

# Human Cytomegalovirus Immediate-Early Protein IE2 Tethers a Transcriptional Repression Domain to p53\*

(Received for publication, October 10, 1995, and in revised form, December 7, 1995)

Hsiu-Lan Tsai‡§, Guang-Hsiung Kou§, Shan-Chun Chen‡, Cheng-Wen Wu‡, and Young-Sun Lin‡¶

From the ‡Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan, and the §Department of Zoology, National Taiwan University, Taipei 10764, Taiwan

**The IE2 gene of human cytomegalovirus has been implicated in the development of coronary restenosis, and the gene product appears to inhibit p53-dependent transactivation. Here we describe an analysis of the IE2-p53 interaction. Repression of p53 function by IE2 requires two separable domains of IE2. The N terminus of IE2 interacts with p53. IE2 has little effect on the ability of p53 to bind specific DNA sequences. Reduction of the transactivation activity of p53 is caused by a transcriptional repression function contributed by the C-terminal domain of IE2. These findings suggest that IE2 may function as a transcriptional repressor, which is recruited to p53's target genes by interacting with p53.**

The tumor suppressor p53 protein is an important negative regulator of cell proliferation (1–5). Loss of p53 function results in genome instability (6, 7) and eliminates growth arrest at the G<sub>1</sub> phase in response to inadequate or detrimental growth conditions (8–10). p53 functions as a typical eukaryotic transcription factor; it binds to specific DNA sequences termed p53-responsive elements (or PRE)<sup>1</sup> (8, 11–16) and stimulates transcription of the target genes (11, 17–19). Paradoxically, p53 also represses transcription of many viral and cellular genes, which apparently do not have PRE (20–22). This function, probably reflecting general negative effects on cellular growth via the induction of WAF1/CIP1 by p53 (23), requires the transactivation activity of p53. In structure, p53 is organized into three functional domains: an N-terminal domain, involved in transcriptional activation; a central domain, mediating specific DNA binding; and a C-terminal domain, responsible for oligomerization, transcriptional repression, and non-specific DNA binding (3, 24, 25).

All of the major classes of small DNA tumor viruses that replicate in mammalian nuclei encode immediate-early gene products to overcome the negative effects of p53 on cell proliferation. For instance, the E1b protein of adenovirus, the E6 protein of papillomavirus and the large T antigen of papovaviruses each eliminates p53 function by interacting with a distinct domain of p53 (Ref. 3). The inactivation of p53 function by the viral immediate-early proteins results in promoting cell

growth and in increasing the available pool of deoxyribonucleotides, which leads to the enhancement of viral replication.

Herpesviruses are the largest among the DNA tumor viruses. Although the replication strategy of herpesviruses must be fundamentally different from that of small DNA viruses, it seems logical that they still have to deal with the negative effect imposed by p53 on cell proliferation so that host cells can enter the S-phase of the cell cycle and thus promote viral replication. Indeed, it has been shown that the immediate-early protein BZLF1 of Epstein-Barr virus, a member of the herpes group, disrupts p53 function by binding directly to the carboxyl-terminal portion of the protein (26).

Human cytomegalovirus (HCMV), another member of the herpesvirus family, contains a double-stranded DNA genome of 229,354 base pairs with a potential to encode for more than 200 proteins (27). HCMV infection promotes DNA synthesis and causes proliferation of a variety of cells (28–30). More recently, HCMV has been found to be involved in the development of coronary restenosis (31) by inducing smooth muscle cell proliferation. A number of immediate-early (IE) proteins of HCMV are expressed following entry of the virus into cells (32). Among them, the IE2 86K protein (referred as IE2, hereinafter) is the most studied. IE2 appears to be a promiscuous transactivator of viral and cellular gene expression (Ref. 33 and references therein). However, to maximally stimulate transcription, both IE1 and IE2 proteins are required (34). Also, IE2 autoregulates its own expression by binding to a short nucleotide sequence, termed the *cis* repression signal, located immediately downstream of the TATA box (35, 36). To date, only a limited number of studies have been performed to assess the functional domains of IE2. Recent evidence has demonstrated that the C terminus of IE2 is involved in such protein activities as activation, autoregulation, and binding to retinoblastoma protein, TBP, and TFIIB (Ref. 33 and references therein).

The fact that immediate-early proteins of both large and small DNA viruses can associate with p53 (3, 26) naturally leads to the prediction that HCMV, by analogy, should employ a similar mechanism to inactivate p53 function. Actually, the IE2 protein of HCMV has been implicated in the disruption of the transactivation function of p53 (31). Evidence collected so far suggests that the interaction between IE2 and p53 is of critical importance to cell growth and HCMV replication. Thus, to further understand the biological significance of the interaction between IE2 and p53, it is important to define the interaction domains of the two proteins and to elucidate the molecular mechanism underlying this interaction. This report demonstrates that two regions of IE2 are required to inactivate p53 function. The N-terminal portion of IE2 interacts with p53. The specific DNA-binding activity of p53, however, seems unaffected by IE2. Repression of the transactivation activity of p53 requires, in addition to the N-terminal end of IE2, a transcriptional repression activity conferred by the C terminus of IE2.

\* This work was supported by grants from the Academia Sinica and the National Science Council of Taiwan (to G. H. K., C. W. W., and Y. S. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 886-2-7899133; Fax: 886-2-7853569.

<sup>1</sup> The abbreviations used are: PRE, p53-responsive element(s); HCMV, human cytomegalovirus; IE, immediate-early; TBP, TATA-binding protein; TFIIB, transcription factor IIB; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; RCA, relative CAT activity.

