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African swine fever virus encodes two genes which share significant homology with the two largest subunits of DNA-dependent RNA polymerases

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ABSTRACT

A random sequencing strategy applied to two large *SalI* restriction fragments (SB and SD) of the African swine fever virus (ASFV) genome revealed that they might encode proteins similar to the two largest RNA polymerase subunits of eukaryotes, poxviruses and *Escherichia coli*. After further mapping by dot-blot hybridization, two large open reading frames (ORFs) were completely sequenced. The first ORF (NP1450L) encodes a protein of 1450 amino acids with extensive similarity to the largest subunit of RNA polymerases. The second one (EP1242L) codes for a protein of 1242 amino acids similar to the second largest RNA polymerase subunit. Proteins NP1450L and EP1242L are more similar to the corresponding subunits of eukaryotic RNA polymerase II than to those of vaccinia virus, the prototype poxvirus, which shares many functional characteristics with ASFV. ORFs NP1450L and EP1242L are mainly expressed late in ASFV infection, after the onset of DNA replication.

INTRODUCTION

Viruses which replicate in the cytoplasm of eukaryotic cells probably have no access to the host transcriptional machinery. Two families of large DNA viruses share this cytoplasmic location: poxviruses, and a yet unnamed group of which African swine fever virus (ASFV) is the only known member. Poxviruses overcome this problem by encoding their own enzymes for transcription and RNA modification. Transcription of vaccinia virus is carried out by a multimeric RNA polymerase composed of two large and many small subunits, all of which seem to be virus encoded (reviewed in (1)). This multisubunit enzyme resembles the RNA polymerases of eukaryotic cells, and the two largest and the smallest subunits of vaccinia virus have similar amino acid sequences to their cellular counterparts (2–4). The replicative cycle of ASFV, consisting first of a nuclear stage followed by a longer cytoplasmic phase of DNA replication (5), is more complex than that of poxviruses, secluded to the cell cytoplasm (reviewed in (6)). Both ASFV and vaccinia virus particles carry a DNA-dependent RNA polymerase which is not sensitive to a-amanitin (1, 7), a toxin which strongly inhibits cellular RNA polymerase II (8). The RNA polymerase activities of ASFV and vaccinia virus particles share a number of properties regarding substrate requirements and pattern of inhibition by rifampicin derivatives (7, 9). Moreover, both viruses encode proteins similar to the eukaryotic transcription factor SII (TFIIS; (10, 11)). Taken together, these data suggest that ASFV might encode an RNA polymerase similar to that of vaccinia virus. The recent description of the ASFV gene coding for mRNA guanylyltransferase (12) suggests that the RNA modification system of ASFV particles (13) could also be virus-encoded, as previously shown for vaccinia virus (1).

Here we report the identification, sequencing and transcriptional expression of the genes encoding the putative two largest RNA polymerase subunits of ASFV. The deduced amino acid sequences of the two gene products show obvious similarities to their poxvirus, eukaryotic and bacterial counterparts. Because of considerable conservation of RNA polymerase domains and critical sites we suggest that the two identified genes correspond to RNA polymerase subunits of ASFV. This finding strongly suggests that ASFV transcription is carried out by a multisubunit virus-encoded RNA polymerase similar to those of vaccinia virus and eukaryotic cells.

MATERIALS AND METHODS

Plasmid clones

The cloning of different restriction fragments of the BA71V strain of ASFV in plasmid vectors has been previously described (14). The basic *Eco*RI (R) collection was cloned in pBR325 (p5), while *SalI* (S) fragments were cloned in pBR322 (p2). See Fig. 1 for identification of the various DNA fragments described in the next paragraphs.

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DNA sequencing

Fragments RK and RT (Fig. 1) were fully sequenced at random as follows. The plasmid clones p5RK and p5RT were sheared by sonication, end-repaired with nuclease S1 and Klenow polymerase, and fragments 300–600 bp-long were cloned into *Sma*I-cut M13mp10. Single-stranded DNA from the recombinants was sequenced by the dideoxynucleotide chain termination method (15) using the M13 universal primer. The nucleotide sequence of the left end of fragment RK, and the random sequencing of fragment RN′ have been previously described (16–18).

