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General method for quantifying base adducts in specific mammalian genes

(*Escherichia coli* UvrABC excision nuclease/psoralen/*cis*-Pt(II)/4-nitroquinoline oxide/Ha-*ras*)

DAVID C. THOMAS*, ANDREW G. MORTON*, VILHELM A. BOHR†, AND AZIZ SANCAR*

*Department of Biochemistry, CB 7260, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7260; and †Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **A general method has been developed to measure the formation and removal of DNA adducts in defined sequences of mammalian genomes. Adducted genomic DNA is digested with an appropriate restriction enzyme, treated with *Escherichia coli* UvrABC excision nuclease (ABC excinuclease), subjected to alkaline gel electrophoresis, and probed for specific sequences by Southern hybridization. The ABC excinuclease incises DNA containing bulky adducts and thus reduces the intensity of the full-length fragments in Southern hybridization in proportion to the number of adducts present in the probed sequence. This method is similar to that developed by Bohr *et al.* [Bohr, V. A., Smith, C. A., Okumoto, D. S. & Hanawalt, P. C. (1985) *Cell* 40, 359–369] for quantifying pyrimidine dimers by using T4 endonuclease V. Because of the wide substrate range of ABC excinuclease, however, our method can be used to quantify a large variety of DNA adducts in specific genomic sequences.**

It is becoming increasingly clear that DNA damage by carcinogenic and chemotherapeutic agents is not distributed randomly in the mammalian genome. Similarly, it appears that the location of the DNA adducts greatly influences their rate of repair (1, 2). In recent years methods of various degrees of resolution have been developed to study the distribution of DNA damage and repair at a subgenomic level (3–8). In one of these methods, which is specific for pyrimidine dimers, the DNA is digested with a restriction enzyme, and the parental DNA is isolated and treated with phage T4 endonuclease V to generate strand breaks at pyrimidine dimer sites. This digest is then analyzed by denaturing Southern gels and probing for specific sequences of interest (8). Using this ESS (enzyme-sensitive sites) assay, Bohr *et al.* (9) demonstrated that rodent cells [Chinese hamster ovary (CHO) line], which remove dimers inefficiently compared to human cells, repaired an actively transcribing, essential gene, encoding dihydrofolate reductase (DHFR), as efficiently as did human cell lines. They also found that genes were repaired more efficiently when actively transcribed (10, 11). Recently, it was shown that the preferential repair of dimers in the *DHFR* gene in CHO cells is due to the nearly exclusive repair of the transcribed strand (12).

Here we describe the most general method to date for quantifying adducts in specific genes. This approach is similar to the method of Bohr *et al.* (8), but by using *Escherichia coli* UvrABC excision nuclease (ABC excinuclease) instead of the dimer-specific T4 endonuclease V, we have greatly expanded the spectrum of adducts that can be quantified. This enzyme incises DNA damaged by many dissimilar (but mainly bulky) agents (13) and, therefore, can be used to detect adducts made by the majority of carcinogens and chemotherapeutic drugs. The excision nuclease has been shown to remove adducts caused by benzo[*a*]pyrene, mitomycin C, psoralens, 4-nitroquinoline oxide (4-NQO), platinum drugs, doxorubicin, nitrogen and sulfur mustards, nitrous acid, polycyclic aromatic hydrocarbons, carbodiimide, and both pyrimidine dimers and pyrimidine(6–4)pyrimidone photoproducts (14). In this study we have used ABC excinuclease to quantify DNA adducts induced *in vitro* by UV, psoralen, *cis*-[(±)-*trans*-1,2-diaminocyclohexane]dichloroplatinum(II) [*cis*-$PtCl_2$(dach)], and 4-NQO in the human homologue of the Ha-*ras* oncogene and the human β-globin gene. The enzyme was also used to measure repair of UV photoproducts in the *DHFR* gene of CHO cells *in vivo*. When compared to parallel measurements made with T4 endonuclease V, it is clear that ABC excinuclease is very suitable for repair measurements of adducts in specific sequences.