## MATERIALS AND METHODS

**Plasmid Construction**—pIE2, which expresses the HCMV IE2 86-kDa protein, was constructed by inserting the HCMV Towne strain IE2 cDNA (37) between the *Hind*III and *Bam*HI sites of pSG424 (38). pIE2ID(136–289) and pIE2ID(45–289) were constructed by deleting the *Sma*I-*Xho*I and *Hpa*I-*Xho*I fragments of pIE2, respectively. pIE2CD189 and pIE2CD80 were constructed by deleting the *Dra*III-*Bam*HI and *Bst*XI-*Bam*HI fragments of pIE2, respectively. Plasmids expressing GAL4-IE2 derivatives, pGAL4-IE2, pGAL4-IE2(290–579), pGAL4-IE2(290–390), pGAL4-IE2(391–579) and pGAL4-IE2(1–289), were cloned by inserting the corresponding DNA fragment of IE2 between the *Eco*RI and *Bam*HI sites of pSG424. Plasmids pSVp53ND50, pSVp53V143A, pSVp53V143ACD30, and pSVp53V143ACD55, all of which express derivatives of p53 (25) and p53V143A (39), were cloned by inserting the corresponding p53 cDNA between the *Hind*III and *Bam*HI sites of pSG424. pGEX1-p53 and pGEX1-p53CD55 were made by inserting the corresponding p53 DNA fragment into the *Sma*I site of pGEX1 (40). Plasmids p3PREcCAT, pCMVTag, pLexA-VP16, pG5TKCAT, and pL6EC were described previously (25, 41–43). pL6EP1C was constructed by inserting one copy of a consensus p53-binding site oligo (25) into the *Sma*I site of pL6EC. pGEM4IE2, pGEM4IE2ID(136–289), pGEM4IE2(1–289), and pGEM4IE2(290–579) were created by inserting the DNA fragment encoding the corresponding IE2 peptide between the *Eco*RI and *Bam*HI sites of pGEM4 (Promega).

**Cell Culture, Transfection, and CAT Assay**—Saos-2 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Approximately  $1.5 \times 10^6$  cells were seeded 12 h before transfection. Calcium phosphate-mediated DNA transfection was performed as described previously (25). Typically, the transfection lasted 12 h. CAT activity was measured 48 h after transfection and quantitated as described previously (25). For temperature shift assays, the incubation temperature was switched to 30 °C for 24 h after incubation at 37 °C for 36 h.

**Western Immunoblotting**—An equal amount (approximately 50 µg) of proteins from extracts of transfected cells was boiled in a sample buffer (125 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 20% glycerol, 0.005% bromophenol blue) for 5 min and then loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon membrane (Millipore). p53, IE2, and GAL4-IE2 derivatives were detected with antibodies against p53 (25), IE2 (DuPont), and GAL4 (UBI), respectively, using the ECL system (Amersham Corp.) according to the manufacturer's instructions.

**In Vitro Translation of Proteins**—*In vitro* transcription/translation was performed with the TNT system (Promega) according to the manufacturer's instructions. The templates were pGEM4IE2, pGEM4IE2ID(136–289), pGEM4IE2(1–289), and pGEM4IE2(290–579).

**GST Fusion Proteins and Pull-down Assay**—GST, GST-p53 and GST-p53CD55 were expressed in and purified from *Escherichia coli* XA90 strain according to standard protocols (40, 44, 45). The ligand concentrations, using bovine serum albumin as a standard, were 6.2, 0.8, and 1.3 mg/ml of resin for GST, GST-p53, and GST-p53CD55, respectively. Aliquots (25 µl) of the GST, GST-p53, and GST-p53CD55 beads were incubated for 2 h at 4 °C with *in vitro* translated, [<sup>35</sup>S]methionine-labeled IE2 derivatives. After being washed with buffer D (46), bound proteins were eluted from beads with buffer D containing 0.1 M glutathione and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

## RESULTS

**Two Regions of IE2 Are Required for Repression of p53 Function**—A peptide sequence comparison between IE2 and other known viral p53-binding proteins, such as adenovirus E1b protein, SV40 T antigen, HPV E6 protein, and Epstein-Barr virus BZLF1 protein, did not reveal any significant homology. To determine the region(s) of IE2 required to interact with p53, we sought to establish a system for the analysis of the effect of IE2 on the transactivation function of p53. The inclusion of IE2 in a transient co-transfection assay caused a moderate reduction of p53 activity, which probably reflected the fact that the expression level of IE2 was too low to optimally interact with p53 (data not shown). p53 is known to inhibit the expression of genes lacking PRE in the promoter (20–22), which may be responsible for the low expression level of IE2. To overcome this obstacle, a temperature-sensitive p53 mutant, p53V143A (39),

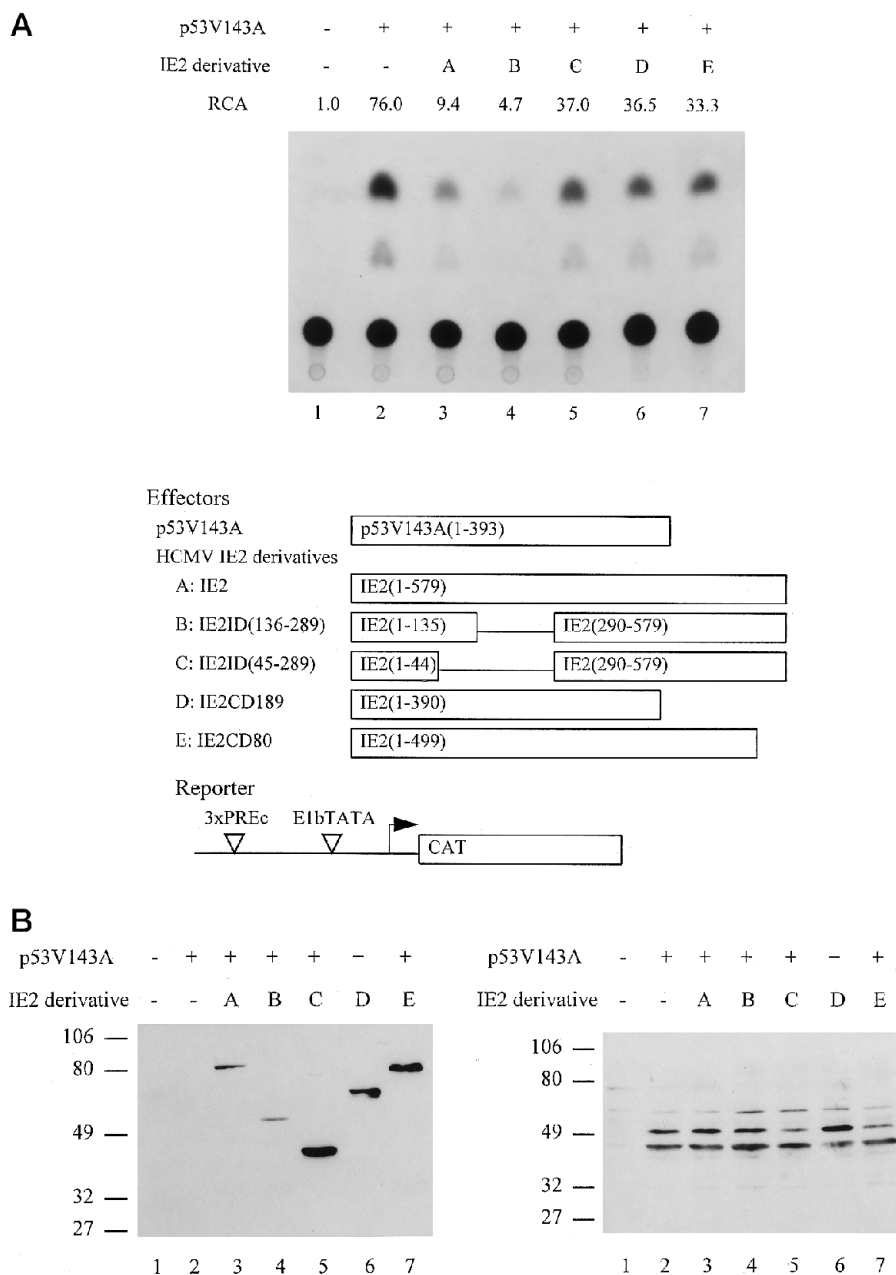
was substituted. As expected, the expression of IE2 was dramatically increased with the p53V143A system; in addition, once IE2 was made, it was no longer sensitive to repression by the wild-type p53 function (data not shown). There seemed one more merit to the choice of the temperature-sensitive p53 mutant for study; p53 protein is almost undetectable within cells under physiological conditions. Although the level of p53 protein can be dramatically increased by DNA damage (3), the entry of linear Epstein-Barr virus DNA, whose ends seem to resemble damaged DNA, alone is not sufficient to induce p53 expression; induction of p53 synthesis is achieved only after the expression of viral immediate-early proteins (47). Likewise, the linear HCMV genome would not be interpreted by cells as a damaged genome and thus should not provide a stimulus for the induction of p53. Rather, the up-regulation of p53 synthesis may require viral immediate-early gene expression. Thus, use of this temperature-sensitive mutant should closely imitate physiological conditions; IE2 is expressed first, and p53 function is then induced by a shift of the assaying temperature. In this sense, p53V143A should not only provide an easy but also a biologically relevant approach to the study of IE2-p53 interaction.

