Cockayne syndrome (CS) is a human disease characterized by sensitivity to sunlight, severe neurological abnormalities, and accelerated aging. CS has two complementation groups, CS-A and CS-B. The CSB gene encodes the CSB protein with 1493 amino acids. We previously reported that the CSB protein is involved in cellular repair of 8-hydroxyguanine, an abundant lesion in oxidatively damaged DNA and that the putative helicase motif V/VI of the CSB may play a role in this process. The present study investigated the role of the CSB protein in cellular repair of 8-hydroxyadenine (8-OH-Ade), another abundant lesion in oxidatively damaged DNA. Extracts of CS-B-null cells and mutant cells with site-directed mutation in the motif VI of the putative helicase domain incised 8-hydroxyadenine in vitro less efficiently than wild type cells. Furthermore, CS-B-null and motif VI mutant cells accumulated more 8-hydroxyadenine in their genomic DNA than wild type cells after exposure to γ-radiation at doses of 2 or 5 Gy. These results suggest that the CSB protein contributes to cellular repair of 8-OH-Ade and that the motif VI of the putative helicase domain of CSB is required for this activity.
mass spectrometry (LC/MS) with the isotope dilution techni-que (17).

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Cell lines were derived from CS1AN.S3.G2, a SV40-transformed human fibroblast in which CSB is disrupted. These cells were described previously (23). CS1AN.S3.G2 was transfected with pcDNA3.1 carrying the wild type CSB, mutant CSB altered in the putative helicase motif VI (Q942E and R946A) or the vector pcDNA3.1 (pc3.1) (Invitrogen) (Fig. 2). The reason we applied Q942E and R946A is that these cell lines are the most deficient ones in repair of 8-OH-Gua among the 8 stably transfected cell lines established in this laboratory with site-directed mutation(s), which are dis-tributed in various motifs of the helicase domain (22). Construction of the mutants and cell lines were described previously (22, 24).

Exposure was carried out as follows. Cells attached on 10-cm² dishes were washed with phosphate-buffered saline and then irradiated at the indicated doses with Gammacell 40 Exactor137Cs (Nordian NTB binding domain (25). Construction of the CSB gene product in 8-hydroxyadenine repair was described previously (23). CSBR946A is constructed by replacing an arginine residue highly conserved in the SNF2 family with alanine.

phosphate buffer (pH 8.0), and incubated with alkaline phosphatase (5 units) at 37 °C for 24 h. The sample was filtered by centrifugation at 6000 × g for 30 min through an ultrafiltration membrane with a molecular mass cutoff of 5 kDa (Millipore Corp., Bedford, MA). An aliquot (5 μl) of the filtered sample was analyzed by LC/MS and found to contain 8-OH-[15N5]dAdo on the basis of the previously reported LC/MS analysis of 8-OH-Ado (17). Preparative separation was used to isolate 8-OH-[15N5]dAdo from an irradiated and dephosphorylated sample of [15N5]dATP using a Supelcosil LC-8 DB reversed-phase column (25 × 1 cm inner diameter, 5-μm particle size) (Supelco, Bellefonte, PA). The solvents and the elution gradient were as previously described (17), except that a flow rate of 2 ml/min was used. The column was kept at room temperature. Under the experimental conditions used, 8-OH-[15N5]dAdo eluting at 18.5 min was completely separated from [15N5]dAdo, which eluted at 17.3 min. This is the same elution order previously described for the unlabeled analogues of these compounds using an analytical LC column (17). The fractions corresponding to 8-OH-[15N5]dAdo were collected. At least 30 injections of 100 μl were performed. Collected fractions were combined, dried in a SpeedVac under vacuum, and then dissolved in 200 μl of water. The absorption spectrum of the solution was recorded between the wavelengths of 210 and 350 nm. The spectrum was identical to the absorption spectrum of authentic 8-OH-dAdo (30).

