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Induction of a Cellular Enzyme for Energy Metabolism by Transforming Domains of Adenovirus E1a

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Brain creatine kinase is a major enzyme of cellular energy metabolism. It is overexpressed in a wide range of tumor cell lines and is used as a tumor marker. We reported recently that the promoter of the human gene has a strong sequence similarity to the adenovirus E2E promoter. This similarity suggested that the brain creatine kinase gene may be regulated by the viral activator E1a. Experiments reported here showed that both enzyme activity and mRNA levels were induced by the oncogenic products of the E1a region of adenovirus type 5, but unlike the viral E2E promoter, which is induced predominantly by E1a domain 3, brain creatine kinase induction required domains 1 and 2. These domains are important for transformation and for the association of E1a with the retinoblastoma gene product and other cellular proteins. The induction by an oncogene of a cellular gene for energy metabolism may be of significance for the metabolic events that take place after oncogenic activation.

The creatine kinases (CKs) are a family of enzymes which are involved in the maintenance of ATP at sites of cellular work (2). Three cytoplasmic isoenzymes of CK have been identified in human tissue. These isoenzymes are dimeric molecules with two dissociable subunits designated as M (muscle) or B (brain) type that can reassociate to form the electrophoretically distinct MM, BB, or MB isotype. The amino acid sequences of these two subunits are encoded by distinct genes (28–30). The basis for the tissue-specific expression of these isozymes is not known. The MM dimer is the major form in skeletal muscle and myocardium; the MB exists in myocardium, while the BB isozyme exists in embryonic and many other tissues at low levels, but most predominantly in the brain and neuronal tissues.

The brain isozyme has specific features which form the background for our investigations. For example, elevated levels of brain CK (CKB) have been detected in tumor samples obtained from patients with small-cell lung carcinoma (10) and prostate and breast cancers (7, 31, 37). As a result of its elevated level, CKB is used as a diagnostic marker for small-cell lung carcinoma.

We have previously isolated a genomic clone for the human B isozyme and have done preliminary characterization of its promoter (4). The results of our initial investigations have demonstrated a strong sequence relationship between the promoter region of the human B isozyme and that of the adenovirus E2E gene (4). The E2E region of the adenovirus encodes a 72-kilodalton (kDa), single-stranded, DNA-binding protein which is involved in virus replication (9). The expression of this gene is highly dependent on the adenovirus oncogenic products of the E1a region (1). The unexpected relationship of the two promoter sequences motivated us to investigate a possible functional relationship.

We show here that the expression of CKB is also regulated by the oncogenic E1a products of the virus. Domains 1 and 2, which are associated with transformation, induction of DNA synthesis, and suppression of some enhancer-driven promoters, are also important for the induction of CKB expression. In contrast, the primary viral transactivation domain is not required. The induction of CK might point to metabolic events that take place after oncogenic activation.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were collected from various laboratories, and the monocytic cells were obtained from either the Massachusetts Institute of Technology (MIT) tissue culture collection or Duke University (the MIT line expressed high levels of CKB, while the Duke University monocytes expressed undetectable levels with our probes). The rest of the cell lines were obtained from the American Type Culture Collection. The attached cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, and those that grew detached in suspension were propagated in Iscovets medium–10% fetal calf serum. Viral stocks were prepared by standard procedures (15). All viruses were propagated and titers were determined on 293 cells, which express wild-type E1a proteins (13).

Infection with adenovirus and mutants. Cells were grown close to confluency, washed with serum-free medium, and infected for 1 h (usually 10 PFU/cell) in a 1-ml volume with continuous rocking. 1-β-D-Arabinofuranosylcytosine (AraC) was added at a concentration of 20 μg/ml postinfection and was replenished every 8 to 10 h. Mock-infected plates had similar treatment but lacked infection with virus. At several times postinfection, cells were washed and harvested for protein by three cycles of freeze-thawing or for total cellular RNA (see below).

