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26. Protocols for animals used in this study were reviewed and approved by institutional animal care and use committees. When anesthesia was required by the protocol (average of once per month or less), animals were lightly anesthetized with Ketamine HCl at 10 mg per kilogram of body mass.

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High Spontaneous Intrachromosomal Recombination Rates in Ataxia-Telangiectasia

M. Stephen Meyn

Ataxia-telangiectasia (A-T) is an inherited human disease associated with neurologic degeneration, immune dysfunction, and high cancer risk. It has been proposed that the underlying abnormality in A-T is a defect in genetic recombination that interferes with immune gene rearrangements and the repair of DNA damage. Recombination was studied in A-T and control human fibroblast lines by means of two recombination vectors. Unexpectedly, spontaneous intrachromosomal recombination rates were 30 to 200 times higher in A-T fibroblast lines than in normal cells, whereas extrachromosomal recombination frequencies were near normal. Increased recombination is thus a component of genetic instability in A-T and may contribute to the cancer risk seen in A-T patients.

Ataxia-telangiectasia is an autosomal recessive disease characterized by progressive cerebellar ataxia, immune defects, progeric changes of the skin, endocrine disorders, gonadal abnormalities, and a high incidence of cancer (*1*, *2*). Heterozygote carriers may also be at increased risk for cancer, particularly carcinoma of the breast (*2*). Patients with A-T are classified into four complementation groups (A, C, D, and E) on the basis of heterodikaryon complementation studies; however, there are no consistent phenotypic differences between complementation groups (*3*). The underlying defect in A-T is unknown, but A-T cells exhibit abnormalities consistent with a defect that involves DNA metabolism or the maintenance of genomic integrity: for example, frequent chromosome aberrations, sensitivity to the killing effects of ionizing radiation, and radiation-resistant DNA synthesis (*3*). Other A-T abnormalities may be the consequence of aberrant immune gene rearrangements. During normal development, the immunoglobulin (Ig) heavy chain gene and T cell receptor (TCR) genes undergo rearrangements that result in the production of IgA, IgE, IgG2, and IgG4 and the expression of α/β chain TCRs (*4*). Patients with A-T have deficiencies in these Ig classes (*1*), a relative lack of T cells expressing α/β chain TCRs (*5*), elevated frequencies of aberrant interlocus TCR gene rearrangements (*6*), and a high frequency of lymphocytes that contain chromosome translocations involving sites near immune genes [reviewed in (*7*)]. Several investigators have suggested that a defect in genetic recombination resulting in an inability to productively rearrange and repair genes would provide a unifying explanation for these immune defects and would also account for the karyotypic abnormalities and radiation sensitivity in A-T (*5*, *7*, *8*). We tested this hypothesis by measuring spontaneous mitotic recombination between directly repeated genes contained in vectors that had been integrated into the chromosomal DNA of A-T, xeroderma pigmentosum (XP), and control human fibroblasts.

Recombination first was measured by means of the retroviral vector pNeoA (Fig. 1A). This vector contains three antibiotic resistance genes driven by independent promoters: a hygromycin resistance gene (*hph*), used to select for stable transformants, surrounded by two mutant genes encoding neomycin resistance (*neo*) that are each defective because of different 8–base pair (bp) linker insertions. A 299-bp deletion of transcriptional enhancer elements in the 3′ long terminal repeat (LTR) of the vector is transferred to the 5′ LTR during infection, rendering the integrated pNeoA provirus transcriptionally silent. As a result, there is no vector mRNA production in infected human cells that might confound interpretation of recombination data. Gene conversions, unequal sister chromatid exchanges (SCEs), and reciprocal intrachromatid exchanges between the *neo* genes can be detected when they result in the reconstitution of a wild-type gene and expression of G418 resistance (Fig. 1B).

