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Baculoviruses contain a gene for the large subunit of ribonucleotide reductase

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In the genomes of two baculoviruses, *Spodoptera exigua* and *S. littoralis* multicapsid nucleopolyhedroviruses (SeMNPV and SpliMNPV, respectively), an open reading frame (ORF) encoding the large subunit of ribonucleotide reductase (RR1) was identified. The predicted amino acid sequences of SeMNPV and SpliMNPV RR1 showed high homology to RR1 proteins from eukaryotes (ca. 70% and 80% similarity, respectively). The amino acid residues thought to be involved in catalytic function were conserved in the baculoviral RR1 ORFs. The RR1 ORFs in SeMNPV and SpliMNPV were located in different genomic positions. In SeMNPV, the RR1 ORF was located upstream of the polyhedrin gene, in an anti-genomic orientation. In SpliMNPV, the RR1 ORF preceded the *p74* gene. By searching databanks, sequences homologous to the N terminus of RR1 were also detected upstream of the polyhedrin gene of three other baculoviruses, *Mamestra brassicae* multicapsid NPV, *Panolis flammea* multicapsid NPV and *Orgyia pseudotsugata* single nucleocapsid NPV. The baculovirus type species, *Autographica californica* multicapsid NPV, however, does not encode RR. A 2·7 kb transcript could be detected throughout infection with SeMNPV, classifying SeMNPV *rr1* as an early gene. Primer extension analysis revealed several early and late start sites. None of the major start sites showed similarity to previously characterized baculoviral transcriptional start motifs. Phylogenetic analysis of prokaryotic, eukaryotic and viral RR1 proteins suggested that SeMNPV and SpliMNPV acquired the gene for RR1 from a eukaryotic source, but independently from each other.

Introduction

Ribonucleotide reductase (RR) is a key enzyme in the biosynthesis of deoxyribonucleotides, catalysing the reduction of ribonucleotides (for reviews see: Thelander & Reichard, 1979; Reichard 1988, 1993; Elledge *et al.*, 1992). The RR (class I) enzyme is present in all eukaryotes and in some prokaryotes, such as *E. coli* grown under aerobic conditions (Reichard, 1993). The *E. coli* RR enzyme is considered to be the prototype and functions as a heterodimer consisting of two large (RR1) and two small (RR2) subunits encoded by different genes. The actual reduction of ribonucleotides takes place at the large subunit. The small subunit supplies the reducing capacity; it contains an active iron centre and provides electrons via a tyrosyl radical. The reduction of ribonucleotides into deoxyribonucleotides is subject to complex allosteric control exerted by binding of nucleotides to the large subunit. The overall activity level of the enzyme is regulated by the binding of ATP and dATP to the large subunit. Expression of ribonucleotide reductase genes is tightly regulated during the cell cycle (Reichard, 1988; Elledge *et al.*, 1992).

Members from several groups of large DNA viruses, such as the poxviruses, herpesviruses, T-even bacteriophages and African swine fever virus (ASFV), encode their own RR. In the animal viruses, RR also acts as a virulence factor. Deletion usually results in an attenuated phenotype with slightly impaired growth in cultured cells and increased restrictions in tissue specificity. RR-encoding viruses can apparently circumvent the strict regulation of RR activity by the cellular enzyme, as well as bypass the compromised host regulation machinery (Reichard, 1988; Conner *et al.*, 1994; Slabaugh *et al.*, 1984; Child *et al.*, 1990; Cunha & Costa, 1992; Howell *et al.*, 1993; Heineman & Cohen, 1994; Huszar & Bacchetti, 1981;

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The nucleotide sequences reported in this paper are available in the EMBL/GenBank database under accession numbers X97578 and X98924.

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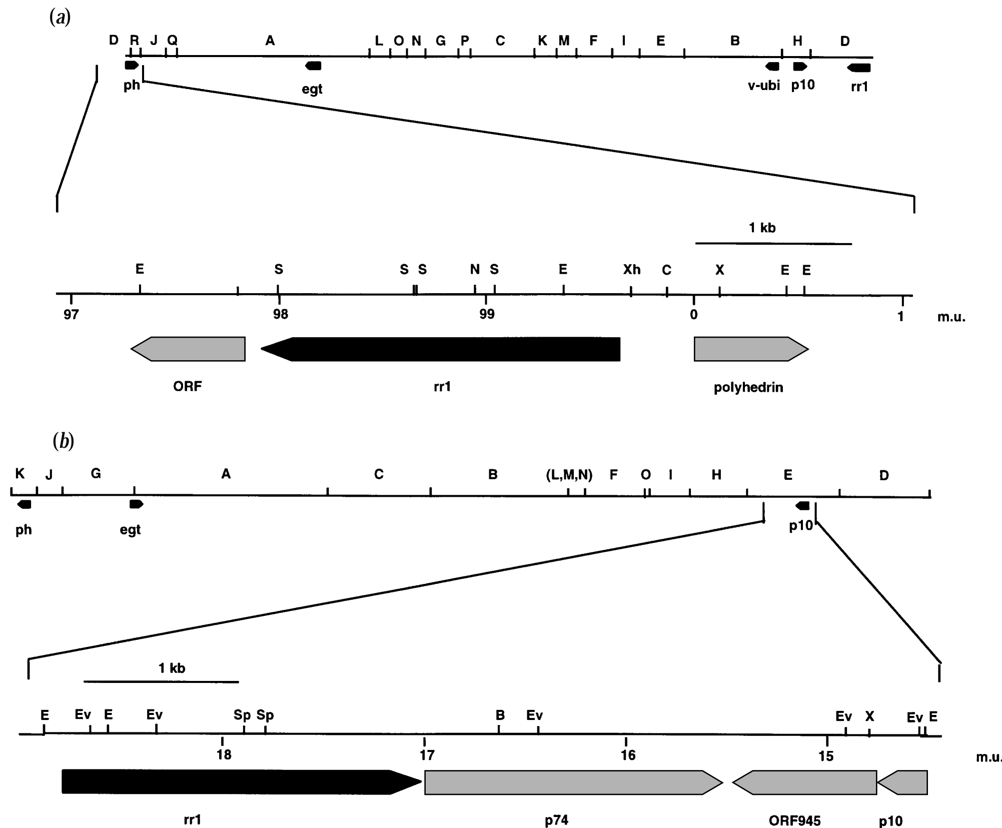

Fig. 1. Location and restriction endonuclease recognition sites of the large subunit of the *rr1* gene region on the physical map of the SeMNPV (*a*) and SpliMNPV (*b*) genome. The location and orientation of the *rr1*, polyhedrin, p10 and *egt* genes are indicated (arrows). Map units (m.u.) are also given. (*a*) Physical map of SeMNPV. E, *Eco*RI; X, *Xba*I; N, *Nru*I; Xh, *Xho*I; S, *Sal*I; C, *Cla*I. (*b*) Physical map of SpliMNPV. E, *Eco*RI; Ev, *Eco*RV; Sp, *Sph*I; X, *Xba*I. The presentation of the SpliMNPV map is reversed compared to previous publications (Croizier *et al.*, 1989; Faktor *et al.*, 1997).

Goldstein & Weller, 1988). RR is an often sought target in the search for antiviral, antimicrobial and cancer therapies, either suppressing enzyme activity exploited by the pathogen during infection, or inhibiting the elevated RR enzyme activity of rapidly growing cancer cells, which are no longer subject to normal cell cycle control (Conner *et al.*, 1994; Lori *et al.*, 1994; Reichard, 1988).