To sequence the left end of fragment RE′, overlapping *Eco*RI/*Pst*I and *Hin*dIII M13 subclones were generated (see Fig. 1). These subclones were sequenced with the M13 universal primer, synthetic oligonucleotides corresponding to internal sequences and dideoxynucleotide chain terminators (15). Fragment RY′ had not been detected in our previous mapping of the ASFV genome (19), and it has now been sequenced with synthetic oligonucleotides in a *Pst*I M13 clone which also overlaps parts of RK and RE′ (Fig. 1). The nucleotide sequence of the left end of fragment RO, up to the first *Xba*I site, has been previously reported (20). To sequence the rest of RO, the entire fragment was cloned in M13 and sequenced as described for the RE′ subclones.

The contiguity in the viral DNA of all sequences obtained from subclones sharing only a restriction site was assessed by sequencing around the corresponding restriction site in an overlapping clone.

Computer analysis

The fragmentary DNA sequences from the random sequencing procedure were compiled using programs DBAUTO and DBUTIL (21). General analyses of DNA and protein sequences were made with the programs of the UWGCG (22). Searches against DNA and protein databases were made with programs TFASTA and FASTA (23). The multiple alignments were generated with programs PILEUP (22) and Clustal V (24). Percentages of identity and similarity between proteins in the multiple alignments were calculated with program DISTANCES (22).

RNA hybridization

Northern blot hybridizations were carried out with total RNA isolated from cultured cells. Vero cells were mock-infected or infected with 20 pfu/cell ASFV BA71V for 18 hr. Infections were also carried out in the presence of 100 μg/ml cycloheximide or 40 μg/ml cytosine arabinoside for 7 hr. Total RNA was prepared as described (25). 15 μg RNA were separated in denaturing agarose, blotted onto nylon filters and hybridized with specific probes according to standard protocols (26). The probe for NP1450L RNA was the synthetic oligonucleotide 5′-GGTGGTT-CCCATTCGCGCATCATAGATACC-3′, complementary to nucleotides 130 to 159 of ORF NP1450L. The probe for EP1242L RNA was the oligonucleotide 5′-GAGTTTACTGC-AGCGGAGATAAAGCTCAGC-3′, complementary to nucleotides 78 to 107 of the ORF. The oligonucleotides were end-labeled with 30 μCi [γ-^{32}P]ATP (26).

RESULTS

Sequencing of the genes coding for the two largest RNA polymerase subunits

A random cloning and sequencing procedure (27) allowed the mapping of different genes within two large *Sal*I fragments of the ASFV genome, SB and SD. Briefly, M13 clones bearing small subfragments of SB and SD were obtained and sequenced as described under Materials and Methods for fragments RK and RT. The DNA sequences were translated in all six frames, and the derived amino acid sequences were compared with a database containing poxvirus protein sequences, and the NBRF protein database, using program FASTP (28). A few M13 recombinants included inserts whose derived amino acid sequences strongly resembled different regions of the two largest RNA polymerase subunits of poxviruses and eukaryotic organisms. The inserts from four such M13 recombinants (AS22 and AS136 for the largest subunit, AS1 and AS90 for the second largest subunit; see Fig. 1) were used to map the corresponding ASFV genes by dot-blot hybridization. The mixed probes for the largest subunit hybridized with fragment SD, and more specifically, with the smaller subclones RT and RN′; the mixture of probes for

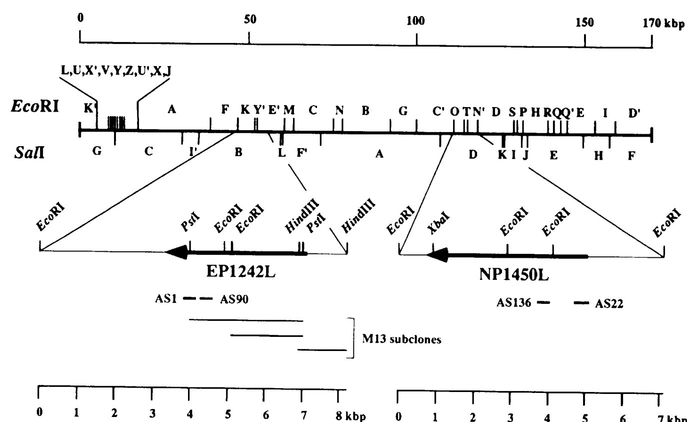

Figure 1. Map position of ORFs NP1450L and EP1242L within the ASFV genome. The *Eco*RI and *Sal*I maps (19) of the BA71V strain of ASFV are shown. ORFs are indicated by arrows. The position of the inserts from M13 clones AS22, AS136, AS1 and AS90 is shown. Subfragments cloned in M13 for sequencing by oligonucleotide walking are also indicated. Only relevant restriction sites are indicated in the expanded regions.

