

**Figure 2.** Screening and genotyping of rare short PATRR22 variants. (A) Genomic structure of the PATRR22 region. LCR-specific primers are designed to amplify the PATRR22 (arrows). Proximal region represents the 'AT-rich region-HSAT1-Alu' cassette repeated three or four times (6,29). Sites for the forward primer (F) carry sequence variations among the AT-rich region polymorphic alleles. In the B and C alleles, the PCR product originates from the closest F site, whereas it can anneal with the second closest site in the A allele. (B) LCR-specific PCR for amplifying the PATRR22. Upper panel represents the LCR-specific PCR for the PATRR22, whereas the lower panel indicates the genotyping of the AT-rich region flanking the PATRR22. Genotypes of the AT-rich region are given at the bottom. The PATRR22-specific PCR product from the A allele is much longer than the others for the reason described above. M, size markers. (C) Rare variants. Lane 1, standard B/C heterozygote; lane 2, compound heterozygote for B/C rare variants; lane 3, C/C homozygote; lane 4, compound heterozygote for C rare variants.

B allele (Case 5). Although the substitution slightly reduces the symmetry of the B allele, the difference did not appear to alter the allelic preference for the C allele.

To investigate the potential role of secondary structure on translocation frequency, we calculated  $\Delta G$  and the  $\Delta G$  value normalized with size. It is assumed that PATRRs with a lower  $\Delta G$  value would show a greater propensity for secondary structure leading to a higher translocation frequency. However, these values did not appear to correlate with the translocation frequency (Table 3).

#### Identification of rare variants that produced t(11;22) less frequently

To elucidate the effect of size and symmetry more completely, we attempted to identify individuals carrying shorter or more asymmetric PATRR22 variants, which are rare. The LCR22s share 97–98% sequence homology with each other. Therefore, polymorphisms of the PATRR22 are difficult to analyze because PCR amplification is always accompanied by multiple PCR products emanating from other LCR22s (6). PCR primers located at sites with subtle nucleotide difference among the LCR22s allowed us to reduce the background amplification (Fig. 2A and B). Occasionally, the PCR amplified non-specific products from other LCR22s, such that we could not use this system to accurately determine the PATRR22 genotype. However, we could utilize this PCR for the identification of rare size variants of the PATRR22 by screening individuals who did not carry the standard ~597 bp PATRR22 on either allele. Over 200 male volunteers were screened and we eventually identified two individuals (Fig. 2C).

One B/C heterozygote (Case 15) was found to carry two short PATRR22s. Sequence analysis revealed that both the B and C alleles manifest a symmetric structure and the sizes were 553

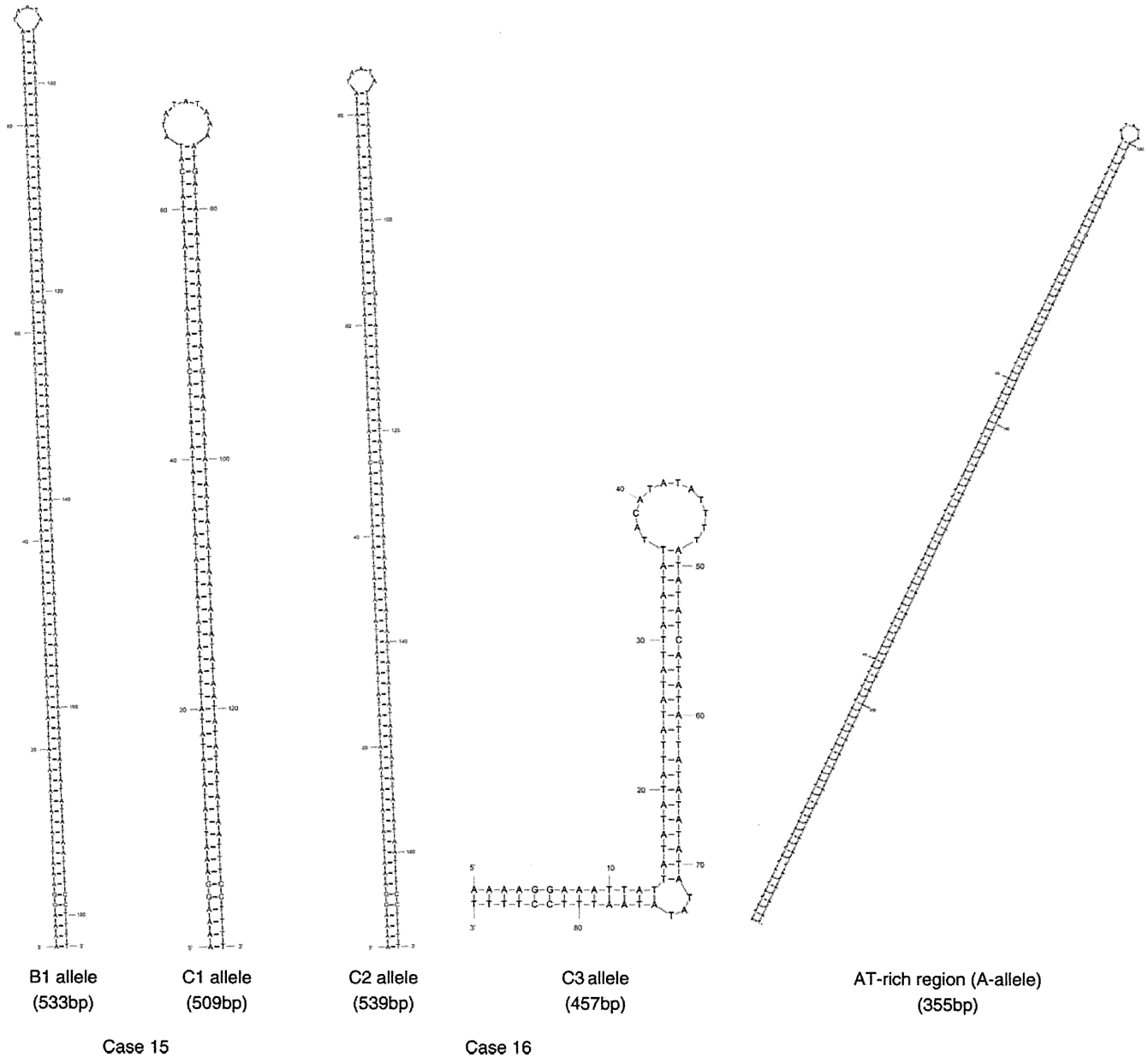
and 509 bp, respectively (Table 4). The total translocation frequency for this male was similar to that of others, but the shorter 509 bp variant produced fewer translocations than the 553 bp variant (18 versus 82%). This result clearly indicates that the size of the PATRR22 affects the translocation frequency. Another case, a C/C homozygote (Case 16), was also found to carry two short PATRR22s, the sizes of which are 539 and 457 bp (Table 4). The 539 bp allele was found to be symmetric, whereas the 457 bp allele manifests an asymmetric structure. The total translocation frequency for this male was also similar to that of others, but the translocations were exclusively derived from symmetric 539 bp PATRR22. Predicted secondary structures for these alleles are shown in Figure 3. The 457 bp PATRR22 manifests an asymmetric structure with a large single-stranded loop at the center. However, we did not determine whether size or symmetry affected the allelic bias.

#### The AT-rich region flanking the PATRR22 also produces translocations

During the course of analysis of the *de novo* t(11;22)s derived from A/C heterozygotes, we unexpectedly identified a variant der(22) product, which originated from the A allele (Fig. 4). The breakpoint on chromosome 11 was located at the center of the PATRR11, which is consistent with the standard t(11;22) breakpoints. However, the breakpoint on chromosome 22 was located at the center of the 355 bp flanking AT-rich region of the A allele (AB538239). The 355 bp AT-rich region forms a quasi-palindromic structure similar to the PATRR. When the entire 355 bp was analyzed, the homology between the arms was 89.3% (Table 3). However, when the central 229 bp region was analyzed separately, the homology was 93% forming an almost perfect palindrome (Fig. 3).

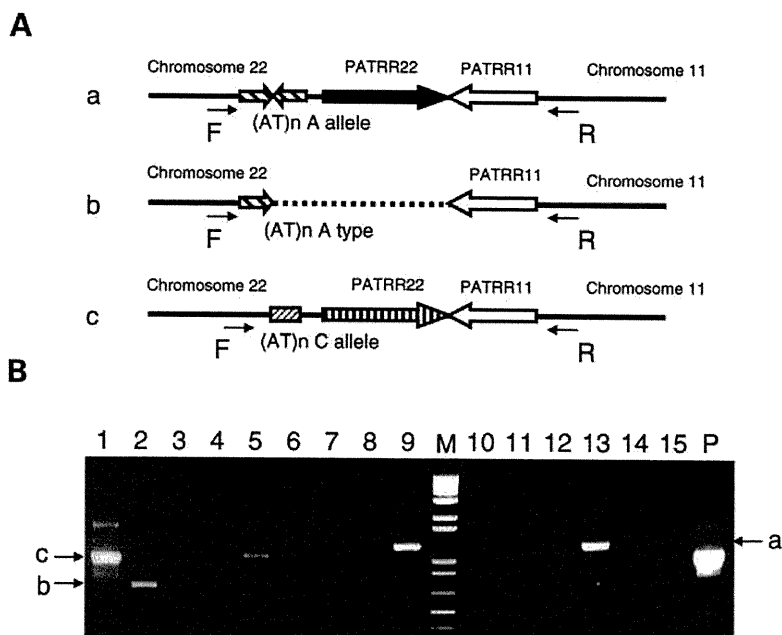
**Table 4.** *De novo* translocation frequency in sperm from two individuals with rare short PATRR22 on both alleles

Case	Genotype of AT-rich region	PATRR11 genotype	Genotype of PATRR22	Template DNA (ng/μl)	<i>De novo</i> translocation Positive PCR	Allelic origin	Frequency
15	B/C	L/L	B1 (553 bp, symmetry) C1 (509 bp, symmetry)	5	28/96	23 (82%) 5 (18%)	$1.66 \times 10^{-4}$ $3.24 \times 10^{-5}$
16	C/C	L/L	C2 (539 bp, symmetry) C3 (457 bp, asymmetry)	10	14/58	14 (100%) 0 (0%)	$2.62 \times 10^{-5}$ 0



**Figure 3.** *M*-fold analysis of the secondary structure of rare short variants of the PATRR22 and proximal AT-rich region flanking the PATRR22 (A allele). For the AT-rich region flanking the PATRR22, only the central 229 bp region is shown.

