Amplification of the ribonucleotide reductase small subunit gene: analysis of novel joints and the mechanism of gene duplication in vaccinia virus

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ABSTRACT

Amplification of the M2 gene encoding the small subunit of ribonucleotide reductase (EC 1.17.4.1) was analyzed in a collection of vaccinia virus (VV) isolates selected for resistance to 5 mM hydroxyurea (HU). Most of the mutants harbored tandem direct repeat arrays of the M2 gene, but several had duplicated M2 as an inverted repeat by genomic rearrangements involving the chromosomal termini. Novel joints formed by direct repeats were mapped, amplified in vitro, and sequenced. The junctions were simple fusions between DNA downstream and upstream of the M2 gene. Lack of sequence homology at the breakpoints indicated that the initial genomic rearrangements leading to gene amplification were due to nonhomologous recombination events.

INTRODUCTION

Hydroxyurea is an inhibitor of DNA synthesis by virtue of its ability to inactivate the enzyme ribonucleotide reductase. Cells which survive selection in cytotoxic concentrations of HU have amplified the gene which encodes the smaller of the two subunits of this enzyme, M2 (1). Vaccinia virus, which encodes most if not all of the enzymes required for viral DNA synthesis, including both subunits of ribonucleotide reductase, is sensitive to growth inhibition by HU; like mammalian cells, the virus can achieve resistance to this drug by amplifying the M2 gene (2,3).

Gene amplification in higher eukaryotes is a poorly understood phenomenon which often culminates in complex rearrangements involving flanking genetic loci as well as the gene under selection. Amplified sequences are variously arranged as direct or inverted repeats, as arrays within the chromosome at the position of the original single copy, or as extrachromosomal elements. Understanding the replicative and/or recombinational mechanisms by which eukaryotic genes are amplified has been

hampered by the difficult task of isolating the products of the
initial steps in the process. Although only a few cellular
amplification joints have been examined at the nucleotide level,
sequence features such as short homologies (4,5,6), potential
stem-loop structures (6), and Alu repeat sequences (6) have been
noted. The study of gene amplification in vaccinia virus, whose
DNA and RNA metabolism resembles that of eukaryotic cells in many
respects (7), facilitates examination of a large number of
independent amplification events.

A preliminary study of two HU-resistant VV mutants (3)
suggested that the gene amplification process involves two steps:
(1) an initial gene duplication event resulting in creation of a
novel joint, and (2) expansion of the array by homologous
recombination. In the present study, we have investigated the
structure of additional amplification mutants of VV to determine
whether the initial duplication event involves identifiable
sequence features.

MATERIALS AND METHODS
Cells and virus.
The African green monkey kidney cell line BSC40 was
maintained in modified Eagle medium supplemented with 5% heat-
inactivated fetal calf serum. A single plaque of wt VV (strain WR)
was expanded to ca. 4×10^9 pfu and a crude stock of virus
prepared as described (8).
Isolation of HU-resistant VV.
Thirty-nine confluent cultures of BSC40 cells in 100-mm
dishes (cultures 1-20) or 60-mm dishes (cultures 21-39) were
infected at multiplicity of infection 0.1 and passaged once
(cultures 1-20) or twice (cultures 21-39) in the presence of 5 mM
HU, a concentration of drug which decreases the 48 h virus yield
to approximately one pfu per input pfu (2). A total of ca. 3×10^7
progeny viruses were subjected to plaque isolation under an
agarose overlay containing 5 mM HU. HU-resistant viruses were
recovered from 95% of cultures passaged twice in HU (18 isolates)
and from 45% of cultures passaged once (9 isolates). Each isolate
was plaque-purified three times and viral DNA was prepared (9,10).
Southern blots.
DNA from wt and variant viruses was cut with restriction
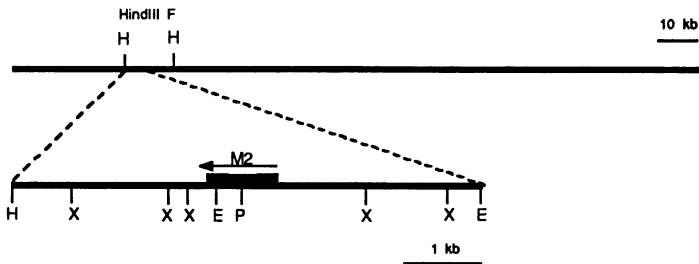enzymes, and the fragments were transferred to nylon membranes

