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Insertion elements and transitions in cloned mouse mammary tumour virus DNA: further delineation of the poison sequences

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ABSTRACT

The provirus of mouse mammary tumour virus (MMTV) is reputed to contain sequences within the viral gag gene that prevent or inhibit its propagation as a recombinant DNA clone in Escherichia coli. Here we report the successful isolation of several λ and plasmid clones comprising the 5' virus-host DNA junction fragments from integrated MMTV proviruses in BR6 mice. Although the λ clones appeared intact, almost all of the plasmids were found to contain the bacterial insertion sequences IS1 or IS2 within a small region of the gag gene. One non-disrupted clone was recovered which had undergone multiple G to A transitions, some of which created stop codons in gag. These results have provided more precise information as to the location of the poison sequences and are discussed in relation to possible explanations for the phenomenon.

INTRODUCTION

In early attempts to clone the DNA provirus of mouse mammary tumour virus (MMTV), several laboratories reported either the anomalous behaviour or non-recovery of the required recombinants in otherwise representative libraries (1-5). These repeated observations led to the suggestion that certain MMTV sequences, for which the term "poison sequence" was coined, might be inimical to the growth of prokaryotic hosts, a concept that has been sustained by subsequent anecdotal reports and has until recently frustrated attempts to complete the characterisation of the MMTV genome (1-11).

The majority of studies on MMTV proviral DNA exploit the single EcoRI site close to the mid-point of the genome (Fig.1). Thus, typical cloning strategies have involved construction of libraries from partial or total EcoRI digests of DNA from infected cells. In the latter situation, the resultant clones comprise the junctions between viral and cellular DNA and

statistically the numbers of 5' and 3' ends recovered should be roughly equal. In practice, particularly when dealing with milk-transmitted MMTV, the number of 3' junctions obtained far exceeds 5' junctions, and it was from this observation that the notion of a poison sequence was originally formed (1-5). Indeed, there have been only sporadic successes in the isolation of 5' junction fragments, and most of those have involved endogenous MMTV proviruses (8,10,12-17). Curiously, in extensive analyses of the milk-transmitted MMTV proviruses characteristic of the BR6 strain of mice, we have reproducibly isolated 5' and 3' ends with roughly equal frequency, in apparent contradiction to earlier reports. However, closer inspection of these clones has revealed consistent rearrangements during transfer of the DNA into plasmid vectors. Significantly, most of the abnormalities map within a 250 base pair region in the MMTV gag gene as predicted from previous studies on the poison sequence. These observations are discussed in the context of our recent delineation of the protein coding domains of the MMTV genome (11).

MATERIALS AND METHODS

Preparation and analysis of DNA

High-molecular-weight DNA was prepared from tissues of BR6 mice by pronase digestion and phenol extraction as described elsewhere (18). A similar procedure was adopted for isolation of DNA from log-phase cultures of the E.coli strains LE392, DH1 and HB101 and for λgtWES.λB. Our methods for restriction enzyme digestion, agarose gel electrophoresis and Southern blotting have been presented in previous publications (18,19). Radioactive probes were prepared by nick translation of gel-eluted fragments, hybridisations were performed in 50% formamide at 42°C, and the hybridised filters were washed in 0.1 x SSC/0.1% SDS at 65°C prior to autoradiography (18).

Cloning of MMTV proviral DNA

BR6 mammary tumour DNA was digested to completion with EcoRI, and ligated into the separated arms of λgtWES (20). Packaged phage were plated on E.coli LE392 and screened by standard procedures using probes specific for the MMTV LTR, the MMTV 5' or 3' sequences, or in some instances with probes for

the int-1 and int-2 genes (18,19,21,22). Positive phage were plaque purified and propagated in LE392 as liquid lysates (20). For small scale DNA preparations, phage were recovered from 20-30 ml lysates by high speed centrifugation and extracted by treatment with pronase and SDS followed by deproteinisation with phenol and chloroform. Samples (1 to 2 μg) of λ DNA were then digested with EcoRI and ligated directly into EcoRI-cut and phosphatase-treated plasmid DNA (20). Most of the clones described were in pAT153, though in some instances the EcoRI fragments were inserted into the pSP65 vector. Plasmids were transfected into E.coli HB101 or DH1 by normal procedures and colonies were screened by transfer to nitrocellulose and hybridisation to nick translated DNA probes (23).

