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S71 Is a Phylogenetically Distinct Human Endogenous Retroviral Element with Structural and Sequence Homology to Simian Sarcoma Virus (SSV)

THOMAS WERNER,*,[1] RUTH BRACK-WERNER,† CHRISTINE LEIB-MÖSCH,‡ HORST BACKHAUS,§ VOLKER ERFLE,† AND RÜDIGER HEHLMANN¶

*GSF-Institut für Säugetiergenetik, †GSF-Abteilung für Molekulare Zellpathologie, and §GSF-Institut für Strahlenbiologie, Ingolstädter Landstrasse 1, D-8042 Neuherberg, ‡Medizinische Poliklinik der Universität, D-8000 München, and ¶III. Medizinische Klinik Mannheim der Universität Heidelberg, 6800 Mannheim, Federal Republic of Germany

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Human endogenous retroviral element S71 had previously been shown to contain gag- and pol-related regions and a 3′ LTR-like sequence. The nucleotide sequence of S71 was determined and compared with the corresponding regions of SSV and its helper virus SSAV. The 1.48-kb S71 gag region consists of matrix protein p15 (MA)-, capsid protein p30 (CA)-, and nucleocapsid protein p10 (NC)-related sections and the 1.82-kb pol region of tether, RNase H (RH), and endonuclease/integrase (IN) sections. The S71 nucleotide sequence contains a 167 amino acid open reading frame encompassing MA. The boundaries of the S71 element are delimited by direct repeats and the entire element is 5.4 kb long. Similarity between S71 and the v-sis-bearing, defective SSV provirus also covers overall structural organization, including the presence of presumably nonretroviral sequences. Both the gag and the pol regions of S71 contain sequences highly conserved in numerous retroviruses. Phylogenetic analysis with conserved CA, RH, and IN sequences showed that of all other (C-type) human retroviral elements available for comparison, S71 is most closely related to infectious primate and murine retroviruses. This suggests that S71 represents a phylogenetic subgroup of its own. In addition we identified short ranges of conserved amino acid sequences within C-type retroviral gag and pol genes sufficient for phylogenetic analysis. Use of these may facilitate large-scale phylogenetic evaluation of C-type retroviral elements and allow rapid classification of new elements.

INTRODUCTION

About 10% of the human genome is thought to have arisen by reverse transcriptase-mediated processes. Sequence information generated in this manner has been given the general term retroposon (Temin, 1985). Retroposons constitute a relatively large, heterogeneous group which can be divided into nonviral and viral sequences (Weiner *et al.*, 1986). The group of nonviral sequences is composed of LINES (long interspersed nuclear sequences; for review, see Skowronski and Singer, 1986), SINES (short interspersed nuclear sequences), processed genes, and pseudogenes. The group of viral retroposons consists of DNA sequences related to infectious retroviruses. On the basis of copy number and length, one can calculate that these retroviral elements make up at least 0.1% of the human genome. Although some of these elements show extensive structural similarity to full-length infectious animal proviruses, the presence of these elements is not the result of a singular somatic infectious process limited to one generation and to a specific cell type. Rather, they are an endogenous part of the human genome and are transmitted from one generation to the next in a stable Mendelian fashion. Sequence similarity with C-type mammalian retroviruses on the one hand and A-, B-, and D-type retroviruses on the other allows division of human endogenous retroviral elements into two groups designated class I and class II, respectively (Callahan, 1988).

Retroviral polymerase genes consist of at least three regions coding for separate activities: the reverse transcriptase (RT), RH, and IN. The approximate location of these sections within the polymerase gene has been mapped with the help of bacterial expression clones containing various regions of retroviral polymerase genes and subsequent testing for retrovirus-specific activities (Hansen *et al.*, 1988; Hizi and Hughes, 1988; Levin *et al.*, 1988). These experimental data agree with previous computer analysis predicting the structural organization of retroviral polymerase genes (Johnson *et al.*, 1986). The major region of homology between various retroviruses is located within their pol genes (Chiu *et al.*, 1984). Multiple alignments of retroviral pol amino acid sequences show that the RNase H domain and the endonuclease region of retroviral pol genes contain highly conserved sequences which may be essential for their functionality (Johnson *et al.*, 1986; Doolittle *et al.*, 1989). Therefore sequences from these sections of the pol gene should be suitable for phylogenetic analysis of class I elements by comparison with each other as well with C-type animal retroviruses. As-

[1] To whom requests for reprints should be addressed.

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suming that these results reflect actual evolutionary relationships, one would expect congruent data from highly conserved sequences located in other C-type retroviral genes. Since numerous class I retroviral elements also contain gag-related regions (Brack–Werner *et al.*, 1989a,b), we decided to look for the presence of highly conserved sequences suitable for phylogenetic analysis in C-type retroviral gag genes.

Molecular clone S71 contains a class I element isolated by low stringency hybridization with recombinant probes derived from the C-type simian sarcoma-associated virus (SSAV). Hybridization analysis with SSAV probes and limited sequence data showed that S71 is an incomplete proviral element consisting of gag–pol-related sequences (Leib-Mösch *et al.*, 1986). At its 3′ terminus it contains a 535-bp sequence with features characteristic of retroviral LTRs (Brack–Werner *et al.*, 1989a). The putative control regions of the S71 LTR-like sequence show a higher degree of sequence similarity to infectious murine and primate proviral LTRs than to other human endogenous retroviral LTRs. The detailed structural analysis of S71 reported here shows that numerous other features of C-type retroviruses are also conserved in the S71 element. To determine whether our initial observation of closer relationship of S71 to primate murine and infectious proviruses is reflected by the complete S71 element we decided to utilize conserved sequences of the gag and pol regions for phylogenetic analysis. Since, in many cases, only fragmentary sequence data are available, phylogenetic analysis of endogenous class I elements was carried out with short conserved sequence stretches (40–50 amino acids) and compared with longer sequence stretches (150–230 amino acids). Our results indicate that S71 is more closely related to the infectious murine and primate proviruses AKV, BaEV, and SSV than to the other class I human endogenous retroviral sequences ERV1, ERV3, 4-1. Therefore S71 may represent a phylogenetically distinct subgroup of class I endogenous retroviral elements.

MATERIALS AND METHODS

Nomenclature

Retroviral proteins are designated using the nomenclature proposed by Leis *et al.* (1988). Subregions of proteins are referred to by an additional lower case letter in parenthesis (e.g., CA(s), CA short region).

Oligonucleotide preparation

Specific sequencing primers (oligonucleotides) were synthesized on an Applied Biosystems Model 381 A DNA synthesizer and purified either by electrophoresis in 14% polyacrylamide gels containing 8 mol/liter urea and subsequent desalting as specified in Brack–Werner *et al.* (1989a) or with OPC cartridges (Applied Biosystems, England).

DNA sequence analysis

S71 subclones were generated by cloning restricted S71 DNA fragments in pUC vectors (Vieira and Messing, 1982). Dideoxy sequencing of double-stranded plasmid DNA was carried out as described by Chen and Seeburg (1985) using either Klenow fragment (Pharmacia, Sweden) or the modified T7 DNA polymerase (Tabor and Richardson, 1987) Sequenase (United States Biochemical Corporation). Both strands were sequenced as shown in Fig. 1.

Computer alignments and construction of phylogenetic trees

Dot matrix analysis (Fig. 3) was carried out with MacGene Plus software (Applied Genetic Technology, Inc.) on a Macintosh Plus computer. Sequence alignments were performed with the GENALIGN program (GENALIGN is a copyrighted software product of IntelliGenetics, Inc.; the program was developed by H. Martinez of the University of California at San Francisco) and the program package for phylogenetic analysis provided by R. F. Doolittle (Feng and Doolittle, 1987; McClure *et al.*, 1988). The phylogenetic trees shown in Fig. 6 were obtained with the latter program package since it provides a full analysis. Sequences were taken from the EMBL database release 10. The particular versions of sequences employed are human endogenous retroviral elements 4-1 (Repaske *et al.*, 1985), ERV3 (O'Connell *et al.*, 1984), and ERV1 (Bonner *et al.*, 1982); baboon endogenous virus (BaEV; Kato *et al.*, 1987), SSV (Devare *et al.*, 1983), SSAV (Brack–Werner *et al.*, 1989c), AKV (Etzerodt *et al.*, 1984), human immunodeficiency virus (HIV-1; Ratner *et al.*, 1985), mouse mammary tumor virus (MMTV; Moore *et al.*, 1987), squirrel monkey virus (SMRV; Chiu *et al.*, 1984), Rous sarcoma virus (RSV; Schwartz *et al.*, 1983), and COPIA (Mount and Rubin, 1985). The sequence of the chimpanzee endogenous virus is to our knowledge published only in the database (accession No. K02915).

RESULTS

S71 sequences related to the SSV gag gene

The gag gene of SSV codes for a 65-kDa gag precursor protein ($pr65^{gag}$) which is processed to yield MA, $p12^{gag}$, CA, and NC (Teich, 1984). Within the gag gene the sequences coding for these proteins are arranged 5′-MA-$p12^{gag}$-CA-NC-3′ (Devare *et al.*, 1983). In the S71 element sequences related to the SSV gag encoding gene extend from position 498 to position 1975 (Fig.

