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A Novel Retroviruslike Family in Mouse DNA

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In the course of structural analysis of VL30 DNA elements, a recombinant retroviruslike element was encountered that contained non-VL30 long terminal repeats (LTRs) flanking internal VL30 sequences. With the aid of this novel LTR sequence probe, we cloned several DNA elements that were apparently members of a new retroviruslike family. A particular DNA element representative of this family (designated GLN) was characterized. It was approximately 8 kilobase pairs long and contained LTRs that are 430 base pairs long. It possessed an unusual primer-binding site sequence that corresponds to $tRNA^{Gln}$ and a polypurine tract primer that is adjacent to the 3′ LTR. The nucleotide sequences of the LTRs and their adjacent regions (which together housed all *cis*-acting retroviral functions) were different from those of known retroviruses and retroviruslike families. The comparison of three different GLN LTR sequences revealed a marked heterogeneity of U_3 sequences relative to the homogeneity of R and U_5 sequences. We estimate that approximately 20 to 50 copies of GLN elements are dispersed in all species of mice. GLN-related LTRs, however, are present in a much higher copy number (1,000 to 1,500 per genome). Nucleotide sequences that are more distantly related to GLN DNA are present in multiple copies in DNAs of other rodents but not in nonrodent genomes.

Chromosomes of normal, uninfected animal cells contain DNA sequences closely related to those of infectious retroviruses. These genetic elements, called endogenous proviruses, are thought to have become permanent residents of the animal genome as a result of germline infections (for a review, see reference 4). There are, however, other sequence elements present in the cell genome that possess retroviruslike properties but do not have nucleic acid sequence homology with any known retrovirus. The genetic origins of these cellular retroviruslike elements are not known. Retroviruslike elements are integrated in different cellular sites in a small direct repeat of the cell DNA. The overall structure of retroviruslike elements resembles the structure of retrovirus proviruses. They contain long terminal repeats (LTRs) that are bounded by short inverted repeats. The LTRs have a U_3-R-U_5 sequence arrangement (that is, the LTR units are assembled from sequences that reside at both ends of the RNA template, which is itself terminally redundant). Adjacent to the 5′ and 3′ LTRs, respectively, are two element-encoded reverse transcription primers, a primer-binding site complementary to the 3′ end of a cellular tRNA (PBS) and a polypurine tract. All of these structural features, which are the hallmarks of reverse transcription, are also shared by retrotransposons of lower eucaryotic organisms (26, 27).

Certain retroviruslike families might also display additional retroviruslike phenotypes. For example, murine intracisternal A particles (IAP) form intracellular particles resembling retroviral nucleocapsids (13), and murine VL30 genes possess a *cis*-acting packaging signal that is recognized by retroviral proteins and is responsible for the efficient rescue of VL30 30S RNA into C-type virions (1, 7). These features of IAPs and VL30s aided in their initial detection and subsequent isolation by molecular cloning (11, 15).

The lack of nucleic acid sequence homology with known retroviruses hampers the discovery of additional resident retroviruslike families, in particular those which lack identifiable phenotypes of retroviruses. In this paper we report the isolation and characterization of an apparently novel murine retroviruslike family.

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MATERIALS AND METHODS

DNA clones. A genomic library was constructed from embryonic BALB/C DNA fragments, obtained by partial *Eco*RI digestion in a charon 4A lambda vector. Cloning from the genomic library was carried out by standard procedures (17). Subgenomic DNA fragments were subcloned in a pUC13 plasmid.

Structural analysis of retroviruslike DNAs. Heteroduplex analysis was carried out as described by Davis et al. (5). DNA fragments were end labeled for sequencing by digesting the DNA with a combination of restriction enzymes that allowed the incorporation of certain [^{32}P]dNTPs exclusively at one end (by the use of the large fragment of DNA polymerase I). DNA sequencing was carried out essentially by the method of Maxam and Gilbert (19). Blot hybridization of genomic DNAs was carried out as previously described (12).