DNA sequence analysis

Selected restriction fragments were cloned in defined orientations into the M13 vectors mp8 and mp9 (24). In some instances, fragments were further digested with HaeIII or AluI to generate smaller segments and cloned into the SmaI site of the M13 vectors. DNA sequences were determined using the chain termination method of Sanger, primed by a synthetic oligonucleotide, and the sequences were compiled using the DBUTIL programme of Staden (25,26).

RESULTS

Isolation of recombinant clones of MMTV proviral DNA

The studies we describe here evolved out of our interest in the sites of proviral integration in BR6 mouse mammary tumours, particularly with reference to the putative cellular oncogenes int-1 and int-2 that are common targets for insertional mutagenesis by MMTV (18,19,21,22). The clones characterised in most detail were therefore those of the milk-transmitted MMTV of BR6 mice inserted into either of these loci, but many others were recovered of proviruses in otherwise anonymous regions of the genome. We also obtained clones comprising the endogenous, germ-line-transmitted MMTV sequences characteristic of this mouse strain, designated Mtv-8, Mtv-17 and Mtv-21 (10 and Fig.1).

All of these recombinants were generated from EcoRI-digested DNA and therefore contained either 5.8 kb of DNA from

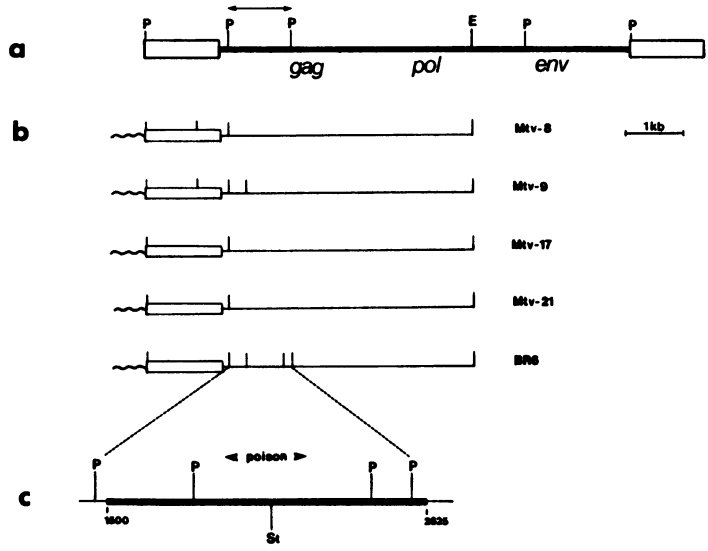

Figure 1. Physical map of the MMTV provirus and location of poison sequences. a) A prototype MMTV provirus is depicted, indicating the sites of cleavage for the restriction enzymes PstI (P) and EcoRI (E) and the 1.1 kb PstI fragment to which the poison sequences were previously mapped (3,4,6,7). b) Polymorphism of MMTV proviruses is illustrated with reference to the PstI sites within the 5' ends of four endogenous loci (Mtv-8, 9, 17 and 21) and the milk-transmitted virus of BR6 mice. ~~ represents flanking cellular DNA. c) The relevant region of the BR6 virus is expanded in order to relate the segment whose sequence is displayed in Fig.4 (bold line) to the physical map of the provirus. The cleavage sites for PstI (P) and StuI (St) and the apparent location of the poison sequences are shown.

the 5' portion of the virus or 4.1 kb from the 3' end, together with variable extents of flanking cellular DNA (11 and Fig.1). Since the average size of these fragments was around 8 to 10 kb, they were ideal for cloning into the λgtWES vector (20) and should have been recovered in approximately equal numbers. However, our normal strategy for characterising the recombinants was to transfer the EcoRI inserts directly from mini-preps of λDNA into the plasmid vectors pAT153 or pSP65. Curiously, the efficiency with which this transfer was achieved was extremely variable and in many instances necessitated high density screening of bacterial colonies in order to identify rare recombinants. In line with our expectations regarding the poison sequences, the difficulties were largely confined to clones

Table 1. Designation of MMTV DNA clones and nature of disruption.

CLONE NUMBER	5' OR 3' JUNCTION	LOCATION	INSERTION/ORIENTATION	
705	5'	int-2	IS1	←
707	5'	Mtv-21	IS2	N.D.
709	5'	int-2	IS1	→
711	5'	int-2	IS1	→
712	5'	Mtv-21	IS2	N.D.
719	5'	Mtv-17	IS1	N.D.
903	5'	int-1	IS1	←
924	5'	int-2	IS2	→
1101	5'	int-2	IS2	←
501	5'	N.D.	Multiple G to A transitions	
503	3'	N.D.		
507	5'	N.D.	Intact provirus (ref. 11)	
502	3'	N.D.		

