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# Comparison of Endonuclease-Sensitive Sites by T4 Endonuclease V and UvrABC Nuclease Treatments Followed by Formamide or Sodium Hydroxide Denaturation

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**Endonuclease-sensitive sites detected by T4 endonuclease V or UvrABC nuclease treatments were compared in the dihydrofolate reductase gene of UV-irradiated Chinese hamster ovary B-11 cells. The number of endonuclease-sensitive sites detected by T4 endonuclease V treatment followed by NaOH denaturation was twice that of formamide denaturation. Repeated treatment of damaged genomic DNA with T4 endonuclease V resulted in no further increase in the number of endonuclease-sensitive sites detected. The numbers of endonuclease-sensitive sites detected by UvrABC nuclease using each denaturation condition were similar. Sequential treatment with the two endonucleases using formamide denaturation resulted in twice the number of endonuclease-sensitive sites detected by treatment of each nuclease alone. Due to a lack of AP endonuclease activity these results suggest the presence of T4 endonuclease V-sensitive sites which could be complemented by alkaline gel separation or by UvrABC nuclease treatment.**

**Keywords:** Endonuclease-sensitive sites, T4 endonuclease V, UV-irradiated DNA, UvrABC nuclease.

## Introduction

UV-light has been used extensively as a DNA damaging agent for DNA repair and damage studies. Two major principal photoproducts in UV (254 nm)-irradiated DNA are pyrimidine dimer (PD) and pyrimidine-pyrimidone (6-4). The relative induction of both of these products has a wide variation (Mitchell *et al.*, 1991). The importance of measuring DNA damage/repair in specific gene regions rather than at the overall genome level has been emphasized along with development of methods. For the measurement of DNA damage, single-strand breaks (SSB) due to a substrate-specific endonuclease with specific subsequent denaturing conditions have been intensively used (Tang *et al.*, 1994). The two best known endonucleases for these types of study are T4 endonuclease V (T4 endo V) and UvrABC nuclease.

T4 endo V is a small (18 kDa), PD-specific enzyme with a distinct sequential two-step cleavage reaction of glycosylase and apyrimidinic (AP) endonuclease activities. These features provide an endonucleolytic cleavage at PD sites (Haseltine, 1983). However, these two reactions are frequently uncoupled so that many uncleaved alkali-labile AP sites are generated after the T4 endo V treatment of UV-irradiated DNA (McMillan *et al.*, 1981; Nakabeppu and Sekiguchi, 1981). UvrABC nuclease is composed of three large components (UvrA: 114 kDa, UvrB: 84 kDa, and UvrC: 74 kDa) and acts by making dual incisions around a DNA adduct and subsequently releasing a 12–13 base-long oligonucleotide containing the adduct. UvrABC nuclease is effective over a broad range of DNA damage and recognizes a conformational distortion in the secondary structure of double-stranded DNA (Sancar, 1996).

Whether the broad spectrum of substrate-specificity of UvrABC covers all of the endonuclease sensitive sites (ESS) of T4 endo V is controversial. In the meantime, the possibility that T4 endo V and UvrABC incise different photoproducts, even though the numbers of ESS due to T4 endo V and UvrABC treatments are fortuitously the same, was discussed (Tang *et al.*, 1994). In this study, based on a quantification of ESS, the substrate specificities of T4 endo V and UvrABC were compared in the 5′-end region (14 kb) of the dihydrofolate reductase (DHFR) gene of UV-irradiated Chinese hamster ovary (CHO) B-11 cells.

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## Materials and Methods

**Cell culture and UV-irradiated genomic DNA** CHO B-11 cells carrying multiple copies of the DHFR gene were grown in Ham's F-12 medium (Gibco BRL, Gaithersburg, USA) supplemented with 10% dialyzed fetal calf serum. Selection pressure for amplification of the DHFR gene was maintained using 500 nM methotrexate in dimethylsulfonate. At approximately 70% confluence, the cells were irradiated using a Westinghouse G15T8 germicidal lamp at an incident rate of 0.8 J/m$^2$/s. The genomic DNA was completely digested overnight at 37°C with the restriction endonuclease Asp718.

**T4 endonuclease V and UvrABC nuclease treatment** T4 endo V was prepared by the method of Friedberg *et al.* (1980). Asp718-digested DNA (3 mg) was incubated with T4 endo V (0.1 nmol was designated as 1× T4 endo V) and 3 ng of an internal standard of linearized pBR322 DNA in 200 ml of a high salt reaction buffer (100 mM NaCl, 1 mM EDTA, and 5 mM Tris, pH 7.7) at 37°C for 60 min. The UvrABC nuclease complex was prepared by the method of Tang *et al.* (1994). Equal amounts of UvrA, UvrB, and UvrC proteins (2.6 pmol of each was designated as 1× UvrABC) were added to a sample DNA (3 mg) with an internal standard pBR322 (3 ng) in 200 ml of reaction buffer (50 mM Tris, pH 7.5, 100 mM KCl, 1 mM ATP, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The reaction mixtures were incubated at 37°C for 60 min. After nuclease treatment, DNA was purified by phenol/chloroform extraction and ethanol precipitation. For repeated and sequential nuclease treatments, the first incubation of nuclease was followed by DNA purification prior to the second incubation.