We increased the number of PCRs to estimate the frequency of this rare translocation. This rare translocation was observed twice in a total of 400 PCRs ( $1.52 \times 10^{-7}$ ). On the other hand, we did not identify any translocations from the 457 bp asymmetric PATRR22 in 400 PCRs ( $<7.59 \times 10^{-8}$ ; data not shown). The fact that the 355 bp symmetric AT-rich region



**Figure 4.** Rare der(22) junction derived from an A allele. (A) Schematic representation of the organization of junction from the der(22) from an A/C heterozygote. The A/C heterozygote produces three types of der(22); a standard der(22) with the breakpoint at the center of the PATRR22 of the A allele (a), a rare der(22) with the breakpoint at the center of the AT-rich region of the A allele (b), and a standard der(22) with the breakpoint at the center of PATRR22 of the C allele (c). (B) The der(22)-specific PCR. The allelic origin of the der(22) can be determined by its size. Lanes 1 and 5, a der(22) derived from a C allele; lanes 9 and 13, a der(22) derived from an A allele; lane 2, a rare der(22) with the breakpoint at the center of AT-rich region of the A allele. M, size markers; P, DNA from a t(11;22) translocation carrier served as a positive control.

produced translocations, but the 457 bp asymmetric PATRR22 did not indicate that not only size but also symmetry is an important factor in determining the translocation frequency.

## DISCUSSION

The data obtained in this study demonstrate that variation of the PATRR22 does not affect the total frequency of *de novo* t(11;22) generation in sperm. This was clearly demonstrated by the observation of a similar total translocation frequency even in compound heterozygotes of short variants. This is in contrast to the finding obtained for the PATRR11 (11). Translocation frequency appears to be stable in sperm samples from the same individual obtained at different times, suggesting that the frequency is not influenced by environmental factors (15). In the present study, when the translocation frequency was compared among individuals with the same PATRR11 genotype, which would be likely to affect the frequency, the overall frequency was similar among the group with different PATRR22 genotypes. One likely reason is that PATRR22 variation is less pronounced relative to the insertion/deletion polymorphisms of the PATRR11 resulting in less change in the translocation frequency.

Another reason might be that variation of the translocation frequency based on the genotype of the PATRR22 was so subtle that the effects of variation in other genetic factors obscured any association. Such factors might include inter-individual differences in genetic predisposition of fidelity in DNA repair (16). For example, although the A allele produced

a significantly smaller number of translocations than C alleles, one rare A/A homozygote, which was predicted to produce translocations less frequently, was actually found to manifest a frequency comparable to others such as the C/C homozygotes (Table 1). In this subject, a certain genetic factor that promotes generation of translocations possibly raised his translocation frequency in spite of the adverse effect of the A/A genotype.

In contrast, comparison of the allelic origin of translocations within an individual allowed us to compare the translocation frequency within a similar genetic background, which clearly demonstrated allelic preference. (i) The A allele (583 bp) produced less translocations than either the B or the C allele (595 and 597 bp, respectively). (ii) A short rare variant (509 bp) produced less translocations than the opposing allele (553 bp). (iii) An asymmetric short variant (457 bp) did not produce any translocations. (iv) An AT-rich flanking region (A allele) that manifested a symmetric palindrome produced translocations despite its short length (355 bp). Taken together, our data indicate that size and symmetry of the PATRR22 affect the translocation frequency, which is consistent with the findings obtained for the PATRR11.

The mechanism of PATRR-mediated chromosomal translocation in humans is still unknown. Analyses of several other recurrent and non-recurrent translocations demonstrated the presence of PATRR-like genomic structures at the breakpoint region, suggesting that at least a certain subset of translocations are mediated by the PATRRs (17,18). Palindromic DNA can generate intra-strand base pairing, which produces

a single-stranded hairpin structure or a double-stranded cruciform structure (19,20). Such secondary structures are likely to be generated during the DNA replication and likely form hotspot for genomic rearrangement (21–23). It is possible that the PATRRs also adopt a cruciform structure that induces genomic instability leading to the recurrent translocations (24). *In vitro* analysis has demonstrated that longer and more symmetric PATRR variants show a greater potential to adopt a cruciform conformation (25). The data that the size and symmetry of the PATRR11 and PATRR22 affect the translocation frequency indirectly provide a strong suggestion that the PATRRs form cruciforms in living cells and that the cruciform secondary structure mediates the recurrent chromosomal translocations. We recently established a plasmid-based model system for PATRR-mediated translocations in human cell lines and demonstrated that the DNA secondary structure was necessary for initiation of rearrangement between the PATRRs (26). A more thorough analysis of PATRR-mediated translocations would reinforce the hypothesis that DNA secondary structure governs the gross chromosomal rearrangements.

## MATERIALS AND METHODS

### Genotyping of the PATRR22

All human samples were provided from individual volunteers in a Japanese population after obtaining the appropriate informed consent. The study was approved by the Ethical Review Board for Human Genome Studies at Fujita Health University. For the analysis of polymorphisms of the PATRR, genomic DNA was extracted from blood samples or cheek swabs using PureGene (Gentra). The PATRR11 was genotyped by PCR using primers described previously (11). For the PATRR22, the size polymorphisms of the AT-rich region flanking the PATRR22 (A allele, 355 bp; B allele, 179 bp; C allele, 121 bp) were first determined. This polymorphism is known to link to polymorphisms of the PATRR22 (6).

Next, the PATRR22s were amplified using LCR-specific primers designed for this study. Primer –469F: 5'-CCATA TGCAGTTATAAATATGTTTCATGGTTAG-3', +440R: 5'-ACAAGTAAACAGTTTTCAAAGCT-3'. The purified PCR products were cloned into pT7Blue vector (Novagen). We used the SURE strain (Stratagene) to maintain the unstable PATRR insert. The plasmid inserts were sequenced as described previously (27). Sequences were determined from at least three plasmid clones to avoid misinterpretation as a result of PCR artifacts.

### Assessment of *de novo* translocation frequency

Sperm samples were provided from individual volunteers with various genotypes of the PATRR22. DNA was extracted using PureGene according to the manufacturer's protocol. Translocation-specific PCR was performed with the appropriate primers as described previously (4). PCR was performed using a protocol of 40 cycles of 10 s at 98°C and 5 min at 60°C. The frequency of *de novo* translocation events was calculated based on the presence of positive PCRs (10). Briefly, we amplified multiple batches of 100 ng sperm DNA each

containing 33 000 haploids ( $n$ ). We counted the number of positive PCRs per total reactions ( $p$ ). The translocation frequency ( $q$ ) was calculated on the basis that the probability of a positive PCR corresponds to a total sum of a binomial series of the translocation frequency calculated using the equation  $p = 1 - (1 - q)^n$  as described previously.

The frequency of *de novo* t(11;22) was determined using the der(22)-specific PCR so that the allelic origin of the translocation could be easily assessed (Fig. 1). The PCR was performed using one primer located flanking the PATRR11 (11 + 290R2: 5'-CTTCTTAACATAGCTTCTAC-AC-3') and the other flanking the PATRR22 (22 – 394F: 5'-TCAGTTTATT CCCAACTCCCAAAT-3'). In the case of an A/C heterozygote, the allelic origin of the translocation is easily identified by the size of the PCR products (Fig. 1B). In the case of a B/C heterozygote, a second PCR was performed using primer pair, 22 – 394F and 22b (5'-CTGCATCCTTCAAC GTTCCATC-3'; Fig. 1B).

### *In silico* cruciform extrusion assay

The potential secondary structure of the PATRR22 as well as of the rare variants identified in the current study (Table 3) was analyzed using the *m*-fold server (<http://www.bioinfo.rpi.edu/applications/mfold>). A free energy value ( $G_{\text{CRUCIFORM}}$ ) was obtained (28). Similarly, a free energy value for the same sequence annealed to its complementary strand ( $G_{\text{DS}}$ ) was obtained and then halved. Free energy for the formation of secondary structure ( $\Delta G$ ) is the  $G_{\text{DS}} - G_{\text{CRUCIFORM}}$  difference, and this value was normalized by the nucleotide number ( $\Delta G/\text{nucleotide}$ ). The most negative free energy value was used for subsequent analyses when numerous values were obtained.

### Statistical analysis

Statistical comparisons between groups were performed using the Student's *t*-test and one-way analysis of variance (ANOVA). Differences were considered to be significant at  $P < 0.05$ .

*Conflict of Interest statement.* None declared.

