DNA Strand Scission by Neocarzinostatin and its Relation to the Inhibition of Cell Cycle Traverse and DNA Synthesis

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ABSTRACT. The effect of neocarzinostatin (NCS) on the cell cycle traverse of BHK cells was examined with flow microfluorometry and [³H]-thymidine incorporation. At a low concentration (1 µg/ml) of NCS a large number of cells were arrested at the G₂ phase. At a high concentration (10 µg/ml) cell cycle traverse from the G₁ to the S phase and DNA synthesis in the middle S phase were inhibited.

Chain elongation, strand scission and repair of DNA were determined by alkaline sedimentation analysis. In the presence of 10 µg/ml NCS, parental DNA was degraded to the size of the replication unit (1.1×10^8 dalton). DNA elongated at most to the size of the replication unit, before the parental DNA was degraded to the same size. DNA strand scission took place even at the low concentration of NCS that induced G₂ arrest. The DNA strand scission induced by 10 µg/ml NCS was repaired only slightly by 5 h after the removal of NCS.

A short exposure of cells to NCS was sufficient to cause the inhibition of DNA synthesis and cell cycle traverse, as well as DNA strand scission.

NCS, isolated from a filtrate of *Streptomyces carzinostaticus* (15), is an acidic protein with a molecular weight of 10,700 (23). The drug is effective against various experimental tumor cells (4, 20, 21, 27) and human cancers (26, 31). NCS has been reported to inhibit DNA synthesis, but not RNA and protein syntheses (13). Cellular DNA chain breakage also is induced by NCS (2, 32). We (16, 17) and others (1, 3) have shown that NCS has endonucleolytic activity *in vitro*. Thymine, accompanied by DNA chain breakage, was released from DNA (16, 29), and the resulting 3' and 5' termini were phosphoryl ends (18, 29). NCS caused DNA chain breakage mainly at the thymidylic acid residues and, to a lesser extent, at the deoxyadenylic acid residues (11). NCS-treated DNA could not be used as the template-primer for *E. coli* DNA polymerase (18), and could not be sealed by polynucleotide ligase (29). Experiments with the wild type *E. coli* and the mutant *UVR*⁻ or *rec*⁻ showed that inhibition of the growth of *E. coli* by NCS resulted from DNA damage that could be repaired by the *recA* system (34).

NCS has been reported to inhibit the cell cycle traverse both at the G₁/S boundary

* To whom all correspondence should be addressed. The abbreviations used are: BHK, baby hamster kidney; DME, Dulbecco's modified Eagle's medium; FMF, flow microfluorometry; HU, hydroxyurea; NCS, neocarzinostatin; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid.

and in the G₂ phase (7). Is G₂ arrest caused by DNA chain breakage? Some evidence has been reported that DNA damage causes G₂ arrest or growth inhibition. Cells, heavily labeled with [³H]-thymidine, were arrested in the G₂-M phase (24); there was a linear relation between chromosome damage and G₂ arrest (30).

In this report, we tried to determine the relation between DNA strand scission and the other biological effects of NCS, *e.g.* inhibition of DNA synthesis and G₂ arrest. Thus, we investigated the effect of NCS on cellular DNA synthesis, DNA chain elongation, cell cycle traverse, and DNA strand scission. Our results suggest that the inhibition of DNA synthesis and G₂ arrest, respectively, are caused by a high and low degree of DNA strand scission.

MATERIALS AND METHODS

Material. Purified neocarcinostatin (Lot No 750563) was obtained from Kayaku Antibiotics Research Co., Ltd., Tokyo, Japan.

Cell lines. The cell line from the baby hamster kidney, BHK21, was grown on Falcon petri dishes in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum with medium changes at 3 day intervals. The cultures were maintained in a humidified atmosphere containing 10% CO₂ at 33°C. The lymphoid cell line, P3HR-1, was originally derived from Burkitt lymphoma (12). Stationary suspension cultures of P3HR-1 were maintained at 37°C in RPMI-1640 medium supplemented with 10% fetal calf serum.

