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Breakpoints of t(4;11) Translocations in the Human *MLL* and *AF4* Genes in ALL Patients are Preferentially Clustered Outside of High-Affinity Matrix Attachment Regions

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Abstract Chromosomal translocations t(4;11) are based on illegitimate recombinations between the human *MLL* and *AF4* genes, and are associated with high-risk acute leukemias of infants and young children. Here, the question was asked, whether a correlation exists between the location of translocation breakpoints within both genes and the location of S/MARs. In ''halo mapping experiments'' (to define *SARs*), about 20 kb of *MLL* DNA was found to be attached to the nuclear matrix. Similar experiments performed for the translocation partner gene *AF4* revealed that SARs are spanning nearly the complete breakpoint cluster region of the *AF4* gene. By using short DNA fragments in ''scaffold reassociation experiments'' (to define *MARs*), similar results were obtained for both genes. However, Distamycin A competition experiments in combination with ''scaffold reassociation experiments'' revealed specific differences in the affinity of each tested DNA fragment to bind the isolated nuclear matrix proteins. When the latter data were compared with the known location of chromosomal breakpoints for both genes, an unexpected correlation was observed. DNA areas with strong MAR affinity contained fewer translocation breakpoints, while areas with weak or absent MAR affinity showed a higher density of chromosomal breakpoints. J. Cell. Biochem. 82: 299–309, 2001.

Key words: *MLL* gene; *AF4* gene; leukemia chromosomal translocation t(4;11); SAR/MAR elements

A number of chromosomal translocations are known to initialize malignant transformation of hematopoietic cells leading to the development of myeloid and lymphoblastic leukemias or lymphomas [Rabbitts, 1994]. Thus, it is of interest to investigate molecular mechanisms that may lead to these translocations. One hypothesis is that the inhibition of the scaffold protein DNA topoisomerase II mediates illegitimate recombination events [Domer et al., 1995; Broeker et al., 1996], presumably by leading to DNA fragmentation at specific SAR or MAR elements (S/MARs) [Käs and Laemmli, 1992]. In general, S/MARs are AT-rich DNA stretches of variable size located in specific chromatin regions. They define the attachment sites of the genomic DNA to the protein backbone of the chromosomes, and therefore, are implicated in the loop domain organization of the chromatin [Mirkovitch et al., 1984; Gasser and Laemmli, 1987; Saitoh and Laemmli, 1994]. Evidence in support of this hypothesis was derived from experiments showing specific DNA fragmentation of the Drosophila histone gene cluster at mapped S/MARs after treatment with teniposide (VM26) [Käs and Laemmli, 1992]. Similar experiments have been performed for the human *MLL* gene, and cytotoxic substances including the topoisomerase II inhibitors VP16 or VM26 induced double-stranded DNA breaks in a distinct area of the breakpoint cluster region [Aplan et al., 1996; Stanulla et al., 1997]. This site of

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preferential drug-induced DNA breakage is located in the 3′-part of the *MLL* breakpoint cluster region, in vicinity of exon 12 of the *MLL* gene [nomenclature according to Nilson et al., 1996; Marschalek et al., 1997], in an area that is part of a mapped SAR [Broeker et al., 1996]. The same region was shown to be sensitive to DNase I treatment, indicating a highly sensitive chromatin structure [Strissel et al., 1998a]. The assumption that a site with particular high sensitivity to induced DNA breakage is located in the *MLL* breakpoint cluster region was further supported by the finding that breakpoints in therapy-induced acute myeloid leukemias (t-AML) were located within the same area of the human *MLL* gene [Domer et al., 1995].

Recombinations may occur within S/MAR elements via aberrant cleavage by topoisomerase II in vivo. For instance, "hot spots" for cleavage by topoisomerase II were identified in the S/MAR of the mouse immunoglobulin kappa gene that correspond to breakpoints of chromosomal translocations [Perlmutter et al., 1984; Shapiro and Weigert, 1987; Sperry et al., 1989]. Other examples for the potential involvement of topoisomerase II-mediated recombination processes were the deletion of a S/MAR of the rabbit Ig kappa chain gene that occured during evolution [Emorine and Max, 1983; Sperry et al., 1989] and the recombination junction of the human ring chromosome 21 that contains S/MARs at the fusion site [Sperry et al., 1989; Wong et al., 1989]. Intra-chromosomal deletions in cases of human thalassemias were also mapped within or adjacent to the S/MAR of intron 2 (IVS2) of the human β-globin gene [Anand et al., 1988; Jarman and Higgs, 1988; Cunningham et al., 1994]. Breakpoints of deletions within the short arm of human chromosome 9 were frequently identified in human leukemias and gliomas [Cairns et al., 1994] and were associated with mapped S/MARs [Diaz et al., 1988; Strissel et al., 1998b].

Therefore, the attractive hypothesis was proposed that recombination processes may preferentially occur in DNA sequence regions rich in S/MAR activity [Cockerill et al., 1987; Sperry et al., 1989; Broeker et al., 1996]. To test this hypothesis, breakpoint cluster regions of recombination partner genes need to be cloned and genomic DNA fragments must be analyzed for their binding affinity to nuclear matrix proteins. However, cloned DNA fragments from breakpoint cluster regions so far are available only for the *MLL* gene [Gu et al., 1994; Marschalek et al., 1995] and the translocation partner genes *AF4* [Reichel et al., 1999], *AF-6* [Saito et al., 1998], and *AF-9* [Strissel et al., 2000].

