McCord, J. M., and Fridovich, I. (1969), *J. Biol. Chem. 244,* 6049–6055.
Nakamura, Y., Otsuka, H., and Tamaoki, B. (1966), *Biochim. Biophys. Acta 122,* 34–42.
Okunuki, K., and Nozaki, M. (1956), in Koso Kenkyuho, Vol. 1, Akabori, S., Ed., Tokyo, Asakura Publishing Co., pp 670–684.
Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1966), *Arch. Biochem. Biophys. 117,* 660–673.
Ovadi, J., Libor, S., and Elödi, P. (1967), *Acta Biochim. Biophys. Acad. Sci. Hung. 2,* 455–458.
Shirakawa, M. (1953), in Hyojun Seikagaku Jikken, Egami, F., Ishimoto, M., Maruo, B., Miura, Y., Sekine, Y., Suda, M., and Tamiya, N., Ed., Tokyo, Bunkodo, pp 321–327.
Suhara, K., Takemori, S., and Katagiri, M. (1972), *Biochim. Biophys. Acta 263,* 272–278.
Tagawa, K., and Arnon, D. I. (1962), *Nature (London) 195,* 537–543.
Tagawa, K., and Arnon, D. I. (1968), *Biochim. Biophys. Acta 153,* 602–613.
Thomé-Beau, F., Lé-Thin-Lan, Olomucki, A., and Thoai, N. v. (1971), *Eur. J. Biochem. 19,* 270–275.
Tu, S.-C., and McCormick, D. B. (1973), *J. Biol. Chem. 248,* 6339–6347.
Vallee, B. L., and Riordan, J. F. (1969), *Annu. Rev. Biochem. 38,* 733–794.
Weil, L., Buchert, R. A., and Mahler, J. (1952), *Arch. Biochem. Biophys. 40,* 245–252.
Weil, L., Tames, S., and Buchert, R. A. (1953), *Arch. Biochem. Biophys. 46,* 266–278.
Weil, L., and Seibles, S. T. (1955), *Arch. Biochem. Biophys. 54,* 368–377.
Yagi, K., and Matsuoka, Y. (1956), in Koso Kenkyuho, Vol. 1, Akabori, S., Ed., Tokyo, Asakura Publishing Co., pp 687–698.

Enzymatic Determination of Nonrandom Incorporation of 5-Bromodeoxyuridine in Rat DNA†

Stephen A. Schwartz

ABSTRACT: Secondary cultures of normal rat embryo cells were synchronized by a double thymidine block and pulsed with 10^{-7} M 5-[^{3}H]bromodeoxyuridine (BrdUrd) or 10^{-7} M [^{3}H]thymidine during an entire S phase (7.5 h). To examine the pattern of [^{3}H]BrdUrd incorporation in rat genomes as compared to [^{3}H]thymidine, DNA was immediately extracted and purified at the completion of the S phase. CsCl density gradient centrifugation revealed that substitution for thymine by bromouracil was less than 7%. Single-strand specific nucleases obtained from *Aspergillus oryzae* and *Neurospora crassa* were allowed to react with native and partially depurinated (24–29%) [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled rat DNA samples, and the products were assayed by hydroxylapatite column chromatography. Approximately 4–6% of the native, nondepurinated rat DNA was hydrolyzed by both nucleases. However, 24–28% of the partially depurinated, [^{3}H]thymidine-labeled rat DNA was hydrolyzed by both enzymes as determined by loss of mass as well as radioactivity. Whereas comparable levels of depurinated, [^{3}H]BrdUrd-labeled DNA were physically hydrolyzed by both nucleases, nearly 65% of the radioactivity was not recovered. Native, as well as depurinated, enzyme-treated DNA samples were sequentially and preparatively reassociated into highly repetitive, middle repetitive, and nonrepetitive nucleotide sequence components. The absolute and relative specific activities of each subfraction of native [^{3}H]thymidine-labeled DNA were comparable. [^{3}H]BrdUrd was differentially concentrated in the middle repetitive sequences as compared to other reiteration frequency types. When depurinated, nuclease-treated DNA samples were similarly fractionated, [^{3}H]thymine moieties were uniformly distributed throughout all sequences. However, a differential loss of [^{3}H]BrdUrd moieties was detected predominantly from the middle repetitive nucleotide fraction. Melting profiles of the renatured DNA samples were characteristic of each respective DNA subfraction regardless of isotopic precursor. These results suggest that [^{3}H]BrdUrd may be differentially incorporated into A + T rich clusters of rat DNA, especially in the moderately repeated chromosomal elements.

Halogenated pyrimidine analogues have been widely used in the study of differential gene expression and regulation in eukaryotic cells. In particular, the thymidine analogue 5-bromodeoxyuridine (BrdUrd)[1] has been extensively utilized in many experimental biological systems to determine molecular mechanisms and schedules for phenotypic and biochemical differentiation (Coleman et al., 1970; Rutter et al., 1973; Turkington et al., 1971; Walther et al., 1974). Moreover, BrdUrd and 5-iododeoxyuridine have been shown to be potent activators of RNA and DNA tumor virus-specific expression from selected animal cells (Hampar et al., 1974; Lowy et al., 1971; Schwartz et al., 1974b; Verwoerd and Sarma, 1973). In order for most of these events to occur, it is necessary that the thymidine analogues be incorporated into the chromosomal DNA of the respective cells (Levitt and Dorfman, 1973; Rutter et al., 1973; Teich et al., 1973). A search for a molecular mechanism for pyrimidine analogue induced alterations of

† From the Department of Pathology, University of Chicago, Chicago, Illinois 60637. *Received February 5, 1976.* Supported by American Cancer Society Research Grant No. 75-35 and U.S. Public Health Service Research Grants CA-14898 and CA-14599.

[1] Abbreviations used are: BrdUrd, bromodeoxyuridine; PBS, phosphate buffered saline; SSC, standard saline-citrate.

genetic regulation must therefore include investigation into the nature of the DNA–pyrimidine analogue interactions.

In some experimental systems, it has been shown that BrdUrd need only substitute a small fraction of the total nuclear thymine moieties in order to differentially suppress or activate specific gene products (Levitt and Dorfman, 1972; Schwartz et al., 1975; Weintraub et al., 1972). Particular regions of eukaryotic DNA may be primarily responsible for the sequential regulation of gene expression (Davidson and Britten, 1973; Paul, 1972). Hence, a distinct pattern of analogue incorporation and distribution within nuclear DNA may correspondingly reflect this genetic regulatory organization.

We have shown that BrdUrd need only substitute into a small subpopulation of rat cell DNA in order to activate maximal latent type C RNA virus-specific expression (Schwartz et al., 1975). Moreover, we have demonstrated that virogenic, radiolabeled BrdUrd moieties were incorporated into middle repetitive nucleotide sequences to a relatively greater degree than into other DNA subfractions (Schwartz and Kirsten, 1974; Schwartz et al., 1974a). This work further describes the nonrandom nature of virogenic BrdUrd localization in rat embryo cell DNA as determined by the use of two different single-strand specific nucleases.