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## Review

## The constitutional t(11;22): implications for a novel mechanism responsible for gross chromosomal rearrangements

Kurahashi H, Inagaki H, Ohye T, Kogo H, Tsutsumi M, Kato T, Tong M, Emanuel BS. The constitutional t(11;22): implications for a novel mechanism responsible for gross chromosomal rearrangements. Clin Genet 2010; 78: 299–309. © John Wiley & Sons A/S, 2010

The constitutional t(11;22)(q23;q11) is the most common recurrent non-Robertsonian translocation in humans. The breakpoint sequences of both chromosomes are characterized by several hundred base pairs of palindromic AT-rich repeats (PATRRs). Similar PATRRs have also been identified at the breakpoints of other nonrecurrent translocations, suggesting that PATRR-mediated chromosomal translocation represents one of the universal pathways for gross chromosomal rearrangement in the human genome. We propose that PATRRs have the potential to form cruciform structures through intrastrand-base pairing in single-stranded DNA, creating a source of genomic instability and leading to translocations. Indeed, *de novo* examples of the t(11;22) are detected at a high frequency in sperm from normal healthy males. This review synthesizes recent data illustrating a novel paradigm for an apparent spermatogenesis-specific translocation mechanism. This observation has important implications pertaining to the predominantly paternal origin of *de novo* gross chromosomal rearrangements in humans.

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Key words: cruciform – gross chromosomal rearrangements – non-B DNA – palindrome – translocation

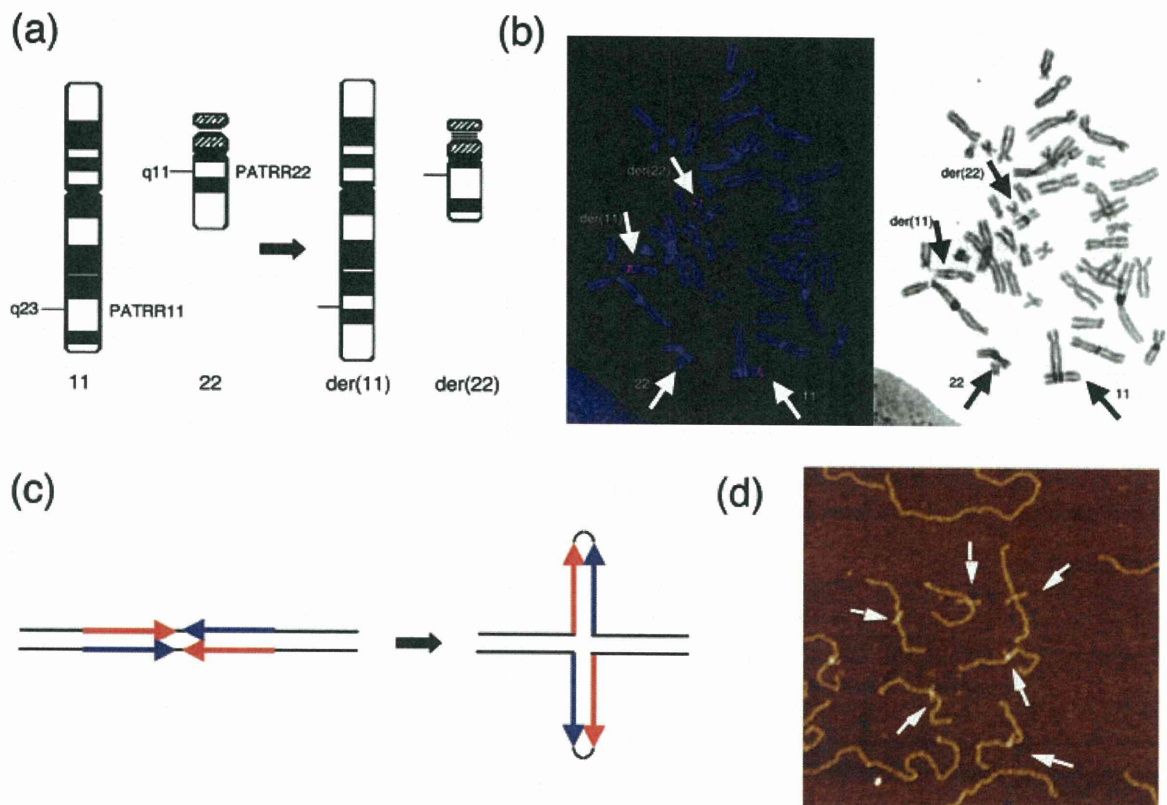
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The constitutional t(11;22)(q23;q11) is the most frequent recurrent non-Robertsonian translocation in humans (Fig. 1a). Similar to Robertsonian translocations or many other nonrecurrent constitutional translocations, balanced carriers of the t(11;22) usually manifest no clinical symptoms, because the rearrangement does not disrupt functional genes. However, translocation carriers often have reproductive problems, such as male infertility, recurrent spontaneous abortions, and the birth of offspring with a chromosomal imbalance. Severely affected offspring often have supernumerary-der(22)t(11;22) syndrome (Emanuel

syndrome, MIM# 609029), as a result of 3:1 meiotic malsegregation of the der(22) (1). The syndrome is characterized by severe mental retardation, preauricular tag or sinus, ear anomalies, cleft or high-arched palate, micrognathia, microcephaly, kidney abnormalities, heart defects, and genital abnormalities in males (2–4). Most of the t(11;22) carrier individuals are identified subsequent to the birth of an individual with Emanuel syndrome.

Translocation is one of the most frequently occurring gross chromosomal rearrangements in humans. Translocation can potentially cause



**Fig. 1.** Palindrome-mediated translocation in humans. **(a)** Schematic representation of the  $t(11;22)(q23;q11)$ . The PATRR11 and PATRR22 are located at the breakpoints on 11q23 and 22q11, respectively. **(b)** FISH analysis of metaphase chromosomes derived from a balanced  $t(11;22)$  carrier using a BAC spanning the breakpoint on chromosome 11. The signal from the chromosome 11 BAC is split by the translocation and appears on both the der(11) and the der(22) (left). The same FISH image was inverted to greyscale to show the location of the relevant chromosomes in the metaphase spreads (right). **(c)** Predicted secondary structure for the palindromic sequence. Short palindromic sequences have the potential to form double-stranded cruciform structures by intrastrand-base pairing in single-stranded DNA. DNA sequences indicated by blue arrows are complementary to those indicated by red arrows. **(d)** Cruciform extrusion of the plasmid harboring the PATRR11. A plasmid bearing the PATRR11 insert was fixed with psoralen treatment followed by ultraviolet exposure. The PATRR11 fragments were released with restriction enzyme digestion and were visualized using AFM. AFM, atomic force microscopy; BAC, bacterial artificial chromosome; FISH, fluorescent *in situ* hybridization; PATRR, palindromic AT-rich repeat.

devastating disorders when it disrupts an important genomic element. The somatic translocations that are commonly identified in cancers or leukemias result in the disruption of proto-oncogenes, leading to the production of chimeric transcripts with oncogenic potential. Likewise, although constitutional translocations occasionally cause specific genetic diseases, most of them are harmless and do not disrupt essential genes. However, the offspring of individuals harboring a balanced translocation have a potential risk of unbalanced translocation, resulting in pregnancy loss or birth of a child with a congenital anomaly syndrome.

The formation of translocation is essentially dependent on two distinct processes; double-strand-breaks (DSBs) and an error in DSB repair (5). DSBs can result from exogenous agents such as ionizing radiation and chemotherapeutic

drugs, and also from endogenously generated reactive oxygen species and mechanical stresses on the chromosomes (6, 7). DSBs can impair cellular function and eventually cause cell death by triggering apoptosis. To counteract such deleterious effects, DSBs are usually repaired through the activity of error-free repair systems, such as homologous recombination (8). However, translocations occasionally result from the activity of an error-prone repair system, such as non-homologous end joining (NHEJ), with or without microhomology (9, 10). Experimental induction of two DSBs is sufficient to produce translocations in mammalian cells (11). Indeed, NHEJ is suggested to be involved in translocation junction formation both in this system and in translocations identified in human disease patients as nonrecurrent translocations (12, 13).

Chromosomal translocations can be random events with nonrecurrent breakpoints, indicating the occurrence of random DSBs followed by an error in the DSB repair pathway. However, a subset of translocations shows recurrent manifestation, suggesting increased susceptibility of one of these two elements at specific loci. Regarding constitutional translocation, the recurrent translocation observed most frequently is the Robertsonian translocation. Breakpoint analyses indicated homologous recombination between pericentromeric repeats that commonly occur on the short arm of acrocentric chromosomes (14). This mechanism is known as non-allelic homologous recombination (NAHR) and is indicative of not only translocations, but also other recurrent gross chromosomal rearrangements, such as deletions and inversions (15–18).

Another example of recurrent translocation is t(4;8)(p16;p23). Both translocation breakpoints have been mapped within the olfactory receptor gene clusters at 4p16 and 8p23, suggesting that NAHR between olfactory receptor genes on different chromosomes is also responsible for the translocation (19). Interestingly, both *de novo* Robertsonian translocations and *de novo* t(4;8) arise preferentially during maternal gametogenesis (20, 21). Although rare, t(4;11)(p16.2;p15.4) is also an example of recurrent translocation characterized by breakpoint homology (22). In sharp contrast is t(11;22)(q23;q11), which is an example of recurrent constitutional translocation that may be caused by susceptibility to DSBs at specific loci.

#### Identification of the PATRR sequences at the breakpoints of constitutional t(11;22)

Fluorescent *in situ* hybridization (FISH) studies using multiple probes on chromosomes 11q23 and 22q11 have shown that in individuals with t(11;22), including *de novo* cases, the t(11;22) breakpoints are confined to same narrow intervals on both chromosomes (1, 23, 24) (Fig. 1b). The recurrent nature of the t(11;22) prompted us to examine the translocation breakpoints in detail to identify the specific genomic structure associated with the chromosome 11 and 22 breakpoints.

