MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6664–6673
0270-7306/90/126664-10$02.00/0

Vol. 10, No. 12

Maintenance of Cellular Proliferation by Adenovirus Early Region 1A in Fibroblasts Conditionally Immortalized by Using Simian Virus 40 Large T Antigen Requires Conserved Region 1

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Received 7 May 1990/Accepted 4 September 1990

Various mutants of adenovirus E1A were assayed for their ability to complement the growth defect at the nonpermissive temperature for the cell line tsa14 which was isolated by immortalizing rat embryo fibroblasts with the thermolabile large T antigen of tsA58. This cell line grows indefinitely at the permissive temperature but undergoes rapid growth arrest upon shift up to the nonpermissive temperature. Since this growth arrest can be overcome by introduction of wild-type simian virus 40 large T antigen, human papillomavirus 16 E7, and adenovirus E1A, the tsa14 cells provided an excellent system for defining regions of E1A necessary for complementation of the growth defect. We demonstrate that conserved region 1 (CR1) is the region of E1A required for complementation. While CR2 of E1A has been shown to be required for the immortalization of primary cells and is also necessary for the binding of the 105-kDa retinoblastoma protein, mutations within this region did not abrogate complementation of the growth defect. However, since both CR1 and CR2 have previously been shown to be absolutely required for immortalization of primary cells by adenovirus E1A, this evidence suggests that the tsa14 system assays for the maintenance of proliferation and that this requires CR1.

The human adenovirus type 5 early region 1A (E1A) encodes two major proteins of 289 and 243 residues which are derived from the differentially spliced 13S and 12S transcripts, respectively. The 289-residue protein comprises two regions that are shared in common with the 243-residue protein, designated exon 1 and exon 2. In addition, there is a region unique to the larger protein between residues 140 and 189. These regions appear to encode certain activities which may contribute to the pleiotropic effects of the E1A molecule.

The activities of E1A include transactivation of both viral (1, 2, 26, 27, 42) and cellular genes (30, 43, 55) and inhibition of certain transcriptional enhancers (5, 20, 21, 61). E1A is also able to repress the expression of the cellular genes c-*myc*, JE, stromolysin, and collagenase (58, 59). The transactivation domain maps to the unique region of the 289-residue protein (25, 34, 35, 38), whereas transcriptional inhibition maps to exon 1 (34, 35, 52).

E1A proteins also induce cellular division and immortalization as well as transformation of embryonic cells in conjunction with early region 1B (E1B) proteins (7, 16, 22, 26, 51, 54). The regions of E1A responsible for these activities have been less clearly defined than those for transactivation and transcriptional inhibition, but at least two regions within exon 1 are required (34, 35, 49, 52, 63). Of the different serotypes of adenovirus, these regions are conserved between residues 40 to 60 (conserved region 1 [CR1]) and 120 to 140 (conserved region 2 [CR2]). In addition, a region close to the amino terminus has recently been shown to be required for transformation (56).

In human cells transfected with or transformed by adenovirus, the E1A proteins form complexes with cellular polypeptides of 65 and 68 kDa and with cellular phosphoproteins of 105, 107, and 300 kDa (10, 11, 19, 65). The 105-kDa E1A-binding protein is the product of the RB1 gene which is absent or altered in retinoblastomas and other human cancers (12, 13). The RB1 gene encodes a nuclear phosphoprotein (33) which has DNA binding activity and can restore a normal phenotype to retinoblastoma cells when introduced exogenously (23), supporting the hypothesis that RB1 gene inactivation is a critical event in tumorigenesis.

