The Adenoviral E1B 55-Kilodalton Protein Controls Expression of Immune Response Genes but Not p53-Dependent Transcription[▽]

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The human adenovirus type 5 (Ad5) E1B 55-kDa protein modulates several cellular processes, including activation of the tumor suppressor p53. Binding of the E1B protein to the activation domain of p53 inhibits p53-dependent transcription. This activity has been correlated with the transforming activity of the E1B protein, but its contribution to viral replication is not well understood. To address this issue, we used microarray hybridization methods to examine cellular gene expression in normal human fibroblasts (HFFs) infected by Ad5, the E1B 55-kDa-protein-null mutant Hr6, or a mutant carrying substitutions that impair repression of p53-dependent transcription. Comparison of the changes in cellular gene expression observed in these and our previous experiments (D. L. Miller et al., *Genome Biol.* 8:R58, 2007) by significance analysis of microarrays indicated excellent reproducibility. Furthermore, we again observed that Ad5 infection led to efficient reversal of the p53-dependent transcriptional program. As this same response was also induced in cells infected by the two mutants, we conclude that the E1B 55-kDa protein is not necessary to block activation of p53 in Ad5-infected cells. However, groups of cellular genes that were altered in expression specifically in the absence of the E1B protein were identified by consensus k-means clustering of the hybridization data. Statistical analysis of the enrichment of genes associated with specific functions in these clusters established that the E1B 55-kDa protein is necessary for repression of genes encoding proteins that mediate antiviral and immune defenses.

The genomes of human subgroup C adenoviruses, such as adenovirus type 5 (Ad5), encode more than 20 proteins that are made prior to the onset of viral DNA synthesis in infected cells (reviewed in reference 1). The great majority of these immediate-early and early viral gene products interact with cellular proteins to optimize expression of viral genes or to block potentially deleterious host responses to infection. The 289R and 243R E1A proteins are prime examples of the former class. The larger E1A protein binds via a unique internal sequence to a specific subunit of the host cell mediator, a component of the RNA polymerase II transcriptional machinery, to stimulate transcription from viral early promoters by this enzyme *in vitro* and in infected cells (7, 77, 85). The 243R E1A protein interacts with the Rb protein and the related family members p107 and p130 to liberate transcriptional regulators of the E2F family, which are required for efficient transcription from the viral E2 early promoter (2, 13). This interaction is also crucial for the mitogenic and transforming activities of the E1A proteins. Adenoviral proteins that protect infected cells against antiviral defenses include E3 gene products, such as the 19-kDa glycoprotein that sequesters major histocompatibility complex class I molecules in the endoplasmic reticulum and several small proteins that prevent induction of apoptosis in response to external signals (20). The two

major E1B proteins, either of which can cooperate with E1A proteins to transform rodent cells (4, 50), can also block apoptosis. The E1B 19-kDa protein was the first viral homologue of a cellular antiapoptotic protein to be identified. It is similar in sequence and function to the cellular Bcl-2 protein and sequesters the proapoptotic proteins Bak and Bad (see reference 86). Consequently, the E1B 19-kDa protein can prevent induction of apoptosis in response to external signals. In contrast, the E1B 55-kDa protein can counter apoptosis that is induced by signals that originate within a cell, upon accumulation and activation of the cellular tumor suppressor p53.

When activated in response to DNA damage or other types of genotoxic stress, this crucial cellular regulator stimulates transcription of many genes that encode proapoptotic proteins, including Bak, Bad, and Puma, or components of the caspase activation machinery, such as Apaf-1 (see reference 22). It has been known for some time that the E1A proteins can induce apoptosis by both p53-dependent and p53-independent mechanisms (14, 45, 47, 56, 65, 82). Induction of p53-dependent apoptosis is accompanied by accumulation of the p53 protein and requires the E1A sequences that are also necessary for transformation and mitogenesis (11, 54, 55, 89). However, the E1B 55-kDa protein can counter these effects by several mechanisms. In cells in which the viral E4 Orf6 protein is also made, such as Ad5-infected cells, this E1B protein binds to the E4 Orf6 protein to form a virus-specific ubiquitin ligase that contains the cellular cullin 5, elongins B and C, and Rbx-1 proteins (32, 64). The p53 protein is a substrate of this enzyme *in vitro* (32, 64) and in cells producing the E1B 55-kDa and E4 Orf6 proteins (10). Mutations that eliminate synthesis of the E1B protein or prevent or impair its interactions with the p53 or E4 Orf6 protein lead to accumulation of p53 (6, 8, 57, 59, 63, 64, 67, 72, 76). It is therefore generally agreed that p53 is targeted

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FIG. 1. Effects of the sub17 mutations on accumulation of the E1B 55-kDa protein and protein V. (A) The coding sequences of the E1B proteins are shown to scale below the arrow representing the major E1B mRNA species, with different reading frames in white, gray, and black. As summarized above the mRNA, deletion of bp 2437 in the Hr6 genomes results in a shift of reading frame and termination of translation a short distance downstream. The C-terminal sequences of the wild-type and sub17 E1B 55-kDa proteins are shown between the expansion lines below, with the substitutions indicated in bold. (B) The E1B 55-kDa protein was examined 36 h after infection with Ad5 or Ad5E1B-sub17 (S17) or in mock-infected cells (M) by immunoblotting as described in Materials and Methods. Numbers at left are molecular masses in kilodaltons.

for proteasomal degradation by the action of the virus-specific ubiquitin ligase.

A second mechanism that operates in transformed cells that contain the viral E1A and E1B proteins is sequestration of the p53 protein by the E1B 55-kDa protein in perinuclear cytoplasmic structures (5, 93). Such sequestration requires interaction of the E1B and p53 proteins (38, 93) and has been correlated with the rapid proliferation of cells that express the E1A and E1B genes (28). The E1B 55-kDa protein can block the action of p53 by a second, E4 Orf6-independent mechanism: its binding to the N-terminal activation domain of p53 inhibits p53-dependent transcription in *in vitro* reactions and transient expression assays (48, 90, 92). Such inhibition is thought to be mediated by a repression domain present in the E1B protein, which was identified by virtue of the ability of a Gal4 DNA-binding domain–E1B 55-kDa fusion protein to repress transcription from several promoters that contained Gal4 binding sites (49, 92). Although the mechanism of repression remains incompletely understood (see reference 2), this function of the E1B protein has been implicated in transformation. Insertion of four amino acids near the C terminus impaired both repression of transcription by the Gal4-E1B protein fusion and transformation by the E1B protein in cooperation with E1A (49, 92), as did substitution at three C-terminal sites of phosphorylation (Ser490, Ser491, and Thr495) or at the two serine residues (80, 81). These substitution mutations also prevented inhibition of p53-dependent apoptosis by the E1B 55-kDa protein (80). More recently, it has been reported that a single amino acid substitution that prevents modification of the E1B 55-kDa protein by addition of Sumo-1 to Lys104 also reduces the transforming activity of the protein and its inhibition of p53-dependent transcription (18). Conversely, substitutions that block nucleocytoplasmic shuttling of the E1B protein were observed to both stimulate transformation and increase repression of p53-dependent transcription (17). These observations indicate that repression of transcription of p53-responsive genes makes an important contribution to the ability of the

E1B 55-kDa protein to cooperate with E1A proteins in transformation of rodent cells, although other activities of this viral protein are also required (33).