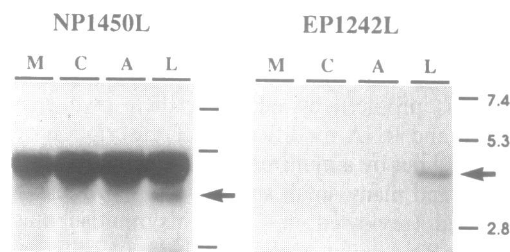

Figure 2. Analysis of the expression of ORFs NP1450L and EP1242L by Northern blot. Total RNA from Vero cells mock-infected (M) or infected in the absence (L) or the presence of either cycloheximide (C) or cytosine arabinoside (A) was subjected to RNA hybridization with probes specific for the NP1450L and EP1242L RNAs. The sizes of molecular mass markers are shown on the right side. Arrows indicate virus-specific RNAs. The probe for NP1450L RNA shows also a non-specific hybridization with the 28S ribosomal RNA.

the second largest subunit recognized fragment SB, and fragment RK within the former one (data not shown).

Fragments RT, RN′ and RK were sequenced as described under Materials and Methods. An analysis of the open reading frames (ORFs) encoded within these fragments revealed that two large genes had been partially sequenced. Comparisons of the derived amino acid sequences of these gene fragments with the RNA polymerase subunits of vaccinia virus and eukaryotic organisms (not shown) revealed that fragments RT and RN′ encode respectively the central and 5′ regions of the gene for the largest subunit, which should be continued in fragment RO. The right end of RK encodes the 3′ half of the gene for the second largest subunit, which should start within fragment RE′.