MATERIALS AND METHODS

Materials. Tritiated 4′-hydroxymethyl-4,5′,8-trimethylpsoralen ([^{3}H]HMT; 10 Ci/mmol; 1 Ci = 37 GBq) was purchased from HRI Associates (Emeryville, CA), tritiated *cis*-$PtCl_2$(dach) (1.2 Ci/mmol) was obtained from Steve Chaney (University of North Carolina), and diacetyl-4-hydroxyaminoquinoline 1-oxide was a gift of I. Walker (University of Western Ontario). The DNAs used as probes were obtained from the following sources: human Ha-*ras* homologue (*HRAS*) and human β-globin gene from pT24-C3 (15) and pSB64B6 (16), respectively, and the CHO *DHFR* probe from pMB5 (17). The ABC excinuclease and T4 endonuclease V were purified as described (18, 19).

Methods. High molecular weight DNA from human lymphocytes was isolated essentially by the method of Bell *et al.* (20). The DNA was digested with *Bam*HI (5 units/μg of DNA) and then subjected to damaging treatments. DNA containing UV photoproducts was prepared by irradiating 50-μl droplets with 254-nm light at 20–200 J/m^2 from a General Electric germicidal lamp at a rate of 0.1 $Jm^{-2}\cdot sec^{-1}$. The photoproducts were quantified by the transformation assay with pBR322 that was irradiated under identical conditions (21). DNA containing 4-NQO adducts was prepared by the method of Panigrahi and Walker (22). The 4-NQO adducts were quantified by reading the absorbance of the treated DNA at 354 nm and using $\varepsilon = 14.4 \times 10^3\ M^{-1}\cdot cm^{-1}$. DNA modified with HMT was prepared by incubating DNA (160 μg/ml) with various amounts of [^{3}H]HMT, first in the dark for 30 min and then in 366-nm light at 5 mW/cm^2 for 10 min with Sylvania F8/BLB black light as the light source. *cis*-$PtCl_2$(dach)-modified DNA was prepared by incubating

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Abbreviations: 4-NQO, 4-nitroquinoline oxide; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; *cis*-$PtCl_2$(dach), *cis*-[(±)-*trans*-1,2-diaminocyclohexane]dichloroplatinum(II); ESS, enzyme-sensitive sites; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; ABC excinuclease, *E. coli* UvrABC excision nuclease.

tritiated *cis*-$PtCl_2$(dach) at 4 μM with DNA at 160 μg/ml in 10 mM Tris·HCl, pH 8.0/1 mM EDTA (TE buffer) for 16 hr at 37°C. The frequencies of psoralen or *cis*-$PtCl_2$(dach) adducts were determined by quantifying the radioactivity covalently associated with DNA. DNA damaged *in vivo* with UV was obtained from the CHO cell line CHO-B11, which contains an amplified *DHFR* gene (8).

Treatment of DNA with ABC Excinuclease. The DNAs were treated with ABC excinuclease in a 40-μl reaction mixture containing 50 mM Tris·HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 10 mM dithiothreitol, 5 μg of DNA, and 4 μg each of the UvrA, UvrB, and UvrC subunits, unless otherwise specified. The reaction mixture was incubated at 37°C for 15 min, and the reaction was stopped by the addition of 2 μl of proteinase K (25 μg/ml of 5% sodium dodecyl sulfate) and incubated at 37°C for 1 hr. The proteinase K-treated samples were then dialyzed against TE buffer in a microdialysis apparatus (Health Products, Rockford, IL), placed in 100 mM NaOH/1 mM EDTA/2.5% Ficoll/0.025% bromocresol green (final concentrations), and incubated 20 min at room temperature before alkaline electrophoresis.