Although several characteristic motifs are known for S/MARs, no unique consensus S/MAR sequence has been identified so far [Boulikas, 1993; Kramer et al., 1996; Van Drunen et al., 1999], and therefore, it is not yet possible to unambiguously identify a functional S/MAR solely by analyzing known stretches of genomic DNA. Instead, DNA sequences displaying S/MAR function can only be identified with biochemical methods, either after preparation of nuclear halo's in combination with Southern blot experiments (to define *SARs* in "halo mapping experiments") [Mirkovitch et al., 1984], or by DNA binding assays using isolated nuclear matrix proteins and genomic DNA fragments (to define *MARs* by "scaffold reassociation experiments") [Cockerill and Garrard, 1986]. Both types of experiments were performed here for a 75 kb contig of the human *MLL* gene and a 52 kb contig of the human *AF4* gene. S/MARs were found for both genes and shown to overlap with their known breakpoint cluster regions, indicating that these two translocation partner genes contain genomic regions with a common chromatin structure.

MATERIALS AND METHODS

Chromosomal Translocation Breakpoints From t(4;11) Patients

All chromosomal breakpoint sequences have previously been published [Reichel et al., 1998, 1999, 2000; Gillert et al., 1999] and the corresponding data have been deposited in public databases (Genbank accession codes: AJ000166–AJ000180, Y16596–Y15599, AJ23-5330–AJ235380, AJ408891–AJ408956).

PAC-DNA

The PAC clone 583j12 (RCPI 1.3–5) was isolated by the German Resource Center, Berlin (RZPD) by using a specific probe encoded by the breakpoint cluster region of the human *AF4* gene. Terminal sequence analysis indicated that the insert of this PAC clone is about 140 kb in length and encodes a portion of the human *AF4* gene (introns 2–18).

Isolation of cDNA Probes Hybridizing With Major Parts of the Human MLL Gene

Six different cDNA probes were cloned and used for hybridization experiments. The cDNA nomenclature corresponds to the published full length cDNA of the human *MLL* gene (Genbank accession code: 1490270). The first cDNA clone codes for exons 3–6 (nt 498–3,597) and is able to hybridize with four different EcoRI fragments of *MLL* genomic DNA. The second cDNA clone contains exons 6–16 (nt 3,499–4,984) and is able to hybridize with EcoRI restriction fragments D–F (Fig. 1). The third cDNA clone encodes *MLL* nucleotides 5,167–5,948 and hybridizes to the EcoRI restriction fragment I. The fourth cDNA clone encodes the nucleotides 5,826–7,476 and hybridizes with fragments J–L. The fifth and sixth cDNA clones are encoding nucleotides 7,036–9,582 and 9,491–12,126 of *MLL*, respectively. The latter clones are hybridized to fragments M and N–P, respectively (Fig. 1). Using these six cDNA's, a total of 16 different restriction fragments have been analyzed, spanning ~75 kb of the human *MLL* gene and encompassing exons 3–37.

Isolation of Genomic DNA Probes Hybridizing to the Breakpoint Cluster Region of the Human *AF4* Gene

The PAC clone 583j12 has been shown to encompass the central and 3′-portions of the human *AF4* gene [Reichel et al., 1999], including the complete *AF4* breakpoint cluster region (Genbank accession code: AJ238093). Isolated Pac 583j12 DNA was used to amplify independent DNA probes that were: (1) applicable for DNA hybridization experiments, because they do not represent repetitive DNA and (2) hybridize to EcoRI restriction fragments of the breakpoint cluster region of the human *AF4* gene. Nine different genomic probes were isolated that hybridized to a contig of 49,347 bp spanning the *AF4* breakpoint cluster region (data not shown). Only two small EcoR1 fragments of the *AF4* gene can not be identified by using these probes: a 414 bp fragment located between fragments 3 and 4, and a 715 bp fragment located between probes 4 and 5. Probes 5–9 recognized two adjacent EcoR1 restriction fragments each. Cloned DNA fragments and corresponding oligonucleotides are listed in Table I.

Halo Mapping Experiments

Nuclear scaffold preparations were performed as described by Mirkovitch et al. [1984] with the following modifications: digestions of nuclear halo's were performed twice with 1,000 U/ml EcoRI restriction enzyme (NEB, Schwalbach, Germany) for at least 8 h, or double digested with 1,000 U/ml EcoRI and BamHI (NEB, Schwalbach, Germany) once. Nucleic

