
Increased activity of polynucleotide ligase in 5-iodo-2'-deoxyuridine and mitomycin C-pretreated simian virus 40 (SV40)-infected monkey kidney cells

M. Mezzina, H.G. Suarez, R. Cassingena and A. Sarasin

Institut de Recherches Scientifiques sur le Cancer, B.P. 8, 94802 Villejuif Cedex, France

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ABSTRACT

The DNA ligase activity has been studied in 5-iodo-2'-deoxyuridine (IUdR), mitomycin C (MMC) and IUdR + MMC-treated SV40-infected or mock-infected CV1Cl1 monkey kidney cells. The results have shown that : 1. The level of enzyme activity is about two or three times higher in IUdR or MMC-treated non-infected cells ; 2. SV40 infection doubles the level of ligase activity in the untreated cells ; 3. There is an additional increase of ligase activity in IUdR or MMC-treated SV40-infected cells ; 4. When infected or mock-infected cells are treated with IUdR + MMC, there is no modification of the results obtained with each drug alone ; 5. Two peaks of different S values are detected in a partial purification of the drug(s) or viral induced enzyme(s).

The increase in the ligase activity in drug-treated cells is independent of semi-conservative DNA synthesis. The drug(s) and viral-induced enzyme(s) is the consequence of a "de novo" synthesis of proteins, as shown by cycloheximide treatment.

INTRODUCTION

It has been shown that, in mammalian cells as well as in T4 phage and *E. coli*, polynucleotide ligase(s) acts in the semi-conservative DNA replication mode by joining Okazaki-type fragments to produce the mature DNA strand (1-3). This enzyme(s) also plays a role in the DNA excision repair mechanism by sealing the newly patch repair DNA to the parental strand (4). The participation of DNA ligase in such a process, in the absence of semi-conservative DNA synthesis, has been established in conditional lethal mutants of bacteriophages (5), *E. coli* (3) and yeast (6), defective in ligating activity. It has not yet been the case in mammalian cells, essentially because of the unavailability of such a type of mutant. The ligase activity varies during the cell cycle increasing in exponentially growing monkey kidney or human cells (7, 8), and in other cellular systems after stimulation of DNA synthesis by different methods (9-15). We have recently shown that the ligase activity is

enhanced after UV-irradiation of cultures of monkey kidney (7) and human cells (8), suggesting a direct role of the enzyme in the final step of the DNA excision-repair mechanism, by sealing the repaired strand.

Treatment of cells with 5-iodo-2'-deoxyuridine (IUdR) and mitomycin C (MMC), induces DNA damages different from pyrimidine dimers and other lesions produced by UV-irradiation. IUdR, an halogenated thymidine analogue, is incorporated into viral, bacterial or mammalian DNA (16), inhibiting its replication ; MMC, a bifunctional alkylating agent, induces the formation of intra- and inter-strand DNA cross-links, interfering with the replication process (17-19).

Infection of resting cells with simian virus 40 (SV40) or polyoma (Py) virus, leads to a new round of cellular DNA synthesis and a rise in the activity of several enzymes involved in this process (20-22). Different authors (12, 13) have also described that the infection of several cellular systems with SV40 or Py enhances the ligase activity. However, it has not been possible to show , in those experiments, that the increase is caused by the appearance of a viral coded enzyme.

IUdR and MMC are inducers of virus production in SV40- and Py virus-transformed cells (17, 23). It has also been reported that, the pretreatment of monkey kidney (CV1C11) and Chinese hamster cells with the drugs, may augment their permissiveness toward the replication in lytic infection of SV40 (16, 24). IUdR and MMC act at different levels to modify the permissiveness of cells to the virus (24). Moreover, Sarasin et al. have shown that the treatment of CV1C11 cells with MMC before infection with UV-irradiated SV40, increases significantly the reactivation factor and the mutagenesis of viral progeny (25). The precise mechanisms underlying these events are at present unknown.

With the aim of further analyzing these mechanisms, we have studied the ligase activity in drug-treated SV40-infected or mock-infected CV1C11 cells.

