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Expression Strategy of *Aedes albopictus* Densovirus

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The transcription map of the *Aedes albopictus* densovirus (AalDNV) brevidensovirus was identified by Northern blotting, rapid amplification of cDNA ends (RACE) analysis, and RNase protection assays. AalDNV produced mRNAs of 3,359 (NS1), 3,345 (NS2), and 1,246 (VP) nucleotides. The two overlapping P7/7.4 NS promoters employed closely located alternate transcription initiation sites, positioned at either side of the NS1 initiation codon. All NS mRNAs coterminated with VP mRNA. All promoters, explored using luciferase assays, were functional in insect and human cell lines.

Invertebrate parvoviruses (densoviruses [DNVs]) are subdivided into four genera: *Densovirus*, *Pefudensovirus*, *Iteravirus*, and *Brevidensovirus* (1–3). Brevidensoviruses have a 4.1-kb single-stranded DNA (ssDNA) genome with three open reading frames (ORFs) on the same strand (about 790 [NS1], 360 [NS2], and 350 [VP] amino acids). The genome of *Aedes albopictus* densovirus (AalDNV) (GenBank accession no. NC_004285) has terminal, T-shaped hairpins (4). Brevidensoviruses were isolated from medically important mosquito vectors, such as *Aedes albopictus* (AalDNV) (4), *Aedes aegypti* (AaeDNV) (5), and *Anopheles gambiae* (AgDNV) (6). AalDNV was isolated from *Aedes albopictus* C6/36 cells (7) but is infectious for *Aedes aegypti* larvae (8, 9).

AaeDNV expression has been studied to some extent (10, 11). ORF_{NS1} of AaeDNV was reported to have a 57-amino-acid N-terminal extension compared to that of AalDNV (4, 5). β-Galactosidase fusion proteins with the three ORFs were enzymatically active, except for NS1 (12) unless NS1-β-gal was constructed downstream of the corresponding AalDNV AUG_{NS1} (11). Primer extension demonstrated that AaeDNV VP transcription started at nucleotide (nt) 2402 (10). Here, AalDNV transcription was analyzed by Northern hybridization, 5′ and 3′ rapid amplification of cDNA ends (RACE), amplicon mapping, and RNase protection assays (RPAs). AalDNV promoter activities were also determined.

AalDNV transcripts were obtained after transfection of pCR2.1-AalDNV (containing the AalDNV genome of 4,176 nt [4] between its EcoRI sites), using Lipofectamine (Invitrogen protocol), in permissive C6/36 cells (7) grown in RPMI medium supplemented with 5% fetal bovine serum (FBS). After 48 h, RNA was isolated using the Stratagene Absolutely RNA miniprep kit.

Northern blotting. ^{32}P-labeled RNA probes for Northern blots targeted overlapping domains of the NS1 and NS2 ORFs or VP ORF. PCR-amplified products (primers in Table 1) were transcribed *in vitro* with [α-^{32}P]UTP and T7 RNA polymerase (NEB) (13). Northern blots using 10 μg of total RNA (13, 14) revealed transcript sizes of 3.4 and 1.3 kb with the VP-specific probe and a 3.4-kb transcript with the NS-specific probe (Fig. 1A).

RACE experiments. The FirstChoice RLM RACE kit (Invitrogen) was used to identify the 5′ starts and 3′ ends of the polyadenylated transcripts. The 5′ adapter primer (IP) and the 3′-anchored primer (AP) (in the kit) were used in PCR with AalDNV-specific primers (Table 1), as shown in Fig. 1B. Amplicons obtained with A626R/IP (~325 bp), A497R/IP (~200 bp), A2740R/IP (~350 bp), A2540R/IP (~150 bp), A3162F/AP (~600 bp), and A3461F (~300 bp) (data not shown) were sequenced. Sequencing revealed that NS1 transcription started at nt 329 (AGTA), 6 nt upstream of AUG_{NS1}, and that VP transcription started at nt 2441 (CAGTCG), 158 nt upstream of AUG_{VP} (Fig. 1B) (sequence data not shown). Sequencing of the 300- and 600-bp amplicons showed a transcription termination position at 3680, 18 nt downstream of the polyadenylation signal at position 3662 (Fig. 1C) (sequence data not shown).

