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Amplification and Chromosomal Dispersion of Human Endogenous Retroviral Sequences

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Endogenous retroviral sequences in humans have undergone amplification events involving both viral and flanking cellular sequences. We cloned members of an amplified family of full-length endogenous retroviral sequences. Genomic blotting, employing a flanking cellular DNA probe derived from a member of this family, revealed a similar array of reactive bands in both humans and chimpanzees, indicating that an amplification event involving retroviral and associated cellular DNA sequences occurred before the evolutionary separation of these two primates. Southern analyses of restricted somatic cell hybrid DNA preparations suggested that endogenous retroviral segments are widely dispersed in the human genome and that amplification and dispersion events may be linked.

Human chromosomal DNA contains a number of families of DNA segments which are distantly homologous to endogenous retroviruses isolated from other mammalian species (1, 7, 8, 11, 13, 17). We previously characterized two distinct families of these sequences: a typical full-length retroviral structure which includes retroviral genes present in replication-competent viruses flanked by long terminal repeats (LTRs) (12) and a truncated sequence which retains *gag* and *pol* sequences but not *env* or LTR homologous sequences (17). The truncated sequences are bordered by multiple, tandem direct repeats which are unrelated to LTRs. The sum of these retroviral sequences in the human haploid genome is approximately 35 to 50 copies for each of the two families (17). The number of primary germline proviral DNA integrations is probably far less than would be suggested by this copy number, since many of the endogenous retroviral structures appear to have arisen by a process of DNA amplification in which a large DNA unit, including both the retroviral sequence and the flanking cellular sequences, was amplified. Our evidence for this type of amplification in the class of truncated endogenous retroviral structures is based primarily on two observations. First, screening a genomic library with a cellular DNA probe that immediately abuts the 5′ end of one of the truncated retroviral clones yielded multiple but distinct clones, all containing the same type of shortened retroviral structure (13). Second, the degree of nucleotide sequence homology between respective 5′ or respective 3′ flanking tandem repeats of different clones is greater than that between 5′ and 3′ repeats within the same clone, suggesting that a sequence drift between the 5′ and 3′ repeats occurred, followed by an amplification process (17).

A similar type of amplification occurred that involved the class of full-length human endogenous retroviral sequences; this event was inferred from genomic blotting data which suggested the presence of conserved restriction sites in the DNA flanking certain full-length retroviral DNA segments (17). In this report, we present further evidence for this hypothesis by characterizing cloned members of an amplified family of full-length retroviral sequences and by using a subclone of the conserved flanking DNA as a hybridization probe in Southern blot analyses of human genomic DNA. Studies of restricted chimpanzee DNA reveal a similar array of related retroviral and flanking cellular sequences, indicating that an amplification event occurred before the divergence of hominids from the common ancestor with African apes. The 35 to 50 copies of each retroviral family are shown here to be dispersed to multiple human chromosomes by an analysis of a panel of rodent × human somatic cell hybrids; several of these sequences were chromosomally mapped.

MATERIALS AND METHODS

Derivation of clones. Clones containing full-length endogenous retroviral sequences were initially selected by screening a bacteriophage λ Charon 4A human genomic DNA library (5) with an internal 1.0-kilobase (kb) *Eco*RI fragment (Fig. 1, probe C) derived from a cloned, truncated human endogenous retroviral segment designated 51-1 (13). Probe c in Fig. 1 contains sequences homologous to the *pol* gene of Moloney murine leukemia virus (13). Because this probe hybridizes to both full-length and truncated families of human retroviral sequences (17), secondary screening of the initial group of positive clones was carried out with a human endogenous LTR probe (specific for the full-length family of sequences [12]). Restriction mapping of new clones was carried out with previously defined subgenomic human endogenous retroviral probes (12) under standard (stringent) hybridization conditions (4, 16). A 450-base pair (bp) *Bam*HI-*Eco*RI fragment containing 3′ flanking cellular sequences was subcloned from phage λ21-1. Its map position is indicated in Fig. 3.

