Novel Sp Family-like Transcription Factors Are Present in Adult Insect Cells and Are Involved in Transcription from the Polyhedrin Gene Initiator Promoter*

Aruna Ramachandran‡§¶, Anjali Jain§¶‖, Puneeta Arora, Murali Dharan Bashyam‡§,
Udayan Chatterjee‡ ‡‡, Sudip Ghosh§, Veena K. Parnaik**, and Seyed E. Hasnain‡§ §§**

*From the ‡Eukaryotic Gene Expression Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India, the §Laboratory of Molecular and Cellular Biology, Centre for DNA Fingerprinting and Diagnostics, ECIL Road, Nacharam, Hyderabad 500076, India, and the **Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India*

We earlier documented the involvement of a cellular factor, polyhedrin (polh) promoter-binding protein, in transcription from the *Autographa californica* nuclear polyhedrosis virus polh gene promoter. Sequences upstream of the polh promoter were found to influence polh promoter-driven transcription. Analysis of one such region, which could partially compensate for the mutated polh promoter and also activate transcription from the wild-type promoter, revealed a sequence (AcSp) containing a CACCC motif and a loose GC box resembling the binding motifs of the transcription factor Sp1. AcSp and the consensus Sp1 sequence (cSp) specifically bound factor(s) in HeLa and *Spodoptera frugiperda* (*Sf*9) insect cell nuclear extracts to generate identical binding patterns, indicating the similar nature of the factor(s) interacting with these sequences. The AcSp and cSp oligonucleotides enhanced *in vivo* expression of a polh promoter-driven luciferase gene. *In vivo* mopping of these factor(s) significantly reduced transcription from the polh promoter. Recombinant viruses carrying deletions in the upstream AcSp sequence confirmed the requirement of these factor(s) in polh promoter-driven transcription in the viral context. We demonstrate for the first time DNA-protein interactions involving novel members of the Sp family of proteins in adult insect cells and their involvement in transcription from the polh promoter.

The temporally regulated and hyperactivated polyhedrin (polh)[1] gene promoter of the *Autographa californica* nuclear

¶ These authors contributed equally to this work.

‖ Present address: Howard Hughes Medical Inst., UCLA School of Medicine, Los Angeles, CA 90024-1662.

‡‡ Present address: Dept. of Molecular Biology, Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037.

§§ To whom correspondence should be addressed. Tel.: 91-40-7155604; Fax: 91-40-7155610; E-mail: ehtesham@www.cdfd.org.in.

[1] The abbreviations used are: polh, polyhedrin; *Ac*NPV, *Autographa californica* nuclear polyhedrosis virus; bp, base pair(s); PPBP, polh promoter-binding protein; EMSA, electrophoretic mobility shift assay; kb, kilobase pair(s); PCR, polymerase chain reaction; ORF, open reading frame; OP, 1,10-*o*-phenanthroline.

polyhedrosis virus (*Ac*NPV) belongs to the class of initiator promoters (1). The primary determinant of polh promoter function is the 8-bp sequence TAAGTATT, which encompasses the transcriptional start point and is absolutely necessary for transcription initiation (2, 3). The minimal polh promoter with all of the essential *cis*-acting elements is defined as an 18-nucleotide region encompassing the initiator sequence (4). A hexanucleotide sequence motif AATAAA, present within the minimal promoter immediately 5′ to the octanucleotide motif TAAGTATT, has been demonstrated, along with the octamotif, to be the target for binding of an unusual 30-kDa cellular transcription factor, the polh promoter-binding protein (PPBP) (5). PPBP is a phosphoprotein that binds with very high affinity and specificity and plays an important role in transcription from this promoter (6), probably acting as an initiator binding protein involved in the recruitment of the transcription machinery (7). PPBP can also specifically bind to the coding strand of the promoter (8) with increased affinity, compared with the duplex promoter, thus maintaining the promoter at the initiation point in a "melted" state allowing for increased rounds of transcription.

Several *Ac*NPV genes have been identified on the basis of their *trans*-regulatory activity in transient expression assays (9). Many of these, including a late gene that encodes a putative protein with motifs conserved in RNA polymerases (10), have been demonstrated to be activators of late and very late viral gene expression by virtue of their effects on early events in the viral infection cascade (9, 11). A virus-encoded four-component RNA polymerase has been recently isolated from baculovirus-infected cells (12). However, reconstitution of polymerase activity has not been demonstrated in a cell-free system using the individually purified proteins. A viral factor, VLF-1, has been shown to transactivate the polh and p10 promoters, supposedly by interacting with the 3′-untranslated ("burst") sequences of these two very late promoters (13). Thus far, except for the host factor PPBP, no other protein that binds to the very late polh promoter and is directly involved in transcription has been identified (6).

Sp1 was first discovered in mammalian (HeLa) cells as an activator of transcription from the SV40 early promoter (14). Sp1 is part of a larger Sp superfamily along with other members, sharing structural and (sometimes) functional homology (reviewed in Refs. 15 and 16). Sp family members bind to GC and GT box sequence motifs present in a variety of cellular and viral promoters (17–19) via three highly conserved C_2H_2 zinc finger motifs present in the C-terminal region of the protein (19). The N-terminal glutamine-rich domains of Sp1, which are

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more divergent among the family members, are essential for transcriptional activation function (20, 21). Interaction between the glutamine-rich activation domains of Sp1 and TATA-binding protein-associated cofactors plays an essential role in the activation of TATA-less promoters (22). The polyhedrin promoter also being a TATA-less initiator promoter prompted us to investigate the requirement of Sp-like factors in transcription from this promoter.

In this report, we present the analyses of DNA sequences upstream of the polh gene promoter and of the factors binding to identified elements within these regions *vis-à-vis* their effect on transcription from the polh promoter in *Spodoptera frugiperda* (*Sf*9) cells. Two regions that influence transcription from the polh promoter were identified. One of these regions has Sp1-binding motifs, can bind to probable Sp family-like factors from *Sf*9 cells, and can complement the lack of initiator promoter-based transcription. Sequestering *in vivo* of the Sp family-like factor(s), which is distinct from PPBP, significantly reduced transcription from the polh promoter, confirming its involvement in polh promoter-driven transcription. Recombinant viruses with deletions in the polh promoter upstream sequences underscored the importance of these factor(s) in the viral context as well and indicated that the enhancement of reporter gene expression was not merely due to an enhancement of viral replication.

EXPERIMENTAL PROCEDURES

Cells and Virus—*Sf*9 cells were cultured in TNMFH (Life Technologies, Inc.) medium supplemented with 10% fetal calf serum as described (23). Wild-type *Ac*NPV strain C6 was used for cell infection in transient expression assays.

Recombinant viruses were constructed by first cloning the polh-driven luciferase gene with intact upstream sequences (pBacMAluc), an 800-bp upstream deletion (pBacΔluc), or a deletion substituted by the AcSp sequence (pBacAcSpluc) into the pBacPAK8 baculovirus transfer vector (CLONTECH Laboratories Inc., Palo Alto, CA). Each plasmid was then transfected into *Sf*9 cells along with BacPAK6 viral DNA (*Bsu*36I digest), and recombinant viruses were constructed, purified, and titrated as per the manufacturer's instructions (CLONTECH). Viral infection was carried out with a multiplicity of infection of 10 of each virus. To ascertain that equal amounts of viral DNA from the different recombinants had entered the insect cells, equal amounts of the reaction mixtures were fixed by dot-blot onto a nylon membrane after the luciferase assay, followed by probing with the *luc* cDNA and densitometric scanning.