RR has not yet been described for baculoviruses, another major group of large DNA viruses. Baculoviruses are pathogenic for arthropods and have a large (80–160 kbp) circular dsDNA genome (Murphy *et al.*, 1995). They replicate in the nucleus of the infected cell. Genes encoding RR have not been reported for the type species of the *Baculoviridae*, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (Ayres *et al.*, 1994).

Here, we present evidence that two other members of the baculovirus family, i.e. *Spodoptera exigua* and *S. littoralis* multicapsid nucleopolyhedrovirus (SeMNPV and SpliMNPV, respectively), contain an open reading frame (ORF) with a high degree of homology to the large subunit of RR1 of eukaryotic and viral origins. Expression of the SeMNPV *rr*1 gene in infected insect cells was investigated by transcriptional analysis. To determine the ancestry of the two baculoviral RR1s, a phylogenetic tree was constructed using parsimony.

Methods

■ **Virus, insects and cells.** The SeMNPV field isolate (SeMNPV/US) (Gelernter & Federici, 1986) was kindly provided as polyhedra by B. A. Federici (Department of Entomology, University of California, Riverside, USA). The polyhedra were propagated in fourth instar *S. exigua* larvae (Smits *et al.*, 1988). Budded virus (BV), used in time-course infection experiments, was obtained from the supernatant of UCR-Se1 (Gelernter & Federici, 1986) or IZD-Se-2109 cells (a gift from B. Moeckel, Institute of Zoology, Technical University, Darmstadt, Germany) which had been infected with haemolymph from SeMNPV-infected fourth instar larvae. The *S. exigua* cell lines were maintained at 27 °C in plastic tissue-culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum. BV titres were determined by the end-point dilution method and expressed as $TCID_{50}$ units/ml. Cells were infected with an m.o.i. of 5 $TCID_{50}$ per cell.

SpliMNPV type-B isolate E15 was plaque-purified from haemolymph of field-infected *S. littoralis* larvae in SPC-Sl-52 cells (Mialhe *et al.*, 1984), maintained at 27 °C in TNM-FH medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum.

■ **DNA analysis.** Viral DNA was extracted from SeMNPV occlusion body derived virions purified by sucrose gradient centrifugation essentially as described by Caballero *et al*. (1992). Viral DNA from SpliMNPV was purified from viral occlusion bodies propagated in third instar *S. littoralis* larvae.

■ **Localization and DNA sequencing of the *rr1* genes.** SeMNPV fragment *Xba*I-D, containing N-terminal sequences of the polyhedrin gene (van Strien *et al*., 1992), was cloned into pUC18. Subfragments upstream of the polyhedrin gene were isolated from agarose gels using the freeze–squeeze method (Sambrook *et al*., 1989) and subcloned into plasmids pTZ19R (Promega) or pBluescriptKS(+) (Stratagene). Sequencing of SeMNPV inserts with standard sequencing and custom-designed primers (Eurogentec) was performed with *Taq* polymerase using the chain termination method.

SeMNPV *rr*1 gene DNA fragments were [α-^{32}P]dATP-labelled and hybridized under non-stringent conditions (Sambrook *et al*., 1989) to a Southern blot of SpliMNPV viral DNA on Hybond-N filters (Amersham). Hybridizing SpliMNPV DNA fragments were purified from agarose gels with the Jetsorb kit (Genomad), (sub)cloned into pUC19 and sequenced according to the chain termination method with standard sequencing primers using the Sequenase kit (United States Biochemical).

■ **Sequence analysis of the *rr1* genes.** Sequences were analysed with the UWGCG computer programs (Devereux *et al*., 1984); DNA and deduced amino acid sequences were compared with the updated GenBank/EMBL, SWISSPROT and PIR databases using the FASTA program and BLAST network service (Altschul *et al*., 1990). Phylogenetic analysis was performed with the PAUP 3.1 program (Swofford, 1993), using GCG pileup to produce input files of aligned protein sequences. Bootstrap analysis (Felsenstein, 1993), included in the PAUP package, was used to assess the integrity of the produced phylogeny.

■ **Isolation of total RNA and Northern blot hybridization.** For Northern blot analysis and primer extension, total RNA was isolated from SeMNPV-infected UCR-Se1 or IZD-Se-2109 cells at various times post-infection (p.i.), as described by van Strien *et al*. (1992). Total RNA was denatured, electrophoresed in agarose gels and blotted onto Hybond-N nylon membrane (van Strien *et al*. 1992). To identify *rr1* transcripts, the blot was hybridized for 16 h at 65 °C with an [α-^{32}P]ATP-labelled *rr1* specific probe. After hybridization, the filter was washed for 5 min with 2× SSC, 0·5% SDS at room temperature, 30 min with 2× SSC, 0·1% SDS at 65 °C and 30 min with 0·1× SSC, 0·1% SDS at 65 °C. The filter was exposed to Kodak XAR film.

■ **Primer extension.** To identify the transcriptional start site(s) of SeMNPV *rr1*, 15 ng of an oligonucleotide (5′ CAGACTATTCAAGA-GCAGAG 3′), complementary to the *rr1* mRNA, was labelled at the 5′ end with [γ-^{32}P]dATP by T4 polynucleotide kinase (Gibco-BRL) in 50 mM Tris–HCl pH 9·5, 10 mM $MgCl_2$, 5 mM DTT, 5% glycerol for 45 min at 37 °C followed by heat denaturation at 90 °C for 10 min. The labelled oligonucleotide was purified on a 1 ml Sephadex G25 column. Labelled primer was added to 10 μg of total infected-cell RNA and the mixture was denatured at 90 °C for 5 min and annealed at 54 °C for 15 min. Reverse transcription was carried out at 48 °C for 1 h in a volume of 15 μl, containing 5 mM of each of the dNTPs and 1 μl Superscript reverse transcriptase (Gibco-BRL) in a buffer supplied by the manufacturer. The reaction was stopped by addition of 5 μl 'stop' buffer containing 95% (v/v) formamide, 0·01% xylene cyanol and 0·01% bromophenol blue. The reaction mixture (6 μl) was analysed in a 6% denaturing polyacrylamide gel.

■ **Accession numbers.** The nucleotide sequences of the SeMNPV and SpliMNPV *rr1* genes are available in the EMBL/GenBank database under accession numbers X97578 and X98924, respectively.

Results

Location and sequence of the *rr1* genes of SeMNPV and SpliMNPV

In the course of sequencing the region upstream of the SeMNPV polyhedrin gene (van Strien *et al*., 1992), a putative ORF of 2310 nt was found (Fig. 1*a*), potentially encoding a protein of 770 amino acids with a molecular mass of 87 kDa. This ORF, which was anti-genomic in orientation, had a high degree of homology to RR1 from eukaryotic and prokaryotic organisms and viruses and will be further referred to as the SeMNPV *rr1* gene.