RESULTS

(i) Isolation of the new element. In the course of the structural analysis of VL30-associated LTRs, we came across a VL30 element whose LTR units were completely unrelated to the previously sequenced VL30 LTRs. The lack of homology with VL30 DNA extended further to the sequences adjacent to the LTRs (see below for actual sequence data and for homology search). The majority of the internal sequences of this element, however, were VL30 sequences. This sequence relationship is displayed in a heteroduplex formed between this DNA element (designated VL8) and a prototypic VL30 element (Fig. 1A). As shown schematically, VL8 and VL30 shared a contiguous stretch of 4.7 kilobases (kb) of DNA. The LTR units in this particular heteroduplex resided in the nonduplexed regions (see the legend of Fig. 1 for details). We suspected that VL8 DNA might be a recombinant VL30 element in which the LTRs and some adjacent sequences were acquired from another retrovirus-like element. We attempted, therefore, to clone

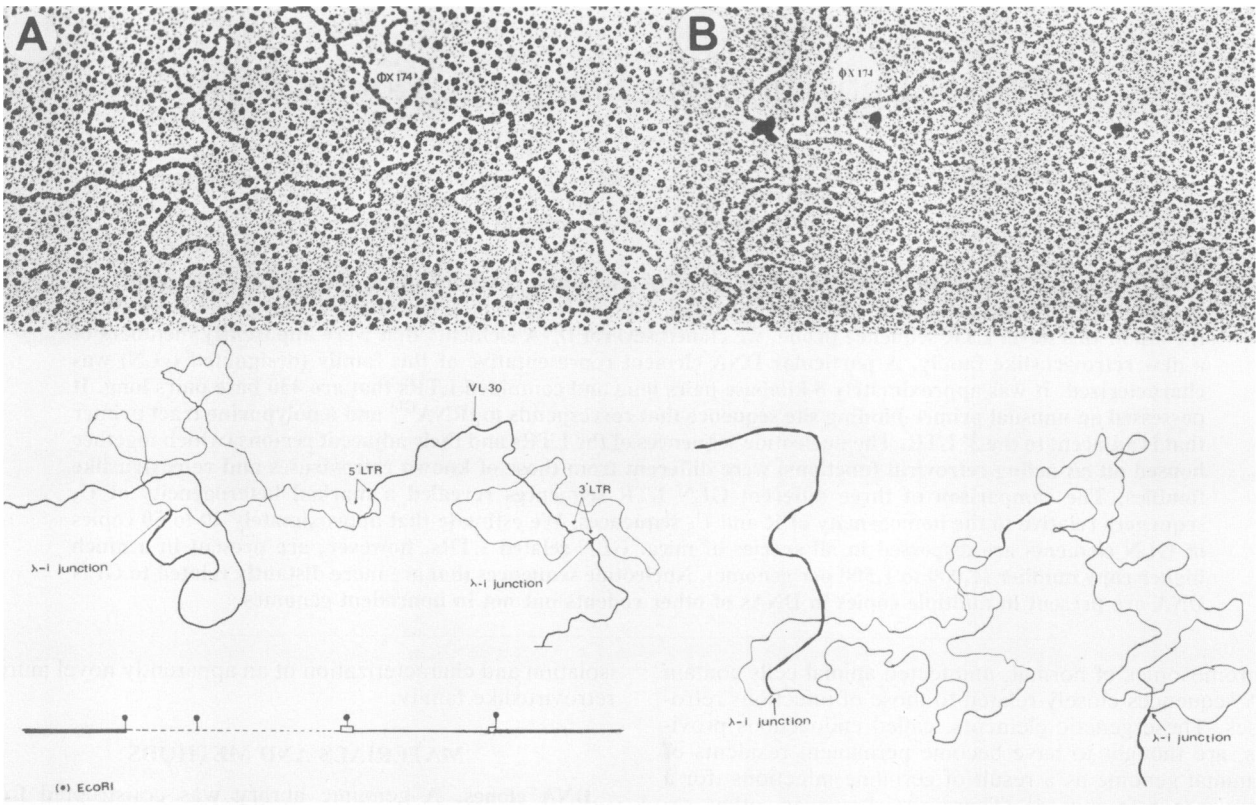

FIG. 1. Heteroduplexes between a prototype VL30 DNA and retroviruslike elements that contain VL30-related segments. DNAs were inserted in a charon 4A lambda vector. The vector-insert junctions (λ-i) are indicated. The VL30 clone used contained 5.6 kb of VL30 DNA. This DNA was heteroduplexed with VL8 (A) or GLN-3 (B) DNA. The scheme at the bottom of the figure indicates the locations of the LTRs on the physical map of VL8 DNA. The *Eco*RI site at the 3′ insert-vector junction corresponds to position 181 of the 3′ LTR. The length of the complete LTR (□) is 397 bp. The unique sequences of the VL8 element (——) are 4.9 kb long, and the 5′ flanking sequences (-----) are 8.4 kb long. Length measurements of heteroduplexes allowed to assign different regions as follows. The single-stranded VL8 DNA at the 3′ side (ca. 0.3 kb) contains 181 bp of the LTR and some additional unique sequences. The double-stranded DNA region (ca. 4.7 kb) is composed of unique VL8 DNA sequences that are homologous to VL30. The single-stranded region at the 5′ side should include, therefore, the 5′ LTR of VL8 DNA. The physical map of GLN-3 DNA, indicating the location of LTR units and sizes of 5′ and 3′ flanking sequences, is shown in Fig. 2A.

