RESEARCH ACCOUNT

A bifunctional baculovirus homologous region (*hr*1) sequence: Enhancer and origin of replication functions reside within the same sequence element

Saman Habib and Seyed E. Hasnain

Eukaryotic Gene Expression Laboratory, National Institute of Immunology, New Delhi 110 067, India

Analysis of the synergy between transcription regulation and DNA replication as well as the mechanisms of enhancer action has been of interest to us. Our studies on the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) have revealed that the viral homologous region sequence, *hr*1, can function as an enhancer of polyhedrin promoter-driven transcription as well as an origin of DNA replication in transfected host insect cells. Minimal sequence requirements for both these activities of *hr*1 have been delineated. A host factor that interacts at multiple sites within *hr*1 has also been implicated in the enhancer function of this sequence. While demonstrating the dual function of *hr*1, our observations also indicate the importance of host factor(s) in regulating crucial processes in the viral infection cycle.

For the past several years, research at the Eukaryotic Gene Expression Laboratory of the National Institute of Immunology has been focussed on the biology baculoviruses. The baculovirus expression vector system, which utilizes the prototype baculovirus AcMNPV (*Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus), has emerged as the system of choice for the expression of many genes of prokaryotic and eukaryotic origin. The advantages offered by this system include the highly-restricted host range of baculoviruses (AcMNPV infects only 39 species of moths), the eukaryotic environment of the host insect cells that allows disulphide bond formation, proper folding, glycosylation, oligomerization and/or other post-translational modifications required for the biological activity of some eukaryotic proteins, as well as the presence of strong polyhedrin (*polh*) and p10 gene promoters that can drive the expression of foreign genes during the very late phase of infection of the virus.

The AcMNPV genome is a 133,894 bp long double-stranded, covalently closed, circular DNA molecule. The genome consists largely of unique sequences with the expressed AcMNPV genes distributed as non-overlapping, contiguous sequences (single exons). Interspersed in the genome are eight homologous regions or *hr*s (*hr*1, *hr*1a, *hr*2, *hr*3, *hr*4A, *hr*4B, *hr*4C and *hr*5) that vary in length from about 0.2 to 1 kb and contain one to eight 28 bp palindromes with an *Eco*RI site at the centre of each palindrome[1,2]. AcMNPV genes are expressed sequentially in a temporally-regulated fashion during the viral infection cycle. The process of infection can be divided into three phases: the early, late and very late phase. The early phase proceeds for the first six hours of infection and precedes viral DNA replication. The late phase which is characterized by extensive viral DNA replication and late gene expression extends from 6 h p.i. to about 20–24 h p.i. The very late phase begins around 20 h p.i. It extends for about two days during which the two major proteins of the occlusion phase, the polyhedrin protein and the p10 protein, are synthesized. An interesting feature of AcMNPV transcription is that early genes require an α-amanitin (an RNA pol II inhibitor) sensitive host RNA polymerase II while late and very late gene transcription is mediated by an α-amanitin and tagetitoxin (an RNA pol III toxin) insensitive, virally encoded and/or modified RNA polymerase[3,4].

AcMNPV *hr*s have been analysed for their role in transcription regulation and replication of the viral genome. *Hr*5 has been demonstrated to enhance expression of reporter genes under the control of early baculovirus promoters[5]. Recent studies have shown that plasmids containing AcMNPV *hr*s can replicate in an infection-dependent manner after transfection into *Spodoptera frugiperda* (Sf9) cells[6], thus suggesting their putative role as origins of replication (*ori*) of the viral genome. With some information on the enhancer functions of *hr*s available to us, we decided to investigate the role of the *hr*1 element in the AcMNPV infection cycle in host Sf9 cells. *Hr*1 is defined as an 880 bp *Cla*I fragment upstream to the polyhedrin gene (*polh*) promoter. It contains four *Eco*RI minifragments – one each of 158 and 89 bp and two of 90 bp and five 28 bp imperfect palindromes (Figure 1). Our interest in *hr*1 arose from the fact that it is located ~3.7 kb upstream of the *polh* promoter, the major promoter used in the baculovirus expression vector system. Given the hypertranscription from the *polh* promoter during the very late phase

