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Single-stranded DNA from oncornavirus-infected cells enriched in virus-specific DNA sequences

(hydroxyapatite chromatography/avian myeloblastosis virus/molecular hybridization/provirus DNA transcription)

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ABSTRACT **We previously found that a minor fraction of single-stranded DNA (ss-DNA) isolated from native nuclear DNA of normal chicken embryonic cells and cells of other species hybridized with bulk nuclear DNA or cellular RNA in great excess. At least one-third of ss-DNA belonging to the nonrepetitious part of the cell genome could be hybridized to homologous RNAs. In the present work, similar results were obtained with ss-DNA from cells of chickens infected by avian myeloblastosis virus (AMV). To investigate whether this enrichment of ss-DNA in transcribed DNA sequences involves provirus DNA, radioactive AMV RNA and cDNA copies of AMV RNA were used. Most of the 70S AMV RNA hybridized much faster to ss-DNA from productively infected leukemic cells than to bulk DNA. cDNA, either double-stranded or single-stranded, made in the presence of actinomycin D hybridized to total nuclear DNA with similar kinetics. In contrast, about half of the double-stranded cDNA molecules hybridized 40–50 times faster to ss-DNA than to total DNA, indicating that only one of the provirus DNA strands seems to be present in ss-DNA. This was confirmed by the fact that relatively insignificant amounts of the ss-cDNA molecules made in the presence of actinomycin D could be annealed to ss-DNA as compared with bulk DNA. These results indicate that actively transcribed DNA sequences can be selectively distributed in the ss-DNA fraction, probably because of single strand breaks in the vicinity of transcription sites.**

The provirus theory of Temin (1), which postulates that the replication of RNA tumor viruses proceeds via DNA copies of viral RNA integrated in the host genome, has been substantiated by the discovery of successful transfection mediated by reverse transcriptase (2, 3), through an intermediate of viral DNA isolated from oncornavirus-transformed cells (4–6) and molecular hybridization between the viral genome and cell nucleic acids (7, 8). In productively infected cells, the integrated provirus DNA appears to be more actively transcribed than most of the cellular genes (9).

We have isolated, from the nDNA of various species, a minor fraction of single-stranded DNA (ss-DNA) and demonstrated that about one-third of the ss-DNA from cultured normal embryonic chicken cells (10) or human RD cells (11) can hybridize to homologous mRNAs. These and other characteristics suggested that ss-DNA arises from duplex molecules destabilized during RNA synthesis and probably split in the course of DNA isolation. If this is true, then ss-DNA of oncornavirus productive cells must be enriched in provirus DNA sequences as compared with total DNA of the same cells and ss-DNA of nonproductive cells. The present work demonstrates that this is indeed the case and provides evidence for preferential single strand breaks in the vicinity of transcribed DNA sites.

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MATERIALS AND METHODS

Cells and Labeling Procedures. Leukemic cells collected at the terminal stage of leukemia from the blood of 3- to 4-week-old chickens infected with avian myeloblastosis virus (AMV), BAI strain A, were washed three times with 199 medium. Approximately 2.5×10^9 cells in 50 ml of 199 enriched medium (12) were transferred to a 500-ml bottle, and [^{3}H]-thymidine (50 μCi/ml; specific activity, 46 Ci/mmol) or [^{3}H]uridine (50 μCi/ml; specific activity, 30 Ci/mmol) was added. The cells were incubated for 24 hr at 37° with slow rotation. The pH of the medium was maintained at 7.4 during the course of incubation. The primary cell cultures prepared from 10-day-old chick embryos were obtained as described (13).

Preparation of DNA from Leukemic or Normal Cells. Cell nuclei were purified by using nonionic detergent (14), resuspended in 0.01 M Tris·HCl, pH 8.3/0.01 M EDTA, and lysed by incubation for 30 min at 37° in the presence of 0.1% sodium dodecyl sulfate and Pronase (100 μg/ml). The concentration of sodium dodecyl sulfate was increased to 1% and the nuclei were reincubated for 30 min at 37°. After addition of sodium perchlorate to 1.0 M, the DNA was extracted three times with chloroform/isoamyl alcohol, 99:1 (vol/vol), precipitated with ethanol, and redissolved in 0.1× standard citrate–saline solution (SSC). Pancreatic RNase (20 μg/ml) and RNase T_1 (20 units/ml) were added and the solution was incubated for 30 min at 37°. After reextraction the DNA was finally precipitated with ethanol and redissolved in and dialyzed against 0.014 M NaCl/0.01 M Tris, pH 8.3/0.002 M EDTA.

