Much work has been focused on the pathways that restore the integrity of the genome after different kinds of lesions, especially double-strand breaks. A classical method to investigate double-strand break repair is the incubation of a DNA substrate with cell-free extracts. In these end-joining assays, the DNA is efficiently ligated by the proteins present in the extract, generating circular molecules and/or multimers. In contrast, using a similar in vitro system, we detected DNA cleavage rather than end ligation. When comparing our results with previous works, a paradox emerges: lower amounts of DNA become multimerized instead of degraded and higher amounts of DNA are degraded rather than multimerized. Here, we have demonstrated that when the DNA/protein ratio is low enough, the DNA-binding proteins of the nuclear extract protect the DNA substrate, avoiding DNA degradation and vice versa. Therefore, the variation of the DNA/protein ratio is enough to switch the outcome of the experiment from a DNA cleavage assay to a typical end-joining assay.

The function of the genome in the cell nucleus depends on a balance between DNA damage and repair. Much work has been focused on the pathways that restore the integrity of the DNA after different kinds of lesions, especially double-strand breaks (DSBs) (1). Because DSBs are one of the most common injuries affecting DNA (2), the repair of these breaks is essential for the equilibrium of the genome (3). Therefore, the knowledge of the processes involved both in DNA cleavage, including DSB generation, and DNA repair are crucial to understanding the stability of the genome. There are two main mechanisms that participate in DSB removal in higher eukaryotes: the non-homologous end-joining (NHEJ) and the homologous recombination (4, 5). NHEJ is a non-conservative pathway that achieves DSB repair without the need of extensive homology between the DNA ends to be joined. It frequently involves DNA end-joining by Ku70 and Ku80 proteins, which activate the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) by stabilizing its interaction with DNA ends (6). This facilitates rejoining by a DNA ligase IV-XRCC4 heterodimer (6). In addition, NHEJ is known to be involved in V(D)J recombination and immunoglobulin class switching in mammals (7).

A classical method to investigate DNA repair is the incubation of a DNA substrate containing a specific DSB with cell-free extracts (8, 9). Using this system, Baumann and West (10) developed the first in vitro end-joining assay that demonstrated a requirement for factors such as Ku70, Ku80, DNA-PKcs, DNA ligase IV, and XRCC4, some of them known to be implicated in NHEJ by previous genetic studies. Later on, similar assays were carried out to investigate the involvement of other factors such as BRCA-1 (11), DNA polymerase α (12), or human polynucleotide kinase (13) in NHEJ. In these end-joining assays, the DNA was always efficiently ligated by the proteins present in the extract, generating circular molecules and/or multimers of the original substrate (10–17). Surprisingly, little or no degradation of the DNA substrate was observed in the outcome of these assays, although endogenous nuclease activities should be present in those extracts.

We have developed an in vitro assay that also implies incubation of a DNA fragment with nuclear extracts. This assay was primarily designed for the detection and location of DNA breaks in the human minisatellite MsH42 region (18), which has been demonstrated to enhance in vitro intramolecular homologous recombination (19, 20). In contrast to other assays, our in vitro system mainly promotes DNA substrate degradation instead of its multimerization. Why are DNA substrates preferentially degraded in our assay, whereas they are end-ligated in other in vitro end-joining assays? In the present work, we have investigated the differences between these end-joining assays and our assay. Our results revealed the effect of the DNA-binding proteins present in the cell-free extract on the outcome of in vitro DNA cleavage/end-joining experiments.

EXPERIMENTAL PROCEDURES

DNA Substrates and Nuclear Extracts—In our assays, the DNA used as substrate was an 880-bp DNA fragment (Ms fragment) that comprises the human minisatellite MsH42 region (18). This fragment was obtained by EcoRI digestion of a pGem-T-Easy plasmid that harbors this region and was purified by electrophoresis, phenol-chloroform extraction, and ethanol precipitation. For some assays, an aliquot of the Ms fragment was end-labeled by the exchange reaction of T4 polynucleotide kinase (Invitrogen) using [γ-32P]ATP (10 Ci/mM, 3000 Ci/mM; Amer sham Biosciences) following the protocol of the manufacturer. The end-labeled DNA was purified with the QIAquick nucleotide removal kit (Qiagen), yielding a specific activity of ~2.8 × 107 cpm/μg. On the other hand, nuclear extracts were prepared from 2-month-old (~300 g) Sprague-Dawley rat testes as described elsewhere (21). The aliquots of nuclear extracts were snap-frozen in liquid nitrogen and quantified by the Bradford assay (Bio-Rad).

