Adenovirus E1A Proteins Regulate Phosphoenolpyruvate Carboxykinase Gene Transcription through Multiple Mechanisms*

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Recently, Kalvakolanu et al. (Kalvakolanu, D. V. R., Liu, J., Hanson, R. W., Harter, M. L., and Sen, G. C. (1992) J. Biol. Chem. 267, 2530–2536) showed that E1A inhibited the basal and cAMP-stimulated transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK). This inhibition was mediated by the conserved region 1 (CR1) domain of E1A, which has been shown by other laboratories to bind to the cellular transcriptional adapter proteins, p300 and cAMP response element binding protein (CREB)-binding protein (CBP). The PEPCK gene promoter contains a functional cAMP-response element, through which CREB and, therefore, CBP modulate transcription, and a consensus p300 DNA binding sequence is also present in a distal protein binding site of the promoter. We hypothesized that E1A might inhibit PEPCK gene transcription by binding to p300 and/or CBP. Surprisingly, we found that E1A consistently stimulated basal transcription from the PEPCK promoter in transfection assays in adenovirus (Ad)-infected HepG2 hepatoma cells or E1A-expressing, stably transduced 3T3 fibroblasts and nuclear run-on assays in Ad-infected H4IE hepatoma cells. E1A also enhanced the stimulation of PEPCK gene transcription by BtCAMP. In transfection assays, wild type Ad5 expressing both 243R and 289R forms of E1A or a mutant virus expressing the 289R form alone stimulated transcription from the PEPCK promoter by approximately 5-fold 20 h postinfection. However, no stimulation was observed in cells infected with a virus expressing either the 243R protein alone or a 289R protein from which conserved region 3 (CR3) was mutated. Mutation or deletion of CR1 of E1A had no significant effect on transcription from the PEPCK promoter. Mutations within conserved region 2 (CR2) of E1A that inhibit the binding of E1A to the retinoblastoma gene product (pRb) further enhanced the stimulation of transcription from the PEPCK promoter by 2-3-fold compared with wild type E1A. These findings suggested that the normal function of pRb is to stimulate PEPCK gene transcription, and that this process is inhibited by the binding of E1A to pRb. This hypothesis was confirmed by overexpressing pRb in HepG2 cells, which stimulated transcription from the PEPCK promoter. Our findings indicate that Ad E1A regulates PEPCK gene transcription through a stimulatory mechanism involving CR3, and by attenuating a stimulatory effect of pRb through CR2.

The nuclear phosphoproteins encoded by the adenovirus (Ad) E1A gene are essential for efficient Ad replication (1). The E1A gene consists of two exons encoding 12 and 13 S mRNAs, which arise through alternative splicing of a single pre-E1A RNA transcript (2). In Ad serotypes 2 and 5 (Ad2 and Ad5) these transcripts translate 243R and 289R E1A proteins that are identical except for a unique internal stretch of 46 amino acids present in the larger protein. DNA sequence homology between Ads of different groups is limited to conserved regions (CRs) located entirely within the first exon of E1A (3). Two CRs, conserved regions 1 (CR1) and 2 (CR2), are present in both the 243R and 289R proteins, whereas the third domain, conserved region 3 (CR3), is unique to E1A 289R.

Mutational analysis of the E1A gene has linked the CRs to many of the biological activities of E1A (i.e. transcriptional activation of both viral and cellular genes, enhancer-mediated transrepression, cellular transformation, etc.) (4). Amino acids encoded within the CRs of E1A interact with specific cellular proteins, thereby transducing the numerous biological activities of E1A. For example, many of the transcriptional transactivation functions associated with E1A are mediated by CR3-encoded amino acids interacting with specific cellular transcription factors like TFIIID (5, 6), ATF2 (6–8), and activator protein 1 (AP-1) (9, 10). On the other hand, CR1- and CR2-encoded amino acids serve as binding sites for the protein product of the retinoblastoma gene (pRb), thereby releasing the cellular transcription factor, E2F, in a transcriptionally active, non-pRb-bound form (11). The enhancer-mediated transrepression function of E1A has been ascribed to both CR1 and CR2 (4).

