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A DNA End-Binding Factor Involved in Double-Strand Break Repair and V(D)J Recombination

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We have identified a nuclear factor that binds to double-stranded DNA ends, independently of the structure of the ends. It had equivalent affinities for DNA ends created by sonication or by restriction enzymes leaving 5′, 3′, or blunt ends but had no detectable affinity for single-stranded DNA ends. Since X rays induce DNA double-strand breaks, extracts from several complementation groups of X-ray-sensitive mammalian cells were tested for this DNA end-binding (DEB) activity. DEB activity was deficient in three independently derived cell lines from complementation group 5. Furthermore, when the cell lines reverted to X-ray resistance, expression of the DEB factor was restored to normal levels. Previous studies had shown that group 5 cells are defective for both double-strand break repair and V(D)J recombination. The residual V(D)J recombination activity in these cells produces abnormally large deletions at the sites of DNA joining (F. Pergola, M. Z. Zdzienicka, and M. R. Lieber, Mol. Cell. Biol. 13:3464–3471, 1993, and G. Taccioli, G. Rathbun, E. Oltz, T. Stamato, P. Jeggo, and F. Alt, Science 260:207–210, 1993), consistent with deficiency of a factor that protects DNA ends from degradation. Therefore, DEB factor may be involved in a biochemical pathway common to both double-strand break repair and V(D)J recombination.

X rays and oxidative metabolism induce a number of different DNA lesions, including base damage, single-strand breaks, and double-strand breaks. In response to this onslaught, cells have evolved specific biochemical pathways for repairing X-ray damage. The search for the genetic basis of these pathways has been greatly aided by the development of a number of easily cultured rodent cell lines that are hypersensitive to the lethal effects of X rays. Cell fusion experiments have shown that these cell lines fall into at least nine genetic complementation groups (10). Three of these groups are defective in the repair of DNA double-strand breaks (DSBs). Presumably, one or more of these genes encode a protein involved in protecting and resolving the free DNA ends generated by X rays.

Another process that requires the resolution of free DNA ends is V(D)J recombination, a process that rearranges germ line DNA to assemble exons encoding immunoglobulin and T-cell receptor proteins (44). Early steps in the process involve the lymphocyte-specific recombination-activating genes *RAG-1* and *RAG-2*. Cotransfection of both genes confers V(D)J recombination activity to nonlymphoid cells; therefore, *RAG-1* and *RAG-2* initiate V(D)J recombination in some still-undefined way (31, 39). Endonucleases recognize and cleave the DNA at sites defined by conserved signal sequences. Other proteins must resolve the free DNA ends generated by cleavage and rejoin the ends, forming a coding join and a signal join. The result is a rearranged immunoglobulin or T-cell receptor gene.

A mutant mouse model provided the first indication that DSB repair and V(D)J recombination share common gene products. Mice homozygous for the *scid* mutation suffer from severe combined immunodeficiency, with an absence of functional B and T cells that leads to extreme susceptibility to infections. When *RAG-1* and *RAG-2* are cotransfected into cells derived from the *scid* mouse, deficient joining of coding ends and 80% precise joining of signal sequences are observed (28). The *scid* mutation also confers hypersensitivity to X rays and a deficiency in DSB repair (2).

The mutant cell lines from each of the three DSB repair-deficient complementation groups (groups 4, 5, and 9) are also deficient in V(D)J recombination (37, 43). When the DSB repair mutant cells are reverted to wild-type X-ray resistance by human chromosome transfer, treatment with azacytidine, or cell fusion, the cells regain the wild-type capacity for V(D)J recombination (43). Residual recombination activity in the mutant cells is characterized by abnormally large deletions of nucleotides in coding joins (group 9) or both coding and signal joins (groups 4 and 5). These results suggest that at least three gene products are involved in a pathway common to both V(D)J recombination and DSB repair. Furthermore, the defects in groups 4 and 5 may render free DNA ends susceptible to exonuclease degradation.

The gel mobility shift assay is a powerful method for analyzing crude cell extracts for the presence of proteins that bind to specific DNA sequences. The formation of a specific complex between the DNA sequence and a protein in the extract may be revealed by titrating away effects from nonspecific DNA-binding proteins; this is done by adding an excess of judiciously chosen unlabeled competitor DNA to the binding reaction mixture (40). We have adapted this assay for the detection of proteins that bind to altered DNA structures rather than to DNA sequence. For example, a UV-damaged DNA probe was used to identify xeroderma pigmentosum group E binding factor (XPE-BF) in crude nuclear extracts, and a cisplatin-damaged DNA probe detected a different factor in both cytoplasmic and nuclear extracts (7). In both cases, the unlabeled competitor DNA was a mixture of undamaged sonicated salmon sperm DNA and the alternating copolymer poly(dI-dC).

To search for a factor that might be involved in DSB repair, the mobility shift assay was adapted to detect binding activity specific for free DNA ends. In particular, undamaged DNA probes were incubated with cell extracts in the presence of

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excess unlabeled competitor DNA, consisting of supercoiled plasmid lacking DNA ends. This paper describes the identification of a nuclear factor that binds to double-stranded DNA ends, independently of the detailed structure of the ends. Furthermore, the analysis of X-ray-sensitive rodent cells for expression of this DNA end-binding (DEB) factor suggests that this factor is involved in both DSB repair and V(D)J recombination.