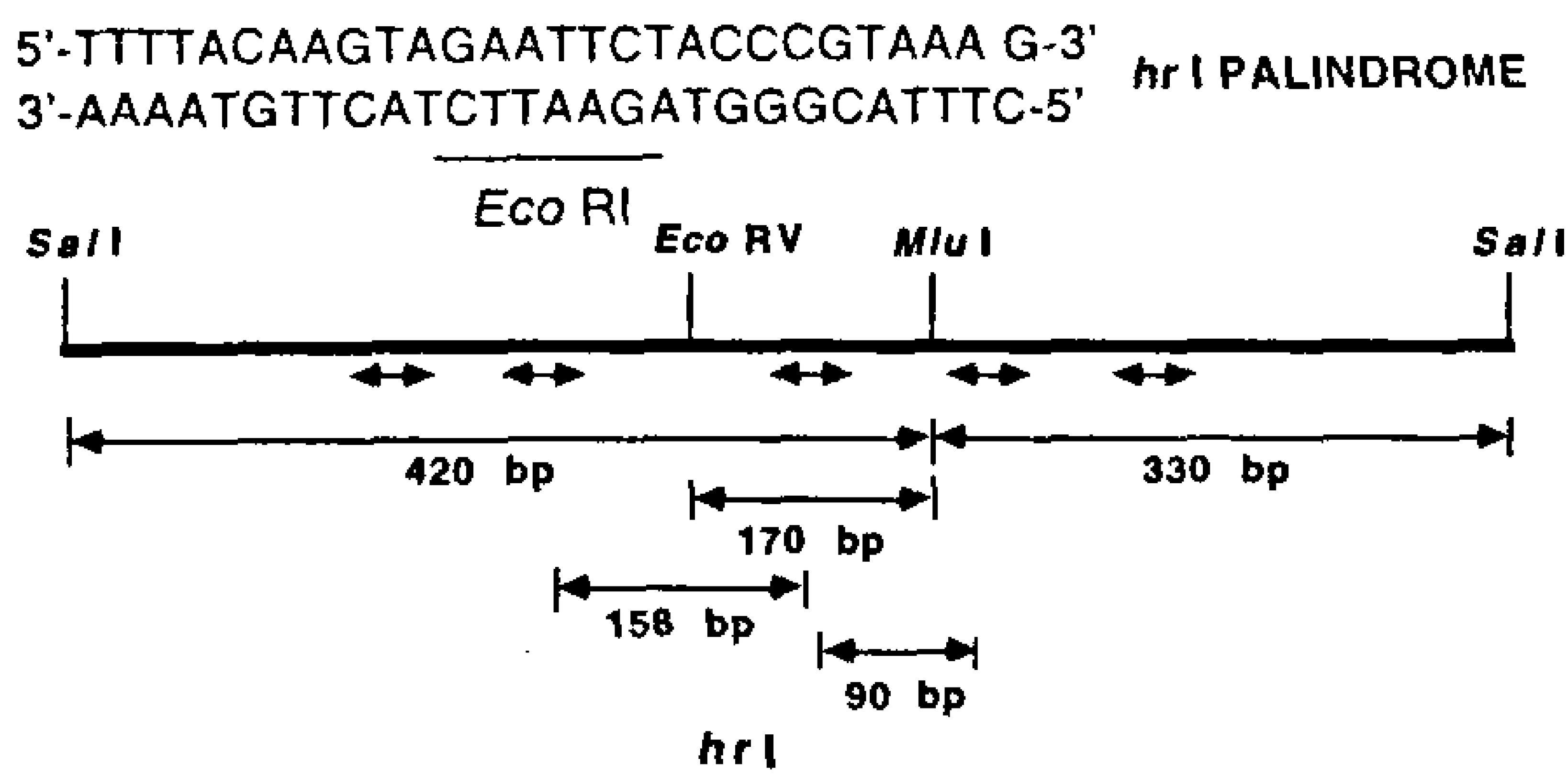

Figure 1. Physical map of the *hr*1 sequence. The five 28-bp core palindrome units are shown as filled double arrows. Each palindrome contains an EcoRI site at its centre (from Habib *et al.*[20]).

of the infection cycle, it seemed plausible that this promoter may be additionally influenced by enhancer-like sequences placed nearby. We suspected that *hr*1, by virtue of its relative proximity to the polyhedrin gene, may serve as an enhancer of transcription from the polyhedrin promoter. The mechanism of regulation of transcription from the polyhedrin promoter is also of considerable interest. Previous investigations in our laboratory had revealed the presence of a ~30 kDa host protein that interacts with high affinity and specificity to transcriptionally essential motifs of the *polh* promoter[7–9]. Although essential determinants of *polh* promoter function have been delineated, the regulatory role of upstream *cis*-elements on promoter activity has not been the subject of detailed study.

A synergy between transcriptional enhancement and DNA replication has been established by elegant studies with different viruses and a role for transcription in the vicinity of viral origins of replication as a prerequisite for initiation of DNA replication has been demonstrated[10]. Alternatively, DNA replication has been shown to facilitate the action of transcriptional enhancers of simian virus (SV40) in transient expression assays without causing an increase in DNA copy number[11]. Moreover, origins of replication in viruses such as Epstein Bar virus (EBV), Bovine Papilloma virus (BPV) and SV40 are associated with transcriptional enhancers and often the two overlap. The nature of the association between DNA replication and late gene transcription is not well understood in AcMNPV. It has been shown that disruption of the viral encoded proliferating cell nuclear antigen (PCNA) delays DNA replication as well as late gene expression[12]. Moreover, a mutation in the *hel* gene, which has homology to helicases is defective in both DNA replication and late DNA replication[13]. In the context of *hr* structure and known *hr* function, an attempt to study the nature of coupling between DNA replication and late and very late gene expression seemed worthwhile.

*Hr*1 acts as a position- and orientation-independent enhancer of polyhedrin promoter-driven transcription

DNA sequences that control gene regulation by RNA polymerase II and are binding sites for transcriptional factors are frequently divided into two major groups – promoters and enhancers. Promoters are located proximal to the mRNA start site and determine where transcription begins and enhancers function distal to the start site in a position- and orientation-independent manner relative to the gene. Certain conserved sequence motifs can be defined for eukaryotic promoters. On the other hand, enhancers lack a well-defined conserved sequence motif but contain multiple protein-binding sites. Since the first identified enhancers were observed in viral genomes[14–18], the properties of these viral transcriptional regulatory sequences now constitute the definition of an enhancer element[19]. By and large, the properties of enhancers include the ability to: (a) increase transcription from *cis*-linked promoters; (b) operate in an orientation-independent manner; (c) exert their influence over large distances independent of position and, (d) enhance expression from heterologous promoters.

In order to analyse the enhancer function of *hr*1, we amplified the *hr*1 sequence by PCR using the AcMNPV genome as template and cloned it in expression plasmids. The amplified 750 bp *hr*1 sequence was cloned upstream to the *polh* promoter-luciferase reporter cassette in the basal construct pSH*luc* to generate the plasmids pSH*luc*-*hr*-U_1 and pSH*luc*-*hr*-U_2 which represented *hr*1 cloned in opposite orientations. *Hr*1 was also cloned downstream to the promoter reporter cassette in the construct pSH*luc*-*hr*-D_1. These plasmids were analysed for luciferase expression levels in a transient expression assay using Sf9 cells. Cells were first transfected with the reporter plasmids using lipofectin and then infected with AcMNPV to maintain appropriate levels of viral factors required for late gene transcription. Our analysis of the effect of *hr*1 on expression from the AcMNPV very late polyhedrin promoter revealed that *hr*1 can exert its enhancement effect in a position- and orientation-independent manner. Although enhancement was consistently observed, the level of enhancement when *hr*1 was placed upstream to the promoter in the orientation opposite of its normal orientation relative to the promoter in the wild type genome was lower (~7-fold) than when it was placed in the normal orientation (~11-fold)[20]. The position of the *hr*1 element with respect to the promoter (5′ or 3′) did not significantly alter the enhancement level. The enhancement of expression was also a direct result of a corresponding increase in transcription from the promoter as revealed by RNAase protection assay of *luc* transcripts

from transiently-transfected cells. Transcription in the presence of *hr*1 was found to be mediated by the viral-encoded or modified α-amanitin-resistant RNA polymerase and followed the normal very late activation profile. Moreover, initiation of transcription in the presence of *hr*1 is at the authentic transcription start point within the TAAG sequence of the TAAGTATT motif of the polyhedrin promoter[20]. In order to study the effect of *hr*1 on AcMNPV late promoter activity, we cloned the *hr*1 sequence downstream to the *cor* promoter (a late promoter of AcMNPV) that drove the expression of the luciferase reporter. A 3-fold enhancement over expression from the basal construct (pSH*cor*P-*luc*) was observed in the presence of *hr*1 (pSH*cor*P-*luc*-*hr*1) and the temporal expression profile expected for the late promoter was observed (manuscript in preparation).

It is significant that in addition to enhancing transcription from the polyhedrin promoter, *hr*1 can also stimulate expression from immediate early IE–N and delayed early 39 K promoter[5,21], a heterologous *Drosophila hsp*70 promoter (Venkaiah *et al.*, unpublished observation), and a AcMNPV late *cor* gene promoter. Additionally, the observation that *hr*1 can also exert its influence in a distance-independent manner even when placed ~6 kb upstream to the polyhedrin promoter (Venkaiah *et al.*, unpublished observation) qualifies the categorization of the *hr*1 sequence as a classical enhancer element.