Materials and Methods

Cell Cultures. Cells were obtained from a highly inbred colony of Wistar/Furth rats. Fetuses were aseptically removed on the 15th or 16th day of gestation and immediately rinsed in sterile phosphate-buffered saline (PBS, 0.14 M NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 1% phenol red) at 5 °C. The tissues were finely minced and incubated for 1 h at 37 °C with constant stirring in a sterile 0.25% trypsin–PBS solution. Following concentration by low-speed centrifugation and three PBS washes, the cell pellets were gently disrupted, resuspended, and dispersed into several 32-oz glass bottles containing 25 ml of medium 199 (Grand Island Biological Company) fortified with 10% fetal calf serum, 100 U/ml penicillin–streptomycin, and 1% L-glutamine. The bottles were incubated in the dark at 37 °C in a humidified, 5% CO_2 atmosphere. After 24 h, the culture fluids were drained and the adherent cells were gently washed three times with sterile PBS, and refed with 25 ml of fortified 199. The rat cell monolayers were usually confluent following 3–5 days of further incubation. The cells were subcultured into 32-oz bottles as before, and prepared for synchrony. Only mycoplasma-free cell cultures were used for subsequent experimentation.

Synchrony of Cell Growth. Rat cell cultures were synchronized by a double thymidine block as previously described (Mueller and Kajiwara, 1969; Schwartz et al., 1975). Briefly, subconfluent, secondary rat embryo cells in the log phase of growth were exposed to 25 ml of fortified medium 199 made 2 mM in unlabeled thymidine (Calbiochem). After 16 h, the cells were thoroughly rinsed with sterile PBS and released from blockade with 25 ml of medium 199 containing 10^{-5} M unlabeled adenosine, cytosine, and guanosine (release medium) for 8 h. The monolayers were then refed with 2 mM thymidine in medium 199 and incubated an additional 16 h. Maximal synchrony was then achieved when the cultures were finally refed with 25 ml of release medium.

DNA Labeling and Extraction. To prepare radiolabeled DNA, cell cultures were released from the second thymidine block with release medium containing either 10^{-7} M [^{3}H]-thymidine (18.3 Ci/mmol, New England Nuclear Corp.) or 10^{-7} M [^{3}H]BrdUrd (12.7 Ci/mmol, New England Nuclear Corp.). At the completion of the DNA synthesis (S) phase, the radioactive fluids were removed from each bottle, and immediately replaced with 10 ml of SSC buffer (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) made 1% in sodium dodecyl sulfate and β-2-mercaptoethanol (Schwartz and Kirsten, 1974; Schwartz et al., 1975). The cell lysates were combined, chilled to 4 °C, and extracted extensively with equal volumes of redistilled phenol saturated in SSC buffer. When the interface was free of flocculent material, the aqueous fraction was extracted 3 times for 15 min with equal volumes of chloroform–isoamyl alcohol (24:1, v/v) at 4 °C. The purified nucleic acids were then precipitated for 16 h at −20 °C in 3 vol of absolute ethanol. The precipitates were collected by centrifugation for 30 min at 10 000g, and dissolved in 1–2 ml of 0.08 M sodium phosphate buffer (pH 6.8) containing 20 μg/ml ribonuclease A (preheated to 90 °C for 15 min, Worthington Biochemicals) and incubated at 37 °C. After 1 h, the reaction mixture was extracted twice for 5 min with equal volumes of chloroform–isoamyl alcohol, and the nucleic acids were precipitated as before. In order to further purify the DNA, the precipitates were dissolved in 10–20 vol of 0.08 M phosphate buffer and dialyzed extensively against the same buffer at 5 °C. The DNA samples were then adsorbed to a column of hydroxylapatite (Bio-Rad Laboratories, Inc.) previously equilibrated at 60 °C with 0.08 M phosphate buffer (Schwartz and Kirsten, 1974; Schwartz et al., 1975). Single-stranded molecules were eluted with 5 column volumes of 0.18 M phosphate buffer and discarded; double-stranded DNA was subsequently eluted in 0.40 M phosphate buffer (Schwartz and Kirsten, 1974; Schwartz et al., 1975). The A_{260}/A_{280} ratio of the purified DNA solutions always exceeded 2.0.

Buoyant Density of DNA. The radiolabeled, purified DNA samples were centrifuged to equilibrium through neutral CsCl density gradients in order to determine the buoyant density. Approximately 25–50 μg of DNA was mixed together with CsCl (optical grade, EM Labs Inc.) in 0.08 phosphate buffer to an overall average density of 1.70 g/ml (Schwartz and Kirsten, 1974). The DNA solutions were centrifuged to equilibrium in a Spinco SW-50.1 rotor at 42 000 rpm for 48 h at 20 °C. The centrifuge tubes were punctured from the bottom, and each gradient fraction was assayed for A_{260}, radioactivity, and refractive index (Schwartz and Kirsten, 1974).

Partial Depurination of DNA. Radiolabeled, purified DNA samples in 2-ml volumes were subtotally depurinated following extensive dialysis against several changes of sodium acetate buffer (0.3 M NaCl, 0.03 M sodium acetate, pH 4.5) at 4 °C for 24 h (Schwartz, 1975). This procedure did not diminish the radioactivity of [^{3}H]thymidine- or [^{3}H]BrdUrd-labeled rat embryo DNA.

Enzymatic Hydrolysis of DNA. [^{3}H]Thymidine- and [^{3}H]BrdUrd-labeled DNA samples were allowed to react with the single-strand specific nuclease S_1 from *Aspergillus oryzae* (Ando, 1966; Shichido and Ando, 1972) obtained from Miles Laboratories, Inc. For the S_1 nuclease bioassay, DNA samples were dialyzed against 0.3 M NaCl, 0.03 M sodium acetate, 1 mM $ZnCl_2$ (pH 4.5) for 24 h at 4 °C (Ando, 1966; Schwartz, 1975; Shichido and Ando, 1972). Reaction mixtures (0.1–0.6 ml, 50–100 μg of DNA) contained 1 unit (313 units/mg of protein) of S_1 nuclease/μg of DNA, and were incubated at 37 °C for 30 min (Schwartz, 1975). The reaction was terminated by the addition of 25 ml of 2 mM EDTA in ice-cold 0.08 M phosphate buffer. The reaction mixtures were extensively dialyzed against 0.08 M phosphate buffer at 4 °C in preparation for hydroxylapatite column chromatography. For the *Neurospora crassa* endonuclease reaction (Miles Laboratories, Inc.) native and depurinated radiolabeled DNA samples were

dialyzed against Tris-HCl buffer (0.1 M Tris-HCl, 0.01 M $MgCl_2$, pH 8.0) at 5 °C for 24 h (Linn and Lehman, 1965a,b). Final enzymatic reaction mixtures (0.1–0.6 ml, 50–100 μg of DNA) contained 1 unit (819 units/mg of protein) of endonuclease/50 μg of DNA in the Tris-HCl buffer (pH 8.0) and were incubated at 37 °C for 30 min (Schwartz, 1975). The reactions were terminated by the addition of 25 ml of 2 mM EDTA in ice-cold 0.08 M phosphate buffer, and dialyzed against 0.08 M phosphate buffer as before. Heat denatured, single-stranded calf thymus DNA was completely digested by both nucleases under the respective optimal conditions described for each enzymatic bioassay.

