Selective Inhibition of Nuclear DNA Synthesis by 9-β-D-Arabinofuranosyl Adenine in Rat Cells Transformed by Rous Sarcoma Virus

(circular extrachromosomal DNA/CsCl–propidium diiodide centrifugation/rat embryo fibroblasts)

CHARLES SHIPMAN, JR.*, SANDRA H. SMITH†, AND JOHN C. DRACH‡

* Department of Oral Biology, School of Dentistry, and Department of Microbiology, School of Medicine; † Department of Oral Biology, School of Dentistry; ‡ Department of Oral Biology, School of Dentistry, and Interdepartmental Graduate Program in Medicinal Chemistry; The University of Michigan, Ann Arbor, Mich. 48104.

ABSTRACT   The drug, 9-β-D-arabinofuranosyl adenine, selectively inhibits the synthesis of nuclear DNA without affecting extrachromosomal DNA synthesis in rat cells transformed by Rous sarcoma virus (B-mix K-44/6). The inhibition was linear with respect to drug concentration over the range of 37–600 μM. Mitosis and total synthesis of DNA per cell were also depressed. DNA synthesis was determined by measurement of [³H]thymidine incorporation into DNA. Covalently-closed circular DNA was extracted by the Hirt procedure and separated from residual chromosomal DNA by buoyant density gradient ultracentrifugation in CsCl–propidium diiodide. On the basis of buoyant density and sedimentation velocity centrifugation, the covalently-closed circular DNA formed in the presence of the drug was indistinguishable from that formed in its absence.

To explore the role of extrachromosomal DNA in virus-transformed cells, we have begun to examine circular DNA from a line of mammalian cells transformed by Rous sarcoma virus (RSV). B-mix K-44/6 cells were subcloned from a population of embryonic rat cells transformed *in vitro* by the Prague strain of RSV (1). These large epithelioid cells are malignant for homologous rat hosts and have been shown by both *in vivo* experiments (1) and cell fusion studies with chick-embryo fibroblasts (Shipman, unpublished observations) to contain the genome of RSV. Circular DNA represents a very small fraction of the total DNA of a mammalian cell. Therefore, to aid in the isolation of circular DNA it would be desirable to inhibit nuclear DNA synthesis without affecting the replication of circular DNA. A selective inhibition of this nature has been accomplished in prokaryotic cells. Helinski and Clewell (2) have described a preparation using chloramphenicol, where Col E₁ plasmid DNA synthesis occurs in the absence of chromosomal DNA synthesis. Similarly, in synchronous cultures of *Chlorella*, synthesis of nuclear DNA was inhibited by more than 90% in the presence of 15 μM cycloheximide, whereas the inhibitor had little effect on satellite DNA synthesis (3). Although the synthesis of cytoplasmic DNA in HeLa cells is somewhat resistant to inhibition by high concentrations of hydroxyurea (4), the selective inhibition of nuclear DNA synthesis has not been described in mammalian cells.

Abbreviations: ara-A, 9-β-D-arabinofuranosyl adenine; ara-ATP, the 5'-triphosphate of ara-A; Col E₁, colicinogenic factor E₁; HBS, HEPES-buffered saline (see ref. 12); HEPES, 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid; PI, propidium diiodide; RSV, Rous sarcoma virus; SDS, sodium dodecyl sulfate.

One of the antibiotics examined in an attempt to selectively inhibit nuclear DNA synthesis while permitting the synthesis of circular DNA was 9-β-D-arabinofuranosyl adenine (ara-A), a known inhibitor of DNA synthesis. Ara-A, a nucleoside antibiotic, is an isomer of adenosine. It differs from adenosine in that the glycosyl linkage is to D-arabinose rather than to D-ribose. The chemistry and biochemistry of the D-arabinosyl nucleosides, including ara-A, have been reviewed (5–8). The compound possesses some activity against transplantable animal tumors and exhibits broad-spectrum activity against DNA viruses (9).

In this communication, we present direct experimental evidence for the specific inhibition by ara-A of chromosomal, but not circular, DNA synthesis in RSV-transformed rat cells.

