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Functional analysis of *Spodoptera frugiperda* nucleopolyhedrovirus late expression factors in Sf9 cells

Marcelo F. Berretta · M. Gabriela López · Oscar Taboga · Alicia Sciocco-Cap · Víctor Romanowski

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Abstract We used transient expression assays to assess the function of the baculovirus *Spodoptera frugiperda* M nucleopolyhedrovirus (SfMNPV) homologs of *Autographa californica* MNPV (AcMNPV) factors involved in late gene expression (*lefs*), in the Sf9 insect cell-line, which is permissive for both viruses. It is well-established that nineteen AcMNPV *lefs* support optimal levels of activity from a late promoter-reporter gene cassette in this assay. A subgroup of SfMNPV *lefs* predicted to function in transcription-specific events substituted the corresponding AcMNPV *lefs* very efficiently. When all SfMNPV *lefs* were assayed, including replication *lefs*, activity was low, but addition of two AcMNPV *lefs* not encoded in SfMNPV genome, resulted in augmented reporter activity. SfMNPV IE-1 was able to activate an early promoter *cis*-linked to an *hr*-derived element from SfMNPV but not from AcMNPV. However, the level of early promoter activation with SfMNPV IE-1 was lower compared to AcMNPV IE-1.

Keywords Late expression factors · AcMNPV · SfMNPV

M. F. Berretta (✉) · A. Sciocco-Cap
Instituto de Microbiología y Zoología Agrícola (IMYZA), INTA Castelar, Castelar, Argentina
e-mail: mberretta@cnia.inta.gov.ar

M. G. López · O. Taboga
Instituto de Biotecnología (IB), INTA Castelar, Castelar, Argentina

V. Romanowski
Instituto de Biotecnología y Biología Molecular (IBBM, CONICET-UNLP), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

Introduction

Baculoviruses are large DNA viruses pathogenic to insects. At the late stage of infection, they produce distinct proteinaceous structures known as occlusion bodies (OBs) that embed the virions responsible for the horizontal transmission of the virus between susceptible larval hosts. According to the shape and size of OBs, baculoviruses were traditionally classified as nucleopolyhedroviruses (NPV) or granuloviruses (GV). A recent revision of the taxonomy of the family determined four groups at the genus level: *Alphabaculovirus* (lepidopteran NPV), *Betabaculovirus* (lepidopteran GV), *Gammabaculovirus* (hymenopteran NPV), and *Deltabaculovirus* (dipteran NPV) [1]. With around 60 baculovirus genomes sequenced to date, there is a critical volume of information to outline baculovirus diversity. Almost 900 different orthologous genes have been identified; in contrast, only 31 genes, referred to as core genes, are present in all genomes [2]. About one-third of core genes are genes related to DNA replication or processing and expression of late and very late genes. Most of our knowledge on baculovirus gene expression comes from studies on the type species *Autographa californica* M nucleopolyhedrovirus (AcMNPV), and a few other phylogenetically related species within group I of the alphabaculoviruses, as defined by Zanotto et al. [3]. Species belonging to group II of the alphabaculoviruses have more heterogeneous gene content and have been less studied with regard to molecular events of the viral infection cycle.

Baculoviruses express their genes in three temporally regulated phases: early, late, and very late. Transcription of early genes depends on the cellular RNA polymerase II. Expression of immediate-early (*ie-*) genes proceeds independently of viral factors, while expression of delayed early genes requires transactivation by other viral factors. IE-1 is a major transactivator of early gene expression in

AcMNPV although at least two other *ie-* genes also activate early promoters [4]. IE-1-mediated transactivation of early promoters increases when IE-1 binds to enhancers known as homologous regions (*hrs*) [5]. *Hrs* also serve as origins of DNA replication (*ori*) in assays of virus-induced plasmid replication [6, 7]. Baculovirus late and very late genes are transcribed from the conserved promoter motif TAAG [8] by an RNA polymerase encoded by the virus [9, 10]. They are the only DNA viruses which replicate in the nuclei of cells that encode their own DNA-directed RNA polymerase. Transcription of late genes requires viral DNA replication; therefore, genes necessary for late expression, collectively known as late expression factors (*lefs*), include not only genes encoding the RNA polymerase subunits and some other factors that are not well characterized but also those encoding the replication machinery [11]. AcMNPV *lefs* were discovered using different approaches including marker rescue experiments with temperature-sensitive mutants, and transient expression assays conducted in the *Spodoptera frugiperda*-derived Sf21 cell-line (reviewed in [11]). In the latter, a plasmid containing a reporter gene driven by a late promoter, and an *hr* sequence that functions as *ori*, was cotransfected into cells along with AcMNPV genomic fragments that were progressively shortened to eventually identify individual genes required for optimal reporter gene activity [12]. Nineteen AcMNPV *lefs* were defined in this manner [13] (Table 1), while nine of these genes were identified as replication factors in plasmid replication assays [14, 15]. Some *lefs* are implicated in determining host specificity in cell culture [16, 17]. The knock-out of several *lefs* has provided complementary information about their actual requirement in the context of the virus replication cycle [18].