MATERIALS AND METHODS

Enzymes

T4-infected E. coli polynucleotide ligase was prepared according to the method previously described (26). T4-infected E. coli polynucleotide kinase was purchased from P.L. Biochemicals (USA) and from Biolabs New England (UK). Bacterial alkaline phosphatase and alcohol dehydrogenase were purchased from Sigma (USA).

Cells

CV1C11, a clone of CV1 cells (16), was cultured in Eagle's minimal essential medium (MEM, Eurobio, Paris) with 10 % calf serum, in a CO₂ incubator at 37°C.

Virus

A large plaque strain (SVLP) of SV40 virus was grown in CV1C11 cells and virus yields were determined by plaque titration on CV1C11 monolayers as described (16).

IUDR and MMC-treatment and infection with virus

CV1C11 cells, suspended in growth medium were seeded (6×10^6 cells/100 cm² flasks), in the presence or absence of 100 µg of IUDR per ml and incubated at 37°C. After four days, the medium was removed and after three washings with serum free medium the cultures were exposed (or not) to MMC. After three hours of incubation at 37°C, the medium was removed and the cultures were rinsed ; after two hours in drug-free medium, cultures were infected with SV40 virus at a multiplicity of infection (MOI) of 0.1 or 10 plaque forming units (PFU) per cell as previously described (16). All experiments were carried out in the dark. Cultures were then washed again, MEM with 2 % calf serum was added and, at designated times after infection or mock-infection, control and treated cultures were trypsinized, resuspended in MEM with 20 % calf serum plus 10 % glycerol, and frozen at -70°C. When cycloheximide (CH) was used (5 µg/ml), it was added immediately after IUDR, MMC treatment or SV40-infection and was present until the cultures were harvested.

Plating efficiency

Samples (10^5 cells) of each set of cultures were harvested before freezing, counted, and known numbers of cells were plated in complete culture medium (MEM plus 10 % calf serum) in 60 mm Petri dishes. Fifteen days later the colonies were fixed and stained with Unna blue and the plating efficiency determined.

Incorporation of ³H-thymidine (³H-TdR) into cellular DNA

CV1C11 cells were seeded and treated as described above. At different times after treatment, labelling was performed by addition of 4 µCi of ³H-TdR per ml for periods of 2 hours. The incorporation of ³H-TdR into cellular DNA was determined after perchloric acid hydrolysis of 5 % trichloroacetic acid-washed cellular pellets. To determine the amount of cellular DNA in a given sample, the optical density was measured at 260 nm ($1 D_{260} = 50 \mu\text{g of DNA}$).

Chemicals

γ -³²P-adenosine triphosphate (ATP ~ 3000 Ci/mmol) was obtained from NEN

Corp. (USA) ; oligo-deoxy-thymidilate (12-18 nucleotides long) was purchased from P.L. Biochemicals (USA) and polydeoxyadenilate (about 500 nucleotides long) from Miles (USA) ; IUdR was obtained from Schwarz-Mann, Orangesburg (USA) ; MMC was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio (USA) ; CH was from Sigma, St. Louis (USA).

Cellular crude extracts

Frozen samples of each experiment were rapidly thawed, suspended (3×10^7 cells/ml) in 0.5 M KCl, 20 mM Tris (hydromethyl)-amino-methane-HCl pH 7.5, 2 mM dithioerythritol (DTE), 0.5 % Triton X-100 and lysed by sonication in ice (2 x 15 sec) with 1 min cooling interval in between. The crude lysate was centrifuged at $105\ 000 \times g$ for 1 hour at 2°C and immediately assayed for DNA ligase activity. Proteins contents in the crude extracts were determined according to the method of Lowry (27).