Genomic blotting and somatic cell hybrids. Preparation of human and chimpanzee DNAs was carried out as previously described (2). Electrophoresis of 5- to 10-μg samples of restricted cellular DNA was performed through 0.6% agarose gels. Nick-translated probes with specific activities of 1×10^8 to 3×10^8 cpm/μg were used in Southern blot hybridizations as previously outlined (4, 16).

Somatic cell hybrids were derived by polyethylene glycol-mediated fusion of fresh human lymphocytes to rodent cells (mouse RAG or Chinese hamster E36) that lack the hypo-

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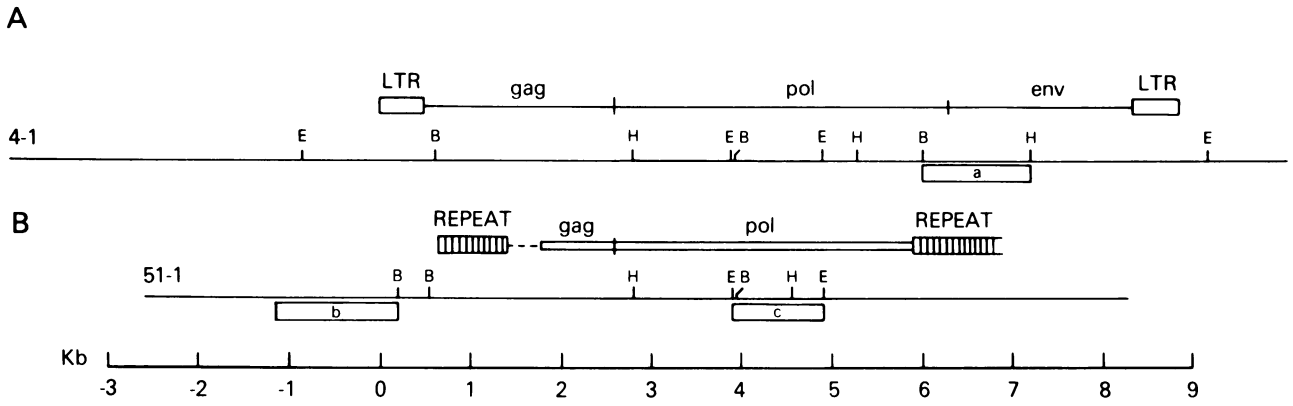

FIG. 1. Schematic representation of full-length (A) and truncated (B) retroviral sequences. A *pol-env* probe (a) from clone 4-1 is specific for full-length sequences. A 5′ flanking probe (b), derived from clone 51-1, specifically hybridizes to the truncated retroviral sequences. A *pol* probe (c), also isolated from clone 51-1, reacts with members of both retroviral families. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