As is common for other densoviruses, NS2 could be translated from nt 411 by leaky scanning (13) on the NS1 transcript. The short untranscribed region of NS1 (UTR_{NS1}), in spite of a consensus AnnAUGG sequence for NS1 initiation, could favor it (13). A canonical initiator Inr_{NS2} sequence (CAGT) is located at nt 342. After TfiI digestion (NEB) at nt 338 of the A831R/IP PCR product, followed by a nested PCR (A626R/IP or A497R/IP) for specific amplification of putative NS2 transcripts, bands of 330 and 200 nt were produced (data not shown). Their sequencing revealed that NS2 transcription started at nt 343 (sequence data not shown). Thus, NS1 and NS2 AalDNV transcription starts were separated by 14 nt on either side of AUG_{NS1}.

Amplicon mapping. In order to confirm that NS and VP transcripts all coterminated at position 3680, primer A2380F (upstream of the VP transcript) and two reverse primers, A3650R (upstream) and A3744R (downstream), of the VP transcript end were designed for reverse transcription (RT)-PCR amplification (Table 1). Only a band of about 1,300 nt was obtained using the A2380F/A3650R set of primers for NS transcripts (Fig. 1C).

RNase protection assays. RPAs were employed to confirm NS1, NS2, and VP transcription starts. RPA probes for NS and VP transcripts were prepared by PCR (Table 1; Fig. 2A), and *in vitro* transcription as described for Northern blot probes. For positive controls, a sense RNA was generated by *in vitro* transcription and RPA was performed in parallel: for NS, two positive-control RNAs spanning from nt 329 to 439 (predicted for NS1) and nt 343 to 439 (predicted for NS2) were used, and for VP one from nt 2441 to 2542 was used. The probes and control RNA were purified from polyacrylamide gel and used in the RPA III kit (Invitrogen). Size markers were generated according to the 5′ RACE results. The NS

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TABLE 1 Primers used for Northern blot probes, RACE, amplicon mapping, and RPAs

Primer name[a]	Sequence	Purpose
A639F	GCTCCAGAGCCTCTGAACAGCTTG	NS probe, Northern blot
A1345R (+T7 sequence)	TAATACGACTCACTATAGGGGTTCTGACTCTTGTGCTGTTTC	NS probe, Northern blot
A3106F	CTAGAAACAGTTGCAGCAACCGGAC	VP probe, Northern blot
A3509R (+T7 sequence)	TAATACGACTCACTATAGGCGTACTTGATATCTGAATTTCATG	VP probe, Northern blot
A3372F	AACTACAACATATGCCACGTCAG	3′ RACE
A3461F	ACAAGTTCCAGACGAAACAGG	3′ RACE
A497R	GTTCGTAATTGTTGGCATTCCT	5′ RACE of NS
A626R	GTGGGTAGATGTTATCAACGG	5′ RACE of NS
A831R	CTTGCCTGTGACCCGTTATTATCC	5′ RACE of NS
A2540R	GTGCGTTGTCTTCTTCTTCTATC	5′ RACE of VP
A2740R	GACCAAACATTACGGAAATGG	5′ RACE of VP
A3126F	CGGACCATTAGCACAACAAAC	3′ RACE
A2380F	GAGTATACAACACAGAGAAG	Amplicon mapping
A3650R	TCATAA GGCATACATGCTAC	Amplicon mapping
A3744R	TCTGTCGTGGACATTATCAG	Amplicon mapping
A272F (+UTS)	GCGATGAATGAACACTGAATCCACCACCACATGATCC	RPA NS probe
A329F (+T7 sequence)	TAATACGACTCACTATAGGAGTAGTATGGAATCAG	RPA-positive control for NS1
A343F (+T7 sequence)	TAATACGACTCACTATAGGGTCTGCAGTGAACATTCG	RPA-positive control for NS2
A439R	TCTCCTCCTGGATTTACACTG	RPA-positive control for NS1 and NS2
A439R (+T7 sequence)	TAATACGACTCACTATAGGTCTCCTCCTGGATTTACACTG	RPA NS probe
A2323F (+UTS)	GCGATGAATGAACACTGGCATATGAACGAAACCTCAC	RPA VP probe
A2441F (+T7 sequence)	TAATACGACTCACTATAGGAGTCGGCCACCAGGTCTTGTAG	RPA-positive control for VP
A2542R	ATGTGCGTTGTCTTCTTCTTC	RPA-positive control for VP
A2542R (+T7 sequence)	TAATACGACTCACTATAGGATGTGCGTTGTCTTCTTCTTC	RPA VP probe
A148F	TCCAATTGGAACACACGGAC	P7/7.4 promoter for AalDNV
A333R	CTACTGACTCTCCCTTC	P7/7.4 promoter for AalDNV
A2431F	CAAACTCATCAGTCGGCCAC	P60 promoter for AalDNV
A2597R	CCTCTGCTTCTTCTTTTGC	P60 promoter for AalDNV