Figure 1. Position of the *M2* gene within the VV genome (185 kb), and expanded view of the left end of the HindIII F fragment (6.2 kb). H, HindIII; X, XbaI; E, EcoRI; P,PstI.

(BioRad) and processed as described (11). Blots were probed with oligonucleotides end-labeled with gamma[^{32}P]ATP, or plasmid vectors containing fragments of VV DNA labeled using the random primer method (12). Hybridization conditions for blots probed with oligonucleotides were as descibed (3).

In vitro DNA amplification and sequencing PCR products.

PCR reactions (0.1 ml) contained 50 mM Tris-HCl, pH 8.8, 50mM KCl, 2.5 mM MgCl$_2$, 0.2 mg bovine serum albumin per ml, 0.2 mM dATP, dTTP, dCTP and dGTP, 600 ng each gel-purified oligonucleotide primer, 2.5 U Taq polymerase (New England Biolabs), and 10-100 ng viral DNA. DNA in reaction mixtures was denatured at 93°C, annealed at 41°C, and extended at 65°C for 3-7 min, depending on lengths of expected products. Reaction mixtures were extracted once with phenol:chloroform:isoamyl alcohol (50:49:1) and once with chloroform. PCR products were diluted 5-fold with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and concentrated/desalted twice using Centricon 30 microconcentrators (Amicon). DNA sequencing of PCR products was performed as described by Higuchi et al. (13).

RESULTS

Genomic structures of HU-resistant VV.

As shown in Figure 1, the *M2* gene lies within the HindIII F fragment of VV, approximately 30 kb from the left end of the genome as the viral map is conventionally drawn (3). To see whether HU-resistant VV exhibited genomic rearrangements involving the *M2* gene, DNA from several variants was digested with HindIII and probed with an oligonucleotide homologous to a portion of the
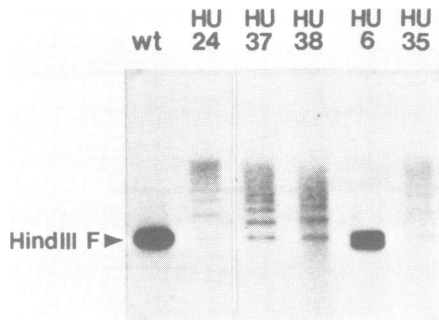
Figure 2. Southern blot of HindIII-restricted DNA from wt VV and five HU-resistant mutants, probed with end-labeled oligo g (homologous to nt 12-31 of the *M2* gene). Sizes of the direct repeats in the mutants shown are HU24, 3.2 kb; HU37, 1.9 kb; HU38, 2.2 kb; HU35, 3.3 kb.

M2 coding sequence. The results, shown in Figure 2, revealed hybridization to a 13.2 kb band that co-migrated with wt HindIII F, as well as hybridization to one or more additional bands. In four cases, the sizes of individual bands in the ladder-like arrays seen in Figure 2 were equal to HindIII F plus unit multiples of additional DNA. Since the HindIII sites bordering fragment F are outside the *M2* coding region, these results suggested that gene amplification involving DNA between the sites had occurred, and that each population derived from a plaque-purified mutant harbored arrays containing from two to ca. twelve copies of a segment of DNA containing *M2* sequences. Therefore, the structure of these mutants was similar to that of two previously described HU-resistant VV isolates, HU1 and 19, that amplified 2.2 and 2.7 kb, respectively, of DNA within HindIII F (3). One isolate, however, exhibited a single new 12.5 kb HindIII band, which hybridized to the probe with the same efficiency as HindIII F itself. This result suggested that HU6 represented a different type of mutant.