MATERIALS AND METHODS

Cell cultures. A. Giaccia kindly provided the cell lines xrs5 (21) (X-ray-sensitive CHO cells from X-ray complementation group 5), AT-5BI (18) (skin fibroblasts from ataxia telangiectasia complementation group D), scid/St and C.B-17 (2) (fibroblasts derived from the *scid* mouse and its parental wild-type strain, respectively), BL-10 (14) and BL-14 (13) (bleomycin-sensitive CHO cells), and SJC-3 (13) (X-ray-sensitive human lymphoblasts). M. Lieber kindly provided the cell lines XR-V15B (47) and XR-V9B (48) (X-ray-sensitive CHO cells from complementation group 5), the corresponding parental lines V79 and V79B, SF-7 (4) (fibroblasts derived from the *scid* mouse), and XR-1 (42) and V-3 (45) (X-ray-sensitive CHO cells from complementation groups 4 and 9, respectively). P. Hanawalt kindly provided the wild-type CHO cell line AA8. Cell lines EM9 (X-ray-sensitive CHO cells from complementation group 1) and wild-type human cell lines HeLa, IMR-90, and Jurkat were obtained from the American Type Culture Collection (Rockville, Md.).

The lymphoblastoid cell lines SJC-3 and Jurkat were grown in RPMI medium with 10% fetal bovine serum (FBS), 1% glutamine, and 1% antibiotic (penicillin and streptomycin). The cell line xrs5 was grown in alpha minimum essential medium with 10% FBS, 1% glutamine, and 1% antibiotic. All other cell lines were grown in Dulbecco's modified Eagle's medium with 10% FBS, 1% glutamine, and 1% antibiotic and were passaged in trypsin-EDTA approximately every 4 days.

Preparation of crude nuclear extract from nuclei. Nuclear extracts were prepared as described previously (33). Briefly, 4×10^6 cells were washed with 1 ml of phosphate-buffered saline (Gibco, Gaithersburg, Md.), pelleted for 5 s at 12,000 × *g*, washed in 1 ml of wash buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 10 mM KCl, 0.5 mM dithiothreitol [DTT]), pelleted again, and suspended in 20 μl of wash buffer containing 1% Nonidet P-40. The cells were lysed during an incubation at 4°C for 10 min with rocking. Nuclei were pelleted for 2 min at 12,000 × *g*, and the supernatant was recovered as the cytoplasmic fraction. The nuclei were extracted in high-salt-concentration buffer (20 mM HEPES, 500 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 1.5 mM $MgCl_2$, 20% glycerol) at 4°C for 15 min and pelleted for 10 min at 12,000 × *g*, and the supernatant was recovered as the nuclear fraction. Protein concentrations were measured by a modification of the Bradford method (5) with protein assay reagent (Bio-Rad, Richmond, Calif.).

Preparation of DNA probes. The DNA fragment f148 consisted of 148 bp from the chloramphenicol acetyltransferase gene as described previously (35). The DNA fragment f320 consisted of 320 bp from the neomycin resistance gene, generated by *Hin*dIII and *Bgl*II cleavage of pRSVneo (15, 41). The fragments were end labeled with the large fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP.

Preparation of competitor DNA substrates. Plasmid pRSVneo DNA was used as competitor DNA either in supercoiled form or after cleavage with restriction enzymes. To analyze DEB activity for single-stranded DNA, linearized plasmid (pRSVneo cut with *Bam*HI) and sonicated salmon sperm DNA (Sigma, St. Louis, Mo.) were heat denatured at 95°C for 5 min, transferred immediately to ice water to minimize reannealing, and added to the binding reaction mixture. Additional competitors were also tested in the binding reaction and included the single-stranded homopolymers poly(dA) and poly(dT), the self-annealing alternating copolymer poly(dA-dT) (Pharmacia, Piscataway, N.J.), circular DNA from the bacteriophages M13 and φX174 in both single-stranded virion and double-stranded replicative forms, and plasmids pUC7 and pEA-1 containing perfect palindromic sequences. These plasmids were used in supercoiled form or were heated at 60°C for 2 h followed by cooling at room temperature to induce cruciform formation. Cruciform structure was confirmed by the loss of restriction enzyme sites at the center of the palindrome.

Electrophoretic mobility shift assay. Binding activity to the DNA probe f148 (or f320) was measured by a gel electrophoretic mobility shift assay described previously (7, 8, 17, 35). A reaction mixture of 10 μl contained 0.2 ng of radiolabeled probe, 0.6 μg of crude nuclear extract, and unlabeled competitor DNA (linearized or supercoiled plasmid DNA) in binding buffer (12 mM HEPES, 5 mM $MgCl_2$, 4 mM Tris [pH 7.9], 100 mM KCl, 0.6 mM EDTA, 0.6 mM DTT, 12% glycerol). For all binding reactions, the protein extract was added last, and the reaction mixture was incubated at room temperature for 30 min, except when indicated. The reaction mixture was resolved by nondenaturing electrophoresis through a 5% polyacrylamide gel in TGE buffer (50 mM Tris [pH 8.5], 380 mM glycine, 2 mM EDTA). The gel was dried on Whatman 3M paper and exposed to Kodak XAR-5 film. Binding activity was quantitated by scanning densitometry.

Azacytidine-induced revertants of xrs5. Azacytidine was used to revert xrs5 cells to X-ray resistance as described previously (20). Briefly, 10^5 cells were seeded into a 25-cm^2 flask, allowed to attach, and then grown for 15 h in medium containing 3 μg of freshly dissolved 5-azacytidine (Sigma, St. Louis, Mo.) per ml. The cells were then grown in normal medium for 4 days and subsequently selected for X-ray resistance by irradiation with 160 rads each day for four consecutive days. For analysis of X-ray resistance, cells were seeded onto 10-cm-diameter plates in 10-fold dilutions and treated with X rays in single doses up to 600 rads. Surviving colonies were then stained with 10% Giemsa stain and counted.

