Replicon fusion in mouse cells is very sensitive to DNA damage induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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Incubation of mouse cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine causes a strong inhibition of DNA replication the extent of which varies with the cell line used. Analysis of the products synthesized in drug-treated cells indicates a particularly severe effect on the joining of replicons while other steps in DNA synthesis like initiation and chain elongation are much less affected. The data indicate that replicon fusion may be extremely sensitive to changes in the topology of DNA induced by the introduction of rare single-strand breaks during repair of *N*-methylated purines produced by incubation of cells with small amounts of the methylating agent

DNA replication *Alkaline sucrose gradients* *MNNG* *Single-strand breaks*

1. INTRODUCTION

Methylating agents, such as the highly mutagenic and carcinogenic *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) cause a variety of lesions in DNA. Some of these are repaired by removal of the modified bases followed by cleavage of the DNA backbone by endonucleases (reviews [1,2]). Studies on the kinetics of the production and the repair of single-strand scissions in the DNA of MNNG-treated mouse cells indicate that breaks appear rapidly and can be detected immediately after incubation of cells with the drug whereas repair is rather slow [3]. The detection of the dose-dependent appearance of nicks in DNA seems to be limited only by the sensitivity of the available methods.

Another notable effect of physically- or chemically-induced damage to DNA is an inhibition of DNA replication (e.g., [4]) which is commonly attributed to a block in the initiation of replicons and/or a reduction or block in the elongation reaction (review [5,6]). These experiments indicate that in MNNG-treated mouse cells the process of replicon convergence is most sensitive to the DNA damage induced by the drug.

2. MATERIALS AND METHODS

2.1. *Cell culture, treatment with MNNG and measurement of DNA synthesis*

3T6 mouse fibroblasts were grown in 60 mm dishes as in [3]. Cells were used when the cultures were near confluence but not dense. Treatment with MNNG was done by adding 5 μl of various dilutions of the compound in formamide directly to the medium to give the final concentration wanted. Controls received 5 μl of formamide. Cells were incubated with the drug for 30 min at 37°C. The medium was then removed and replaced by fresh medium containing 2% calf serum. DNA synthesis was measured by adding [^{3}H]thymidine (1 μCi/ml medium) at various times after MNNG treatment. Labeling was done for 30 min at 37°C. The medium was then removed, the cells were washed twice with phosphate-buffered saline and lysed by addition (2 ml/plate) of 0.1% SDS–0.02 M EDTA (pH 8.0) at room temperature. DNA was precipitated from the lysate with 10% trichloroacetic acid at 0°C, precipitates were collected on Whatman GF/C filters, washed with 5% trichloroacetic acid followed by methanol/ether (1/1), dried and counted.

L5178Y lymphoma cells were grown in suspension culture in Fischer medium containing 10%

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horse serum. Cells were used when they reached late log phase (2–5 × 10^5 cells/ml). About 10^6 cells were collected by centrifugation, suspended in 5 ml pre-warmed Fischer medium and treated with MNNG for 30 min. Cells were then recovered by centrifugation, washed once with medium and resuspended in medium containing 2% horse serum. Labeling was carried out for 30 min by adding [^{3}H]thymidine (1 μCi/ml medium) to the suspension. Cells were then recovered, washed and the incorporated radioactivity measured as described for 3T6 cells.

Friend erythroleukemic cells were grown in suspension culture in Dulbecco-Eagle medium containing 10% fetal calf serum. About 10^6 cells were used for each experiment. Treatment with MNNG and measurement of DNA synthesis were done as described for the lymphoma and 3T6 cells, except for the difference in media used.

2.2. *Alkaline sucrose gradient centrifugation*

Pulse-labeled (30 min) or pulse-chase labeled cells were recovered (by centrifugation in case of lymphoma and Friend cells, by trypsinization in case of 3T6 cells) and washed with phosphate-buffered saline. The cell pellet (~ 10^6 cells) was suspended in 0.5 ml 0.15 M NaCl–0.01 M EDTA (pH 8.0). A 5 μl aliquot of the suspension was used to determine the radioactivity incorporated.

