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Anoxia-Inducible Rat VL30 Elements and Their Relationship to *ras*-Containing Sarcoma Viruses

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VL30 elements are associated with cancer by their overexpression in rodent malignancies, their induction in a fibroblast response to anoxia which shares features with the malignant phenotype, and their presence recombined into Harvey murine sarcoma virus (HaSV) and Kirsten murine sarcoma virus. These sarcoma viruses contain *ras* oncogenes flanked on both sides by retrotransposon VL30 element sequences, in turn flanked by mouse leukemia virus sequences. Three very basic questions have existed about the VL30 element sequences found in sarcoma viruses: (i) how did they become recombined, (ii) what are their exact boundaries, and (iii) why are they there? To help decipher the nature of VL30 elements in sarcoma viruses, we examined VL30 clones isolated from an anoxic fibroblast cDNA library and independently by polymerase chain reaction cloning from rat cell DNA. Sequence comparisons with HaSV revealed that HaSV was formed by the substitution of 0.7 kb of VL30 sequences by 0.9 kb of c-Ha-*ras* sequences, with this event possibly facilitated by the presence of an identical *Alu*-like repeat found upstream of the 5′ recombination point in both the VL30 element and c-Ha-*ras*. Recombination occurred 42 bases beyond the *Alu*-like sequences in VL30 and 1596 bases beyond them in c-Ha-*ras*, at position 926 of HaSV. The 3′ *ras*-VL30 recombination event in HaSV occurred within a seven-base region of shared sequence identity, between HaSV bases 1825 and 1831. Recombination between Moloney leukemia virus (MoLV) and VL30 appears to have occurred at a point corresponding to base 218 or 219 of MoLV and was near a TAR-like VL30 sequence; such recombination at the 3′ end was between positions 7445 and 7456 of MoLV (HaSV positions 4694 to 4703). Kirsten murine sarcoma virus was found to be closely analogous to HaSV, and limited similar features were also seen with Rasheed sarcoma virus.

VL30 elements are a family of retrotransposons present at about 50 copies per haploid genome in rats and mice (2, 3, 23, 24); these elements share features with endogenous retroviruses, including regions of sequence similarity to retroviral *gag* and *pol* genes and long terminal repeat regions (27, 35). Retroviral packaging signals and primer binding sites allow them to be efficiently packaged by retroviruses and copied to DNA by retroviral reverse transcriptase. Pseudotyped VL30 elements integrate at new genomic sites, confirming their functional retrotransposon nature (8). VL30 elements are often found expressed at high levels in malignancies and tumor-derived cell lines, particularly of rat origin, although they have not been found expressed in benign tumors (2, 15, 21).

VL30 sequences were first found recombined in the Harvey and Kirsten murine sarcoma viruses (HaSV and KiSV). These viruses arose upon passaging of Moloney leukemia virus (MoLV) and Kirsten leukemia virus, respectively, in rats (22, 25), and each contains 5′- and 3′-terminal sequences derived from the parental mouse leukemia viruses, a *ras* oncogene, and rat VL30 sequences flanking both sides of the *ras* oncogene (2, 17, 41). Disruption of VL30 sequences 3′ to the *ras* oncogene of HaSV eliminates its ability to induce solid tumors in vivo; possible explanations have included loss of enhancer sequences or removal of a functional open reading frame (3, 5, 18, 40). HaSV-derived vectors retaining VL30 sequences are in widespread use, and at least in the case of mammary tumorigenesis induced by the *neu* oncogene, such VL30 sequences may have significantly affected oncogenicity in transgenic animals (7, 28).

Sarcoma virus-specific VL30 elements have been characterized only in the rat. The existent clones of mouse VL30 elements have minimal overall sequence similarity to sarcoma virus-specific rat VL30, show different regulatory patterns, have never been found incorporated in mouse-derived sarcoma viruses, but are still often seen expressed in mouse tumors (4, 15, 20). Extensive similarities between the malignant phenotype and a response of normal rat fibroblasts to anoxia, in which VL30 is strongly induced, have suggested that loss of control of expression of the anoxic response might contribute to malignant conversion during tumor progression (3, 5).

To help clarify the nature of VL30 elements induced by anoxia, we constructed an anoxic cDNA library and isolated VL30 clones. Sequencing, analysis, and further polymerase chain reaction (PCR)-based cloning revealed several features of how VL30 elements came to be incorporated in the highly oncogenic *ras*-containing retroviruses and suggested possible new roles for VL30 sequences in such viruses.