Preparation of 5'-³²P-labelled oligo(dT)₁₂₋₁₈ substrates and DNA ligase assay

Olivera's method (28) was used with slight modifications : 0.3 μ moles/ml of oligo(dT)₁₂₋₁₈ were dephosphorylated by incubation at 37°C in the presence of 5 units of alkaline phosphatase for 30 min. The reaction was stopped by two treatments with 1 volume of iso-amyl alcohol-chloroform (1:24), followed by three phenol and three ether treatments. This dephosphorylated oligo(dT) was 5'-labelled with γ -³²P-ATP at a concentration of $1-3 \times 10^{-4}$ μ mol/ml (specific activity ~ 3000 Ci mmol) in the presence of 60 mM Tris-HCl pH 7.6, 6 mM MgCl₂, 6 mM β -mercaptoethanol and 10 units of polynucleotide kinase at 37°C. At 30 min intervals, 4 units of fresh enzyme was added to incubation mixture (0.5 ml). A two μ l aliquot of the reaction mixture was removed every 30 min and acid insoluble ³²P material was precipitated with 10 % trichloroacetic acid in the presence of 200 μ g/ml of carrier yeast RNA. When the plateau of ³²P incorporation was reached, the reaction was stopped by one phenol and ether treatment. The specific activity of 5'-³²P-oligo(dT) obtained was 2×10^7 cpm/nmol.

DNA ligase assay was carried out as described (7). One unit of enzyme is defined as the amount converting 1 nmol of 5'-³²P dT residues into alkaline phosphatase resistant form in 1 hour at 37°C. The specific activity of 5'-³²P dT residues in the ligase mixture assay was $1-3 \times 10^5$ cpm/nmol.

Gradients

Aliquots of each crude extract corresponding to 1.2 mg of proteins were applied onto 5-20 % linear sucrose gradients in 20 mM Tris-HCl pH 7.5, 2 mM DTE, 0.5 M KCl, 1 mM EDTA, 0.8 mM PMSF. Centrifugations were performed in a Beckman rotor type SW41 at 37000 rpm for 40 hours at 4°C. The gradients were

collected from the top of the tubes. Ten μ l aliquot of each fraction (0.2 ml) was removed and incubated in ligase assay mixture for 30 min. Bovin serum albumin and alkaline phosphatase were used as standard references to calculate the coefficient of sedimentation (S^{20}_W values : 4.4 and 6.8).

RESULTS

We have previously shown (16, 24) that the optimal conditions for obtaining the maximum increase of SV40 replication with IUDR on CV1C11 cells (and other cellular systems), were a 96 hours pretreatment of the cultures with 100 μ g/ml of the drug. Smaller doses of IUDR or shorter treatments with that dose, were shown to be less effective ; higher doses or longer treatments with 100 μ g/ml, were extremely toxic for the cells (16). Consequently, all the experiments in this paper were done respecting the experimental conditions cited above.

Our earlier experiments showing that MMC-pretreatment enhances the yield of SV40 virions, were carried out in a cellular system other than CV1C11 (18, 24). Consequently, to establish the optimal conditions to obtain a maximum effect in CV1C11 cells, cultures were treated with different doses of MMC (from 0.5 to 20 μ g/ml), virus infected and the virions production determined 72 hours later. In parallel mock-infected cells, the effect of the same doses of the drug on ligase activity was studied. From the results presented in Table I, we decided in all the following experiments to treat the cells with 1 μ g/ml of MMC, a dose producing the optimal effect both on virus production and on ligase activity.

The effect of the drug treatment on the synthesis of cellular DNA in CV1C11 cells, was studied by measuring the incorporation of ^3H -TdR. The results obtained are summarized in Table II. The following points emerge from the experiments : 1. up to 48 hours IUDR or MMC-treatment caused a drastic inhibition of the cellular DNA-synthesis ; 2. at 72 hours the precursor incorporation was of the same order in treated and control cells, probably due to the fact that at about 60 hours the controls reached confluence (data not shown) and stopped growing. Examination of the cultures under the light microscope revealed a significant enlargement of the cells after the IUDR or MMC-treatment, indicating an unbalanced growth of the cells. Indeed, we have previously reported that drug-treated cultures in spite of the markedly reduced ability to replicate DNA exhibit continuing RNA and protein synthesis with highly diminished cell division (16, 18).