As shown in Fig. 1A, IE2 repressed p53 function efficiently (compare lanes 2 and 3). Repression was specific for IE2 because another HCMV IE protein, IE1, had no effect on the transactivation function of p53 (data not shown). Removal of IE2 residues 136–289 consistently resulted in a small increase in the protein's repression activity toward p53 (compare lane 4 to lane 3). However, a further deletion of IE2 residues 45–135 largely reduced the protein's repression activity (compare lane 5 to lane 2). We thus concluded that a region encompassing IE2 residues 45–135 was required for the inhibition of p53 function. Besides, Fig. 1A also indicates that the C terminus of IE2 was also involved in the repression, because both C-terminal truncation mutants, IE2CD80 and IE2CD189, weakly repressed p53 activity (Fig. 1A, compare lanes 6 and 7 to lane 2), suggesting that the intactness of IE2's C terminus was required for full repression activity. (Further supports for this point were provided in the experiment of Fig. 4A.) Note that the inability of IE2 derivatives in repressing p53 function was not due to a low level of protein being made. In fact, all three inactive IE2 mutants were more abundant than the other two active derivatives (Fig. 1B, left panel, compare lanes 5, 6, and 7 to lanes 3 and 4). Neither could the low CAT activity be attributed to a fluctuation in the level of p53 (Fig. 1B, right panel), indicating that IE2 derivatives had little effect on the expression of p53.

**The C-terminal End of p53 Is Involved in the IE2-p53 Interaction**—We demonstrated in the above experiments that two regions of IE2 were required for the inactivation of p53 function. Next, we performed experiments to map the region(s) of p53 required for the IE2-p53 interaction. As shown in Fig. 2, the transactivation function of both p53V143A and p53V143ACD30, a derivative lacking the most C-terminal 30 residues of p53V143A, was repressed by IE2 to a certain degree (compare lanes 2 and 4 to lanes 3 and 5). However, a further deletion of 25 residues from the C terminus of p53V143ACD30 resulted in a complete loss of responsiveness to IE2 (compare lane 6 with lane 7). The observation that p53V143ACD30 had higher transactivation activity than the other two p53 derivatives (Fig. 2, compare lane 4 with lanes 2 and 6) was consistent with previous reports that removal of C-terminal 30 residues enhances the transactivation activity of p53, whereas removal of C-terminal 55 residues results in a p53 derivative with transactivation activity similar to that of full-length p53 (25, 48). Like that of p53V143A (Fig. 1B), the expression of C-terminal deletion mutants of p53V143A appeared unaffected

**FIG. 1. Two IE2 regions are required for repression of p53 function.**

**A**, repression of the transactivation activity of p53 by IE2 derivatives. 5  $\mu$ g of reporter, 1  $\mu$ g of p53V143A expression plasmid, and 10  $\mu$ g of each plasmid expressing IE2 derivatives were transfected per sample. Experiments were repeated 3 times. 1  $\mu$ g of plasmid pCH110 (Pharmacia Biotech Inc.) containing a functional LacZ gene was used as an internal control to monitor transfection efficiency. After transfection, cells were incubated at 37 °C for 36 h, and the temperature was then shifted to 30 °C for another 24-h incubation. An autoradiogram of a typical experiment is shown. Diagrams of the structure of the effectors and reporter are shown below the autoradiogram. The presence (+) or absence (–) of p53V143A, the IE2 derivative and relative CAT activity (or RCA) are indicated above each track of the autoradiogram. RCA is the mean -fold stimulation of transcription compared with basal activity. The standard deviations were 0,  $\pm 8.0$ ,  $\pm 1.1$ ,  $\pm 0.8$ ,  $\pm 7.1$ ,  $\pm 5.2$ ,  $\pm 5.6$  for lanes 1–7, respectively. **B**, protein levels of IE2 derivatives (left panel) and p53V143A (right panel). Transient transfection was performed as in Fig. 1A. Proteins of Saos-2 cells transfected with the vector alone (lane 1), with p53V143A (lane 2), or with p53V143A and IE2 derivatives (lanes 3–7) were fractionated on a 10% SDS-polyacrylamide gel. IE2 derivatives (left panel) and p53V143A (right panel) were detected by immunoblotting. The position of p53V143A is indicated by arrow-head (right panel). The presence (+) or absence (–) of p53V143A as well as the IE2 derivative are indicated above each track of the immunoblot. The positions of molecular mass markers in kilodaltons are shown on the left. The effectors are the same as in Fig. 1A; therefore, their diagrams are omitted.