the retrovirus-like element that is the presumptive donor of the LTR units possessed by VL8 DNA. A specific probe was prepared from the 5′ LTR of VL8 DNA. This probe spanned roughly the 3′ half of the LTR, from the *Eco*RI site at position 181 of the LTR to a *Bgl*II site present 15 nucleotides downstream of the 397-base-pair (bp) LTR. This hybridization probe was used in screening a genomic library prepared from BALB/c mouse embryos. Hybridizing clones were subsequently tested for hybridization with the synthetic oligonucleotide 5′ ACCTCCAAGGTGACTCTA 3′. This oligonucleotide is complementary to the primer-binding site of VL8 and was chosen because it is different from the common VL30 PBS sequences and from PBS sequences of other retroviruslike elements (see below). Positive clones were selected for further characterization. The structural analysis of a representative clone, designated GLN-3, is described below.

A heteroduplex between GLN-3 DNA and a prototypic VL30 clone (the same VL30 DNA used in Fig. 1A) is shown in Fig. 1B. As can be seen, GLN-3 contained less VL30-related sequences than did VL8. Homology with VL30 was confined to a segment of approximately 0.9 kb of DNA. Reciprocal blot hybridization experiments with GLN-3 and VL30 DNAs have shown that the homologous DNA segment was located close to the 5′ LTR (Fig. 2B). It is not known whether the limited homology between GLN-3 and VL30 represented a limited genetic relatedness between the two families or, alternatively, whether GLN-3 DNA was also a recombinant that had acquired some VL30 sequences. In either case, GLN-3 DNA was clearly a member of a gene family that is distinct from the VL30 family.

(ii) Retroviruslike features of GLN-3 DNA. The GLN-3 element was approximately 8 kb long and contained LTRs 430 bp long (Fig. 2A). To demonstrate the retroviruslike nature of GLN-3 DNA, we focused on the structural organization of the LTR units, on their junction with flanking cellular sequences, and on the putative primer-binding sites PBS and PPT. Results of sequence determinations are schematically shown in Fig. 2D. GLN-3 DNA was flanked on both sides by the sequence CTCT. This is probably the consequence of a four-nucleotide-base-pair duplication that occurred upon integration of GLN-3 DNA; analysis of the unoccupied locus is required, however, to establish this point. The sequence arrangement at the cell-element junctions is consistent with a mechanism of integration from within (i.e., the loss upon integration of the LTR terminal dinucleotides AA [from 5′ LTR] and TT [from 3′ LTR]). This mechanism, common to all retrovirus-like elements, places the nucleotides TG. . .CA at the exact ends of the integrated element. These nucleotides are also present in the same