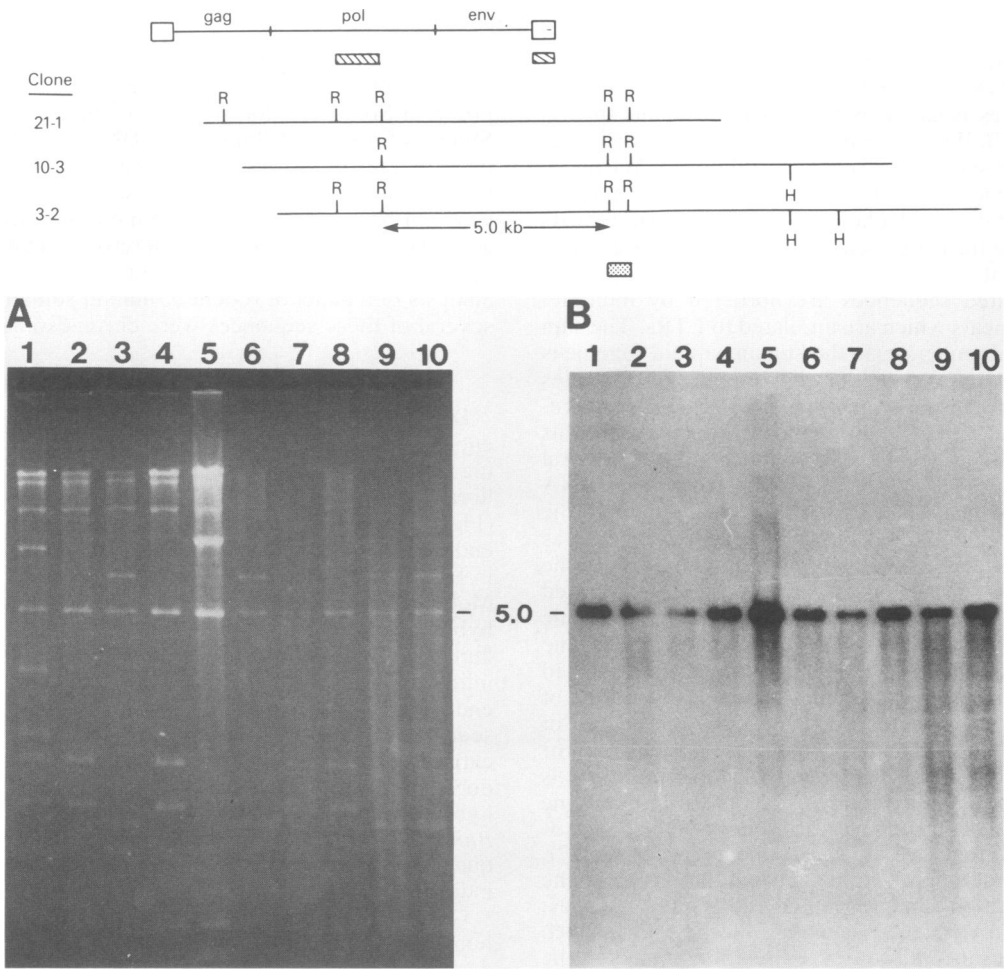

FIG. 2. Digestions of amplified human endogenous retroviral segments. (Top) Three clones isolated from a human genomic library and containing sequences characteristic of the full-length retroviral family are restriction mapped and aligned both with one another and with a schematic drawing showing the locations of the *pol* (▨) and LTR (▧) probes (12) used for their isolation. Note the presence of conserved *Eco*RI sites in the 3′ viral flanking sequence defining the conserved 5.0-kb viral-cell junction and the 400-bp cellular flanking *Eco*RI fragment (▩). Abbreviations: R, EcoRI; H, *Hin*dIII. (The complete *Hin*dIII map is shown in Fig. 3). (Bottom) Samples (0.3 μg) of 10 independent lambda phage clones were digested with *Eco*RI (A), electrophoresed through 0.6% agarose gels, and stained with ethidum bromide (0.5 μg/ml). Clones 21-1, 10-3, and 3-2 are shown in lanes 3, 4, and 6, respectively. DNA from the gel shown in panel A was then transferred to a nitrocellulose membrane and hybridized to the LTR probe shown at the top (B). The conserved 5.0-kb junction fragment is indicated.

xanthine guanine phosphoribosyltransferase gene, thus allowing selection in hypoxanthine-aminopterin-thymidine medium. All hybrids were derived from a single human female (8). A panel of 60 hybrid cell lines was selected based on the high-frequency retention of low numbers of different human chromosomes (8, 9). Hybrids were genetically characterized by G-trypsin banding, G-11 staining, and enzyme typing of 20 to 28 isozyme markers previously assigned to human chromosomes at the same passage from which high-molecular-weight DNA was extracted (8–10). Genomic DNA was digested with *Kpn*I, electrophoresed through 0.4% agarose gels, and subjected to Southern analysis (16) with probes specific for the full-length retroviral family (*pol-env*), the truncated family (5′ flanking DNA), and both families (*pol*). The probes were designated a, b, and c, respectively, in Fig. 1.