RNA preparation and analysis. Total cellular RNA from infected plates was prepared by the guanidinium-CsCl method (38). For primer extension analysis, the ^{32}P-end-labeled oligonucleotides were hybridized overnight (at 30°C) to 30 μg of total RNA, and the hybrids were precipitated and washed. Extension with avian myeloblastosis virus reverse

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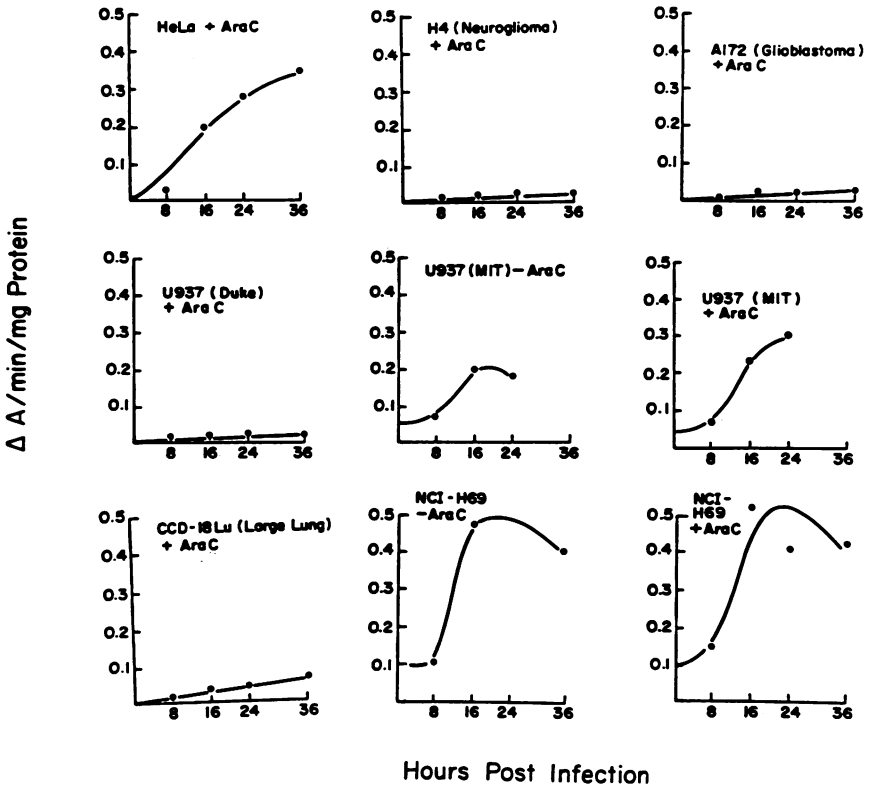


FIG. 1. Induction of CK enzymatic activity in human cells. HeLa cells, H4 neuroglioma, A172 glioblastoma, two monocytic cell lines U937 (MIT, Duke), large lung cells CCD-18Lu, and small-cell lung carcinoma NCI-H69 were infected with adenovirus 5 at 20 PFU/cell in the presence or absence of 20 μg of AraC per ml as indicated. At the indicated times, cells were harvested, washed, and lysed by three cycles of freeze-thawing. The supernatant was checked for CK activity.

transcriptase in the presence of unlabeled nucleotides was carried out (at 42°C) for 90 min. This was followed by RNase A digestion, two phenol-chloroform extractions, and precipitation. Products were analyzed on 8% acrylamide gels.