VL30 cDNA clones were prepared from RNA isolated by the method of Godowski and Knipe (19) from 16-h anoxic FRE normal rat embryo fibroblast cells (1, 5). cDNAs were synthesized from the anoxic polyadenylated mRNA with the aid of a cDNA synthesis kit from Amersham (code RPN.1256). The cDNAs were cloned into *Eco*RI-digested lambda gt10 arms by using a cDNA cloning system kit from Amersham (code RPN.1257). The lambda gt10 anoxic cDNA library was screened by using the protocols for preparing replica filters and filter hybridization provided in the Amersham cDNA cloning kit (code RPN.1257). Hybridization was carried out at 65°C by using a VL30-specific DNA probe, pVL30DS (4), which is a 1.3-kb *Pvu*II fragment derived from VL30-specific sequences

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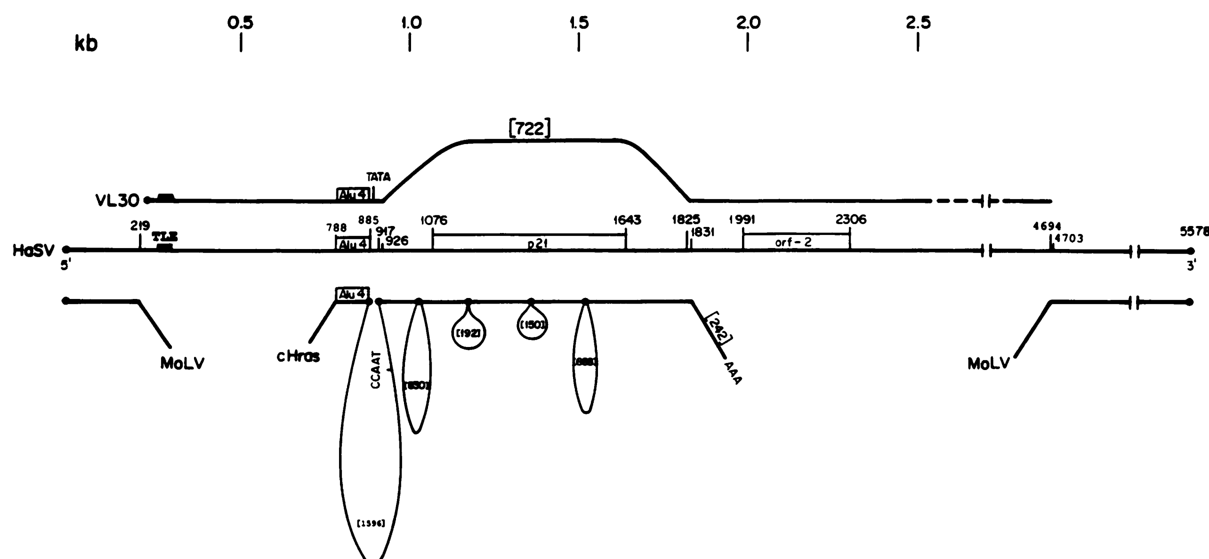

FIG. 1. Schematic overview of the sequence alignments of VL30 cDNA clones and the c-Ha-*ras* gene relative to HaSV. A VL30-derived TLE extends from bases 265 to 299. Recombination of c-Ha-*ras* into VL30 may have been facilitated by *Alu*-4 sequences present in each, although recombination itself occurred 32 bases downstream of the *Alu*-4 in VL30.

in KiSV 3.0 kb downstream of the 5′ long terminal repeat, cloned into the *Pvu*II site of pBR322. The probe insert was separated from the vector sequences on a 1% agarose gel, electroeluted, and random prime labeled with [α-^{32}P]dTTP.

Fifty-one positive clones were picked from secondary lifts, and the bacteriophage DNA was subsequently purified (36). These DNAs were digested with *Eco*RI to remove the vector arms, electrophoresed on a 1% agarose gel containing 0.5 μg of ethidium bromide per ml, and compared with a 1-kb ladder (Bethesda Research Laboratories). The inserts were subcloned into the *Eco*RI site of pUC13. Plasmid DNA was isolated by alkaline hydrolysis as described by Sambrook et al. (36). DNA from each of the plasmids was then transferred to Zetabind membranes (Cuno) and hybridized as described by Church and Gilbert (10). As we were particularly interested in VL30 elements closely related to HaSV, these blots were hybridized to 5′-end-labeled oligonucleotide GA-1, an antisense 51-mer to nucleotides 2245 to 2295 of HaSV. These sequences represent VL30 sequences from an open reading frame within HaSV which is in a region associated with sarcoma induction (40).

