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A novel approach to cloning transcriptionally active retrovirus-like genetic elements from mouse cells

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ABSTRACT

A family of dispersed, moderately repeated mouse genetic elements is expressed as retrovirus-like 30S RNA species (VL30 RNA) which can be transmitted to other cells when packaged as a pseudovirion complex by murine leukemia viruses (MuLV). Using the endogenous reverse transcriptase reaction of VL30 RNA-containing MuLV particles, full-length VL30 DNA was synthesized and cloned in pAT153. Analysis of a number of clones identified long terminal repeat structures (LTRs) characteristic of retrovirus proviruses and transposable genetic elements. Whilst the unique region of all clones was identical, the LTRs displayed some heterogeneity. Comparison of the unique region of cloned VL30 DNA with mouse genomic VL30 sequences showed the retrovirus-derived clones to be encoded by only a few members of the divergent VL30 gene family. These findings thus demonstrate a method for cloning a defined sub-class of retrovirus-like cellular genes which are both transcriptionally active and transmissible by a retrovirus.

INTRODUCTION

When murine leukemia viruses are propagated on rat or mouse cells the progeny virus particles are often found to contain cellular RNAs which share many structural properties with retrovirus genomic RNAs [1-4]. These virus-like 30S RNAs (VL30 RNAs) can also be transmitted to other cells when packaged as a pseudovirion complex by a murine leukemia virus [5]. By analogy with retroviruses, infection is probably accomplished by reverse transcription of VL30 RNA to produce "proviral" VL30 DNA which then integrates into host cell DNA. VL30 RNA thus resembles a defective retrovirus genome although nucleic acid hybridization and oligonucleotide mapping studies have not revealed any significant homology with known murine leukemia virus genomes [2-4,6].

In the mouse, VL30 RNA is encoded by about 150 dispersed genetic elements which exhibit a considerable sequence divergence [7]. Some of these sequences have been cloned from BALB/c mouse genomic libraries and have been shown to contain long terminal repeats (LTRs) characteristic of retrovirus proviruses [8-11]. However, as it is not known whether all members of the VL30 family are (a) capable of being transcribed, and (b) capable of being packaged and

transmitted by retroviruses, it is difficult to assess the potential biological significance of VL30 clones obtained by this method. To overcome these problems we have developed an alternative strategy for cloning retrovirus-transmitted VL30 genes. This exploits the ability of detergent-disrupted retrovirus particles to synthesize abundant quantities of retroviral DNA [12,13]. In this report we show that VL30 DNA can also be synthesized in such endogenous reactions. Furthermore we report the cloning of *in vitro* synthesized VL30 DNA and demonstrate that it is encoded by only a sub-set of the VL30 sequences present in mouse genomic DNA.

MATERIALS AND METHODS

Cells and Virus

Growth of NIH-3T3 cells and characterization of the NIH-3T3-derived Kirsten murine leukemia virus (Ki-MuLV) producer cell line A1, have been described previously [14].

Analysis of Viral RNA

Ki-MuLV-encapsidated RNA was resolved on a horizntal 1.0% (w/v) agarose gel in 50% de-ionized formamide, 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 0.005% diethylpyrocarbonate, pH 7.0. Sample preparation and conditions of electrophoresis were as described previously [6], except that RNA was heated at 65°C for one minute then rapidly cooled in ice prior to loading on the gel. Following electrophoresis the gel was washed in water then stained with ethidium bromide (1 μg ml^{-1}) for 30 min. Bands of RNA were visualized under UV light. Transfer of RNA from the gel to nitrocellulose paper was performed essentially as described previously for Southern blotting of DNA (15).

Source of Viral DNA

Purification of low mol. wt. DNA from NIH-3T3 cells recently infected with Ki-MuLV was performed essentially as described by Norton *et al.* [15].

Ki-MuLV for endogenous reactions was purified from the tissue culture fluid of A1 cells by membrane filtration (1.2 μm, Millipore) and concentrated by centrifugation. Further purification was achieved by pelleting virus through a 4 ml 20% (w/v) sucrose cushion in 50 mM Tris HCl (pH 7.5) at 240,000 g for 1.5 h at 4°C. Viral pellets were resuspended in a minimum volume of 50 mM Tris HCl (pH 7.5) and either used immediately or after storage at -70°C.