Spodoptera frugiperda M nucleopolyhedrovirus (SfMNPV) is a member of group II alphabaculoviruses that has potential to be developed as biocontrol agent against the fall armyworm, *S. frugiperda*, an agricultural pest of economic importance in the Americas [19, 20]. SfMNPV is a host-specific virus, and in cell culture it infects productively the Sf21 cell-line [21–23] and its clonal derivative Sf9 [24]. Early studies of pulse-labeled proteins of SfMNPV-infected Sf21 cells, showed a program of protein synthesis throughout the infection cycle similar to that observed in this cell-line infected with AcMNPV [23, 25]. SfMNPV has been studied for many years although it was not until recently that the complete sequence of SfMNPV genomes became available [26–28]. Of the 19 *lefs* identified in AcMNPV, only 16 homologs were found in SfMNPV genome (Table 1). Being smaller in number, genes predicted as *lefs* may have functionally evolved in this species to fulfill late gene expression. Alternatively, other genes may affect this process in SfMNPV, either directly or indirectly. Likewise, in AcMNPV additional genes may be considered as *lefs* [11]. For baculovirus species belonging to group II alphabaculoviruses there are few studies aimed to characterize *lefs* involved in replication [29, 30], but there is a lack of experimental evidence with regard to the functionality of the complete set of genes homologous to AcMNPV *lefs* in a permissive cellular environment. In this study, we assessed the ability of SfMNPV *lefs* to support late gene expression in transient assays, in Sf9 cells. For this purpose, we adapted the system originally developed to study AcMNPV *lefs*, and found that only the subgroup of SfMNPV *lefs* corresponding to *lefs* considered involved in transcription-specific activities, substituted for their AcMNPV counterparts very efficiently, while SfMNPV replication *lefs* exhibited low-level functionality. Since *ie-1* is included in the group of replication *lefs*, we further investigated its functionality in an early gene expression assay. Results presented here are discussed in terms of the utility of our system for screening SfMNPV genome for genes potentially influencing gene expression in this species and possibly in other group II alphabaculoviruses.

Materials and methods

Cells and viruses

Spodoptera frugiperda Sf9 cells obtained from American Type Culture Collection (ATCC) were cultured at 27 °C as a monolayer in Sf900 II insect medium (Invitrogen) supplemented with 1 % fetal bovine serum (Internegocios, Argentina) and 1 % antibiotic–antimycotic solution (GIBCO). The SfMNPV stock used in this study, SfMNPV-AR, was originally obtained from a single *S. frugiperda* larva and was characterized as a single isolate [31].

Plasmid constructions

Plasmids utilized to assess late gene expression contained luciferase as reporter gene under control of late promoter of AcMNPV *vp39* capsid gene *cis*-linked to a fragment of the specified *hr* sequence. In reporter plasmid pAc*hr5*CAPluc, the *hr* fragment (AC *hr*) corresponds to AcMNPV *hr5* [32], and in pSf*hr1*CAPluc, the *hr* (Sf *hr*) corresponds to a fragment of SfMNPV-AR *hr1* that encompasses five palindromic repeats. pSf*hr1*CAPluc was constructed in three steps. First, a 616-bp fragment of SfMNPV-AR *hr1* was amplified with primers Fwd 5′-TTTTCATTAACTAACCACCATCA-3′ and Rev 5′-CCCGGGGATTGCAAAGATTAACTGTAACAAAC-3′ (*Xma*I site underlined); the PCR product was cloned in vector pCR®4-TOPO (Invitrogen), then removed by digestion with *Not*I and *Xma*I and gel-purified. In parallel, a reporter plasmid containing CAT under AcMNPV *vp39*

Table 1 Comparison of AcMNPV and SfMNPV *lefs*

ORF name	ORF number		Amino acid residues		Amino acid identity (%)	AcMNPV LEF function
	AcMNPV	SfMNPV[a]	AcMNPV	SfMNPV[a]		
lef-1	14	18	266	239	40	DNA primase
lef-2	6	16	210	213	45	Primase accessory protein
lef-3	67	90	385	388	28	ssDNA binding protein
lef-4	90	76	464	459	47	Capping enzyme, RNA polymerase complex
lef-5	99	68	265	277	54	Transcription factor
lef-6	28	128	173	155	32	Transcription factor
lef-7	125	21	226	351	Not sig	Possible ssDNA binding protein
lef-8	50	113	876	881	61	RNA polymerase complex
lef-9	62	97	490[b]	498	65	RNA polymerase complex
lef-10	53a	106	78	75	48	Transcription factor
lef-11	37	120	112	133	37	Replication/transcription factor
lef-12	41	–	181	–	–	Transcription factor
ie-1	147	136	582	681	31	Transactivator, *hr*-binding protein
ie-2	151	–	408	–	–	Transactivator, cell-cycle control
dnapol	65	92	984	1,040	45	DNA polymerase
p143	95	72	1,221	1,228	41	DNA helicase
39K	36	121	275	309	33	Transcription factor
p47	40	116	401	399	54	RNA polymerase complex
p35	135	–	299	–	–	Apoptosis inhibitor

ssDNA single-stranded DNA

[a] Values correspond to GenBank accession HM595733

[b] Value corresponds to the protein product translated from the second ATG of the ORF

promoter, pCAPCATΔHNΔSE [32], was digested with the same enzymes to separate Ac *hr*, and the resulting 4,740 bp backbone was ligated to Sf *hr*. Finally, this construct was digested with *Sac*I and *Bgl*II, and the 1,187 bp fragment containing Sf *hr cis*-linked to AcMNPV *vp39* promoter was purified and ligated to the backbone of pAc*hr5*CAPluc previously treated with the same enzymes to remove the tandem Ac *hr-vp39* promoter.