Isolation of ss-DNA. The modification of the method for hydroxyapatite chromatography used in these studies has been described (15). Briefly, elution was carried out at 56° with pH 7.85 phosphate buffer and, instead of column chromatography, a batch procedure was used. Under these conditions the separation of the ss- and double-stranded (ds)-DNA is greatly improved. The ss-DNA and ds-DNA fractions were dialyzed against 0.1× SSC and sonicated with a Branson sonifier B_{12} for 20 sec in an ice bath at position 6 to obtain DNA fragments of 5–7 S as measured by alkaline sucrose gradient centrifugation.

Synthesis of AMV [^{3}H]cDNA. The standard endogenous RNA-dependent DNA polymerase reaction of Leis and Hurwitz (16, 17), which provides DNA copies (cDNA) representing more than 60% of the AMV genome, was used with and without addition of actinomycin D (AMD), which is known to inhibit the DNA-dependent reaction (18). The reaction mixture con-

Abbreviations: ss-DNA, single-stranded DNA; AMV, avian myeloblastosis virus; SSC, standard citrate–saline solution (0.15 M NaCl/0.015 M Na citrate, pH 7); ds-DNA, double-stranded DNA; AMD, actinomycin D; C_0t, nucleotide concentration (mol/liter) × time (sec).

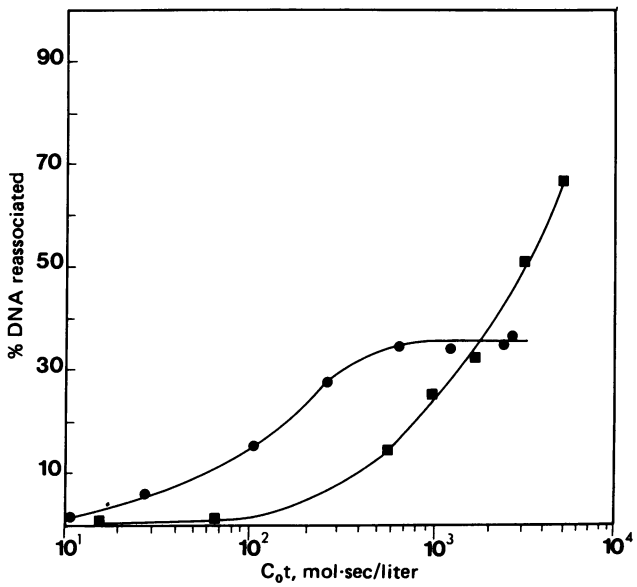

FIG. 1. Reassociation kinetics of ss-DNA from chicken leukemic cells. [^{3}H]Thymidine-labeled ss-DNA (●) was incubated at 66° in 2× SSC containing 0.1% sodium dodecyl sulfate, for different times. The amounts reassociated were determined by measuring the resistance to S_1 nuclease as indicated in *Materials and Methods*. As control (■), nonrepeated DNA sequences, selected by hydroxyapatite chromatography of bulk nuclear DNA renatured at C_0t=300 mol·sec/liter after alkaline denaturation, were reassociated under the same conditions.

tained (per ml): 55 mM Tris·HCl, pH 8.3; 40 mM KCl; 12.5 mM $MgCl_2$; 30 mM dithiothreitol; 0.1% Nonidet P-40; 10 μM dGTP; 10 μM dTTP; 400 μCi of [^{3}H]dCTP (specific activity, 25.5 Ci/mmol); 100 μg of AMD, and about 2 mg of AMV protein. After incubation for 4 hr at 37°, dATP, dCTP, dGTP, and dTTP (each 0.025 μm) were added and the mixture was incubated for 16 hr at 37°. In the absence of AMD, the same reaction mixture was used except that it contained 0.80 μM dGTP, 0.80 μM dATP, 37.5 μCi of [^{3}H]dCTP (specific activity, 25 Ci/mmol) and 60 μCi of [^{3}H]dTTP (specific activity, 16.6 Ci/mmol) per ml and was incubated for 2 hr at 41°.