The retrovirus endogenous reaction relies on the viral reverse transcriptase for synthesis of double-stranded DNA copies of encapsidated RNA. The reaction takes place within the virion which is partially lysed to allow the entry of deoxyribonucleoside triphosphates. Using optimum conditions

large quantities of full-length proviral DNA can be obtained [12,13,15]. For Ki-MuLV maximum synthesis of full-length DNA was obtained at a virus concentration of 4 mg ml^{-1} in the presence of 0.02% (v/v) Triton X-100. Otherwise, reaction conditions were as described by Benz and Dina [12]. Endogenous reaction mixtures were incubated at 37°C for 12 h (longer incubations resulted in degradation of reaction products). In vitro synthesized DNA products were deproteinized and purified by S1 nuclease treatment followed by preparative gel electrophoresis, essentially as described previously [15].

Analysis of proviral DNA by restriction enzyme digestion and the Southern blotting technique has been described in an earlier publication [15].

Detection of Ki-MuLV and VL30 Nucleic Acid by Filter Hybridization

Filter hybridization, post hybridization washing and autoradiography were performed as described previously [15]. Ki-MuLV/VL30 cDNA probe (specific activity 10^9 cpm μg^{-1}) was synthesized from purified virion RNA templates as described by Avery et al. [16], except that 125 to 250 μ Ci ^{32}P dCTP of specific activity 2 to 3 x 10^6 Ci m mol^{-1} were routinely used. "Nick translation" of DNA (specific activity 10^8 cpm μg^{-1}) was performed following the method of Rigby et al. [17].

Cloning of VL30 cDNA

Molecular linkers containing the Bam HI recognition site (Bethesda Research Laboratories) were phosphorylated with polynucleotide kinase (Collaborative Research) and blunt-end ligated to VL30 cDNA species using methods described elsewhere (18). The DNA was then cleaved with Bam HI and linker fragments removed by Sephadex G-200 (superfine) chromatography. VL30 DNA was then ligated into Bam HI-linearized pAT153 vector DNA which had been treated with calf intestinal alkaline phosphatase (Boehringer-Mannheim) to reduce self-circularization. The resulting recombinants were used to transform E. coli HB101 cells. Ampicillin-resistant, tetracycline-sensitive colonies were grown up and plasmid DNA isolated by the cleared lysate procedure [19]. VL30 inserts were recognized by their length (approx. 5 kbp), and the ability to hybridize with Ki-MuLV/VL30 cDNA probe.

All cloning manipulations were carried out in accordance with guidelines published by the Genetic Manipulation Advisory Group.

RESULTS

VL30 DNA Synthesized in Virus-Infected Cells

Figure 1(a) shows an agarose gel profile of the virion RNA species of Ki-

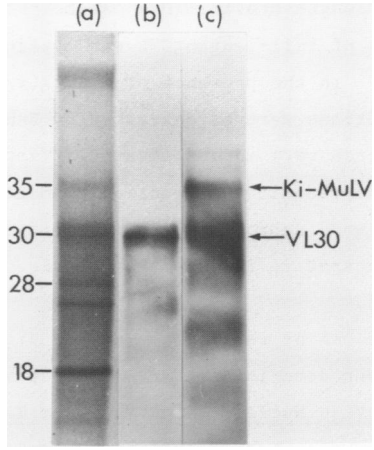

Figure 1. RNA species encapsidated by Ki-MuLV particles. Purified virions were lysed by addition of SDS to 0.5%. Nucleic acid was deproteinized by phenol : chloroform extraction and precipitated by ethanol. Following denaturation by heating to 65°C, RNA species were resolved by electrophoresis through a 1.0% (w/v) agarose/formamide gel. Size estimation was by comparison with mouse ribosomal RNA markers. (a) Ethidium bromide-stained gel photographed under UV light. (b) Northern blot of (a) annealed to ^{32}P-labelled "nick-translated" VL30 clone NVL-3 DNA. (c) The same filter re-annealed to ^{32}P-labelled cDNA synthesized from total poly (A)-containing Ki-MuLV RNA. In lane (a) the bands at positions corresponding to 28S and 18S markers represent virion-associated ribosomal RNA.

MuLV particles purified from the tissue culture fluid of NIH-3T3 mouse cells. Two major bands of RNA are visible; a slower migrating species of approx. 35S and a faster migrating RNA of approx. 30S (see Clewley and Avery [6]). The larger RNA is the genomic RNA of Ki-MuLV. The smaller, (30S) RNA species is identical in size and properties to RNAs described in other cell lines [2] that are designated VL30 RNAs [4,6]. The heterogeneous band pattern of the VL30 RNA seen in fig. 1(a) indicates that more than one species of VL30 RNA is packaged in Ki-MuLV particles.