The construction of the AcMNPV *lef* library in plasmid pHSEpiHisVI$^+$ has been described [13]. In this vector, each *lef* was cloned under *Drosophila* heat shock protein (hsp) 70 promoter and fused to HA and His tags at the N-terminus. To clone the sixteen SfMNPV *lefs* individually in the same vector, we amplified by PCR each *lef* ORF from the second codon to the stop codon with specific forward and reverse primers listed in Table 2, and using SfMNPV-AR DNA as template. The PCR products were digested with *Bgl*II and *Not*I sites (except for *ie-1* and *p47*, which were digested with *Bam*HI and *Not*I) and cloned in the respective sites of pHSEpiHisVI$^+$. All constructs were verified by nucleotide sequencing. Plasmid Sf*ie0*H-X consists in an *Xba*I-*Hin*dIII SfMNPV-AR genomic fragment of approximately 7,250 bp (see Fig. 1c) cloned in the respective sites of plasmid pcDNAII (Invitrogen), extending from 228 bp upstream of *exon-0* ORF to 757 bp downstream of stop codon of *ie-1* ORF. Sf*ie-1*S-X is a subclone of Sf*ie0*H-X that was obtained by first digesting the latter with *Sac*I and *Hin*dIII to remove *exon-0* ORF; then, the linearized vector was blunt-ended and religated. The resulting clone retained *ie-1* including 515 bp upstream of the ORF and downstream sequence as in Sf*ie0*H-X.

Reporter plasmid pBAS35K-Luc/28*mer*-up+/PA has been described [33], and was used in this study to assess early gene expression. In this plasmid luciferase is driven by the TATA box-containing basal promoter of AcMNPV *p35* gene (nucleotides −30 to +12, where position +1 corresponds to the transcriptional start site) with a 28-*mer* imperfect palindromic repeat from AcMNPV *cis*-linked upstream to the basal promoter. The reporter plasmid pBAS35K-Luc/46*mer* contains a 46-*mer* imperfect palindrome corresponding to the third repeat of SfMNPV-AR *hr1* that matches exactly the homologous repeats from two sequenced SfMNPV isolates (GenBank accession numbers EF035042 [27] and HM595733 [28]). This plasmid was constructed by first digesting pBAS35K-Luc/28*mer*-up+/PA with *Bgl*II and *Mlu*I to remove the 28-*mer* sequence between these restriction sites and then by ligation of the backbone to annealed oligonucleotides 5′-GATCTTCAAA

Table 2 Oligonucleotides used for amplification of SfMNPV *lefs*

Oligonucleotide[a]	Sequence[b]
Sf***lef1***N-*Bgl*II	GAAGATCTGCATCATTCTTATCCTGCATTCA
Sf***lef1***C-*Not*I	GAATGCGGCCGCTTAAGATTTGGTAGTAGTTAGTAAATACGA
Sf***lef2***N-*Bgl*II	GAAGATCTGCGGCGGAACAGTTACTGTCGT
Sf***lef2***C-*Not*I	GAATGCGGCCGCTCAATAGTTACAAATAGGATTAGTTCCT
Sf***lef3***N-*Bgl*II	GCAGATCTTCTTTGTCTACGGAAATGCCTAC
Sf***lef3***C-*Not*I	GAATGCGGCCGCTTAAAAACCTTCAAAGTTATCAGT
Sf***lef4***N-*Bgl*II	GAAGATCTGTTGTCGAAAACGAAATTTCTTA
Sf***lef4***C-*Not*I	GAATGCGGCCGCTTAATTAGGCACCAAACGATCT
Sf***lef5***N-*Bgl*II	GCAGATCTTCTAAAGCTAACGCATGCCAAGT
Sf***lef5***C-*Not*I	GAATGCGGCCGCTTAGTTAACCGTGACGATGCGGT
Sf***lef6***N-*Bgl*II	GCAGATCTTATGTGTTTTATATTAACGGTACT
Sf***lef6***C-*Not*I	GAATGCGGCCGCTTATTTTTTATCATAAAGTTTATCA
Sf***lef7***N-*Bgl*II	GCAGATCTTGTGTTCGTGTAGTATATAAACGGT
Sf***lef7***C-*Not*I	GAATGCGGCCGCTTAATAATCATCAAAACTTCCCA
Sf***lef8***N-*Bgl*II	GAAGATCTACGGACGTGATTGTCGATTTTAA
Sf***lef8***C-*Not*I	GAATGCGGCCGCTTATCTCATAATTGTATTATTACAGT
Sf***lef9***N-*Bgl*II	GAAGATCTATCGACATCATGTCGTCGGCGA
Sf***lef9***C-*Not*I	GAATGCGGCCGCTCAATCCAAAAACATGTCTAACA
Sf***lef10***N-*Bgl*II	GTAGATCTTCTTCAGTGTCCATCGCGGAT
Sf***lef10***C-*Not*I	GAATGCGGCCGCTCATAGTGTTTGGTCACTTTGCA
Sf***lef11***N-*Bgl*II	GAAGATCTGATAAACAATCGTCGAGGCAGCA
Sf***lef11***C-*Not*I	GAATGCGGCCGCTCAGTTAAAGCGTTCGTTGTGGT
Sf***ie1***N-*Bam*HI	CGGGATCCCACACTCTCAACGACAACGCCAA
Sf***ie1***C-*Not*I	GAATGCGGCCGCCTAACAGTTGTCGTTGTCCTGT
Sf***dnapol***N-*Bgl*II	GAAGATCTGTGGCGTTTTCGTTGCTCGA
Sf***dnapol***C-*Not*I	GAATGCGGCCGCTCAACAATCCCTTTCGCATTTGA
Sf***p143***N-*Bgl*II	GAAGATCTGCGACTGCGGAGATAAGTGTTGA
Sf***p143***C-*Not*I	GAATGCGGCCGCCTAACATATAAATTCAGGTTGATT
Sf***p47***N-*Bam*HI	CGGGATCCGGTTTTGCGCGTTTTTACGAGA
Sf***p47***C-*Not*I	GAATGCGGCCGCTCAATATAATGTTACAAGTCTTTGA
Sf***39K***N-*Bgl*II	GAAGATCTAACACCACAACGAACGCTTTAACT
Sf***39K***C-*Not*I	GAATGCGGCCGCTTAACTATATGACATTTGTGTGT

[a] Oligonucleotide name includes the corresponding *lef* name italicized and in boldtype. N and C denote forward and reverse oligonucleotide, respectively

[b] Restriction endonuclease recognition sites are underlined

CTTTGCTTTCCGCGAAACACTTTACCGAAAGCAAAGATCGAA-3′ and 5′-CGCGTTCGATCTTTGCTTTCGGTAAAGTGTTTCGCGGAAAGCAAAGTTTGAA-3′.

