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A 30-kDa host protein binds to two very-late baculovirus promoters

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A 30-kDa host factor (polyhedrin-promoter-binding protein; PPBP) specifically binds to sequences critical for transcription from the baculovirus polyhedrin (p29) gene initiator promoter [Burma, S., Mukherjee, B., Jain, A., Habib, S. & Hasnain, S. E. (1994) *J. Biol. Chem. 269*, 2750–2757; Mukherjee, B., Burma, S. & Hasnain, S. E. (1995) *J. Biol. Chem. 270*, 4405–4411]. A host factor also binds, in gel shift assays, to the very-late p10 gene promoter through DNA sequence motifs similar to the PPBP · p29 interaction. The p10 · host factor complex was specifically competed out with oligonucleotides containing p29 cognate sequence motifs AATAAA and TAAGTATT, but this did not occur when these motifs were replaced with random sequences. From ultraviolet cross-linking analysis, the molecular mass of this host factor was estimated to be approximately 30 kDa. Experiments were performed to investigate if this host factor displayed any differences in affinity and turnover with respect to the p29 and p10 untranslated leader sequences known to be important for temporal fine tuning and the late burst of transcription. Half-life determination of the p10-binding protein revealed similar binding affinities for the initiator elements of both the promoters, but higher affinity for the p10 5′-untranslated region (≈30 min versus ≈10 min). The involvement of a similar host factor binding to both the p10 and p29 promoters indicates the possibility of a similar mode of transcription initiation from these two very-late promoters.

Keywords: baculovirus; host factor; polyhedrin-promoter-binding protein; transcription; very-late p10 and p29 gene promoters.

The *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin gene is transcribed at high levels very late in the infection cycle and involves a virus-specific or a virus-modified host RNA polymerase that is resistant to α-amanitin [1–3]. Many viral genes have been identified, based on subtractive library hybridization approach [4–7], that are believed to be involved in late and very-late gene transcription, although the direct role of any of these late expression factor (*lef*) genes has not been demonstrated. Many of these late gene factors regulate processes upstream to p29 gene expression in the temporal cascade of viral gene transcription and may be indirectly involved in p29 regulation. Although the involvement of some cellular factors in this process has been speculated [5], the molecular mechanism of transcription regulation from this or other very-late promoters remains unclear.

We have identified a 30-kDa cellular factor, the p29 promoter-binding protein or PPBP [8, 9], that binds to a hexanucleotide AATAAA and an octanucleotide TAAGTATT motif surrounding the mRNA start site. These two PPBP motifs essentially constitute the minimal polyhedrin promoter [10] sufficient for the basal level of transcription. PPBP could bind to the initiator promoter only in its phosphorylated form [8] and possessed both dsDNA and ssDNA binding activities. PPBP · ssDNA binding activity was dependent upon ionic interactions unlike dsDNA binding and exhibited relatively higher affinity and a longer half life indicating its possible involvement at a crucial step during and after transcription initiation [11]. Further, this host factor was present ubiquitously but in varying amounts in other insect cell lines [12] that expressed different levels of heterologous genes. Analyses of DNA · protein interactions [12] with nuclear extracts from a *Bombyx mori* (Bm5) cell line (which does not promote transcription from the AcNPV p29 promoter) and co-purification, on an affinity matrix [9], of another protein factor of a similar size suggested the existence of PPBP-associated factors possibly of viral origin that may have important role in transcription.

The p10 promoter regulating transcription of another very-late gene encoding a 10-kDa protein, which has also been used for foreign gene expression [13, 14], is also transcribed at high levels by an α-amanitin-resistant form of RNA polymerase [15]. The p10 promoter exhibits sequence similarity to the p29 promoter around the mRNA initiation site [16]. Like the p29 promoter, it is also A+T rich and the untranslated leader region is not only required for promoter activity but is also important for the very-late burst of transcription [17–19]. Though essentially very late in their time of activation post infection, subtle differences between these two promoters have been reported in terms of the precise activation time and relative strength [20]. There are suggestions that despite the above dissimilarities these two baculovirus very-late promoters might follow a common regulatory pattern.

We initiated studies to ascertain the involvement of host factor in p10 transcription. In this paper, we show that a PPBP or PPBP-like 30-kDa cellular factor also binds the p10 promoter and this binding involves motifs similar to the PPBP cognate

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Abbreviations. AcNPV, *Autographa californica* nuclear polyhedrosis virus; Bm5, *Bombyx mori* insect cell line; EMSA, electrophoretic mobility shift assays; HSV, herpes simplex virus; IBP, initiator-binding protein; *lef*, late expression factor; PPBP, polyhedrin-(p29)-promoter-binding protein; Sf9, *Spodoptera frugiperda* insect cell line; TBP, TATA-binding protein.

