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Transcriptional Mapping of a Late Gene Coding for the p12 Attachment Protein of African Swine Fever Virus

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The transcriptional characterization of the gene coding for the p12 attachment protein of the African swine fever virus is presented. The results obtained have been used to generate the first detailed transcriptional map of an African swine fever virus late gene. Novel experimental evidence indicating the existence of major differences between the mechanisms controlling the transcription of late genes in African swine fever virus and poxviruses is provided.

African swine fever virus (ASFV) is the only member of an, as yet, unnamed group of icosahedral deoxivirus (8). ASFV replication is host restricted, infecting only soft ticks of the *Ornithodoros* genus and different members of the Suidae family (for a review, see reference 24). Infection of domestic pigs with ASFV causes a severe disease, the African swine fever (for a review, see reference 13), for which an effective vaccine is not yet available. Although the morphology of ASFV particles is very akin to that of the members of the *Iridoviridae* family (4), both the genome structure and some biochemical properties of ASFV have provided the basis for speculating that ASFV might be a distant relative of the poxviruses (23). The ASFV genome is formed by a single molecule of double-stranded DNA of approximately 170 kb (22) which associates with several proteins to form an electron-dense nucleoid structure, known as the virus core, which is enclosed within a large icosahedral capsid. The outer surface of the virions is formed by a lipoprotein envelope (10, 16) which is important during the first events of the infection cycle. The adsorption of ASFV to susceptible cells is mediated by the interaction of a structural virus protein, located within the outer envelope of the virion, with a specific cell receptor (2, 9). This protein, designated p12, is encoded by the O61R open reading frame (ORF) which is located within the central region of the virus genome (Fig. 1) (1). The accumulation of the p12 protein in infected cells is detectable only during the late phase of the infectious cycle (1). Thus, it seemed likely that the p12 gene belongs to the late gene class of ASFV. ASFV gene expression appears to follow a cascade mechanism similar to that described for poxviruses (15), where immediately upon infection the expression of a large subset of genes, known as early genes, begins. The transcriptional silencing of most members of this subset is coincidental with the onset of virus DNA replication, when the transcription of a second gene class, the late genes, is initiated (3, 18). Very little is known about the specific regulatory mechanisms controlling the expression of ASFV genes. We have therefore sought to characterize the transcription of the p12 gene in the hope that this gene could be used as a model to study the regulatory mechanisms involved in ASFV late gene expression.

For this study, a set of the different types of virus-specific RNAs synthesized in Vero cell cultures infected with the BA71V strain of ASFV (11) was obtained: total RNA isolated at 7 h postinfection from infected cultures maintained in the presence of either the inhibitor of protein synthesis cycloheximide (immediate-early RNA) or the inhibitor of DNA replication cytosine arabinoside (early RNA) and RNA isolated at 18 h postinfection (late RNA). As a control for all the experiments, total RNA isolated from mock-infected Vero cells was used. The study was initiated by analyzing, through Northern (RNA) blot hybridization (21), the presence of p12-specific transcripts during the replicative cycle of ASFV. A total of 10 μg of each of the different RNAs was subjected to denaturing agarose electrophoresis, blotted onto nitrocellulose paper, and then hybridized to a ^{32}P-end-labeled oligonucleotide (5′-AATGATTGCTACGATAAG TAATGTTTCTAC-3′) complementary to the region between nucleotides 37 and 66 of the coding strand of the O61R ORF (Fig. 1). The results of this hybridization (Fig. 2A) show that the oligonucleotide probe specifically hybridizes to transcripts found only in late RNA. Two radioactive bands were detected (Fig. 2A, lane L): a very intense band corresponding to a transcript of approximately 1.25 kb and a second one, much fainter, corresponding to a transcript of approximately 0.55 kb.