Analytical LC/MS was carried out to confirm the identity of 8-OH-[15N5]dAdo and its purity. The isolated compound was pure and did not contain any detectable unlabeled 8-OH-dAdo. The elution time of 8-OH-[15N5]dAdo was the same as that of 8-OH-dAdo, and its mass spectrum was similar to that of 8-OH-dAdo (17). As expected, however, the masses of the typical ions of 8-OH-[15N5]dAdo were shifted by 5 Da to greater masses, i.e. m/z 157 (the protonated base ion (BH+)), 273 (the protonated molecular ion (MH+)), and 295 (the sodium adduct ion (MNa+)). The concentration of the solution of 8-OH-[15N5]dAdo was determined by UV spectrophotometry using the absorption coefficient of 12764 mol−1 cm−1 at 270 nm (30) and by LC/MS using the mass of 8-OH-dAdo as an internal standard. Both measurements yielded essentially identical results. The concentration of the solution of 8-OH-[15N5]dAdo was 0.037 ± 0.003 μM.

8-OH-dAdo was measured in DNA samples spiked with 2 pmol of 8-OH-[15N5]dAdo per 70 μg of DNA. The concentration of DNA samples was determined by UV spectrophotometry. DNA samples were hydrolyzed with nuclease P1, phosphodiesterase I, and alkaline phosphatase as described (17) and filtered by centrifugation at 6000 × g for 30 min using an ultrafiltration membrane with a molecular mass cutoff of 5 kDa. An aliquot of 20 μl of the filtered samples containing 20 μg of hydrolyzed DNA was injected on the LC column. Characteristic ions of 8-OH-dAdo at m/z 157 (BH+), 268 (MH+), and 280 (MNa+) and those of 8-OH-[15N5]dAdo at m/z 157 (BH+), 273 (MH+), and 295 (MNa+) were recorded during LC/MS analysis using the selected-ion-monitoring (SIM) mode at the retention time period, when these compounds eluted.

Statistics—Groups were compared using one-way analysis of variance tests. Duncan’s multiple range test was used for post-hoc comparison of means. Differences were considered significant when p < 0.05.

RESULTS

In this study, we investigated the possibility that CSB is involved in cellular repair of 8-OH-Ado, a major lesion in oxidatively damaged DNA. 8-OH-Ado repair was assessed and compared in wild type, CS-B-null, and putative CS-B helicase motif VI mutant cells. Glycosylase/apurinic lyase activity in CS-B cell lines was quantified by measuring incision of an oligonucleotide with a single 8-OH-Ado residue. Fig. 3A shows
the incision activity of wild type, CS-B-null, and two CS-B mutant cell lines, and the results are summarized in Fig. 3B.

Fig. 4, A and B show similar results, the only difference being that we used the substrate with C opposite the 8-OH-Ade lesion to possibly generate maximal nick-forming activity (25, 26). CS-B-null and mutant cell lines incise 8-OH-Ade less efficiently than wild type cells in both situations. The activity of CS-B-null cells was 3-fold lower than wild type cells (p < 0.05), and the activity of motif VI mutants (CSQ942E and CSBR946A) was 2-fold lower than wild type cells (p < 0.05). There were no differences in uracil or 5-hydroxycytocine incision of WCE of the tested cell lines (data not shown) (22).

The role of CSB in repairing 8-OH-Ade was also examined by measuring the level of 8-OH-Ade in DNA of cells exposed to γ-radiation at doses of 2 or 5 Gy. 8-OH-Ade was measured as its nucleoside 8-OH-dAdo in wild type, CS-B-null, motif VI mutant cells using LC/IDMS, a recently developed assay for identification and quantification of oxidatively modified DNA nucleosides (17). DNA samples isolated from cells were hydrolyzed to nucleosides by endo- and exonucleases. Prior to hydrolysis, an aliquot of 8-OH-[15N5]dAdo was added as internal standard to the DNA samples. LC/IDMS was carried out in SIM mode to monitor the characteristic ions of 8-OH-dAdo and 8-OH-[15N5]dAdo at the appropriate retention time period, when these compounds eluted. The BH2, MH+, and MNa+ ions of both compounds were simultaneously recorded. As expected, no difference between the retention times of these analogues was observed. Fig. 5 shows the ion-current profiles at m/z 152 (BH2+), m/z 290 (MNa+) of 8-OH-dAdo and at m/z 157 (BH2+), m/z 295 (MNa+) of 8-OH-[15N5]dAdo, recorded during the LC/IDMS-SIM analysis of the enzymic hydrolysate of a DNA sample isolated from CSBQ942E cells following γ-radiation at 2 Gy. The results showed unequivocal identification of 8-OH-dAdo in DNA from all cell lines used in this study. The quantification was achieved by the integration of the signals of the monitored ions such as those in Fig. 5 and the calculation of the level of 8-OH-dAdo on the basis of the known amount of 8-OH-[15N5]dAdo added to the DNA samples as an internal standard prior to enzymic hydrolysis.