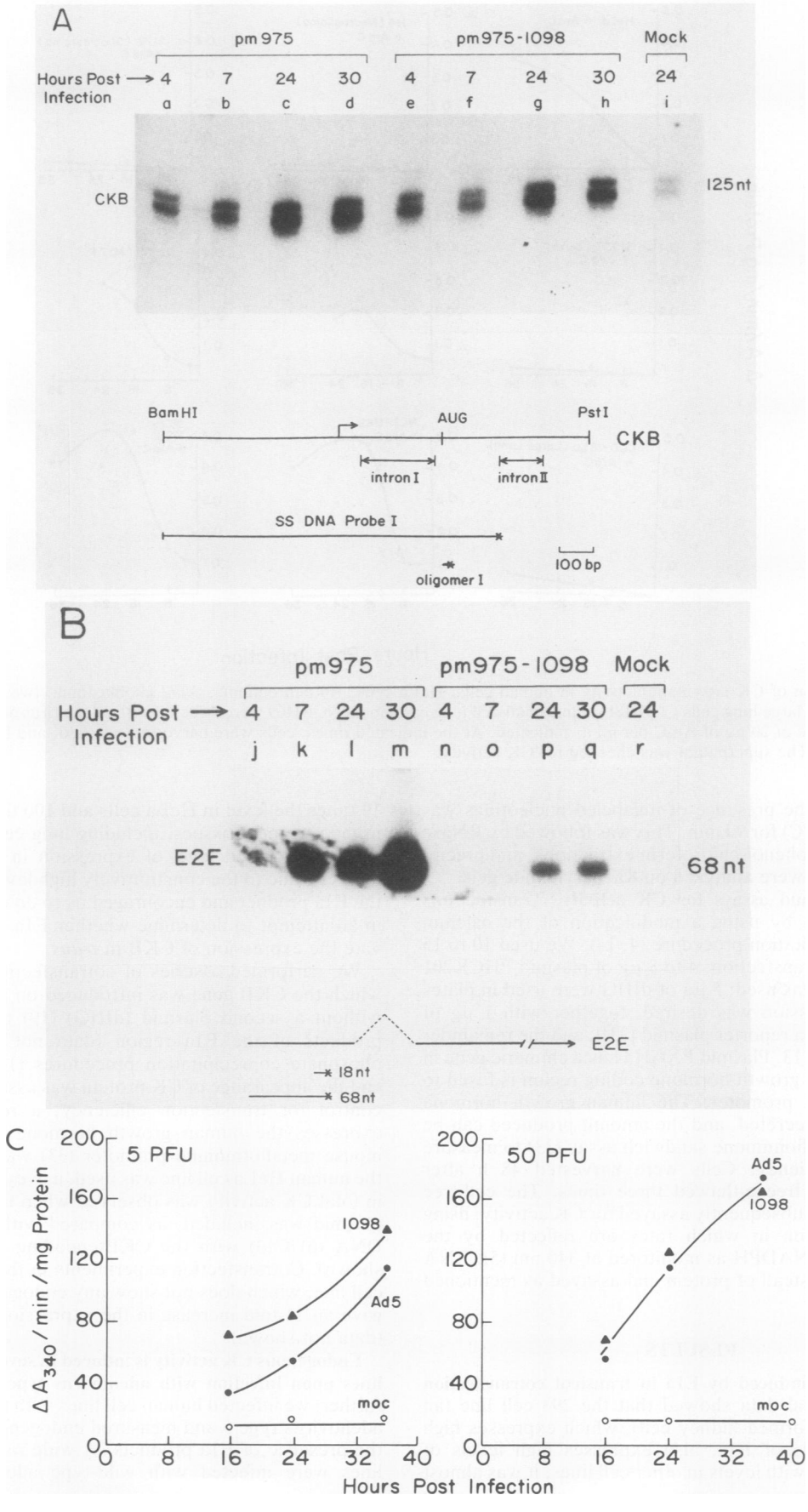
Transfections and assays for CK activity. Transfections were carried out by using a modification of the calcium phosphate precipitation procedure (4, 14). We used 10 to 15 μg of DNA per transfection: 4 to 8 μg of plasmid PHCK201 (CKB gene [4]) was used; 5 μg of dIIIG were used in plates when E1a expression was desired, together with 1 μg of plasmid PXGH5 (a reporter plasmid [33]); and the remainder was plasmid PUC13. Plasmid PXGH5 has a chimeric gene in which the human growth hormone coding region is fused to the mouse MT-1 promoter. The human growth hormone gene product is secreted, and the amount produced can be analyzed by a radioimmune sandwich assay (33) to measure transfection efficiency. Cells were harvested 48 h after transfection and freeze-thawed three times. The cell-free supernatant was subsequently assayed for CK activity, using a coupled reaction in which rates are reflected by the accumulation of NADPH as monitored at 340 nm (5). RNA was harvested instead of protein and assayed as mentioned previously.

RESULTS

CK activity is induced by E1a in transient cotransfection experiments. Initial data showed that the 293 cell line (an adenovirus-transformed kidney cell), which expresses high constitutive levels of E1a, also expresses high levels of CKB. Compared with levels in other cell lines, it was almost 10 times the level in HeLa cells and 100 times that observed in many human tissues, including lung cells. This indicated that the elevated level of expression in the 293 cell lines might be due to the constitutively high level of expression of the E1a product and encouraged us to do direct experiments in an attempt to determine whether E1a products can activate the expression of CKB in *trans*.

We performed a series of cotransfection experiments in which the CKB gene was introduced on a plasmid, with or without a second plasmid (dIIIG) (19) that expresses the products of the E1a region (data not shown). Calcium phosphate coprecipitation procedures (14) were followed, and the appearance of CK protein was assayed 48 h later. To control for transfection efficiency, a reporter gene that expresses the human growth hormone derived from the mouse metallothionine promoter (33) was included. When the human HeLa cell line was used, a five- to sixfold increase in total CK activity was observed when the E1a-expressing plasmid was included, as compared with adding a carrier DNA (pUC13) with the CKB-encoding plasmid (data not shown). Cotransfection experiments in the mouse NIH 3T3 cell line, which does not show any endogenous CK activity, gave an 11-fold increase in the expression of CKB activity (data not shown).