Flow microfluorometry. P3HR-1 cells were sedimented at 1,000 rpm for 5 min then stained with 2 ml of propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate) (19). BHK cells were directly stained with propidium iodide solution after removal of the medium as described previously (10). Samples were kept at 4°C in the dark and analyzed within 24 h in a model 4801 Cytofluorograf (Bio/physics Systems, Inc.)

Cell synchronization of BHK 21 cells.

1) Serum starvation: Asynchronously growing cultures were washed once with Tris-buffered saline then incubated in DME containing 0.25% calf serum for 72 h. After 72 h, the medium was changed to DME containing 10% calf serum to allow cell cycle traverse (6).

2) Hydroxyurea treatment: Cells first were grown with DME containing 0.25% calf serum for 48 h, then with an isoleucine free (ileu⁻) medium containing 5% dialyzed calf serum for 24 h, after which they were maintained for 16 h with complete medium containing 2 mM hydroxyurea (HU). The cell cycle traverse was allowed to start by removing HU-containing medium, washing with Tris-buffered saline followed by the addition of normal growth medium (33).

Estimation of the rate of DNA synthesis. Cells were labeled with 1 μCi/ml [³H]-thymidine (40–60 Ci/mmol, New England Nuclear). Labeled cells were washed once with cold Tris-buffer, pH 7.5, then lysed with a solution containing 0.05 M Tris, pH 7.5, 1 mM EDTA, 0.15 M NaCl and 0.6% SDS. After 10 min at room temperature, TCA was added to the lysate at a final concentration of 10%. Acid-insoluble material was collected on glass fiber filters (Whatman GF/C, 2.4 cm) previously soaked with 10% TCA. The filters were washed with 5% TCA then dried, after which the radioactivity was counted in the toluene base scintillation fluid.

Alkaline sedimentation analysis.

1) DNA chain elongation: Labeled cells were washed twice with a cold solution containing 0.15 M NaCl, 10 mM EDTA, and 250 μg/ml cold thymidine, then treated at 4°C with 1 ml of a solution containing trypsin and cold thymidine. After centrifugation at 1,000 rpm for

5 min at 4°C, the pellet was resuspended in 0.2 ml of solution containing 0.15 M NaCl and 10 mM EDTA, then lysed in 0.2 ml of lysis solution (0.4 N NaOH, 1% sarkosyl, 20 mM EDTA) layered on the top of a 5–20% alkaline sucrose gradient (0.3 N NaOH, 0.7 M NaCl, 1 mM EDTA) that contained 1 ml of 70% sucrose cushion. The gradients were kept at 4°C for 17 ± 3 h, then centrifuged at 23,000 rpm for 5 h at 20°C in a Beckman SW40Ti rotor. Fractions of 0.5 ml were taken from the bottom of each tube, and 100 μ l of each fraction was collected on glass fiber filters (Whatman GF/C) as described above. The filters were washed with 5% TCA and dried, then the radioactivity was counted.

2) DNA strand scission and its repair: BHK and P3HR-1 cells, that had been previously labeled with 0.1 μ Ci and 0.4 μ Ci [14 C]-thymidine (40–60 Ci/mmol, New England Nuclear) for 15 h, were treated for the alkaline sucrose gradient centrifugation as described above except that cold thymidine was not used, and the centrifugation was performed in a Beckman SW40Ti rotor or a Hitachi RPS40 rotor at 20°C.

3) Calculation of molecular weight: The molecular weight of DNA was calculated from the empirical relationships derived Burgi and Hershey (5), $(D_2/D_1) = (M_2/M_1)^{0.35}$, using 3×10^7 and 1×10^8 as the respective molecular weights for λ and T₄ phage DNA.