[a] In the primer names, "UTS" represents the unspecific target sequence and "R" and "F" represent sense and antisense, respectively. The numbers in the primer names indicate the 5′ end of the primer sequence in AalDNV. The underlined sequences represent the UTS or T7 sequences.

probe protected the expected 96 and 110 nt of the NS transcript 5′ ends (Fig. 2B), confirming NS1 and NS2 mRNA start positions. Similarly, the VP probe confirmed the VP transcript 5′ extremity by protecting 101 nt (Fig. 2B). A protected band at 219 nt, slightly smaller than the entire probe (236 nt, including 17 nt of unspecific sequence), showed the coexistence of NS transcripts.

Promoter activity. NS (P7/7.4) and VP (P60) promoter regions were amplified by PCR (primers in Table 1) and cloned into

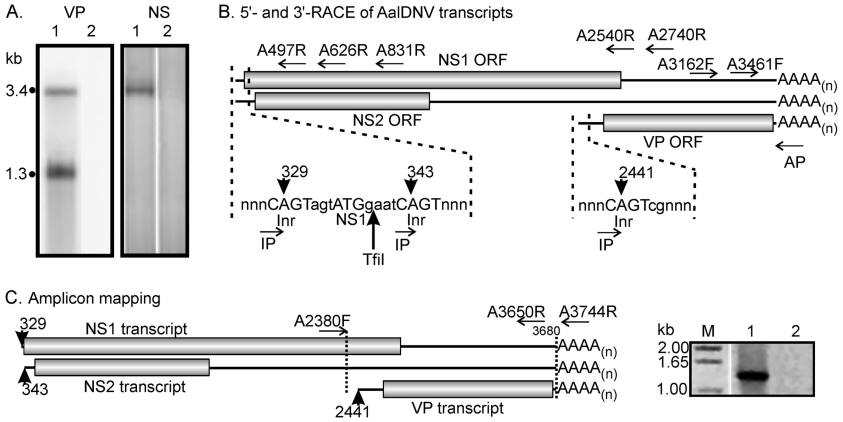


FIG 1 (A) Northern blot analysis of AalDNV transcription 48 h after transfection with vectors with virus-specific inserts (lanes 1) or without inserts (lanes 2). The VP probe also detected bands corresponding to the size of NS transcript indicating that VP and NS transcripts may coterminate. (B) Strategy of 5′ and 3′ RACE. Above the ORFs are the specific primers that were designed, and below are the ORFs and IP and AP primers from the Invitrogen kit (indicated with arrows). We took advantage of the TfiI restriction site between the putative Inr of NS1 and NS2 to distinguish between these transcripts. (After digestion, only amplicons from the second Inr would be obtained.) (C) Confirmation of termini of NS transcripts of AalDNV. The diagrams represent transcript map results by 5′ and 3′ RACE. Forward primers that do not recognize VP transcripts and reverse primers, both upstream and downstream of the VP transcript end (3′ RACE), were used in a PCR (indicated in the diagrams). As expected, only the inboard reverse primer (A3650R in lane 1) gave a product. M, markers; lane 2, A2380F/A3744R.