In order to follow the kinetics of DNA ligase activity from 24 to 72

Table I

MMC ($\mu\text{g/ml}$)	Virus yield (PFU/ 10^6 treated cells) (1)	Ligase activity (U/mg proteins) (2)
0	7.5×10^2	8.3
0.5	3.6×10^3	18.6
1	1.0×10^4	22.8
3	1.2×10^4	16.6
5	2.0×10^3	18.5
10	1.0×10^3	17.1
20	4.0×10^2	15.6

Virus production in MMC-treated CV1C11 cells after SV40 infection and ligase activity in MMC-treated uninfected CV1C11 cells. After treatment with MMC, parallel sets of cultures were : 1) infected with SV40 (MOI 0.1 PFU/cell) and virus production determined 72 hours after infection ; 2) used to measure ligase activity.

hours after the treatment, seven sets of parallel cultures were handled in each experiment as described in Materials and Methods : a. control ; b. IUDR-treated ; c. MMC-treated ; d. IUDR + MMC-treated ; e. untreated, SV40-infected ; f. IUDR-treated, SV40-infected and g. MMC-treated, SV40-infected.

Data presented in Figure 1 show : 1. a fairly constant level of enzyme activity in the control cultures, up to 72 hours ; 2. that the kinetics in the IUDR or MMC-treated cultures are similar for the two drugs, reaching a maximum increase in ligase activity (3 and 2 times respectively) at 48 hours after the end of treatment ; 3. that in IUDR + MMC-treated cells there is no modification of the results obtained with each drug alone. Figure 1 also summarizes data concerning the effect of SV40-infection on ligase activity, in IUDR or MMC-pretreated cells. Results presented show : 1. that in untreated cells, SV40-infection increases by a factor of 2 the enzyme activity, confirming results previously reported by Sambrook and Shatkin (12) ; 2. that the IUDR or MMC-pretreated virus-infected cultures presented an additive increase of ligase activity, reaching at 48 hours a maximum of 4 and 3 times respectively compared to controls.

The treatment of cells with 5 $\mu\text{g/ml}$ of CH, an inhibitor of protein synthesis, blocks the ligase induction indicating that the increased activity

Table II

Treatment	Hours after treatments	CPM/ μ g cellular DNA	% of control
NONE	24	8300	100
	48	5700	100
	72	1000	100
IuDR	24	3200	39
	48	1600	28
	72	1000	100
MMC	24	4000	48
	48	1700	30
	72	1200	120

Time-course of the incorporation of ^3H -TdR in IuDR or MMC-treated and control cells. The cultures were labelled for 2 hours with 4 $\mu\text{Ci/ml}$ of ^3H -TdR and the acid soluble radioactivity and the concentration of cellular DNA were determined at different times after the end of the treatment.

observed in IuDR or MMC-treated and in virus-infected cultures, probably represents a de novo protein synthesis rather than an activation of preformed enzymes (Table III). In this table, data concerning the effect of CH on survival of IuDR or MMC-treated cells is also shown. CH at a concentration of 5 $\mu\text{g/ml}$ has little effect on plating efficiency of untreated CV1C11 cells. On the other hand, the same dose of the drug potentiates cell killing by IuDR, MMC or IuDR + MMC, as measured by plating efficiency. These data, together with the inhibition of the increase of ligase activity, are consistent with the hypothesis that in CV1C11 cells CH inhibits a process of repair of IuDR or MMC-damaged DNA.

A partial purification of the enzyme was carried out by sedimentation of 48 hours post-treatment crude extracts, through 5-20 % sucrose gradients. Figure 2 shows the profiles of enzyme activity in each gradient fraction for the different samples studied. Control cells present a principal peak of enzyme activity, whose sedimentation value was calculated to be about 6.5 S and a higher peak with an S value of 5 S (Figure 2A). In SV40-infected cells, these

