parable position with respect to the dimers that protect them. The parts of the 3'-terminal sequence that were never found on the filter were positioned in the spaces between the first and second dimer and between the second and third dimer, thus being thought susceptible to ribonuclease attack. Site 2 has been drawn melted out in order to make spanning of the second dimer possible. This is not purely speculative since addition of a few coat protein subunits to RNA 4 results in a considerable decrease of ethidium bromide binding capacity (Srinivasan & Jaspars, 1982). It is quite possible that sites 1 and 3 have also lost most of their secondary structure upon binding of coat protein, thus permitting a long-range interaction with the internal coat protein binding site.

Since we know that infection by alfalfa mosaic virus only takes place if each of the three genome RNAs has bound some coat protein molecules (Smit et al., 1981), it is of much interest to investigate complex formation with these RNAs, too. If well-defined complexes are formed and can be separated, the problem of the minimum number of coat protein dimers sufficient for biological activation can be solved.

A priori the four alfalfa mosaic virus RNAs seem to have a common basis for complex formation, since the sequence of 150 nucleotides at their 3' termini is largely homologous (Pinck & Pinck, 1979; Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980). Indeed it appeared that the coat protein protects part of this sequence in the three genome RNAs against ribonuclease degradation. A further similarity with the protein binding by RNA 4 is that certain internal sites of the genome RNAs are protected as well (D. Zuidema et al., unpublished results).

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Nuclease S_1 Sensitive Sites in Parental Deoxyribonucleic Acid of Cold- and Temperature-Sensitive Mammalian Cells†

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ABSTRACT: Temperature-sensitive mutants of 3T3 cells (H6-15) express the transformed phenotype at 33 °C and the normal phenotype at 39 °C. Cold-sensitive mutants of Chinese hamster ovary cells (cs4-D3) express the transformed phenotype at 39 °C and the normal phenotype, along with a G_1 block, at 33 °C. When either cell type is under conditions such that it is normal and in a G_0 state, the number of S_1-sensitive sites in purified DNA, labeled in parental chains only, is zero. When the normal cells are stimulated by 10% serum, the number of S_1 sites per 10^5 base pairs increases slightly, to 0.7 in cs4-D3 and 1.1 in H6-15. Under conditions permitting the expression of the transformed phenotype, but not proliferation, the maximum number of S_1 sites per 10^5 base pairs is 5 in cs4-D3 and 44 in H6-15. When the stationary transformed cells are stimulated by 10% serum, the number of S_1 sites per 10^5 base pairs increases to 6 in cs4-D3 and 43 in H6-15. Furthermore, the DNA from the stimulated transformed H6-15 cells contains at least twice as many S_1 sites as the total number of breaks (nicks plus gaps), raising the possibility of the acquisition of stable looped or cruciform structures as the cells are stimulated.

Single-stranded regions in DNA have been demonstrated in numerous systems, but their nature and function are unknown at present (Painter & Schaefer, 1969; Habener et al., 1970; Schlegel & Thomas, 1972; Tan & Lerner, 1972; Collins, 1974, 1977, 1979; Case & Barker, 1975; Hoffman & Collins, 1976; Collins et al., 1977; Henson, 1978). Crick (1971) has proposed a model for their involvement as conformation-dependent regulatory signals. During replication, nucleosomal DNA is more sensitive to DNase I (85%) than when in bulk nucleosomes, suggesting a destabilized conformation (Seale, 1977). All of the current models of replication predict various structural changes in DNA such as nicks, unwound regions,

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and gaps (Champoux, 1978; Hand, 1978). We have previously reported that purified DNA pulse labeled for 0.5 min can be extensively digested by S_1 nuclease (i.e., 60%), which is not surprising as 44% of the chains were entirely single stranded by hydroxylapatite chromatography (Collins, 1979). S_1 digestion removed 18% of the radiolabel of ^{14}C parental chains of S-phase DNA (Collins, 1977). When WI-38 cells in G_0 were stimulated to proliferate (Collins, 1977), the number of S_1-sensitive sites/10^5 base pairs of parental chains increased from 0 (0 h) to 6.5 (4 h), 22 (6 h), and 41 (12 h), while the cells were moving from G_0 through the G_1 phase (2–12 h) to the S phase (onset at 12 h). The value of 41 sites/10^5 base pairs for late G_1 DNA (Collins, 1977) corresponds to a total of 2.7×10^6 sites/cell, assuming a DNA content of 7.2 pg for a G_1 WI-38 cell (Collins et al., 1980). This number is very close to the estimated number of replicons of WI-38 (Collins et al., 1980) (i.e., 10^5–10^6). Long single chains of parental DNA of (2–4) $\times 10^7$ daltons, comprising about 2% of the total DNA, were demonstrated in Raji cells (Bjursell et al., 1979).