DNA transfections

Sf9 cells (3.2×10^5) were seeded in each well of 12-well plates and were transfected with the indicated amounts of DNA. Total DNA to be transfected per well was standardized by adding lambda DNA when needed, and mixed with 3 µl of Cellfectin® (Invitrogen) reagent in serum free Sf900 II media. Cells were incubated at 27 °C for 4 h and after that, the transfection mixture was replaced by fresh media containing 1 % fetal bovine serum. The transfected cells were incubated at 27 °C for the period required for each assay.

Transient expression assays

For late gene expression assays, cells seeded in 12-well plates were transfected with 0.8 µg of reporter plasmids and 0.2 µg of each plasmid expressing the *lefs* that were included in each experiment. Cells were incubated at 27 °C for 48 h post-transfection. Luciferase activity was measured with Luciferase Assay System (Promega). In brief, cells were lysed for 5 min in 400 µl of Reporter Lysis Buffer (Promega) at room temperature and then transferred into ice-chilled 1.5 ml tubes, vortexed and

centrifuged 30 s to pellet cell debris. Lysate (20 µl) and Luciferase Assay Reagent (80 µl, Promega) were mixed in 96-well opaque plates and luminescence was measured with a Veritas™ Microplate Luminometer (Turner Biosystems), according to the manufacturer's instructions.

For early gene expression assays, cells seeded in each of 12-well plates were transfected with 1.0 µg of reporter plasmids carrying the *p35* promoter elements and 0.5 µg of plasmid expressing AcMNPV *ie-1* or equimolar amounts of plasmids expressing SfMNPV *ie-1*. Cells were incubated at 27 °C for 24 h post-transfection and luciferase activity was measured as described for late gene expression assays.

When viral DNA was used in the assays, 0.5 µg of the specified DNA was included in the transfection mix. All transfections were repeated in triplicate at different times, and the average of these experiments is presented.

Immunoblotting

To compare the levels of expression between AcMNPV *ie-1* and SfMNPV *ie-1* cloned in vector pHSEpiHisVI$^+$, the same cell extracts that were measured for luciferase activity were treated with 2× cracking buffer (0.13 M Tris pH 6.8, 4 % sodium dodecyl sulfate [SDS], 20 % glycerol, 0.14 % bromphenol blue, 2 % 2-mercaptoethanol) and boiled for 2 min. Equal volumes of lysates were resolved by 12 % SDS–polyacrylamide gel electrophoresis (PAGE). The resolved proteins were transferred to a nitrocellulose membrane (Whatman) and immunolabeled with 1:500 dilution of anti-HA monoclonal antibody (Invitrogen) and 1:1,000 dilution of goat anti-mouse IgG-HRP (horseradish peroxidase) (SIGMA) and detected by chemiluminescence with ECL western blot reactives (Pierce, Thermo Scientific).

Results and discussion

Transactivation of a late promoter by SfMNPV *lefs* and complementation by AcMNPV *lefs*

To construct the collection of clones we designated as SfMNPV *lef* library, we cloned individually all SfMNPV *lefs* listed in Table 1, fused to HA and His tags, under *Drosophila* hsp 70 promoter, in the expression vector pHSEpiHisVI$^+$ [13] (see "Materials and methods" section for details). This vector was originally developed to express constitutively AcMNPV *lefs* in Sf21 cells. The AcMNPV *lef* library (previously designated pHSEpiHis *lef* library [13]), including all AcMNPV *lefs* listed in Table 1, has been found to support expression of a reporter gene under control of the late promoter of AcMNPV *vp39* capsid gene, in transient assays, in Sf21 cells. In the present study, we adapted this system to assess the functionality of SfMNPV *lefs* to support late gene expression, in Sf9 cells. To this end, the complete SfMNPV *lef* library was cotransfected into cells along with a reporter plasmid containing AcMNPV *vp39* promoter-driven luciferase, *cis*-linked to an *hr* fragment. This promoter was responsive to activation by both AcMNPV and SfMNPV, in the context of Sf9 cells transfected with viral DNA (Fig. 1b). This is consistent with the high level of sequence identity of *vp39* promoter regions of both viruses, including two conserved TAAG motifs proximal to the ATG translational start codon, probably due to overlap with the 5′ end of *lef-4* gene (which is encoded in the complementary DNA strand). We tested *hr* sequences from each virus in the reporter plasmid since, in transient assays, *hrs* have been shown to have species-specific effects on both virally-induced replication of plasmids [29, 34] and transactivation of a late gene promoter [35].