MATERIALS AND METHODS

9-β-D-Arabinofuranosyl adenine was provided through the courtesy of Dr. Robert Hodges of Parke, Davis and Co., Ann Arbor, Mich. Col E₁ 23S DNA was the gift of Dr. Don B. Clewell. B-mix K-44/6 cells were kindly supplied by Dr. Jan Svoboda. Propidium diiodide (PI) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Calbiochem, San Diego, Calif. [*Methyl*-³H]thymidine was obtained from New England Nuclear, Boston, Mass.

Cell Culture. B-mix K-44/6 RSV-transformed rat cells were grown in Eagle's minimal essential medium supplemented with 10% tryptose phosphate broth (Difco, Detroit, Mich.) and 10% unheated calf serum. The cultures were grown in disposable 75-cm² Falcon flasks in a humidified atmosphere of 3% CO₂–97% air.

Labeling of DNA. DNA was labeled with tritium by replacing the medium of early-log-phase cells with a medium containing 10 μCi of [³H]thymidine/ml (6.7 Ci/mmol). After 22 hr the radioactive medium was removed. The cells were washed twice and incubated with fresh medium for 1 hr to exhaust the intracellular pool of radioactive thymidine and thymidine phosphates (10).

Selective Extraction of Covalently-Closed Circular DNA. The procedure developed was modified from that described by Hirt (11). After incubation for 1 hr with fresh medium, the cell monolayer was washed twice with HEPES-buffered saline (HBS) (12) containing 2.5 mM EDTA (pH 7.3 at 22°) (HBS–EDTA). 10 ml of this buffer was added to each
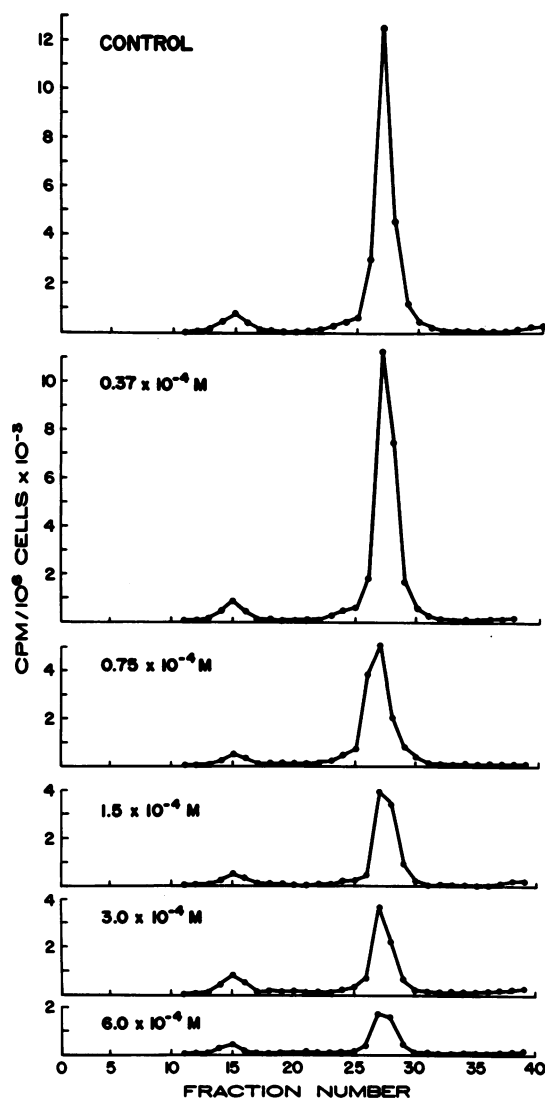
FIG. 1. Effect of ara-A on DNA from RSV-transformed rat cells extracted by the Hirt procedure. The supernatant fraction from the Hirt procedure was mixed with CsCl and PI in Tris-EDTA-NaCl buffer and centrifuged in a Ti 50 rotor (15°) at 40,000 rpm for 60 hr. 9-drop fractions were collected from the bottom of the tube and the acid-precipitable radioactivity was determined in 50-μl aliquots. *Fractions 13–17* represent covalently-closed circular DNA, whereas *fractions 25–30* represent residual linear and nicked circular fragments.

