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Vaccinia Virus-Encoded Ribonucleotide Reductase: Sequence Conservation of the Gene for the Small Subunit and Its Amplification in Hydroxyurea-Resistant Mutants

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The vaccinia virus gene that encodes the small subunit of ribonucleotide reductase was localized to the *Hin*dIII F fragment by using degenerate oligonucleotide probes. DNA sequencing revealed a leftward-reading open reading frame that predicted a protein of 37 kilodaltons whose amino acid sequence was much more homologous to the mouse and clam M2 sequences (~80%) than to the corresponding herpesvirus (~27%) or procaryotic (~19%) gene products. Vaccinia virus mutants selected for the ability to grow in high concentrations of a specific inhibitor of ribonucleotide reductase, hydroxyurea, amplified the M2 gene and harbored tandem arrays (2 to 15 copies) of the gene within the *Hin*dIII F region. RNA isolated at early times after infection with wild-type virus and probed with an internal fragment of the M2 gene indicated one major (1.2 kilobases) and two minor (4.0 and 2.1 kilobases) transcripts. S1 nuclease analysis and primer extension experiments identified an RNA start site 12 nucleotides upstream of the putative initiation ATG codon.

The importance of the de novo biochemical route in supplying precursors for DNA synthesis is underscored by the fact that two classes of viruses with large DNA genomes, the T-even bacteriophages and the herpesviruses, encode ribonucleotide reductase (EC 1.17.4.1). This enzyme, which consists of two nonidentical subunits, designated M1 and M2 in mammalian cells, carries out direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides. In eucaryotes, ribonucleotide reductase activity is cell cycle dependent, and the variation in holoenzyme activity is regulated by synthesis and turnover of the M2 subunit (11).

We have investigated whether vaccinia virus (VV), the prototypic poxvirus, might also have acquired the ability to reduce ribonucleotides, since these viruses replicate in the cytoplasm of infected cells and show no dependence on the cellular S phase (17). A marked increase in reductase activity was in fact detected after synchronous infection of primate cells with VV (35). In contrast to the bacteriophage T4 and herpes simplex virus (HSV) ribonucleotide reductases, which are insensitive to many of the regulatory signals that control and direct cellular reductases (1, 4), the VV-induced enzyme exhibits both allosteric inhibition by dATP and dTTP and a stringent requirement for a positive activator (36).

To obtain genetic evidence that the induced enzyme was of viral origin, we have isolated hydroxyurea-resistant variants of VV (37). This drug interferes with DNA synthesis by destroying an essential tyrosyl-free radical in the small subunit of ribonucleotide reductase (18), leading rapidly to a depletion of DNA precursors. The drug-resistant viruses overproduce ribonucleotide reductase activity and a 34-kilodalton (kDa) polypeptide synthesized early in infection, which we hypothesized was the viral counterpart to the small subunit (M2).

In this report, we describe how we used the hydroxyurea-resistant mutants and mixed oligonucleotide probes to map the M2 gene within the *Hin*dIII F region of the VV genome. The amino acid sequence of the M2 protein, deduced from DNA sequencing, is strikingly homologous to the sequences reported for the clam (39), mouse (42), and *Saccharomyces cerevisiae* (10) M2 proteins. Additionally, we found that the hydroxyurea-resistant mutants have extensively amplified the M2 gene, adding as much as 40 kilobases (kb) of additional DNA to the 180-kb viral genome.

MATERIALS AND METHODS

Preparation of viral DNA. BSC_{40} cells in 100-mm plates were infected with wild-type (wt) VV (0.1 PFU per cell) or hydroxyurea-resistant mutant HU1 or HU19 (1 PFU per cell). Hydroxyurea (5 mM) was present during growth of mutant viruses. After 48 h, viral DNA was purified from infected cells by procedures previously described (8, 12). Yields from HU1 and HU19 infections were approximately 10 and 25%, respectively, of those routinely achieved with wt virus.

Oligonucleotide probes and hybridization conditions for Southern blots. Mixed oligonucleotide pool MS1 [(C/T)T C(A/G)AA(A/G)AA(A/G)TT(C/T/A/G)GT] and oligonucleotide RD1(CATAAGTATCGATCAAA) were synthesized by the Central Service Laboratory, Center for Gene Research and Biotechnology, Oregon State University. Oligonucleotides were gel purified and end labeled at 37°C for 30 min in a 25-μl reaction mixture containing 50 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 20 pmol of oligonucleotide, 20 pmol of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp.), and 20 U of polynucleotide kinase (New England BioLabs, Inc., or Promega Biotec). Restriction fragments in agarose gels were partially depurinated (two times for 15 min each in 0.25 N HCl), neutralized, and transferred to nylon membranes (Bio-Rad Laboratories) with a transfer buffer of 1 M ammonium acetate–0.02 N NaOH. Blots were prehybridized and hybridized in 5× SSPE (0.9 M NaCl, 50 mM sodium phosphate [pH 7.8], 5 mM EDTA)–1% sodium dodecyl sulfate–0.5% Carnation powdered milk–0.5 mg of denatured salmon sperm DNA per ml. The end-labeled probe was heated and added to the hybridization solution immediately before use (25 μl of end-labeling reaction mix-

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ture per 10 ml of solution). The blots were hybridized for 12 to 16 h at temperatures indicated for the individual experiments, washed four times for 10 min each at room temperature in wash solution (5× SSPE, 0.1% sodium dodecyl sulfate) and one time for 10 min at the hybridization temperature, and exposed to X-ray film with an intensifying screen at −80°C for appropriate times (2 to 24 h).

DNA sequencing. A clone of the *Hin*dIII F fragment in pBR322 was originally obtained from B. Moss. DNA subfragments were inserted into M13 vectors and sequenced by the dideoxynucleotide chain termination method (31, 41), using either Klenow fragment (New England BioLabs) or Sequenase (United States Biochemical Corp.).

Northern blotting (RNA blotting). Total early viral RNA (10 μg), isolated as described previously (44), was fractionated in a formaldehyde-agarose gel as described previously (20). The RNA was transferred to a nylon membrane (Bio-Rad) and hybridized to viral DNA that had been ^{32}P labeled by the hexamer primer method (13).

S1 nuclease mapping and primer extension. S1 mapping using 5′-end-labeled DNA fragments was performed as previously described (30). For primer extension, 20 μg of total RNA was annealed to 3 to 5 ng of a 5′-end-labeled synthetic oligonucleotide, RD1, in a 15-μl reaction mixture containing 10 mM Tris (pH 8.0), 0.25 M KCl, and 1 mM EDTA. After being heated to 60°C, the mixture was cooled to 42°C and incubated for 1 h. Reverse transcription was performed in a 45-μl reaction mixture containing 10 mM Tris (pH 8.3), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM deoxynucleoside triphosphate, and 9 U of reverse transcriptase (Promega Biotec) at 37°C for 1 h. The nucleic acid was then precipitated with 0.2 M sodium acetate and ethanol.