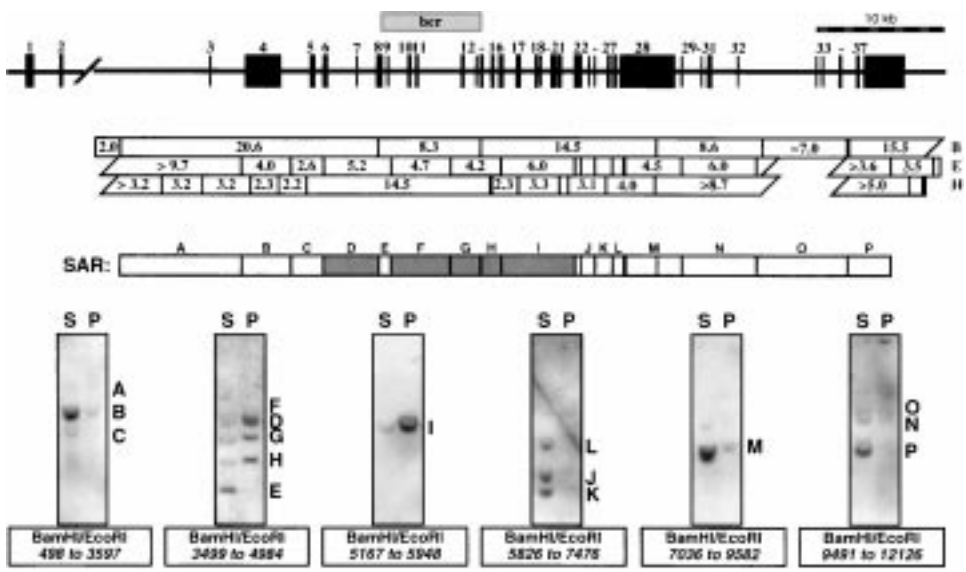

Fig. 1. SARs identified by halo mapping in the human *MLL* gene. Top: Exon/intron structure of the human *MLL* gene (exons 1–37). bcr: breakpoint cluster region. Center: restriction map for BamHI (B), EcoRI (E), and HindIII (H). A–P: restriction fragments of the 75 kb contig (exons 3–37). Dark grey boxes: restriction fragments with SAR function. Bottom: Southern blots using the six *MLL* cDNA probes. Each restriction fragment is marked with corresponding letters A–P. S, P: supernatant and pellet fractions.

TABLE I. Cloned AF-4 Gene Fragments Used for DNA Hybridization Experiments

DNA probe	lgth in bp	Oligonucleotides (5′-3′) used for PCR amplification
1	687	FelC·31 TAGAGCATACATTCTGTCACTG FelC·53 AACTGGGAACATACAGCTAAC
2	314	Bo·31 CTTTTTCCTCCGTAGCTCATG Bo·5 GCCTCTGAAGTGCAGGTGAC
3	277	IPE·3 CGAAGCAAGTATTTGCTGTTGC IPH·5 CTGAGTCTTTCTTTCCTATTAGC
4	807	EP·3 TTAATCTCCTTTGGAGGCTGC PX·5 GAAAAAGCAGCAGGATGATAC
5	206	600·31 GGAGAATCTTGCTCATAAGCAC 7800·5 GAATCCACTTCTCACTGTAGTC
6	1200	8100·31 ATTTAGGCAACTACTCTTATAGG I27·52 CCTGGGTGTGCCTACTTCAG
7	551	3600·3 TCATCGTGGACTCCAAAAGCAT 2600·51 GTTTGTAAGTCCTTCTCTGGC
8	359	2300·3 CAGAAGTAGTCTTGGAACGTA 4000·5 CTTCAAAGGTAAGTCTAAAGATGA
9	942	4400·32 AGTTTGCCGGGCAGGTCTGA AF4·51 GGCCATGAATGGGTCATTTC

acids in the supernatant and pellet fractions were purified and digested by Proteinase K (100 μg/ml). After phenol- and chloroform-extraction, nucleic acids were ethanol-precipitated and dissolved in TE buffer. After size-separation on 1% agarose gels, Southern blot analysis was performed by using the *MLL*- and *AF4*-specific probes listed above.

Scaffold Reassociation Experiments

To assay DNA binding to nuclear matrices, nuclear matrix protein preparations were performed from human Raji cells (ATCC#: CCL-86) as described by Cockerill and Garrard [1986]. Isolated nuclear matrix proteins from $1-1.3 \times 10^7$ Raji cells, were incubated for 2 h at room temperature with different amounts of sheared *E. coli* competitor DNA (100–500 μg/ml; Sigma Aldrich, Dassel, Germany) and 50 ng of (γ-^{32}P)-ATP labeled DNA fragments. Specific competition experiments were performed by using different amounts of sheared *E. coli* competitor DNA in the presence of the specific minor groove binding competitor Distamycin A (Sigma Aldrich, Dassel, Germany) [Romig et al., 1994].

DNA fragments *MLL* a–f and *AF4* A–K (see Table II) were prepared by long-range PCR amplification [Reichel et al., 1999] by using either genomic DNA or PAC 583j12 DNA together with specific pairs of oligonucleotides as listed in Table II. After incubation, DNA present in the pellet- and supernatant fraction was separated in a single centrifugation step. Pellet fractions were washed once in binding buffer [according to Cockerill and Garrard, 1986] and digested with Proteinase K (400 μg/ml overnight at 37°C). After phenol- and chloroform-extraction, nucleic acids were ethanol-precipitated and dissolved in TE buffer. Supernantants were ethanol-precipitated and dissolved in TE buffer. All samples were loaded on 1% agarose gels and after electrophoretic separation, gels were dried and exposed to X-ray film.

