Chlorella Virus NY-2A Encodes at Least 12 DNA Endonuclease/Methyltransferase Genes

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The 380-kb chlorella virus NY-2A genome is highly methylated; 45% of the cytosines are 5-methylcytosine (5mC) and 37% of the adenines are N6-methyladenine (6mA). Based on the sensitivity/resistance of NY-2A DNA to 80 methylation-sensitive DNA restriction endonucleases, the virus is predicted to encode at least 10 DNA methyltransferases: 7 6mA-specific methyltransferases, M.CviQI (GTmAC), M.CviQII (RmAR), M.CviQIII (TCGmA), M.CviQIV (GmATC), M.CviQV (TGCA), M.CviQVI (GmANTC), and M.CviQVII (CAGT); and 3 5mC-specific methyltransferases, M.CviQVIII [RGmC(T/C/G)], M.CviQIX (CCC), and M.CviQXI (CGR). Five of the 6mA methyltransferase genes, M.CviQI, M.CviQII, M.CviQVI, M.CviQ VII, were cloned and sequenced. In addition, 2 site-specific endonuclease activities, R.CviQI (G/TAC) and NY2A-nickase (R/AG), were detected in cell-free extracts from NY-2A virus-infected chlorella. Therefore, the NY-2A genome contains at least 12 DNA methyltransferase and endonuclease genes which, altogether, compose about 3±4% of the virus genome.

INTRODUCTION

The genomes of most animal and plant viruses lack modified bases. Exceptions include the two iridoviruses, frog virus 3 (Willis and Granoff, 1980) and fish lymphocytosis disease virus (Wagner et al., 1985), in which 20 to 22% of the cytosines exist as 5-methylcytosine (5mC), human papillomavirus (Danos et al., 1980; Burnett and Sleeman, 1984), and the chlorella viruses which are members of the Phycodnaviridae family (Van Etten, 1995). In fact, 5mC and 6-methyladenine (6mA) are common in chlorella virus genomes; 5mC concentrations range from 0.1 to 47% of the total cytosines and 6mA concentrations vary from 0 to 37% of the total adenines (Van Etten et al., 1985; Nelson et al., 1993). Furthermore, the chlorella viruses can be distinguished from one another by the sensitivity or resistance of their DNAs to methylation-sensitive restriction endonucleases. That is, each virus expresses a characteristic DNA modification phenotype. The discovery of methylated bases in these viral genomes ultimately revealed that these viruses encode multiple DNA methyltransferases and DNA site-specific (restriction) endonucleases, some of which resemble bacterial restriction-modification (R-M) enzymes (e.g. Xia et al., 1986; Xia and Van Etten, 1986; Nelson et al., 1993; Que et al., 1997).

This paper describes the DNA methyltransferases and DNA site-specific endonucleases encoded by virus NY-2A, which is the chlorella virus with the highest concentration of methylated bases in its genome (45% 5mC and 37% 6mA) (Schuster et al., 1986). The 380-kb NY-2A genome (Rohozinski et al., 1989) encodes at least 10 DNA methyltransferases and 2 DNA site-specific endonucleases, making the NY-2A genome one of the most concentrated sources of R-M genes known.

RESULTS

Virus NY-2A DNA methylation phenotypes

High performance liquid chromatography analysis of the chlorella virus NY-2A genome indicated that 5mC comprises 45% of its cytosines and 6mA accounts for 37% of its adenines (Schuster et al., 1986). The DNA modification phenotype of the NY-2A genome was deduced from the resistance/sensitivity of the DNA to 80 5mC and 6mA methylation-sensitive restriction endo-
nucleases (McClelland et al., 1994). NY-2A DNA was at least partially resistant to 76 of these 80 endonucleases. In fact, only Dral, Ndel, SspI, and Sau3A cleaved NY-2A DNA to apparent completion. From these experiments we predict that NY-2A DNA contains 6mA in at least seven DNA sequences, GTmAC, RmAR, TCGmA, GmATC, TGCmA, GmANTC, and CmATG, and 5mC in at least three DNA sequences, RGmC(T/C/G), mCC, and mCGR (Table 1). Assuming a random sequence distribution, the seven 5mC modifications can account for all of the 45% 5mC in NY-2A DNA; the RGmC(T/C/G) methylation contributes 9.6% (1.6% of 5mC), mCC methylation contributes 25% (6.8% of 5mC), and the mCGR methylation contributes 12.5% (2.4% of 5mC) for a total of 47% 5mC.