Enhancement is independent of the *ori* function of *hr*1

*Hr*1 has been described as a putative viral *ori* based on the *Dpn*I-sensitivity assay but the ability to detect this function of *hr*1 has been shown to depend upon the transfection and infection regime[22]. It was therefore, necessary to ascertain whether the enhancement of reporter expression was a reflection of DNA copy number change due to the replication of *hr*1-containing plasmids in transfected cells. DNA dot-blot analysis of Sf9 cells immediately after transfection and 60 h p.i., in the absence or presence of inhibitory amounts of aphidicolin, a DNA replication inhibitor, was carried out. It was apparent from these results that the *hr*1-containing plasmids did not register any increase in copy number over what was observed immediately after transfection. Complementary experiments where replication of these *hr*1-plasmids was blocked by aphidicolin did not result in a decrease in copy number of these plasmids when their amounts were checked 60 h p.i. As expected, aphidicolin also inhibited viral DNA replication, resulting in a drastic fall in reporter expression in transient expression assays[20]. The influence of *hr*1 on *luc* expression, therefore, seemed to be independent of *hr*1-mediated DNA replication of the reporter plasmid.

We sought confirmation of this result by utilizing the information that a circular form of transfected DNA carrying an AcMNPV *ori* sequence is necessary for replication and that linear fragments do not replicate in host cells[22]. Sf9 cells, transfected with reporter plasmids linearized at a unique restriction site, were assayed for reporter expression 60 h p.i. and compared with expression from control circular plasmids. It was apparent that the fold-enhancement of expression from *hr*1-containing plasmids over that from the basal plasmid was similar in the circular and linearized plasmid transfection sets.

The third set of evidence came from direct *Dpn*I sensitivity assay of replication of *hr*1 plasmids and the control plasmid pUC18. The restriction enzyme *Dpn*I cleaves the GATC sequence only when the A residue is methylated by *Dam* methylase. Unlike bacterial cells, eukaryotic cells lack *Dam* methylase and therefore, all input plasmid DNA that has replicated in bacterial cells will be sensitive to *Dpn*I while plasmid DNA that has replicated in insect cells will not be methylated at the GATC site and will be resistant to *Dpn*I digestion. In our experiments, all plasmid DNA isolated from transfected Sf9 cells was found to be sensitive to *Dpn*I. Even when 20 µg (the DNA amount used for transient expression) of the *hr*1-plasmid was transfected, no *Dpn*I-resistant form was detected. These results unequivocally demonstrated that *hr*1 did not induce template DNA replication in our transfection regime and the enhancement of transcription from *polh* promoter was a direct result of the enhancer-like function of *hr*1.

Kool *et al.*[22] demonstrated that the transfection and infection regime followed for transient replication of plasmids detected by *Dpn*I sensitivity assays determines the ability to detect replicated *hr*-plasmids. When they tested the *Hin*dIII-F fragment of AcMNPV, containing *hr*1, by first transfecting and following it immediately by AcMNPV infection, they failed to detect replication of the *hr*1-containing plasmid. It is important to reiterate that identical transfection and infection regimes for the transient expression assays were followed in our study. Replicated forms of transfected plasmids were, however, detected when cells were first transfected and, 24 h later, infected with AcMNPV[22]. This could be reproducibly seen by us in *Dpn*I assays with *hr*1 constructs. Using the altered transfection regime, we could detect replicated (unmethylated and thus *Dpn*I-resistant) form of *hr*1-plasmids in virus-infected cells while the plasmid did not replicate in uninfected cells. Leisy and Rohrmann[23] could also detect *hr*1-mediated replication using another transfection regime, thereby complementing the observation that replication of *hr*-plasmids is infection-dependent[24]. The reason for this discrepancy in the replication status and its dependence upon the transfection regime is not clear. It is possible that lipofectin–DNA complexes do not dissociate early enough for the DNA to be available for interaction with viral factors

required for replication. A 24 h gap between transfection and infection circumvents this problem and the plasmid DNA carrying the putative *ori* replicates. Since the transient expression assay for polyhedrin promoter-driven luciferase reporter was carried out much later (60 h p.i.), expression of the reporter was detected even when cells were transfected with reporter plasmids and immediately infected with AcMNPV. This transient transfection protocol enabled the detection of the enhancer function of *hr*1 independent of its *ori* activity. Alternatively, a 24 h gap between transfection and infection in the altered regime could detect replicated forms of *hr*1-plasmids.

Overlap of transcriptional regulatory sequences and replication origin sequences at *hr*1

Regions of the *hr*1 sequence carrying five 28 bp core palindromes and palindrome-flanking sequences, were analysed for their enhancer activity in transient expression assays as well as their ability to act as *oris* in transfected cells. The analysis of six *hr*1-deletion constructs in transient expression assays revealed that *hr*1-driven enhancement is a function of the number of palindromes and flanking sequence units[20]. Enhancement by *hr*1 can thus be seen as an additive effect of *hr*1 'modules'. Our results also demonstrated that an intact palindrome along with the flanking sequence functions as the minimal enhancer 'module' in transient expression assays. On the other hand, a palindrome alone seems to be both necessary and sufficient for the *ori* function of *hr*1 as revealed by *Dpn*I sensitivity assays of constructs carrying segments of *hr*1 (ref. 20). Although there is an overlap in the sequence requirements for *ori* and enhancer activity of *hr*1, there seem to be differences in the minimal sequence determinants for these functions.

Activation of transcription by DNA replication is a general phenomenon for genes linked to an enhancer. Moreover, enhancer-independent activation signals, such as SV40 T antigen *trans*-activation, function independently of DNA replication. This synergy between enhancement and DNA replication is seen with a number of enhancers including synthetic sequences constructed from multimerized binding sites of a single enhancer-binding factor[11].

Studies on viruses have established a role for transcription in the vicinity of viral *oris* as a prerequisite for the initiation of DNA replication. The discovery of a transcriptional activator in the function of a replication origin in *Saccharomyces cerevisiae*[25] has extended this relationship to eukaryotes. Although there is a large amount of evidence that has established the role of transcription in DNA replication, there have been relatively few reports on the role of DNA replication in transcriptional control. Studies on the latter include the observation that heterochromatin and fragile sites are among the last sequences to be replicated in a genome, although the cause and effect relationship between repression and late replication is unknown[26]. Genetic experiments have also established that a specialized *ori* in *S. cerevisiae* plays a critical role in regulating gene expression[27]. Wilson and Patient[11] demonstrated an enhancer-dependent general role for DNA replication in activation of transcription in transient transfection assays. This effect was observed for a wide variety of genes, in a range of cell types, and was independent of DNA copy number. By using a variety of synthetic and natural enhancers in their study, they proposed that replication modifies chromatin to facilitate enhancer action.