The phosphoenolpyruvate carboxykinase (PEPCK) gene encodes the rate-determining enzyme in gluconeogenesis, and its transcription can be stimulated or repressed in response to a number of extracellular stimuli as well as by tissue-specific and developmental factors (12–15). Recently, Kalvakolanu et al. (16) demonstrated that E1A inhibited both basal and cAMP-stimulated transcription of the PEPCK gene. These data indicated that CR1-encoded amino acids, which are required for the interaction of E1A with the cellular nuclear proteins, p300 and CBP, were required for the inhibition of PEPCK gene trans-

1 The abbreviations used are: Ad, adenovirus; PEPCK, phosphoenolpyruvate carboxykinase; CR, conserved region; AP-1, activator protein 1; CAT, chloramphenicol acetyltransferase; CRE, cAMP-response element; CBP, CREB-binding protein; CREB, cAMP response element binding protein; CEBP, CCAAT enhancer binding protein; MOL, multiplicity of infection.
cription by E1A. A functional CRE that binds CREB, and therefore CBP, has been identified in the PEPCK gene promoter (12, 17). Sequence analysis of the PEPCK gene promoter also shows that a region of the promoter (P6 (Ref. 12) or AF-1 (Refs. 18 and 19)) that plays important roles in glucocorticoid and retinoic acid responsiveness (18, 20) contains a consensus p300 binding sequence (21). These preliminary findings led us to hypothesize that E1A could inhibit PEPCK gene transcription by interacting with p300 and/or CBP and blocking their transcriptional adaptor function. Alternatively, E1A could inhibit PEPCK gene transcription by binding to p300, with subsequent binding of the E1A-p300 complex to the P6 region of the PEPCK gene promoter.

We report here that E1A, unexpectedly, stimulates basal PEPCK gene transcription even under conditions identical to those reported by Kalvakolanu et al. (16). The stimulation of PEPCK gene transcription was mediated by CR3 of E1A. Transcription from the PEPCK promoter was further stimulated by a mutant E1A protein from which CR2 was deleted as well as with mutant E1A proteins containing point mutations in CR2 that abolish the interaction of E1A with pRb. Overexpression of pRb alone or in the presence of E1A stimulated transcription from the PEPCK gene promoter. These findings indicate that E1A regulates PEPCK gene transcription through two mechanisms. First, E1A stimulated transcription from the PEPCK promoter through CR3, presumably by interacting with a transcription factor(s) that binds to the PEPCK gene promoter. Secondly, E1A appears to attenuate a stimulatory effect of pRb on PEPCK gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**

The AdS deletion mutant dl343 (22) was provided by Thomas Shenk (Princeton University, Princeton, NJ). The Ad5 E1A mutant, d520 (23) expresses only the L5S form of E1A, and pM975 (24), the Ad5 L5S E1A only-expressing virus, was provided by Arnold Berk (University of California, Los Angeles, CA). E1A-I104 and E1A-I108 (25), which are Ad5 E1A CR1 and CR2 deletion mutants, respectively, were provided by Stanley Bayley (McMaster University, Hamilton, Ontario). DL1 and DL2 (26), which were constructed from pM975, contain linker scanning mutated forms of CR1 and CR2, respectively, and were provided by M. Fahnestock (University of California, Los Angeles, CA). The Ad5 E1A mutants E1A-RG2, 13S-928, and RG2–928 (27, 28) were obtained from Elizabeth Moran (Temple University, Philadelphia, PA). The E1A product encoded by E1A-RG2 contains a single amino acid substitution (R to G at position 2), which abolishes interaction of E1A-p300 binding. 13S-928 encodes a 13S form of E1A with a single amino acid substitution (cysteine 124 to glycine) that abolishes binding to the retinoblastoma gene product (pRb). Wild type AdS was grown and titered in 293 cells. All the Ad E1A mutants were grown and titered in 293 cells. The cells from which the viral stocks were grown were screened for contamination by mycoplasma by the Mycotect assay (Life Technologies, Inc.) and were negative. The eukaryotic expression vector for wild type, human pRb, pRWT-HA/SVE (29) was provided by Robert Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). [α-32P]UTP was purchased from Dupont NEN. Vanadylribonucleosides, ATP, GTP, CTP, and RNase-free DNase were purchased from Life Technologies, Inc. Luciferase assay reagents were obtained from Analytical Luminescence Laboratory (San Diego, CA), and chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay kits were obtained from Boehringer Mannheim.

