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Cis Effect of the Type 5 Adenovirus E1A Gene Enhancer Element on Cellular Transformation

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Mutants of type 5 adenovirus that lack all or part of the early region 1A (E1A) gene enhancer element transform rodent embryo fibroblast (CREF) cells at higher efficiencies than wild-type virus. An analysis of viral E1A cytoplasmic mRNA levels in mutant and wild-type virus-infected CREF cells revealed no differences in the levels of the E1A mRNAs. This implies that a decrease in the rate of viral E1A gene expression was not responsible for the transforming properties of the enhancer-less viruses. Unlike wild-type virus, however, the mutant viruses were able to replicate their genomes in the normally nonpermissive CREF cells. This change in viral DNA template concentration further resulted in an increase in early gene mRNA concentrations in mutant-virus-infected CREF cells. These studies suggest several possible mechanisms that could be responsible for the increased transforming potentials of these viruses, including 1) a cis effect of removing the viral E1A enhancer element on the efficiency of viral DNA integration, 2) viral DNA replication, or 3) an increase in the levels of the viral E1A and E1B mRNAs owing to viral DNA replication in the virus-infected CREF cells.

Key words: DNA replication, virus mutants, CREF cells, mutant viruses, fibroblasts

The type 5 adenovirus early region 1A and 1B genes have been extensively analyzed, because the proteins encoded by these genes contribute to the regulation of a wide variety of cellular events, including transcriptional and posttranscriptional control of viral and cellular gene expression, regulation of viral and cellular DNA synthesis, and cellular immortalization and morphological transformation (for a review, see Ginsberg [1]). Expression of the viral E1A gene prior to the onset of viral DNA replication results in the production of two differentially spliced mRNAs of 13S and 12S in size, which encode nuclear phosphoproteins of 289 and 243 amino acids (R), respectively [2–5]. The 289R protein has been shown to stimulate the rate of transcriptional initiation from a wide variety of viral and cellular gene promoters [6–9]. The 243R protein lacks the

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amino acid sequences unique to the 289R protein, which are responsible for this transactivating function. The 243R protein however, is capable of initiating the events leading to cellular transformation [10,11]. Interestingly, the transformants derived from viruses expressing only the 243R protein display a partially transformed morphology (the cells remain fibroblastic as opposed to becoming epithelioid), and they are cold-sensitive for the maintenance of this phenotype [10,12]. Both proteins share domains that suppress the rate of transcription from certain viral and cellular gene promoters [13,14] and are responsible for the induction of cellular DNA synthesis and cell proliferation [15,16]. It has therefore been suggested that transformation by adenovirus may be achieved by the suppression of cellular genes that regulate cell growth [15,17], although activation of certain genes may also contribute to this process.

Transcription from the viral E1B gene results in two predominant mRNA species of 22S and 13S in size [2–4,18]. The 22S mRNA encodes two proteins of 495 and 175 amino acids [19]. The larger protein has been shown to play a role in the nuclear-cytoplasmic transport of late viral mRNAs and the shut-off of host protein synthesis [20–22]. Expression of this protein in adenovirus-transformed rodent cells contributes to the phenotypic characteristics of the transformed cell, including morphological characteristics, anchorage-independent growth, and serum dependence [23]. The 175R protein appears to play an indirect role in viral DNA replication by blocking the entry of cellular nucleases into the infected cell nucleus [24]. Viruses that fail to express the wild-type 175R protein are transformation-defective, even though viral E1A gene expression is normal [23,25,26]. This observation has led to studies showing that this protein and the E1A proteins are both necessary to elicit the cascade of cellular events leading to the generation of the transformed cell [27].

Although considerable effort with various mutagenic schemes and recombinant adenoviruses has led to many of the conclusions concerning the role of individual proteins in the process of cellular transformation, very little is known about how these proteins function mechanistically. To begin to study these mechanisms, an understanding of how the rate of viral E1A and E1B gene expression affects the efficiency of transformation is necessary.

