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 $CsSO_4$ density sedimentation of the nascent DNA labeled with ^{3}H-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 $(SV)_{40}$ (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 Gautschi 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 [^{3}H]thymidine (TdR) uptake pretreatment with 5-Fluoro-deoxyuridine (FUdR), (*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% CO_2. 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 FUdR 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 μCi/OD_{260}) from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind., and $CsSO_4$ from Garrard and Schlesinger. Samples of ^{3}H-labeled yeast RNA (1,000 cpm/μg), ^{3}H-labeled *Bacillus subtilis* DNA (1.13×10^5 cpm/μg), and Fd bacteriophage DNA were kindly provided by, respectively, Doctors O. Pogo (New York Blood Center), D. Dubnau, L. Day (of this Institute), and W. Szer (New York University Medical Center).

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

Cells were plated at a density of 6×10^6 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 10^7 per plate, M^{-6} 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 μCi/ml of [^{3}H]TdR (sp act 13 or 21 Ci/mmol) in a solution containing 60 mM Tris-HCl pH 7.4, 60 mM NaCl, 11 mM glucose, and 2 mM $MgCl_2$. Incorporation of [^{3}H]TdR was terminated by lysing the cells upon the addition of four times the volume of a solution containing 0.5% sodium dodecyl sulfate (SDS), 0.05 M sodium phosphate pH 6.8, 5 mM EDTA. A crude nascent DNA fraction was obtained from the lysate by sedimenting the bulk DNA by means of ultracentrifugation at 20°C for 2 h at 45,000 rpm as described by Fox et al. (7). Further purification of the rapidly labeled and bulk DNA was achieved by means of hydroxyapatite column chromatography. Both DNA species were first sheared by extrusion several times through 18 and 23 gauge syringe needles then absorbed onto hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) columns, 7 $cm^2 \times 10$ cm. After adsorption, columns were washed with 500 ml of 0.05 M sodium phosphate pH 7.0, and the elution of DNA was carried out by increasing the buffer molarity stepwise from 0.1 to 0.3 M. The acid-insoluble radioactivity was measured throughout the elution pattern by precipitating 50-μl aliquots of the fractions with 10% trichloroacetic acid (TCA) and the acid-insoluble material trapped on Millipore (Millipore Corp., Bedford, Mass.) was counted in 10 ml of a mixture containing toluene-Triton (2:1) and 5.5 g 2,5-diphenyloxazole (PPO) and 0.1 g POPOP/liter of toluene in a Beckman scintillation spectrometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

For the analysis by gel electrophoresis, rapidly labeled single-stranded (ss) DNA was extracted with a mixture of phenol-*m*-cresol and hydroxyquinoline after the procedures of Kirby (12), as described by Oishi (14) for nascent DNA.

Isolation of [^{3}H]Uridine Rapidly Labeled DNA

Monolayer cultures were grown to confluence in MEM supplemented with 10% FCS. After removal of the nutrient medium, cells were incubated at 25°C for 15 min in a mixture of 10^{-6} M TdR, 10^{-6} M dCyt, 60 mM Tris HCl pH 7.4, 60 mM NaCl, 11 mM glucose, 2 mM $MgCl_2$ (solution A) to which were added 200 μCi/ml of [^{3}H]UdR (40 Ci/mmol). At the end of incubation, cells were lysed with 0.25% Nonidet P_{40} (NP_{40}, Shell Chemical Corp., New York, N. Y.), mixed in solution A with 30 μg/ml of polyvinylsulfate and 0.2% diethylpyrocarbonate (Eastman Kodak Co., Rochester, N. Y.). The last two chemicals were added to all solutions during preparation of rapidly labeled DNA to minimize endogenous RNase activity. Freed nuclei were sedimented by centrifugation at 1,200 rpm for 5 min, washed once with solution A, then lysed by 0.5% SDS in 0.05 M sodium phosphate, pH 6.8 and 5 mM EDTA. Rapidly labeled DNA fractions were separated and purified on hydroxyapatite columns by the procedure used with [^{3}H]TdR rapidly labeled DNA cited above.