The unintegrated proviral DNA found in cells recently infected by a retrovirus is predominantly linear in the cytoplasm and circular in the nucleus [15,20]. We therefore examined the low molecular weight fraction of DNA from cells 18-20 hrs after infection with VL30-containing Ki-MuLV to see whether unintegrated linear and circular VL30 DNA could be found. Fig. 2(a) lane (i) shows the result of Southern blot analysis of cytoplasmic DNA. Both Ki-MuLV and VL30 DNA species were detected using a cDNA probe synthesized from virion RNA. In addition some hybridization of probe to contaminating high

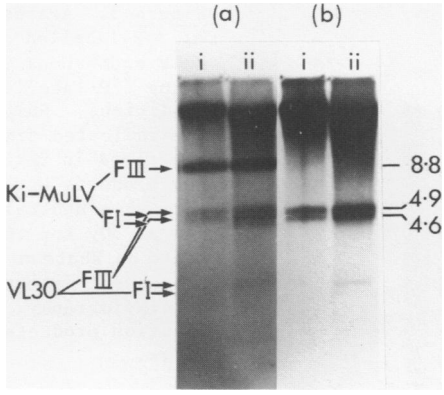

Figure 2. Unintegrated forms of Ki-MuLV and VL30 DNA. Low mol. wt. DNA was extracted from the cytoplasm or nucleus of NIH-3T3 mouse fibroblasts after recent infection with Ki-MuLV. Following electrophoresis through a 1.0% (w/v) agarose gel, DNA species were immobilized on nitrocellulose and hybridized with (a) Ki-MuLV/VL30 cDNA probe, and later (b) "nick-translated" VL30 clone NVL-3 DNA probe. Lanes (i) and (ii) cytoplasmic and nuclear DNA, respectively. Abbreviations used in this figure: FI, Form I DNA (supercoiled closed circular DNA); F III, Form III DNA (linear DNA). Sizes of DNA species are indicated in kilobase pairs.

molecular weight mouse DNA can be seen. Two major size classes of DNA (8.8 kb and 4.6-4.9 kb) were found. The larger of these is the Ki-MuLV linear proviral DNA which we have characterized in detail elsewhere (manuscript in preparation). The smaller species is of a size commensurate with that of the virion 30S RNA and its appearance as a doublet reflects the heterogeneity of the VL30 RNA. When the nuclear DNA fraction was examined, a more complex profile was found (fig. 2(a), lane (ii)). This was due partly to the presence of cytoplasmic DNA in the nuclear DNA preparation and also to the fact that closed circular Ki-MuLV DNA co-migrates with the linear VL30 DNA species. The inferred identities of the various species are depicted in fig. 2. For clarity we have shown the same Southern blot hybridized with a cloned VL30 probe (see later) in fig. 2(b). The nuclear fraction (lane (ii)) can be seen to contain both the linear cytoplasmic VL30 DNA species and a doublet migrating in a position expected for closed circular VL30 DNA. Thus, the unintegrated proviral DNA intermediates of VL30 appear to be exactly analogous to those found with retroviruses.

Synthesis and Characterization of in vitro VL30 DNA

When detergent-disrupted retrovirus particles are incubated in the presence of high concentrations of deoxyribonucleoside triphosphates, large

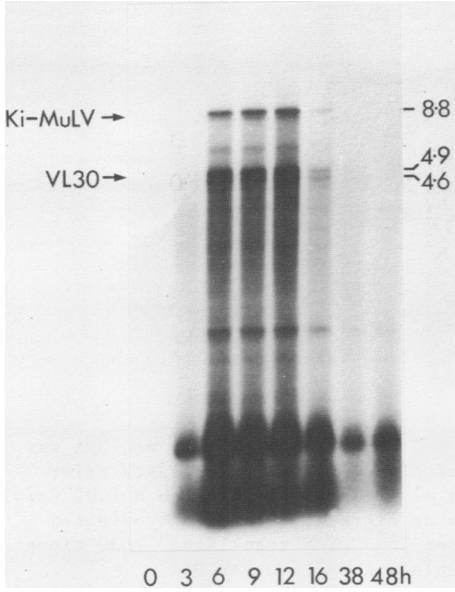

Figure 3. Agarose gel electrophoresis of ^{32}P-labelled in vitro DNA. A Ki-MuLV endogenous reaction was performed using ^{32}P-labelled dCTP under standard conditions. Samples were removed at the indicated times and purified as described in methods. Double-stranded DNA products were electrophoresed through a neutral 0.8% (w/v) agarose gel at 30v for 16.5 h. The gel was dried on Whatman DE-81 paper for subsequent detection of DNA species by autoradiography. Sizes of major reaction products are given in kilobase pairs.

amounts of full-length retroviral DNA are synthesized by the endogenous reverse transcriptase [12,13]. Such in vitro synthesized DNA is identical to that found in virus-infected cells [12,13,15] and has been shown to possess biological integrity by infectivity assays [13,21].