All of the chlorella virus DNA methyltransferases characterized to date are monospecific, i.e., each methyltransferase has a single 2- to 4-bp recognition sequence (e.g., Nelson et al., 1993; Que et al., 1997). Therefore, we predict that NY-2A encodes at least 10 separate DNA methyltransferase genes. In accordance with the nomenclature of Smith and Nathans (1973), the 7 Chlorella virus (Cvi) adenine methyltransferases and 3 cytosine methyltransferases were designated M.CviQI to M.CviQV and correspond to the DNA modification phenotypes listed in Table 1.

Cloning NY-2A DNA methyltransferase genes

E. coli clones which expressed the M.CviQI (GTmAC), M.CviQIII (TCGmA), M.CviQV (TGmCA), and M.CviQVI (GmANTC) DNA methyltransferase genes were selected from an NY-2A DNA library by digestion of the pooled plasmid DNAs with restriction endonucleases Rsal (GT/AC), TaqI (TG/CA), CviRI (TG/CA), or HinfI (G/ANTC), respectively, prior to a second round of transformation into E. coli (Lunnen et al., 1988). Because Rsal, TaqI, CviRI, and HinfI do not cleave DNAs which contain 6mA in their restriction sites (McClelland et al., 1994), plasmid DNAs resistant to these four enzymes were expected to express sequence-specific DNA methyltransferases which methylate GTmAC, TCGmA, TGmCA, or GmANTC sites, respectively. The M.CviQVII (CmATG) gene was cloned using a slightly different method. A 4.6-kb DraI fragment of NY-2A DNA, which hybridized to the cloned M.CviAII methyltransferase (CmATG) gene from chlorella virus PBCV-1 (Zhang et al., 1992) was eluted from an agarose gel and cloned into the EcoRV site of pBluescript (SK-).

Attempts to clone the other five predicted DNA methyltransferases by expression in E. coli were unsuccessful. Expression of either the NY-2A M.CviQII (RmAR) methyltransferase gene or the M.CviQVI gene should produce plasmid DNA resistance to HinfI. However, only one HinfI resistant clone was obtained and this clone contained the M.CviQVI gene (see below). Several attempts to clone the NY-2A-encoded M.CviQIV (GmATC) methyltrans-
Characterization of the M.CviQI (GTmAC) methyltransferase gene

Digestion of the NY-2A DNA library with RsaI endonuclease yielded one RsaI-resistant plasmid clone, pJN6, which contained a 1.0-kb insert DNA. pJN6 was also resistant to cleavage by KpnI (GGTACC), ScaI (AGTACT), and RsaI (GTAC) when its insert DNA was cloned in the opposite orientation. pJN6 was cleaved by other restriction endonucleases, including MboI (GATC), MboII (AGTACG), and SphI (CTGCAG). SphI cleaves GTmACG, but not CGTACG, sequences (McClelland et al., 1994). Therefore, pJN6 likely contains the M.CviQI gene whose gene product M.CviQI methylates adenine in GTAC sequences.

Sequencing pJN6 revealed an ORF predicted to encode a protein of 267 amino acids with a molecular mass of 30,919; this putative protein contains some of the sequence motifs expected for a 6mA-specific methyltransferase (Fig. 1A). Malone et al. (1995) recently reported that bacterial 6mA methyltransferases contain nine motifs (named I, II, III, IV, V, VI, VII, VIII, and X), with motifs I and IV being the most highly conserved. Furthermore, they separated the 6mA methyltransferases into three groups, α, β, and γ, based on the sequential order of these conserved motifs. The pJN6-encoded ORF contains at least five of these nine motifs, including the two most highly conserved motifs, an EPsaGdG sequence at positions 32 to 38 (motif I) and an NPPF sequence at positions 47 to 53 (motif IV) (Klimasauskas et al., 1989; Malone et al., 1995). The positions of motif I and motif IV indicate that M.CviQI is a member of the γ family of 6mA methyltransferases and that its target recognition domain (TRD) is near the carboxyl terminus of the protein.

