Isolation of Novel Human Retrovirus-Related Sequences by Hybridization to Synthetic Oligonucleotides Complementary to the tRNA^Pro Primer-Binding Site

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Synthetic oligonucleotides complementary to putative retroviral primer-binding sites were used as hybridization probes to detect novel retroviruslike sequences. An 8.1-kilobase element with structural features of a retroviral provirus was isolated from a human genomic library by this approach. Nucleotide sequence analysis of its 600-base-pair long terminal repeats revealed characteristic motifs known as regulatory signals for RNA polymerase II transcription: CCAAT, TATA, and ATTAAA. In addition, a putative *pol* gene displays apparent homologies to conserved regions of retroviral reverse transcriptase. The 5' long terminal repeat is flanked at its 3' end by a putative primer-binding site for reverse transcription with homology to tRNA^Pro. This element is therefore termed HuRRS-P (human retrovirus-related sequence–proline). There are 20 to 40 copies of HuRRS-P homologous sequences in DNAs of human and simian origin.

The chromosomal DNA of eucaryotes harbors several families of retroviral proviruses and retrovirus-related sequences (34). It was shown recently that transposable elements of yeasts transpose via a reverse transcriptase-mediated mechanism (3). These elements, called retrotransposons (1), might constitute a link between retroviruses and transposable elements, and retroviruses can be seen as a class of retrotransposons with an additional extracellular phase. Whether transposons were ancestors of retroviruses or vice versa remains to be shown (30). Both transposons and retroviruses are probably the major source of mutagenic activity in the genome of lower eucaryotes (27). The insertion of a mobile element may result in an activation or inactivation of cellular genes. Examples in vertebrates are the activation of the c-*onc* genes by an insertion of avian leukosis virus or an intracisternal A particle element or the inactivation of the α-collagen gene owing to an integration of a Moloney leukemia virus (9, 11, 13). In view of their possible pathogenic effects, there were many attempts to identify retrotransposons in human DNA. There were two incidental findings in flanking sequences of other genes (14, 15); the only other approach used to search for such sequences was that of DNA hybridization of known retroviral sequences to human DNA. Owing to sequence conservation of some parts of the retroviral genome, low-stringency hybridization led to the isolation of new retrovirus-related sequences from human genomic libraries (4, 5, 17–20, 29). In this paper we describe a successful application of a novel approach which overcomes the apparent limitations of the previous method.

Retroviruses and retrotransposons use tRNA molecules as primers for reverse transcription (34). A 18- to 23-base-pair (bp) segment located downstream of a 5' long terminal repeat (LTR) constitutes the primer-binding site complementary to 3' terminal nucleotides of a tRNA molecule. Several tRNA species were found to serve as primers, but a given species is characteristic for each virus. We used synthetic oligonucleotides homologous to several tRNA primer-binding sites to screen a human genomic library. The isolation and char-

acterization of new human retrotransposonlike sequences are reported here.

MATERIALS AND METHODS

Synthetic oligonucleotides and DNA clones. Oligonucleotides were synthesized as 22-mers by the method of Beaucage et al. (2). Their sequences were derived from known mammalian tRNAs (28). A cloned Moloney murine sarcoma virus DNA was kindly provided by G. Vande Woude. The human genomic library cloned in lambda EMBL3 and the human genomic library cloned in cosmid pcos2EMBL vectors were generous gifts of H. Lehrach and A. Frischauf (8, 21). We are indebted to K. Dörries for the gift of human DNAs of different tissues. High-molecular-weight DNAs of simian origin were prepared from different cell lines by standard laboratory methods. African green monkey DNA was isolated from Vero cells. MLA 144, a gibbon lymphoma cell line, and 1670, a marmoset cell line, were kindly provided by B. Fleckenstein. ZUG-4, a cell line derived from baboons, was a gift of H. Wolf.