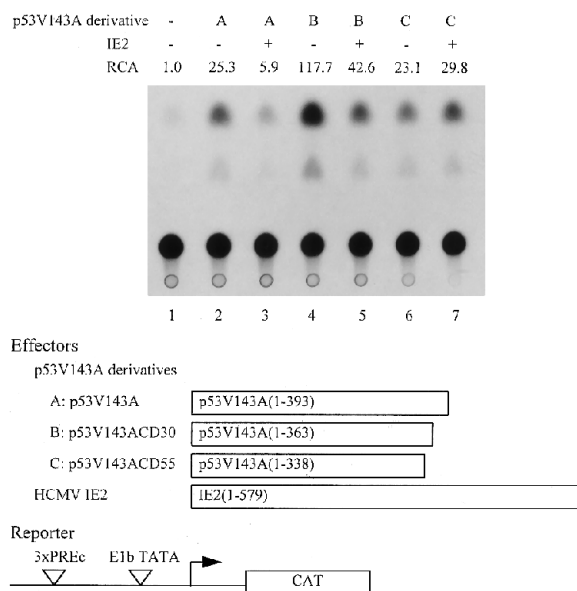
by IE2 (data not shown). On the basis of these results, we concluded that a region encompassing residues 339–363 of p53 was involved in the IE2-p53 interaction. Since transactivation by p53V143ACD55 was not affected by IE2 (Fig. 2, compare lane 7 to lane 6), we could further conclude that neither the inhibition of p53-dependent transactivation by IE2 was caused by the competition for (or squelching of) a transcription factor required for p53 function by IE2, nor was the inhibition of transcription due to the binding of IE2 to a putative *cis* repression signal located around the transcription initiation site of the assaying promoter.

**IE2 Does Not Block the Specific DNA-binding Activity of p53**—Since the C terminus of p53 was involved in the IE2-p53 interaction (Fig. 2), we speculated that IE2 might not affect the p53 specific DNA-binding activity, which is located in the central region of the protein. To test this idea, an *in vivo* DNA binding assay (42) was performed. Briefly, one copy of PRE was placed immediately downstream of the reporter's TATA box. Thus, binding of a p53 derivative to the PRE of the reporter should block assembly of the transcription initiation complex

on the promoter and therefore reduce CAT activity. p53ND50, a derivative providing only p53 DNA-binding activity and thereby causing no negative effect on the expression of IE2 (3, 23), reduced transcription of the reporter containing PRE (Fig. 3, compare lane 3 with lane 2, and also lane 12 with lane 11). The reduction was specific because transcription of a corresponding reporter without PRE was not affected (Fig. 3, compare lane 8 with lane 7). The SV40 large T antigen, known to inhibit the DNA-binding activity of p53 by forming a large T-p53 complex, restored CAT activity in a dose-dependent manner (Fig. 3, compare lanes 12, 13, and 14 with lane 11). In contrast, IE2 showed little activity to eliminate the transcriptional block (Fig. 3, compare lane 4 with lane 3), although IE2 was expressed to a level comparable with that of the p53V143A system (data not shown). A reasonable interpretation for these observations was that the p53 derivative was capable of binding PRE in the presence of IE2. Alternatively, another plausible explanation was that IE2 indeed inhibited p53 to bind PRE; in addition, IE2's failure to restore CAT activity simply reflected that the p53 derivative, under the influence of IE2,

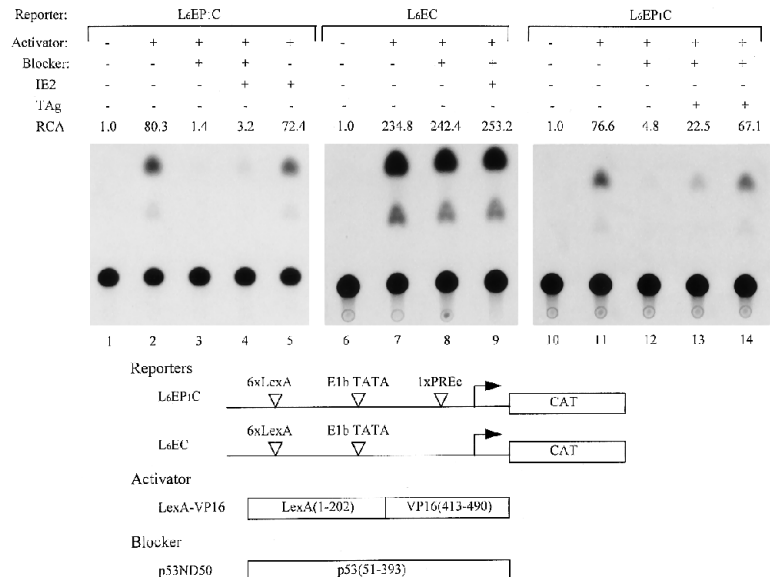
might somehow function as a nonspecific transcriptional repressor. This plausibility gained some support from the fact that IE2 functioned as a direct transcriptional repressor (Fig. 4A) and that the repression domain of p53 (24, 25) was still left intact in the p53 derivative used for the *in vivo* DNA binding assay. However, control experiments revealed that IE2 could not significantly inhibit transcription of a promoter nonspecifically (Fig. 3, lane 5; also see Fig. 4A, lane 10), nor did the p53 derivative, in the presence of IE2, exhibit any negative effect on the transcription of the promoter lacking PRE (Fig. 3, lane 9). Taken together, the transcriptional block by the p53 derivative was PRE-dependent (Fig. 3, compare lanes 2 and 3 with lanes 7 and 8), implying that binding of PRE by the p53 derivative was responsible for the negative effect. Moreover, the presence of IE2, unlike that of SV40 large T antigen, had little detrimental effect on the specific DNA-binding activity of p53 (Fig. 3, compare lanes 3 and 4 with lanes 12 and 14).