To map the 5′ end of the transcripts detected by Northern blot hybridization precisely, two complementary experimental approaches, primer extension (14) and S1 nuclease analysis (6), were used. The primer extension was carried out as previously described (19) by using, as the primer, the oligonucleotide previously used for the Northern blot analysis. After hybridization of the primer to 10 μg of the appropriate RNA, the samples were incubated for 1 h at 40°C in the presence of avian myeloblastosis virus reverse transcriptase and then subjected to electrophoresis under denaturing conditions. As shown in Fig. 2B, extension of the ^{32}P-end-labeled primer was only observed in the sample corresponding to late RNA (lane L). In this sample, an extended DNA product of 77 nucleotides in length was detected, thus showing the existence of a major transcriptional initiation site located 11 nucleotides upstream of the O61R ORF (Fig. 3A). The S1 nuclease analysis to map the 5′ ends of these transcripts was performed as previously described (19). A total of 7.5 μg of the appropriate RNA was hybridized to 5 × 10^3 cpm of a 548-nucleotide ^{32}P-3′-end-labeled probe which

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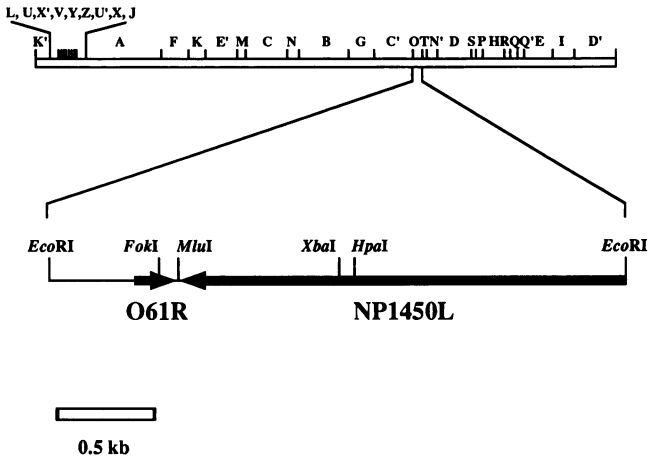

FIG. 1. p12 gene transcriptional mapping strategy. The diagram shows the relative position of the O61R and the NP1450 ORFs within genome of the BA71V strain of ASFV. The ORF O61R codes for p12. The lettering in the upper part of the figure represents the *Eco*RI restriction fragments within the virus genome (5). The location within the *Eco*RI O fragment of the oligonucleotide used for the Northern blot and primer extension analyses, the single-stranded ^{32}P-end-labeled probes used for the S1 mapping of the p12 gene, and the positions of the endonuclease restriction sites used for their generation are indicated.