RESULTS

Cloning and characterization of a family of amplified full-length human endogenous retroviral sequences. Human chromosomal DNA contains both full-length and truncated endogenous retroviral sequences (17). After the isolation of a few full-length retroviral clones (12), a partial *Alu*I-*Hae*III human gene library in phage λ Charon 4A was successively screened with a 1.0-kb *pol* probe (Fig. 1, probe C) and a 550-bp LTR probe (12) to obtain several additional representatives of the 8.8-kb human endogenous retroviral family. Ten of the newly isolated full-length clones contained a common 5.0-kb *Eco*RI fragment which hybridized to the LTR probe (Fig. 2B) and which extended 1.3 kb into the 3′ flanking cellular DNA. On further inspection, five of these new clones, although containing similar restriction sites in the 3′ flanking DNA, such as a pair of *Eco*RI sites that defined the 400-bp fragment shown in Fig. 2, were distinct from each other as shown by scattered restriction site polymorphisms. The initial results suggest that, like some of the class of truncated endogenous retroviral segments, some members of the full-length human retroviral family were also amplified and physically associated with similar flanking cellular DNA sequences.

This interpretation was verified by using a cloned, 450-bp *Bam*HI-*Eco*RI segment of 3′ flanking DNA as a hybridization probe in Southern blots of genomic DNA (Fig. 3). This 3′ flanking DNA probe hybridized to the 5.35-kb *Bam*HI and the 6.35- or 8.25-kb *Hin*dIII restriction fragments which span the 3′ viral-cell DNA junction in the cloned members of the putative amplified family (Fig. 3). If many members of this amplified family exist in human DNA, then Southern blotting of genomic DNA cleaved by *Bam*HI or *Hin*dIII should clearly reveal bands of the same size as those seen in the cloned DNA. The intensity of these bands would be increased in proportion to the copy number of endogenous retroviral sequences which are members of this particular amplified family and thus contain the predicted restriction fragments. In fact, the human genome did contain the expected intensely reactive *Bam*HI and *Hin*dIII fragments (Fig. 3, lane 1).

Results of experiments recently done in our laboratory (Rabson et al., manuscript in preparation) indicate that the full-length endogenous retroviral segments, all of which contain a distinctive tRNA glutamic acid primer-binding site (14, 17), are present in many nonhuman higher primates. We therefore thought it would be of interest to examine these primate DNAs for the presence of intensely reactive (i.e., amplified) cleavage fragments capable of hybridizing to the