When the AcMNPV *lef* library was cotransfected with reporter plasmid pAc*hr5*CAPluc, containing an AcMNPV-derived *hr* (Ac *hr*), maximum levels of reporter activity were obtained (Fig. 1a, column 3, positive control). The ability of the AcMNPV *lef* library to transactivate the late promoter was specific with regard to the *hr* sequence; when an SfMNPV-derived *hr* (Sf *hr*) was present, as in reporter plasmid pSf*hr1*CAPluc, reporter activity dropped to about 4 % of the positive control (Fig. 1a, column 4). Comparatively, the SfMNPV *lef* library cotransfected with pSf*hr1*CAPluc resulted in 2 % the activity of the positive control (Fig. 1a, column 6). Even though this value is very low, it is above background levels obtained with the SfMNPV *lef* library cotransfected with pAc*hr5*CAPluc (Fig. 1a, column 5). It is worth to mention that Sf *hr* spans five palindromes of the *hr1* region of SfMNPV, while Ac *hr* contains only two palindromes from AcMNPV *hr5* (see "Materials and methods" section). In relation to the role of *hrs* as enhancers of early genes, the strength of the transactivation correlates primarily with the number of palindromes in the *hr* [36]. We do not know whether the *hr* length could affect activation of the late promoter noticeably, in the assay. In any case, the lower activity obtained with the SfMNPV *lef* library compared to that obtained with the AcMNPV *lef* library does not correlate with the length of the *hr* in the reporter plasmids.

In these experiments, AcMNPV *p35* (see Table 1) was cotransfected with the SfMNPV *lef* library. There is evidence that the functional contribution of *p35* to the AcMNPV *lef* library is to prevent apoptosis of transfected cells [37, 38]. Therefore, since SfMNPV lacks a *p35* homolog, the AcMNPV gene was included in both libraries to avoid artifacts that could arise due to cell viability. In parallel experiments conducted without *p35* in the transfection mix, the activity of the reporter obtained with the

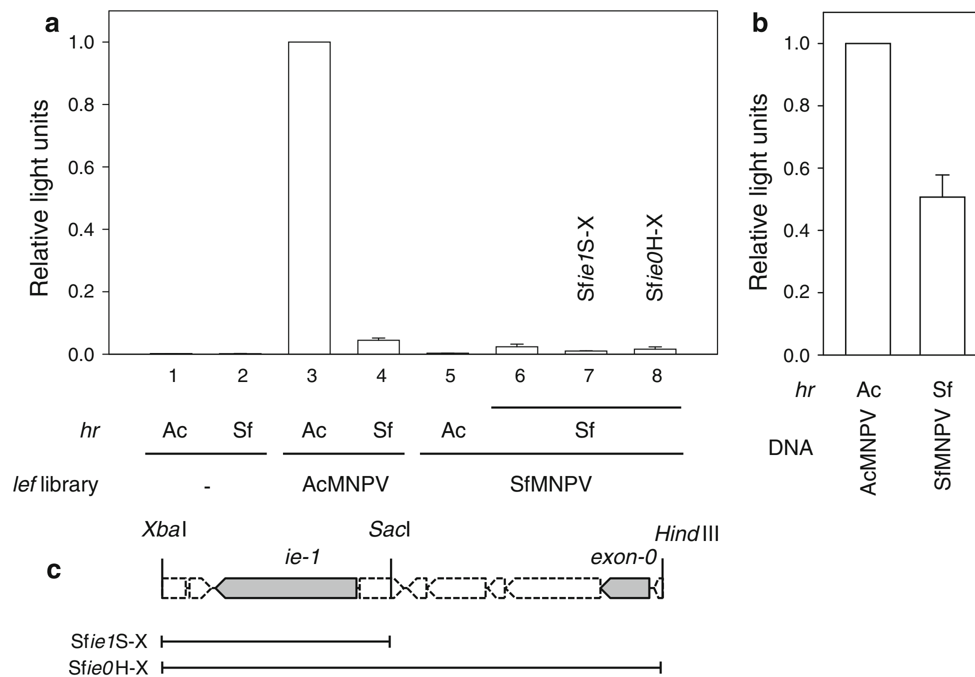

Fig. 1 Late gene expression in the presence of SfMNPV *lefs* and SfMNPV DNA in Sf9 cells. **a** Sf9 cells were cotransfected with AcMNPV or SfMNPV *lef* libraries (0.2 μg of each *lef*) and reporter plasmids (0.8 μg) pAc*hr5*CAPluc, containing a fragment of AcMNPV *hr5* (Ac *hr*), or pSf*hr1*CAPluc, containing a fragment of SfMNPV *hr1* (Sf *hr*). SfMNPV *ie-1* was replaced in SfMNPV *lef* library by genomic clones Sf*ie1*S-X (column 7) or Sf*ie0*H-X (column 8). Cells were harvested 48 h post-transfection and lysates were analyzed for luciferase activity. Luciferase activity from cells cotransfected with AcMNPV *lef* library and reporter plasmid pAc*hr5*CAPluc (column 3) was set to 1.0. **b** Transactivation of the indicated reporter plasmids by cotransfection with AcMNPV or SfMNPV viral DNA (0.5 μg). **c** Schematic diagram of the SfMNPV genomic region encompassing *ie-1* and *exon-0* ORFs (*shaded arrows*). Restriction fragments cloned in plasmids Sf*ie1*S-X and Sf*ie0*H-X are indicated

SfMNPV *lef* library decreased by as much as 40 % (data not shown).

Results presented here indicate that SfMNPV *lefs*, as a group, have very low late promoter transactivation capacity in the assay. The difference observed in reporter activation by AcMNPV and SfMNPV *lef* libraries is in contrast to more similar activities observed when genomic DNA of each virus was cotransfected with the indicated reporter plasmid (Fig. 1b). One possible explanation is that SfMNPV factors other than those represented in the *lef* library are required to stimulate late gene expression.