Hybridization and screening methods. Radioactive labeling of the DNA probes, filter transfer, and hybridizations were carried out by the methods described by Maniatis et al. (16). After hybridization with labeled oligonucleotides, filters were washed in 6× SSC–0.05% sodium PP$_i$ (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The temperature was adjusted to a level between 50 and 65°C, dependent on the stringency and the oligonucleotide used. Filters hybridized with nick-translated probes were washed several times in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C, 0.2× SSC–0.1% sodium dodecyl sulfate at 60°C, and 1× SSC–0.1% sodium dodecyl sulfate at 60°C for high-, medium-, and low-stringency conditions, respectively. Positive lambda or cosmid clones were plaque or colony purified at least twice, characterized by digestion with restriction enzymes, and subcloned in pUC12 vectors.

DNA sequencing. DNA fragments were subcloned in M13mp18 or M13mp19 vectors and sequenced by the M13 dideoxy method described by Sanger et al. (23). The sequences presented were determined on both strands and with overlapping clones. Computer-assisted comparisons of

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        LTR                      PBS
5'  [T G...      ...C A] T T T G G G G G C T C G T C C G G G A T   3'  MoMSV

         3'  [g t a a a c c] c c g a g c a g g c c c t a    5'  Pro

         3'  [g t a a a c c] a t c g t c t c c t a c c a a  5'  Met

         3'  [g t a a a c c] a c a g t c t t c a c c c t a  5'  Leu
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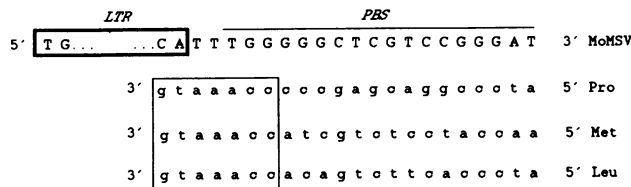
FIG. 1. Design of oligonucleotides used as hybridization probes. The top row shows the LTR (bold box) and the primer-binding site (*PBS*) of Moloney murine sarcoma virus (MoMSV). Lower-case letters in the following rows indicate the sequences of different synthetic oligonucleotides: Pro, murine tRNA^{Pro}-derived sequences; Met, human tRNA^{Met}-derived sequences; Leu, human tRNA^{Leu}-derived sequences. The boxed nucleotides are common to all probes used and are not a part of the tRNA genes.

nucleotide sequences were performed with the University of Wisconsin Genetics Computer Group software and the EMBL data base, release 8.

RESULTS

Strategy of the design of hybridization probes. Possible homologies of tRNAs to putative primer-binding sites were exploited to search for new retrotransposonlike sequences. Our choice of specific tRNA-derived sequences as probes was directed by the primer usage of known human retroviruses: tRNAs specific for Ile, His, Arg, Lys, and Glu for endogenous retroviruses (14, 15, 18, 19, 29) and Pro or Lys for HTLVI/II and the human immunodeficiency virus, respectively (34). Three 22-mer oligonucleotides with homology to putative primer-binding sites of tRNA^{Pro}, tRNA^{Leu} and tRNA^{Met} were prepared, because these tRNAs are not known to be used by endogenous human retroviruses. All of them start with the sequence 3'-GTAAACC. . .5' (Fig. 1). The GT corresponds to CA, the LTR-terminal nucleotides of the majority of transposable elements and retroviruses. The following two nucleotides, AA, correspond to TT, which in many cases border the 5' LTRs of retroviruses (34). The ACC sequence represents the 3' terminus of all tRNA molecules, which is not encoded in tRNA genes, but is added posttranscriptionally. The complement of ACC is a part of the retroviral primer-binding sites. It means that of the 22 nucleotides of the synthetic probe, only 15 nucleotides are homologous to a tRNA gene. This difference was used to adjust the stringency of hybridization to avoid a cross-hybridization with tRNA genes and tRNA-like repetitive sequences in the genome (7). The tRNA^{Pro}-homologous 22-mer oligonucleotide is identical to the primer-binding site and its 5'-end-adjacent nucleotides of the Moloney murine sarcoma virus. Therefore, medium- or low-stringency hybridization conditions for this oligonucleotide were determined experimentally by using a cloned Moloney murine sarcoma virus DNA (see Materials and Methods).