In this report, we have used S_1 nuclease as a probe for destabilized or single-strand regions (Collins, 1977) in DNA isolated from two cell lines that are reversible for the transformed phenotype. The H6-15 line was derived from the mouse 3T3 line, via SV40 transformation, by Renger & Basilico (1972). By most in vitro criteria, they express the transformed phenotype in a temperature-dependent manner. The virus rescued from these transformed cells behaved like wild-type SV40, suggesting that a cellular mutation exists (Renger & Basilico, 1972, 1973; Basilico et al., 1974). At the nonpermissive temperature of 39 °C, the H6-15 cells behave almost essentially like 3T3 cells. 3T3 cells have characteristics typical of normal cells in culture including low saturation density (Todaro & Green, 1963; Pollack et al., 1968), contact inhibition for growth and DNA synthesis (Dulbecco, 1970), and dependence upon serum concentrations for growth (Dulbecco, 1970). 3T3 cells have been used to represent normal cells in studies on transformation (Todaro et al., 1961; Todaro & Green, 1964). At 39 °C, H6-15 cells have low saturation density, contact inhibition of growth and DNA synthesis and greater sensitivity to serum requirements and lack the ability to form colonies on a contact-inhibited monolayer of 3T3 cells (Renger & Basilico, 1972). In addition, H6-15 cells maintained at 39 °C are able to enter a true quiescent state, e.g., G_0 (Basilico & Zouzais, 1976; Zouzais & Basilico, 1979). The ability for a cell line to reach a state of viable "G_1" arrest under conditions restrictive for growth has been used as another criterion distinguishing normal cells from SV40 transformants (Nilausen & Green, 1965; Bartholomew et al., 1976). G_0 is the special designation given to these resting cells which are metabolically quiescent, and thus different from G_1 cells. G_0 cultures are further defined as having 95% of the cells with a G_1 complement of DNA (Baserga, 1978). These resting, nonproliferating cells remain viable and metabolically active. However, they contain only low amounts of biosynthetic enzymes and appear to be in a "maintenance state" (Thorpe et al., 1974). Whereas cultures of G_0 and G_1 cells have the same DNA content and hence cannot be distinguished by flow cytometric measurements, they can be distinguished by their respective low and high rates of RNA synthesis (Baserga, 1968, 1978). G_0 cells can be induced to proliferate when returned to conditions optimal for growth (Pardee, 1978). The effect of high temperature on H6-15 is fully reversible. About 48 h after being shifted to low temperature, the cells are as though they had always been at low temperature (Renger & Basilico, 1972). On the other hand, the properties of H6-15 grown at the permissive temperature (33 °C) are common to transformed cells and compare favorably with those of the standard SV40 transformant SV3T3 (Renger & Basilico, 1972, 1973; Pollack et al., 1968; Todaro et al., 1964). H6-15 cells maintained at 33 °C are characterized by the loss of the susceptibility to contact inhibition, the ability to reach high saturation densities in culture, the growth in culture as a multilayer, and the ability to grow on colonies of normal 3T3 (Renger & Basilico, 1972). At the permissive temperature, these cells are also less dependent upon serum requirements for growth (Zouzais & Basilico, 1979). Unlike normal cells in culture, virally transformed cells cannot enter a true quiescent state with 95% of the cells in G_0 (Bartholomew et al., 1976). However, these transformed cells will become stationary when nutrients are limited in the culture medium. Both H6-15 at 33 °C and SV3T3 cells will become stationary (Basilico & Zouzais, 1976; Zouzais & Basilico, 1979; Ide & Baserga, 1976). The effect of low temperature is fully reversible. Reversal of transformation after shift up requires about 24 h (Renger & Basilico, 1973).

