Gene conversion and evolution of Xq28 duplicons involved in recurring inversions causing severe hemophilia A

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Inversions breaking the 1041 bp *int1h*-1 or the 9.5-kb *int22h*-1 sequence of the *F8* gene cause hemophilia A in 1/30,000 males. These inversions are due to homologous recombination between the above sequences and their inverted copies on the same DNA molecule, respectively, *int1h*-2 and *int22h*-2 or *int22h*-3. We find that (1) *int1h* and *int22h* duplicated more than 25 million years ago; (2) the identity of the copies (>99%) of these sequences in humans and other primates is due to gene conversion; (3) gene conversion is most frequent in the internal regions of *int22h*; (4) breakpoints of *int22h*-related inversions also tend to involve the internal regions of *int22h*; (5) sequence variations in a sample of human X chromosomes defined eight haplotypes of *int22h*-1 and 27 of *int22h*-2 plus *int22h*-3; (6) the latter two sequences, which lie, respectively, 500 and 600 kb telomeric to *int22h*-1 are five-fold more identical when in *cis* than when in *trans*, thus suggesting that gene conversion may be predominantly intrachromosomal; (7) *int1h*, *int22h*, and flanking sequences evolved at a rate of about 0.1% substitutions per million years during the divergence between humans and other primates, except for *int1h* during the human-chimpanzee divergence, when its rate of evolution was significantly lower. This is reminiscent of the slower evolution of palindrome arms in the male specific regions of the Y chromosome and we propose, as an explanation, that intrachromosomal gene conversion and cosegregation of the duplicated regions favors retention of the ancestral sequence and thus reduces the evolution rate.

[Supplemental material is available online at www.genome.org. The sequence data from this study have been submitted to GenBank under accession nos. AY619998–AY620001 and AY781298–AY781308.]

One in 30,000 males is born with an inversion breaking the factor VIII gene (*F8*) and causing severe hemophilia A. In 90% of these patients the break is in intron 22 of *F8* and affects a 9.5-kb sequence called *int22h*-1 (Fig. 1) which is also present in inverted orientation 500 kb (*int22h*-2) and 600 kb (*int22h*-3) more telomerically (Naylor et al. 1992, 1993, 1995; Lakich et al. 1993; P.M. Green, N. Waseem, R.D. Bagnall, and F. Giannelli, unpubl.).

In the remaining 10% of patients the break is in intron 1 and affects a 1041-bp sequence called *int1h*-1 (Fig. 1; GenBank accession no. AY619998), which is duplicated in inverted orientation 140 kb more telomerically (*int1h*-2; GenBank accession no. AY781298) (Bagnall et al. 2002; P.M. Green, N. Waseem, R.D. Bagnall, and F. Giannelli, unpubl.).

The above inversions result from frequently recurring homologous recombination between the above sequences in the *F8* gene and their more telomeric copies on the same DNA molecule (Naylor et al. 1995; Bagnall et al. 2002).

The sequences of both the *int22h* and *int1h* copies are >99.9% identical; hence they either duplicated very recently or are undergoing concerted evolution.

Aradhya et al. (2002) showed that a probe containing part of *int22h* hybridized to three DNA segments in blots of chimpanzee and gorilla DNA and to two segments in blots of orangutan and pygmy chimpanzee DNA, thus suggesting that duplication of *int22h* might not be recent. Furthermore, analysis of the hemophilia A mutation in the Chapel Hill colony of hemophilic dogs (Lozier et al. 2002) provides evidence of two copies of the *F8A* gene, which is a segment of *int22h*. Thus, at least for *int22h*, the hypothesis of recent duplication seems less likely than concerted evolution. In this article we demonstrate that both *int1h* and *int22h* have duplicated at least 25 million years ago (Mya) and that gene conversion is the process responsible for high copy identity. Our results also suggest how intrachromosomal gene conversion may sometimes appear to have a bias in favor of restoration to the ancestral sequence.

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Results

Int1h

DNA from a male chimpanzee (*Pan troglodytes*), African Green monkey (*Cercopithecus aethiops*), and Rhesus monkey (*Macaca mulatta*) was used as a template for PCR reactions that amplify human *int1h*-1 and *int1h*-2. These reactions yielded sequences homologous to *int1h*-1 and *int1h*-2 from all the above DNAs (Supplemental Fig. 1; GenBank accession nos. AY781299–AY781304), thus showing that the duplication of *int1h* has occurred before the split between the human and Rhesus or African Green monkey and hence more than 25 Mya (Purvis 1995). Interspecies comparisons of *int1h* (Table 1 and Supplemental Fig. 1) and flanking sequences (Table 2 and Supplemental Fig. 2) showed rates of evolution close to 0.1% base substitutions per million years for all regions and during each interspecies diver-

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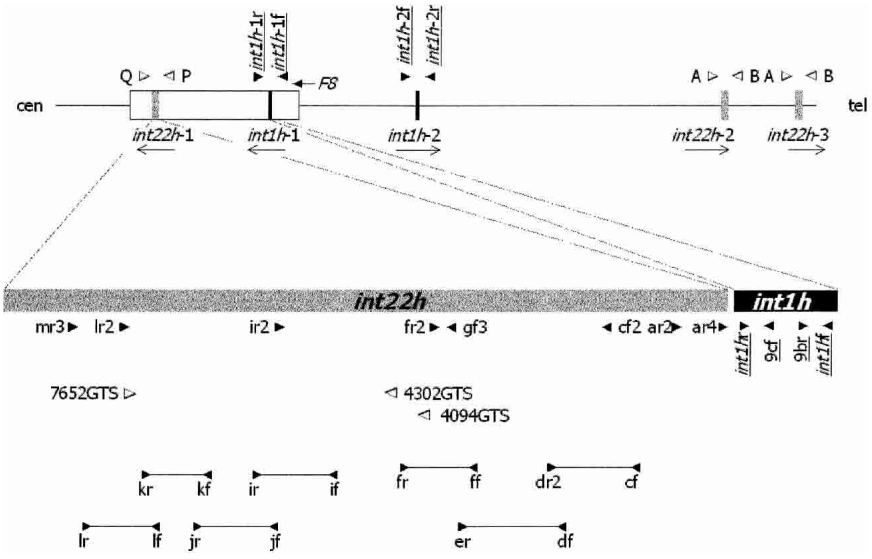