In contrast, relatively little is known about how inhibition of the transcriptional function of p53 facilitates adenovirus replication. The C-terminal insertion and substitution mutations described in the previous paragraph have been reported to reduce the efficiency of viral replication in HeLa cells by 2 to 3 orders of magnitude (80, 81). Whether this phenotype is a consequence of failure to repress p53-dependent transcription is not known, but it seems unlikely; HeLa cells produce the human papillomavirus type 18 E6 protein (71), which in conjunction with a cellular protein acts as a ubiquitin ligase that targets p53 for proteasomal degradation (see reference 70). We therefore wished to investigate the role of transcription repression by the E1B 55-kDa protein during productive infection and have used microarray hybridization methods to compare alterations in cellular gene expression in normal human cells infected by Ad5 and E1B mutant viruses.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFFs) and Ad5-transformed 293 cells (27) were maintained in culture as described previously (24). Ad5 and the E1B mutants were propagated in 293 cells, and the concentration of infectious particles was determined by plaque assay (88) on these same cells.

The sub16 and sub17 mutations that introduce substitutions at C-terminal sites of phosphorylation of the E1B 55-kDa protein (80, 81) were introduced in the viral genome by using a modification of the AdEasy system (34). A transfer plasmid containing the E1A and E1B transcription units was first constructed. The Ad5 sequence from bp 322 to bp 3942 in the viral genome was amplified from purified Ad5 DNA by PCR with appropriate primers and high-fidelity DNA polymerase (pfuUltra High-Fidelity DNA polymerase; Stratagene) and cloned between the NotI and MfeI sites of the pShuttle plasmid of the AdEasy system (34). The structure of the resulting transfer plasmid, pShuttle-E1, in which the E1A and E1B sequences were reconstructed, was confirmed by restriction endonuclease digestion and sequencing of the inserted sequence (Genewiz Inc.). The sub16 and sub17 mutations (Fig. 1A) were introduced into pShuttle-E1 by using the QuikChange II site-directed mutagenesis kit with the protocol recommended by the manufacturer (Stratagene). Plasmids carrying the desired mutations were identified by the presence of a new restriction endonuclease site, and

the presence of the mutations was confirmed by sequencing the E1B gene (Genewiz Inc.). The mutations were recovered into the viral genome by homologous recombination with pAdEasy (34) in *Escherichia coli* BJ5183 cells, and the presence of the mutations was confirmed by sequencing. Viral genomes were released from the plasmids by digestion with PacI (34) and introduced into complementing 293 cells by using Transfectin lipid reagent (Bio-Rad Laboratories), and viruses recovered were subjected to two cycles of plaque purification in 293 cells, prior to amplification. The presence of the mutations in the mutant virus genomes was reconfirmed by sequencing of viral DNA (Genewiz Inc.).

Immunoblotting. HFFs were infected with 30 PFU/cell of Ad5, Hr6, or the substitution mutant Ad5E1B-sub16 or Ad5E1B-sub17, or mock infected, and harvested during the late phase of infection, 36 h after infection (24). Cells were washed with phosphate-buffered saline (Gibco-BRL); resuspended in 0.05 M Tris-HCl, pH 6.8, containing 10% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate, 0.05% (wt/vol) bromophenol blue, and 2% (vol/vol) β -mercaptoethanol; and sonicated twice for 30 s each. The steady-state concentration of the E1B 55-kDa protein was examined by immunoblotting with the 2A6 (69) monoclonal antibody, as described previously (25).

Analysis of cellular gene expression. HFFs that had been maintained in culture for 4 days after reaching confluence were infected with 30 PFU/cell of Ad5, Hr6, or Ad5E1B-sub17 or mock infected. Cells were harvested at the end of the 1-h adsorption period or 30 h after infection. Total cellular RNA was purified, amplified, labeled with Cy5-CTP, and hybridized to Agilent Whole Human 44K microarrays exactly as described previously (51). The reference RNA for the competitive hybridization was the same preparation of Cy3-CTP-labeled mixture of human RNAs isolated from five different cell lines as that used in our previous experiments (51). Array hybridization, washes, and data acquisition were performed as described previously (51). Raw data were extracted using the Agilent Feature extraction software and loaded onto the Princeton University PUMA database (<http://puma.princeton.edu>) for storage and spot quality filtering, as described previously (51). Processing of filtered $\log_2$ dye ratio data, including zero transformation and visualization, and analyses using significance analysis of microarrays (SAM), consensus clustering, and Gene Ontology (GO) Term Finder were performed as described previously (51). The dye-normalized and background-corrected data set is available for download at <http://puma.princeton.edu/>. Processed data (spot quality filtered, $\log_2$ transformed, and zero transformed) are available for download at the website http://genomics-pubs.princeton.edu/Ad5vsMut_HFF. All figures containing expression data can also be downloaded and searched interactively on the website http://genomics-pubs.princeton.edu/Ad5vsMut_HFF.

RESULTS

Isolation and of the Ad5E1B-sub17 mutant. As described in the introduction, previous studies have established that the Ad5 E1B 55-kDa protein contains a C-terminal domain that represses transcription in *in vitro* reactions and transient expression assays. Phosphorylation of three residues located close to the C terminus, Ser490, Ser491, and Thr495, is critical for this activity: substitutions that simultaneously replaced all three sites with alanine or that replaced Ser490 and Ser491 impaired repression of p53-dependent transcription and of transcription of a reporter gene with a promoter containing Gal4 binding sites when the E1B proteins were fused to the Gal4 DNA-binding domain (80, 81). As we wished to examine specifically the contribution of this activity of the E1B 55-kDa protein to viral replication, we isolated mutant viruses carrying substitutions for these phosphorylation sites.