RESULTS

Halo Mapping Experiments Using Partial Contigs of the Human *MLL* and *AF4* Genes

Nuclear halo preparations were digested with BamHI and EcoRI. Pellet (SAR-containing) and supernatant fractions (loop-fraction) were subsequently purified and equal amounts of genomic DNA of both fractions were separated by electrophoresis in agarose gels. Southern blots were prepared and hybridized with the six different radiolabeled *MLL* cDNA probes. A continuous contig (*MLL* intron 2–exon 37) represented by the EcoRI restriction fragments A–P was analyzed (Fig. 1). *MLL* restriction fragments D and F–I fractionated to the pellet, while all other fragments of the *MLL* gene remained in the supernatant. This led to the conclusion that the two *MLL* gene regions located between exons 6–8 and exons 10–22 are SARs (dark grey boxes in Fig. 1) [nomenclature of *MLL* exons according to references Nilson et al., 1996; Marschalek et al., 1997]. Thus, major parts of the *MLL* breakpoint cluster region (exons 8–14) are overlapping with the mapped SARs and are creating a long scaffold attachment region of about 20 kb in the central region of the human *MLL* gene. Only a small 1.2 kb BamHI/EcoRI fragment between

TABLE II. Amplification of MLL- and A4- Specific DNA Fragments Using Long Range PCR

Fragment	lgth in bp	Oligonucleotides (5′-3′) used for PCR
MLL a	3900	6·3 CCTGTCACTAGAAACAAGGC 8·5 GGAGGAGGCTCACTACTGC
MLL b	2185	8·3 CCCAAAACCACTCCTAGTGAG 10·5 GTTCAAAGTGCCTGCATTCTC
MLL c	1854	10·3 CAGCAGATGGAGTCCACAG I11·52 ATCTTTAGGACTTCATATTTGCC
MLL d	5036	I11·CB ATGTCCATGACATATCACTGAG 15·5 CATCATAACATTTGTCACAGAG
MLL e	3496	15·3 ATCGCTGGGTCCATTCCAA 19·5 TTGCTTGATACTTTATTTGGC
MLL f	3356	19·3 CAAATGGAACGTGTTTTTCCA 22·5 CCATATGCACATTCTTTAGTGA
AF4 A	5258	AF4x1 CTTCTATGCAGAGTACCATGCTTTAGCACCAAG AF4y3 CTACTCCATTGTTGACAGCAGATGCACTGGTG
AF4 B	4916	AF4x4 GGCACCAGTGCATCTGCTGTCAACAATGGAG AF4y7 TAAGATTGTGTCATAATCTTCTACCTTATGTTGG
AF4 C	5578	AF4x7 TTTCCTTGTGGCCTCTTGTCACATCATGTTAGG AF4y10 AGGAACAGTGTTTACCTACTTCTGTCTCCAGAG
AF4 D	6927	AF4x11 TCTGGAGACAGAAGTAGGTAAACACTGTTCCTG AF4y14 TTAACCAGTCTTTCCTCCATGATATGAGGACAG
AF4 E	5040	AF4x15 TAGTATTATGACGCAGTGCCGCTATCAAAGTAG AF4y17 TGGAGTGTGCATGCACATGCATAAATGCATACG
AF4 F	5574	AF4x17 GCCATTGCAGCCGCCAGCACACGAGACAG AF4y20 CCACATGAGGCCCCATGAAGCCCATTGTGG
AF4 G	5965	AF4x21 CTTAATCATTCATGGCTGCTGTGCCCACAATGG AF4y24 TCAAGAAAATCTAGTTTGTAAGTCCTTCTCTGG
AF4 H	6483	AF4x25 GTCATGTTAAAGTCAGTGTCTATACCATTGAGG AF4y27 CGTCAAGAGTAAGAGCTGGAATGGACCCTGG
AF4 I	5324	AF4x28 ATGCCCATTTATGATTTGGAAATCATAACTTGG AF4y30 GGTTTTGGGTTACAGAACTGACATGCTGAGAG
AF4 K	5223	AF4x31 CAGCATGTCAGTTCTGTAACCCAAAACCAAAG AF4y33 GCAGATTCTACTCCTTACCAGCCATCAACTGG

exon 8 and intron 9 of the *MLL* breakpoint cluster region (1.2 kb; fragment E in Fig. 1) was not part of this 20 kb SAR area. The 5′- and 3′-portions of the *MLL* gene (fragments A–C and J–P) displayed no SAR function in these experiments.

The breakpoint cluster region of the human *AF4* gene was similarly analyzed. Probes 1–9 were used to identify SAR containing areas within the breakpoint cluster region of this gene (Fig. 2). All DNA fragments except fragment B were found in the pellet fraction, indicating that SAR-containing areas were spanning a major portion of the breakpoint cluster region of this gene. Fragment B was found only in the supernatant fraction, indicating that this small 596 bp fragment lacked SAR function as detected in this assay. Fragments E (414 bp) and G (715 bp) cannot be identified with the probes used for this experiment and therefore, no conclusion can be reached for these two fragments. A positive (*MLL* cDNA encoding exon 8–14) and a negative control (*MLL* exon 4) were carried along in parallel to verify the correct distribution of "SAR"- and "Loop"-DNA fractions. From the results we concluded that almost the complete DNA sequence of the breakpoint cluster region of the *AF4* gene contributes to SAR function, either as part of one very large or several small SAR elements.