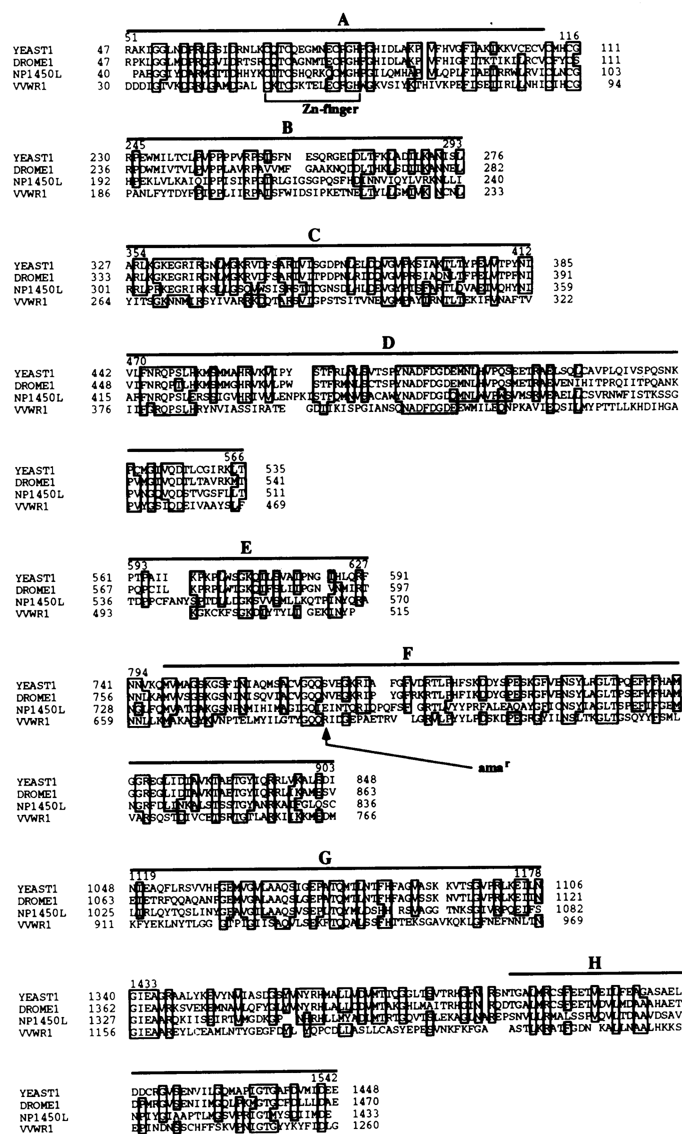

Figure 3. Multiple alignment of conserved regions of the largest subunits. Domains shared with the *E.coli* β' subunit are called A to H as described (34, 50); domain E is absent in *E.coli* (50). Boxes enclose identical amino acids in at least three out of the four sequences. Numbers on top of the alignment refer to the position in the multiple alignment, while numbers at the beginning and end of the sequences refer to the position in the protein sequence. The Zn-finger (according to (42)), and the position responsible for amanitin resistence (ama^r) in mouse (47) are also indicated. YEAST1 and DROME1: largest subunits of *S.cerevisiae* and *D.melanogaster* RNA polymerase II; VVWR1: largest subunit of vaccinia virus strain WR RNA polymerase.

To complete the sequence of the gene coding for the largest subunit, the nucleotide sequence of fragment RO was determined by oligonucleotide walking in M13mp18 clones bearing that fragment. The complete ORF encoding this subunit is 4350 bp-long and, according to the nomenclature proposed for ASFV ORFs (11), it has been called NP1450L (Fig. 1). ORF NP1450L is tightly packed between the genes coding for the attachment protein of ASFV to Vero cells (20) and DNA ligase (18), with spacers less than 100 bp-long. The deduced molecular mass and isoelectric point of protein NP1450L are 163.8 kDa and 8.8, respectively.

Overlapping M13 clones bearing the left end of fragment RE′ and fragment RY′ (Fig. 1) were also sequenced, using synthetic oligonucleotides, to complete the sequence of the gene coding for the second largest subunit. The ORF encoding this subunit,

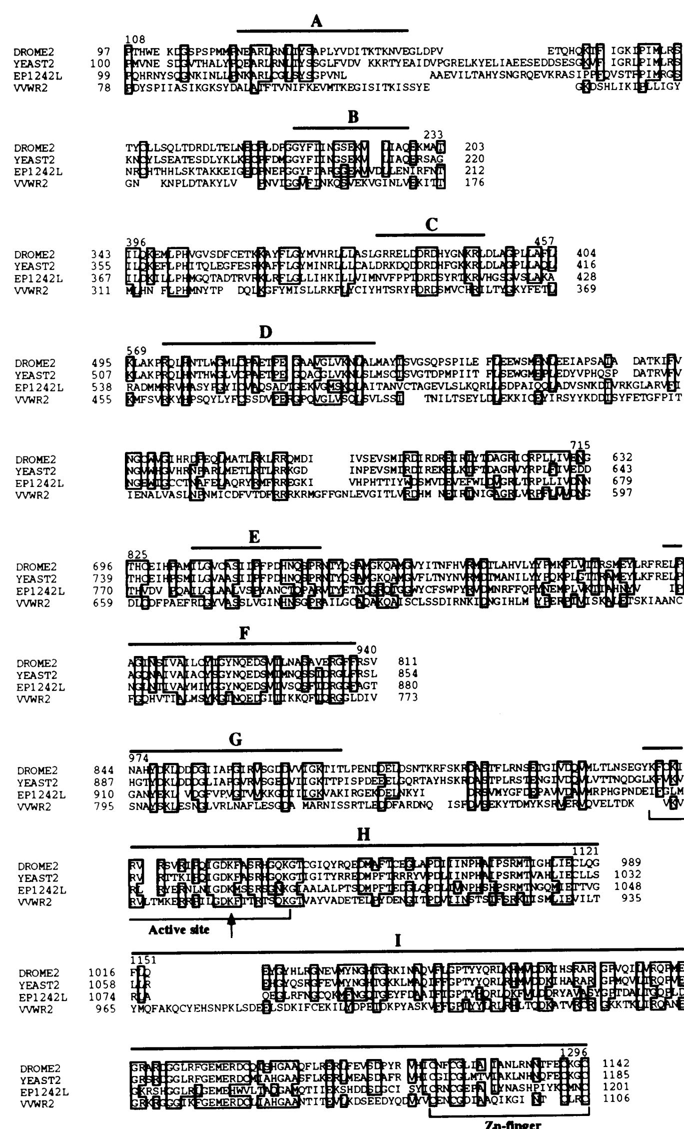

Figure 4. Multiple alignment of conserved regions of the second largest subunits. A to I represent the domains conserved between eukaryotic and *E.coli* subunits (36, 37). Residues identical in at least three out of the four sequences are boxed. Numbering is as in Fig. 3. The active site ((44); the most probably labeled amino acid is marked with an arrow) and the Zn-finger (according to (42)) are indicated. Abbreviations are as in Fig. 3, with a ‘2’ instead of a ‘1’.