Figure 1. Map of *int1h* and *int22h* duplicons. (*Top*) *F8* gene (clear box), with arrow indicating direction of transcription, plus sequence of *int1h* (black boxes) and *int22h* (gray boxes) with arrows indicating orientation. Clear and solid arrow heads indicate location and orientation of primers flanking *int22h* copies and *int1h* copies, respectively (for primer sequences see Liu et al. 1998 and Bagnall et al. 2002). (*Bottom*) *Int22h* and *int1h* showing location and orientation of primers listed in Supplemental Table 3. These were primers required for completing the sequence of the chimpanzee's *int22h* sequences (solid arrow heads), or for allele-specific PCR in *int22h* (clear arrow heads), or for amplification of DNA for FSPCCM analysis (lines terminating with arrow heads). Other primers for sequencing *int22h* were from Naylor et al. (1995) (not shown). Primers for sequencing *int1h* (solid arrow heads and underlined names) are from Bagnall et al. (2002).

gence except for *int1h* during human–chimpanzee divergence when a significantly lower rate of evolution was observed (Table 2). This intriguing exception will be discussed later. However, in contrast with the interspecies comparisons, the two *int1h* copies of each species showed much greater similarity than could be expected after 25 Myr of separate existence as they showed only 1-, 0-, 6-, and 9-nucleotide (nt) differences and hence divergences of 0.096%, 0%, 0.57%, and 0.86% in humans, chimpanzee, African Green, and Rhesus monkey, respectively (Table 1 and Supplemental Fig. 1).

To see if this high intraspecies identity was the result of gene conversion, the test developed by Balding et al. (1992) was used. This test compares the duplicated sequences in pairs of species, considering in particular the frequency of instances where the two species differ by the same base substitution at the same site of both repeats. These instances, called "co-doubles," are expected to be rare in independently evolving duplicates, whereas they can be readily produced, in each species, by gene conversion as this process can copy a mutation of one duplicate into the other of the same species. The Balding et al. (1992) test provided strong evidence of gene conversion in all species comparisons, as the null hypothesis of independent evolution was shown to be improbable, $P < 0.001$ for human + chimpanzee and even more improbable $P \ll 0.001$ for all other pairs, namely, human + African Green monkey, human + Rhesus monkey, chimpanzee + African Green monkey, chimpanzee + Rhesus monkey, and African Green + Rhesus monkey.

The distribution of co-doubles along the *int1h* sequence was not uniform (Supplemental Table 1), as a modest excess of co-doubles relative to Poisson expectation was observed in the central region (nt 521–624) and also toward the ends of the duplicates that are farthest apart, although the last 30 nt of these ends appear also rich in singles (Supplemental Fig. 1 and Supplemental Table 1).

Human *int1h* sequence variation was analyzed by examin-

Table 1. Divergence between *int1h* repeats of humans and nonhuman primates

***Int1h* nucleotide**	**5**	**44**	**62**	**107**	**108**	**153**	**159**	**178**	**190**	**233**	**248**	**296**	**310**	**339**	**354**	**438**	**450**	**476**	**488**	**521**	**540**	**549**	**561**	**563**	**592**	**608**	**611**	**653**	**665**	**679**
human *int1h*-1	T	C	T	G	T	G	C	A	G	C	G	C	A	C	T	G	T	C	G	A	G	C	T	C	C	A	G	C	T	C
human *int1h*-2	T	C	T	G	T	G	C	A	G	C	G	C	A	C	T	G	T	C	G	A	G	C	T	C	C	A	G	C	T	C
chimpanzee *int1h*-1	T	C	T	G	T	G	C	A	G	C	G	C	A	C	T	G	T	C	G	A	G	C	T	C	T	A	G	C	T	C
chimpanzee *int1h*-2	T	C	T	G	T	G	C	A	G	C	G	C	A	C	T	G	T	C	G	A	G	C	T	C	T	A	G	C	T	C
AGM *int1h*-1	A	T	T	A	C	G	T	G	A	T	T	T	G	T	C	A	A	T	A	G	C	T	C	G	T	A	T	T	C	A
AGM *int1h*-2	T	T	T	A	C	G	T	G	A	T	T	T	G	T	C	A	A	T	A	G	C	T	C	G	T	A	T	T	C	A
Rhesus *int1h*-1	A	T	G	A	C	A	C	G	A	T	T	C	G	T	C	A	T	C	A	G	C	T	C	G	T	C	T	T	C	G
Rhesus *int1h*-2	T	T	T	A	C	G	C	G	A	T	T	C	G	T	C	A	T	C	A	G	C	T	C	G	T	C	T	T	C	G

***Int1h* nucleotide**	**687**	**726**	**733**	**769**	**789**	**790**	**798**	**816**	**823**	**834**	**855**	**856**	**880**	**930**	**944**	**950**	**960**	**975**	**982**	**991**	**1000**	**1001**	**1002**	**1008**	**1011**	**1023**	**1024**	**1028**	**1031**	**1038**
human *int1h*-1	C	C	C	C	A	G	C	G	C	T	C	G	A	G	T	C	G	A	C	G	C	A	A	A	G	G	T	C	A	A
human *int1h*-2	C	C	C	C	A	G	C	G	C	T	C	G	A	G	T	C	G	A	C	G	C	A	A	C	G	G	T	C	A	A
chimpanzee *int1h*-1	C	C	C	C	A	G	C	G	C	T	C	G	A	G	T	C	G	A	C	G	C	A	A	A	G	G	T	C	A	A
chimpanzee *int1h*-2	C	C	C	C	A	G	C	G	C	T	C	G	A	G	T	C	G	A	C	G	C	A	A	A	G	G	T	C	A	A
AGM *int1h*-1	C	T	T	T	C	T	G	C	T	C	C	A	C	C	T	T	A	G	G	C	C	G	G	G	G	G	A	A	G	A
AGM *int1h*-2	C	T	T	T	C	T	G	C	T	C	C	A	C	C	T	T	A	G	G	C	T	G	G	A	G	A	T	A	A	A
Rhesus *int1h*-1	T	T	C	T	C	T	G	G	T	C	T	A	C	C	G	T	A	G	G	C	C	A	G	G	A	G	T	A	G	A
Rhesus *int1h*-2	T	T	C	T	C	T	G	G	T	C	T	A	C	C	G	T	A	G	G	C	C	G	G	A	G	A	T	A	A	C

Int1h residue number is shown on top.

Table 2. Frequency of base substitutions observed in *int1h* and flanking sequences of different pairs of species

Species pairs	*Int1h*			Flanking sequence			*P* value
	d	nt	pro	d	nt	pro	
H + C	3	2082	0.00144	51	6941	0.00735	<0.001*
H + A	99	2080	0.0476	200	3516	0.0568	0.131
H + R	96	2082	0.0461	233	4409	0.0528	0.239
C + A	96	2080	0.0461	185	3465	0.0534	0.260
C + R	93	2082	0.0447	218	4366	0.0499	0.390
A + R	29	2092	0.0138	59	3516	0.0168	0.460

(H) human, (C) chimpanzee, (A) African Green monkey, (R) Rhesus monkey; (d) number of divergences, (nt) number of aligned nucleotides excluding sites of insertion/deletion mutations, (pro) proportion of divergence; *P* value is result of Normal test of equal proportions involving *int1h* and flanking sequences; *statistically significant difference.

ing 57 X chromosomes and this showed that nt 1008 was always A in *int1h*-1 and C in *int1h*-2, while nt 698 of *int1h*-2 was G in 9 and C in 48 X chromosomes (Bagnall et al. 2002).