Analyses of DNA in alkaline sucrose gradients as well as calibration of these gradients were done as in [7,8]. Aliquots (50 μl) of the cells suspensions (~ 10^5 cells) were applied onto the lysis mixture on top of 5–20% alkaline sucrose gradients and 30 min allowed for cell lysis. Gradients were then centrifuged for 130 min at 30 000 rev./min in a SW50.1 rotor at 20°C. After centrifugation, gradients were fractionated from the bottom and radioactivity was determined by scintillation counting after addition of 5 ml Triton–toluol scintillator.

3. RESULTS

3.1. *Sensitivity of DNA replication in mouse cells to MNNG*

Incubation of 3T6 mouse fibroblasts with MNNG resulted in a dramatic inhibition of DNA replication already at very low drug doses (table 1). In these experiments, DNA replication was measured by 30 min pulse labeling of cells pretreated for 30 min with different concentrations of MNNG followed by 30 min incubation in fresh medium. MNNG at 0.2 μM already led to a significant inhibition of DNA synthesis in 3T6 cells (not shown). Much to our surprise we found that DNA replication in two other mouse cell lines, lymphoma and Friend erythroleukemia cells, was much less sensitive to MNNG (table 1). Here, no inhibition was observed in cells treated with < 10 μM MNNG. In both cell lines, 10 μM MNNG was found to cause the production of breaks detectable by the sensitive alkaline elution method ([9] and unpublished).

Table 1

Inhibition of DNA replication in mouse cells by pretreatment with MNNG

[MNNG]	Incorporation of [^{3}H]thymidine (%)		
(μmol/l)	3T6	L5178Y	Friend
0	100	100	100
1	55	103	102
2	49	108	102
5	44	109	101
10	39	96	84
20	30	67	65

Cells were treated for 30 min with MNNG at the final concentrations indicated. Medium was then changed and cells were pulse-labeled 30 min later. Incorporation of radioactivity was measured as in section 2: 100% equals in cpm/10^6 cells: 119 234 for 3T6; 44 188 for L5178Y; 57 612 for Friend cells

3.2. *Replicon fusion is the step of DNA replication most sensitive to MNNG*

To gain some insight into the mechanism by which lesions introduced into DNA by MNNG treatment of cells interfered with DNA replication, DNA of treated and untreated 3T6 cells was analysed by sedimentation in alkaline sucrose gradients using a pulse and pulse-chase labeling protocol (fig.1). The results show that the sedimentation properties of replication intermediates of 30 min pulse labeled DNA from control cells and from MNNG-treated cells were nearly identical