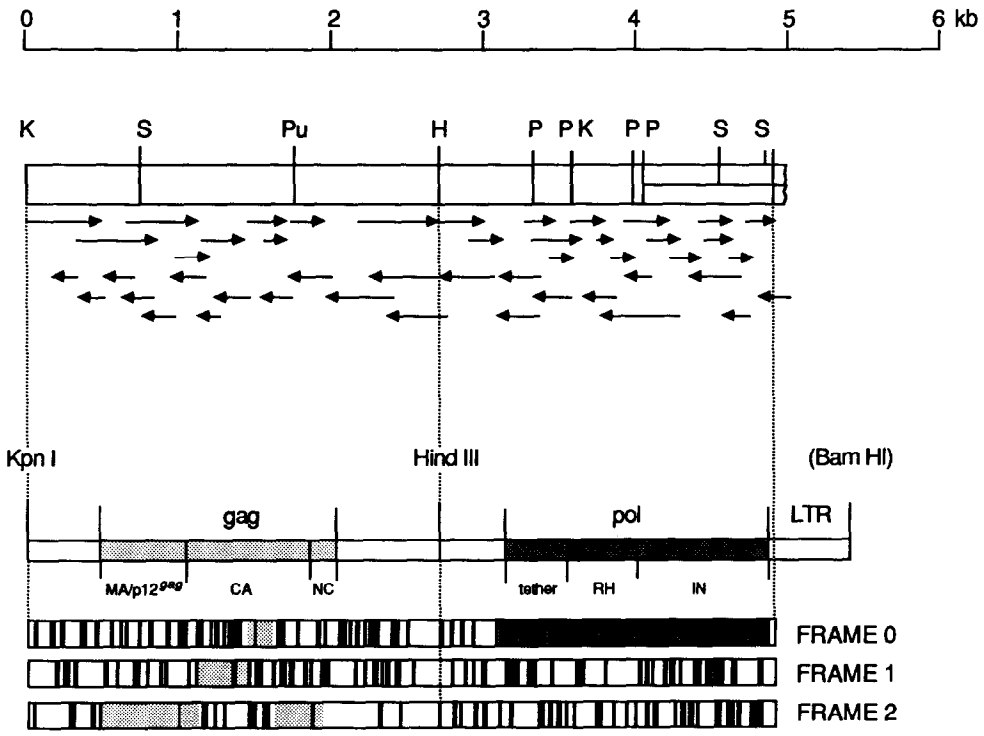


FIG. 1. Nucleotide sequencing strategy of S71 and organization of retrovirus related sequences. A physical map marking the positions of restriction sites relevant in the generation of S71 subclones is depicted at the top. H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pu, *Pvu*II; S, *Sac*I. Arrows indicate the extent and direction of nucleotide sequences obtained from one or more sequencing experiments with an individual subclone of S71. Parallel arrows covering the same sequence range indicate sequencing runs carried out with various overlapping subclones. The range of retrovirus-related sequences in S71 was determined by alignment with the corresponding SSV/SSAV sequences (see text). Vertical solid lines mark the boundaries of MA/p12^{gag}, CA, and NC sections of the gag region and the tether, RH, and IN sections of the pol region. Stippled regions in the three reading frames deduced from the S71 nucleotide sequence indicate the relative location of SSV gag (light stippled) and SSV/SSAV pol (dark stippled) amino acid sequences. The positions of stop codons in each frame are marked by vertical lines.

2). Translation of this region of S71 shows that the deduced amino acid sequences contain extended regions of similarity with the SSV MA, CA, and NC sections and less similarity in the p12^{gag} corresponding region (Figs. 2 and 3). The largely uninterrupted diagonal in the computer-generated dot matrix analysis indicates extensive collinearity of both sequences.

The SSV gag-related amino acid sequences in S71 are dispersed among various reading frames (Fig. 1), indicating that multiple frame shifts preclude the synthesis of the full-length gag precursor protein. However, a possible open reading frame extending from position 498 to 1004 (168 AA), marks the beginning of the S71 gag-related region and encompasses the entire MA-related section (Fig. 2). This reading frame begins with a methionine codon which matches in 7 out of 9 positions with the consensus sequence CC A/G CCAUG(G) established by Kozak (1984).

Oroszlan *et al.* (1977, 1981) found the NH_2-terminal region of the major internal virion protein of C-type retroviruses (CA) to contain the following highly conserved sequence: PLR(X)$_{7-15}$YWPFS(X)SDLYNWK. The deduced amino acid sequence of S71 between positions 1041 and 1135 is identical to the above conserved sequence in 11 out of 15 positions (67%, underlined above), indicating that this region of S71 constitutes the 5′ terminus of the CA-related sequences. The largely collinear alignment of the S71 CA region with the corresponding CA region of SSV (Fig. 3) locates the 3′ terminus of S71 CA-related sequences around position 1804 (Fig. 2). Sequences related to the NC coding region of the SSV gag gene extend from position 1805 to 1975 in S71. The amino acid sequences of C-type retroviral NC proteins contain a highly conserved, cystein-rich structure implicated in nucleic acid binding (Covey, 1986; Fütterer and Hohn, 1987). In the S71 NC this $CX_2CX_4HX_4C$ nucleic acid binding motif is fully conserved (positions 1913–1954).

The deduced amino acid sequence of the S71 gag region shows an overall identity of about 40% with the amino acid sequence of the SSV gag gene (underlined amino acids in Fig. 2). From the computer-generated dot matrix analysis it is evident that similarity is lowest in the p12^{gag} region (Fig. 3), which is also reflected in the relatively large number of gaps obtained in a Needleman–Wunsch alignment of these sequences (data

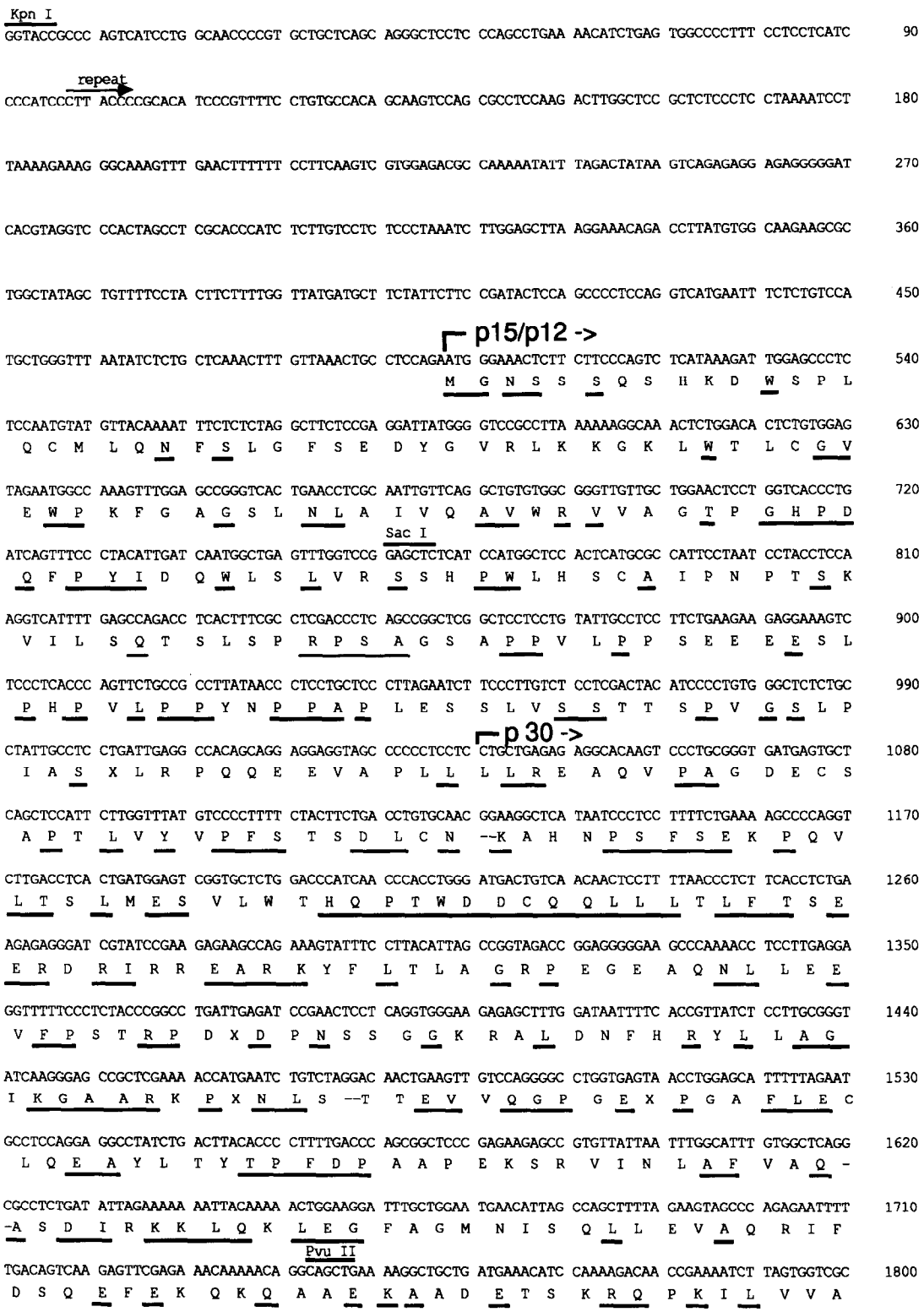
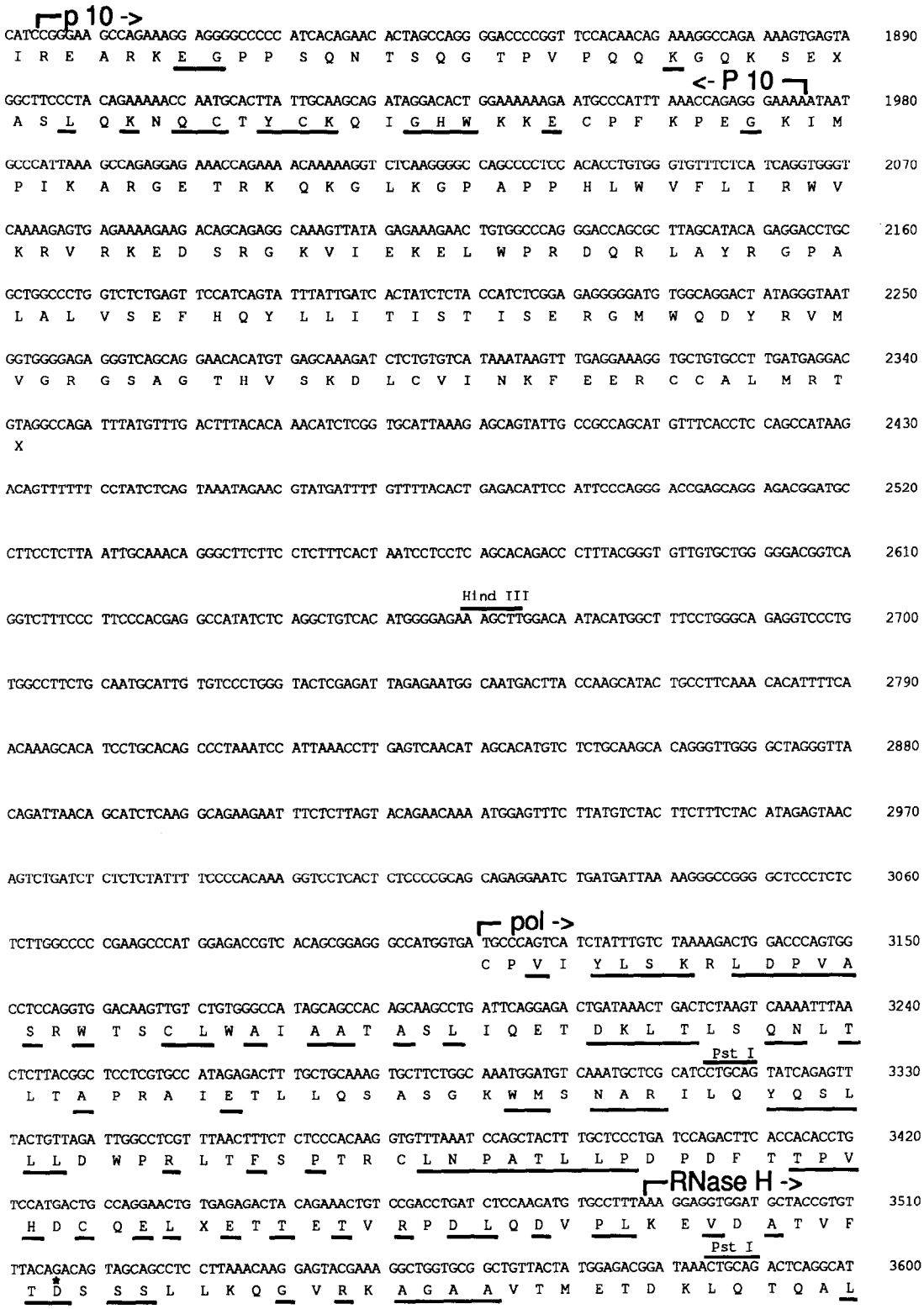