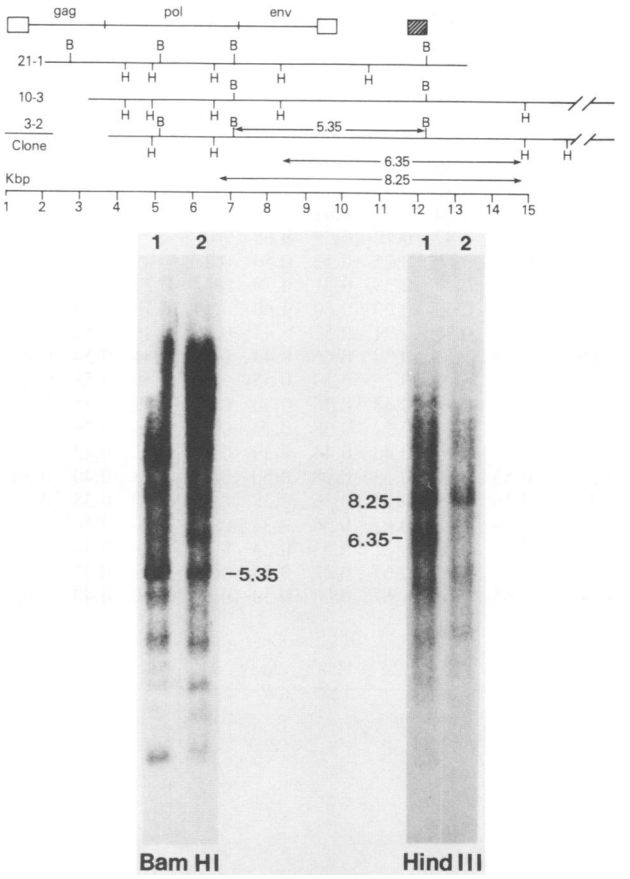

FIG. 3. Amplification of similar retroviral DNA segments in humans and chimpanzees. (Top) The *Hin*dIII and *Bam*HI maps of three human retroviral clones are aligned, and the map position of a 450-bp *Bam*HI-*Eco*RI 3′ flanking cellular DNA segment (▨), subcloned from λ21-1 DNA, is indicated. The locations of the 5.35-kb *Bam*HI and the 6.35- and 8.25-kb *Hin*dIII junction fragments are also shown. Abbreviations: B, *Bam*HI; H, *Hin*dIII. (Bottom) Autoradiograms of Southern blots of human (lane 1) and chimpanzee (lane 2) DNAs, cleaved with *Bam*HI and *Hin*dIII.

450-bp *Bam*HI-*Eco*RI cellular DNA probe. Our results indicate that restricted chimpanzee DNA contained the 5.35-kb *Bam*HI and 8.25-kb *Hin*dIII fragments predicted from the restriction maps of the human endogenous retroviral clones which contain conserved viral and 3′ flanking cellular sequences (Fig. 3, lane 2). A smear of hybridization in both species suggests many other closely related sequences exist. A similar experiment conducted with cleaved African green monkey DNA failed to generate intensely reactive bands (data not shown). The results therefore suggest that a class of endogenous retroviral sequences is shared by humans and chimpanzees and that similar retroviral flanking DNA units were amplified in both primate families. Whether these units (of as yet undetermined size) are tandemly organized is not known, although we have not encountered such an arrangement within a single phage clone (average insert size, 15 kb).

Chromosomal mapping with somatic cell hybrids. The chromosomal organization of truncated and full-length retroviral families in the human genome was examined by a Southern analysis of DNAs prepared from a panel of 40 rodent × human somatic cell hybrids that segregate human chromosomes in different combinations (8, 9). Cellular DNAs, prepared from the hybrids, were digested with *Kpn*I,

TABLE 1. Frequency of discordant hybrids between 21 human endogenous retroviral sequences

Retroviral sequence	T1	T2	T3	T4	T5	T6	T8	T9	T10	T13	T15	T17	T18	T20	T21	F3	F4	F5	F6	F9	F21
T1	—																				
T2	0.44	—																			
T3	0.44	0.39	—																		
T4	0.71	0.50	0.61	—																	
T5	0.41	0.24	0.55	0.41	—																
T6	0.28	0.47	0.17	0.55	0.68	—															
T8	0.44	0.50	0.25	0.52	0.50	0.21	—														
T9	0.58	0.47	0.33	0.41	0.39	0.44	0.22	—													
T10	0.44	0.33	0.00	0.60	0.60	0.14	0.18	0.33	—												
T13	0.44	0.17	0.21	0.57	0.41	0.30	0.46	0.54	0.23	—											
T15	0.39	0.56	0.38	0.46	0.44	0.39	0.46	0.54	0.41	0.42	—										
T17	0.39	0.44	0.25	0.59	0.55	0.26	0.50	0.58	0.27	0.29	0.13	—									
T18	0.28	0.39	0.48	0.38	0.30	0.38	0.42	0.55	0.46	0.26	0.35	0.48	—								
T20	0.59	0.27	0.52	0.38	0.15	0.73	0.50	0.26	0.55	0.39	0.64	0.68	0.35	—							
T21	0.35	0.53	0.42	0.44	0.39	0.35	0.42	0.42	0.43	0.46	0.09	0.22	0.30	0.61	—						
F3	0.55	0.60	0.53	0.08	0.60	0.38	0.38	0.40	0.50	0.69	0.25	0.38	0.43	0.64	0.29	—					
F4	0.36	0.30	0.73	0.36	0.29	0.60	0.50	0.38	0.77	0.40	0.64	0.79	0.14	0.20	0.50	0.29	—				
F5	0.46	0.10	0.62	0.36	0.33	0.57	0.50	0.50	0.55	0.29	0.71	0.71	0.23	0.23	0.71	0.31	0.12	—			
F6	0.31	0.07	0.22	0.53	0.24	0.28	0.28	0.44	0.19	0.17	0.59	0.41	0.33	0.38	0.63	0.56	0.41	0.20	—		
F9	0.39	0.39	0.67	0.21	0.25	0.57	0.47	0.35	0.69	0.38	0.47	0.64	0.07	0.24	0.27	0.23	0.05	0.18	0.41	—	
F21	0.65	0.62	0.67	0.06	0.50	0.61	0.47	0.44	0.63	0.72	0.56	0.72	0.41	0.44	0.56	0.13	0.38	0.28	0.52	0.26	—
No. of hybrids	19	18	24	24	24	24	24	24	23	24	24	24	24	23	24	22	21	21	24	25	