The substitutions that result in replacement of Ser490, Ser491, and Thr495 with alanine (sub16) or replace just the two serines (sub17) (Fig. 1A) were introduced into the viral genome by homologous recombination in *E. coli*, as described in Materials and Methods. The presence of the desired mutations in the mutant viral genomes was confirmed by sequencing of viral DNA. The E1B 55-kDa protein appears to be relatively sensitive to even small changes in its sequence: a number of 4-amino-acid insertions have been shown to destabilize the protein or prevent its efficient entry into infected cell nuclei

(25, 42). We therefore first examined the effects of the sub16 and sub17 mutations on the steady-state concentration attained by the E1B 55-kDa protein-infected cells. As will be described elsewhere (M. Mashiba et al., submitted for publication), this protein could not be detected in either HFFs or HeLa cells infected by Ad5E1B-sub16. In contrast, the accumulation of the E1B protein late in infection was very similar in HFFs infected by Ad5 and in those infected by Ad5E1B-sub17 (Fig. 1B), as was that of the related 84R E1B protein that is not affected by the sub17 mutations (Fig. 1A). Synthesis of similar quantities of the E1B 55-kDa protein in HeLa cells infected by Ad5 and the equivalent mutant was also reported previously (80). These observations indicate that the Ser490Ala and Ser491Ala substitutions do not decrease the stability of the protein.

Overview of microarray hybridization data. We performed two-color hybridizations using Agilent 44K Whole Genome microarrays to examine changes in the concentrations of cellular RNA species in HFFs infected with Ad5, the E1B 55-kDa null mutant Hr6, or the substitution mutant Ad5E1B-sub17 or mock infected. Three independent samples of infected cells were harvested immediately after virus adsorption (time zero) and 30 h after infection, and duplicate samples of mock-infected cells were collected at 0, 24, and 48 h. Total cellular RNA was purified, amplified, and labeled as described in Materials and Methods. Labeled cRNAs prepared from Ad5-, mutant-, or mock-infected samples (red channel) were hybridized competitively with approximately equal concentrations of a common reference cRNA (green channel). The reference cRNA was prepared from a mixture of RNAs isolated from a diverse set of human cells and cell lines (51). For each hybridization, variations in the input of labeled cRNA were corrected by a standard computational dye normalization. Prior to data filtering and analysis, the final expression values for each probe were zero transformed: the $\log_2$ expression values for mock, Ad5, and Ad5E1B-sub17 infections were linearly transformed by subtracting the mean values of the corresponding time zero samples.

To establish the robustness of our experimental design, we compared the changes in gene expression in response to Ad5 infection to those detected in our previous, high-resolution time course analysis of the changes in gene expression induced by Ad5 infection of HFFs (51). Using SAM (84), we identified the set of genes that exhibited significant alterations in expression 30 h after Ad5 infection and yet remained unchanged after mock infection. We then isolated the resulting 1,113 significantly upregulated and 505 significantly downregulated mRNAs (false discovery rate [FDR] = 0.9%) from a joined data set containing the wild-type expression data from both experiments and clustered them hierarchically. This comparison revealed broad correspondence between the downregulated and upregulated genes (data not shown; available at http://genomics-pubs.princeton.edu/Ad5vsMut_HFF/) in the two experiments. As virus infection, cell handling, and RNA isolation and processing, as well as the microarray hybridizations, were performed by separate investigators (D.L.M. and B.R.) in the two experiments, these results indicate a high level of reproducibility between the independent microarray hybridization assays that we have performed.

To isolate a core set of probes that showed specific changes

FIG. 2. Clusters of coregulated cellular RNAs 30 h after infection with Ad5 or E1B mutants. The expression profiles of significantly regulated genes were clustered into eight distinct groups with similar expression response profiles across the different Ad5 genotypes as described in Materials and Methods. Each cluster was searched for statistically significant enrichment of genes that code for proteins with common functions (*P* values are Bonferroni corrected for multiple hypothesis testing). GO terms enriched in clusters 2, 3, and 4 are shown at right, with the corresponding *P* values. Genes that increased and decreased in expression are shown in yellow and blue, respectively, as indicated on the scale bar. hpi, hours postinfection.

in cellular gene expression in response to infection, we applied the following intensity filters to the data: probes were required to exhibit a log₂ expression value of ≥ 1 (equivalent to twofold change) in at least three arrays in the infected series and a log₂ expression value of ≤ 0.4 (equivalent to 1.3-fold change) in no more than two arrays in the mock-infection series. A total of 4,200 probes corresponding to 2,924 unique genes met these criteria. This data set can be accessed at the Princeton University Microarray database (PUMA) (see Materials and Methods). To gain an overview of the extent to which E1B mutations altered the gene expression changes induced by Ad5

infection, we used an agglomerative consensus k-means clustering method (52) to resolve the filtered data set into eight sets of probes defined by common expression profiles across the three virus genotypes (Fig. 2). In this way, we identified several sets of genes that differed strikingly in expression following infection with Ad5 or the E1B mutants. The most dramatic differences in RNA concentrations were grouped in clusters 2 and 5, in which RNA concentrations increased and decreased 2- to 16- and 2- to 6-fold, respectively, in Hr6-infected cells but remained largely unchanged after infection with Ad5. Together clusters 2 and 5 contain probes corre-

FIG. 3. Expression of immune response genes is specifically altered by infection with Hr6 specifically. Application of SAM identified 339 RNAs that are altered in concentration in response to infection with the E1B-null mutant (Hr6), compared to infection with Ad5 (FDR = 0.1%). Functional classes of RNAs significantly overrepresented among Hr6-specific RNAs are indicated at the right (*P* values are Bonferroni corrected for multiple hypothesis testing). hpi, hours postinfection.

sponding to 647 unique genes (324 downregulated and 323 upregulated).