RESULTS

Mapping the M2 gene. A mixed oligonucleotide probe for the VV M2 gene was designed based on an examination of the predicted amino acid sequences of four ribonucleotide reductase small-subunit proteins: *Escherichia coli* protein B2, a 38-kDa protein from HSV type 1, a 34-kDa protein from Epstein-Barr virus, and a 42-kDa protein from clams (33). The probe, designated MS1, was designed to detect a conserved region five amino acids long (Thr-Asn-Phe-Phe-Glu) present in the three eucaryotic sequences near the carboxy termini of the proteins. Using VV DNA cut with various restriction enzymes (Fig. 1A), Southern blot analyses were performed at several temperatures. At 37°C and under standard conditions for oligonucleotide hybridization (5× SSPE), the probe hybridized strongly to two sites within the viral genome (Fig. 1B). With reference to VV genomic maps (9), these sites were contained within the left ends of *Hin*dIII-D (*Bgl*I-A, *Hin*dIII-D, *Kpn*I-G, or *Kpn*I-H) and *Hin*dIII-F (*Bgl*I-D, *Hin*dIII-F, *Kpn*I-G, or *Kpn*I-H). Hybridization to *Hin*dIII-F was preferentially eliminated by a higher-stringency wash (37°C, 1× SSPE).

A computer search of the DNA sequence of the 16-kb *Hin*dIII D fragment (27) identified the probable site of MS1 hybridization within open reading frame (ORF) D2, which putatively encodes a 17-kDa protein of unknown function. A dissociation temperature of 40°C was calculated for an MS1-ORF D2 hybrid, which would contain one mismatch. However, the reading frame of the ORF D2 sequence did not contain the conserved amino acid sequence represented by the MS1 probe.

To map the site of MS1 hybridization within the 13.5-kb *Hin*dIII F fragment, the end-labeled oligonucleotide was hybridized to restriction enzyme-digested pBR322-F. Hybridization to a 2.75-kb *Eco*RI fragment, 2.35-kb *Xba*I fragment, 2.8-kb *Bgl*II fragment, and 3.6-kb *Cla*I fragment (Fig. 2B) located the site approximately 2.6 kb to the right of the *Hin*dIII K-F fragment boundary. Note that an additional *Cla*I site (indicated by an asterisk in Fig. 2B) was not cut in viral DNA propagated in *E. coli*, presumably because of overlap with a *dam* methylation site (determined by DNA sequencing). Use of the mixed probe as the sequencing primer and the 2.75-kb *Eco*RI subfragment cloned in M13 as the template confirmed the location of the gene; the amino acid sequence immediately upstream of the MS1 primer was highly homologous to the M2 sequence deduced from clam DNA (39). The MS1 probe mixture contained a sequence perfectly complementary to its intended target in the VV M2 gene; however, 12 of 15 base pairs in the Thr-Asn-Phe-Phe-Glu-coding sequence were A · T, resulting in a less stable hybrid than the spurious hybridization to ORF D2.

Hybridization of the blot shown in Fig. 1A and B to a nondegenerate oligonucleotide probe, RD1, which was designed after sequencing of the gene was partially completed, is shown in Fig. 1C. RD1 detected the M2 gene uniquely, and its target sequence was present in genomic fragments of 3.4 kb (*Eco*RI), 12 kb (*Bam*HI), 5.6 kb (*Pst*I), and 2.35 kb (*Xba*I) (lanes 4, 5, 6, and 8, respectively). This probe spans the aforementioned *Cla*I site within the M2 gene and therefore gave no signal from *Cla*I-digested viral DNA (lane 7).

Structure of the M2 gene in hydroxyurea-resistant mutants. We have recently described hydroxyurea-resistant VV mutants which were selected stepwise in increasing concentrations of the drug (37). The 50% inhibitory dose for variants capable of growth in 5 mM hydroxyurea is approximately 10-fold greater than for wt virus. The mutants overproduce an early 34-kDa protein and ribonucleotide reductase activity. The drug resistance phenotype is markedly unstable in the absence of selective pressure. Together, these observations suggest gene amplification as the mechanism underlying hydroxyurea resistance. To investigate whether hydroxyurea-resistant mutants exhibited gross alterations in genomic organization, DNA was prepared from wt VV and two independently derived mutants, HU1 and HU19, digested with various restriction enzymes, and electrophoresed on an agarose gel (Fig. 3A, left panel). Three differences between wt and mutant DNA were observed: (i) one or two bands which were barely detectable in wt DNA appeared prominent in mutant DNA (e.g., *Hin*dIII digests, arrowheads); (ii) one band in each digest appeared to be substoichiometric in mutant DNA (squares); and (iii) a faint ladder of new bands was present in mutant DNA, extending upward from the fragments identified as substoichiometric. Control experiments in which uninfected BSC_{40} cells were subjected to the viral DNA preparation procedure demonstrated that the prominent bands were of cellular origin, presumably mitochondrial, and their preponderance in mutant DNA preparations was an artifact resulting from the low per-cell yield of HU1 and HU19 DNA (data not shown).

Gene amplification involving the M2 gene was revealed by hybridization of the RD1 probe to a blot prepared from the agarose gel shown in Fig. 3A. The small-subunit gene was present in mutant DNA not only on a fragment identical in size to that in wt DNA but also in a series of fragments which increased in size by increments up to a maximum of approximately 50 kb (estimated from the mobility of the 50-kb *Hin*dIII A fragment in Fig. 3A, left panel) (Fig. 3A, right panel). A second group of restriction enzymes, *Bgl*I, *Eco*RI, and *Pst*I, generated a single extrastoichiometric band from