Screening of a human genomic library with oligonucleotide probes. By screening a representative human genomic library in 600,000 lambda phages with radioactively labeled tRNA^{Pro}-derived oligonucleotide, seven positive signals were obtained under low-stringency hybridization conditions; eight positive clones were obtained with the tRNA^{Leu}-derived oligonucleotide under similar conditions. No hybridization signals were detected with the tRNA^{Met}-derived probe. Only the tRNA^{Pro}-specific clones were further characterized. Six of the seven clones were eliminated by using medium-stringency conditions during the hybridization procedures. The resulting clone PλV1 was mapped and subcloned

in pUC vectors. A 1-kilobase (kb) region surrounding the putative primer-binding site was sequenced by the M13 dideoxy method. The sequence shows a 18/22 match to the tRNA^{Pro} specific oligonucleotide, containing a perfect primer-binding site. We found features characteristic of LTR sequences within the upstream region. In detail, there are two CCAAT boxes, a TATA box, and an ATTAAA box in the correct order and distance (33). Although clone PλV1 contains about 13 kb of human sequences downstream of this 5' LTR-like sequence, we did not detect another LTR in the 3' direction by hybridization experiments. Therefore, PλV1 probably represents a truncated member of endogenous retroviruslike elements. To find a full-length element, we screened a human cosmid library with a probe derived from LTR sequences of the clone PλV1.

Isolation of HuRRS-P. A 540-bp PstI-HinfI fragment of PλV1, containing part of the LTR and 3' flanking sequences, served as a radioactively labeled probe to screen a human genomic library in 150,000 cosmids. A total of 22 positive clones were isolated. The cosmids were cut with PstI, the enzyme which generates the 5' end of the LTR probe, transferred to nitrocellulose, and hybridized with the same probe. Of these 22 cosmids, 5 clones contain two LTR-hybridizing restriction fragments, indicating a possible full-length proviruslike structure. The clone cos29 was further analyzed by restriction mapping. Figure 2 shows an 8.1-kb elementlike structure with two direct repeats separated by a 6.9-kb stretch of internal sequence. The element was termed HuRRS-P (Human retrovirus-related sequence–proline).

Nucleotide sequence analysis of HuRRS-P. Nucleotide sequence analysis of HuRRS-P was performed with parts of the clone cos29, containing the 5' and 3' LTRs and surrounding sequences (Fig. 3). The assumption that the 8.1-kb element originated from an integration event was confirmed by the finding of two direct repeats, ATACC in front of the 5' LTR and ATACT following the 3' LTR. One mismatch within these target site duplications is probably due to a point mutation. At 631 bp in length, the 5' LTR is 7 bp longer than the 3' LTR; the nucleotide sequence homology between these two LTRs and the PλV1 LTR is about 87%. Further characteristic features include (i) short inverted repeats, TGA. . ./. . .TCA, bordering the two LTRs; (ii) motifs known as promoter sequences of RNA polymerase II, the CCAAT box and the TATA box at positions 373 and 423, respectively; and (iii) a putative polyadenylation signal, ATTAAA, at position 531. ATTAAA is a variation of the AATAAA that is present in about 12% of natural mRNA precursors (35). The CCAAT box, as well as the TATA box, is usually found in a distinct order and at a distinct distance in relation to the guanosine residue at the mRNA cap site. These distances, which are reported to be 70 to 80 bp and 20 to 30 bp, respectively, upstream of the transcription initiation site, parallel perfectly the situation in HuRRS-P. The appropriate position of the cap site would be the guanosine residue at nucleotide 446. This site, together with the 5' end of the LTR, defines a U3 region of 445 bp. The cap site itself represents the start of the R region, whose end is defined by the dinucleotide CA 20 to 30 bp downstream of the polyadenylation signal (33). This dinucleotide is found at position 555; the R region is therefore 110 bp in length. The remaining LTR sequences constitute U5 region of about 76 bp. The lengths of U3, R, and U5 regions are in accord with reported dimensions of other retroviral LTRs (6).