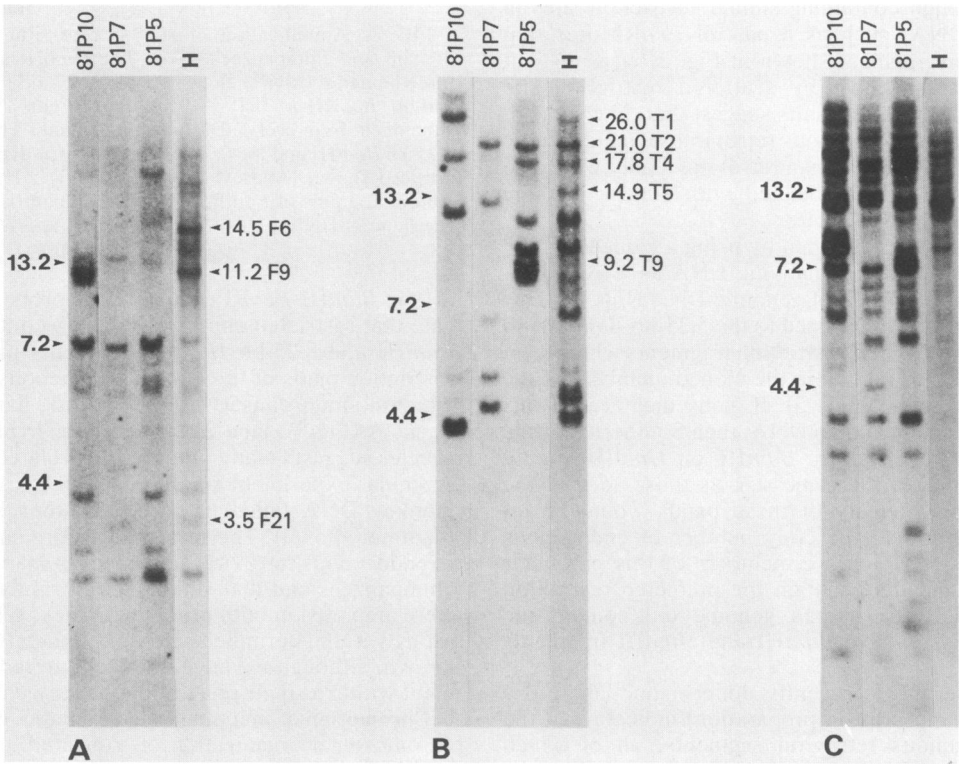

FIG. 4. Representative Southern blot hybridizations of restricted rodent × human somatic cell hybrid DNAs. DNAs (20 to 40 μg) from somatic cell hybrids were digested with *Kpn*I and hybridized to probes a, b, or c of Fig. 1 after electrophoresis and transfer to nitrocellulose membranes, as shown in panels A, B, and C, respectively. A sample (10 μg) of the parental human leukocyte DNA was also digested with *Kpn*I and analyzed in lane H of each panel. Molecular sizes are in kilobases. Bands in panels A and B that were assigned a chromosomal location are indicated by the designations in Tables 1 and 2.