**Cell Lines and Transfection and Infection Procedures**

HepG2 human hepatoma cells and HA11E rat hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD). 3T3-neo and the 12 S (MT 12-1) and 13 S (MT 13-2) E1A-expressing cell lines (30) were obtained from James Cook (National Jewish Center, Denver, CO). HepG2 and H11E cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% calf serum. 3T3 cell lines were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 5% calf serum.

Plates of HepG2 or 3T3 cells were grown to approximately 80% confluency and transfected with the indicated plasmids by calcium phosphate-DNA coprecipitation as described by Park et al. (17). Immediately following transfection, HepG2 cells were infected with the indicated Ads as described previously (31).

Luciferase assays were performed on a Monolight 2010 luminometer using the enhanced luciferase assay kit (Analytical Luminescence Laboratory, San Diego, CA) according to the supplier's directions. CAT assays were performed with CAT enzyme-linked immunosorbent assay kits (Boehringer Mannheim) according to the manufacturer's instructions. Transfection efficiencies were normalized by cotransfecting the cells with a plasmid containing a chimeric Rous sarcoma virus long terminal repeat–β-galactosidase gene, and β-galactosidase levels were measured as described previously (17). All experiments were repeated three times, and consistent results were obtained in all cases.

**Nuclear Run-on Transcription Assays**

H41IE cells were infected with wild type Ads or the indicated mutant Ads as described above. Twenty hours after infection, the cells were harvested, their nuclei were isolated, and nuclear run-on transcription assays were performed as described by Granner et al. (32). PEPCK DNA was hybridized to a 600-base pair HindIII fragment of the PEPCK coding region exused from the plasmid, pBH1.2 (33). Levels of PEPCK transcription were corrected for differences in β-actin gene transcription by hybridizing RNA to an 1150-base pair fragment of the rat β-actin gene exused from the plasmid, p5′-actin.

**Quantitation of E1A Proteins and pRb**

Quantitation of E1A Proteins—3 × 10⁶ cells were infected for 24 h (unless otherwise noted) as described previously with the indicated Ad (MOIs of 100–200 plaque-forming units/ml as shown). The Ad-infected cells were lysed in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentrations of radioimmunoprecipitation supernatants were determined by the BCA protein assay (Pierce), and equal amounts of protein from each cell lysate were separated on 10% polyacrylamide-SDS gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h in PBS containing 5% dry milk, incubated with the anti-E1A monoclonal antibody, M73, supplied by Edward Harlow (Massachusetts General Hospital, Boston, MA). Following extensive washing with PBS (PBS containing 0.05% Triton X-100) the membranes were incubated with 125I-labeled protein A (ICN, Costa Mesa, CA) for an additional hour and subjected to three additional washes in PBS. The bands were visualized by autoradiography.

Quantitation of pRb—Extracts of infected and/or transfected cells prepared as described above were resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. The blots were blocked with PBS containing 5% dry milk and then treated with mouse monoclonal anti-pRb antibody (Upstate Biotechnology, Inc., Lake Placid, NY). The blots were washed three times in PBS and then treated with anti-mouse IgG followed by protein A–alkaline phosphatase conjugate. After the blots were washed, specific immune complexes were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**RESULTS**

Optimization of E1A Protein Expression—To study the effect of E1A on PEPCK gene transcription, we determined the optimal conditions for wild-type and mutant E1A protein expression in Ad-infected HepG2 cells. The Ads used in this and subsequent experiments, the E1A proteins they express, and the binding of the E1A proteins to the cellular proteins p300, p105Rb, and p107 are described in Table I. Following infection with wild-type Ads (100 plaque-forming units/ml) levels of E1A proteins were barely detectable at 4 h postinfection and continued to increase in a time-dependent manner, reaching a maximum approximately 16–24 h postinfection (data not shown). Similar kinetics of expression were observed with Ads expressing various mutant E1A proteins (data not shown). When cells were infected with Ads expressing mutant E1A proteins, MOIs of approximately 200 were generally required to produce levels of mutant E1A protein comparable with wild type E1A levels at an MOI of 100 (data not shown). Unless otherwise noted, the effects of E1A on PEPCK gene transcrip-
HepG2 cells were treated with dibutyryl-cAMP (Bt2cAMP), transcription from the PEPCK promoter. When uninfected approximately 20-fold in Ad5-infected H4IIE (data not shown). HepG2 cells, basal PEPCK gene transcription was stimulated using the PEPCK gene promoter-luciferase reporter gene in cells is poorly transcribed. Consistent with our observations transcription assays since the endogenous PEPCK gene in HepG2 cells (data not shown). Unexpectedly, wild type Ad5 E1A consistently stimulated basal transcription from the PEPCK promoter by 5-fold as compared with transcription levels in mock-infected cells (Fig. 1). No significant increase in transcription was observed in cells infected with an Ad that does not express E1A (dl343), indicating that the stimulation of transcription by wild type Ad5 was mediated by E1A.