Electrophoretic Mobility Shift Assays (EMSAs)—Crude nuclear protein extracts from *Sf*9 cells were prepared as described (24). The consensus Sp1-binding oligonucleotide (cSp, TATTCGATCGGGGCGGGGCGAGCC) was obtained commercially from Promega Inc., and the AcSp oligonucleotide (TAATGGGGTGTATAGTACCGCTGCGCATAGTC) was chemically synthesized (Rama Biotechnology, Hyderabad, India). Complementary synthetic oligonucleotides were annealed and labeled with T4 polynucleotide kinase using $[\gamma\text{-}^{32}P]ATP$. The binding reaction consisted of ~ 5 μg of nuclear extract and 1 ng of labeled annealed oligonucleotide ($\sim 10^4$ cpm). For EMSAs, the crude nuclear extract was incubated in the presence of the binding buffer (10 mM Tris-HCl, pH 7.5, 0.7 mM Hepes-KOH, pH 7.7, 30 mM KCl, 1 mM EDTA, 50 mM EGTA, 0.8 mM $MgCl_2$, 7 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.05% Nonidet P-40, 10% glycerol) and 1 μg of poly(dI-dC) for 10 min at 25 °C followed by incubation of ^{32}P-labeled oligonucleotide for 20 min. The DNA-protein complexes were resolved at 4 °C in an 8% (75:1 acrylamide/bisacrylamide) nondenaturing polyacrylamide gel in 0.5× TBE buffer (0.045 M Tris borate, 0.001 M EDTA) at 200 V for 3 h. The gel was dried, covered with plastic wrap, and exposed overnight to Hyperfilm MP (Amersham Pharmacia Biotech) at −70 °C. For competition analyses, a 400-fold excess of the appropriate unlabeled, double-stranded DNA was added along with the labeled DNA in the binding reaction. Radiolabeled oligonucleotide used as a probe is marked by an *asterisk* in all EMSA figures. For EMSAs using the pure Sp1 protein (Promega Inc.), 1 footprinting unit of Sp1 (~ 25 ng of protein) was used in the binding reaction without the addition of poly(dI-dC). The remaining conditions were identical with all other EMSAs.

Plasmids and Constructs—All DNA manipulations were carried out as described (25). For the construction of pAJpolluc (Fig. 1) harboring

the wild-type polh promoter, the 92-bp *Eco*RV-*Bam*HI promoter fragment was obtained from the transfer vector pVL1393 (26) and cloned at the *Hin*cII-*Bam*HI site of plasmid pAJluc (a derivative of pUC18 carrying the 1892-bp *luc* gene (26) ligated at the *Bam*HI site), placing it upstream from the luciferase reporter gene. pAJmHluc (Fig. 2) carried a synthetic 65-mer polh promoter with the mutated hexanucleotide motif (CCGCCC instead of AATAAA) cloned at the *Hin*dIII-*Sal*I site of pAJluc driving the *luc* gene. pKNluc (Fig. 1) was constructed by cloning the *luc* gene at the *Bam*HI site downstream of the polh promoter within the transfer vector pVL1393. A 2.77-kb *Sal*I-*Hin*dIII fragment from pKNluc was ligated at the *Sal*I-*Hin*dIII sites of pUC18 to obtain the construct pKN603luc (Fig. 1).

pAcSp·pol·luc and pcSp·pol·luc vectors were constructed by cloning the AcSp oligonucleotide and the cSp oligonucleotide, respectively, at the *Pst*I-*Hin*dIII site in pAJpolluc and confirmed by dideoxy sequencing. pAR1 and pAR2 vectors used for the *in vivo* mapping experiments were constructed by cloning the AcSp and cSp oligonucleotides, respectively, at the *Pst*I-*Hin*dIII site in pUC19.

pBacMAluc was constructed by cloning the *luc* gene at the *Bam*HI site of the pBacPAK8 transfer vector. pAcSp·pol·luc and pAJpolluc were digested with *Pvu*II and *Sac*I to release the promoter-luciferase cassette with and without the AcSp oligonucleotide, respectively, and end-filled using the Klenow fragment of *Escherichia coli* DNA polymerase I (New England BioLabs, Beverly, MA). The AcSp-containing fragment was cloned into the *Mlu*I-*Xho*I sites of pBacPAK8 (after end-filling the digested vector fragment first), whereas the other *Pvu*II-*Sac*I fragment was cloned into the *Mlu*I-*Sma*I site of pBacPAK8. The clones so obtained were called pBacAcSpluc and pBacΔluc, respectively.

Site-directed Mutagenesis—For site-directed mutagenesis experiments, the 2.77-kb *Sal*I-*Hin*dIII fragment from pKNluc was cloned within the polylinker region of the 3.0-kb phagemid pBS$^+$ to generate the construct, pAJ$_{pBS}$603-luc (Fig. 2). The plasmid construct pAJ$_{pBS}$603-luc was transformed into competent TG1 cells and infected with the phage M13KO7, and single-stranded template DNA was isolated and used for site-directed mutagenesis using standard protocols (25). The quality of the template was checked by sequencing with a T7 primer. Five picomoles of a 24-mer oligonucleotide (CATCTCGCACCCCCCTAAGTATTT), spanning the region to be mutated and harboring the mutated version of the hexamotif (underlined), was phosphorylated with 5 mM ATP for 30 min at 37 °C. It was annealed to the single-stranded DNA template followed by extension and ligation reactions in the presence of Klenow and T4 DNA ligase enzymes. The reaction mixture was transformed into competent TG1 cells to generate pAJ$_{pBS}$603mH-luc (Fig. 2), and the mutant clones were identified by colony hybridization using the 24-mer oligonucleotide as a probe. The mutation was confirmed by dideoxy sequencing.

In Vivo Luciferase Expression Assays—The expression of luciferase in *Sf*9 cells transfected with the reporter plasmids was carried out as described (27). Light emission was monitored with a manual luminometer (model 1250; Bio-Orbit Oy, Turku, Finland) over an integration period of 10 s. All of the transfections were repeated, in duplicate, at least three times. To ascertain that equal amounts of plasmid DNA from the different constructs had entered the insect cells, equal amounts of the reaction mixture after the luciferase assay were fixed by dot-blot on a nylon membrane followed by probing with the *luc* cDNA and densitometric scanning. All transfections included appropriate negative controls, *viz.* mock-transfected *Sf*9 cells and cells subjected only to viral infection without plasmid transfection.

In vivo mapping (28) was monitored on a Lumicount microplate luminometer (Packard Instrument Company, Meriden, CT) according to the manufacturer's instructions. For this, 2 μg of the reporter plasmid was used (pAcSp·pol·luc or pcSp·pol·luc) with or without 18 μg of specific competitor (pAR1 or pAR2) or nonspecific competitor (pUC19).

UV Cross-linking—The binding reaction was carried out as described for the EMSAs, but after incubation with the labeled probes the tubes were exposed to short-wave UV light for half an hour using a hand-held UV monitor (model UVGL-58; UVP, Inc., San Gabriel, CA), and the DNA-protein complexes were resolved as described earlier (5).

DNase I Protection Assays—A 140-bp PCR product was generated using forward and reverse primers termed FPL and FPR, corresponding to the coding and noncoding strands of *Ac*NPV, respectively, containing the AcSp motif approximately in the center of the amplicon. The primer sequences are: FPL, TATGTATCTATCGTATAGAG, and FPR, ACACACTCCGAAGAACTACC. 5 ng of pKN603luc was used as the template for PCR. 200 ng of each primer was radiolabeled with T4 polynucleotide kinase (New England BioLabs) and $[\gamma\text{-}^{32}P]ATP$ and used separately in a PCR reaction along with an equal amount of unlabeled opposite primer to generate a radiolabeled coding or noncoding strand.

Sp-like Factors and Baculovirus Polyhedrin Transcription

Plasmid constructs

Luciferase activity
(relative light units)