Hydroxylapatite Column Chromatography. The products of the single-strand specific nuclease digestions were made 0.08 M in phosphate buffer by extensive dialysis at 4 °C and assayed by passage through columns of hydroxylapatite equilibrated in the same buffer at 60 °C. Single-stranded DNA was eluted in 3 column volumes of 0.18 M phosphate buffer. Double-stranded DNA was eluted in 0.40 M phosphate buffer and subsequently prepared for reassociation kinetics (Schwartz and Kirsten, 1974; Schwartz et al., 1975).

DNA–DNA Reassociation. Native double-stranded DNA solutions, as well as those obtained from nuclease digestion and hydroxylapatite column chromatography, were diluted to 0.1 M phosphate buffer and made 1 mM in EDTA (Schwartz and Kirsten, 1974; Schwartz et al., 1975). This solution was passed twice through a Ribi-Sorvall Cell Fractionator under a pressure of 50 000 psi (Britten et al., 1975; Schwartz and Kirsten, 1974; Schwartz et al., 1975). This mechanical shearing procedure consistently produced double-stranded DNA fragments 400–450 nucleotides in length as determined by the alkaline banding method of Studier (1965). The uniformly sheared DNA was then dialyzed extensively against 0.08 M phosphate buffer at 4 °C, and repurified as before over thermal hydroxylapatite columns. In order to quantitatively subfractionate the radiolabeled, sheared DNA according to nucleotide sequence repetition frequency, the purified samples were prepared for DNA–DNA reassociation. DNA reaction mixtures were made 0.40 M in phosphate buffer, 1 mM in EDTA (Britten et al., 1975; Schwartz and Kirsten, 1974; Schwartz et al., 1975), and sealed into glass ampules. The ampules were totally immersed in boiling water for 7–10 min (Britten et al., 1975; Schwartz and Kirsten, 1974; Schwartz et al., 1975). After thermal denaturation of DNA, the reaction vessels were briefly submerged in ice–water and then totally immersed in a 60 °C water bath (Britten et al., 1975; Schwartz and Kirsten, 1974; Schwartz et al., 1975). The reassociation was permitted to proceed until a C_0t (moles of nucleotides liter^{-1} × seconds) value of 1 was attained (Britten et al., 1975), whereupon the ampules were immediately opened, and the contents rapidly diluted into 40 vol of deionized, distilled water at 4 °C. The resultant DNA solution was then adsorbed to a column of hydroxylapatite equilibrated in the same buffer at 60 °C (Schwartz and Kirsten, 1974; Schwartz et al., 1975). Single-stranded nucleic acids were totally eluted in sufficient 0.18 M phosphate buffer. Double-stranded DNA was eluted in the same manner with 0.40 M phosphate buffer. The double-stranded fraction (highly repetitive nucleotide sequences) was stored. The single-stranded DNA was concentrated, made 0.40 M in phosphate buffer, and prepared for further reassociation as before to a C_0t of 100 (Schwartz and Kirsten, 1974; Schwartz et al., 1975). After subsequent column fractionation, the double-stranded DNA (middle repetitive nucleotide sequences) was retained for further study. The single-stranded DNA was reprepared as before for reassociation to a C_0t of 10 000. The resultant double-stranded molecules constituted the nonrepetitive (unique) nucleotide sequences. In this manner, each [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled DNA sample was totally fractionated into its respective nucleotide sequence subpopulations. The C_0t determinations were calculated and adjusted according to the Na^+ concentration (Britten et al., 1975; Schwartz and Kirsten, 1974). Recovery of radiolabeled DNA in all instances was greater than 90% following the multiple subfractionations.

DNA Thermal Stability Determination. The renatured duplex DNA was assayed for thermal stability in order to determine the fidelity of base pairing (Britten et al., 1975; Schwartz and Kirsten, 1974). The highly repetitive, middle repetitive, and nonrepetitive double-stranded DNA fractions were separately dialyzed against 0.18 M phosphate buffer, at 4 °C. The samples were adsorbed to columns of hydroxylapatite equilibrated in the same buffer at 60 °C (Schwartz and Kirsten, 1974). The columns were washed with 3 column volumes of 0.18 M phosphate buffer at each point, as the temperature was raised in small increments to 95 °C. A representative aliquot of each wash was assayed for radioactivity in a Packard scintillation spectrometer. To ascertain that the hydroxylapatite was free of bound radioactivity following the final 95 °C wash, each column was rinsed with 3 vol of 0.40 M phosphate buffer (Schwartz and Kirsten, 1974).

Results

Cell Synchrony. In order to equally expose all rat DNA sequences to radiolabeled thymidine and BrdUrd, cultures of rat cells were pulse-labeled during a single, synchronous S phase. When released from the second thymidine block, cells resumed the cell cycle at the G_1/S interface (Mueller and Kajiwara, 1969; Schwartz et al., 1975). The combined (S + G_2) phase lasted approximately 8.5 h following release. A subsequent wave of mitosis immediately followed and persisted for nearly 1 h (Schwartz et al., 1975). The combined cell-cycle events were calculated to be (S + G_2) + M + G_1 = 8.5 + (0.5–1.0) + 2.5 = 12 h doubling time (Schwartz et al., 1975). Greater than 90% of the cells were in close synchrony as determined by autoradiographic analysis of S phase. Only low-passage cell cultures were eventually pulse-labeled for radioactive DNA preparations, as the relative divergence and deviations from synchrony reproducibility increased with multiple passages in vitro.

Characterization of DNA. [^{3}H]Thymidine- and [^{3}H]-BrdUrd-labeled rat DNA were analyzed by isopycnic centrifugation to equilibrium through linear, neutral CsCl gradients (Schwartz and Kirsten, 1974; Schwartz et al., 1975). [^{3}H]Thymidine-labeled rat DNA banded as a single peak of absorbance and radioactivity with a buoyant density of approximately 1.70 g/ml (Figure 1A). The [^{3}H]BrdUrd-labeled rat DNA similarly banded in CsCl as a single peak of A_{260} and radioactivity (Figure 1B). The increase in specific gravity to nearly 1.72 represented <7% substitution of total thymine residues by bromouracil (Rownd, 1967). The initial specific activities of the exogenous labeled DNA precursors were similar, as were the subsequent specific activities of the extracted, purified DNA samples. These findings suggested that inherent cellular metabolic pathways for the general utilization and incorporation of [^{3}H]thymidine and [^{3}H]BrdUrd into DNA were quantitatively comparable.