FIG. 2. Nucleotide sequence of human retroviral element S71. The relative positions of restriction sites shown in the schematic in Fig. 1 are indicated. The S71 retroviral element is delimited by direct 7-bp repeats (arrows). The sequence of the 3′ LTR-like region extending from position 4975 to 5509 has been published previously (Brack–Werner *et al.*, 1989b). The S71 nucleotide sequence was translated and retrovirus-related amino acid sequences were identified by a Needleman-Wunsch alignment with SSV/SSAV and AKV protein sequences. The SSV/SSAV-related deduced amino acid sequence of S71 is depicted below the nucleotide sequence and amino acid residues in common with SSV/SSAV are underlined. Interruption of underlining between adjacent amino acid residues marks gaps in the S71 amino acid sequence required for maximum similarity with the SSV/SSAV protein sequence. Termination codons are marked with an X and dashes indicate frame shifts. The borders of the MA, CA, and NC gag regions were inferred from alignment with the gag gene of SSV. Additional confirmation of the 5′ border of the S71 CA



region was obtained by the presence of a sequence highly conserved in the NH_2 terminal region of C-type retroviral gag proteins (see Results). Assignment of the S71 pol amino acid sequences to the tether, RH, and IN sections of the polymerase gene is based on the data of Johnson *et al.* (1986). In the RH region, 11 highly conserved amino acid residues (Levin *et al.*, 1988) and their relative locations in the S71 sequence are marked with asterisks. In the IN region, asterisks mark the relative locations of conserved amino acid residues identified by Johnson *et al.* (1986).

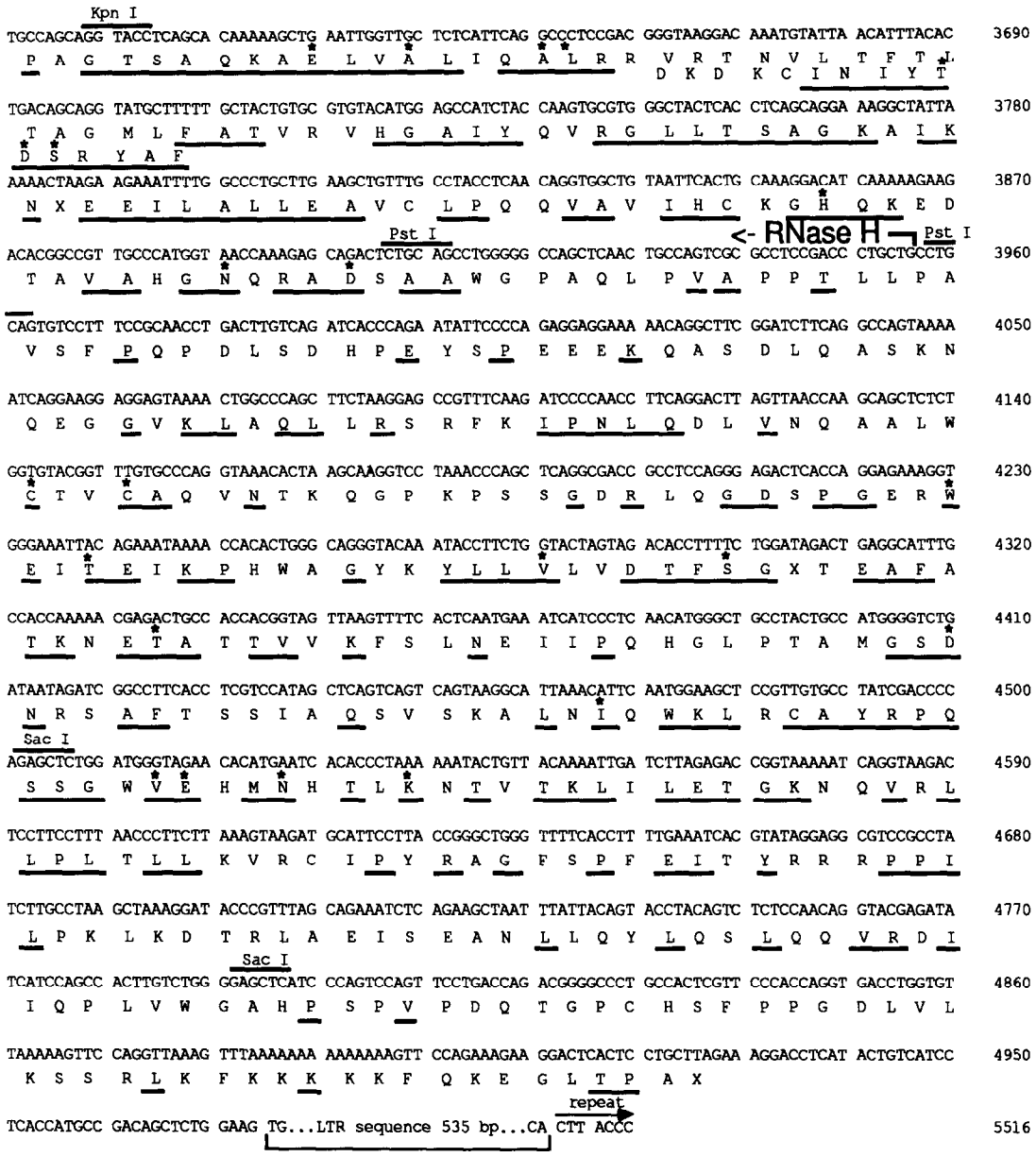


FIG. 2—*Continued*

not shown). Within the S71 gag region, the CA sequences show an amino acid identity of 47% (119/253 amino acids), indicating that this region is most highly conserved.

S71 sequences related to the SSAV pol gene

We had previously identified a 1.2-kb SSAV pol probe which yields a relatively strong hybridization signal with S71 (Leib-Mösch *et al.*, 1986). Sequence analysis of this fragment and part of the adjacent (3′) fragment procuring an overlap with the published SSV sequence was carried out (Fig. 4; Brack-Werner *et al.*, 1989c). This allowed generation of a 2205 nucleotide SSAV/SSV composite pol sequence structured 5′-tether-RH-IN-3′ (Fig. 4) for comparison with the S71 pol region.