Scale:
1 map unit = 1286.5 bp

pAJpolluc   37

ORF603

pKN603luc   90

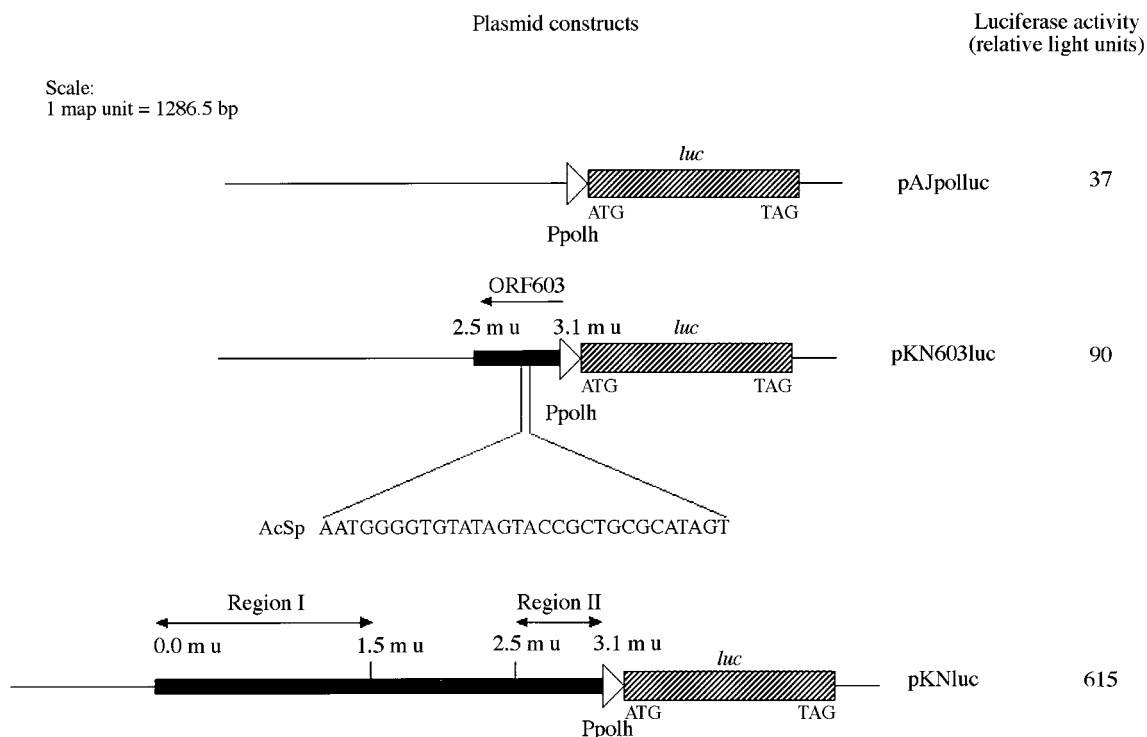AcSp   AATGGGGTGTATAGTACCGCTGCGCATAGT

pKNluc   615

FIG. 1. **Polyhedrin upstream sequences enhance reporter gene expression.** Schematic representation of the plasmid constructs pAJpolluc, pKN603luc, and pKNluc. pKNluc has ~4 kb upstream, and pKN603luc has ~766 bp upstream, whereas pAJpolluc has no sequences upstream from the polh promoter. The luciferase activity values of the constructs after being used in transient expression assays are indicated on the *right*.

The PCR products were gel-purified and concentrated to a final volume of about 100 μl. 2 μl was used to take Cerenkov counts in a scintillation counter. About 25,000 cpm was used per reaction. The DNase I reaction was carried out at ambient temperature as follows. The binding reaction was carried out using 4 footprinting units (~100 ng) of pure recombinant human Sp1 (Promega Inc.), as per the Sp1 EMSA conditions in a volume of 40 μl followed by the addition of 40 μl of a 5 mM CaCl$_2$, 10 mM MgCl$_2$ mix. 30 s later, 1.3 μl of 1:3 diluted RNase-free DNase I (Promega Inc.) was added and incubated for 2 min, followed by the addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS). The DNA was extracted twice with an equal volume of 1:1 phenol:chloroform, and glycogen was added to a final concentration of 20 mg/ml; the mixture was precipitated with ethanol at −70 °C for 3 h, washed twice with 70% ethanol, and resuspended in 5 μl of sequencing loading buffer containing 90% formamide. Control DNase I reactions were carried out under identical conditions except that no protein was added. A + G ladders were generated for both strands using formic acid and piperidine according to the method of Maxam and Gilbert (29). Equal counts of all reactions were loaded onto an 8% denaturing acrylamide gel containing 1× TBE and 8 M urea and resolved at 75 watts for 2 h, after which the gel was fixed for 10 min in 10% acetic acid and 20% methanol, covered with plastic film, dried under vacuum, and subjected to autoradiography at −70 °C.

RESULTS

A 4-kb Sequence Upstream from the Polyhedrin Promoter Modulates Polyhedrin Basal Transcription—A series of progressive Bal31 exonuclease deletion constructs of pNEluc (30), encompassing the complete 4-kb sequence upstream from the polh gene promoter within the *Ac*NPV *Eco*RI-I fragment, were generated and used in luciferase-based transient expression assays (31). This deletion analysis identified two regions, region I and region II, spanning map units 0–1.5 and 2.5–3.12, respectively, on the *Eco*RI "I" fragment of the viral genome. Deletion of these sequences resulted in a drastic reduction of reporter gene expression in comparison with the original pNEluc plasmid construct (31). Having identified the approximate boundaries of upstream sequences influencing polh promoter activity, three clones with defined upstream sequences

were constructed and used in luciferase-based transient expression assays to evaluate the effect of such *cis*-acting sequences on the minimal promoter-driven expression (Fig. 1). pAJpolluc displayed basal luciferase expression, pKN603luc consistently showed 2–3-fold increased expression above pAJpolluc, and pKNluc displayed about a 20-fold increase in basal expression. The results complement our earlier observations that the 4-kb sequence upstream from the polh promoter does contain sequence elements that enhance basal polh expression.

Region II Can Compensate for the Absence of the Hexanucleotide Motif within the Polyhedrin Initiator Promoter—Having demonstrated the stimulatory role of the upstream 766-bp sequence on reporter gene expression from the polh basal promoter, we evaluated the importance of this sequence *vis-à-vis* the essential determinants of this promoter. We previously showed that the hexamotif (AATAAA) present within the initiator region of the polyhedrin promoter is critical for PPBP binding (5, 8) and subsequent expression from this promoter (6). To further extend this observation, the expression of luciferase in pAJmHluc was compared with pAJ$_{pBS}$603mH-luc. Luciferase expression from the construct pAJ$_{pBS}$603-luc (carrying an intact hexamotif and the ORF603 sequence) was used as a control. pAJ$_{pBS}$603mH-luc exhibited reduced *luc* expression compared with pAJ$_{pBS}$603-luc, but this activity was still significantly higher than pAJmHluc, which did *not* carry the ORF603 upstream sequence. In pAJmH−luc (where the hexamotif was mutated *without* the presence of any upstream sequences), luciferase activity above the background cut-off limit of 10 mV was not detected. These data demonstrate that the hexamotif is critical for promoter function; however, its absence can be compensated, albeit to a lesser extent, by sequence elements within the upstream region II containing ORF603.

An Sp1-like GC Box and CACCC Motif Is Present within ORF603—To arrive at the sequence determinants involved in
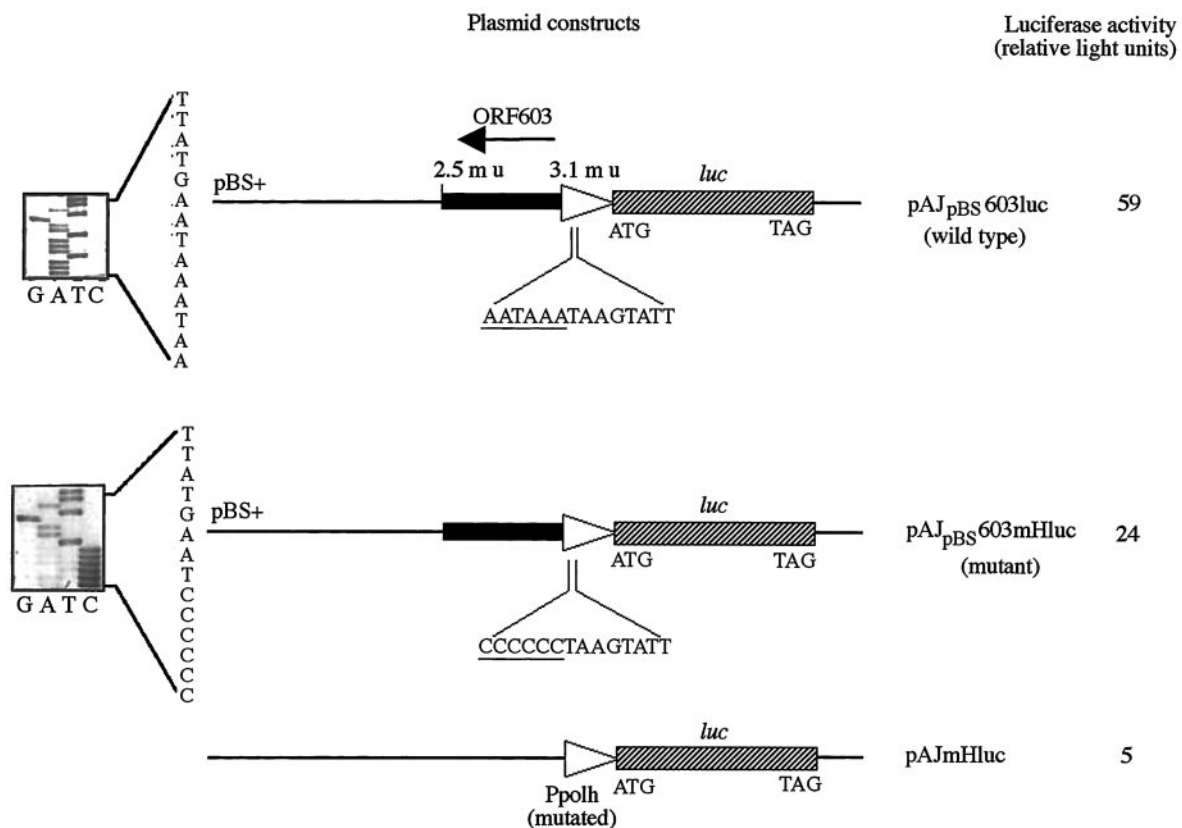
FIG. 2. **The upstream ORF603 region partially compensates for the mutated polyhedrin promoter.** Schematic representation of the plasmid constructs pAJpBS603-luc with the unmutated hexamotif AATAAA, pAJpBS603mH-luc carrying the mutated hexamotif CCCCCC in place of AATAAA, and pAJmHluc carrying the mutated promoter with no upstream sequences, along with their respective luciferase activity values.