N.D. = Not determined.

The clones were numbered systematically such that 701-719 were all from library No. 7, etc., and were separated into 5' and 3' junctions based on hybridisation to specific MMTV probes (10). Further analysis with site specific probes identified those proviruses located within the int-1, int-2, Mtv-17 or Mtv-21 loci. The 501/503 and 507/502 pairs represent the matched 5' and 3' ends of single proviruses that have permitted complete characterisation of the MMTV provirus (11). The presence and orientation of bacterial insertion sequences IS1 and IS2 in the various clones are indicated.

comprising 5' junction fragments, but were encountered with both the milk-transmitted and endogenous proviral sequences. Nevertheless, we have successfully characterised a number of 5' junctions, recovered from DNA libraries of four separate BR6 tumours (see Table 1).

Characterisation of cloned MMTV DNA

The EcoRI fragments recovered as recombinant plasmid clones were characterised by detailed restriction mapping (10). The

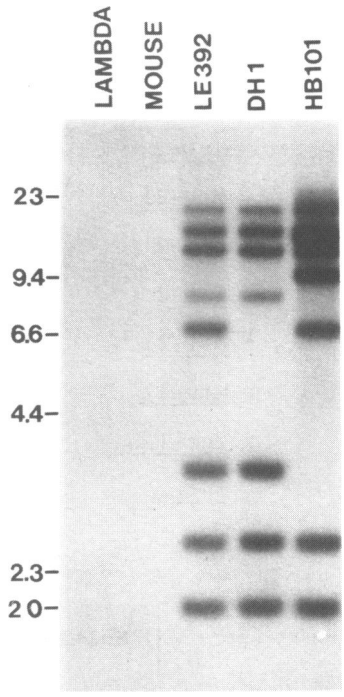

Figure 2. Origin of inserted sequences in MMTV DNA clones. The figure shows a Southern blot of DNA from the indicated sources, digested with PstI, and probed with a 1.0 kb HinfI fragment derived from the additional DNA in clone 924. Numbers on the left indicate the size (kb) and positions of HindIII-digested lambda DNA markers.

sizes of the various digestion products were calculated relative to a series of DNA standards of known sequence and the derived maps were estimated to have an accuracy of around 50 base pairs. In the course of these analyses it became apparent that almost all of the 5' junction fragments had inconsistencies within the viral sequences which could not be accounted for by errors in measurement, co-migration of bands, or partial digestion. More significantly, some of the plasmid DNAs yielded mixed restriction patterns so that we were able to attribute the problem to insertion of DNA within the MMTV provirus.

As several of the clones had insertions of roughly the same size, around 1.3 kb, we were interested in determining the origin of this additional DNA, and whether the same DNA was present in

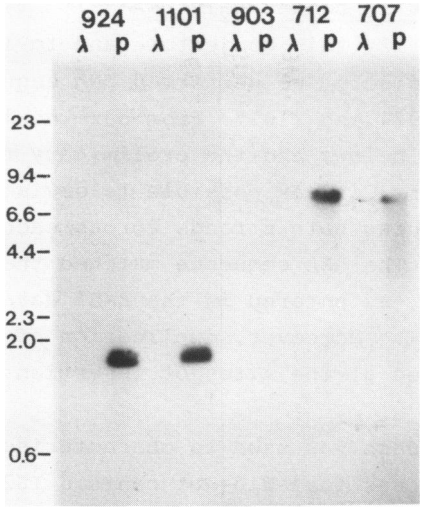

Figure 3. Acquisition of inserted sequences in multiple MMTV DNA clones. Approximately 1 μg of either the (λ) or plasmid (p) DNA for each of the numbered MMTV 5'-end clones (Table 1) was digested with PstI, blotted onto nitrocellulose and hybridised to the 1.0 kb HinfI fragment specific for the additional DNA in the 924 plasmid. Numbers on the left indicate the positions and sizes of marker DNAs. Both the 924 and 1101 clones contain DNA from the BR6 milk-borne virus in which the insertions map within the 0.65 kb PstI fragment whereas 707 and 712 contain the 5' end of Mtv-21 in which the disrupted fragment would be 4.3 kb in size (see Fig.1). The 903 plasmid was subsequently shown to contain a different insertion sequence (see text).