#### The C Terminus of IE2 Contains a Transcriptional Repres-



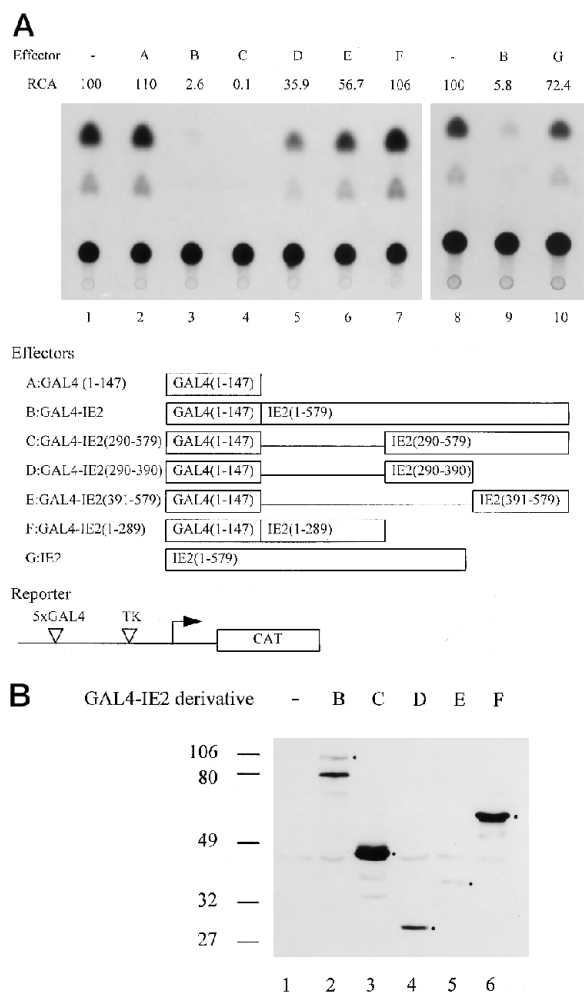
**FIG. 2. The C terminus of p53 is required for the interaction with IE2.** Transfection was performed as described in Fig. 1A, except that effectors were p53V143A, p53V143ACD30, p53V143ACD55, and IE2. Otherwise as in Fig. 1A. The standard deviations were 0,  $\pm 3.5$ ,  $\pm 0.6$ ,  $\pm 14.5$ ,  $\pm 6.9$ ,  $\pm 3.7$ ,  $\pm 5.5$  for lanes 1–7, respectively.

**FIG. 3. IE2 does not inhibit the specific DNA-binding activity of p53.** 1  $\mu$ g of reporter pL6EP1C (lanes 1–5 and 10–14) or pL6EC (lanes 6–9) was cotransfected with each expression plasmid for activator (1  $\mu$ g), blocker (2  $\mu$ g), and IE2 (6  $\mu$ g). The amount of expression plasmid for SV40 large T antigen (TA) is 3  $\mu$ g (lane 13) and 6  $\mu$ g (lane 14). The presence (+) or absence (–) of activator, blocker, IE2, and large T antigen as well as RCA are indicated above each track of the autoradiogram. After transfection, cells were incubated at 37 °C for 48 h, and CAT activity was measured. Diagrams of the structure of the activator, blocker, and reporters are shown below the autoradiogram. Otherwise as in Fig. 1A. The standard deviations were 0,  $\pm 16.5$ ,  $\pm 0.3$ ,  $\pm 0.6$ ,  $\pm 16.1$ , 0,  $\pm 43.7$ ,  $\pm 5.2$ ,  $\pm 40.6$ , 0,  $\pm 10.7$ ,  $\pm 0.8$ ,  $\pm 4.5$ ,  $\pm 12.6$  for lanes 1–14, respectively.



**sion Domain**—Data collected so far indicate that inhibition of p53 function by IE2 appeared not to be caused by a loss of the specific DNA binding activity of p53. In light of this, it is worth noting that adenovirus E1b also has no effect on the DNA binding activity of p53 (43). E1b represses the p53's target gene transcription by tethering, via its binding to the N terminus of p53, a transcriptional repression domain to the promoter (43). To examine whether IE2 contains a transcriptional repression domain, we performed repression studies as described previously (43). In brief, chimeric proteins between the DNA binding domain of GAL4(1–147) and IE2 derivatives were first constructed, and then their repression activity was assayed with the reporter pG5TKCAT, which bears five GAL4 binding sites upstream of the thymidine kinase promoter. The reason for choosing thymidine kinase promoter as a reporter was that this promoter, unlike the E1b TATA promoter used in the above studies, possesses detectable transcriptional activity, which is necessary for the assay of repression. Two lines of evidence indicate that there was probably little change in the configuration and activity of IE2 when fused to the GAL4 DNA binding domain. First, GAL4-IE2 and IE2 exhibited comparable activity in repressing p53 function, and second, GAL4-IE2 derivatives behaved like their corresponding IE2 derivatives, regarding the repression of p53 function (data not shown). Accordingly, it should be appropriate to transfer conclusions drawn from studies of the GAL4-IE2 fusion protein to IE2.