A conventional positional cloning strategy allowed us to identify both constitutional t(11;22) breakpoints, although the work was challenging due to the inherent genomic instability in the breakpoint regions. We first identified a breakpoint cluster region on 11q23, which was approximately 450 bp in length with a high AT content (93%). The breakpoint on 11q23 constitutes a nearly perfect palindromic structure, with 98% identity

between its proximal and distal arms (Fig. 1c). We designated this configuration as a PATRR on 11q23 (PATRR11) (25, 26). The cloning of the 22q11 breakpoint was also an extraordinarily challenging. Eventually, a similar PATRR was identified within one of the unclonable gaps unresolved by the human genome project (27). The size of the PATRR on 22q11 (PATRR22) was approximately 590 bp, which was slightly larger than PATRR11. In spite of their similarity with regard to AT-richness, no substantial homology was observed between PATRR11 and PATRR22 (58% identity).

In addition, we examined junction fragments originating from more than 50 independent balanced t(11;22) carriers to localize the breakpoints precisely (28). The breakpoints on both chromosomes were located at the center of the PATRRs. Only a small number of identical nucleotides were found at the point where the original two sequences were joined, and there were always small deletions (no greater than 50 nucleotides) at the breakpoint regions of both PATRRs (26, 27). Collectively, these data suggested that the mechanism for this recurrent chromosomal translocation involved the occurrence of DSBs at the center of the two PATRRs, followed by repair via the NHEJ pathway.

These findings were confirmed by two other research groups who tested different subsets of translocation carriers and showed similar breakpoints (29, 30).

#### PATRR-mediated chromosomal translocation as one of the universal pathways for gross chromosomal rearrangements in humans

In the human genome, the PATRR22 site appears to be extremely susceptible to breakage. The chromosome 22q11 has been designated as a hotspot for translocation breakpoints, because cytogenetic studies have shown that a large number of translocation breakpoints cluster at 22q11. FISH mapping has indicated that the breakpoints of numerous translocations involving 22q11 cluster within the same interval that includes the PATRR22 (31–35). These data suggest the involvement of PATRR22 in the etiology of all 22q11-related translocations.

We analyzed two unrelated individuals with constitutional t(17;22)(q11;q11) that presented with neurofibromatosis type 1 (NF1) (32, 36). Each translocation disrupted the *NF1* gene on 17q11, resulting in patients displaying the NF1 phenotype. As was expected, FISH analysis localized the 22q11 breakpoints within the same interval where the breakpoint of t(11;22) resides. Further



analyses of the 17q11 breakpoints within the *NFI* gene revealed the presence of an approximately 200 bp PATRR sequence within intron 31 of the *NFI* gene (PATRR17) (36, 37). Subsequent molecular cloning of other translocation breakpoints has shown similar palindromic, and often AT-rich, sequences on partner chromosomes, such as 4q35.1, 1p21.2 and 8q24.1 (38–40). Hence, palindrome-mediated chromosomal translocation appears to be one of the possible universal pathways for creating human genomic rearrangements. This subset of translocations appears to occur in a nonrandom fashion and to be possibly mediated by the genomic instability of palindromic DNA.

Small palindromic DNAs like the PATRRs have the potential to form stem-loop structures through intrastrand-base pairing within single-stranded DNA. As a consequence, they form a specific structure consisting of a single-stranded hairpin or a double-stranded cruciform (Fig. 1c). We analyzed the tertiary structure of the cloned PATRR11 *in vitro* (41). A plasmid containing the PATRR11 adopts a non-B DNA conformation under negative superhelicity. Using atomic force microscopy (AFM), we were able to visualize the cruciform extrusion from the PATRR11 plasmid directly (Fig. 1d). Thus, we propose that the secondary structure of the PATRRs induces genomic instability leading to both recurrent and nonrecurrent chromosomal translocations in humans.

#### Translocation-specific polymerase chain reaction (PCR) detects *de novo* t(11;22)s in sperm from normal healthy males

We established a t(11;22)-specific PCR system utilizing sequence data from the junction fragments. PATRR-flanking primers were designed both on 11q23 and 22q11 to amplify the der(11) and the der(22) junction fragments (Fig. 2a). This PCR approach successfully amplified the der(11) and der(22) junction fragments from balanced t(11;22) carriers as well as the der(22) junction fragments of patients with Emanuel syndrome (28).

To determine the prevalence of t(11;22), we examined the frequency of *de novo* translocations in DNA derived from sperm samples obtained from normal, healthy male volunteers (42). Translocation-specific PCR was performed using conditions that would allow for the detection of a single molecule of target DNA. Multiple aliquots were amplified from each sperm sample (Fig. 2b). When 100 ng aliquots of sperm DNA, each containing 33,000 haploids as templates, were amplified, translocation-specific PCR products were detected in a substantial number

of reactions (42). The presence of both positive and negative PCRs clearly indicates the *de novo* origin of the translocation (Fig. 2b). Translocation-specific PCR products were never detected in DNA from blood or cheek swab DNA from the same donors, essentially excluding the possibility that the positive PCR results seen in sperm were the result of contamination or PCR artifacts.

The frequency of sperm *de novo* translocation events was calculated based on the presence of positive PCRs. Using the number of positive PCRs expressed relative to the total number of PCRs performed, the frequency was calculated on the basis that the probability of observing a positive PCR corresponded to the total sum of a binomial series of the translocation frequency calculated as described previously (42). For an initial analysis, we examined sperm samples from four randomly selected healthy male volunteers, and the estimated frequency of the translocation was approximately  $1 \times 10^{-5}$ , which is unexpectedly high. The frequencies for der(11) and der(22) were approximately equal, suggesting that *de novo* translocation occurs as a reciprocal rearrangement. This observation is in agreement with the observation that the majority of patients with the supernumerary-der(22) syndrome are the offspring of a balanced translocation carrier, rather than representing a *de novo* event (2–4).

#### PATRR polymorphisms affect the *de novo* translocation frequency

In the course of our PATRR analyses, we identified that the PATRRs on several chromosome 22 partner chromosomes are hypervariable among individuals (37, 43). Size polymorphisms due to deletion, insertion and duplication, including the center region, are common, supporting the idea that the center of a PATRR is fragile. The central modifications often resulted in asymmetric palindromic structures (Fig. 2c). This observation prompted us to test the hypothesis that PATRR polymorphism might affect the frequency of *de novo* t(11;22)s.

We examined sperm samples from normal healthy males with various PATRR11 genotypes (43). Homozygotes for the long symmetric PATRR11 (L-PATRR11), the most frequent allele, produced *de novo* translocations at a frequency ranging between  $1.52 \times 10^{-5}$  and  $1.57 \times 10^{-4}$ . Heterozygotes for the L-PATRR11 and symmetric short PATRR11 alleles (S1, S2) produced *de novo* translocations at an overall frequency similar to that detected for L-PATRR11 homozygotes. However, the translocation products derived from the symmetric short PATRR were observed less