To survey the entire collection, DNA from each mutant was restricted with PstI, an enzyme which cuts within the VV *M2* gene (see Figure 1). As shown in Figure 3, each of the mutants exhibited an aberrant PstI pattern, as compared to wt. In 25 cases, a new band was generated which stained more intensely with ethidium bromide than did similar-sized bands in the same lane.
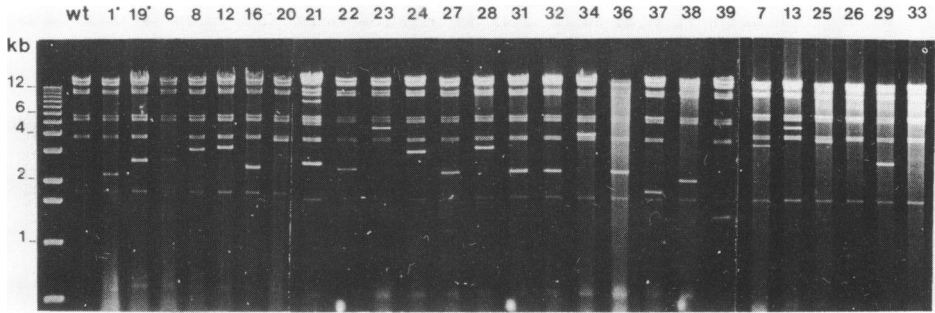
Figure 3. PstI digests of wt VV DNA and DNA from 26 HU-resistant mutants. (HU5, HU9 and HU35 are not shown.)

This result suggested that these genomes contained multiple copies of a DNA segment containing a PstI site. A single band of this type appeared in each case; therefore, we concluded that the amplified DNA segments were arranged as direct, or head-to-tail, repeats. Since the sizes of the new PstI bands in HU24, 35, 37, and 38 corresponded to the incremental increases observed in the HindIII F bands in Figure 2, and Southern blot analysis of several other mutants of this type confirmed that the new PstI bands contained M2 sequences (not shown), we concluded that the lengths of the new PstI fragments were equal to the lengths of the repeated DNA. The sizes of these fragments ranged from 1.5 kb (HU39) to 5.7 kb (HU26 and 33).
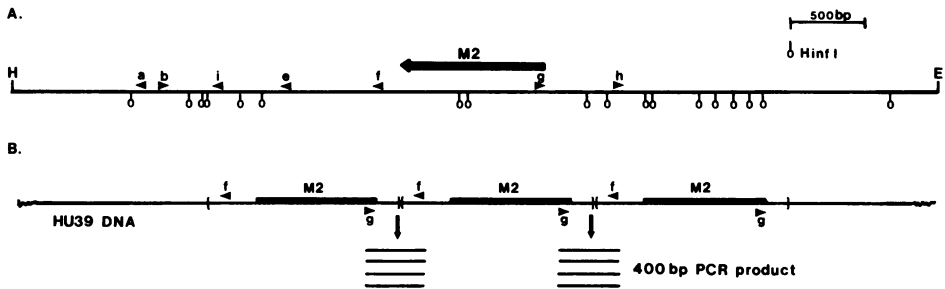