Partial Depurination of DNA. To detect a differential qualitative pattern of [^{3}H]BrdUrd incorporation, rat DNA was partially depurinated in order to produce potential substrate sites for single-strand specific nucleases. In this manner,

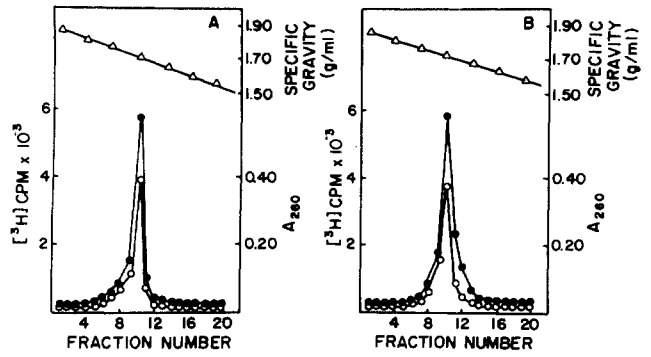

FIGURE 1: Buoyant density determination of [^{3}H]thymidine- and [^{3}H]-BrdUrd-labeled rat DNA. DNA samples (20–50 μg) were centrifuged to equilibrium in neutral CsCl gradients for 48 h at 42 000 rpm in a Spinco SW-50.1 rotor at 20 °C. Gradient fractions were measured for A_{260} (○), radioactivity (●), and refractive index (△).

dissimilar losses of radioactivity would reflect the respective patterns of distribution between the two labeled DNA precursors. Table I demonstrates the levels of depurination achieved when DNA samples in neutral SSC buffer were extensively dialyzed against an acidic sodium acetate buffer (pH 4.5) for 24 h at 4 °C. Radiolabeled guanosine and adenosine were added to untreated and 10^{-7} M BrdUrd-treated rat embryo cell cultures. DNA was extracted and subjected to partial depurination to determine whether the presence of bromouracil influenced the relative loss of adenine or guanine. Although the physical recovery of DNA from all samples was essentially complete as determined by A_{260}, 24–29% of the radioactivity was missing following dialysis. No alterations in specific activity were observed whenever these DNA samples were similarly dialyzed against neutral 0.08 M phosphate buffer. Conversely, no substantial reductions in radioactivity were detected when [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled DNA samples were subjected to dialysis against the acidic acetate buffer. Therefore, the extent of depurination between native and BrdUrd-treated DNA samples was similar, as was the respective loss of adenine and guanine.

Single-Strand Specific Nuclease. In order to determine whether bromouracil was differentially localized or clustered into particular nucleotide sequences, [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled, depurinated DNA samples were allowed to react with two different single-strand specific nucleases and assayed over hydroxylapatite columns. Table II shows the nature of the enzymatic products obtained following digestions with S_1 nuclease from *Aspergillus oryzae*. Nearly 74% of the mass and 78% of the radioactivity of depurinated, [^{3}H]thymidine-labeled rat DNA remained double stranded following digestion with S_1 nuclease. On the other hand, 73% of the mass, but only 38% of the radioactivity of [^{3}H]BrdUrd-labeled rat DNA was recovered as double-stranded molecules following S_1 nuclease digestion. This finding accounted for a net decrease in BrdUrd-labeled DNA specific activity of approximately 50%.

To corroborate the dissimilar digestion patterns of [^{3}H]-thymidine-and [^{3}H]BrdUrd-labeled DNA generated by S_1 nuclease, a second single-strand specific nuclease was sought. Linn and Lehman (1965a,b) characterized an endonuclease from *Neurospora crassa* which has a substrate specificity similar to that of the S_1 enzyme. Moreover, the in vitro reaction conditions are optimal in Tris-HCl (pH 8.0) as compared to pH 4.5 for the *Aspergillus* enzyme (Linn and Lehman, 1965a,b). Therefore, subtotally depurinated DNA samples were dialyzed extensively against the Tris-HCl buffer (pH 8.0) prior to enzymatic hydrolysis. Table III shows that about 28% of the mass and 21% of the radioactivity of [^{3}H]thymidine-labeled, depurinated rat DNA was not recovered as double-stranded molecules following *N. crassa* endonuclease digestion and hydroxylapatite fractionation. Of the subtotally depurinated [^{3}H]BrdUrd-labeled DNA, a similar 24% was missing following digestion. However, 65% of the [^{3}H]bromouracil residues was absent from the corresponding material. This differential loss of isotope corresponded to a decrease in specific activity of [^{3}H]BrdUrd-labeled DNA of nearly 47%. To confirm the substrate specificity of both nucleases, 5.0 A_{260} units of heat-denatured calf thymus DNA were allowed to react with each enzyme under the respective optimal conditions of bioassay in vitro. After extensive extraction with chloroform–isoamyl alcohol and dialysis against 0.08 M phosphate buffer, no A_{260} units could be detected from either reaction mixture.

Nondepurinated, native DNA labeled with [^{3}H]thymidine or [^{3}H]BrdUrd were similarly allowed to react with the *N. crassa* endonuclease to determine whether naturally occurring single-stranded regions existed in the rat genome. Purified, native DNA samples were dialyzed extensively against the Tris-HCl buffer (pH 8.0), digested with the appropriate amount of enzyme, and analyzed over hydroxylapatite (Table IV). Nearly all of the DNA was ultimately recovered as double-stranded forms when assayed over hydroxylapatite and monitored for both A_{260} and radioactivity. The overall loss of DNA averaged between 4 and 6% for [^{3}H]thymidine- as well as [^{3}H]BrdUrd-labeled samples. Analogous reactions between S_1 nuclease and nondepurinated DNA yielded similar results.

DNA–DNA Reassociation. The single-strand specific nu-

TABLE I: Partial Depurination of Radiolabeled Rat DNA Samples following Dialysis against Sodium Acetate Buffer.[a]

	[^{3}H]Guo-Labeled DNA	[^{3}H]Guo-Labeled + 10^{-7} M BrdUrd DNA	[^{3}H]Ado-Labeled DNA	[^{3}H]Ado-Labeled + 10^{-7} M BrdUrd DNA
Before dialysis				
DNA mass	59.2 μg	66.2 μg	68.1 μg	57.4 μg
Sp act.	940 cpm/μg	2180 cpm/μg	1350 cpm/μg	1180 cpm/μg
After dialysis				
DNA mass	60.0 μg	64.7 μg	69.1 μg	55.8 μg
Sp act.	710 cpm/μg	1550 cpm/μg	980 cpm/μg	890 cpm/μg
% depurination	24	29	28	24

[a] ^{3}H-Labeled rat DNA samples in SSC buffer (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) were dialyzed against sodium acetate buffer (0.3 M NaCl, 0.03 M sodium acetate, pH 4.5). The level of depurination was determined by relative loss of specific activity of DNA sample following dialysis. (The specific activity standard deviation is ±4%.)