Scaffold Reassociation Experiments With Specific DNA Fragments of the Human *MLL* and *AF4* Genes

Specific PCR fragments of the *MLL* (MLL a–f; Fig. 3A and C) and *AF4* breakpoint cluster regions (*AF4* A–K; Fig. 3B and D) were generated. To determine the specificity of nuclear matrix proteins bound to *MLL* and *AF4* PCR fragments, control experiments were performed by using a cloned and well characterized fragment of the human interferon-β-S/MAR as a positive control (Fig. 4A) [Bode and Maass, 1988; Mielke et al., 1990; Kay and Bode, 1994; Benham et al., 1997]. The PCR amplified DNA fragments *MLL* a–f were used to verify the results obtained in the halo mapping experiments. Each of the latter fragments were incubated after radioactive end-labeling with isolated nuclear matrix proteins. High-affinity binding was observed for *MLL* fragments c and d, while fragments a, e, and f had reduced affinities (Fig. 4B). Only fragment b failed to bind in the presence of 200 μg/ml competitor DNA, in agreement with the result of the halo mapping experiments (see above). From these

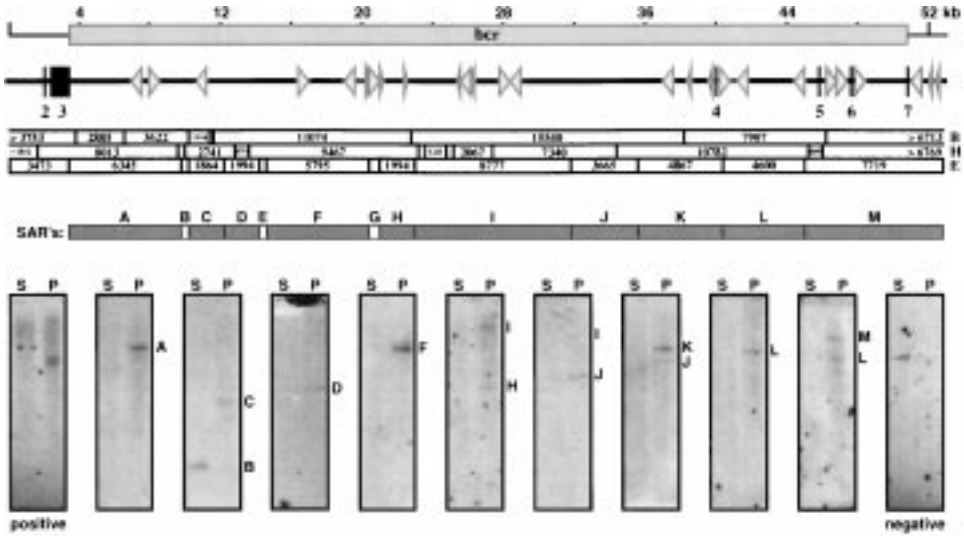

Fig. 2. SARs identified by halo mapping in the human *AF4* gene. Top: size marker and exon/intron structure of the breakpoint cluster region of the human *AF4* gene (introns 1b-7; light grey triangles indicate the presence and orientation of Alu repetitve DNA elements). Center: restriction map for BamHI (B), HindIII (H), and EcoRI (E). A–M: restriction fragments of the 49 kb contig (exon 3–inton 7). Dark grey boxes: restriction fragments with SAR function. Bottom: Southern blots using the probes 1–9 and two cDNA probes of the human *MLL* gene that were used as positive (left) and negative controls (right; see Material and Methods). Each restriction fragment is marked with corresponding letters A–M. S, P: supernatant and pellet fractions.