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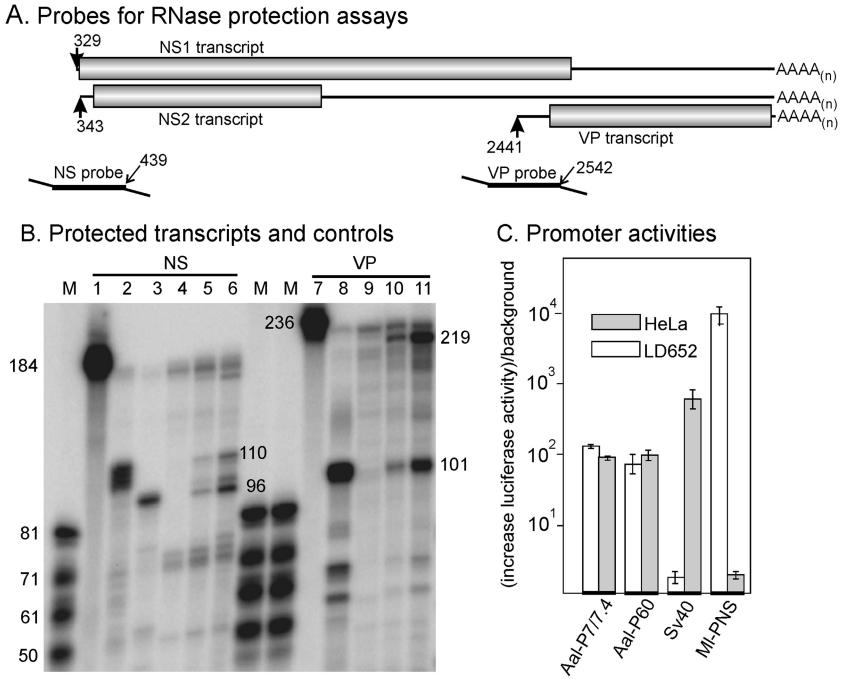


FIG 2 (A) Diagram of AalDNV expression and location of probes. (B) RNase protection assay of starts of NS and VP transcripts of AalDNV. Lanes: 1, NS probe with a specific length of 184 nt and short nonspecific extra terminal sequences; 2, positive control for NS1 with specific size obtained with RACE experiments; 3, positive control for NS2 with specific size obtained with RACE experiments; 4, total RNA from C6/36; 5, total RNA from AalDNV-infected C6/36 (15 μg); 6, total RNA from AalDNV-infected C6/36 (40 μg); 7, VP probe; 8, positive control for VP; 9, total RNA from C6/36; 10, total RNA from AalDNV-infected C6/36 (15 μg); 11, total RNA from AalDNV-infected C6/36 (40 μg). The band at 101 nt confirmed the VP start, and the band at 219 nt represented protection of the VP probe on the NS transcript. (C) Luciferase activity 40 h after transfection with different promoter constructs as fold increase over background (transfected pGL4 without insert). Inverse orientation of the promoters did not increase activity significantly over background. SV40 (not shown) and the insect virus MlDNV NS promoter (Ml-NSP) had a strong preference for cells from vertebrates and invertebrates, respectively. Surprisingly, AalDNV had significant activity in both types of cells.

EcoRV in pGL4.20, upstream of the luciferase gene (Promega; GenBank accession no. DQ188840) to estimate their functionality. Those with the reverse promoter orientation, shown by sequencing, served as controls. The *Mythimna loreyi* (MlDNV) NS promoter (an insect virus replicating in LD652 cells) (15) and the simian virus 40 (SV40) promoter were also cloned into pGL4.20 vectors to serve as positive controls. Transfection of LD652 using DOTAP (Roche) and HeLa cells using Lipofectamine (Invitrogen) in 24-well plates was performed according to the suppliers' instructions. Cells were harvested 40 h posttransfection and resuspended in 100 μl of Bright-Glo lysis buffer (Promega), and relative luciferase activity was determined according to Promega's instructions. The AalDNV P7/7.4 and P60 promoters were functional both in insect cells and in human cells, in contrast to constructs lacking a promoter or with promoters in the reverse orientation, whereas the SV40 promoter preferred HeLa cells and the MlDNV promoter preferred insect cells (Fig. 2C).