Figure 3 shows the time course of an endogenous reaction using purified VL30 RNA-containing Ki-MuLV particles. Discrete products can be seen to appear after 3 h and to accumulate up to a maximum of 12 h. Thereafter degradation occurs. The sizes of the two major reaction products (8.8 kb and 4.6-4.9 kb) are identical to the in vivo synthesized Ki-MuLV and VL30 DNA species; furthermore the in vitro VL30 DNA again appears as a doublet. We have estimated the yield of Ki-MuLV and VL30 DNA based on band intensity in ethidium bromide-stained gels to be about 100 ng and 200 ng respectively for an initial input of 4 mg virus (data not shown). This is comparable to data presented for other retroviruses published by ourselves [15] and others [12,13], and represents an approximately one thousand fold greater yield than that achieved in vivo. Unlike the latter source, in vitro-synthesized DNA is also essentially free of contaminating cell DNA.

In addition to the major reaction products shown in fig. 3, other DNA species can be seen. The sizes and relative amounts of these minor products are similar to those observed by workers with other retroviruses [12,13]. Such species arise as intermediates in the formation of the final endogenous

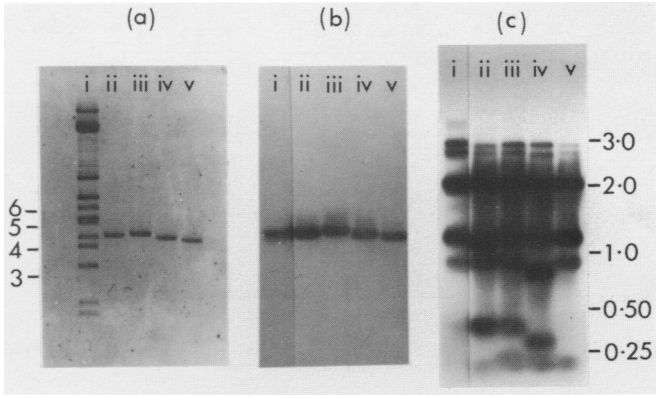

Figure 4. Characterization of VL30 cDNA clones. (a) Ethidium bromide-stained 0.6% agarose gel visualized under UV light. Lanes (ii) to (v) undigested VL30 clones NVL-1 to NVL-4, respectively. Molecular size reference in lane (i) provided by .Eco RI plus .Hind III restriction fragments. (b) Southern blot of a similar gel; lane (i) in this case contains uncloned VL30 cDNA for comparison. (c) Southern blot of Sac I restriction fragments of the samples shown in (b), following electrophoresis through a 1.5% agarose gel. Both (b) and (c) were hybridized with "nick-translated" VL30 clone NVL-3 DNA probe. Sizes of DNA species are given in kilobase pairs.

reaction products [22,23].

When gel-purified VL30 DNA was digested with restriction enzymes, the complexity of the resulting fragment patterns indicated that the VL30 DNA was composed of a relatively homogeneous population of molecules. An example of this is shown in fig. 4(c) lane (i). Here, in vitro VL30 DNA Sac I restriction fragments were separated by gel electrophoresis and detected after Southern blotting with a cloned VL30 DNA probe. This confirms earlier observations based on oligonucleotide mapping of virus-encapsidated VL30 RNA which similarly showed it to possess limited sequence heterogeneity [6].