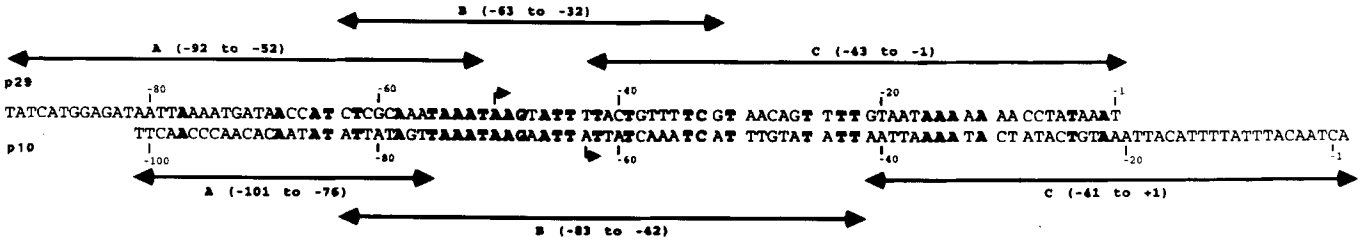

Fig. 1. Sequence alignment of the nucleotide sequences of the AcNPV polyhedrin (p29) and p10 gene promoters [19, 24]. The sequences are aligned with respect to the TAAG motif. The transcription start point, marked with an extending arrow, is at position −50 for the p29 promoter and at −63 for the p10 promoter. Common bases within the two sequences are shown in bold. The relative boundaries (indicated by numbers) of domains A−C for both the promoters are demarcated above and below the p29 and p10 sequences.

sequences. Significant differences exist in the affinity and turnover of PPBP with respect to the 5′-untranslated leader sequence of the two very-late genes.

EXPERIMENTAL PROCEDURES

Materials. Grace's insect cell culture medium was purchased from Gibco-BRL and fetal bovine serum was obtained from Sigma. [γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham. All other chemicals were molecular biology grade and were available commercially.

Gel mobility shift assays. Sf9 (*Spodoptera frugiperda* insect cell line) cells were grown in Grace's insect cell culture medium with 10% fetal bovine serum [21]. Crude nuclear protein extracts were prepared according to the method described earlier [8, 9]. Complementary synthetic oligonucleotides were annealed and labeled by T4 polynucleotide kinase using [γ-^{32}P]adenosine triphosphates. 2−4 μg crude nuclear extract and 1 ng labeled (≈10^4 cpm) annealed oligonucleotides were used in a binding reaction at 25°C for 15 min in the presence of 10 mM Hepes/NaOH, pH 7.5, 200 mM NaCl, 0.5 mM dithiothreitol, and 1 μg poly[d(I · C)]/poly[d(A · T)] [8]. The DNA · protein complex was separated by electrophoresis at 4°C in a 5% (29:1 acrylamide/bisacrylamide) non-denaturing polyacrylamide gel in buffer A (7 mM Tris/HCl, pH 7.5, 3 mM sodium acetate, 1 mM EDTA). The gel was then dried and exposed overnight to Hyperfilm MP (Amersham, UK) at −70°C. For cold competition analyses, an excess of appropriate unlabeled, dsDNA was added along with the labeled DNA in the reaction mixture.

Ultraviolet cross-linking of the DNA · protein complex. After the binding reaction, the reaction mixture was kept on ice and irradiated with ultraviolet light (254 nm) for 30 min at a distance of 1 cm [22] using a hand held short wave UV-254 nm Mineralight lamp (model UVG-11, UVP Inc., USA), submitted to SDS/PAGE in a 15% polyacrylamide gel [23], dried, and autoradiographed.

RESULTS

A host factor with cognate sequence recognition motifs similar to PPBP binds to the p10 promoter. Alignment of polyhedrin and p10 promoters identified a consensus TAAG motif that was used as a reference in designing p10-promoter-specific oligonucleotides (Fig. 1) for electrophoretic mobility shift assays (EMSA). Assays carried out with the B domain of the p10 promoter using nuclear extracts from uninfected (Fig. 2, lane 2) or infected (Fig. 2, lane 8) Sf9 cells revealed a DNA · protein complex that could be specifically competed out with a 25-fold molar excess of unlabeled p10B domain oligonucleotide (Fig. 2, lanes 3 and 9). Nonspecific DNA could not displace the p10B