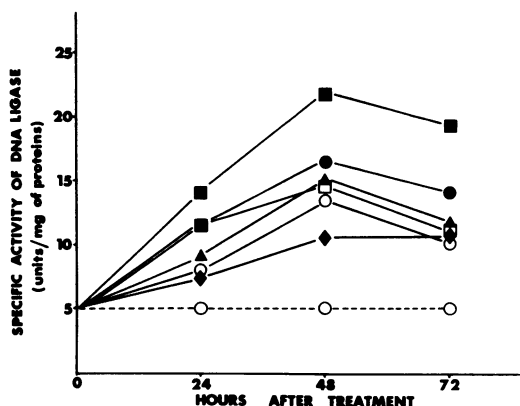


Figure 1 Kinetics study of DNA ligase activity (U/mg of proteins) in crude extracts from CV1C11 cultures, either in : controls (--- O ---), or in cells treated with : IuDR (100 µg/ml, 96 h : — □ —), MMC (1 µg/ml, 3 h : — ○ —), IuDR + MMC (100 µg/ml, 96 h + 1 µg/ml, 3 h : — ▲ —), SV40 (MOI 10 PFU/ cell : — ◆ —), MMC + SV40 (— ● —), IuDR + SV40 (— ■ —). Data are mean values of 6 experiments.

Table III

Samples	Ligase activity		Plating efficiency	
	- CH	+ CH	- CH	+ CH
Controls	5.1	4.6		83.0
IuDR	14.5	4.5	34.0	1.4
MMC	13.4	3.9	67.0	37.0
IuDR + MMC	15.0	5.3	29.0	1.0
SV40	10.5	4.6		
IuDR + SV40	21.6	6.1		
MMC + SV40	16.5	5.3		

Specific activity of DNA ligase (U/mg of proteins) in crude extracts from CV1C11 cultures, harvested 48 hours after IuDR or MMC treatment and/or infection with SV40 as described. CH (5 µg/ml) was added immediately after treatment and was present until the cultures were harvested. The effect of CH on survival of treated cells (plating efficiency) is expressed as % of colonies formed in the control untreated culture.

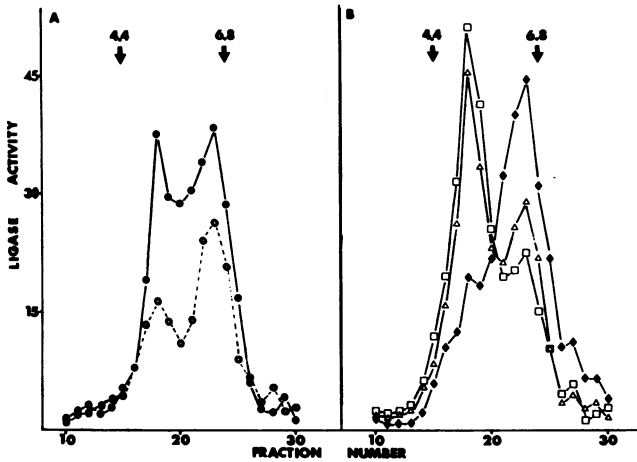


Figure 2 Partial purification of ligase in 5-20 % sucrose gradients. Ligase activity (U/mg of proteins) was determined in each fraction as described in Materials and Methods. 1. Control (--- O ---) ; 2. SV40 (MOI 10 PFU/cell : —◆—) ; 3. MMC (1 µg/ml, 3 h : —△—) ; 4. IUDR (100 µg/ml, 96 h : —□—) ; 5. Artificial mixture of MMC-treated + untreated SV40-infected crude extracts : —●—). Arrows indicate the S value of the following markers : bovin serum albumin (4.4), alkaline phosphatase (6.8). Gradients were collected from the top (40 fractions).

two peaks of ligase activity are also found but the heavy form has a two-times higher specific activity compared to controls (Figure 2B). The profiles of enzyme activity in IUDR or MMC-treated cells show a principal 5 S peak with about three times the ligase activity of the corresponding peak in the control. A smaller heavy peak is also seen, with the same value of enzyme activity as the 6.5 S peak of the control (Figure 2B). An artificial reconstruction experiment was carried out by mixing MMC-treated and untreated SV40-infected extracts. Two different peaks with approximately the same specific activity were detected in this sample : a light one corresponding to the 5 S peak of the controls and MMC-treated cells and a heavy one corresponding to the 6.5 S peak of the control and SV40-infected cells (Figure 2A). These data eliminate the possibility that the two forms of ligase are the consequence of a proteolytic activity converting a heavy into a light form of enzyme. The IUDR or MMC-treated SV40-infected samples show profiles of enzyme activity resembling those found in the artificial mixture of MMC-treated + untreated SV40-infected extracts (data not shown).