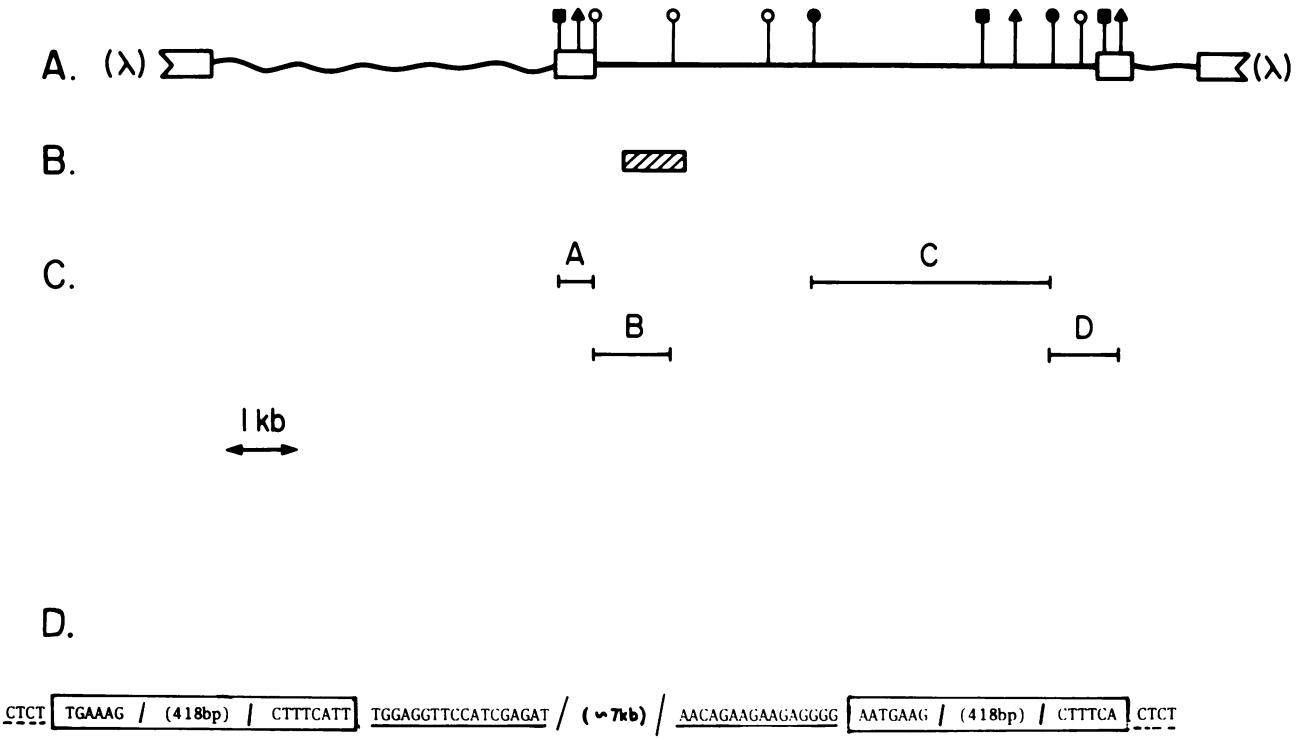

FIG. 2. Genomic organization of GLN-3 DNA. (A) Physical map of GLN-3 DNA. The element (——), LTRs (□), and flanking DNA (〰) are indicated. Restriction sites: ■, *Sma*I; ▲, *Sac*I; ○, *Bgl*II; ●, *Eco*RI. (B) Region of homology (▨) with VL30 DNA, as determined by measurement of heteroduplexes of the type shown in Fig. 1B and by blot hybridization experiments. (C) Subcloned fragments used as probes in experiments described in the text: A, an LTR-specific *Sma*I-*Bgl*II fragment; B, a 1.1-kb *Bgl*II fragment; C, a 3.4-kb *Eco*RI fragment; D, a 0.9-kb *Eco*RI-*Sac*I fragment. (D) Sequence organization at the LTR boundaries. LTRs are schematically shown as boxes. The terminally inverted LTR sequences are indicated. PBS and PPT sequences are indicated by a solid underline, and the duplicated flanking sequences are indicated by a broken underline. Drawing is not in scale.

location in many eucaryotic cellular movable genetic elements. The 18 nucleotide base pairs that followed the 5′ LTR unit of GLN-3 corresponded almost perfectly to the 3′ end of rat $tRNA^{Gln}$. Adjacent to the 3′ LTR unit was the polypurine tract 5′ AAACAGAAGAAGAGGGGAA 3′, which presumably served as the primer-binding site for the synthesis of the + strand of DNA.

The LTR units of GLN-3 DNA were distinguished by terminal inverted repeats (Fig. 3, dotted underlines). Both 5′ and 3′ LTRs were sequenced and were found to be identical. The LTRs contained a potential CAT box (the sequence CCAAT at positions 190 through 194), a potential TATA box (the sequence TATAAA at positions 239 through 244) and a potential polyadenylation signal (the sequence ATTAAA at positions 322 through 327). There were three CA residues (a preferred polyadenylation site) in the region 17 to 24 bp downstream of the polyadenylation signal. It remains to be shown, however, that these sequences actually function as transcriptional promoter and polyadenylation signals. The division of the LTR into U_3, R, and U_5 domains (Fig. 3) should, therefore, be considered as tentative.

(iii) GLN-3 DNA is distinct from known retroviruslike elements. GLN-3 DNA is apparently a member of a multicopy retroviruslike family (see below). Members of this family are distinguished by the PBS sequence 5′ TGGAG GTTCCACTGAGAT 3′. This PBS differs by only one nucleotide from a major $tRNA^{Gln}$ isoacceptor species (29). This unusual PBS sequence has recently been found in a subfamily of endogenous murine leukemia virus (MuLV)-related proviruses (20). Blot hybridization experiments have shown, however, that GLN-3 DNA has no significant homology with MuLV-related proviruses (data not shown). This conclusion was confirmed by direct comparison of available nucleotide sequences.