rescuing basal transcription in the presence of the mutant polh initiator, the ORF603 sequence was scanned for binding motifs specific for known transcription factors. A region from −438 to −468, termed as AcSp, which was GC-rich in the otherwise AT-rich baculovirus genome, was identified within this sequence stretch (Fig. 1). Analysis of this sequence showed some similarity to the GC-rich sequences that bind the general transcription factor Sp1, which is known to play an important role in the initiator-mediated mechanisms of transcription (32–34). Comparison of the AcSp sequence with the Sp1-binding motif consensus (defined by comparing sequences from 36 sources) (35, 36), (G/T)(G/A)GGCG(G/T)(G/A)(G/A)(G/T), identified two putative Sp family binding motifs, TACCGCTGC, with about 70% homology with the consensus GC box, and a consensus CACCC motif, which is bound by some Sp family proteins. This analysis and the fact that the polh promoter is an initiator promoter pointed to the possibility of the involvement of an Sp1-mediated mechanism in polh promoter regulation.

An Sp-like Host Factor(s) Is Present in Insect Cell Nuclear Extracts—EMSAs were carried out to ascertain the presence of *trans*-acting factor(s) in *Sf*9 cells that could bind to the AcSp motif. The oligonucleotides corresponding to the cSp sequence and the AcSp sequence were used as probes in EMSAs with nuclear extracts prepared from uninfected and *Ac*NPV-infected *Sf*9 cells (24). It is evident that at least one complex of similar mobility is obtained with both uninfected (Fig. 3*a*, *lanes 3* and *4*) and infected (*lanes 5* and *6*) nuclear extracts using both probes. In addition, a faster mobility complex is also evident under these binding conditions with uninfected cell nuclear extract (*lanes 3* and *4*) with both of the probes. These results demonstrate the presence of a host factor(s) in the insect cell nuclear extracts that can bind to the Sp1-like sequence motif

present within ORF603 of region II (AcSp) as well as cSp.

The Consensus Sp1 Sequence Can Effectively Compete with the AcSp Sequence for Binding to Sp-like Factor(s) Present in Sf9 Cells—The Sp-like complex generated with uninfected *Sf*9 nuclear extract was subjected to cross-competition analyses in EMSAs using authentic Sp1-binding motifs. Fig. 3*b* shows AcSp binding to (uninfected) *Sf*9 nuclear extract in the absence of any cold competitor (*lane 2*) and in the presence of AcSp, cSp, and pUC18 cold competitors (*lanes 3*, *4*, and *5*, respectively). It is interesting to note that in certain instances the consensus Sp1 sequence can compete even better than the homologous competitor for the AcSp probe. This is understandable because the factor(s) present could well have a higher affinity for the Sp1 cognate sequence defined by cSp rather than the AcSp sequence. pUC18 did not compete for the binding, further pointing to the specificity of the complexes formed.