In the S71 element a section approximately 1800 nucleotides in length (Fig. 2, position 3111–4928) shows extended similarity with the SSV/SSAV sequence in dot-matrix analysis (Fig. 3). Under the conditions chosen for this analysis (see legend to Fig. 3) similarity between both sequences continues through several stretches in a collinear fashion and seems to taper off towards the end of both sequences. S71 sequences between nucleotide 1975 and 3111 or downstream of nucleotide 4928 showed no similarity to the SSV/SSAV

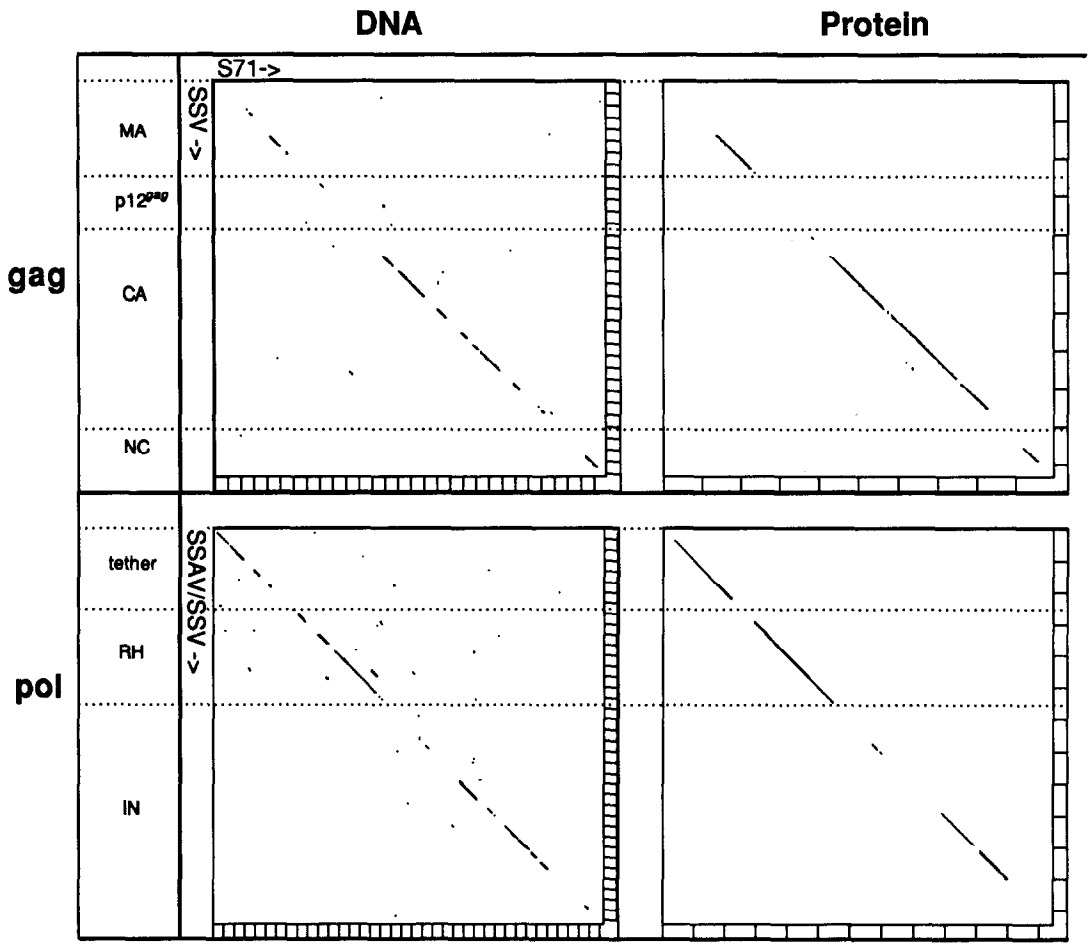


FIG. 3. Comparison of the DNA and deduced amino acid sequences of the S71 gag and pol regions with the corresponding SSAV/SSV sequences. The diagonal lines shows the extent of similarity and collinearity of the S71 gag and pol regions with the SSV gag gene and the composite SSAV/SSV pol sequence. No similarity was observed beyond these regions. Dots in both protein and DNA sequence comparisons signify a minimal match of 56% in a window size of 20 residues. gag region: *X* axis, S71 nucleotide position 498–1978 (DNA); 491 amino acid residues (protein). *Y* axis, SSV nucleotide positions 1398–2936 (Devare *et al.*, 1983); 513 amino acid residues. pol region: *X* axis, S71 nucleotide position 3111–4928 (DNA); 606 amino acid residues (protein). *Y* axis, SSAV/SSV composite pol sequence (Brack-Werner *et al.*, 1989c) nucleotide position 239–2044; 602 amino acid residues (encompasses SSV nucleotide position 2903–3785; 294 amino acid residues).

sequence or any other retroviral polymerase gene available for comparison.

Organization of S71 pol sequences

The deduced amino acid sequence of the S71 pol region showing a high degree of similarity with the SSAV/SSV pol protein sequence is contained largely in one frame interrupted by three termination codons (Fig. 1). Dot matrix comparison of both protein sequences indicates a relatively high degree of similarity (Fig. 3), and the overall amino acid identity is about 46%. Comparisons of the overall S71 polymerase sequences with human endogenous provirus 4-1 (Repaske *et al.*, 1985) and with AKV (Etzerodt *et al.*, 1984) were also carried out (data not shown), confirming our previous results of sequence similarity between all three sequences (Leib-Mösch *et al.*, 1986).

Computer analysis of various retroviral polymerase genes indicates that these are composed of five sections organized 5′–PR–RT–tether–RH–IN–3′ (Johnson *et al.*, 1986). By comparison with polymerase genes from other retroviruses such as AKV, the SSAV/SSV pol sequence can be deduced to contain the tether–RH–IN sections (Fig. 4). The high degree of similarity between the SSAV/SSV and S71 amino acid sequences and collinearity of both sequences over more than 600 amino acids indicate that the S71 pol sequences are also organized 5′–tether–RH–IN–3′. The S71 pol sequence ends about 30 nucleotides upstream of the beginning of the envelope reading frame in SSV (Devare *et al.*, 1983).

The deduced amino acid sequence of the S71 tether section (Fig. 2 positions 3111–3489) is 126 amino acids in length and of these 66 (52%) are identical to

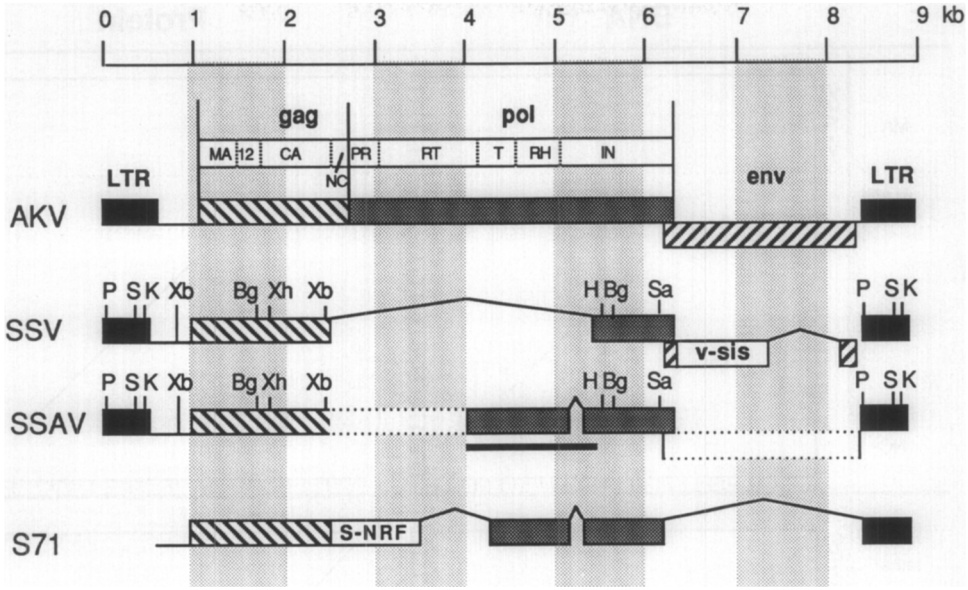


FIG. 4. Organization of retrovirus-related sequences in S71 as compared to full-length proviruses (AKV, SSAV) and a transforming defective provirus (SSV). The genomic organization of the murine endogenous AKV provirus was taken from the viral nucleotide sequence (Etzerodt *et al.*, 1984) and supplemented by comparison with the MoMuLV sequence (Shinnick *et al.*, 1981). Division of the AKV pol gene into PR, RT, tether, RH, and IN was adopted from Johnson *et al.* (1986). The organization of the truncated SSV provirus was determined from the published nucleotide sequence (Devare *et al.*, 1983). The relative location of SSV sequences in the SSAV genome was inferred from restriction sites common to both proviral sequences (Gelmann *et al.*, 1981; Devare *et al.*, 1983). The region of the SSAV pol gene sequenced for comparison with S71 is underlined in boldface. Where retroviral genes are either partially or completely missing (SSV and S71 pol, env) the remaining sequences are aligned with the corresponding AKV sequences and contiguous sequences are connected by slanted lines. S-NRF, S71-nonretroviral reading frame. B, *Bgl*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. ■, LTR; ▧, gag gene; ■, polymerase gene; ▨, envelope gene; ⬚ in the SSAV provirus, unsequenced regions not contained in SSV.

the SSAV/SSV sequence. Comparisons of the amino acid sequences of various tether sections have suggested that this region is relatively poorly conserved between different retroviral polymerase genes (Johnson *et al.*, 1986) and is the fastest changing entity of retroviral polymerase genes (McClure *et al.*, 1988). However, we find the number of amino acid identities between the tether sections of AKV, BaEV, SSAV, and S71 to lie in the same range as the amino acid identities between the respective RH regions (Table 1) previously shown to be relatively highly conserved between various polymerase genes (Johnson *et al.*, 1986). This suggests that, within the group of C-type retroviruses, the tether region is as well conserved as other sections of the polymerase gene and may be of more significance than just a spacer separating the reverse transcriptase and RH domains.