In this study, we have attempted to determine how the rate of viral E1A gene expression exhibited by recombinant adenoviruses influences the frequency of transformation using a cloned rat embryo fibroblast (CREF [28]) cell line. Based on the studies of Hearing and Shenk [29,30], we reasoned that we could decrease the rate of viral E1A gene transcription and protein expression in CREF cells using recombinant adenoviruses that lacked all or part of the viral E1A enhancer element and then correlate these changes in E1A expression directly with the efficiency of cellular transformation. Although we did observe an increase in focus formation as a function of the lack of viral enhancer sequences, we were surprised to find that the E1A enhancer element was not fully functioning to stimulate E1A expression in CREF cells. Rather, additional studies suggest that the occurrence of viral DNA replication and/or a cis effect of removing these viral enhancer sequences is the factor contributing to the transforming properties of these viruses.

MATERIALS AND METHODS

Cells and Viruses

Monolayer cultures of CREF (a cell line derived as a single-cell clone from Fischer rat embryo cells [28]) cells and Hela cells were maintained in Dulbecco's modified

Eagle's medium, supplemented with 5% fetal bovine serum or 10% supplemented bovine calf serum, respectively. Viral stocks were obtained by infection of monolayer cultures of human 293 cells (human embryonic kidney cell line that constitutively expresses the viral E1A and E1B genes [31]) with the appropriate virus and the preparation of crude cellular lysates by repeated cycles of freezing and thawing. Titers of viral stocks were determined as previously described [12].

Transformation of CREF Cells

Transformation assays using the viruses shown in Figure 1 and CREF cells were performed as previously described [27]. To determine the effect of various viruses on cell survival following virus infection of CREF cells, a clonal survival assay as previously described was performed [27].

Assays for Viral Early Gene Expression

The production of stable cytoplasmic mRNAs from the viral E1A, E1B, E2A, and E4 early genes was scored by hybridizing 20 μg of total cytoplasmic RNA to uniformly labeled (^{32}P-UTP) antisense RNA probes (7.5×10^5 CPM) and analyzing the RNAse T2-digested products on 5% denaturing acrylamide gels. The sp6 RNA polymerase assays for the viral E1A, E1B, and E4 genes have been previously described [27] and are noted in Figure 2; these result in protected RNAs of 183 and 195 nucleotides for E1B; 487, 347, and 111 nucleotides for E1A; 291 nucleotides for E4; and 240 nucleotides for E2A.

Assays for Viral DNA Replication

Viral DNA replication was monitored by two independent assays. Using the same E1B antisense RNA probe described earlier and cytoplasmic RNA harvested from virus-infected (20 p.f.u./cell for 16 h P.I.) CREF cells, we were able to score the pIX mRNA, which is expressed only following the onset of viral DNA replication [32]. Viral DNA was also monitored and quantitated by labeling virus-infected CREF cells for 20 min with ^{3}H-thymidine (100 μCi/ml) at the time indicated in the legend to Figure 4 and hybridizing total cell DNA to nitrocellulose filters containing 10 μg of denatured In340 viral DNA. To distinguish between viral DNA replication and the incorporation of labeled thymidine by DNA repair mechanisms, CREF cells infected by In340 virus were treated with cytosine arabinoside (40 μg/ml) during the virus infection and the subsequent labeling periods to block viral DNA replication.

RESULTS

Viruses That Lack All or Part of the E1A Enhancer Element Transform CREF Cells at Greater Efficiencies Than Ad5wt

Hearing and Shenk [29,30] previously localized the cis-acting DNA element that positively regulates the rate of viral E1A gene transcription, using a variety of recombinant viruses. The E1A enhancer element has been localized between nucleotides -305 and -141, relative to the start site of E1A gene transcription, and has been shown to overlap with the DNA packaging signal sequences. To determine how the rate of viral E1A gene expression would influence the efficiency of focus formation by enhancer-mutant viruses, a CREF cell transformation assay was performed. In340-A5 virus has a deletion of half of the E1A enhancer element; In340-2 lacks all of the E1A enhancer

Transformation frequency of Viral Mutants using CREF cells

Virus	Experiment 1	Experiment 2	Avg	fold
In340	18, 17, 17	20, 20, 19, 23	19	-
In340-2	87, 84, 79	65, 80, 87, 74	79	4
In340-11	42, 62, 55	54, 59, 63, 57	56	3
In340-A5	29, 28, 20	51, 39, 30, 33	33	1.7