Int22h

Naylor et al. (1995) reported the sequence of *int22h*-1, its flanking regions, and the regions flanking either of both *int22h*-2 and *int22h*-3. They also detected some differences between *int22h*-1 and *int22h*-2 plus *int22h*-3; however, they could not independently sequence *int22h*-2 and *int22h*-3 because clones containing only one or the other of these repeats had not yet been identified. We used such clones (Hassock 2000) to sequence the two distal *int22h* copies and also resequenced *int22h*-1 from a British male as the sequence published by Naylor et al. (1995) contained 31 undefined residues. The revised sequence of *int22h*-1 and the sequences of *int22h*-2 and *int22h*-3 are available at GenBank accession nos. AY619999, AY620000, and AY620001, respectively.

Long-range PCR experiments on primate male DNA showed that human sequence primers flanking *int22h*-1 and either of both *int22h*-2 and *int22h*-3 readily amplified the corresponding sequences of chimpanzee DNA. The PCR reaction that amplifies *int22h*-2 and *int22h*-3 yielded products that had no site showing the presence of two different nucleotides. Therefore, because the results of Aradhya et al. (2002) had shown that the chimpanzee has three copies of *int22h*, we assume that, in the chimpanzee we examined, *int22h*-2 and *int22h*-3 were identical. The African Green monkey DNA did not sustain the amplification of the full *int22h* sequence, but when a primer internal plus one external to *int22h*-1 were used simultaneously, a 6.5-kb product was obtained consisting of 1 kb homologous to the human sequence flanking *int22h*-1 on the telomeric side followed by 5.5 kb homologous to nt 1–5553 of human *int22h* (GenBank accession no. AY781307). Similarly, a 6.6-kb product was obtained with a primer external to both telomeric *int22h* sequences and one internal. This PCR product was homologous to human *int22h* from nt 1261 to its telomeric end and terminated with 40 bp of sequence homologous to the sequence flanking either of the two human telomeric *int22h* copies on their telomeric side (GenBank accession no. AY781308). However nucleotides homologous to human 6027–7842 were absent in African Green monkey, presumably due to a deletion/insertion event.

These data provide further proof that duplication of *int22h* predates the human–African Green monkey split.

The sequence of the human and chimpanzee's *int22h* copies were compared (Table 3 and Supplemental Fig. 3). The chimpanzee's *int22h* differed from the human by nine insertions/deletions, of which two affected only *int22h*-1, replacement of human nt 7854–8549 with an inverted copy of nt 7866–7911 and the tandem duplication of nt 8755–8802 (GenBank accession nos. AY781305 and AY781306). These insertions/deletions reduce the length of *int22h* common to the human and chimpanzee DNA samples to 8854 nt.

This alignment of the human and chimpanzee's sequences showed 102 base substitutions between the *int22h*-1 sequences, 95 between the *int22h*-2, and 94 between the *int22h*-3 (Table 3). This indicated an evolution rate of about 0.1% base substitutions per million years of divergence. In contrast, human *int22h*-1 differed from *int22h*-2 and *int22h*-3 at 12 and 13 sites, respectively, while the latter two *int22h* sequences differed at a single site. Similarly, the chimpanzee's *int22h*-1 differed at 24 sites from both *int22h*-2 and *int22h*-3, as the latter two appeared to be identical.

The *int22h*-1 and *int22h*-2 or *int22h*-3 sequences of humans and chimpanzee were then examined using Balding et al's (1992) test of gene conversion after disregarding the variation at nt 4350 between the human *int22h*-2 and *int22h*-3. The vast majority of base differences were found to be co-doubles, and the Balding test provided very strong evidence of gene conversion as the probability of the null hypothesis of independent evolution of the *int22h* copies was extremely small ($P \ll 0.001$). The distribution of co-doubles along the *int22h* repeat was nonhomogeneous because when the sequence was divided into 19 segments of 466 bp, the frequency of co-doubles departed significantly from Poisson expectation in several segments (Supplemental Table 2). An excess of co-doubles was observed over a wide region comprising nt 4198 to 6532, while co-doubles were relatively rare near to the ends of the duplicated sequence. These results suggest a broad peak of gene conversion events in the central region (nt 4195–6524) and a dearth of gene conversion at the periphery (nt 1–466 and 8581–9512) of the human and chimpanzee's *int22h* sequence alignment.

The alignment of the human *int22h* copies with the sequence we obtained from the African Green monkey (see Supplemental Fig. 4) gives data in keeping with the results of the human–chimpanzee comparison because it shows 616 base substitutions in 11,835 pairs of nucleotides or a divergence of 5.2%, equivalent to an evolution rate of 0.1% per million years of divergence. In contrast, the sequence available on the *int22h* duplicates of the African Green monkey showed only 29 base substitutions in 4468 nucleotide pairs or a divergence of 0.064%. Furthermore, the base differences between the *int22h* duplicates of humans and African Green monkey are mostly in the form of co-doubles (i.e., 209 co-doubles, 31 singles and two doubles). Thus, during the human–African Green monkey divergence,

Table 3. Divergence between *int22h* repeats of humans and chimpanzee

Int22h residue	8	49	193	459	472	683	742	1113	1319	1437	1450	1602	1701	2086	2133	2170	2478	2520	2798	2820	2834	3156	3223	3546	3777	3867	4094	4098	4099	4157	4178	4247	4257	4302	4303	4331
Human *h*-1	G	T	T	**a**	G	C	T	T	G	A	G	A	T	G	T	T	A	A	A	C	T	C	A	G	G	T	**a**	G	G	C	T	G	T	C	G	C
Human *h*-2 + 3	G	**c**	T	G	G	C	T	T	G	A	G	A	T	G	T	T	A	A	A	C	T	C	A	G	G	T	G	G	G	C	T	G	T	**g**	G	C
Chimp h-1	**a**	T	T	G	**a**	A	C	C	T	G	T	C	C	C	C	G	G	G	C	T	C	T	G	A	A	C	G	T	A	G	G	A	C	C	T	T
Chimp *h*-2 + 3	G	T	**c**	G	G	A	C	C	T	G	T	C	C	C	C	G	G	G	C	T	C	T	G	A	A	C	G	T	A	G	G	A	C	C	T	T