(i) Promoter 7/7.4 elements. The RACE experiments and RPA (Fig. 2) revealed the location of Inr1 and Inr2 at either side of the NS1 protein initiation codon (Fig. 3C). Consequently, the NS1 transcript could potentially be translated into both NS1 and NS2 proteins, whereas the NS2 transcript could only code for the NS2 protein. Different constructs, leaving Inr1 and its upstream elements intact (for NS1 transcription) but with mutations in the NS2 promoter and NS1/2 translation elements, were made with a luciferase reporter gene (Fig. 3B and C). Constructs containing the intact luciferase AUG (F1 to F7) served as a positive control for transcription, whereas those lacking the luciferase AUG (F1− to F7−) showed the impact of the various mutations.

P7/7.4 consisted of three segments (Fig. 3B). The KpnI-SacI segment I contained upstream promoter elements, the SacI-PstI segment II included both TATA boxes, ATG_{NS1}, and both Inrs and, finally, the PstI-HindIII segment III (about 20 nt for the NS1 ORF and about 70 nt for the NS2 ORF) contained the downstream promoter elements (DPE) and ATG_{NS2}. These segments were connected in pBluescript (PCR with primers containing appropriate restriction sites and pCR2.1-AalDNV template) and were transferred as a whole (or after mutation) to the pGL4.20 luciferase reporter. The diagram in Fig. 3C delineates the different constructs and knockouts (using the Transformer kit according to Clontech's instructions) (Fig. 3C). Certain ATGs were mutated to TTG, Inr (CATG) was mutated to GCCG, and the TATA box of NS2 was mutated to GCTCGAG. In addition, alternates of F1 to F7 that lacked the luciferase initiation codon (F1− to F7−) as well as two constructs from which the NS2 core promoter was mutated (F2* and F5−*) were obtained. C6/36 and 293T (as for HeLa) cells were transfected, and luciferase activity was determined. Except for F2* and F5−*, all constructs were expected to yield both NS1 and NS2 mRNAs. The observed luciferase activity in C6/36 and 293T cells (Fig. 3E) corresponded well with the expected luciferase activities, summarized in Fig. 3D, from (i) fusion proteins with NS1 (F1 and F1−) and NS2 (F5 and F5−), (ii) directly from the luciferase

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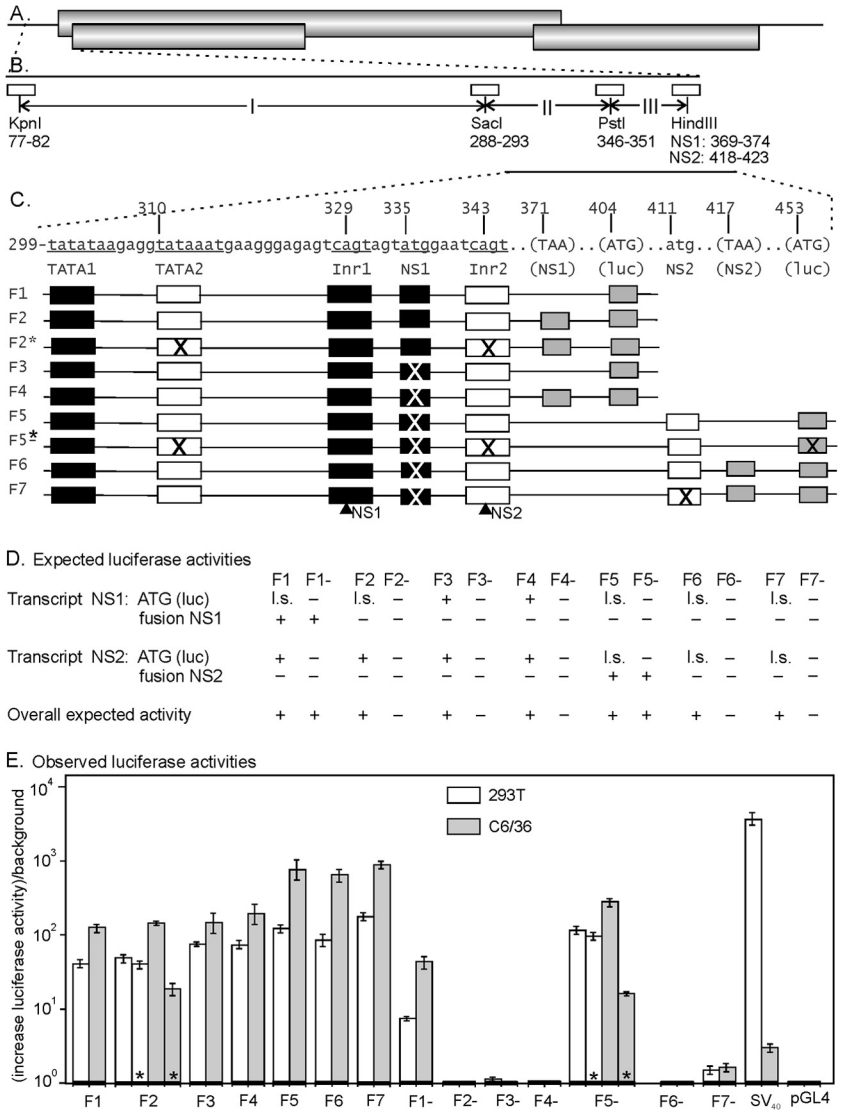