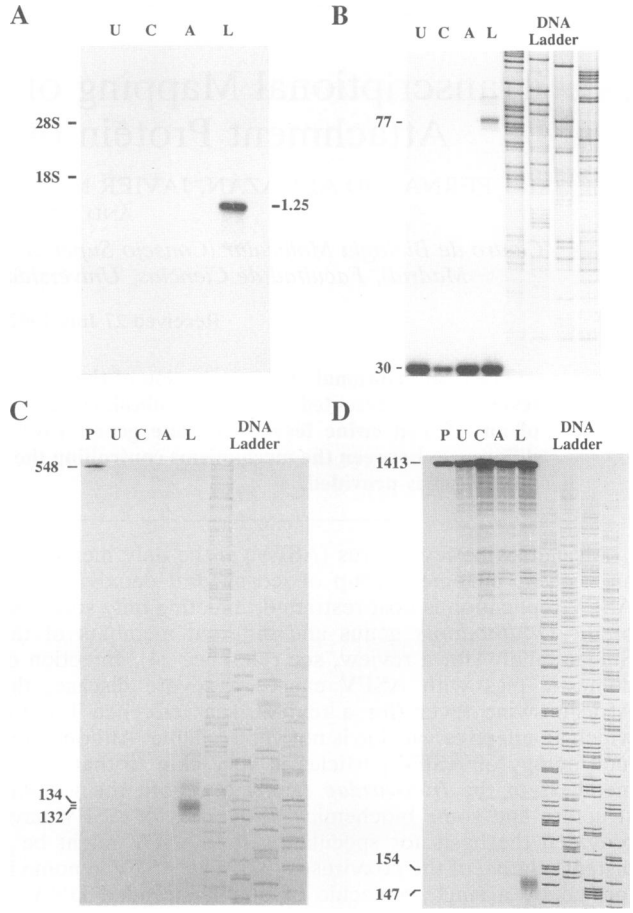

FIG. 2. Transcriptional mapping of the p12 gene. Autoradiograms of the Northern blot hybridization (A), primer extension analysis (B), 5′ end mapping by S1 nuclease analysis (C), and 3′ end mapping by S1 nuclease analysis (D) of four types of RNA, mock-infected (lanes U) and ASFV virus-induced immediate-early (lanes C), early (lanes A), and late (lanes L), are shown. In the autoradiogram corresponding to the Northern blot hybridization, the positions of the 28S and 18S rRNAs and the size (in kilobases) of the most abundant transcript are shown. The samples corresponding to the primer extension and S1 nuclease analyses were electrophoresed alongside an irrelevant DNA sequencing reaction (DNA ladder) used as a size marker. In the autoradiograms corresponding to the S1 analyses, the migration of the undigested probe is indicated (lane P). Numbers at the side of the autoradiograms correspond to the size (nucleotides) of the relevant DNA fragments.

was generated by digestion with the restriction enzyme *Fok*I, treatment with calf intestinal phosphatase, labeling with [γ-^{32}P]ATP in the presence of polynucleotide kinase, and digestion with *Eco*RI (Fig. 1). The resultant DNA-RNA hybrids were digested with S1 nuclease for 1 h at 20°C and then subjected to electrophoresis under denaturing conditions (19). This analysis revealed the existence of three major bands of 132 to 134 nucleotides, corresponding to S1 nuclease-protected DNA fragments, in the late RNA sample (Fig. 2C). This result shows that transcripts spanning the genomic region covered by the probe are synthesized during the late phase of the infectious cycle and initiate 10 to 12 nucleotides upstream of the initiator AUG of the O61R ORF (Fig. 3B).

The mapping of the 3′ end of the p12 transcripts was carried out by S1 nuclease analysis. Initially, two 5′-end-labeled probes covering a DNA region of approximately 1 kb which includes the last 116 nucleotides of the O61R ORF (Fig. 1) were used. Therefore, an additional ^{32}P-end-labeled probe of 1,414 bases was generated by *Xba*I digestion, labeling with [α-^{32}P]dTTP in the presence of Klenow enzyme, and consequent digestion with *Eco*RI (Fig. 1). After digestion of the DNA-RNA hybrids with S1 nuclease for 1 h at 20°C, a series of bands corresponding to protected DNA fragments of 147 to 154 bases were generated (Fig. 2D). Similar results were obtained by using other periods (30 and 45 min) and temperatures (30 and 37°C) of incubation. The results obtained demonstrate that the 3′ ends of the transcripts initiating immediately upstream of the O61R ORF map approximately 980 nucleotides downstream of the termination codon of the ORF (Fig. 3A and C).

The primer extension and S1 nuclease analyses described clearly show that the p12 gene is transcribed only during the late phase of the infection, giving rise to mRNAs of approximately 1.18 kb before the addition of the 3′ end poly(A) tail, a size which is consistent with that of the most abundant mRNA species detected by Northern blot hybridization (Fig. 2A). With both types of analysis, a small number of transcripts initiating several nucleotides upstream of the major transcriptional initiation site were detected (Fig. 2A and B, lanes L). However, it is important to note that the primer extension analysis did not reveal the synthesis of high-molecular-weight primer-extended products that could correspond to the presence of poly(A) heads which are found in poxvirus late mRNAs (7, 20) at the 5′ end of the p12 transcripts. Surprisingly, the presence of an initiation or a termination site(s) that could account for the less abundant 0.55-kb transcript detected by Northern blotting has not been detected. This could be explained by the lower sensitivity of the primer extension and/or the S1 nuclease analyses compared with the Northern blot hybridization method or, alternatively, by the hybridization in the Northern blot of