Int22h residue	4340	4350	4353	4360	4364	4378	4414	4610	4612	4640	4759	4769	4776	4827	4859	4860	4869	4870	4943	5040	5076	5150	5259	5346	5431	5470	5640	5674	5725	5748	5785	5845	5876	5880	5909	5971	6017	6083
Human *h*-1	**a**	C	C	C	G	G	A	C	G	G	G	C	G	T	C	A	G	A	A	T	**g**	G	C	C	C	A	C	C	A	A	C	G	C	A	A	G	G	G
Human *h*-2 + 3	G	**C**	C	C	G	G	A	C	G	G	G	C	G	T	C	A	G	A	A	T	A	G	C	C	C	A	C	C	A	A	C	G	C	A	A	G	G	G
Chimp h-1	G	T	**g**	T	**c**	C	G	G	C	A	A	G	T	C	G	G	A	G	G	G	A	C	T	G	T	G	T	T	G	G	G	C	G	G	G	T	T	A
Chimp *h*-2 + 3	G	T	**t**	T	G	C	G	G	C	A	A	G	T	C	G	G	A	G	G	G	A	C	T	G	T	G	T	T	G	G	G	C	G	G	G	T	T	A

Int22h residue	6157	6233	6275	6373	6420	6428	6456	6499	6565	6685	6702	6718	6976	7145	7192	7245	7278	7411	7609	7638	7652	7667	7733	7765	7779	7830	8579	8726	8731	8742	8746	8854	9085	9217	9243	9479	9502	9503
Human *h*-1	G	C	T	C	C	C	C	A	C	G	G	T	C	C	G	C	C	G	G	T	**a**	A	A	A	C	C	A	T	C	C	A	A	C	G	G	T	A	C
Human *h*-2 + 3	G	C	T	C	C	C	C	A	C	G	G	T	C	C	G	C	C	G	G	T	G	A	A	A	C	C	A	T	C	C	A	A	C	G	G	T	A	C
Chimp h-1	A	G	C	T	G	G	T	G	T	A	A	G	T	T	**c**	A	C	T	A	T	G	G	T	T	**t**	**t**	**g**	**g**	**t**	C	A	A	G	G	**t**	T	**g**	**t**
Chimp *h*-2 + 3	A	G	C	T	G	G	T	G	T	A	A	G	T	T	G	A	**t**	T	A	**c**	G	G	T	T	C	C	A	T	C	**t**	**g**	**g**	G	**c**	G	**c**	A	C

Int22h residue number is shown on top. Base divergences representing co-doubles are highlighted gray while singles and doubles are in bold lower case. Base difference between human *int22h*-2 (C) and *int22h*-3 (T) at residue 4350 is indicated by showing the C of *int22h*-2 in bold uppercase.

int22h has evolved at a rate similar to that observed during the human–chimpanzee split and the high similarity in the segment of the *int22h* duplicates that we were able to study in African Green monkey is due to gene conversion.

The variation of the *int22h* sequence among humans was examined by investigating 19 normal British males and 16 hemophilia A patients with inversions involving *int22h* (i.e., six inversions due to recombination of *int22h*-1 with *int22h*-2 and 10 due to recombination of *int22h*-1 with *int22h*-3). The *int22h*-1 sequences of the 19 control males showed eight different haplotypes, defined by the association of alleles at 12 variable sites (Table 4). *Int22h*-2 and *int22h*-3, which cannot be individually amplified, were identical in six control males and different at a single site in nine. In the remaining four control males more

Table 4. Haplotypes of *int22h*-1

	Chimpanzee h1	459	4094	4302	4340	4341	4350	4619	5076	5880	7652	8564	8878	*int22h*-1 haplotype number
		G	**G**	**C**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	**A**	**G**	
Control individual	C1 h1	A	A		A		C		G					1
	C2 h1	A	A		A		C		G					1
	C3 h1	A	A		A		C		G					1
	C4 h1	A	A		A		C		G					1
	C5 h1	A	A		A		C		G					1
	C6 h1	A	A		A		C		G					1
	C7 h1	A	A		A		C		G					1
	C8 h1	A	A		A		C		G					1
	C9 h1	A			A		C		G					2
	C10 h1	A			A		C		G			G		3
	C11 h1	A			A		C		G		G			4
	C12 h1	A		G			C							5
	C13 h1	A		G		C							T	6
	C14 h1			G			C	G						7
	C15 h1			G			C	G						7
	C16 h1			G			C	G						7
	C17 h1			G			C	G		A	G			8
	C18 h1			G			C	G		A	G			8
	C19 h1			G			C	G		A	G			8

Int22h-1 human nucleotide number at sites of polymorphism is shown at the top. Chimpanzee nucleotides at these sites are shown in bold. Empty spaces are identical to ancestral (chimpanzee). Novel alleles are in upper case.

differences were found and in order to distinguish the haplotypes of the two sequences it was necessary to use allele-specific primers so as to examine the association of nucleotides at the sites of divergence (Table 5A). In the hemophilia A patients the unrecombined distal sequence (either *int22h*-3 or *int22h*-2) can be specifically amplified and its haplotype can be directly determined (Table 5B,C). The 54 distal *int22h* sequences examined showed 27 different haplotypes (Table 6), which fall clearly into two groups: haplotypes 1–16 and haplotypes 18–27. These are distinguished by the presence or absence of a nonancestral allele (i.e., divergent from chimpanzee) at nt 49, 1007, 1477, 1567, and 4619 and a G or A at nt 8509. Haplotype 17 appears more similar to the 18–27 group but does not differ from the 1–16 group at nt 1567 and 8509. Human *int22h*-1 differs from *int22h*-2 and *int22h*-3 at three sites (nt 3, 10, and 12) that appear nonpolymorphic (Supplemental Fig. 3) and at a further 23 sites that are polymorphic among the individuals we have examined (Tables 4 and 6).

The two distal *int22h* sequences on the same X chromosome (in *cis*) usually showed the same haplotype, and the average number of differences between these sequences was 1.32 (95% confidence interval obtained from bootstrapping with individuals resampled was 0.68–2.11). This is fivefold smaller than the number of differences between all possible pairs formed by the unrecombined *int22h*-2 and *int22h*-3 sequences specifically amplified from the inversion patients, which was 6.67 (95% confidence interval obtained from balanced bootstrap, with 6 *int22h*-2 and 10 *int22h*-3 chosen for each sample, was 3.63–9.60). Similarly the average number of differences between all possible pairs formed by one or the other of the distal *int22h* sequences of one control male with one or the other distal *int22h* of the remaining 18 controls was 5.98 (95% confidence interval 3.82–6.79). Both these two average numbers of differences are significantly greater than the differences between distal *int22h* sequences on the same chromosome as shown by the nonoverlapping confidence intervals.

It is clear from Tables 4 and 6 that the nonancestral alleles at nt 4094, 4302, 4340, 4341, 4350, 4619, 5076, 5880, and 7652 of human *int22h*-1 and the other two *int22h* copies may represent co-doubles when considered in relation to the chimpanzees' sequences. Thus the above variants may reveal gene conversion events that occurred during the history of the *int22h* sequences examined.