The AcSp Sequence Can Compete with the cSp Oligonucleotide—Having shown that the *Sf*9 Sp-like factor(s) binding to the AcSp sequence can be effectively competed out by the consensus Sp1 sequence, the reverse experiment was carried out using radiolabeled cSp oligonucleotide as a probe in EMSAs. It is apparent that the binding (Fig. 3*c*, *lane 3*) was abolished after homologous cold competition (*lane 4*), but when cold AcSp was used as a competitor (*lane 5*), the complex with reduced mobility was competed out rather inefficiently. This is further evident after comparison of the competition with the nonspecific competitor pUC18 (*lane 6*). Interestingly, the complex of higher mobility (lower shift) was better competed. *Lanes 1* and *3* show cSp mobility without and with the nuclear extract, respectively. *Lane 2* shows the binding of AcSp with the nuclear extract to compare the similar nature of the shifts obtained with both of the probes.
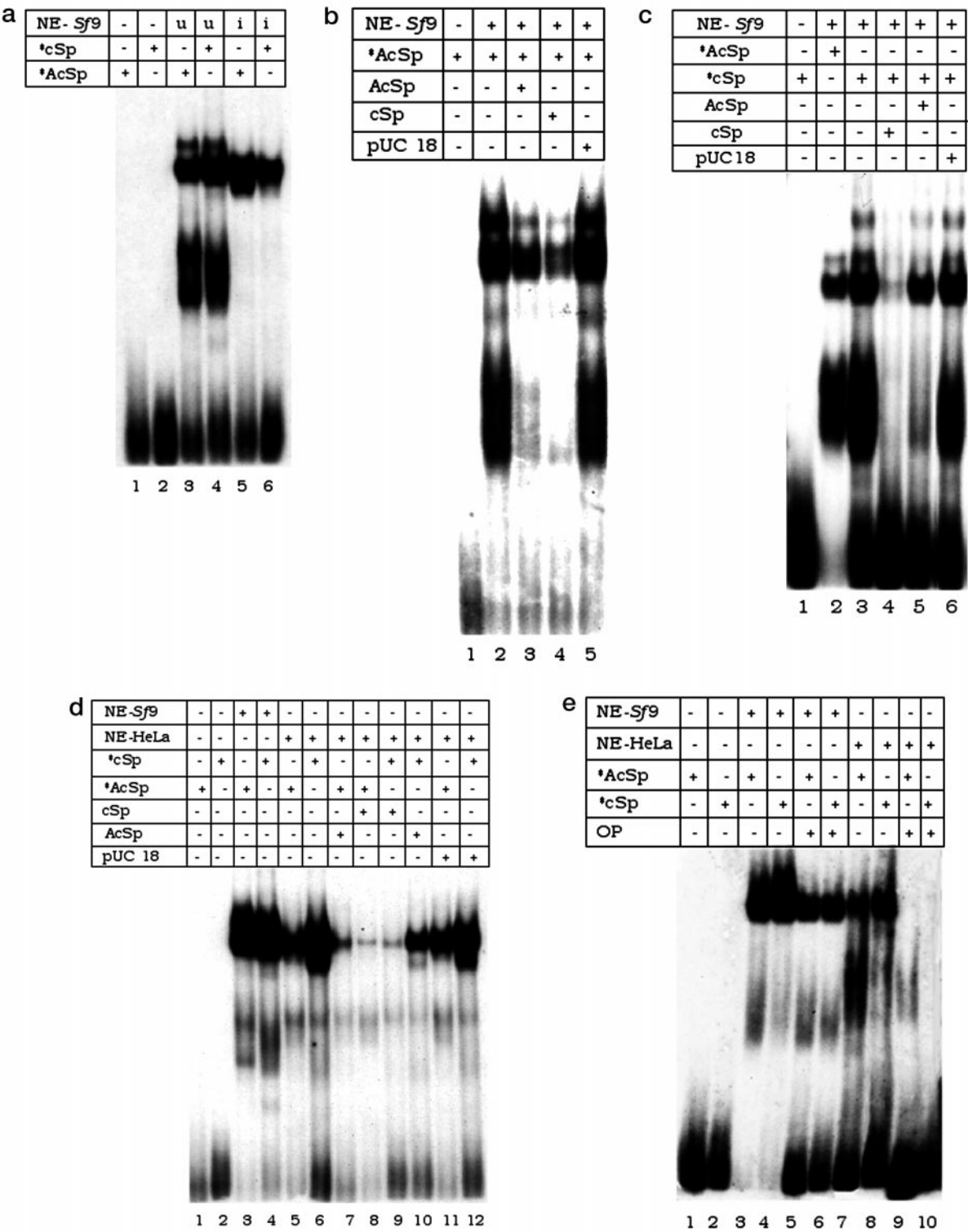
FIG. 3. *a*, an Sp family-like factor(s) is present in insect cell nuclear extracts. An EMSA using oligonucleotides carrying the cSp and AcSp sequences is shown. *Sf*9 nuclear extracts from uninfected (*u*) cells (*lanes 3* and *4*) or virus-infected (*i*) cells (*lanes 5* and *6*) were used in binding reactions. *b*, cSp can effectively compete with AcSp for binding to the Sp family-like insect factor(s). An EMSA with the AcSp sequence motif is shown. *Lane 1*, free probe without any protein extract; *lane 2*, binding in the presence of *Sf*9 nuclear extract; *lanes 3*, *4*, and *5*, binding in the presence of cold competitors AcSp, cSp, and pUC18 DNA, respectively. *c*, AcSp competes with cSp for binding to the *Sf*9 Sp family-like factor(s). An EMSA was carried out using cSp as a probe. *Lane 1*, free probe; *lanes 3–6*, binding in the presence of *Sf*9 nuclear extract alone (*lane 3*) or in the presence of unlabeled cSp (*lane 4*), AcSp (*lane 5*), and pUC18 DNA (*lane 6*). *Lane 2* shows binding with AcSp as a probe for comparing the nature of the complexes obtained with both probes. *d*, HeLa Sp family factor(s) bind to the AcSp sequence. An EMSA using HeLa (*lanes 5–12*) and *Sf*9 (*lanes 3* and *4*) nuclear extracts. AcSp probe was used in the absence of any extract (*lane 1*) or with *Sf*9 nuclear extract (*lane 3*) and HeLa cell nuclear extract (*lane 5*) is shown. *Lanes 2*, *4*, and *6* show the corresponding results using the cSp probe. *Lanes 7* and *8* show competition with homologous cold AcSp and heterologous cSp competitors, respectively. *Lanes 9* and *10* similarly show competition with homologous cold cSp and heterologous AcSp competitors, respectively. Competition using pUC18 is shown in *lanes 11* and *12*. *e*, the HeLa and *Sf*9 Sp family-like protein(s) require zinc for DNA binding. An EMSA performed with labeled AcSp and cSp probes is shown. *Lanes 1* and *2*, free AcSp and cSp probes, respectively. Binding of both probes with *Sf*9 and HeLa cell nuclear extract is shown in *lanes 3* and *4* and *lanes 7* and *8*, respectively. *Lanes 5* and *6* show binding in the presence of 20 mM OP using *Sf*9 nuclear extract, whereas *lanes 9* and *10* represent binding of the two probes in the presence of HeLa cell nuclear extract and 20 mM OP.
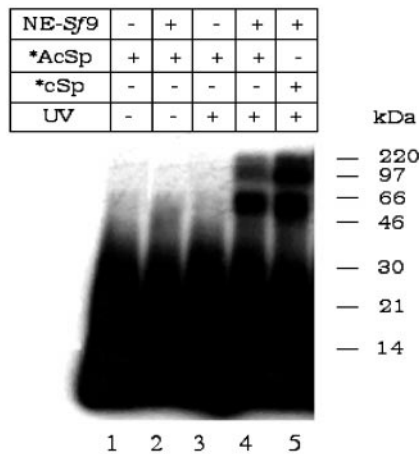
NE-Sf9	-	+	-	+	+
*AcSp	+	+	+	+	-
*cSp	-	-	-	-	+
UV	-	-	+	+	+

FIG. 4. **UV cross-linking with cSp and AcSp probes.** *Lanes 1* and *2* show AcSp probe without and with *Sf*9 nuclear extract, respectively, without UV exposure. *Lane 3* shows free AcSp probe after UV exposure. *Lanes 4* and *5* show proteins cross-linked to the AcSp and cSp probes, respectively, using *Sf*9 nuclear extract.

*B domain	+	+	+	+	+
NE-Sf9	-	+	+	+	+
B domain	-	-	+	-	-
AcSp	-	-	-	-	+
cSp	-	-	-	+	-

FIG. 5. **The *Sf*9 Sp-like factor(s) is distinct from PPBP.** EMSA was carried out with labeled polyhedrin promoter B domain (*lane 1*), *Sf*9 nuclear extract (*NE-Sf9*), and competitions with a 25-fold excess of unlabeled B domain (*lane 3*), cSp (*lane 4*), and AcSp (*lane 5*) oligonucleotides.

The Sp Family Factor(s) Present in HeLa Cell Nuclear Extract Also Binds to the AcSp and cSp Sequences—The data presented above established the presence of factor(s) present in *Sf*9 cells, which specifically bind to AcSp as well as to the Sp1 consensus sequence, indicating that the insect factor(s) behaved like the Sp family of proteins in terms of cognate sequence recognition and cross-cold competitions. It was therefore pertinent to investigate whether the *Sf*9 Sp family complex is the same as the well characterized Sp family of factors present in HeLa cells. Fig. 3*d* shows the binding of AcSp and cSp with *Sf*9 and HeLa nuclear extracts. *Lanes 3* and *5* show the binding of factor(s) present in *Sf*9 and HeLa extracts to radiolabeled AcSp, respectively. *Lanes 4* and *6* show the binding of similar factors present in *Sf*9 and HeLa extracts to the cSp probe. Interestingly, a similar complex is obtained using both probes and either of the extracts. The HeLa cell factor(s) binds to the AcSp oligonucleotide to generate a complex with reduced intensity as compared with that obtained with cSp (compare *lane 5* with *lane 6*), possibly because the AcSp sequence is not 100% identical to the Sp1 consensus. The fact that the HeLa extracts containing the Sp family of factors generate a complex similar to that of *Sf*9 extract with either of the probes directly points to the possibility of *Sf*9 cells also harboring Sp family-like transcription factors. Interestingly, the competition pattern seen with the HeLa extracts mirrors that seen with *Sf*9 extracts (shown earlier in Fig. 3, *b* and *c*) in that cSp can compete very efficiently with the AcSp probe, but AcSp competitor cannot compete as efficiently as cSp for binding to the cSp probe (compare *lane 8* with *lane 10*). Homologous cross-cold competitions expectedly abolished binding with both probes (*lanes 7* and *9*), whereas a nonspecific competitor, pUC18, did not affect binding with either probe (*lanes 11* and *12*). These results convincingly demonstrate that whereas *Sf*9 cell nuclear extracts harbor Sp family-like transcription factors, the corresponding factors from HeLa cells can also bind to the viral AcSp motif.

The Sp Family-like Proteins Both in HeLa and Sf9 Nuclear Extracts Require Zinc for Binding to the Cognate Nucleotide Sequence Motif—Binding of the mammalian Sp family proteins to DNA involves zinc fingers (19). We investigated the requirement of zinc by the *Sf*9 Sp family-like factor(s) with regard to its interaction with the cSp or AcSp motif. Inhibition of binding of these factors from the HeLa cell nuclear extract to cSp and AcSp in the presence of 1,10-*o*-phenanthroline (OP), a known chelator of zinc, was used as a reference. In an EMSA where OP
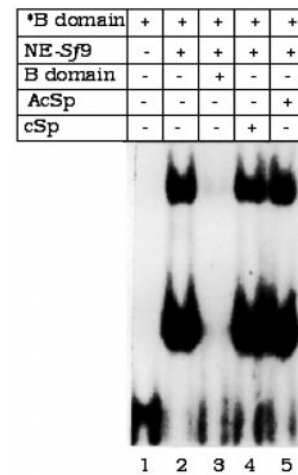
is added to the reaction mixture, binding of the *Sf*9 Sp-like factor(s) is seen to be affected (Fig. 3*e*). The binding of both AcSp and cSp probes with *Sf*9 nuclear extract (*lanes 3* and *4*) was significantly reduced by the addition of 20 mM OP (*lanes 5* and *6*), and the binding with the HeLa nuclear extract (*lanes 7* and *8*) was completely abolished (*lanes 9* and *10*) under similar conditions. The inhibition of binding exhibited by HeLa cell Sp family factors in the absence of zinc is consistent with our knowledge of the Sp family proteins; the same effect seen for the *Sf*9-derived Sp-like factor(s) strengthens the Sp family-like characteristics of the *Sf*9 factor(s).