Molecular Cloning of VL30 DNA

The relatively large quantities of pure VL30 DNA obtained from the endogenous reaction provide a convenient source of cDNA suitable for cloning. Since the enzyme Bam HI fails to cleave the majority of in vitro VL30 cDNA species (data not shown), we inserted the cDNA into the plasmid vector pAT153 using molecular linkers containing the Bam HI recognition site. VL30-containing recombinants were propagated in E. coli HB101 cells. The approximately 5 kb inserts were identified by their ability to hybridize with the Ki-MuLV/VL30 cDNA probe. In addition, VL30 clones hybridize specifically to virion VL30 RNA. Figure 1 shows, for example, the virion RNA species

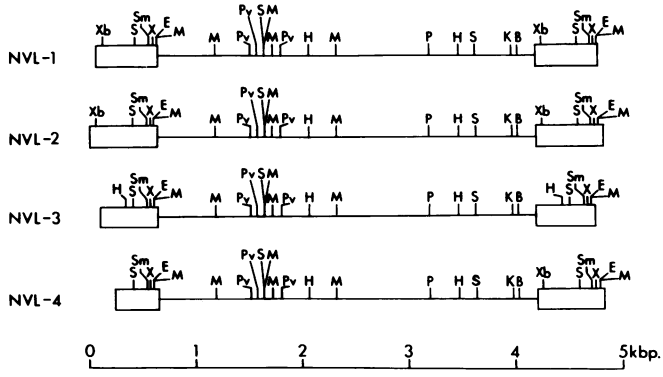

Figure 5. Physical map of VL30 cDNA clones NVL-1 to NVL-4. Restriction enzyme abbreviations used: Xb, Xba I; S, Sac I (Sst I); Sm, Sma I; X, Xho I; E, Eco RI; M, Msp I; Pv, Pvu II; H, Hind III; P, Pst I; K, Kpn I, B, Bgl II. Boxed areas indicate LTR sequences. Enzymes Bam HI and Sal I do not cleave NVL clone DNA. The scale indicates length of DNA in kilobase pairs.

hybridized to either a VL30 clone (b) or the cDNA probe (c). Thus, the cloned VL30 species do not represent truncated Ki-MuLV molecules, a conclusion which is supported by restriction enzyme analysis of Ki-MuLV DNA (data not shown). Also, in fig. 2(b) a VL30 clone can be seen to hybridize to both size classes in the doublet of in vivo VL30 DNA. Similar results were obtained with other VL30 clones (data not shown).

A total of 12 independently isolated clones obtained in two separate cloning experiments could be assigned to only four different classes on the basis of size and restriction fragment patterns.

The range of sizes displayed by the four types of VL30 is shown in fig. 4. Section (a) is an ethidium bromide-stained gel. Section (b) is a Southern blot on which the VL30 clones have been run in parallel with uncloned DNA for comparison. Figure 4(c) demonstrates that the VL30 clones are representative of the uncloned in vitro VL30 DNA since common Sac I restriction fragments are detected by Southern blotting.

Detailed restriction maps of the four classes of VL30 clones are presented in fig. 5. We have adopted the nomenclature "NVL" (from NIH VL30). Of the twelve NVL clones isolated, eight were of the type designated NVL-3, two were of the NVL-2 type and NVL-1 and NVL-4 were each represented once. It can be seen from the relative positions of restriction enzyme sites that all possess long terminal repeats (LTRs), wherein reside the only apparent differences between the four VL30 types. The lengths of these sequences vary

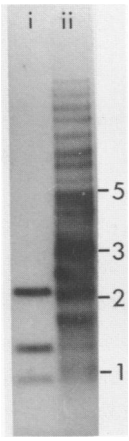

Figure 6. Detection of VL30 clone NVL-3-related sequences in NIH-3T3 DNA. DNA samples were digested to completion with Sst I and run on a 0.8% agarose gel. Following transfer of restriction fragments to nitrocellulose, the filter was hybridized with "nick-translated" VL30 clone NVL-3 DNA probe. (i) Approx. 275 pg VL30 clone NVL-3 DNA added to 10 µg Eco RI-digested calf thymus high mol. wt. DNA. (ii) 10 µg high mol. wt. NIH-3T3 DNA. Sizes of DNA species are given in kilobase pairs.

from approximately 550 to 630 bp. NVL-4 possesses an incomplete LTR at one end of the molecule; this probably arose as a consequence of aberrant reverse transcription. Such molecules can be predicted to occur from a consideration of models of retrovirus reverse transcription [22,23]. The unique region of sequence which is bounded by the LTRs appears to be identical for all four types of clone.