DNA Cleavage Assay—The standard assay was performed by incubating 1.5 μg of the Ms fragment (2.8 pmol) with 12 μg of testes nuclear extract in 50 μl of 20 mM Tris-HCl, pH 7.5, 10 mM MgSO4, 1 mM ATP, and 0.1 mM of each dNTP. After incubation at 37°C for 25 min, reactions were stopped by quick freezing at ~70°C; followed by phenolchloroform extraction and ethanol precipitation. The samples were analyzed by electrophoresis through 2% agarose gels in 1× TAE buffer. In some experiments, increasing amounts (0.01–1 μg) of purified histone H1 (H1) (Roche Applied Science) were added to the reactions. In
other assays, the same amounts of H1 were preincubated at 37 °C for 10 min with 1 μg of recombinant prothymosin α (ProTα), and then this mixture was added to the reaction. Recombinant ProTα (22) was kindly provided by Dr. Fernando Domínguez. In the experiments with different DNA/protein ratios, 3 ng (5.6 fmol) of end-labeled Ms fragment (~84000 cpm) plus increasing amounts of unlabeled Ms fragment (0–2.8 pmol) were employed as substrate. In these assays, agarose gels were blotted onto Hybond-N membranes (Amersham Biosciences), followed by autoradiography.

RESULTS

In previous works, we have shown that the human minisatellite MsH42 region is able to enhance intramolecular homologous recombination in vitro (19, 20). Because many recombinational processes are triggered by DNA damage, we set up an in vitro DNA cleavage assay to study where DNA lesions could be taking place in the MsH42 region. To this end, we incubated the Ms fragment that contains the minisatellite MsH42 plus its flanking sequences with rat testes nuclear extracts. After incubation, the processed DNA was separated by electrophoresis in agarose gels. As shown in Fig. 1 (third lane from the left), we obtained a pattern of smaller fragments derived from the nuclease activity on the Ms substrate, and the degree of end ligation was almost negligible. When these results are compared with those obtained in similar assays designed to study DNA end-joining, there is a striking difference: most of the end-joining assays employed to analyze NHEJ show a remarkable absence of DNA degradation (10–17), indicating that the DNA is somehow protected against the endogenous nuclease activities, whereas this protection is undetectable in our assay.

We first investigated whether under our in vitro assay conditions we could promote multimerization by protecting the Ms fragment. To obtain this protection, we incorporated into the reaction mixture growing amounts of H1, which enhances intermolecular end ligation in vitro (23). The results of these experiments revealed that the addition of increasing amounts of H1 produces a complete switch in the result of the assay, blocking DNA degradation and enhancing DNA multimerization (Fig. 1). It is worth noting that at elevated concentrations of H1 the end ligation of the Ms fragment is reduced (Fig. 1, lanes 9 and 10), suggesting that the DNA fragment becomes totally covered by the histone, avoiding its multimerization. To corroborate the effect of H1 on the DNA cleavage assay, we carried out experiments in which this protein was preincubated with ProTα before its addition to the reaction mixture. Because ProTα was demonstrated to be an H1-binding protein (24–26),
it was expected that the addition of this protein would counteract the protection of the DNA fragment by H1. Fig. 2 shows that the presence of ProTα provoked a rise in the quantity of H1 needed to avoid the formation of the cleavage products as well as the hindrance to its multimerization (Fig. 2, compare with same amounts of H1 in Fig. 1). In our standard assay, the DNA/ratio employed was 234 fmol/μg protein, whereas in similar assays (10–17) it was much lower (0.25–5.8 fmol/μg protein). If we take into consideration the results obtained in each type of assay, multimerization versus degradation, with the different quantities of DNA substrate, a paradox emerges: lower amounts of DNA become multimerized instead of degraded and higher amounts of DNA are degraded rather than multimerized. The results obtained with H1 provide the clue to explain this apparent paradox. Thus, low amounts of DNA substrate would be protected against degradation by the DNA-binding proteins of the extract, whereas protection becomes insignificant when large quantities of DNA are present. To verify this hypothesis, several reactions with different DNA substrate/protein ratios were carried out by adding increasing amounts of unlabeled Ms fragment (0–2.8 pmol) with 3 ng (5.6 fmol) of end-labeled Ms fragment (Fig. 3). The DNA/protein ratio in these reactions ranges from 0.47 to 234 fmol of Ms fragment/μg of protein nuclear extract. The results of these experiments showed that reactions with the lower DNA/protein ratio, similar to that used in several end-joining assays (10–17), are characterized by reduced degradation and enhanced ligation (mainly intramolecular) of the Ms fragment. The reactions with a high DNA/protein ratio, similar to the standard assay, show a pattern of bands derived from specific nuclease activities and much less end-joining activity. Altogether, these results are bona fide proof that the variation of the DNA/protein ratio in the in vitro assay is enough to switch the outcome of the experiment from a DNA cleavage assay to a typical end-joining assay. Therefore, when the DNA/protein ratio is sufficiently low, the DNA-binding proteins in the nuclear extract are able to cover the DNA substrate, thus avoiding its degradation.