Dideoxynucleotide sequencing was carried out by using protocols for Sequenase (U.S. Biochemical Corp.) or the BST sequencing kit (Bio-Rad Industries) or by using a double-stranded DNA cycle sequencing system (Bethesda Research Laboratories) (37). Searches for similar sequences were carried out as described previously (27, 31), using the GenBank data base.

Of the 51 VL30 cDNA clones that we isolated from the anoxic fibroblast cDNA library by using the KiSV-derived VL30-specific probe, pVL30DS, the three largest cDNA fragments were termed VL30p47 (4.5 kb), VL30p33 (2.0 kb), and VL30p40 (2.7 kb). A Southern blot of the three plasmid DNAs was screened with an oligonucleotide probe specific to the biologically significant region including open reading frame ORF-2 in the VL30-derived sequences of HaSV, which spans nucleotides 1991 to 2306. Since both VL30p47 and VL30p33 included this region but VL30p40 did not, these two cDNAs were chosen for sequencing and further nucleotide analysis.

The first 701 nucleotides of VL30p33 were found to be 99% homologous to nucleotides 229 to 926 of HaSV. VL30p33 nucleotides 1429 to 2005 were 97% similar to HaSV nucleotides 1821 to 2399. VL30p47 sequences starting at nucleotide 391 and continuing to the 3′ end of the clone were 92% similar to nucleotides 1825 to 2545 of HaSV. Sequence alignments were found by inserting gaps to maximize the number of matching nucleotides, using the local homology algorithm of Smith and Waterman (39). The gap weight used was 5.000, and the length was 0.300 for nucleotide alignments and 3.00 and 1.00 for amino acid alignments. A summary illustrating the overall interrelationships of VL30, MoLV, c-Ha-*ras*, and HaSV is shown in Fig. 1; data establishing the recombination points are presented below.

The regions of VL30p33 and VL30p47 not similar to those of HaSV (VL30p33 positions 702 to 1428 and VL30p47 positions 1 to 390) share close similarity with each other and appeared to represent a region of VL30 which was replaced by *ras* sequences during the recombinational events creating HaSV. This finding is consistent with electron microscopy heteroduplexing results by Young et al., in which a single rat VL30 was studied (43), and recent sequence analysis of a single MoLV-VL30 recombinant (26). Alternatively, the differences might reflect differences between VL30p33, VL30p47, and another rat VL30 element which was the progenitor of HaSV, with HaSV being a simple insertion of *ras* into a VL30 instead of a substitution of *ras* for some VL30 sequences. To evaluate this possibility, PCR analyses were carried out with rat cell DNA and HaSV-derived VL30 primers to precisely define the recombination points of VL30 in this virus (Fig. 2). VL30 primers which flank *ras* in HaSV (BF4 and BF5) would produce an amplified product of about 220 bases if a simple insertion event occurred, while a product of about 940 bases would be seen if all rat VL30 elements which could have given rise to HaSV had a structure closely related to those VL30p33 and VL30p47, with 0.7 kb of VL30 being replaced by *ras* during the genesis of the sarcoma virus. The sole such amplified product obtained was of about 0.9 kb, and sequencing of this amplified fragment showed close homology to VL30p33 and