Table I. Percent identity and similarity (in parentheses) between the largest subunits according to the multiple alignment.

YEAST1	DROME1	NP1450L	VVWR1	
100 (100)	51.0 (67.5)	27.0 (45.5)	20.4 (39.4)	YEAST1
	100 (100)	26.5 (45.2)	20.4 (41.0)	DROME1
		100 (100)	19.8 (37.5)	NP1450L
			100 (100)	VVWR1

Percentages of identity and similarity were calculated with program DISTANCES (22), using threshold values of 1.5 and 0.6, respectively.

Table II. Percent identity and similarity (in parentheses) between the second largest subunits according to the multiple alignment.

DROME2	YEAST2	EP1242L	VVWR2	
100 (100)	59.3 (73.6)	29.0 (47.6)	21.0 (38.0)	DROME2
	100 (100)	29.0 (46.2)	20.5 (39.7)	YEAST2
		100 (100)	18.4 (36.8)	EP1242L
			100 (100)	VVWR2

Percentages of identity and similarity were calculated as described in Table I.

named EP1242L, is 3726 bp-long. The stop codon of this ORF is included in a set of tandem repeats which also encompasses the 3′ end of a preceding ORF with the opposite transcriptional orientation (not shown). Protein EP1242L has a deduced molecular mass of 139.9 kDa, and an isoelectric point of 7.3.

ORFs NP1450L and EP1242L are expressed late in infection

The expression of ORFs NP1450L and EP1242L was analyzed by RNA hybridization. RNA was obtained from uninfected or ASFV-infected Vero cells. RNA was also prepared from cells infected in the presence of either cycloheximide or cytosine arabinoside, which would allow the detection of RNAs synthesized in the absence of protein synthesis and DNA replication, respectively (29).

The probe for NP1450L RNA recognized a specific band of 4.15 kb in the late RNA sample, prepared after the beginning of DNA replication (Fig. 2). Recent studies on some ASFV mRNAs suggest that they start a short distance (less than a 100 bp) upstream of the corresponding ORF (30–32), and that they end at runs of seven or more consecutive thymidylate residues (7T motif; 30, 31). The distance from the beginning of ORF NP1450L to the first 7T motif downstream of the ORF is 4.46 kb. We confirmed that the NP1450L RNA has an apparent size of 4.15 kb by hybridization with a second oligonucleotide probe (not shown), so we consider that this RNA might have an anomalous electrophoretic mobility which would cause an underestimation of its size.

The probe for EP1242L RNA hybridized to a single band of 4.18 kb in late RNA (Fig. 2); a band of the same size could also be seen in cytosine arabinoside RNA after overexposition of the film (not shown), indicating a low level of expression of ORF EP1242L in the absence of DNA replication. The distance from the beginning of ORF EP1242L to the first 7T motif downstream of the ORF is 3.94 kb, in rather good agreement with the size determined for this RNA.

NP1450L and EP1242L are more similar to the eukaryotic RNA polymerase II subunits than to those of vaccinia virus

FASTA searches in SWISSPROT and NBRF protein databases with the derived amino acid sequences of NP1450L and EP1242L gave high scores against the largest and second largest subunits of eukaryotic, poxviral and bacterial RNA polymerases, respectively (not shown). Eukaryotic cells have three different RNA polymerases of similar multimeric structure, whose largest and second largest subunits share a high amino acid sequence similarity (reviewed in (33)). Pairwise comparisons of NP1450L and EP1242L with the largest and second largest subunits of *Saccharomyces cerevisiae* RNA polymerases I, II and III, respectively, show that the ASFV subunits are more similar to the equivalent subunits of RNA polymerase II than to those of RNA polymerases I and III (not shown). The alignment of the largest subunit of yeast RNA polymerase II with NP1450L pointed out the absence of a repetitive C-terminal domain (C-TD) in the ASFV protein. Lower, but significant, percentages of identity were obtained when NP1450L and EP1242L were compared with the equivalent subunits of *E.coli* RNA polymerase, β' and β (not shown). This is consistent with the recognized similarity of the two largest RNA polymerase subunits of eukaryotic and bacterial cells (34–37).