RESULTS

Identification of DEB activity. Since X rays induce DSBs in DNA, the electrophoretic mobility shift assay was used to search for a protein that binds specifically to DNA ends. Crude nuclear extracts were incubated with a linear ^{32}P-labeled DNA probe in the presence of excess supercoiled plasmid competitor DNA. Extracts from wild-type Chinese hamster ovary (CHO) cells produced a mobility shift of the DNA probe to bands B1, B2, and B3 (Fig. 1A). When additional competitor DNA was added to the binding reaction mixture, there was loss of binding activity in B3 but no loss in B1 and B2 if the DNA remained intact as a supercoiled plasmid, even when as much as 1,000 ng of plasmid DNA was added. However, when the plasmid was cut once with a restriction enzyme, there was an 80 to 90% decrease in bands B1 and B2, but not B3, when as little as 20 ng of DNA was added to the binding reaction mixture (Fig. 1A). The degree of competition did not depend on the type of free DNA ends generated: 5′ overhangs (*Bam*HI or *Hin*dIII), 3′ overhangs (*Aat*II), or blunt ends (*Sma*I). Instead,

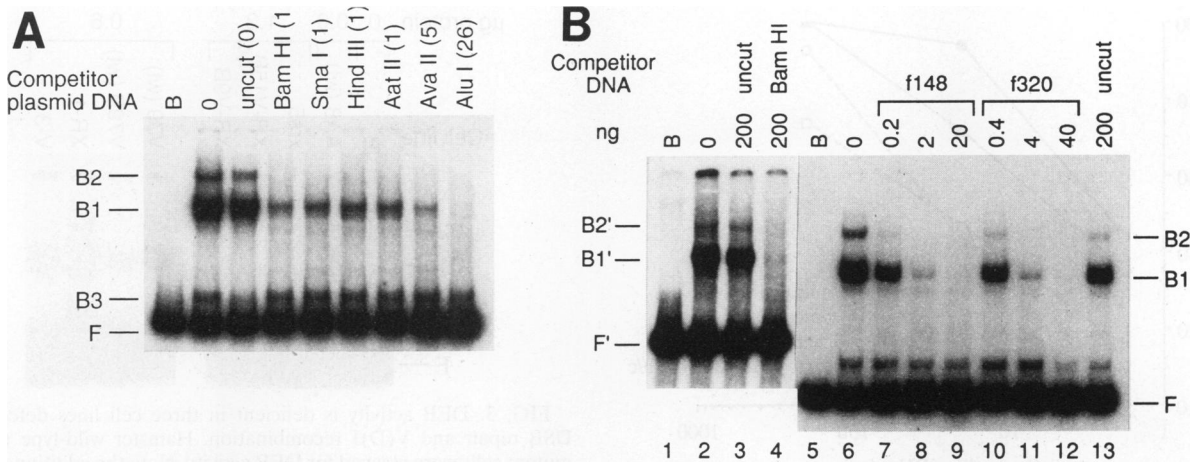

FIG. 1. Identification of DEB activity. (A) Competition for binding activity depends on the number of DNA ends but not on the restriction enzyme used to generate the ends. The electrophoretic mobility shift assay was performed by adding nuclear extract from Chinese hamster ovary cells (AA8) to a reaction mix containing DNA probe f148 and 40 ng of supercoiled pRSVneo DNA. Some reaction mixtures contained no additional DNA (0) or an additional 20 ng of pRSVneo, either as supercoiled plasmid (uncut) or cleaved by *Bam*HI, *Sma*I, *Hin*dIII, *Aat*II, *Ava*II, or *Alu*I. Numbers in parentheses indicate the number of restriction sites present for each enzyme in pRSVneo. The differences in binding activity among the single-cutting enzymes were not significant since they were not seen consistently in four independent experiments. The lane marked B was loaded with a binding reaction mixture lacking extract. Positions of the free f148 probe (F) and protein-DNA complexes (B1, B2, and B3) are indicated. (B) Two different DNA probes detect DEB activity. The binding reaction was performed with either f320 (lanes 1 through 4) or f148 (lanes 5 through 13). Positions of the free f320 probe (F′) and its end-specific protein-DNA complexes (B1′ and B2′) are indicated. Competitor DNA was added as pRSVneo DNA either as supercoiled plasmid (uncut) or after cleavage with *Bam*HI. Alternatively, competitor DNA consisted of unlabeled f148 or f320, titrated so that the amounts represented equivalent numbers of DNA ends. The lanes marked B were loaded with a binding reaction mixture lacking extract, and those marked 0 contain no additional DNA.

it increased stoichiometrically as the number of free DNA ends increased. Furthermore, when pRSVneo plasmid DNA was cleaved with *Sau*3AI, which cuts at 31 sites and leaves 5′ overhangs identical to those generated by *Bam*HI, the degree of competition again increased proportionally to the number of free DNA ends. Thus, the binding activity in bands B1 and B2, but not B3, was specific for DNA ends.