There is coupling between late and very late gene transcription and viral genome replication in AcMNPV and both cycloheximide and aphidicolin block transcription of late and very late genes[3]. Disruption of the viral-encoded PCNA delays DNA replication as well as late gene expression[12,28]. Also, the ts8 mutant with mutation in the helicase (*hel*) gene with homology to helicases, is defective in both DNA replication and late gene expression[13]. The nature of the dependence of late and very late gene transcription on replication remains to be defined. Our results demonstrate that *hr*1 enhances transcription from the late *cor* promoter and the very late polyhedrin promoter in transient expression assays. In light of the relationship between replication and transcriptional activation demonstrated for other systems, *hrs* of AcMNPV, that function as both viral replication origins and transcriptional enhancers, may be considered as sequences that establish a link between viral replication and late and very late gene transcription *in vivo*.

Sequence organization of the *hr*1 enhancer element

Enhancers are composed of multiple genetic elements, or modules (reviewed by Dynan[29]); the mixture of sequence motifs comprising a particular enhancer determine the enhancer effect. Insight into the relationship between enhancer function and enhancer modules has come from elegant studies on the extensively characterized SV40 and immunoglobulin (Ig) gene enhancers[30-32]. Refined genetic analysis has shown that in many cases, as in the SV40 enhancer, discrete modules can be further divided into basic units of enhancer structure called enhansons. The SV40 enhancer is composed of 15 to 20 bp long enhancer elements or modules that cooperate with one another or with duplicates of themselves to enhance transcription. Enhancer elements or modules are bipartite, being composed of subunits called enhansons, that can be duplicated or interchanged[31]. Enhansons probably correspond to individual binding sites for *trans*-activating protein factors.

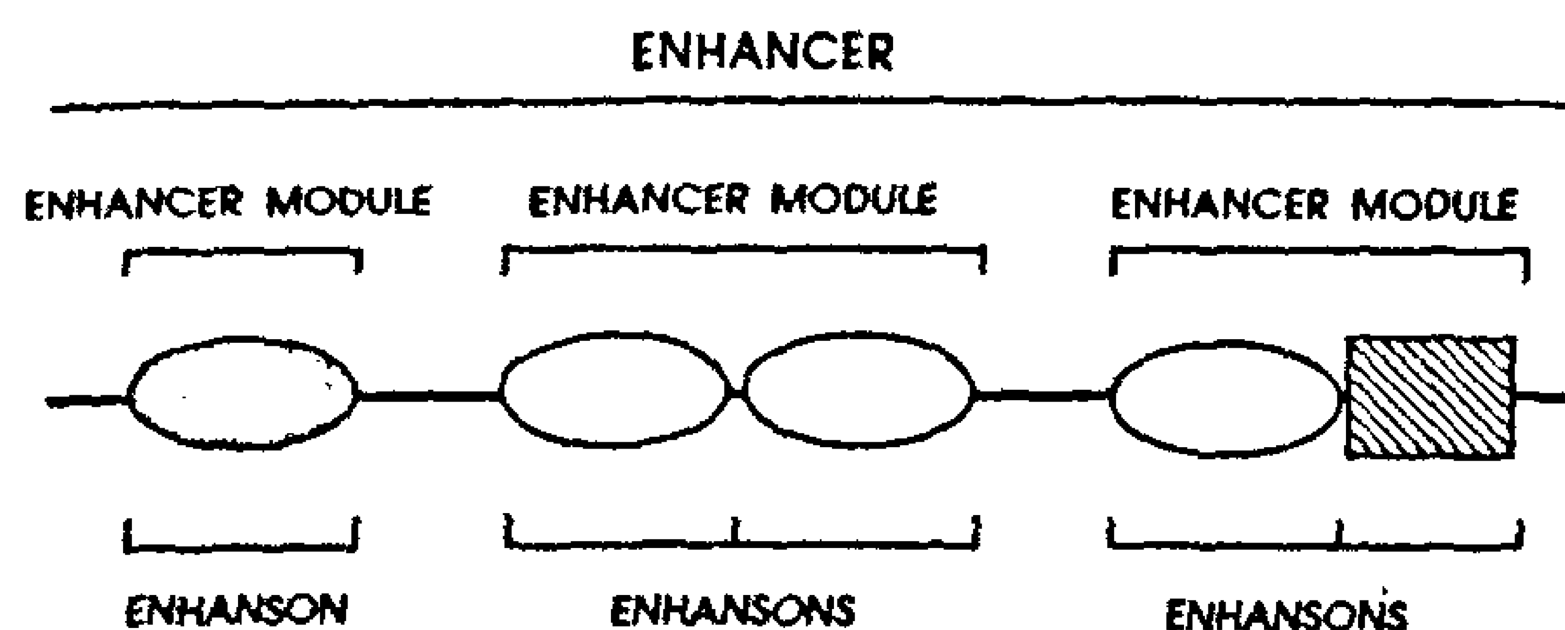

Figure 2. General organization of an enhancer element based on the structure of the SV40 enhancer (adapted from Dynan[29]).