Figure 4. (A) HinfI map of leftmost 6.2 kb of HindIII F fragment of wt VV. The position and orientation of the M2 gene (large arrow) and 20-mer oligonucleotides used in PCR and sequencing reactions (arrowheads) are shown. (B) Strategy for in vitro amplification of recombination joint present in HU39 DNA.
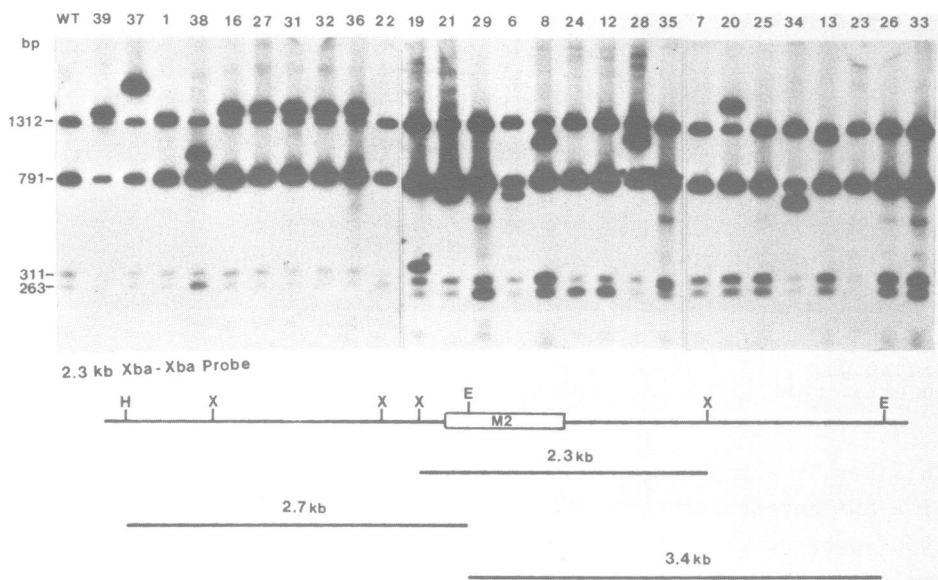
Figure 5. Southern blots of HinfI-restricted DNA from wt VV and 27 HU-resistant mutants, probed with the 2.3-kb XbaI fragment. The same blots were stripped and re-probed with the 2.7-kb HindIII-EcoRI fragment and the 3.4 kb EcoRI-EcoRI fragment (results not shown).

Mapping the endpoints of amplified DNA.

Our strategy for obtaining the sequence of the amplification joints present in the mutant collection required mapping the endpoints of the duplicated segments. To facilitate this task, we determined 6.2 kb of wt DNA sequence surrounding the *M2* gene in HindIII F (Roseman, N. and Slabaugh, M., manuscript in preparation). The 18 HinfI sites distributed throughout the sequenced region are shown in Figure 4A. DNA from wt and mutant viruses was digested with HinfI, and the novel fragments (that is, those not present in wt DNA) were detected by Southern analysis using three probes which spanned the 6.2-kb region we had sequenced. Representative results, obtained with the 2.3-kb probe, are shown in Figure 5. By re-probing blots with each of the three probes, HinfI junction fragments were detected in 25 isolates, indicating that these mutants had at least one endpoint within the leftmost 6.2 kb of HindIII F. Similar-sized novel HinfI fragments from HU16, 27, 31, 32, and 36 suggested that these mutants might
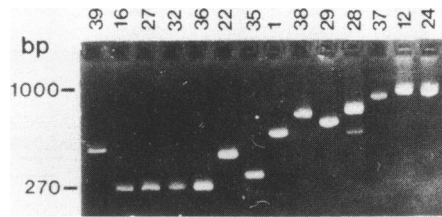
Figure 6. Products (270-1000 bp) produced by 25 cycles of PCR using 10-100 ng viral DNA isolated from the indicated mutants as templates. Primers: HU37, HU38, HU39, f/g; HU12, HU16, HU22, HU24, HU27, HU29, HU32, HU36, e/h; HU1, e/g; HU28, a/g; HU35, a/h; not shown, HU19 and HU31, e/h.

be identical. The approximate location of each amplification endpoint was deduced from the repeat size determined by PstI digestion and the size of the HinfI junction fragment. This approach enabled us to map recombination sites for mutants in which both endpoints fell within the region of known sequence.

Sequencing novel amplification joints.

To obtain the DNA sequence across the recombination joints, in vitro DNA amplification using the polymerase chain reaction (PCR) was employed. Oligonucleotides homologous to the positions indicated by small arrowheads in Figure 4A were designed such that pairwise combinations would flank putative joint sequences. As illustrated in Figure 4B for HU39, the oligonucleotides prime divergently on a wt DNA template, but are expected to generate a discrete PCR product if recombination has occurred. PCR products of the expected sizes were generated from mutant templates (Figure 6).