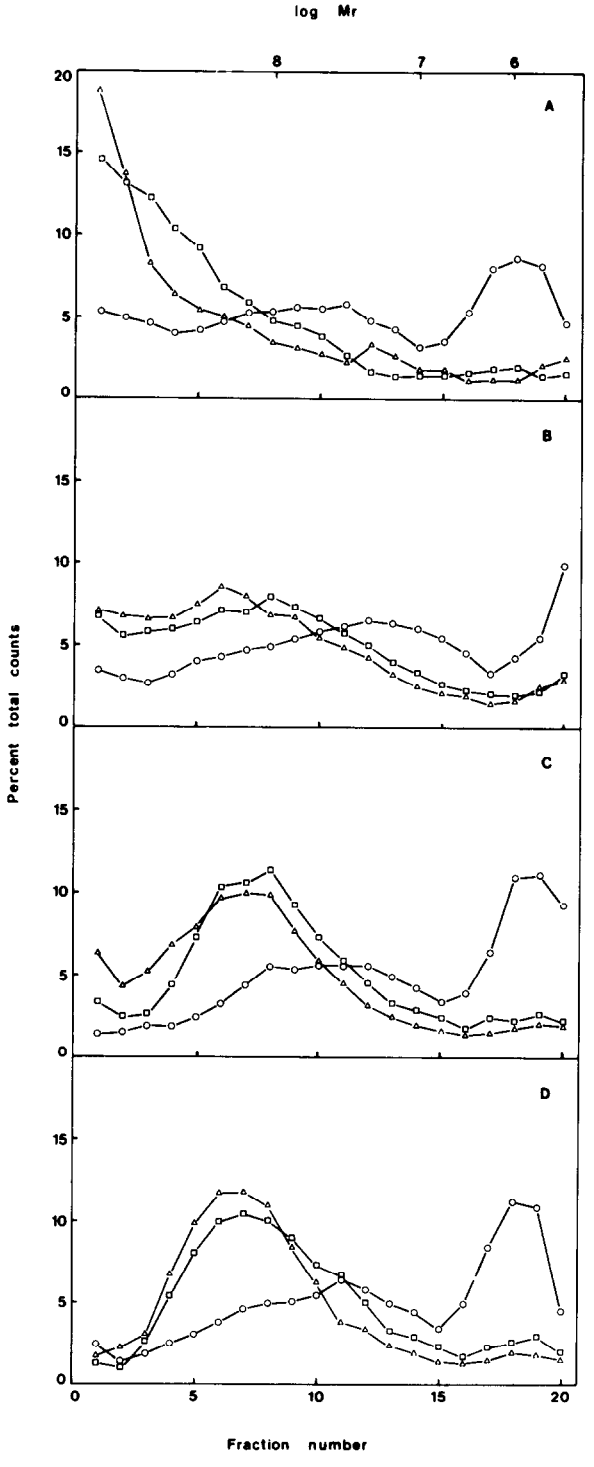

Fig.1. Effect of preincubation of 3T6 cells with MNNG on the sedimentation of pulse and pulse-chase labeled DNA in alkaline sucrose gradients: (A) control cells, not treated with MNNG; (B) cells pre-treated for 30 min with 0.5 μM MNNG; (C) cells pre-treated with 1 μM MNNG; (D) cells pre-treated with 5 μM MNNG. 30 min after MNNG treatment, cells were pulse labeled with [^{3}H]thymidine (2 μCi/ml medium, 30 min) and used without chase (○) or after 1 h (□) or 2 h (△) chase in fresh medium. Total counts/gradient were between 29 600 and 67 100 cpm. Sedimentation was from right to left. The relative molecular mass (M_r) of molecules in the gradients is given in the scale at the top of the figure.

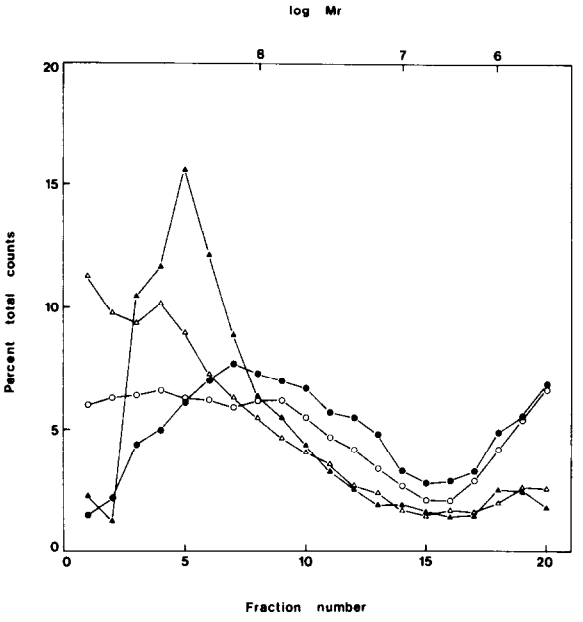

Fig.2. Effect of preincubation of Friend erythroleukemic cells with MNNG on the sedimentation properties of pulse and pulse-chase labeled DNA in alkaline sucrose gradients: (open symbols) control cells not treated with MNNG, (○) pulse labeled for 30 min, no chase; (△) after 2 h chase; (closed symbols) cells treated for 30 min with 5 μM MNNG; 30 min later they were pulse labeled and used without chase (●) or after 2 h chase in fresh medium (▲) Total counts per gradient were between 12 700 and 15 600 cpm. Sedimentation was from right to left.

except for the absence of DNA molecules of very high M_r in the profiles from treated cells. A major difference in this part of the gradients could be seen after chase periods of 1 or 2 h. Whereas DNA of control cells was under these conditions converted into material with very high M_r exceeding the size of replicons, DNA of MNNG-treated cells could only be chased into molecules sedimenting at about the rate of completed replicons. The replicon size of mouse cells is 100–200 μm [10], which corresponds to $2–4 \times 10^8$ daltons for double-stranded DNA. It should be noted that a partial block of replicon convergence could already be observed after treatment with 0.5 μM MNNG. Higher drug concentrations (1 μM and 5 μM) inhibited DNA synthesis 45% and 56%, respectively (table 1) but caused a complete inhibition of the fusion of replicons (fig.1). It is worth noting that the optimal size of replication intermediates obtained under these conditions was independent of the amount of MNNG used (cf. fig. 1C,D).