To confirm the observations using the PEPCK gene promoter-luciferase reporter gene in HepG2 cells, transcription of endogenous PEPCK gene was also measured by nuclear run-on transcription assays in mock or Ad5-infected H4IIE rat hepatoma cells. H4IIE cells were selected for nuclear run-on transcription assays since the endogenous PEPCK gene in HepG2 cells is poorly transcribed. Consistent with our observations using the PEPCK gene promoter-luciferase reporter gene in HepG2 cells, basal PEPCK gene transcription was stimulated approximately 20-fold in Ad5-infected H4IIE (data not shown).

We have also found that Ad E1A enhanced cAMP-stimulated transcription from the PEPCK promoter. When uninfected HepG2 cells were treated with dibutylryl-cAMP (Bt2cAMP), PEPCK promoter-driven transcription was stimulated by approximately 5-fold (Fig. 2). However, treatment of Ad5-infected cells with Bt2cAMP produced a 12-13-fold increase in transcription from the PEPCK promoter. Ad5 infection alone produced a 5-fold increase in transcription from the PEPCK promoter as previously observed. No further increase in Bt2cAMP-stimulated transcription was seen in cells infected with dl343, which does not express E1A proteins (data not shown).

The ability of Ad5 infection to stimulate transcription from series of luciferase reporter vectors containing the full-length PEPCK promoter in which individual protein binding sites had been mutated (12, 17, 34-36) was used to define E1A-responsive regions of the PEPCK promoter. We found that mutations in several protein binding sites including the 3' portion of the P3 element (P3(I), Ref. 12), which binds C/EBP proteins (17, 35, 36), the P6 element, which has been shown to be involved in glucocorticoid and retinoic acid responsiveness (18, 20), the P1 site (12), which binds nuclear factor 1/CCAAT box transcription factor (NF-1/JCTF), and C/EBP proteins (36), or the distal CAMP-response element (CRE-2, Ref. 12), which also binds C/EBP proteins (36), inhibited E1A-stimulated transcription by 30% and 75% (data not shown). Mutations in other protein binding sites (i.e. TATA box, CRE-1, P2, p3(SI), P4, and P5) had no significant effect on E1A-stimulated transcription.

E1A CR3 Stimulates Basal PEPCK Gene Transcription—The E1A gene encodes two major transcripts, the 12 and 13 S mRNAs, which translate the 243R and 289R E1A proteins, which are identical except for a unique internal stretch of 46 amino acids present in the larger protein, which comprises CR3 of E1A. PEPCK transcription was stimulated approximately 5-fold in cells infected with either wild type Ad5 or pm975, an Ad5 E1A mutant that expresses only the 289R form of E1A (Fig. 3A). In contrast, no stimulation of transcription was noted in cells infected with the Ad5 mutant, dl520, which expresses only the 243R form of E1A or DL1, which was constructed from pm975 and contains a linker scanning mutation encompassing CR3.

