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Transcription of the Baculovirus Polyhedrin Gene Reduces the Levels of an Antisense Transcript Initiated Downstream

BENG GUAT OOI† AND LOIS K. MILLER*

Departments of Entomology and Genetics, University of Georgia, Athens, Georgia 30602

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A late 3.2-kilobase (kb) RNA initiated approximately 2 kb downstream of the 3′ end of the *Autographa californica* nuclear polyhedrosis virus polyhedrin-coding sequence and traversed the polyhedrin gene in an antisense direction. This RNA was sense RNA for the two open reading frames flanking the polyhedrin gene. A mutant virus, vXpoly, which differs from wild-type virus only at the essential RNA initiation site in the polyhedrin promoter, exhibited higher levels of the 3.2-kb RNA than did wild-type virus during the polyhedrin transcriptional phase. Thus, transcription of the polyhedrin gene down regulates the levels of this 3.2-kb RNA.

Transcription of individual genes within the large DNA genome of baculoviruses often involves the synthesis of multiple, overlapping, unspliced RNAs that may be of different temporal classes and may be both sense and antisense (for a review, see reference 3). The possibility that extended and antisense transcripts contribute to baculovirus gene regulation is an attractive hypothesis because genes of different temporal classes are interspersed in the baculovirus genome.

Polyhedrin gene regulation is of particular interest because this promoter is used in many baculovirus expression vector systems (for a review, see reference 5). An overlapping set of RNAs is transcribed from the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) polyhedrin gene (2). Three RNAs in this set (1.2, 3.4, and 4.9 kilobases [kb]) comprise the vast majority of polyhedrin transcripts and appear to be coordinately regulated as very late RNAs with a common 5′ end. Other minor late RNAs are also observed in Northern (RNA) blots probed with polyhedrin cDNA (2).

While mapping the transcripts of genes flanking the polyhedrin gene (Fig. 1), we observed a 3.2-kb RNA traversing the polyhedrin gene in the antisense direction (Fig. 2B). In wild-type (wt)-infected cells, maximal levels of this RNA were observed 12 h postinfection (p.i.), decreasing rapidly thereafter. Low levels of a 3.2-kb RNA were also observed in the presence of the protein synthesis inhibitor cycloheximide, which effectively blocks late but not early Ac*M*NPV gene transcription (1, 9–12). These characteristics indicate that the 3.2-kb RNA is primarily transcribed as a late class RNA, although it is also transcribed at low levels as an early RNA.

To map the 3.2-kb RNA further, Northern blot analysis with a strand-specific probe of the downstream-flanking 1629 open reading frame (ORF) revealed a 3.2-kb RNA which was regulated identically to the 3.2-kb polyhedrin antisense RNA (Fig. 2C). The major RNA observed in this region was a 2-kb RNA which is known to cover the entire 1629 ORF and terminate in the intergenic region between the polyhedrin and 1629 ORFs (S. Howard, R. Possee, and D. H. L. Bishop, unpublished data). The 2- and 3.2-kb RNAs were regulated identically. The sharp maximum at 12 h and the rapid decline in levels of these two RNAs at 18 h p.i. reflect a unique regulatory pattern for late baculovirus transcripts studied to date. The data suggest that these two RNAs may be coordinately regulated from a common 5′ terminus. A minor 1.5-kb RNA may also be present in this region at low levels (Fig. 2C). Cycloheximide treatment reduced the levels of the larger two RNAs, again indicating a late component in transcriptional regulation. The similarity in the size and regulation of the 3.2-kb RNA observed by using the 1629 and polyhedrin antisense probes (Fig. 2B and C) suggested that the same RNA was detected by both probes.

To prove that the polyhedrin 3.2-kb antisense RNA extends from within the polyhedrin gene through the 1629 ORF and to determine the 5′ end of this RNA, a 5′-end-labeled probe extending from within the polyhedrin ORF to the *Eco*RI site approximately 3 kb downstream was used in a nuclease protection assay (Fig. 3B). Three size classes of protected fragments which exhibited late regulation, 2.1, 1.7, and 1.3 kb, were observed. The maximal level of the RNA(s) protecting these fragments was found at 12 h p.i., consistent with Northern blot analyses. Late Ac*M*NPV RNAs are generally not spliced, so it is likely that these three protected fragments represented either 5′ ends of RNA or S1-sensitive regions. The sizes of the larger two protected DNAs place the 5′ end(s) just beyond the 5′ end of the 1629 ORF; this position is similar if not identical to the 5′ end of the 2-kb 1629 ORF-specific RNA mapped by Howard et al., unpublished data). A smaller (0.6-kb) protected fragment was also observed with this probe (Fig. 3B) and reflected an RNA which was regulated as a very late RNA. The 5′ end of this RNA mapped near the 3′ end of polyhedrin RNA. We have not studied this RNA further; it may be a short RNA, a minor RNA, or an artifact of the mapping procedure, since an additional very late RNA was not observed in Northern blots (Fig. 2B).