TABLE II: Hydroxylapatite Fractionation of Subtotally Depurinated, Double-Stranded Rat DNA Samples following Hydrolysis with S_1 Nuclease.[a]

DNA Label Type	S_1 Nuclease	Double-Stranded DNA (μg)	Double-Stranded DNA (cpm)	Double-Stranded DNA Lost (%)	Double-Stranded cpm Lost (%)	Double-Stranded Sp Act. (cpm/μg)
[^{3}H]Thymidine	−	89.0	124 000			1400
[^{3}H]Thymidine	+	65.2	98 300	26	21	1500
[^{3}H]BrdUrd	−	92.0	185 300			2000
[^{3}H]BrdUrd	+	67.0	69 700	27	62	1000

[a] Approximately 100 μg of double-stranded, depurinated rat DNA was allowed to react with S_1 nuclease as described in the text. Digestion products were analyzed over hydroxylapatite for residual double-stranded DNA. (The specific activity standard deviation is ±4%.)

TABLE III: Hydroxylapatite Fractionation of Subtotally Depurinated, Double-Stranded Rat DNA Samples following Hydrolysis with *N. crassa* Endonuclease.[a]

DNA Label Type	*N. crassa* Nuclease	Double-Stranded DNA (μg)	Double-Stranded DNA (cpm)	Double-Stranded DNA Lost (%)	Double-Stranded cpm Lost (%)	Double-Stranded Sp Act. (cpm/μg)
[^{3}H]Thymidine	−	153.7	433 400			2800
[^{3}H]Thymidine	+	110.8	341 300	28	21	3000
[^{3}H]BrdUrd	−	107.0	350 500			3200
[^{3}H]BrdUrd	+	79.2	123 300	24	65	1500

[a] Approximately 150 μg of [^{3}H]thymidine-labeled DNA and 100 μg of [^{3}H]BrdUrd-labeled DNA were depurinated and allowed to react with *N. crassa* single-strand specific endonuclease as described in the text. Reaction mixtures were subsequently assayed over hydroxylapatite for double-stranded DNA. (The specific activity standard deviation is ±4%.)

TABLE IV: Hydroxylapatite Fractionation of Nondepurinated, Double-Stranded Rat DNA Samples following Hydrolysis with *N. crassa* Endonuclease.[a]

DNA Label Type	*N. crassa* Nuclease	Double-Stranded DNA (μg)	Double-Stranded DNA (cpm)	Double-Stranded DNA Lost (%)	Double-Stranded cpm Lost (%)	Double-Stranded Sp Act. (cpm/μg)
[^{3}H]Thymidine	−	150.0	202 400			1300
[^{3}H]Thymidine	+	140.0	193 000	6	4	1300
[^{3}H]BrdUrd	−	196.6	438 300			2200
[^{3}H]BrdUrd	+	188.3	420 600	4	4	2200

[a] Approximately 150 μg of native [^{3}H]thymidine-labeled rat DNA and 200 μg of native [^{3}H]BrdUrd-labeled rat DNA were dialyzed against Tris-HCl buffer (pH 8.0) and digested with the *N. crassa* single-strand specific endonuclease as described in the text. Reaction mixtures were made 0.08 M in sodium phosphate buffer and ultimately reassayed over hydroxylapatite. (The specific activity standard deviation is ±4%.)

clease digestion patterns (Tables II–IV) suggested that [^{3}H]BrdUrd moieties were distributed within rat nucleotide sequences in a pattern distinct from the random [^{3}H]thymidine localization. We previously reported that [^{3}H]BrdUrd was incorporated into middle repetitive rat DNA sequences to a greater extent than was [^{3}H]thymidine under similar conditions of synchronous cell growth (Schwartz et al., 1975). Therefore, DNA–DNA reassociation studies were performed as before (Schwartz and Kirsten, 1974; Schwartz et al., 1975) in order to quantitatively subfractionate the native as well as depurinated, nuclease-treated, double-stranded DNA according to base sequence reiteration frequency (Britten et al., 1975). The determination of the respective specific activities of highly repetitive and nonrepetitive DNA subpopulations as compared to the complete, initial specific activity would reflect the distribution of the isotopes throughout the rat genome. As seen in Table V, the overall initial specific activity of each DNA type was compared to the specific activity of each respective nucleotide sequence subpopulation. Following denaturation and sequential reassociation of native DNA according to repetition frequency, it appeared that [^{3}H]thymidine moieties were randomly and uniformly distributed throughout all sequence types. As before (Schwartz and Kirsten, 1974; Schwartz et al., 1975), [^{3}H]bromouracil moieties were incorporated to a relatively greater degree in the moderately repeated nucleotide sequence elements of rat DNA. The nonrepeated (unique) DNA fraction contained comparatively less radioactivity. When the depurinated, S_1 nuclease-treated DNA samples were similarly renatured according to reiteration frequency (Table VI), a random and uniform pattern of

TABLE V: Fractionation of Native Rat Embryo DNA by Sequential Reassociation and Hydroxylapatite Column Chromatography.[a]

	[^{3}H]Thymidine-Labeled DNA Sp Act.		[^{3}H]BrdUrd-Labeled DNA Sp Act.	
	Absolute (cpm/μg)	Relative	Absolute (cpm/μg)	Relative
Complete DNA reaction mixture	1070	1.00	990	1.00
Highly repetitive DNA subfraction	1010	0.95	1020	1.03
Middle repetitive DNA subfraction	1110	1.04	1310	1.32
Nonrepetitive DNA subfraction	1010	0.95	660	0.66

[a] DNA samples were sheared, heat denatured, and reassociated in solution according to text. The DNA samples were sequentially and quantitatively fractionated into highly repetitive (C_0t 0-1), middle repetitive (C_0t 1-100), and nonrepetitive (100-10 000) nucleotide sequences. The specific activity of each subpopulation was recorded and expressed in absolute units (cpm/μg) as well as relative to the corresponding unfractionated sample. (The specific activity standard deviation is ±4%.)