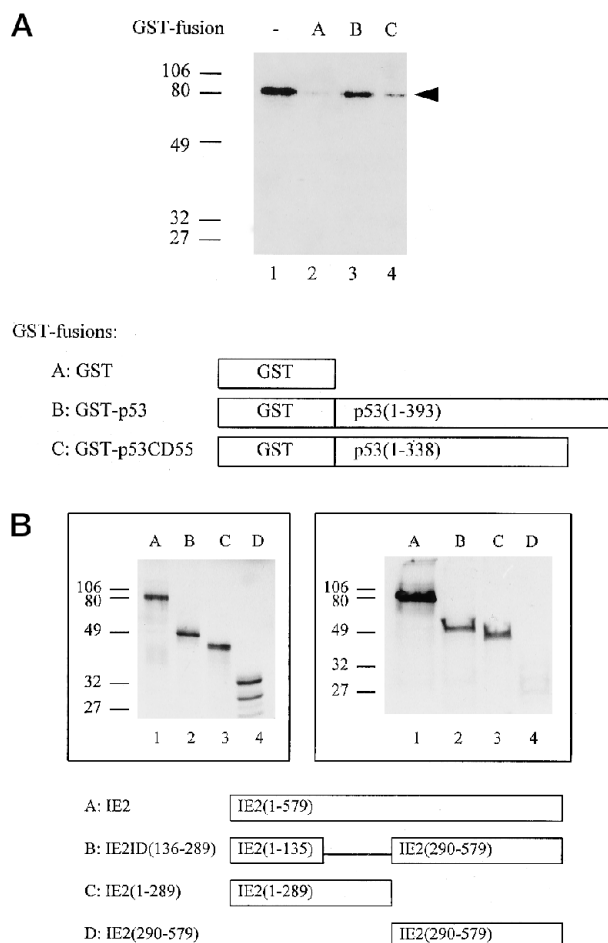
As shown in Fig. 4A, GAL4-IE2 repressed transcription of the reporter pG5TKCAT (compare lane 3 with lane 1). The repression was dependent upon both the GAL4 and IE2 modules of the chimeric protein, since neither the GAL4 DNA binding domain nor the IE2 alone was able to significantly repress transcription from the promoter of G5TK (compare lanes 2 and 10 with lanes 1 and 8). The repression domain was mapped to the C-terminal half of IE2, whereas the N-terminal half of IE2 possessed no detectable repression activity (compare lanes 4 and 7 with lane 1). The entire C-terminal half of IE2 seemed to be required for full repression activity, because a further reduction in length of the IE2 module from GAL4-IE2(290–579) resulted in a large decrease in the repression activity (compare lanes 5 and 6 with lane 4). A Western blot shown in Fig. 4B demonstrated that most of the chimeric proteins were expressed to a similar level. Proteins GAL4-IE2(290–579) and GAL4-IE2(1–289) were two exceptions. The former's high level of expression, in conjunction with the removal of an activation domain located in the N terminus of IE2



**FIG. 4. GAL4-IE2 functions as a direct transcriptional repressor.** *A*, GAL4-IE2 represses CAT activity driven by a thymidine kinase promoter with five upstream GAL4-binding sites. 5  $\mu$ g of reporter and 5  $\mu$ g of each GAL4 derivative were transfected per sample. The effector and RCA are indicated above each track of the autoradiogram. The CAT activity of the reporter alone was set at 100. Diagrams of the structure of the effectors and the reporter are shown below the autoradiogram. Otherwise as in Fig. 3. The standard deviations were 0,  $\pm 18.2$ ,  $\pm 0.5$ ,  $\pm 0.03$ ,  $\pm 6.4$ ,  $\pm 9.4$ ,  $\pm 21.1$ , 0,  $\pm 0.8$ ,  $\pm 12.8$  for lanes 1–10, respectively. *B*, protein levels of GAL4-IE2 derivatives. Transfection was performed as in Fig. 4A. The GAL4-IE2 derivatives are the same as in Fig. 4A; thus, their diagrams are omitted. GAL4 derivatives were detected with an anti-GAL4 antibody. Each of the positions of GAL4-IE2 derivatives was indicated by a dot. Otherwise as in Fig. 1B.

(33) might be responsible for its strong repression activity toward reporter pG5TKCAT (Fig. 4A, lane 4 and Fig. 4B, lane 3), whereas the latter's high level of expression demonstrated that the N terminus of IE2 indeed had little repression activity (Fig. 4A, lane 7, and Fig. 4B, lane 6).

**The N Terminus of IE2 Interacts with p53 *in Vitro***—To determine whether IE2 and p53 can interact *in vitro*, as well as to delineate the IE2 domain mediating this interaction, the ability of GST-p53 fusion proteins to interact with *in vitro* translated  $^{35}$ S-labeled IE2 was investigated by a protein pull-down assay (Fig. 5). GST alone showed little affinity toward IE2 (Fig. 5A, compare lane 2 with lane 1). The intact 393-amino acid p53 protein (linked to GST) retained labeled IE2 protein to glutathione-linked beads (Fig. 5A, compare lane 3 with lane 1). Removal of the C-terminal 55 amino acids from p53 (p53CD55), which knocked out IE2-p53 interaction *in vivo* (Fig. 2), significantly reduced the retention of IE2 (Fig. 5A, compare lane 4 with lane 3). The N-terminal portion of IE2 was sufficient for



**FIG. 5. GST-p53 fusion protein retains  $^{35}$ S-labeled IE2 protein in pull-down assays.** *A*, retention of IE2 to wild-type p53 fusion protein (GST-p53) but not mutant p53 fusion protein (GST-p53CD55). Lane 1, input IE2 protein; lanes 2, 3, and 4, retention of IE2 protein by GST, GST-p53, and GST-p53CD55, respectively. The position of IE2 protein is indicated by an arrowhead. The GST protein ligand, whose structure is shown below the autoradiogram, is indicated above each track of the autoradiogram. The positions of molecular mass markers in kilodaltons are indicated on the left. *B*, retention of IE2 derivatives by GST-p53 protein. The left panel shows one-hundredth each of the input IE2 derivatives directly loaded onto the gel. The right panel shows retention of IE2 derivatives by GST-p53 protein. The IE2 derivative whose structure is shown below the autoradiogram is indicated above each track of the autoradiogram. The positions of molecular mass markers in kilodaltons are indicated on the left.

its interaction with p53 *in vitro*, since derivatives containing IE2 residues 1–289 were capable of binding to p53, whereas a derivative containing IE2 residues 290–579 failed to do so (Fig. 5B, right panel, compare lane 3 with lane 4). Furthermore, IE2(1–289) and IE2ID(136–289) seemed to interact with GST-p53 equally well (Fig. 5B, right panel, lanes 2 and 3). The overlapping region between the two IE2 derivatives encompasses IE2 residues 1–135, implying that this region of IE2 contains a p53-interacting domain. We could not rule out, however, that other interacting domains for p53 might exist in IE2, because, for example, IE2 had higher affinity toward p53 than IE2ID(136–289) or IE(1–289) did (Fig. 5B, right panel, compare lanes 1, 2, and 3). Nonetheless, these results, together with those obtained from *in vivo* co-transfection assays (Fig. 1), demonstrated that the N terminus of IE2 interacted with p53.

#### DISCUSSION

Previous studies have demonstrated a functional as well as a physical interaction between HCMV IE2 and p53 proteins (31).