Suggestions of gene conversion associated with recombination were obtained from the analysis of the 16 hemophilia A patients mentioned above and one of seven patients with the inversion involving *int1h*. In this latter patient the nonancestral nt C observed at site 1008 of only *int1h*-2 in all 57 control human X chromosomes analyzed (Bagnall et al. 2002) was found in both recombined *int1h* sequences, thus suggesting gene conversion of the region containing this nucleotide. Analysis of the patients with the *int22h*-related inversion was more complex. Since the haplotypes of the *int22h* sequences prior to the inversion were not known, the events that occurred during the recombination event causing the inversion could only be indirectly reconstructed. To do this all combinations of known haplotypes of the relevant sequences (either *int22h*-1 plus *int22h*-2 or *int22h*-1 plus *int22h*-3) were considered for each patient and then probable combinations were selected with the help of the following criteria:

1. That the distal *int22h* of each likely combination should be as close a match as possible to the patient's unrecombined distal *int22h*, because of the close similarity of these sequences observed in control X chromosomes.
2. That the combination of haplotypes was capable of yielding the recombined *int22h* sequences seen in the patient either directly or only with the help of gene conversion as the observation of de novo mutations arising during the recombination event was considered unlikely.

The vast majority of haplotype combinations thus selected were found to require gene conversion in order to account for the haplotypes of the patients' recombined *int22h* sequences. The length of the gene conversion tract, of course, varied according to the combination considered and, for the purpose of illustration, Figure 2 shows for each patient the combination of haplotypes that best fits the above two selection criteria and requires the shortest gene conversion tract to account for the patient's recombined *int22h* sequences. The gene conversion events proposed in Figure 2 are usually in the region between nt 4094 and 5880, which lies within the region of *int22h*, showing a broad peak of co-doubles. This is the region thought to have experienced most gene conversion events during the evolution of *int22h* in humans and chimpanzees.

Discussion

The results presented above show that *int1h* as well as *int22h* duplicated more than 25 Mya. During species divergence these sequences evolved at substitution rates close to those expected for noncoding DNA (Ebersberger et al. 2002), i.e., about 0.1% per million years except for *int1h* during the human–chimpanzee split. However, the copies of *int1h* and *int22h* within each species analyzed are more than 99% identical. This suggests that they undergo a process of intraspecies homogenization and that their identity is not due to functional constraints strong enough to result in marked suppression of interspecies divergence. In fact no essential function has so far been assigned to either *int1h* or *int22h*. However, the latter contains the intronless *F8A* gene that encodes a protein found to coprecipitate with huntingtin (Levinson et al. 1992; Peters and Ross 2001). This coding sequence represents 1.1 kb of *int22h* and shows less human–chimpanzee divergence than the rest of *int22h* (0.45% vs. 1.3%; $P < 0.01$). Nevertheless, in humans the loss of one *F8A* gene appears to be well tolerated, as deletions of the *F8* gene resulting in loss of *int22h*-1 do not cause other phenotypes than hemophilia A (Casula et al. 1990).

The process causing homogenization of the duplicated *int1h* and *int22h* sequences appears to be gene conversion, as our results demonstrate that in most regions of *int1h* and *int22h* all interspecies divergences are in the form of co-doubles. In these regions gene conversion events must have been frequent enough to allow either restoration of the ancestral sequence or duplication of any mutation that occurred in one copy into the (or one) other copy.

The distribution of co-doubles in *int22h* shows that gene conversion between *int22h*-1 and the two distal *int22h* sequences is most frequent in the central region and most rare near the ends. The overlap between the region of *int22h* with the greatest co-double density and the region of putative gene conversion in hemophilia A inversion patients suggests that the same region of *int22h* is a focus for recombination as well as gene conversion not associated with recombination ("pure" gene conversion) between *int22h*-1 and the distal *int22h*. This is in keeping with the

Table 5. *Int22h*-2 and *int22h*-3 polymorphic sites and sites of divergence from *int22h*-1

	Chimpanzee h2/h3	3	10	12	49	459	880	1007	1168	1477	1567	4069	4094	4302	4340	4341	4350	4619	5076	5880	7652	8444	8449	8509	8564	8878	8802–8850
		A	**T**	**T**	**T**	**G**	**G**	**G**	**A**	**C**	**A**	**C**	**G**	**C**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	—	—	—	**A**	**G**	**Y**
A. Control individuals	C1 h2/3				C								A	G			C			A		*C*	*A*	*G*			N
	C1 h2/3				C								G	G			T			A							N
	C2 h2/3							A	G	G	G						C	G			G	*T*	*G*	*A*			Y
	C2 h2/3							A	A	G	G						C	G			G	*T*	*G*	*A*			Y
	C3 h2/3				C								A	G						A	G						N
	C3 h2/3				C								G	G						A	G						N
	C4 h2/3				C									G			C		G	A	G						N
	C4 h2/3				C									G			C		A	A	G						N
	C5 h2/3				C									G						A							N
	C5 h2/3				C									G						A							N
	C6 h2/3				C								A	G			C			A	G						N
	C6 h2/3				C								A	G			C			A	G						N
	C7 h2/3						A	A		G	G			G			C	G							*A*		Y
	C7 h2/3						G	A		G	G			G			C	G							*A*		Y
	C8 h2/3				C								A	G						A							N
	C8 h2/3				C								G	G						A							N
	C9 h2/3				C									G						A	G						N
	C9 h2/3				C									G						A	G						N
	C10 h2/3				T			A		G				G			C			G	A						N
	C10 h2/3				C			G		C				G			C			A	G						Y
	C11 h2/3				C									C	A		C			A	G						N
	C11 h2/3				C									G	G		T			A	G						N
	C12 h2/3							A		G	G			G			C	G				*T*		*A*			N
	C12 h2/3							A		G	G			G			C	G				*C*		*A*			N
	C13 h2/3				C								A	G						A	G	*C*	*A*				N
	C13 h2/3				C								G	G						G	A	*T*	*G*				N
	C14 h2/3							A		G	G			G			C	G					*G*	*A*			Y
	C14 h2/3							A		G	G			G			C	G					*A*	*A*			Y
	C15 h2/3							A		G	G	G		G			C	G			G			*A*			Y
	C15 h2/3							A		G	G	C		G			C	G			G			*A*			Y
	C16 h2/3				C								A	G			C			A	G						N
	C16 h2/3				C								A	G			C			A	G						N
	C17 h2/3							A		G	G			G			C	G				*T*		*A*			Y
	C17 h2/3							A		G	G			G			C	G				*T*		*A*			Y
	C18 h2/3				C									G						G	G						N
	C18 h2/3				C									G						A	G						N
	C19 h2/3				C									G						A	G						N
	C19 h2/3				C									G						A	G						N
B. Proximal inversions	a h3				C									G		C					G						N
	b h3							A		G	G			G			C	G				*T*		*A*			Y
	c h3				C								A	G			C	G		A	G						N
	d h3							A		G	G			G			C	G				*T*		*A*			Y
	e h3				C									G		C					G						N
	f h3							A		G	G			G			C	G				*T*		*A*			Y
C. Distal inversions	UKA181 h2				C									G			C			A	G						N
	UKA36 h2				C									G			C		G	A	G						N
	UKA502 h2				C									G			C			A	G						N
	UKA57 h2				C									G		C					G						N
	UKA657 h2				C									G						A	G						N
	UKA667 h2				C									G					G	A	G						N
	UKA695 h2				C									G			C			A	G						N
	UKA697 h2				C												C			A	G						N
	UKA708 h2				C									G			C				G						N
	UKA744 h2				C									G						A	G						N