Endogenous CK activity is induced in several human culture lines upon infection with adenovirus type 5. To investigate further, we infected human cell lines with the wild-type virus adenovirus type 5 and measured endogenous CK activity in the presence of E1a products. A wide range of human cell lines were infected with wild-type adenovirus, and CK


A
pm975
pm975-1098
Mock
Hours Post Infection
4 7 24 30 4 7 24 30 24
a b c d e f g h i
CKB
125nt
BamHI
AUG
PstI
CKB
intron I
intron II
SS DNA Probe I
oligomer I
100 bp
B
pm975
pm975-1098
Mock
Hours Post Infection
4 7 24 30 4 7 24 30 24
j k l m n o p q r
E2E
68nt
E2E
18nt
68nt
C
5 PFU
50 PFU
1098
Ad5
moc
ΔA340 / min / mg Protein
Hours Post Infection
0 40 80 120 160 200
0 8 16 24 32 40

activity was monitored for up to 36 h postinfection. Infections were carried out in the presence and absence of AraC, a compound which inhibits the replication of the virus and thereby maintains a high level of E1a (12). Control experiments established that AraC alone has no effect on expression of CKB in mock-infected HeLa cells (data not shown). HeLa cells, the monocytic U937 (MIT tissue culture collection), and an established SCLC line (NCI-H69) showed induction (3- to 12-fold) of CK activity by 16 to 24 h postinfection (Fig. 1). Each of these cells has a level of endogenous CK activity that was detected by our assay even in the absence of adenovirus infection. There was no significant induction of expression upon infection with adenovirus of the H4 neuroglioma, A172 glioblastoma, CCD-18Lu large-cell lung carcinoma, or U937 (Duke University collection) cell types (Fig. 1). In these cases we also could not detect any endogenous CK activity in the absence of adenovirus infection. (It is recognized that CK expression may be so low that even with a severalfold induction we would not be able to measure any activity.) We demonstrated that the induction of CKB was due to infection with the virus and not to the serum added by mock infecting HeLa cells in parallel (data not shown). The products of the E1a region are important for induction because infecting at high multiplicities with the mutant virus *dl*312 (an E1a deletion mutant) showed no effect on CKB expression (see below).

The proteins encoded by the E1a region are necessary for efficient viral replication in permissive human cell lines and for viral transformation of nonpermissive rodent cells. The major proteins are nuclear phosphoproteins of 243 and 289 amino acids, which have the same amino and carboxy termini and differ only by the presence of 46 additional amino acids in the 289-amino-acid polypeptide (12). These are products of 12S and 13S mRNA species, respectively. The E1a proteins contain three distinct domains that are strongly conserved among adenovirus subgroups and species (17, 39). The vast majority of domain 3 is unique to the 289-amino-acid protein and is important for transactivation of early viral promoters (1, 11, 16, 20–22, 26, 32). Domains 1 and 2 are required for transcriptional repression, transformation, and induction of DNA synthesis but not for activation of transcription (3, 11, 12, 16, 20–24, 26, 27, 32, 40, 45). To define regions of E1a important for activation of the cellular CKB gene, we investigated the effect of mutations in different domains of the E1a protein on CKB expression. The parent molecule that was used for generating the mutants in domain 3 used in the experiments described below was *pm*975. This is a construct of the virus that can express only the wild-type 289-amino-acid protein (22).

Point mutations in domain 3 that are defective for activation of E2E expression can activate CKB expression. The transactivation domain 3 is unique to the 13S product and is considerably more basic than the overall E1a proteins. A Gly→Asp point mutation in domain 3 (mutant *pm*975–1098) dramatically impairs E1a activation of E2E and of the early viral promoters (20). However, this mutation has no effect on the ability of E1a to activate expression of CK in HeLa cells at a wide range of multiplicities of infection (see below). By primer extension of end-labeled oligonucleotides, it was possible to analyze CKB and E2E mRNAs in the same infected HeLa dishes. The induction of CKB mRNA levels by wild-type *pm*975 (Fig. 2A, lanes a to d) and mutant *pm*975–1098 virus (Fig. 2A, lanes e to h) contrasts with the behavior of E2E RNA synthesis. In the latter case, the *pm*975–1098 mutation reduced dramatically E1a activation of E2E RNA synthesis (cf. lanes j to m [wild type] and lanes n to q [mutant] in Fig. 2B). Figure 2C shows that CK activity was induced to similar levels upon infection with both wild-type and 1098 mutant, at multiplicities of infection of 5 and 50 PFU/cell.