**DNA denaturation and gel electrophoresis** DNA was denatured by either formamide pretreatment or NaOH treatment during gel electrophoresis. Fresh deionized formamide (90%, v/v) was added to nuclease-treated DNA and the mixtures were incubated at 37°C for 60 min (Tang *et al.*, 1994). Immediately after incubation, the mixtures were electrophoresed at 5 V/cm for 3 h in a 0.5% agarose horizontal gel in TBE buffer (50 mM Tris, pH 7.9, 50 mM borate, and 5 mM EDTA) containing 0.5 mg/ml of ethidium bromide. For NaOH gel electrophoresis, the sample was loaded onto a 0.5% agarose gel containing 30 mM NaOH and 1 mM EDTA (Bohr *et al.*, 1985). Southern blot hybridization with $^{32}$P-labeled random primed probes of pMB5 and quantification of autoradiograms followed the method of Tang *et al.* (1994).

## Results & Discussion

**NaOH effect on T4 endo V treated DNA** Based on the fact that formamide denatures double-stranded DNA completely (Tang *et al.*, 1994), and that NaOH denaturation produces nicks at AP sites (Ross and Tang, 1985), it is important to know whether UvrABC can recognize most of the T4 endo V-sensitive sites. T4 endo V treatment followed by NaOH denaturing conditions (Fig. 1A) resulted in a lower intensity of the 14 kb fragments of the DHFR gene than did the treatment followed by formamide denaturing conditions. However, UvrABC treatment followed by either set of denaturing conditions resulted in no difference due to the conditions.

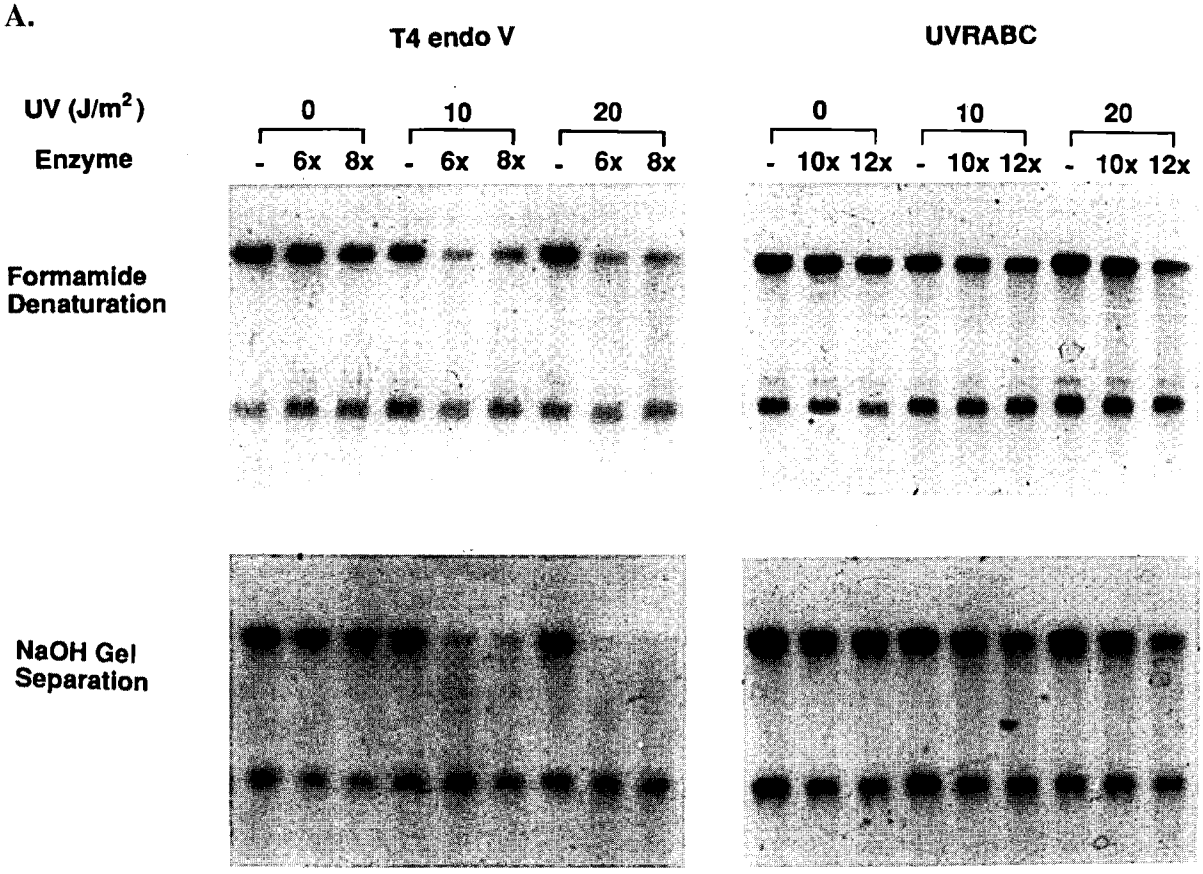
Densitometric analysis of autoradiograms (Fig. 1B) indicated that NaOH gel separation increased the number of ESS due to T4 endo V treatment to twice the number resulting from neutral formamide denaturation. There was, however, no effect on the ESS number due to UvrABC treatment. These data suggest that T4 endo V treatment of damaged DNA produces alkali-labile AP sites as well as SSB. AP sites were caused by glycosylase activity without coupling the AP endonuclease activity of T4 endo V, which could not be detected as an SSB in neutral formamide denaturing conditions, but could be detected in NaOH denaturation conditions. Because of the dual incision (nick) due to DNA phosphodiesterase activity (Sancar, 1996), ESS formation due to UvrABC treatment is independent of the denaturing conditions.