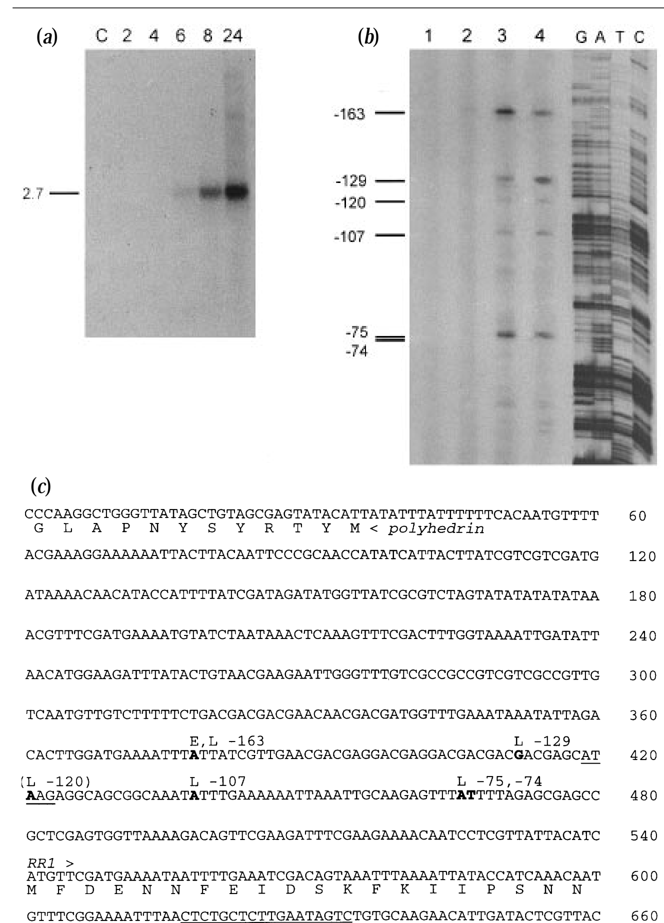

Fig. 2. Transcriptional analysis of the SeMNPV *rr1* gene region. (*a*) Northern blot analysis of total RNA extracted from uninfected (lane C) and SeMNPV-infected UCR-Se cells at 2, 4, 6, 8, 12 and 24 h p.i. for *rr1* transcripts. (*b*) Primer extension analysis of *rr1* transcripts performed with a 20-mer oligonucleotide complementary to the *rr1* RNA, ^{32}P-labelled at the 5′ end. The oligonucleotide was annealed to total RNA from uninfected (lane 1) and SeMNPV-infected IZD-Se cells isolated at 4, 12 and 24 h p.i. (lanes 2, 3 and 4) and elongated by reverse transcription. Sizes of the extension products were determined by comparison with a sequence ladder run alongside (lanes G, A, T and C) obtained from an SeMNPV *rr1*-containing plasmid clone and the 20-mer oligonucleotide as a sequence primer. Numbers indicate the position of the 5′-terminal nucleotides. (*c*) Nucleotide sequence of the SeMNPV *rr1* promoter region. Transcriptional start sites as determined by primer extension analysis are indicated in bold. The location of the oligonucleotide primer is double underlined.

Table 1. Percentage identity and similarity (in bold) of SeMNPV and SpliMNPV RR1 with RR1 proteins from several organisms and viruses

See Fig. 3 legend for abbreviations.

	SpliM NPV	Hs	Ce	Sp	Pf	VV	ASFV	HSV-1	HVS	Ec
SeMNPV	50·1	51·3	51·2	48·9	48·9	51·3	38·3	29·8	27·0	26·9
	69·1	**71·6**	**70·7**	**69·4**	**69·4**	**70·9**	**59·2**	**54·4**	**50·8**	**49·8**
SpliMNPV		67·8	64·4	61·4	58·7	61·7	39·6	28·3	27·2	28·0
		80·5	**78·8**	**78·1**	**73·7**	**78·3**	**58·4**	**49·5**	**51·2**	**50·1**

Baculovirus early (CAGT) and late (TAAG) consensus transcriptional start sites (Blissard & Rohrmann, 1990) were observed 42 and 120 nt upstream of the translational start codon, respectively (Fig. 2*c*). No TATA box sequences could be identified. Immediately after the stop codon the nucleotide sequence was relatively A/T rich. A consensus poly(A) signal (AATAAA) was first seen 639 nt downstream of the translational stop codon (data not shown). The presumed translational start codon was in agreement with the Kozak consensus rule (Kozak, 1983). The putative start codons of the SeMNPV *rr1* and polyhedrin gene were separated by an intergenic region of 516 nt. The SeMNPV *rr1* stop codon and a putative downstream ORF (data not shown) were separated by 117 nt.

An *rr1* gene was also identified in SpliMNPV by hybridization with an SeMNPV *rr1* DNA probe (data not shown). The SpliMNPV *rr1* ORF was localized on the *Hin*dIII-E fragment of the SpliMNPV genome (Croizier *et al*., 1989) and cloned and sequenced (Fig. 1*b*). The SpliMNPV *rr1* ORF consisted of 2345 nt with the potential to encode a protein of 781 amino acids with a predicted molecular mass of 87 kDa. Upstream of the presumed translational start site no consensus late baculoviral transcription start site (Blissard & Rohrmann, 1990) could be detected. A baculovirus consensus early CAGT sequence, without a preceding TATA box, was observed 79 nt in front of the translational start codon. Following the translational stop codon, three consensus poly(A) signals (AATAAA) were found. The ATG of the SpliMNPV *rr1* ORF was in a favourable translational context (Kozak, 1983). SpliMNPV *rr1* preceded an ORF with homology to the AcMNPV *p*74 gene (Kuzio *et al*., 1989). The SpliMNPV *rr1* stop codon and the presumed SpliMNPV *p*74 start codon were separated by 101 nt.

Transcriptional activity of the SeMNPV *rr1* gene

To investigate transcription of the SeMNPV *rr1* gene, a Northern blot with total RNA isolated from SeMNPV-infected cells at several times p.i. was hybridized to an *rr1*-specific probe (Fig. 2*a*). The SeMNPV *rr1* gene transcript was approximately 2·7 kb in size and present throughout infection, as observed after overexposure of the autoradiogram (data not shown), thus classifying *rr1* as an early gene. The amount of transcript increased at later time-points.

The transcriptional start of the SeMNPV *rr1* gene was determined by primer extension (Fig. 2*b*). At early (4 h) and late (12, 24 h) times p.i., several transcriptional start sites were used. The major early start site was located at the A of the sequence TTT**A**TT at nt −163 with respect to the translational start codon. At late times p.i. this site was also used. In addition, major late transcriptional start sites were observed at GAC**G**AC (nt −129), at GGC**A**AA (nt −107) and at TTT**AT**TT (nt −74 and −75). The consensus late promoter sequence AT**A**AG (nt −120) was also used late in infection, although much less frequently than the other late start sites. Overexposure of the primer extension autoradiogram showed no indication of the use of this site at 4 h p.i., which also showed that the observed bands in the primer extension analysis were not due to preliminary stops. Comparable results were obtained with primer extension experiments at different annealing temperatures (data not shown). The length of the SeMNPV *rr1* transcript (2·7 kb) was in reasonable agreement with the length of the ORF (2·3 kb), assuming a poly(A) tail of normal length (0·2 kb), a 5′ leader of 74–163 nt and transcription termination near the translational stop codon.

Comparison of baculoviral RR1 proteins with those from other sources

The amino acid sequences of SeMNPV and SpliMNPV RR1 were compared with those from other organisms and viruses. The overall homology (identity and similarity) is given in Table 1. The baculovirus RR1s have the highest homology to RR1s from eukaryotic organisms. The homology of SpliMNPV RR1 to these RR1s is higher than that of SeMNPV RR1. The homology between SeMNPV and SpliMNPV RR1, 69%, is essentially the same as that between SeMNPV and eukaryotic RR1s. The homology with RR1s from other DNA viruses is usually lower.