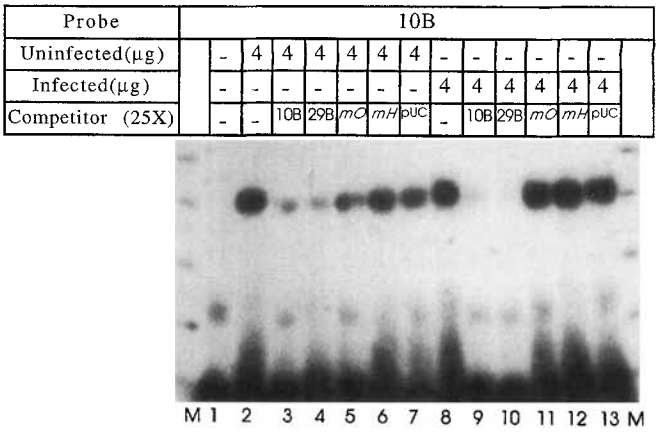

Fig. 2. Binding of the host factor PPBP to the p10 gene promoter. End-labeled domain B of the p10 promoter was incubated alone (lane 1), with 4 μg (lanes 2−7) of nuclear extract from uninfected Sf9 cells, or with 4 μg (lanes 8−13) of nuclear extract from Sf9 cells infected with AcNPV. The complex obtained with the uninfected and infected extract (lanes 2 and 8) was competed with 25 ng unlabeled p10B (lanes 3 and 9), p29B (lanes 4 and 10), *mutOct* (lanes 5 and 11), *mutHex* (lanes 6 and 12), or pUC18 (lanes 7 and 13). M refers to the labeled ϕX/TaqI molecular size marker. *mutHex* (*mH*) oligonucleotide (CTCGCACCGCCCTAAGTATTTTACTGTTTTCG) and *mutOct* (*mO*) oligonucleotide (CTCGCAAATAAAGCCTGCGGTTACTGTTTTCG) carried replacements of the PPBP cognate motifs, AATAAA and TAAGTATT respectively, with random sequences shown underlined.

domain complex (Fig. 2, lanes 7 and 13). The complex could also be competed out in the presence of unlabeled oligonucleotide representing the polyhedrin B domain (Fig. 2, lanes 4 and 10). However, mutated versions of the polyhedrin B domain carrying sequences different from the PPBP cognate motifs (*mutHex* where AATAAA has been replaced with CCGCCC and *mutOct* where TAAGTATT has been replaced with GCCTGCGG) on the polyhedrin promoter could not compete for binding to the p10B domain (Fig. 2, lanes 5, 6, 11, and 12) thus confirming that the factor that binds the p10 promoter recognizes the same sequence motif as that recognized by PPBP [8]. The same factor is also present in the uninfected and infected insect cell extracts (Fig. 2, lanes 2−7 and lanes 8−13, respectively), which suggests that it is a host factor. These results clearly indicate that the host factor that binds to transcriptionally important motifs of the p10 promoter is similar to the host factor PPBP in terms of cognate sequence recognition motifs.

The same 30-kDa factor binds to the p10 promoter. To further establish that the p10-promoter-binding factor is the same as PPBP that binds to the p29 promoter, ultraviolet cross-linking analyses were performed with radiolabeled oligonucleotides