Novel joint sequences.

To identify recombination points, PCR products were sequenced as described, and sequences determined from mutant templates were compared to the wt DNA sequence. In Figure 7, the sequences surrounding recombination joints have been aligned with homologous parental sequences upstream (wt right) and downstream (wt left) of the M2 gene. Each joint appeared to be the product of a simple recombination event between two regions of wt DNA. In no case had non-parental nucleotides been added at the joint. The extent of the amplified region present in each mutant is indicated in Figure 8. Although several endpoints were clustered (e.g. the

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            WT left     AATATCATGTATGATATGgcatctacaaaatca
              HU28      AATATCATGTATGATATGATAGTACTAATAACa
          WT right      gtcgataatttaatTATGATAGTACTAATAACa

            WT left     TACTAGACTGTACCAAAatatttcattgtgata
              HU37      TACTAGACTGTACCAAATCCGAATGGGAGGCGT
          WT right      tgcgtaacatttgtgAATCCGAATGGGAGGCGT
                                         ————→      ←————

            WT left     TAGTATATCTCATCGGTggatggacgaacaatg
               HU1      TAGTATATCTCATCGGTACTATCGTTAAAAATT
          WT right      cgacgtttaatcggttTACTATCGTTAAAAATT
                                   ————→

            WT left     TGTTGAGCGTTGGTTCCacgggatgctgcttg
              HU19      GGTTGAGCGTTGGTTCCTACTATGAATATATCT
          WT right      ggagtcatcgttacaaCTACTATGAATATATCT
                                              ————→  ←————

          WT left       CAACATGAATATTAACTcaccagttagatttgt
           HU12/24      CAACATGAATATTAACTAAAACGCGTTAATATC
          WT right      tatatatcatcattcaTAAAACGCGTTAATATC
                                         ————→  ←————

             WT left    ATCAAACAATTGGATTccaattcctccgatgaa
     HU16/27/31/32/36   ATCAAACAATTGGATTATCTTTCGCGCTTAATG
          WT right      tacgatgcgaaataacATCTTTCGCGCTTAATG
                                   ————→     ←————

            WT left     CTGTTGAGCGTTGGTTccacgggatgctgctt
              HU22      CTGTTGAGCGTTGGTTATGCGAAATAACATCTT
          WT right      gcgaagttaaaatacgATGCGAAATAACATCTT

            WT left     AGTGTTACATGATGGGtatagaatacggactat
              HU38      AGTGTTACATGATGGGACTAACGTGTCACATAT
          WT right      acatatttgtacccacACTAACGTGTCACATAT
                                   ————→            ←————

            WT left     GGATACCCGCTAACAAtaaattatacgtagtag
              HU29      GGATACCCGCTAACAAAAGGTTATAGCGCTAGC
          WT right      ttggtattataatgatAAGGTTATAGCGCTAGC
                                            ————→

            WT left     AGGAGATCAAGGGTTTggatcaacaggacttag
              HU35      AGGAGATCAAGGGTTTTTTCGCGCTTAATGGTT
          WT right      gatgcgaaataacatcTTTCGCGCTTAATGGTT
                                   ————→

            WT left     CGTCGTCAATTTATTAcgtgcttctatattaac
              HU39      CGTCGTCAATTTATTAGCCCGCTTCCCACAACA
          WT right      ataattcatcctccatGCCCGCTTCCCACAACA
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Figure 7. DNA sequences from 11 recombination joints aligned with the regions upstream and downstream of the *M2* gene from which the junctions were derived (wt-left and wt-right, respectively). Sequences present in the recombination joints are uppercase; direct repeats at the crossover points are boldface; short dyad symmetries in parental sequences are indicated by arrows.

upstream HU16, 22, and 35 breakpoints are within 15 nucleotides of each other), there was no compelling evidence for "hotspots".