To determine whether the 3′ end of the 3.2-kb RNA extends into the 603 ORF lying upstream of the polyhedrin gene, Northern blots were hybridized with a probe covering most of this ORF (Fig. 2A). A 3.2-kb RNA, regulated in a fashion similar to that of the polyhedrin 3.2-kb RNA, was observed in this region also. A minor (approximately 1-kb) RNA was observed from 6 through 24 h p.i. and in the presence of cycloheximide.

To confirm that the 3.2-kb RNA crossing the 603 ORF extends across the *Eco*RV site into the polyhedrin-coding sequence, a probe, 5′ end labeled at the *Mlu*I site in the 603 ORF and extending through the *Hin*dIII site in the polyhe-

* Corresponding author.
† Present address: Human and Molecular Genetics, Baylor College of Medicine, Houston, TX 77030.

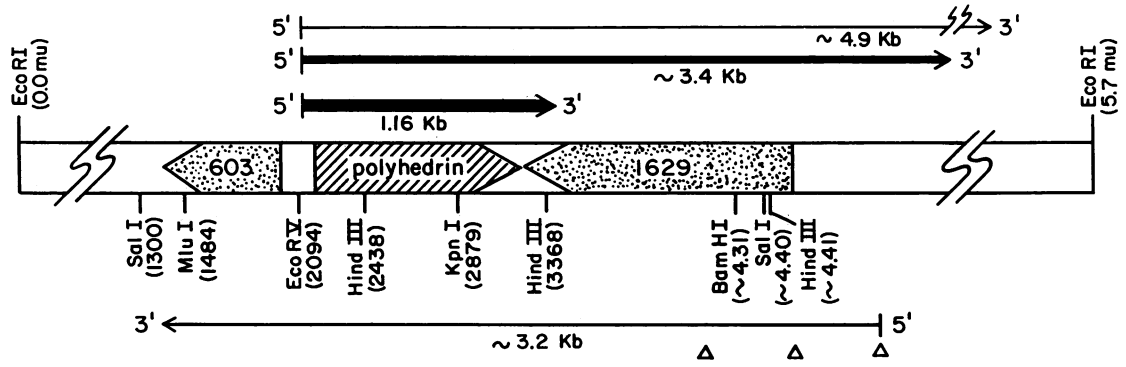

FIG. 1. Restriction map and ORFs in the region of the Ac*M*NPV genome that encodes the polyhedrin gene. The region shown lies within the 7.3-kb *Eco*RI I fragment which maps from the origin (0.0 map unit [mu]) of the 128-kb Ac*M*NPV DNA genome to 5.7 map units. Symbols: ▨, position of the polyhedrin ORF; ▩, positions and directions of the two ORFs which flank the polyhedrin gene (R. Possee, personal communication), designated 603 and 1629; ⟶, ➡, and ➡, direction and approximate lengths of the polyhedrin sense transcripts (2); ⟵, position of the 3.2-kb RNA mapped in this report; △, the three possible 5′ ends identified by S1 nuclease mapping analysis. The positions of key restriction sites are shown, and the precise or approximate nucleotide numbers at the center of the site are provided in parentheses; these numbers are based on the coordinates and sequence of Matsuura et al. (4).

drin-coding sequence, was used in an S1 nuclease protection analysis. These experiments confirmed that a major transcript extended across this entire region at 12 h p.i. (data not shown). By using RNA from 18 h p.i., S1 nuclease protection analysis also revealed a protected fragment mapping near the TAAG site of the polyhedrin promoter. The TAAG site is an essential component of the polyhedrin promoter and the site of polyhedrin RNA initiation (5a). Additional

