Histochemical detection of DNA strand scissions in mammalian cells by in situ nick translation

HISTOCHEMICAL DETECTION OF DNA STRAND SCISSIONS IN MAMMALIAN
CELLS BY IN SITU NICK TRANSLATION

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ABSTRACT

A method to visualize in situ of single strand scissions of DNA
in fixed mammalian cells has been developed. Using the nuclear nick
translation with biotin-labeled dUTP followed by binding to avidin-
biotin-peroxydase complex, the nuclei of HeLa cells which had been
treated with a DNA-damaging antibiotic bleomycin were specifically
stained, implicating that the histochemical detection of single
strand scissions (nicks) of DNA in fixed cells was completed without
destroying the morphology, and without using autoradiography.

INTRODUCTION

A quantitative measurement of single strand scissions of
cellular DNA is essential to investigate the effects of DNA-damaging
agents on mammalian cells, as well as the process of DNA repair by
the cells. For this purpose, ultracentrifugation of DNA in an
alkaline sucrose gradient has been most commonly used (McGrath and
Williams, 1966; Lett et al., 1967). Recently, several methods which
are more sensitive in detecting single strand scissions have been
developed, such as alkaline elution (Kohn and Grimek-Ewig, 1973) and
nucleoid sedimentation (Cook and Brazell, 1976). However, all these
techniques require destruction of cells for in vitro analysis so that
the morphology of the tissue or cells are lost and one cannot
distinguish particular cells with DNA nicks from neighboring cells
without nicks. Therefore, developent of an in situ method to detect
DNA strand scissions on fixed culture cells or tissue sections is
essential.

Recent advances in biochemical technology seem to throw light on
this problem. Nick translation reaction, originally described by
Kelly et al. (1970) has become popular in radioisotope-labeling of
DNA probes (Rigby et al., 1977). The principle of this technique is
based on the activity of E.coli DNA polymerase I to bind to a nick
containing 3'-OH terminus and substitute the nucleotides one by one
with externally added radioactive nucleotides in 5' to 3' direction.
The amount of this reaction is dependent on the number of nicks and

hence on the action of DNAse I which is usually added to the reaction
mixture. Kerem et al. (1983) applied this technique for in situ nick
translation of the fixed mammalian metaphase chromosomes to show that
heterochromatin of inactivated X chromosomes, which is less
susceptible for digestion by DNAse I, contained less grains on
autoradiography after nick translation than transcriptionally active
chromatin did. On the other hand, Langer et al. (1981) synthesized
biotin-labeled dUTP and demonstrated that it is incorporated into
DNA in place of TTP in the nick translation reaction. Thus, it has
become possible to combine histochemical methods to visualize labeled
DNA probe without autoradiography. Among the histochemical methods,
the use of avidin-biotin-peroxidase complex developed by Hsu et al.
(1981) seems to be the most sensitive way to detect labeled probe.
    In this report we exploited these recent techniques for in situ
detection of the single strand scissions in HeLa cells induced by an
antitumor antibiotic, bleomycin.