Exponentially growing H6-15 cells at both the permissive and nonpermissive temperatures are T-antigen positive (Basilico & Zouzais, 1976; Zouzais & Basilico, 1979). Stationary cultures of H6-15 at 33 °C are also T-antigen positive. However, when cells maintained at 39 °C were grown on low serum, they became T-antigen negative (Basilico & Zouzais, 1976; Zouzais & Basilico, 1979).

The cs4-D3 line was derived from the normal Chinese hamster ovary (CHO) line, via mutagenesis with ethyl methanesulfonate, by Farber & Unrau (1975). This cold-sensitive mutant was described by Crane & Thomas (1976). At the nonpermissive temperature of 33 °C, the cells arrest in G_1, from which they can be stimulated to enter the S phase by simply placing them at 39 °C. At 33 °C, they will not reactivate chick erythrocytes upon fusion, but will do so at 39 °C (Tsutsui et al., 1978). They will not exhibit contact inhibition at 39 °C. They are considered to have the normal phenotype at 33 °C and the transformed phenotype at 39 °C (Crane & Thomas, 1976). A curious property of these cells is that they have a fibroblast shape at 39 °C and an epithelial shape at 33 °C. The epithelial shape at 33 °C can be reversed by the addition of dibutyl-cAMP without subsequent proliferation (Crane & Thomas, 1976).

Experimental Procedures

Cell Cultures. H6-15 and cs4-D3 cells were kindly supplied by Dr. C. Basilico, Department of Pathology, New York University School of Medicine, and Dr. R. Baserga, Department of Pathology, Temple University School of Medicine, respectively. The H6-15 cells were grown in Dulbecco's medium supplemented with 10% calf serum as described by Farber & Unrau (1975). The cs4-D3 cells were maintained on basal Eagle's medium supplemented with 10% fetal calf serum, as described for culture of WI-38 cells (Collins, 1977).

Reagents. All tissue culture supplies were obtained from Flow Laboratories. [^{14}C]Thymidine (54 mCi/mmol), [^3H]-thymidine (20 Ci/mmol), and [^3H]UdR (45 Ci/mmol) were purchased from New England Nuclear and contained greater than 99% of the radioactivity in thymidine when examined by paper chromatography (Fink & Adams, 1966). Pancreatic ribonuclease and Pronase were supplied by Sigma. Propidium iodide was supplied by Calbiochem. R6K [^3H]DNA (a bacterial plasmid DNA containing 51S and 39S components) was the kind gift of Dr. Francis Macrina of the Medical College of Virginia Campus of Virginia Commonwealth University.

^{14}C-Labeled Parental DNA. Cell cultures were subcultured to one-half saturation density into tissue culture media containing 10% serum and 1 μCi/mL [^{14}C]thymidine. The cells were maintained on this medium (with one media change) for 5 days. The radioactive media were removed, and the cells were rinsed and again subcultured to one-half saturation density. Subculturing was continued so as to obtain eight subcultures from an original culture exposed to ^{14}C. For some experiments, these cells were then placed on media containing 0.5% serum to cause them to enter the G_0 state. The cells were then stimulated by the addition of fresh media containing 10% serum, and the DNA was extracted as described below.

Purification of DNA. DNA was purified essentially according to Marmur (1961), as modified by us (Collins, 1977). No protein could be detected in the final sample by the microbiuret method of Zamenhof (1957); hence, protein concentration is less than 1 μg/mL when DNA concentration is 100 μg/mL. No material banding as RNA was detected in CsCl gradients.

S_1 Nuclease Digestion. DNAs were digested with S_1 nuclease, as previously described in detail (Collins, 1977). After 20 min, 91% of heat-denatured DNA was rendered acid soluble by the enzyme preparation used for these experiments. This enzyme will hydrolyze single-stranded regions but not nicks or double-stranded regions, as evidenced by its lack of activity with the open circular form of R6K plasmid DNA (data not shown; Collins, 1977).

Sucrose Gradient Centrifugation of DNA. Duplex DNA was sedimented in 5-mL sucrose gradients, as previously described (Collins, 1974). The molecular weights in neutral and alkaline gradients were determined by using R6K [^{3}H]DNA as a marker, according to the equation of Studier (1965). The resulting data were analyzed by a general nonlinear least-squares computer program to quantitate the amounts of radioactivity in various Gaussian distributions. The details have been described elsewhere (Collins, 1978).