FIG 3 Analysis of AalDNV P7/7.4 promoter elements using a luciferase reporter gene. (A) Diagram of ORFs in AalDNV, where nt 77 to 423 contain typical sequences of core promoter elements for NS1 and NS2. (B) Three segments (I to III) were connected via pBluescript in pGL4 and could be individually mutated and swapped. (C) In the constructs, filled boxes represent elements thought to direct NS1 mRNA expression and open boxes those thought to direct NS2 mRNA expression, filled triangles represent transcript starts that were established, crosses represent knockouts, and gray boxes represent replaced sequences. Additionally, mutants were made for all constructs in which the initiation codon of luciferase was knocked out (F1− to F7−), and in 2 constructs TATA2 and Inr2 (for NS2 transcripts) were knocked out (indicated by *). (D) Expected activities using the luciferase reporter gene in the pGL4 vector. ("l.s." represents luciferase activity if leaky scanning occurs.) (E) The observed luciferase activity matched the expected activity, except for the very low F7− activity (none expected). These results indicated that the NS initiation codons, and hence the two sets of promoter elements for transcription, were individually important for expression but nevertheless leaky scanning on the NS1 transcript could also contribute to the expression of NS2.

initiation codon (F1 to F7), or (iii) after leaky scanning (for F1, F2, and F5 to F7). Therefore, individual elements of both NS1 and NS2 promoters contributed to mRNA expression.

Nevertheless, NS2 could be generated on the NS1 transcript by leaky scanning. Blocking NS2 transcription by mutating its core promoter in F5−* yielded an ~90% drop in luciferase activity in C6/36 cells compared to F5−. The remaining activity could be ascribed to a fusion protein from ATG_{NS2} on the NS1 transcript since the introduction of an NS2 stop codon in frame after the NS2 initiation codon in F6− or knocking out ATG_{NS2} in F7− abolished luciferase activity. Consequently, for F2* the remaining luciferase activity was due mainly to leaky scanning on the NS1 transcript and initiation from ATG_{luc}. In conclusion, although NS1 and NS2 mRNAs have their own promoter elements in p7/7.4, NS1 mRNA contributes significantly through leaky scanning to NS2 expression.

In conclusion, Northern blotting, RACE, amplicon mapping, and RPA results were all in agreement. AalDNV used one promoter region with closely overlapping elements to start transcription of NS1 and NS2 at positions that are just 14 nt apart at either side of ATG_{NS1}. No clear TATA-like motif sequences were found upstream of the initiator sequence CAGT of the VP of AalDNV and AaeDNV, suggesting that these promoters were under the control of DPE (16, 17). This regulatory circuit is likely to be one

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means by which insect virus networks can transmit transcriptional signals, such as those from DPE-specific and TATA-specific enhancers, via distinct pathways (18), to regulate NS and VP expression.

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