TABLE 2. Assignment of retroviral restriction fragments to human chromosomes

Human chromosome	% Discordancy with retroviral restriction fragments							
	FRV1 (F6)	FRV2 (F9)	FRV3 (F21)	TRV1 (T1)	TRV2 (T2)	TRV3 (T4)	TRV4 (T5)	TRV5 (T9)
1	55	27	38	50	25	38	20	48
2	44	24	21	53	29	23	14	39
3	27	14	42	32	24	46	[9][a]	38
4	36	24	62	33	33	40	26	35
5	46	26	26	44	25	33	27	48
6	55	10	44	41	38	32	23	35
7	41	23	25	44	38	33	19	44
8	48	[0]	39	39	38	41	24	43
9	39	14	42	33	24	45	18	24
10	32	14	29	32	35	40	15	48
11	[13]	50	64	50	[6]	55	26	54
12	52	26	[9]	69	47	[4]	36	33
13	48	28	32	50	33	37	37	[9]
14	40	20	52	[11]	41	68	40	46
15	46	27	44	47	35	35	39	25
16	52	38	36	53	35	38	33	42
17	48	14	26	47	25	30	14	36
18	38	17	42	47	25	40	30	52
19	48	19	36	44	19	32	13	27
20	48	32	52	42	44	48	33	30
21	33	33	35	33	41	46	25	54
22	48	30	48	35	44	55	25	32
X	41	60	71	44	61	57	68	54

[a] Boxed numbers indicate assignment of the restriction fragment to the respective chromosome.

an enzyme which does not cut or cuts the retroviral sequence a single time (13, 14, 17). After transfer to nitrocellulose membranes, the restricted DNAs were hybridized (Fig. 4) with the following segments: (i) a *pol-env* segment (Fig. 1, probe a) specific for the full-length family (panel A), (ii) a 5′ flanking cellular DNA segment (a 1.4-kb *Taq*I-*Bam*HI fragment; Fig. 1, probe b) specific for the truncated family (panel B), and (iii) a *pol* fragment (Fig. 1, probe c) common to both human retroviral families (panel C). Restriction fragments were scored as present or absent in at least 18 hybrids (Table 1). Each restriction fragment was compared with the others by calculating a frequency of discordance. This frequency is equal to the number of hybrids which contain one but not the other restriction fragment divided by the number of hybrids which contain both or neither of the fragments. Integrations located on the same chromosome would be expected to have a frequency of 0.0. Data on eight of the bands shown in Table 1 are shown in Table 2. The percent discordancy, defined as the sum of the hybrids which contain either a particular chromosome or a particular restriction fragment but not both, divided by the total number of hybrids scored for the presence of the fragment, multiplied by 100, is shown (Table 2). Low percentages for one chromosome allowed the assignment of a restriction fragment to that chromosome, as indicated by the boxes in Table 2.

The *pol-env* probe reacted with 27 bands in human DNA, designated F1 through F27 in descending order of size (Fig. 4A), six of which were well-delineated in several of the hybrid cell DNAs. Most of the *pol-env* reactive bands segregated in different combinations in the somatic cell hybrids, indicating dispersal to multiple human chromosomes (Table 1). Three of these full-length retroviral segments, designated FRV in Table 2, could be assigned to human chromosomes based on concordance of the fragment and the human chromosome scores (Table 2). These segments were FRV1 (F6, 14.5 kb) which was assigned to chromosome 11, FRV2 (F9, 11.2 kb) which was assigned to chromosome 8, and FRV3 (F21, 3.5 kb) which was assigned to chromosome 12.