Purification of [^{3}H]cDNA. After incubation, the reaction mixtures were adjusted to 0.25% in sodium dodecyl sulfate and 100 μg/ml in Pronase and were incubated at 37° for 30 min. The products were extracted twice with an equal volume of chloroform/isoamyl alcohol after addition of 150 μg of yeast RNA as carrier, precipitated at −20° in the presence of 0.2 M NaCl with 2 volumes of ethanol, centrifuged at 20,000 × *g* for 15 min, resuspended in 0.01 M phosphate buffer, pH 7.85, and chromatographed on hydroxyapatite. The greatest part of cDNA (specific activity, 1×10^7 cpm/μg) synthesized in the presence of AMD eluted as ss-DNA and only this part was used for hybridization experiments. The main cDNA fraction synthesized in the absence of AMD eluted as ds-DNA and only this fraction was used for hybridization. Each cDNA fraction was dialyzed against 0.1× SSC, adjusted to 0.1 M NaOH, incubated for 18 hr at 37°, neutralized by addition of 1 volume of cold 2 M NaH_2PO_4, dialyzed against 0.1× SSC, and precipitated with ethanol.

Hybridization of AMV cDNA and AMV RNA. Aliquots of AMV [^{3}H]cDNA were mixed with unlabeled cell DNA as indicated in Figs. 2 and 3. After heating for 10 min at 100° and fast cooling, hybridization was carried out by incubation at 66° in 2× SSC containing 0.1% sodium dodecyl sulfate, for different time periods. The extent of hybridization was determined by digestion with S_1 nuclease. Aliquots (5 or 10 μl) were diluted to a final volume of 250 μl in S_1 buffer (0.03 M Na acetate, pH 4.5/0.0018 M $ZnCl_2$/0.15 M NaCl) plus 15 μg of denatured calf thymus DNA with or without S_1 nuclease in excess. After incubation for 30 min at 40°, each sample was precipitated with trichloroacetic acid and filtered (Whatman glass-fiber, GF/B), and the filter was assayed for radioactivity in a liquid scintillation spectrometer. Results of hybridization were plotted as the percentage of S_1-resistant [^{3}H]cDNA versus the C_0t value [nucleotide concentration (mol/liter) × time (sec)]. Hybridization of 70S AMV [^{3}H]RNA prepared as described (8, 12) was performed under similar conditions, except that RNases were utilized instead of S_1 nuclease and all solutions were pretreated with 1% diethylpyrocarbonate and heated for 10 min at 80°.

RESULTS

Isolation and Characterization of ss-DNA. With the improved method of hydroxyapatite chromatography, about 2% of the native nuclear DNA from leukemic chicken cells was isolated as ss-DNA. Similar proportions of ss-DNA had been previously found in other cells including chicken embryonic cells (11, 15, 19, 20). Whatever its origin, the ss-DNA could be entirely degraded by DNase or S_1 nuclease and was resistant to RNase or alkaline treatment. When centrifuged in a CsCl density gradient, its mean buoyant density was the same as that of denatured bulk DNA. The mean sedimentation coefficient of ss-DNA, run through alkaline sucrose gradient, was 9–10 S, corresponding to 1–1.2 kilobases, whereas that of bulk DNA was about 18 S, corresponding to 6 kilobases (21). The molecular complexity of ss-DNA was analyzed by comparing its self-reassociation kinetics with those of the nonrepetitious portion of bulk DNA. As shown in Fig. 1, 30–32% of ss-DNA consisted of complementary chains reassociated at a mean C_0t value of approximately 100 mol·sec/liter. The rest of the ss-DNA remained single stranded even after reaching C_0t values of 3000–4000 mol·sec/liter, sufficient to allow renaturation of more than half of the nonrepeated sequences of bulk DNA. Similar results, obtained with three different preparations of DNA, indicated that the main part of ss-DNA seems to consist of fragments from only one of the DNA double strands. The complementary sequences of these non-self-reassociable fragments do exist in the cell genome because, like ss-DNA from other cells (10, 11), the ss-DNA from AMV-producing cells became hybridized to bulk nuclear DNA with the kinetics of nonrepeated DNA sequences. In addition, up to 35–36% of the ss-DNA from leukemic cells could be annealed with polysomal RNA as compared with 6–7% for bulk DNA [results not shown were similar to those obtained with ss-DNA from other cells (10, 11)].