To demonstrate that GLN-3 DNA is a member of a multicopy family, we aligned the experimentally determined sequences that surround PBS in three different genomic clones that we have isolated. The three clones were very similar to one another (Fig. 4). The main differences among the three elements in the region shown were short deletions in the U_5 regions and duplications of short segments 3′ to the PBS. For example, the sequence CTAAGTGT was tandemly duplicated (in VL8) or triplicated (in GLN-3), and GLN-2 contained a tandem duplication of 36 bp. Clearly, however, the similarities among GLN-3, GLN-2, and VL8 indicate that they are members of the same family.

To determine whether GLN-3 is related to any known retrovirus, we compared the sequences shown in Fig. 4, as well as the sequences of the entire LTRs (Fig. 3), with available mammalian and viral sequences in the Genetic Sequence Data Bank. The survey included the following elements: MuLVs of different strains, AKV MuLV, mink cell focus-forming viruses, the subclass of endogenous MuLV-related proviruses that contain PBS^{Gln} (20), mouse mammary tumor virus-related proviruses, VL30 elements, IAP elements, proviruses of nonmouse origins, and murine repetitive DNA families. No significant homology was detected with any of these elements. Some homology was detected between GLN-3 DNA and a member of a group of very recently described retrovirus-related DNA sequences

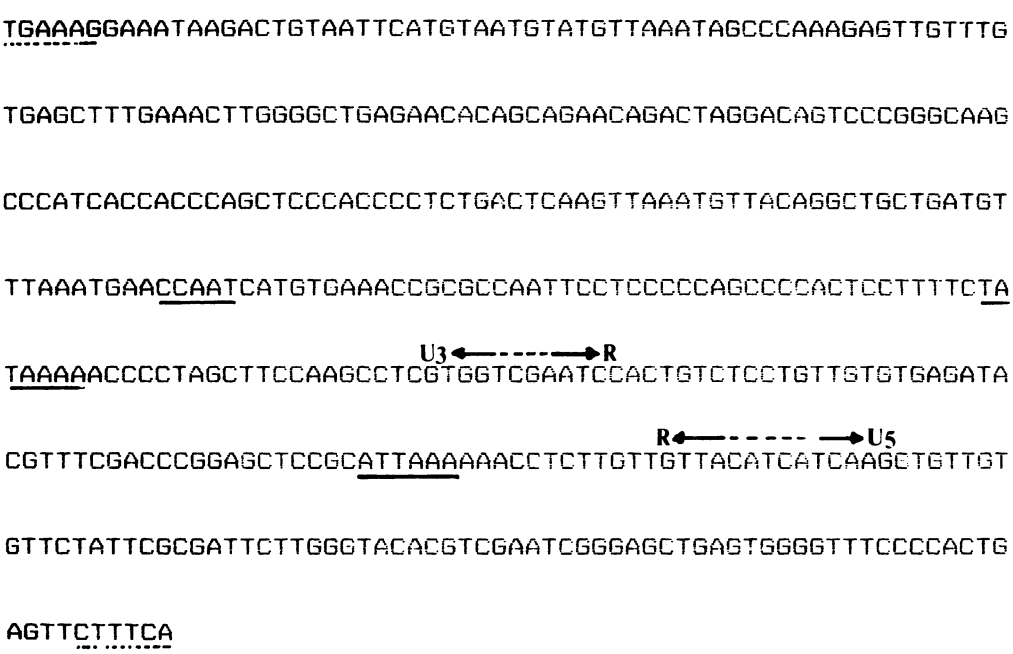

FIG. 3. Nucleotide sequences of GLN-3 LTRs. The terminally inverted repeats are indicated by a broken line. A potential CAT box, a TATA box, and a polyadenylation signal are underlined. Accordingly, U_3, R, and U_5 boundaries were tentatively assigned, as shown.

(24). The latter element, however, is significantly shorter, has different restriction maps, and contains the usual PBS sequence of $tRNA^{Pro}$ (24). Thus, GLN-3 represents a novel retroviruslike family which we call the GLN family.