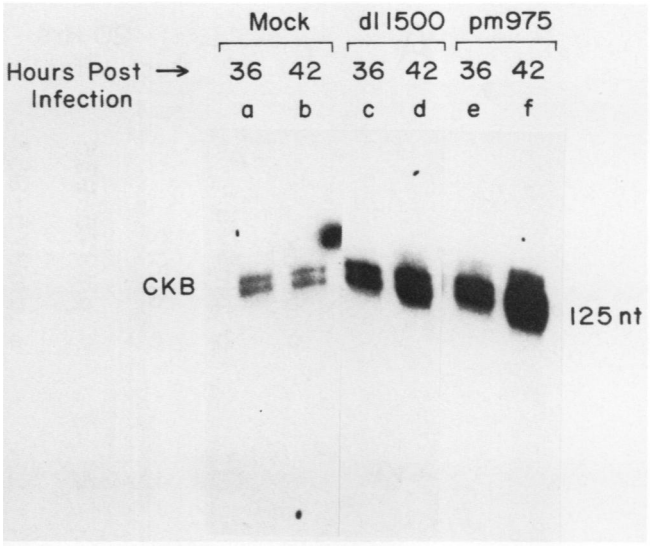


FIG. 3. Activation of expression of CKb by the 12S product of E1a, which lacks domain 3. HeLa cells were infected at 20 PFU/cell with virus expressing the 12S (*dl*1500) or 13S (*pm*975) products (22, 23) in the presence of AraC. Total cellular RNA was harvested at 36 and 42 h postinfection, and 30 μg was probed for CKB mRNA by using the primer extension probe shown in Fig. 2A. Lanes a and b show results for a mock infection, lanes c and d are for RNA that was harvested from *dl*1500-infected plates, and lanes e and f show results of probing RNA that was harvested from cells infected with the *pm*975 virus. nt, Nucleotides.

FIG. 2. Induction of expression of CKB by a point mutation in domain 3 (*pm*975–1098) that is defective for induction of E2E. HeLa cells were infected (at 20 PFU/cell) with wild-type *pm*975 adenovirus type virus (encodes the 13S product) or with the *pm*975–1098 mutant which has a point mutation in domain 3 (20) as described in Materials and Methods. AraC was added at a concentration of 20 μg/ml postinfection and was replenished every 8 to 10 h. Mock-infected plates had similar treatment but lacked infection with virus. At 4, 7, 24, and 30 h postinfection, total cellular RNA was harvested and purified, and 30 μg was hybridized to an end-labeled probe that is specific for CKB (oligomer I, panel A) or for E2E (18 nucleotides [nt], panel B). The positions of these probes relative to the two genes are indicated below each autoradiogram and they give primer-extended bands of 125 (A) and 68 (B) nucleotides, respectively. (A) Induction of CKB mRNA (lanes a to d are for the wild-type virus and lanes e to h are for the mutant). Lane i depicts the endogenous level of CKB in the HeLa cell line that was used (24-h mock-infected plate), and the level does not change with time (data not shown). (B) Induction of E2E RNA (lanes j to m are for wild-type virus and lanes n to q are for the mutant). Lane r is an analysis of the RNA isolated from the mock infection and shows no endogenous E2E RNA. (C) Results of measurements of CK activity when cells were infected with wild-type and mutant virus at 5 and 50 PFU/cell in the presence of AraC. The cells were harvested for protein at 16, 24, 36, and 40 h postinfection and assayed for CK activity (Materials and Methods). Activity is reported as a change in A_{340} per minute per milligram of protein. Symbols: ○, CKB activity in mock-infected plates; ●, in wild-type *pm*975 virus-infected plates; ▲, in plates infected with *pm*975–1098 mutant. Ad5, Adenovirus type 5.