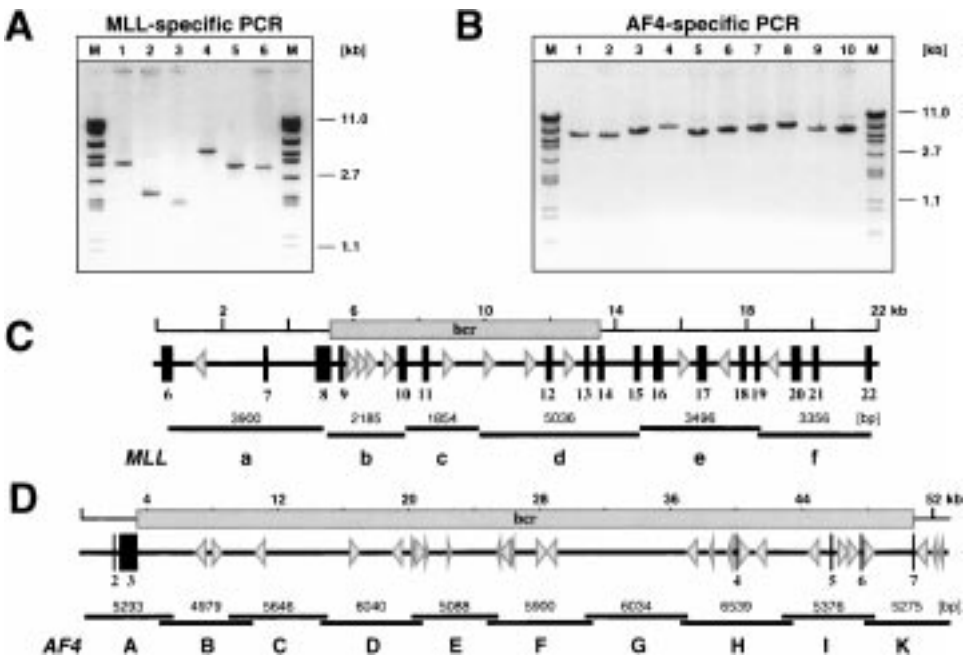

Fig. 3. PCR amplified probes of the *MLL* and *AF4* genes used for scaffold reassociation experiments. **A:** Agarose gel showing the six PCR amplification products specific for the *MLL* gene (exons 6–22). **B:** Agarose gel showing the 10 PCR amplification products specific for the *AF4* gene (introns 1b-7). **C:** Top. Size marker. bcr: breakpoint cluster region. Scheme of the PCR amplified regions of the *MLL* gene (black boxes: exons with corresponding numbers; light grey triangles: Alu repetive DNA elements and their orientation; black lines: amplified products (a–f) and their sizes in basepairs. **D:** Top. Size marker. bcr: breakpoint cluster region. Scheme of the PCR amplified regions of the *AF4* gene (black boxes: exons with corresponding numbers; light grey triangles: Alu repetive DNA elements and their orientation; black lines: amplified products (A–K) and their sizes in bp.

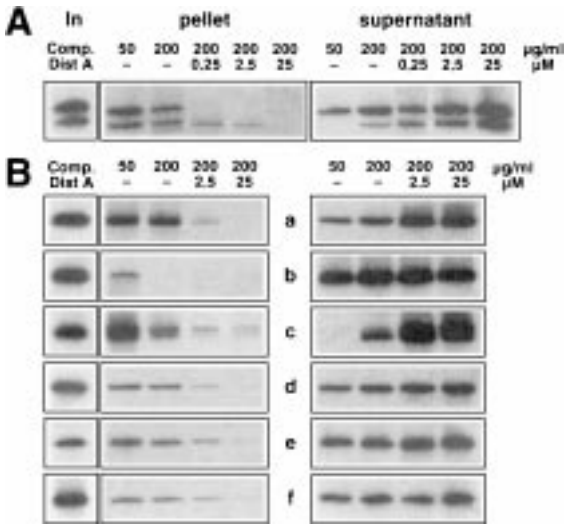

Fig. 4. Scaffold reassociation experiments with PCR amplified DNA fragments of the human *MLL* gene. **A:** Control experiment as described in Materials and Methods using a 2.2 kb EcoRI fragment from the human IFN-β upstream SAR element that was cloned into the polylinker of plasmid pTZ18R. In: DNA input for each reaction. Pellet: amount of DNA that was pelleted after the centrifugation step. Supernatant: amount of unbound DNA after the centrifugation step. Comp.: amount of unspecific *E. coli* competitor DNA in µg/ml. Dist A: amount of the specific minor-groove binding competitor Distamycin A in micromolar. Upper band: pTZ18R vector DNA fragment (internal control); lower band: human beta interferon SAR 2200 DNA fragment. **B:** Results of the experiments using the *MLL* PCR fragments a–f. Legend similar as for panel A.

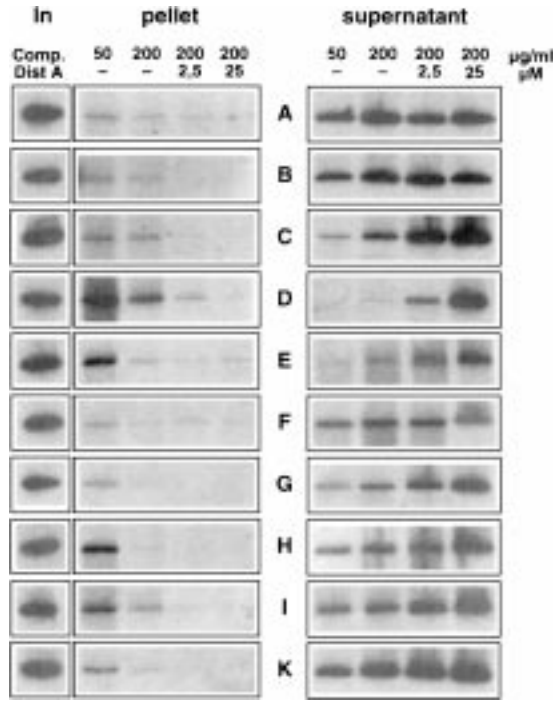

Fig. 5. Scaffold reassociation experiments with PCR amplified DNA fragments from the human *AF4* gene. Results of the experiments using the *AF4* PCR fragments A–K. In: DNA input for each reaction. Pellet: amount of DNA that was pelleted after the centrifugation step. Supernatant: amount of unbound DNA after the centrifugation step. Comp.: amount of unspecific *E. coli* competitor DNA in µg/ml. Dist A: amount of the specific minor-groove binding competitor Distamycin A in micromolar.

results it was concluded that the *MLL* gene contains a large MAR area with an interruption in the centromeric portion of the breakpoint cluster region.