Figs. 3 and 4 show multiple alignments of various regions of NP1450L and EP1242L with the corresponding subunits of *S.cerevisiae* and *D.melanogaster* RNA polymerase II, and vaccinia virus RNA polymerase. The domains conserved in the eukaryotic and *E.coli* subunits are basically maintained in the ASFV proteins. The importance of these conserved regions is heightened by the fact that many different mutations impairing the function of the yeast enzyme map within them, even though some others lie outside the conserved domains (38–41). Also within conserved regions are the Zn-fingers of both subunits ((42), and refs. therein), already shown to bind Zn (43), and the active (nucleotide-binding) site of the second largest subunit (44). All these sites are strictly conserved in the ASFV subunits (Figs. 3 and 4).

The multiple alignments whose fragments are shown in Figs. 3 and 4 allowed us to obtain optimized calculations of the similarity existing among the yeast, *Drosophila*, vaccinia virus and ASFV RNA polymerase subunits. The results, shown in Tables I and II, demonstrate that the ASFV proteins are more similar to the eukaryotic RNA polymerase II subunits than to those of vaccinia virus.

DISCUSSION

The presence in the ASFV genome of genes encoding proteins similar to the two largest RNA polymerase subunits of poxviruses and eukaryotic organisms makes it very likely that ASFV transcription is carried out by a multimeric, virus-encoded, RNA polymerase. The putative largest subunit of ASFV RNA polymerase has no repetitive C-terminal domain, thus resembling the poxviral subunit (2), but its amino acid sequence is more similar to those of the largest subunits of eukaryotic RNA polymerases II than to the vaccinia virus subunit. The sequence of the second largest subunit of ASFV is also more similar to that of eukaryotic RNA polymerases II. Moreover, ASFV and vaccinia virus RNA polymerases might have a slightly different

subunit composition, because ASFV encodes a protein similar to the sixth subunit of yeast RNA polymerase II (RPB6; J.M.Rodríguez, R.J.Y., J.F.Rodríguez and E.V., unpublished), while no similar protein is encoded in the genome of vaccinia virus. Taken together, all these data suggest that ASFV RNA polymerase could be more similar to the eukaryotic RNA polymerase II than to the poxviral enzyme, both in subunit composition and in the amino acid sequences of the individual subunits. The mechanism of action of the ASFV protein similar to the eukaryotic TFIIS and to the vaccinia virus protein *rpo30* could shed light on this matter, considering that the eukaryotic protein is an RNA polymerase-associated transcription factor, while *rpo30* is considered a true enzyme subunit due to its tight association to the viral enzyme (10).

The eukaryotic RNA polymerase II is very sensitive to α-amanitin (reviewed in (8)), an inhibitor of RNA elongation (45, 46). The mutation responsible for α-amanitin resistence in a BALB/c 3T3 mouse cell line is an Asn to Asp change at position 793 in the largest subunit (47). This Asn residue is highly conserved in RNA polymerases II (see the multiple alignment in (48)). In fact, as has previously been noticed (47, 48), the changes at that position of RNA polymerase II correlate very well with the partial or complete resistence to α-amanitin of the enzyme from *S.cerevisiae* (49), *S.pombe* (48), vaccinia virus (1) and ASFV (7), which have Ser, Ile, Arg and Glu, respectively, at the corresponding position ((47, 48); see also Fig.3).

The genes encoding the putative two largest subunits of ASFV RNA polymerase are mainly expressed late in the virus infectious cycle, after replication of viral DNA has begun. This fact implies that most, if not all, the ASFV RNA polymerase activity present in the infected cell at the early stage was carried by the infecting virus particle(s) (7). On the other hand, the RNA polymerase molecules synthesized during the late phase are probably responsible of further transcription of late genes, and are finally incorporated into ASFV particles.

In summary, we have identified two ORFs which encode RNA polymerase subunits with considerable similarity to their cellular counterparts. The capacity of ASFV to encode such proteins suggests that ASFV transcription is carried out by a multimeric enzyme whose subunits are encoded in the viral genome. Studies to characterize the RNA polymerase of ASFV and identify additional subunits are underway.

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