FASTA analysis revealed that the M.CviQI amino acid sequence has 24% amino acid identity with a portion of chlorella virus SC-1A M.CviSIII (TGCmA) methyltransferase (Que et al., 1997) and 23% amino acid identity with M.HincII (GTYRmAC) from Haemophilus influenzae (Ito et al., 1990) (Table 2). This is the first time a 6mA methyltransferase with GTAC sequence specificity has been cloned from any organism.

Characterization of the M.CviQIII (TGCmA) methyltransferase gene

Incubating the NY-2A DNA library with TaqI yielded many TaqI-resistant plasmid clones. One clone, pKM120, containing a 2.9-kb insert DNA, was chosen for further study. Plasmid pKM120 was also resistant to Clal (ATCGAT), Xhol (CTCGAG), and Sall (GTCGAC) endonucleases, but was cleaved by PstI (CTGCAC), Sphi (GCA-TGC), RsaI (GTAC), and HinfI (GANTC).

The DNA sequence of pKM120 revealed an ORF of 1107 nucleotides which could encode a polypeptide of 386 amino acids with a predicted molecular mass of 42,158. The amino acid sequence of this ORF contains the nine sequence motifs found in many 6mA methyltransferases (Malone et al., 1995) including the two most highly conserved motifs, an EPscGtG sequence at positions 47 to 53 (motif I) and a NPPF sequence at positions 112 to 115 (motif IV) which is involved in catalysis (Fig. 1B). M.CviQIII is a member of the γ group of 6mA methyltransferases and its TRD is predicted to be near the carboxyl terminus of the protein.

Comparison of the M.CviQIII amino acid sequence to other chlorella virus-encoded and bacterial 6mA methyltransferases revealed that it is most similar to two isomethylomers M.CviBIII (75% amino acid identity) and M.CviSIII (51% amino acid identity) from chlorella viruses NC-1A (Narva et al., 1987) and SC-1A (Que et al., 1997), respectively (Table 2). M.CviQIII has 34 to 37% amino acid identity with three chlorella virus 6mA methyltransferases that recognize TGCA sequences, M.CviSI (Que et al., 1997), M.CviRI (Stefan et al., 1991), and M.CviQV (see below). M.HincII (GTYRmAC) from H. influenzae (Ito et al., 1990) is the bacterial 6mA methyltransferase with the greatest similarity to M.CviQIII (FASTA score of 211) while the bacterial isomethylomer M.TaqI (Slatko et al., 1987) has a FASTA score of only 143.

Characterization of the M.CviQV (TGCmA) methyltransferase gene

Incubation of the NY-2A DNA library with CviRI yielded one CviRI-resistant plasmid clone, pKM102, containing a 2.0-kb insert DNA. pKM102 DNA was resistant to CviRI when its insert DNA was cloned in the opposite orientation and it was also resistant to PstI (CTGCAG) and Nsil (ATGCGAT), but was cleaved by MboI (GATC), TaqI (CTCGA), Sall (GTCGAC), Sphi (GCA-TGC), and ApaLI (GTCGAC).
ApaLI is insensitive to 6mA methylation in its recognition sequence (McClelland et al., 1994).

DNA sequencing of pKM102 revealed an ORF of 1149 nucleotides which could encode a polypeptide of 382 amino acids with a molecular mass of 43,397. The predicted amino acid sequence of this ORF contains the nine 6mA methyltransferase motifs, including the two most highly conserved motifs, an EPtcGtG sequence at positions 53 to 59 (motif I) and a NPPF sequence at positions 115 to 118 (motif IV) which is involved in catalysis (Fig. 1C). M. CviQV (TGCmA) is in the group of 6mA methyltransferases and its TRD is predicted to be in the carboxyl terminal portion of the protein.