In addition to the features listed above, which are shared by HuRRS-P and other retroviral sequences, we found the expected primer-binding site adjacent to the 5' LTR. Despite
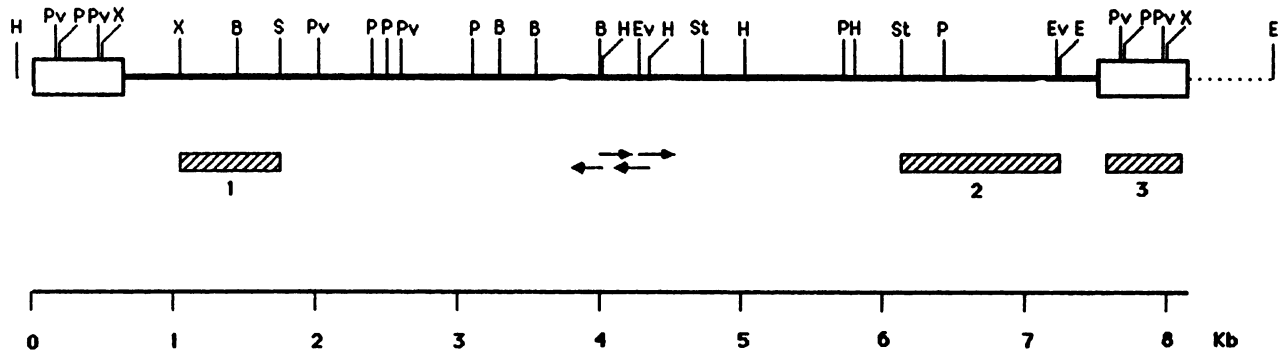
FIG. 2. Restriction map of HuRRS-P. Symbols: ▭, LTRs, ▨ (boxes 1 and 2), positions of fragments used as putative *gag* and *env* probes; ☐ (box 3), *Hin*fI-*Bst*NI fragment used as LTR probe (cleavage sites not shown). The arrows correspond to a sequenced region of putative *pol* origin. Abbreviations: B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I; St, *Stu*I; X, *Xba*I.

two mismatches and a 1-bp insertion, the primer-binding site proved to be most closely related to the complement of the murine tRNA^{Pro}, when compared with all known tRNA sequences (28). To ascertain that the sequences between the two putative LTRs are indeed retrovirus-related genes, we sequenced part of the internal region (Fig. 2) and compared the deduced amino acid sequence with that of other retroviruses. The best homology (44%) was found between HuRRS-P and the Moloney leukemia virus *pol* gene (Fig. 4). The homology is even better (62%) if conservative exchanges are taken into account. The homologous regions belong to highly conserved *pol* sequences previously described (31). The sequences of the LTRs and the length and the restriction map of the isolated element, as well as the

usage of a tRNA^{Pro} primer-binding site, clearly distinguish HuRRS-P from recently published human sequences of retroviral origin. Computer-assisted comparisons of HuRRS-P LTR sequences and the retroviruslike sequences isolated by Steele et al. (29), Bonner et al. (4), O'Connell and Cohen (18), Paulson et al. (20), Mager and Henthorn (15), Ono (19) and Maeda (14) reveal no apparent homologies. A family of 300 human elements related to baboon endogenous retrovirus isolated by Noda et al. (17) is not sequence characterized. This type of elements is distinguishable from HuRRS-P by copy number, LTR length, or restriction map. Callahan et al. (5) described another family of human endogenous retroviruses with homology to mouse mammary tumor virus, which was distinguished from mammalian type C viruses and HuRRS-P by comparison of *pol* sequence data.