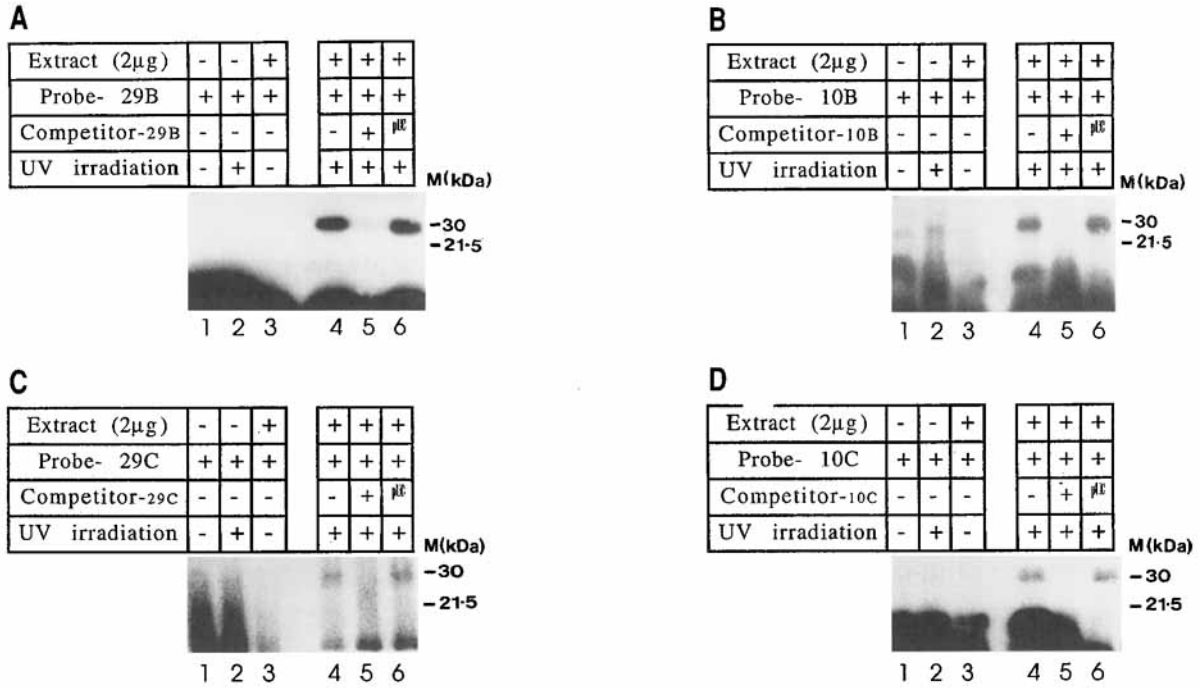

Fig. 3. The same 30-kDa factor binds both the very-late promoters. Labeled p29B (A), or p10B (B), or p29C (C), or p10C (D) was either not subjected to ultraviolet irradiation (lane 1) or ultraviolet irradiated alone without nuclear extract (lane 2) or with 2 μg nuclear extract without irradiation (lane 3) to serve as negative controls. In experimental sets, the incubation mixture was irradiated for 30 min without any competitor DNA (lane 4). Competition of the cross-linked complex was performed with 25 ng of the respective homologous domain (lane 5) or 25 ng pUC18 (lane 6). Protein molecular size markers (kDa) are indicated on the right.

(Fig. 3A–D, lane 1) representing the initiator (B domain) and the 5′ leader region (C domain) of both genes. As expected, a cross-linked complex with a molecular mass of 30 kDa [8] was obtained with labeled p29B domain oligonucleotide (Fig. 3A, lane 4). Labeled p10B domain also revealed a complex having a molecular mass of 30 kDa (Fig. 3B, lane 4). The faint bands of approximately 30 kDa observed in lanes 1 and 2 in Fig. 3B represent artifacts generated by the oligonucleotide alone that disappear upon the addition of nuclear extract (Fig. 3, lane 3). These complexes could be competed away with an excess of the respective unlabeled domains (Fig. 3A and B, lane 5), but were unaffected in the presence of an excess of pUC18 DNA (Fig. 3A and B, lane 6) confirming the specificity of the cross-linked complex. A complex with a molecular mass of 30 kDa was again evident for both the p29C and the p10C domains (Fig. 3C and D, lane 4) representing the untranslated leader regions. The specificity of the complex was checked in a homologous cold competition assay with an excess of the respective unlabeled domain (Fig. 3, lane 5). The complex could not be competed out in the presence of a non-specific competitor, pUC18 (Fig. 3, lane 6). In control experiments (Fig. 3, lanes 1–3), DNA · protein complex was not obtained with the probe alone (Fig. 3, lane 1), even after ultraviolet irradiation (lane 2) or when the probe and the nuclear extract were not irradiated (lane 3). These results demonstrate that a host factor with a molecular mass of 30 kDa, which is similar to PPBP, binds both the polyhedrin and p10 promoters. However, to demonstrate that the p10 and p29 promoter-binding proteins are the same, direct evidence such as a partial proteolytic profile of the two purified host proteins is desirable. Furthermore, this host factor also has the ability to independently contact the initiator and also the 5′-untranslated leader region.

The host protein that binds the polyhedrin and p10 promoters is sequence specific and is not subject to competition by poly[d(A · T)] sequences. Given the fact that the polyhedrin and the p10 promoters are A+T rich and also that the apparent commonality between the B and C domains of the p10 promoter is their A+T richness, it was logical to determine if the host factor binding was simply a function of the A+T rich sequence of the various domains. Gel mobility shift assays were carried out in presence of 1 μg poly[d(I · C)] (Fig. 4A) or 1 μg poly[d(A · T)] (Fig. 4B) using nuclear extracts prepared from Sf9 insect cells. The DNA · protein complex generated with labeled p10B domain (Fig. 4, lane 2) could be specifically competed with 25-fold excess of unlabeled p10B domain (Fig. 4, lane 3), or p10C domain (Fig. 4, lane 4). The same complex could not be competed in the presence of a 25-fold excess of poly[d(I · C)] (lane 5) or poly[d(A · T)] (lane 6) or an A+T rich oligonucleotide, $(A \cdot T)_{11}$ (Fig. 4, lane 7). These results clearly demonstrate that PPBP specifically recognizes similar binding motifs within the B and C domains of the p10 promoter and not just any A+T rich sequence.