Comparison of the M.CviQV amino acid sequence to other chlorella virus-encoded and bacterial 6mA methyltransferases (Table 2) revealed 79% amino acid identity with its isomethylomer M. CviRI from chlorella virus XZ-6E (Stefan et al., 1991). Interestingly, it only has 32% amino acid identity to another isomethylomer M. CviSI from chlorella virus SC-1A (Que et al., 1997) which is about the same homology observed with chlorella virus TCGA methyltransferases M. CviBIII (Narva et al., 1987), M. CviQIII (this paper), and M. CviSIII (Que et al., 1997). The bacterial methyltransferase with the highest similarity to M. CviQV was M. TaqI (T CGmA) with a FASTA score of 197.

FIG. 1. The predicted amino acid sequences of M.CviQI (A), M.CviQIII (B), M.CviQV (C), M.CviQVI (D), and M.CviQVII (E) DNA methyltransferases. Each of the five virus-encoded 6mA methyltransferases contain most of the nine sequence motifs (underlined) present in 6mA methyltransferases (Malone et al., 1995).
Characterization of the M.CviQVI (GmANTC) methyltransferase gene

Incubation of the NY-2A DNA library with Hinfl resulted in one Hinfl-resistant plasmid clone, named pJN7. Plasmid pJN7, which contained a 11-kb DNA insert, was resistant to HhaI (GANTC), but was sensitive to Mbol (GATC), Rsal (GTAC), and Nialll (CATG). pJN7 was also resistant to Hinfl digestion when the insert DNA was cloned in the opposite orientation.

Sequencing the 11-kb insert DNA from pJN7 identified a 786 base ORF, which could encode a protein with 261 amino acids and a predicted molecular mass of 31922. This ORF has the nine motifs characteristic of 6mA methyltransferases, including the two most highly conserved motifs, an EPFaGga sequence at positions 31 to 37 (motif I) and a DPPY sequence at positions 177±180 (motif IV) (Fig. 1D). The order of the nine motifs places M.CviQVI in the α group of 6mA methyltransferases and predicts that its TRD is in the center of the protein.

M.CviQVI has 82% amino acid identity with its iso- methylomer M.CviBII from chlorella virus NC-1A (Kan et al., 1992) (Table 2). M.CviQVI also has about 30% amino acid identity with several bacterial DNA methyltransferases, 33 and 29% identity with the H. influenzae (Fleischmann et al., 1995) and E. coli (Brooks et al., 1983) Dam methyltransferases (GmATC), respectively, and 27% identity with M.DpnII (GmATC) from Streptococcus pneumoniae (Mannarelli et al., 1985) (Table 2). Surprisingly, the homology between M.CviQVI and its two bacterial isomethylomers, M.Hinfl (Chandrasegaran et al., 1988) and M.HhaI (Schoner et al., 1983) is poor, displaying only 14 and 15% amino acid identity, respectively. Furthermore, the amino acid sequence of M.Hinfl places it in the β group of adenine methyltransferases (Malone et al., 1995), whereas M.CviQVI is a member of the α group.

Characterizing the M.CviQVII (CmATG) methyltransferase gene

We reported previously that NY-2A DNA hybridized weakly to the M.CviAII (CmATG) DNA methyltransferase gene from chlorella virus PBCV-1 (Zhang et al., 1997). Because both NY-2A and PBCV-1 DNAs are methylated at CmATG sites, we suspected that a 4.6-kb Dral fragment from NY-2A DNA, which hybridized to the M.CviAII gene contained the M.CviQVII (CmATG) gene. This Dral fragment was cloned into the pBluescript (SK+) EcoRV site and named pyZ137. pyZ137 was resistant to CviAII (CATG), Nialll (CATG), and SpfI (GATGC) endonucle-
ases, but was cleaved by several other restriction endonucleases including Rsal (GTAC), TaqI (TCGA), MboI (GATC), and CviRI (TCGA). pYZ137 DNA was also resistant to NlaIII when its insert was cloned in the opposite orientation.

