Identification of Two Independent Transcriptional Activation Domains in the *Autographa californica* Multicapsid Nuclear Polyhedrosis Virus IE1 Protein

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The *Autographa californica* multicapsid nuclear polyhedrosis virus immediate-early protein, IE1, is a 582-amino-acid phosphoprotein that regulates the transcription of early viral genes. Deletion of N-terminal regions of IE1 in previous studies (G. R. Kovacs, J. Choi, L. A. Guarino, and M. D. Summers, J. Virol. 66:7429–7437, 1992) resulted in the loss of transcriptional activation, suggesting that this region may contain an acidic activation domain. To identify independently functional transcriptional activation domains, we developed a heterologous system in which potential regulatory domains were fused with a modified *Escherichia coli* Lac repressor protein that contains a nuclear localization signal (^NLacR). Transcriptional activation by the resulting ^NLacR-IE1 chimeras was measured with a basal baculovirus early promoter containing optimized Lac repressor binding sites (*lac* operators). Chimeras containing IE1 peptides dramatically activated transcription of the basal promoter only when *lac* operator sequences were present. In addition, transcriptional activation by ^NLacR-IE1 chimeras was allosterically regulated by the lactose analog, isopropyl-β-D-thiogalactopyranoside (IPTG). For a more detailed analysis of IE1 regulatory domains, the M1 to T266 N-terminal portion of IE1 was subdivided (on the basis of average amino acid charge) into five smaller regions which were fused in various combinations to ^NLacR. Regions M1 to N125 and A168 to G222 were identified as independent transcriptional activation domains. Some ^NLacR-IE1 chimeras exhibited retarded migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. As with wild-type IE1, this aberrant gel mobility was associated with phosphorylation. Mapping studies with the ^NLacR-IE1 chimeras indicate that the M1 to A168 region of IE1 is necessary for this phosphorylation-associated effect.

The baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) is a large double-stranded DNA virus that encodes over 150 major open reading frames (1). Viral gene expression is subdivided into early and late phases. Early-phase genes are transcribed by the host RNA polymerase II (20) and encode proteins that are required for DNA replication and late-gene transcription. After DNA replication, late-phase genes are transcribed by a virus-specific RNA polymerase (for reviews, see references 3, 42, and 50). A protein that appears to be pivotal in regulating the infection cycle is the 67-kDa immediate-early protein 1 (IE1). IE1 was initially characterized as a potent transcriptional activator of early genes (16) and is essential for both DNA replication and late transcription. IE1 homologs have been identified in *Orgyia pseudotsugata* MNPV, *Bombyx mori* NPV, *Choristoneura fumiferana* MNPV, and *Helicoverpa zea* NPV (12, 21, 32, 56). *ie1* gene expression is detected throughout infection, due to the presence of early and late viral promoters (18, 29, 47). The persistence of IE1 may reflect its multiple functions during the course of infection. A temperature-sensitive IE1 mutant of AcMNPV was used to demonstrate the role of IE1 in regulating the transcription of the PE38 and ETL genes during AcMNPV infection (48). In transient expression studies, the IE1 protein has been shown to promiscuously activate the expression of a heterologous group of promoters, including a number of early viral promoters (5, 16, 45) and a host cell promoter (39). Transcriptional activation by IE1 may occur by two distinct mechanisms: (i) sequence-independent activation

that requires only basal promoter motifs and does not appear to involve specific DNA sequence recognition by IE1 (4) and (ii) sequence-dependent activation of enhancer-associated promoters (17). Enhancers of early transcription in AcMNPV are tandem arrays of imperfect palindromes which are called homologous repeats (hr) and are distributed throughout the viral genome (1, 11, 15). IE1 binds as a dimer to the 28-bp imperfect palindrome subunit of AcMNPV hr DNA (28, 49). IE1 may also play a role in stimulating viral DNA replication, as hr sequences can function as replication origins when they are transfected into virus-infected cells (26, 27, 46). Because IE1 may also function as a negative regulator of viral transcription (7, 30), it is referred to as a *trans*-regulator. It was recently reported that IE1 negatively regulates transcription from the pe38 and ie2 promoters by binding specifically to an 8-bp consensus sequence near the transcriptional start sites of these genes (35).

Studies of truncations of the wild-type IE1 protein have suggested the potential locations of several functional domains (28). Deletions of 52 to 145 residues from the N terminus of IE1 reduced or eliminated transcriptional activation but did not affect binding to hr DNA. In contrast, the deletion of ≥25 amino acids from the C terminus of IE1 severely reduced or eliminated IE1 binding to hr DNA. Although IE1 is phosphorylated (9, 10) and is found primarily in the nucleus (55a), phosphorylation sites and domains required for nuclear localization have not yet been mapped. It is therefore not yet clear how deletions in the wild-type IE1 protein may affect the nuclear localization, phosphorylation, or dimerization of the native IE1 protein. Because N-terminal deletions (which affected transcriptional activation) removed a cluster of negatively charged amino acids (28), and because acidic domains

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are characteristic of transcriptional activators such as GCN4, GAL4, and VP16 (6, 19, 57), it was suggested that the N terminus of IE1 may contain an acidic activation domain (28, 56).