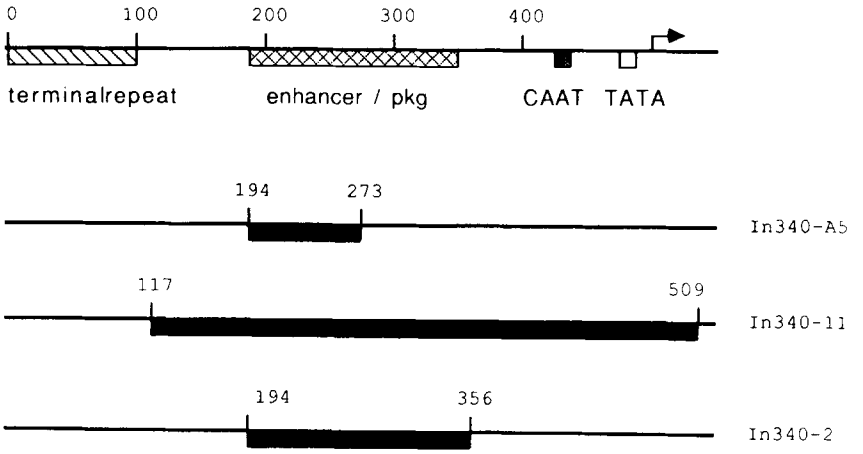

Fig. 1. Comparative transformation of CREF cells by enhancer mutant viruses and In340 virus. CREF cells were infected with 15 p.f.u. of the appropriate virus per cell, and, following adsorption, the cells were resuspended and replated at 10^5 cells per 60 mm plate for 6 weeks at 37°C. "Avg" is the mean number of transformed colonies from both experiments and represents the quantitation of six to ten plates in each experiment. "Fold" represents the increase in the average number of foci for both experiments for each of the viruses shown relative to wild-type In340 virus. At bottom is a schematic representation of the E1A gene promoter proximal and enhancer regions, relative to the start site for E1A transcription and the extreme left of the viral genome. The viral enhancer and DNA packaging signal (pkg) have been shown to map to the same DNA region [29]. The viral genomes shown at the bottom contain various mutations affecting E1A expression, and the isolation and characterization of these viruses has been described [29]. Solid boxes represent DNA deletions, with the nucleotide location of these deletions shown in base pairs.

sequences [29] (see Fig. 1). The In340-11 virus lacks all the enhancer, promoter proximal, and E1A capsite sequences and expresses wild-type E1A proteins by initiating transcription in the terminal DNA repeat sequences [29] (see Fig. 1). All of these mutations were recombined into an In340 virus genome [29], which complements the viral DNA packaging defect.

CREF cells were infected with 15 p.f.u./cell of each virus, and, following a 6 week incubation period at 37°C, cells were fixed and stained, and the foci per dish were quantitated. As is shown in Figure 1, the virus that lacked all of the E1A enhancer

element, In340-2, transformed CREF cells at a fourfold higher frequency than wild-type parental virus, In340. Removing the distal half of the enhancer element also led to a twofold increase in focus formation. The foci that developed due to these viruses and In340 virus appeared to be completely transformed based on their epithelioid morphology. Furthermore, several clonally derived transformed cell lines established using each of the viruses shown in Figure 1 displayed similar cloning efficiencies in soft agar (data not shown). We have previously shown that different viruses display different degrees of cytotoxicity when introduced into CREF cells [27]. However, none of the viruses used in this study displayed any differences in cytotoxicity (data not shown), suggesting that this did not contribute to the transformation phenotypes observed. It should also be noted that the appearance of foci in In340-2- and In3409-A5-infected CREF cells was accelerated by 2 weeks when compared to In340 virus-induced foci. In340-11 virus also lead to a threefold increase in focus formation, although there was no acceleration in our ability to discern foci when compared to In340 virus. Based on the observations of Hearing and Shenk [29,30], these results suggested that, when the rate of viral E1A gene transcription (and corresponding E1A protein levels) is decreased, the frequency of focus formation is increased.