RESULTS

Effect of NCS on cell cycle traverse in asynchronous BHK and P3HR-1 cells. Flow microfluorometric analysis was carried out to determine the effect of NCS on the cell cycle traverse in asynchronous BHK and P3HR-1 cells. In the presence of 1 μ g/ml NCS, the progressive accumulation of BHK cells with a G₂-M DNA content accompanied by a decrease in the number of cells with a G₁ DNA content was observed (Fig. 1). After 30 h, almost all the cells contained G₂-M DNA. Cells with G₂-M DNA were in the G₂ or late S phase as there was no accumulation of mitotic cells (data not shown). After exposure of the cells to 0.1 and 10 μ g/ml NCS for 30 h, the distribution of the cellular DNA content was similar to that in untreated cells. NCS at 0.1 μ g/ml did not inhibit DNA synthesis, but 10 μ g/ml NCS inhibited DNA synthesis to a considerable degree, as shown below (Fig. 7). After 30 h, the numbers of cell increased in the presence of 0.1 μ g/ml NCS but not with 10 μ g/ml (data not shown). These results show that 0.1 μ g/ml NCS does not inhibit the cell cycle traverse and 10 μ g/ml NCS stops it.

The same effect of NCS on cell cycle traverse was seen in P3HR-1 cells, although the NCS concentration that arrested the cells at the G₂-M phase was 0.1 μ g/ml (Fig. 2).

Effect of NCS on cell cycle traverse in synchronized BHK cells at the early S phase. To investigate the blocking point of NCS during the S phase, we synchronized cultures of BHK at the start of the S phase (35) with a low serum, ileu⁻ medium followed by HU treatment. The traverse of cells through S phase was examined with fluorometric analysis. In the absence of NCS, cells traversed synchronously through the S phase after the removal of HU (Fig. 3). At 8 h after HU release, most cells were in the late S or G₂ phase. In the presence of 1 μ g/ml NCS, the rate of cell cycle traverse was reduced; cells reached the G₂ phase after 10 h and remained there. In the presence of 10 μ g/ml NCS, however, the rate of cell cycle traverse was extensively reduced; most cells remained in the early S phase even at 10 h after HU release. FMF analysis showed clearly that NCS has different effects on the traverse of the S phase, depending on the concentration.

Fig. 1.

Fig. 2.

Fig. 3. DNA distribution histograms for synchronized BHK cells. Cells were seeded at 4×10^4 /50 mm dish, then synchronized with low serum, the ileu⁻ and HU treatment described in Materials and Methods. After removal of HU, the DNA histograms were examined at 0, 2, 4, 6, 8, 10 and 24 h as described in Materials and Methods. A, not exposed to NCS; B, exposed to 1 μ g/ml NCS; C, exposed to 10 μ g/ml NCS.

Effect of NCS on the entry from G_1 to S phase. To confirm quantitatively the results of FMF analysis, we examined the effect of NCS on the cell cycle traverse by estimating the activity of DNA synthesis. Cell synchronization was carried out by serum starvation, which arrests cells in G_1 (G_0). BHK cells started DNA synthesis 12 h after the addition of serum, and the rate of DNA synthesis was at a maximum

Fig. 1. DNA distribution histograms for BHK cells exposed to NCS. Cells were prepared for FMF analysis as described in Materials and Methods. Channel numbers indicate the increasing content of DNA per cell. Channel number 30 corresponds to the G_1 DNA content. A₁–A₃, histograms of cells not exposed to NCS after 5, 10 and 30 h of culture. B₁–B₃, C₁–C₃, D₁–D₃: histograms of cells exposed to 0.1, 1 and 10 μ g/ml of NCS for 5, 10 and 30 h, respectively.

Fig. 2. DNA distribution histograms for P3HR-1 cells exposed to various concentrations of NCS. Cells were cultured in the absence [A, B, G] and in the presence of NCS at 0.01 (C, H), 0.1 (D, I), 1 (E, J), and 10 μ g/ml (F, K), for 0 h (A), 24 h (B–F), and 48 h (G–K).

Fig. 4. Effect of NCS on entry into the S phase. BHK cells were seeded at 1×10^5 /35 mm dish, then synchronized on the following day with DME containing 0.25% calf serum for 72 h as described in Materials and Methods. Immediately after the addition of DME containing 10% calf serum, 1 μ g/ml of NCS (●—●), 10 μ g/ml of NCS (▲—▲), or no NCS (○—○) was added. DNA synthesis was estimated by [3 H]-thymidine (1 μ Ci/ml) labelling for 30 min at various times after the addition of DME containing 10% calf serum (0 h). Incorporated radioactivity was determined as described in Materials and Methods.

at 24 h (Fig. 4). When 10 μ g/ml NCS was added immediately after the addition of serum, BHK cells could not start DNA synthesis.