TABLE VI: Fractionation of Depurinated, S_1-Treated, Denatured Rat DNA by Sequential Reassociation and Hydroxylapatite Column Chromatography.[a]

	[^{3}H]Thymidine-Labeled DNA Sp Act.		[^{3}H]BrdUrd-Labeled DNA Sp Act.	
	Absolute (cpm/μg)	Relative	Absolute (cpm/μg)	Relative
Complete DNA reaction mixture	970	1.00	1020	1.00
Highly repetitive DNA subfraction	1040	1.06	940	0.92
Middle repetitive DNA subfraction	870	0.90	740	0.72
Nonrepetitive DNA subfraction	1040	1.07	900	0.89

[a] DNA samples were partially depurinated, allowed to react with S_1 nuclease, and prepared for reassociation into reiteration frequency subfractionations as in Table V. (The specific activity standard deviation is ±4%.)

[^{3}H]thymidine distribution was again observed throughout. On the other hand, [^{3}H]BrdUrd residues were most conspicuously depleted from the middle repetitive DNA fraction. The relative specific activity of this particular component was only 72% that of the complete genome. This finding is in contrast to the distribution of [^{3}H]BrdUrd in the nondepurinated, nondigested rat DNA where the middle repetitive sequences were relatively enriched in the analogue.

Thermal Stability. The comparative contamination or overlap of the fractionated DNA subpopulations with each other was minimal as before (Schwartz and Kirsten, 1974; Schwartz et al., 1975). Figure 2 demonstrates the thermal stability of each purified, reassociated DNA fraction. The double-stranded molecules which reannealed by a C_0t of 1 (highly repetitive DNA) melted similarly on hydroxylapatite irrespective of labeled precursor. The T_m of these duplex molecules was approximately 80 °C. A slight difference in thermal stability was observed between [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled C_0t (1-100) (middle repetitive) DNA. In this instance, 50% of the analogue-treated sequences were single stranded at 77 °C, as compared to 74.5 °C, for the [^{3}H]thymidine-labeled counterpart. A similar pattern was observed when C_0t (100-10 000) (nonrepetitive) duplex DNA was heat denatured over hydroxylapatite. The melting temperatures of [^{3}H]BrdUrd-labeled and [^{3}H]thymidine-labeled sequences were 84 and 82 °C, respectively.

Discussion

The effects of halogenated thymidine analogues on animal cell behavior in vitro are multifold and complex (Coleman et al., 1970; Levitt and Dorfman, 1973; Lowy et al., 1971; Rutter et al., 1973; Turkington et al., 1971). Under controlled experimental conditions, particular cell-specific phenotypes and functions can be differentially activated (Hampar et al., 1974; Lowy et al., 1971; Schwartz et al., 1974a) or repressed (Levitt and Dorfman, 1973; Rutter et al., 1973; Stellwagen and Tomkins, 1971), reversibly (Coleman et al., 1970; Davidson and Horn, 1974; Stellwagen and Tomkins, 1971) or irreversibly (Levitt and Dorfman, 1972; Morris, 1973). Although exact molecular mechanisms for these observations are still unknown, in most instances the analogue must be incorporated into nuclear DNA (Levitt and Dorfman, 1973; Stellwagen and Tomkins, 1971; Teich et al., 1973). The localization and distribution of these compounds in host chromosomal DNA may reflect the genetic organization of certain regulatory elements. Therefore, a distinct and reproducible pattern of virogenic BrdUrd incorporation and localization in DNA may reveal an inherent nuclear genomic arrangement for regulation of type C virus expression. We have previously demonstrated that BrdUrd activated type C virus-specific function in normal rat cells in vitro (Schwartz et al., 1974a). Furthermore, we have shown by several techniques that virogenic [^{3}H]BrdUrd residues were incorporated into rat embryo DNA in a nonrandom fashion, as compared to [^{3}H]thymidine (Schwartz and Kirsten, 1974; Schwartz et al., 1974b, 1975; Schwartz, 1975). This work characterizes further the dissimilar pattern of thymidine and BrdUrd distribution in rat nucleotide sequences as determined by single-strand nuclease digestion of partially depurinated DNA.

Normal rat cells were synchronized in order to equally expose all replicating nucleotide sequences to each radiolabeled DNA precursor. This experimental procedure has been shown to be most advantageous for the maximal elicitation of latent type C virus from normal rat cell cultures with minimal amounts of BrdUrd (Schwartz et al., 1975). Moreover, the high degree of synchrony assured that BrdUrd was incorpo-

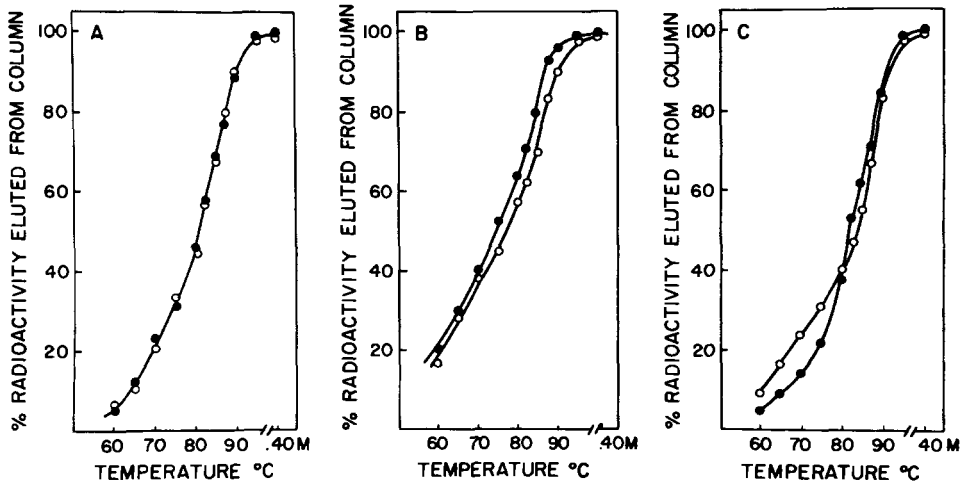

FIGURE 2: Thermal stability of reassociated rat DNA subpopulations as determined by hydroxylapatite fractionation. Subtotally depurinated, S_1-treated, sequentially reassociated DNA samples (150–200 μg) were made 0.18 M in phosphate buffer, and adsorbed to hydroxylapatite columns at 60 °C. As the temperature was increased in small increments, each wash of 0.18 M phosphate buffer was assayed for radioactivity. A final wash of 0.40 M phosphate buffer was performed to confirm that no radioactivity remained on the column after the 95 °C elution. The respective T_m of each [^{3}H]thymidine-labeled (●) and [^{3}H]BrdUrd-labeled (○) DNA fraction was determined for highly repetitive (A), middle repetitive (B), and nonrepetitive (C) nucleotide sequences.

rated only within a single strand of each purified DNA molecule after the completion of the S phase. The low-passage rat cells were easily and reproducibly synchronized by the double thymidine block technique. However, variability and divergence from synchrony were more pronounced whenever higher passage rat cell strains were used. For this reason, only secondary cultures were synchronized and ultimately prepared for the DNA depurination and enzymatic degradation studies. Moreover, these experimental, synchronous conditions of labeling are consistent with the observation that only one strand of DNA need be substituted by BrdUrd in order for certain analogue-mediated events to occur (Rutter et al., 1973; Schwartz et al., 1975).