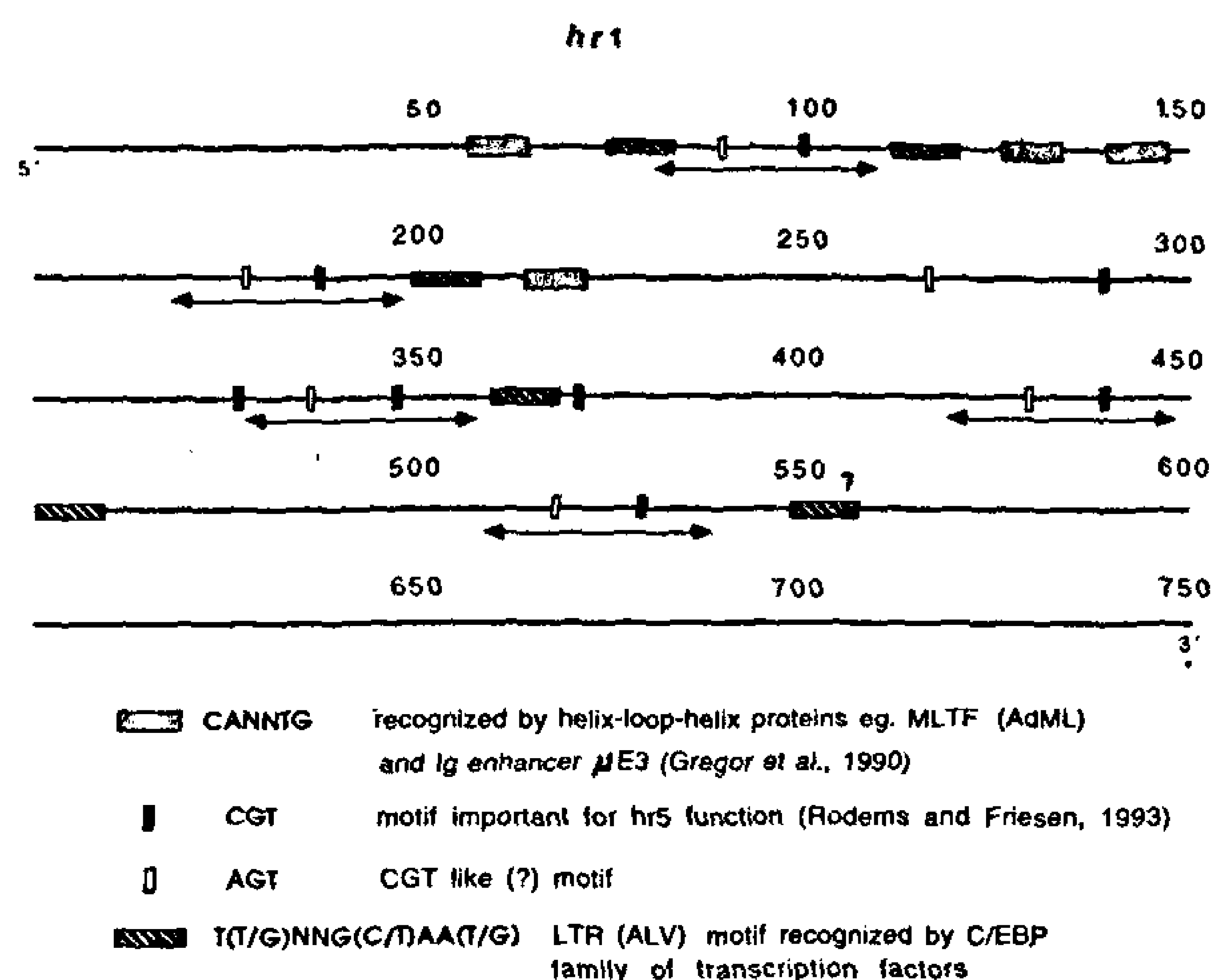

Figure 3. Location and distribution of different sequence motifs in *hr*1 that specifically interact with previously-reported enhancer binding proteins and transcription factors (from Habib *et al.*[20]).

The general organization of an enhancer, based on arrangements found in the SV40 enhancer, is depicted in Figure 2 (adapted from Dynan[29]). An enhancer element consists of enhancer modules which are in turn comprised of enhansons. One type of enhanson forms a functional enhancer module when a single copy of the enhanson is present (Figure 2, left). The second type of module is comprised of two copies of a tandemly repeated enhanson (centre). The third type forms a functional module in combination with a nonidentical enhanson (right). At present, it is not clear whether the difference between enhansons is due to differences in the proteins that interact with them, or whether there is another explanation, such as differences in the relative affinities of the cognate enhanson motifs for the protein factors. Other enhancers, such as the Ig heavy chain enhancer and the enhancers associated with the IE genes of *Herpes simplex* virus, exhibit an apparent functional redundancy with no one site being crucial for enhancer function[32,33].

A search for sequence motifs that interact with known enhancer-binding proteins and transcription factors in other viral and animal systems revealed that a few of these motifs were present in *hr*1 (Figure 3). The most interesting were the motifs with high homology to the consensus enhancer element motif [T(T/G)NNG (C/T)AA(T/G)] recognized by the C/EBP family of transcription factors in the LTR enhancer of avian leukosis virus[34]. This motif was repeated (with one or two base pair changes) 3′ to each palindrome in *hr*1. This incidentally is also the region in the flanking sequences that has a high level of homology and forms of the repeating structural units of *hr*1. This observation may be of relevance when seen in the context of the structural organization of viral enhancers such as the SV40 enhancer.

The *hr*1 sequence carries five 28 bp core palindromes with a single base mismatch in the consensus palindrome sequence. The *Eco*RI restriction enzyme, that cuts at the centre of each palindrome, cleaves *hr*1 into fragments of 89 bp, 158 bp, 90 bp, and 90 bp length from 5′ to 3′, respectively. The 5′ and 3′ flanking sequences of the 750 bp *hr*1 sequence are 85 bp and 207 bp long, respectively. Sequence comparison of palindrome intermediate sequences revealed a high level of homology between them; the homology was maximum in the sequences immediately adjacent to the palindromes. The palindromes and palindrome-flanking sequences can be interpreted as repeating structural units in the *hr*1 element. *Hr*1-mediated enhancement is a function of the number of palindrome core and core-flanking sequences with maximum enhancement achieved with the full set of five *hr*1 palindromes; a corresponding reduction in enhancement with three and two palindrome and flanking sequence units was observed. A single core palindrome or palindrome-intermediate sequences without an intact palindrome could not enhance expression. Thus, functional analysis of the *hr*1 deletion constructs also suggests that a palindrome together with the flanking sequence is the minimal functional element of the *hr*1 enhancer.

Interaction of host factors at transcriptional regulatory elements of the polyhedrin gene

All enhancer sequences identified thus far serve as binding sites for one or more *trans*-acting factors. The enhancer effect depends not only upon the mixture of sequence motifs comprising a particular enhancer but also upon the assortment of *trans*-acting factors present within a particular cell type or at different stages of development. This second level of complexity is particularly interesting because a number of enhancer motifs bind to more than one *trans*-acting factor, some of which may in turn be developmentally restricted in their distribution. Variations in the arrangement of binding

sites within an enhancer provide the potential to create new DNA–protein complexes by forming heterodimers among or within different families of transcription factors[35]. These interactions may result in enhancer specificity, additional regulatory controls, and a high level of transcription. It is possible that enhancer activity is the result of the additive effects of a single redundant enhancer motif. Alternatively, multimerized enhancers could become nucleation sites for the various factors required for enhancer activity.

In order to determine the factors involved in *hr*1 interactions, we carried out gel retardation assays with nuclear extract prepared from uninfected cells and AcMNPV-infected cells 3, 6, 12, 36 and 50 h p.i. With the 330 fragment of *hr*1 (this fragment carries one full and one half palindrome with the intermediate sequence) as probe, a stronger shift of higher mobility and a weaker shift of lower mobility were detected at all time points[36]. There was no visible difference in the complexes obtained with extracts from uninfected and infected cells, thereby indicating the involvement of host factor(s) in *hr*1 interactions.

There are reports of both cellular host and viral proteins binding to viral enhancer elements. The active enhancer–transactivator complexes in the SV40 enhancer include interactions with several motifs in the enhancer element and there are a number of reported pairs of bound factors that may serve as functional enhancer element complexes. The HeLa cell nuclear extracts, for instance, contain an SphI and SphII motif binding factor that interacts more tightly with the SphII motif[30]. Also, the purified factors AP-2 and AP-3 may represent factors that cooperate to form an active SV40 enhancer element[36,37]. A nuclear protein, called EF-C, binds to functionally important sites in the polyomavirus (Py) and hepatitis B virus (HBV) enhancer regions and has been found to correspond to an additional activity, RFX, which is a family of related transcription factors that form homo- or heterodimers and bind to the conserved functionally important X box in the MHC class II antigen promoter regions. The Py and HBV enhancer regions have evolved high affinity binding sites for dimeric EF-C/RFX-1 and the interaction of EF-C with an intact inverted repeat is required for functional activity of viral enhancers[38]. Host and tissue-specific factors that interact with distinct regions of the enhancer sequences of the U3 region of the rous sarcoma virus LTR have been identified[39]. These factors share homology among themselves and with Y-box (inverted CCAAT) factors[40].