We performed experiments to examine whether there is some special point in the G_1 phase, from which NCS can not inhibit the start of cellular DNA synthesis. Cultures of BHK cell were synchronized in G_1 (G_0) as above. After the serum addition, the cultures were treated with 10 μ g/ml NCS for 30 min every 2 h during the G_1 period; their ability to synthesize DNA after 24 h was estimated by pulse labeling with [3 H]-thymidine. 30 min of NCS treatment should be sufficient for the effect of NCS to show up since we found that a brief treatment of asynchronous BHK cells with NCS was enough to inhibit DNA synthesis (Fig. 7). Table 1 shows that the ability to synthesize DNA was significantly reduced by every NCS treatment from 0 to 10 h after the serum addition. This indicates that all the 10 μ g/ml NCS treatments from early G_1 to late G_1 inhibited the entry of cells into the S phase.

The inhibition of the cell cycle traverse during the S phase by NCS was dependent on the concentration (Fig. 3). We then examined the effect of the NCS concentration on the cell cycle traverse from the G_1 to S phase. Cultures of BHK were synchronized with a low serum medium as above. Immediately after the addition of serum, various concentrations of NCS were added. The ability to synthesize DNA 24 h after the serum addition did not change at 0.1 μ g/ml NCS (Fig. 5). A 50% reduction was observed at 0.4–0.6 μ g/ml NCS, but at concentrations above 8 μ g/ml, NCS caused more than a 95% reduction of the control level.

Effect of NCS on DNA synthesis in the middle of the S phase. Ebina *et al.* (7) reported that in the middle of the S phase, NCS did not inhibit DNA synthesis. To confirm their results, we added NCS at the middle of the S phase. Cultures of BHK then were

Fig. 5.

Fig. 6.

Fig. 5. Effect of NCS on entry into the S phase. BHK cells (1×10^5 /35 mm dish) were synchronized at the G₁ phase with low serum as described in Materials and Methods. Various concentrations of NCS were added immediately after the addition of DME containing 10% calf serum. DNA synthesis was estimated by 1 μ Ci [³H]-thymidine labelling for 30 min, 24 h after the addition of serum. Acid-insoluble radioactivity was determined as described in Materials and Methods.

Fig. 6. Inhibition of DNA synthesis in the middle S phase of BHK cells by NCS. Cells were seeded at 10^5 /35 mm dish, then synchronized at the G₁ phase with low serum as described in Materials and Methods. At the 21st hour after the addition of the complete medium various concentrations of NCS were added. DNA synthesis was estimated by cumulative labelling with 1 μ Ci/ml [³H]-thymidine; acid-insoluble radioactivity was determined as described in Materials and Methods. ●—●, no NCS; ▲—▲, 0.1 μ g/ml; ■—■, 1 μ g/ml; △—△, 5 μ g/ml; ○—○, 10 μ g/ml.

synchronized with a low serum medium. At 21 h after the serum addition, when most cells were in the S phase, various concentrations of NCS were added. DNA synthesis was estimated by cumulative labeling with [³H]-thymidine. DNA synthesis in the middle of the S phase also was inhibited by NCS (Fig. 6). In the presence of a high concentration of NCS, 5 to 10 μ g/ml, DNA synthesis was linear for the first 2 h although the rate of DNA synthesis was reduced, and reached a plateau about 4 h after the NCS addition.