**DISCUSSION**

*In vitro* end-joining assays are a widely employed technique to study the mechanisms of NHEJ (8–17), one of the most important pathways for DNA repair in higher eukaryotes (4, 5). In the present work, we have developed an *in vitro* assay to study the specific cleavage of a minisatellite-containing DNA fragment by the nuclear extract. We have shown that the interaction between the DNA substrate and H1 in our assay drastically modifies the result of the experiment. The binding of H1 to DNA not only abrogates the nuclease activity but also promotes its linear multimerization (Fig. 1). This is in agree-
ment with previous work reporting that H1 enhances DSB-ligation reactions, probably facilitating the end to end connection of DNA molecules by the formation of long nucleoprotein complexes that lead to DNA assembled structures (23). Because these nucleoprotein complexes are probably unsuitable substrates for the degradation activity, multimerization goes along with DNA protection. Because ProTα is able to bind free H1 (25), its addition provokes a decrease in the effective concentration of H1 that can associate to the DNA fragment. The weakening of the H1 effect produced by preincubation with ProTα confirms the protective role played by H1 in our assays (Fig. 2). The mild attenuation of the H1 effect produced by PreTα could be explained because this protein may not be capable of releasing H1 bound to DNA (25), and hence all H1

Fig. 3. The ratio of DNA/protein conditions the outcome of DNA cleavage assays. Assays with different DNA/protein ratios were performed by adding increasing amounts (0.175–2.8 pmol) of unlabeled Ms fragment to 5.6 fmol of end-labeled Ms fragment. The upper panel shows the electrophoretic analysis of the assay products; the middle panel shows the autoradiogram of the same blotted gel. The second lane in the upper panel corresponds to a standard reaction. The bottom of the figure shows a scheme of the processes taking place on the Ms fragment. Ms, M, and scheme keys are as in Fig. 1. Circles represent DNA-binding proteins.
molecules that get joined to DNA remain stably associated to it. The results presented here provide strong evidence that DNA-binding proteins in the cell-free extracts are protecting DNA from degradation and favoring its ligation in end-joining/DNA cleavage assays. HMGB1 and HMGB2 are abundant nuclear proteins that bind to the nucleosome linker DNA of the chromatin in a competitive manner with H1 (27). It has been reported that these proteins stimulate in vitro DSBR-ligation reactions (28), promoting intramolecular rather than intermolecular ligation (23). This finding is consistent with our results in the experiments with a low DNA/protein ratio (Fig. 3). Thus, suppression of DNA degradation is achieved and intramolecular ligation is predominant, suggesting that nuclear DNA-binding proteins different from H1 may be involved in the protection of DNA against nuclease activity in these assays. The fact that DNA protection appears at low DNA/protein ratios indicates the shielding of DNA against nucleases is fully accomplished only if enough protein is present per DNA molecule. The DNA/protein ratio in several end-joining assays (10–17) ranges from 0.25 to 5.8 fmol of DNA substrate/μg protein. The low DNA/protein ratio of 0.47 fmol of DNA/μg protein employed in this work is a value included within such a range. All these values are much lower than those in our standard assays (234 in this work is a value included within such a range. All these are much lower than those in our standard assays (234