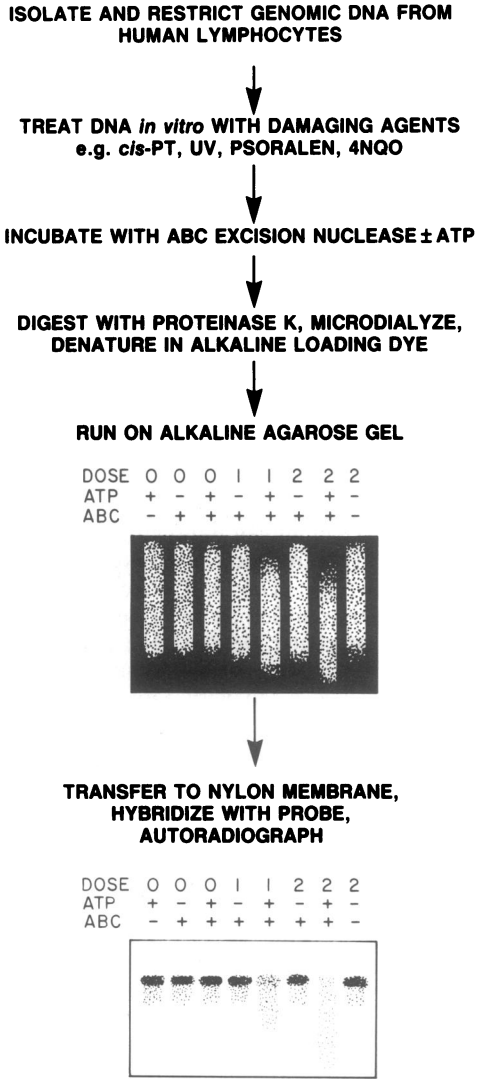

FIG. 1. Scheme for measuring DNA adducts in specific genomic sequences with ABC excinuclease (ABC). This scheme is for the reconstruction studies conducted in this paper. For studying DNA repair, steps 1 and 2 should be reversed. The schematic drawings are for the appearances of an ethidium bromide-stained gel (upper gel) and for an autoradiogram of the same gel after probing with a specific sequence (lower gel). *cis*-Pt, *cis*-$PtCl_2$(dach).

Analysis of Incision on Alkaline Agarose Gels. The samples were loaded onto a 0.6% agarose gel in 30 mM NaOH/1 mM EDTA, run, and processed as described (8). ESS were quantified after transfer and hybridization either by densitometry of the autoradiograms or by excising the appropriate part of the nylon membrane and determining radioactivity directly by scintillation counting, both techniques yielding similar results. The number of ESS and therefore the number of adducts incised were calculated from the zero term of the Poisson distribution, ESS = $\ln P_0$, where P_0 is the fraction of DNA that remains unincised. In using this calculation for ABC excinuclease, two corrections need to be made. First, one needs to account for the nonspecific incisions made by this enzyme. These ranged from 0 to 0.03 incision per kbp in the experiments presented here. Second, the efficiency of the enzyme cutting is <100% and depends on the amount of DNA in the reaction mixture. Under the conditions reported here, we find this efficiency to be reproducibly about 30% with 5 μg of DNA and about 50% with 1 μg of DNA in the reaction mixture.

RESULTS

Methods for Measuring Adducts in Specific Genomic Sequences. To establish the utility of ABC excinuclease as a probe for many DNA damaging agents of dissimilar structures (13), we first conducted reconstruction experiments using purified human DNA that was damaged by various agents to predetermined levels. In Fig. 1 we summarize our overall experimental protocol for these reconstruction experiments. Briefly, DNA was isolated from human lympho-

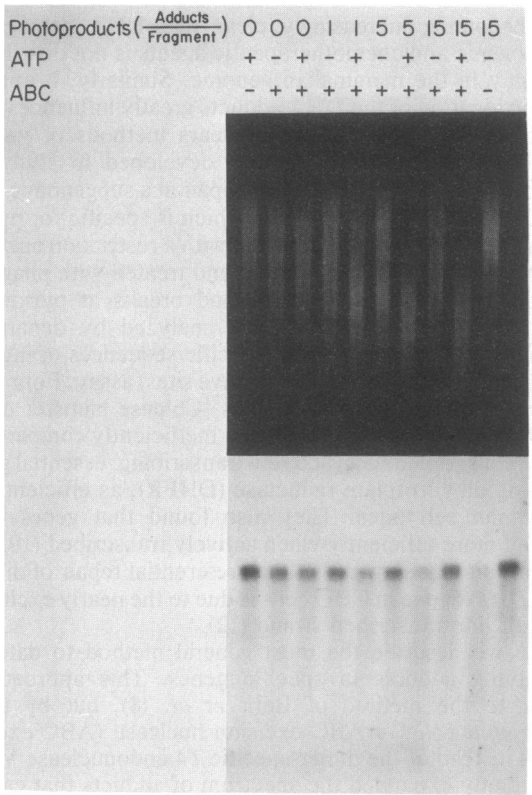

FIG. 2. Quantification of UV photoproducts in human Ha-*ras* by ABC excinuclease (ABC). Five micrograms of *Bam*HI-digested human DNA containing the indicated number of photoproducts was incubated with ABC excinuclease and the fragments were separated on a 0.6% alkaline agarose gel. (*Upper*) Photograph of ethidium bromide-stained gel. (*Lower*) Autoradiogram of a Southern blot of the same gel probed with Ha-*ras*. The indicated modification levels were determined independently as described.