An extensive homology was detected between GLN LTRs and a solitary LTR element that has recently been isolated from the mouse c-*mos* locus. This LTR element is nested in another type of repeated element (called a *Bgl*II repeat) and is flanked by a 4-bp duplication of cellular sequences (22). It is thus possible that this solitary LTR is a descendant of the GLN-3-type retroviruslike element. The relationships between this LTR element (which is designated here as MS LTR) and LTR units that are associated with two members of the GLN family are shown in Fig. 5. The three LTRs shared very similar U_5 and R domains but differed extensively in their U_3 regions. Interestingly, MS LTR contained a U_3 region that was approximately 200 bp longer than the U_3 region of GLN LTRs. This difference may be due to an insertion in MS LTR or, alternatively, to a deletion in GLN-3 LTR (Fig. 5, left). A different relationship exists between GLN-3 LTR and VL8 LTR. These LTRs contained a nonhomologous substitution at their U_3 region (Fig. 5, right). The nature of the genetic alterations that have occurred in GLN genomes and have led to the profound differences in LTR sequences is not understood. (Note that in both GLN-3 and VL8, the 5′ LTR and the 3′ LTR are identical.)

(iv) Multiple copies of GLN elements are present in mouse DNA, and related sequences are found in DNAs of other rodents. To estimate the copy number of GLN elements, we

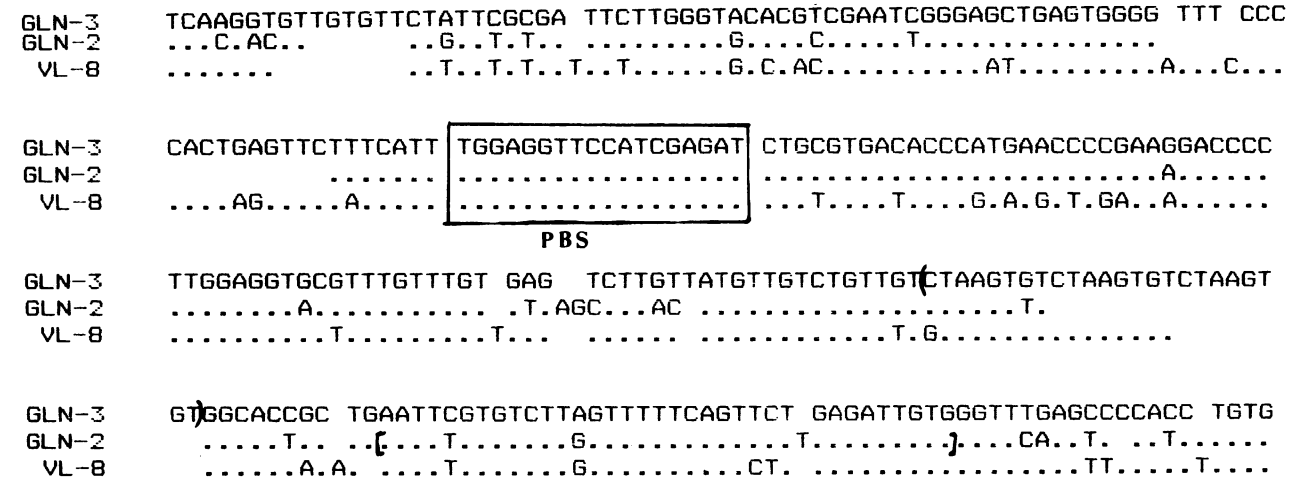

FIG. 4. Nucleotide sequences of PBS and surrounding regions in three different GLN clones. PBS sequences are boxed. Dots indicate identical nucleotides. The bracketed sequence in GLN-2 was tandemly duplicated but was shown only once (for illustrative reasons). The bracketed sequence in GLN-3 is a tandem triplication of the sequence CTAAGTGT.

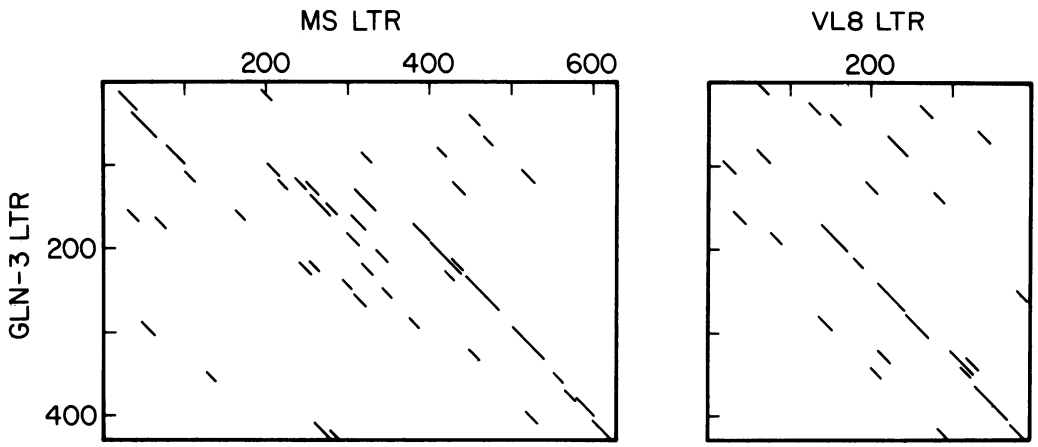

FIG. 5. Comparison of different GLN-LTRs by dot matrix analysis. LTRs were subjected to a dot matrix comparison by using the system described by Larson (14) with some modifications. A stringency of 8 of 10 matches was used. Comparisons shown are for GLN-3 LTR, VL8 LTR, and the LTR element found by Propst and Vande Woude (22) in the c-*mos* locus (and designated here as MS LTR).