Int22h-2 and *int22h*-3 polymorphic sites and sites of divergence from *int22h*-1 are shown at top. Chimpanzee nucleotides at these sites are in bold. Empty spaces are identical to ancestral (chimpanzee) sequence or to bases found in reference human DNA and absent in chimpanzee (italics). Novel sequence variations are in upper case. Column 8802–8850 corresponds to presence (Y) or absence (N) of polymorphic tandem duplication. (*A*) Data from normal individuals (i.e., C1 to C19); each h2/3 row represents one or the other of the distal repeats of the controls. (*B*) Data from hemophilia A patients with inversions resulting from recombination of *int22h*-1 with *int22h*-2. (*C*) Data from hemophilia A patients with inversions resulting from recombination of *int22h*-1 with *int22h*-3. a–f and UKA numbers are patients' codes; h2 and h3 refer to *int22h*-2 and *int22h*-3, respectively.

Table 6. Haplotypes of two distal *int22h* sequences

Haplotype number	49	880	1007	1168	1477	1567	4069	4094	4302	4340	4341	4350	4619	5076	5880	7652	8444	8449	8509	8802–8850	No. observed
Chimpanzee	**T**	**G**	**G**	**A**	**C**	**A**	**C**	**G**	**C**	**G**	**G**	**T**	**T**	**A**	**G**	**A**	—	—	—	**Y**	**1**
1	C								G						A		*C*	*A*	*G*	N	4
2	C								G						A	G				N	9
3	C								G			C			A	G				N	5
4	C								G							G				N	1
5	C								G		C					G				N	3
6	C								G			C				G				N	1
7	C									A		C			A	G				N	1
8	C											C			A	G				N	1
9	C							A	G						A					N	1
10	C							A	G			C			A					N	1
11	C							A	G						A	G				N	2
12	C							A	G			C			A	G				N	4
13	C							A	G			C	G		A	G				N	1
14	C								G								*T*	*G*		N	1
15	C								G			C		G	A	G				N	2
16	C								G					G	A	G				N	1
17			A		G				G			C								N	1
18			A		G	G			G			C	G						*A*	Y	2
19		A	A		G	G			G			C	G						*A*	Y	1
20			A		G	G	G		G			C	G			G			*A*	Y	1
21			A		G	G			G			C	G			G			*A*	Y	1
22			A		G	G			G			C	G					*G*	*A*	Y	1
23			A		G	G			G			C	G						*A*	N	1
24			A		G	G			G			C	G				*T*		*A*	N	1
25			A		G	G			G			C	G				*T*		*A*	Y	5
26			A		G	G						C	G			G	*T*	*G*	*A*	Y	1
27			A	G	G	G						C	G			G	*T*	*G*	*A*	Y	1

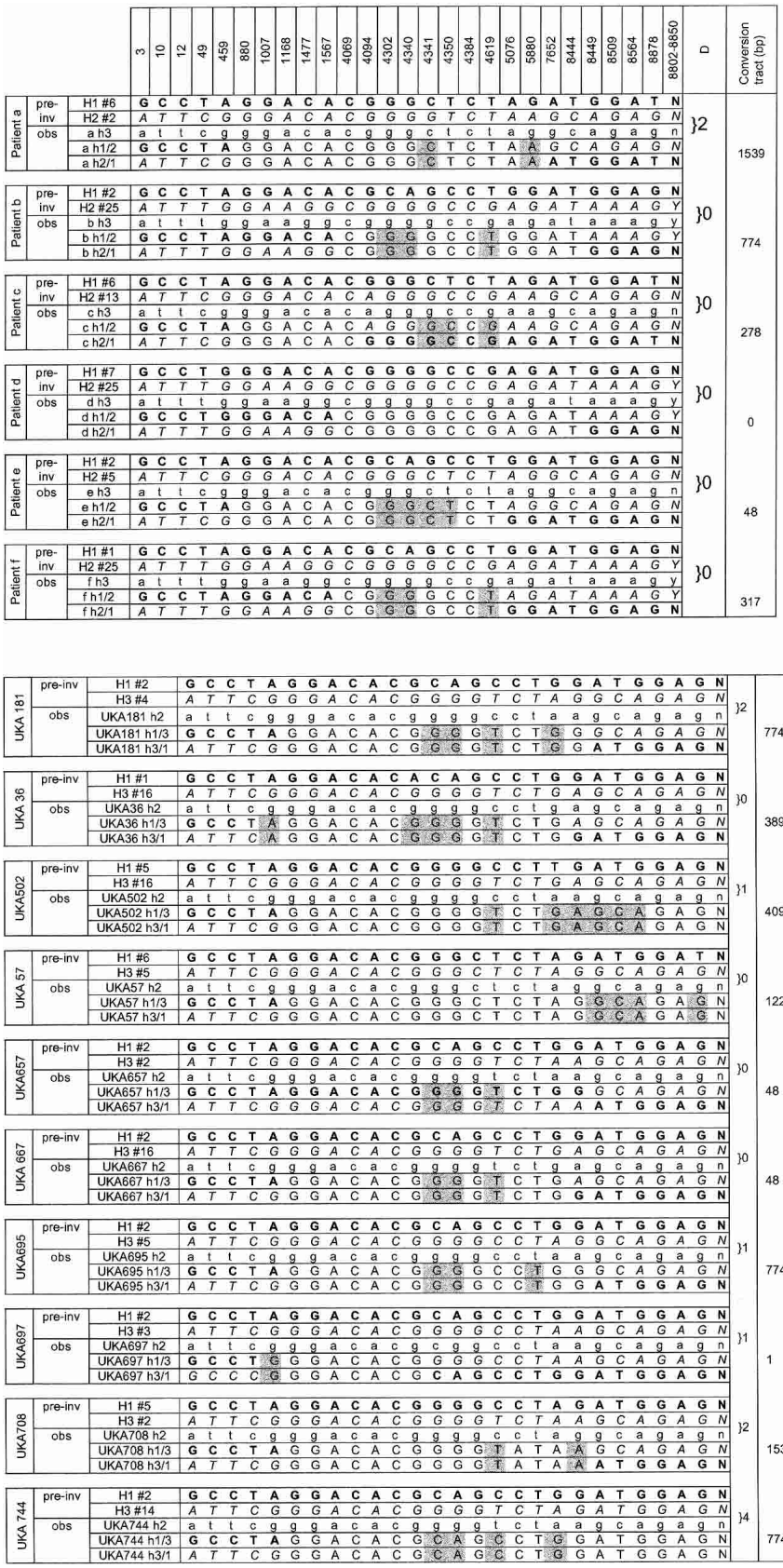
Distal *int22h* human nucleotide number at sites of polymorphism is on the top. Empty spaces indicate that base is identical to that of chimpanzee's sequence or to bases found in the reference human DNA and absent in chimpanzee (italics). Novel alleles are in upper case. Column 8802–8850 shows presence (Y) or absence (N) of polymorphic tandem duplication.