Alignment of baculoviral RR1 with an array of cellular and viral RR1s indicated that the overall length of SeMNPV and SpliMNPV RR1 fell within the range observed for other RR1s

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SeMNPV   : -----------MFDENNFEIDSKFKIIPSNNVSENLTLLLNSLCKNIDTRYVDVPKLVHK :  49
SpliMNPV : ----------MLPKRDGRKPDVSVRKIKYR---------IEKQCYGLDMYFVNEASLTRR :  41
Hs       : ---------MHVIKRDGRQERVMFDKITSR---------IQKLCYGLNMDFVDPAQITMK :  42
Ce       : ---MQRYNSTYVVKRDGRKEDVHFDKITSR---------IQKLSYGLNMDFVDPVAVAIK :  48
Sp       : ---------MFVYKRDGRQEKVAFDKITAR---------VSRLCYGLDSDHVDPVEITQK :  42
Pf       : ---------MYVLNRKGEEEDISFDQILKR---------IQRLSYGLH-ELVDPARVTQG :  41
VV       : ---------MFVIKRNGYKENVMFDKITSR---------IRKLCYGLNTDHIDPIKIAMK :  42
ASFV     : --------------------------------------------------MENFFIVKK :   9
HSV1     : FLGDAVNREPALMLEYFCRCAREETKRVPPRTFGSPPRLTEDDFGLLNYALVEMQRLCLD : 388
HVS      : ------------------------------------MSQET---IISNLIDMLKVSAG :  19
T4       : ------MQLINVIKSSGVSQSFDPQKIIK---------VLSWAAEG---TSVDPYELYEN :  42
Ec       : -----MNQNLLVTKRDGSTERINLDKIHR---------VLDWAAEGLHNVSISQVELRSH :  46

SeMNPV   : VVAES-GDIGFDKIKLYCADV-----------AASMTYVHYDYALLAGRILVEDLHSKVS :  97
SpliMNPV : VVQGIYPGVTTVELDNLIAET-----------AASMTIDHSDYSLLAARLAVSNLHKETK :  90
Hs       : VIQGLYSGVTTVELDTLAAET-----------AATLTTKHPDYAILAARIAVSNLHKETK :  91
Ce       : VISGLYKGVTTVELDNLAAET-----------AASMTTQHPEYALLAARIAVSNLHKKTN :  97
Sp       : VISGVYPGVTTIELDNLAAET-----------AATMTTKHPDYAILAARIAVSNLHKQTE :  91
Pf       : VINGMYSGIKTCELDELAAQT-----------CAYMATTHPDFSILAARITTDNLHKNTS :  90
VV       : VIQGIYNGVTTVELDTLAAEI-----------AATCTTQHPDYAILAARIAVSNLHKETK :  91
ASFV     : LASATYGKALNVDLNKLLQALNHHSLQGLISYCSALTILHYDYSTLAARLSVYLLHQSTA :  69
HSV1     : VPPVPPNAYMPYYLREYVTRLVNGFKPLVSRS--ARLYRILG-VLVHLRIRTREASFEEW : 445
HVS      : WDREA---------NEISGRLFHKLMDMSSTETISQYMSLFG-PLLEPHILEFIQNYEQ- :  68
T4       : IKSYLRDGMITTDDIQTIVIKAA----------ANSISVEEPDYQYVAARCLMFALRKHVY :  92
Ec       : IQFY--DGIKTSDIHETIIKAA----------ADLISRDAPDYQYLAARLAIFHLRKKAY :  94

SeMNPV   : SCFVTIANDLFS---------RNVLSDEFNQ-----LVKKHGAILNNEIKHERDYN---- : 139
SpliMNPV : DLFFNVIVDMYEAIDQKTGAATEMISDFHYG-----IIAANADRLNSAIMHDRDFN---- : 141
Hs       : KVFSDVMEDLYNYINPHNGKHSPMVAKSTLD-----IVLANKDRLNSAIIYDRDFS---- : 142
Ce       : KVFSEVMKTLHEFHHPHTGKHAPMISDETWA-----IIEKNADKLNSAIVYDRDYS---- : 148
Sp       : KVFSTVVQQLHDYVNPKTDKPAPMISDKIYD-----IVMKHKDELDSAIIYDRDFT---- : 142
Pf       : DDVAEVAEALYTYKDVR-GRPASLISKEVYD-----FILLHKDRLNKEIDYTRDFN---- : 140
VV       : KLFSEVMEDLFNYVNPKNGKHSPIISSITMD-----IVNKYKDKLNSVIIYERDFS---- : 142
ASFV     : SSFSKAVSLQAAQSC-------SRLSSHFVD-----VVYKYKAIFDSYIDYSRDYK---- : 113
HSV1     : LRSKEVALDFGLTERLREHEAQLVILAQALDHYDCLIHSTPHTLVERGLQSALKYEEF-- : 503
HVS      : -EIDEVCLEYRASYDFMCLRNCGILPAKRF--YDTYVLPPRTEMNG-------KYESIPH : 118
T4       : GQYEPRSFIDHISYCVNAGKYDPELLSKYSA-----EEITF---LESKIKHERDME---- : 140
Ec       : GQFEPPALYDHVVKMVEMGKYDNHLLEDYTE-----EEFKQ---MDTFIDHDRDMT---- : 142

SeMNPV   : -YKYFGFKTLTNGYLLK--IDDVIMERPQHMLMRVALAIHGD----D-VKSVIETYQLMS : 191
SpliMNPV : -YDYFGFKTMQRSYLFK--INGITVERPQYMMMRVAIGIHGR----D-IDAAIETYNLMS : 193
Hs       : -YNYFGFKTLERSYLLK--INGKVAERPQHMLMRVSVGIHKE----D-IDAAIETYNLLS : 194
Ce       : -YTYFGFKTLERSYLLK--INKEIVERPQQMLMRVSIGIHGD----D-ITSAIETYNLMS : 200
Sp       : -YNFFGFKTLERSYLLR--IDGKVAERPQHMIMRVAVGIHGE----D-IEAAIETYNLMS : 194
Pf       : -YDYFGFKTLERSYLLR--INNKIIERPQHLLMRVSIGIHID----D-IDKALETYHLMS : 192
VV       : -YNYFGFKTLEKSYLLK--INNKTVERPQHMLMRVAVGIHQW----D-IDSAIETYNLLS : 194
ASFV     : -LSLLGIETMKNSYLLKN-KDGVIMERPQDAYMRVAIMIHGMGRVVN-MKMILLTYDLLS : 170
HSV1     : YLKRFGGHYMESVFQMYTRIAGFLACRATRGMRHIALGREG-----SWWEMFKFFFHRLY : 558
HVS      : FFARIAAYCAWNCI-MCEPLKDTLVYVQKRD-WNVEIKTD--------MQIFKYFYKVIS : 168
T4       : -FTYSGAMQLKEKYLVKDKTTGQIYETPQFAFMYTIGMALHQDEPVD-RLKHVIRFYEAVS : 198
Ec       : -FSYAAVKQLEGKYLVQNRVTGEIYESAQFLYILVAACLFSNYPRETRLQYVKRFYDAVS : 201

                                   *
SeMNPV   : LGTFTHASPTLFSAGTRRQQMCSCFLQSIKDDSVDG-IYKTLHE-SALISNLGGGLGLSA : 249
SpliMNPV : NGYFTHASPTLFSAATPKAQMSSCFLVAIKEDSIEG-IYDTLKQ-CAMISKSGGGIGFHV : 251
Hs       : ERWFTHASPTLFNAGTNRPQLSSCFLLSMKDDSIEG-IYDTLKQ-CALISKSAGGIGVAV : 252
Ce       : ERYMTHASPTLFNSGTCRPQMSSCFLLTMSEDSILG-IYDTLKQ-CALISKSAGGIGLNV : 258
Sp       : QRYFTHASPTLFNAGTPRPQLSSCFLVTMKDDSIEG-IYDTLKM-CAMISKTAGGIGIYI : 252
Pf       : QKYFTHATPTLFNSGTPRPQMSSCFLLSMKADSIEG-IFETLKQ-CALISKTAGGIGVAV : 250
VV       : EKWFTHASPTLFNAGTSRHQMSSCFLLNMIDDSIEG-IYDTLKR-CALISKMAGGIGLSI : 252
ASFV     : RHVITHASPTMFNAGTKKPQLSSCFLLNV-NDNLEN-LYDMVKT-AGIISGGGGGIGLCL : 227
HSV1     : DHQIVPSTPAMLNLGTRNYYTSSCYLVNPQATTNKATLRAITSN-VSAIILARNGGIGLCV : 617
HVS      : SQLVCCATPVMRSAGVAGENLSSCFIIAPTLDTEKSTISSIFGE-LAPLLASRSGVGVDV : 227
T4       : TRQISLPTPIMAGCRTPTRQFSSCVVIE-AGDSLKS-INKASASIVEYISKRA-GIGINV : 255
Ec       : TFKISLPTPIMSGVRTPTRQFSSCVLIE-CGDSLDS-INATSSAIVKYVSQRA-GIGINA : 258

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Fig. 3. For legend see p. 2372.