DEB activity was also observed with the probe f320, which has a sequence completely different from that of f148 (Fig. 1B). The f320 probe detected a binding activity that was also inhibited by competition with linearized plasmid DNA but not with supercoiled plasmid DNA. Furthermore, the binding activity detected by f148 was inhibited by competition by both f148 and f320 with equal efficiency when the competitor DNAs were added in amounts that gave equivalent numbers of DNA ends. When supercoiled plasmid pRSVcat DNA, which contains the sequences in f148, was added to the binding reaction mixture, no competition was observed (data not shown). Thus, binding activity did not depend on the DNA sequence of the probe.

Sonication cleaves DNA at a variety of positions in the polydeoxyribose backbone and leaves ends with a range of different overhangs. Nevertheless, inhibition of binding occurred with DNA either cut with restriction enzymes or cleaved by sonication (Fig. 2A). The inhibition was equivalent for linearized pRSVneo (5.7 kb) and for sonicated salmon sperm DNA (average of 1.5 kb), when the fourfold difference in length was taken into account.

DEB factor has affinity for double-stranded but not single-stranded DNA ends. Binding activity was significantly greater for double-stranded than for single-stranded ends. Linearized plasmid and sonicated salmon sperm DNA both showed decreased competition for binding following heat denaturation (Fig. 2A). In each case, a 10-fold increased amount of denatured competitor DNA was required to produce the same level of inhibition as that produced by native DNA. The residual competition with denatured DNA may have been due to incomplete denaturation, partial reannealing of the DNA, or the formation of secondary structures in the single-stranded DNA that are recognized by DEB factor.

To further test the affinity of DEB factor for single-stranded DNA ends, competition experiments were performed with the homopolymers poly(dA) and poly(dT). Both homopolymers are obligate single strands of DNA that cannot self-anneal to form secondary structures. There was no detectable inhibition of DEB activity with either poly(dA) or poly(dT) (Fig. 2B). However, when the homopolymers were mixed together and allowed to anneal to form double-stranded DNA, there was inhibition of binding. The self-annealing alternating copolymer poly(dA-dT) also produced inhibition of binding. Thus, the DEB activity was specific for double-stranded rather than single-stranded DNA ends but was independent of the detailed structure of the double-stranded ends.

It is noteworthy that the average length of the self-annealing poly(dA-dT) (2,600 nucleotides [nt]) was greater than the length of the poly(dA) (300 nt) and poly(dT) (174 nt). Nevertheless, the poly(dA-dT) was more active as a competitor, perhaps because it is capable of forming secondary structures.

DEB factor has affinity for some DNAs without ends. To further examine the affinity of DEB factor for secondary structure, DNAs from the bacteriophages M13 and φX174 were tested as competitors for binding activity. Both bacteriophages have circular genomes in either single-stranded virion or double-stranded replicative forms containing sequences near their origins of replication capable of forming complex secondary structures. Circular M13 DNA successfully competed for binding activity in both single-stranded and double-stranded forms (Fig. 2C). Competition was unaffected by treatment of M13 replicative form with topoisomerase I (data not shown). Competition was significantly greater than it was