Sequencing about 1800 bases at one end of the 4.6-kb DNA insert from pYZ137 revealed a 975 base ORF, which encoded a protein of 324 amino acids with a predicted molecular mass of 37,224 (Fig. 1E). This ORF has the nine sequence motifs characteristic of 6mA-specific methyltransferases, including a DLFaGsc sequence at positions 34 to 40 (motif I) and a DPPY sequence at positions 205-208 (motif IV), which are the two most highly conserved motifs (Fig. 1E). The order of the nine motifs places M.CviQVII in the α group of 6mA methyltransferases and the TRD is predicted to be in the center of the protein.

M.CviQVII has 80% amino acid identity with its two chlorella virus isomethylomes, M.CviSI and M.CviAI from viruses SC-1A (Que et al., 1997) and PBCV-1 (Zhang et al., 1992), respectively, and 37% amino acid identity with its bacterial isomethylomer M.NlaIII (CmATG) from Neisseria lactamica (Labbe et al., 1990) (Table 2). M.CviQVII is also similar to the N-terminal portion of several large (>600 amino acids) type IIIs bacterial 6mA methyltransferases. It has 38% amino acid identity with M.FokI (GGmATG/CmATCC) from Flavobacterium okeano-

Isolation and characterization of DNA site-specific endonucleases

Two site-specific endonuclease activities were detected in cell-free extracts prepared from NY-2A-infected chlorella cells at 16 h p.i. The first activity, named NY2A-nickase, which eluted from a heparin sepharose CL-6B column at 0.5 to 0.6 M NaCl, cleaved pUC19 DNA into many small fragments. The second activity, which eluted at 0.7 to 0.8 M NaCl, cleaved pUC19 DNA into two fragments and is the previously described CviQI site-specific endonuclease (Xia et al., 1987). CviQI, originally named CviII (Xia et al., 1987), cleaves GTAC sequences between the G and T. Both endonuclease peaks were collected and purified further by phosphocellulose and hydroxylapatite chromatography.

NY2A-nickase cleaves between purine and A in 5'-RAG-3' sequences. However, it does not cleave complementary 5'-CTY-3' sequences (Fig. 2). Thus the NY2A-nickase creates double-stranded breaks only when two 5'-RAG-3' sequences are close together on opposite strands. Consequently NY2A-nickase does not produce a completely stable DNA digestion pattern. In this re-
donucleases. Extrapolating from the 330,740-bp chlorella virus PBCV-1 genome, which contains 377 putative coding sequences (Li et al., 1997), the 380-kb NY-2A genome is predicted to contain 434 coding sequences. As a minimum, the NY-2A genome contains 12 R-M genes or 1 out of every 36 NY-2A genes is a R-M gene, making the NY-2A genome one of the most concentrated sources of R-M genes known. Several large dsDNA-containing bacteriophages, such as E. coli P1 (Humbelin et al., 1988; Coulby and Sternberg, 1988), E. coli T4 (Hattman et al., 1985; Krabbe and Carlson, 1991), and Bacillus subtilis phages (Behrens et al., 1987), encode 2 to 4 different R-M genes. A few bacteria, such as Nostoc PCC7524 (Reaston et al., 1982), Dactylococcus salina (Laue et al., 1991), and Neisseria gonorrhoeae (Stein et al., 1995) encode 5 to 14 R-M systems. Presumably these bacterial genomes are at least 5 to 10 times larger than the NY-2A genome. However, the number of R-M genes that exist in a single bacterial genome increased dramatically with the recent report that Helicobacter pylori encodes 3 type I, 1 type II, 3 type III, and 4 type IV R-M systems. In addition to these complete R-M systems, 7 adenine-specific and 4 cytosine-specific DNA methyltransferases and 1 of unknown specificity were found, each one of which may have a companion restriction endonuclease (Tomb et al., 1997). Thus, R-M genes comprise 34 to 45 of the predicted 1590 coding sequences in the H. pylori genome or 1 out of every 35 to 47 genes is a R-M gene.