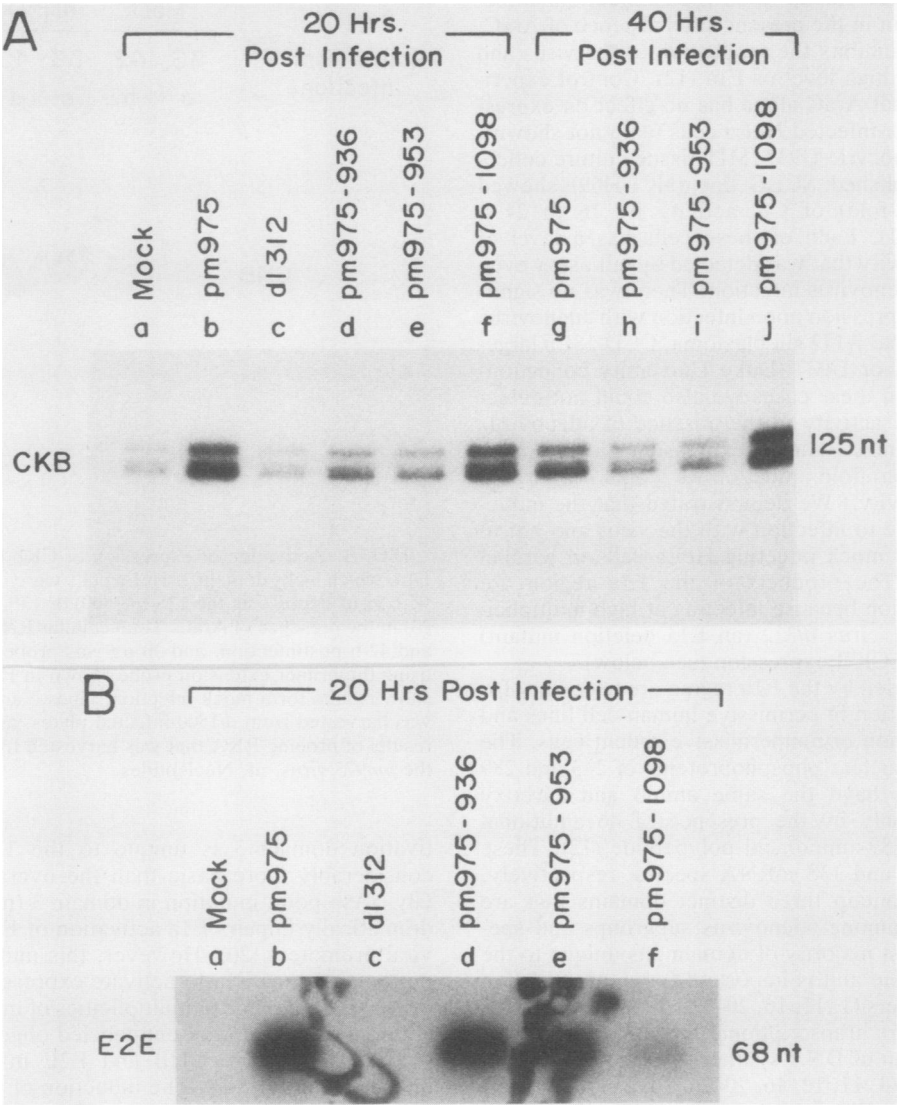
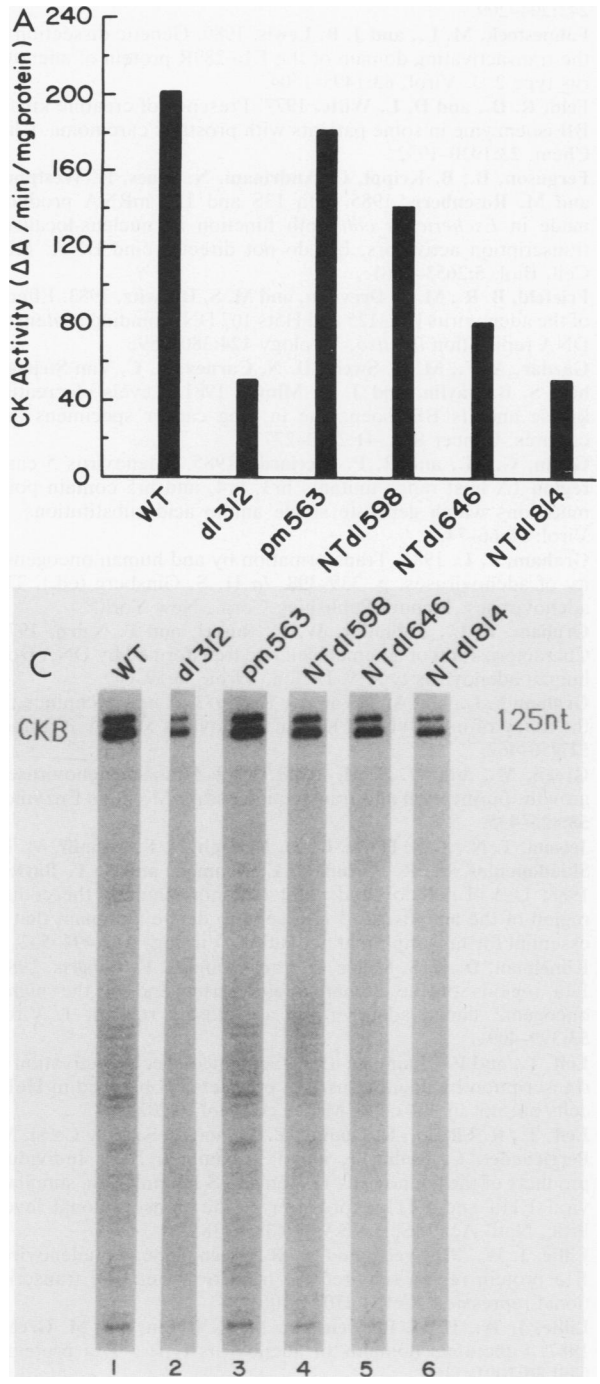