After chromatography on hydroxyapatite, further purification was carried out using nitrocellulose columns to remove any free-RNA from the RNA bound to rapidly labeled DNA (20). The nitrocellulose RS ¼ s columns (Randolph Co., Houston, Texas), 1.1 $cm^2 \times 4.6$ cm, were prepared as described by Boezi and Armstrong (2). They were first standardized with ^{3}H-labeled yeast RNA and [^{3}H-]TdR-rapidly labeled HeLa cell DNA. The bound material was eluted successively with 0.4 M NaCl and 1 mM EDTA in 0.05 M Tris-HCl buffer pH 6.75 (TSE-buffer) and then with 0.5% SDS in the TSE-buffer (SDS-TSE-buffer). The flow rate was maintained at 5 ml/h during sample application and elution, and fractions of 0.5 ml were collected. 50-μl aliquots were used for determination of the acid-insoluble cpm in the fractions, as previously described. About 80% of the bound radioactivity could be recovered from the 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 dialysed for 2 days at 4°C against 3 liters of distilled water for further analysis in $CsSO_4$ 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-μl 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 [^{3}H]TdR incorporation was expressed as a percentage of the total.

TABLE I

Distribution of [^{3}H]TdR in Rapidly Labeled and Bulk DNA Fractions after Pulsing and Chasing

	Supernatant rapidly labeled DNA	Pellet (Bulk DNA)
	% of total label	*% of total label**
1-min pulse	77.5	22.5
1-min pulse, 10-min chase	0.5	99.5

Monolayers containing 8 × 10^6 cells were labeled with 100 μCi/ml of [^{3}H]TdR (13 Ci/mmol) in 1-min pulses. Some cultures received medium with 0.1 mM thymidine immediately after the pulse, and incubation was continued for 10 min more. Incorporation of [^{3}H]TdR into the rapidly labeled and bulk DNA was determined as described in Materials and Methods.

* Percent of the total TCA-precipitable cpm.

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. [^{14}C]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 $CsSO_4$. All samples were heat-denatured at 100°C for 5 min followed by rapid cooling on ice, and to each was added 1 OD_{260} of bacteriophage Fd ss DNA as carrier. The sample was mixed with saturated $CsSO_4$ in a final volume of 10.8 ml of 5 mM EDTA at a ρ 1,506 g/cm^3, and centrifugation was performed at 20°C for 60 h at 38,000 rpm in a 50 Ti rotor (Beckman Instruments). When [^{3}H-]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

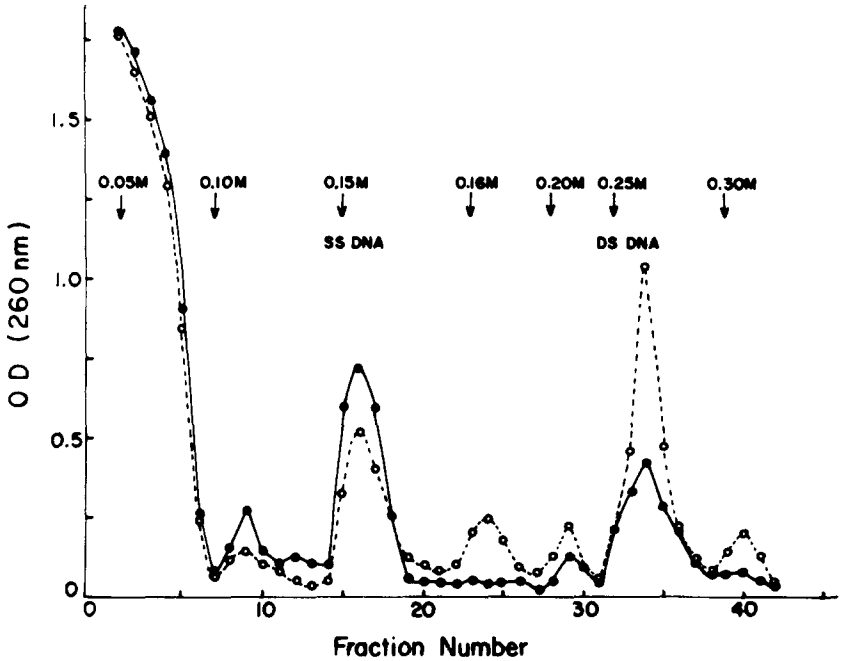

FIGURE 1 Monolayers containing 8 × 10^6 cells were incubated as described in Materials and Methods. Supernate (rapidly labeled) and bulk DNA isolated from lysates were separated on hydroxyapatite columns after adsorption. The absorbance at 260 nm was measured after alkaline hydrolysis. (●—●) rapidly 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 PO_4	Percent of total	Specific radioactivity
	M	*cpm*	*cpm/μg*
Rapidly labeled DNA	0.15 ss DNA	42.4	51
	0.25 ds DNA	57.6	78
Bulk DNA	0.15 ss DNA	0	—
	0.25 ds DNA	100	10

Monolayers containing 8 × 10^6 cells were incubated with 50 μCi/ml of [^{3}H]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 [^{14}C]tRNA, 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. [^{3}H]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 Mg^{++} 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 [^{3}H-]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 [^{3}H]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.