Fig. 6 shows the level of 8-OH-dAdo in cells following exposure to ionizing radiation. Cells were irradiated and allowed 30 min to recover and repair radiation-induced DNA damage. The level of 8-OH-dAdo was similar (~0.7 molecules/10⁶ DNA nucleosides) in genomic DNA of non-irradiated cells regardless of genotype. No change in the 8-OH-dAdo level was observed in γ-irradiated wild type cells, indicating complete and rapid re-

### Table I

| Oligonucleotide* | Specificities |
|-----------------|--------------|
| 5′-GCTCTAGGCC(8-OH-Ade)AGCTTGATCTGCCAGTT-3′ | 8-OH-Ade containing substrate |
| 5′-GCTCTAGGCCCTGATCTGCCAGTT-3′ | Normal A-T pair for control |
| 5′-AACTGGCACATCAAGCTTGGCCTAGGC-3′ | Complement strand for 8-OH-Ade-T pair |
| 5′-AACTGGCACATCAAGCTGCGCCCTAGGC-3′ | Complement strand for 8-OH-Ade-C pair |

*Oligonucleotides were prepared by Midland Certified Reagent Co., Midland, TX.
pair of this lesion. The time of complete repair within 30 min is in agreement with the recently reported repair kinetics of 8-OH-Ade in human cells (18). In contrast, significantly greater levels of 8-OH-dAdo were observed in /H9253-irradiated CS-B-null and motif VI mutant cells. In motif VI mutants, exposure to /H9253-radiation at 5 Gy followed by a 30-min incubation resulted in a higher level of 8-OH-dAdo than exposure to 2 Gy. These results demonstrate that 8-OH-dAdo accumulates in a dose-dependent manner in irradiated mutant cells but does not accumulate in irradiated wild type cells.

**DISCUSSION**

Previous studies have shown that mutations in CSB cause a deficiency in cellular repair of 8-OH-Gua (22). The present study shows that CS-B-null and motif VI mutant cells are also deficient in incision of 8-OH-Ade. Consistent with this observation, 8-OH-Ade accumulates more in genomic DNA of CS-B-null and motif VI mutant cells than in wild type cells following exposure of cells to γ-radiation at low doses. These results suggest that CS-B-null and motif VI mutant cells are deficient in cellular repair of 8-OH-Ade. This in turn indicates that CSB plays an important role in cellular repair of 8-OH-Ade and that the putative helicase motif VI of CSB is important for this DNA repair function.

CSB is highly homologous to proteins of the SWI/SNF2 family (2, 3, 31). SWI/SNF proteins participate in a wide variety of cellular functions including DNA repair, regulation of transcription, maintenance of chromosome stability, and chromatin remodeling (5, 31–33). SWI/SNF2 proteins contain seven highly conserved motifs for DNA or RNA helicase activity (2, 32), but so far, none has been shown to have this function. Previous experiments were carried out to characterize the cellular response of various CS-B mutant cell lines to different challenges (Table II) (22, 24, 34). We have also mapped the CSB putative helicase motifs and determined their role in cellular repair of 8-OH-Gua (Table II). Those experiments showed that motifs V and VI are essential for the cellular response to oxidative stress and for cellular repair of 8-OH-Gua in genomic DNA. The results presented here show that CSB motif VI also plays an important role in cellular repair of 8-OH-Ade.