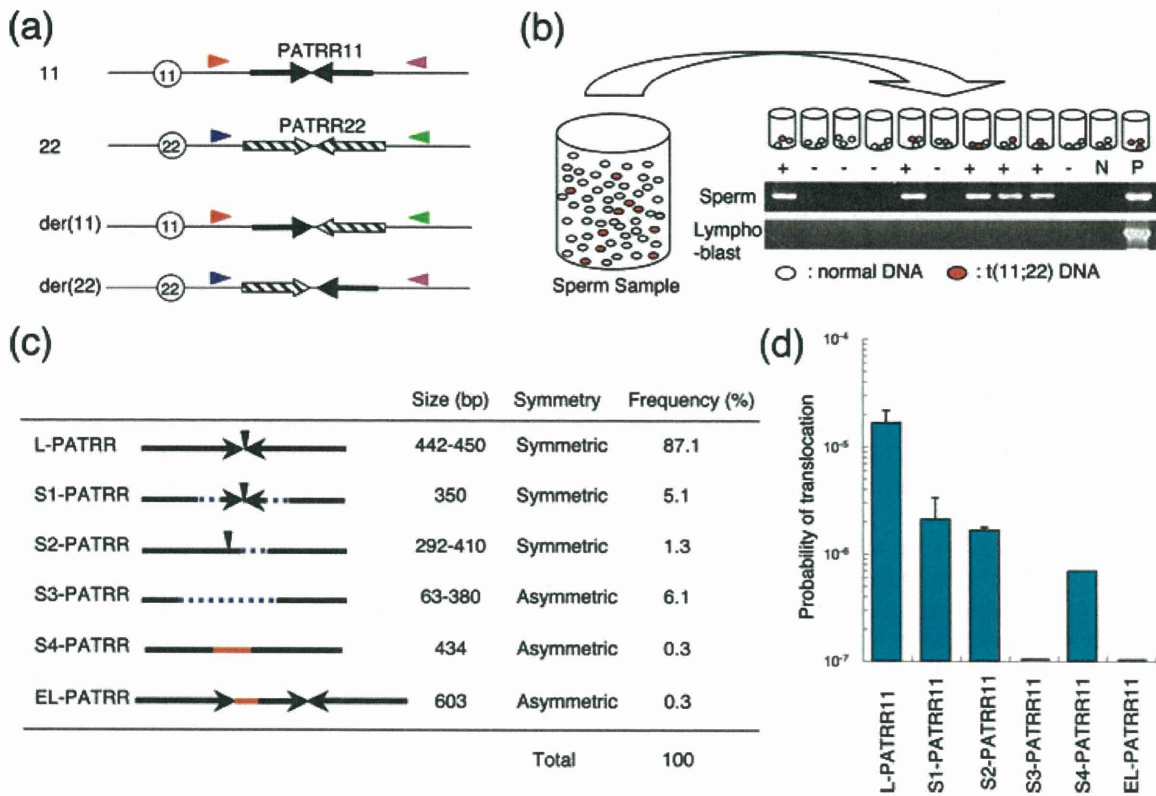


Fig. 2. Polymorphisms of the palindrome affect the *de novo* translocation frequency. (a) Location of PCR primers. Arrows indicate each arm of the PATRR11 (solid arrows) and PATRR22 (hatched arrows). Size polymorphisms of the PATRR11 were examined by PCR using primers indicated by red and pink triangles. Translocations were detected using one of the primers flanking the PATRR11 (red or pink triangles) and with one of the primers flanking the PATRR22 (blue or green triangles). Centromeres are represented by circles. (b) Strategy for estimation of translocation frequency by PCR. Genomic DNA was extracted from sperm samples. Translocation-specific PCR was performed using multiple batches of template DNA. The translocation frequency was calculated using the equation,  $q = 1 - (1 - p)^{1/n}$ ; with  $n$  = number of haploid genomes per aliquot,  $p$  = the probability that an aliquot contains a translocation, product and  $q$  = the probability that one randomly selected haploid genome in a given aliquot sustained a translocation. The gel images show representative PCR results. The upper panel shows results derived from sperm DNA, whereas the lower panel presents results from lymphoblast DNA. Lane M, size marker; lane N, negative control; lane P, genomic DNA from a t(11;22) balanced carrier serving as a positive control. (c) Characterization of the PATRR11 variants. Arrows indicate each unit of inverted repeats. Vertical arrowheads indicate the center of the palindromic sequence. Dotted blue lines show the deleted region, while red lines indicate the insertion of sequences of unknown origin. Other characteristics and the frequency in the general population are shown on the right. (d) A histogram showing the probability of *de novo* translocation by allele types. Each bar represents the mean value and the vertical bar indicates standard deviation on a log scale. The allele types are abbreviated. PCR, polymerase chain reaction; PATRR, palindromic AT-rich repeat.

frequently ( $\sim 10^{-6}$ ) suggesting that the frequency is dependent on the size of the PATRR (Fig. 2d). However, although individuals heterozygous for an L-PATRR11 and an asymmetric short PATRR11 (S3) produced *de novo* translocations at a similar overall frequency, the asymmetric short PATRR did not produce any *de novo* translocations. These observations clearly show that PATRR polymorphisms affect the frequency of translocations. Collectively, these data, including the data for rare genotypes, indicate that the size and symmetry of the center of the PATRR11 determines the frequency of *de novo* t(11;22)s (Fig. 2d). Indeed, the

translocation frequency reflects the propensity of each polymorphic allele for secondary structure formation (44). PATRR22 is slightly longer than other PATRRs, which might explain why all of the known PATRR-mediated translocations involve PATRR22 (27). Thus, it is reasonable to propose that the potential for adopting a secondary structure is probably to contribute to the susceptibility to translocation development.

#### The t(11;22) is independent of DNA replication

Curiously, translocation-specific PCR has never detected a *de novo* translocation event in any

tissues other than sperm. Diverse human tissues such as peripheral leukocytes, lymphoblasts, and skin fibroblasts were consistently negative for *de novo* translocation by PCR analysis (42). We also tested various cultured somatic cell lines derived from human cells (HEK293, HeLa, HepG2 and THP-1), but all were negative (0 positive PCR in 40 reactions,  $<7.67 \times 10^{-7}$ ) (45). The fact that only sperm samples produce *de novo* t(11;22)s supports the idea that PATRR-mediated translocations occur primarily during gametogenesis. These findings are unusual and appear to be inconsistent with the established mechanisms pertaining to the instability of palindromic DNA sequences.

The instability of palindromic DNA has been extensively investigated over the last 20 years. Susceptibility to deletion within palindromic regions has been consistently shown in many experimental organisms, including *Escherichia coli* (46–48), *Saccharomyces cerevisiae* (49, 50), and mice (51, 52). Such deletions appear to be primarily mediated by the stalling of DNA replication at a region that has formed a secondary structure. This blockade appears to be resolved by ‘slippage’ or ‘strand switch’. Another important pathway for palindromic DNA deletions is endonuclease cleavage of such a secondary structure during replication. The PATRR11 was found to be deleted from the BAC encompassing the breakpoint region (25, 26). Furthermore, the fact that PATRR22 is underrepresented in BAC/PAC/YAC libraries also suggests that PATRRs are highly unstable in bacteria and yeast. Indeed, translocations have been induced in regions containing palindromic or inverted repeats in vegetative yeast when DNA replication is compromised (53).

Combined with data supporting the sperm-specific occurrence of *de novo* translocations, PATRR-mediated translocations may result from palindrome instability facilitated by the high number of cell divisions and DNA replications during spermatogenesis. Paradoxically, we analyzed sperm samples from 10 male donors and found no age-dependent increase in the frequency of *de novo* t(11;22)s (54). We also obtained samples from the same donors after a 6-year interval, and no age-dependent increase in translocation frequency was observed (54). If the translocation occurs during DNA replication, the samples from older donors that have undergone greater number of germline divisions should theoretically include greater number of translocations. Thus, these findings may invoke a novel paradigm regarding palindrome instability that is independent of DNA replication.

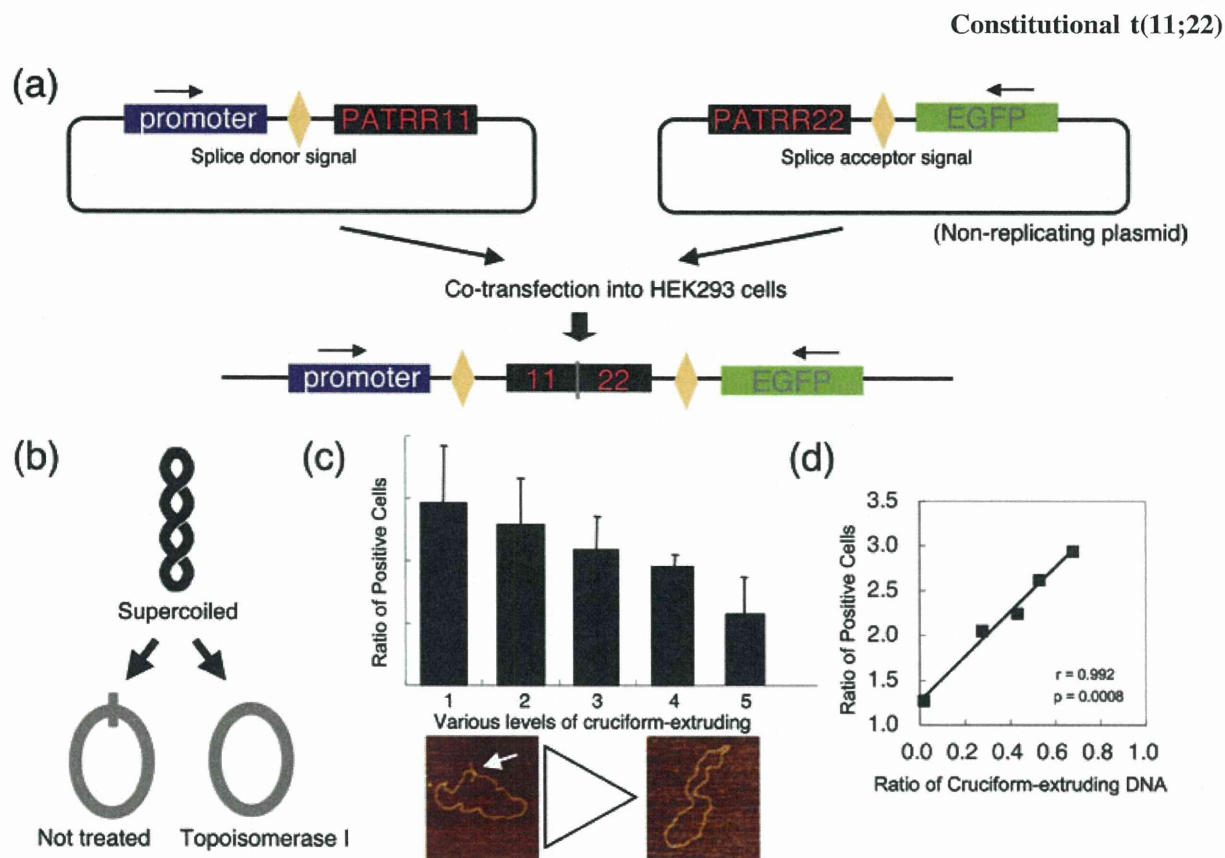
The consequence of replication-related palindromic instability is often a deletion. Indeed,

PATRRs often manifest themselves as size polymorphisms among individuals due to deletions, insertions and duplications, as mentioned in the previous section (43). However, our recent breakpoint analyses clearly distinguished the mechanisms of deletion from those of translocation (55). Microhomology identified at the deletion endpoints is significantly longer than that at translocation breakpoints, suggesting that PATRR-mediated deletions develop in a homology-directed manner. Another interesting finding is that all of the insertions that were identified within the deletion junctions were AT-rich sequences, whereas insertions found at translocation junctions were non-AT-rich DNA sequences. Thus, it could be hypothesized that rearrangements within the PATRR, such as deletions, insertions and duplications, are induced by secondary structure formation during DNA replication followed by repair through a homology-dependent pathway. In contrast, PATRR-mediated translocation may be driven by a completely different mechanism. Indeed, inhibition of DNA replication in cultured human cells induces deletions within the PATRR11, but not translocations between different PATRRs (45). Thus, these findings lend support to the possibility of replication-independent translocations.

### Establishment of a model system

To gain a better understanding of how DNA secondary structure contributes to palindrome-mediated genomic instability, there have been ongoing attempts to establish a model system that recapitulates PATRR-mediated translocation. Establishment of such a model system would also provide information on the underlying mechanisms of PATRR-mediated gross chromosomal rearrangements.