REFERENCES

1. Pastink, A., Becker, J. C., and Lohman, P. H. (2001) Mutat. Res. 480–481, 37–50
2. Chu, G. (1997) J. Biol. Chem. 272, 26196–26205
3. Kanaar, R., Hoeijmakers, J. H. J., and van Gent, D. C. (1998) Trends Cell Biol. 8, 483–489
4. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwi, Y., Shinozuka, A., and Takeda, S. (1998) EMBO J. 17, 5497–5506
5. Richardson, C., and Jasim, M. (2000) Mol. Cell. Biol. 20, 9068–9075
6. Karran, P. (2000) Curr. Opin. Genet. DeV. 10, 144–150
7. Singleton, B. K., and Jeggo, P. A. (1999) in DNA Recombination and Repair (Smith, P. J., and Jones, C. J., eds), pp. 16–37, Oxford University Press, Oxford
8. North, P., Ganesh, A., and Thacker, J. (1999) Nucleic Acids Res. 26, 6203–6210
9. Nicolas, A. L., and Young, C. S. H. (1994) Mol. Cell. Biol. 14, 170–180
10. Baumann, P., and West, S. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14066–14070
11. Zhong, Q., Boyer, T. G., Chen, P-L., and Lee, W-H. (2002) Nucleic Acids Res. 30, 3277–3288
12. Chappell, C., Hanahaki, L. A., Karimi-Busheri, F., Weinfeld, M., and West, S. C. (2002) EMBO J. 21, 2827–2832
13. Masson, R. M., Thacker, J., and Fairman, M. P. (1996) Nucleic Acids Res. 24, 4946–4953
14. Sathees, C. R., and Raman, M. J. (1999) Mutat. Res. 433, 1–13
15. Feldmann, E., Schiemann, V., Goedecke, W., Reichenberger, S., and Pfeiffer, P. (2000) Nucleic Acids Res. 28, 2585–2596
16. Odersky, A., Panyutin, I. V., Panyutin, I. G., Schunck, C., Feldmann, E., Goedecke, W., Neumann, R. D., Ohe, G., and Pfeiffer, P. (2002) J. Biol. Chem. 277, 11756–11764
17. Boim, F., Gonzalez, A. I., Rodriguez, J. M., and Gomez-Mambrasar, J. (1997) FEBS Lett. 418, 251–257
18. Boim, F., Rodriguez, J. M., and Gomez-Mambrasar, J. (1998) J. Mol. Biol. 278, 499–505
19. Bezerra, V., Redlich, F., and Rodriguez, J. M. (2000) J. Biol. Chem. 275, 13841–13847
20. Bezerra, V., Redlich, F., and Rodriguez, J. M. (2000) J. Biol. Chem. 275, 13841–13847
21. Abmayr, S. B., and Workman, J. (1987) Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 12–3–12–4, Wiley Interscience, New York
22. Vega, F., Vidal, A., Hellman, U., Wernstedt, C., and Dominguez, F. (1998) J. Biol. Chem. 273, 10147–10152
23. Yamanaka, B., Katsuya, E., Yoshioka, K., Nagaki, S., Yoshida, M., and Teraoka, H. (2002) Biochem. Biophys. Res. Commun. 292, 268–273
24. Gomez-Mambrasar, J., and Rodriguez, F. (1998) Biochem. J. 333, 1–3
25. Karayiannakis, A., Sandulache, P., Frangou-Lazaridou, M., Lai, C.-Y., Tsolas, O., Becker, P. B., and Papamarkakis, T. (1998) Nucleic Acids Res. 26, 3111–3118
26. Segade, F., and Gomez-Mambrasar, J. (1999) Int. J. Biochem. Cell Biol. 3, 1241–1246
27. Ogawa, Y., Aizawa, S., Shirakawa, H., and Yoshida, M. (1995) J. Biol. Chem. 270, 9272–9280
28. Nagaki, S., Yamamoto, M., Yunoto, Y., Shirakawa, H., Yoshida, M., and Teraoka, H. (1998) Biochem. Biophys. Res. Commun. 256, 137–141

Acknowledgments—We thank Dr. F. Domínguez for providing the purified ProTis and Paula Barros for help in some experiments.