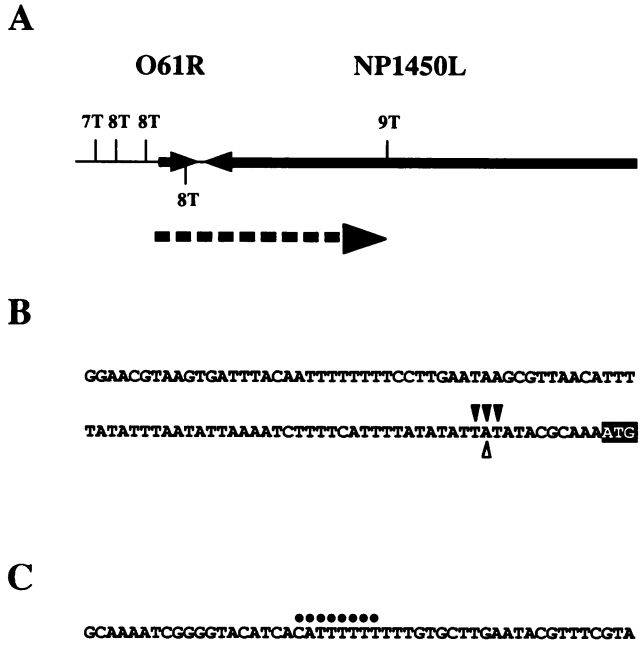

FIG. 3. (A) Map of the p12 mRNA. The diagram shows a map of the O61R mRNA, indicated by a dashed arrow, within the *Eco*RI O fragment of the ASFV genome. The positions of the O61R and NP1450 ORFs and the distribution of stretches of seven or more consecutive T residues (T) in either strand are also shown. (B) Precise location of the p12 transcriptional initiation site. The first ATG of the O61R ORF, indicated by a box, and the upstream nucleotide sequence are shown. The locations of the transcriptional initiation sites detected by S1 nuclease (solid triangles) and primer extension analysis (empty triangles) are indicated. (C) Precise location of the p12 transcriptional termination site. The diagram shows the nucleotide sequence surrounding the detected 3′ ends, indicated by circles, above the sequence of the mRNAs corresponding to the p12 gene.

the oligonucleotide to virus-induced mRNA unrelated to p12.

We have recently shown (3) that the 3′ ends of several ASFV early genes map within a conserved sequence motif formed by at least seven thymidylate residues (7T). Interestingly, the 3′ ends of the p12 transcripts map within the first run of several consecutive thymidylate (9T) residues found in the downstream region of the ORF (Fig. 3A and C). This result strongly suggests that the 7T motif might be involved in the 3′ end formation of both early and late ASFV mRNAs. The distribution of the 7T motif within the *Eco*RI O fragment (Fig. 3A) also suggests that this sequence might play an important role in preventing transcriptional interference among neighboring genes. Thus, the presence of several 7T motifs immediately upstream of the transcriptional initiation site of the p12 gene could be explained as a mechanism to prevent transcriptional arrest of p12 by run-through of upstream transcripts. Similarly, the 7T motifs found within the coding strand of both O61R and NP1450L (25) ORFs (Fig. 3A) may be involved in preventing the generation of RNA-RNA hybrids formed by symmetrical transcription of opposing genes. Experiments to test these hypotheses are currently under way.

In conclusion, because some of the biochemical attributes of ASFV, including the existence of a virus-encoded enzymatic machinery responsible for the synthesis of mature virus mRNAs, strongly resemble those of the poxviruses (23), it seemed likely that both ASFV and poxviruses could share similar transcriptional mechanisms. However, our results demonstrate that transcripts corresponding to the p12 gene, a bona fide ASFV late gene, are structurally different from those corresponding to poxvirus late mRNAs. Poxvirus late mRNAs are heterogeneous in size and contain 5′ poly(A) heads (for a review, see reference 15). In contrast, the p12 transcripts have a defined length and do not possess 5′ poly(A) heads. Additionally, it is also important to note the lack of the sequence motif TAAAT within the proximity of the transcriptional initiation site of the p12 gene (Fig. 3B). The TAAAT motif is an essential element of most poxvirus late promoters (12, 17).

Although the mapping of more ASFV late genes will be required to obtain a general picture of ASFV late transcription, the results presented here indicate that the mechanisms controlling the expression of the ASFV late genes might have evolved independently from those of poxviruses.

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