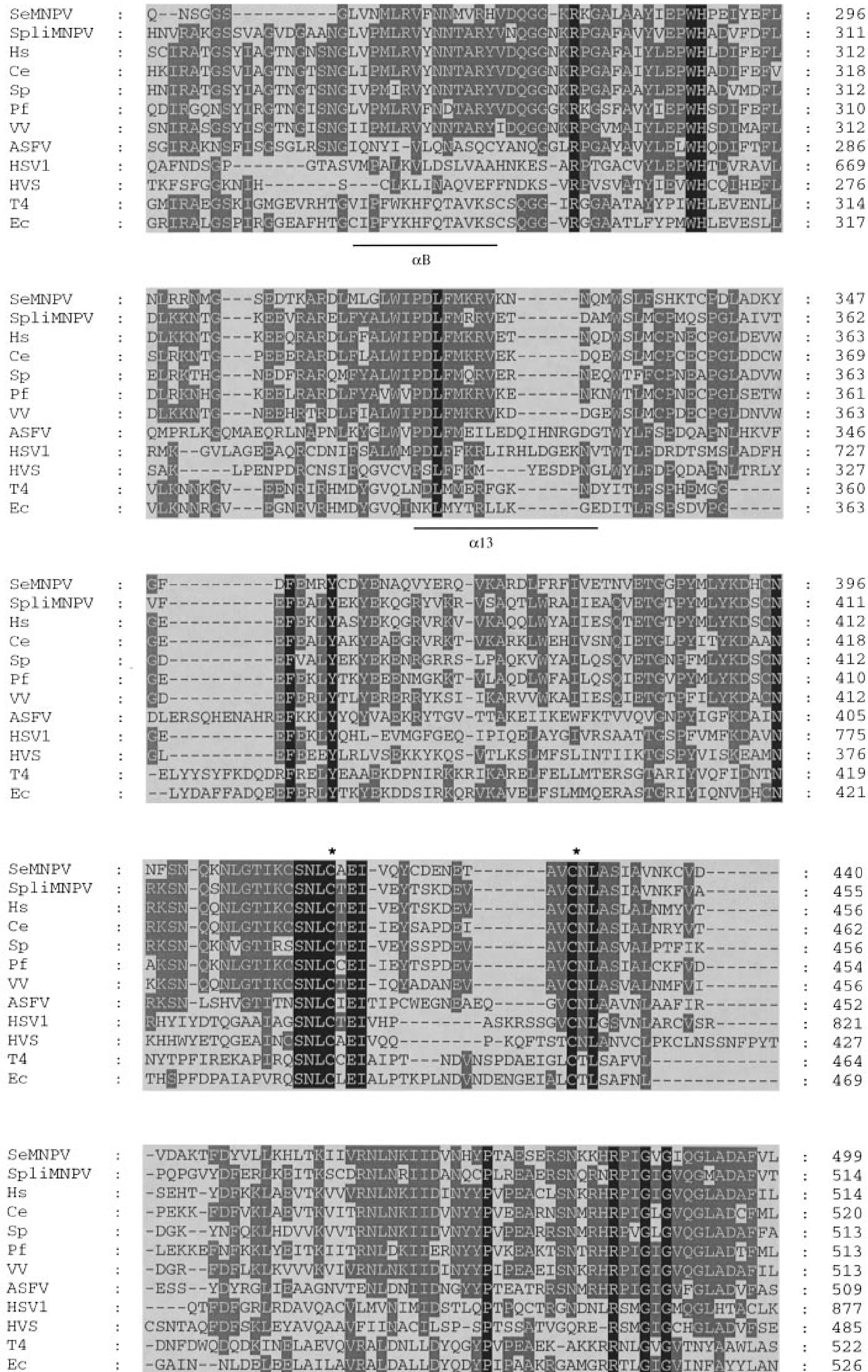

Fig. 3. For legend see p. 2372.

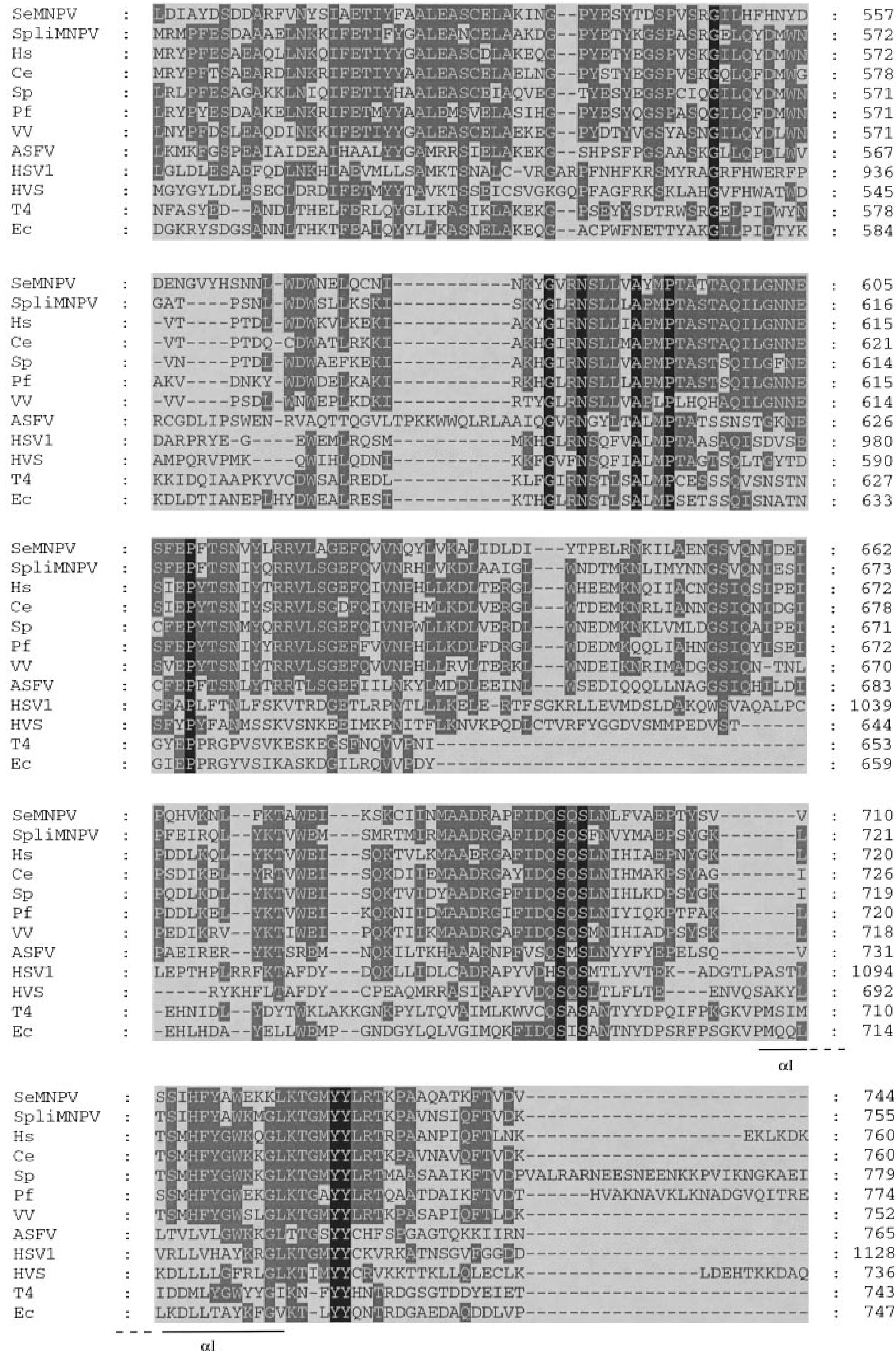

Fig. 3. For legend see p. 2372.