To independently identify activation domains from transcriptional activator proteins, putative activation domains can be fused to the DNA binding domain from a well-characterized protein such as LexA or Lac repressor (LacR) (2, 6, 33, 37). By the measurement of the activity of such chimeras on promoter constructs containing the appropriate DNA binding site, transcriptional activation domains can be identified and dissected in isolation from other functions or activities of the wild-type protein. In the current study we used the LacR protein to generate LacR-IE1 chimeras. LacR is an extensively characterized transcriptional regulatory protein (14). Functional domains of LacR have been identified by genetic studies and X-ray crystallography (13, 36). The potential of chimeric LacR constructs as allosterically regulated transcriptional activators has been previously established (2, 33, 37). For this study, we generated a construct (^NLacR) encoding the DNA binding and dimerization domains of the *Escherichia coli* LacR protein and the nuclear localization signal (NLS) from the simian virus 40 (SV40) large-T antigen. To map transcriptional regulatory domains from IE1, we generated ^NLacR chimeras containing portions of the AcMNPV IE1 protein. To quantify transcriptional activation by LacR-IE1 chimeras, we constructed a target promoter-reporter plasmid containing LacR binding sites upstream of a previously characterized baculovirus basal promoter (5) and a chloramphenicol acetyltransferase (CAT) reporter gene. Using this system, we identified two independent transcriptional activation domains from the IE1 protein. In addition, two potential transcription inhibitory domains and a phosphorylation-associated region within the IE1 protein were mapped.

MATERIALS AND METHODS

Cells and antisera. *Spodoptera frugiperda* cells from Sf21 and Sf9 cell lines (58) were maintained in TNM-FH (18a) medium (Gibco) supplemented with 10% fetal bovine serum as described previously (4). Anti-LacR antiserum was generated in a Flemish Giant Chinchilla Cross rabbit by three inoculations with 100 µg of purified LacR protein (Promega) and 0.5 ml of complete-incomplete (1:2) Freund's adjuvant per injection, at 2-week intervals.

Plasmid constructs. The *lacI* gene (encoding the LacR protein) was amplified by PCR from plasmid pKSM715 (41) with the primers LLP (5'-GGCCCGGCGAAGTACTATGAACCAAGTAAC-3') and LRP (5'-CGTTAATTAAGTTGCGTCACTGCC-3'). Primer LLP included a mutation (indicated in lowercase) that changed the bacterial *lacI* GTG translational start site to ATG. *XmaI* and *PacI* sites were engineered into the ends of the *lacI* PCR product. Sequences encoding the NH₂-MPKKRKRV-COOH residues of the SV40 large-T antigen NLS were added to the 5' end of the *lacI* construct by synthesizing two complementary primers (5'-CCGATCCTAGACTATGACGCAACCTAAGAAGAAGAGGAAGGTTTC-3' and 5'-CCGGGAACCTCTCTCTCTCTAGGTTGCGTCATAGTCAGGAT-3') with engineered *XmaI* site overhangs. Annealed NLS primers were ligated into an *XmaI* site, upstream and in frame with the *lacI* gene, and the protein product resulting from the NLS-Lac repressor fusion is referred to as ^NLacR. The resulting ^NLacR-encoding plasmid, p166NLSlacI, contained OpMNPV gp64 early promoter sequences (-166 to -6) (5), a Kozak consensus sequence ATG (31), the ^NLacR open reading frame, and the AcMNPV IE1 polyadenylation signal.

^NLacR-IE1 fusion constructs were generated by PCR amplifying *ie1* gene regions and ligating them into the *KasI* site in the *lacI* gene, which results in fusion after P333 of the LacR protein and removal of the 28 C-terminal amino acids of LacR. *KasI* and *XhoI* sites and stop codons were engineered into PCR primers so that *ie1* gene fragments were flanked by a *KasI* site on the N-terminal end and by a stop codon and an *XhoI* site on the C-terminal end. *ie1* gene PCR fragments were all cloned as *KasI/XhoI* fragments, and each cloned PCR fragment was verified by DNA sequencing.

To assay the function of ^NLacR-IE1 fusion proteins, we cloned a CAT reporter gene under the control of a basal promoter and two *lac* operator (LacO) sequences. A perfectly palindromic analog of the partially palindromic wild-type *E. coli* LacR binding site (O₁ LacO) was previously shown to bind LacR more tightly than the wild-type LacO (51, 54). A self-complementary 22-nucleotide oligomer (5'-GATCGAATTGTGAGCGCTCACAATTC-3') encoding the LacO analog se-

quence was annealed and ligated as a concatameric double LacO insertion into the *SmaI* site of plasmid pBS-BglII to generate plasmid pBS-BglII-LacO2. A fragment containing a CAT reporter gene under the control of a basal promoter was then inserted 33 bp downstream of the LacO sequences. The basal promoter, CAT gene, and polyadenylation signal were excised from plasmid p64CAT-166 (5) as a *MluI-HindIII* fragment and ligated into the *HincII-HindIII* sites of pBS-BglII-LacO2 to produce the plasmid p64CAT-92LacO. The p64CAT-92LacO construct was identical to plasmid p64CAT-92 (5) except that it contained two tandem LacO sites 33 bp upstream of the basal promoter (Fig. 1C).