cytes and digested with a restriction enzyme, damaged with the appropriate treatments, and then incubated with ABC excinuclease. After deproteinization, the DNA was separated on an alkaline agarose gel, transferred to a nylon support membrane, and then probed with a sequence of interest. For most of the studies reported here we probed for the Ha-*ras* gene, which has been implicated in a number of cancers induced by chemical carcinogens (16). ABC excinuclease is a complex enzyme made up of three subunits that requires ATP for its function and does not turnover in the absence of additional proteins (23, 24). Under optimal conditions (5 μg of DNA and 4 μg of each subunit in a 40-μl reaction mixture incubated 15 min at 37°C), only 30% of the adducts can be removed. This limitation is an artifact of *in vitro* conditions and is not due to the enzyme's recognizing a specific subset of total adducts, as similar limitations are seen when using defined substrates (25). Furthermore, we have shown that excision frequency is proportional to the base-modification level regardless of neighboring sequences (13).

Quantification of UV Photoproducts and Psoralen, *cis*-$PtCl_2$(dach), and 4-NQO Adducts in the Ha-*ras* Gene. We first conducted reconstruction experiments using human lymphocyte DNA that had been modified *in vitro* with several doses of UV, *cis*-$PtCl_2$(dach), HMT, or 4-NQO—all of which are well-characterized substrates for ABC excinuclease. The modified DNA was incubated with the enzyme and analyzed for incision in the Ha-*ras* gene by alkaline gel electrophoresis and subsequent Southern hybridization. The results are shown in Figs. 2–5. The average molecular weight of the DNA was decreased as a result of ABC excinuclease. The autoradiograms further show that the intensity of the signal [a 6.6-kilobase-pair (kbp) *Bam*HI fragment] decreased as a function of modification level in ABC excinuclease-treated samples. We also probed the 1.9-kbp *Bam*HI fragment carrying part of the human β-globin gene (Fig. 4 *Bottom*); at

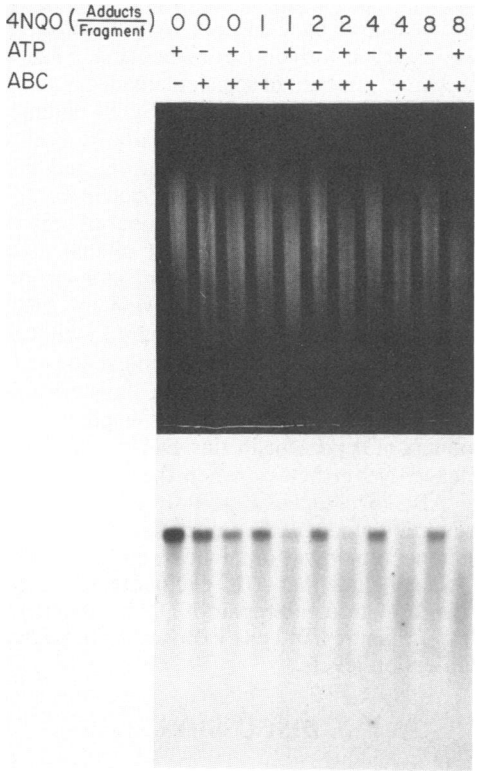

FIG. 3. Incision of human DNA containing 4-NQO adducts by ABC excinuclease (ABC). *Bam*HI-digested DNA was treated with 4-NQO and processed as in Fig. 2. (*Upper*) Photograph of ethidium bromide-stained gel. (*Lower*) Autoradiogram of the same gel after probing with Ha-*ras*.