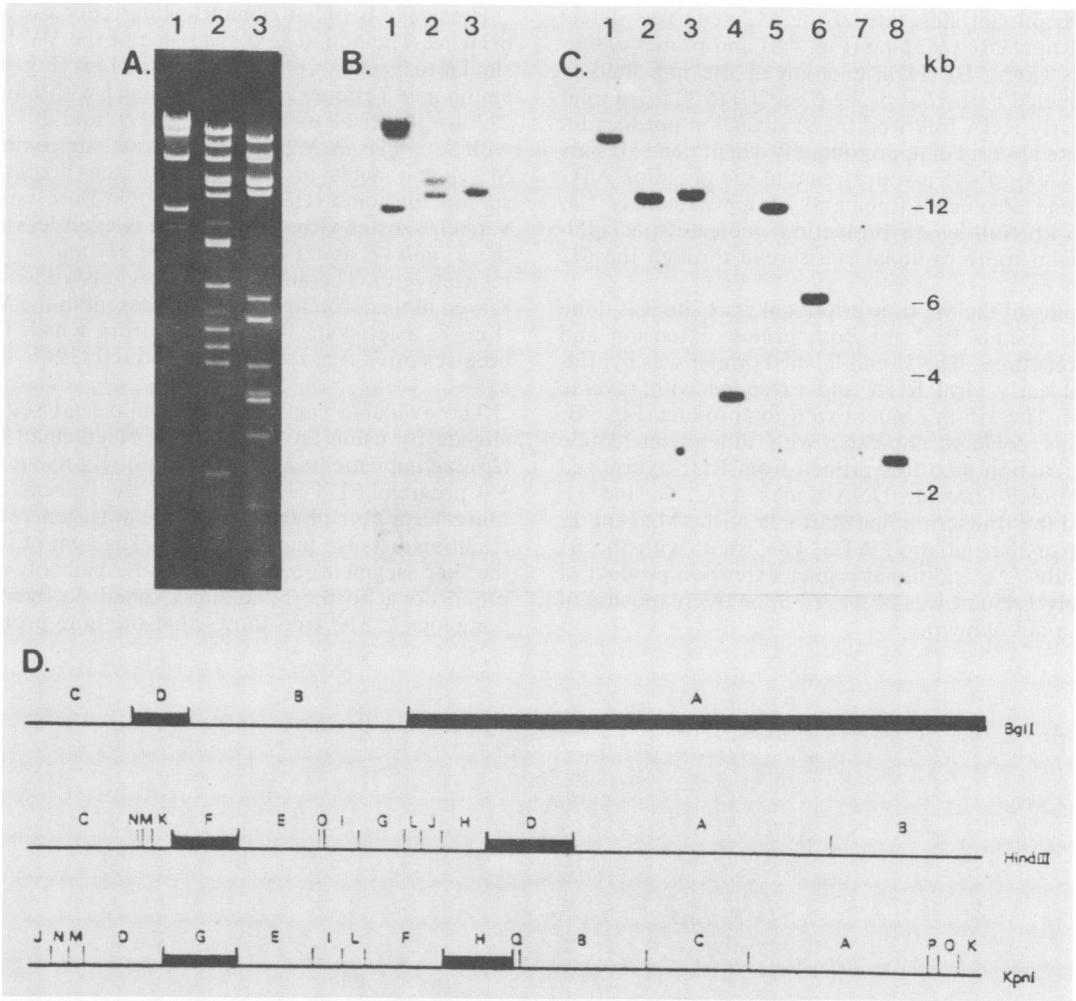

FIG. 1. Mapping of the VV M2 gene using oligonucleotide probes. (A) Ethidium bromide-stained agarose gel of purified viral DNA digested with *Bgl*I, *Hin*dIII, and *Kpn*I (lanes 1 to 3, respectively). (B) Southern blot of the gel shown in panel A probed at 37°C with end-labeled oligonucleotide pool MS1. (C) Southern blot of the gel lanes shown in panel A, plus digestions with *Eco*RI, *Bam*HI, *Pst*I, *Cla*I, and *Xba*I (lanes 4 to 8, respectively), probed at 40°C with end-labeled oligonucleotide RD1. (D) Diagrammatic representation of hybridization of MS1 to the VV genome. Solid bars indicate hybridizing fragments.

HU1 and HU19 DNA (Fig. 3B, left panel, open circles). As expected, the RD1 probe revealed that these bands contained M2 gene sequences (Fig. 3B, right panel), and the sizes of the new bands, 2.2 and 2.7 kb, corresponded to the increments in the ladders seen in Fig. 3A. We concluded that *Hin*dIII, *Kpn*I, and *Bam*HI cut VV DNA outside of the region amplified in HU1 and HU19, whereas *Bgl*I, *Eco*RI, and *Pst*I each cut once within the amplified segments. Furthermore, the amplifications must involve tandem direct repeats, since several different restriction enzymes generated DNA fragments of the same length. The largest repeat array was estimated to consist of 14 to 15 U, adding 35 to 40 kb of DNA to the viral genome. Our model for the structure of genomic DNA in wt and hydroxyurea-resistant VV is shown in Fig. 4.

Careful examination of the ethidium bromide-stained gel shown in Fig. 3A indicated that the restriction fragments containing the cross-linked termini of the viral chromosome were shorter by approximately 300 bp in HU1 DNA (indicated by a T) than in either wt or HU19 DNA. Whether the structure of the HU1 termini was responsible for the low yields of progeny virus we routinely obtained with this mutant remains to be investigated.

Nucleotide sequencing. To obtain the nucleotide sequence of the M2 gene, appropriate subfragments of *Hin*dIII-F were cloned into M13 vectors and sequenced by the dideoxynucleotide chain termination method by using either commercially available M13 primers or synthetic oligonucleotide primers (Fig. 5). For approximately 90% of the sequence shown, the sequence of both strands was determined.

The sequence reveals a 957-nucleotide (nt) ORF reading from right to left. Directly upstream and downstream of the complete ORF are incomplete ORFs also reading leftward. The complete ORF has the capacity to encode a protein with an M_r of 36,943.

Transcript identification and mapping. Transcripts encoded within the M2 gene region were identified by Northern blot analysis (Fig. 6). A 634-nt *Eco*RI-*Eco*RV DNA fragment located entirely within the M2 gene hybridized to a major transcript of 1.2 kb and two minor transcripts of 2.1 and 4.0 kb. The 1.2-kb transcript is sufficient to encode the viral M2 protein.

The transcriptional start site of the M2 gene was determined by S1 nuclease mapping (Fig. 7A) and primer extension analysis (Fig. 7B). Hybridization of the indicated 5′ single end-labeled *Pst*I-*Xba*I probe (Fig. 7C) to 30 μg of total immediate-early RNA followed by S1 digestion produced a major protected hybrid of approximately 490 nt (lane 3). This maps the RNA start site just upstream of the initiation ATG of the M2 gene. Protected fragments of approximately 1.4, 1.5, and 1.6 kb (full-length protection) indicate that additional upstream transcriptional starts read through the M2 gene.

Fine mapping of the M2 transcriptional start site was done by using the primer RD1 for both primer extension and sequencing reactions. The 5′-end-labeled primer was hybridized to total early viral RNA and extended with reverse transcriptase. The 355-nt primer extension product (Fig. 7B, lane PE) was coelectrophoresed with dideoxynucleotide sequencing reaction mixtures primed from RD1 hybridized to single-stranded template DNA (lanes A, C, G, and T). This mapped the transcriptional start site of the M2 gene 12 nt upstream of the initiation ATG, consistent with the S1 nuclease results. An additional primer extension product of approximately 1,450 nt was observed upon overexposure of the film (data not shown).