PPBP binds with different affinities to the 5′-untranslated leader regions. After demonstrating that a PPBP-like host factor also binds to the p10 5′-untranslated region, we designed experiments to examine if PPBP displayed differences in binding to this region so important for promoter function as well as late burst of transcription. This was of interest since this region does not share the extent of sequence similarity apparent for the initiator region. EMSA experiments coupled with cross-cold-competition analyses were performed with the C domains (Fig. 5) of the polyhedrin (−43 to −1) and the p10 promoter (−41 to +1). It was apparent that the 29C · PPBP complex (Fig. 5A, lane 2) could be specifically competed by a 25-fold molar excess of 29C, 29B, 10C, or 10B domains (Fig. 5, lanes 3–6) indicating a somewhat similar affinity of this factor for the four domains. An excess of non-specific DNA could not compete for complex formation (Fig. 5, lane 9). Further, *mutHex* (lane 7) and *mutOct*

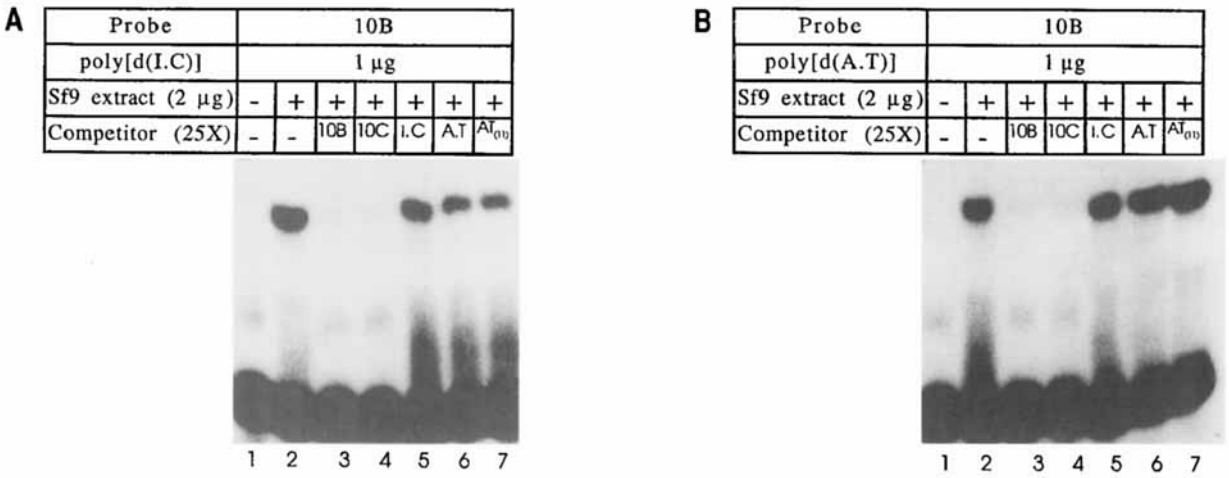

Fig. 4. PPBP binding is sequence specific and is not subject to competition by poly[d(A · T)] sequences. The binding reaction with labeled p10B domain was either carried out in presence of 1 µg poly[d(I · C)] (A, lanes 1–7) or 1 µg poly[d(A · T)] (B, lanes 1–7). The DNA · protein complex obtained (lane 2) was competed in the presence of 25 ng p10B (lane 3), or p10C (lane 4), or poly[d(I · C)] (lane 5), or poly[d(A · T)] (lane 6), or $(A \cdot T)_{11}$ (lane 7). In lane 1, labeled p10B domain is incubated alone without any nuclear extract.