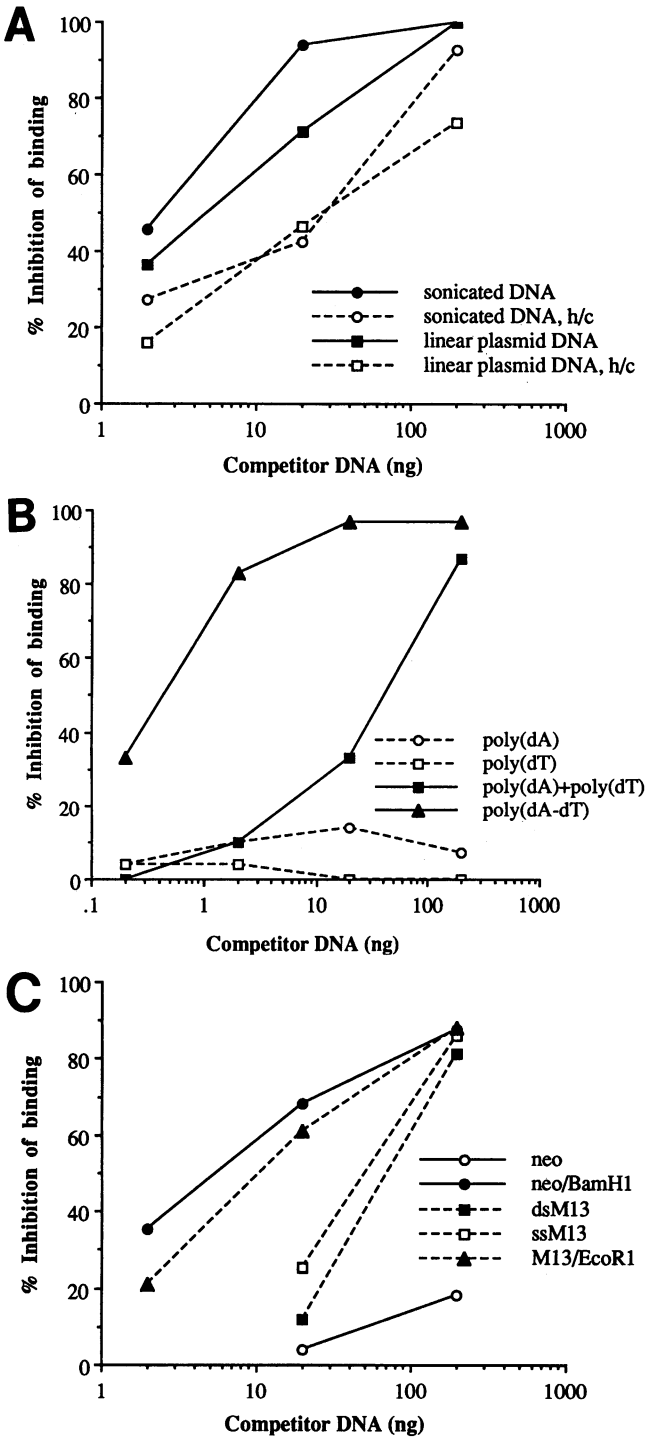

FIG. 2. DEB factor binds to DNA ends created by sonication or to secondary structures. (A) Competition with sonicated salmon sperm DNA or plasmid DNA in either the native or the denatured state. Sonicated salmon sperm DNA or plasmid pRSVneo DNA linearized with *Bam*HI was used as the competitor for binding to the DNA probe. Competitor DNA was denatured by heating for 5 min at 95°C followed by immediate cooling in ice water (h/c). The binding reaction time was reduced to 15 min to minimize the time for reannealing. (B) Competition with homopolymers. Synthetic homopolymers poly(dA) and poly(dT), either alone or mixed in a 1:1 weight ratio, and alternating copolymer poly(dA-dT) were used as competitors for binding activity. (C) Competition with M13 phage DNA. Both single-stranded (ss) and double-stranded (ds) circular M13 DNAs were used as competitors and compared with plasmid pRSVneo DNA (neo) either uncut or cleaved by *Bam*HI or ds M13 DNA cleaved by *Eco*RI.

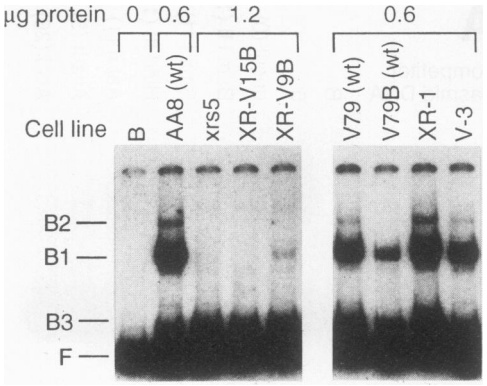

FIG. 3. DEB activity is deficient in three cell lines defective for DSB repair and V(D)J recombination. Hamster wild-type (wt) and mutant cells were assayed for DEB activity. Note the addition of 1.2 μg of protein extract for the xrs5, XR-V15B, and XR-V9B mutant cells, which had abnormally low levels of DEB activity, compared with 0.6 μg of extract for the other cell lines. Positions of the free probe (F) and protein-DNA complexes (B1, B2, and B3) are indicated.

for supercoiled plasmid pRSVneo but was less than it was for linearized DNA derived by cutting pRSVneo or M13 replicative form once with *Bam*HI or *Eco*RI, respectively. Similar results were obtained with ϕX174 DNA (data not shown).

To examine the possibility that DEB factor might recognize hairpin loops, plasmids containing perfect palindromes were also tested. The plasmids pUC7 and pEA-1 contain perfect palindromic sequences of 48 and 380 bp, respectively. Both plasmids failed to compete for binding activity as supercoiled plasmids, as relaxed plasmids following topoisomerase I treatment, or as cruciform plasmids following heat denaturation and slow reannealing.

DEB factor is a moderately abundant nuclear protein. Binding activity was susceptible to proteases (proteinase K and pronase) but not to RNase A and was found predominantly in nuclear rather than cytoplasmic extracts. The bulk of DEB activity was contained in B1, and if band B1 represents one binding event, extracts contained 140,000 (±50%) molecules of DEB factor per CHO AA8 cell and 200,000 molecules per HeLa cell. These estimates represent lower limits since band B2 may represent additional binding events and since the binding reactions were done with crude extracts and a large excess of competitor DNA, which may have titrated away some fraction of the binding activity.

DEB activity is deficient in X-ray-sensitive cell lines defective for DSB repair and V(D)J recombination. Wild-type and X-ray-sensitive cell lines were assayed for the presence of DEB activity. Figure 3 shows results with wild-type cells and mutant cells defective for DSB repair. Significant binding activity was seen in the mutants XR-1 and V-3. By contrast, there was an absence of detectable binding activity in the mutants xrs5 and XR-V15B and greatly reduced activity in XR-V9B. These deficiencies in DEB activity were confirmed by multiple independent extractions and binding assays. Significant binding activity was present in the wild-type cells AA8, V79, and V79B, from which V-3 and xrs5, XR-V15B, and XR-V9B were derived, respectively. Note that all cell extracts contained

TABLE 1. DEB activity in wild-type and mutant cell lines[a]

Cell line	Cell type	X-ray resistance (group)	V(D)J recombination activity		DEB activity (units/μg)
			Signal	Coding	
HeLa	Human, cervical carcinoma	R			2.2
IMR-90	Human, lung fibroblast	R			1.7
AT5BI	Human, skin fibroblast, AT-D	S			2.4
Jurkat	Human, T-cell lymphoma	R			4.4
SJC3	Human, mutated lymphoblast	S			4.3
NIH 3T3	Mouse, fibroblast	R			1.9
C.B-17	Mouse, fibroblast	R			2.1
scid	Mouse, fibroblast	S (9)[b]	80% precise[c]	Reduced	1.2[d]
V79	Hamster, CHO	R	wt	wt	1.0
XR-V15B	Hamster, CHO	S (5)[b]	Reduced[e]	Reduced[e]	<0.02
V79B	Hamster, CHO	R	wt	wt	0.5
XR-V9B	Hamster, CHO	S (5)[b]	Reduced	Reduced	<0.02[f]
AA8	Hamster, CHO	R	wt	wt	1.6
EM9	Hamster, CHO, EMS sensitive	S (1)	wt	wt	2.0
BL-10	Hamster, CHO, bleo sensitive	R			1.8
BL-14	Hamster, CHO, bleo sensitive	S			2.2
XR-1	Hamster, CHO	S (4)[b]	Reduced	Reduced	1.3
V-3	Hamster, CHO	S (9)[b]	80% precise[c]	Reduced	1.0
xrs5	Hamster, CHO	S (5)[b]	Reduced	Reduced	<0.02
xrs5(rev)	Hamster, CHO, revertant	R			1.0–1.4