hybridized a GLN-specific probe to a lambda phage library of BALB/c genomic DNA and determined the frequency of hybridizing clones. The probe used was a 3.4-kb *Eco*RI fragment of internal GLN-3 sequences (Fig. 2, fragment C). Sensitive blot hybridization experiments with cloned MuLV and VL30 DNAs showed that this probe did not detect VL30 or MuLV-related proviruses. From the frequency of the hybridizing genomic clones, we estimated that 20 to 50 GLN elements were present in mouse DNA (data not shown). Since this figure might be biased as a result of a nonrepresentative genomic library, we also estimated the frequency of GLN elements relative to the frequency of VL30 elements in duplicate replicas obtained from the same plating of the genomic library. (The copy number of VL30 elements has been estimated as 100 to 200 per genome by a measure independent of the cloning frequency [12]). In support of the above figure, we estimated that the reiteration frequency of GLN DNA was one-half to one-third that of VL30 elements (data not shown). A remarkably different result was obtained when a GLN-3 LTR-specific probe was used. The LTR probe used was a *Sma*I-*Bgl*II fragment that spans the majority of the LTR (Fig. 2C, probe A). With this probe, a copy number of 1,000 to 1,500 was estimated. This result indicates that the majority of GLN-type LTRs might be present either in other retroviruslike contexts or as solitary LTRs.

Retroviruslike elements are often specific to a particular animal species but may also contain related sequences in other species. We determined the evolutionary conservation of GLN DNA by screening genomic DNA blots from rodents other than *Mus musculus* and from nonrodents. The probe used for this survey was a 0.9-kb *Sac*I RI fragment that corresponds to the 3′ end of the element (Fig. 2C, probe D). A short DNA segment was used as a probe to minimize the potential problem of nonelement DNA sequences that might be embedded in GLN DNA. Also, the location of the probe sequences close to the element terminus allowed the detection of junction fragments. GLN DNA sequences were present in multiple copies in *Mus musculus* (Fig. 5). In other experiments, we detected multiple bands in other species of the genus *Mus* (data not shown). Similar results were obtained when a hybridization probe that corresponds to the 5′ end of the element was used (data not shown). Related sequences were also present in other rodents such as rat, Okomys, and guinea pig (Fig. 6). Extensive exposure times were required, however, to visualize GLN-related sequences in nonmouse DNAs. This suggests that only a partial homology exists between GLN DNA and the related sequences present in other rodents. The multitude of the hybridizing bands in rat, okomys, and guinea pig DNAs suggests that these organisms contain multiple copies of the related retroviruslike family. In nonrodent (chicken and human) DNAs, homologous DNA sequences could not be detected.

DISCUSSION

In this paper we describe the isolation and preliminary characterization of a novel murine retroviruslike family which we call the GLN family. DNA sequences of the new family were initially encountered as one of the components in an apparently recombinant retroviruslike element. Several retroviruslike genomes isolated from murine and human cells contain DNA segments that are related to more than one family of retroviruses or retroviruslike elements. These genetic units are thought to have arisen through recombination between members of the different families from which they are composed. Examples are retroviruslike elements that are composed of both MuLV-related and VL30 segments (8), the infectious M432 retrovirus that contains IAP-related sequences (3), and MuLV-related proviruses whose LTR units contain inserts derived from a family of solitary LTRs (10). A striking example of a resident retrovirus element that is a mosaic of DNA sequences of different origins is an element recently isolated from human DNA that contains DNA sequences related to D-, C-, B-, and A-type particles (2). The occurrence of these putative recombinant elements suggests that there have been genetic interactions between different retroviral classes during evolution. Specifically, resident retroviruslike elements may exchange DNA sequences with other cellular retroviruslike elements or with incoming exogenous retroviruses. This putative information transfer among retroviruslike elements makes it difficult to determine the evolutionary history of a particular recombinant element. As with other retrovirus-like families, the genetic origin of GLN elements is not known. If GLN DNA sequences are descendants of a once-competent retrovirus, then introduction of GLN sequences into mouse DNA must have occurred before speciation, and the progenitor retrovirus is probably extinct. Alternatively, GLN elements could have evolved from cellular DNA sequences.