independent clones. A 1.0 kb HinfI fragment was therefore isolated from clone 924 for use as a probe specific for the inserted DNA. Figure 2 shows the results of Southern blotting analysis on DNA from normal mouse tissue, the λ vector used in cloning, and from the commonly used E.coli hosts LE392, HB101 and DH1. These data indicated that the additional DNA in clone 924 was derived from the E.coli chromosome. Moreover, different strains of E.coli contained multiple copies at several different locations, some of which were apparently conserved between strains (Fig.2). However, the key observation, illustrated in Figure 3, was that the same sequence was present in four independent plasmid clones, though not in the λ clones from which they were derived. Taken together, these findings suggested that the discrepancies in the proviral maps may have been caused by bacterial insertion sequences (IS elements).

Nature of insertions in cloned MMTV DNA

In order to verify this conjecture and to locate the insertions more precisely, we performed DNA sequence analysis on two of the clones, 924 and 1101. From our knowledge of the MMTV provirus (10,11 and below) and the preliminary restriction mapping of the insert, it was possible to devise an effective strategy for sequencing both strands across each of the insertions (not shown). The DNA sequence matched that of the transposable element IS2, as entered in the EMBL data bank, in all but 4 positions (27). Moreover, duplication of 5 base pairs of MMTV DNA had occurred at the sites of insertion as expected for IS2 (28).

A similar approach was used to characterise inserted sequences in the clones that did not contain IS2. Here the DNA sequence was only obtained across one or other end of the IS element, but in all cases the derived sequence matched that of IS1, as summarised in Table 1 (29). To date, we have not encountered any other IS elements in clones which have been analysed in any detail.

Position of IS elements in the MMTV genome sequence

We have recently established the complete nucleotide sequence of an integrated MMTV provirus from one of the BR6 mouse mammary tumours (11). The success of this venture relied on sequencing the 5' junction directly from a λ clone (507 in Table 1) and we have not succeeded in transferring this particular EcoRI insert into a plasmid vector. It was therefore of considerable interest to determine exactly where in the MMTV genome the various IS elements were located in different clones. Figure 4 presents the DNA sequence from nucleotides 1500 to 2625 of the MMTV provirus in which the N-terminal region of the MMTV gag gene precursor, Pr77, is depicted in single-letter amino acid code (11). Superimposed on this sequence are the known locations and orientations of IS1 and IS2 elements in the numbered clones discussed in Table 1, as determined by DNA sequence analysis across one or both ends of each element. While there is no apparent sequence or directional specificity for the insertions, it is noticeable that most are clustered within a 150 base pair region between the PstI site at nucleotide 1809

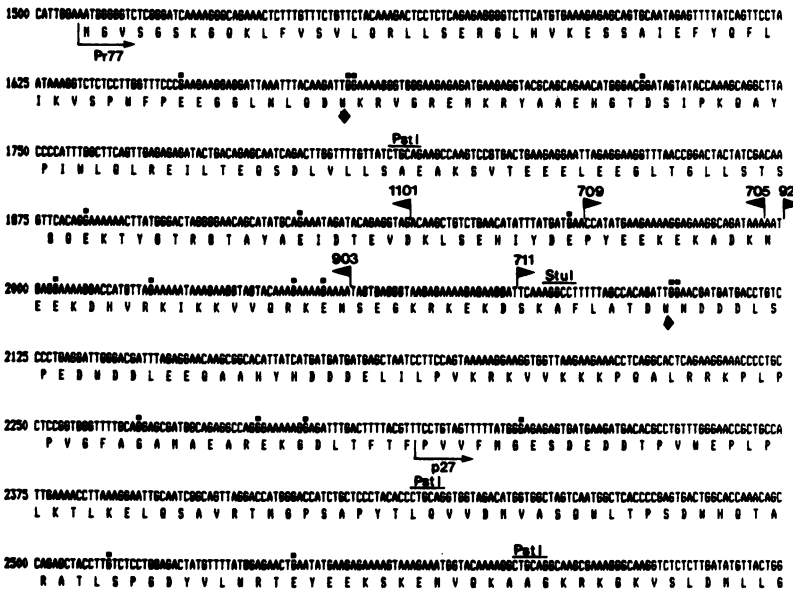

Figure 4. Location of IS elements and transitions in the MMTV gag sequence. The DNA sequence of clone 507 is presented, extending from nucleotides 1500 to 2625 of the provirus, together with the predicted amino acid sequence of the gag gene in single letter code. Superimposed on this sequence are the specific G to A transitions detected in clone 501 (■) with the resultant stop codons depicted as ◆. The positions of PstI and StuI restriction sites and the N-termini of the Pr77gag precursor and major core protein p27 are as indicated. The arrowheads portray the location and orientation of bacterial IS elements in the numbered MMTV DNA clones (see Table 1).

and the StuI site at 2085 (Figs. 1 and 4). However, some insertions, such as in clones 707, 712 and 719, occurred 3' to the StuI site. The positions of these were not determined by sequence analysis, but from restriction enzyme analysis (not shown) we estimate that they must occur within 1-200 base pairs of the StuI site.