Transfections and CAT assays. For each experiment, transfections were performed in 24-well plates (188 mm²/well) with 3.5×10^5 Sf21 or Sf9 cells per well and 3 µg of supercoiled plasmid DNA per well as described previously (5). After transfection, cells were incubated in TNM-FH medium at 27°C. At 36 h post-transfection, cells were washed with phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 2.7 mM KCl, and 120 mM NaCl [pH 7.4]), resuspended in 500 µl of PBS, and lysed by three cycles of freezing and thawing. Extracts were heated at 65°C for 15 min, and insoluble cellular debris was removed by centrifugation. Supernatants were removed and stored at -70°C for CAT assays. CAT assays were performed by the two-phase fluor diffusion assay (44) as described previously (4) with 20 µl of each cell extract. CAT activity was calibrated with a purified CAT enzyme (Sigma).

Immunofluorescence. For analysis of nuclear localization by immunofluorescence microscopy, Sf21 cells (10^5 cells) were transfected with 1 µg of plasmid DNA by CaPO₄ precipitation. At 36 h posttransfection, the cells were washed with PBS and then fixed for 30 min in 4% paraformaldehyde (Sigma) in PBS. The cells were permeabilized by incubation in 0.3% Triton X-100 (Sigma) in PBS for 10 min. Anti-LacR polyclonal antiserum (Promega) was diluted 1:500 in blocking buffer (PBS, 1% bovine serum albumin, 1:500 normal goat serum [Sigma]) and applied to the cells for 2 h. After two 5-min washes in blocking buffer, a secondary antibody [goat F(ab')₂ anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (Pierce)] was diluted 1:500 in blocking buffer and applied for 2 h. Cells were washed three times with blocking buffer and mounted onto glass slides in 90% glycerol-2.5% DABCO (1,4-diazobicyclo[2.2.2]octane). Immunofluorescence imaging was done by confocal scanning microscopy (model MRC600; Bio-Rad) on a Zeiss Axiocvert 10 microscope with a 40× objective.

SDS-PAGE and Western blot analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, Sf9 or Sf21 cells (3×10^5 cells) were washed with PBS before lysis in 500 µl of 1× disruption buffer (125 mM Tris-HCl, 1% SDS, 2.5% mercaptoethanol, 10% glycerol, 0.2% bromophenol blue). After denaturation (at 95°C for 5 min), proteins were electrophoresed on SDS-PAGE gels (1.5×10^4 cells per lane) and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Blots were incubated for 1.5 h in anti-LacR polyclonal antiserum diluted at 1:4,000 in PBS-T (0.05% Tween 20 in PBS), washed three times in PBS-T, then incubated for 1.5 h in a 1:10,000 dilution of secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham). Following three washes in PBS-T, horseradish peroxidase-immunolabeled proteins were visualized by enhanced chemiluminescence (Amersham).

Dephosphorylation assays. Sf9 cells (10^6 cells) were washed and suspended in 500 µl of chilled Tris-buffered saline (137 mM NaCl, 2 mM KCl, and 25 mM Tris base [pH 7.4]). Cell suspensions were separated into 2 equal volumes and pelleted. The cells were resuspended and then lysed by freezing and thawing in either 250 µl of phosphatase inhibitor buffer (PIB; 1 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 200 mM NaV [pH 7.4]) or 250 µl of phosphatase buffer (PB; 200 mM ZnSO₄, 1 mM MgCl₂, 50 mM Tris base [pH 8.5]). Also present in all of the lysates was 1× proteinase inhibitor mix (1 mM pepstatin, 1 mM leupeptin, and 4 mM Pefabloc SC) (Boehringer Mannheim). A 50-µl aliquot of the PB lysate was removed, and 5 µl of 1% SDS-25 mM dithiothreitol was added. This was followed by incubation at 95°C for 5 min, followed by cooling to 4°C and the addition of 5 U of calf intestinal phosphatase (CIP; Promega). This sample was called the PB + CIP lysate. The PIB, PB, and PB + CIP lysates were each incubated for 1.5 h at 37°C. An equal volume of 2× SDS-PAGE disruption buffer was then added to each lysate, and the proteins were denatured at 95°C for 5 min. During the incubation at 95°C in SDS-PAGE disruption buffer, 5 U of CIP was added to each of the PIB and PB lysates so that SDS-PAGE gel protein loadings were equivalent. CIP added in this manner was immediately inactivated. Protein from 2.5×10^4 cells was loaded into each lane of an SDS-6.5% PAGE gel. After electrophoresis, proteins were immunoblotted and probed with anti-LacR antiserum as described above.