After pNeoA was transfected into the mouse retroviral packaging cell line PA317,

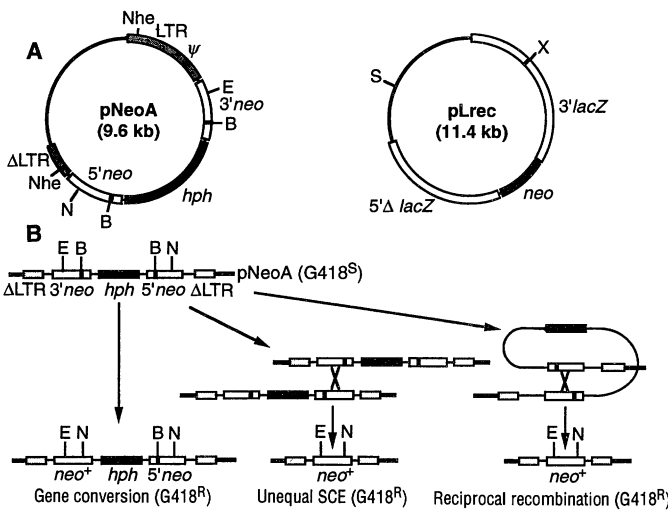

Fig. 1. The pNeoA and pLrec recombination vectors. (**A**) Vector maps. The pNeoA was constructed as described (*9*) by the insertion of two copies of *neo* from pCMIneo-(polA) along with the *hyg* from pHyg into the polylinker region of the retroviral vector pB2d (*27*). The Nae I site of the left-hand *neo* and the Eag I site of the right-hand *neo* have been destroyed by the insertion of 8-bp Bam HI linkers. The pLrec was constructed as described (*15*) by the insertion of an 8-bp Xho I linker into the Hpa I site 1351 bp from the start of the *lacZ* of pCH110 (*28*) and then the insertion of *neo* from pCMIneo(polA) and a 3-kb Hpa I–Bam HI *lacZ* fragment of pCH110 into the Bam HI site of the modified pCH110 plasmid. Restriction sites: B, Bam HI; E, Eag I; N, Nae I; Nhe, Nhe I; S, Sca I; and X, Xho I. Abbreviations: ψ, retroviral packaging signal sequence; ΔLTR, LTR containing a 299-bp deletion; and G418S, G418 sensitivity. (**B**) Intrachromosomal recombination of the integrated pNeoA vector. When integrated into genomic DNA, the pNeoA vector can undergo several different recombinational events that lead to reconstitution of wild-type *neo*, which confers G418 resistance (G418R): nonreciprocal transfer of information from one *neo* to another (gene conversion) and reciprocal exchanges that involve loss of the duplication and deletion of sequence between the points of exchange (unequal SCE and reciprocal intrachromatid exchange). The integrated pLrec vector can undergo the same rearrangements, leading to reconstitution of a wild-type *lacZ*.

Departments of Genetics and Pediatrics, Yale University School of Medicine, New Haven, CT 06510.

clones that produced retroviral particles containing pNeoA RNA were isolated, and conditioned media from these clones was used to infect SV40-transformed human fibroblasts derived from control individuals, XP patients, and A-T patients (9). A third A-T line, AT2K, was transfected with an Nhe I–cut restriction fragment of pNeoA DNA that lacked functional retroviral LTR sequences (9). Human cell lines containing a single integrated copy of the pNeoA vector were identified and used for recombination studies (*10*). Rates of spontaneous intrachromosomal recombination between the mutant *neo* genes that resulted in G418 resistance were determined by fluctuation analysis of the occurrence of recombinants during the growth of multiple parallel populations of cells (*11*). Fluctuation tests were performed on the two human fibroblast lines that served as controls: GM637 (normal) and GM847 (Lesch-Nyhan); an XP cell line from complementation group A: GM4429C; and three A-T cell lines: GM5849, GM9607, and AT2K (*12*). Three independent transformants representing different vector integration sites, as determined by Southern (DNA) blot analysis of vector-genome junction fragments, were studied from the control lines, four were studied from the XP cell line, and six from the A-T lines (Table 1). Spontaneous rates of intrachromosomal recombination for the control and XP cell lines were similar, averaging 0.52×10^{-5} event per locus per cell generation for GM637, 0.45×10^{-5} for GM847, and 0.56×10^{-5} for GM4429C. These rates are of the same magnitude as those reported by Bhattacharya *et al.* (*13*) for recombination in control and XP human fibroblasts and demonstrate that the defect in DNA repair associated with XP does not affect mitotic recombination rates.