On the whole these results suggest that, in CV1C11 cells, two forms of

enzyme may exist and either one could predominate depending on : a) the physiological conditions of the cells ; b) the treatment to which they are submitted (see discussion).

DISCUSSION

In an attempt to better understand the mechanism(s) by which IUDR or MMC modify SV40 replication in CV1C11 cells (16, 25), we have studied how DNA ligase activity is affected by the drug treatment in virus-infected and mock-infected cells. The results have shown that :

1. The level of enzyme activity is about two to three times higher in IUDR or MMC-treated non-infected cells as compared to untreated cells.
2. When the cells are treated both with IUDR and MMC, there is no increase in the ligase activity compared to the results obtained with each drug alone.
3. In agreement with the results of other authors (12), SV40-infection enhances twice the level of ligase activity in CV1C11 cells.
4. There is an additive effect in the increase of ligase activity in IUDR or MMC-treated SV40-infected cells. This effect is reproducibly more important in IUDR-treated cells.
5. Two peaks of different S values are detected in a partial purification of the drug- or viral-induced enzyme(s).

Several cellular functions may be induced after treatment with agents which block DNA synthesis or give rise to DNA lesions such as IUDR or MMC. Among these functions a de novo synthesis of DNA ligase may allow the treated cells to more rapidly complete DNA repair. In parallel studies carried out with our IUDR, MMC or IUDR + MMC-treated non-infected cells, it has been found that constant levels of DNA polymerase α , β and γ activities exist up to 72 hours following the end of the treatment (Wicker, personal communication). These data reveal a different mode of regulation between DNA ligase and DNA polymerizing activities after the cell treatment, as we have already reported for UV-irradiated cells (7). In contrast, in SV40 and polyoma-infected cells, polymerase α and ligase functions seems to be regulated in coordination (Wicker, personal communication and 12).

Our present and previous results (16-18) suggest that the increase of ligase activity observed in treated cells is not linked to a revival of semi-conservative DNA synthesis after removal of the drugs and that it requires a synthesis of new proteins which is inhibited by CH treatment. This strongly suggests a direct involvement of a ligating activity in a DNA repair process in CV1C11 cells, as previously postulated in other eukaryotic systems (8, 11).

In our experiments, the ligase activity detected in IUDR or MMC-pretreated virus-infected cells is identical or higher than the sum of the ligase activities induced by each treatment alone. This result suggests that drug treatment and SV40-infection induce different stimuli which could act on the regulation of two different ligase species. At present, there is no evidence that the increase in ligase activity observed in SV40 or Py-infected cells, is caused by the appearance of a viral coded enzyme (12, 13). Moreover, in the DNA sequence of the SV40 genome there is no room for an unidentified protein which could be a viral DNA ligase. The possibility exists that in mammalian cells different ligases are specifically involved in DNA replication and DNA repair processes. In fact, evidence for the existence of two different DNA ligases (ligase I and II) has been reported previously by several authors in the different cellular systems (14-15, 29-31) : DNA ligase I could be associated with the replication process (15, 29) and DNA ligase II does not appear to correlate with cell mitotic activity (9, 30-31) and recently it has been reported that its activity is increased after the treatment of mouse cells with alkylating agents (32).

These findings together with our data of the sucrose gradients, provide evidence for the presence in untreated-infected and treated-uninfected samples of two different DNA ligase activities : a heavy form closely related to DNA replication and a light form concerned with DNA repair processes in the cells.

Finally, from the data presented in this paper a tempting hypothesis would be that the enhancement by the drugs of SV40 replication in CV1C11 cells and other cellular systems (16, 18, 24) is the consequence of an increased activity of some cell coded enzymes (such as DNA ligase) involved in DNA synthesis and DNA repair.

Experiments are in progress to : 1. purify the DNA ligase(s) from CV1C11 drug-treated or SV40-infected cells in order to establish the existence, or not, of two different enzymes ; 2. determine the role and the nature of the cellular factors that activate the viral replication apparatus.

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