flask, and the cells were incubated at 37°. After 15 min of incubation, the flasks were shaken and the cells were decanted into centrifuge tubes. Centrifugation for 5 min at 250 × *g* readily sedimented the cells. The supernatant fraction was discarded, and each pellet was resuspended in 1 ml of HBS-EDTA. 1 ml of 1% sodium dodecyl sulfate (SDS) in 0.01 M HEPES (pH 7.4)–0.01 M EDTA was added to the cell suspension and gently mixed. After 20 min incubation at room temperature, 5 M NaCl was added to make a final concentration of 1 M, and the sample was mixed by slowly inverting the tube 10 times. The sample was stored at 4° overnight, after which it was centrifuged at 17,000 × *g* for 30 min at the same temperature. 95–99% of the nuclear DNA sedimented along with the SDS during this last step, whereas the circular DNA remained in the supernatant
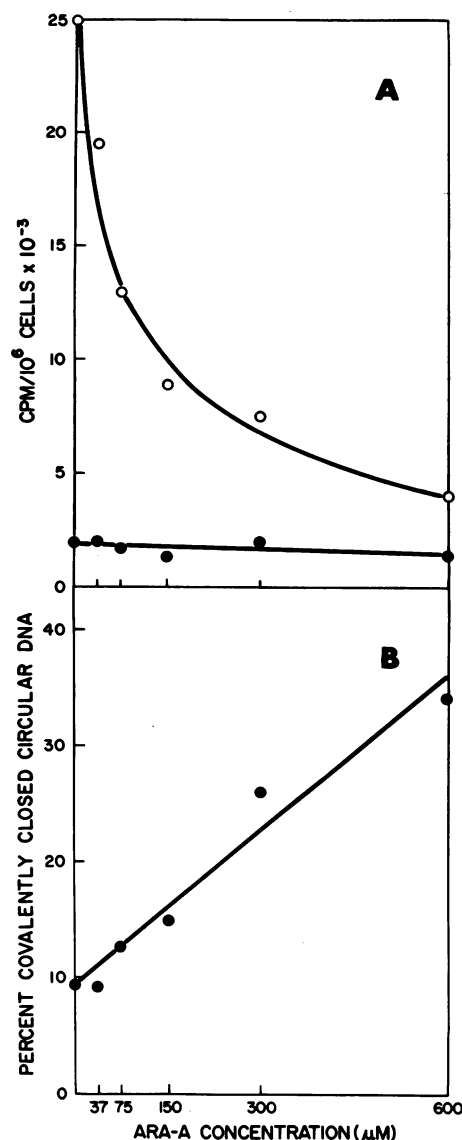

FIG. 2. Selective inhibition of nuclear DNA synthesis by ara-A in RSV-transformed rat cells. The total cpm represented by each peak in Fig. 1 was calculated and expressed as a function of concentration of ara-A. (*A*) Amount of residual linear (O——O) and covalently-closed circular (●——●) DNA synthesized in the presence of different concentrations of ara-A. The line of best fit for the data points for covalently-closed circular DNA was computed by a method of least squares and resulted in a linear plot (correlation coefficient = +0.98) with a slope of −0.07. (*B*) Amount of covalently-closed circular DNA synthesized at different drug concentrations. The amount of covalently-closed circular DNA is expressed as a percentage of the total DNA in the supernatant fraction of the Hirt procedure.

fraction. In those cases where total radioactivity was determined, the 17,000 × *g* pellet was resuspended in 5 ml of warm (37°) HBS-EDTA and sonicated for 3 min at 20 kHz on a Bronwill sonicator set to deliver 70 W of acoustical power through a needle probe.

CsCl-Propidium Diiodide (PI) Buoyant Density Ultracentrifugation. Closed circular DNA was separated from nicked circular and linear DNA fragments, essentially by the method of Radloff *et al.* (13). PI was substituted for ethidium

bromide since PI produces a density separation 1.8 times larger than ethidium bromide (14).

The supernatant sample from the modified Hirt extraction procedure (about 2 ml) was mixed with 7.75 g of CsCl, 4.4 ml of 0.03 M Tris (pH 8.0)–5 mM EDTA–0.05 M NaCl, and 1.5 ml of PI (1 mg/ml). In early experiments 0.1 ml of B-mix K-44/6 chromosomal [^{14}C]DNA was added as a marker. The CsCl solution, in either cellulose nitrate or polyallomer tubes, was overlaid with light mineral oil. The experiments were performed in Ti 50 rotors in a Beckman Spinco model L3-50 preparative ultracentrifuge at 15°. Occasionally a Ti 60 rotor was used for the preparation of larger amounts of labeled circular DNA. After centrifugation at 105,000 × g for 64 hr, fractions were removed from the bottom of the tubes by a Buchler polystaltic pump and an LKB fraction collector.