In contrast, four independent transformants of GM5849, an A-T line from complementation group D, yielded spontaneous intrachromosomal recombination rates whose average was >100 times those of the control cell lines: 79.5×10^{-5} event per locus per cell generation (range, 51×10^{-5} to 121×10^{-5}). Recombination rates in GM9607, a fibroblast line established from another group-D A-T patient, also were elevated: 27×10^{-5} event per locus per cell generation. A pNeoA transformant of AT2K, an A-T line whose complementation group is unknown (*14*), also expressed high rates of recombination: 87.5×10^{-5} event per locus per cell generation.

Spontaneous intrachromosomal recombination rates were next measured in a control cell line (GM847) and an A-T cell line (GM5849) by means of pLrec, a nonretroviral vector (Fig. 1A). This vector has geometry similar to that of pNeoA but uses mutant *Escherichia coli lacZ* genes as its recombination substrates (*15*). An SV40 promoter drives the left-hand *lacZ* gene, which contains a Bam H1 linker insert 1351 bp from the start of the coding region, whereas the first 727 bp have been deleted from the right-hand *lacZ* gene. Recombination events involving the *lacZ* genes result in the expression of β-galactosidase, which is detectable by histochemical staining. Sca I–linearized pLrec was transfected into A-T and control SV40-transformed human fibroblasts by means of electroporation, and cell lines containing a single integrated copy of the pLrec vector were identified from among the G418-resistant clones (*15*). Rates of spontaneous intrachromosomal recombination between the mutant *lacZ* genes that resulted in expression of β-galactosidase were determined by fluctuation analysis (Table 2). Analysis included clone 67, a derivative of GM5849 that acquired radioresistance after transfection with normal human genomic DNA (*16*). Two independent transformants representing different integration sites of the vector were studied for each cell line. Spontaneous rates of intrachromosomal recombination in the control cell line were similar to those observed with the pNeoA vector, averaging 0.51×10^{-5} event per locus per cell generation. Spontaneous recombination rates again were >100 times higher in the A-T cell line, averaging 165×10^{-5} event per locus per cell generation. Spontaneous recombination rates in clone 67 cells averaged 18×10^{-5} event per locus per cell generation, almost one-tenth the rates in its parent, GM5849, but still more than 20 times higher than the control cell line.

As with the pNeoA transformants, recombination rates between pLrec clones representing different integration sites varied by no more than threefold, demonstrating that differences seen between A-T and control cell lines were not attributable to effects of integration site or the use of a retroviral vector. It is possible that high recombination rates in SV40-transformed A-T fibroblasts are not an intrinsic aspect of the A-T phenotype but result from a unique interaction between the SV40 T antigen

Table 1. Spontaneous intrachromosomal recombination rates of *neo* in human fibroblasts containing pNeoA. For each fluctuation test, 5 to 20 dishes were seeded in parallel to yield fewer than 500 colonies per dish. These parallel cultures were then expanded to a final population of 1×10^6 to 4×10^6 cells per dish. A total of 0.5×10^6 to 4×10^6 cells from each culture were then plated and grown in the presence of G418 (200 to 400 μg/ml). At the same time, representative samples of each culture were plated and grown without G418 to determine cloning efficiency and estimate the total number of cells screened. After 3 weeks, selected $G418^R$ colonies were cloned for molecular analysis, the plates were stained with Giemsa, and surviving colonies of 50 cells or more were counted. Spontaneous recombination rates were derived from the total number of $G418^R$ colonies obtained, and the total number of cells screened by means of fluctuation analysis tables (*11*).