Analysis of cellular functions targeted by Ad5 and E1B mutants. We have previously shown that Ad5 infection induces changes in expression of genes associated with common functions, including cell cycle control, DNA repair, nucleocytoplasmic transport, chromatin assembly, and ribosome biogenesis (51). Furthermore, genes that clustered together based on the similar kinetics of changes in their expression following infection were found to encode proteins that participate in common cellular pathways. It was therefore possible that the genes differentially expressed in cells infected by Ad5 and the E1B mutants specify products that function in common biological functions. To test this hypothesis, we searched each of the eight clusters for significant enrichment of genes associated with specific cellular functions. We used a local implementation of GO Term Finder (see Materials and Methods), which maps each gene in a query list to a node in the “biological process ontology” of the GO Consortium and computes a probability for the preponderance of each function in the query list (*P* values are Bonferroni corrected for multiple hypothesis testing).

Three of the eight clusters of significantly altered transcripts were enriched for those that function in common cellular processes. RNAs that increased strongly in concentration by 30 h after infection, irrespective of E1B status, were grouped in cluster 4 and showed a significant enrichment for those specifying proteins that participate in mRNA and noncoding RNA processing and splicing, ribosome biogenesis (all with *P* values of <10^{−8}), and mitochondrial protein targeting (*P* < 10^{−4}) (Fig. 2). The probes in cluster 3 correspond to genes that are also activated in response to Ad5 and Ad5E1B-sub17 infection, albeit less strongly, but increased in expression to a significantly higher degree in Hr6-infected cells. Transcripts that fall into this group are significantly enriched for those that code for proteins functioning in gene expression (*P* = 10^{−7}) and RNA processing (*P* = 10^{−5}). Remarkably, genes that are strongly and specifically activated only in response to infection with the E1B 55-kDa-protein-null mutant virus Hr6 (cluster 2) are heavily enriched for those encoding proteins that participate in immune responses and innate antiviral defense (*P* = 10^{−10} and *P* = 10^{−8}, respectively) (Fig. 2). For example, HLA-B, HLA-E, HLA-C, and HLA-F, four members of the

HLA class I heavy chain paralogues, are coordinately upregulated in response to Hr6 infection, as are the genes for the interferon-inducible proteins Ifi35, Gbp, Gbp2, Ifi1, Ifi16, and Gtpbp1; interleukin 29 and interleukin 6; the Notch ligand Dll1; and the inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon, Ikbke. Transcripts of genes associated with virus-specific cellular responses were also coinduced in Hr6-infected cells. Among them are transcripts of the MX2 gene, the interferon gene IFNA4, TRIM5, and the interferon-regulated genes IRG7 and IFIH1. Furthermore, cluster 2 is significantly enriched in transcripts of genes associated with the regulation of apoptosis (*P* = 10^{−5}). These include STAT1, RIPK2, CHK2, CASP1, CASP10, and FAS1, as well as several negative regulators of apoptosis like CFLAR, the caspase 1 inhibitors CO1 and INCA, and the protease inhibitor SERPINB9.

A more rigorous isolation of genes specifically altered after Hr6 infection using SAM resulted in a set of 339 unique genes (FDR, 0.1%) that are activated following Hr6 infection compared to Ad5. A survey of this group for overrepresentation of functional classes using GO Term Finder identified several immune-related and antiviral response terms with high confidence (Fig. 3; see Table 2), including NF-κB pathway regulation and the interferon response, confirming our initial analysis. In the absence of the E1B 55-kDa protein, expression of these genes was increased 2- to over 25-fold (Table 1).

A notable, and unexpected, conclusion of this analysis is that the majority of aberrations in the transcription of cellular genes associated with E1B mutant virus infection were independent of the previously described transcriptional function of the E1B 55-kDa protein (see the introduction). For virtually all probes isolated on the basis of differential expression in Ad5- and Hr6-infected HFFs, the signals observed following Ad5E1B-sub17 infection were indistinguishable from those detected in Ad5-infected cells (Fig. 2). This result indicated that the transcriptional alterations induced by the absence of the E1B 55-kDa protein cannot be recapitulated with point mutations that eliminate repression of transcription by the E1B protein. However, it remained possible that infection with Ad5E1B-sub17 led to some alterations of cellular transcription compared to Ad5 infection. In fact, analysis of the differences between Ad5- and Ad5E1B-sub17-infected cells using SAM resulted in the isolation of a very small number of unique

TABLE 1. Immune and antiviral response genes repressed by the E1B 55-kDa protein^b

Gene ID	Unigene ID	Gene name	Hr6/Ad5 ^a
APOBEC3G	Hs.660143	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G	3.8
CD274	Hs.521989	CD274 molecule	5.3
CD47	Hs.446414	CD47 molecule	2.5
CTSS	Hs.181301	Cathepsin S	2.6
GBP1	Hs.62661	Guanylate binding protein 1, interferon inducible, 67 kDa	8.5
GBP2	Hs.386567	Guanylate binding protein 2, interferon inducible	4.6
GBP3	Hs.656774	Guanylate binding protein 3	3.7
GBP4	Hs.409925	Guanylate binding protein 4	18.7
GBP5	Hs.513726	Guanylate binding protein 5	26.4
HLA-E	Hs.650174	Major histocompatibility complex, class I, E	2.2
IFI16	Hs.380250	Gamma interferon-inducible protein 16	2.7
IFI35	Hs.632258	Interferon-induced protein 35	3.4
IFIH1	Hs.163173	Interferon induced with helicase C domain 1	5.1
IFIT2	Hs.437609	Interferon-induced protein with tetratricopeptide repeats 2	20.0
IFIT3	Hs.47338	Interferon-induced protein with tetratricopeptide repeats 3	14.1
IFNA4	Hs.1510	Alpha interferon 4	8.3
IKBKE	Hs.321045	Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon	3.1
IL-29	Hs.406745	Interleukin 29 (lambda interferon 1)	4.6
IL-6	Hs.654458	Interleukin 6 (beta interferon 2)	7.3
IRF1	Hs.436061	Interferon regulatory factor 1	4.4
IRF7	Hs.166120	Interferon regulatory factor 7	3.2
LAG3	Hs.409523	Lymphocyte activation gene 3	2.5
LGP2	Hs.55918	Likely ortholog of mouse D11lgp2	3.1
MASP2	Hs.655645	Mannan-binding lectin serine peptidase 2	1.7
MX2	Hs.926	Myxovirus (influenza virus) resistance 2 (mouse)	2.9
MYD88	Hs.82116	Myeloid differentiation primary response gene -88	4.1
PLSCR1	Hs.130759	Phospholipid scramblase 1	2.8
PSMB9	Hs.654585	Proteasome (prosome, macropain) subunit, beta type, 9	4.7
PSME2	Hs.434081	Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	2.8
RAG1	Hs.73958	Recombination-activating gene 1	2.4
SAMHD1	Hs.580681	SAM domain and HD domain 1	4.2
SECTM1	Hs.558009	Secreted and transmembrane 1	3.3
TAP1	Hs.352018	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	7.1
TLR2	Hs.519033	Toll-like receptor 2	5.7
TRIM5	Hs.370515	Tripartite motif-containing 5	3.4
XBP1	Hs.437638	X-box binding protein 1	2.3

^a Ratios of average raw expression values of Hr6- and Ad5-infected cells at 30 h after infection.