Lack of Viral E1A Enhancer Sequences Does Not Influence the Accumulation of E1A mRNAs in the Cytoplasm of CREF Cells

In CREF cells and Hela cells infected with the viruses shown in Figure 1, the accumulation of viral early cytoplasmic mRNAs was analyzed by RNA protection assays using a series of gene-specific ^{32}P-labeled antisense probes. Cytoplasmic RNA was isolated from CREF cells and Hela cells infected with 20 p.f.u./cell at 6 h and 8 h postinfection, respectively. As was previously shown in Hela cells [29], removing various amounts of the E1A enhancer element leads to a decrease in cytoplasmic E1A mRNA accumulation (see Fig. 2A). Quantitative analysis of these findings, as determined by densitometric scanning of the data shown in Figure 2, and additional experiments, revealed that E1A mRNA levels were 5% (In340-2), 10% (In340-11), and 40% (In340-A5) relative to In340 virus. The reduction in E1A mRNA expression observed in enhancer-mutant virus infected Hela cells led to a corresponding decrease in viral E1B and E4 gene expression, suggesting that the level of E1A 289R protein directly influences the rate of viral E1B and E4 gene expression.

When cytoplasmic E1A mRNA accumulation was measured in virus-infected CREF cells, a surprising result was observed. E1A mRNA levels were similar for all the viruses (Fig. 2B). These results and the results obtained in virus-infected Hela cells were shown to be due to transcriptional control, as opposed to differential mRNA stability, as determined by in vitro nuclear run-on assays (data not shown). It should be noted that the rate of E1A gene transcription in In340 virus-infected CREF cells is tenfold less than that observed in In340 virus-infected Hela cells on a per cell basis (from quantitative analysis of data shown in Fig. 2 and additional experiments). Hence, the E1A enhancer element is not functioning optimally to increase the rate of E1A gene transcription in virus-infected CREF cells. The steady-state levels of the viral mRNAs from early regions E1B and E4 were the same for all the viruses in CREF cells (see Fig. 2B). This confirms that the levels of E1A 289R protein were similar in enhancer-mutant and wild-type virus-infected CREF cells.