To confirm the stimulation of PEPCK transcription by 289R E1A in the absence of viral infection, luciferase production from the PEPCK promoter was measured in MT 12-1 and 13-2, 3T3 cells that stably express either the 243R or 289R E1A proteins, respectively. Transcription from the PEPCK promoter was approximately 24-fold higher in 13-2 cells compared with control.

| Mutant               | E1A expressed | Mutation                        | P300 | P107 | P105Rb |
|---------------------|---------------|---------------------------------|------|------|--------|
| Ad5                 | 13 S, 12 S    | None                            | +    | +    | +      |
| dl343               | None          | E1A deleted                      | +    | +    | +      |
| pm975               | 13 S          | No 12 S expression              | +    | +    | +      |
| dl520               | 12 S          | No 13 S expression              | +    | +    | +      |
| E1A-1104            | 13 S, 12 S    | Amino acids 48-60 deleted       | -    | +    | -      |
| E1A-1108            | 13 S, 12 S    | Amino acids 124-127             | -    | +    | -      |
| DL1                 | 13 S          | CR3 deleted                     | +    | +    | +      |
| DL2                 | 13 S          | CR2 deleted                     | +    | -    | -      |
| 135-928             | 13 S          | Cys-124 to Gly                  | +    | +    | -      |
| E1A-RG2-928         | 13 S, 12 S    | Arg-2 to Gly, Cys-124 to Gly    | -    | +    | -      |
| Sub1008             | 13 S          | CR1 deleted                     | -    | +    | +      |

FIG. 1. Ad E1A stimulates PEPCK gene transcription. HepG2 cells were transfected with a plasmid containing the full-length PEPCK gene promoter linked to the luciferase reporter gene (~490pPCLuc). The cells were then infected with the indicated Ads for 20 h. Luciferase activity in cell lysates was measured as described under "Experimental Procedures." Levels of transcription are shown relative to transcription levels in mock-infected cells.
3T3-neo cells (Fig. 3B). No stimulation of transcription was observed in MT12–1. These data indicate that E1A stimulates PEPCK gene transcription in a CR3-dependent manner.

Role of E1A CR1 in Modulating Basal PEPCK Transcription—Kalvakolanu et al. (16) demonstrated in their report that CR1 of E1A was required for the inhibition of PEPCK gene transcription and that mutations in CR1 abolished the ability of E1A to inhibit PEPCK gene transcription. However, we have found that mutations in CR1 have little or no effect on the ability of E1A to regulate transcription from the PEPCK gene promoter. The E1A protein encoded by E1A-1104 contains a deletion in residues 48–60 within CR1, resulting in an inability to bind the cellular protein, p300, without significantly decreasing either p105 or p107 binding (Table I). Infection of HepG2 cells with E1A-1104, which expresses both 12 and 13 S forms of E1A, did not significantly change transcription from the PEPCK promoter from basal levels (Fig. 4). Similar results were observed with the Sub 1008 virus that expresses a 13 S protein from which CR1 is deleted.

Role of E1A CR2 in Modulating Basal PEPCK Transcription—Next, we measured the ability of E1A proteins containing mutations in CR2 to regulate transcription from the PEPCK promoter. Infection of HepG2 cells with Ad5 stimulated transcription from the PEPCK promoter by approximately 5-fold, whereas infection with E1A-1108, which expresses 243R and 289R E1A proteins containing a deletion of residues 124–127 in CR2, increased transcription by 11–14-fold (Fig. 5). Likewise, PEPCK promoter-driven transcription was stimulated by 11–14-fold in cells infected with DL2, which expresses a 289R E1A protein containing a linker scanning mutation that deletes CR2. By comparison, transcription was stimulated only 5-fold in cells infected with the native 289R-expressing virus, pm975. These data, using matched sets of viral mutants (Ad5 versus E1A-1108, and pm975 versus DL2), demonstrate that the E1A-dependent stimulation of PEPCK transcription can be partially inhibited by CR2-encoded residues of E1A.

The previous mutations in CR2 abolish E1A binding to a number of cellular proteins (p107, p60 (cyclin A), pRb) (Table I). However, the “superstimulation” of transcription from the PEPCK promoter was also observed in cells expressing mutant E1A proteins that are unable to bind pRb alone. The Ad mutant 13S-928, which contains a point mutation in CR2 specifically blocking E1A binding to pRb without affecting p107 or p60 binding stimulated transcription from the PEPCK promoter by 13–14-fold compared with the 5-fold increase with pm975 (Fig. 5). The lack of E1A-p300 binding in modulating PEPCK transcription is further illustrated by noting that RG2–928, which encodes E1A proteins (13 and 12 S) unable to bind both p300 and pRb does not increase the level of transcription from the PEPCK promoter more than cells infected with E1A-1108, which interacts with p300 normally. These data cannot be explained by the disparate levels of mutant E1A proteins in infected cells. Comparisons of matched sets of E1A mutants (E1A 13 S only expressing Ad: pm975, 13S928, DL2; E1A 12 and 13 S expressing Ad, Ad5, E1A-1104, E1A-1108, RG2–928)
demonstrate equivalent levels of E1A proteins (data not shown) but consistently higher levels of PEPCK transcription with CR2 mutants that do not bind Rb.