Homology between ribonucleotide reductase small-subunit proteins. The amino acid sequence of the VV M2 protein is similar to its eucaryotic cellular homologs (Fig. 8). Of the 319 amino acid residues, 258 are identical with mouse M2 (42), 227 are identical with clam M2 (39), and 207 are identical with *S. cerevisiae* M2 (10). In contrast, approximately 85 VV M2 amino acids are shared with the herpesvirus small-subunit proteins (HSV [22], Epstein-Barr virus [15], and varicella-zoster virus [26]). There is even less identity with the *E. coli* (7) and T4 (34) proteins (77 and 46 amino acids, respectively). The amino acids previously identified as conserved in this protein (33) are all present in the VV sequence (Fig. 8, asterisks). The tyrosine from which the hydroxyurea-sensitive free radical is generated (19) is boxed in Fig. 8.

The available small-subunit amino acid sequences were aligned by using an algorithm of Needleman and Wunsch (25), as implemented by Gotoh (16). By using the PHYLIP 3.0 program FITCH, distributed by Felsenstein, a Fitch-Margoliash (14) phylogenetic tree was generated from the resulting distance matrix (Fig. 9). The sum of the lengths of the line segments connecting any pair of sequences is proportional to the relatedness based on overall sequence homologies. The tree shows that the nine proteins fall into

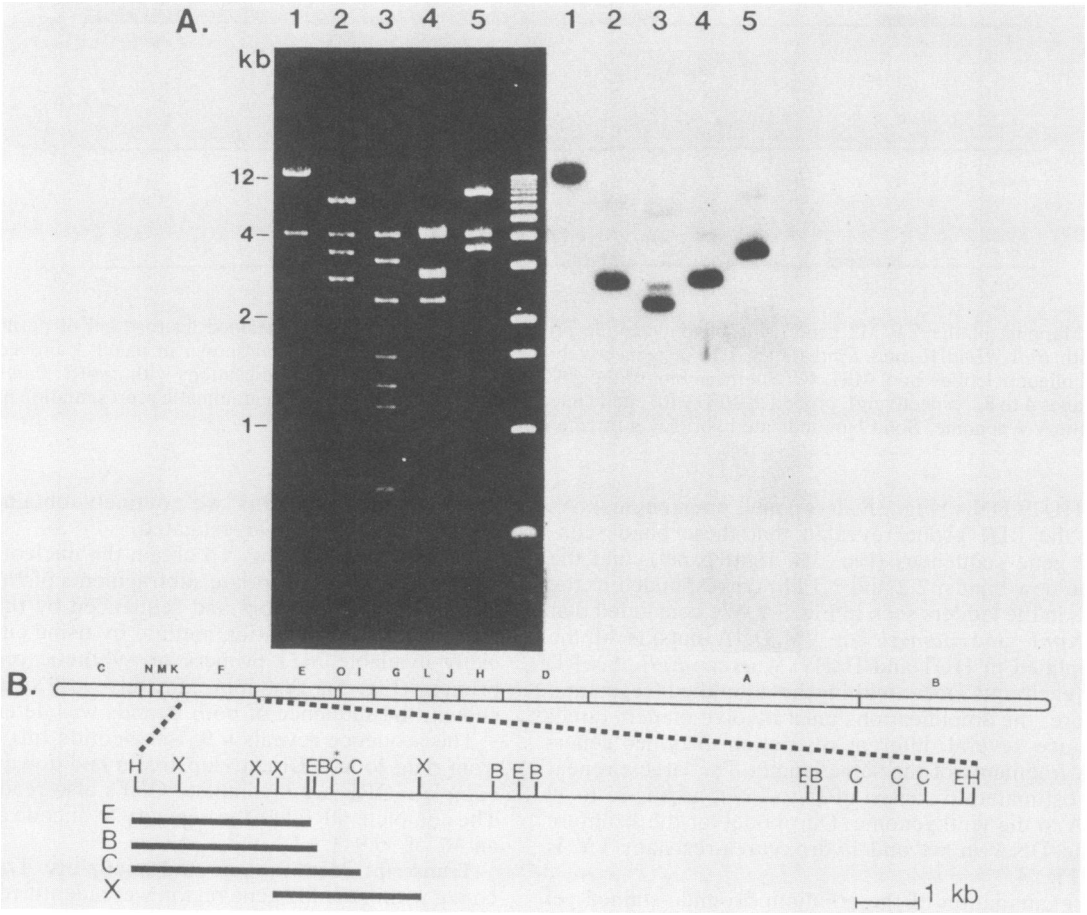

FIG. 2. Fine mapping of the VV M2 gene within the 13.5-kb *Hin*dIII F fragment. (A) Ethidium bromide-stained agarose gel and Southern blot probed at 37°C with oligonucleotide pool MS1. Lane 1, Plasmid pSG124 containing HSV type 1 ribonucleotide reductase genes; lanes 2 to 5, pBR322-F. DNAs were digested with *Eco*RI (lanes 1 and 2), *Xba*I (lane 3), *Bgl*II (lane 4), and *Cla*I (lane 5). (B) Diagrammatic representation of hybridization of MS1 within *Hin*dIII-F. Solid bars indicate hybridizing fragments. The *Hin*dIII map of VV is shown for reference. E, *Eco*RI; B, *Bgl*II; C, *Cla*I; X, *Xba*I. The asterisk indicates a *Cla*I site, as explained in the text.