RESULTS

The N terminus of the AcMNPV IE1 protein is highly acidic, and deletions from the N terminus of the wild-type IE1 protein indicated that regions within the N terminus are necessary components of a fully functional transcriptional activator protein (28). To determine if the N-terminal regions alone were sufficient to function as transcriptional activation domains, we developed a heterologous system in which potential transcriptional activation domains of IE1 could be assayed in isolation

FIG. 1. Strategy for mapping transcriptional activation domains with chimeric LacR-IE1 proteins. (A) An SV40 NLS was fused to the N terminus of LacR to generate the protein ^NLacR. ^NLacR-IE1 chimeras were generated by fusing portions of the IE1 protein to ^NLacR at Pro 333 of LacR. A chimeric protein containing the M1 to T266 region of AcMNPV IE1 fused to the C terminus of ^NLacR is illustrated schematically and labeled ^NLacR-IE1(M1-T266). (B) The subcellular localization of proteins LacR, ^NLacR, and ^NLacR-IE1(M1-T266) in transfected Sf21 cells was determined by immunofluorescent staining with an anti-LacR polyclonal antiserum and confocal microscopy. Mock-transfected cells received only vector plasmid (pBS) DNA. Cells were fixed, permeabilized, and stained at 36 h posttransfection and then examined by confocal microscopy. (C) Reporter constructs p64CAT-92 (5) and p64CAT-92LacO are illustrated. Both constructs contain a 92-bp basal early promoter and a CAT reporter cassette. p64CAT-92LacO also contains two LacR binding site sequences (LacO) 125 bp upstream of the translational start point. (D) Sequence-specific transcriptional activation by the ^NLacR-IE1 chimera. Plasmids encoding either the ^NLacR protein (^NLacR) or a ^NLacR-IE1 chimera [^NLacR-IE1(M1-T266)] were each cotransfected with either the p64CAT-92 or the p64CAT-92LacO reporter plasmid. In addition, cotransfections with plasmid p64CAT-92LacO were performed and maintained in the presence of 10 mM IPTG. Transfected Sf9 cells were harvested at 36 h posttransfection, and CAT activity was determined from cell lysates. CAT activity measurements are indicated as the average of four replicate cotransfections, and error bars represent standard error.

from other potential functions or interactions of the wild-type IE1 protein. For these studies, portions of the IE1 protein were fused to the C terminus of the *E. coli* LacR protein. To ensure that LacR-IE1 chimeras were efficiently translocated to the nucleus, the NLS of the SV40 large-T antigen (22) was fused to the N terminus of LacR to produce ^NLacR (Fig. 1A). Using an anti-LacR antiserum and confocal microscopy, we compared the cellular localization of wild-type LacR, ^NLacR, and an ^NLacR-IE1(M1-T266) chimera in transfected Sf21 insect cells (Fig. 1B). Whereas the wild-type *E. coli* LacR protein was detected predominantly in the cytoplasm, the ^NLacR construct and the ^NLacR-IE1(M1-T266) chimera were detected primarily within the nuclei (Fig. 1B). Thus, the SV40 NLS was sufficient to target both the ^NLacR construct and the larger LacR-IE1(M1-T266) chimera to the nuclei of Sf21 cells.

To analyze transcriptional activation by ^NLacR-IE1 chimeras, we constructed a target promoter-reporter plasmid containing optimized LacR binding sites (LacO), a basal early

promoter derived from a baculovirus (5), and a CAT reporter cassette (Fig. 1C). Consistent with the results of previous studies of the basal promoter (5, 24), transcription measured by transient expression assays of this target promoter-reporter (p64CAT-92LacO) was very low, and cotransfection of p64CAT-92LacO with the unfused Lac repressor construct (^NLacR) did not result in reporter activation (Fig. 1D, mock versus ^NLacR). When the N-terminal M1 to T266 portion of IE1 was fused to ^NLacR, the resulting chimeric protein, ^NLacR-IE1(M1-T266), activated the expression of the operator-containing construct (p64CAT-92LacO) but not that of the operatorless basal promoter construct (p64CAT-92) [Fig. 1D, ^NLacR-IE1(M1-T266)]. This result indicates that activation by the chimeric LacR-IE1 construct was dependent on and mediated by LacR-IE1 binding to the operator. To confirm this conclusion, the same experiment was performed in the presence of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG), which binds to LacR and prevents its interaction with operator

FIG. 2. Amino acid charge profile of the N terminus of AcMNPV IE1 protein. The 582-amino-acid IE1 protein is represented as a bar with the acid-base charge profile of the N terminus shown below (acidic regions are black; basic regions are white). Average charge was calculated from a 10-residue window. Five charge regions were designated as follows: A (M1-A65), B (A65-N125), C (G123-A168), D (A168-G222), and E (G222-T266). Region A was further divided into two subregions, ¹A (M1-S17) and ²A (S17-A65). Amino acid sequences from each region (¹A to E) of the AcMNPV IE1 protein are shown below, with acidic amino acids (black boxes) and basic amino acids (white boxes).

sequences. In the presence of IPTG, p64CAT-92LacO activation by the ^NLacR-IE1 chimera was greatly diminished [Fig. 1D, ^NLacR-IE1(M1-T266)]. We also observed that ^NLacR could competitively inhibit ^NLacR-IE1 activation when ^NLacR, ^NLacR-IE1(M1-T266), and p64CAT-92LacO were cotransfected into insect cells (data not shown). As expected, the promiscuous wild-type IE1 protein activated transcription from both reporter constructs, and activation was not inhibited by IPTG (data not shown). The promiscuous transcriptional activation by wild-type IE1 contrasts with the sequence-specific activation observed from ^NLacR-IE1 chimeras. For all subsequent ^NLacR-IE1 fusion constructs, the requirement for LacO sequences for transcriptional activation and the inhibition by IPTG were verified (data not shown). In these experiments, we demonstrated that the ^NLacR-IE1(M1-T266) chimera activated the reporter gene in a LacO-dependent fashion, and we can conclude that the N-terminal portion of IE1 (M1 to T266) contains at least one domain that is sufficient for the activation of transcription, independent of the C-terminal region of the IE1 protein.