FIG. 4. Evidence that point mutations in domain 2 are impaired for E1a-induced expression of CKB. HeLa cells were infected with wild-type or mutant viruses, and RNA was harvested at 20 and 40 h postinfection and probed for CKB (A) or E2E (B) by use of primer extension probes (see legend to Fig. 2). Infections were done with the following viruses: lane a, mock infection; lanes b and g, wild-type *pm*975 virus; lane c, mutant *dl*312 virus (an E1a deletion mutant); lanes d, e, h, and i, mutant viruses *pm*975–936 and *pm*953, each of which has a point mutation in domain 2; lanes f and j, mutant virus *pm*975–1098, which has a point mutation in domain 3.

The 12S product can activate CKB expression. Infection with the *dl*1500 virus, which expresses the E1a 12S product lacking domain 3, also resulted in induction of CKB expression (Fig. 3). Compared with mock-infected cells (lanes a and b), significant induction of CKB was observed in *dl*1500-infected plates (lanes c and d). The induction was slightly lower than that seen with wild-type *pm*975 (lanes e and f) and required a longer period (which may be a result of low-level expression of the 243-amino-acid E1a product) (20). Mutant *dl*312 had no effect on CKB expression, even at longer time points (data not shown). Collectively, the experiments of Fig. 2 and 3 demonstrate that domain 3 of E1a, which is important for the induction of E2 and early viral genes, is not essential for induction of CKB expression.

Two mutations in domain 2 are defective for CKB induction. Domain 2 of E1a is small (19 amino acids), acidic, and essential for the ability of E1a to perturb the cell cycle and induce DNA synthesis (21, 24–26, 42). Amino acid substitutions within this domain impair the ability of the E1a products to immortalize primary cells and to cooperate with the *ras* oncogene to transform these cells (20, 26, 32, 45). None of these mutations interferes with the ability of the 13S product to transactivate other viral early genes. Many transforming proteins from other DNA tumor viruses show considerable similarity to domain 2 (34, 36). This domain has been shown to be important for the association of E1a with some cellular proteins, including the retinoblastoma gene product (41, 43).

Two point mutations in domain 2 (*pm*975–936 [Glu-126→Gly] and *pm*975–953 [Ser-132→Gly]) render the E1A 289-amino-acid gene product defective for collaboration with the activated *ras* oncogene in transforming primary rat



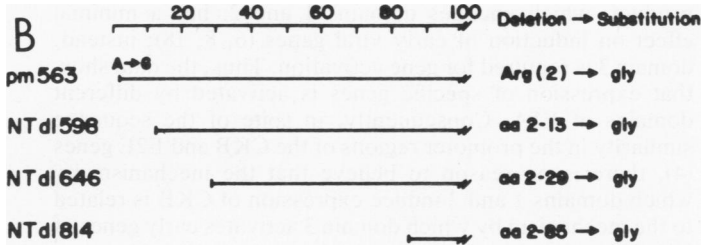


FIG. 5. Evidence that amino-terminal deletions of E1a obliterate the induced expression of CKB. HeLa cells were infected with wild-type virus or mutant viruses with E1a amino-terminal deletions generated by Whyte et al. (42). Cells were harvested for protein (see Materials and Methods), and CK activity was determined and represented by bars (A). Panel B shows the amino acids deleted in each mutant (right), with the names given to them by Whyte et al. (42) to the left. Panel C represents cell infected in a similar fashion to panel A but harvested for RNA and probed with a CKB-specific probe (see legend to Fig. 2). nt, Nucleotides.

kidney cells (20). These mutants are also poor inducers of DNA synthesis in growth-arrested cells, but they have no affect on the induction of the early viral genes.

The increase in CKB mRNA was severely impaired in HeLa cells infected with either of the domain 2 mutant viruses (Fig. 4A, lanes d and e). The mock infection (lane a) and infection with the *dl*312 deletion mutant (lane c) showed no increase of CKB RNA above the endogenous amount in these particular cells. Infection with wild-type *pm*975 and domain 3 mutant (*pm*975–1098) virus gave the expected induction (cf. lanes b and f). The same observations are made when RNA is harvested at 40 h postinfection (Fig. 4A, lanes g to j).