Table 1. Characterization of cDNA probes

	AMV cDNA synthesized, % of nonhydrolyzed	
Hydrolysis	Without AMD	With AMD
None	100	100
Alkali	94.5	100
DNase	2.9	3.7
S_1 nuclease	61.8	33.5
Denatured and S_1	6.4	2.5

Characterization of AMV [^{3}H]cDNA. The radioactive products of the endogenous DNA polymerase reactions were degraded by DNase but were resistant to RNases and alkali. The results of S_1 nuclease digestion (Table 1), confirmed by hy-

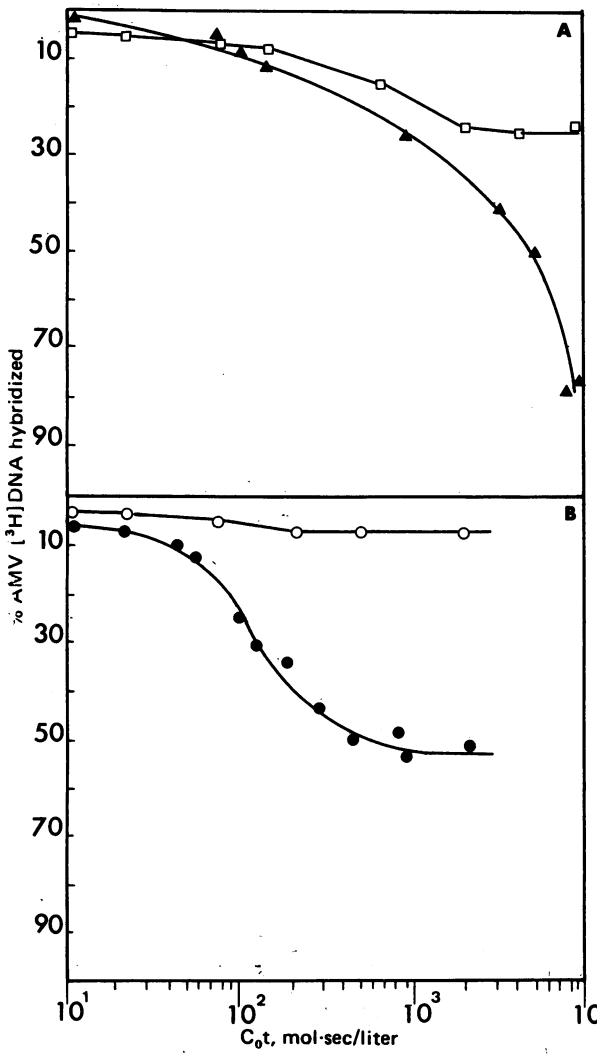

FIG. 2. Hybridization kinetics of AMV ds-[^{3}H]cDNA (synthesized in absence of AMD) with cell DNA. (*A*) AMV cDNA hybridized to total nuclear DNA of uninfected cells (□) or of productively infected leukemic cells (▲) at a cell DNA-to-cDNA ratio of 7×10^5. (*B*) AMV cDNA hybridized to ss-DNA of the same uninfected (○) or productively infected (●) cells. The amounts hybridized were determined by measuring the resistance of [^{3}H]cDNA to S_1 nuclease digestion.

droxyapatite chromatography, showed that the major part of the AMV cDNA synthesized in the presence of AMD consisted of ss-DNA chains and, in the absence of AMD, of ds-chains. No more than 40–45% of the latter cDNA probe hybridized to AMV 70S RNA in excess, as compared with 80–85% for the former. The fact that cDNA synthesized in the presence of AMD was not entirely digested by S_1 nuclease or totally hybridized to viral RNA is in agreement with the recent report (22) that AMD does not obligatorily inhibit all of the DNA-directed DNA synthesis.