Sucrose Velocity Sedimentation Ultracentrifugation. Pooled fractions from the CsCl–PI buoyant density ultracentrifugation experiments were dialyzed against 1000 volumes of 2.5 mM Tris (pH 8.0)–1 mM EDTA for at least 4 hr. The dialyzed samples were overlayed on 5–20% neutral sucrose in Tris–EDTA–NaCl buffer, and centrifuged at 189,000 × g for 45 min. Col E₁ 23S DNA was used as an internal velocity sedimentation marker. The experiments were performed in SW50.1 rotors.

Determination of Radioactivity in DNA. 50- or 100-μl aliquots of fractions containing labeled DNA were spotted on small numbered squares of Whatman no. 1 filter paper. After they were dried, the squares of filter paper were washed once with ice-cold 5% Cl$_3$CCOOH, twice with 95% ethanol, and once with anhydrous diethyl ether. The dried squares of filter paper were immersed in 10 ml of toluene–PPO (2,5-diphenyloxazole), and the samples were counted in a Beckman model LS-250 liquid scintillation counter.

RESULTS

The effect of ara-A on the synthesis of covalently-closed circular DNA was studied by addition of various concentrations of the drug to cells in medium containing [^{3}H]thymidine. After nearly all of the nuclear DNA was pelleted by the Hirt procedure, the supernatant fractions were centrifuged to equilibrium in a CsCl gradient containing PI to

isolate covalently-closed circular DNA. When corrected for cell number (Figs. 1 and 2*A*), the synthesis of residual nuclear DNA in the Hirt supernatant fraction decreased dramatically, while the synthesis of covalently-closed circular DNA appeared to be unaffected. When the amount of covalently-closed circular DNA was expressed as a percent of total DNA in the Hirt supernatant fraction, a linear relationship with the concentration of ara-A was noted (Fig. 2*B*). At 600 μM ara-A, about one-third of the DNA extractable by the Hirt procedure was covalently closed.

To insure that the decrease in the amount of linear DNA in the supernatant fraction accurately reflected an inhibition of all nuclear DNA synthesized, total DNA synthesis was examined by measurement of Cl$_3$CCOOH-insoluble radioactivity in both the supernatant fluid and pellet resulting from the Hirt extraction procedure. Table 1 illustrates that total DNA synthesized also decreased as a function of drug concentration. When total DNA synthesis was adjusted to a per cell basis, DNA synthesized per cell decreased linearly as a function of the concentration of ara-A (Fig. 3).

After inhibition by various concentrations of ara-A, the circular DNA recovered from the CsCl–PI gradients was sedimented through 5–20% neutral sucrose. Based on the behavior of an internal 23S Col E₁ marker, the sedimentation velocity values of the covalently-closed circular DNA did not vary significantly. The sedimentation profiles of circular DNA from cells grown in the absence and presence of the highest concentration of ara-A are compared in Fig. 4.