The conditions for depurination were sufficient to allow a 24–29% loss of [^{3}H]guanine and [^{3}H]adenosine, but little or no loss of [^{3}H]bromouracil or [^{3}H]thymine from rat DNA. This mild acidic pretreatment was intended to generate single-stranded regions of DNA which were appropriate substrates for two different single-strand specific nucleases. The relatively comparable extent of depurination of all DNA samples argues against any dissimilar loss of radiolabeled adenine or guanine due to incorporation of bromouracil. Furthermore, only by depurination was rat DNA made sensitive to enzymatic hydrolysis to any substantial extent. Nondepurinated DNA was only minimally digested under similar conditions of bioassay using the S_1 and *Neurospora* nucleases. Even though naturally occurring single-stranded regions have been detected in native sea urchin morula DNA (Case and Baker, 1975a), similar chromosomal structure and organization have not been observed in DNA from higher vertebrate organisms (Davidson and Britten, 1973; Sheid et al., 1975). Furthermore, our DNA purification technique of hydroxylapatite fractionation assured that the majority of rat DNA was most likely in a double-stranded form prior to depurination.

Previous studies on the mechanisms of action of BrdUrd have revealed that the analogue need not substitute a great many available thymine moieties in DNA for substantial bioactivity to occur. Indeed, maximal inhibition of cell-specific gene product synthesis has occurred subsequent to a 2% level of replacement (Levitt and Dorfman, 1972). We have shown that 10^{-7} M [^{3}H]BrdUrd was sufficient to activate type C RNA virus-specific expression from synchronized rat embryo cells (Schwartz et al., 1975) in which fewer than 7% of thymine moieties were replaced by bromouracil. As determined by sequential DNA–DNA reassociation and fractionation, the virogenic [^{3}H]BrdUrd was incorporated to a greater extent in middle repetitive nucleotide sequences than was [^{3}H]thymidine. Similar nonrandom concentration of [^{3}H]BrdUrd into middle repetitive sequences of avian limb bud DNA was recently observed by others (Strom and Dorfman, 1976). Case and Baker similarly detected a difference in the sites of incorporation of BrdUrd and thymidine in differentiating sea urchins (Case and Baker, 1975a). Henderson and Strauss recently characterized several human cell lines which lacked the ability to incorporate BrdUrd into more than a single strand of replicating DNA (Henderson and Strauss, 1975). Altogether, these observations suggest that BrdUrd is often utilized and incorporated into eukaryotic DNA in a manner distinctly different from thymidine under certain experimental conditions.

The enzymatic digestion patterns of slightly depurinated rat DNA further confirmed the nonrandom nature of [^{3}H]-BrdUrd incorporation. That the amount of [^{3}H]thymidine residues lost following treatment with both nucleases corresponded closely with the physical loss of rat DNA implied that the label was indeed randomly and uniformly substituted throughout the genome. On the other hand, the differentially greater loss of [^{3}H]BrdUrd from nuclease-treated rat embryo DNA is in agreement with the previous observations of nonrandom and nonuniform localization of this analogue. Since all rat DNA samples were similarly depurinated irrespective of previous isotopic label or BrdUrd treatment, subsequent digestion patterns by the *Neurospora* and *Aspergillus* nucleases most likely disclosed dissimilar incorporation. The decreases in mass of depurinated DNA following nuclease digestion were comparable regardless of isotopic precursor as well. However, the dissimilar loss of radioactivity from [^{3}H]BrdUrd-labeled DNA was significant following reaction with both nuclease types. This finding suggests that [^{3}H]-bromouracil moieties were situated in close proximity to each other in purine-rich elements made labile to both nucleases.

Therefore, clusters or concentrations of [^{3}H]BrdUrd in A + T rich regions of the rat genome would be highly susceptible to single-strand specific nuclease attack following subtotal removal of A residues by mildly acidic depurination. Our results are in agreement with such an explanation.

The differentially greater loss of [^{3}H]bromouracil from the middle repetitive nucleotide sequences was not unexpected, as this particular DNA subfraction originally contained relatively more of the radioactive analogues. The apparent lability of middle repetitive rat DNA to single-strand specific nucleases following mild depurination is unclear. This property may be accounted for by a high A + T content, as was recently described for mouse repeated DNA sequences (Straus and Birnboim, 1974). Long eukaryotic-specific pyrimidine runs in repetitive DNA sequences of sea urchin embryos were recently reported as well (Case and Baker, 1975b). Britten et al. (1975) observed that the exonuclease and endonuclease functions of the *Aspergillus* enzyme were sufficient to degrade a portion of reassociated intermediate repetitive DNA. This observation may reflect a particular feature common to this component of eukaryotic genomes. Since the [^{3}H]thymidine- and [^{3}H]BrdUrd-labeled samples were digested prior to reassociation into DNA subfractions, production of nuclease-sensitive sites was not the result of inaccurate base pairing during renaturation.

The melting profiles of the reassociated, purified DNA subpopulations indicate excellent fidelity of base pairing following depurination and enzymatic hydrolysis. The nonrepetitive (single copy) DNA samples melted 1–3 °C below the T_m reported for native rat DNA (Holmes and Bonner, 1974; Schwartz and Kirsten, 1974). The middle repetitive nucleotide sequences melted with broader, less homogenous profiles and lower melting temperatures. This finding may be attributed to the extreme shortness of the double-stranded repetitive regions in rat DNA as described by Bonner and coworkers (1973). Unfortunately, the base composition of rat middle repetitive DNA sequences (approximately 20% of the genome) is currently unknown (Holmes and Bonner, 1974). The exact A + T ratio of these particular elements may account for their melting profile as well as their apparent enrichment in BrdUrd residues. The highly repetitive DNA sequences in mammalian genomes have not been as thoroughly characterized as the middle and nonrepetitive classes. Their homogenous melting profiles with a T_m of 81 °C may indeed reflect an inherent similarity of base sequences, plentiful "fold-back" DNA, or a high G + C content.

Although others have described preferential incorporation of BrdUrd in eukaryotic DNA (Haut and Taylor, 1967; Strom and Dorfman, 1976), the rat embryo system is dose dependent. We previously reported that when bromouracil replacement exceeded 80%, the pattern and extent of localization of BrdUrd in DNA were indistinguishable from that of thymidine (Schwartz and Kirsten, 1974). The nonrandom distribution was most pronounced when fewer than 7% of the thymine moieties were substituted. The rat DNA in this work was labeled during a single S phase with a low concentration (10^{-7} M) of analogues, immediately extracted at the conclusion of S phase, and maintained in dark vessels throughout the course of experimentation. These methods and precautions hopefully minimized alterations in BrdUrd-substituted DNA caused by cellular repair and ultraviolet irradiation induced excision of bromouracil (Ehrlich and Riley, 1974), respectively. Therefore, the data most likely reflect nonrandom incorporation rather than nonrandom degeneration of bromouracil in rat DNA. Moreover, the preferential incorporation of BrdUrd may be a consequence of the complexity of the eukaryotic genome, since random replacement by bromouracil for thymine in *Escherichia coli* DNA is well known (Rudner et al., 1962).