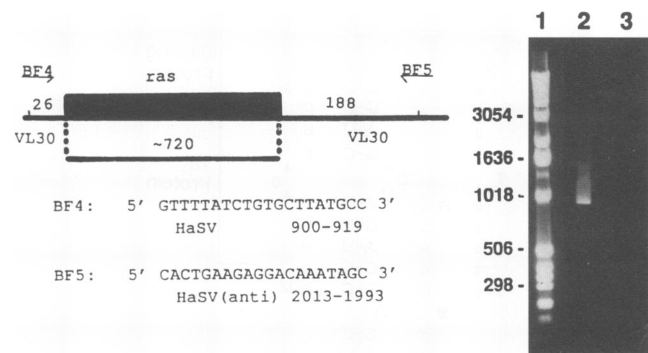

FIG. 2. PCR analyses of HaSV, compared with endogenous VL30 elements within the rat genome, indicate that a substitution process inserted *ras* into VL30. The 5′ primer (BF4) corresponds to VL30 sequences at HaSV positions 900 to 919, and the 3′ primer (BF5) corresponds to VL30 sequences at HaSV positions 1993 to 2013. PCRs were carried out with 20 ng of template FRE rat genomic DNA and 300 ng of each of the amplimers in the presence of 250 μM deoxynucleoside triphosphates, 0.05 M KCl, 0.01 M Tris (pH 8.4), 0.015 M $MgCl_2$, 0.1 mg of gelatin per ml, and 1 U of *Taq* DNA polymerase. Each PCR underwent 30 cycles of amplification as follows: 90°C for 30 s, 55°C for 30 s, and 70°C for 1 min. After amplification, the reaction mixtures were electrophoresed on 1.2% agarose gels in Tris-borate-EDTA containing 0.5 μg of ethidium bromide per ml. The DNA was transferred to Zetabind membranes (Cuno) and hybridized to VL30p33 random prime labeled with [α-^{32}P]dCTP. Lanes: 1, molecular weight standards (1-kb ladder; Bethesda Research Laboratories); 2, rat DNA amplification with BF4 and BF5; 3, no-DNA control with BF4 and BF5.

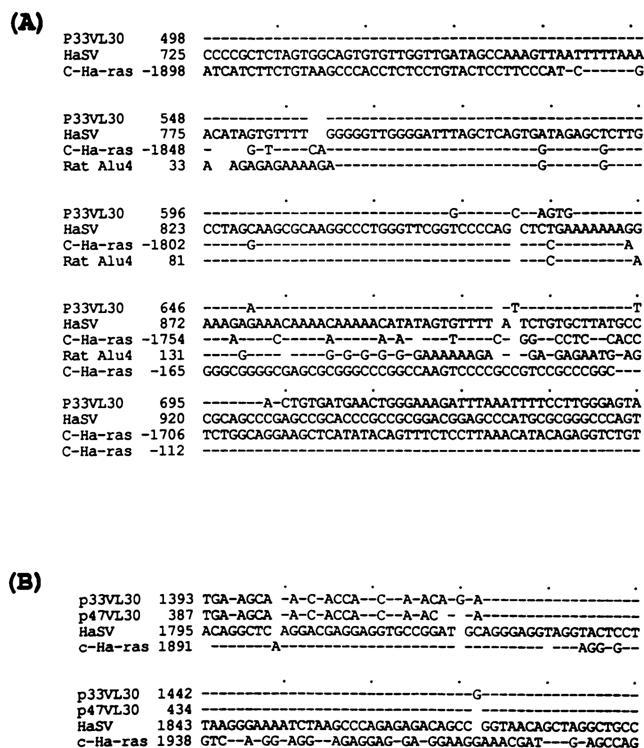

FIG. 3. Sequence homologies are associated with the recombination points inserting *ras* into VL30 during the genesis of HaSV. (A) At the 5′ junction, VL30, c-Ha-*ras*, and HaSV v-*ras* sequences all contain a rat repetitive element, rat type III *Alu*-4. The repetitive region from each of the three sequences was aligned with the HaSV sequence as the reference standard. Nucleotides identical to those in the HaSV sequence are depicted by dashes, nucleotides differing from the HaSV sequence are indicated, and missing nucleotides are represented by blank spaces. (B) The 3′ junction of *ras* and VL30 reveals that recombination occurred within a seven-base region of identity extending from HaSV bases 1825 to 1831.

confirmed its VL30 nature. These data establish that HaSV had to have arisen via a mechanism substituting *ras* for a similarly sized region of VL30.