The joint sequences of HU16, 27, 31, 32, and 36 were identical, as were those from HU12 and 24. To determine whether these joints were present in the starting stock of VV, we subjected wt DNA prepared from the starting stock to PCR amplification, reasoning that a band of predictable size would be
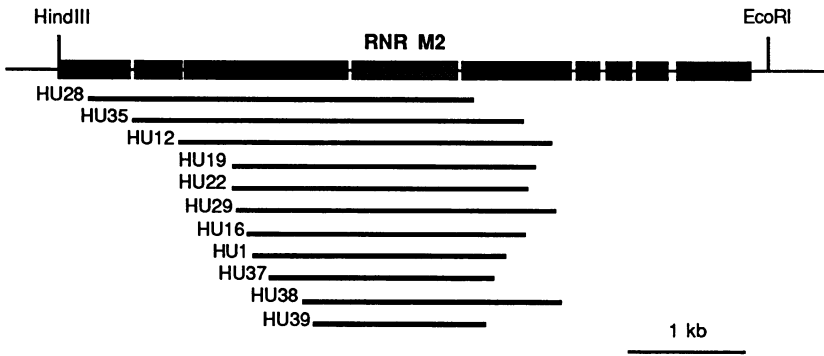
Figure 8. Regions of HindIII F amplified as direct repeats in HU-resistant VV mutants. Endpoints were determined by sequencing recombination joints and comparing the results to the wt VV DNA sequence. Shown on the map are the open reading frames identified by computer analysis of the DNA sequence (boxes). The gene that encodes the small subunit of ribonucleotide reductase is designated RNR M2.

generated even if the joint was initially present at very low abundance. As shown in Figure 9, bands that co-migrated with the prominent PCR products generated from amplification mutants HU31 (lane 2) and HU24 (lane 4) were indeed produced from the wt template (lanes 3 and 5), but not from an unrelated stock of wt DNA from another laboratory (not shown). To see whether other
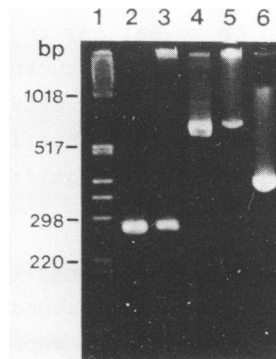

Figure 9. PCR amplification of multiply-isolated recombination joints from mutant and wt DNA. Lane 1, size markers (BRL); lane 2, HU31 DNA, primers h/e; lane 3, wt DNA, primers h/e; lane 4, HU24 DNA, primers h/i; lane 5, wt DNA, primers h/i; lane 6, wt DNA, primers b/i.

mutants in our collection pre-existed in the starting stock, we
challenged wt DNA with each pair of primers used to generate the
products shown in Figure 6. One additional novel joint was
detected (HU28, not shown). These results indicated that at least
three events resulting in *M2* gene duplication occurred during
expansion of a single plaque to the starting stock prior to
passage in HU.

DNA sequence features at recombination sites.
        In five mutants, the precise recombination point could not
be identified due to the presence of one- to four-bp repeats
present in the parental sequences (Figure 7, boldfaced
nucleotides). In prokaryotes, short sequence homologies of less
than 15 bp are strongly associated with both deletion (14) and
amplification (15) junctions. In eukaryotes, however, sequence
repeats at the few joints which have been sequenced are either
very short (1-3 nucleotides) or lacking (4,5,16,17,18). The
presence of short direct repeats at the frequency we observed was
not statistically significant. Additionally, there was no evidence
of "patchy homologies" in parental sequences--regions of broken
direct repeats that are hypothesized to align nonhomologous
recombining regions in prokaryotes (15).

        Some authors have argued that sequence features such as dyad
symmetries capable of forming stem-loop structures may promote
nonhomologous recombination in eukaryotes (6). A computer search
of the DNA sequences 200 bp upstream and downstream of each VV
recombination site failed to reveal any regions of dyad symmetry
capable of forming stable stem-loop structures. However, as
indicated by arrows in Figure 7, short dyad symmetries of four to
seven bp flanked the recombination points in about half of the
parental sequences. The statistical significance of this
observation was not evaluated.