results of the direct analysis of meiotic crossover hotspots in human sperm, which were found also to be active sites of pure gene conversion (Jeffreys and May 2004). However, the concerted evolution of the *int1h* duplicates and of *int22h*-1 relative to the two distal *int22h* sequences is completely unrelated to sequence interactions resulting in recombination because these are expected to yield unviable mutations, namely inversions found to cause severe hemophilia A, if the recombinations are between duplicates on the same DNA molecule, or dicentric and acentric fragments, if recombinations are between duplicates not on the same DNA molecules (i.e., intersister chromatids, interhomologous chromatids, interhomologous chromosomes).

We found that human *int22h*-2 and *int22h*-3 sequences are fivefold more similar to each other when in *cis* than when in *trans* (i.e., on different chromosomes). This is interesting for two reasons. First, this is because it shows that the fivefold excess of inversions caused by recombination of *int22h*-1 with *int22h*-3 relative to those caused by recombination of *int22h*-1 with *int22h*-2 among patients with hemophilia A (Antonarakis et al. 1995) must be explained by factors other than differences in sequence similarity between *int22h* duplicates; for example, the effect of chromatin structure on recombination that has been noted in yeast (Paques and Haber 1999). Second, this is because the above finding suggests that pure gene conversion, at least between *int22h*-2 and *int22h*-3, is more often intrachromosomal than interchromosomal as only the former homogenizes sequences in *cis* without affecting sequences in *trans*.

In fact intrachromosomal gene conversion may help to explain the significant deficit of divergences we observed between human and chimpanzee *int1h* (see Table 2) if we assume that *int1h*-1 and *int1h*-2 are tightly linked in humans and chimpanzee. This assumption is likely to be correct at least in humans, where these sequences are only 140 kb apart. In chimpanzee the distance between the two *int1h* sequences is likely to be similar to that found in humans but firm data are not yet available as gaps remain in the sequence of this region of the chimpanzee's X chromosome. We argue that, in the presence of tight linkage, intrachromosomal gene conversion should favor the retention of the ancestral sequence because mutations duplicated by gene conversion and cosegregating will be lost from the population through genetic drift at a rate similar to that of single mutations. Thus the duplication of an *int1h* mutation by intrachromosomal gene conversion will not adequately compensate the alternative event, resulting in conversion of the mutation back to the ancestral sequence. Clearly if *int1h* in humans and chimpanzees tend to retain the sequence of the common ancestor they should show a reduced rate of interspecies divergence. Of course, interchromosomal gene conversion such as conversion between sequences on homologous chromosomes or chromatids should not contribute to the above effect because the copies of the mutations duplicated by these types of gene conversion do not cosegregate.

Intrachromosomal gene conversion and cosegregation of converted sequences may also explain Rozen et al.'s (2003) ob-



servation that the human–chimpanzee sequence divergence is significantly lower in the arms of palindromes than in other male specific Y chromosome regions. The male specific part of the Y chromosome contains 5.7 Mb of euchromatin, showing an imperfect palindromic structure (Skaletsky et al. 2003). The arms of these palindromes measure from 9 kb to 1.45 Mb and the between arm spacers are 2–170 kb long. The arms have sequence identity greater than 99%, and this is thought to result from gene conversions, which probably occur at a rate of at least 600 nt per Y chromosome per generation and retard the evolutionary decay of testis-specific genes located in the arms of palindromes (Rozen et al. 2003). Thus in the Y chromosome gene conversion acquires an important functional role.

In contrast to the above data on the *int1h* duplicates, which are expected to be fairly tightly linked, and those on the arms of the palindromes of the Y chromosome, which are absolutely linked (Rozen et al. 2003) the *int22h* sequences of humans and chimpanzee show a substitution rate per million years (0.115%) appropriate for noncoding DNA and not significantly different ($P = 0.071$) from that of 4818 bp of sequence flanking *int22h*-1 (base substitution rate = 0.0809% per million years; see Supplemental Fig. 5). As *int22h*-1 is 500 and 600 kb away from *int22h*-2 and *int22h*-3, respectively, recombination between *int22h*-1 and the two distal *int22h* sequences may so reduce cosegregation of regions that experienced intrachromosmal gene conversion as to prevent the bias in favor of the retention

Figure 2. Observed and possible pre-inversion *int22h* haplotypes for patients with *int22h*-related inversions. For each patient a set of proposed pre-inversion haplotypes (pre-inv) are shown. The haplotype of the proposed pre-inversion proximal and distal *int22h* sequence are in bold and italics, respectively, and the haplotype number is indicated (e.g., H1#2 is haplotype 2 of *int22h*-1 and H3#23 is haplotype 23 of distal *int22h*). The observed haplotypes (obs) show the nonrecombined *int22h* in lower case and the recombined haplotypes. The distribution of bold and italic characters in the latter two haplotypes shows how the pre-inversion haplotypes have rearranged. Standard capital letters indicate noninformative sites. Proposed gene conversions are highlighted gray. h1/3 and h1/2 are the recombined sequences at proximal location while h2/1 and h3/1 are those at distal location. Column "D" indicates number of differences between the patient's non-recombined *int22h*-2 or *int22h*-3 sequence and the distal *int22h* haplotype chosen to represent the pre-inversion distal *int22h*. The distance between the first and last allele that requires a gene conversion event to account for the observed recombined sequences is the gene conversion tract (last column).

of the ancestral sequence and hence, the suppression of human–chimpanzee interspecies divergence.