We have recently developed a plasmid-based model system of PATRR-mediated translocation (56). This system utilizes two plasmids harboring either PATRR11 or PATRR22 sequences, which act as substrates for rearrangement within mammalian nuclei. The first plasmid includes a promoter and a splice donor sequence upstream of PATRR11, whereas the second plasmid contains the PATRR22 sequence followed by a splice acceptor sequence and then the green fluorescence protein (GFP) gene coding sequence (Fig. 3a). Translocation-like rearrangements between the plasmids were detected by PCR 24 h after co-transfection into human cell lines. The junction sequences were found to be analogous to the human t(11;22) junction sequences, suggesting that this model system recapitulates the



**Fig. 3.** Plasmid-based model system of palindrome-mediated translocation. **(a)** Schematic representation of the detection system. When the two plasmids harboring PATRR11 and PATRR22 are rearranged within each PATRR region, the transcript from the fusion product splices out the intervening junction sequence and expresses the downstream GFP gene product. **(b)** Strategy for preparing PATRR plasmids with or without cruciform extrusion. Plasmid purified from *Escherichia coli* is negatively supercoiled (upper). The PATRR plasmid with negative superhelicity energetically favors cruciform extrusion forming intrastrand-base pairing. The positive free energy of cruciform formation is offset by relaxation of the negative superhelical density (left). If the negative superhelicity is abrogated by incubation with topoisomerase I prior to cruciform extrusion, a relaxed plasmid without the extruded cruciform can be obtained (right). **(c)** The incidence of translocation-like rearrangements following the use of differently preparative techniques for PATRR plasmids. GFP-positive cells were counted after co-transfection with various levels of cruciform-extruding PATRR plasmids. The plasmids were prepared by the alkaline-SDS method (1) and by the Triton method followed by either incubation in various NaCl concentrations (2, 10 mM; 3, 50 mM; 4, 200 mM) or topoisomerase I treatment (5). Representative AFM images of the PATRR plasmid are indicated as follows: arrow indicates cruciform extrusion of the PATRR plasmids. **(d)** The degree of cruciform in the transfected plasmids affects the levels of rearrangement. The degrees of cruciform extrusion observed by AFM correlate with the ratio of GFP-positive cells (right;  $r = 0.992$ ,  $p = 0.0008$ ). AFM, atomic force microscopy; GFP, green fluorescence protein; PATRR, palindromic AT-rich repeat; SDS, sodium dodecyl sulfate.

PATRR-mediated t(11;22) translocations, which occur in humans.

To examine the contribution of DNA secondary structure to this translocation-like rearrangement, we prepared plasmid samples that contained differing proportions of cruciform-extruding plasmids (Fig. 3b). The plasmids were then transfected into human cell lines and the rearrangement frequency was quantified by GFP reporter gene expression as detected using flow cytometry. The plasmids isolated using the alkali-method, which contained an abundance of cruciform-extruding plasmid DNA, showed the greatest number of GFP-positive cells (Fig. 3c). In contrast, plasmids treated with topoisomerase I, which had fewer

cruciform-extruding plasmids, resulted in smaller numbers of GFP-positive cells. The proportion of cruciform-extruding plasmids monitored by AFM was correlated with the number of GFP-positive cells (Fig. 3c,d). These data show that cruciform conformation of the PATRR contributes to this translocation-like event.

Furthermore, our data provide indirect evidence for the presence of DNA secondary structures in living cells, and also indirectly but strongly support the hypothesis that the PATRR sequences adopt a cruciform conformation that induces genomic instability, leading to the translocation. In this model system, no translocation was detected between endogenous PATRRs or between PATRR

plasmids and endogenous PATRRs. These results indicate that all the enzymatic activity that is required for translocation to occur is present in human somatic cell lines with the exception of the capacity for cruciform extrusion of the PATRR. These results also support the hypothesis described in the next section.

#### **Hypothesis: underlying mechanism dictating spermatogenesis-specific translocation**

As mentioned previously, PATRR-mediated translocation appears to be specific to meiotic cells. During mammalian meiosis, there are two types of physiological DNA breakage. A substantial number of DSBs occur as an initiating step for meiotic recombination. SPO11, a meiosis-specific endonuclease, mainly functions in this step (57). Subsequently, repair of the DSBs by homologous recombination progresses by a RAD52-mediated homology detection and RAD51/DMC1-mediated strand invasion. Holliday junctions (HJs), which form as intermediates during this process, are finally resolved by endonuclease activity. The four-way junction of a cruciform DNA structure is analogous to this HJ structure and could represent a substrate for the HJ resolvase, although such an enzyme has not been definitely identified in higher organisms such as yeast or mammals (58). Either of these steps might provide a good 'candidate process' for the generation of the DSBs leading to translocation events (59, 60).

To prove the presence of such meiosis-specific cruciform resolution, it is necessary to determine whether female germ cells can also produce *de novo* t(11;22) translocations. Toward this end, it might be reasonable to survey female germ cells for the presence of *de novo* t(11;22)s using similar translocation-specific single molecule detection PCR. However, performing this experiment is limited by the finite number of human oocytes available for examination. Even with the existing evidence of a high frequency of *de novo* translocations in sperm, female germ cells cannot be analyzed using a similar strategy.

As an alternative, we obtained samples from eight individuals with a *de novo* t(11;22) translocation together with samples from their parents to determine the parental origin of the translocation. Because the PATRR shows sequence variation among individuals, we theorized that determination of a parental origin for an individual translocation was possible through comparison of the sequence of translocation junction fragments on the der(11) and the der(22) with the PATRR11 and PATRR22 sequences on normal chromosomes

11 and 22 in the parental samples. Indeed, segregation analysis has shown that the *de novo* events are exclusively of paternal origin in our patient population, although to date only a finite number of samples have been examined (61). This finding implies that it is not necessarily a meiotic mechanism, but rather a spermatogenesis-specific mechanism that permits the development of the t(11;22) translocation.

DNA breakage might occur during late spermatogenesis when DNA is packaged into dense chromatin. The successive transition of chromatin components from histones to protamines causes dynamic changes in chromatin structure (62, 63). During this process, release from nucleosomes may contribute to the release of free negative supercoiling. With nucleosome withdrawal, temporary and local accumulation of an excess of negative superhelicity is possible, potentially exacerbating secondary structure formation within PATRR sequences with subsequent formation of strand breaks. A similar mechanism has been proposed for triplet-repeat expansion, which has been shown to be of postmeiotic origin (64).

All of the hypotheses described above relate to the causes of frequent DSBs at the PATRR sequences. However, the factors that facilitate such high efficiency DSB repair and aberrant joining between two PATRRs are still an enigma that remains to be elucidated. The role of spatial proximity between the two PATRRs in meiotic and postmeiotic cells deserves further investigation (65). Thus, there are still numerous factors surrounding the mechanism of translocation formation that remain to be elucidated.

#### **What the t(11;22) research tells us**

Chromosomal abnormalities in humans often manifest a gender bias with respect to parental origin. Aneuploidy, or a numerical abnormality, is more probably to arise in maternal gametogenesis (66). In contrast, 80% of known structural chromosomal abnormalities are of paternal origin (67). This is not surprising because late spermatids and sperm, which are non-dividing haploid cells, cannot undergo homologous recombination. However, components of NHEJ repair are expressed at low levels in these cells (68). It is formally possible that such structural abnormalities arise when DSBs induced by endogenous or exogenous mutagens are repaired by error-prone NHEJ system. However, our current hypothesis points to the importance of postmeiotic physiological events during spermatogenesis for the generation of the translocation. This hypothesis has important implications

of the predominantly paternal origin of *de novo* gross chromosomal rearrangements in humans.

To elucidate the precise mechanism of palindrome-mediated translocation, it will be necessary to establish a mouse model system for t(11;22) research. Because the mouse genome does not possess PATRR-like sequences, one could postulate that the introduction of two copies of PATRRs will be sufficient for the creation of palindrome-mediated translocations in a mouse. Further characterization of the PATRR dynamics in a mammalian germline should aid the future elucidation of the complex biology of non-B DNA-mediated translocations.

### Future prospects

PATRR-mediated translocation provides the opportunity for novel insight into mechanisms of chromosomal translocations. This concept could also be applied to translocations that develop in somatic cells. The recurrent nature of translocations observed in cancers or leukemias has been long thought to be random events resulting from a selective growth advantage. However, some translocation breakpoints have the potential for non-B DNA conformation (69). For example, the breakpoint regions of translocation partners in immunoglobulin gene-related translocation are reported to form Z-DNA or triplex DNA (70–72). Increasing research efforts are being applied toward elucidation of the molecular components leading to the DSB formation at these non-B DNAs, such as structure-directed nucleases.

Considerable progress has been made toward understanding the molecular mechanisms underlying the generation of recurrent translocations. However, questions still remain pertaining to the mechanisms of nonrecurrent translocations, which have also been long thought to be random events. A novel molecular mechanism has also been proposed to explain the occurrence of another subset of gross chromosomal rearrangements, deletions and duplications. This proposed mechanism explaining nonrecurrent gross chromosomal rearrangements is not based on the occurrence of DSBs, but is associated with DNA replication (73, 74). In brief, a free DNA end is generated by a stall during DNA synthesis, which restarts after switching to the template DNA on a different chromosomal site, resulting in gross chromosomal rearrangement. This mechanism, called replication Fork Stalling and Template Switching (FoSTeS) and microhomology-mediated break-induced replication (MMBIR), might be involved in some non-recurrent chromosomal translocations.