Inverted *M2* gene duplication.
        Four mutants in the collection, HU5, 6, 7, and 9, did not
exhibit structural evidence of tandem-repeat gene amplification
since their novel PstI joint fragments appeared to be present only
once per genome. However, in these cases, certain other PstI bands
appeared to be present at twice the wt abundance, suggesting
duplication of large segments of the genome (see Figure 3, HU6 and
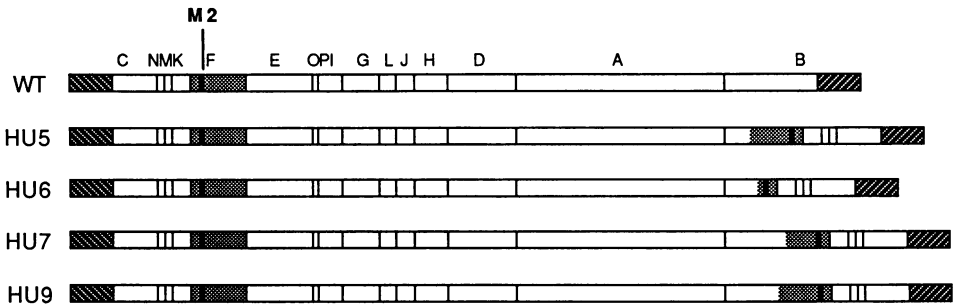7). Since a PstI map of the entire VV genome is not available,

Figure 10. HindIII restriction map of the 185-kb wt VV genome and four HU-resistant mutants which encode two copies of M2 in an inverted orientation. The 10-kb inverted terminal repetitions characteristic of wt VV are indicated by diagonal lines; HindIII F is stippled; the M2 gene is shown as a black box.

digestion of wt and mutant DNAs with SalI was utilized to characterize these viruses. The results of the restriction mapping indicated that 29-36 kb of DNA derived from the left terminus of the viral chromosome had been fused to a truncated right terminus in an inverted orientation in these mutants. As diagrammed in Figure 10, the M2 gene was included in the duplicated left terminus, generating viruses encoding two copies of M2. The inverted orientation, however, had apparently precluded further increases in gene copy number by homologous recombination. Since most of the recombination points involved in formation of the inverted duplication mutants fell outside of the part of HindIII F we sequenced, we were unable to identify their novel joint sequences by using PCR.

In addition to the four terminal transposition mutants that had duplicated M2 as described, four other mutants exhibited altered termini which did not involve the M2 gene because less DNA had been transposed (data not shown). Three cases (HU35, 37, and 38) were left-hand transpositions; one case (HU26) was a right-hand transposition. In these mutants, the M2 gene was present in tandem repeat units within HindIII F (see Figure 2).

The structure of HU21, a mutant exhibiting extensive M2 amplification as judged by Southern analysis (not shown) is unique in our collection, and is more complex than either the simple tandem-repeat mutants or the inverted-duplication mutants (see

PstI digests, Figure 3). HU21 was not investigated further in the present study.

DISCUSSION

     We analyzed 22 early-stage amplification mutants of VV and found that the majority of them resulted from joining of DNA upstream and downstream of the selected gene to produce a direct repeat. The repeat size, which was characteristic of individual isolates, ranged from 1.5 to 5.7 kb in length, and in several cases included genes flanking M2. Eleven joints were sequenced and the parental recombination points identified in wt DNA. We examined parental sites for sequence features that might promote recombination, but found none. Each plaque-purified direct-repeat mutant contained a unique novel joint which was as amplified as the selected gene, indicating that further rearrangements did not occur during the rounds of viral replication between plaque isolation and DNA preparation. We concluded from these results that the predominant mode of gene amplification in VV is generation of a direct repeat by nonhomologous recombination and expansion of the array by homologous recombination.

     DNA rearrangments leading to gene amplification are being studied in a number of cellular systems due to the association of this phenomenon with both drug resistance and malignancy (reviewed in 19). There is accumulating evidence that different processes give rise to the primary event, which occurs in a single cell and is therefore not directly amenable to analysis, and subsequent events, which often result in structures exhibiting a variety of rearrangements including indirect repeats and highly amplified novel joints not present at earlier times. For example, a well-studied locus in methotrexate-resistant CHO cells contains a large number of DHFR gene copies as tandem repeats of an inverted-repeat unit which is itself a rearranged subset of sequences initially generated by a direct repeat (20).