**Repeated and sequential nuclease treatments** Repeated treatment with T4 endo V using formamide denaturation showed the same level of ESS (0.89) as for a single treatment (0.89). Repeated treatment with T4 endo V followed by NaOH gel separation also showed a similar level of ESS (1.65) as for a single treatment (1.70). These data suggest that a lack of AP endonuclease activity is not caused by an insufficient amount of T4 endo V, but is probably due to preferred recognition sites. Repeated treatment by UvrABC showed a similar number of ESS (0.78) as did a single treatment (0.73), which excludes the possibility that the UvrABC activity observed in this study was incomplete or subsaturated.

Sequential treatment with both endonucleases using formamide denaturation doubled the number of ESS (1.60–1.66) compared with single treatments with each endonuclease (0.73–0.89). Furthermore, sequential treatment followed by NaOH denaturation showed similar levels of ESS (1.53–1.55) as for formamide denaturation. These results suggest that incomplete endonuclease activity due to T4 endo V can be complemented by UvrABC, or *vice versa*, because these endonucleases probably have some different incision sites.

This interpretation is consistent with the report of Nakayama *et al.* (1992) that a double mutant defective in both endonucleases is more sensitive than a single mutant defective only in UvrABC. A supplementation relationship between these two endonucleases was suggested by Bockrath *et al.* (1988), who reported that cells containing UvrABC activity transformed with T4 endo V require an even larger influence of UV-irradiation. For a short fragment of UV-irradiated DNA, it has been suggested that a broad specificity of UvrABC does not cover all of the T4 endo V sensitive-sites, even with an overall correspondence between the two nucleases (Tang *et al.*, 1994; Sancar, 1996). Therefore, there seem to be two sets of ESS due to T4 endo V treatment. The first set is

A.




B.

(unit: ESS/14 kb)

| Denaturing Condition | T4 endo V | | UvrABC | |
|---|---|---|---|---|
| | 10 J/m$^2$ | 20 J/m$^2$ | 10 J/m$^2$ | 20 J/m$^2$ |
| Formamide | 0.45 | 0.89 | 0.45 | 0.73 |
| NaOH | 0.79 | 1.70 | 0.44 | 0.72 |

**Fig. 1.** Measurement of endonuclease-sensitive sites due to T4 endonuclease V and UvrABC nuclease treatment followed by formamide or NaOH denaturing conditions. A. Damaged genomic DNA of CHO B-11 cells at two dosages of UV-irradiation (10 and 20 J/m$^2$) was treated using two levels of each nuclease (6× and 8× for T4 endo V, 10× and 12× for UvrABC). Controls were: no UV-irradiated DNA (0) and no nuclease treatment (−). B. Densitometric quantification of endonuclease sensitive sites in a 14 kb band of the DHFR gene and normalization with a 4.4 kb band of linearized pBR322 as an internal standard were performed using a Poisson distribution equation.

**Table 1.** Repeated and sequential treatments of 20 J/m$^2$ UV-irradiated genomic DNA of CHO B-11 cells with T4 endo V and UvrABC nucleases followed by formamide or NaOH denaturing conditions.

(unit: ESS/14 kb)

| Denaturing Condition | Nuclease Treatments | | | |
|---|---|---|---|---|
| | T4/T4 | ABC/ABC | T4/ABC | ABC/T4 |
| Formamide | 0.89 | 0.78 | 1.66 (0.79) | 1.60 (0.69) |
| NaOH | 1.65 | N.D. | 1.55 | 1.53 |

( ): 10 J/m$^2$ UV-irradiation; N.D.: not determined;
T4: T4 endonuclease V; ABC: UvrABC nuclease.

completely processed by a two-step reaction of T4 endo V producing SSB, which is then separated by either NaOH or formamide denaturing conditions. The second set is processed incompletely only by the DNA glycosylase activity of T4 endo V and becomes AP sites, which are not detected as SSB by formamide denaturing conditions, but are detected either by NaOH gel separation or by sequential UvrABC treatment.

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