UV Cross-linking Experiments of the Sf9 Sp Family-like Protein(s) Binding to AcSp and cSp Reveal Identical Complexes—Fig. 4 shows UV cross-linking experiments carried out with 0.5 µg of *Sf*9 nuclear extract and AcSp and cSp probes. *Lanes 1–3* represent controls to rule out any nonspecific interactions that may be observed. *Lanes 4* and *5* show cross-linking with the AcSp and cSp probes, respectively, to generate DNA-protein complexes of similar molecular sizes.

The Sf9 Sp Family-like Factor(s) Is Distinct from PPBP—We previously demonstrated the requirement of host factors in regulating polh promoter-driven transcription (5, 6, 8, 24, 28, 37, 38). It is therefore important to show that the Sp family-like factor(s) present in *Sf*9 cells described above is distinct from the well characterized PPBP. Fig. 5 shows the binding of PPBP to the polh promoter B domain oligonucleotide (5) carrying the basal promoter determinants. The complex generated by the binding of PPBP, present in uninfected *Sf*9 nuclear extract, to the labeled B domain oligo (*lane 2*) can be specifically competed out only by the presence of homologous cold competitor (*lane 3*) and *not* with cSp (*lane 4*) or AcSp (*lane 5*), indicating that the Sp-like factor(s) present in *Sf*9 extract is distinct from the host factor PPBP involved in polh gene transcription.

Recombinant Human Sp1 Protein Binds Weakly to the AcSp Sequence in EMSAs—To substantiate the authenticity of the cSp probe, EMSAs were carried out with pure recombinant human Sp1 (Fig. 6*a*). cSp expectedly binds to pure hSp1 (*lane 6*) under the reaction conditions used. However, the mobility of the shift is less than that with the *Sf*9 extract (*lane 5*), indicating that the insect proteins binding to cSp are not classical Sp1 but a different member of the Sp family. AcSp probe also shows a weak shift with pure Sp1 (*lane 3*) as compared with the *Sf*9 extract (*lane 2*). These results demonstrate that the AcSp sequence is recognized by the human Sp1 protein and that the
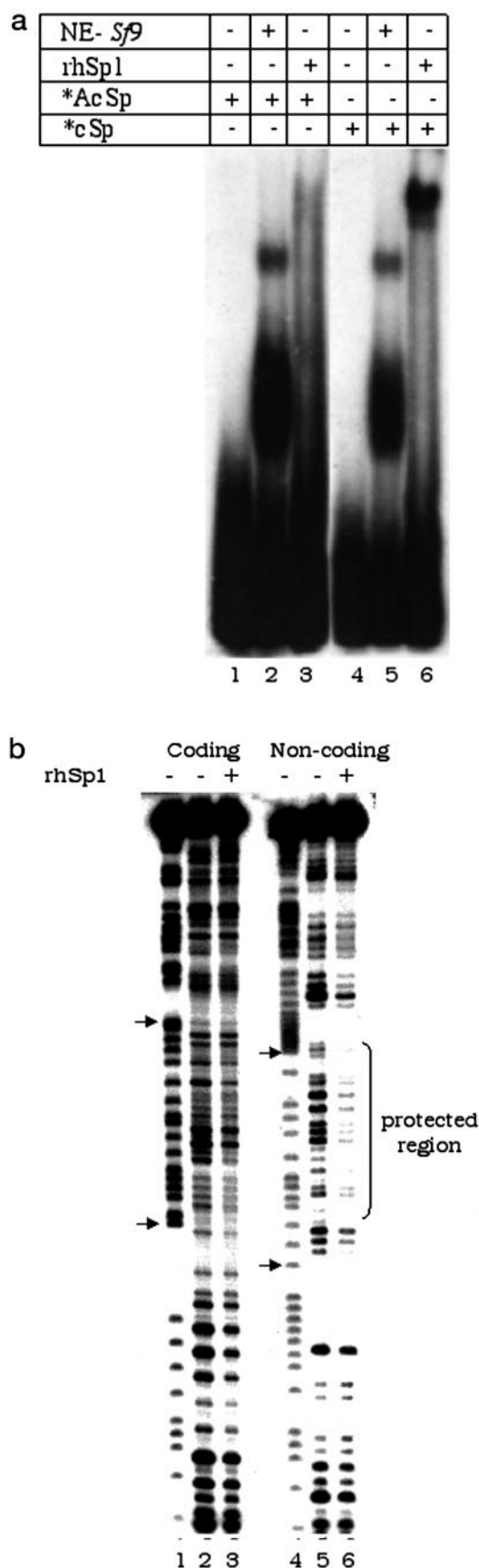
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insect cell Sp factor(s) is different from classical Sp1.

Sp1 Protein Binds to the AcSp Noncoding Strand—DNase I footprinting assays were carried out (Fig. 6*b*) using a 140-bp viral polh upstream region as a probe containing the AcSp sequence motif in the center and pure Sp1 protein (Promega Inc.). The coding strand does not show any protection in the AcSp region. However, practically the entire AcSp sequence is significantly protected on the noncoding strand, confirming the ability of the AcSp element to bind to members of the Sp family. The boundaries of the AcSp sequence are marked by *arrows*.

The Insect Sp Family-like Protein(s) Is Involved in the Enhancement of Reporter Gene Expression in Vivo—Transient transfection experiments were carried out using the plasmids pAcSp·pol·luc, pcSp·pol·luc, and pKN603luc (described under "Experimental Procedures"). It was observed that all three constructs enhanced luciferase expression to about the same extent (data not shown), indicating that AcSp is perhaps the critical motif involved in the transcription enhancement exhibited by the upstream ORF603 sequence. *In vivo* mopping experiments (28) were carried out using pAcSp·pol·luc and pcSp·pol·luc as reporters in the presence of varying amounts of competitor plasmids. pUC19 served as a nonspecific competitor, whereas pAR1 (AcSp construct) and pAR2 (cSp construct) were used as specific competitors (described under "Experimental Procedures"). The results (Fig. 7) demonstrate a dramatic drop in reporter expression in the presence of competitor plasmid (pAR1 or pAR2; *second*, *third*, *fifth*, and *sixth lanes*). The nonspecific competitor pUC19 failed to show a similar effect (*first* and *fourth lanes*). The *seventh* and *eighth lanes* depict the luciferase activity obtained with 20 μg of pAcSp·pol·luc and pcSp·pol·luc, respectively. These results indicate that the transcription enhancement is indeed due to the involvement of the Sp family-like factor(s) and not merely a function of the *cis* sequence.

The AcSp Sequence Is Functionally Significant in the Viral Context—Three viruses were constructed carrying the luciferase reporter driven by the polh promoter with varying upstream sequences (Fig. 8*a*) to establish the requirement of the AcSp motif in the viral context. vMAluc has all of the upstream sequences intact including the upstream hr1 enhancer (28), vΔluc has an immediate upstream 800-bp deletion, and vAcSpluc has the 800-bp deletion replaced with the AcSp oligonucleotide. Luciferase activity was assayed after infecting *Sf*9 cells at a multiplicity of infection of 10 (Fig. 8*b*). Uninfected cells (*Sf*9) or cells infected with wild-type *Ac*NPV virus showed no luciferase expression, whereas infection with vΔluc showed extremely low expression. Infection with vAcSpluc increased luciferase expression 1000-fold compared with the construct carrying no AcSp sequence (vΔluc), and infection with vMAluc, which contains all upstream sequences intact, showed a 10,000-fold increase in luciferase activity as compared with vΔluc. Fig. 8*c* shows the dot-blot analysis of cells infected in duplicate by the three recombinant viruses probed with the radiolabeled *luc* gene. Densitometric scanning revealed that the levels of infection of the three viruses were comparable. It is therefore clear that the identified AcSp sequence functions *in vivo* in the viral context.