[a] The cells have either wild-type resistance (R) or increased sensitivity (S) to X rays. For the sensitive cells, the X-ray complementation group is indicated in parentheses when known. V(D)J recombination activity at the signal and coding joins is shown when it has been measured (37, 43). DEB activity was measured by electrophoretic mobility shift assay, quantitated by scanning densitometry, and expressed as units of binding activity per microgram of cell extract. One unit was defined by the shift of 33% of the DNA probe. The xrs5(rev) cells were xrs5 cells induced to revert to X-ray resistance by treatment with azacytidine as described in the legend to Fig. 4. Errors in DEB activity were ±30%. Abbreviations: AT, ataxia telangiectasia; bleo, bleomycin; CHO, Chinese hamster ovary; EMS, ethyl methanesulfonate; wt, wild type.

[b] Deficient in DSB repair.

[c] Signal joins occurred at normal frequency but were only 80% precise (28, 37).

[d] DEB activities were 1.1 and 1.4 in two mouse *scid* lines, SF7 and scid/St, respectively.

[e] See reference 36.

[f] DEB activity was 0.1 units/μg in the original heterogeneous population obtained from M. Lieber but was <0.02 units/μg when XR-V9B was subcloned as an X-ray-sensitive cell line (see the text).

nonspecific binding activity in band B3, which served as an internal control.

To explore the origin of the low but positive level of DEB activity in XR-V9B, subclones were derived from single cells. DEB activity was found to be missing in most subclones but expressed at normal levels in other subclones, apparently as the result of spontaneous reversion during passage. Furthermore, the subclones with absent DEB activity were markedly sensitive to X rays, and the subclones with normal DEB activity had wild-type X-ray resistance. Thus, DEB activity was absent in all three group 5 cell lines tested: xrs5, XR-V15B, and XR-V9B.

A survey of wild-type and mutant cell lines is summarized in Table 1. DEB activity was present in mouse, hamster, and human cells. DEB activity was consistently in the range of 1.0 to 2.4 units/μg, with a few notable exceptions. The CHO line V79B showed a mild, reproducible deficit (0.5 units/μg), despite a wild-type phenotype. The two lymphoid cell lines expressed approximately twofold-higher levels. DEB activity was specifically absent in mutant cells from X-ray complementation group 5.

Absence of DEB activity was not caused by inhibitory factors. When extracts from group 5 cells (xrs5) were mixed with wild-type extract (AA8) in the binding assay mixture, no decrease in wild-type binding activity was observed (data not shown).

When xrs5 cells are reverted to X-ray resistance with azacytidine, DEB activity is also restored. Treatment with azacytidine will induce xrs5 cells to revert to X-ray resistance at a frequency of about 1% (20). Azacytidine acts as a competitive inhibitor of methyltransferase to demethylate previously methylated, and therefore silenced, genes. Thus, azacytidine may be used to induce expression of a silent wild-type allele when the active allele has been inactivated by mutagenesis. When xrs5 cells were treated with azacytidine and selected with X rays, pooled colonies (data not shown) and cloned cell lines had wild-type X-ray resistance (Fig. 4A). Significantly, 23 independent clones selected for X-ray resistance all showed restored DEB activity (Fig. 4B), further supporting the involvement of DEB factor in resolving the free DNA ends generated by X-ray-induced DSBs and V(D)J recombination. Pooled cells from XR-V15B were induced to revert at a similar rate by treatment with azacytidine with the same restoration of DEB activity.

DEB factor does not bind to V(D)J signal sequences. Because DEB factor appears to be involved in V(D)J recombination, competition experiments were performed with V(D)J recombination signal sequences. The signal sequences consist of a heptamer separated from a nonamer by either a 12-base or a 23-base spacer. Plasmids containing both signal sequences in tandem or inverted orientations are active as substrates for V(D)J recombination (28). In experiments testing the affinity of DEB factor for signal sequences, supercoiled plasmids containing the 23-base signal sequence either alone or together with the 12-base signal sequence in both tandem and inverted orientations failed to compete for DEB activity, suggesting that a different factor is required for signal sequence recognition.