     In an attempt to characterize the initial events in CAD gene amplification, 33 independent drug-resistant mutants which had amplified the target gene only a few-fold were examined as soon as possible after selection (21). The results of this study were most consistent with a mechanism in which unequal sister chromatid recombination events generated a small number of very large

(~10,000 kb) amplified regions. Subsequent selection in higher drug concentrations yielded derivatives in which *CAD* gene copy number had been increased but the amount of co-amplified DNA had decreased (22). Therefore, the primary event may create a DNA structure which is capable of rapid evolution to a more highly amplified and rearranged organization.

The novel DNA junctions present in amplified DNA are expected to provide clues to the mechanisms giving rise to the rearrangements. To date, fewer than ten cellular amplification joint sequences have been presented (5,6,16,23,24). Although the authors identified various sequence features at parental sites, the collective results indicate that the events do not require sequence homology nor any particular sequence feature. It should be noted that most of the novel joints sequenced thus far are those present within an inverted repeat. Although inverted repeats are a prevalent feature of highly amplified structures (25,26) and evidence has been presented that they may represent a primary event in gene amplification (16), the fact that they are frequently located near a selected gene has facilitated their isolation and analysis. The termini of cellular tandem repeats, especially those resulting from primary events, on the other hand, may be hundreds of kilobases from the selected gene (21,27).

The present study, utilizing VV as a model system, examined primary events in gene amplification. Similar to a recent study of bacterial *NEO* gene amplification in human cells (28), we concluded that direct-repeat gene duplication was the initial event leading to tandem reiteration of the selected gene. Such a model predicts that the duplication can be readily expanded to an array, or reduced to a wt locus containing a single copy of the sequence by homologous recombination between out-of-register repeat units. In agreement with this, we found that tandem-repeat VV mutants (but not inverted-repeat mutants) responded rapidly to increased or decreased selection pressure (present study,3). Similarly, amplification of the *CAD* gene was unstable in all first- and second-step mutants tested but stable in highly amplified multi-step mutants (22).

The breakage and rejoining reactions underlying gene amplification may be related or identical to those that generate

chromosomal deletions, translocations, and inversions (19). The lack of sequence features at VV amplification breakpoints is similar to the results of a recent study of 16 *C. elegans* deletion breakpoints (28).

How agents that inhibit DNA synthesis or damage DNA affect the gene amplification process is an unresolved issue. An increased rate of *DHFR* gene amplification in CHO cells treated with HU has been reported (30,31); however, the drug failed to stimulate amplification of a bacterial *NEO* gene stably integrated into human cells (28). The present study was not designed to permit quantitative analysis of mutation rates; however, if the failure to PCR-amplify novel joints from starting stock DNA was a valid indication of mutants that did not exist prior to passage in HU, then the rate of mutation to *M2* amplification was much greater in the presence of the drug than in its absence (3-10 events per 4 x 10^9 replications without HU compared to 10-20 events per 3 x 10^7 replications in the presence of HU). Additionally, eight mutants (30%) in our collection exhibited rearrangements of the viral termini. Viable terminal transposition mutants of this type have been described in several poxviruses, albeit at much lower frequencies than observed in this study (32,33,34,35,36). The prevalence of this class of mutants in our collection may reflect a general effect of HU on genomic rearrangement. One hypothesis is that intermediates in the rearrangement reaction, *e.g.* breaks in DNA, are generated with increased frequency under conditions where replication fork movement is severely slowed.

If, as our results suggest, breakage and ligation reactions generate rearranged VV chromosomes, it will be important to determine which viral or cellular enzymes are involved. One candidate is the topoisomerase I encoded by this virus, which was recently reported to promote illegitimate excision of lambda prophage when expressed in *E. coli* (37).

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