(Fig. 3). SeMNPV RR1 had diverged in several regions of the protein and showed a few gaps and amino acid insertions when compared with eukaryotic RR1s.

Several consensus amino acid residues (Mao *et al.*, 1992; Stubbe *et al.*, 1990; Uhlin & Eklund, 1994) could be recognized in the baculoviral RR1. These included cysteine residues

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SeMNPV     :  ------ETVASCSKKRKMNDDDDNVVCVSCHA  :  770
SpliMNPV   :  ------NAAAATAATAAAAFAVSSSDCTSCSS  :  781
Hs         :  EKVSKEEEEKERNTAAMVCSLENRDECLMCGS  :  792
Ce         :  ----NALKTNQQAETPATVAESQDEGCLMCSG  :  788
Sp         :  SAEPTKEEIDIYNEKVLACSIKNPEACEMCSA  :  811
Pf         :  VSRETISTESTVTQNVCPLRRNNDEQCLMCSG  :  806
VV         :  -------------DKIKPPVVCDSEICTSCSG  :  771
ASFV       :  ------------------SEKACNADCEACLL  :  779
HSV1       :  -----------------------NIVCMSCAL  : 1137
HVS        :  IVLADLARELPDSHKTEDACPLDQSECIACQ-  :  767
T4         :  ---------------------PKADDCAACKL  :  754
Ec         :  ------------------SIQDDGCESGACKI  :  761
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Fig. 3. Alignment of the predicted amino acid sequences of the RR1 proteins of the baculoviruses SeMNPV and SpliMNPV with those of *Homo sapiens* (Hs; Parker *et al*., 1991), *Caenorhabditis elegans* (Ce; Sulston *et al*., 1992), *Plasmodium falciparum* (Pf; Rubin *et al*., 1993), *Schizosaccharomyces pombe* (Sp; Fernandez-Sarabia *et al*., 1993), vaccinia virus (VV; Tengelsen *et al*., 1988), African swine fever virus (ASFV; Boursnell *et al*., 1991), herpes simplex virus type 1 (HSV1; Nikas *et al*., 1986), herpesvirus saimiri (HVS; Nicholas *et al*., 1992), bacteriophage T4 (Tseng *et al*., 1988) and *E. coli* (Ec; Nillson *et al*., 1988). Gaps introduced to optimize alignment are indicated by dots. Shading is used to indicated the occurrence (black, 100%; grey, at least 60%) of identical amino acids. Asterisks indicate essential cysteine residues. Alpha helices involved in dimerization are underlined (see text).

```
SeMNPV     :  ----MFDENNFEIDSKFKIIPSNNVSENLTLLLNSLCKNIDTRYVDVPKLVHKVVAES-G  :   55
SpliMNPV   :  ------------MLPKRDGRKEDVSVRKIKYRIEKQCYGLDMYFVNPASLTRRVVQGIYP  :   48
MbMNPV     :  -----------MYVVKRDGRLEQVSSSKLYQRIHRLCHDLNSQFVHPRAVSLKVIKGLSD  :   49
PfMNPV     :  -----------MYVVKRDERLEQVSPSKLHNRIHRLCHDLNSQFVHPKAVSLKVIKSLSD  :   49
OpSNPV     :  MNDNSVNVNNKLYVVKRDGRKQAVYLDKITSKLRRLSYGAHVASGQNDIPG---------  :   51
Hs         :  -----------MHVIKRDGRQERVMFDKITSRIQKLCYGLNMDFVDPAQITMKVIQGLYS  :   49
Ce         :  -----MQRYNSTYVVKRDGRKEDVHFDKITSRIQKLSYGLNMDFVDPVAVAIKVISGLYK  :   55

SeMNPV     :  DIGFDKIKLYCADVAASMTYVHYDYALLAGRILVEDLHSKVSSCFVTIANDLFS------  :  109
SpliMNPV   :  GVTTVELDNLIAETAASMTIDHSDYSLLAARLAVSNLHKETKDLFFNVIVDMYEAIDQKT  :  108
MbMNPV     :  NTTTEDIDIYAASVAATLTYKHYDYDTLAGRLLVTNMHKYVDESFTKVVQELHK------  :  103
PfMNPV     :  RTTTENIDIYAASVAATLTYKHYDYDMLAGRLLVTNMHKYVDESFTKVVQRLHK------  :  103
OpSNPV     :  ------------------------------------------------------------  :    -
Hs         :  GVTTVELDTLAAETAATLTTKHPDYAILAARIAVSNLHKETKKVFSDVMEDLYNYINPHN  :  109
Ce         :  GVTTVELDNLAAETAASMTTQHPEYALLAARIAVSNLHKKTNKVFSEVMKTLHEFHHPHT  :  115

SeMNPV     :  ---RNVLSDEFNQLVKKHGAILNNEIKHERDYNYKYFGFKTLTNGYLLKIDDVIMERPQH  :  166
SpliMNPV   :  GAATPMISDFHYGIIAANADRLNSAIMHDRDFNYDYFGFKTMQRSYLFKINGITVERPQY  :  168
MbMNPV     :  ---HNLVSDELLSVTIKHSKIIEENIDMKLDLDYNYFGYQTLKNGYLIKINEKVAPTIQH  :  160
PfMNPV     :  ---HNLVSDELLLVTIKHSKIIEENINMKLDLDYNYFGYQTLKNGYLIKINEKVAER---  :  157
OpSNPV     :  ------------------------------------------------------------  :    -
Hs         :  GKHSPMVAKSTLDIVLANKDRLNSAIIYDRDFSYNYFGFKTLERSYLLKINGKVAERPQH  :  169
Ce         :  GKHAPMISDETWAIIEKNADKLNSAIVYDRDYSYTYFGFKTLERSYLLKINKEIVERPQQ  :  175

SeMNPV     :  MLMRVALAIHGDDVKSVIETYQLMSLGTFTHASPTLFSAGTRRQQMCSCFLQSIKDDSVD  :  226
SpliMNPV   :  MMMRVAIGIHGRDIDAAIETYNLMSNGYFTHASPTLFSAATPKAQMSSCFLVAIKEDSIE  :  228
MbMNPV     :  MHMRIALGIFGDDIDSAIKSYKFLSRKMYTHASPTMFAAGTLTPQTSLFSVDHGKRxHSR  :  220
PfMNPV     :  ------------------------------------------------------------  :    -
OpSNPV     :  ------------------------------------------------------------  :    -
Hs         :  MLMRVSVGIHKEDIDAAIETYNLLSERWFTHASPTLFNAGTNRPQLSSCFLLSMKDDSIE  :  229
Ce         :  MLMRVSIGIHGDDITSAIETYNLMSERYMTHASPTLFNSGTCRPQMSSCFLLTMSEDSIL  :  235

SeMNPV     :  GIYKTLHESALISNLGGGL  :  245
SpliMNPV   :  GIYDTLKQCAMISKSGGGI  :  247
MbMNPV     :  HLxHIKRLRHDFxTRHGG-  :  238
PfMNPV     :  -------------------  :    -
OpSNPV     :  -------------------  :    -
Hs         :  GIYDTLKQCALISKSAGGI  :  248
Ce         :  GIYDTLKQCALISKSAGGI  :  254
```

Fig. 4. Alignment of predicted amino acid sequences of the N terminus of the RR1 proteins of SeMNPV, SpliMNPV, *Homo sapiens* and *Caenorhabditis elegans* with those of the baculoviruses MbMNPV, PfMNPV and OpSNPV. Shading indicates the occurrence of identical amino acids.