After it was established that the N-terminal 266 amino acids of IE1 were sufficient to convert ^NLacR to a transcriptional activator, the 266-residue N-terminal portion of IE1 was subdivided into five regions (approximately 60 amino acids each) based on the amino acid charge profile. These regions, illustrated in Fig. 2, are designated A (M1-A65), B (A65-N125), C (G123-A168), D (A168-G222), and E (G222-T266) according to the relative position of each region. Region A was further divided into subregions ¹A (M1-S17) and ²A (S17-A65) for a more detailed examination of that region. Consequently, region A is referred to as region ^{1,2}A. Sequences encoding individual regions (Fig. 2) or groups of these regions were amplified from the IE1 gene by PCR and fused to the ^NLacR construct at P333 of LacR. Each ^NLacR-IE1 chimera was named according to the IE1 protein region or group of regions included in the construct. For example, the largest chimera, ^NLacR-IE1(M1-T266), is referred to as ^{1,2}ABCDE. To identify independent transcriptional activation domains and to examine potential interactions between regions, we generated three series of constructs representing N-terminal truncations, C-

FIG. 3. Transcriptional activation by ^NLacR-IE1 chimeras containing N-terminal truncations of IE1 charged regions. (A) N-terminal truncations of the M1 to T266 portion of IE1 were fused to P333 of ^NLacR. Construct names (left) correspond to the charged regions of IE1 present in each ^NLacR-IE1 construct. Bars represent the relative sizes of the IE1 region fused to ^NLacR, and numbers below the diagram indicate IE1 amino acid positions. (B) Sf21 cells were cotransfected with the CAT reporter plasmid p64CAT-92LacO and each ^NLacR-IE1 chimera containing an IE1 N-terminal truncation. Promoter activation by each chimera is indicated as fold CAT activity above reporter alone. Each bar represents an average of three transfections, and error bars represent standard error. Controls for each experiment included transfection with p64CAT-92LacO alone, cotransfection of each ^NLacR-IE1 construct with p64CAT-92, and cotransfection of each ^NLacR-IE1 construct with p64CAT-92LacO in the presence of IPTG (data not shown). (C) Western blot analysis of chimeric ^NLacR-IE1 proteins. Proteins from Sf9 cells transfected with plasmids encoding ^NLacR-IE1 chimeras were separated by electrophoresis on SDS-PAGE gels and examined by Western blot analysis with an anti-LacR antiserum.

FIG. 4. Transcriptional activation by ^{N1}LacR-IE1 chimeras containing C-terminal truncations of IE1 charged regions. (A) C-terminal truncations of the M1 to T266 portion of IE1 were fused to P333 of ^{N1}LacR. Construct names (left) correspond to the charged regions of IE1 present in each ^{N1}LacR-IE1 construct. Bars and labels are represented as in Fig. 3A. (B) CAT reporter activation by ^{N1}LacR-IE1 chimeras containing IE1 C-terminal truncations. Cotransfections and controls are as described in Fig. 3. Each bar represents the average of three transfections, and error bars represent standard errors. (C) Western blot analysis of chimeric LacR-IE1 proteins was performed as described for Fig. 3C.

terminal truncations, and small (approximately 60 residues) individual regions from the M1 to the T266 portion of IE1. All were fused to ^{N1}LacR, and expression of each chimera was verified by Western blot analysis.

N-terminal truncations of the M1 to T266 portion of IE1 in the ^{N1}LacR-IE1 chimeras are shown in Fig. 3. Removing the N-terminal 17 amino acids from the N terminus of ^{1,2}ABCDE to produce ²ABCDE reduced CAT activity by over 60%. Deletion of the entire 65-amino-acid ^{1,2}A region to produce BCDE further reduced CAT activity to near-background levels. Of the remaining N-terminal deletion mutants (CDE, DE, and E), only DE produced measurable transcriptional activation above background levels. Therefore, these data indicated an important role for the N-terminal 65 amino acids of IE1.