TABLE 1

AMINO ACID IDENTITIES OF THE TETHER (T) AND RNASE H REGION OF pol (%)

	S71		SS(A)V		BaEV		AKV	
	T	RH	T	RH	T	RH	T	RH
S71	—	—	52	49	49	47	49	53
SS(A)V	—	—	—	—	65	61	64	61
BaEV	—	—	—	—	—	—	71	65

By alignment with various other C-type retroviral RH sequences (see below) we found the S71 RH domain to extend from position 3489 to 3956 (Fig. 2). 82/156 amino acids in the S71 pol frame (Fig. 1) are identical with SSAV/SSV. Amino acid identity between both sequences is interrupted in a short stretch between position 3660 and 3707 in the S71 pol frame (frame 0). However, in this region the −1 reading frame (Fig. 1, frame 2) contains 11 contiguous amino acids identical with the SSAV/SSV pol sequence (Fig. 2). This increases the amino acid identities between both sequences to 60%. Levin *et al.* (1988) report the presence of conserved amino acids in the RH domain. All of these are also conserved in the S71 RH sequence (marked with an asterisk in Fig. 2) and three of these are located between position 3660 and 3707 in the −1 frame.

The sequences between position 3957 and 4938 constitute the IN section. Out of 21 invariant amino acid residues pointed out by Johnson *et al.* (1986), 13 are conserved in both the S71 and the SSAV/SSV se-

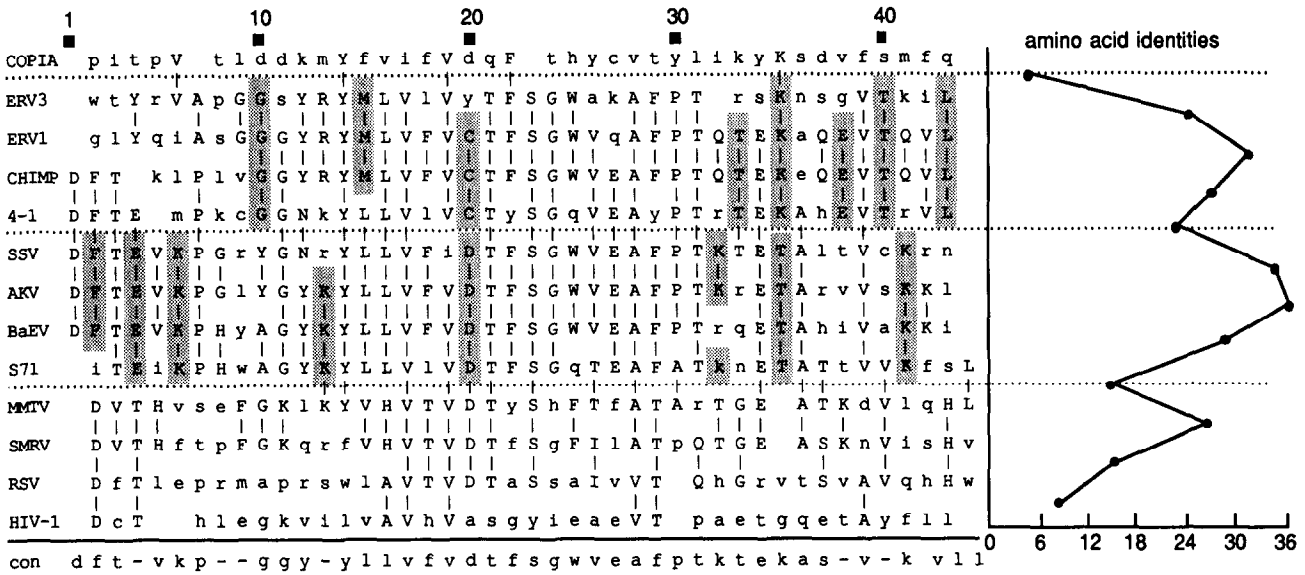


FIG. 5. Multiple alignment of a short conserved region within the endonuclease section of 13 retroviral polymerase sequences (GENALIGN): COPIA (aa 491–530), ERV3 (4980–5111), ERV1 (25–151), CHIMP (44–169), 4-1 (5365–5490), SSV (2936–3064), AKV (4996–5124), BaEV (4908–5036), S71 (4236–4364), MMTV (6006–6131), SMRV (202–328), RSV (4408–4533), HIV-1 (3999–4115). All numbers correspond to nucleotide positions, except COPIA (aa = amino acids). For references see Materials and Methods. Subgroup-specific conserved amino acids are shaded. The graph on the right shows amino acid identities between consecutive sequence pairs (from top to bottom).

quence (marked by an asterisk in Fig. 2). The overall amino acid identity of the S71 IN sequence with the SSAV/SSV pol sequence is about 40%.

Overall structure of S71

Upon retroviral integration host cell sequences are duplicated so that the provirus is flanked by short direct repeats. A direct repeat of the 7-bp sequence immediately adjacent to the S71 LTR-like region is located 400 bp upstream of the gag-related region (positions 98–104 in Fig. 2). Assumption that this 7-bp duplication is a result of retroviral integration results in a length of 5406 nucleotides for the S71 element. Next to the gag (1478 nucleotides) and the pol regions (1818 nucleotides), the S71 element contains two regions with no discernible similarity to any retroviral sequences available for comparison. The first region spans the 394-bp between the 5′ direct repeat and the gag-related region. The second retrovirus unrelated sequence is 1132 bp long and separates the S71 gag and pol regions (positions 1979–3110 in Fig. 2). Translation of this sequence suggests a possible open reading frame extending from a methionine codon at position 1979 to a termination codon at position 2344 (Fig. 2) and corresponding to 121 amino acids (13 kDa).

Phylogenetic relationship of S71 to other retroviruses/retroviral elements

One approach to obtain information about phylogenetic relationships is to look for common mutations in a set of similar sequences. Composition of such sets is often limited by the number of available sequences. The IN section of retroviral polymerase genes seemed to be a likely candidate for this type of analysis since it has previously been shown to contain a number of highly conserved residues (Johnson *et al.*, 1986). Within the endonuclease section we chose a region comprising about 40 amino acids for which sequence data were available for 13 different retroviral elements. Multiple alignments were carried out using the GENALIGN program and the results are shown in Fig. 5.

The SSV, AKV, BaEV, and S71 sequences all contain an Asp (D) residue at position 20 and Thr (T) at position 35 while ERV3, ERV1, CHIMP, and 4-1 exhibit a Cys (C) (ERV3 Tyr, Y) and a Lys (K) in the respective positions. This suggests that these C-type retroviral sequences can be divided into two subgroups. Careful analysis of the alignment reveals subgroup-specific amino acid preferences at 14 positions (2, 4, 6, 10, 13, 15, 20, 32, 33, 35, 38, 40, 41, and 43) at which identical amino acid residues are conserved in at least three of four members of a putative subgroup (shaded in Fig. 5).

S71 shares 7 out of the 8 amino acids specific for the SSV, AKV, BaEV subgroup, while it contains none of the conserved amino acids specific for the other subgroup. This suggests that S71 is more closely related to sequences AKV, BaEV, and SSV than to the other human endogenous retroviral elements ERV3, ERV1, and 4-1.

pol

endonuclease region (IN)

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                          * **   * **   *  *        *
AKV    IN   IDFTEVKPGLYGYKYLLVFVDTFSGWVEAFPTKRETARVVSKK
BaEV   IN   IDFTEVKPHYAGYKYLLVFVDTFSGWVEAFPTRQETAHIVAKK
SSV    IN   VDFTEVKPGRYGNRYLLVFIDTFSGWVEAFPTKTETALTVCKR
S71    IN    ITEIKPHWAGYKYLLVLVDTFSGXTEAFATKNETATTVVKF
ERV1   IN    GLYQI ASGGGYRYMLVFVCTFSGWVQAFPTQTEKAQEVTQV
Chimp  IN    DFTKL PLVGGYRYMLVFVCTFSGWVEAFPTQTEKEQEVTQV
4-1    IN   VDFTEM PKCGGNKYLLVLVCTYSGQVEAYPTRTEKAHEVTRV
ERV3   IN    WTYRVAPGGSYRYMLVLVYTFSGWAKAFPTRSKNSXEVTKI
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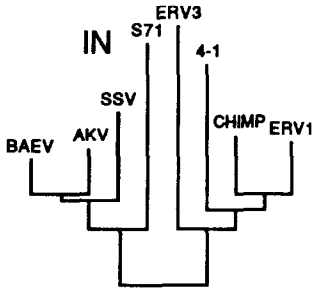