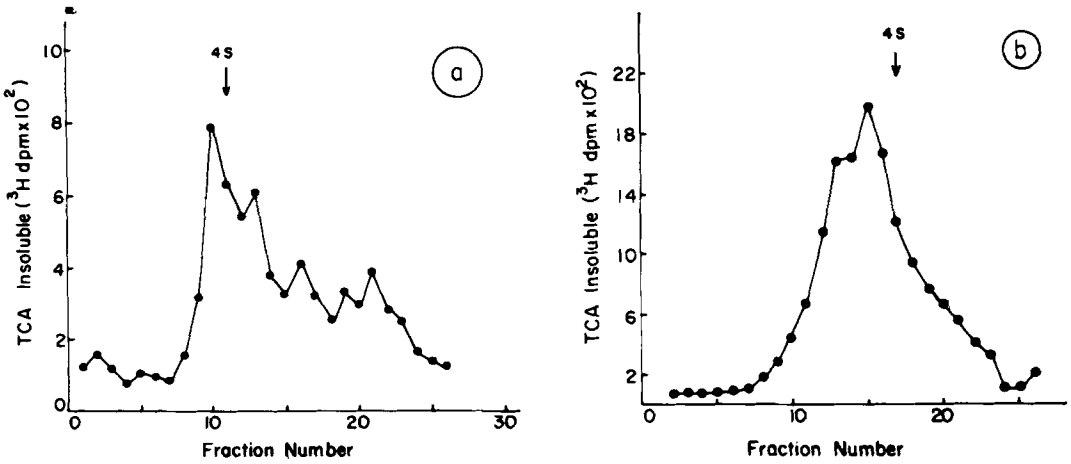

FIGURE 2 Monolayers containing 8 × 10^6 cells were incubated with 50 μCi/ml of [^{3}H]TdR (sp act 21 Ci/mmol) for 1 min. Rapidly labeled ss DNA fractions were purified on hydroxyapatite according to Fig. 1 and centrifuged at 20°C for 6 h in (*a*) or 7 h in (*b*) at 56,000 rpm in neutral gradients containing 5–20% wt/vol sucrose 1 M NaCl, 10 mM Tris-HCl pH 7.5, and 1 mM EDTA. [^{14}C]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.