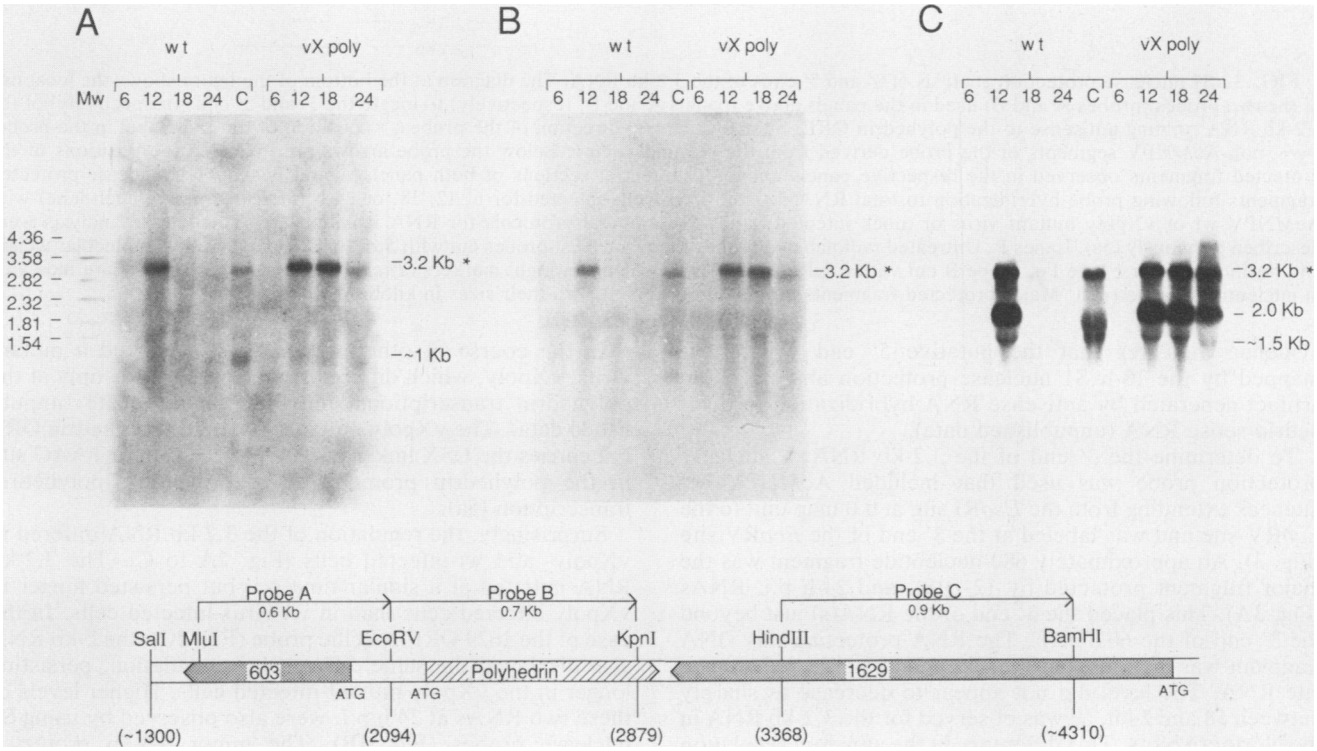

FIG. 2. Northern blots of RNA isolated from wt- or vXpoly-infected cells and hybridized to three different probes within and flanking the polyhedrin gene. Along the bottom of the figure is a restriction map of the region showing the positions of the three probes (probes A, B, and C) corresponding to panels A, B, and C, respectively. These strand-specific RNA probes were prepared by T3 or T7 RNA polymerase transcription of the appropriate plasmid DNA, and the arrows indicate the direction of probe transcription. All Northern blots show RNA isolated from cells after 6, 12, 18, or 24 h (indicated above each lane) of infection with either wt Ac*M*NPV or the polyhedrin promoter mutant vXpoly. Lanes C, RNA from wt-infected, cycloheximide-treated cells isolated at 12 h p.i. Details concerning cell growth, RNA isolation, and blot hybridization are found elsewhere (5a). Each lane contains 40 μg of glyoxalated total RNA. The gels were 1% agarose, and molecular weight markers (Mw), derived from glyoxalated pBR322 DNA fragments, were hybridized separately and are shown realigned on the left. Approximate molecular sizes of the RNAs are shown on the right of each panel. The 3.2-kb RNA, common to all blots, is marked by an asterisk.