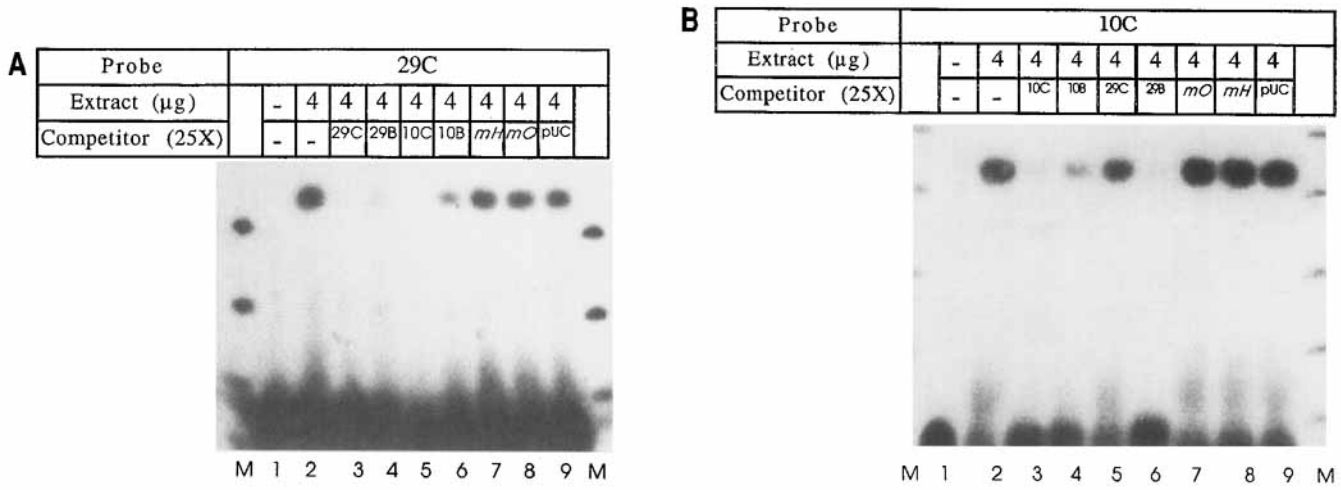

Fig. 5. PPBP binding to the 5′-untranslated region of the very-late genes. Labeled p29C domain (A) was either incubated alone (lane 1) or in the presence of 4 µg Sf9 nuclear extract (lanes 2–9). The DNA · protein complex obtained (lane 2) was competed in the presence of 25 ng p29C (lane 3), or p29B (lane 4), or p10C (lane 5), or p10B (lane 6), or *mutHex* (lane 7), or *mutOct* (lane 8), or pUC18 (lane 9). Labeled domain p10C 5′-untranslated leader region (B) was either incubated alone (lane1) or in the presence of 4 µg Sf9 nuclear extract (lanes 2–9). The DNA · protein complex obtained (lane 2) was competed in the presence of 25 ng p10C (lane 3), or p10B (lane 4), or p29C (lane 5), or p29B (lane 6), or *mutOct* (lane 7), or *mutHex* (lane 8), or pUC18 (lane 9). M is the DNA molecular size marker.

(lane 8) polyhedrin promoter domains, or non-specific competitor DNA (Fig. 5, lane 9) could not compete for the complex confirming the authenticity of the PPBP complex. In a complementary experiment, the 10C · PPBP complex (Fig. 5B, lane 2) could be specifically competed by a 25-fold molar excess of unlabeled 10C (lane 3), 10B (lane 4), and 29B domains (Fig. 5, lane 6). However, a 25-fold molar excess of unlabeled 29C domain (Fig. 5, lane 5) could not compete for 10C · complex formation to the same extent as compared to the homologous 10C competitor. This complex was again not affected by competition using the mutated versions of the polyhedrin promoter or excess nonspecific competitor DNA (Fig. 5, lanes 7–9). These results clearly indicated that PPBP bound to the 10C domain relatively strongly as compared to the 29C domain as a result of a higher affinity for the p10 5′-non-coding region than for the corresponding sequence of the p29 promoter.

The p10 5′-leader · PPBP complex has a longer half-life than the p29 5′-leader · PPBP complex. To confirm the observed PPBP affinity differences within the 5′-non-coding leader domains described above, the half-life of PPBP with respect to the B and C domains of the two promoters was determined [11, 25]. Preformed p29 · PPBP (Fig. 6A) or p10 · PPBP complexes (Fig. 6B) were challenged with an excess of unlabeled p29 or p10 untranslated leader sequences, respectively, and reactions were loaded onto a running gel over a time period extending from 0 min to 60 min. The extent of loss of radioactivity from the original complexes was quantitated by phosphorimage analysis (Molecular Imager Bio-Rad, USA, model GS-250) and the percentage of maximal binding was plotted against time (Fig. 6C). The half-life of p29 untranslated leader · PPBP complex was estimated to be only approximately 10 min whereas that of the p10 untranslated leader · PPBP complex was about