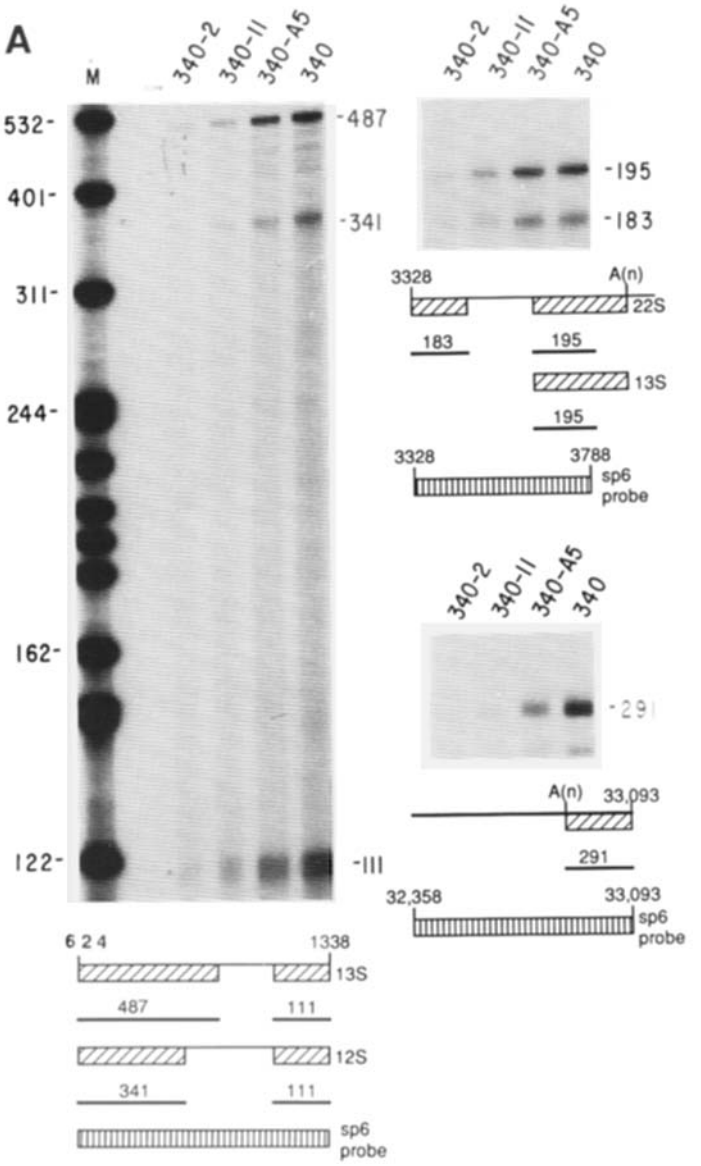

Fig. 2. Steady-state RNAs in virus-infected CREF and Hela cells. Hela (**A**) and CREF (**B**) cells were infected with 15 p.f.u./cell with the viruses shown above each lane in the autoradiogram and scored for the presence of E1A, E1B, and E4 RNAs by RNAseT2 protection assays. CREF cells were infected for 8 h, and Hela cells were infected for 6 h. Total cytoplasmic RNA (20 μg) was hybridized to ^{32}P-UTP-labeled antisence RNA probes (7.5×10^5 CPM) for each of the transcription units, and the RNase T2-resistant products were analyzed on 5% denaturing acrylamide gels. Autoradiographic exposures were 12 h for Hela cells and 50 h for CREF cells. The lengths of the protected RNAs for each transcription unit were determined by comparison with labeled DNA markers, and these lengths are shown at the bottom of each panel and to the left or right of each panel.

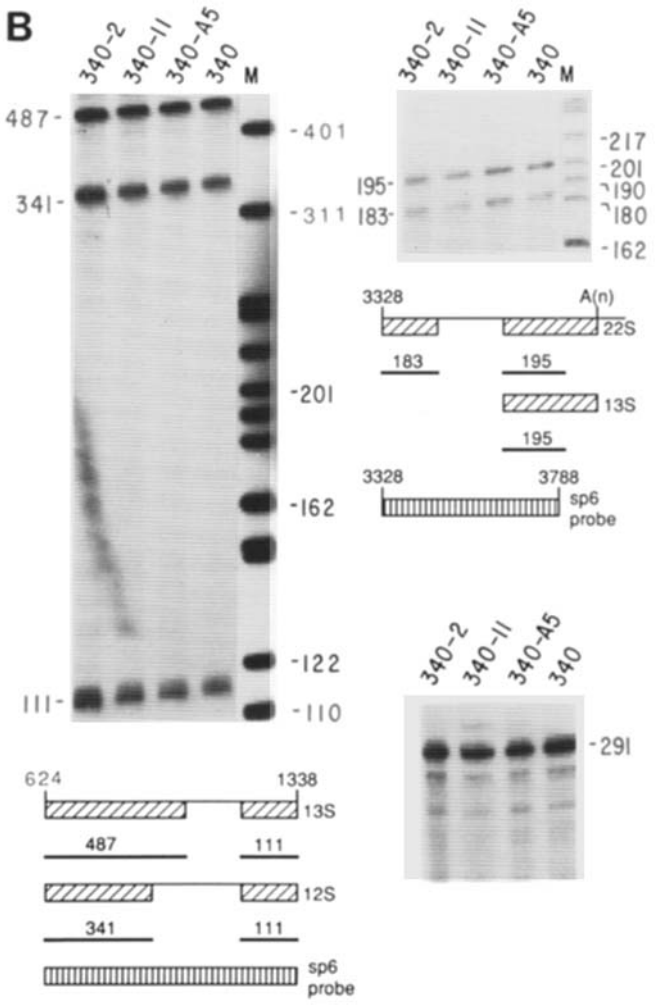

Fig. 2 (continued)

Viral DNA Replication Is Observed in In340-2 and In340-11 Virus-Infected CREF Cells

Using a wide variety of experimental approaches, we previously determined that wild-type virus-infected CREF cells do not support detectable levels of viral DNA replication (H.S. Ginsberg and L.E. Babiss, unpublished observation). In one series of experiments, we sought to determine whether a prolonged virus infection of CREF cells would result in higher intracellular E1A protein levels that would then produce an increase in viral early gene expression. CREF cells were infected (20 p.f.u./cell) with each of the viruses shown in Figure 1, for 16 h, at which time cytoplasmic RNA was isolated and viral E1B and E2A mRNA levels were assayed. To our surprise, E1B and E2A cytoplasmic mRNA levels were three- to fourfold greater in In340-2 and In340-11 virus-infected CREF cells (see Fig. 3). We also observed pIX mRNAs in these virus-infected cells, which is not normally expressed until the onset of viral DNA