A role for moderately repeated, nontranscribed DNA sequences in regulation of eukaryotic phenotypic expression has been previously proposed (Britten et al., 1975; Davidson and Britten, 1973). More specifically, Bonner and co-workers (Bonner et al., 1973) have demonstrated the interspersion of middle repetitive sequences between single-copy DNA sequences in the rat genome. Paul (1972) has further proposed that this family of DNA constitutes "address sites" with an affinity for regulatory proteins which modulate gene activity. More recently, Sevall and colleagues (1975) isolated a population of rat nonhistone chromosomal proteins which have a distinctive binding capacity for middle repetitive DNA. Consequently, one conceivable model for a mechanism of BrdUrd effects on rat genetic expression may involve an altered binding relationship between a particular middle repetitive nucleotide sequence and its corresponding regulatory protein(s) (David et al., 1974). Certainly, an analogous example has already been reported between BrdUrd and a well-characterized, prokaryotic regulatory system (Lin and Riggs, 1972). Accordingly, the activation of a specific gene product (type C RNA virus) by BrdUrd can be considered as the result of suppression of synthesis of a specific repressor protein(s). Indeed, Aaronson and Dunn (1974) have reported the similar activation of latent RNA tumor virus synthesis from mouse cells by the use of compounds which suppress protein synthesis. The exact mechanisms for these observations and the manner by which BrdUrd is differentially incorporated into particular genetic elements, however, remain to be clarified.

Acknowledgment

The author thanks Dr. Godfrey Getz for his suggestions and critical appraisal of this manuscript. The excellent technical assistance of Miss Dorcas Joslin is also gratefully acknowledged.

References

Aaronson, S. A., and Dunn, C. Y. (1974), *Science 183*, 422–424.

Ando, T. (1966), *Biochim. Biophys. Acta 114*, 158–168.

Bonner, J., Garrard, W. T., Gottesfield, J., Holmes, D. S., Sevall, J. S., and Wilkes, M. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38*, 303–310.

Britten, R. J., Graham, D. E., and Neufeld, B. R. (1975), *Methods Enzymol. 29*, 363–418.

Case, S. T., and Baker, R. F. (1975a), *Nature (London) 253*, 64–66.

Case, S. T., and Baker, R. F. (1975b), *J. Mol. Biol. 98*, 69–82.

Coleman, A. W., Coleman, J. R., Kankel, D., and Werner, I. (1970), *Exp. Cell Res. 59*, 319–328.

David, J., Gordon, J. S., and Rutter, W. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2808–2812.

Davidson, E. H., and Britten, R. J. (1973), *Q. Rev. Biol. 48*, 565–613.

Davidson, R. L., and Horn, D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 3338–3342.

Ehrlich, M., and Riley, M. (1974), *Photochem. Photobiol. 20*, 159–165.

Hampar, B., Derge, J. G., Nanoyama, M., Chang, S.-Y., Tagamets, M. H., and Showalter, S. D. (1974), *Virology 62*, 71–89.

Haut, W. F., and Taylor, J. H. (1967), *J. Mol. Biol. 26*,

389-401.
Henderson, E. E., and Strauss, B. (1975), *Cell 5,* 381-387.
Holmes, D. S., and Bonner J. (1974), *Biochemistry 13,* 841-848.
Levitt, D., and Dorfman, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1253-1257.
Levitt, D., and Dorfman, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2201-2205.
Lin, S.-Y., and Riggs, A. D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2574-2576.
Linn, S., and Lehman, I. R. (1965a), *J. Biol. Chem. 240,* 1287-1293.
Linn, S., and Lehman, I. R. (1965b), *J. Biol. Chem. 240,* 1294-1304.
Lowy, D. R., Rowe, W. P., Teich, N., and Hartley, J. W. (1971), *Science 174,* 155-156.
Morris, J. E. (1973), *Dev. Biol. 35,* 125-142.
Mueller, G. C., and Kajiwara, K. (1969), *Fundam. Tech. Virol.,* 21-27.
Paul, J. (1972), *Nature (London) 238,* 444-446.
Rownd, R. (1967), *Biochim. Biophys. Acta 134,* 464-467.
Rudner, R., Shapiro, H. S., and Chargaff, E. (1962), *Nature (London) 195,* 143-146.
Rutter, W. J., Pictet, R. L., and Morris, P. W. (1973), *Annu. Rev. Biochem. 42,* 601-646.
Schwartz, S. A. (1975), *Biochem. Biophys. Res. Commun. 65,* 1081-1087.
Schwartz, S. A., Horio, D., and Kirsten, W. H. (1974a), *Biochem. Biophys. Res. Commun. 61,* 927-933.
Schwartz, S. A., and Kirsten, W. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3570-3574.
Schwartz, S. A., Panem, S., and Kirsten, W. H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1829-1833.
Schwartz, S. A., Panem, S., Stefanski, E., and Kirsten, W. H. (1974b), *Cancer Res. 34,* 2255-2259.
Sevall, J. S., Cockburn, A., Savage, M., and Bonner, J. (1975), *Biochemistry 14,* 782-789.
Sheid, B., Pedrinan, L., Lu, T., and Nelson, J. H. (1975), *Biochem. Biophys. Res. Commun. 66,* 1131-1133.
Shichido, K., and Ando, T. (1972), *Biochim. Biophys. Acta 287,* 477-484.
Stellwagen, R. H., and Tomkins, G. M. (1971), *J. Mol. Biol. 56,* 167-182.
Straus, N. A., and Birnboim, H. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2292-2995.
Strom, C. M., and Dorfman, A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1019-1023.
Studier, F. W. (1965), *J. Mol. Biol. 11,* 373-381.
Teich, N., Lowy, D. R., Hartley, J. W., and Rowe, W. P. (1973), *Virology 51,* 163-173.
Turkington, R. W., Majumder, G. C., and Riddle, M. (1971), *J. Biol. Chem. 246,* 1814-1819.
Verwoerd, D. W., and Sarma, P. S. (1973), *Int. J. Cancer 12,* 551-562.
Walther, B. T., Pictet, R. L., David, J. D., and Rutter, W. J. (1974), *J. Biol. Chem. 249,* 1953-1964.
Weintraub, H., Campbell, G. L., and Holtzer, H. (1972), *J. Mol. Biol. 70,* 337-350.