We also examined C-terminal truncations of the M1 to T266 portion of IE1 (Fig. 4). C-terminal truncations produced a complex pattern of activation. Removing the 44-amino-acid region E from the C terminus of ^{1,2}ABCDE to produce ^{1,2}ABCD resulted in an approximately four- to fivefold increase in CAT activity from the original ^{1,2}ABCDE construct and a total activity approximately 1,900-fold above that of the p64CAT-92LacO reporter alone (Fig. 4B, ^{1,2}ABCDE versus ^{1,2}ABCD). Removing region D from ^{1,2}ABCD to produce ^{1,2}ABC led to a reduction of approximately 75% of p64CAT-92LacO transcriptional activation compared with that of ^{1,2}ABCD. However, the approximately 450-fold activation level of ^{1,2}ABC was similar to the activation level for the entire ^{1,2}ABCDE construct. Further C-terminal deletions to produce ^{1,2}AB increased CAT activation to 1,200-fold above that for the reporter alone. Comparison of constructs ^{1,2}AB and

^{1,2}ABCD in several experiments (data not shown) revealed that ^{1,2}ABCD consistently produced 1.5- to twofold greater CAT activity than ^{1,2}AB did. Surprisingly, region ^{1,2}A alone induced comparatively little CAT activity, although the level of activation measured was approximately 40-fold above that for the reporter alone (Fig. 4B, ^{1,2}A). The lower activity from the ^{1,2}A region was unexpected, since deletion of the ^{1,2}A region from ^{1,2}ABCDE in the N-terminal deletion experiments resulted in a striking reduction in CAT activation (Fig. 3, BCDE). However, these experiments show that the ^{1,2}AB region comprises a strong activation domain (Fig. 4, ^{1,2}AB).

To further examine the activities of the M1 to T266 portion of IE1, we examined isolated regions (^{1,2}A, ²A, B, C, D, and E) for independent transcriptional activation. When regions ^{1,2}A, ²A, B, C, D, and E were individually fused to ^{N1}LacR, an unexpected pattern of p64CAT-92LacO transcriptional activation was observed (Fig. 5). ^{N1}LacR fusions of regions ^{1,2}A, ²A, and B produced relatively low levels of reporter activation (approximately 41-, 23-, and 28-fold, respectively) compared to the approximately 1,200-fold activation observed from ^{1,2}AB (Fig. 4). Interestingly, the deletion of 17 amino acids from the N terminus of ^{1,2}A, producing ²A, resulted in an approximately 50% reduction in CAT activity. This result was consistent with those of earlier experiments in which an approximately 60% reduction in CAT activity resulted from the removal of subregion ¹A from a larger construct (Fig. 3, ^{1,2}ABCDE versus ²ABCDE). Of the six independent regions fused to ^{N1}LacR, only region D resulted in relatively high levels of CAT activity (approximately 360-fold above the activity for p64CAT-92LacO alone). Thus, only region D functioned as a highly

FIG. 5. Transcriptional activation by ^{N1}LacR-IE1 chimeras containing single charged regions from IE1. (A) Single charged regions from the M1-T266 portion of IE1 (Fig. 2) were fused to P333 of ^{N1}LacR. Construct names (left) correspond to the charged region(s) of IE1 present in each ^{N1}LacR-IE1 construct. Bars and labels are represented as in Fig. 3A. (B) CAT reporter activation by ^{N1}LacR-IE1 chimeras containing single charged regions from IE1. As a control, construct ^{1,2}ABCDE was included. Cotransfections and additional controls are as described for Fig. 3B. Each bar represents the average of three transfections, and error bars represent standard errors. (C) Western blot analysis of chimeric LacR-IE1 proteins was performed as described in the legend for Fig. 3C.

FIG. 6. Comparisons of predicted and measured sizes of LacR-IE1 chimeras and correlation with phosphorylation. (A) Measured (open triangles, solid line) and predicted (open circles, dashed line) sizes for each chimeric ^NLacR-IE1 protein examined in this study are shown. Protein mass (in kilodaltons) is indicated on the left y axis, and the IE1 region present in each ^NLacR-IE1 chimera is indicated on the x axis. Bars indicate the degree of transcriptional activation detected from each construct, with fold activation of the p64CAT-92LacO reporter indicated on the right. (B) Effects of dephosphorylation on the mobility of ^NLacR (lanes 1 to 3) and 1,2ABCD (lanes 4 to 6) proteins in SDS-PAGE gels. Cell lysates were incubated in PIB, PB, or PB + CIP, as described in Materials and Methods. Proteins were detected by Western blot analysis as described above. Molecular masses (in kilodaltons) measured from SDS-PAGE gels are indicated on the right, and the positions of standards are indicated on the left.

active independent transcriptional activation domain. The role of the activation domain in region D was also observed in C-terminal deletion constructs. The deletion of region D from construct 1,2ABCD, producing 1,2ABC, resulted in a reduction of activity of about 75% (Fig. 4B, 1,2ABCD versus 1,2ABC). No detectable CAT reporter activity above background level was detected from region C or E (Fig. 5B). In addition, the presence of region C or E correlated with a negative effect on activation (Fig. 4B, 1,2ABCDE versus 1,2ABCD and 1,2AB versus 1,2ABC). These results are consistent with possible inhibitory roles for regions C and E.