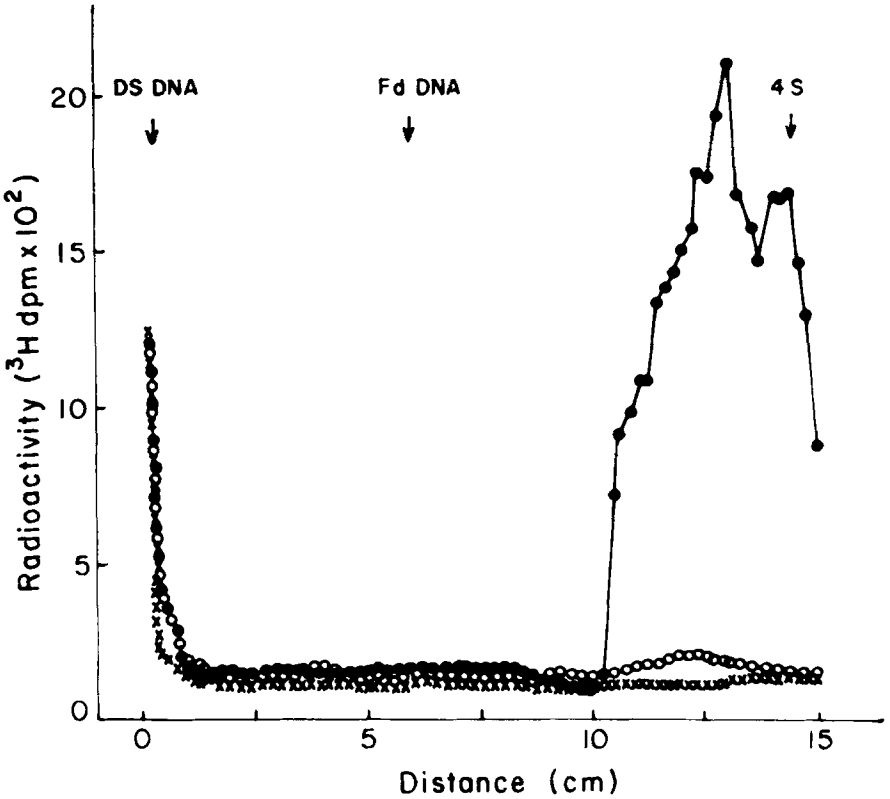

FIGURE 3 Rapidly labeled DNA fractions, isolated as described in Materials and Methods, were extracted with phenol and electrophoresed in a mixture of 0.5% agarose and 1.7% acrylamide according to procedures described in Materials and Methods. The external markers added were: ds calf thymus DNA, Fd bacteriophage 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. (●—●) [^{3}H]TdR pulse-labeled for 1 min; (O—O) [^{3}H]TdR pulse-labeled for 1 min, chased for 10 min; and (×—×) [^{3}H]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 insusceptible. 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. 2 *a* 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. 2 *a*). 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. 2 *b*).

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

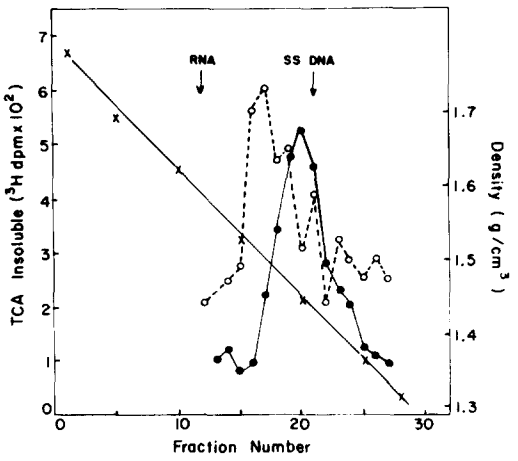

FIGURE 4 Rapidly labeled ss DNA, isolated and purified by hydroxyapatite chromatography, was heated for 5 min at 100°C to denature any ds DNA, then either mixed directly with saturated $CsSO_4$ or first subjected to alkali hydrolysis in 0.3 M NaOH at 100°C for 5 min before mixing with $CsSO_4$. Centrifugation was carried out in a 50 rotor for 60 h at 38,000 rpm at 20°C. The ss calf thymus DNA and yeast tRNA markers were treated in the same manner. Acid-precipitable cpm were determined on individual fractions. (O---O) alkali-untreated; and (●—●) alkali-treated; (×—×) density.

labeled nascent ss DNA was covalently linked to primer RNA, [^{3}H]TdR pulse-labeled DNA was sedimented to equilibrium in $CsSO_4$ 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 [^{3}H]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 [^{3}H]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 $CsSO_4$, became distributed among two peak fractions, one with a density somewhat greater than that of ss *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