Other Procedures. Determination of radioactivity has been described elsewhere (Collins, 1977). Determination of the amount of DNA per cell by fluorescence measurements with a flow cytometry machine and subsequent computer analysis of the proportions of cells in the S phase of the cell cycle have been described in detail (Collins et al., 1980).

Results

Preliminary Experiments. The uptake of [^{3}H]TdR and [^{3}H]UdR into trichloroacetic acid soluble radioactivity was not grossly different in cs4-D3 at 33 and 39 °C, nor in H6-15 at 33 and 39 °C; hence, permeability to nucleosides is not temperature dependent (data not shown). Also, the specific activity of purified [^{14}C]DNA, labeled in parental chains only, was not grossly dependent on the temperature of cultivation of H6-15 (cs4-D3 has a G_1 block at 33 °C and must be grown at 39 °C). Typical specific activities were from 600 to 2000 cpm/μg.

Cell Cycle Phase Durations. The DNA content per cell distributions obtained from flow cytometry measurements of DNA/propidium fluorescence of cs4-D3 and H6-15 cells (Collins et al., 1980) are not shown for the sake of brevity. Computer analysis of these DNA distributions (Collins et al., 1980) will yield the relative G_1, S, and G_2M phase transit times as well as the percentages of cells in the G_1, S, and G_2M phases. The product of T_c (the total division cycle time) and the relative transit times (expressed as a fraction of the total division cycle time) yields the duration of the phases in hours. For example, a T_c of 13 h and a relative G_1 transit time of 0.40 indicate a G_1-phase duration of 5.2 h (Collins et al., 1980). Preliminary analysis of log-phase cultures of cs4-D3 at 39 °C yielded the following values for phase durations: G_1 = 5.2 h, S = 4.2 h, and G_2M = 3.6 h.[1] The durations for H6-15 at 33 °C were G_1 = 12.8 h, S = 7.5 h, and G_2M = 4.2 h.[2] The corresponding values for H6-15 at 39 °C were G_1 = 8.2 h, S = 7.8 h, and G_2M = 2.0 h.[3]

Normal cs4-D3 in G_0 and G_1. The DNA contents per cell for cs4-D3 grown at 39 °C and then shifted to 33 °C on 0.3% serum for 48 h corresponded to 92% G_1, 3% S, and 5% G_2M. The proportions for parallel cultures shifted to 33 °C on 10% serum were 90% G_1, 6% S, and 4% G_2M. However, the latter culture (on 10% serum) yielded acid-stable counts of 7700 cpm/10^6 cells following a 15-min pulse with [^{3}H]UdR whereas the cultures on 0.3% serum yielded only 600 cpm/10^6 cells. Thus, the cultures on 0.3% serum can be considered to be in a biochemically quiescent state [i.e., G_0; see Collins (1977)] and are termed "normal G_0", while the cultures on 10% serum have the characteristics of the G_1 phase (e.g., RNA synthesis) and are termed "normal G_1". Hence, when cs4-D3 is cultured at 33 °C on 0.3% serum, the 92% cells with a DNA content equivalent to that of G_1 cells are in the G_0 state. Following a 15-min exposure to [^{3}H]TdR, the amount of acid-stable counts per minute per 10^6 cells was minimum, i.e., 180 for normal G_0 and 310 for normal G_1, as expected.

Transformed cs4-D3 in Stationary and G_1 Phases. The cellular DNA contents of cs4-D3 at 39 °C on 0.3% serum corresponded to 82% G_1, 8% S, and 10% G_2M. As transformed cells apparently lack the ability to enter the G_0 state (Baserga et al., 1973), these cultures are referred to as "transformed stationary" cultures. Six hours after the addition of 10% serum, the DNA contents per cell corresponded to 80% G_1, 10% S, and 10% G_2M. These are termed "transformed G_1-phase" cultures. This designation is supported by the observation that 16 h after the addition of 10% serum the cells appeared to be in log phase with DNA contents per cell corresponding to 40% G_1, 28% S, and 32% G_2M. The acid-stable counts following a 15-min exposure to [^{3}H]TdR were 340, 410, and 11 000 cpm/10^6 cells for the transformed stationary, the transformed G_1, and the log phase, respectively.