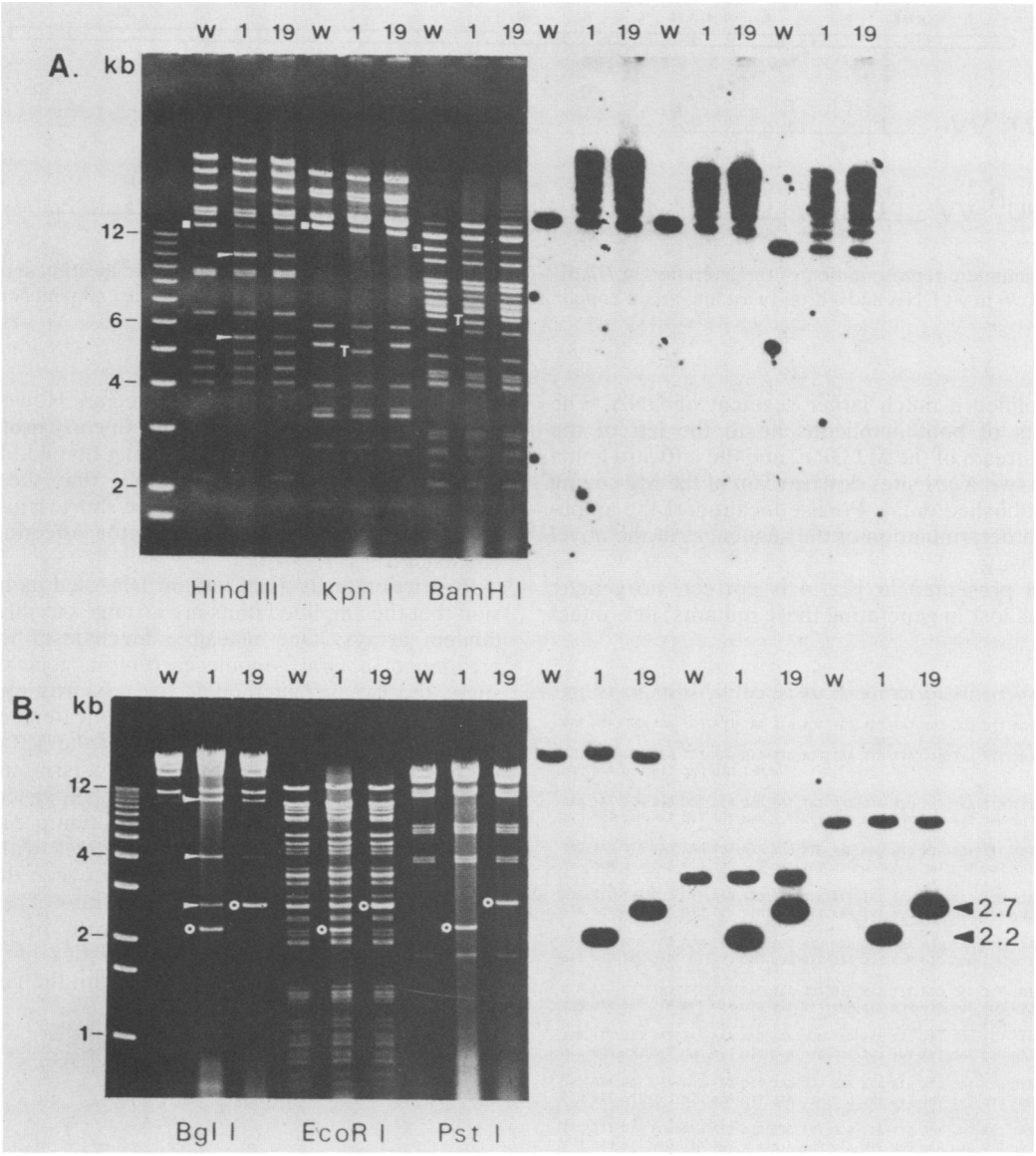

FIG. 3. Determination of the structure of amplified DNA containing M2 genes. Ethidium bromide-stained agarose gels (left panels) and Southern blots probed with end-labeled oligonucleotide RD1 (right panels) containing purified viral DNAs digested with restriction enzymes that cut outside (A) (*Hin*dIII, *Kpn*I, and *Bam*HI) and inside (B) (*Bgl*I, *Eco*RI, and *Pst*I) the amplified region. The 2.2- and 2.7-kb amplicons are indicated by small circles (stained gel) and arrowheads (autoradiogram). The squares and T's are explained in the text. W, wt W DNA; 1, HU1 DNA; 19, HU19 DNA.

three groups: herpesviruses, eucaryotes, and procaryotes. It also indicates that the four eucaryotic proteins are more closely related to each other than are the three representatives of the herpesvirus group.

DISCUSSION

The amino acid sequence of a 37-kDa protein encoded within the *Hin*dIII F fragment of VV was inferred from the DNA structure. The protein was found to be ~80% identical to the M2 subunit of eucaryotic cellular ribonucleotide reductases. Evidence that this viral gene is expressed to produce an active enzyme subunit was obtained by comparing DNA isolated from hydroxyurea-resistant mutants with DNA from wt virus. The mutants exhibited an aberrant *Hin*dIII F fragment, shown in this study to harbor amplified DNA containing the small-subunit gene.

Amplification of DNA is a widespread phenomenon in cultured cells and tumors. The resultant increase in gene dosage may foster a growth advantage or allow the cell to survive drug treatment. Despite intensive investigations in a number of laboratories, the mechanisms underlying gene amplification are not well understood (for reviews, see references 32 and 40). We believe that the present report is the first describing gene amplification in response to drug selection in a DNA virus. Gene amplification in bacteriophage T4 in response to selective growth conditions was recently described (46). The ability to generate gene amplifications in the relatively manipulatable VV genome may prove to be a valuable tool in the study of this important genetic aberration.

Although the coding portion of the M2 gene encompasses 957 nt, both hydroxyurea-resistant mutants characterized in

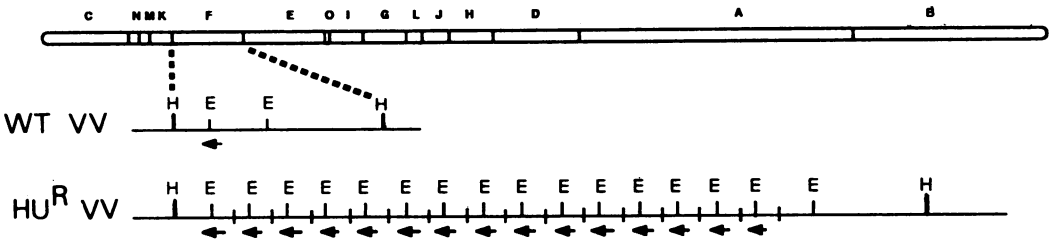

FIG. 4. Diagrammatic representation of the alteration in *Hin*dIII-F as a result of M2 gene amplification. The location and orientation of the M2 gene (arrow) in wt DNA and a drug-resistant variant containing highly amplified M2 DNA are shown. The endpoints of the amplicons are estimated.

this study amplified a much larger segment of DNA. The rightward limits of both amplicons lie to the left of the *Bam*HI site upstream of the M2 ORF, and the leftward limits are beyond the two *Xba*I sites downstream of the M2 coding sequence (unpublished data). Precise location of the amplicon termini and determination of the sequences at the novel joints is under way.

If the model presented in Fig. 4 is correct, no genetic information was lost in generating these mutants; i.e., intact wt viral DNA flanks the amplified region. However, the HU1 virus displays multiple aberrations in early protein synthesis and replicates poorly (37), suggesting that additional genetic lesions may be present. The fact that the cross-linked terminal regions of HU1 DNA were shorter than those of wt DNA may be a contributing factor affecting replication efficiency.