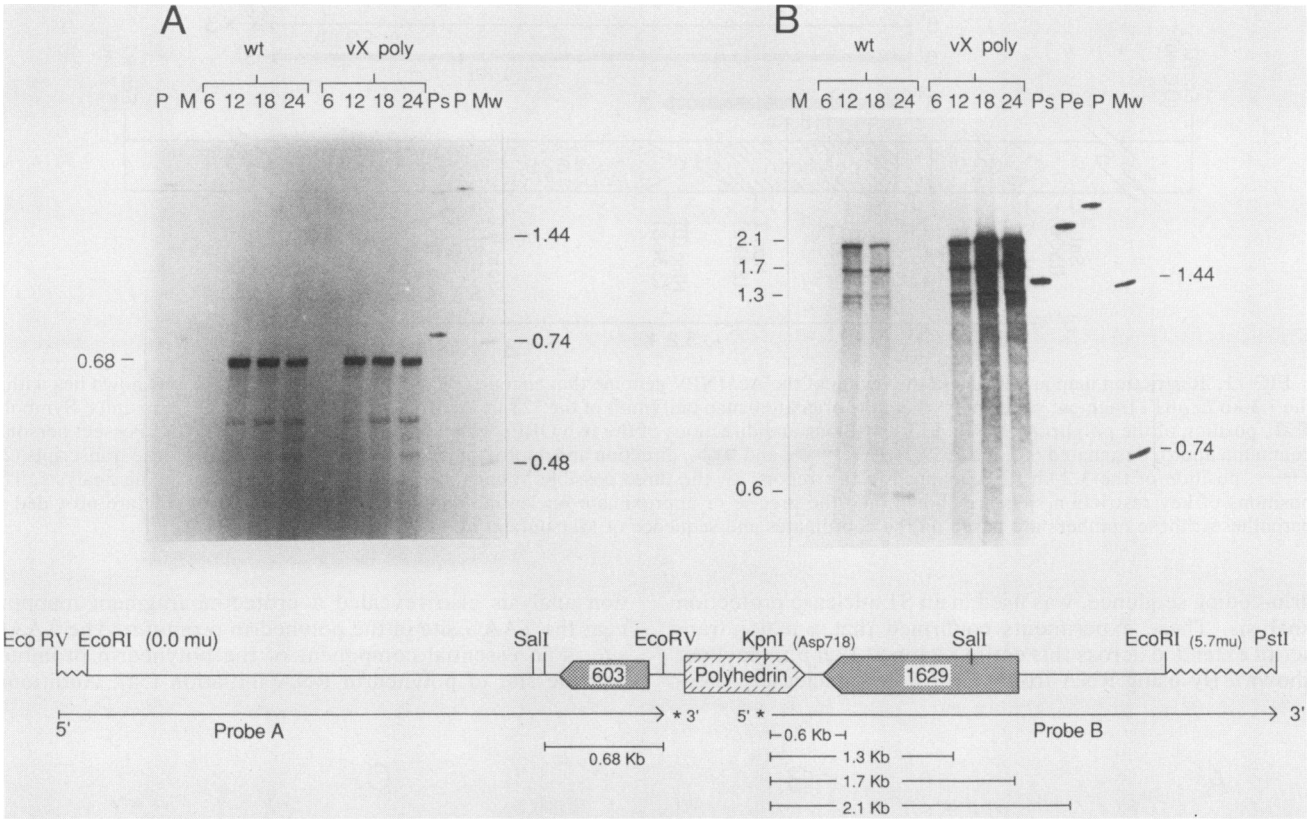

FIG. 3. S1 nuclease protection analysis of 3′ and 5′ ends of the 3.2-kb RNA. The diagram at the bottom of the figure shows the locations of the two probes (probes A and B) used in the panels above (panels A and B, respectively) to locate the 3′ and 5′ ends (respectively) of the 3.2-kb RNA running antisense to the polyhedrin ORF. Symbols, ⟶, direction of the probes; *, position of the radiolabel in the probe; ∿∿∿, non-Ac*M*NPV segments of the probe derived from the plasmid vector. Below the probe arrows are indicated the positions of the protected fragments observed in the respective panels above. The central sections of both panels A and B show S1 nuclease-protected fragments following probe hybridization to total RNA isolated from cells infected for 6, 12, 18, or 24 h (indicated above each lane) with Ac*M*NPV wt or vXpoly mutant virus or mock infected (lanes M). General protocols for RNA isolation and S1 nuclease analysis were described previously (5a). Lanes P, Untreated radiolabeled probe alone; lanes Ps, probes cut with *Sal*I to provide additional molecular weight and position markers; lane Pe, probe B cut with *Eco*RI; lanes Mw, molecular weight markers (*Taq*I digests of pUC19). Sizes are indicated in nucleotides to the right. Major protected fragments are noted on the left with their sizes in kilobases.

evidence indicates that the putative 5′ end which was mapped by the 18-h S1 nuclease protection analysis is an artifact generated by antisense RNA hybridization to polyhedrin sense RNA (unpublished data).