MATERIALS AND METHODS

    Cell culture and drug treatment. HeLa-S3 cells were grown on
cover slips (18 x 18mm) in Eagle's minimum essential medium plus 10%
fetal calf serum in a humidified, 5% CO_2 atmosphere. Bleomycin
(Nippon Kayaku Co., Inc.) was stored in -80°C and was dissolved into
the medium just prior to the use. At an exponentially growing state,
the cells were treated with bleomycin at 37°C. Then the cover slips
were removed, were rinsed with phosphate buffered saline and were
fixed for 10 min in acetone/ethanol (1:1) at room temperature.
    In situ nick translation. After fixation of the cells, the
cover slips were incubated at room temperature with 15μl nick
translation mixture containing 50mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 10mM
2-mercaptoethanol, 240 U/ml of E. coli DNA polymerase I (Boeringer
Manheim, endonuclease-free), 400 pg/ml of activated pancreatic DNAse
I (Worthington Diagnostic Systems, Inc.), 30μM each of dATP, dGTP,
dCTP (Sigma Biochemical Co.) and either TTP or biotin-labeled dUTP
(Enzo Biochemical Co.). For monitoring the amount of reaction, 1.5μM
^{3}H-dCTP (62 Ci/mmol, Amersham International plc) was added to the
mixture. Depending on the cases, DNA polymerase I and/or DNAse I
were withheld from the reaction mixture. The reaction was terminated
by rinsing the cover slips with 50mM Tris-HCl pH 7.5. For the
measurement of the incorporation of ^{3}H-dCTP into macromolecules, the
cover slips were incubated for 5 min in 5% TCA, for 5 min in 2.5% TCA
and for 5 min in ethanol at 4°C. After being dried, the acid-
insoluble materials were dissolved into 1N NaOH, were adsorbed on
glass fiber filters and were counted for radioactivity in a toluen-
based scintillation solution.
    Histochemical staining. For the visualization of incorporated
biotin-labeled dUMP, the cover slips were incubated with a solution
containing avidin and a biotin-conjugated horse radish peroxidase
(Vector Laboratories Inc.) in 50mM Tris pH 7.5 for 45 min at room
temperature. After being rinsed, the cover slips were incubated for
5 min with a peroxidase substrate solution containing 0.05% hydrogen

peroxide and 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in
50mM Tris-HCl pH 7.5. Then the cover slips were rinsed, were stained
briefly with methyl green solution (1% in 0.2M acetate buffer pH 4.0)
, were differentiated in tertially butanol, then in xylene and were
mounted on glass slides for microscopic observations.

RESULTS

     In situ nick translation of fixed HeLa cell nuclei. First, we
attempted to confirm the in situ nick translation reaction on the
nuclei of fixed HeLa cells in terms of the incorporation of dCMP into
macromolecules. The results are shown in Fig. 1. With the reaction
mixture containing neither DNA polymerase I nor DNAse I, the
incorporation was very low, indicating that a non-specific adsorption
of radioactive nucleotides or an uptake of nucleotides due to the
endogenous DNA polymerase activity in fixed cell nuclei is
negligible. In the presence of E.coli DNA polymerase I in the
mixture, a considerable amount of incorporation was observed during
the incubation, reaching the plateau at 2 hr. As DNAse I was further
added to the mixture, the incorporation was stimulated by 2-3 folds.
Thus, in agreement with the characteristics of nick translation, the
reaction was dependent both on the presence of E. coli DNA polymerase
I and the nicks induced by DNAse I. Considerable incorporation in
the absence of DNAse I seems to be due to the nicks naturally
existing in the intact HeLa cell nuclei, but the possibility of the
nick formation in the process of cell fixation and enzyme reaction
can not be ruled out. In this regard, using DNA polymerase I free
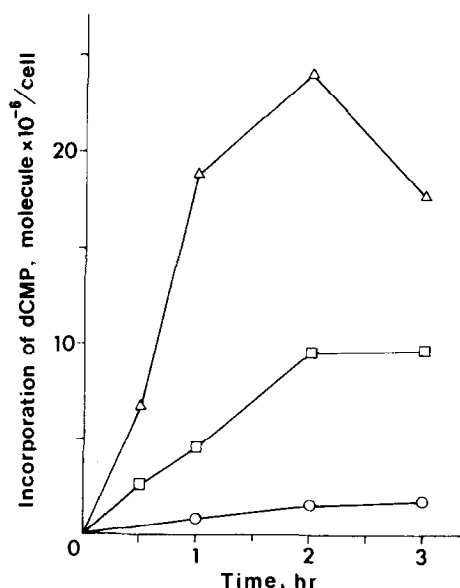from endonuclease contamination is essential in this system.