Although the sensitivity to MNNG of DNA replication in lymphoma and in erythroleukemic cells was considerably lower (table 1), 5 μM MNNG had the same effect on replicon convergence in these cell lines as in 3T6 cells. This is shown for Friend erythroleukemia cells in fig.2. The size of the intermediates accumulated in pulse-chase experiments was very close to that in similarly treated 3T6 fibroblasts.

4. DISCUSSION

Most DNA-damaging agents have a profound influence on DNA replication in affected cells. Depending on the lesion introduced this can result in a block at the replication fork, a decrease in the rate of chain elongation or an inhibition of the initiation of replication [5,6]. Here, we have described experiments which strongly indicate that MNNG induces lesions which interfere primarily with the convergence of replicons to larger chromosomal units. Although the M_r of DNA 'fragments' of the size of replicons can no more be determined precisely by sucrose gradient centrifugation, it is striking that sedimentation properties of chased DNA in MNNG-treated cells correspond approximately to replicon sizes determined for mouse cells by other means [10]. Also, this size does not change with the concentration of MNNG used for treatment (fig.1) as long as it is below that required to detect breaks in parental DNA by gradient centrifugation, which is 50 μM [3]. The difference to the sedimentation of untreated labeled and chased DNA is quite striking.

Experiments on the replication of SV40 DNA indicate that replicon completion is one of the rate-limiting steps in this process [11]. As the organization and mode of replication of papovavirus chromatin has much in common with that of cellular chromatin, one could envisage a similar rate-limiting step in the replication of cellular DNA. Indeed, experiments with mouse embryo cells support such a view [19]. The reason for the fact that convergence of replicons is slow may lie in a specific topological requirement for this reaction. Agents like MNNG, which cause the production of single-strand breaks, would then have a strong effect on this reaction. This should hold also for many other DNA-damaging conditions like irradiation with UV. Most studies on the effect of UV light on DNA replication consider primarily the inhibition of fork progression by pyrimidine dimers [5,6]; less attention has been paid to the influence of single-strand breaks transiently produced during the repair reaction [6,12]. Whenever this reaction is considered, however, it is assumed to be the cause of the inhibition of replicon initiation observable in UV-treated cells [5,13]. Our results suggest that replicon fusion is much more sensitive to nicks in DNA than is initiation. This apparent discrepancy may primarily lie in the methods employed:

(1) In the majority of studies using alkaline sucrose gradient centrifugation to analyse DNA synthesis products after treatment of cells, only pulse labeling for various lengths of time was employed; pulse-chase experiments were rarely carried out.

(2) Most importantly, DNA was usually fragmented to a size of $\sim 10^8$ daltons by X-irradiation [14] or by extended incubation in alkaline solution [15] prior to gradient centrifugation. The maximum size of the replication intermediates observable, therefore, cannot exceed that of replicons; replication steps leading to larger products cannot be analysed under these conditions.

DNA replication in the S phase of the cell cycle is an ordered process in which groups of replicons,

arranged close to each other on the chromosome, are initiated and replicated simultaneously at a certain time [10,16,17]. This order might be controlled by the requirement that one set of replicons must be completed and joined before another set is initiated. If this were the case, inhibition of the fusion of one set of replicons will eventually lead to an inhibition of initiation of other ones. This, together with the above methodology to analyse DNA fragments, could explain why in some cases an effect on initiation was observed when in fact the event primarily affected was replicon joining.

More information on this late, hitherto rather neglected step in DNA replication should promote our understanding of regulatory processes in the S phase. These include such important questions as to why each replicon is initiated once and only once in a normal S phase. Interference with the underlying controls (e.g., by a slow-down of replicon fusion) might increase the chance for re-initiation at already replicated stretches of DNA leading to saltatory replication which is one mechanism proposed to explain gene amplification [18]. Experimental systems are at hand to test such questions.

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