^b Genes differentially regulated in Hr6-infected cells and antiviral responses were isolated by SAM comparing Hr6 and Ad5 infection (FDR = 0.1%). ID, identifier.

genes, 39 (even with a highly permissive FDR of 10%), that were differentially expressed. Not surprisingly, the expression profiles of these genes were very similar in cells infected by Ad5E1B-sub17 and in cells infected by Hr6 (data not shown). This subset of genes represents a potential target of the previously described transcriptional regulation function of the E1B 55-kDa protein. Nevertheless, we observed that the great majority of changes induced by infection in the absence of the E1B 55-kDa protein do not take place in Ad5E1B-sub17-infected cells (Fig. 2).

Analysis of p53-responsive genes. In our previous study, we demonstrated that Ad5 infection of normal human cells results in very effective suppression of the transcriptional activity of p53. A set of primary p53 target genes identified by Kannan and colleagues (41) exhibited no evidence of activation at any point during the course of Ad5 infection examined in that study (12 to 60 h after infection). We reasoned that this effect may be due to the E1B 55-kDa protein and its ability to prevent p53 accumulation and/or activation of p53-dependent transcription. In order to test this hypothesis, we isolated the same set of p53 target genes from our data set, excluding only those probes that displayed nonspecific transcriptional responses in mock-infected cells. As observed previously, infec-

tion of HFFs with Ad5 fully suppressed the p53 transcriptional program (Fig. 4): the great majority of primary p53 target genes exhibited either no response to Ad5 infection or a reversal of the p53-induced changes. Surprisingly, with the exception of ATF3 (see below), these same responses were observed in the absence of the E1B 55-kDa protein (Hr6) or after infection with Ad5E1B-sub17, which encodes an altered E1B protein that cannot repress transcription (Fig. 4; Table 2). We also examined expression of 35 additional genes represented in our data set that have been reported to be transcriptionally activated or repressed by p53 (16, 74, 75). In all cases, the change in expression in response to p53 was suppressed or reversed in both Ad5- and Hr6-infected cells (Table 2). These observations indicate that the Ad5-induced suppression of the p53 transcriptional program is not dependent on the presence of the E1B 55-kDa protein.

The concentration of ATF3 transcripts was increased some sixfold, specifically in Hr6-infected cells (Table 2). This gene encodes a member of the cyclic AMP-dependent activity transcription factor/cyclic AMP response element-binding protein family that represses transcription when homodimeric (30, 31). Its expression is induced in response to a variety of stresses, including endoplasmic reticulum stress, amino acid starvation,

FIG. 4. The expression of p53 target genes in cells infected by Ad5 or E1B mutants. The gene expression responses of direct p53 target genes were isolated and clustered hierarchically based solely on the expression profiles in response to infection. The rightmost column is a qualitative representation of p53-dependent expression (41), in which yellow and blue represent p53-activated and -repressed genes, respectively. hpi, hours postinfection.

DNA damage, and ischemia (30, 31, 40). More recently, ATF3 has been implicated in regulation of inflammatory responses (23), and its expression in primary monocytes and macrophages has been reported to be stimulated by delta interferon and other inflammatory factors (35). This gene therefore appears to represent another example of those associated with immune responses that are repressed only when the E1B 55-kDa protein is made in infected cells (Fig. 2).

The E1B 55-kDa proteins and alterations in expression of cell cycle genes. Recent reports have implicated E1B gene products in the regulation of expression of cell cycle-related genes. Rao et al. identified 345 genes that varied in expression by twofold or greater when normal lung fibroblasts (WI38 cells) were infected with an Ad5 mutant virus (Adhz60) defective for production of all E1B proteins compared to infection with the wild-type virus (66). A major class of such E1B-dependent genes were found to be cell cycle related, and it was noted that infection by the E1B null mutant failed to activate expression of CYCLIN E1, CDC25A, CDK5R, and PPAT. With the exception of CDK5R (transcript levels of both isoform 1 and isoform 2 remain unaltered), these genes were also found to be strongly activated upon Ad5 infection of primary HFFs (CYCLIN E1, 2.5-fold; CDC25A, 10-fold; PPAT, three-fold). However, our data revealed no dependence of these responses on the E1B 55-kDa protein: the concentrations of these cellular RNAs in Hr6-infected cells were the same as, or even higher than, those observed in Ad5-infected cells (data not shown). Even though the differential expression of cell cycle-dependent genes in E1B mutant-infected cells was not reproduced in our experiments, we wished to determine the degree of overlap between the sets of E1B-dependent changes

in transcript concentration identified in our system and those identified by Rao et al. From the 345 genes deemed significantly altered by these authors, we isolated the expression data of the 181 unique genes also present in our data set (excluding all those that showed responses to mock infection or low data quality; see Materials and Methods), joined them with the wild-type/mutant ratios published by Rao et al. (66), and clustered the composite data set hierarchically. This comparison indicated that the differential expression patterns of the genes reported by Rao et al. were not reproduced in our data (Fig. 5). Further, by comparison to the list of 339 unique genes that exhibited infection-specific and significantly different expression levels in Ad5- and Hr6-infected cells (Fig. 3), we determined that only 1% of the genes reported by Rao et al. are also present in the gene set that we identified.

Some of these differences may be explained by the different genotypes of the mutant viruses (see Discussion) or the elimination of changes also detected in mock-infected cells in our experiments. Nevertheless, we would expect the changes induced upon infection with Adhz60, which cannot express any E1B gene products (66), to include those observed in the absence of only the E1B 55-kDa protein in Hr6-infected cells. Furthermore, the substantial increases in expression of cellular genes associated with immune responses to viral infection seen in Hr6-infected cells (Fig. 2) were not evident in Adhz60-infected WI38 cells. This discrepancy might be the result, at least in part, of differences in microarray hybridization platforms or other technical differences, although many of the increases in RNA concentration in Hr6-infected cells were sufficiently large that they would be readily detectable independently of the analysis platform. Other possible explanations include infection of different types of host cells or analysis of cellular gene expression at different times after infection, during the late phase of infection (reported here), or at 12 h postinfection (66).