Restriction analysis of mutant DNA led us to the conclusion that the amplified units are arranged as direct repeats in tandem arrays. One plausible mechanism for the initial creation of a viral genome carrying a gene duplication is suggested by current models for poxvirus replication (23, 24). A large deletion event within a concatemeric replication intermediate could bring one copy of the M2 gene into the vicinity of an M2 gene with the same polarity in another unit genome. Telomere resolution would then generate a mutant with a direct duplication. Under selection pressure favoring overexpression of gene products encoded within the duplication, homologous recombination between the progeny of such mutants would generate larger arrays of repeats, as well as genomes carrying only a single copy of the gene. The phenotypic and genotypic characteristics of HU1, HU19, and their derivatives are consistent with the last part of this

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  1 CCA TGA ATG TCG ATA ATT TAA TTA TGA TAG TAC TAA TAA CAA TGC TAT CAA TAA TAC TTG

 61 TAA TTA TTG TAG TGA TTG CGG CGA TAT CGA TGT ACA AAA GAT CCA AGT ACA GGC ATA TAG

121 ATA ACT GAA AAA AAA TTT ATT GTT ATT GTT AAT TTA GTT ATG GAA CCC ATC CTT GCA CCA
                                              MET Glu Pro Ile Leu Ala Pro

181 AAT CCA AAT AGA TTT GTT ATT TTC CCA ATC CAA TAT TAT GAC ATC TGG AAC ATG TAT AAA
    Asn Pro Asn Arg Phe Val Ile Phe Pro Ile Gln Tyr Tyr Asp Ile Trp Asn Met Tyr Lys

241 AAG GCA GAG GCA TCA TTT TGG ACA GTG GAA GAA GTA GAT ATA TCT AAA GAT ATC AAT GAT
    Lys Ala Glu Ala Ser Phe Trp Thr Val Glu Glu Val Asp Ile Ser Lys Asp Ile Asn Asp

301 TGG AAT AAA CTA ACA CCA GAC GAA AAA TAT TTT ATA AAA CAT GTA TTG GCG TTT TTT GCA
    Trp Asn Lys Leu Thr Pro Asp Glu Lys Tyr Phe Ile Lys His Val Leu Ala Phe Phe Ala

361 GCC AGT GAC GGA ATA GTG AAT GAA AAT TTG GCG GAA CGA TTT TGT ACA GAA GTA CAG ATT
    Ala Ser Asp Gly Ile Val Asn Glu Asn Leu Ala Glu Arg Phe Cys Thr Glu Val Gln Ile

421 ACC GAG GCT AGA TGT TTC TAC GGA TTT CAG ATG GCC ATT GAA AAC ATT CAT TCG GAA ATG
    Thr Glu Ala Arg Cys Phe Tyr Gly Phe Gln Met Ala Ile Glu Asn Ile His Ser Glu Met
                  RD1
481 TAT AGT CTT TTG ATC GAT ACT TAT GTT AAA GAT AGT AAT GAA AAA AAC TAT CTC TTT AAT
    Tyr Ser Leu Leu Ile Asp Thr Tyr Val Lys Asp Ser Asn Glu Lys Asn Tyr Leu Phe Asn

541 GCC ATA GAA ACG ATG CCT TGT GTA AAA AAG AAG GCC GAT TGG GCT CAA AAG TGG ATA CAT
    Ala Ile Glu Thr Met Pro Cys Val Lys Lys Lys Ala Asp Trp Ala Gln Lys Trp Ile His

601 GAC AGC GCC GGT TAT GGA GAG AGA CTT ATT GCC TTT GCT GCA GTA GAA GGA ATC TTC TTT
    Asp Ser Ala Gly Tyr Gly Glu Arg Leu Ile Ala Phe Ala Ala Val Glu Gly Ile Phe Phe

661 TCT GGA TCA TTC GCT TCC ATA TTT TGG CTT AAA AAG CGT GGC CTA ATG CCC GGA CTC ACG
    Ser Gly Ser Phe Ala Ser Ile Phe Trp Leu Lys Lys Arg Gly Leu Met Pro Gly Leu Thr

721 TTT TCC AAC GAA CTA ATT AGT AGA GAC GAG GGT CTG CAC TGC GAT TTC GCA TGT TTG ATG
    Phe Ser Asn Glu Leu Ile Ser Arg Asp Glu Gly Leu His Cys Asp Phe Ala Cys Leu Met

781 TTT AAA CAT TTA TTG CAT CCA CCG AGT GAA GAA ACC GTT AGA TCT ATT ATA ACA GAT GCG
    Phe Lys His Leu Leu His Pro Pro Ser Glu Glu Thr Val Arg Ser Ile Ile Thr Asp Ala

841 GTA TCC ATT GAA CAA GAA TTT CTT ACT GCG GCT CTT CCA GTT AAA CTT ATA GGA ATG AAT
    Val Ser Ile Glu Gln Glu Phe Leu Thr Ala Ala Leu Pro Val Lys Leu Ile Gly Met Asn

901 TGT GAA ATG ATG AAA ACA TAT ATA GAA TTC GTC GCG GAT AGA TTG ATT TCT GAA TTG GGA
    Cys Glu Met Met Lys Thr Tyr Ile Glu Phe Val Ala Asp Arg Leu Ile Ser Glu Leu Gly

961 TTT AAA AAA ATT TAT AAT GTT ACC AAT CCG TTT GAT TTC ATG GAA AAT ATA TCA TTG GAA
    Phe Lys Lys Ile Tyr Asn Val Thr Asn Pro Phe Asp Phe Met Glu Asn Ile Ser Leu Glu
                MS1
1021 GGA AAA ACT AAT TTT TTC GAA AAA CGT GTG GGT GAA TAC CAA AAA ATG GGA GTT ATG TCT
     Gly Lys Thr Asn Phe Phe Glu Lys Arg Val Gly Glu Tyr Gln Lys Met Gly Val Met Ser

1081 CAA GAA GAT AAT CAT TTT TCT TTA GAT GTT GAC TTT TAA AGA AAC ATA AAT GCC GAT ATT
     Gln Glu Asp Asn His Phe Ser Leu Asp Val Asp Phe TER

1141 TGT TAA TAC TGT GTA CTG TAA GAA TAT ATT AGC ATT GTC TAT GAC TAA GAA ATT CAA AAC

1201 AAT TAT TGA TGC TAT AGG TGG CAA TAT AAT AGT CAA TTC TAC GAT ATT GAA AAA GTT ATC

1261 TCC TTA CTT TCG CAC ACA TTT ACG TCA AAA
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FIG. 5. Nucleotide sequence of VV DNA encoding the small subunit of ribonucleotide reductase. The RNA start site as determined by S1 nuclease mapping and primer extension is marked by an arrowhead. The positions of oligonucleotides referred to in the text are shown.

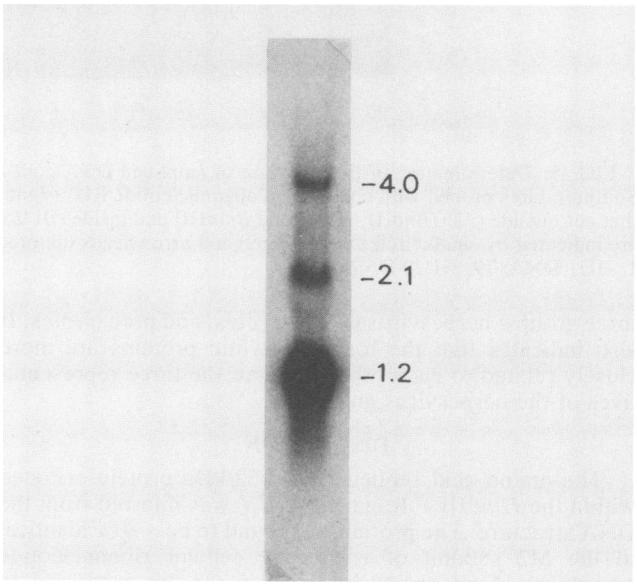

FIG. 6. Northern blot analysis. Early viral RNA (10 μg) was electrophoresed on a formaldehyde-agarose gel and transferred to a nylon filter. A hexamer primer-labeled DNA fragment from within the M2 gene was used as a probe. The numbers at the right indicate calculated sizes in kilobases as determined by coelectrophoresis of RNA markers (Bethesda Research Laboratories, Inc.).