The effect of the inhibitor on cell division was also examined. In the absence of ara-A, the cells underwent 2.2 divisions during the 22-hr labeling period, resulting in a cell
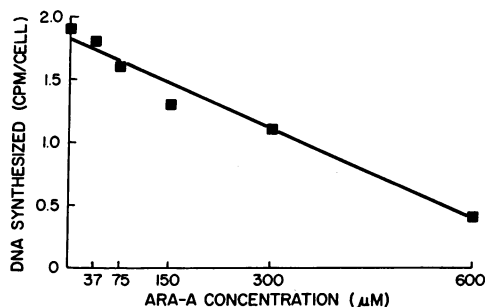
Fig. 3. Effect of different concentrations of ara-A on total DNA synthesized per cell by RSV-transformed rat cells. Total DNA was determined by combining total counts in the supernatant and pellet fractions from the Hirt procedure (see Table 1). Counts in the 17,000 × g pellet were measured after resuspension of the pellet in warm HBS–EDTA, and solubilization of the DNA by sonication.
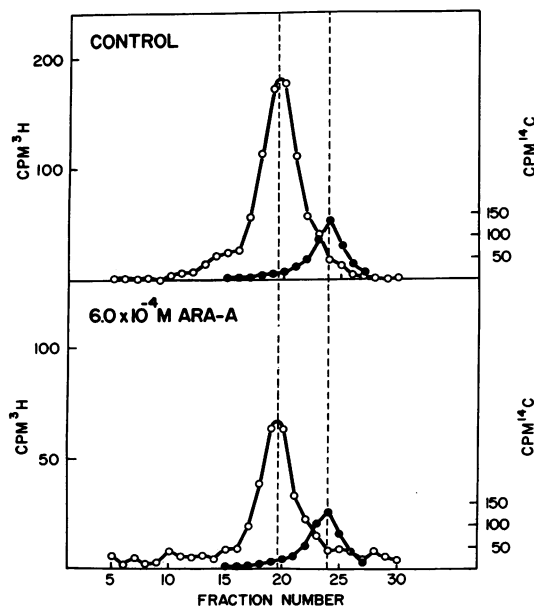
Fig. 4. Sedimentation analysis of covalently-closed circular DNA isolated from B-mix K-44/6 cells. A 0.2-ml aliquot of the dialyzed pooled circular DNA (Fig. 1, fractions 13–17) diluted in Tris–EDTA–NaCl buffer was mixed with 0.1 ml of Col E₁ 23S marker [^{14}C]DNA. Sedimentation (from *right* to *left*) was through 5–20% neutral sucrose gradients in a SW50.1 rotor (15°) at 45,000 rpm for 45 min. ^{3}H-labeled covalently-closed circular DNA from B-mix K-44/6 cells (O———O) grown in the absence (*top*) and presence (*bottom*) of 600 μM ara-A; ^{14}C-labeled Col E₁ DNA (●———●).

TABLE 1. *Effect of ara-A on total DNA synthesis and cell division in RSV-transformed rat cells*

Concentration ara-A (μM)	Cell divisions*	DNA extracted by modified Hirt procedure [cpm/fraction ($\times 10^{-6}$)]			Total DNA synthesis/cell† (cpm/cell)
		Supernatant	Pellet	Total	
0	2.2	0.26	12.3	12.6	1.9
37	2.2	0.21	12.4	12.6	1.8
75	2.0	0.27	9.7	9.9	1.6
150	1.9	0.18	7.0	7.2	1.3
300	1.9	0.20	5.7	5.9	1.1
600	1.3	0.10	1.3	1.4	0.4

* Calculated on the basis of an initial cell inoculum of 1.5×10^6 cells/75-cm^2 flask.
† Total DNA synthesized/cell as a function of drug concentration is illustrated in Fig. 4.

density of 6.8×10^6 cells per flask. In contrast, only 1.3 divisions occurred in the presence of 600 μM ara-A. This corresponds to a cell density of 3.7×10^6 cells/flask.

Since it has been reported that the synthesis of cytoplasmic DNA in HeLa cells is somewhat resistant to inhibition by hydroxyurea (4), this drug was evaluated in B-mix K-44/6 cells. The dramatic selective inhibition seen with ara-A was not observed. When used at the highest concentration of ara-A tested (600 μM), hydroxyurea did not produce a selective inhibition of linear DNA. The maximum selective effect (at any concentration of hydroxyurea tested) was an 8% increase in the relative amount of covalently-closed circular DNA in the supernatant fraction from the Hirt procedure. At a drug concentration of 10 mM, synthesis of both covalently-closed circular DNA and linear DNA was virtually abolished.

DISCUSSION

Experiments presented in this communication demonstrate that in B-mix K-44/6 cells, a line of RSV-transformed rat cells, the drug ara-A depresses cell division and selectively inhibits the incorporation of [³H]thymidine into chromosomal, but not into circular, DNA. The circular DNA synthesized in the presence of the inhibitor was indistinguishable from that synthesized in control cells, based upon buoyant density and sedimentation velocity centrifugation studies.