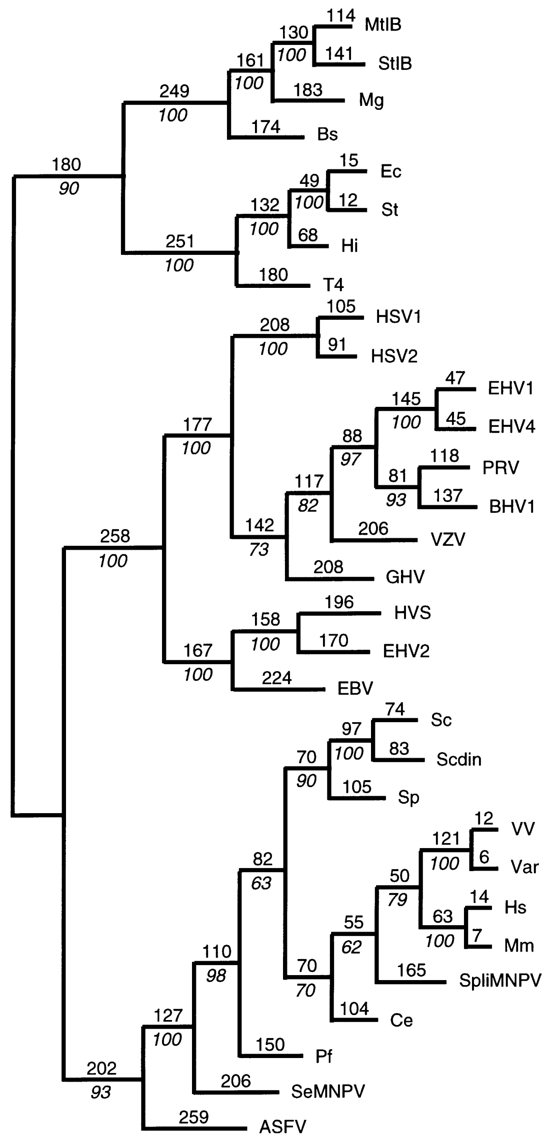

Fig. 5. Bootstrap analysis (100 replicates) of an unrooted phylogenetic tree of RR1 proteins constructed with PAUP heuristic search algorithm. Numbers at the branches indicate branch length (normal print) and frequency of cluster (italics). MtlB, *Mycobacterium tuberculosis* nrdE (Yang *et al*., 1994); StlB, *Salmonella typhimurium* nrdE (Jordan *et al*., 1994); Mg, *Mycoplasma genitalium* (Fraser *et al*., 1995); Bs, *Bacillus subtilis* (accession no. Z68500); Ec, *Escherichia coli* nrdA (Nillson *et al*., 1988); St, *Salmonella typhimurium* nrdA (Jordan *et al*., 1994); Hi, *Haemophilus influenzae* (Fleischmann *et al*., 1995); T4, bacteriophage T4 (Tseng *et al*., 1988); HSV1, herpes simplex virus type 1 (Nikas *et al*., 1986); HSV2, herpes simplex virus type 2 (Swain & Galloway, 1986); EHV1, equine herpesvirus type 1 (Telford *et al*., 1992); EHV4, equine herpesvirus type 4 (Riggio & Onions, 1994); PRV, pseudorabies virus (Kaliman *et al*., 1994); BHV1, bovine herpesvirus type 1 (Simard *et al*., 1995); VZV, varicella zoster virus (Davison & Scott, 1986); GHV, gallid herpesvirus 2 (Darteil *et al*., 1995); HVS, herpesvirus saimiri (Nicholas *et al*., 1992); EHV2, equine herpesvirus type 2 (accession no. U20824); EBV, Epstein–Barr virus (Baer *et al*., 1984); Sc, *Saccharomyces cerevisae* (Elledge & Davis, 1990); Scdin, *Saccharomyces cerevisae* DNA damage inducible subunit (Yagle & McEntee, 1990); Sp, *Schizosaccharomyces pombe* (Fernandez *et al*., 1993); VV, vaccinia virus (Tengelsen *et al*., 1988); Var, variola virus (Shchelkunov *et al*., 1993); Hs, *Homo sapiens* (Parker *et al*., 1991); Mm, *Mus musculus* (Caras *et al*., 1985); Ce, *Caenorhabditis elegans* (Sulston *et al*., 1992); Pf, *Plasmodium falciparum* (Rubin *et al*., 1993); ASFV, African swine fever virus (Boursnell *et al*., 1991).

known to be involved in the formation of essential thiols in the *E. coli* RR1 protein, i.e. Cys-225, Cys-439, Cys-462, Cys-754 and Cys-759 (*E. coli* numbering), as well as residues surrounding Cys-225, Cys-439 and Cys-462 in the active site and two adjacent tyrosine residues (Tyr-730 and Tyr-731) presumably involved in electron transfer from the small subunit.

Structural analysis of the *E. coli* RR1 protein (Uhlin & Eklund, 1994) and previous research (Davis *et al*., 1994; Mao *et al*., 1992) revealed areas responsible for dimerization of RR1 and holoenzyme formation with RR2 subunits. The dimerization domains (two alpha helices, αA and αB) were well-conserved in SpliMNPV RR1 and slightly less conserved in SeMNPV RR1. Holoenzyme formation involves binding of the ultimate C-terminal residues of RR2 to two RR1 alpha helices (αI and α13). The baculoviral RR1s showed reasonable sequence homology to the corresponding eukaryotic regions.

Databank searches revealed the occurrence of RR1-like sequences upstream of the polyhedrin gene in *Mamestra brassicae* multicapsid NPV (MbMNPV; Cameron & Possee, 1989), *Panolis flammea* multicapsid NPV (PfMNPV; Oakey *et al*., 1989) and *Orgyia pseudotsugata* single nucleocapsid NPV (OpSNPV; Leisy *et al*., 1986). The predicted amino acid sequences in these viruses showed homology to the N terminus of RR1 (Fig. 4). MbMNPV and PfMNPV RR1 showed the same gap around position 110 in amino acid alignment as SeMNPV RR1 in comparison with eukaryotic RR1. However, the available sequences from MbMNPV and PfMNPV showed a somewhat higher homology to the eukaryote RR1 N terminus than did SeMNPV RR1.

Phylogenetic reconstruction of the origin of the baculoviral *rr1* genes

In order to investigate the origin of the *rr1* gene in SeMNPV and SpliMNPV, a phylogenetic tree was constructed with amino acid sequences of 30 RR1 proteins from prokaryotes, eukaryotes and viruses. An unrooted parsimonious tree was calculated with the PAUP heuristic search algorithm, followed by bootstrap analysis to assess the variability of the produced phylogeny (Fig. 5).