Sequence analyses of VL30 helped define the *ras* oncogene recombination points in HaSV. The 5′-most 701 nucleotides of VL30p33 were found to be 99% similar to nucleotides 229 to 926 of the HaSV, at which point similarity completely ceases (Fig. 3). The c-Ha-*ras* sequences have been long assumed to start at nucleotide 981 in HaSV (28, 34), but similarity between VL30p33 and HaSV ended 55 nucleotides prior to this point at nucleotide 926, and similarity between HaSV and c-Ha-*ras* began at nucleotide 917, with a nine-base overlap existing between *ras* and VL30. This result closely parallels those for a recently described MoLV-VL30 recombinant (26). A GenBank data base search of the VL30p33 sequence revealed a 99-nucleotide rat repetitive element, rat type III *Alu*-like repetitive sequence 4 (*Alu*-4), farther upstream between nucleotides 561 and 659. The same rat repetitive sequence occurs in the 5′ region upstream of the c-Ha-*ras* gene and is also found in HaSV, although in c-Ha-*ras* it is 1.66 kb upstream of the *ras* coding sequences whereas in HaSV it is only 21 nucleotides upstream of sequences derived from the untranslated first exon of *ras* at bases 788 to 885. Sequence comparison between VL30p33, Harvey murine v-*ras*, and c-Ha-*ras Alu*-like repeats indicates that the point of recombination between c-Ha-*ras* and VL30 sequences did not occur within the *Alu* repeat itself but instead was after 32 more VL30 nucleotides 3′ to the *Alu* repeat at HaSV position 917. The region of similarity between c-*ras* and VL30 provided by the repetitive sequence has the potential to have facilitated the substitution of *ras* for 0.7 kb of VL30, the recombination event that produced the interior region of HaSV. Two previous studies have detected sequence similarity in the region of the *Alu* repeat but failed to recognize it as such. One suggestion was made that it represented a partial duplication of c-Ha-*ras* (9, 11, 27).

The 3′ *ras*-VL30 junction in HaSV was found to be relatively straightforward. Starting with nucleotide 1041 of the VL30p33 sequence and continuing to the end of the VL30p33 clone, VL30p33 and VL30p47 were found to be 95% similar. The 5′-most 390 nucleotides of VL30p47 along with nucleotides 702 to 1428 of VL30p33 are totally dissimilar to the HaSV sequence and reflect where its *ras* oncogene was inserted. Starting at HaSV nucleotide 1825, VL30p47 begins similarity and VL30p33 resumes similarity to HaSV. A seven-base region of shared identity with c-Ha-*ras* extends from HaSV positions 1825 to 1831, at which point similarity to c-Ha-*ras* ceases. Recombination of *ras* with VL30 evidently occurred within this seven-base region of identity (Fig. 3B). Beyond this point, HaSV is clearly of VL30 origin, with the remainder of the VL30p33 sequence 97% similar to HaSV nucleotides 1823 to 2399 and the remaining 810 nucleotides that have been sequenced from VL30p47 92% homologous to HaSV nucleotides 1823 to 2545.

The 5′ recombination junction of VL30 and MoLV sequences has been previously placed in the region of HaSV bases 206 to 219 (42). A comparison of VL30p33 with HaSV showed VL30 identity extending down to base 229, and comparison of MoLV with HaSV showed nearly complete

(A)

```
VL30 Amp.   1                         ----------------------------
p33VL30     1                                    -----------------
HaSV      196 CCACCGGGAGGCAAGCCGGCCGGCGTTTGTCTTGTCTGTTGTGTCTTGTC
MoLV      196 -----------T----T----A--AAC-TATC---G-C-GTCCGA-----

VL30 Amp.  29 --------------------------------------------------
p33VL30    18 --------------------------------------------------
HaSV      246 CTGTGAACGATCGATCAATAGGCTCAGATCTGGGGACTATCTGGGCGGGC
MoLV      246 TA---TCTATGACTGATT-TATGCGCCTG-GTC--TACTAG-TA--TAA-
```

(B)

```
VL30        94 -------------------------------- --T-------------
HaSV      4586 AGACTTTTTAGCTGACCTCCAAGAATACCTAACCTCCCTCTCAGAGGTAG
MoLV      7336 -A-A-CAA-CT--A----AG--A-G-CT-TC--T-----G--T--A--T-

VL30       143 -------A----AG-----------------------------G--GT
HaSV      4636 TCCTTCAGAATAGAAAAAGATTAGACCTGATATTCCTTAAAGAAAGAAC
MoLV      7386 ----A-------C---GGG-CC--------T----T--A------G--GGG

VL30       192 ----------TCTG--AG--   A--------------A-A-----TT--
HaSV      4685 CTGTGTGCTACACTAA  AA   GAATGTTGCTTCTATGCGGACCACACA
MoLV      7436 ---------GCT----   --GAA--------------------------

VL30       239 ---G--A-T-A----TC---AAAT---C-T-A-A-A---T AG-CA-AGA
HaSV      4730 GGACTAGTGAGAGACAGCATGGCCAAATTGAGAGAGAGGC GAATCAGAG
MoLV      7484 ----------------------------------------TT--------
```