In a previous study, it was shown that IE-1 of *Lymantria dispar* MNPV (LdMNPV), other member of the group II alphabaculoviruses, did not support transient plasmid replication when expressed in Ld652Y cells along with the remaining LdMNPV replication factors. In contrast, IE-0, a variant of IE-1, was required to obtain replication of the reporter plasmid [39]. In species in which they were studied experimentally, IE-1 differs from IE-0 in that the latter has an extended N-terminus, since it is translated from a spliced transcript that encodes portion of *exon-0* ORF in frame with *ie-1* ORF [39–41]. Although these products are functionally similar they may exhibit differential properties in different contexts [39, 42, 43]. Since replication of the reporter plasmid is necessary for activation of the late promoter in the assay, we assessed the complementing capacity of an SfMNPV genomic fragment from which *ie-0* could be expressed to explore possible requirement of an SfMNPV IE-0 homolog for promoter activation. Plasmid Sf*ie0*H-X (Fig. 1c) was constructed to contain a 7.2 kbp *Hin*dIII-*Xba*I genomic fragment that spans from sequences upstream *exon-0* to downstream *ie-1* ORFs. This fragment is homologous to regions that function as templates for synthesis of the spliced *ie-0* mRNA in other baculoviruses. In addition we tested plasmid Sf*ie1*S-X, a subclone of Sf*ie0*H-X that represents a *Sac*I-*Xba*I genomic fragment containing only *ie-1* along with a few hundred base pair flanking sequences. SfMNPV *ie-1* in the *lef* library was replaced by each *ie-1*-containing genomic clone and the resulting libraries were cotransfected with reporter plasmid pSf*hr1*CAPluc. Reporter activity did not increase in the presence of Sf*ie0*H-X in the library (Fig. 1a, column 8) compared to that with the original *lef* library. A similar result was obtained with Sf*ie1*S-X (Fig. 1a, column 7). Since we did not confirm expression of IE-0 from Sf*ie0*H-X, these results preclude us from making definitive conclusions about IE-0 requirement in the assay.

Besides *p35*, two other AcMNPV *lefs* are not present in the genome of SfMNPV: *ie-2* and *lef-12* (see Table 1). *ie-2* is considered stimulatory for DNA replication in a cell-line-specific manner [17], while *lef-12* is thought to have specific influence in transcription [44]. According to current knowledge, the remaining AcMNPV *lefs* are also predicted to function mainly through their involvement in either replication- or transcription-specific events. In the experiments presented below, we defined the replication group of *lefs* including: *lef-1*, *lef-2*, *lef-3*, *lef-7*, *lef-11*, *ie-1*, *dnapol* and *p143*; and the transcription group containing: *lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *p47*, and *39K*. To investigate a possible link between low reporter gene activity and lack of functionality of either subgroup of SfMNPV *lefs*, we replaced each subgroup of genes in the SfMNPV *lef* library with the corresponding subgroup of the AcMNPV *lef* library, and viceversa. AcMNPV *ie-2* and *lef-12* were assessed separately to evaluate their contribution in different contexts. Results shown in Fig. 2 indicate that there are two independent principal components of the assorted libraries that maximize reporter activity: AcMNPV replication *lefs* (Fig. 2, compare columns 1–4 to columns 5–8), and AcMNPV-specific *lefs ie-2* and *lef-12* (Fig. 2, compare odd columns to even columns). This implies that SfMNPV transcription *lefs* substituted for the corresponding AcMNPV *lefs* activity very efficiently, from 76 to 95 %, depending of the library context (Fig. 2, compare column 3–1, and column 8–6, respectively). Controls with only one subgroup of *lefs* of either library were omitted for simplicity, since they resulted in background levels of reporter gene activity.

In addition to the experiments presented here, we tested the combination of AcMNPV replication and SfMNPV transcription *lefs*, with or without *ie-2*/*lef-12* (as indicated for columns 3 and 4 of Fig. 2) in cotransfections with the reporter plasmid pSf*hr1*CAPluc. Reporter activity resulted in 1.5 and 0.5 % the activity of positive control, respectively (data not shown). These results suggest that the specificity of the interaction with the *hr*, in the context of the late promoter activation, is primarily restricted to the replication *lefs*.

AcMNPV *ie-2* and *lef-12* stressed the ability of SfMNPV *lefs* to support late promoter activity. They augmented reporter activity from 3- to 6-fold depending whether the library contained either AcMNPV or SfMNPV replication *lefs*, respectively (Fig. 2, e.g. compare column 3–4, and column 5–6, respectively). *ie-2* is missing in the whole lineage of group II alphabaculoviruses while *lef-12* is present in some members of this group. It is not known whether SfMNPV encodes functional homologs of these genes.

In order to explore further the limitation posed by SfMNPV replication *lefs* to attain substantial activation of the late promoter, there are same questions that need to be addressed. For example, substitutions of individual *lefs* may lead to the identification of a specific *lef* being the limiting factor. Also, the dosage effect should be analyzed for *lefs* exhibiting low protein expression levels. In its current state, we proposed our system as a useful tool to systematically interrogate SfMNPV genome for genes able to mimic the stimulatory effect of *ie-2 and lef-12* on the SfMNPV *lef* library. Besides their fundamental role in the