DISCUSSION

Our previous global analysis of the effects of Ad5 infection on expression of human genes in normal human fibroblasts identified changes in some 10% of the genes examined and in genes associated with a variety of specific cellular processes or programs (51). For example, the great majority of E2F-responsive genes increased substantially in expression by 12 h after infection, and Ad5 infection reversed the quiescence program (12) and induced the core serum response. In contrast to results obtained with other viruses, including several herpesviruses and retroviruses (see reference 39), genes encoding proteins that participate in innate and adaptive immune defenses to viral infection were not significantly enriched in any of the clusters of genes defined on the basis of the kinetics of the changes in the corresponding RNA concentrations following Ad5 infection (51). It is now clear from the data presented here that the expression of many such genes is repressed in Ad5-infected cells, by a mechanism that requires the E1B 55-kDa protein: consensus k-means clustering and GO term analysis established that the set of genes that were increased in expression only in cells infected by the E1B 55-kDa-protein-null mutant Hr6 were heavily enriched in those associated with innate and adaptive immune responses to viral infection (Fig.

TABLE 2. Expression of p53 target cells in Ad5- and Hr6-infected HFFs^d

Gene ID	Unigene ID	Gene name	Avg raw expression ^a		p53 ^b
			Ad5	Hr6	
Genes identified by Kannan et al. (41)					
ABCA3	Hs.26630	ATP-binding cassette, subfamily A (ABC1), member 3	0.9	1.1	Up
ACTA2	Hs.500483	Actin, alpha 2, smooth muscle, aorta	0.8	0.6	Up
ADFP	Hs.3416	Adipose differentiation-related protein	0.7	0.8	Up
ATF3	Hs.460	Activating transcription factor 3	0.6	6.3	Up
BAK1	Hs.485139	BCL2-antagonist/killer 1	1.6	1.8	Up
BBC3	Hs.467020	BCL2 binding component 3	0.7	0.8	Up
CDKN1A	Hs.370771	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.8	0.7	Up
CES2	Hs.282975	Carboxylesterase 2 (intestine, liver)	0.8	0.8	Up
CTDSP2	Hs.524530	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2	0.6	0.5	Up
DMWD	Hs.515474	Dystrophia myotonica, WD repeat containing	0.9	0.9	Up
ENG	Hs.76753	Endoglin (Osler-Rendu-Weber syndrome 1)	1.0	1.0	Up
EXT2	Hs.368404	Exostoses (multiple) 2	0.9	0.9	Up
FEZ1	Hs.224008	Fasciculation and elongation protein zeta 1 (zyglin I)	0.9	0.8	Up
GADD45A	Hs.80409	Growth arrest and DNA damage inducible, alpha	0.6	0.7	Up
HRAS	Hs.37003	V-Ha-ras Harvey rat sarcoma viral oncogene homologue	1.4	1.3	Up
IGFBP6	Hs.274313	Insulin-like growth factor binding protein 6	0.9	1.7	Up
KIAA0247	Hs.440025	KIAA0247	1.0	1.1	Up
LCAT	Hs.387239	Lecithin-cholesterol acyltransferase	0.9	0.7	Up
MAN2B1	Hs.356769	Mannosidase, alpha, class 2B, member 1	0.7	0.7	Up
NDN	Hs.50130	Necdin homologue (mouse)	0.9	0.7	Up
NEFL	Hs.521461	Neurofilament, light polypeptide 68 kDa	1.0	0.9	Up
NHLH2	Hs.46296	Nescient helix-loop-helix 2	0.9	1.0	Up
NINJ1	Hs.494457	Ninjurin 1	1.1	1.7	Up
PDIA4	Hs.93659	Protein disulfide isomerase family A, member 4	1.1	1.3	Up
PDLIM4	Hs.424312	PDZ and LIM domain 4	1.4	1.1	Up
PRKAB1	Hs.6061	Protein kinase, AMP activated, beta 1 noncatalytic subunit	1.0	1.0	Up
RPS8	Hs.512675	Ribosomal protein S8	0.8	0.8	Up
SCRIB	Hs.436329	Scribbled homologue (<i>Drosophila melanogaster</i>)	1.4	1.6	Up
SMAD7	Hs.465087	SMAD family member 7	0.8	0.7	Up
SMTN	Hs.149098	Smoothelin	1.1	0.6	Up
SPAG1	Hs.591866	Sperm-associated antigen 1	3.0	3.8	Up
SURF1	Hs.512464	Surfeit 1	0.7	0.4	Up
TAX1BP3	Hs.12956	Tax1 (human T-cell leukemia virus type 1) binding protein 3	0.9	0.5	Up
TST	Hs.474783	Thiosulfate sulfurtransferase (rhodanese)	1.0	0.5	Up
WDR68	Hs.410596	WD repeat domain 68	0.9	0.6	Up
ZNF7	Hs.493218	Zinc finger protein 7	0.9	0.4	Up
FAS	Hs.244139	Fas (tumor necrosis factor receptor superfamily, member 6)	0.9	3.0	Up
FHL2	Hs.443687	Four and a half LIM domains 2	0.8	0.7	Up
HIST1H2AC	Hs.484950	Histone cluster 1	0.8	0.8	Up
POMZP3	Hs.488877	POM (POM121 homologue, rat) and ZP3 fusion	1.1	1.4	Up
DGKA	Hs.524488	Diacylglycerol kinase, alpha 80 kDa	0.7	0.5	Up
ARL4A	Hs.245540	ADP-ribosylation factor-like 4A	1.4	2.1	Down
C4orf10	Hs.398178	Chromosome 4 open reading frame 10	1.0	0.7	Down
CCNE1	Hs.244723	Cyclin E1	4.0	2.6	Down
COL18A1	Hs.517356	Collagen, type XVIII, alpha 1	0.9	0.8	Down
EIF4A1	Hs.129673	Eukaryotic translation initiation factor 4A, isoform 1	1.5	2.0	Down
FOSL2	Hs.220971	FOS-like antigen 2	0.7	0.7	Down
INA	Hs.500916	Internexin neuronal intermediate filament protein, alpha	1.9	3.3	Down
LAMC2	Hs.591484	Laminin, gamma 2	1.0	0.9	Down
MGC5370	Hs.484551	Hypothetical protein MGC5370	0.9	1.2	Down
PMP22	Hs.372031	Peripheral myelin protein 22	0.6	0.6	Down
PURA	Hs.443121	Purine-rich element binding protein A	0.9	1.0	Down
TMEM97	Hs.199695	Transmembrane protein 97	2.0	2.2	Down
Other genes ^c					
ABCB1	Hs.489033	ATP-binding cassette, subfamily B (MDR/TAP), member 1	1.2	1.3	Up
APAF1	Hs.708112	Apoptotic peptidase activating factor 1	1.0	1.6	Up
BAI1	Hs.194654	Brain-specific angiogenesis inhibitor 1	1.0	1.0	Up
BAX	Hs.631546	BCL2-associated X protein	0.9	0.7	Up
BBC3	Hs.467020	BCL2 binding component 3	0.7	0.8	Up
BID	Hs.591054	BH3-interacting domain death agonist	1.9	2.2	Up
CASP6	Hs.654616	Caspase 6, apoptosis-related cysteine peptidase	0.9	0.8	Up