A number of factors interacting with cellular enhancers have been identified. The well-characterized cellular Ig heavy and light chain enhancers, which are functionally redundant regions of 500–700 bp, contain multiple copies of an 'E' motif as well as recognition sites for the NF-κB and cell-specific octamer binding proteins[32]. The sites defined by protein binding are important for the functional activity of Ig enhancers with those restricted in their cellular distribution playing the most important roles. The 38 kDa *hr*1-binding host factor is the only *hr*-binding host protein of AcMNPV identified so far. The other *hr*-binding factor, IE-1, is a viral immediate-early gene product whose interaction with *hr*5 and *hr*1a has been analysed[2,41]. Interestingly, transcriptional enhancer activity of *hr*5 requires dual palindrome half-sites that mediate binding of an IE-1 dimer[42]. Studies on protein interactions with both *hr*5 and *hr*1a have failed to detect a specific host factor–*hr* interaction, although the possibility of host factor(s) interacting with *hr*1a of AcMNPV has not been completely negated[2]. Another host factor, the 30 kDa PPBP, interacts with transcriptionally important sequences within the polyhedrin gene promoter. PPBP exhibits both single and double-stranded DNA-binding activity, interacts with the promoter with very high specificity and affinity and requires phosphorylation for binding[7,8]. It is of significance that two host proteins may be involved in mediating transcription from the hyperactive and very late polyhedrin gene promoter of AcMNPV.

The *hr*1-binding host factor binds at multiple sites within the *hr*1 element. We consistently obtained specific complexes with the uninfected nuclear extract irrespective of the region of *hr*1 being used as probe. The number of complexes obtained with different *hr*1 fragments depend upon the length of the probe and the nature of sequences therein. The 420 bp probe carries three full palindromes, 9 bp of the fourth palindrome and four palindrome-flanking sequences and generates three complexes. The 330 bp probe carries two palindromes with two flanking inter-core sequences and forms two complexes; the 170 bp fragment has a single intact palindrome with two flanking sequences and also forms two complexes; the 158 bp and 90 bp probes which carry 128 bp and 90 bp inter-core sequences, respectively form a single complex each. On the other hand, a palindrome alone does not bind the factor, suggesting that sequences flanking the palindrome core are important for binding and a palindrome alone is not sufficient for this interaction. The minimal functional enhancer element, as revealed by analysis of *hr*1 deletion constructs in transient expression assays, is also a core palindrome together with the core-flanking sequences. The host protein binding sites within *hr*1 thus define the minimal functional enhancer elements of this sequence.

The nature of the palindrome-flanking sequences required for *hr*1–host factor interaction was revealed by using nine overlapping double-stranded oligodeoxyribonucleotides encompassing different regions of an *hr*1 palindrome and its 5′ and 3′ flanking sequences as competitors in a binding reaction with the 170 bp fragment of *hr*1[36]. Results of these experiments demonstrated that short segments representing both 5′ and 3′-flanking se-

quences are required for the DNA–protein interaction. A half-palindrome, a palindrome alone, or flanking sequences alone without the palindrome are not sufficient for the interaction. Moreover, an oligonucleotide carrying an intact palindrome with shuffled flanking sequences could act at an efficient competitor of protein binding, indicating promiscuity in the sequence requirement of the 9-bp 5′ and 3′ palindrome-flanking segments required for binding of the *hr*1-binding protein (*hr*1-BP).

The position and intensity of the retarded DNA–protein complex obtained with *hr*1 in gel retardation assays does not change throughout, suggesting that either the factor is not modified during the infection cycle or that its DNA-binding activity is not drastically altered by the modification[36]. Phosphorylation is required for binding and the dephosphorylated host protein is incapable of binding *hr*1. The cellular factor binds *hr*1 with high specificity and affinity. Though there is a slight concentration-dependent decrease in complex formation in the presence of salt, a complex is obtained even when 2 M NaCl is added to the binding reaction. This suggests that ionic interactions do not play a major role in complex formation. The host protein approaches *hr*1 through the minor groove of the DNA double helix[36]. The host factor–*hr*1 complex is also not disrupted in the presence of a high concentration of EDTA (up to 100 nM) indicating that divalent cations are not required for binding. A K_d value of $6.5\ (\pm 0.2) \times 10^{-11}$ M for the host factor–*hr*1 complex demonstrates the unusually high affinity of the factor for *hr*1. An extremely low K_d value ($\sim 3.7 \times 10^{-12}$ M) has also been reported for the other host protein, PPBP, that interacts with transcriptionally-important motifs within the polyhedrin promoter[7]. The high affinity of these cellular factors for viral sequences may be crucial for the virus to recruit them from their normal sites of action in the host cell.

Involvement of the *hr*1-binding protein in the enhancer function of *hr*1

To provide evidence for the functional role of the *hr*1-binding protein (*hr*1-BP), an *in vivo*-mopping assay[36] was developed. In this assay the binding of *hr*1-BP to the reporter constructs pSH*luc* and pSH*luc*-*hr*-U$_1$ was completed in the presence of increasing concentrations of the pUC18-based plasmid carrying the *hr*1 sequence alone (pSH*hr*1). This construct pSH*hr*1, which carries only the *hr*1 sequence cloned in pUC18, when used as competitor to sequester the *hr*1-BP present in the cells, would gradually render *hr*1-BP unavailable for binding to the *hr*1 sequence present in *cis* to the polyhedrin promoter-driven luciferase reporter in pSH*luc*-*hr*-U$_1$. Sf9 cells co-transfected with the reporter construct as well as different amounts of the competitor plasmid were assayed for luciferase expression 60 h p.i[36]. As expected, the presence of pSH*hr*1 did not have any effect on the expression of pSH*luc*, which did not carry the *hr*1 element. The expression from pSH*luc*-*hr*-U$_1$, which had been enhanced several-fold by the enhancer effect of *hr*1, was reduced proportionately as the amount of competitor was increased to 1, 5, and 10 µg. In the presence of 10 and 20 µg of pSH*hr*1 competitor, the reporter expression level was brought down to that of the basal plasmid pSH*luc*. This demonstrated that the non-availability of *hr*1-BP due to the binding of this factor to an *hr*1 sequence present in *trans* abolishes the enhancement effect. The enhancer-binding host factor is therefore important for mediating the enhancer function of *hr*1 *vis-a-vis* the *polh* promoter of AcMNPV.