A property which E1A proteins share with the simian virus 40 (SV40) large T antigen (LT Ag), the large T antigen of polyomavirus, and the E7 gene of human papillomavirus 16 (HPV16) is the ability to establish continuous in vitro proliferation of primary cells (24, 29, 47, 48, 50, 51). Moreover, these same viral proteins have also been shown to associate with the RB1 protein (8, 9, 63). In adenovirus E1A, two regions appear to be necessary for binding of RB1 (CR1 and CR2). CR2 bears significant homology with regions in SV40 LT Ag and HPV16 E7. Indeed it has been demonstrated that RB1 binding and transformation activity is maintained if the region homologous to CR2 of SV40 LT Ag replaces the CR2 of E1A (39). Thus these conserved regions are implicated in the transforming activity of the respective viral proteins (28, 35, 40, 41, 65).

The observation of a functional similarity between these viral transforming proteins has been extended by the use of a biological assay (24). This assay utilizes a cell line derived from rat embryo fibroblasts that have been conditionally immortalized using the temperature-sensitive mutant SV40 LT Ag derived from tsA58 (57). These cells grow indefinitely at the permissive temperature (33°C) but rapidly undergo growth arrest upon shift up to 39.5°C, the nonpermissive temperature for the mutant LT Ag. In the original study (24), the introduction of the 243-residue E1A protein into the tsa14 cell line enabled the cells to overcome the growth arrest and to maintain proliferation at the nonpermissive

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temperature. Subsequently the E7 protein of HPV16 has also been shown to complement the growth defect (62).

In consideration of these results, we were interested in determining whether or not the ability of the E1A molecule to complement the growth defect of the tsa14 cell line mapped to the region that was implicated in binding the RB1 protein. The results presented here suggest that in this system, the ability of E1A to complement the growth defect is distinct from RB1 binding and is dependent upon CR1.

MATERIALS AND METHODS

Construction of pCMVCR1 and pCMVCR1N+. To express E1A-specific oligopeptides containing only CR1, two plasmids, pCMVCR1 and pCMVCR1N+ (Fig. 1a), were constructed by using the vector pORFX11 such that expression of the CR1 sequences was regulated from the strong immediate-early cytomegalovirus promoter (3). This was done by first removing the synthetic translation initiation codon and the frameshift polylinker of pORFX11 by a *Kpn*I-*Hin*dIII digest and replacing it with a synthetic 74-bp *Kpn*I-*Hin*dIII directional oligonucleotide to yield the intermediate vector p22. This insert contained an *Nco*I site whose ATG corresponds to amino acid 15 of the E1A protein, followed by sequences up to the *Pvu*II site of E1A, followed by a *Nar*I site, a 5-amino-acid nuclear transport signal, and a translational termination codon. In addition, each end of this minigene was flanked by *Bam*HI sites to facilitate further manipulation. The recombinant pCMVCR1 was constructed from the vector p22 by inserting the 190-bp *Pvu*II-to-*Nar*I fragment derived from the wild-type E1A plasmid pCE (54) into *Pvu*II-*Nar*I-digested p22. This fragment encodes the CR1 of the E1A protein between amino acids 23 and 86. Recombinant pCMVCR1N+ was constructed from pCMVCR1 by inserting a 73-bp oligonucleotide encoding amino acids 1 to 22 of the E1A protein. This was done by synthesizing this oligonucleotide with *Kpn*I and *Pvu*II restriction sites at the ends such that it could be inserted into *Kpn*I-*Pvu*II-digested pCMVCR1. The *Nco*I site in CR1 is lost in this process.

Cell culture and transfection. All cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and 100 μg of penicillin and streptomycin per ml. Untransfected tsa14 cells were maintained at the permissive temperature of 33°C, and transfected cells were maintained at either 33 or 39.5°C as described in the text. E1A recombinant plasmids were cotransfected into tsa14 cells on 60-mm-diameter dishes in a 10:1 ratio with the pBS+hygro selectable marker plasmid by the calcium phosphate procedure of Graham and van der Eb (15) as modified by Parker and Stark (46). The cells were not subjected to a glycerol shock but instead were treated with chloroquine diphosphate (100 μg ml^{-1}) for 12 h (37). After 24 h, the cells were passaged 1:8 and selected in hygromycin B (Calbiochem-Behring, La Jolla, Calif.) at a final concentration of 100 μg ml^{-1} for 48 h. Half the dishes corresponding to each treatment were then shifted up to the nonpermissive temperature. Hygromycin selection was maintained until colonies were clearly visible. Culture plates were then either stained with 2% methylene blue in 50% ethanol and scored or used to isolate representative colonies for subsequent expansion and characterization of transfected cell lines.