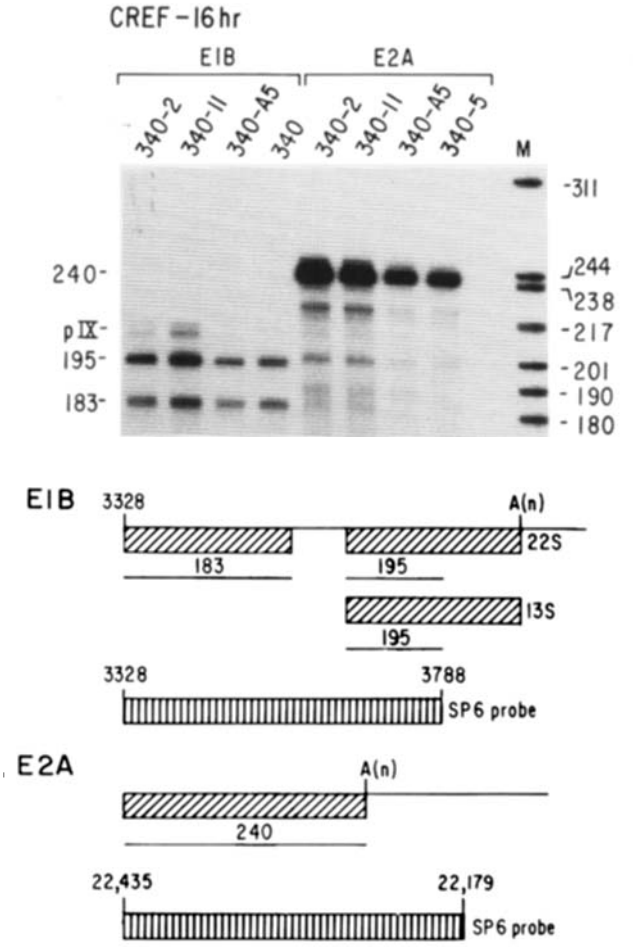

Fig. 3. Steady-state RNAs in virus-infected CREF cells. CREF cells were infected with 15 p.f.u./cell of the virus shown above each lane for 16 h, at which time total cytoplasmic RNA was harvested. See the legend to Figure 2 for details of the RNase T2 assay and product analysis. The presence of a novel protected RNA, 205 nucleotides in length, in the E1B assay is indicative of the pIX mRNA.

replication. This observation suggested that the increase in early gene expression seen in In340-2 and In340-11 virus-infected CREF cells at 16 h was not due to a change in the rate of viral E1A gene expression but more likely to an increase in the viral DNA template concentrations.

Since it was possible that pIX transcription could be occurring in the absence of viral DNA replication, it was necessary to analyze carefully the kinetics of viral DNA replication in virus-infected CREF and Hela cells. Replicate monolayer cultures of CREF and Hela cells were infected with the appropriate viral mutant, or In340 virus at 20 p.f.u. per cell. At various times during the virus infection, the cells were labeled with ^{3}H-thymidine (100 μCi/ml), and total infected cell DNA was isolated and hybridized to filters containing excess denatured In340 viral DNA. The results of this analysis are shown in Figure 4 and reveal that In340 virus replicates its DNA efficiently in Hela cells and rather poorly, if at all, in CREF cells. This correlated with previous studies, in which