It is not clear what mechanism underlies the role of CSB in the repair of 8-OH-Gua and 8-OH-Ade. The enzymes involved specifically in repair of 8-OH-Ade have not yet been identified (reviewed in Ref. 19), but some evidence suggests that repair of 8-OH-Ade is mechanistically different from repair of 8-OH-Gua. Among the bacterial and mammalian DNA glycosylases, which were investigated for their activity on lesions in oxidatively damaged DNA, only *Escherichia coli* formamidopyrimidine glycosylase (Fpg) exhibited a low activity on 8-OH-Ade in DNA containing multiple modified bases (35, 36). However, this activity was insignificant when compared with the activity of Fpg on 8-OH-Gua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 4,6-diamino-5-formamidopyrimidine (FapyAde), which are the principal substrates of Fpg. The inactivity of Fpg was suggested to result from the absence of a C6-keto group in 8-OH-Ade (37). A recent study on the cellular repair of modified DNA bases showed that 8-OH-Ade is efficiently repaired in human cells with kinetics similar to the repair kinetics of pyrimidine-derived lesions rather than to...
that of purine-derived lesions (18). This suggests that human cells possess enzyme(s) to repair 8-OH-Ade. However, it is not known whether this repair activity involves BER or NER, or both. A yeast functional homolog of E. coli Fpg encoded by the OGG1 gene of Saccharomyces cerevisiae (yOgg1) was shown to excise 8-OH-Gua and FapyGua, but not FapyAde or 8-OH-Ade (38, 39). Human homologues of yOgg1 were recently isolated (reviewed in Ref. 40). Two polymorphic forms of hOgg1 namely 

| Mutated motif (Fig. 1) | Cellular sensitivities (22, 24, 35) | RNA recovery after UV (24, 35) | Glycosylase/apurinic lyase activity in WCE (22) | Levels of 8-OH-dGua in DNA after γ-ray (22) |
|-----------------------|-----------------------------------|--------------------------------|----------------------------------------------|------------------------------------------|
|                       | UV 4-NQOa | γ-Ray | 8-OH-Gua | 5-OH-Cyb | Uracil | NA | NA | NA |
| WT                    | +       | +     | +++      | +++      | +++   | +  | NA  | NA |
| Null                  | ++      | +     | +++      | +++      | +++   | +  | NA  | NA |
| Ia                    | +       | +     | +        | +        | +     | +  | NA  | NA |
| II                    | ++      | +     | +        | +        | +     | +  | NA  | NA |
| III                   | +       | +     | +        | +        | +     | +  | NA  | NA |
| V                     | +++     | +     | +        | +        | +     | +  | NA  | NA |
| VI                    | +++     | +     | +        | +        | +     | +  | NA  | NA |
| NTB                   | +       | +     | +        | +        | +     | +  | NA  | NA |

a 4-NQO: 4-nitroquinoline-1-oxide.

b 5-OH-Cyt: 5-hydroxycytosine.
c ±, ++ and +++ are the relative responses referring to the column titles.
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|-----------------------|-----------------------------------|--------------------------------|----------------------------------------------|------------------------------------------|
|                       | UV 4-NQOa | γ-Ray | 8-OH-Gua | 5-OH-Cyb | Uracil | NA | NA | NA |
| WT                    | +       | +     | +++      | +++      | +++   | +  | NA  | NA |
| Null                  | ++      | +     | +++      | +++      | +++   | +  | NA  | NA |
| Ia                    | +       | +     | +        | +        | +     | +  | NA  | NA |
| II                    | ++      | +     | +        | +        | +     | +  | NA  | NA |
| III                   | +       | +     | +        | +        | +     | +  | NA  | NA |
| V                     | +++     | +     | +        | +        | +     | +  | NA  | NA |
| VI                    | +++     | +     | +        | +        | +     | +  | NA  | NA |
| NTB                   | +       | +     | +        | +        | +     | +  | NA  | NA |

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|-----------------------|-----------------------------------|--------------------------------|----------------------------------------------|------------------------------------------|
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| WT                    | +       | +     | +++      | +++      | +++   | +  | NA  | NA |
| Null                  | ++      | +     | +++      | +++      | +++   | +  | NA  | NA |
| Ia                    | +       | +     | +        | +        | +     | +  | NA  | NA |
| II                    | ++      | +     | +        | +        | +     | +  | NA  | NA |
| III                   | +       | +     | +        | +        | +     | +  | NA  | NA |
| V                     | +++     | +     | +        | +        | +     | +  | NA  | NA |
| VI                    | +++     | +     | +        | +        | +     | +  | NA  | NA |
| NTB                   | +       | +     | +        | +        | +     | +  | NA  | NA |

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The Cockayne Syndrome Group B Gene Product Is Involved in Cellular Repair of 8-Hydroxyadenine in DNA

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