Reversibility of NCS treatment. To examine whether the effect of NCS on DNA synthesis and cell cycle traverse is reversible or irreversible, we treated asynchronous cultures of BHK with NCS for 5, 10 and 30 min, then compared the effect of NCS with that of the whole period treatment. Based on the results shown in Figs. 1, 3 and 7, we used 1 μ g/ml and 10 μ g/ml NCS as the respective concentrations at which G₂ arrest and the inhibition of DNA synthesis could be induced. DNA synthesis was

TABLE 1. EFFECT OF NCS ON THE ENTRY FROM THE G₁ TO S PHASE

		cpm/dish	%
A	Control		
	0 h	130	1
	10 h	160	1
	24 h	29,600	100
B	Cells treated with NCS		
	0 h	620	2
	2 h	630	2
	4 h	690	2
	6 h	660	2
	8 h	880	3
	10 h	1,770	6

BHK cells (2×10^5 /dish) were synchronized with DME containing 0.25% calf serum as described in Materials and Methods. DNA synthesis was estimated by [3 H]-thymidine labelling for 30 min as described in Materials and Methods. For A, DNA synthesis was estimated at the indicated times after the addition of serum. For B, cells were treated with $10 \mu\text{g/ml}$ NCS for 30 min at the indicated times after the addition of serum. DNA synthesis was estimated 24 h after the addition of serum.

Fig. 7. Inhibition of DNA synthesis as a function of the exposure time to NCS: Asynchronously growing BHK cells, 2×10^5 /35 mm dish, were treated with $10 \mu\text{g/ml}$ NCS for 5, 10 and 30 min, then washed twice with Tris-buffered saline. The cells were resuspended in DME medium containing 10% calf serum. DNA synthesis was estimated at the indicated times by cumulative labelling with $1 \mu\text{Ci}$ [3 H]-thymidine. Acid-insoluble radioactivity was counted as described in Materials and Methods. Treatment with NCS for 0 min (●), 5 min (■), 10 min (▲), 30 min (□), and the whole period (△).

Fig. 8. G_2 arrest as a function of the exposure time to NCS. BHK cells were seeded at 2×10^5 /50 mm dish and allowed to grow for 48 h, after which they were processed immediately for FMF analysis (A) and at 30 h (B). The other cells were treated with $1 \mu\text{g/ml}$ NCS for 10 min (C), 30 min (D) and the whole period (E). FMF analysis was performed after 30 h as described in Materials and Methods.

followed by cumulative labeling with $[^3\text{H}]$ -thymidine and G_2 arrest was followed by FMF analysis. Short treatment of cells with both concentrations of NCS was sufficient to produce the same degree of the effect as the whole period treatment did (Fig. 7 and 8). This indicates that the effect of NCS should be irreversible.

Effect of NCS on DNA chain elongation. We then examined whether the inhibition of DNA synthesis is due to the inhibition of DNA chain elongation. Asynchronous cultures of BHK, that previously had been labeled with $0.1 \mu\text{Ci}$ of $[^{14}\text{C}]$ -thymidine for 15 h, were pulse-labeled with $50 \mu\text{Ci/ml}$ of $[^3\text{H}]$ -thymidine for 5 min. The cultures then were harvested immediately, or were chased with cold thymidine. The size of the newly synthesized DNA was determined by alkaline sucrose gradient centrifugation. The molecular weight of the DNA synthesized for 5 min was $6\text{--}8 \times 10^7$ dalton (Fig. 9A). After a chase for 30 min, almost all the newly synthesized DNA was sedimented at the bottom (Fig. 9B), but in the presence of $10 \mu\text{g/ml}$ NCS, the DNA chain elongated to a molecular weight of at most 1.1×10^8 dalton (Fig. 9C–E).

The same inhibition pattern of DNA chain elongation was observed after HU

Fig. 9. Alkaline sucrose gradient centrifugation analysis of DNA chain elongation. BHK cells (1×10^5 /35 mm dish), that previously had been labeled with $0.1 \mu\text{Ci/ml}$ [^{14}C]-thymidine for 15 h, were labeled with $50 \mu\text{Ci/ml}$ [^3H]-thymidine for 5 min. The labeled cells (A) were washed with Tris-buffered saline containing $50 \mu\text{g/ml}$ cold thymidine then chased in culture medium containing $50 \mu\text{g/ml}$ cold thymidine in the presence (C–E) or absence (B) of $10 \mu\text{g/ml}$ NCS. Alkaline sucrose gradient centrifugation was performed as described in Materials and Methods. Chase for 30 min (B, C), 1 h (D), and 2 h (E). Positions of T_4 and λ DNA are shown. Sedimentation is from right to left. ○—○, [^{14}C]; ●—●, [^3H].

treatment. The DNA chain (about 6×10^6 dalton) synthesized in the presence of HU could elongate to 1.1×10^8 dalton at most in the presence of $10 \mu\text{g/ml}$ NCS (data not shown).