Fig. 1. Time course of in situ
nick translation reaction. The
amount of reaction is indicated
as the number of total
incorporated dCMP molecules per
cell which was caulculated by
the uptake of radioactivity
from ^{3}H-CTP involved at 1/20 of
total dCTP in the reaction
mixture. The reaction mixture
contained neither DNA polymer-
ase I nor DNAse I (o——o),
DNA polymerase I but no DNAse I
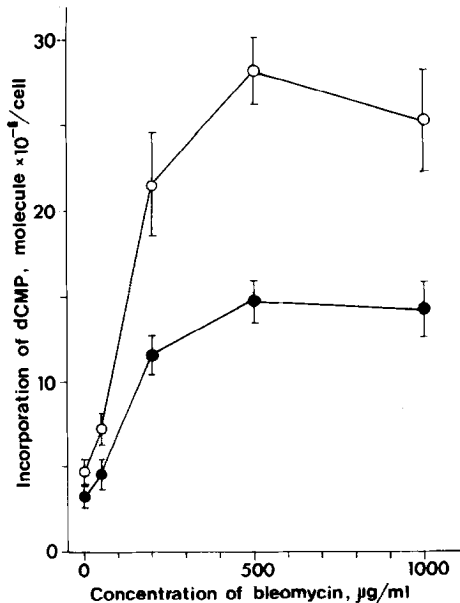(□——□) or both DNA polymerase
I and DNAse I (△——△).

Fig. 2. Effect of pretreatment of cells with bleomycin on in situ nick translation reaction. The cells were treated with the indicated concentrations of bleomycin before fixation and then were nick translated for 1 hr with a reaction mixture containing DNA polymerase I but not DNAse I, in the presence of either 30μM TTP (o——o) or 30μM biotinylated dUTP (●——●). The amount of reaction was measured in the same way as in Fig. 1. Bars indicate standard deviations.

Effect of the pretreatment of HeLa cells with bleomycin. The results in Fig. 1 suggest that by performing in situ nick translation in the absence of DNAse I, it is possible to detect the nicks with 3'-OH termini which existed before fixation of the cells. To confirm this, cells were treated with an antitumor drug, bleomycin. Bleomycin, a glycopeptide antibiotic isolated from Streptomyces verticillus by Umezawa et al. (1966), has been used widely for clinical purposes. The primary lethal action of this drug seems to be the single strand scission of cellular DNA (Suzuki et al., 1969) which is mediated by the release of free radicals by this drug in the presence of Fe(II) and oxygen (Sugiura and Kikuchi, 1978). In the in vitro study, the final form of the chain breakage of DNA by bleomycin is accompanied by a release of free bases and, therefore, may not be a simple nick at the phosphodiester bond, but at least the 3' terminus of the breakage is not phosphorylated (Tien Kuo and Haidle, 1973). Following the pretreatment of cells with various concentrations of bleomycin, the cells were fixed and were nick translated for 1 hr without DNAse I in the presence of either TTP or biotinylated dUTP. The amount of the reaction was monitored by the incorporation of ^{3}H-dCTP as in Fig. 1. The results are shown in Fig. 2. Depending on the concentration of bleomycin, the incorporation was markedly stimulated, reaching 5-6 times that of control at the concentration 500 μg/ml. The reaction with biotinylated dUTP was considerably lower than that with TTP. Thus, it was shown that in situ nick translation can detect the single strand scission of DNA induced by the treatment of cells with bleomycin before fixation.

Visualization of nicks. One method to visualize the result of in situ nick translation is autoradiography following the