The NY-2A DNA methyltransferase genes have not been mapped on the virus genome. As a practical matter, it will be difficult to construct a NY-2A DNA restriction map because NY-2A is resistant to cleavage by most restriction endonucleases. However, the M.CviQV (TGCmA) and M.CviQVII (CmATG) genes are close to one another in the NY-2A genome because DNA sequences flanking these two genes overlap. These two 6mA DNA methyltransferase genes are separated by 370 nucleotides which encode two small ORFs. One of these small ORFs has 68% amino acid identity with a 75 codon ORF, named A687R, of unknown function from the prototype chlorella virus PBCV-1 (Li et al., 1997). R-M genes are widespread in the chlorella viruses and their presence leads to two obvious questions: Where did the genes come from? What is their biological function? Several observations suggest that the bacterial and chlorella virus R-M enzymes have evolved from a common progenitor. (i) Amino acid sequence motifs characteristic of bacterial 5mC and 6mA DNA methyltransferases are also present in the chlorella virus methyltransferases. (ii) Some of the chlorella virus methyltransferases have up to 37% amino acid identity with portions of bacterial methyltransferases. (iii) Chlorella virus restriction endonuclease genes (Zhang et al., 1992; Swaminathan et al., 1996; Li et al., 1997), like bacterial restriction endonuclease genes (Wilson and Murray, 1991), are adjacent to companion DNA methyltransferase genes. (iv) Many chlorella virus methyltransferase genes have upstream regions which function as promoters in E. coli (e.g., Narva et al., 1987; Stefan et al., 1991). All five of the NY-2A virus methyltransferases described in this report have a sequence which resembles the bacterial -35 (TTGACA) promoter sequence (only one base different in each case) (Table 2). However, the similarity and the 16 to 19 nucleotide spacing to a consensus -10-like (TATAAT) sequence is poor for these five genes.

Although the evidence suggests that the bacterial and chlorella virus DNA methyltransferases probably have common evolutionary origins, the amino acid homology data and the promoter sequence data suggest an ancient association between the chlorella viruses and the methyltransferase genes. A comparison of the G + C content of the five NY-2A methyltransferase genes with total NY-2A DNA supports this hypothesis. If the G + C contents of these five genes differ significantly from total NY-2A DNA, then the DNA methyltransferase genes are probably "alien" (Anton et al., 1997). In contrast, if the G + C contents are similar to the total genomic DNA, the genes have probably existed in the genome for a long time. The M.CviQI, M.CviQIII, M.CviQV, M.CviQVI, and M.CviQVII genes have 38, 37, 42, 38, and 41% G + C contents, respectively, which are close to the 41.5% G + C content of the entire NY-2A genome (J. L. Van Etten, unpublished results).

The biological function(s) of the chlorella virus-encoded R-M enzymes is unknown. However, the common occurrence of the DNA methyltransferase and site-specific endonuclease genes in these viruses suggest that these enzymes have an important function in the natural history of the viruses. Bacterial R-M enzymes allow foreign DNAs and DNA viruses to be recognized and degraded enzymatically (e.g., see Wilson, 1988). In fact, the name "restriction" refers to their role in excluding foreign DNA. Conversely, bacterial DNA methyltransferases serve to prevent self-digestion of bacterial DNA by a cognate isospecific endonuclease. Assuming the chlorella virus R-M enzymes have an essential biological function, then they must either serve a role similar to that of bacterial R-M enzymes or else they must have some other function in the virus life cycle. For example, the endonucleases may help degrade host DNA, providing deoxynucleotides that are recycled into virus DNA (Xia et al., 1986). Methylation of nascent DNA by the cognate methyltransferase would protect it from self-digestion (Xia and Van Etten, 1986). Alternatively, the virus-encoded enzymes may prevent infection by a second virus. Experimental tests of these two hypotheses have yielded ambiguous results (Chase et al., 1989; Burbank et al., 1990). At present, it is not even known if the chlorella virus-encoded site-specific endonucleases cleave host DNA in situ. Experiments designed to address this issue are
in progress. Regardless of their biological role, the 12 DNA R-M genes encoded by chlorella virus NY-2A represent one of the most concentrated sources of R-M genes described to date.