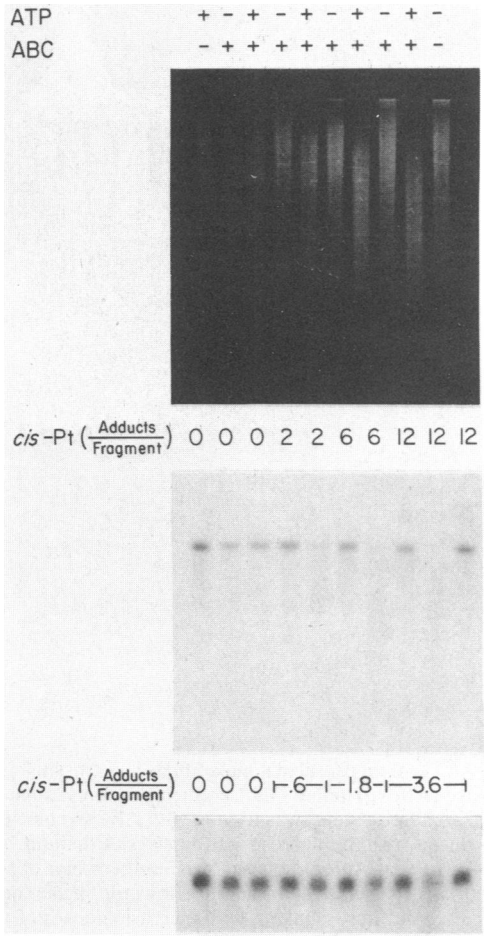

FIG. 4. Incision of human DNA containing *cis*-$PtCl_2$(dach) (*cis*-Pt) adducts by ABC excinuclease (ABC). *Bam*HI-digested DNA containing adducts at the indicated frequencies was treated with ABC excinuclease, and fragments were separated on an alkaline gel. After transfer to a nylon support membrane, fragments were first probed with the Ha-*ras* gene, autoradiographed, deprobed, and then probed with the human β-globin gene. (*Top*) Ethidium bromide-stained gel. (*Middle*) Autoradiogram of Ha-*ras*-probed gel (fragment size is 6.6 kbp). (*Bottom*) Autoradiogram of β-globin gene-probed gel (fragment size is 1.9 kbp). Note the appearance of some crosslinks at the higher doses.

higher levels of modification, adducts in this relatively small fragment could be quantified, allowing relatively fine measurements of damage distribution and repair in narrow regions surrounding and within genes.

At high doses of *cis*-$PtCl_2$(dach) and HMT, interstrand crosslinks were formed, giving rise to a higher molecular weight DNA band that corresponded to the Ha-*ras* duplex on alkaline gels (Figs. 4 and 5). Upon treatment with ABC excinuclease, the intensities of the main 6.6-kbp band (which contained monoadducts only) as well as that of the higher molecular weight band (which contained both interstrand crosslinks and some monoadducts) decreased. At high modification levels, the decrease in intensity of the crosslinked DNA could be due to incisions either at crosslinks or at monoadducts in the crosslinked molecular subpopulation. However, at low modification levels (about one adduct per fragment), the probability of the crosslinked molecules containing a second adduct is low; therefore, the decrease in intensity of these bands is mainly due to incision of ABC excinuclease at interstrand crosslinks (24).

The number of incisions made by ABC excinuclease was calculated from the decrease in Ha-*ras* and β-globin gene band intensities in Figs. 2–5. These values were plotted as a

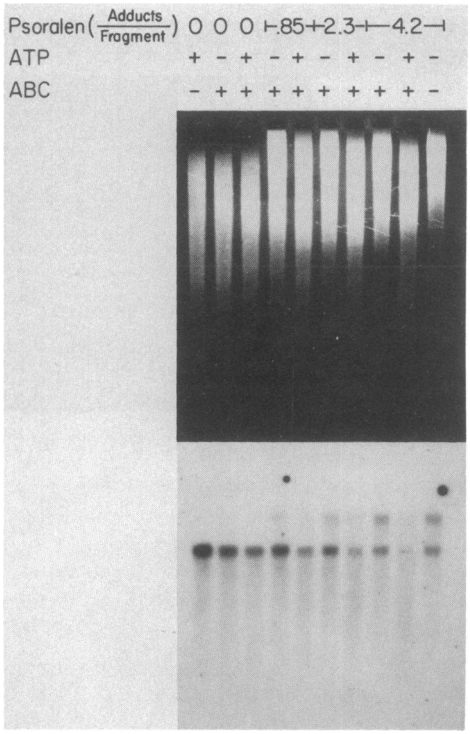

FIG. 5. Incision of psoralen monoadducts and crosslinks by ABC excinuclease (ABC). Human DNA containing the psoralen adducts at the indicated frequencies was treated with ABC excinuclease and analyzed on an alkaline agarose gel. (*Upper*) Ethidium bromide-stained gel. (*Lower*) Autoradiogram of a Southern blot of the same gel probed with Ha-*ras*. Note the shift towards higher molecular weight as a result of crosslinking with increasing doses of psoralen in *Upper*. In addition to the main Ha-*ras* band, a second band of lower mobility is evident in *Lower*. This band represents crosslinked Ha-*ras*, and the fraction of *ras* in this form increases with the number of total adducts (and, therefore, the average number of crosslinks).