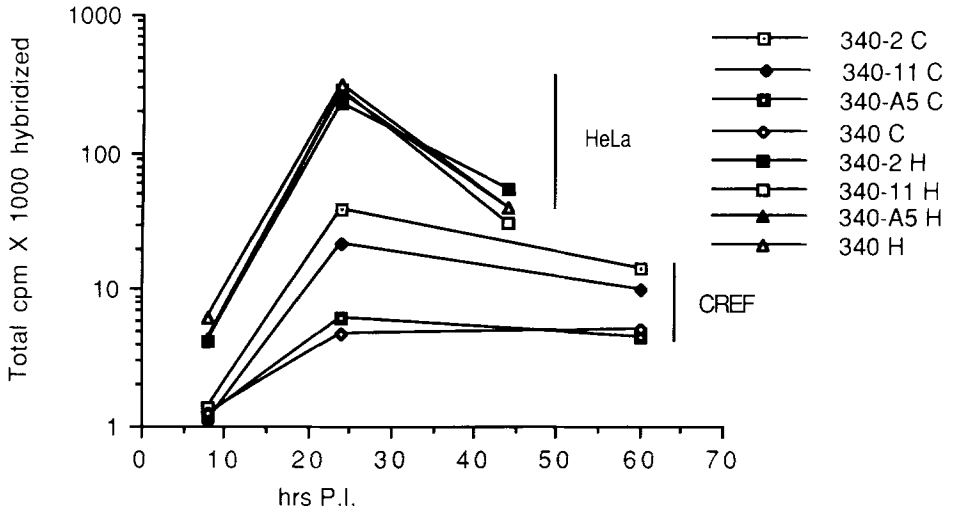

Fig. 4. Analysis of viral DNA replication by enhancer mutant viruses and In340 virus in CREF and Hela cells. Monolayer cultures of Hela and CREF cells were infected with 15 p.f.u./cell of the appropriate virus, and total cell DNA was labeled with ^{3}H-thymidine for 20 min at the times indicated. Infected-cell DNA was isolated and hybridized to nitrocellulose filters containing 10 μg denatured In340 virus DNA. All counts were corrected for background and nonspecific hybridization and normalized for the efficiency of hybridization as determined using ^{32}P-labeled In340 virus DNA. The results for each virus (represented by a symbol) in both cell types are shown at right, with C representing virus-infected CREF cells and H representing virus infected Hela cells.

we observed that the hybridizable counts measured in wild-type virus-infected CREF cells were also the result of small amounts of viral DNA replication and random incorporation of labeled thymidine into the parental viral genomes by cellular DNA repair mechanisms (H.S. Ginsberg and L.E. Babiss, unpublished observation). This result was obtained by incubating virus-infected CREF cells with ^{3}H-thymidine in the presence of chemicals (hydroxyurea or AraC) that inhibit viral DNA replication.

All of the viral mutants replicated equally well in Hela cells compared to wild-type In340 virus. This was surprising in that a lag in the expression of the viral early gene proteins involved in viral DNA replication would have been anticipated, particularly in In340-2 virus-infected Hela cells. An analysis of cytoplasmic E1A mRNA levels in In340 and In340-2 virus-infected Hela cells at 10 h postinfection revealed similar levels of the E1A mRNAs (data not shown). Thus the E1A enhancer appears to have a profound effect on E1A transcription *only* at a very early stage of the viral replicative cycle, as was originally suggested by Hearing and Shenk [29,30].

As the pIX promoter expression result had indicated, the In340-2 and In340-11 viruses did replicate their DNAs in CREF cells. This is the first observation that CREF cells can support this amount of viral DNA replication. The integrity of the progeny viral DNA was determined by alkaline sucrose gradient analysis, which revealed no gross degradation of the progeny viral DNA (data not shown). Although a significant amount of hybridizable counts could be measured in In340 and 340-A5 virus-infected CREF cells, this was largely the result of DNA repair rather than viral DNA replication (data not shown).

DISCUSSION

In this paper we have shown that viruses lacking all or part of the adenovirus E1A enhancer element transform CREF cells at a frequency exceeding that observed with wild-type virus. Although these observations might have suggested that reducing the rate of viral E1A gene expression was the probable cause for this virus transformation phenotype, this was shown not to be the case. An analysis of viral E1A gene expression by all the viruses (see Fig. 1), upon infection of CREF cells, revealed that E1A mRNA levels were not influenced by the presence of the viral E1A enhancer element (see Fig. 2A,B). Therefore, the transforming properties of the viral mutants were not defined at this level. An analysis of progeny viral DNA levels in mutant and wild-type virus-infected CREF cells suggested that the In340-2 and In340-11 viruses were able to replicate their DNA. This was based on three independent assays that 1) scored the synthesis of nascent progeny viral DNA (see Fig. 4); 2) measured the transcriptional activity from a viral promoter, which is functional only following viral DNA replication (see Fig. 3); and 3) analyzed the viral genome organization in clonally derived virus-transformed cell lines (data not shown). This is the first observation of significant viral DNA replication in CREF cells and might suggest that this replicative property contributes to the transforming phenotypes observed. However, our inability to detect viral DNA replication in In340-A5-infected CREF cells (twofold increase in transformation by this virus compared to In340 virus) suggests that DNA replication alone may not be the only mechanism contributing to cellular transformation.