FIG. 6. *a*, recombinant human Sp1 binds weakly to the AcSp sequence in EMSAs. Binding of radiolabeled AcSp (*lanes 1–3*) and cSp (*lanes 4–6*) oligonucleotides with pure recombinant human Sp1 protein (*rhSp1*, *lanes 3* and *6*) and *Sf*9 nuclear extract (*NE-Sf9*, *lanes 2* and *5*) is shown. *b*, Sp1 protein binds to the AcSp noncoding strand. *Lanes 1–3* represent DNase I protection analysis using the radiolabeled 140-bp coding strand of the *Ac*NPV genome containing the AcSp sequence approximately in the center. *Lane 1* depicts the A + G sequencing ladder. *Lanes 2* and *3* show DNase I treatment in the absence and the presence, respectively, of recombinant human Sp1 (*rhSp1*) protein. *Lanes 4–6* represent corresponding lanes using the labeled noncoding strand. The boundaries of the AcSp sequence are marked by *arrows* on both labeled strands.
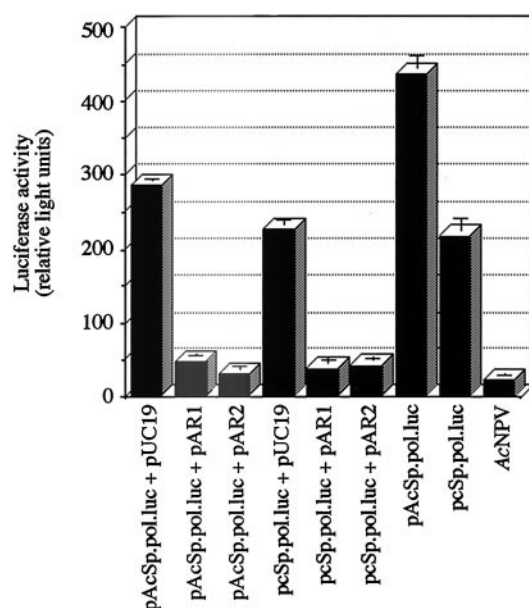
FIG. 7. **Mopping of the insect Sp family protein(s)** *in vivo* **causes a reduction in polh promoter-driven reporter gene expression.** Luciferase expression levels using 20 µg of pAcSp·pol·luc (*seventh lane*) and pcSp·pol·luc (*eighth lane*) plasmids after transfection into *Sf*9 cells were compared in the presence of specific or nonspecific co-transfected competitor plasmids. The *first* and *fourth lanes* show luciferase expression using the reporter plasmids pAcSp·pol·luc and pcSp·pol·luc, respectively, with pUC19 used as a nonspecific competitor. The *second* and *third lanes* depict luciferase expression using pAcSp·pol·luc in the presence of competitor plasmids pAR1 or pAR2, respectively. Likewise, the competition with pAR1 or pAR2 using pcSp·pol·luc as reporter is shown in the *fifth* and *sixth lanes*, respectively. The *ninth lane* depicts *Ac*NPV infection carried out in the absence of any transfected plasmid.

DISCUSSION

A battery of viral proteins have been implicated in transcription from the polh promoter (9–12, 39) either directly or because of a replication effect. However, reports from our laboratory have brought into focus the categoric involvement of host proteins as well in this process (5, 6, 28, 37). PPBP has been shown to be involved in binding to the polh promoter and in transcription from it, and another protein, hr1BP, binds to the hr1 viral enhancer sequence to bring about transcription enhancement. Our report on the presence of host Sp family-like proteins, while documenting for the first time the existence of novel members of this family in adult insect cells, also confirms their functional relevance in transcription from the polh promoter.

Region II, which enhances basal transcription, contains an open reading frame, ORF603, encoding a putative 201-amino acid protein product. However, the enhancement is not mediated through its protein product as conclusively demonstrated by deletion experiments (40) within this region. The enhancement caused by the region II sequence therefore pointed to the possibility of this sequence *per se* being essential and not the putative protein product. We identified a nucleotide sequence stretch, named AcSp, within the ORF603 that resembled the motifs recognized by the cellular transcription factor Sp1.

Sp1, the first member of the Sp superfamily to be isolated and characterized, has been found to recognize asymmetric GC and GT boxes present in a wide variety of cellular and viral promoters (41). A comparison of 36 different binding sites revealed a range of binding affinities, differing by at least 10–20-fold, with individual binding sites displaying a remarkable degree of sequence variation (35, 36, 41). The AcSp sequence within ORF603 of region II has a close resemblance to the Sp1

consensus sequence and could bind to Sp family-like proteins in the *Sf*9 and HeLa cell nuclear extracts. Further, both the HeLa and insect proteins binding to the AcSp and consensus Sp sequences required zinc for DNA binding, highlighting the Sp-like characteristics of the *Sf*9 factor(s).

Sp1 has been purified from human T cells, placental tissue, and from several other organisms like mouse, rat, chicken, etc. The human placenta-derived Sp1 is found to be ~40 kDa (42). The molecular size of the insect Sp family-like factor(s) appears to be around 60 or 90 kDa. The ~90-kDa protein correlates well with the known molecular mass range of the Sp family (~95–105 kDa), whereas the ~60-kDa protein could reflect the known size heterogeneity displayed by these factors (42). It is evident that the *Sf*9 factor(s) binding to AcSp and cSp does not represent classical Sp1; indeed, pure Sp1 protein binds extremely weakly to AcSp in EMSAs and has a different mobility from the insect protein-DNA complex (Fig. 6*a*). However, the AcSp sequence is capable of binding to pure Sp1 as revealed by DNase I footprinting analysis (Fig. 6*b*). The Sp1-AcSp complex may be prone to dissociation under the conditions used for study, a problem that can be circumvented by carrying out DNase I protection analyses where the interaction need be stable only for a short time in solution before DNase I treatment. We observed no supershifting or immunodepletion with anti-Sp1, anti-Sp3, or anti-Sp4 antibodies (data not shown). It is therefore apparent that although this protein(s) is a member of the Sp family, as observed by its recognizing an Sp-related sequence and demonstrating a requirement for zinc for DNA binding, it is not Sp1.

This particular family of zinc finger proteins is one of the most diverse and populous known (15, 16), given the steady flow of reports documenting the presence of new and related members in a wide range of eukaryotic systems. A recent report even offers evidence of functional Sp1-binding sites in a basidiomycete fungus, *Cryptococcus neoformans* (43). Other proteins that belong to the Sp superfamily include the Krüppel-like factors, including BKLF, EKLF, IKLF, etc. (15, 16); the BTEB proteins (BTEB1 and 2); CPBP proteins (CPBP, Zf9, UKLF); TIEG (TIEG 1 and 2) proteins (15, 16); and the protein products of the huckebein (*hkb*) (44), buttonhead (*bth*) (45), and *D-Sp1* (46) genes that are expressed in *Drosophila* embryos only during the blastoderm stage. There are no reports, however, of such factors being present in adult insect tissue (20, 47, 48).

Sp2, Sp3, and Sp4, the other closely related members of the Sp1 subgroup of the Sp family of proteins (49–51), are structurally similar, recognizing similar but not always identical GT- and GC-rich sequences, and act as activators or repressors of transcription depending on their intrinsic properties and the promoter context (50, 52). Sp1 is known to cross-talk with a large number of cellular as well as viral transactivators. The Sp family members are also known in several instances to bind concomitantly and synergistically (20) to sequences upstream from various promoters, thereby effecting transcriptional activation (53) or repression (52) in a context-dependent fashion.