Inhibition of DNA synthesis by ara-A was first noted by Cohen in a purine auxotroph of *Escherichia coli* (15). Brink and LePage (16) and Doering *et al.* (17) later observed an inhibition of DNA, but not RNA or protein, synthesis in mouse ascites tumors and mouse L cells. The conversion of ara-A to its triphosphate has been demonstrated (16, 18, 19), and three mechanisms have been proposed for the inhibition of DNA synthesis based on the action of ara-ATP (the 5'-triphosphate of ara-A) (20): (*i*) the fraudulent nucleotide may be incorporated into DNA and function as a chain terminator (21); (*ii*) ribonucleoside diphosphate reductase might be inhibited by ara-ATP, thereby depriving the cell of deoxynucleoside triphosphates (22, 23); and (*iii*) the synthesis of DNA may be inhibited directly by the action of ara-ATP on DNA polymerase (20, 22).

The inhibition of chromosomal DNA synthesis in B-mix K-44/6 cells also may involve these mechanisms. Why circular DNA synthesis remains refractory to inhibition is unknown. Preliminary electron microscopy studies (Hascall and Shipman, unpublished observations) indicate that, on the basis of contour length, virtually all of the circular DNA molecules from B-mix K-44/6 cells are mitochondrial in origin. Although the formal possibility exists that the proviral form of Rous sarcoma virus could exist as a DNA plasmid in RSV-transformed virogenic cells, there is no experimental evidence to substantiate this hypothesis. Thus, assuming that the majority of closed circular DNA is mitochondrial, several possibilities for the selective inhibition by ara-A exist. Mitochondrial membranes may be impermeable to the inhibitor. This seems somewhat unlikely, however, as several nucleosides and nucleotides are known to be transported into a mitochondrion (24, 25). Enzymes involved in mitochondrial DNA synthesis, e.g., DNA polymerase, which differs from the nuclear enzyme (26), may be insensitive to the inhibitor. A possible analogy exists with *E. coli*, where the drug has no effect on DNA polymerase I (27). Since DNA polymerase I does not appear to be an obligatory component of the DNA replication machinery of *E. coli* (28), subsequent experimentation with the replicative polymerases of *E. coli* will be of interest.

The possibility that DNA synthesis only appeared to be inhibited selectively has been considered. The apparent inhibition of chromosomal DNA synthesis might have resulted from a blockade of [³H]thymidine uptake by the cell or from an inhibition of thymidine kinase. The labeling of circular DNA in the presence of ara-A and the inhibition of cell division provide internal controls that eliminate these possibilities. An apparent selective inhibition also might result if chromosomal and mitochondrial DNAs were synthesized and degraded at sufficiently different rates. Mitochondrial (circular) DNA is generally synthesized more rapidly than nuclear DNA, and both species are replicated semiconservatively (29, 30). Consequently, inhibition of mitochondrial DNA synthesis should be more, not less, readily observed than inhibition of nuclear DNA synthesis.

Data from studies on selective inhibition may be subject to misinterpretation if tissues are used in which only a small percentage of cells are undergoing division. Kimberg and Loeb (31) reported that cortisone inhibited nuclear DNA synthesis to a greater degree than mitochondrial DNA synthesis in rat liver. They point out, however, that this may not be a selective effect on DNA synthesis; rather, only that proportion of cells engaged in nuclear DNA synthesis may be sensitive to the inhibitor. This question is not involved in the present study with ara-A. Under the experimental conditions used, virtually all B-mix K-44/6 cells grown in culture are rapidly dividing.

While this manuscript was in preparation, another report on the resistance of DNA species to inhibitors appeared.

Bell (32) found that in the presence of ethidium bromide—a known inhibitor of mitochondrial DNA synthesis (33)—several drugs produced different degrees of selective inhibition of DNA synthesis in explants of embryonic chick muscle. Hydroxyurea inhibited the synthesis of I-DNA [informational DNA, a reported species of cytoplasmic DNA (34)] to a greater extent than nuclear DNA, whereas 5-fluorodeoxyuridine and arabinosyl cytosine had the reverse effect.

The unique inhibition of nuclear DNA synthesis by ara-A and the inhibition of mitochondrial DNA synthesis by ethidium bromide provide powerful tools for the discriminate study of DNA synthetic processes in eukaryotic cells.

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