the RNases.

DISCUSSION

Previous studies have shown that newly synthesized HeLa DNA is in the form of short, partially ss segments (17). Moreover, Habener et al. (10) observed that newly formed DNA, while within the nucleus, has the capacity to assume either the ds or the ss conformation, depending on the conditions of DNA extraction. They reported that very little ss nascent DNA was present when DNA was extracted with phenol. Under our experimental conditions not involving phenol extraction, we observed that rapidly labeled DNA was in both ss and ds conformations. Those results are in agreement with the findings of Painter and Schaefer (17) and Fox et al. (7) who also avoided phenol.

Furthermore, the rapidly labeled ds DNA was more susceptible to Neurospora crassa endonuclease than was bulk DNA, suggesting the presence of ss stretches in this material even though the whole molecule behaves like ds DNA during hydroxyapatite chromatography. As concerns the size of rapidly labeled DNA, Schandl and Taylor (24) found that nascent DNA of Chinese hamster cells had a sedimentation coefficient of 4.2S. This value is in agreement with our data on HeLa cells and the findings of Fox et al. (7) on human lymphocytes. Incidentally, the size of these fragments was not significantly different regardless of whether extraction was made with phenol or not.

The controversial results obtained in the search for RNA-DNA hybrids may stem from the very different methods used for detecting such hybrids. Several factors may have been of paramount importance in the successful outcome of our current investigation including: (a) the use of slowly growing cells in a nutrient medium which was almost completely depleted of TdR; (b) the ability to slow down DNA synthesis by incubation at suboptimal temperature before administering a 1-min pulse with isotopic precursors of high specific radioactivity; and (c) the avoidance of alcohol precipitation so as to maintain the integrity of RNA molecules and improve the DNA yield.

The negative results reported by others may...
have been due to any one or a combination of the above factors. It now seems most plausible that initiation of DNA synthesis in mammalian cells, like that of DNA in prokaryotic systems and animal viruses, most probably involves an RNA primer. However, the possibility that in the animal cell the RNA-DNA hybrid may have a shorter half-life or greater instability should not be overlooked, and it could be the basis for the heretofore contradictory findings in different laboratories.

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Note added in Proof: Since the acceptance of this paper, Mendelsohn et al. (1975. Biochim. Biophys. Acta. 407:283) showed that some of the DNA-RNA complexes isolated in CsSO4 may be artifactual binding. However, they found that it is possible to eliminate such binding by placing formaldehyde in the density gradient. Under these conditions the RNA remaining associated with DNA represents 20% of the nucleic acid content of the molecule.

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