Normal H6-15 in G_0 and G_1 Phases. The cellular DNA contents per cell of H6-15 at 39 °C on 0.3% serum corresponded to 83% G_1 (really G_0, see below), 10% S, and 7% G_2M. These cultures possessed only 1200 acid-stable cpm/10^6 cells following a 15-min pulse with [^{3}H]UdR, and are termed normal G_0. Six hours after the addition of 10% serum, the DNA contents per cell corresponded to 84% G_1, 9% S, and 7% G_2M. The acid-stable counts following a 15-min exposure to [^{3}H]UdR were 8200 cpm/10^6 cells. Hence, these are termed normal G_1 cultures. Eighteen hours after the addition of 10% serum, the cellular DNA contents corresponded to those of typical log-phase cultures, with 51% G_1, 38% S, and 11% G_2M. Hence, the 6-h cultures can be considered to represent mainly G_1 cells. The acid-stable counts per minute per 10^6 cells following a 30-min exposure to [^{3}H]TdR were 380, 420, and 12 000 for the normal G_0, the normal G_1, and the log phase, respectively.

Transformed H6-15 in Stationary and G_1 Phases. The DNA contents per cell of H6-15 cultures at 33 °C on 0.3% serum were 82% G_1, 8% S, and 10% G_2M. This is termed a

[1] Assuming T_c = 13 h (Farber & Unrau, 1975; M. S. Glock, unpublished results).

[2] Assuming T_c = 24 h (Renger & Basilico, 1972; M. S. Glock, unpublished results).

[3] Assuming T_c = 18 h (Renger & Basilico, 1972; M. S. Glock, unpublished results).

Table I: S_1 Nuclease Sensitive Sites of DNA from Cells Reversible for the Transformed Phenotype

H6-15						cs4-D3					
	s value		no. of S_1 sites/ 10^5	culture conditions			s value		no. of S_1 sites/ 10^5	culture conditions	
phenotypic description	$-S_1$	$+S_1$	bp[a]	T (°C)	serum (%)	phenotypic description	$-S_1$	$+S_1$	bp	T (°C)	serum (%)
normal G_0	69	69	0.0	39	0.3	normal G_0	69	69	0	33	0.3
normal G_1	69	40	1.1	39	10	normal G_1 – block	62	40	0.7	33	10
transformed stationary	53	12 (60%)	44.3	33	0.3	transformed stationary	46	24 (47%)	5.2	39	0.3
		46 (40%)	0.5					46 (63%)			
transformed G_1	34	12	43	33	10	transformed G_1	46	23	6.0	39	10
						transformed stationary + Bu_2cAMP	52	29	2.8	33	10

[a] (Number of S_1 sites per duplex/number of base pairs per duplex) × 10^5. The number of S_1 sites per duplex was calculated as (M_r in the absence of S_1 nuclease/M_r in the presence of S_1 nuclease) – 1.

transformed stationary culture. Six hours after the addition of 10% serum, the DNA contents per cell corresponded to 80% G_1, 11% S, and 9% G_2M. These are termed transformed G_1 cultures. This designation is supported by the finding that 18 h after the addition of 10% serum, the cultures had DNA contents per cell of 53% G_1, 30% S, and 17% G_2M, typical of log-phase cultures. The acid-stable counts per minute per 10^6 cells following a 30-min exposure to [^{3}H]TdR were 620, 780, and 18 000 for transformed stationary, transformed G_1, and transformed log phase, respectively.

Single-Stranded Character of ^{14}C-Labeled Parental DNA. When DNA isolated from cs4-D3 and H6-15 grown under conditions whereby both are normal G_0 was digested by S_1 nuclease, the percentages of radioactivity released were about 1% and 3%, respectively. However, when the same cells were grown under conditions whereby they were normal G_1, the percentages digested increased to 3% (cs4-D3) and 8% (H6-15).