incorporation of radioactive nucleotides. However, this usually
takes weeks of exposure to detect tritium-labeled DNA, and the
localization of grains is not always reliable enough to allow
detailed cytological analysis. Therefore, we substituted TTP with
biotinylated dUTP in the reaction mixture. As shown in Fig. 2, the
amount of nick translation reaction with biotinylated dUTP was
somewhat lower than that with TTP, but the stimulation effect by
bleomycin was apparent in both cases. Visualization was performed
by binding the incorporated biotin-dUMP with avidin-biotin-peroxidase
complex, followed by staining with enzyme substrate dye (Fig. 3). In
the control where the cells were nick translated in the absence of
DNA polymerase I, the nuclei were negative to the histochemical
stain. When the cells without pretreatment with bleomycin were nick
translated in the presence of DNA polymerase I, the nuclei showed
only a trace of brown tint after the DAB treatment (Fig. 3-a).
However, when the cells which had been treated with bleomycin
(500 µg/ml) before fixation were nick translated, the nuclei were
clearly stained brown (Fig. 3-b). On light microscopic observation,
the nuclear stain has a granular appearance and the nucleoli are
left free from stain, suggesting that the fine localization of the
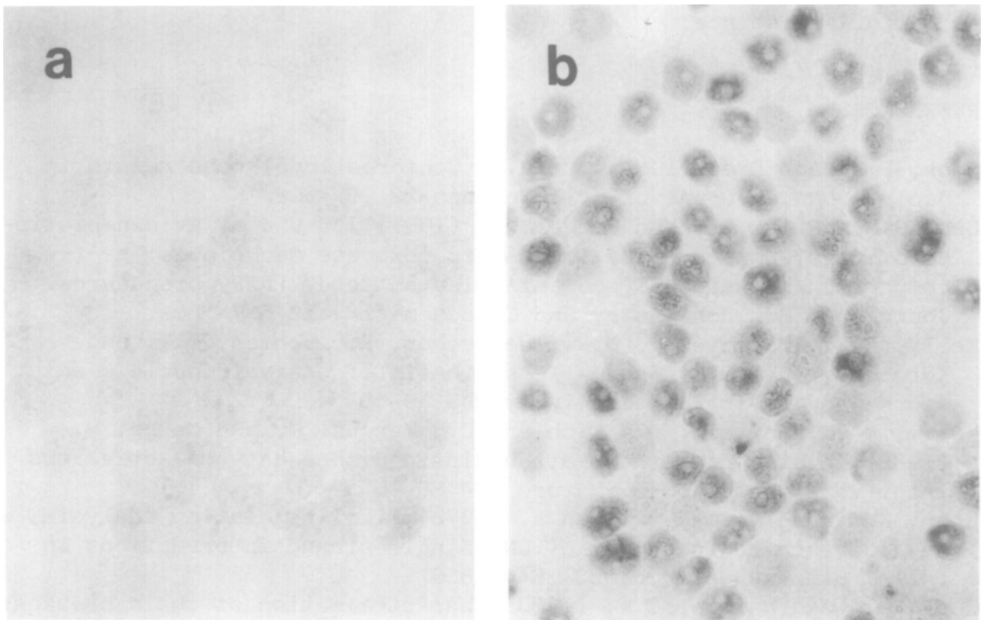DNA nicks is visualized on the nucleus.

Fig. 3. Visualization of in situ nick translation. After being nick
translated in situ with biotinylated dUTP, the cells were treated
with avidin-biotin-peroxidase complex, stained with DAB and were
counter-stained with methyl green. The reaction mixture contained
DNA polymerase I but not DNAse I. a) Cells without pretreatment.
b) Cells pretreated with 500 µg/ml of bleomycin. Photograph with a
green filter.   ×396

DISCUSSION

In this report we showed that the nuclei of HeLa cells which had been treated with bleomycin were quantitatively nick translated in situ and that the result could be visualized histochemically, using biotinylated dUTP. The actual number of nicks detectable by this technique is yet to be decided, but at least the concentration of bleomycin 50 µg/ml, which caused a significant increase in the nick translation reaction (Fig. 2), is comparable to the concentration used in other reports in which bleomycin-induced nicks were detected significantly by means of alkaline sucrose gradient (Suzuki et al., 1969, Saito and Andoh, 1973).

Although this report dealt with only a specific case of DNA strand scission in cultured cells caused by an antitumor drug, the described technique of detecting DNA nicks in situ without destruction of morphology seems to be applicable to tissue sections. If the sensitivity of this technique is sufficient, it may be possible to use it as a tool to highlight DNA nicking not only in cancerous tissues treated with DNA-damaging agents but also in normal tissues under certain physiological conditions.

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