The tree showed that prokaryotes and eukaryotes, as expected, were located on separate branches. Viral RR1 sequences were found in many different locations in the tree. The herpesvirus RR1s were all clustered, implying that they diverged from a common ancestor. Bootstrap analysis supported the phylogenetic separation of the herpesviruses into α- and γ-herpesviruses.

Both SeMPNV and SpliMNPV grouped with eukaryotic organisms. The phylogeny of this branch was reasonably well-supported by bootstrap analysis. Tree data suggest that SeMNPV and SpliMNPV RR1 do not share a recent common

ancestor, but that these viruses acquired the gene for RR1 independently from each other as well as from other DNA viruses.

Discussion

The complete nucleotide sequence of the *rr1* gene was determined in the baculoviruses SeMNPV and SpliMNPV. Transcriptional activity was investigated after RNA isolation from SeMNPV-infected cultured insect cells. Both Northern blotting and primer extension showed low transcriptional activity early in infection, which increased at later stages. The early transcription of the SeMNPV *rr1* gene is in good agreement with its presumed role in deoxyribonucleotide synthesis required for DNA replication. Herpesvirus and vaccinia virus *rr* genes are also transcribed as early genes (Schmitt & Stunnenberg, 1988; Swain & Galloway, 1986).

The sequence surrounding the major transcriptional start sites showed no homology to previously characterized start sites of other baculovirus genes, for instance those involved in DNA replication, such as helicase (Lu & Carstens, 1992) and DNA polymerase (Tomalski *et al.*, 1988). No homology between the sequences 5′ of the translational start of SeMNPV, SpliMNPV and other baculovirus *rr1* genes could be detected.

The alignment of SeMNPV and SpliMNPV RR1 with other RR1s (Fig. 3) showed that amino acid residues known to be involved in enzymatic activity were conserved. This suggests that the baculoviral *rr1* gene could code for a functional constituent of an RR enzyme. SeMNPV RR1 had a lower homology to eukaryotic RR1 than SpliMNPV RR1. In comparison to the *E. coli* structural regions (Uhlin & Eklund, 1994), regions from eukaryotic and specifically viral RR1 which diverged from the *E. coli* sequence, often map to loops separating α-helices and β-barrels. One of the gaps in the SeMNPV RR1 alignment was located around amino acid 250. A similar situation was observed in several other viral RR1s. In *E. coli*, this region forms a loop (L1, amino acids 259–277) between the dimerizing domains of RR1. It has been suggested that this and other loops in the dimerization region may change from a flexible to a fixed position at subunit formation, hence stabilizing the holoenzyme (Uhlin & Eklund, 1994). The SeMNPV enzyme may thus show a stability different from that of the cellular enzyme.

Sequences with homology to the RR1 N terminus were found in the baculoviruses MbMNPV, PfMNPV and OpSNPV (Fig. 4). This suggests that RR1 is not only encoded by SeMNPV and SpliMNPV, but is widespread among baculoviruses. The *rr1* gene in MbMNPV, PfMNPV and OpSNPV baculoviruses had a similar genomic location to that from SeMNPV, i.e. positioned upstream of the polyhedrin gene. Phylogenetic trees based on polyhedrin gene sequences (Zanotto *et al.*, 1993; Cowan *et al.*, 1994) placed SeMNPV, MbMNPV and PfMNPV in subgroup IIA. The position of OpSNPV was variant depending on which algorithms and DNA or amino acid sequences were used. OpSNPV was either in group IIB, together with SpliMNPV, or in IIA. OpSNPV might belong to subgroup IIA, based on the observed homology to the RR1 N terminus and its genomic location next to the polyhedrin gene.

Bootstrap analysis of the parsimonious phylogenetic tree (Fig. 5) confirmed the independent ancestry of the *rr1* gene in SeMNPV and SpliMNPV. This conclusion is in agreement with the different genomic location of the *rr1* gene in these two viruses, as well as with the observed lower homology of SeMNPV RR1 with eukaryotic RR1s. Alternatively, if the common ancestor of SeMNPV and SpliMNPV already encoded RR1, the implication would be that the *rr1* gene evolved much faster in SeMNPV than in SpliMNPV. This assumption seems questionable and is not supported by determination of the evolutionary rates of the polyhedrin genes of SeMNPV and SpliMNPV (Zanotto *et al.*, 1993; Cowan *et al.*, 1994), nor by comparison of other genes of SeMNPV and SpliMNPV (data not shown). However, the phylogenetic reconstruction may yield a different result when full-length sequences of other baculovirus *rr1* genes become available.

The positioning of SeMNPV and SpliMNPV in the eukaryote branch of the tree suggests that each virus derived RR1 independently from an eukaryotic source, for example their host. The polyphyletic origin of baculovirus RR1 contrasted with the observed monophyletic grouping of the herpesviruses and of the two poxviruses. Only RR1 from SpliMNPV and poxviruses grouped inside a cellular clade, whereas RR1 from SeMNPV, ASFV, herpesviruses and bacteriophage T4 diverged more than their presumed ancestors did. This suggests that viral RR1 usually diverges faster than cellular RR1.

RR1 proteins need to associate with RR2 dimers in order to form a functional enzyme. In most large DNA viruses (with the exception of vaccinia virus) the genes for RR1 and RR2 are located adjacent to each other. A different situation exists in β-herpesviruses such as human cytomegalovirus, which only contain a RR1-like ORF (Chee *et al.*, 1990). However, unlike the baculoviral RR1 ORFs, the RR1-like ORFs in β-herpesviruses show only very limited homology to other RR1s and do not encode the amino acid residues known to be involved in ribonucleotide reduction. It has been suggested that these highly diverged genes may have acquired another function during the β-herpesvirus life cycle (Conner *et al.*, 1994). In SeMNPV, homology to RR2 could not be detected either in the 2·5 kb sequence upstream or in the 14 kb sequence downstream of the SeMNPV RR1 ORF (data not shown). However, RR2 might be located elsewhere on the genome. As yet, we do not know if SeMNPV and SpliMNPV encode their own RR2, or alternatively, combine with cellular RR2 proteins. Such a heterologous association is known in yeast, where two RR enzymes are recognized. One enzyme results from the combination of RR1 and RR2, whose normal expression is

restricted to certain phases of the cell cycle. The other enzyme is induced by DNA damage and is a combination of the same RR2 and a different RR1 (Elledge *et al.*, 1992).

Virus-encoded RR enzymes from poxviruses, herpesviruses, ASFV and phage T4 are not essential and are distinct from the cellular enzyme in several aspects. It remains to be elucidated if similar characteristics hold for baculoviruses encoding or lacking RR.

Not all large DNA viruses encode RR. As can be concluded from sequence analysis of the complete genome, the baculovirus type species AcMNPV does not encode RR (Ayres *et al.*, 1994) and neither does the closely related *Bombyx mori* NPV (accession number L33180). This explains our failure in attempts to hybridize an SeMNPV *rr1* DNA probe to AcMNPV DNA (data not shown). The herpesvirus channel catfish virus (Davison, 1992) also does not encode RR. The reasons for this are unknown. The lack of *rr1* in AcMNPV will allow the study of SeMNPV *rr1* in an AcMNPV recombinant expressing this gene.

During the course of our experiments, Ahrens *et al.* (1997) published the entire sequence of the *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) genome and also identified an *rr1* gene, which potentially encoded a relatively short protein of 593 amino acids. In juxtaposition with the OpMNPV *rr1* gene, a gene with low sequence homology to RR2 was found in this baculovirus. Preliminary phylogenetic analysis indicated that the OpMNPV *rr1* gene did not share a recent common ancestor with either SeMNPV or SpliMNPV, but was acquired independently in these three baculoviruses.

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