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Detection of Carcinogen-Induced DNA Breaks by Nick Translation in Permeable Cells

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A nick-translation reaction with E. coli DNA polymerase I (pol. I) was used to detect in situ DNA breaks produced by chemical carcinogens. Normal human fibroblasts treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in various doses were permeabilized with lysolecithin, and were nick translated in the presence of [^{3}H]dCTP and pol. I. The radioactivity incorporated increased with MNNG concentration, and was directly proportional to the poly(ADP-ribose) synthetase activity. Other DNA-damaging agents such as bleomycin or 4-nitroquinoline 1-oxide also caused the nick translation rate to increase. When MNNG-treated cells were cultured in fresh medium containing no MNNG, the increase in the rate of nick translation in permeable cells became less and this decrease was abolished by addition of aphidicolin or cytosine arabinoside. The nick translation method described here may be a useful means for estimating intrinsic DNA breaks in cells treated with carcinogens.

Radiation and chemical carcinogens modify cellular DNA and induce strand breaks. This DNA damage is considered responsible for mutation and carcinogenesis. DNA strand breaks are usually detected by alkaline sucrose gradient centrifugation (1) or an alkaline elution method (2). Some researchers have used the nucleoid sedimentation technique for this purpose (3). Under alkaline conditions, however, no discrimination can be made between alkaline-labile DNA modification and DNA strand incision. In making an analysis by nucleoid sedimentation, most of the nuclear proteins are removed from DNA by high salt.

DNA damage is repaired by cellular enzymes, and the processes involved are known to be significantly affected by chromatin proteins (4-7). Thus, it

Abbreviations : MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; pol. I, E. coli DNA polymerase I.

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is important to detect intrinsic DNA strand breaks which may be accessible to repair enzymes. We attempted to measure the DNA breaks in permeabilized cells using nick translation with DNA polymerase I.

MATERIALS AND METHODS

Normal human fibroblasts, WI-38 at population doublings of 35∿40, were cultured as described previously (7). Cells in a confluent state were harvested with trypsin, and suspended in 0.25 M sucrose, 0.1 M Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 0.5 mM dithiothreitol (8). Lysolecithin was added to a final concentration of 100 μg/ml. The cell suspension was kept on ice for 5 min, and 98∿100% of cells became permeable to erythrosin B under these conditions both with the control and carcinogen-treated cells.

Aliquots of the cell suspension were spun down at 800 g for 5 min, and permeabilized cells were suspended either in a nick translation mixture or in an assay mixture for poly(ADP-ribose) synthetase at a cell density of $5∿10\times10^4$ cells/40μl.

The nick translation mixture contained 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml bovine serum albumin, 0.05 mM each of dATP, dGTP, and dTTP, and 2 μCi/ml [^{3}H]dCTP (19 Ci/mmole). E. coli DNA polymerase I (pol. I, New England Biolab, 12,000 units/ml) was added at 6 units/ml.

The mixture for poly(ADP-ribose) synthetase contained 35 mM Tris, pH 7.9, 30 mM $MgCl_2$, 0.6 mM ethylenediamine tetracetic acid, 20 mM mercaptoethanol, 40 μM NAD, and 0.25 μCi/ml [U-^{14}C-adenine]NAD (9). Reactions were carried out at 20°C for 10∿20 min unless otherwise stated and terminated by the addition of cold 5% trichloroacetic acid. Samples were sonicated, and filtrated through GF/C filters. The radioactivity retained on filters was measured.

RESULT AND DISCUSSION

Fig. 1 illustrates the time course of DNA synthesis in permeabilized WI-38 cells. The incorporation of [^{3}H]dCTP increased linearly for 30 min at a rate two- to four-fold higher in cells treated with MNNG (20 μg/ml) for 1 hr than that in untreated cells. When pol. I was not included in the reaction mixture, incorporation of [^{3}H]dCTP occurred to only a small extent.

A similar increase in the nick translation rate was also observed for cells pretreated with DNA-damaging agents such as bleomycin or 4-nitroquinoline-1-oxide, or permeable cells treated with DNase I (Table I). The increase in the nick translation rate in bleomycin-treated cells was less than that in cells treated with other agents, probably as a result of modified 3'-termini which are not used as a primer by pol. I (18, 19). MNNG did not affect the permeability of the cells, as judged by staining with erythrosin B (data not shown).