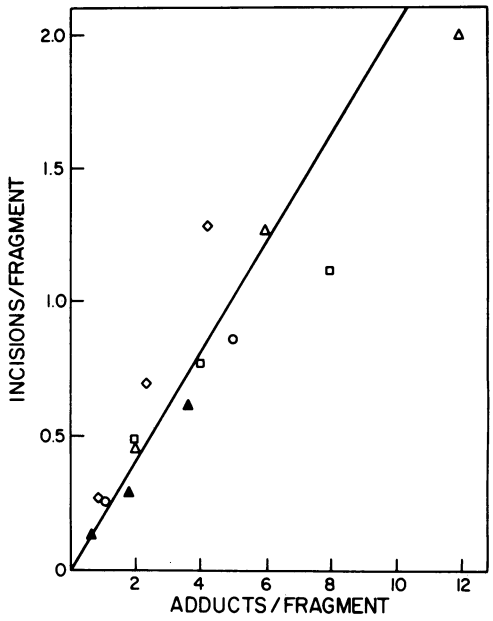

FIG. 6. Effects of the number and type of adducts on incision frequency of ABC excinuclease. The number of specific incisions made in the Ha-*ras* and β-globin genes as a function of the number of adducts per fragment carrying these genes is plotted. The number of adducts per fragment was determined as described in *Materials and Methods*. ○, UV photoproducts in Ha-*ras*; □, 4-NQO adducts in Ha-*ras*; △, *cis*-$PtCl_2$(dach) adducts in Ha-*ras*; ▲, *cis*-$PtCl_2$(dach) adducts in the β-globin gene; ◇, HMT adducts in Ha-*ras*.

function of adduct frequency per fragment to obtain Fig. 6. Two significant conclusions can be drawn from this figure. First, under our optimal conditions, ABC excinuclease incised the damaged DNA with only about 30% efficiency in both gene sequences. Therefore, to obtain the absolute number of adducts, the number of incisions needs to be multiplied by a factor of 3.3. Second, even though there was a slight variation in cutting efficiency depending upon the damaging agent (the psoralen adducts being cut the most efficiently), all bulky adducts appeared to be roughly equally susceptible to ABC excinuclease, suggesting that the standard curve we have generated with these four different damaging treatments may be generally applicable to other bulky lesions recognized by the enzyme, regardless of the sequence being measured (14).

Measurement of Differential Repair in CHO Cells by Using ABC Excinuclease. Having established the usefulness of ABC excinuclease for quantifying DNA adducts in reconstruction experiments, we proceeded to use it to study DNA repair in the *DHFR* gene of CHO cells. Like other rodent cells, these cells repair pyrimidine dimers poorly compared to human cells, yet they are not more sensitive to UV than human cells. Bohr *et al.* (8), using T4 endonuclease V as a probe, recently showed that the explanation for this paradox may be that CHO cells repair essential genes (such as *DHFR*) as efficiently as do human cells (≈70% in 24 hr) even though the overall repair of pyrimidine dimers was only 20% of that of human cells. We wished to confirm these conclusions using ABC excinuclease and to demonstrate the use of this enzyme for probing damage and repair in specific sequences. CHO-B11 cells (25) were UV-irradiated at 20 J/m^2 and allowed to repair for 0, 8, and 24 hr. DNA was isolated, and the dimers in the *DHFR* gene were quantified with ABC excinuclease or T4 endonuclease V. There was no significant decrease at 8 and 24 hr in the level of overall genome adducts as tested by both enzymes (Fig. 7 *Upper*). When the repair of the *DHFR* gene was measured by Southern hybridization, extensive repair was observed with both enzymes (Fig. 7 *Lower*). At all time points, the numbers of incisions made by ABC excinuclease were smaller than those made by T4 endonuclease V (Fig. 7). The numbers of incisions made by both enzymes were calculated from the data in Fig. 7, and a duplicate experiment and the results are summarized in Table 1. Under these experimental conditions, the number of incisions made by ABC excinuclease was about half of that made by T4 endonuclease V (which presumably cuts at every pyrimidine dimer). The higher efficiency of incision by ABC excinuclease seen in these experiments compared with those above is at least in part due to (*i*) the ability to use lower DNA and enzyme concentrations in this amplified system and also (*ii*) the fact that this enzyme recognizes a greater variety of photoproducts (13) present in this DNA than does the UV endonuclease. Nevertheless, when the number of incisions made by ABC excinuclease at different time points was compared to that made by the same enzyme at zero time, the same fractional repair was obtained as with T4 endonuclease V. Thus, we conclude that ABC excinuclease can be used to monitor the formation and repair of DNA adducts in specific sequences of mammalian cells exposed to biologically relevant modification levels.