DEB activity is not induced by X rays. To test whether DEB activity is induced by X rays, subconfluent AA8 CHO cells were exposed to either 100 or 200 rads, a dose range known to

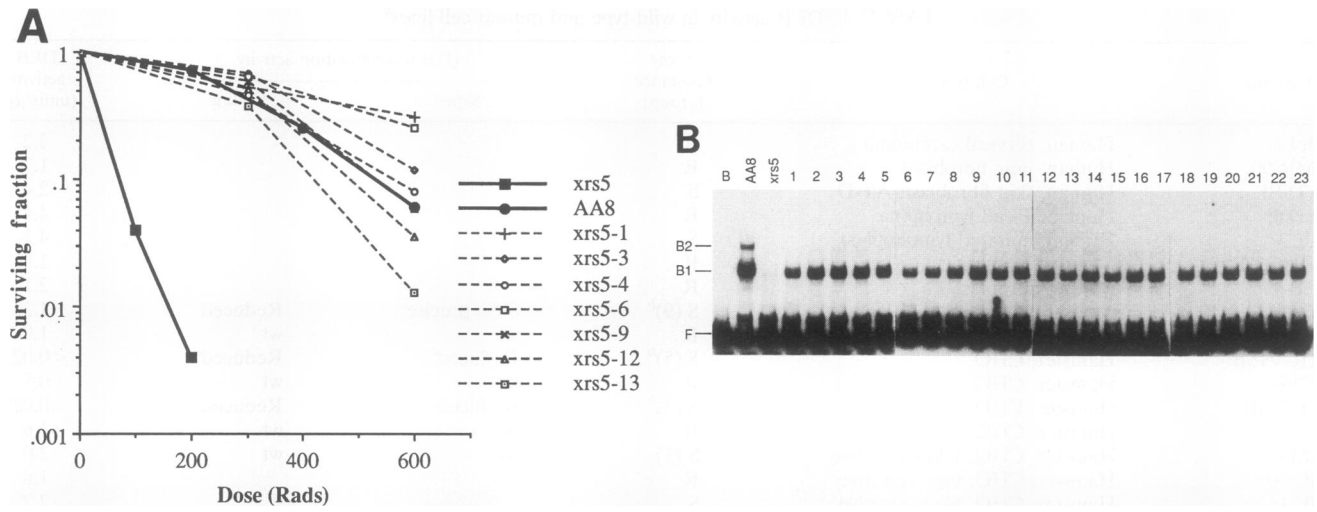

FIG. 4. Reversion of xrs5 cells to X-ray resistance also restores DEB activity. (A) X-ray resistance of cloned xrs5 revertants. Wild-type (AA8), mutant (xrs5), and revertant cell clones were plated in triplicate in 10-fold dilutions and treated with X-ray doses of 100, 200, 300, 400, or 600 rads. Surviving cells were grown as colonies, which were stained with Giemsa stain and counted. (B) DEB activity in xrs5 revertants. AA8, xrs5, and 23 cloned X-ray-resistant revertant cell lines (numbered 1 to 23 above the lanes) were tested for DEB activity. Positions of the free probe (F) and protein-DNA complexes (B1 and B2) are indicated.

be optimal for increasing the expression of a number of X-ray-induced proteins (3). Extracts were made before and 2, 4, 8, and 24 h after X-irradiation and were found to contain equivalent amounts of DEB activity. However, CHO cells might have an altered response to X rays, since they fail to induce the GADD45 gene (12). Therefore, the same experiment was repeated for the primary human fibroblast line IMR-90, a subline of WI-38, which is known to have normal induction of both p53 and GADD45 after X-irradiation (23). Again, DEB activity was not induced by X rays (data not shown).

DISCUSSION

The electrophoretic mobility shift assay was used to identify a DNA end-binding factor that is moderately abundant and localized to the nucleus. The DEB activity was detected in a broad range of cells, including those derived from skin, lungs, ovaries, lymphocytes, lymphomas, and cervical carcinomas. It bound to double-stranded DNA ends, independently of the detailed structure of the ends, but had no detectable affinity for single-stranded DNA ends. The DNA structures recognized by DEB factor were quite varied and included blunt ends, 5′ overhangs, and 3′ overhangs created by restriction enzyme cleavage as well as sonicated (11) DNA ends. This would be expected of a protein capable of recognizing the diverse DSBs generated by X rays (22). DEB factor also had affinity for circular single- and double-stranded DNA from the bacteriophages M13 and φX174, both of which contain sequences capable of forming complex secondary structures (24). A model for how DEB factor is able to recognize its different substrates is shown in Fig. 5. Thus, the apparent versatility of binding may be explained if the site of protein binding is at the fork representing the transition of double-stranded DNA to two single strands.

Despite its abundance and affinity for DNA ends, DEB factor has not generally interfered with mobility shift assays designed to study other DNA-binding proteins. Such assays have typically included excess unlabeled linear DNA or poly(dI-dC), thus inhibiting DEB activity by competition.

Because DNA DSBs are a major type of lesion produced by both X rays and the anticancer drug bleomycin, a number of X-ray- and bleomycin-sensitive cell lines were tested for the expression of DEB activity. DEB activity was notably deficient in three X-ray-sensitive cell lines: xrs5, XR-V15B, and XR-V9B. The xrs5 and XR-V15B cell lines have been unambiguously assigned to X-ray complementation group 5 (10). XR-V9B was originally assigned to a new complementation group (48) but has now been reassigned to group 5 (46). Thus, DEB activity was specifically absent in three independently derived mutant cell lines from group 5, one of the three complemen-

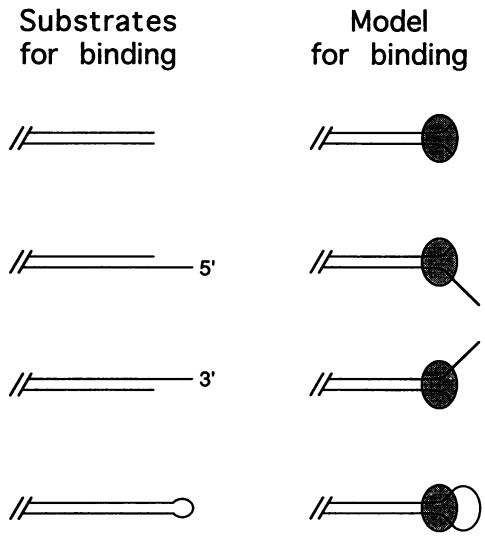

FIG. 5. Model for DEB activity for its DNA substrates. Substrates for binding include DNA with blunt, 5′-overhang, 3′-overhang, or sonicated ends as well as circular DNA with secondary structure. A potential structure common to all these substrates is the DNA fork at the point of transition from one double strand to two single strands, which may be the structure recognized by DEB factor (shaded oval).

tation groups defective in both DSB repair and V(D)J recombination.