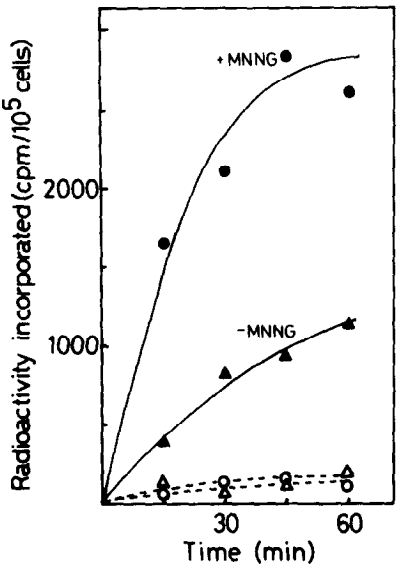

Fig. 1. Time course of nick translation. Cells in a confluent stage were treated with MNNG (20 μg/ml) for 1 h (circles) or untreated (triangles) and then permeabilized. Permeable cells were incubated in the nick translation mixture in both the presence (closed symbols) and absence (open symbols) of pol. I at 15°C. The radioactivity in cold trichloroacetic acid fractions was measure.

It was not possible to determine directly whether the increase in nick translation was due to an increase in the number of initiation sites or an increase in the rate of elongation. However, the above results may indicate that DNA lesions can be recongnized by pol. I in permeable cells and that DNA gaps are used as primers.

Table I. Effects of DNA-damaging Agents on Nick Translation Rate

Treatment	cpm/10^5 cells/10 min		
	-pol I.	+pol. I	Δ
-	480	793	373
bleomycin 100 μg/ml	574	1267	693
MNNG 20 μg/ml	559	2003	1444
4-nitroquinoline-1-oxide 3×10^{-5} M	173	3543	3370
DNase I 10 μg/ml	560	3009	2450

Cells were treated with chemicals for 1 hr, and then permeabilized. Permeable cells were incubated in the nick translation mixture in both the absence and presence of pol. I. In the case of DNAse I treatment, untreated cells were permeabilized and incubated with DNase I at 15°C for 5 min. DNase I treated cells were collected by centrifugation, and then incubated in the nick translation mixture.

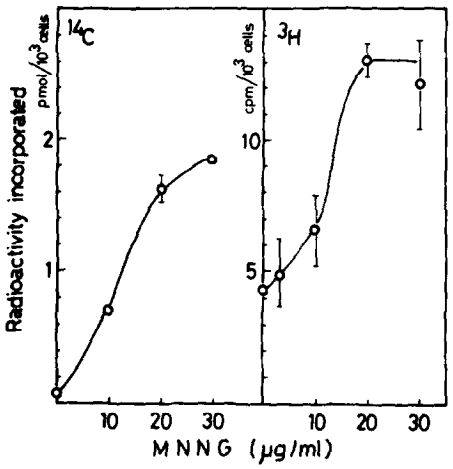

Fig. 2. Effects of MNNG on poly(ADP-ribose) synthetase activity and the nick translation rate. Cells were treated with varing concentrations of MNNG. Permeabilized cells were incubated in the poly(ADP-ribose) assay mixture (left) or in the nick translation mixture (right) at 20°C for 10 min. In case of nick translation, the radioactivity of [^{3}H]dCTP incorporated in the absence of pol. I was subtracted from that incorporated in the presence of pol. I.

Pol. I requires DNA gaps with free 3'-OH, and radiation-induced single strand breaks have been reported to contain inhibitory 3'-terminal phosphate (10). We treated permeable cells from a MNNG-treated culture with alkaline phosphatase and nick translated the cells, but there was essentially no increase in the rate of nick translation (data not shown).

It is well known that poly(ADP-ribose) synthetase requires DNA nicks for its activity (8) which increases when the cells are treated with DNA-damaging agents such as radiation or chemical carcinogens (11,12). This increase in activity can thus be regarded as indicative of DNA breaks in nuclei. The results of Fig. 2 show the dose-dependence of the increase in poly(ADP-ribose) synthetase activity and nick translation rate in permeable cells. These increases accompanied each other when the cells were treated with relatively high concentrations of MNNG (10∿20 μg/ml).