A recent report confirmed the preferential paternal origin of *de novo* nonrecurrent translocations using flow-sorted derivative translocation chromosomes followed by segregation analysis. Interestingly, development of the translocation appeared to be associated with increased paternal age (75). Based on the fact that male gametogenesis undergoes much higher number of mitotic divisions than that in female, this observation is in agreement with a replication-based mechanism of the translocation formation. This result is in clear contrast with the existing data on t(11;22) translocations and the possible PATRR-mediated mechanism (54). Further detailed studies and accumulated breakpoint information for nonrecurrent translocations are needed to elucidate the molecular mechanisms leading to gross chromosomal rearrangements.

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### Conflict of interest

We declare no conflict of interest.

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RESEARCH

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# DNA secondary structure is influenced by genetic variation and alters susceptibility to *de novo* translocation

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## Abstract

**Background:** Cumulative evidence suggests that DNA secondary structures impact DNA replication, transcription and genomic rearrangements. One of the best studied examples is the recurrent constitutional t(11;22) in humans that is mediated by potentially cruciform-forming sequences at the breakpoints, palindromic AT-rich repeats (PATRRs). We previously demonstrated that polymorphisms of PATRR sequences affect the frequency of *de novo* t(11;22)s in sperm samples from normal healthy males. These studies were designed to determine whether PATRR polymorphisms affect DNA secondary structure, thus leading to variation in translocation frequency.

**Methods:** We studied the potential for DNA cruciform formation for several PATRR11 polymorphic alleles using mobility shift analysis in gel electrophoresis as well as by direct visualization of the DNA by atomic force microscopy. The structural data for various alleles were compared with the frequency of *de novo* t(11;22)s the allele produced.

**Results:** The data indicate that the propensity for DNA cruciform structure of each polymorphic allele correlates with the frequency of *de novo* t(11;22)s produced ( $r = 0.77$ ,  $P = 0.01$ ).

**Conclusions:** Although indirect, our results strongly suggest that the PATRR adopts unstable cruciform structures during spermatogenesis that act as translocation hotspots in humans.

**Keywords:** Polymorphism, Palindrome, Secondary structure, Hairpin structure, Cruciform structure, Breakpoint, Translocation

## Background

Accumulating evidence indicates that alternative DNA structures (non-B DNA) cause a diversity of genomic rearrangements [1,2]. It is well known that a subset of repeat sequences such as trinucleotide repeats sustain dynamic mutations via DNA secondary structure intermediates leading to their expansion or contraction [3]. The finding that the t(14;18) translocation observed in follicular lymphoma might result from instability of triplex DNA at the breakpoint implies that gross chromosomal rearrangements can also be mediated by non-canonical DNA structures [4,5]. A large-scale survey

demonstrates that translocation breakpoints or deletion endpoints in human genetic diseases are consistently found in proximity to predicted non-B DNA structures [6].

Chromosomal translocations have long been thought to be random events. However, recent findings have highlighted two distinct mechanisms that lead to recurrent translocations in humans [7]. A subset of recurrent translocations arises between two homologous regions located on different chromosomes. Robertsonian translocations are mediated by highly repetitive regions on the short arms of the five acrocentric chromosomes, while t(4;8)(p16;p23) translocations result from exchange between two clusters of olfactory-receptor genes on 4p and 8p presumably via homologous recombination [8,9]. Another mechanism is the so-called palindrome-

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mediated chromosomal translocation [10]. Palindromic AT-rich repeats (PATRRs) were first identified at the breakpoints of the recurrent constitutional t(11;22)(q23;q11) [11-13]. All of the translocation breakpoints are located within the 450 bp PATRR on 11q23 (PATRR11) and the 590 bp PATRR on 22q11 (PATRR22), which do not share sequence homology with one another [14]. The majority of the breakpoints are located at the center of the PATRRs, suggesting that genomic instability of the palindrome center is the etiology of the recurrent translocation [15]. PATRRs also contribute to other recurrent and non-recurrent translocations such as the t(17;22)(q11;q11) [16,17], t(4;22)(q35;q11) [18], t(1;22)(p21.2;q11) [19], and t(8;22)(q24.13;q11.21) [20,21]. Translocation-specific PCR can frequently detect *de novo* t(11;22)s in sperm from normal healthy males [22].

Recently, we also identified *de novo* PATRR-mediated t(8;22)s as well as t(8;11)s by a similar PCR method, suggesting that a considerable proportion of the translocations result from a palindrome-mediated mechanism [21].

Palindromic DNA has the potential to form a secondary structure, an extruded DNA cruciform, through the intra-strand base pairing of adjacent inverted repeat units. A number of palindromic sequences have been identified in the human genome [23], but not all of the palindromes behave as sites for translocation breakpoints. The translocation-associated PATRRs reported so far, share a common structure, 1) a nearly perfect palindrome of several hundred base pairs in length, 2) an AT-rich center and a non-AT-rich region at both ends, 3) another nearby AT-rich region on one side of the PATRR, all of which invoke cruciform structure forming propensity [24]. Indeed, the cloned PATRRs identified at the translocation breakpoints assume a cruciform conformation *in vitro* [25,26]. We propose that the PATRR also adopts a cruciform conformation in living cells, which induces genomic instability leading to translocation formation in humans. In fact, the propensity for secondary structure of the PATRRs on chromosomes 11, 17 and 22 reflects the relative incidence of the relevant chromosomal translocations [27].

In our previous study, we demonstrated that the PATRR11 at the translocation breakpoint often manifests size polymorphisms due to central deletions within the PATRR11, and that this polymorphism affects the frequency of *de novo* t(11;22)s in sperm samples from normal healthy males [28]. Subsequently, we demonstrated that PATRR22 polymorphisms also impact *de novo* translocation frequency [29]. To determine whether PATRR polymorphisms influence secondary structure leading to variation in their translocation frequency, we investigated the secondary structure forming potential of each polymorphic PATRR11 and compared

it with its relevant translocation frequency. The results suggest that propensity for secondary structure formation is reflected in the rate of translocations formed.

## Results

### Size and symmetry of the palindromes affect *de novo* translocation frequency

To better understand how polymorphic variants of the palindromic sequence affects *de novo* translocation frequency in sperm, we classified the polymorphic PATRR11s into three categories based on the size and symmetry of the palindromic sequences (Figure 1A). The most frequent allele is characterized by a nearly perfect palindromic sequence of 442-450 bps (L-PATRR11). We further grouped minor short variants into symmetric short and asymmetric short PATRR11s (SS-PATRR11 and AS-PATRR11). The size of the SS-PATRR11s and AS-PATRR11s were 212-434 bp.

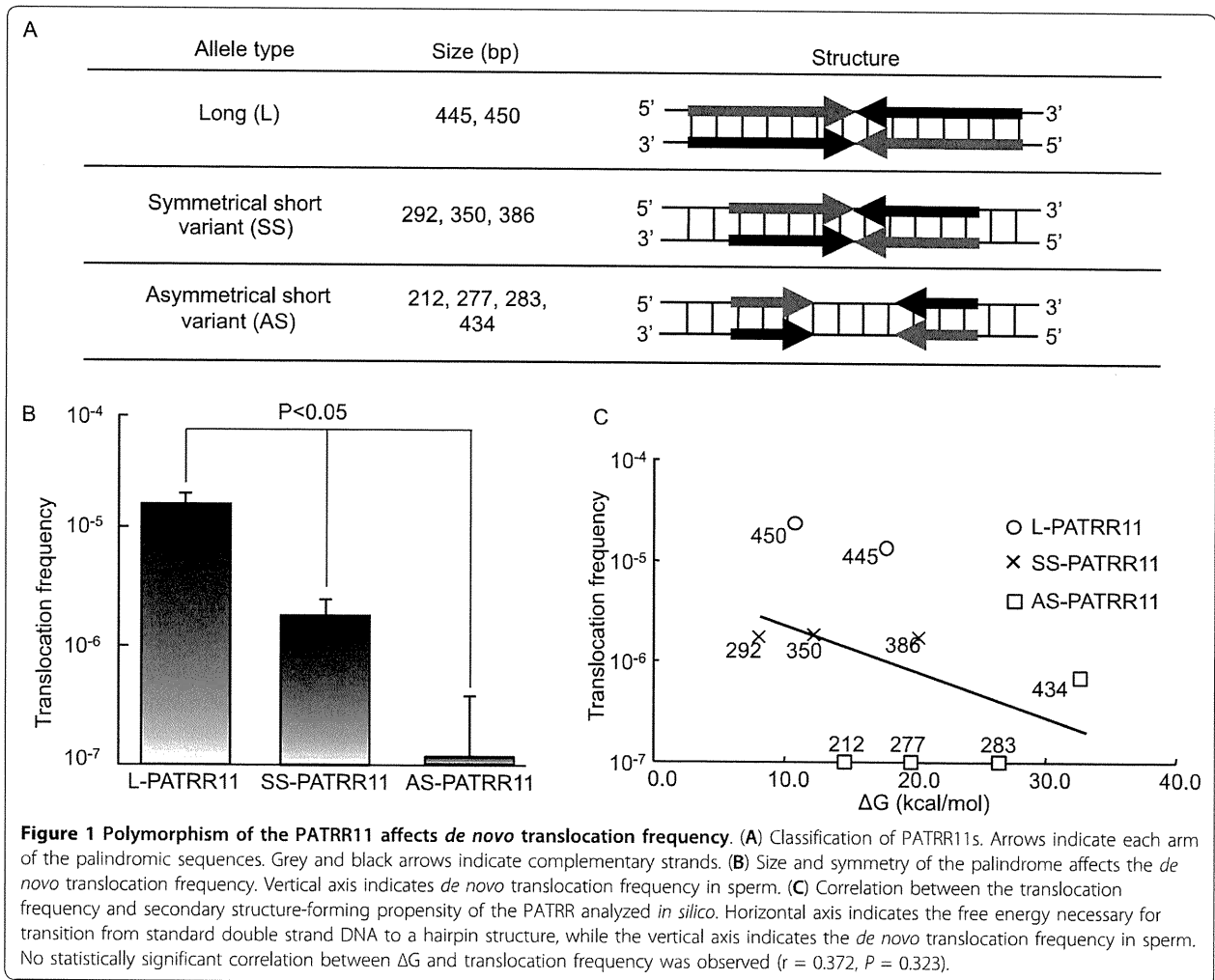
We attempted to estimate the frequency of *de novo* translocations originating from each PATRR allele. To distinguish the allelic origin of translocation products, we selected individuals heterozygous for PATRR11 polymorphisms for analysis. L-PATRR11 produces *de novo* translocations in approximately  $10^{-5}$  gametes ( $1.30-2.11 \times 10^{-5}$ ). On the other hand, variant PATRR11s generally produce translocations at a lower frequency. For SS-PATRR11, the translocation frequency is about 10-fold lower than that of L-PATRR11 ( $1.71-1.82 \times 10^{-6}$ ), while AS-PATRR11s rarely produce *de novo* translocation products ( $\leq 6.81 \times 10^{-7}$ ) (Table 1, Figure 1B). The differences in translocation frequency were statistically significant between the three groups ( $P = 0.01$ ).