RNase H region (RH)

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                *                                  * *  ** *   **
AKV    RH    PDADHTWYTDGSSFLQ EGQRKAGAAVTTETEVIWARALPAGTSAQRAELIALTQALKMAEGKR
BaEV   RH    PDADHTWYTDGSSYLD SGTRRAGAAVVDGHNTIWAQSLPPGTSAQKAELIALTKALELSKGKK
SSAV   RH    PGV PAWYTDGSSFIA EGKRRAGAAIDDGKRTVWASSLPEGTSAQKAELVALTQALRLAEGRD
S71    RH    KEVDATVFTDSSSLLK QGVRKAGAAVTMETDKLQTQALPAGTSAQKAELVALIQALRRVRTNV
4-1    RH     SVDWELYVDGSSFVNPQEERCAGYAVVTLDTVAEARSFPQGTSTQKAELIALIRALELSEGKT
HIV    RH    IVGAETFYVDGAAN   RETKLGKAGYVTNKGRQKVVPL TNTTNQKTELQAIYLALQ DSGLE

                                        *          *  *     *        *
AKV    RH    LNVYTDSRYAFATAHIHGEIYRRRGLLTSEGREIKNKSEILALLKALFLPKRLSIIHCLGHQKGD
BaEV   RH    ANIYTDSRYAFATAHTHGSIYERRGLLTSEGKEIKNKAEIIALLKALFLPQEVAIIHCPGHQKGQ
SSAV   RH    INIYTDSRYAFATAHIHGAIYKQRGLLTSAGKDIKNKEEILALLEAIHLPKRVAIIHCPGHQKGN
S71    RH    LTFTLTAGMLFATVRVHGAIYQVRGLLTSAGKAIKNXEEILALLEAVCLPQQVAVIHCKGHQKED
4-1    RH    VNIYTDSXYVFLTLQVHGALCKEKGLLNSGGKDIKYQQEILQLLEAVWKPHKVAVIHCGGHQXAS
HIV    RH    VNIVTDSQYALGIIQAQPD  KSESELVNQIIEQLIKKEKVYL   AWVPAHKGI   GGNEQVD

               *
AKV    RH    SAEARGNRLADQAAREAAI        KTPPDTSTLL
BaEV   RH    DPVAVGNRQADRVARQAAMAEVLTLATEPDNTSHIT
SSAV   RH    DPVATGNRRADEAAKQAAL         STRVLAETTK
S71    RH    TAVAHGNQRADSAAWGPA          QLPVAPPTLL
4-1    RH    TLVGLGNSCTDLEAQKAAS         ALPGISDSPP
HIV    RH    KLVSAGIR                    KILFLDGIDK
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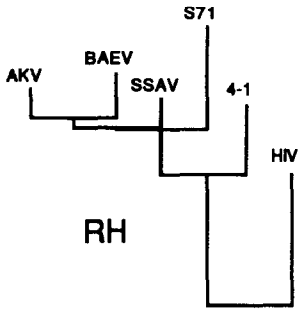

FIG. 6. Phylogenetic analysis of primate and murine retroviral sequences. Multiple alignments of amino acid sequences in two retroviral genes and corresponding phylogenetic trees shown on the right side: CA(s) (short): AKV (1296–1497), BaEV (1296–1457), ERV3 (1323–1484), SSV (2064–2225), S71 (1022–1282), 4-1 (1744–1908). CA(l) (long): BaEV (1266–1937), SSV (2034–2705), AKV (1306–1977), S71 (1092–1762), 4-1 (1717–2391), HIV-1 (807–1473). IN: BaEV (4908–5033), AKV (4996–5121), SSV (2936–3061), S71 (4236–4358), CHIMP (44–166), ERV1 (25–148), 4-1 (5365–5487), ERV3 (4980–5108). RH: AKV (4153–4623), BaEV (4077–4568), SSAV (614–1081), S71 (3489–3956), 4-1 (4534–5004), HIV-1 (3429–3809). All numbers correspond to nucleotide positions. For references see Materials and Methods. In the alignments a dash indicates a frameshift, an X represents a stop codon. Amino acids that are conserved in all sequences are marked by an asterisk.

For additional confirmation, eight of the sequences most similar to S71 in Fig. 5 were used for phylogenetic analysis according to the method of Feng and Doolittle (1987). The corresponding phylogenetic trees clearly divide these sequences into the subgroups defined by common mutations in the GENALIGN alignment (Fig. 6, IN). However, the rather short range of the sequences compared could result in a biased alignment, as both methods use similar algorithms.

Another region known to be conserved is the RH domain of the polymerase (about 160 amino acids; Johnson *et al.*, 1986). The clustering order of AKV, BaEV, SSAV, S71, and 4-1 obtained with this much longer stretch of amino acids agrees with that of the IN sequences (Fig. 6, RH). Unfortunately, sequences of this region are not available for the other human endogenous elements ERV1 and ERV3. Furthermore, RH and IN are both part of the same gene known to be most highly conserved among retroviruses (Chiu *et al.*, 1984; McClure *et al.*, 1988). Therefore we decided to extend this analysis to sequences derived from an independent gene. For this purpose we used a conserved segment in CA for which sequence data were available for six of the eight elements used in the endonuclease alignment. The resulting phylogenetic tree (Fig. 6, CA(s)) shows essentially the same branching order as the two pol-derived trees (RH and IN). Furthermore, sequences 4-1 and ERV3 are clustered into a separate subgroup from S71, as in the endonuclease tree (IN). The relative order of the AKV, BaEV, SSV/SSAV, S71 and Chimp, ERV1, ERV3, 4-1 sequences, respectively, was found to be identical for all four multiple alignments. Extension of analysis to a much longer gag region (about 230 amino acids, including the highly conserved region) yielded essentially the same relative branching order as the short conserved CA region and the pol RH domain, the only difference being that SSV clustered with BaEV instead of AKV (Fig. 6). This sug-

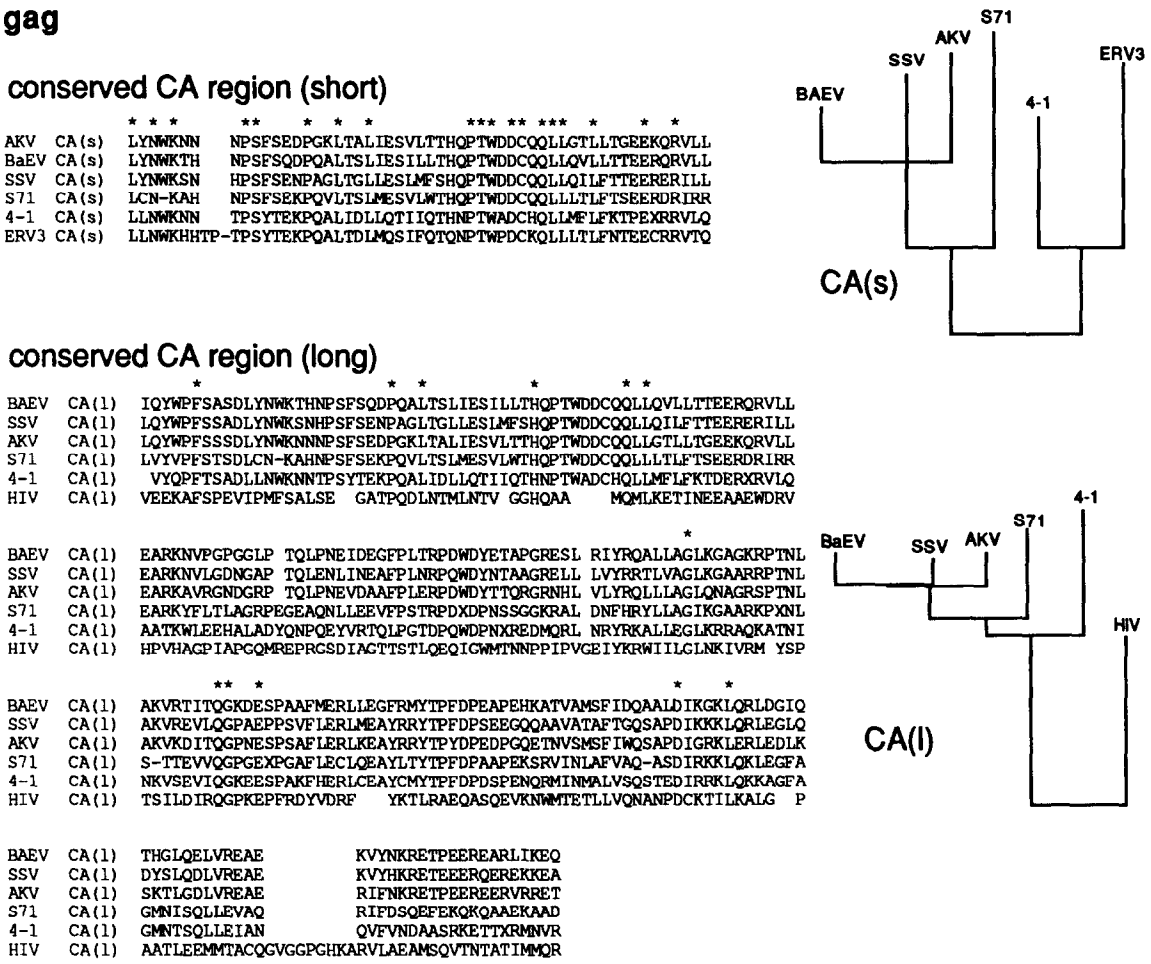

FIG. 6—*Continued*

gests that, at least in our case, these short ranges of amino acid sequences (IN, CA) reflect the overall relationship obtained with three to four times larger regions (RH, CA).