When the first report of gene conversion in higher eukaryotes (Slightom et al. 1980) was followed by many other examples (Liebhaber et al. 1981; Bentley and Rabbitts 1983; Mellor et al. 1983; Michelson and Orkin 1983; Weiss et al. 1983; Hardison and Margot 1984; Stoeckert et al. 1984) Powers and Smithies (1986) asked whether pure gene conversion represented a distinct process from recombination. This question has not yet been fully answered, but as the number of duplicated sequences known to undergo concerted evolution increases, the pervasive effect of gene conversion on the genome becomes clearer.

Here we have shown how pure gene conversion has maintained the identity of two different duplicated sequences of the X chromosome that predispose to inversions breaking the *F8* gene and causing hemophilia A. This gene conversion is of an intensity reminiscent of the palindromic regions of the Y chromosome and thus suggests that gene conversion may be an important common factor in the concerted evolution of intrachromosomally duplicated sequences. Some of our results suggest a prevalence of intrachromosomal gene conversion events and we propose that when these involve closely linked regions they favor the preservation of the ancestral sequence and thus reduce the rate of evolutionary change in the region involved.

Methods

DNA was extracted using standard procedures (Miller et al. 1988) from (1) 10 mL of blood donated (with informed consent) by 19 normal British males and 25 males with hemophilia A (9 with the *int1h* related inversion, 6 with inversions due to recombination of *int22h*-1 with *int22h*-2 and 10 with inversions due to recombination of *int22h*-1 with *int22h*-3); (2) cultured fibroblasts (Coriell Cell Repositories, cell strain GM03452) from a male chimpanzee; (3) brain from a male African Green monkey; and (4) peripheral lymphocytes of a male Rhesus monkey. LLNL human clones U100A9, containing *int22h*-2, and U214E7, containing *int22h*-3, were obtained from the MRC UK HGMP Resource Centre and DNA extracted according to the supplier's protocol.

PCR amplification of *int1h* duplicates and flanking regions was performed as previously described (Bagnall et al. 2002).

Int22h duplicates were PCR amplified from 100 ng of genomic DNA or 10 ng of clone DNA using 1 µL 10× Expand Long PCR polymerase buffer (Roche Diagnostics), 0.5 mM of each dATP, dCTP, and dTTP, 0.25 mM dGTP, 0.25 mM deaza dGTP (Roche Diagnostics), 7.5% DMSO, 50 ng of each oligonucleotide primer, and 1 U Expand Long PCR DNA polymerase (Roche Diagnostics). Ten cycles of PCR were performed (94°C 30 sec, 68°C 12 min) and were immediately followed by 20 further cycles of PCR (94°C 30 sec, 68°C 12 min plus 20 sec per cycle). Primer sequences for amplification of human and chimpanzee *int22h* sequences were as previously described (Liu et al. 1998). African green monkey *int22h*-1 and distal *int22h* segments were amplified using, respectively, primer pairs P (Liu et al. 1998) plus IR and CF (Supplemental Table 3) plus B (Liu et al. 1998).

Allele-specific PCR of distal *int22h* sequences was performed using the products of the PCR directed by primers A and B of Liu et al. (1998). The reactions comprised 1 µL of PCR product, 1 µL 10× Expand Long PCR polymerase buffer, 0.5 mM of each dATP, dCTP, and dTTP, 0.25 mM dGTP, 0.25 mM deaza dGTP, 7.5% DMSO, 50 ng of each oligonucleotide primer, and 1 U Expand Long PCR DNA polymerase. Thirty cycles of PCR were performed (94°C 30 sec, 68°C 12 min). Allele-specific PCR primer sequences are listed in Supplemental Table 3.

Sequencing of *int1h* and *int22h* duplicates and flanking regions was performed according to the manufacturer's instructions using the BigDye v3.1 dye terminator kit (ABI Perkin-Elmer) on 4-µL aliquots of PCR product incubated with 2 µL ExoSAP-It (USB Bioproducts) at 37°C for 15 min followed by heating at 80°C for 15 min. The products of the sequencing reactions were analyzed on an ABI 3100 DNA sequencer. Primers for sequencing *int1h* and *int22h* have been previously described (Naylor et al. 1995; Bagnall et al. 2002), and additional primers required for sequencing the chimpanzee *int22h* copies are shown in Supplemental Table 3.

To analyze variation of the *int22h* sequences among normal males and hemophilia A patients, *int22h*-1 and the distal *int22h* duplicates were amplified. *Int22h* nucleotides 1–1100, 4100–5100, and 8400–9512 were sequenced directly from PCR products as described above whereas nucleotides 1100–4100 and 5100–8400 were analyzed by fluorescent solid phase chemical cleavage of mismatches (FSPCCM) as follows: DNA for FSPCCM was prepared by initially amplifying, with 10 cycles of PCR, *int22h*-1 or both distal duplicates. A 1-µL aliquot of the primary PCR was further amplified using 2.5 µL 10× Amplitaq reaction buffer (Perkin-Elmer), 1.5 mM $MgSO_4$, 200 ng of each nested oligonucleotide primer, 0.5 mM of each dNTP, and 2.5 U Amplitaq DNA polymerase (Perkin-Elmer). Thirty cycles of PCR were performed at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 2 min. The seven primer pairs for amplification of mismatch target sequences are listed in Supplemental Table 3. Identical biotinylated fluorescent mismatch probes were amplified directly from a human clone (U100A9), which contains *int22h*-2, using 25 PCR cycles. Probe sequences were purified from a 1% agarose gel using GeneClean (Bio101) according to the manufacturer's instructions. FSPCCM analysis was performed as previously described (Waseem et al. 1999).

Recombined *int22h*-1, recombined distal *int22h,* and unrecombined *int22h* sequences were specifically amplified from inversion patient DNA using, respectively, primer pairs PB, AQ, and AB (Liu et al. 1998). *Int22h* nucleotides 1–1100, 4100–5100, and 8400–9512 were sequenced directly from PCR products as described above. Constant regions comprising nucleotides 1100–4100 and 5100–8400 were not analyzed in inversion patients.

The presence of gene conversion was tested using the method of Balding et al. (1992) for synonymous sites in codons, as the calculations developed there for fourfold degenerate sites are applicable to introns where a constant rate of mutation across the region and no selection is expected.

Uniformity of the occurrence of co-double sites and other types of sites (doubles, which differ from co-doubles because the divergent bases are not the same in both repeats, and singles, where a divergence occurs in only one of the repeats) was assessed via the Poisson approximation to the binomial distribution. The overall probability of a type (co-double, double, single) was estimated by the observed proportion of that type for the entire region. The region was then divided into smaller regions of length m and the Poisson probability of observing k sites of the given type out of m potential sites was calculated.

Significance of two divergence rates was determined via a Normal test of proportions (implemented in Minitab v14, Minitab Inc.) Confidence intervals for pairwise average differences were obtained by the method of bootstrapping (e.g., Manly, 1997) using 1000 samples.

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