Digestion of H6-15 ^{14}C-labeled parental DNA by S_1 nuclease revealed that the increasing order of radioactivity removed was normal G_0 (3%), normal G_1 (8%), transformed stationary (12%), and transformed G_1 (18%) (data not shown). The corresponding values for cs4-D3 were much less, i.e., normal G_0 (1%), normal G_1 block (2%), transformed stationary (4%), and transformed G_1 (7%). As DNA from cells in the S phase would be expected to contain a large amount of parental strand S_1 nuclease sensitive regions, due to unfilled gaps involving nascent strands, data with log-phase cultures are not reported. The remainder of the experiments described herein deal with the number of S_1-sensitive sites of the purified DNA preparations under conditions where very few cells were in the S phase (Table I).

S_1-Sensitive Sites. DNA containing ^{14}C only in the parental chains was treated with S_1 nuclease and sedimented through neutral sucrose gradients, with untreated DNA serving as a control (Figure 1). For the sake of brevity, the cs4-D3 data are not shown. Normal G_0 H6-15 DNA possesses no, or very few, S_1 sites (Figure 1A). Normal G_1 H6-15 DNA possesses a few S_1 sites (Figure 1C). Transformed stationary H6-15 DNA contains two populations of molecules, one with no (or few) sites and one with many sites (Figure 1B). Transformed G_1 H6-15 contains many S_1 sites (Figure 1D). For ease of presentation, the number of S_1 sites per 10^5 base pairs (bp) in H6-15 and cs4-D3 DNAs is given in Table I. This number increases in DNA from H6-15 cells in the order normal G_0 (0.0), normal G_1 (1.1), transformed stationary (60% have 44 and 40% have 0.5, for an average of 27), and transformed G_1 (43). The number of S_1 sites in DNA from cs4-D3 cells is much lower than in the DNA from H6-15 cells. For example, the number in transformed G_1 cs4-D3 cells is only 6/10^5 bp

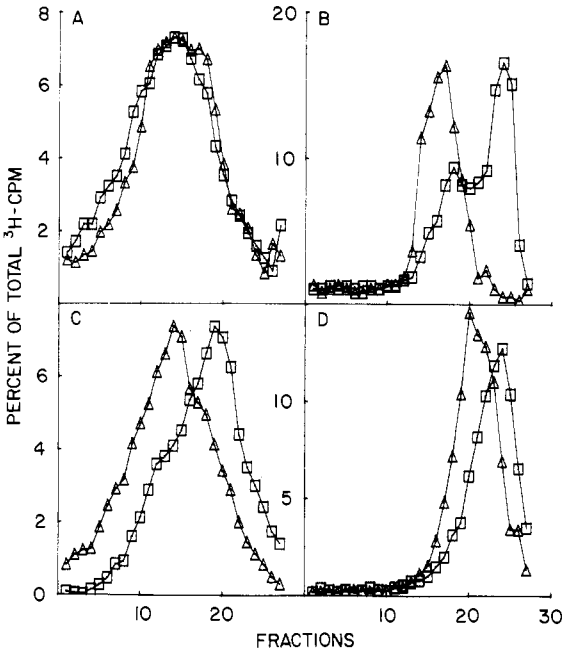

FIGURE 1: Sedimentation of DNA after S_1 nuclease digestion. Approximately 0.5 mL of [^{14}C]DNA purified from H6-15 cells was layered onto 5–20% neutral sucrose gradients. The tubes were centrifuged at 40 000 rpm at 4 °C for 2 h in an SW 50.1 rotor. The gradients were punctured, and the radioactivity of the subsequent fractions was determined. Approximately 20 μg of DNA was used for each experiment: control undigested DNA (△); S_1-digested DNA (□). The cultures used were (A) normal G_0, (B) transformed stationary, (C) normal G_1, and (D) transformed G_1.

compared to 43/10^5 bp in transformed G_1 H6-15 cells (Table I). It has been reported that when dibutyl-cAMP is added to cs4-D3 cells at the nonpermissive temperature of 33 °C, the cell shape changes to that characteristic of cells at the permissive temperature of 39 °C (Crane & Thomas, 1976). When dibutyl-cAMP was added to cs4-D3 cultures at 33 °C and on 10% serum for 4 h, the cell shape indeed changed. The DNA purified from these cells was found to contain 2.8 S_1 sites/10^5 bp compared to only 0.7/10^5 bp for the corresponding cultures without dibutyl-cAMP (normal G_1, Table I). In control experiments, H6-15 cells in the normal G_0 state which contained ^{14}C were mixed with transformed G_1 cells prior to purification of the DNA. In each case, the [^{14}C]DNA subsequently isolated had an S_1 sensitivity of 3% and had zero S_1 sites, as expected (data not shown). These experiments tend to rule out the differential presence of diffusible nucleases as an explanation of our data.