Each tree shown in Fig. 6 is based on calculations from the distance matrix derived from binary alignments. While the overall topology remains unaltered over a considerable range of distances, the individual branch lengths are highly dependent on the assumption of common rates of amino acid changes for the sequences compared. Since defective endogenous sequences do not face continuous functional selection, a common rate of amino acid changes with active viral sequences cannot be assumed. The branch lengths in Fig. 6 are plotted according to the calculated distances in the depicted trees without further adjustments. Although this may not correlate with the real evolutionary distances, it should not interfere with the overall topology of the trees.

DISCUSSION

The organization of retrovirus-related sequences in the S71 element was determined by nucleotide sequence analysis and comparison with the corresponding SSV and SSAV/SSV sequences. The S71 truncated proviral element contains gag- and pol-related sequences followed by a 3′ LTR-like sequence. The S71 gag region is of roughly the same length as the SSV gag gene (1478 vs 1539 nucleotides) and shows distinct sequence similarity to all sections of the SSV gag gene except $p12^{gag}$. A similar situation can also be observed for the gag region of the human endogenous C-type provirus 4-1 which also exhibits least homology to MoMuLV gag within the $p12^{gag}$ section (Repaske *et al.*, 1985). On the basis of the alignment of the amino terminal CA sequences, Oroszlan *et al.* (1977, 1981) divided mammalian C-type retroviruses into four subgroups. Comparison of the above region in S71 with the sequences characteristic for each of the four subgroups shows that the S71 element clearly falls into the SSAV/GaLV subgroup (Fig. 7).

The S71 pol region consists of tether–RH- and IN-related sections. The retrovirus-related sections within the gag and pol regions of S71 are arranged in the relative order typical of a number of murine and primate retroviral genomes. However, the S71 element is lack-

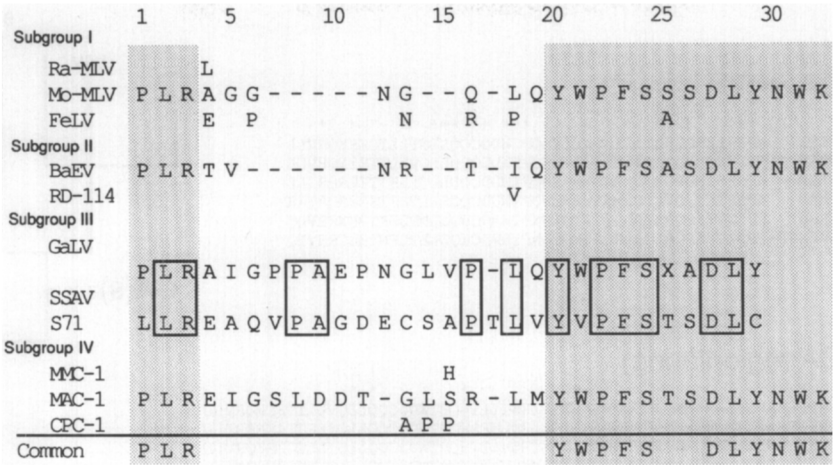

FIG. 7. Comparison of CA amino terminal amino acids according to Oroszlan *et al.* (1977, 1981) in Weiss *et al.* (1985). Ra-MLV, Rauscher murine leukemia virus; Mo-MLV, Moloney murine leukemia virus; FeLV, feline leukemia virus; RD-114, feline endogenous virus; GaLV, Gibbon ape leukemia virus; MMC-1, endogenous type C virus of *Macaca mulatta;* MAC-1, endogenous type C virus of *M. arcotoides,* CPC-1, endogenous type C virus of *Colobus polytomos.* X, unidentified amino acid.

ing over half of the polymerase gene, encompassing the protease coding region and the complete reverse transcriptase domain (Fig. 4).

A prevalent feature of transducing retroviruses is the partial or complete lack of genes required for retroviral multiplication, as is the case in S71. The sequence relatedness of S71 with the v-sis-bearing defective SSV provirus in the gag and endonuclease regions suggests comparison of the overall organization of retroviral sequences in both proviral elements. Both elements contain a complete gag gene, whereas both are missing a large part of the polymerase gene, including the entire reverse transcriptase domain, and most or all of the envelope gene (Fig. 4). Notably, in both elements part of a retroviral gene has been replaced by a presumably nonretroviral sequence. In SSV part of the envelope gene is replaced by the sis oncogene which is essential for the SSV transforming activity (Robbins *et al.,* 1982). Transcription of v-sis can occur in the absence of the 5′ LTR and donor splice signals suggesting that appropriate signals reside in the SSAV sequences preceding v-sis (Devare *et al.,* 1983). As mentioned above, part of the pol gene in S71 is replaced by a presumably nonretroviral sequence with a 121 amino acid open reading frame (S71-nonretroviral reading frame, S-NRF; Fig. 4). We have recently obtained evidence for the expression of related sequences in human placenta and are currently investigating the possibility that S-NRF may have a cellular counterpart not associated with retrovirus-related sequences (manuscript in preparation). Therefore, similarities between S71 and SSV are not confined to sequence level but can be expanded to include their general structure implicating analogous mechanisms for the generation of both truncated proviral sequences.

Direct repeats flanking integrated proviruses are known to be a hallmark of the retroviral integration mechanism. Although the exact number of duplicated nucleotides cannot be determined for S71, their presence and location in S71 suggests that S71, like SSV, is a proviral element resulting from a retroviral integration event. In this case the absence of cis sequences in S71 required for integration makes it conceivable that at some point there existed a full-length counterpart to S71 analogous to SSAV/SSV.

Sequence comparison of S71 with SSV/SSAV by dot matrix analysis indicates a higher degree of conservation on amino acid level than on nucleotide level (Fig. 3). This was analyzed in more detail for the short conserved CA and IN regions (Table 2). Only one amino acid substitution in the gag region and none in the endonuclease region is caused solely by a nucleotide change in the third position. The distribution of nucleotide substitutions among the three positions of the respective codons therefore are clearly biased towards silent third position changes. This bias suggests func-

TABLE 2

NUCLEOTIDE SUBSTITUTIONS VERSUS AMINO ACID CHANGES IN S71 AS COMPARED TO SSV

Region:	1. Position	2. Position	3. Position
CA	16 (25%)	11 (18%)	35 (56%)
IN	14 (29%)	9 (18%)	27 (55%)
Total:	Nucleotide substitutions	Changed codons	Amino acid substitutions
CA	62	37	19
IN	51	32	16

tional constraints effective on protein level indicating transient functionality of these proteins.

A critical factor in phylogenetic analysis with short range sequences is the choice of sequence regions used for comparison. We have identified two regions leading to results that reflect the overall situation as correctly as alignments with much longer sequences at least for C-type retrovirus-related sequences. Since our analysis demonstrates the reliability of these short sequence ranges they can be used as targets for polymerase chain reactions (PCR) for rapid classification of newly isolated sequences (Shih *et al.*, 1989). The initial phylogenetic evaluation of new elements could be carried out with these amplified sequences suggesting that the effort of large scale sequencing may no longer be necessary for this purpose.

Extension of this type of phylogenetic analysis to include sequences derived from the envelope gene would be a logical continuation. However, phylogenetic trees derived from env-related sequences differ widely from the largely congruent trees obtained with retroviral enzyme sequences such as RH and IN (McClure *et al.*, 1988) as well as with the capsid protein-derived sequences analyzed in this study (Fig. 6). This suggests that, during retrovirus evolution, these three proteins have faced similar pressure for functional selection which is different from that governing evolution of envelope proteins (Doolittle *et al.*, 1989). The rapid evolutionary change of envelope-related proteins is apparently at least in part caused by major genetic recombination events (McClure *et al.*, 1988). Furthermore, exposure of envelope proteins to the immune system of the host calls for adaptive changes in these proteins, as reflected by the high rates of change in the envelope sequences of different HIV isolates.

We previously reported that the control regions of the S71 3′-LTR-like sequence are most homologous to infectious murine and primate proviruses (Brack–Werner *et al.*, 1989a). All phylogenetic trees obtained with highly conserved retroid sequences within S71 also point out that S71 is more closely related to murine and primate infectious proviruses than to ERV1, ERV3, Chimp, and 4-1. Moreover the endonuclease (IN) and the short CA region (CA(s)) alignments show an even more distinct pattern clustering S71 with AKV, BaEV, and SSV into one subgroup and the other three (two) human endogenous retroviral sequences into another subgroup (Fig. 6). The relative order of all human endogenous elements compared to the murine and primate infectious proviruses is reproduced in each of the four alignments. We have reported the SSAV-related subgroup to comprise about 35 copies per human haploid genome (Brack–Werner *et al.*, 1989b). Low stringency hybridization of human DNA with a specific S71 probe derived from the tether–RH region revealed at least 15 strongly hybridizing fragments (data not shown). On the basis of these findings, S71 may represent a subgroup of human endogenous retroviral elements which is distinct from the ERV1, ERV3, 4-1 subgroup. Sequencing of further endogenous retroviral elements is required to shed more light on the evolutionary relationship of human retroposons.

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REFERENCES

BONNER, T. I., O'CONNELL, C., and COHEN, M. (1982). Cloned endogenous retroviral sequences from human DNA. *Proc. Natl. Acad. Sci. USA* **79,** 4709–4713.