DISCUSSION

Recent research indicates that mammalian DNA exposed to genotoxic agents is not damaged or repaired uniformly (1, 2), or both, and that these distributions may have important implications to cell survival and mutagenesis. During the past 5 years, a number of methods have been developed to quantitify DNA adducts in specific sequences by Southern

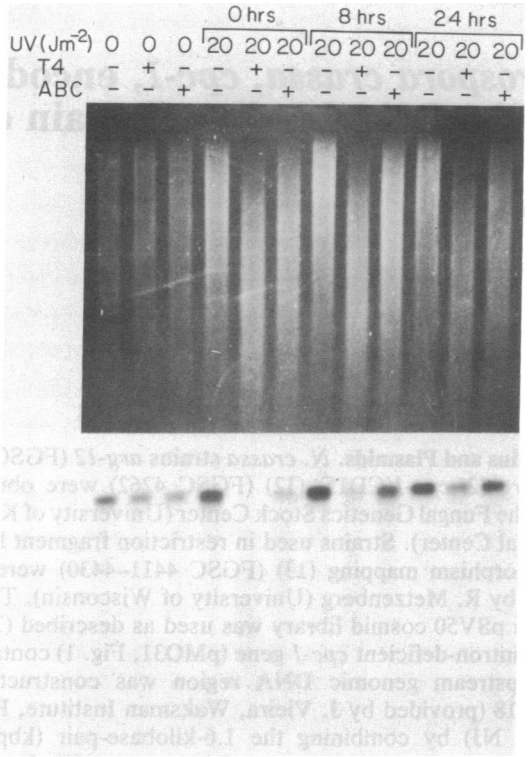

FIG. 7. Repair of UV photoproducts in *DHFR* of CHO-B11 cells as measured by T4 endonuclease V and ABC excinuclease. CHO cells were irradiated with 254-nm light at 20 J/m^2 and DNA was isolated from the cells and subjected to digestion by the repair enzymes after cleavage with *Kpn* I restriction endonuclease. For each lane 1 μg of DNA was digested with either 1 μl of T4 endonuclease V (equivalent to 1.5×10^{10} incisions per min at 37°C) or 1 μg each of the subunits (UvrA, UvrB, and UvrC) of ABC excinuclease for 15 min at 37°C. The samples were denatured and run on a 0.5% alkaline agarose gel, which was photographed after staining with ethidium bromide (*Upper*) and autoradiographed after probing with a *DHFR* probe specific for the 14.1-kbp-long *Kpn* I fragment originating from the 5′ terminus of the *DHFR* gene (*Lower*).

hybridization. Most of these methods are specific for one type of adduct (7, 8, 26–29). In this paper we describe a more general method to quantify DNA adducts in defined sequences in which the damaged DNA is treated with the *E. coli* ABC excinuclease, fragments are separated on an alkaline agarose gel, and specific sequences are probed by DNA blot hybridization. While the method is similar to that of Bohr *et al.* (8) the use of ABC excinuclease expands the scope of this method to measure the damage and repair of DNA treated with about a dozen agents already shown to create substrate for this enzyme and perhaps many more that have not yet been tested.

Table 1. Measurement of repair of UV photoproducts in *DHFR* of CHO cells

Time after UV, hr	T4 endonuclease V		ABC excinuclease	
	Incisions*	% repair	Incisions*	% repair
0	1.84	—	1.10	—
8	1.14	39.7	0.69	37.6
24	0.41	78.3	0.17	84.5

Cells were irradiated with 20 J/m^2. The values given in this table are the averages of two experiments.
*Number per 14.1-kbp *Kpn* I fragment carrying the 5′ part of *DHFR*.

Limitations of this assay are that the enzyme incise *DNA* at <100% efficiency and that most *DNA* damage caused by methylating agents *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and methyl methanesulfonate is not recognized by ABC excinuclease (29). Even with these limitations, we believe this is a most versatile assay to quantify DNA adducts in unique sequences and should enable us to conduct many studies regarding chemotherapy and chemical carcinogenesis that previously were not feasible for lack of a method with sufficient specificity, sensitivity, or generality.

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