In order to ascertain whether the S_1 sites are due to gaps along the chains, we analyzed the DNAs used for Table I by

Table II: Number of Breaks in DNA from Cells Reversible for the Transformed Phenotype

	H6-15				cs4-D3		
	s value		no. of breaks/ 10^5		*s* value		no. of breaks/ 10^5
phenotypic description[a]	neutral	alkaline	bases[b]	phenotypic description[a]	neutral	alkaline	bases[b]
normal G_0	69	74	0.16	normal G_0	57	71	0
normal G_1	80	57	1.3	normal G_1 – block	62	63	0.6
transformed stationary	53	22	16	transformed stationary	49	34	3.1
transformed G_1	57	17	18	transformed G_1	46	36	2.6
				transformed stationary + Bu_2cAMP	52	44	1.5

[a] For culture conditions, refer to Table I. [b] (Number of breaks per strand/number of bases per strand) $\times 10^5$. The number of breaks per strand was calculated as $[(M_r \text{ neutral}/2)/M_r \text{ alkaline}] - 1$.

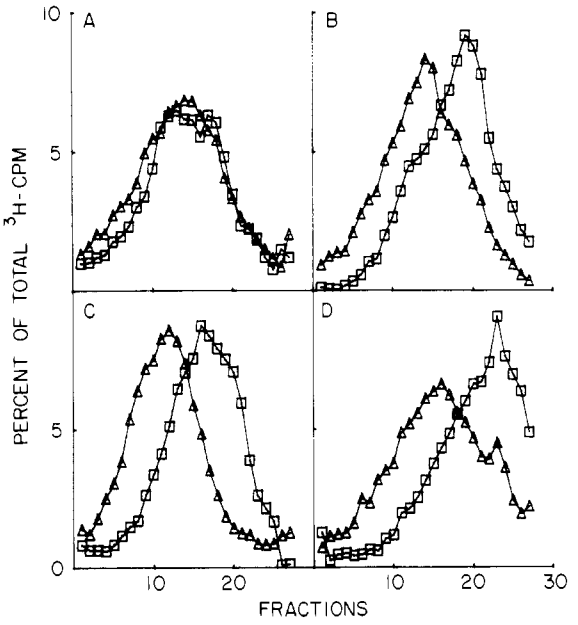

FIGURE 2: Number of breaks in DNA. Approximately 0.5 mL of [^{14}C]DNA purified from H6-15 cells was layered onto 5–20% neutral alakaline sucrose gradients. The conditions were the same as in the legend to Figure 1: neutral gradients (△); alkaline gradients (□). The cultures used were (A) normal G_0, (B) transformed stationary, (C) normal G_1, and (D) transformed G_1.

neutral and alkaline sucrose gradients (Figure 2) to determine the number of breaks (nicks plus gaps). The number of breaks per 10^5 bases in length of H6-15 DNA was 0.2 (normal G_0), 1.3 (normal G_1), 16 (transformed stationary), and 18 (transformed G_1) (Table II). Thus, the number of S_1 sites exceeds the number of breaks of the parental chains by a factor of at least 2 and hence cannot be due to gaps. It should be mentioned that under the conditions used our S_1 nuclease preparations do not hydrolyze nicks (Collins, 1977). It might be noted that the *s* values for DNAs not digested with S_1 nucleases (Table I) are sometimes lower than those for the corresponding DNAs of Table II in neutral gradients. For example, for H6-15, normal G_1 DNA is only 69 S in Table I, whereas it was 80 S in Table II. This can be attributed as the result of shear forces during the lengthy dialysis procedures used prior to S_1 nuclease treatment (Collins, 1977).