Hybridization of AMV cDNA and AMV RNA with Cellular DNA. About 50% of the AMV ds-cDNA (made in the absence of AMD) hybridized to ss-DNA of productively infected leukemic cells at a mean C_0t value of less than 100—that is to say, 40–50 times faster than to total DNA of the same cells (Fig. 2). No more than 25% of the same cDNA probe could be reassociated with total DNA from noninfected chicken cells and much less with ss-DNA. This agrees with recent data on the extent of homology between the provirus DNA sequences of endogenous and exogenous avian oncornaviruses (23). In three

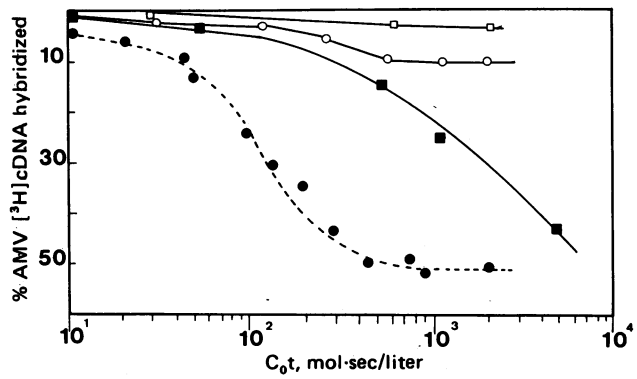

FIG. 3. Hybridization kinetics of AMVss-[^{3}H]cDNA (synthesized in the presence of AMD) with cell DNA. AMV cDNA was hybridized to ss-DNA of noninfected cells (□) or total DNA (■) and ss-DNA (○) of productively infected chicken cells. Experimental conditions were the same as in the legend of Fig. 2. Hybridization of AMV ds-cDNA (synthesized in the absence of AMD) with ss-DNA of chicken leukemic cells (●) is shown to facilitate comparison.

different experiments, half of the AMV ds-cDNA molecules hybridized much faster to ss-DNA than to total DNA of AMV-producing cells but little more than half, even after reaching C_0t values at which 80–85% of the same cDNA probe hybridized to total DNA. As illustrated by Fig. 3, at all C_0t values, a much smaller proportion of the ss-cDNA copies synthesized in the presence of AMD hybridized to ss-DNA, compared with total DNA from leukemic cells, and insignificant amounts hybridized to ss-DNA from normal cells.

The double-stranded structure of cDNA·ssDNA hybrids was verified by hydroxyapatite chromatography and thermal fusion. The melting curve of these hybrids was almost as sharp as that of native cell DNA but their melting temperature was 5° lower. This difference was probably due to the small size of cDNA (4–5 S in sucrose gradient).

The selective enrichment of ss-DNA in transcriptable provirus sequences was confirmed by RNA·DNA annealing experiments (Fig. 4). Even for the low DNA/RNA ratio (1:6000), the major part of the purified 70S AMV RNA hybridized to ss-DNA from productively infected cells (with a $C_0t_{1/2}$ value of about 500) and relatively negligible amounts hybridized to total nuclear DNA or purified ds-DNA under the same conditions.

DISCUSSION

The occurrence in eukaryotic cells of ss-DNA sequences (24–32), generally considered as DNA replication intermediates (25–29), products of alteration caused by cell aging (30, 31), or artifacts of the DNA extraction procedures (32), have been reported. We recently obtained evidence that suggested other mechanisms might be involved. The major part of ss-DNA could not consist of replication intermediates because its lifespan was similar to that of bulk DNA (10, 33). Another possibility that had to be considered is that ss-DNA is related to RNA synthesis. In effect, different transcription mechanisms have been proposed that imply unwinding of the DNA helix (34–37). Studying transcription of a phage DNA in a cell-free system, Bick *et al.* (38) used formamide, at a concentration below that required for partial denaturation of DNA, to visualize opened regions that involve 700–1500 DNA base pairs in the vicinity of the growing RNA chains. Groner *et al.* (39) found that the greatest part of the RNA synthesized in chromatin isolated from chicken leukemic cells was in the form of RNA·DNA hybrids. Our previous data provided evidence for DNA strand separa-