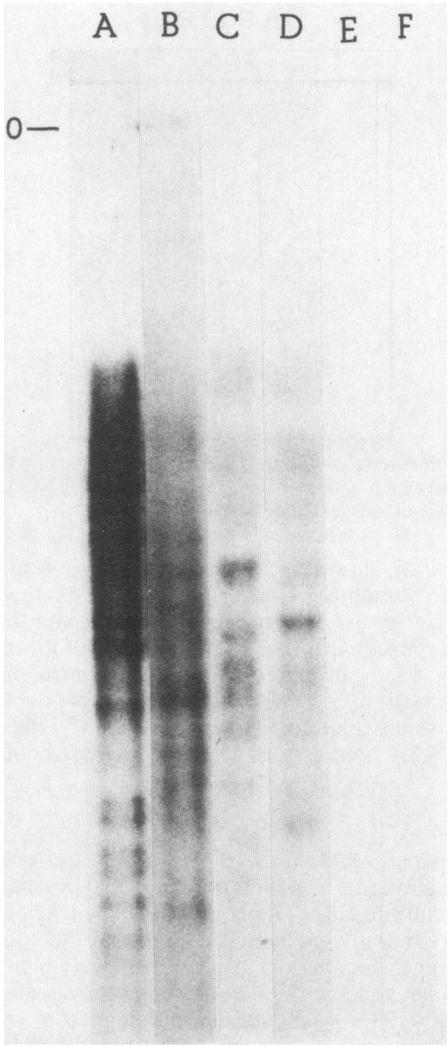

FIG. 6. Occurrence of GLN-related sequences in DNAs of different animal species. DNA (15 μg) from each of the indicated species was digested with *Eco*RI, electrophoresed through an agarose gel, and blot hybridized with a subgenomic GLN-3 DNA fragment (fragment D in Fig. 2C). DNAs: lane A, mouse; lane B, rat; lane C, Okomys; lane D, guinea pig; lane E, chicken; lane F, human. The autoradiogram was exposed for 1 day (lane A) or for 7 days (lanes B through F). O, Origin.

Since no member of the GLN family has been completely sequenced, it is not known to what extent internal GLN genes share homology with other retroviruses or retrovirus-like elements. To demonstrate the uniqueness of GLN DNA, we have focused our study on the nature of the ends of the elements, i.e., on the nucleotide sequences of the U_3, R, and U_5 regions (which together form the LTRs), on the two primer-binding sites, and on a stretch of DNA downstream of the 5′ LTR, where the packaging signal of transmissible viruses resides (18, 28). These genomic domains house all of the *cis*-acting functions required for retroviral transcription, reverse transcription, integration, and encapsidation. In certain replication-defective retroviruses, these are the only retroviral functions that are retained, while the bulk of internal protein-coding sequences are deleted or replaced. As shown above, all of these sequence domains in GLN3 were unique and unrelated to those of familiar retroviruses and retroviruslike elements. Noteworthy is the nature of the PBS sequence, often used to distinguish different classes of retroviruses. Most mammalian retroviruses use $tRNA^{Pro}$; an exception is mouse mammary tumor virus, which uses $tRNA^{Lys}$. However, the spectrum of tRNAs thought to serve as potential primers has recently been broadened with the discovery of additional PBS sequences in resident retroviruslike elements. This includes $tRNA^{Gly}$ (VL30) (6, 9), $tRNA^{Phe}$ (IAP) in murine elements, and $tRNA^{His}$, $tRNA^{Arg}$, and $tRNA^{Glu}$ in different human retroviruslike families (16, 21, 25).

Twenty to 50 copies of GLN elements are present in mouse DNA. LTR-related sequences are present, however, in a much higher copy number (approximately 1,000 to 1,500). We have not analyzed clones which contain LTR-related sequences in molecular contexts other than GLN elements. The homology between GLN LTR and the solitary LTR units found in the c-*mos* locus (22) (Fig. 5) suggests that at least a fraction of those LTRs might be present as solitary LTRs. Solitary LTRs have been found in both retrotransposons and retroviruslike elements, but the ratios of full-sized elements to their solo LTR counterparts are highly variable. In VL30 DNAs, for example, there are at least 20-fold more full-sized elements than solo LTRs (23). The factors that determine this variability are not known.

We are currently studying the transcription patterns of GLN elements and the nature of their coding regions.

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