Organization of HuRRS-P in the human genome and copy number. To examine the organization of HuRRS-P in the human genome and to estimate the copy number, we performed Southern blot hybridizations (Fig. 5). High-molecular-weight human DNA was digested with restriction enzymes and hybridized to three different probes derived from the LTR (lanes A to C), putative *gag* (lane D), and putative *env* (lane E) regions of the HuRRS-P element. To allow a more quantitative evaluation of the copy number, a known LTR fragment equivalent to 1 and 5 copies, respectively, was included. Under medium-stringency hybridization conditions, 10 to 20 bands were counted with the
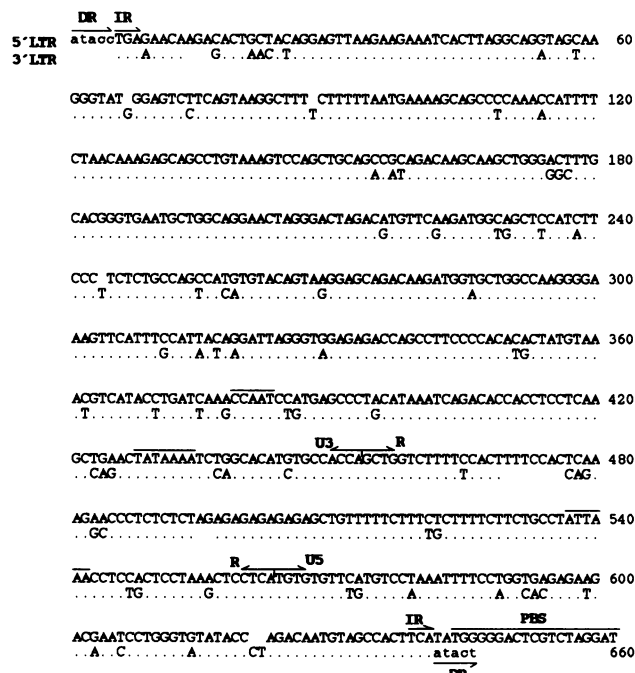
FIG. 3. Nucleotide sequence comparison of HuRRS-P LTRs. Identical nucleotides in both LTRs are marked with dots, different nucleotides are indicated, and deletions appear as gaps. Sequence motifs involved in transcriptional regulation are overlined. Abbreviations: U3, U5, and R, borders of unique regions 3 and 5 and repetitive region, respectively; DR, direct repeats; IR, inverted repeats; PBS, primer-binding site.
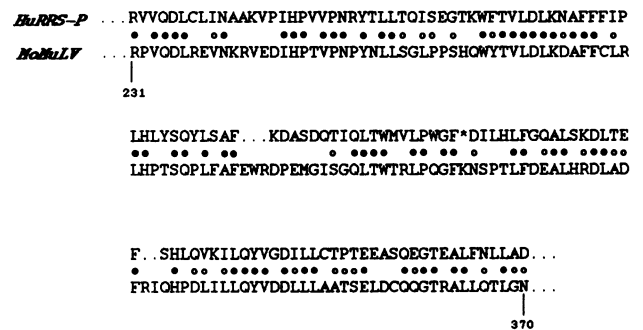
FIG. 4. Alignment of amino acid sequences of HuRRS-P and Moloney murine leukemia virus (MoMuLV) polymerase. The position of HuRRS-P sequences in the entire element is indicated in Fig. 2. Numbering of Moloney murine leukemia virus amino acids corresponds to the first residue of the polymerase-coding region. Symbols: ● and ○, identical and chemically similar amino acids, respectively (26).
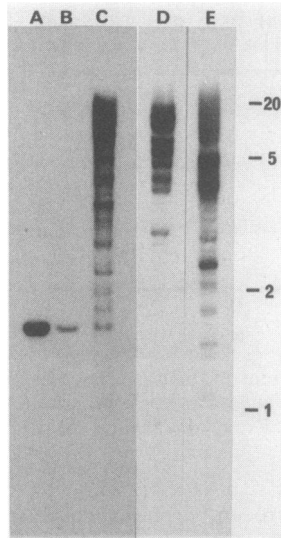
FIG. 5. HuRRS-P homologous sequences in the human genome. Portions (20 μg) of *Eco*RI-digested salmon sperm DNA mixed with 50 pg (equivalent to five copies per haploid genome) (lane A) or 10 pg (equivalent to one copy per haploid genome) (lane B) of the 1.5-kb *Eco*RI fragment containing the 3' LTR of HuRRS-P and 20-μg portions of human DNA digested with *Eco*RI (lanes C and D) or *Stu*I (lane E) were separated on a 0.9% agarose gel, blotted, and hybridized under medium-stringency conditions (see Materials and Methods) with the LTR probe (lanes A to C), the *gag* probe (lane D), or the *env* probe (lane E). The fragments used as probes are defined in Fig. 2. The autoradiograph is shown after a 1-day exposure. Size markers (in kilobase pairs) are indicated.

internal probes. The LTR probe detected slightly more bands. The fact that there are shorter fragments than expected from the restriction map is probably due to truncation or restriction site variation of different elements.