Since NCS is known to cause DNA strand scission, the size of the parental DNA was examined simultaneously. DNA degradation was induced in the presence of NCS, and proceeded with time as shown in Fig. 9 (open circles). After 30 min, the parental DNA had not been degraded to 1.1×10^8 dalton. After 2 h, the parental DNA was degraded in the presence of NCS to the maximal size of the newly synthesized DNA. It is notable that the accumulative molecules of DNA synthesized or degraded in the presence of NCS had molecular weight of 1.1×10^8 dalton which corresponds to the size of the average mammalian replication unit.

DNA strand scission by NCS and its repair. Strand scission of cellular DNA has been reported to be induced by NCS in HeLa cells (2, 28) and L1210 cells (32). However, there is a marked difference between these reports with respect to the concentrations and exposure times of NCS that induce DNA strand scission. If the effects of NCS are due to DNA strand scission, then scission should be detectable at the concentrations of NCS that inhibit DNA synthesis and the cell cycle traverse. The

Fig. 10. DNA strand scission as a function of NCS concentration. A. Asynchronous cultures of P3HR-1 cells ($3 \times 10^5/\text{ml}$) were labeled with $0.4 \mu\text{Ci}/\text{ml}$ [^{14}C]-thymidine for 15 h, then washed once with Tris-buffered saline and incubated in RPMI-1640 medium containing 10% fetal calf serum for 3 h. Cells then were treated with various concentrations of NCS for 10 min. Immediately after treatment, the cells were collected at 4°C by centrifugation at 1,000 rpm for 5 min, and processed for alkaline sucrose gradient centrifugation as described in Materials and Methods. Centrifugation was performed in a Hitachi RPS40 rotor at 34,000 rpm for 1 h. Sedimentation is from right to left. ●—●, control; △—△, 0.1 μg ; ■—■, 1 μg ; ○—○, 10 μg NCS. B. Asynchronous cultures of BHK cells ($2 \times 10^5/35 \text{ mm dish}$), labeled with $0.1 \mu\text{Ci}/\text{ml}$ [^{14}C]-thymidine for 15 h, were treated with NCS. Immediately after NCS treatment, the cells were trypsinized and collected as described above, after which they were processed for alkaline sucrose gradient centrifugation as described in Materials and Methods. Centrifugation was performed in a Beckmann SW40 Ti rotor at 23,000 rpm for 2 h. ●—●, control; △—△, 1 μg ; ○—○, 10 μg NCS.

Fig. 11. DNA strand scission as a function of the exposure time of BHK cells to NCS. Asynchronous cultures of BHK cells (1×10^5 /35 mm dish), that had been labeled with $0.1 \mu\text{Ci/ml}$ [^{14}C]-thymidine for 15 h, were treated with $10 \mu\text{g/ml}$ NCS for various periods. Immediately after washing out NCS, the cells were trypsinized, then collected and processed for alkaline sucrose gradient centrifugation as described in Materials and Methods. Centrifugation was performed in a Beckmann SW40 Ti rotor at 23,000 rpm for 2 h. Exposure time: $\bullet$ — $\bullet$, 0 min; $\blacktriangle$ — $\blacktriangle$, 1 min; $\circ$ — $\circ$, 2 min; $\triangle$ — $\triangle$, 10 min. The percent of radioactivity in the bottom of each tube was 71% (0 min), 54% (1 min) and 41% (2 min).

concentration of NCS that induced G_2 arrest was $0.1 \mu\text{g/ml}$ in P3HR-1 cells and $1 \mu\text{g/ml}$ in BHK cells. Both the treatment of $0.1 \mu\text{g/ml}$ NCS in P3HR-1 cells and $1 \mu\text{g/ml}$ in BHK cells induced DNA strand scission (Fig. 10). In both types of cell, the extent of cellular DNA strand scission depended on the concentration of NCS. DNA strand scission took place even with a short exposure of the cells to NCS (Fig. 11). When DNA repair synthesis was examined in NCS-treated BHK cells, no complete recovery was observed even 5 h after the removal of NCS (Fig. 12).