In these studies, all constructs were verified by DNA sequencing and by analysis of protein products on SDS-PAGE gels (Fig. 3C, 4C, and 5C). We noted that the measured sizes of some ^NLacR-IE1 chimeras (determined in SDS-PAGE gels) varied from the predicted sizes, even though DNA sequencing confirmed that all constructs were correct. Figure 6A shows a comparison of measured versus predicted molecular sizes of ^NLacR-IE1 chimeras. The measured sizes of 1,2ABC, 1,2ABCD, 1,2ABCDE, and 2ABCDE were significantly larger than predicted. Because the phosphorylation of native AcMNPV IE1 was previously reported to result in lower SDS-PAGE mobility (9, 10), we investigated the possibility that phosphorylation might be responsible for the observed effects on the gel mobility of these chimeras. CIP treatment of transfected cell lysates resulted in a significant increase in the mobilities of all of the aberrantly sized ^NLacR-IE1 fusion constructs (data not shown). After CIP treatment, we observed that the smallest fusion protein bands were at or near the predicted sizes of the fusion proteins. Figure 6B shows an experiment in which the LacR-IE1 fusion construct 1,2ABCD (which had the largest size discrepancy) was dephosphorylated by CIP treatment. Transfected cell lysates were prepared in either a phosphatase inhibitor buffer (PIB) or a phosphatase buffer (PB). Lysis in PIB preserved the apparent 80.2-kDa size of the 1,2ABCD fusion construct (Fig. 6B, lane 4), while lysis in PB buffer produced a number of faster-migrating protein bands (Fig. 6B, lane 5). This result suggested that endogenous phosphatase

activity is present in cell lysates. Boiling lysates in PB buffer plus SDS and dithiothreitol, followed by incubation with CIP enzyme, resulted in the most rapidly migrating protein band (measured at approximately 63 kDa), which is near the predicted size (62.4 kDa) for this construct (Fig. 6B, lane 6). As a control, lysates containing the unfused ^NLacR protein were processed similarly (Fig. 6B, lanes 1, 2, and 3). We observed no changes in the apparent migration of ^NLacR when it was incubated with CIP, indicating that the aberrant migration of some ^NLacR-IE1 chimeras resulted from phosphorylation of the IE1 peptide on the chimeric proteins. Aberrant SDS-PAGE migration was detected for only some ^NLacR-IE1 constructs, and this dramatic phosphorylation-associated gel retardation appears to correlate with the presence of region 1,2ABC. Analysis of potential phosphorylation sites reveals a dense cluster of potential phosphorylation sites in region 1,2A. However, regions B and C also appear to be necessary for the observed aberrant gel migration.

DISCUSSION

To identify independent transcriptional activation domains in the baculovirus IE1 protein, we constructed a LacR-based activation system in which nuclear localization, DNA binding, and dimerization were provided by a chimeric ^NLacR protein. LacR binding has been extensively characterized, and the tertiary and quaternary structures of the LacR protein are known. For constructs used in this study, LacR was truncated at proline 333, which removes the LacR tetramerization domain (residues 338 to 356) but retains LacR dimerization domains (13, 36). The dimeric form of LacR remains functional for LacO binding and IPTG regulation (23, 43). Thus, for ^NLacR-IE1 fusions, dimerization and nuclear localization are provided by the ^NLacR portion of the chimeric construct, and the deletion or inclusion of potential IE1 dimerization or nuclear localization domains should not affect the analysis of transcriptional activation. The addition of IPTG also allowed us to confirm for each construct that the observed transcriptional

activation was dependent on the binding of the LacR-IE1 chimera to the LacO sequence and was not a result of promiscuous activation by the chimera. Thus, the LacR-LacO system was used to tether IE1 peptides to DNA sequences adjacent to a TATA box. IE1 domains that activate transcription in this assay are likely to do so by virtue of interactions with an assembled RNA polymerase II complex or by facilitating the assembly of that complex.

Using this ^NLacR-based system, we identified two activation domains within the M1 to T266 portion of IE1. One activation domain, ^{1,2}AB, consists of N-terminal sequences from M1 to N125 and corresponds to the most acidic region of the IE1 protein. The observed independent activity of this domain is consistent with the previously reported loss in activation from N-terminal deletions (of the M1 to V145 region) of the native IE1 protein (28). Analysis of a number of ^NLacR-IE1 chimeras suggests that domains ^{1,2}A and B together constitute a single functional activation domain. ^NLacR-IE1 chimeras containing either domain ^{1,2}A or B showed only low levels (approximately 30- to 40-fold) of transcriptional activation (Fig. 5B). The ^NLacR chimera containing the same segments (^{1,2}AB) together showed exceptionally high (approximately 1,200-fold) levels of transcriptional activation (Fig. 4B).

A second activation domain was located from A168 to G222 (region D [Fig. 5]). Of the individual IE1 regions examined, region D resulted in the highest independent activity (Fig. 5B). Like regions ²A and B, region D is negatively charged, consistent with acidic activation domains identified from other transcriptional activators. Thus, IE1 contains two acidic domains that can each independently activate transcription in a heterologous system. The identification of two activation domains in transcriptional activator proteins from the well-characterized GAL4 protein of yeast has also been reported (40).