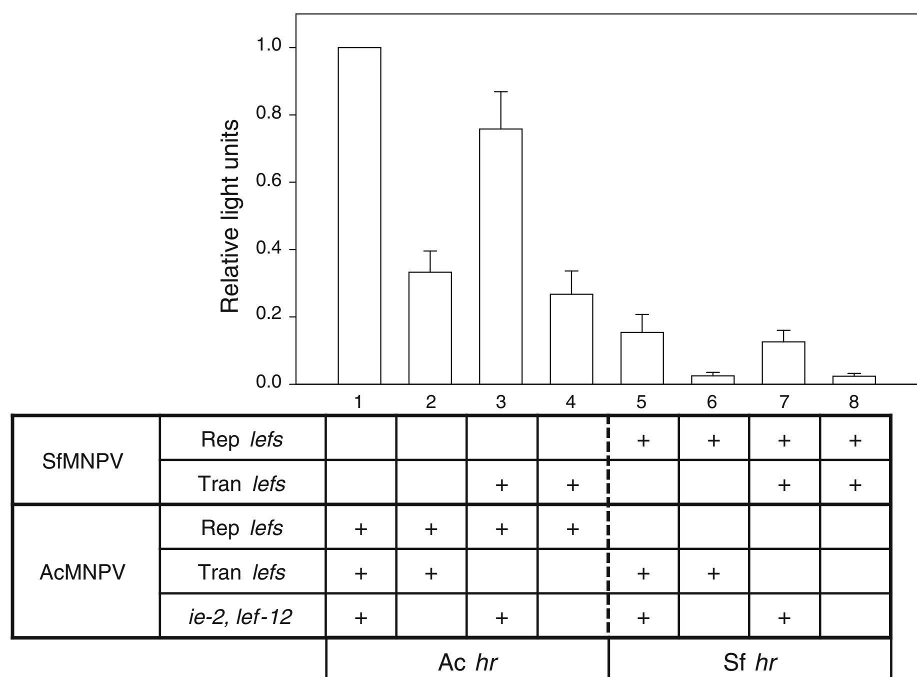

Fig. 2 Late gene expression using combinations of subgroups of SfMNPV and AcMNPV *lefs* involved in transcription or replication. Sf9 cells were cotransfected with *lef* libraries containing different combinations of AcMNPV and SfMNPV *lefs* as indicated (+) and reporter plasmids pAc*hr5*CAPluc (Ac *hr*) or pSf*hr1*CAPluc (Sf *hr*). Replication (Rep) *lefs* include *lef-1* to *-3*, *lef-7*, *lef-11*, *ie-1*, *dnapol*, and *p143*. Transcription (Tran) *lefs* include *lef-4* to *-6*, *lef-8* to *-10*, *39K* and *p47*. Cells were harvested 48 h post-transfection and lysates were analyzed for luciferase activity. Luciferase activity from cells cotransfected with the entire AcMNPV *lef* library and reporter plasmid pAc*hr5*CAPluc (column 1) was set to 1.0

infection cycle, some *lefs* have been involved in determining host range in AcMNPV. To this regard, AcMNPV is considered a generalist virus. Therefore, the identification of possible new *lefs* in a specialist virus like SfMNPV, which is also a representative of a different group of alphabaculoviruses compared to AcMNPV, may contribute to new findings in relation to this trait.

Transactivation of an early promoter *cis*-linked to an AcMNPV- or SfMNPV-derived *hr* element by SfMNPV *ie-1*-containing clones

IE-1 is involved in multiple processes during the virus infection cycle and may exhibit differential properties, including possible differential roles for IE-1 and IE-0 in each process. IE-1 functions have not been studied extensively among members of group II of alphabaculoviruses. As mentioned above, IE-1 of LdMNPV was not active for plasmid replication in Ld652Y cells. Moreover, only IE-0 supported expression from an early promoter in both Ld652Y and Sf9 cell-lines [39]. In contrast, IE-1 of *Spodoptera exigua* MNPV (SeMNPV) was functional in a transient early gene expression assay, in Sf21 cells [35]. Since low reporter activity supported by SfMNPV *lefs* in late expression assays was mainly associated with the group of replication *lefs*, in which *ie-1* is included, we investigated the ability of SfMNPV IE-1 to stimulate early gene expression in a model system. To drive the reporter gene we used the basal portion of the well characterized AcMNPV *p35* early promoter, encompassing the TATA box motif and the transcription initiation site, *cis*-linked to an *hr* element. In previous studies this promoter motifs, linked to an AcMNPV *hr*-derived single palindromic repeat (Ac 28-*mer*), were found to be the minimal elements