HuRRS-P homologous sequences in other species. The existence of homologous sequences in other hominoid or simian species allows us to estimate the appearance of this sequence in the primate evolutionary tree. A Southern blot with *Eco*RI-digested DNAs of human, gibbon, baboon, african green monkey, and marmoset origin was hybridized with the LTR probe. The autoradiograph in Fig. 6 shows positive hybridizing signals with the human LTR probe under medium-stringency hybridization conditions even in the distantly related marmoset DNA. This result indicates that HuRRS-P was already present in the genome of an ancestor of old- and new-world monkeys. Even under low-stringency hybridization conditions, HuRRS-P-related sequences could not be detected in murine DNA (data not shown).

DISCUSSION

Screening of a human genomic library with synthetic oligonucleotides complementary to tRNA^{Pro} led to the identification of a novel element termed HuRRS-P. The main advantage of this novel approach over commonly used hybridization to the conserved regions of already known retroviruses is the potential to discover entirely novel types of elements with no homology to other retroviruses.

Several features justify the classification of HuRRS-P as a retroviruslike element. It has an overall structure of a retroviral provirus with LTRs bounded by 5-bp target site duplications. The LTRs contain characteristic motifs known

as regulatory signals for the RNA polymerase II transcription. The structural preservation of these canonical sequences suggests that they might still be functionally active. The existence of *pol* homology as revealed from the amino acid sequence is another strong evidence for retroviral origin of HuRRS-P.

Several tRNA species have been implicated as primers for the reverse transcription (34). Our choice of a tRNA^{Pro}-derived hybridization probe was directed by the fact that tRNA^{Pro} is the primer of well characterized mouse type C retroviruses and a putative primer of the mouse retrotransposonlike sequences VL30 and murine retrovirus-related sequences (10, 25). Thus the usage of a tRNA^{Pro} by HuRRS-P is of interest. Despite the previous use of type C virus probes, no endogenous human retrovirus with a tRNA^{Pro}-specific primer-binding site was reported (34). Further analysis should clarify possible relationships between HuRRS-P and type C viruses.

The estimation of the copy number of HuRRS-P must be treated with some reservations. Retrovirus and retrovirus-like sequences recombine with a high frequency. The existence of such mixed families in the mouse genome has been reported (12, 22, 24). Thus the estimated copy number might be misleading if a part of a new element used as a hybridization probe is also a part of another sequence family. As an attempt to overcome this problem, three different segments of HuRRS-P were used as probes to calculate the copy number. This number is consistent with the frequency of positively hybridizing signals in the cosmid library.

The existence of HuRRS-P homologous sequences in the DNA of all simian species tested suggests that a progenitor virus of these sequences entered the germ line of a distant human ancestor at least 45×10^6 years ago. It is likely that, as for a majority of other acquired retroviruslike sequences,
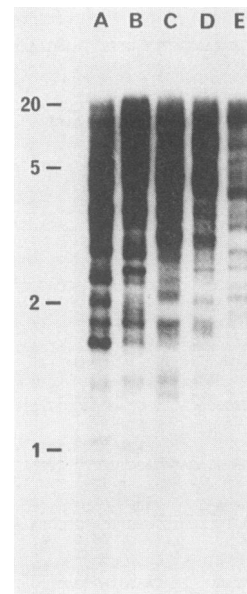

FIG. 6. Detection of HuRRS-P homologous sequences in monkey DNAs. A Southern blot containing 20 μg of *Eco*RI-digested DNAs of human (lane A), gibbon (lane B), baboon (lane C), African green monkey (lane D), and marmoset (lane E) origin was hybridized under medium-stringency conditions (see Materials and Methods) with the LTR-specific probe (Fig. 2). The autoradiograph is shown after a 2-day exposure. Size markers (in kilobase pairs) are indicated.

HuRRS-P underwent multiple alterations that might prevent their expression as infectious viruses. This does not necessarily mean that some parts of their genome could not be expressed; they could even evolve to functional cellular genes. A precedent could be the recently described homology between the intracisternal A particle element major protein and immunoglobulin E-binding factor (32).

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