As a result of viral DNA replication, cytoplasmic mRNA levels for the viral early genes were elevated in In340-2 and In340-11 virus-infected CREF cells. This increase in early gene expression might also explain the transforming properties of these viruses. Several studies have indicated that increasing the rate of expression of viral oncogenes leads to an increase in the frequency of focus formation [33,34]. However, the studies of Raptis et al [35] suggest that the frequency of morphological transformation of rat F-111 cells by polyoma virus, is increased by decreasing the rate of middle T-antigen expression. Similarly, we have recently determined that reducing the rate of E1A gene transcription by a mutant recombinant adenovirus results in a tenfold increase in focus-formation upon infecting CREF cells (G.R. Adami, R.H. Herbst, and L.E. Babiss, manuscript in preparation). However, differences in the kinetics of E1A expression in this latter study, and by In340-2 and In340-11 viruses, do not rule out the possibility that increasing E1A expression at 16 h postinfection of CREF cells results in an increase in focus formation.

Since all of the viruses display some altered transformation phenotype compared to wild-type virus, it is possible that deleting viral sequences in the E1A enhancer region could enhance the efficiency or frequency of viral DNA integration into the host chromosomal DNA sequences. Of course, this would have to be a very specific cis effect, since these sequences are located intact in the right end of the viral genome. To date, there is no convincing evidence that adenovirus DNA integration occurs at preferential locations in the host DNA sequences, so it is unlikely that deleting the viral enhancer sequences now permits sequence specificity for viral DNA integration. We have shown [36] that the bovine papilloma virus (BPV-1) is capable of transforming CREF cells, but, unlike adenovirus, the BPV-1 DNA remains stablely extrachromosomal. Lusky and Botchan [37] have shown that deleting a region of the BPV-1 genome results in transformation-competent viruses, which now integrate their genomes into the host

chromosomal DNA. In this situation, however, the integration of the viral genome appeared to be the result of its inability to replicate, due to a mutation in a viral gene product necessary for replication. We are presently testing whether there is a sequence that might influence the efficiency of the viral DNA integration process.

Viral DNA replication has been shown to influence the efficiency of focus formation by several DNA tumor viruses, including SV40 [38], BPV-1, and adenovirus. In the case of adenovirus, Dorsch-Hassler et al. [39] have shown that a virus (H5ts125) containing a temperature-sensitive lesion in the viral 72 kd DNA-binding protein, transforms rodent fibroblast cells at a higher efficiency (eightfold) at the nonpermissive temperature for viral DNA replication of 39.5°C. That viral DNA replication is contributing to the transforming characteristics of this virus is supported by the observation that full-length viral genomes could be detected in transformants obtained at 39.5°C, whereas incomplete genomes (suggesting that viral DNA replication occurred prior to the viral DNA integration process) were observed at the permissive temperature of 32°C. If viral DNA replication is contributing to the transforming phenotypes of the viruses we describe in this paper, then this would contrast with the H5ts125 studies, where viral DNA replication is decreasing the efficiency of focus formation. The differences between the two studies could be explained by the cells used in the transformation assays. In our studies we used CREF cells, which are a continuous cell line derived from a single cell; Dorsch-Hassler et al. [39] used secondary cultures of Fischer rat embryo cells (which are probably heterologous in their ability to support viral DNA replication). Since the later secondary cultures are composed of a heterogeneous population of cells, the measured transforming and replicative characteristics of adenovirus are less clear.

The inability of the E1A enhancer element to stimulate viral E1A gene transcription in virus-infected CREF cells was surprising. A wide variety of cellular transcriptional factors have been shown to interact with the cis-regulatory elements that modulate E1A gene transcription (for a review, see Jones et al. [40]). We have recently shown that the cellular transcription factor E2f (which binds to the E1A enhancer element and is necessary for enhancer function) is modified properly in CREF cells and has similar binding activities compared to Hela cells [41]. Additional studies have identified a cellular factor in CREF cell nuclei that binds to viral DNA sequences 5′ to the E1A enhancer element (R. Herbst, M. Pelletier, and L. Babiss, manuscript in preparation). This factor is 20 times more abundant in CREF cell nuclei then in Hela cell nuclei and might be functioning in CREF cells to suppress enhancer function.

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