Cell line	Retroviral clone	$G418^R$ colonies* (no.)	Cells screened (in millions)	Rate of conversion to $G418^R$ (events per 10^5 cells per generation)
		Control cell lines		
GM637D	1-S6	58 (20)	2.57	0.76
	1-F11	4 (20)	1.19	0.28
GM847	1-S2	23 (20)	1.81	0.55
		18 (10)	2.41	0.35
		XP cell lines		
GM4429D	1-F2	9 (10)	1.37	0.39
		38 (7)	1.96	0.73
	1-S1	6 (16)	0.59	0.71
		10 (9)	1.91	0.30
		3 (10)	1.58	0.18
	1-S2	11 (10)	1.60	0.38
	2-S1	17 (17)	0.67	1.21
		A-T cell lines		
GM5849	1-F1	1,459 (8)	0.323	83.7
	2-S1	13,036 (20)	2.05	89.7
		460 (5)	0.196	51.0
	2-S6	2,245 (8)	0.313	121
		1,689 (9)	0.353	84.0
		1,262 (12)	0.386	60.0
	2-S8	6,197 (15)	1.41	64.6
		5,998 (8)	1.25	70.6
		2,137 (10)	0.401	90.5
GM9607	1-13	358 (7)	0.286	28.5
		318 (7)	0.278	26.7
AT2K	23	343 (8)	0.210	87.5

*Numbers in parentheses indicate number of parallel populations for each fluctuation experiment.

required for transformation and the A-T fibroblasts. However, this is unlikely, given that the SV40-transformed XP and control cell lines have recombination rates similar to those reported for other mammalian cell lines (*17*), and there are no major effects of SV40 transformation on other aspects of the A-T phenotype, including radiosensitivity, radiation-resistant DNA synthesis, and chromosomal instability (*18*).

For the further characterization of recombination in control and A-T cells, Southern blotting and amplification by the polymerase chain reaction (PCR) were used to analyze DNA from G418^R and LacZ$^+$ recombinant clones (*15*). Recombinational events were confirmed by demonstration of the loss of a Bam HI linker restriction site and restoration of wild-type Nae I or Eag I site at the linker inserts in the *neo* genes for pNeoA and the loss of the Xho I linker restriction site and restoration of the wild-type Hpa I site in *lacZ* for pLrec. The G418^R or Lac$^+$ clones in which one of the linker mutations had converted and *neo* or *lacZ* genes as well as the intervening selectable marker had been retained without other demonstrable rearrangements were considered to represent gene conversions. The proportion of gene conversion events recovered differed between the control and A-T cell lines; more than 85% of events analyzed (12 out of 14) were consistent with gene conversion for the Lesch-Nyhan and XP clones whereas only 62% (16 out of 26) of recombinant A-T clones analyzed were gene conversions. It is possible that these events might represent reversion of the linker inserts, as both reversion and gene conversion could result in the same DNA sequence changes. To eliminate this possibility, we tested the reversion frequency of the *neo* and *lacZ* linker mutations in derivatives of GM637, GM847, and GM5849 stably transformed with vectors carrying only one mutant gene (*9*, *15*). More than 2.5×10^7 cells were screened for each mutant *neo* and 10^8 cells for each *lacZ* mutant. No G418^R or LacZ$^+$ revertants were recovered in these experiments; it is concluded that the pNeoA and pLacZ recombination vectors were not detecting reversions of the linker inserts.