Base transitions in cloned MMTV DNA

In addition to the matched EcoRI fragments (507/502) that permitted sequencing of an intact MMTV provirus (11) we isolated a second pair of junction fragments (501/503) that also represented the 5' and 3' ends of a single provirus. Clone 501 was of particular interest since it was successfully propagated as a

plasmid but showed no evidence of disruption by an IS element. The 501/503 pair were therefore considered ideal templates for DNA sequencing and for preliminary infectivity studies as a reconstructed provirus. However, it quickly emerged that the DNA was unlikely to be infectious due to the presence of stop codons within the presumed gag gene. Subsequent comparison with the apparently normal 507 fragment, sequenced directly from a λ clone, revealed that 501 had undergone 50 G to A transitions, of which 8 gave rise to stop codons in the gag, pro or pol domains (11). As shown in Figure 4, two of these stop codons occurred in the vicinity of the presumed poison sequences, though the significance of this is uncertain since others were distributed fairly randomly throughout the 5' region of the provirus. Curiously, many of the transitions occurred in A-rich regions and gave rise to lysine codons, but it seems likely that they were present in the original provirus rather than being a consequence of cloning since identical transitions were detected within both the 501 and 503 LTRs (not shown).

DISCUSSION

Numerous independent reports have alluded to problems encountered in manipulating MMTV DNA clones, but it is evident that these difficulties may be manifest in different ways. Thus, some laboratories have observed deletions, here we describe insertions and transitions, while in other situations the requisite clones could not be detected at all (1-10). Many of these differences could be explained by variations in procedures, for example the choice of vectors, the strain of MMTV, and the use of integrated versus non-integrated proviral DNA or amplified versus non-amplified libraries. From our experience, we consider it important to avoid amplification of libraries, having observed that recombinants containing the 5' MMTV sequences grow poorly and yield abnormally small plaques. Similarly, transfer of these fragments into a plasmid vector yields bacterial colonies of below average size. Indeed, many of the clones we describe proved extremely reluctant to regrow following transfer to nitrocellulose for high density screening and their growth in liquid culture was initially very slow. We assume therefore that our

tenacity in propagating these colonies exerted strong selective pressure for variants that grew well and that acquisition of an IS element is simply one of several ways in which this might be achieved. Two significant observations support this notion: a) that IS elements were not present in the original λ clones, suggesting that they were acquired upon transfer of the MMTV fragments into plasmids (Fig.3), and b) that we occasionally saw plasmid DNA preparations which were mixtures of the normal and disrupted patterns, suggesting that one evolved from the other during propagation. However, given that IS elements transpose relatively infrequently (10^{-4} to 10^{-7} per generation) and integrate essentially at random (28) we see no obvious reasons for the preponderance of insertions in our clones, rather than deletions or other forms of rearrangement.

What then is the feature that makes the MMTV provirus so inhibitory to bacterial growth? Previous studies on the milk-transmitted viruses of the GR and C3H mouse strains had localised the poison sequences to a 1.1 kb PstI fragment in the viral gag gene (Fig. 1 and refs. 3,4,6,7). Our observations are entirely consistent, given that the BR6 virus has two additional PstI sites in this region (11, and Fig. 1). Unfortunately the DNA sequence in the vicinity is unremarkable (Fig. 4), a fact noted previously by Fasel et al. who, somewhat surprisingly in the light of other experience, succeeded in cloning the 1.1 kb fragment from unintegrated GR viral DNA (6). There are 89 base pair differences between their sequence and ours including a small shift out of and into frame (nucleotides 1638-1662) but the essential features with regards to protein coding potential are conserved. More particularly, only two of the base changes correspond to any of the G to A transitions observed in our BR6 clone 501, and there is no apparent clustering of the differences around the region occupied by IS elements in other clones.