Continued on following page

TABLE 2—Continued

Gene ID	Unigene ID	Gene name	Avg raw expression ^a		p53 ^b
			Ad5	Hr6	
CCNG1	Hs.79101	Cyclin G1	0.7	0.5	Up
CDKN1A	Hs.370771	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.8	0.7	Up
EEF1A1	Hs.586423	Eukaryotic translation initiation factor 1 alpha 1	0.9	0.6	Up
EGFR	Hs.488293	Epidermal growth factor receptor	1.1	1.1	Up
FAS	Hs.244139	Fas (tumor necrosis factor receptor superfamily, member 6)	0.9	3.0	Up
FDXR	Hs.69745	Ferredoxin reductase	0.8	0.8	Up
GADD45A	Hs.80409	Growth arrest and DNA damage inducible, alpha	0.6	0.7	Up
HGS	Hs.514590	Hepatocyte growth factor-regulated tyrosine kinase substrate	1.1	1.0	Up
LRDD	Hs.592290	Leucine-rich repeats and death domain containing	1.2	1.6	Up
MDM2	Hs.567303	Mdm2 p53 binding protein homologue (mouse)	1.0	1.2	Up
ODC1	Hs.467701	Ornithine decarboxylase 1	2.5	2.9	Up
P53AIP1	Hs.160953	P53-regulated apoptosis-inducing protein 1	1.2	1.0	Up
PERP	Hs.520421	PERP, TP53 apoptosis effector	0.8	0.9	Up
PPM1D	Hs.591184	Protein phosphatase 1D magnesium dependent, delta isoform	0.9	1.5	Up
PTEN	Hs.500466	Phosphatase and tensin homologue	0.7	0.7	Up
SFN	Hs.523718	Stratifin	1.0	0.9	Up
THBS1	Hs.164226	Thrombospondin 1	1.0	0.8	Up
TP53	Hs.654481	Tumor protein p53	1.3	1.1	Up
TP53I3	Hs.50649	Tumor protein p53 inducible protein 3	1.2	0.7	Up
TRIAP1	Hs.69499	TP53-regulated inhibitor of apoptosis 1	1.4	2.4	Up
CCND1	Hs.523852	Cyclin D1	0.8	0.8	Down
CRYZ	Hs.83114	Crystallin, zeta (quinone reductase)	0.7	0.5	Down
HSPA8	Hs.702021	Heat shock 70-kDa protein 8	2.1	3.0	Down
IER3	Hs.591785	Immediate-early response 3	0.7	1.1	Down
MYC	Hs.202453	Similar to open reading frame 114	0.8	0.6	Down
SCD	Hs.558396	Stearoyl coenzyme A desaturase (delta-9-desaturase)	1.6	3.6	Down
SOD2	Hs.487046	Superoxide dismutase 2, mitochondrial	1.6	3.6	Down
TNFRSF10B	Hs.521456	Tumor necrosis factor receptor superfamily, member 10b	1.1	2.0	Down

^a Average raw expression values at 30 h after infection, expressed as a ratio to average zero time point values.

^b Regulation of target genes by p53.

^c The gene list was assembled via literature searches and online resources (16, 74, 75).

^d ID, identifier.

2). This set included four HLA class I heavy chain genes, interferon-inducible genes, and genes encoding interleukins and proteins for regulators of the NF- κ B pathway (Fig. 2; Table 1). The reductions of expression of these cellular genes, some quite large (Table 1), were observed 30 h after Hr6 infection, when infected, quiescent HFFs are just entering the late phase of infection (51). This temporal pattern suggests that the reduced concentrations of transcripts of cellular genes associated with immune defenses cannot be ascribed to regulation of mRNA export by the E1B 55-kDa protein (see references 2 and 25), which occurs only during the late phase of infection. The E1B 55-kDa protein therefore appears to fulfill a previously unrecognized function, repression of expression of host cell genes that encode proteins that induce or execute innate and adaptive antiviral defense mechanisms (Fig. 2).

As summarized in the introduction, previous studies using *in vitro* transcription and transient expression assays have demonstrated that the E1B 55-kDa protein can act as a repressor of transcription by RNA polymerase II, an activity that correlates with transforming activity. The mechanism of such repression is not fully understood, but the viral protein is thought to act on the basal transcription machinery via an as-yet-undiscovered cellular corepressor (see reference 2). Alanine substitutions at two C-terminal sites of phosphorylation, Ser490 and Ser491, have been reported to impair both inhibition of

p53-dependent transcription by the E1B 55-kDa protein and repression of transcription by a Gal4 DNA-binding domain–E1B protein fusion (80, 81). However, mutations that introduce these substitutions had no effect on repression of expression of the cellular genes identified in these experiments by the E1B 55-kDa protein: the concentrations of their transcripts were not altered significantly in cells infected by Ad5E1B-sub17 (Fig. 2). This result indicates that the E1B 55-kDa protein represses expression of specific cellular genes by a mechanism distinct from that previously characterized in simplified experimental systems.