The detection of truncated human retroviral family members with the 5′ flanking cellular DNA probe gave a different banding pattern (Fig. 4B). A total of 23 reactive fragments, designated T1 through T23 in descending order of size, were detected, 15 of which were readily delineated. With few exceptions, such as T3 and T10, the discordance of these sequences with each other, as well as with the FRV segments, was high, indicating that they were located on a number of different human chromosomes (Table 1). Five of the truncated human retroviral sequences, designated TRV in Table 2, could be tentatively assigned to human chromosomes (Table 2). These segments were TRV1 (T1, 26.0 kb), assigned to chromosome 14; TRV2 (T2, 21.0 kb), assigned to chromosome 11; TRV3 (T4, 17.8 kb), assigned to chromosome 12; TRV4 (T5, 14.9 kb), assigned to chromosome 3; and TRV5 (T9, 9.2 kb), assigned to chromosome 13.

The 1.0-kb *pol* probe (Fig. 1, probe c) hybridized to at least 37 different *Kpn*I fragments, and these were dispersed in different combinations in the hybrids as well (Fig. 4C). We conclude that the two families of endogenous retroviruses are probably not tandemly arranged in the human genome; rather, they appear to be dispersed, retaining common flanking cellular DNA sequences, to multiple chromosomal locations.

DISCUSSION

The finding that human endogenous retroviral sequences and associated flanking cellular DNA were amplified is similar to the situation observed with endogenous feline leukemia virus sequences, in which restriction sites in flanking cellular DNA are also conserved (15). The mechanism responsible for amplification of endogenous feline leukemia virus segments in the cat genome is unknown. We previously reported the existence of virtually no restriction enzyme polymorphisms involving the numerous endogenous retroviral sequences when several different preparations of human DNA were subjected to Southern analyses (17). The finding of this study, therefore, suggests that continued amplification or rearrangement of the endogenous human retroviral sequences is not presently occurring. Because a similar array of amplified DNA is also present in the chimpanzee genome (Fig. 3), the amplification event(s) we investigated must have occurred prior to the evolutionary divergence of humans and chimpanzees.

The endogenous retroviral sequences are located on several different human chromosomes, as deduced from Southern blot analyses of somatic cell hybrid DNAs. In these analyses, a no-cut or one-cut restriction enzyme such as *Kpn*I was used to generate junction fragments containing both viral and cellular sequences. We were, therefore, surprised to detect multiple, comigrating, intense bands in some of the Southern blots (e.g., Fig. 4C) that were greater than 10 kb in size. The pattern (except for size) was reminiscent of band recruitment after digestion with an internal cutter. The similar electrophoretic mobilities of these *Kpn*I cleavage products precluded the unambiguous mapping of a majority of the reactive bands, thereby effectively minimizing evidence for the dispersal of retroviral segments to many loci in the human genome. Dispersal of these sequences among different human chromosomes could

therefore be more common than we were able to demonstrate. If so, the frequent occurrence of these intensely hybridizing bands which apparently are both amplified and dispersed would support a model linking amplification of proviruses to the dispersion process. This idea is supported by the findings of Hillova et al. (3) which show that recently integrated Rous sarcoma proviral DNA, as well as flanking cellular DNA, underwent both amplification and dispersion to different chromosomes in Chinese hamster cells.

Additional classes of human endogenous retroviral sequences have been reported by other researchers (1, 6, 8, 11). The single copy type C endogenous proviruses, ERV1 and ERV3, have been mapped to chromosome 18 (8) and chromosome 7 (11), respectively. Human retroviral sequences related to retroviruses types B and D have been identified and are present at approximately 50 copies per cell (1). An abundant family of repetitive sequences containing LTR elements, RTVL-H, was initially identified in the β-globin gene cluster and has been estimated to be present at 800 to 1,000 copies per cell (6). It will be of interest to determine if amplification and dispersion of integrated proviruses of these latter two families contribute to their higher copy number.

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