DISCUSSION

Mammalian chromosomal DNA is replicated bi-directionally from multiple replication origins (14). The replication unit varies in size. DNA fiber autoradiography has demonstrated that the average replication unit is $50 \mu\text{m}$ (14) or $120 \mu\text{m}$ (37) in Chinese hamster cells and $60 \mu\text{m}$ in somatic chicken cells (25). Sucrose gradient analysis has shown that the replication unit is 2×10^8 dalton in murine lymphoma cells (22), and 1.4×10^8 dalton in Chinese hamster cells (36).

In the presence of $10 \mu\text{g/ml}$ NCS, the DNA chain could elongate at most to a molecule of 1.1×10^8 dalton which corresponds to the size of the average replication unit in mammalian cells (Fig. 9). This suggests that once synthesized, the DNA chain can elongate to the size of the replication unit in NCS-treated cells, but the ligation of

Fig. 12. Repair synthesis of DNA after the exposure of BHK cells to NCS. Asynchronous cultures of BHK cells (1×10^5 /35 mm dish) that had been labeled with $0.1 \mu\text{Ci/ml}$ for 15 h were either exposed to $10 \mu\text{g/ml}$ NCS for 10 min or not ($\circ$ — $\circ$). After washing out NCS, the cells again were incubated in DME containing 10% calf serum for 0 h, ($\bullet$ — $\bullet$); 3 h, ($\blacktriangle$ — $\blacktriangle$); 5 h, ($\blacksquare$ — $\blacksquare$), after which they were processed for alkaline sucrose gradient centrifugation as described in Materials and Methods. Centrifugation was performed in a Beckmann SW40 Ti rotor at 23,000 rpm for 50 min.

the adjacent, newly synthesized replicons is inhibited. Significantly parental DNA also was degraded into a molecule of 1.1×10^8 dalton when there was long exposure of cells to $10 \mu\text{g/ml}$ NCS. Since *in vitro* NCS mainly breaks DNA at the thymidylic acid residues, not at the specific nucleotide sequence (11), the specific region of cellular DNA, that produced the 1.1×10^8 dalton molecule, might be an AT-rich region or a specific site in the chromatin structure.

Ebina *et al.* reported that NCS inhibited the formation of microtubular paracrystals induced by vinblastine in HeLa cells (8), the formation of cap and the cell spreading in mammalian cells (9). They speculated that NCS affected the microtubular system which was followed by inhibition of DNA synthesis in the S phase and of organization of spindle fibers during G_2 . However, the effect on the microtubular system required a high concentration of NCS (5 – $50 \mu\text{g}$) in their experiments, as compared with the concentration reported by Beerman and Goldberg that inhibited DNA synthesis in HeLa cells (2), and so this effect of NCS probably is not responsible for the inhibitory activity of NCS on DNA synthesis.

NCS at $1 \mu\text{g/ml}$ caused some DNA strand scission; this damage was repaired, but not completely. Thus, the replication of some chromosomal DNA could not be completed due to the damage by NCS although most chromosomal DNA could be replicated to the G_2 -M DNA content. Cells, therefore, might be arrested in the late S phase, rather than in the G_2 phase.

NCS might not affect the progression of G_1 as there is no cellular DNA synthesis in G_1 ; however, we do not know to what extent transcription is disturbed by DNA strand scission. The entry from G_1 to the S phase, as measured by DNA synthesis acti-

vity, was inhibited by NCS to the same degree as that DNA synthesis was inhibited in the middle S phase; this shows that both inhibitions may be induced by damage to same target molecule. Our data suggest that the primary target of NCS might be DNA, and that G₂ arrest and arrest during the S phase appear to depend on the degree of DNA damage caused by NCS.

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