The doses causing an increase in the poly(ADP-ribose) synthetase activity were much higher than those affecting cell survival or DNA strand scissions as determined by alkaline sucrose gradient centrifugation. DNA nicks were detected by conventional alkaline sucrose gradient centrifugation with cells treated with MNNG at a concentration as low as 1 μg/ml (data not shown), but

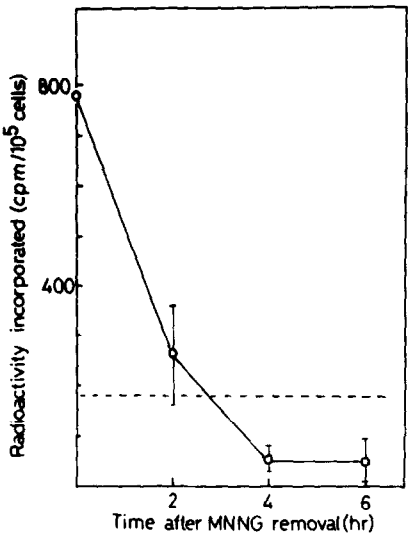

Fig. 3. Repair of nick translation-sensitive sites. Cells treated with MNNG (20 μg/ml, 1 h) were cultured in the absence of MNNG. Cells were permeabilized at certain times and then incubated in nick translation mixture. The radioactivity is shown as described in Fig. 2. The dashed line indicates the control level of untreated cells.

an increase in the nick translation rate was observed only in those cells treated at much higher concentrations. However, the methods employing alkaline conditions for detecting DNA strand breaks could not distinguish between DNA nicks *in situ* and alkaline-labile DNA modifications, since apurinic sites or bases with carcinogen adducts are sensitive to alkali and artificially cleaved during manipulation (13). The fact that high doses of MNNG are required for both the activation of poly(ADP-ribose) synthetase and an increase in nick translation rate may indicate that intrinsic DNA nicks in the cells are induced only when they are treated with such concentrations of MNNG. Alternatively, some DNA nicks are covered with chromatin proteins and thus not accessible to enzymes such as poly(ADP-ribose) synthetase and pol. I, and chemical carcinogens may alter the chromatin conformation.

When MNNG-treated cells were further cultured in the absence of MNNG, the rate of nick translation ceased to increase in permeable cells (Fig. 3), possibly as a result of the sealing of DNA gaps by repair processes or chromatin proteins covering due to the rearrangement of nuclear proteins (15-17). As shown in Table II, the addition of cytosine arabinoside or aphidicolin during post-treatment culture was found to accelerate nick translation in

Table II. Effects of Inhibitors on the Recovery of Nick Translatable Lesions.

Treatment	cpm/10^5cells/ 10 min		
	-pol. I	+pol. I	Δ
-	367	407	40
MNNG 20 μg/ml. 1h.	450	600	150
MNNG 20 μg/ml. 1h. → - MNNC 4h.	373	463	90
MNNG 20 μg/ml. 1h. → - MNNG + aphidicolin 2 μg/ml 4h.	367	1210	843
MNNG 20 μg/ml. 1h. → - MNNG + cytosine arabinoside 4 μg/ml 4h.	330	961	631
aphidicolin 2 μg/ml, 4h.	436	635	199
cytosine arabinoside 4 μg/ml, 4h.	347	487	140

Cells were treated with MNNG for 1 h and assayed for nick translation directly or assayed following cultivation in the absence of MNNG for 4h with and without inhibitors. Portions of untreated cells were cultured for 4h with inhibitors.

permeable cells. These inhibitors alone caused the rate of nick translation to increase to some extent. The finding that these inhibitors prevented the nick translation rate from decreasing during post-treatment cultivation suggests that the decrease shown in Fig. 3 indicates the rejoining of DNA strand breaks.

Noguchi and Kada utilized DNA polymerase I to detect endonuclerase activity with γ-irradiated DNA (14), and Cleaver used exonuclease III and S1 nuclease to determine the patch size of repair-synthesized DNA (13). It was difficult to determine the number of DNA-nicks by the present method, since the nick translation rate depends both on the number of DNA nicks and rate of elongation, but the nick translation method using pol. I as described in the present paper may be useful for estimating the DNA strand breaks induced by DNA damaging agents.

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