Thus, having determined that the size and symmetry of the PATRR11 appear to determine the frequency of *de novo* t(11;22)s, it seemed reasonable to hypothesize that polymorphisms of the PATRR11 might dictate translocation frequency through their secondary structure-forming propensity.

Therefore, we analyzed the secondary structure-forming propensity of the PATRR by calculating the free energy required for a transition from standard linear double-stranded DNA to intrastrand annealing, or a so-called hairpin structure [19] (Table 1). We then analyzed the correlation between the calculated secondary structure-forming propensity of a given PATRR11 and its *de novo* translocation frequency. The translocation frequency did not correlate with the free energy for hairpin/cruciform formation ( $r = 0.37$ ,  $P = 0.32$ ) (Figure 1C).

### *In vitro* analysis of cruciform extrusion of PATRR plasmids

We then analyzed the *in vitro* cruciform-forming propensity of the PATRRs using plasmids having various PATRR11s as inserts [25,27]. First we cloned each



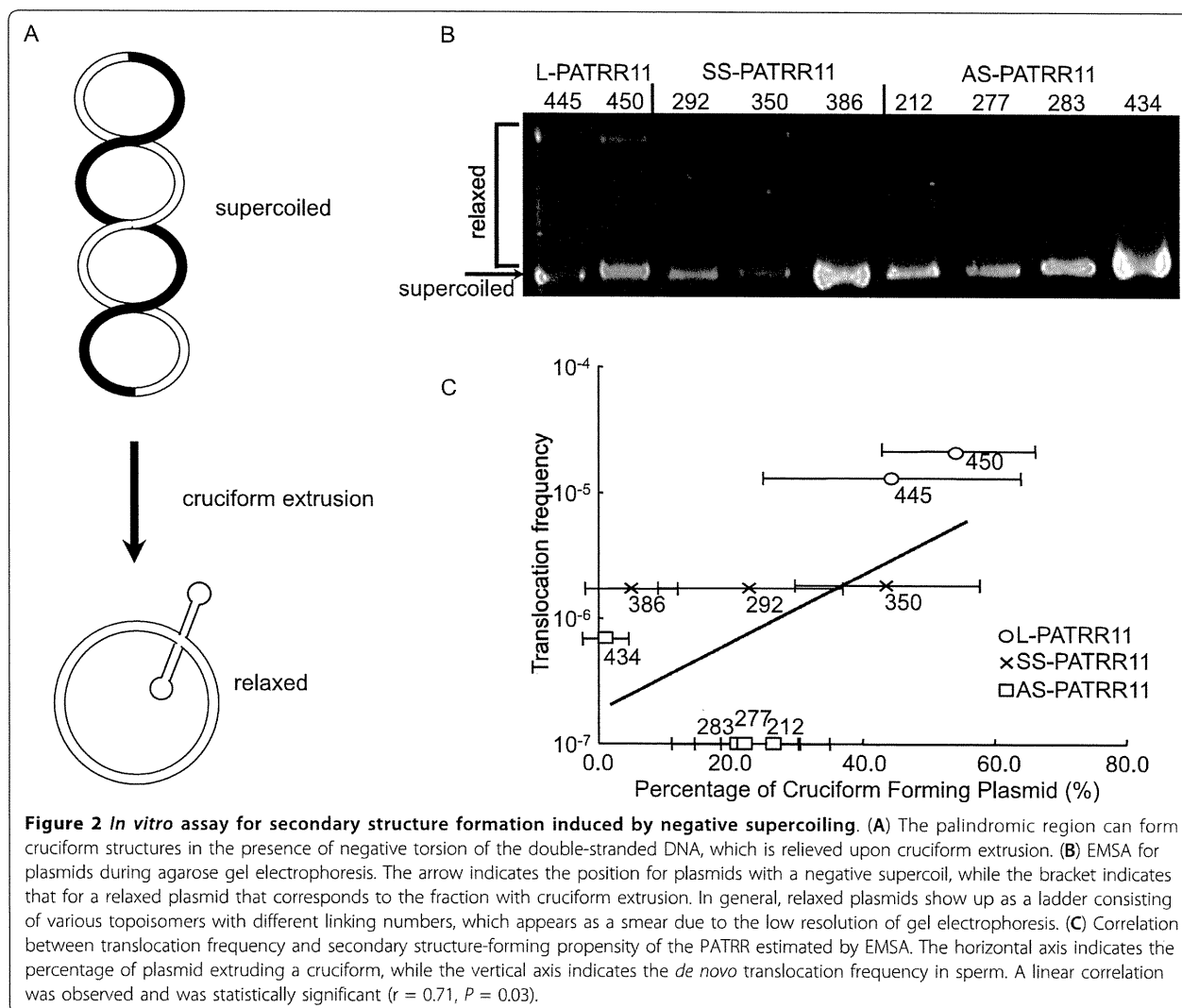
polymorphic PATRR11 into a plasmid vector and analyzed its propensity for cruciform formation by an electrophoresis mobility shift assay (EMSA). This assay is based on the fact that mobility is retarded when negative superhelical density is relieved by cruciform extrusion (Figure 2A). We extracted plasmid DNA using the

triton-lysis method such that cruciform formation during DNA extraction was minimal. To induce cruciform formation, plasmids were incubated for 30 min at 37°C in 100 mM NaCl. The conformation of plasmid DNA was analyzed by band shift on agarose gel electrophoresis.

**Table 1 Potential secondary structure of individual PATRR11 variants by free energy calculation**

Type of PATRR11	Nucleotide bp (Accession No.)	$G_{ds}$ Kcal/mole	$G_{stru}$ Kcal/mole	$\Delta G$ Kcal/mole	Translocation frequency
L-PATRR11	445(AF391129)	-392.5	-178.4	17.9	$1.32 \times 10^{-5}$
	450(AB235178)	-397.5	-187.9	10.9	$2.11 \times 10^{-5a}$
SS-PATRR11	292(AB235183)	-259.8	-121.8	8.1	$1.73 \times 10^{-6}$
	350(AB235180)	-314	-144.7	12.3	$1.82 \times 10^{-6a}$
	386(AB235182)	-335.2	-147.3	20.3	$1.71 \times 10^{-6}$
AS-PATRR11	212(AF391128)	-195.6	-83.1	14.7	$< 4.05 \times 10^{-8a}$
	277(AB235187)	-247.9	-104.2	19.8	$< 1.67 \times 10^{-7}$
	483(AB235186)	-252.9	-99.95	26.5	$< 7.62 \times 10^{-8}$
	434(AB235190)	-380.1	-157.3	32.8	$6.81 \times 10^{-7}$

<sup>a</sup> These values are the mean of 4 (450 bp L-PATRR11 allele), 2 (350 bp SS-PATRR11 allele), and 3 (212 bp AS-PATRR11 allele)samples.



For L-PATRR11, one distinct band with retarded migration was observed accompanied by a ladder of multiple bands (Figure 2B). We confirmed that the plasmids in the retarded band extruded a cruciform by showing that the band disappeared if the plasmid was digested with T7 endonuclease prior to electrophoresis. This enzyme can cut the four-way junction of cruciform DNA (data not shown). Similar results were obtained in the analysis of SS-PATRR11, whereas AS-PATRR11 did not show such retarded bands. To estimate the percentage of cruciform forming plasmids, we summed the intensity of the retarded bands and calculated their ratio to the sum of all of the bands including the band at the standard negative supercoiled position. The ratio correlated to the frequency of *de novo* translocations for each allele ( $r = 0.73, P = 0.03$ ) (Figure 2C). However, inter-assay variability was significant due to difficulty in the quantification of multiple bands.

To estimate the prevalence of cruciform extrusion more accurately, EMSA was performed for the plasmid insert only. Since the PATRR cannot maintain a cruciform conformation as short linear DNA, PATRR plasmids were treated with psoralen and ultraviolet light to form covalent cross-links prior to excision of the plasmid insert by restriction enzyme digestion. We detected a clear retarded band derived from the plasmid insert on standard agarose gel electrophoresis (Figure 3A). We confirmed that the DNAs located in the retarded bands originate from the cruciform by cleavage with T7 endonuclease or by direct observation using atomic force microscopy (AFM) (Figure 3B). The intensity of the retarded band on EMSA correlated well with the translocation frequency ( $r = 0.77, P = 0.01$ ) (Figure 3C).

## Discussion

Our previous study demonstrated that the size and symmetry of polymorphic PATRRs appears to affect the