Regulation of Basal PEPCK Gene Transcription by E1A and pRb—Our results demonstrated that E1A regulates PEPCK gene transcription in part by interacting with the cellular protein, pRb. The interaction of E1A with pRb appears to attenuate the ability of pRb to stimulate transcription from the PEPCK promoter, since higher transcription levels were observed with mutant E1A proteins that cannot bind pRb than with wild type E1A proteins. To determine whether pRb regulates PEPCK promoter-driven transcription, we measured luciferase production from the PEPCK promoter in HepG2 cells transfected with increasing amounts of a pRb expression vector (pRbWT-HA/SVE). Increasing levels of pRb in these cells were confirmed by Western blot analysis (not shown). As hypothesized, expression of pRb stimulated transcription from the full-length PEPCK promoter in a concentration-dependent manner (Fig. 6). Furthermore, transcription from the PEPCK promoter was further stimulated by pRb in cells infected with Ad5. No change in transcription was noted in cells transfected with an empty pRb expression vector.

**DISCUSSION**

Previous studies by Kalvakolanu et al. (16) showed that the E1A proteins inhibited basal and cAMP-stimulated transcription of the PEPCK gene. This process was dependent upon CR1 of E1A, which has been shown to interact with the cellular transcriptional adaptors, p300 and CBP (37–39). Since the PEPCK promoter contains a CREB/CBP binding site and a potential p300 consensus binding site, we hypothesized that the PEPCK promoter might be a valuable system for further analysis of E1A and p300/CBP transcriptional activity. Interestingly, rather than inhibiting PEPCK gene transcription, we have consistently observed that E1A stimulates basal and cAMP-stimulated transcription from the PEPCK gene promoter. Our findings also differ from those reported by Kalvakolanu et al. (16) in that only E1A 289R regulated transcription from the PEPCK promoter, while both 243R and 289R exhibited activity in the other study. In addition, we found that mutations in CR1 had little effect on PEPCK promoter-driven transcription, whereas both CR2 and CR3 influenced PEPCK gene transcription through independent mechanisms.

It is unclear why our findings differ so significantly from those reported by Kalvakolanu et al. (16). The differences between our data and those reported by Kalvakolanu et al. (16) may be due to infection conditions or the PEPCK promoter-CAT reporter gene plasmids used in each laboratory. For example, Kalvakolanu et al. (16) used MOIs that were significantly lower and times of infection that were much shorter than used in our studies. Under the infection conditions reported by Kalvakolanu et al. (16) we were unable to detect significant levels of E1A expression but observed small increases in PEPCK promoter-driven transcription (data not shown). We also found that E1A proteins stimulated transcription from the PEPCK promoter-CAT reporter gene plasmids (generously supplied by Richard Hanson, Case Western Reserve University, Cleveland, OH) used in the studies by Kalvakolanu et al. (16). Therefore, we find that E1A stimulates transcription from the PEPCK gene promoter even under the conditions reported in the other study, and we are currently unable to explain the differences between our data and those reported by Kalvakolanu et al. (16). However, we have consistently observed that E1A stimulates PEPCK gene transcription by multiple techniques and experimental systems. Transcription from the PEPCK promoter was stimulated by E1A 289R as measured by nuclear run-on transcription assays or by luciferase (and CAT) reporter gene analysis. Similar data were obtained in several different cell lines (H4IIE, HepG2, 3T3) and in Ad-infected cells or in stably transfected cells. Therefore, we have concluded, based on the findings of numerous experiments, that E1A 289R stimulates PEPCK gene transcription.