Figure 4B shows the results of probing the same RNA samples for the E2E product. The results reveal that, as reported previously (20), both domain 2 mutants activate expression of E2E to a level similar to that induced by the wild-type virus (cf. lanes d and e with lane b in Fig. 4B), while the domain 3 point mutant (*pm*975–1098) and E1a deletion mutant (*dl*312) are defective for activation (Fig. 4B, lanes f and c).

Amino-terminal deletions in domain 1 obliterate the induction of CKB expression. Whyte et al. (42) have generated a series of deletion mutations spanning the entire protein-coding region of E1a and assayed for their ability to cooperate with an activated *ras* oncogene to induce transformation in primary baby rat kidney cells. They found that amino acids 1 to 85 (which cover domain 1 amino acids 46 to 77) as well as domain 2 are essential for transformation. Deletions of all or part of amino acids 1 to 85 resulted in a loss of transforming activity. These mutants include *pm*563 (an R-to-G mutation at position 2) and the deletion mutations NT*dl*598 (deletion of sequences encoding amino acids 2 to 13), NT*dl*646 (amino acids 2 to 28), and NT*dl*814 (amino acids 2 to 85). All of these mutations were defective in the transformation assays.

We tested the effect of these mutations on the induction of CK expression. HeLa cells were infected with wild type or the above-mentioned deleted viruses. Figure 5A shows a gradual loss of CK induction as region 1 is deleted. A comparable amount of E1a protein was expressed from these mutants except for the reduction in levels of the biggest deletion (NT*dl*814), using Western blots (immunoblots) probed with anti-E1a antibodies (data not shown). Figure 5A shows that mutant NT*dl*646 (which expresses an amount of E1a protein comparable to wild-type *pm*975) is significantly disabled for induction of CKB expression. The same observation was made when we harvested total RNA from infected cells and probed with a CKB-specific primer extension oligonucleotide (Fig. 5C).

DISCUSSION

Data presented here demonstrate that CKB mRNA and protein in many human tissue culture cell lines are induced by the E1a products. Moreover, domain 3 of E1a is not needed for the induction of CKB. Instead, domain 2 is required (Fig. 4) and amino-terminal deletions into domain 1 also lose their ability to induce CKB (Fig. 5). The 12S

product, which encodes domains 1 and 2, has a minimal effect on induction of early viral genes (6, 8, 18); instead, domain 3 is required for gene activation. Thus, the data show that expression of specific genes is activated by different domains of E1A. Consequently, in spite of the sequence similarity in the promoter regions of the CKB and E2E genes (4), there is no reason to believe that the mechanism by which domains 1 and 2 induce expression of CKB is related to the mechanism by which domain 3 activates early genes of adenovirus.

Studies with the E1a-inducible proliferating cell nuclear antigen suggested that the 12S product of the virus can induce its expression (46), although induction of mRNA levels has not been reported. A few cellular genes (such as the hsp70 heat shock protein and thymidylate synthase) have been shown to be induced by E1a, with a possible involvement of the 12S product in the induction of the hsp70 gene (35, 44, 46). At present, CKB is the only example of a cellular gene whose induction has been clearly established to require both transforming domains 1 and 2.

Whyte et al. (41, 43) have identified some cellular proteins that associate with E1a proteins. Such an association might mediate the biological functions of E1a. Three major proteins with molecular weights of 105,000, 107,000, and 300,000 were identified (41, 43). The 105-kDa protein was identified as the anti-oncogene product of the retinoblastoma gene, revealing a novel oncogene anti-oncogene association (41). This interaction requires amino acids 1 to 85 as well as 121 to 126 of the E1a protein. The 107-kDa protein association with E1a requires amino acids 121 to 126, while the 300-kDa protein association requires amino acids 1 to 85.

The induction of CKB does not correlate precisely with the association of E1a with any one specific protein of the three identified above. A mutation (*pm*563) that disrupts interaction with the 300-kDa protein strongly stimulates CKB expression, a deletion (NT*dl*814) capable of binding the 107-kDa protein does not stimulate CKB, and a point mutation (*pm*975–953) capable of binding the retinoblastoma gene product is impaired in stimulation of CKB. The difference in the (in vitro) binding assay and the (in vivo) stimulation assay make comparison of phenotypes difficult, leaving open the possibility that E1a induces CKB via one of these associated proteins. Regardless of the detailed mechanism, the induction of CKB, an enzyme for cellular energy metabolism, by E1a may be related to metabolic events that take place after oncogenic activation.

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