BRACK–WERNER, R., BARTON, D. E., WERNER, T., FOELLMER, B. E., LEIB–MÖSCH, C., FRANCKE, U., ERFLE, V., and HEHLMANN, R. (1989a). Human SSAV-related endogenous retroviral element: LTR-like sequence and chromosomal localization to 18q21. *Genomics* **4,** 68–75.

BRACK–WERNER, R., LEIB–MÖSCH, C., WERNER, T., ERFLE, V., and HEHLMANN, R. (1989b). Human endogenous retrovirus-like sequences. *In* "Modern Trends in Human Leukemia VIII" (R. Neth, Ed.), Springer-Verlag, Berlin/Heidelberg/New York, in press.

BRACK–WERNER, R., WERNER, T., LEIB–MÖSCH, C., HEHLMANN, R., and ERFLE, V. (1989c). Primary structure of the SSAV tether—RNase H—endonuclease (pol) region deleted in SSV. *Nucleic Acids Res.* **17,** in press.

CALLAHAN, R. (1988). Two families of human endogenous retroviral genomes. *Banbury Rep.* **30,** 91–100.

CHEN, E. Y., and SEEBURG, P. H. (1985). Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* **4,** 165–170.

CHIU, I.-M., CALLAHAN, R., TRONICK, S. R., SCHLOM, J., and AARONSON, S. A. (1984). Major pol gene progenitors in the evolution of oncoviruses. *Science* **223,** 364–370.

COVEY, S. N. (1986). Amino acid sequence homology in gag region of reverse transcribing elements and the coat protein gene of cauliflower mosaic virus. *Nucleic Acids Res.* **14,** 623–633.

DEVARE, S. G., REDDY, E. P., LAW, J. D., ROBBINS, K. C., and AARONSON, S. A. (1983). Nucleotide sequence of the simian sarcoma virus genome: Demonstration that its acquired cellular sequences encode the transforming gene product p28(sis). *Proc. Natl. Acad. Sci. USA* **80,** 731–735.

DOOLITTLE, R. F., FENG, D.-F., JOHNSON, M. S., and MCCLURE, M. A. (1989). Origins and evolutionary relationships of retroviruses. *Q. Rev. Biol.* **64,** 1–30.

ETZERODT, M., MIKKELSEN, T., PEDERSEN, F. S., KIELDGAARD, N. O., and JØRGENSEN, P. (1984). The nucleotide sequence of the AKV murine leukemia virus genome. *Virology* **134,** 196–207.

FENG, D.-F., and DOOLITTLE, R. F. (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* **25,** 351–360.

FÜTTERER, J., and HOHN, T. (1987). Involvement of nucleocapsids in reverse transcription: A general phenomenon? *Trends Biochem. Sci.* **12,** 92–95.

GELMANN, E. P., WONG–STAAL, F., KRAMER, R. A., and GALLO, R. C. (1981). Molecular cloning and comparative analyses of the genomes of simian sarcoma virus and its associated helper virus. *Proc. Natl. Acad. Sci. USA* **78,** 3373–3377.

HANSEN, J., SCHULZE, T., MELLERT, W., and MÖLLING, K. (1988). Identification and characterization of HIV-specific RNase H by monoclonal antibody. *EMBO J.* **7,** 239–243.

HIZI, A., and HUGHES, S. H. (1988). Expression of Moloney murine leukemia virus and human immunodeficiency virus integration proteins in *Escherichia coli. Virology* **167,** 634–638.

JOHNSON, M. S., MCCLURE, M. A., FENG, D.-F., GRAY, J., and DOOLITTLE, R. F. (1986). Computer analysis of retroviral pol genes: Assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. *Proc. Natl. Acad. Sci. USA* **83,** 7648–7652.

KATO, S., MATSUO, K., NISHIMURA, N., TAKAHASHI, N., and TAKANO, T. (1987). The entire nucleotide sequence of baboon endogenous virus DNA: A chimeric genome structure of murine type C and simian type D retroviruses. *Japan. J. Genet.* **62,** 127–137.

KOZAK, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acid Res.* **12,** 857–872.

LEIB–MÖSCH, C., BRACK, R., WERNER, T., ERFLE, V., and HEHLMANN, R. (1986). Isolation of an SSAV-related endogenous sequence from human DNA. *Virology* **155,** 666–677.

LEIS, J., BALTIMORE, D., BISHOP, M., COFFIN, J., FLEISSNER, E., GOFF, S. P., OROZLAN, S., ROBINSON, H., SKALKA, A. M., TEMIN, H. M., and VOGT, V. (1988). Standardized and simplified nomenclature for proteins common to all retroviruses. *J. Virol.* **62,** 1808–1809.

LEVIN, J. G., CROUCH, R. J., POST, C., HU, S. C., MCKELVIN, D., ZWEIG, M., COURT, D. L., and GERWIN, B. I. (1988). Functional organization of the murine leukemia virus reverse transcriptase: Characterization of a bacterially expressed AKR DNA polymerase deficient in RNase H activity. *J. Virol.* **62,** 4376–4380.

MCCLURE, M. A., JOHNSON, M. S., FENG, D.-F., and DOOLITTLE, R. F. (1988). Sequence comparison of retroviral proteins: Relative rates of changes and general phylogeny. *Proc. Natl. Acad. Sci. USA* **85,** 2469–2473.

MOORE, R., DIXON, M., SMITH, R., PETERS, G., and DICKSON, C. (1987). Complete nucleotide sequence of the milk transmitted mouse mammary tumor virus: Two frameshift suppression events are required for the translation of gag and pol. *J. Virol.* **61,** 480–490.

MOUNT, S. M., and RUBIN, G. M. (1985). Complete nucleotide sequence of the drosophila transposable element Copia: Homology between Copia and retroviral proteins. *Mol. Cell. Biol.* **5,** 1630–1638.

O'CONNELL, C., O'BRIEN, S., NASH, W. G., and COHEN, M. (1984). ERV3, a full-length human endogenous provirus: Chromosomal localization and evolutionary relationship. *Virology* **138,** 225–235.

OROSZLAN, S., COPELAND, T., SMYTHERS, G., SUMMERS, M. R., and GILDEN, R. V. (1977). Comparative primary structure analysis of the p30 protein of woolly monkey and gibbon type C viruses. *Virology* **77,** 413–417.

OROSZLAN, S., COPELAND, T. D., GILDEN, R. V., and TODARO, G. J. (1981). Structural homology of the major internal proteins of endogenous type C viruses of two distantly related species of old world monkeys: *Macaca arcoides and Colobus polykomos. Virology* **115,** 262–271.

RATNER, L., HASELTINE, W., PATARCA, R., LIVAK, K. J., STARCICH, B., JOSEPHS, S. F., DORAN, E. R., RAFALSKI, J. A., WHITEHORN, E. A., BAUMEISTER, K., IVANOFF, L., PETTEWAY, JR., S. R., PEARSON, M. L., LAUTENBERGER, J. A., PAPAS, T. S., GHRAYEB, J., CHANG, N. T., GALLO, R. C., and WONG–STAAL, F. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313,** 277–284.

REPASKE, R., STEELE, P. E., O'NEILL, R. R., RABSON, A. B., and MARTIN, M. A. (1985). Nucleotide sequence of a full-length human endogenous retroviral segment. *J. Virol.* **54,** 764–772.

ROBBINS, K. C., DEVARE, S. G., REDDY, E. P., and AARONSON, S. A. (1982). *In vivo* identification of the transforming gene product of simian sarcoma virus. *Science* **218,** 1131–1133.

SCHWARTZ, D. E., TIZARD, R., and GILBERT, W. (1983). Nucleotide sequence of Rous sarcoma virus. *Cell* **32,** 853–869.

SHIH, A., MISRA, R., and RUSH, M. G. (1989). Detection of multiple, novel reverse transcriptase coding sequences in human nucleic acids: Relation to primate retroviruses. *J. Virol.* **63,** 64–75.

SHINNICK, T. M., LERNER, R. A., and SUTCLIFFE, G. J. (1981). Nucleotide sequence of Moloney murine leukemia virus. *Nature (London)* **293,** 543–548.

SKOWRONSKI, J., and SINGER, M. F. (1986). The abundant LINE-1 family of repeated DNA sequences in mammals. *Cold Spring Harbor Symp. Quant. Biol.* **2,** I, 457–464.

TABOR, S., and RICHARDSON, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* **84,** 4767–4772.

TEICH, N. (1984). Taxonomy of retroviruses. *In* "Molecular Biology of Tumor Viruses, RNA Tumor Viruses" (R. Weiss, N. Teich, H. Varmus, and J. Coffin, Eds.), 2nd Ed., pp. 25–207, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

TEMIN, H. M. (1985). Reverse transcription in the eukaryotic genome: Retroviruses, pararetroviruses, retrotransposons, and retrotranscripts. *Mol. Biol. Evol.* **2,** 455–468.

VIEIRA, J., and MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19,** 259–268.

WEINER, A. M., DEININGER, P. L., and EFSTRATIADIS, A. (1986). Nonviral retroposons: Genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. *Annu. Rev. Biochem.* **55,** 631–661.

WEISS, R., TEICH, N., VARMUS, H., and COFFIN, J. (Eds.) (1985). Appendix E: Amino acid sequences of retroviral structural proteins. *In* "Molecular Biology of Tumor Viruses, RNA Tumor Viruses," 2nd ed., Supplements and Appendixes, pp. 1215–1216, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.