In addition to activation domains, we identified two potential inhibitory domains: C (G123-A168) and E (G222-T266). Each of these domains consists of a substantial number of positively charged amino acids (Fig. 2). Evidence for their negative roles in transcription is indicated by the decrease in observed transcriptional activation when region C or E was included with peptides that otherwise exhibited high levels of transcriptional activation. For example, when region C was added to ^{1,2}AB to generate ^{1,2}ABC, reporter activation was substantially reduced (Fig. 4B). When region E was added to ^{1,2}ABCD to generate ^{1,2}ABCDE, activation declined similarly (Fig. 4B). In construct CDE, the removal of region C resulted in a small but detectable increase in the level of activation by DE (Fig. 3B). It is possible that the basic charges within regions C and E may negatively affect transcriptional activation. Transcriptional inhibition domains that are constituted from positively charged amino acids are known to exist in transcriptional activators such as the Bel1 transcriptional activator of human foamy virus (8, 34). It has been reported that IE1 negatively regulates transcription from the *ie0*, *ie2*, and *pe38* promoters (7, 30, 35). Therefore, regions C (G123-A168) and E (G222-T266) may play a role in negative regulation by IE1. Mechanisms that may explain the observed inhibition by these basic regions could include (i) direct interactions with components of the assembled RNA polymerase II complex, (ii) indirect interactions through secondary "inhibitor" proteins, or (iii) neutralization of adjacent activation regions on the IE1 protein.

The use of the heterologous LacR system to identify transcriptional activation domains provides several major advantages over approaches involving direct mutagenesis of a functionally complex protein such as IE1. Mutations (deletions) in the IE1 protein may result in subtle or substantial effects on

dimerization, nuclear localization, folding, or (specific or non-specific) DNA binding. Because IE1 is a promiscuous transcriptional activator, mutations in the native IE1 protein may also affect the autoregulation of the promoter that is responsible for the transcription of the mutant IE1 gene. In ^NLacR-IE1 chimeras, dimerization, nuclear localization, and sequence-specific DNA binding functions were provided by the LacR protein. The present study is the first in which the potential transcriptional activation domains of IE1 are analyzed in isolation from other necessary functions of the native protein. Although this heterologous system represents a powerful tool and provides a number of major advantages for the study of complex transcriptional regulatory proteins, caution must be exercised in the interpretation of the results from any heterologous system, since peptide domains are examined in a context that is dissimilar to that of the wild-type protein.

We observed that some ^NLacR-IE1 chimeras were phosphorylated in such a manner that the proteins migrated more slowly in SDS-PAGE gels. The measured sizes of some chimeras were therefore substantially different from their predicted sizes. The aberrant sizes were attributed to phosphorylation, since phosphatase (CIP) treatment reduced the sizes to the approximate predicted sizes (Fig. 6B). Although altered gel mobility resulting from the phosphorylation of wild-type IE1 was previously reported (9, 10), the association of this effect with specific regions in the N terminus of IE1 has not been previously reported. It is not clear whether the altered protein mobility in SDS-PAGE gels is the result of extensive phosphorylation or the result of phosphorylation of only a small number of sites. In the current study, we mapped the IE1 regions necessary for this altered protein mobility. Sequences from regions ^{1,2}A (M1-A65), B (A65-N125), and C (G123-N125) were present in all cases in which dramatic changes in protein mobility were observed (Fig. 6). No single region (^{1,2}A, ²A, B, or C) resulted in a dramatic change in protein mobility. This phosphorylation-associated change in protein mobility did not correlate directly with transcriptional activation, since relatively high levels of activation were observed from constructs that exhibited no altered mobility in SDS-PAGE gels. This result does not suggest that phosphorylation is unimportant in IE1 transcriptional activation. The fact that the aberrant migration of the chimeric proteins was observed only when three regions (^{1,2}A, B, and C) were present suggests several possible explanations. First, the three regions may each contain a number of phosphorylation sites, and phosphorylation at most sites or all sites may result in aberrant migration. Alternatively, these regions together may somehow facilitate or catalyze the phosphorylation of the LacR-IE1 chimera. Other transcriptional activators, including CREB, UBF, and c-Jun (55, 59, 62), have been shown to require phosphorylation to function. The phosphorylation of the adenovirus E1A transcriptional activator protein has been shown to regulate the specificity of transcriptional activation (61). In the case of the GAL4 transcriptional activator of yeast, it was shown that phosphorylation occurs only when GAL4 is localized to the nucleus and when the DNA binding domain is present (52, 53). Phosphorylation has also been shown to alter the function of the SV40 large-T antigen, modulating the role of this protein in transcriptional activation and DNA replication (60). In addition to its role as a regulator of transcription, the IE1 gene is essential for baculovirus replication (25, 38). IE1 binds to hr sequences, which serve both as transcriptional enhancers and as origins of replication. It has been speculated (9) that, like SV40 large-T antigen, IE1 may modulate these regulatory activities through phosphorylation.

By fusing portions of IE1 to ^NLacR, we dissected and inde-

pendently analyzed the activation domains of AcMNPV IE1. We identified two separate activation domains (M1-N125 and A168-G222) and two regions that appear to contain negative regulatory activities (G123-A168 and G222-T266). The identification of two independent transcriptional activation domains in IE1 suggests that IE1 is functionally and structurally complex. The isolation and dissection of IE1 with LacR-IE1 chimeras thus provides a new and powerful tool for understanding this complexity.

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