Mechanisms of enhancer action

Promoters and enhancers are two primary DNA elements that control transcription by RNA polymerase II. Promoters function proximal and upstream to the transcription initiation site and determine where transcription begins. Enhancers stimulate promoters and function distal to the initiation site either upstream or downstream. Enhancers can also function as origins of DNA replication. While the primary function of promoters and origins of replication is to facilitate the assembly of an active initiation complex, the primary function of enhancers is controversial. Two models of enhancer action are generally considered. In the first model, enhancers and promoters are considered to act together in the formation of an active transcription initiation complex with additional transcription factors that can act at a greater distance from the mRNA start site being provided by the enhancers[43,44]. Evidence to support this model is the fact that transcription factors can frequently function as components of either promoters or enhancers. In the second model, promoters and enhancers perform distinct functions; the promoter facilitates formation of an active initiation complex and enhancers act from a distance to relieve repression of weak promoters by altering chromatin structure[45,47]. Support for this model comes from studies on analysis of gene expression in mouse preimplantation embryos[48], transcription in cell free systems[49] and manipulating the activity of enhancers in stably-integrated plasmid constructs[47]. A small number of studies reported recently dispute the model that enhancers increase the rate of transcription from promoters and propose that enhancers increase the number of transcriptionally active templates, i.e. in a population of transfected cells enhancers increase the number of expressing cells but not their level of expression[47].

Of the many proposed mechanisms of enhancer action that include 'looping', 'twisting', 'sliding' or 'tracking',

and 'oozing', the mechanisms of DNA looping and alteration of chromatin structure are most widely accepted (reviewed by Ptashne[50]; Felsenfeld[45]). The mechanism of looping proposes that proteins bound at widely separated sites act by contacting each other, with the intervening DNA bending or looping to allow protein–protein interaction; the interaction between DNA-bound proteins, not looping *per se* regulates gene expression. Amongst other experimental evidence, the support for this model has come from 5 or 15 bp and 10 or 21 bp insertions between the middle segment and the enhancer and the middle segment and the TATA in the SV40 early gene promoter region[51]. The insertion of 5 or 15 bp decreased transcription *in vivo* more drastically than the insertion of 10 or 21 bp. The enhancer functions even when repositioned hundreds of base pairs from the middle segment. A simple interpretation of these experiments is that proteins bound to the enhancer contact other proteins bound to the middle segment and that these in turn contact a protein bound at TATA. When the enhancer is placed at a distance (and in the normal position as well) the DNA presumably loops out to allow protein–protein interaction.

The model for enhancer action involving alteration in chromatin structure has been developed from a series of experiments. Long-range activation of transcription by GAL4-VP16 protein, bound 1300 bp upstream, was dependent on packaging of the template into histone H1-containing chromatin[49]; the chromatin structure of the rat prolactin gene facilitates the occurrence of protein–protein interactions between transcription factors bound to widely-separated regulatory elements[52]; 1-cell mouse preimplantation embryos can utilize enhancer-responsive promoters efficiently without an enhancer, whereas 2-cell embryos require an enhancer to achieve the same levels of expression suggesting that enhancers relieve a repression in 2-cell embryos that is absent in 1-cell embryos[46]. Alteration of chromatin structure by enhancer elements has also been seen in viral enhancers. The DNA in the chromatin encompassing the SV40 enhancer is far more accessible to nucleases than other regions of SV40 DNA[53]. When present in *cis*, the SV40 enhancer can induce a DNAaseI hypersensitive site within the β-globin gene[54]. It has thus been proposed that the primary function of enhancers is to prevent general repression of promoter activity by altering chromatin structure. There are two kinds of mechanisms that disrupt chromatin structure. The first mechanism, dynamic competition, does not require DNA replication to make regulatory regions accessible to transactivators. In this model, *trans*-acting factors can bind at or near the nucleosomes to destabilize or displace the histone octamer. The other mechanism is the pre-emptive competition model. In these enhancers, core histones block the factor-binding sites in such a way that these are accessible only when the site is at least partially exposed at the replication fork before chromatin assembly[45].

The precise mode of temporal activation of the polyhedrin gene promoter is not clear at present. The direct involvement of any of the *lef* genes in transcription from the promoter has not been demonstrated[55]. The observation by Morris and Miller[56] that the basal promoter activity is contained within a 18 bp sequence comprising of the TAAGTATT motif and the upstream AATAAA sequence, which are required for PPBP binding[7,8,57,58] has added a new dimension to the enigma of regulation of transcription from this 'initiator' promoter. *Hr*1 could simply be acting through its enhancer function to aid in the activation of the polyhedrin promoter[59]. The 38 kDa host factor interacts with *hr*1 with very high affinity, requires phosphorylation for binding and approaches the AT-rich *hr*1 sequence through the minor groove of the DNA double-helix. The binding of the host factor to *hr*1 may contribute to low levels of basal transcription relatively early in the infection cycle. Subsequently, as infection proceeds, the host factor may help create an environment which would make overall transcription more responsible to the presence of yet unidentified inducible host or viral proteins bound to nearby sites in *hr*1.

While it is likely that the two functions (*ori* and enhancer-like) are carried out by different sequence elements within *hr*1, some of them may overlap as demonstrated by the fact that a palindrome alone is sufficient for *ori* function but requires additional core flanking sequences for enhancer activity. Although, at present, we only have evidence to implicate the host *hr*1-BP in the enhancer function *hr*1, the possibility that the host factor is involved in the dual function of this sequence cannot be ruled out. Further investigations on the *ori* function of *hr* sequences in intact AcMNPV viruses are critical to an understanding of this element with demonstrated duality of function.

1. Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. and Possee, R. D., *Virology*, 1994, **202**, 586–605.
2. Leisy, D. J., Rasmussen, C., Kim, H. T. and Rohrmann, G., *Virology*, 1995, **208**, 742–752.
3. Huh, N. E. and Weaver, R. F., *J. Gen. Virol.*, 1990, **71**, 195–202.
4. Glocker, B., Hoopes, R. R., Jr., Hodges, L. and Rohrmann, G. F., *J. Virol.*, 1993, **67**, 3771–3776.
5. Guarino, L. A. and Summers, M. D., *J. Virol.*, 1986, **60**, 215–223.
6. Kool, M., Voeten, J. T. M., Goldbach, R. W., Tramper, J. and Vlak, J. M., *J. Gen. Virol.*, 1993, **74**, 2661–2668.
7. Burma, S., Mukherjee, B., Jain, A., Habib, S. and Hasnain, S. E., *J. Biol. Chem.*, 1994, **269**, 2750–2757.
8. Mukherjee, B., Burma, S. and Hasnain, S. E., *J. Biol. Chem.*, 1995, **270**, 4405–4411.
9. Hasnain, S. E., Habib, S., Jain, A., Burma, S. and Mukherjee, B., *Methods Enzymology: RNA Polymerase and Associated Factors* (eds Adhya, S., Abelson, J. N. and Simon, M. I.), Academic Press, New York, 1996, pp. 20–32.