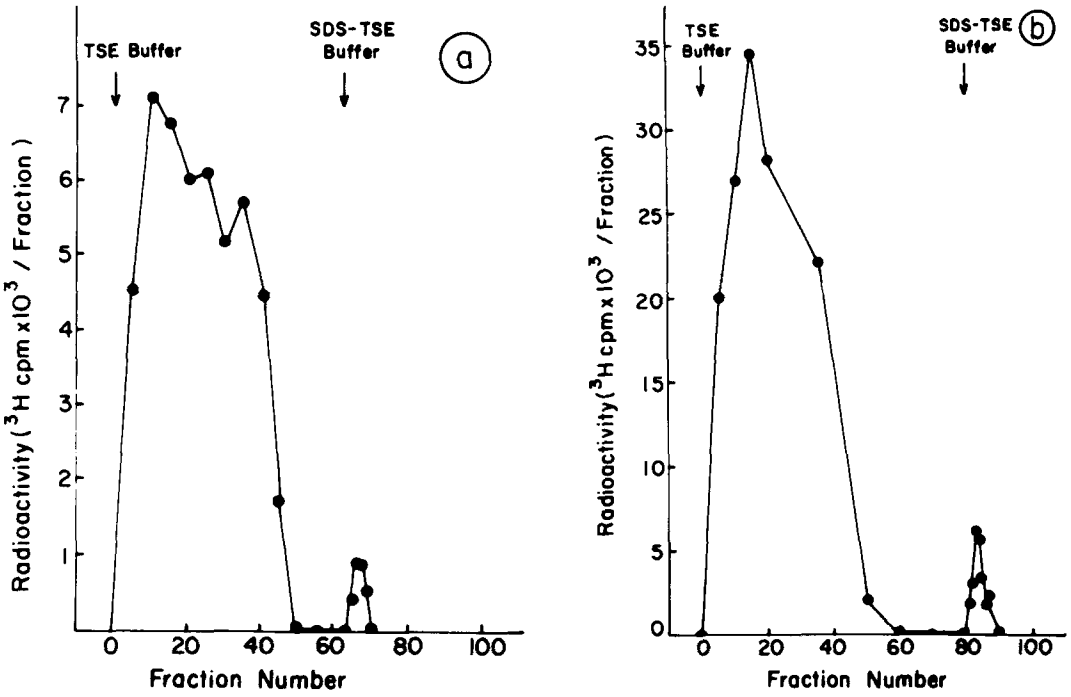

FIGURE 5 *a* and *b* Monolayers containing 8 × 10^6 cells were pulse labeled for 10 min at 25°C with 200 μCi/ml of [^{3}H]UdR (40 Ci/mmol). The rapidly labeled ss DNA fraction, isolated on hydroxyapatite columns, was further purified on 1 cm^2 × 4 cm nitrocellulose columns and eluted successively with 0.05 M Tris-HCl buffer pH 6.75 containing 0.4 M NaCl and 1 mM EDTA (TSE-buffer) and TSE-buffer containing 0.5% SDS (SDS-TSE-buffer). Free RNA is eluted by TSE-buffer and RNA linked to rapidly labeled DNA by SDS-TSE-buffer. In (*a*) chromatography of ss DNA, in (*b*) of ds DNA.

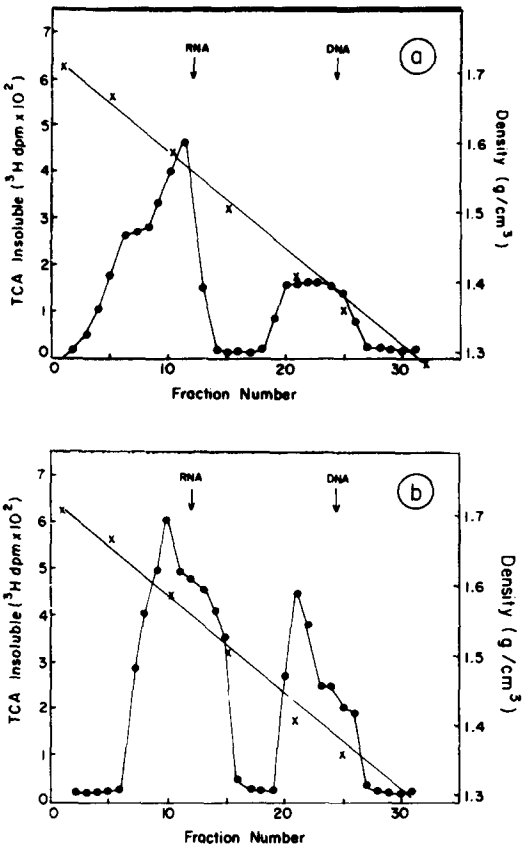

FIGURE 6 *a* and *b* Fractions of rapidly labeled DNA eluted from nitrocellulose columns with 0.5% SDS-TSE buffer were dialyzed against distilled water, and centrifuged at 20°C in a 50 rotor for 60 h at 38,000 rpm in $CsSO_4$ gradients. In (*a*) ss material and in (*b*) ds material. Marker molecules of heat-denatured *B. subtilis* ^{3}H-labeled DNA and ^{3}H-labeled yeast RNA were cocentrifuged in separate tubes. All fractions from the gradient were prepared for counting the acid-precipitable material. (●—●)[^{3}H]dpm; (×—×) density.

columns also became separated into two peak fractions. This material also was resolved by $CsSO_4$ 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 $CsSO_4$ 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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