VL30 Sequences in Mouse Chromosomal DNA

It has been shown previously that at least some VL30 elements in mouse DNA possess a provirus-like structure, where cell DNA sequences are linked to the VL30 LTRs [9,11]. In order to compare the sequence organization of our cloned VL30 elements with related sequences in mouse DNA, use was made of the enzyme Sac I (Sst I) which cleaves all NVL DNAs at four sites (fig. 5). The three internal fragments generated thus serve as markers to identify genomic VL30 elements which are very closely related or identical to our clones. Figure 6 lane (ii) shows the result of annealing cloned VL30 probe to an Sst I digest of NIH-3T3 DNA. Intense bands seen on the autoradiograph represent multiple copies of internal restriction fragments of genomic VL30 elements. Unique restriction fragments such as those containing VL30/cell DNA junction sequences would be lost in the background hybridization "noise". It can be seen from the digest pattern in fig. 6 lane (ii) that Sst I digestion of mouse genomic DNA generates multiple copy restriction fragments which co-migrate with the cloned VL30 DNA marker fragments in lane (i). By comparison of band intensities on the autoradiograph we estimate the copy number of such closely related VL30 elements in mouse DNA to be approximately 2-4 per haploid genome. This figure would represent less than 5% of the total VL30-related sequences

which are estimated to be dispersed throughout mouse DNA [7].

DISCUSSION

Our studies demonstrate that large quantities of pure VL30 DNA can be synthesized in an endogenous reaction using purified MuLV particles. This DNA is similar to that found in recently infected cells, and restriction enzyme analysis shows it to possess a complexity which is comparable to that of virion VL30 RNA. Several clones were obtained using this source of DNA and all were shown to have LTR structures characteristic of retrovirus proviruses and transposable genetic elements [24]. In this respect our NVL clones resemble examples of VL30 elements cloned from BALB/c mouse genomic DNA [9,11]. The 400 to 500 bp-long LTR regions of the latter genomic clones, however, are notably shorter than the 550 to 630 bp NVL LTRs. At present, the significance of this observation is not clear. The NVL clones, nonetheless, would be expected to differ from randomly isolated genomic VL30 elements in two ways; (a) they are complementary to transcriptionally active members of the VL30 family, and (b) they represent VL30 RNAs which are both selectively packaged by murine retroviruses and are templates for reverse transcriptase. Therefore, they are probably transmissible to other host cells [5]. We have recently obtained direct evidence for such transmissibility by the detection of newly integrated NVL DNA in Ki-MuLV-infected rat cells (unpublished data).

We could identify only four types of VL30 cDNA insert by restriction enzyme mapping of our NVL clones. Areas of sequence heterogeneity were confined to the LTRs, whereas the unique region bounded by these structures was common to all clones. The corresponding LTRs of retrovirus proviruses are known to contain sequences involved in the regulation of eukaryotic gene expression [24]. It is possible therefore that differences in VL30 LTRs may result in different levels of transcriptional activity, which could account for the variation in frequency of isolation of the four types of VL30 clone. Alternatively, genomic copy number and the efficiency of viral packaging and reverse transcription may account for the relative abundance of different VL30 elements obtained by this approach.

We have shown by comparison with murine genomic DNA that our VL30 clones represent a sub-set of the divergent VL30 gene family. We estimate that NVL VL30 elements are encoded by a maximum of 5% of the members of this family. Such a figure would be consistent with the notion that only a limited number of VL30 elements may be transcriptionally active. Indeed, studies on the methylation status of VL30 members have shown the majority to be

hypermethylated (unpublished data and [7]).

Recently, Giri et al., [25] showed the existence of discrete regions of homology shared by a rat VL30 element, a VL30 element derived from BALB/c genomic DNA and cloned representatives of both ecotropic and xenotropic MuLV proviruses. This suggests that recombination events with VL30 elements occurred early in MuLV evolution. Although in our analysis of NVL VL30 clones no homology with the ecotropic Ki-MuLV genome was detected, we did not employ the low stringency hybridization conditions used by these workers. It would be of interest to determine whether our retrovirus-transmitted VL30 elements contain similar regions of MuLV-related sequence which might be involved in the co-packaging with MuLV genome RNAs.

No biological role has yet been found for members of the VL30 gene family. However, as discussed earlier these elements do possess LTR structures which by analogy with those of retrovirus proviruses probably contain regulatory sequences for transcription. Since VL30 elements are transmissible by retroviruses, they could therefore play a role in MuLV-induced malignant transformation. Thus, a newly integrated VL30 element could cause activation of an adjacent cellular proto-oncogene by a "downstream promotion" mechanism similar to that proposed for some weakly oncogenic retroviruses [26]. The molecular cloning of such retrovirus-transmissible VL30 elements is a first step toward investigating this possibility.

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