FIG. 4. The junctions of VL30 and MoLV as recombined into HaSV. (A) At the 5′ junction, sequence identity of HaSV and VL30 extends downstream from HaSV position 218 but may extend further upstream; MoLV homology ends at position 219. VL30 Amp. represents the sequence of the BF2-BF3-amplified product. (B) The 3′ junction of VL30 and MoLV lies within a 140-base region of extensive sequence similarity, although actual recombination appears to have occurred between HaSV bases 4694 and 4703.

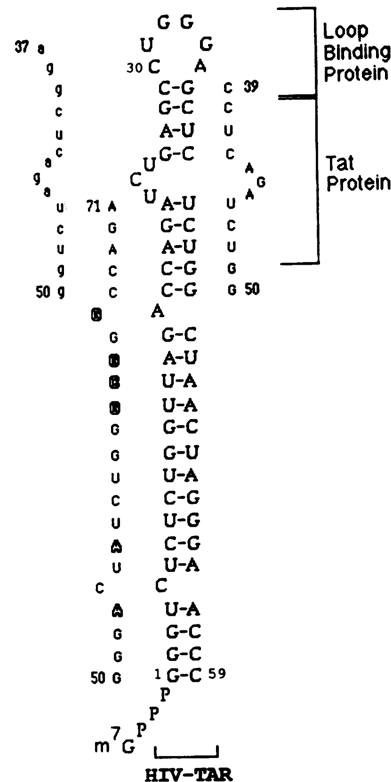

FIG. 5. A domain with similarity to the HIV TAR exists at the 5′ end of VL30p33 and is found recombined into both HaSV and KiSV. VL30 sequences are illustrated alongside the HIV TAR stem-loop. Sequences identical to the sense strand of TAR are shown in capital letters, with mismatches in boldface. A region of VL30-TAR homology on the opposite side of the stem-loop corresponds to VL30p33 bases 37 to 50 and matches antisense sequences 18 to 31 of TAR (lowercase letters).

MoLV identity up to base 219 (Fig. 4A). We carried out PCR analyses by using paired primers BF1 and BF3, corresponding to the 5′ region of VL30p33, with the left-hand primer BF1 extending to its 5′ end, corresponding to HaSV positions 229 to 224. We created another primer, BF2, corresponding to HaSV bases 218 to 233, with only 5 bases of overlap at the 5′ end of VL30p33. BF3 was an antisense primer corresponding to HaSV bases 377 to 360. Use of these primers with rat cell DNA revealed both amplified products, with the BF1-BF3 set producing an amplified product slightly smaller than the product of the BF2-BF3 set. Direct sequencing of these products confirmed that both were homologous to VL30 and HaSV but not MoLV. These results are consistent with VL30 homology extending to base 218 of HaSV, placing the junction of MoLV and VL30 sequences in HaSV at base 218 or 219. However, the primer would not have to have been perfectly complementary to the template to have achieved this result, and some sequence variations from the VL30 progenitor could exist in HaSV in the region from bases 219 to 229.

To localize the 3′ recombination point of MoLV and VL30 in HaSV, sequencing of an additional VL30 cDNA clone, Mbo350, was able to define the 3′ junction to a region between bases 4694 and 4703 on the HaSV genome (Fig. 4B). This occurred within a region of 140 bases which showed extensive overall similarity (73%) between VL30 and MoLV.

An interesting feature was found at the 5′ end of the VL30 p33 clone, namely, a domain with similarity to the transactivation response element (TAR) site of human immunodeficiency virus (HIV). The HIV TAR consists of a structural RNA composed of the first 60 bases of HIV type 1 RNA, which forms a stem-loop including an unpaired UCU which plays a central role in binding the HIV Tat transactivator protein (12, 13). Bases 37 to 71 of VL30 clone p33 showed extensive similarity to the HIV TAR (Fig. 5). Data base searches with the VL30 TAR-like element (TLE) sequence revealed identical sequences in both HaSV and KiSV, with identity to VL30p33 over the entire region (corresponding to HaSV bases 234 to 310 and KiSV bases 1253 to 1329). Related sequences were found only in known lentiviruses (HIV, simian immunodeficiency virus, and human T-cell leukemia virus type I), not in simple retroviruses or in any published mouse VL30 sequences (20).