The same experimental strategy was applied to the PCR fragments *AF4* A–K derived from the *AF4* breakpoint cluster region. These PCR amplimers were of roughly comparable sizes (4.916–6.927 bp and medium length of 5.629 bp). All PCR amplimers were incubated with distinct amounts of unlabeled *E.coli* competitor DNA, the specific minor groove binding competitor Distamycin A and equal amounts of isolated nuclear matrix proteins. The PCR amplimers *AF4* D and E showed higher affinity than all other PCR amplimers (Fig. 5). All other DNA fragments had a lower affinities to the isolated matrix proteins and fragments *AF4* A, F, and K showed very weak matrix protein binding activity. In conclusion, the *AF4* breakpoint cluster region exhibit binding acitivities to isolated nuclear matrix proteins, however, different areas of *AF4* display different affinities as demonstrated by these in vitro experiments. Moreover, the results from the in vitro binding experiments are consistent with the data obtained by the in vivo halo mapping experiments, because all DNA fragments are still binding nuclear matrix proteins in the presence of 200 µg/ml competitor DNA.

Location of Chromosomal Breakpoints Relative to SAR and MAR Elements in the *MLL* and *AF4* Genes

The sequence of the chromosomal breakpoints on both derivative chromosomes of the t(4;11) all patients analyzed here, have recently been published [Reichel et al., 1998, 1999, 2000; Gillert et al., 1999]. This information was used to analyze the location of mapped chromosomal breakpoints of t(4;11) leukemia patients relative to areas of the *MLL* and *AF4* genes for which S/MAR function was observed here in the halo mapping and scaffold reassociation experiments. The density of chromosomal breakpoints was found to be inversely correlated to the

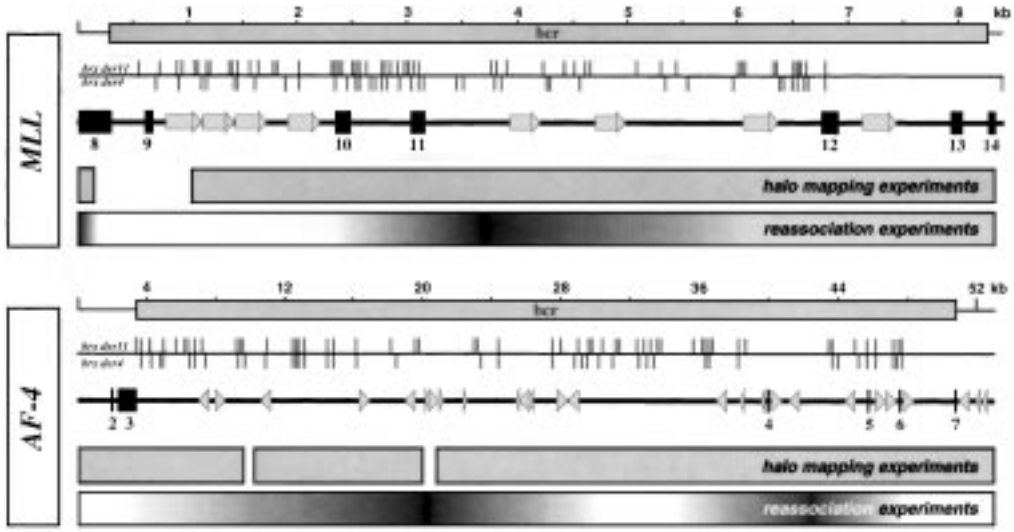

Fig. 6. Comparitive view of chromosomal break sites in the *MLL* and *AF4* genes and the results of the halo mapping and scaffold reassociation experiments. Top row: Scale with breakpoint cluster region; second row: derivative 11 and derivative 4 breakpoints that were mapped for the *MLL* gene; third row: schematic representation of the *MLL* breakpoint cluster region (exons 8–14) showing Alu repetitive elements and their orientation (light grey arrows); fourth and fifth row: results of the halo mapping and scaffold reassociation experiments, respectively. High-affinity binding of nuclear matrix proteins to specific DNA fragments was indicated in deep black; white areas: fragments with weak or undetectable binding affinity. Bottom: Scale with breakpoint cluster region; second row: derivative 11 and derivative 4 breakpoints that were mapped in the *AF4* gene; third row: scheme of the *AF4* breakpoint cluster region (introns 1b-7) with Alu repetitive elements and their orientation (light grey triangles); fourth and fifth row: results of the halo mapping and scaffold reassociation experiments, respectively. DNA fragments binding with high-affinity to nuclear matrix proteins are shown in deep black; fragments with weak or undetectable binding affinity in white.

location of DNA fragments with high-affinity binding for nuclear matrix proteins (summarized in Fig. 6). The chromosomal breakpoints were clustered in the centromeric area of the breakpoint cluster region of the *MLL* gene that was devoid of S/MAR activity (Figs. 1 and 5B: fragment b). Conversely, the telomeric area of the breakpoint cluster region of the *MLL* gene contained fewer translocation breakpoints and overlapped with a high-affinity S/MAR element (Figs. 1 and 5B: fragments *MLL* c and d). Fragments *MLL* e and f were located outside of the *MLL* breakpoint cluster region and consequently no correlation between the occurence of chromosomal breakpoints and S/MAR function could be obtained for this region.

Similar results were obtained for the breakpoint cluster region of the *AF4* gene (Figs. 3 and 7). Fragments *AF4* D and E showed high-affinity binding of nuclear matrix proteins, but displayed a lower density of chromosomal breakpoints than fragments *AF4* A–C and F, G, and K. Fragments *AF4* H and I had higher MAR activities than the surrounding fragments of the *AF4* gene and contained fewer chromosomal breakpoints.