10. DePamphilis, M. L., *Trends Cell Biol.*, 1993, **3**, 161–167.
11. Wilson, A. C. and Patient, R. K., *Nucleic Acids Res.*, 1993, **21**, 4226–4304.
12. O'Reilly, D. R., Crawford, A. M. and Miller, L. K., *Nature*, 1989, **337**, 606.
13. Gordon, J. D. and Carstens, E. B., *Virology*, 1984, **138**, 69–81.
14. Grosschedl, R. and Birnstiel, M. L., *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 7102–7106.
15. Moreau, P., Hen, R., Wasylyk, B., Everett, R. and Gaub, M., *Nucleic Acids Res.*, 1981, **9**, 6047–6069.
16. Fromm, M. and Berg, P., *Mol. Cell. Biol.*, 1983, **3**, 991–999.
17. Tyndall, C., La Mantia, G., Thacker, C. M., Favalero, J. and Kamen, J., *Nucleic Acids Res.*, 1981, **9**, 6231–6250.
18. de Villiers, J. and Schaffner, W., *Nucleic Acids Res.*, 1981, **9**, 6251–6264.
19. Atchison, M. L., *Annu. Rev. Cell Biol.*, 1988, 127–153.
20. Habib, S., Pandey, S., Chatterji, C., Burma, S., Ahmad, R., Jain, A. and Hasnain, S. E., *DNA Cell Biol.*, 1996, **15**, 737–747.
21. Carson, D. D., Summers, M. D. and Guarino, L. A., *J. Virol.*, 1991, **65**, 945–951.
22. Kool, M., Voeten, J. T. M., Goldbach, R. W., Tramper, J. and Vlak, J. M., *J. Gen. Virol.*, 1993, **74**, 2661–2668.
23. Leisy, D. J. and Rohrmann, G. F., *Virology*, 1993, **196**, 722–730.
24. Pearson, M., Bjornson, R., Pearson, G. and Rohrmann, G., *Science*, 1992, **57**, 1382–1384.
25. Marahrens, Y. and Stillman, B., *Science*, 1992, **255**, 817–823.
26. Mc Caroll, R. M. and Fangman, W. L., *Cell*, 1988, **54**, 505–513.
27. Dillin, A. and Rine, J., *Trends Biol. Sci.*, 1995, **20**, 231–235.
28. Crawford, A. M. and Miller, L. K., *J. Virol.*, 1988, **62**, 2773–2781.
29. Dynan, W. S., *Cell*, 1989, **58**, 1–4.
30. Davidson, I., Fromentai, C., Augereau, P., Wildeman, A., Zenke, M. and Chambon, P., *Nature*, 1986, **323**, 544–548.
31. Ondek, B., Gloss, L. and Herr, W., *Nature*, 1988, **333**, 40–45.
32. Lenardo, M., Pierce, J. W. and Baltimore, D., *Science*, 1987, **236**, 1573–1577.
33. Thompson, C. C. and McKnight, S. L., *Trends Genet.*, 1992, **8**, 232–236.
34. Bowers, W. J. and Ruddel, A., *J. Virol.*, 1992, **66**, 6578–6586.
35. Tjian, R. and Maniatis, T., *Cell*, 1994, **77**, 5–8.
36. Habib, S. and Hasnain, S. E., *J. Biol. Chem.*, 1996, **271**, 28250–28258.
37. Mitchell, P. J., Wang, C. and Tjian, R., *Cell*, 1987, **50**, 847–861.
38. David, E., Garcia, A. D. and Hearing, P., *J. Biol. Chem.*, 1995, **14**, 8353–8360.
39. Guntaka, R. V., Kandala, J. C., Shin, B. A., Nambiar, A., Svoboda, J. and Cleavinger, P., *Folia Biologica* (Praha), 1994, **40**, 237–247.
40. Wolffe, A. P., Tafuri, S., Ranjan, M. and Familari, M., *New Biologist*, 1992, **4**, 290–298.
41. Guarino, L. A. and Dong, W., *Virology*, 1994, **200**, 328–335.
42. Rodems, S. M. and Friesen, P. D., *J. Virol*, 1995, **69**, 5368–5375.
43. Carey, M., Leatherwood, J. and Ptashne, M., *Science*, 1990, **247**, 710–712.
44. Schatt, M. D., Rusconi, S. and Schaffner, W., *EMBO J*, 1990, **9**, 481–487.
45. Felsenfeld, G., *Nature*, 1992, **355**, 219–224.
46. Majumdar, S., Miranda, M. and DePamphilis, M. L., *EMBO J.*, 1993, **12**, 1131–1140.
47. Walters, M. C., Margis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groudine, M. and Martin, D. I. K., *Genes Dev.*, 1996, **10**, 185–195.
48. Majumdar, S. and DePamphilis, M. L., *BioEssays*, 1995, **17**, 879–889.
49. Laybourn, P. J. and Kadonaga, J. T., *Science*, 1992, **257**, 1682–1685.
50. Ptashne, M., *Nature*, 1986, **322**, 697–701.
51. Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A. and Zenke, M., *Nature*, 1986, **319**, 121–126.
52. Cullen, K. E., Kladde, M. P. and Seyfred, M. A., *Science*, 1993, **261**, 203–206.
53. Jongstra, J., Reudelhuber, T. L., Oudet, P., Benoist, C. and Chae, C. B., *Nature*, 1984, **307**, 708–714.
54. Enver, T., Brewer, A. C. and Patient, R. K., *Nature*, 1985, **318**, 680–683.
55. Lu, A. and Miller, L. K., *J. Virol.*, 1995, **69**, 975–982.
56. Morris, T. D. and Miller, L. K., *Gene*, 1994, **140**, 147–153.
57. Hasnain, S. E., Jain, A., Habib, S., Ghosh, S., Chatterji, U., Ramachandran, A., Das, P., Venkaiah, B., Pandey, S., Liang, B., Ranjan, A., Natarajan, K. and Azim, C. A., *Gene*, 1997, **190**, 113–118.
58. Habib, S., Burma, S., Chatterjee, U., Das, P., Jain, A., Mukherjee, B., Natarajan, K., Ranjan, A. and Hasnain, S. E., *Current Developments in Animal Virology* (eds Jameel, S. and Wagner, E. K.), Oxford and IBH, India, 1996, pp. 205–210.
59. Venkaiah, B., Habib, S., Hasnain, S. E., US Patent application no. U 011199-O, 1997.

ACKNOWLEDGEMENT. This work was supported by a grant to S.E.H. from DST and the Young Scientist grant to S.H. from the Indian National Science Academy, New Delhi.

Received 16 September 1997; accepted 19 September 1997