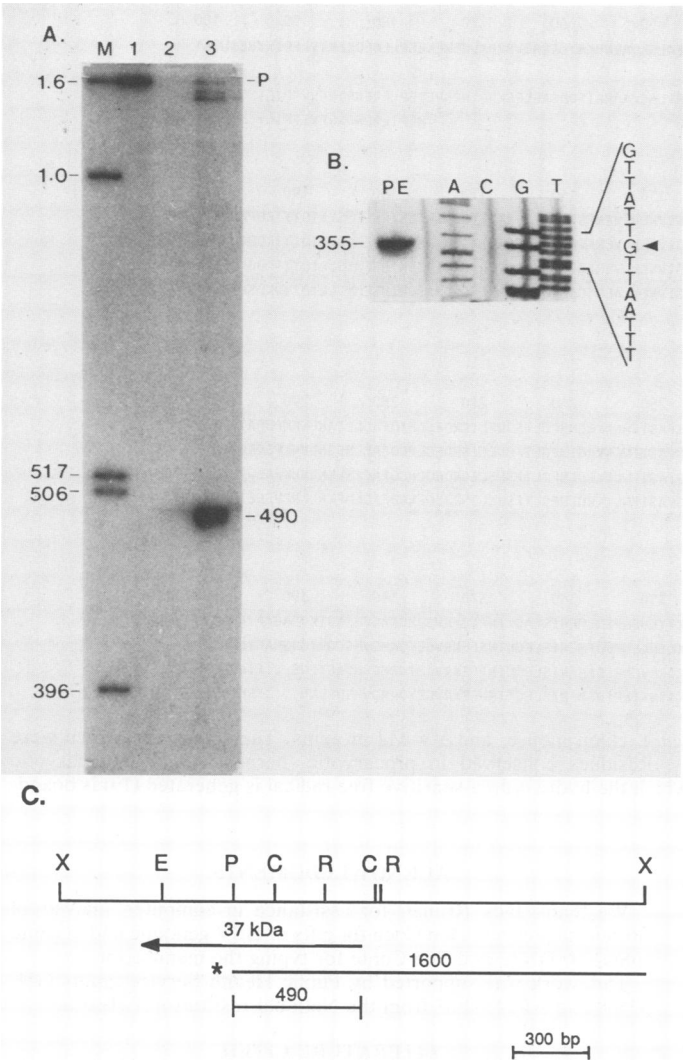

FIG. 7. Fine mapping of the site of transcription initiation. (A) S1 nuclease mapping using a 1,600-nt *Pst*I-*Xba*I DNA fragment 5′ end labeled at the *Pst*I site. The probe was hybridized to the indicated RNA and treated with S1 nuclease. Protected fragments were analyzed on a 4% sequencing gel. Lane M, Labeled markers; lane 1, full-length probe; lane 2, S1 analysis with tRNA as a control; lane 3, S1 analysis with 30 μg of early viral RNA. P, Full-length probe. (B) Primer extension mapping of the mRNA start site, using the oligonucleotide RD1. Lane PE, Primer extension product; lanes A, C, G, and T, dideoxynucleotide sequencing reactions. An arrowhead indicates the mRNA start site in the context of the sequence. The numbers in the margins of panels A and B are sizes in nucleotides, except 1.6 and 1.0, which are kilobases. (C) Diagrammatic representation of the position and orientation of the M2 ORF in the context of a restriction map of the region. The predicted molecular mass of the protein is indicated. The 5′ single end-labeled 1,600-nt *Pst*I-*Eco*RV S1 probe is shown along with the 490-nt S1-resistant hybrid. X, *Xba*I; E, *Eco*RI; P, *Pst*I; C, *Cla*I; R, *Eco*RV.

scenario: the marked instability of the hydroxyurea-resistant character in the absence of drug pressure (37), the presence within individual plaques of viruses displaying variability in hydroxyurea resistance (37), and the existence of amplicon ladders containing up to 15 repeat units of the M2 gene and flanking DNA (this study). Similar plasticity in an artificially constructed λ*tk*λ duplication contained within the VV genome has recently been described (2).

The hydroxyurea-resistant mutants examined in this study were previously shown to overproduce the enzyme activity 4- to 10-fold relative to the wt (37). Since both large and small subunits are required for activity, we reprobed the blots shown in Fig. 3 with an oligonucleotide probe specific for the large subunit of ribonucleotide reductase (L. A. Tengelsen, M. B. Slabaugh, J. K. Bibler, and D. E. Hruby, submitted for publication) to determine whether coamplification had occurred. Hybridization to a single restriction fragment in all instances (data not shown) indicated that HU1 and HU19, similar to cellular mutants selected for hydroxyurea resistance (45), had amplified only the small-subunit gene. This result suggests that either the M2 subunit is limiting in a wt infection or that increased synthesis of M2 stimulates some process leading to elevated levels of M1 as well. In most eucaryotic cell systems examined, the M2 subunit is indeed limiting, whether due to sequestration of maternal M2 mRNA (38) or cell cycle dependence of M2 gene expression (11).

The deduced *S. cerevisiae* clam, mouse, and VV M2 amino acid sequences are remarkably similar. The approximately 80% homology between cellular and VV enzymes is higher than that reported for the thymidine kinase gene (68% for VV/mouse [43]) or the large subunit of RNA polymerase (~26% for VV/*S. cerevisiae* or VV/*Drosophila melanogaster* [6]). Since the VV sequences such as the one characterized in this study resemble a processed version of a cellular gene, a plausible hypothesis is that they were incorporated into the viral genome after fortuitous reverse transcription of a cellular mRNA (3, 21).

Ribonucleotide reductases found in bacteria and eucaryotic cells exhibit complex allosteric regulation, whereas the enzyme encoded by the herpesviruses is essentially unregulated (18). The enzyme induced after VV infection biochemically resembles, but is distinct from, the cellular enzyme (36). The task of ascribing enzymatic characteristics to the presence of specific amino acid residues in the small and large subunits via protein engineering will be facilitated by the continued accumulation of genetic information.