DISCUSSION

The major conclusions drawn from this study were: (a) both the human *MLL* and *AF4* genes contain DNA regions with S/MAR function, (b) S/MAR regions overlapped with the mapped breakpoint cluster regions of both genes, and (c) the majority of chromosomal breakpoints in t(4;11) leukemic cells were found in areas of the *MLL* and *AF4* breakpoint cluster regions that displayed weak or no S/MAR function.

SAR elements identified by halo mapping had already been identified in the breakpoint cluster region of the *MLL* gene, mainly in the area upstream of exon 8 and downstream of exon 11 [Broeker et al., 1996]. Here, the identification of two S/MARs in the *MLL* gene encompassing exons 6–8 and 12–22 confirmed these initial findings (Figs. 1 and 4B) and extended our knowledge about the distribution of SAR elements within the *MLL* gene to larger genomic region.

Furthermore, we raised the question about specific chromatin structures in the translocation partner gene *AF4*. The *AF4* gene was chosen, because structural parts of this gene were known [Nilson et al., 1997] and the translocation t(4;11) is frequently associated with high risk acute lymphoblastic leukemia. More importantly, the *AF4* breakpoint cluster region was cloned [Nilson et al., 1997] and completely sequenced (exons 2–7) [Reichel et al., 1999], and thus, this part of *AF4* was available for the experiments presented here.

S/MAR functions were observed for a large portion of the *AF4* breakpoint cluster region (Figs. 2 and 5). This led to the conclusion that both genes involved in t(4;11) translocations have common structural chromatin features, including S/MAR elements. This demonstrates for the second time that a partner gene involved in 11q23 translocations does exhibit S/MAR function in its breakpoint cluster region. Recently, data were presented for the human *AF9* gene that is involved in the recurrent t(9;11) translocation [Strissel et al., 2000]. In these studies, two breakpoint cluster regions were identified within the *AF-9* gene. Chromosomal breakpoints in nine individual t(9;11) patients were mapped in regions of *AF-9* that were flanked by two high-affinity SARs as defined by halo mapping experiments.

The S/MAR containing regions of both genes are very large as defined by the halo mapping experiments (*MLL*: 4 and 16 kb; *AF4*: 10 and 42 kb) and the scaffold reassociation experiments (*MLL*: 3.9 and 13.7 kb; *AF4*: 32.1 and 11.9 kb). This is highly unusual when compared to other known S/MARs which are normally in the range of 300 to several thousand basepairs [Gasser and Laemmli, 1987; Saitoh and Laemmli, 1994; Benham et al., 1997]. These extended S/MARs may alternatively represent an internal clustering of several shorter S/MAR regions similar to the clusters of attachment sites that are described for the centromeric regions of chromosomes 1 and 16, leading to DNA loops, that are much smaller in size than an average loop [Vogelstein et al., 1980; Gasser and Laemmli, 1987; Barone et al., 1994; Strissel et al., 1996]. Thus, the question arises whether there exists a correlation between specific chromatin structures and the density of chromosomal breakpoints.

One argument in favor of this hypothesis came from experiments using inhibitors of topoisomerase II (epipodophyllotoxins). Topoisomerase II resembles an important component of the chromosomal scaffold and is essentially involved in S/MAR function [Berrios et al., 1985; Gasser et al., 1986; Heck and Earnshaw, 1986; Sperry et al., 1989]. Using the two topoisomerase II inhibitors VP16 and VM26, specific DNA double strand breaks were induced in the telomeric portion of the *MLL* breakpoint cluster region [Aplan et al., 1996; Stanulla et al., 1997; Strissel et al., 1998a]. Moreover, the existence of t-AML patients demonstrates that the therapeutic use of topoisomerase II inhibitors may account for a certain number of tumor patients that aquired chromosomal translocations of the human *MLL* gene after treatment with these inhibitors [Domer et al., 1995].

When the available chromosomal breakpoints in *MLL* and *AF4* [Reichel et al., 2000: data from 60 t(4;11) patients] were compared with the results of the scaffold reassociation experiments, a more detailed picture emerged: the density of chromosomal breakpoints in the breakpoint cluster region of both genes was inversely correlated with the distribution of the high-affinity MAR's as mapped by scaffold reassociation experiments (Fig. 6). Data obtained for the human *AF9* gene [Strissel et al., 2000] led to a similar conclusion, because chromosomal breakpoints between the *MLL* and *AF9* genes were also located outside of the two high-affinity SARs mapped for this particular gene. One SAR was identified within and the other outside of the breakpoint cluster region of the *AF9* gene [Strissel et al., 2000]. These findings may be explained by the assumption that distinct classes of S/MARs might exist [Van Drunen et al., 1999] with differential susceptibility for chromatin breakage. If this interpretation were correct, then a simple model may account for the observations made here. If nuclear matrix proteins are bound with high affinity to certain DNA areas, they presumably make this area more rigid for cellular processes. If this hypothesis were true, then one would expect that high-affinity S/MARs should protect against chromosomal translocations and that low affinity S/MARs or areas without S/MAR function should be more prone to DNA strand breaks. Moreover, the distance between two neighbouring S/MARs in the chromosomal DNA would be critical: short DNA loops located between long S/MARs would statistically

aquire more torsional stress than large DNA loops located between short S/MARs. Further analysis of more translocation partner genes will help to get a more detailed picture about chromatin structures and incidence for chromosomal translocations.

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