Basal levels of polh promoter expression are increased in the presence of sequences containing the consensus Sp1 or the AcSp sequence. Our findings show both an enhancement (in the case of an intact initiator) and a rescue of transcription (in the presence of a mutant initiator) with upstream regions of the polh promoter carrying the AcSp or cSp motifs. In the bovine papillomavirus E2-responsive promoters, the TATA box or the initiator can be functionally replaced by Sp1-binding sites (32). It is known that human TATA-binding protein-associated factor, hTAF$_{II}$130 (54), and its *Drosophila* homolog, dTAF$_{II}$110 (55), interact with the glutamine-rich activation domains of human Sp1, suggesting a role for these interacting
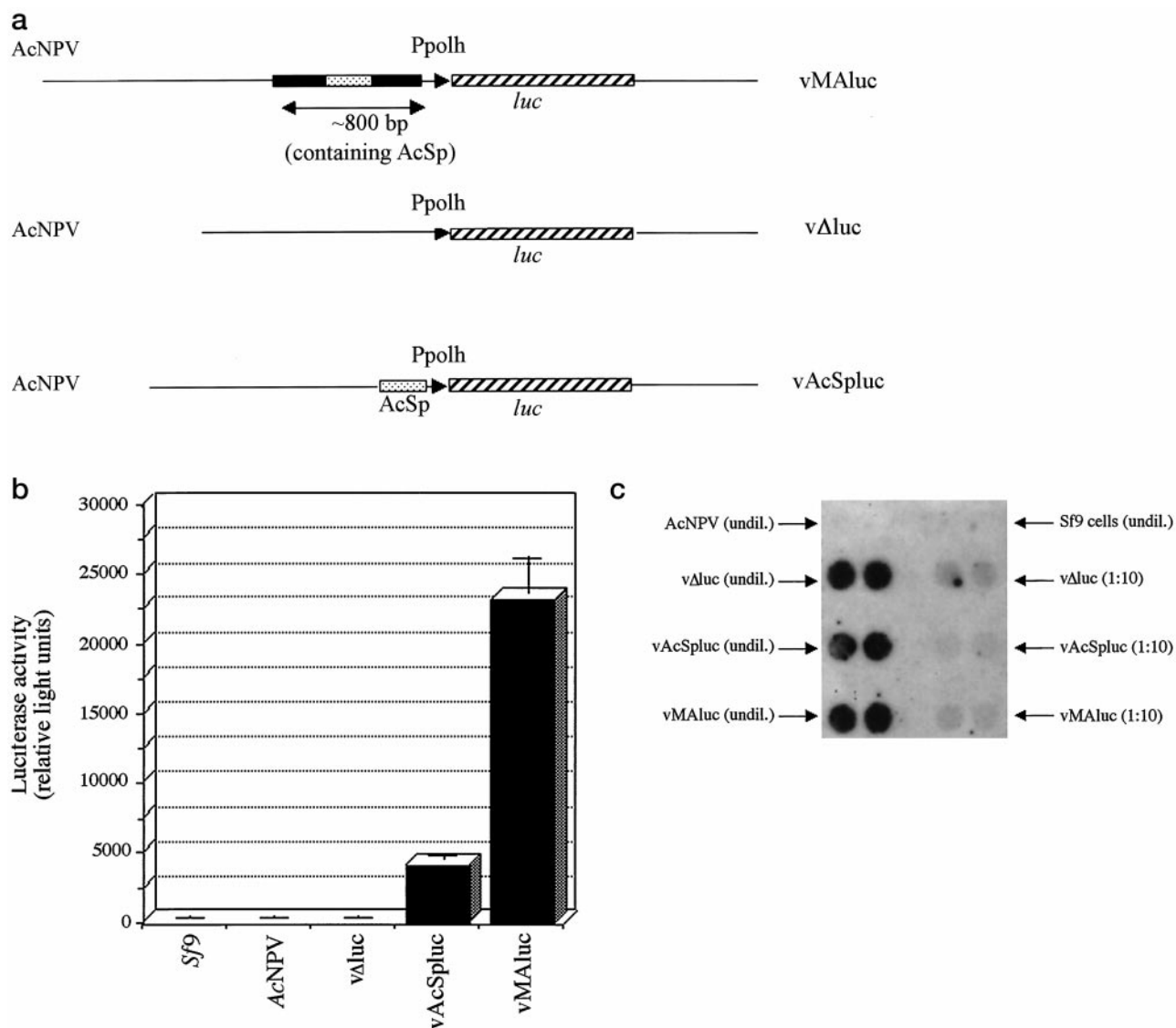
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FIG. 8. **The AcSp motif is necessary for transcription enhancement from the viral polh promoter *in vivo*.** *a*, schematic representation of the recombinant baculoviruses vMAluc, vΔluc, and vAcSpluc carrying the polyhedrin promoter-driven luciferase gene, with varying sizes of upstream sequences. *b*, luciferase levels recorded in *Sf*9 cells or after infection with a multiplicity of infection of 10 for *Ac*NPV or recombinant viruses, assayed 65 h.p.i. *c*, dot-blot of cells infected with vΔluc, vAcSpluc, and vMAluc recombinant viruses at 65 h.p.i. Duplicate wells were dotted without dilution or after 1:10 dilution. The negative controls were uninfected cells (*Sf*9) and wild-type virus-infected cells (*Ac*NPV) dotted undiluted in duplicate. The *luc* gene was used as a probe.

proteins as direct co-activator targets for Sp1. *Sf*9 cells are known to contain TBP, but no studies have been carried out on TATA-binding protein-associated factors present in *Sf*9 cells. By comparison with the human and *Drosophila* models, it is possible that the Sp family-like proteins observed by us may bring about transcription enhancement in an analogous fashion. However, basal transcription from the polh promoter is mediated by a viral RNA polymerase (12), and no cellular factors to date have been identified that take part in this process; the only exception to this was PPBP (6), which was identified in our laboratory as a promoter-binding protein that is critical for polh transcription. Our observation that mutations in the polh promoter and the crucial PPBP-binding motifs are partially restored if region II containing the AcSp sequence is present brings into focus the important role of cellular factors in basal transcription.

Furthermore, mopping of the Sp family *trans*-acting factors significantly reduces transcription from the polh promoter, pointing to the necessity of this DNA-protein interaction in transcription activation. The use of recombinant baculoviruses

provides definitive evidence that the AcSp sequence is required for hypertranscription from the polh promoter *in vivo*. The deletion of an upstream 800-bp region in vΔluc practically abolished luciferase gene expression, whereas the substitution of the AcSp sequence motif (vAcSpluc) increased expression 1000-fold. The virus with completely intact upstream sequences (vMAluc) increased transcription a further 10-fold. Several viral proteins have been shown to play a role directly or indirectly in activated transcription from the polh promoter. The Sp family-like proteins observed by us are the first instance of such factors having a role in transcription in this system.

Interestingly, the increase in transcription using AcSp-carrying plasmids in transient transfections was merely 2–3-fold in contrast to the dramatic 1000-fold increase with the corresponding virus. On further analysis, however, our observations proved to be in concordance with recent models proposing that enhancer-binding elements acting from a promoter-distant position require interaction with proximal sequences near the vicinity of the TATA box to recruit RNA polymerase (56). In

this case, the initiator element possibly requires the Sp family-like proteins (apart from other factor(s) such as PPBP) to help recruit the polymerase. This may explain why even when upstream enhancer elements are present, such as the hr enhancer sequences, the absence of promoter-proximal sequences (as in vΔluc) prevents the efficient recruitment of the RNA polymerase, thereby reducing transcription enhancement. Densitometric scanning of the dot-blot of viral DNA after infection and luciferase assay showed no significant difference in the amounts of viral DNA, confirming that the enhancement of transcription by the AcSp sequence was not merely due to an increase in the replication of the virus.

Host factors are increasingly finding importance in regulating transcription from baculovirus very late promoters (5, 6, 8, 24, 27, 28, 36, 37, 57, 58). In addition to PPBP, we have shown that another host factor hr1BP binds to the hr1 enhancer sequence (27) and is involved in hr1-mediated enhancement of transcription from the polh promoter (28). Our observations on an unusual viral sequence element-binding novel Sp family factor(s) in adult *Sf*9 cells to regulate expression of a gene crucial for baculovirus survival in the environment highlight a new facet of the mechanisms governing transcription from the viral polh promoter. Studies are in progress to further characterize the AcSp-binding factor(s) and elucidate in greater detail their role in transcription. Given that the polh promoter is a TATA-less initiator promoter coupled with the known involvement of members of the Sp family of proteins in initiator-mediated transcription (22), our results establish a vital link between the insect Sp family-like proteins and regulation of baculovirus polyhedrin-initiator transcription.

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