Recombinant clones in which a linker mutation had converted and one *neo* or *lacZ* gene as well as the intervening selectable marker had been lost without additional rearrangements were considered to represent either reciprocal intrachromatid exchanges or unequal SCEs (Fig. 1B). A total of 2 of the 14 control and 8 of the 26 A-T recombinants met this description. Hence, elevations in the rates of recombination in A-T include increases in the rate of reciprocal intrachromatid exchanges or unequal SCEs (or both) as well as gene conversions. The wild-type *neo* was retained in 2 of the 26 recombinant pNeoA A-T clones, but they lost the other *neo* gene as well as the *hph* gene and a flanking LTR sequence, which suggests that a complex rearrangement occurred.

In previous studies of genetic recombination in A-T cells, researchers measured the frequency of extrachromosomal recombination between homologous genes carried by viruses or plasmids and found differences between control and A-T cell lines of not more than threefold (*19*). To study extrachromosomal recombination, we electroporated a Nhe I–cut DNA fragment of the pNeoA vector into a control cell line (GM847) and two A-T lines (GM5849 and GM9607) and incubated representative samples of transfected populations; either hygromycin or G418 was added to the culture medium 48 hours after transfection. The proportion of hyg^R colonies that were also G418^R in the transfected population represents the fraction of cells that recombined the two defective *neo* genes in the pNeoA vector during transfection. This proportion is the frequency of extrachromosomal recombination, averaging 2.40% for the control cell line, 1.58% for GM5849, and 0.85% for GM9607 (Table 3). These relatively minor differences between control and A-T cell lines are similar to those reported in the literature (*19*). Survival and mutagenesis of irradiated shuttle vectors, parvovirus, and herpes simplex virus also have been reported to be normal in A-T cells (*20*). Taken together, these data suggest that the abnormality in A-T specifically affects the integrity of chromosomal DNA rather than extrachromosomal viral or plasmid DNA.

The high spontaneous rates of recombination between repeated genes in two recombination vectors in SV40-transformed A-T fibroblasts adds intrachromosomal genetic instability to a spectrum of in vitro and in vivo genomic instability in A-T, which includes increased frequencies of

Table 2. Spontaneous intrachromosomal recombination rates of *lacZ* in human fibroblasts containing pLrec. For each fluctuation test, dishes were seeded with sufficient numbers of cells to provide 100 to 500 colonies per dish, which were grown until individual colonies contained 500 to 2000 cells each. These were stained for LacZ expression with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (*15*). The total number of LacZ$^+$ cells in each dish was determined by direct enumeration of the stained population under microscopic examination if <10,000. For populations >10,000, the cells were suspended by trypsinization, and then the total number was calculated from the concentration of cells in suspension. Spontaneous recombination rates were derived from the number of LacZ$^+$ cells detected and the total number of cells screened by means of fluctuation analysis tables (*11*).

Cell line	Clone	LacZ$^+$ cells detected (no.)	Cells screened (no.)	Rate of conversion to LacZ$^+$ (events per 10^5 cells per generation)
		Control cell lines		
GM847	LNL6	41	2.1×10^6	0.72
		206	7.6×10^6	0.70
	LNL4	38	4.8×10^6	0.29
		18	2.5×10^6	0.34
		A-T cell lines		
GM5849	5L20	10	3.77×10^3	152
		447	4.30×10^4	226
		50	9.23×10^3	189
	5L21	17	4.63×10^3	175
		75	2.90×10^4	82
Clone 67	B3	283	3.9×10^5	17
	A1	528	5.8×10^5	19

Table 3. Frequency of extrachromosomal recombination of pNeoA in human fibroblasts. For each experiment, 8×10^6 fibroblasts were electroporated with 8 μg of Nhe I–cut pNeoA vector DNA as described (*15*). The majority of cells in each sample were grown in G418 (200 to 400 μg/ml) beginning 48 hours after plating to determine the number of stably transfected colonies containing *neo* that had recombined before selection, whereas the remaining cells were grown in hygromycin (100 to 150 μg/ml). After 3 weeks, the plates were stained, and surviving colonies of 50 cells or more were counted.