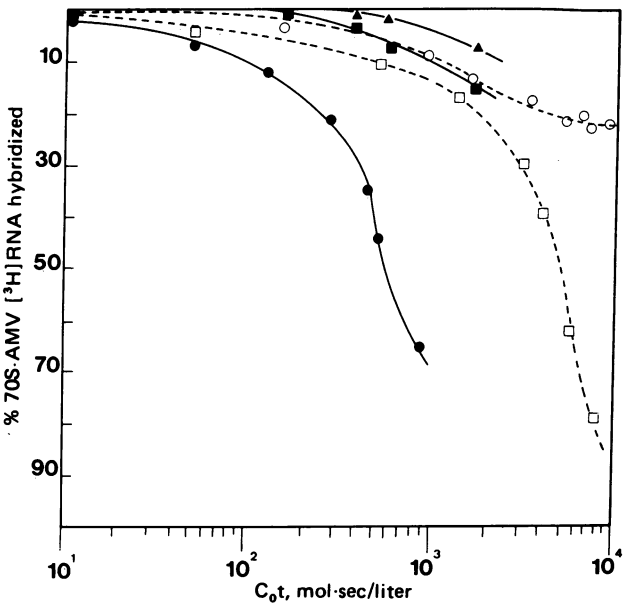

FIG. 4. Hybridization kinetics of purified 70S AMV [^{3}H]RNA. AMV [^{3}H]RNA (8000 cpm; 30 ng) was hybridized to a 6000-fold excess of total nuclear DNA (■), ds-DNA (▲), or ss-DNA (●) from AMV productive myeloblasts. AMV [^{3}H]RNA (20,000 cpm, 75 ng) was hybridized to a 40,000-fold excess of total nuclear DNA from myeloblasts (□) or normal embryonic chicken cells (○). Aliquots were treated with pancreatic RNase (50 μg/ml) and T1 RNase (20 units/ml) and processed for trichloroacetic acid precipitation. Percentage hybridized was determined after subtraction of control values (time 0 or acid-precipitable fraction after thermal melting of the hybrid measured on other samples) that usually did not exceed 4–5% of the input [^{3}H]RNA.

tion in the course of gene activity: one-third of the ss-DNA from normal embryonic chick cells (10) or human RD cells (11) could be hybridized to homologous RNAs.

As shown by hybridization kinetics, ss-DNA contains several thousand different transcription sites. It could therefore be postulated that ss-DNA must be enriched in DNA sequences corresponding to highly transcribed genes. Provirus DNA integrated in the genome of oncornavirus-transformed cells offers a good example of such genes. In fact, as shown above, AMV RNA and half of the AMV ds-cDNA sequences hybridized much faster to ss-DNA than to total DNA of AMV-productive myeloblasts and very little hybridized to ss-DNA of nonproductive cells. In contrast, very little of the AMV ss-cDNA hybridized to ss-DNA of myeloblasts. The reproducibility of these results permitted the conclusion that the ss-DNA from AMV-transformed cells is considerably enriched in virus-specific DNA fragments, mostly, if not exclusively, originating from the transcribed DNA strand. This raised the question of whether this selection of ss-DNA sequences is limited to tumor virus genes. It was reported that viral DNA binds preferentially to single strands of denatured host DNA (40), and, once integrated, single strand breaks can free the viral genome (41).

These data suggested a model for the replication of DNA tumor viruses, based on cellular and viral DNA single-strand interaction (42). In the case of Rous virus, nonintegrated provirus DNA molecules have been isolated from the cytoplasm of transformed cells, at a long time after infection (43). Although the occurrence of free DNA copies of the AMV genome in productively infected cells cannot be excluded, it is not likely that our results are due to such material. In preliminary experiments using nuclear DNA prepared by the Hirt procedure (44), similar results were obtained. The DNA pellet solubilized by moderate pipetting was analyzed by hydroxyapatite chromatography. Its ss-DNA content, which originally was about 0.6%, rose to 1.6% after further deproteinization and RNase treatment. Finally, at least two-thirds of the ss-DNA appeared to represent noncomplementary DNA fragments (see Fig. 1) which hybridize to bulk DNA and homologous RNAs (results not shown). The latter data suggest that not only the virus DNA sequences found in ss-DNA but also most of the ss-DNA fragments derive from the transcriptable DNA strand that codes for cellular mRNAs. This implies preferential single-strand breaks, occurring in the vicinity of DNA transcription sites either *in vivo* or in the course of DNA preparation.

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