In this report, we defined the domains required for the IE2-p53 interaction and analyzed the molecular mechanism underlying this interaction. IE2 can be divided into two domains, regarding the repression of p53-mediated transcriptional activation. Data obtained from *in vitro* protein-protein interaction studies demonstrate that the N terminus of IE2 contains a p53-interacting domain (Fig. 5B). Evidence supporting that the C terminus of IE2 functions as a transcriptional repression domain comes from experiments with derivatives of the GAL4-IE2 fusion protein (Fig. 4A). Importantly, the IE2 domain required for transcriptional repression is mapped to the C terminus containing residues 290–579 of the protein, no matter how the IE2 is brought to the promoter, either by fusion to the GAL4 DNA-binding domain (Fig. 4A) or by interacting with p53 (Fig. 1A). Furthermore, IE2 does not affect the ability of p53 to bind PRE *in vivo* (Fig. 3). Thus, we conclude that IE2 inhibits p53-mediated transcription by tethering a repression domain to p53. We note, however, that IE2ID(136–289) interacts with p53 less strongly *in vitro* (Fig. 5B, lanes 1 and 2) but represses p53 better *in vivo* than IE2 (Fig. 1A, lanes 3 and 4). We currently do not have an explanation for it.

The IE2-p53 interaction shows many parallels with the well established interaction between E1b and p53. First, like E1b (49), IE2 can repress p53-dependent transcriptional activation (Figs. 1A and 2). Second, like E1b (43), IE2 does not affect the ability of p53 to bind specific DNA sequences (Fig. 3); rather, it tethers a transcriptional repression domain to p53 (Figs. 4A and 5B). Despite these similarities, different regions of p53 are involved in the E1b- and IE2-p53 interactions; E1b binds to the N terminus of p53 (3), whereas IE2 targets the C terminus (Figs. 2 and 5A).

p53-dependent transactivation was not completely repressed by IE2 (Figs. 1A and 2). The strength of IE2's repression activity may not be a viable explanation for the incomplete inhibition, because IE2 appears able to efficiently repress transcription when brought to a promoter by fusion to the GAL4 DNA binding domain (Fig. 4A). Thus, some other mechanism has to account for the incomplete repression of p53 activity. We speculate that in the IE2-p53 complex, unlike the E1b-p53 one, the N-terminal activation domain of p53 is not involved in the interaction and is, therefore, probably free to contact the transcription machinery, explaining why IE2 fails to efficiently repress p53 function (Figs. 1A and 2). Alternatively, the incomplete repression of p53 function by IE2 could be caused by the existence of activation domains in the latter (33). Perhaps, a putative interaction between the activation domains of p53 and IE2 could partially overcome the negative effect of IE2 and thus result in incomplete repression. It should be noted, however, that Speir *et al.* (31) observed a good repression of the transactivation activity of p53 by IE2 in primary human coronary smooth muscle cells (31). The reason for the observed discrepancy is not known. However, since the current studies were carried out in a p53-negative tumor cell line, the discrepancy may reflect the physiological differences of the recipient cells used for transient transfection assays.

It is noteworthy that the C terminus of p53 is indispensable for the interaction with IE2 of HCMV and BZLF1 of Epstein-Barr virus, both immediate-early proteins of herpesviruses (Ref. 26 and the present work). Although the C terminus of p53 is rarely involved in tumor mutations (3), this region has been shown to interact with TBP and the hsc-70 protein (3). In addition, this region, which contains two phosphorylation sites, also mediates the oligomerization, transformation, and transcriptional repression activities of p53 (3, 24, 25). By targeting this region, IE2 can potentially affect any of these p53 functions. In contrast, very few functions have been assigned to the

N terminus of IE2 (33). However, an examination of the primary sequence of this IE2 domain reveals several interesting features characteristic of eukaryotic transcription factors: a repeated motif of proline-*N*-proline, a helix-loop-helix-turn-helix structure and stretches of polyglutamic acid and polyserine (50). This observation implies that p53 can potentially interact with nuclear proteins containing such structural motifs.

Both IE2 and p53 interact with a number of cellular proteins, such as TBP and TFIIB (Refs. 33 and 51 and references therein). The possibility that interaction between IE2 and p53 is mediated through some cellular protein(s) has not been totally excluded, since each of the assays used to examine the IE2-p53 interaction either included the reticulocyte lysate or was performed within cells. Nonetheless, it is very unlikely that interaction between IE2 and p53 is mediated through TBP or TFIIB, since TBP and TFIIB interact with the C terminus of IE2 (Ref. 33 and references therein), whereas this region is dispensable for the IE2-p53 interaction (Fig. 5B).

IE2 has been shown to transactivate homologous and heterologous gene expression as well as to negatively autoregulate. The present studies identify a third activity of IE2; it functions as a direct transcriptional repressor when brought to a promoter either by fusing to the GAL4 DNA-binding domain (Fig. 4A) or by interacting with p53 (Figs. 1A and 2). Since p53 possesses a transcriptional repression domain (24, 25) and p53 itself mediates transcriptional repression of heat shock 70 promoter by interacting with the CCAAT-binding factor (52), these raise the question of whether p53 is a cofactor required for IE2 repression. However, the finding that GAL4-IE2 repressed transcription of a target promoter in cells lacking p53 does not favor this idea (Fig. 4A). In light of this and of the fact that IE2 possesses specific DNA binding activity (36), it is important to determine whether IE2 can repress transcription of genes bearing IE2 binding sites in the promoter/enhancer region. Moreover, transcriptional repression domains have been shown to be alanine-rich (53), highly basic (54, 55) and rich in proline and hydrophobic amino acids (56, 57). As a transcriptional repressor, IE2 appears unique in that it bears no similarity to the aforementioned repression domains. Further studies of the repression activity of IE2 should elucidate the molecular mechanisms concerning how direct transcriptional repressors work.

**Acknowledgments**—We thank Dr. J. Y. Chen for the p53V143A clone, Dr. J. C. Alwine for plasmid pCMVTag, and Dr. A. J. Berk for plasmid pG5TKCAT. We also thank Drs. H. F. Yang-Yen, J. Yen, Y. J. Chern, and K. King and Ms. J. Sugden for comments.