To determine the 3′ end of the 3.2-kb RNA, a nuclease protection probe was used that included Ac*M*NPV sequences extending from the *Eco*RI site at 0.0 map unit to the *Eco*RV site and was labeled at the 3′ end of the *Eco*RV site (Fig. 3). An approximately 680-nucleotide fragment was the major fragment protected by 12-, 18-, and 24-h p.i. RNAs (Fig. 3A). This placed the 3′ end of the RNA(s) just beyond the 3′ end of the 603 ORF. The RNA protecting this DNA fragment was maximal between 12 and 18 h p.i., reflecting a late RNA. The level did not appear to decrease as sharply between 18 and 24 h, as was observed for the 3.2-kb RNA in the Northern blots. This difference in the apparent regulation of this RNA may be because the S1 nuclease analysis included both 1- and 3.2-kb RNAs, whereas the Northern blots quantitated these two RNAs separately.

Collectively, the nuclease protection data show that the 3′ and 5′ ends of the 3.2-kb RNA map near the 3′ end of the 603 ORF and 5′ end of the 1629 ORF, respectively. The predicted length is in good agreement with the size of the 3.2-kb RNA observed on Northern blots.

In the course of other work, we constructed a mutant virus, vXpoly, which differed from wt Ac*M*NPV only at the polyhedrin transcriptional initiation (TAAG) site (unpublished data). The vXpoly mutant retains the polyhedrin ORF but carries the LSX linker mutation (5a, 8) at the TAAG site in the polyhedrin promoter, thus preventing polyhedrin transcription (5a).

Surprisingly, the regulation of the 3.2-kb RNA differed in vXpoly- and wt-infected cells (Fig. 2A to C). The 3.2-kb RNA initiated at a similar time p.i. but persisted longer in vXpoly-infected cells than in wt virus-infected cells. In the case of the 1629 ORF-specific probe (Fig. 2C), the 2-kb RNA also displayed the same difference in regulation, persisting longer in the vXpoly mutant-infected cells. Higher levels of these two RNAs at 24 h p.i. were also observed by using S1 nuclease probes (Fig. 3B). The minor 0.6-kb protected fragment, reflecting a very late RNA, was not observed in vXpoly-infected cells, suggesting that polyhedrin transcription positively influences the levels of this fragment.

Thus, in addition to blocking polyhedrin transcription, the vXpoly mutation affected the regulation of RNAs which originated over 2.5 kb downstream. The effects were most dramatic on the 2- and 3.2-kb RNAs, which are probably coordinately regulated. High levels of these two RNAs were

maintained between 18 and 24 h p.i. in mutant- but not wt-infected cells.

The difference in 3.2-kb RNA regulation in the presence and absence of polyhedrin gene sense transcription may be due to (i) the disruption of promoter complexes downstream of the polyhedrin gene by polyhedrin-initiated RNA polymerases traversing this region beginning at 18 h p.i., (ii) an increased rate of RNA turnover between 18 and 24 h p.i. resulting from double-stranded RNA formation with the polyhedrin mRNAs, or (iii) the down regulation of the 1629 ORF promoter by the polyhedrin protein acting as a repressor (polyhedrin was not present or was present in very low amounts in vXpoly-infected cells because of a lack of transcription). We favor the first two hypotheses currently. Polyhedrin is not known to play a regulatory role; viruses lacking the polyhedrin-coding region do not exhibit substantially altered gene regulation (5a, 6, 7). The possibility that the turn-on of the polyhedrin promoter results in promoter competition and lower rates of 3.2-kb RNA transcription has also been considered but seems unlikely unless the 3.2-kb RNA is inherently unstable (unstable in the absence of antisense RNA). Other characterized late Ac*M*NPV RNAs do not show the rapid decline in levels between 18 and 24 h p.i. that is exhibited by the 2- and 3.2-kb RNAs crossing the 1629 ORF. The unique regulation observed for the 2- and 3.2-kb RNAs is more likely due to either the disruption of transcriptional complexes in the 1629 ORF region by polyhedrin promoter-initiated transcriptional complexes or enhanced RNA turnover due to antisense RNA destabilization. The importance of this down regulation of the 3.2-kb RNA by polyhedrin transcription to the Ac*M*NPV infection process and to the use of the polyhedrin gene region for foreign gene expression vector work remains to be determined. Knowledge of the function of the 1629 ORF in Ac*M*NPV infection will be valuable in this regard.

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