Discussion

Many tissues exist in a nonproliferating, biochemically quiescent state, termed G_0, from which they can be induced to once more enter the cell cycle, at G_1, by the appropriate stimulus, for example, lentectomy in the regenerating lens of the newt (Collins, 1972), partial hepatectomy in the regenerating liver (Bucher, 1967), and antigenic stimulation in lymphocytes (Makinodan & Albright, 1967). However, it may be that the equivalent G_0 state does not exist in vitro (Baserga, 1968). Nevertheless, cell cultures are useful model systems, and the availability of cold- and temperature-sensitive mutants permissive for growth control and transformation increases their usefulness.

We have previously reported that the number of S_1 sites in DNA undergoing replication can be extensive, even when the nascent chains were as long as the parental chains. This precludes the possibility that the nuclease was acting on unfilled gaps between the ends of the nascent chains associated with the parental chains (Collins, 1979). The finding reported herein, that S_1 sites are present in G_1-phase DNA of H6-15 at the permissive temperature (33 °C), and that only a maximum of one-half can possibly be ascribed to gaps, thus extends our earlier observations on the S phase to the G_1 phase. It should be stressed that the data of Figures 1 and 2 cannot be ascribed to the presence of low numbers of contaminating S-phase cells in the cultures used herein. The maximum number of S-phase cells in any culture was only 11%, in transformed G_1 cultures of H6-15 (Table I), whereas all of the purified DNA from that culture behaved as though it contained 43 S_1 sites/10^5 base pairs in length and 18 breaks/10^5 bases in length (Tables I and II, respectively). In duplicate experiments, the values for the number of S_1 sites and the number of breaks did not vary from the ones reported here by more than 8%.

The finding that the number of S_1 sites is about 40-fold fewer when H6-15 is at the nonpermissive temperature (39 °C) raises interesting questions about the possibility of regulatory signals in the form of transient stable structures, sensitive to S_1 nuclease, that cannot be answered at this time. Such structures have been proposed by Crick (1971), and it would seem that inverted repeats would be likely candidates as sequences from which cruciforms could be formed, perhaps by a topoisomerase. Such structures, once formed, would contain base pairing along their hairpin lengths and be stable (i.e., not precluded by thermodynamic calculations, as would be underwound regions). Signals for the initiation of replication at replicon origins have been proposed by Hand (1978). Such signals have not yet been found in cellular DNA, but have been "proven" in mitochondria, bacterial viruses, and animal viruses. The S_1 sites in G_1 DNA (Table II), thus, may prove to be involved in the later initiation of DNA synthesis (i.e., the onset of the S phase).

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Ultraviolet Light Induced Preferential Cross-Linking of Histone H3 to Deoxyribonucleic Acid in Chromatin and Nuclei of Chicken Erythrocytes†

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ABSTRACT: Histones have been cross-linked to DNA in chicken erythrocyte nuclei and chromatin by using ultraviolet light irradiation at 254 nm. Following irradiation, cross-linked histone–DNA adducts were isolated and purified by hydroxylapatite chromatography, and the DNA component was subjected to acid hydrolysis. Of several hydrolysis techniques investigated, trichloroacetic hydrolysis of the DNA component of the adducts was found to be most effective. Histones isolated from hydrolyzed histone–DNA adducts were characterized by gel electrophoresis and fingerprint analysis. No histone–histone protein adducts were observed. All histone fractions have been shown to cross-link DNA in nuclei or chromatin by utilizing the technique employed, but with different propensities. The order of observed cross-linking, deduced from kinetic experiments, is H1 + H5, H3 > H4 > H2A ≫ H2B. The preferential binding of the core histone H3, as compared to the other core histones, is discussed in light of recent data concerning histone–DNA interactions and nucleosome structure. The use of the ultraviolet light technique as a conformational probe to study chromatin is also discussed.

The fundamental unit of chromatin structure, termed the nucleosome, consists of about 170 base pairs of DNA associated with a core of histones [see McGhee & Felsenfeld (1980) for a review]. The nucleosome core is composed of two molecules each of four histones (H2A, H2B, H3, and H4) around which is coiled 146 base pairs of DNA. One histone molecule (H1) is bound to DNA where it enters and exits from the nucleosome core. The nucleosome structure of chicken erythrocyte chromatin has been shown to be the same as for other chromatins, except that varying amounts of a unique histone (H5) may replace H1 in the nucleosome (Shaw et al.,

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