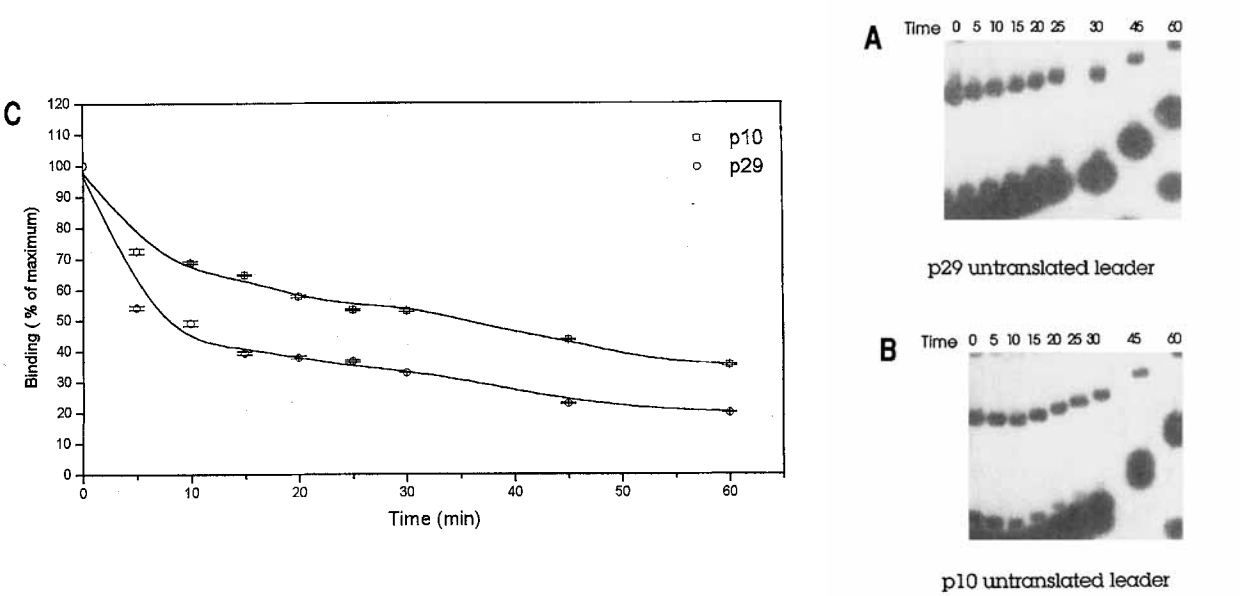

Fig. 6. PPBP has a higher half-life for the p10 5′-untranslated region of the p10 promoter as compared to the p29 leader. Preformed p29C · PPBP complex (A) and p10C · PPBP complex (B) were challenged with 30 ng unlabeled homologous domain. The reactions were loaded onto a running gel over a time period of 0 min to 60 min (shown over each lane). The dissociation of the original complex was plotted as binding (percentage of maximum versus time) (C). The open squares (□) represent the binding with respect to the p10 domain and the open circles (○) represent the binding with the p29 domain. The variance values have been calculated at each time point and are shown as error bars.

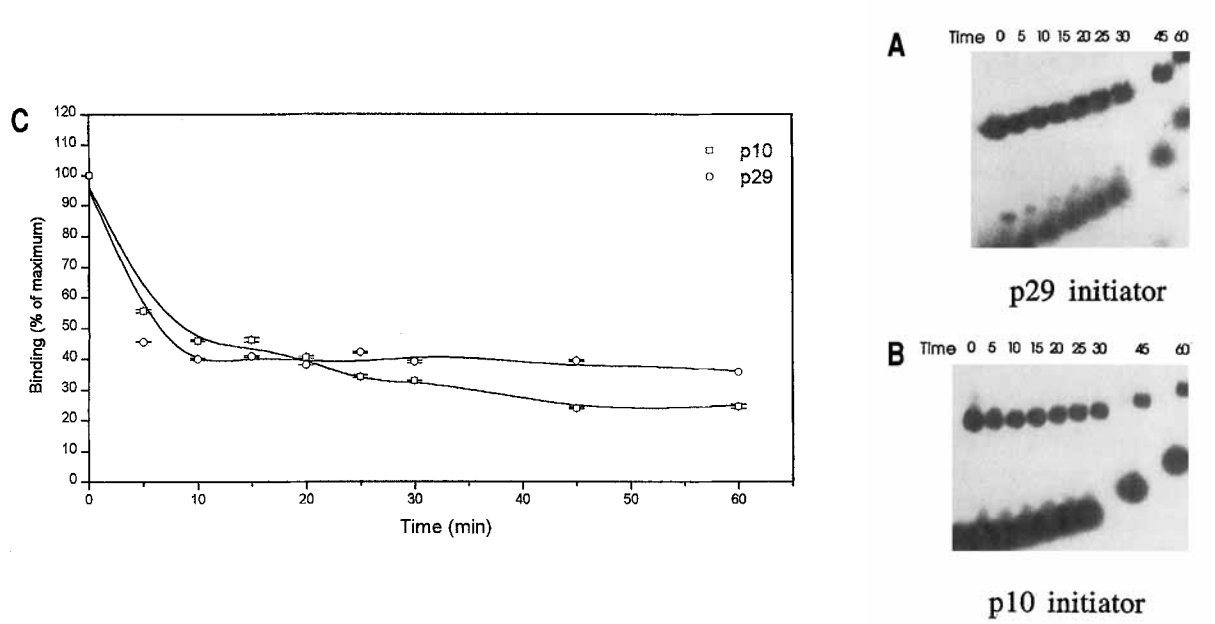

Fig. 7. PPBP has a similar half-life for the initiator region of the p10 and p29 promoters. Preformed p29B · PPBP complex (A) and p10B · PPBP complex (B) were challenged with 30 ng unlabeled homologous domain. The reactions were loaded onto a running gel over a time period of 0 min to 60 min (shown over each lane). The dissociation of the original complex was plotted as binding (percentage of maximum versus time) (C). The open squares (□) represent the binding with respect to the p10 domain and the open circles (○) represent the binding with the p29 domain. The variance values have been calculated at each time point and are shown as error bars.