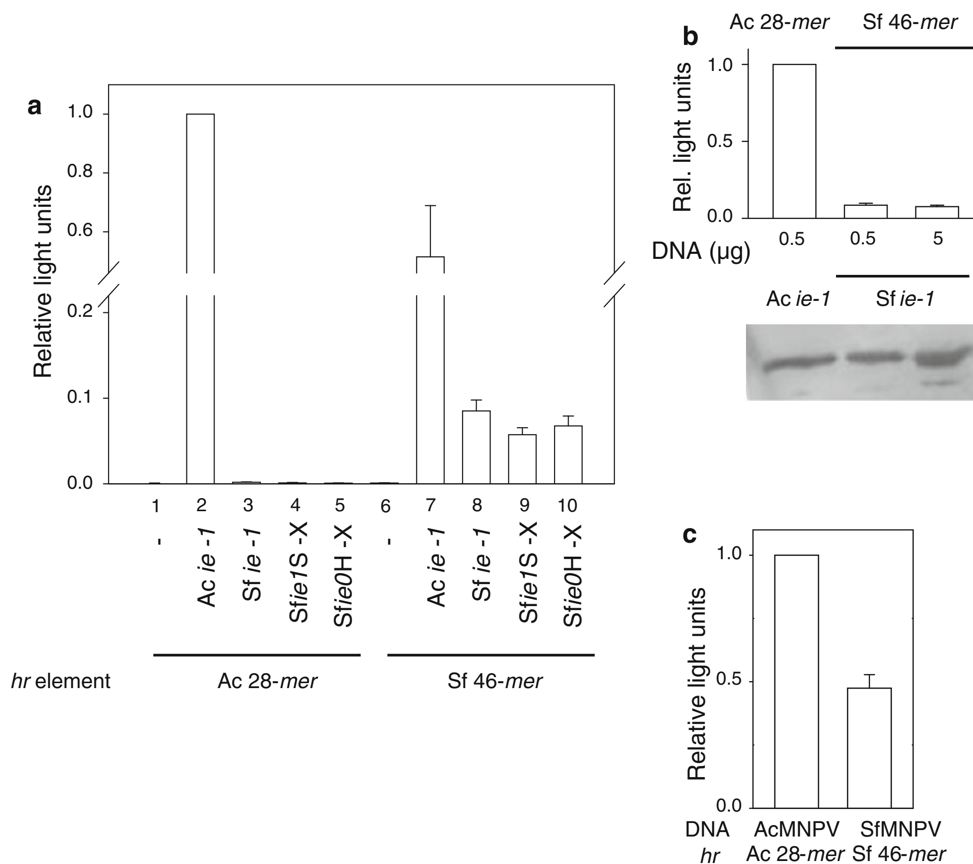

Fig. 3 Transactivation of the early *p35* basal promoter by SfMNPV IE-1. **a** Sf9 cells were cotransfected with 0.5 µg of AcMNPV *ie-1* (Ac *ie-1*), SfMNPV *ie-1* (Sf *ie-1*) or equimolar amounts of plasmids Sf*ie1*S-X or Sf*ie0*H-X, and reporter plasmids (1.0 µg) pBAS35 K-Luc/28*mer*-up+/PA (Ac 28-*mer*) or pBAS35K-Luc/46*mer* (Sf 46-*mer*). Reporter plasmids contained the luciferase gene under control of the *p35* basal promoter, which encompasses the TATA element and RNA start site, *cis*-linked to a palindromic 28-*mer* from AcMNPV *hr5* or a palindromic 46-*mer* from SfMNPV *hr1*, respectively. Ac *ie-1* and Sf *ie-1* express the corresponding IE-1 products fused to HA and His tags, under control of hsp 70 promoter. Cells were harvested 24 h post-transfection, and lysates were analyzed for luciferase activity. Luciferase activity from cells cotransfected with Ac *ie-1* and reporter Ac 28-*mer* (column 2) was set to 1.0. **b** Sf *ie-1* was tested as in panel "**a**" but using the indicated amount of DNA in transfections. Expression was tested using an aliquot of a lysate also used to measure luciferase activity and is shown below each column. **c** Transactivation of the indicated reporter plasmid by cotransfection with AcMNPV or SfMNPV viral DNA (0.5 µg)

required for *hr*-enhanced activation mediated by AcMNPV IE-1 [33]. This construct was also responsive to activation by heterologous IE-1 when Ac 28-*mer* was replaced by an *hr* element from the same viral species as the IE-1 protein [35]. Therefore, in the present study, we used reporter plasmids containing luciferase under control of the *p35* basal promoter with either Ac 28-*mer* or an SfMNPV *hr*-derived palindromic repeat (Sf 46-*mer*) [27] inserted upstream of the promoter. Cotransfection of SfMNPV viral DNA and reporter plasmid having Sf 46-*mer* into Sf9 cells stimulated expression from reporter gene though the efficiency was about 50 % of that obtained by cotransfection of AcMNPV DNA and Ac 28-*mer*-containing plasmid (Fig. 3c).

To test promoter activation by *ie-1*, we cotransfected Sf9 cells with reporter plasmids and *ie-1*-expressing clones from the AcMNPV and SfMNPV *lef* libraries, or the SfMNPV genomic fragments tested in late expression assays (see above). AcMNPV *ie-1* supported the highest level of reporter activity in the presence of the reporter plasmid with Ac 28-*mer* (Fig. 3a, column 2, positive control). In the presence of Sf 46-*mer* in the reporter plasmid, AcMNPV *ie-1* resulted in 51 % reporter activity compared to that of positive control (Fig. 3a, column 7). SfMNPV *ie-1* consistently enhanced expression of the reporter gene in plasmid with Sf 46-*mer* (Fig. 3a, column 8); nevertheless, the level of expression represented at most about 10 % of the positive control. There were no significant differences in activity from this reporter when cotransfected with plasmid Sf*ie0*H-X, which is predicted to express IE-0 (Fig. 3a, column 10). Therefore, we could not propose a preferential role to either IE- product in the activation of the promoter, in our assay. SfMNPV *ie-1* clones failed to stimulate reporter activity from plasmid containing Ac 28-*mer* (Fig. 3a, columns 3–5). This may reflect more strict constraints for the specificity of interaction between SfMNPV IE-1 and *hr* elements in comparison to AcMNPV IE-1. The levels of expression from HA-tagged AcMNPV *ie-1* and SfMNPV *ie-1* were similar as revealed by immunoblotting (Fig. 3b); therefore, lower transcriptional activation observed with SfMNPV *ie-1* was not a consequence of lower amount of protein. In fact, transfection with higher concentration of SfMNPV *ie-1* DNA did not produce an increase in reporter activity.

In comparison, reporter activity obtained with SfMNPV DNA relative to that obtained with AcMNPV DNA was higher than the relative activity of the reporters obtained with the corresponding *ie-1* clones. This suggests that in the context of the viral infection SfMNPV IE-1 may be better expressed or may be the target of modifications that are needed to upregulate the activity of the protein product. This question could be addressed by testing clones of a genomic library of the virus to identify genes influencing positively IE-1 activity as a transactivator of the early promoter in the assay. Alternatively, SfMNPV IE-1 may have a more strict dependence to a native *hr* sequence than AcMNPV IE-1, in order to mediate activation of an early promoter. Native *hrs* have sequences between palindromes that bind cellular transcription factors which cooperate with IE-1-mediated transactivation of early promoters. In support of this, linkage to native *hr* sequences activates early promoters to some extent in insect cells, even in the absence of IE-1 [45].

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