The first two acute transforming retroviruses found carrying *ras* oncogenes were KiSV and HaSV, which had also incorporated rat VL30 element sequences. This finding suggested that VL30 sequences might make a significant and perhaps necessary contribution to *ras*-mediated oncogenesis. Subsequent isolation of Rasheed and BALB sarcoma viruses from rat and mouse, respectively, which contain *ras* oncogenes but no immediately evident VL30, appeared to contradict this pattern (33, 34). We observe a region of sequence similarity between VL30p33 and Rasheed virus which spans 279 nucleotides, between VL30p33 bases 57 and 418. The similarity between VL30p33 and Rasheed virus over the entire region is 70%, although from nucleotides 57 to 137 and 337 to 418 on VL30, it exceeds 94%. The region of similarity between VL30p33 and Rasheed virus is located 5′ to the rat type III *Alu*-4 discussed above and is not related to any known rat or mouse *Alu* sequences. Aside from 14 bases of the TLE sequence, further

searches with the sequences of this region in VL30p33 and Rasheed sarcoma virus have not resulted in significant similarity to other known retrovirus-related sequences. These results combine to confirm that Rasheed sarcoma virus fits the pattern seen with the KiSV and HaSV; namely, it contains recombined *ras* and VL30. Since Rasheed sarcoma virus has not been completely sequenced, it remains to be determined whether additional VL30 sequences are present downstream of *ras* and whether ORF-2 is present. The mouse homolog of rat sarcoma virus-specific VL30 has yet to be identified, leaving it unclear whether the BALB sarcoma virus may not also contain VL30 along with *ras*.

In this study of rat VL30 elements and their comparison with murine sarcoma viruses in which they were incorporated, we have identified the recombination points as regions of shared sequence identity, with a repetitive *Alu* element potentially facilitating *ras* insertion into VL30. In the case of HaSV, our data indicate that recombination of *ras* with VL30 and of VL30 with MoLV could have occurred either entirely at the DNA level or alternatively by a process involving strand switching during reverse transcription. Regions of sequence identity exist at each of the four recombination points; the presence of a packaging signal in VL30 RNA makes strand switching a straightforward possibility for the MoLV-VL30 recombination. Recombination of *ras* and VL30 would appear likely to involve the shared *Alu* sequences near but not at the 5′ recombination junction. However, the *Alu* sequences as well as the immediate downstream sequences in HaSV are both of VL30 origin and represent contiguous VL30 sequences, while as they exist in rat DNA, they are separated from c-Ha-*ras* by 1,596 bases, making some strand-switching process appear more likely. Thus, if this recombination involved strand switching during reverse transcription, it would presumably require packaging in virus particles of an incompletely spliced c-Ha-*ras* mRNA, with MoLV-VL30 recombination having occurred prior to this event. An alternate possibility is that *ras*-VL30 recombination occurred by strand switching during cellular DNA replication; a third possibility is that the *Alu* sequences were not directly involved. The finding of a natural recombinant between MoLV and VL30 indicates that recombination of these two elements could have preceded *ras* recombination into VL30 but does not preclude the converse (26).

The ultimate question is, why did VL30 elements end up recombined in all three isolates of rat-derived *ras*-containing murine sarcoma viruses? The presence of homologous sequences in VL30 and c-Ha-*ras* and in type C leukemia viruses and VL30 could simply have increased the probability of *ras* recombining into a retroviral vector. The absence of *Alu*-related sequences in Rasheed virus might argue against this possibility, although such sequences could have subsequently been lost after the virus first arose. Alternatively, VL30 may be providing sequences with functions directly augmenting *ras*-based oncogenesis; these could include RNA structural features and/or encoded proteins (3, 18, 40). It may prove particularly interesting to characterize the role, if any, of the TLE sequences in VL30 and the sarcoma viruses. Are they vestiges of an ancestral retroelement with a functional TAR, are they still somehow involved in transactivation processes, or might they have some other role?

Nucleotide sequence accession numbers. The VL30 sequences are deposited in the GenBank data base with accession numbers M91235 (VL30p33) and M91234 (VL30p47).

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