30 min. Interestingly, a comparison of the half-life of PPBP with respect to its binding to the p29 and p10 initiators (B domain) was as expected approximately 15 min (Fig. 7) in both cases. These results demonstrate that the PPBP · p10 leader sequence displays a longer half-life than the p29 leader further indicating the possible involvement of this host factor in the late burst of transcription characteristically associated with the p10 and p29 leader sequences.

DISCUSSION

The baculovirus very-late promoter p10 besides having a similar activation profile exhibits conspicuous similarities to the p29 initiator promoter sequence (Fig. 1) and also to the consensus initiator sequence as proposed by Javahery et al. [27] and thus also belongs to the class of initiator promoters [28]. It would therefore be logical to expect a similar mode of regulation

for the two very-late promoters including the requirement of a host factor as an initiator-binding protein (IBP). We previously showed that a host factor binds to transcriptionally important motifs within the p29 promoter. Recent experiments indicate that PPBP · p29 interaction is critical and crucial for transcription from this promoter *in vivo* (Hasnain, S. E., Jain, A., Ghosh, S., Mukherjee, B., Tuteja, N. & Das, P., unpublished results). PPBP is an IBP that is distinct from the TATA-binding protein (TBP) and, in particular, the Sf9 TBP [26] in several respects (Hasnain, S. E., Jain, A., Ghosh, S., Mukherjee, B., Tuteja, N. & Das, P., unpublished results).

Comparison of the two promoters for PPBP cognate motifs shows that the eight-residue motif (TAAGTATT) is about 90% similar, while in the six-residue motif (AATAAA) the last four bases are shared between the two promoters. The ATAAG motif within the initiator promoter is considered to have originated from the host genome [29], which can possibly explain why this motif is recognized by a host factor. Binding of the same host factor to two different promoters has earlier been reported for the herpes simplex virus (HSV) promoter that has similar recognition sequences [30].

The untranslated leader regions of the baculovirus very-late genes are believed to be essential for the very-late burst in transcription [17, 31, 32]. This region also contributes to the relative difference in the efficiency of expression within the very-late promoters [20] and between the late and very-late promoters [10, 17]. It has been suggested [10] that the affinity of the TAAG sequence, which is present at the transcription initiation site of the late and very-late promoters, for as yet unidentified late transcription factors determines the time difference between the two. The data further suggest that the p10 TAAG has a somewhat higher affinity than the p29 TAAG and, therefore, it is activated earlier than the polyhedrin promoter. In the absence of any data demonstrating direct binding of any late expression factors (*lef*) to late or very-late promoters, this argument lacks experimental support. While the affinity of PPBP for the TAAG and the surrounding sequences of both p10 and p29 promoters is similar (half-life of approximately 15 min), it nonetheless shows striking differences in terms of binding to the 5′-untranslated leader sequences. Therefore, it is tempting to propose that the binding of PPBP with a much higher affinity (half life about 30 min) to the p10 5′-non-translated leader sequence probably allows it to reach its activation peak earlier than the polyhedrin promoter.

The involvement of cellular factors in conjunction with viral factors in transcriptional activation has been well documented for several other viral systems[30, 33–39]. Parvoviruses are also dependent on cellular factors [40] both for their replication as well as transcription. YY1, which is a common cellular transcriptional regulator that is an IBP, is also involved in transcription activation from the parvovirus P5 promoter. Adenoviruses make use of the same host cell transcriptional complex as HSV to initiate its infection cycle. In most cases, the viral factors are recruited to the transcription complex by the host factor through protein/protein interactions. It is conceivable that PPBP, which is also a host-encoded factor, works similarly. The requirement of virus infection for very-late gene transcription is well documented and PPBP may act to contact the initiator that further nucleates the assembly of the entire transcription complex.

The purpose of utilizing a host factor by the baculovirus for transcription of its two very important late genes, which is critical for its survival in nature, is probably a very ingenious mechanism that has been adapted by the virus to coordinate and control distinct eukaryotic processes through a common protein factor [41]. Although the natural function of PPBP in the host cell is yet to be determined, PPBP is an example of a host factor with a role in transcription limited not just to one promoter, but extending to other very-late promoters, probably fine tuning the subtle differences between them.

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