Obviously, the mapping of IS1 and IS2 insertions in various clones focuses attention on a much smaller region, extending for only 200-300 base pairs around the StuI site. Within this domain, there appears to be no specificity as to where and in what orientation the IS elements insert (Fig. 4) and no prevalence for either IS1 or IS2 in particular libraries or particular

clones (Table 1). For example 705, 709 and 711 are three independent isolates of the same EcoRI fragment in which IS1 has inserted in different locations and in different orientations. One curious note, however, is that the three clones in which the IS elements were 3' to the StuI site were derived from endogenous MMTV proviruses, while those on the 5' side were from the milk-transmitted BR6 virus (10, and Table 1). The numbers involved are clearly too low to draw firm conclusions, but the distinction between exogenous and endogenous sequences raises an important issue.

Several laboratories, including ours, have repeatedly cloned the 5' end of the endogenous Mtv-8 locus in both lambda and plasmid vectors and with no evidence for rearrangements (8,9,13-16). In contrast, we have only once isolated an Mtv-9 5' end, despite screening several large libraries of BALB/c DNA, though again the derived plasmid clone was apparently intact (10). From BR6 mice we recovered only one copy of the Mtv-17 5' end and three of Mtv-21 out of over 5 x 10^6 recombinant phage, and observed IS disruptions in all but one of the derived plasmid clones. Even at the crude level of restriction enzyme mapping, with PstI for example, it is clear that MMTV proviruses are polymorphic in the vicinity of the poison sequences (Fig. 1 and ref. 10). It seems likely therefore that the potency of the inhibitory elements in different exogenous and endogenous MMTV proviruses may vary considerably as a result of differences in DNA sequence.

Examination of the DNA sequence alone gives no definite clue as to why it should be so inimical to bacterial growth. One possibility is that the poison sequence mimics a binding site for an important E.coli protein, such that normal growth control is perturbed. The potency of such sequences would be dependent on their copy number and accessibility within the cell. Thus the same element carried within lambda DNA may pose less of a problem, particularly as phage infection results in cell lysis irrespective of the presence of the additional DNA. However, since disruption of the region can occur anywhere within 200-300 base pairs, the binding site would have to be unusually large, or complex in terms of protein-DNA interactions.

Alternatively, the poison sequences may mimic a function in bacterial DNA, such as an origin of replication or a transcriptional promoter. A prokaryotic promoter has indeed been detected in MMTV DNA, but this particular example was located in the LTR rather than the poison region (31). However, these effects would only interfere with plasmid replication or expression of drug resistance and could therefore be compensated for *in trans* by a plasmid in which the poison phenomenon had been abrogated. Moreover, disruption of our clones occurred irrespective of orientation of the MMTV sequences in the plasmid (data not shown).

A third, and perhaps the most tenable possibility is that the poison sequences might encode a protein that directly inhibits bacterial growth. Although the exact nature of any protein or oligopeptide product remains entirely speculative, the only substantial open reading frame in the region is that for the viral *gag* gene (11). The major *gag* precursor, Pr77, encompasses at least five low molecular weight proteins, p10, pp21, p8, p27 and p14 (30). The latter two, representing the major core protein and nucleic acid binding protein respectively, have been positioned in the DNA sequence by partial amino acid sequencing of their N-termini (11). Thus, p27 begins with PVVF... at nucleotide 2315, roughly 300 nucleotides distal to the major concentration of IS element insertions and probably downstream of others that have been less accurately mapped (Fig. 4). At present, we have no data that would allow us to locate the termini of pp21 and p8 in the DNA sequence, but have noted that about 40 amino acid residues preceding the start of p27 the sequence is extremely basic, consistent with the apparent properties of p8. Since most of the insertions map upstream of this motif, our conjecture is that they must occur almost exclusively within the coding domain of the virion phosphoprotein pp21 (30).

The existence of stop codons in the relevant domains of clone 501 would obviously support this interpretation (Fig. 4). On the other hand, the endogenous *Mtv-8* locus has been shown to support expression of apparently normal and functional *gag* protein and yet has proved readily clonable in plasmids (9,13, 14). It will therefore be important to compare directly the *gag*

protein sequences encoded by the various endogenous and milk-borne MMTV proviruses. However, the unfortunate dilemma that the poison phenomenon poses is that a precise molecular explanation would be best achieved by recombinant DNA manipulation of the unclonable sequences. Future progress may therefore depend on the development of E.coli strains in which the effect is less pronounced or the exploitation of different vector-host systems.

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