MATERIALS AND METHODS

Viruses, vectors, and host strains

The growth of the host alga, Chlorella strain NC64A, on MBM medium, the production of the plaque-forming virus NY-2A, and the isolation of viral DNA have been described (Van Etten et al., 1983; Schuster et al., 1986). Escherichia coli strains DH5α MCR (mcrA-, mcrB-, mrr-) and ER1470 (dam-) served as the hosts for pUC19 and E. coli Sure (mcrA-, mcrCB-, mrr-) (Stratagene, La Jolla, CA) served as the host for pBluescript (SK-).

Cloning DNA methyltransferase genes

An NY-2A DNA library was created as follows: Sau3A partial digests of viral DNA were ligated into the BamHI site of pUC19 DNA, followed by transformation into E. coli DH5α MCR. Clones which expressed the virus-encoded DNA methyltransferase genes were selected from the DNA library by digestion of pooled-ampicillin-resistant plasmids with two sequential digests with the appropriate restriction endonucleases prior to a second round of transformation into E. coli DH5α MCR (Lunnen et al., 1988). Plasmid DNAs from the resultant ampicillin-resistant bacterial colonies were expected to contain the targeted NY-2A methyltransferase genes.

Enzyme isolation and assays

Chlorella NC64A cells (2 × 10^7 cells/ml, 5 liters total volume) were infected with NY-2A at an m.o.i. of five. Virus-infected cells were collected by centrifugation at 16 h p.i. (Van Etten et al., 1988). Infected cells were disrupted and extracts were prepared as previously described (Xia et al., 1986). Clarified cell extracts were applied to a heparin sepharose CL-6B (Pharmacia, Piscataway, NJ) column, and eluted with a Tris–HCl-buffered linear gradient, pH 7.5. Active fractions were judged by digestion of pUC19 DNA, were combined and eluted as indicated: phosphocellulose (Bio-Rad Lab, Hercules, CA), 0.1 to 1.0 M KCl linear gradient in 0.01 M KPO_4 buffer (pH 7.5); hydroxylapatite (Bio-Rad HTP), 0.01 to 1.0 M KPO_4 linear gradient, pH 7.5. Active fractions were collected, dialyzed against 10 mM Tris±HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA (pH 8.0), 1 mM DTT, 50% glycerol, and stored at -20°C.

DNA site-specific endonuclease activity was assayed for 1 to 2 h at 25°C in 25-μl reaction mixtures containing 50 mM Tris±HCl (pH 8.0), 10 mM MgCl_2, 50 mM NaCl, 1 μg of pUC19 DNA, and 1 to 3 μl of enzyme extract. Digestion products were electrophoresed on 1 to 2% agarose gels in 1 x TBE buffer (80 mM Tris±HAc, 8 mM EDTA) (Maniatis et al., 1982), stained with 0.5 μg/ml ethidium bromide and visualized by midrange ultraviolet illumination.

Other procedures

Viral DNAs were cloned into pBluescript (SK-2) and grown in E. coli Sure for sequencing. Nested deletions of cloned viral DNAs were made with an “Erase-a-Base” exonuclease III kit (Promega, Madison, WI). Appropriate plasmid clones were sequenced on both strands by the procedure of Sanger et al., (1977) as modified by Tabor and Richardson (1987) using a Sequenase kit (version 2.0) from Amersham Life Science Inc. (Arlington Heights, IL). Some DNA fragments were also sequenced at the University of Nebraska Center for Biotechnology core facility using a LiCor (Lincoln, NE) model 4000 DNA sequencer.

Southern transfer of DNA to nylon membranes employed standard protocols (Maniatis et al., 1982). Radioactive DNA probes were prepared by nick translation using a Gibco BRL kit (Gaithersburg, MD). DNA and putative protein sequences were analyzed with the University of Wisconsin Genetics Computer Group package of programs (Genetics Computer Group, 1994).

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