The DSB repair mutants have V(D)J recombination phenotypes that vary among the three complementation groups. These phenotypes are consistent with the specific deficiency of DEB activity in group 5. For example, cells from group 5 (xrs5, xrs6, XR-V15B, and XR-V9B) are impaired for both coding and signal joins (37, 43). Furthermore, in group 5 cells, residual joining activity is characterized by abnormally large nucleotide deletions, consistent with deficiency of an end-binding factor that protects the DNA ends from exonuclease digestion.

Group 9 cells (scid and V-3) had normal levels of DEB activity, as would be expected from their significantly different V(D)J recombination phenotype, a severely decreased frequency of coding joins in the face of a normal frequency of less precise signal joins (27, 37, 43).

It is noteworthy that group 4 cells (XR-1) had normal levels of DEB activity but have a phenotype similar to that of group 5, with impairment of both coding and signal joining activity with large deletions in the residual joins. We hypothesize that XR-1 is deficient in a second protein distinct from, but perhaps interacting with, DEB factor, so that both DEB factor and XR-1 protein participate in the protection of free DNA ends during V(D)J recombination and DSB repair.

The resolution of free DNA ends appears to occur by similar mechanisms for both DSB repair and V(D)J recombination. Thus, when a DSB is created by either X-ray damage or cleavage at recombination signal sequences, a protein must exist to recognize the free DNA ends. We propose that DEB factor is such a recognition protein, acting to bind and protect DNA ends from degradation and serving as a focal point for recruiting other proteins to perform the rejoining reaction. These additional proteins will likely include the gene products corresponding to X-ray complementation groups 4 and 9.

Is DEB factor identical to one of the previously described proteins with end-binding activity? Poly(ADP-ribose) polymerase is a 116-kDa nuclear protein that binds to DNA ends and catalyzes the polymerization of the ADP-ribose moiety of NAD^+ into a homopolymer of repeating ADP-ribose units, which is bound to various nuclear proteins. Enzymatic activity is dependent on the presence of DNA with either single- or double-stranded breaks and appears to play a role in the cellular response to X rays (1). However, poly(ADP-ribose) polymerase has a much higher affinity for heat-denatured calf thymus DNA than for untreated DNA (32), making it clearly distinct from DEB factor. Furthermore, the gene maps to human chromosome 1 (9), while chromosome 2 restores X-ray resistance and V(D)J recombination to group 5 cells (19, 43).

Another protein that binds DNA ends is Ku, which is composed of two subunits of 70 and 86 kDa. It was originally identified as the autoantigen in a patient with polymyositis-scleroderma overlap syndrome (30). Ku has a number of striking similarities with DEB factor: it binds to DNA ends generated by sonication or by restriction cleavage leading to 5′, 3′, or blunt ends (29); it does not bind to the homopolymers poly(dA) or poly(dT) (34); it has affinity for M13 virion DNA; it is localized to the nucleus; and it is moderately abundant, with estimates of 4×10^5 molecules per cell. The Ku 70-kDa subunit has been localized to chromosome 22, but the Ku 86-kDa subunit maps to chromosome 2 (6), making the Ku p86 gene a candidate for the defect in group 5 cells.

Ku also forms a complex with a 350-kDa polypeptide to form a DNA-dependent protein kinase (DNA-PK), which is activated by the presence of free DNA ends (16). DNA-PK phosphorylates a number of proteins, including the tumor suppressor p53 (26). When cells are exposed to X rays, p53 levels rise as a result of protein stabilization, leading to G_1 arrest of the cell cycle (25). If Ku and DEB factor are indeed identical, DEB may play a role in transducing the signal that couples X-ray-induced DNA damage to increased p53 levels and cell cycle arrest.

In conclusion, DEB factor is absent in three X-ray-sensitive cell lines from complementation group 5, which is characterized by defects in both DSB repair and V(D)J recombination, and is restored when the cells revert to X-ray resistance. The loss of a protein that binds to DNA ends is consistent with the cellular defect in DSB repair and the defect in V(D)J recombination leading to large deletions of nucleotides in the residual joins. Although suggestive, these results do not prove that loss of DEB factor is responsible for the phenotype of the group 5 cells. Conclusive proof must await identification of the gene(s) encoding DEB factor and the successful rescue of group 5 cells in DNA transfection experiments. Nevertheless, we believe that further studies of DEB factor promise to yield insight into the biochemistry of DNA repair, V(D)J recombination, and perhaps cell cycle control.

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ADDENDUM

We have recently completed experiments demonstrating that Ku antisera recognize DEB factor in both hamster and human extracts (38). Furthermore, transfection of an expression vector for Ku p86 cDNA confers X-ray resistance and V(D)J recombination activity on XR-V15B cells (40a).

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