## REFERENCES

1. Finlay, C. A., Hinds, P. W., and Levine, A. J. (1989) *Cell* **57**, 1083–1093
2. Levine, A. J., Momand, J., and Finlay, C. A. (1991) *Nature* **351**, 453–456
3. Prives, C., and Manfredi, J. J. (1993) *Genes & Dev.* **7**, 529–534
4. Ullrich, S. J., Anderson, C. W., Mercer, W. E., and Appella, E. (1992) *J. Biol. Chem.* **267**, 15259–15262
5. Vogelstein, B., and Kinzler, K. W. (1992) *Cell* **70**, 523–526
6. Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and Wahl, G. M. (1992) *Cell* **70**, 937–948
7. Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T. D. (1992) *Cell* **70**, 923–935
8. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) *Cell* **71**, 587–597
9. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7491–7495
10. Kessis, T. D., Slebos, R. J., Nelson, W. G., Kastan, M. B., Plunkett, B. S., Han, S. M., Lorincz, A. T., Hedrick, L., and Cho, K. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3988–3992
11. Zambetti, G. P., Bargonetti, J., Walker, K., Prives, C., and Levine, A. J. (1992) *Genes & Dev.* **6**, 1143–1152
12. Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991) *Science* **252**, 1708–1711
13. Bargonetti, J., Friedman, P. N., Kern, S. E., Vogelstein, B., and Prives, C. (1991) *Cell* **65**, 1083–1091
14. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) *Nature Genet.* **1**, 45–49
15. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993) *Genes & Dev.* **7**, 1126–1132

16. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992) *Mol. Cell. Biol.* **12**, 2866–2871
17. Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A. J., and Shenk, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 12028–12032
18. Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. (1992) *Science* **256**, 827–830
19. Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R., and Prives, C. (1992) *Nature* **358**, 83–86
20. Ginsberg, D., Mehta, F., Yaniv, M., and Oren, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9979–9983
21. Mercer, W. E., Shields, M. T., Lin, D., Appella, E., and Ullrich, S. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 1958–1962
22. Santhanam, U., Ray, A., and Sehgal, P. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7605–7609
23. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75**, 817–825
24. Horikoshi, N., Usheva, A., Chen, J., Levine, A. J., Weinmann, R., and Shenk, T. (1995) *Mol. Cell. Biol.* **15**, 227–234
25. Hsu, Y. S., Tang, F. M., Liu, W. L., Chuang, J. Y., Lai, M. Y., and Lin, Y. S. (1995) *J. Biol. Chem.* **270**, 6966–6974
26. Zhang, Q., Gutsch, D., and Kenney, S. (1994) *Mol. Cell. Biol.* **14**, 1929–1938
27. Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., Kouzarides, T., and Martignetti, J. A. (1990) *Curr. Top. Microbiol. Immunol.* **154**, 125–169
28. St Joer, S. C., and Hutt, R. (1977) *J. Gen. Virol.* **37**, 65–73
29. St Joer, S. C., Albrecht, T. B., Funk, F. D., and Rapp, F. (1974) *J. Virol.* **13**, 353–362
30. Albrecht, T., Boldogh, I., Fons, M. P., and Nagy, T. V. (1993) in *Molecular Aspects of Human Cytomegalovirus Diseases* (Becker, Y., Darai, G., and Huang, E. S., eds) Springer-Verlag, New York
31. Speir, E., Modali, R., Huang, E. S., Leon, M. B., Shawl, F., Finkel, T., and Epstein, S. E. (1994) *Science* **265**, 391–394
32. Wathen, M. W., and Stinski, M. F. (1982) *J. Virol.* **41**, 462–477
33. Hagemeier, C., Caswell, R., Hayhurst, G., Sinclair, J., and Kouzarides, T. (1994) *EMBO J.* **13**, 2897–2903
34. Becker, Y., Darai, G., and Huang, E.-S. (1993) *Molecular Aspects of Human Cytomegalovirus Diseases*, Springer-Verlag, Berlin
35. Pizzorno, M. C., and Hayward, G. S. (1990) *J. Virol.* **64**, 6154–6165
36. Lang, D., and Stamminger, T. (1993) *J. Virol.* **67**, 323–331
37. Pizzorno, M. C., Mullen, M. A., Chang, Y. N., and Hayward, G. S. (1991) *J. Virol.* **65**, 3839–3852
38. Lillie, J. W., and Green, M. R. (1989) *Nature* **338**, 39–44
39. Zhang, W., Guo, X. Y., Hu, G. Y., Liu, W. B., Shay, J. W., and Deisseroth, A. B. (1994) *EMBO J.* **13**, 2535–2544
40. Smith, D. B., and Johnson, K. S. (1988) *Gene (Amst.)* **67**, 31–40
41. Lukac, D. M., Manuppello, J. R., and Alwine, J. C. (1994) *J. Virol.* **68**, 5184–5193
42. Martin, K. J., Lillie, J. W., and Green, M. R. (1990) *Nature* **346**, 147–152
43. Yew, P. R., Liu, X., and Berk, A. J. (1994) *Genes & Dev.* **8**, 190–202
44. Lin, Y. S., and Green, M. R. (1991) *Cell* **64**, 971–981
45. Lin, Y. S., Ha, I., Maldonado, E., Reinberg, D., and Green, M. R. (1991) *Nature* **353**, 569–571
46. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
47. Allday, M. J., Sinclair, A., Parker, G., Crawford, D. H., and Farrell, P. J. (1995) *EMBO J.* **14**, 1382–1391
48. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71**, 875–886
49. Yew, P. R., and Berk, A. J. (1992) *Nature* **357**, 82–85
50. Stenberg, R. M. (1993) in *Molecular Aspects of Human Cytomegalovirus Diseases* (Becker, Y., Darai, G., and Huang, E. S., eds) pp. 330–359, Springer-Verlag, Berlin
51. Liu, X., Miller, C. W., Koeffler, P. H., and Berk, A. J. (1993) *Mol. Cell. Biol.* **13**, 3291–3300
52. Agoff, S. N., Hou, J., Linzer, D. I., and Wu, B. (1993) *Science* **259**, 84–87
53. Licht, J. D., Hannarose, W., Reddy, J. C., English, M. A., Ro, M., Grossel, M., Shakhovich, R., and Hansen, U. (1994) *Mol. Cell. Biol.* **14**, 4057–4066
54. Baniahmad, A., Kohne, A. C., and Renkawitz, R. (1992) *EMBO J.* **11**, 1015–1023
55. Saha, S., Brickman, J. M., Lehming, N., and Ptashne, M. (1993) *Nature* **363**, 648–652
56. Han, K., and Manley, J. L. (1993) *Genes & Dev.* **7**, 491–503
57. Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P., and Rauscher, F. J., III (1991) *Science* **253**, 1550–1553