The E1B protein has been reported to interact with cellular proteins that repress transcription, the corepressor complex that contains Sin3A and histone deacetylase I (Hdac I) (61), and the death domain-associated protein (Daxx) (95). Although a central sequence of the E1B protein, between amino acids 156 and 261, binds directly to Hdac I *in vitro*, the viral protein also interacts strongly with Sin3A in transformed 293 and infected HeLa cells (61). Binding to this corepressor complex has been reported to be required for the ability of the viral protein to block repression of transcription by p53 (62). Binding of the E1B 55-kDa protein to Daxx, which also appears to be direct, was observed to inhibit the stimulation of p53-dependent transcription by Daxx in transient expression assays (95). The repression of expression of endogenous genes by the

FIG. 5. The expression of previously described E1B-dependent genes in cells infected by Ad5 or E1B mutants. Shown is a hierarchical clustering of a composite data set in which the gene expression ratios (wild type/mutant) of 181 previously reported E1B-dependent genes (66) are appended to our data. hpi, hours postinfection.

E1B protein described here cannot be attributed to effects on p53. Of the cellular genes present in cluster 2 (Fig. 2), only two have been identified as direct targets of transcriptional regulation by p53 (41). Nor do the C-terminal phosphorylation site substitutions that impair repression of p53-dependent transcription by the E1B protein (81) eliminate repression of expression of these genes following infection of HFFs (Fig. 2). Finally, and perhaps most compelling, the p53 transcriptional program is inhibited as effectively in Hr6-infected HFFs as it is in Ad5-infected cells (Fig. 4; Table 2).

The Daxx protein has also been reported to block the activity of several other transcriptional activators, to interact with Hdac II, and to share sequences in two putative amphipathic α -helices with Sin3A, properties that suggest that this protein can also function as a corepressor (see reference 68). It is therefore possible that the Sin3A- and Hdac I-containing corepressor and/or Daxx contributes to repression of expression cellular genes by the E1B 55-kDa protein in infected cells.

Although the E1B 55-kDa protein is necessary to prevent expression of specific genes by 30 h after infection of normal human cells, the data presented here establish unequivocally that it is not required to block the action of p53 in HFFs: the suppression of the p53 transcriptional program characteristic of Ad5-infected HFFs (Fig. 4) (51) was also complete in cells infected by Hr6 or Ad5E1B-sub17 (Fig. 4; Table 2). It has been reported previously that accumulation of p53 in infected human lung carcinoma (A549) cells resulting from an R239A

substitution in the E1B protein was not accompanied by increased synthesis of two products of p53 target genes, Mdm-2 and p21. Rather, expression of both cellular genes was repressed as effectively as in Ad5-infected cells (36). Similarly, infection of normal human small airway epithelial cells by Ad5 mutants that cannot direct synthesis of any of the proteins made from the E1B 55-kDa open reading frame (Onyx-015) or that encode an E1B 55-kDa protein that cannot bind to p53 (Onyx-053) led to accumulation of p53 but not activation of expression of seven genes transcriptionally activated by p53 (59). Our global analysis extends such findings not only to a significantly larger set of genes that are transcriptionally activated by p53 but also to genes that are repressed by this cellular protein (Fig. 4; Table 2) and to a second type of normal host cell. Thus, there is a growing body of evidence indicating that the E1B 55-kDa protein is not necessary to block the transcriptional function of p53 in normal human cells infected by Ad5.

As p53 accumulates in various normal and transformed host cells infected by E1B 55-kDa-protein-null mutants or by viruses carrying mutations that block the interaction of the E1B protein with p53 or the E5 Orf6 protein (see the introduction), this conclusion implies that activation of p53 is prevented in Ad5-infected cells by one or more additional mechanisms. The activity of p53 is strictly regulated, and its activation requires various posttranslational modifications. One modification that is crucial is acetylation by the histone acetyltransferase p300/Cbp (79), which is bound by the viral E1A proteins (see reference 2). The E1A-p300 interaction has been reported to be required for accumulation of p53 (11). However, p53-dependent transcription has not been observed to increase in cells infected by mutants producing E1A proteins with alteration that impair their interaction with p300 (36, 59). During its activation, p53 is also modified by phosphorylation of specific residues and removal of ubiquitin (see references 58 and 83), raising the possibility that adenoviral proteins may inhibit one or both of these reactions. One possible candidate is the E4 Orf4 protein, which has been shown to interact with protein phosphatase 2A to induce dephosphorylation of viral E1A and cellular SR splicing proteins, as well as decreased activity of cellular transcriptional activators, including JunB and E2f (19, 43, 46, 53). It has been proposed that the modifications required to activate p53 take place in the nuclear structures termed Pml nuclear bodies (Pml oncogenic domains or nuclear domains 10): in response to various forms of stress, p53 associates with Pml nuclear bodies, which contain several enzymes that modify the protein, including Hausp (a p53 deubiquitinase), Hipk2 (a p53 kinase), and the acetyltransferase Cbp/p300 (reviewed in references 3, 21, 26, 29, and 60). The disruption of Pml nuclear bodies and reorganization of their components induced by the viral E4 Orf3 protein (9, 15, 37, 44) could also block activation of p53 in Ad5-infected cells.

Cellular RNAs that specify proteins implicated in progression through the cell cycle and proliferation have been observed to increase substantially in concentration following Ad5 infection of quiescent human foreskin and lung fibroblasts and the diploid fibroblast line WI38 (51, 66, 94). Comparison of the alterations in expression of such genes, particularly as exemplified by cyclin E1, induced by infection with Ad5 or the E1B mutant Adh260 or dl1520 (Onyx-015), led to the conclusion

that the E1B 55-kDa protein open reading frame is required to stimulate expression of cyclin E1 (66, 96). The former mutant lacks all E1B coding sequences, whereas the latter carries a stop codon in place of the second E1B 55-kDa protein codon and consequently cannot direct synthesis of the E1B 55-kDa protein or any of the smaller related proteins (Fig. 1A). In contrast, the Hr6 mutation affects only the E1B 55-kDa protein (87). As we observed similar degrees of stimulation of expression of cyclin E1 and other genes associated with cell cycle progression in Ad5- and Hr6-infected cells, we conclude that the E1B 55-kDa protein is not required for this response to infection. Rather, this effect appears to be a function of one of the smaller, E1B 55-kDa-protein-related proteins, which also fail to be made in dl1520-infected cells. Little is known about the molecular properties and functions of these viral proteins. However, the 156R protein, which shares both N- and C-terminal sequences with the 55-kDa protein (78), can transform rodent cells in cooperation with E1A gene products (73). Furthermore, the rate of proliferation of such transformed cells correlated with the concentration of the E1B 156R protein produced (73), suggesting that this E1B protein might stimulate expression of genes associated with cell cycle progression and proliferation.

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