Our data indicate that E1A 289R regulates basal transcription from the PEPCK promoter by two mechanisms. The first mechanism involves the stimulation of transcription from the PEPCK promoter by CR3. This conclusion is based on the inability of both wild type 243R protein and a mutant 289R E1A protein from which CR3 was deleted to stimulate transcription from the promoter. This finding is consistent with the transcriptional transactivation function normally attributed to CR3 of E1A. For example, E1A has been shown to stimulate gene transcription by binding to ATF2 (6–8), TFIID (5, 6), and the CCAAT box binding factor of the hsp70 promoter (40) through CR3. In other experiments, 289R has been shown to enhance the transcriptional activity of SP1 (6), implicating CR3 in this transcriptional process.

A number of transcription factor binding sites have been identified in the PEPCK promoter through which E1A could
Act. A TATA box, which binds TFIIID (12), as well as a cAMP regulatory element (CRE-1), which binds CREB/ATF and C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds NF-1 (12), are present in the PEPCK promoter. Sites that mediate glucocorticoid (18), thyroid hormone (41), and insulin C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds regulatory element (CRE-1), which binds CREB/ATF and act. A TATA box, which binds TFIIID (12), as well as a cAMP regulatory element (CRE-1), which binds CREB/ATF and C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds NF-1 (12), are present in the PEPCK promoter. Sites that mediate glucocorticoid (18), thyroid hormone (41), and insulin C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds NF-1 (12), are present in the PEPCK promoter. Sites that mediate glucocorticoid (18), thyroid hormone (41), and insulin C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds NF-1 (12), are present in the PEPCK promoter. Sites that mediate glucocorticoid (18), thyroid hormone (41), and insulin C/EBP proteins (17, 36), and a CCAAT/NF-1 site, which binds CREB, thereby blocking cAMP-stimulated transcription. How pRb regulates PEPCK gene transcription is unclear. Like the stimulation of transcription by E1A, the mutation of several protein binding sites in the PEPCK promoter inhibits the effect of pRb on transcription from the PEPCK promoter. Since pRb has not been shown to bind to the PEPCK promoter, pRb may stimulate PEPCK gene transcription by inhibiting the expression or activity of a transcription factor that inhibits PEPCK gene transcription.

Finally, we have shown that Ad E1A also enhances cAMP-stimulated transcription from the PEPCK promoter. This data also differs from the findings of Kalvakolanu et al. (16) who showed that E1A inhibited cAMP-stimulated PEPCK gene transcription through CR1. Their findings are particularly interesting since Arany et al. (38) and Lundblad et al. (39) recently reported that E1A also inhibits cAMP-stimulated transcription from the somatostatin promoter and from a Gal4-responsive promoter in the presence of a chimeric Gal4-CREB protein. This inhibition was also mediated by CR1, which interacts with CBP and p300. Presumably, E1A binds CBP and prevents it from interacting with protein kinase A-phosphorylated CREB, thereby blocking cAMP-stimulated transcription. In preliminary experiments, we have also found that E1A blocks cAMP-stimulated transcription from the somatostatin promoter and with the Gal4-CREB-responsive system. However, E1A consistently enhanced Bt2cAMP-stimulated PEPCK gene transcription in side-by-side assays. Furthermore, in preliminary experiments the dominant negative CREB inhibitor, KCREB (49), inhibited cAMP-stimulated transcription from the PEPCK promoter but did not significantly block the enhanced levels of transcription observed in the presence of both Bt2cAMP and E1A. However, mutation of the proximal CAMP-response element (CRE-1), which binds CREB, completely blocked CAMP-stimulated transcription and the enhanced levels of transcription observed with E1A and Bt2cAMP. These findings suggest that E1A and cAMP may regulate PEPCK gene transcription through a CREB-independent mechanism. Studies by Hanson and colleagues (36) have shown that the proximal CRE of the PEPCK promoter also interacts with C/EBP proteins as well as AP-1. Their data support the hypothesis that C/EBP or other proteins may also mediate the CAMP response of the PEPCK promoter. In the future, we will address the ability of E1A to interact with C/EBP proteins and AP-1 and the effect of these interactions on CAMP-stimulated PEPCK gene transcription.

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Fig. 7. Model of Ad-stimulated PEPCK gene transcription. Wild type and mutant E1A proteins and pRb are indicated. The mechanism of stimulation by E1A and pRb is not known but is indicated by the direct binding of the proteins to the promoter. Levels of transcription are indicated by the thickness of the arrows.
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