As expected from the time course of enzyme induction after wt VV infection (35), M2 transcripts were detected in early RNA. Northern blot analysis indicated the presence of three transcripts hybridizing to a probe which was internal to the M2 coding sequence. The major transcript, 1.2 kb long, is sufficient to encode a 37-kDa protein. Evidence that the minor transcripts of 2.1 and 4.0 kb were the result of readthrough from upstream genes was obtained by S1 nuclease protection analyses and primer extension experiments. The transcriptional start site of the M2 gene was mapped to a G residue 12 nt upstream of the translational start codon. Early transcription of the VV thymidine kinase gene and 7.5-kDa gene initiates at an identical hexanucleotide sequence, TTATTG (29).

A recent report describes leader RNAs fused to the protein-coding sequences of a major late structural polypeptide (5). One of the late leaders isolated as a cDNA clone corresponds in map position and orientation to the M2 ORF but contains sequences upstream of the M2 RNA start site.

Ribonucleotide reductase is a central enzyme in DNA metabolism, providing the only route for de novo synthesis of deoxyribonucleotides. Whether the genes encoding this enzyme are essential for VV replication is of interest, not only for understanding poxvirus replication but also with regard to attenuation of vaccine strains. Perkus et al. (28) have reported on a series of VV insertion mutants, one of which contains the herpesvirus thymidine kinase gene in the

```
                10        20        30        40        50        60        70        80        90       100
YEAST MPKETPSKAAADALSDLEIKDSKSNLNKELETLREENRVKSDMLKEKLSKDAENHKAYLKSHQVHRHKLKEMEKEEPLLNEDKERTVLFPIKYHEIWQAY
CLAM                                                                                                    MY
MOUSE       MLSVRTPLATIADQQQLQLSPLKRLTLADKENTPPTLSSTRVLASKAARRIFQDSAELESKAPTNPSVEDEPLLRENPRRFVVFPIEYHDIWQMY
VV                                                                                  MEPILAPNPNRFVIFPIQYYDIWNMY

               110       120       130       140       150       160       170       180       190       200
YEAST KRAEASFWTAEEIDLSKDIHDWNNRMNENERFFISRVLAFFAASDGIVNENLVENFSTEVQIPEAKSFYGFQIMIENIHSETMSLLIDTYIKDPKESEFL
CLAM  KKAEASFWTAEEVDLSKDMAHWESL.KKEEKHFISHVLAFFAASDGIVNENLVERFSKEVQVTEARCFYGFQIAMENIHSEMYSLLIDTYIKDPQERDFL
MOUSE KKAEASFWTAEEVDLSKDIQHWEAL.KPDERHFISHVLAFFAASDGIVNENLVERFSQEVQVTEARCFYGFQIAMENIHSEMYSLLIDTYIKDPKEREYL
VV    KKAEASFWTVEEVDISKDINDWNKL.TPDEKYFIKHVLAFFAASDGIVNENLAERFCTEVQITEARCFYGFQMAIENIHSEMYSLLIDTYVKDSNEKNYL
                *         *         *                                                 *   *   *

               210       220       230       240       250       260       270       280       290       300
YEAST FNAIHTIPEIGEKAEWARLWIQDADALFGERLVAFASIEGVFFSGSFASIFWLKKRGMMPGLTFSNELICRDEGLHTDFACLLFAHLKNKPDPAIVEKIV
CLAM  FNAIETMPCVKEKADWAMRWINDDSSSYAERVVAFAAVEGIFFSGSFASIFWLKKRGIMPGLTFSNELISRDEGLHCDFACLMFSHLVNKPSQERIHQII
MOUSE FNAIETMPCVKKKADWALRWIGDKEATYGERVVAFAAVEGIFFSGSFASIFWLKKRGLMPGLTFSNELISRDEGLHCDFACLMFKHLVHKPAEQRVREII
VV    FNAIETMPCVKKKADWAQKWIHDSAG.YGERLIAFAAVEGIFFSGSFASIFWLKKRGLMPGLTFSNELISRDEGLHCDFACLMFKHLLHPPSEETVRSII
                          *   *  **                                        * ***  *

               310       320       330       340       350       360       370       380       390       400
YEAST TEAVEIEQRYFLDALPVALLGMNADLMNQYVEFVADRLLVAFGNKKYYKVENPFDFMENISLAGKTNFFEKRVSDYQKAGVMSKSTKQEAGAFTFNEDF
CLAM  DEAVKIEQVFLTEALPCRLIGMNCDLMRQYIEFVADRLLLELKCDKLYNKENPFDFMEHISLEGKTNFFEKRVGEYQKMGVMSGGNTGDSHAFTLDADF
MOUSE TNAVRIEQEFLTEALPVKLIGMNCTLMKQYIEFVADRLMLELGFNKIFRVENPFDFMENISLEGKTNFFEKRVGEYQRMGVMSNSTENS...FTLDADF
VV    TDAVSIEQEFLTAALPVKLIGMNCEMMKTYIEFVADRLISELGFKKIYNVTNPFDFMENISLEGKTNFFEKRVGEYQKMGVMSNEDNH....FSLDVDF
                                                                  *         *
```

FIG. 8. Comparison of amino acid sequences of yeast (*S. cerevisiae*), clam, mouse, and VV M2 proteins. The sequences shown were aligned by eye, and gaps were introduced to maximize identities. Residues conserved in procaryotic, herpesvirus, and eucaryotic small-subunit proteins are indicated by asterisks. The tyrosine from which the hydroxyurea-sensitive free radical is generated (19) is boxed.

leftmost *Bgl*II site in *Hin*dIII-F. Thus, the M2 gene is interrupted in this mutant. The insertion occurs in a relatively nonconserved region of the coding sequence but would separate the carboxy-terminal portion of the protein (with two completely conserved amino acids) from the remainder of the enzyme. It seems likely, therefore, that ribonucleotide reductase is nonessential for VV reproduction in tissue culture cells, probably due to partial complementation by the host cell enzyme. Whether the enzyme is essential for growth in nondividing cells remains to be investigated.

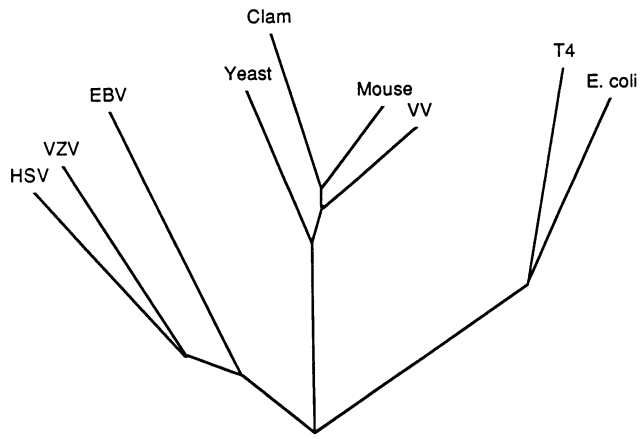

FIG. 9. Phylogenetic tree generated by computer analysis of nine M2 amino acid sequences as described in the text. The data used were derived from the present report and references 10 to 12 and 24 to 28. VZV, Varicella-zoster virus; EBV, Epstein-Barr virus.

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