Cell line	G418^R colonies* (no.)	hyg^R colonies* (no.)	Frequency of G418^R colonies
GM847	78 (80)	945 (20)	0.0206
	6 (89)	27 (11)	0.0274
GM5849	64 (80)	714 (20)	0.0224
	14 (89)	190 (11)	0.0091
GM9607	27 (80)	1192 (20)	0.0056
	64 (89)	688 (11)	0.0115

*Numbers in parentheses are percent of the total population plated.

translocations and other chromosome aberrations in lymphocytes and fibroblasts, micronuclei formation in epithelial cells, and loss of heterozygosity in erythrocytes (*7*, *21*). Hyperrecombination is a specific feature of the A-T phenotype rather than a generic consequence of defective DNA repair in that an XP cell line (which is defective in excision repair) exhibited normal spontaneous recombination rates [Table 1 and (*13*)]. Elevated spontaneous rates of mitotic recombination between homologous chromosomes in vivo could help contribute to the high incidence of cancer in A-T patients by causing loss of heterozygosity at recessive oncogene loci, a key genetic event in tumorigenesis.

Several hypotheses have been offered to explain the pleiotropic A-T phenotype, including defects in DNA recombination and repair (*5*, *7*, *8*, *22*), unusual chromatin or cytoskeletal structure (*23*, *24*), and cell cycle abnormalities (*23*, *25*). Our findings of high spontaneous recombination rates are not consistent with an inability to productively rearrange DNA but may result from defects in damage-sensitive cell cycle checkpoints (*23*, *26*) that allow A-T cells to replicate DNA or enter mitosis before repair of spontaneous DNA damage is complete. These same checkpoint defects could cause aberrant immune gene rearrangements by allowing DNA replication or mitosis to disrupt switch recombination and TCR gene rearrangement, leading to selective Ig deficiencies and low frequencies of T cells expressing α/β TCRs as well as a high frequency of T cells with interlocus TCRs and chromosome translocations.

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Regulation of the Ets-Related Transcription Factor Elf-1 by Binding to the Retinoblastoma Protein

Chung-Yih Wang, Bronislawa Petryniak, Craig B. Thompson, William G. Kaelin, Jeffrey M. Leiden*

The retinoblastoma gene product (Rb) is a nuclear phosphoprotein that regulates cell cycle progression. Elf-1 is a lymphoid-specific Ets transcription factor that regulates inducible gene expression during T cell activation. In this report, it is demonstrated that Elf-1 contains a sequence motif that is highly related to the Rb binding sites of several viral oncoproteins and binds to the pocket region of Rb both in vitro and in vivo. Elf-1 binds exclusively to the underphosphorylated form of Rb and fails to bind to Rb mutants derived from patients with retinoblastoma. Co-immunoprecipitation experiments demonstrated an association between Elf-1 and Rb in resting normal human T cells. After T cell activation, the phosphorylation of Rb results in the release of Elf-1, which is correlated temporally with the activation of Elf-1–mediated transcription. Overexpression of a phosphorylation-defective form of Rb inhibited Elf-1–dependent transcription during T cell activation. These results demonstrate that Rb interacts specifically with a lineage-restricted Ets transcription factor. This regulated interaction may be important for the coordination of lineage-specific effector functions such as lymphokine production with cell cycle progression in activated T cells.

T lymphocyte activation and proliferation in response to signaling through the cell surface T cell antigen receptor (TCR) is a model system for studies of cell cycle–specific transcriptional regulation (*1*). Resting peripheral blood T cells remain in G0 until they encounter an antigen-presenting cell that displays a specific antigenic peptide in conjunction with the appropriate major histocompatibility complex molecule. Antigenic stimulation results in the transcriptional induction of more than 100 new genes, many of which are specific to T cells (*1*). Antigen-stimulated T cells progress through the cell cycle and proliferate concomitantly with these changes in gene expression.

Members of the Ets family of transcrip-