The pBS+hygro selectable marker plasmid was constructed by excising the *Hin*dIII-*Pst*I fragment containing the hygromycin resistance gene from pY3 (4) and subcloning it into pBS+ (Stratagene). This was a generous gift from Robin Brown.

Growth analysis. A total of 10^3 cells of each selected cell line (transfected or wild type) were passaged and plated onto 60-mm-diameter dishes such that triplicate plates for each time point were available. At 10 h after passage, the plating efficiency of the cells was calculated, and representative dishes were shifted to the nonpermissive temperature and grown for the appropriate periods of time.

Immunoprecipitation and gel electrophoresis. Cell lines derived from transfection of tsa14 cells with the various E1A mutants were plated onto 100-mm-diameter dishes and grown to 80% confluence. Cells were incubated with [^{35}S] methionine (100 μCi; Amersham Radiochemicals, Amersham, United Kingdom) in Dulbecco modified Eagle medium without methionine for 2 h and washed twice with phosphate-buffered saline without magnesium and calcium. Cells were lysed in a solution consisting of 50 mM Tris hydrochloride (pH 7.5), 150 mM NaCl, and 1% Triton X-100. Cell lysates were immunoprecipitated with the anti-E1A monoclonal antibody PAbM73 (18) in the presence of 2% protein A-Sepharose (Sigma Biochemicals) at 4°C for 4 h. The anti-polyomavirus large T-antigen monoclonal antibody F4/pA5 (45) was used as a negative control. Immune complexes were washed twice in lysis buffer and three times with lysis buffer without Triton X-100 and then suspended in a solution containing 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue, and 20 mM Tris hydrochloride (pH 6.8). The samples were heated to 100°C for 5 min and fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel.

RESULTS

Complementation of the growth defect of tsa14 cells at the nonpermissive temperature by mutants of E1A. To investigate which regions of the E1A molecule were able to functionally complement the growth defect due to the thermolabile SV40 LT Ag, various mutants of E1A (shown in Fig. 1b) were transfected into tsa14 cells; the plasmid pZipSV776 (denoted as TAg in Table 1 and Fig. 2), which is capable of expressing wild-type SV40 large and small tumor antigens, was used as a positive control. The recombinant E1A plasmids used in this study have been described previously by Schneider et al. (52). The mean results of three separate experiments are shown in Table 1 and Fig. 2. As shown previously, both the 243-residue E1A molecule (mutant JF12) as well as the SV40 tumor antigens complemented the growth defect (24). Additionally, the JN20 construct encoding the 289-residue 13S E1A molecule as well as the wild-type construct (pCE) encoding both E1A proteins also complemented. Together these results demonstrated that both the 12S and 13S E1A molecules were able to functionally substitute for the SV40 LT Ag and that the unique region of E1A was not essential for this activity.

Most of the nonconserved residues between CR1 and CR2 are deleted in the mutant GNC (Fig. 1b). These nonconserved residues were not necessary for rescue of the growth defect, as the GNC mutant rescued at an efficiency comparable with that of the wild-type E1A (Fig. 2).

The conserved regions CR1 and CR2 of E1A have been implicated in cellular immortalization as well as co-operation in cellular transformation. Additionally, CR1 and CR2 regions have also been strongly implicated in the ability of E1A proteins to bind RB1 (11, 64). In this colony assay, we have found that mutants carrying either deletions or substitutions (DCS, GCX, and G3/2) or point mutations (pM933 and pM957) within CR2 had little effect upon the ability to rescue

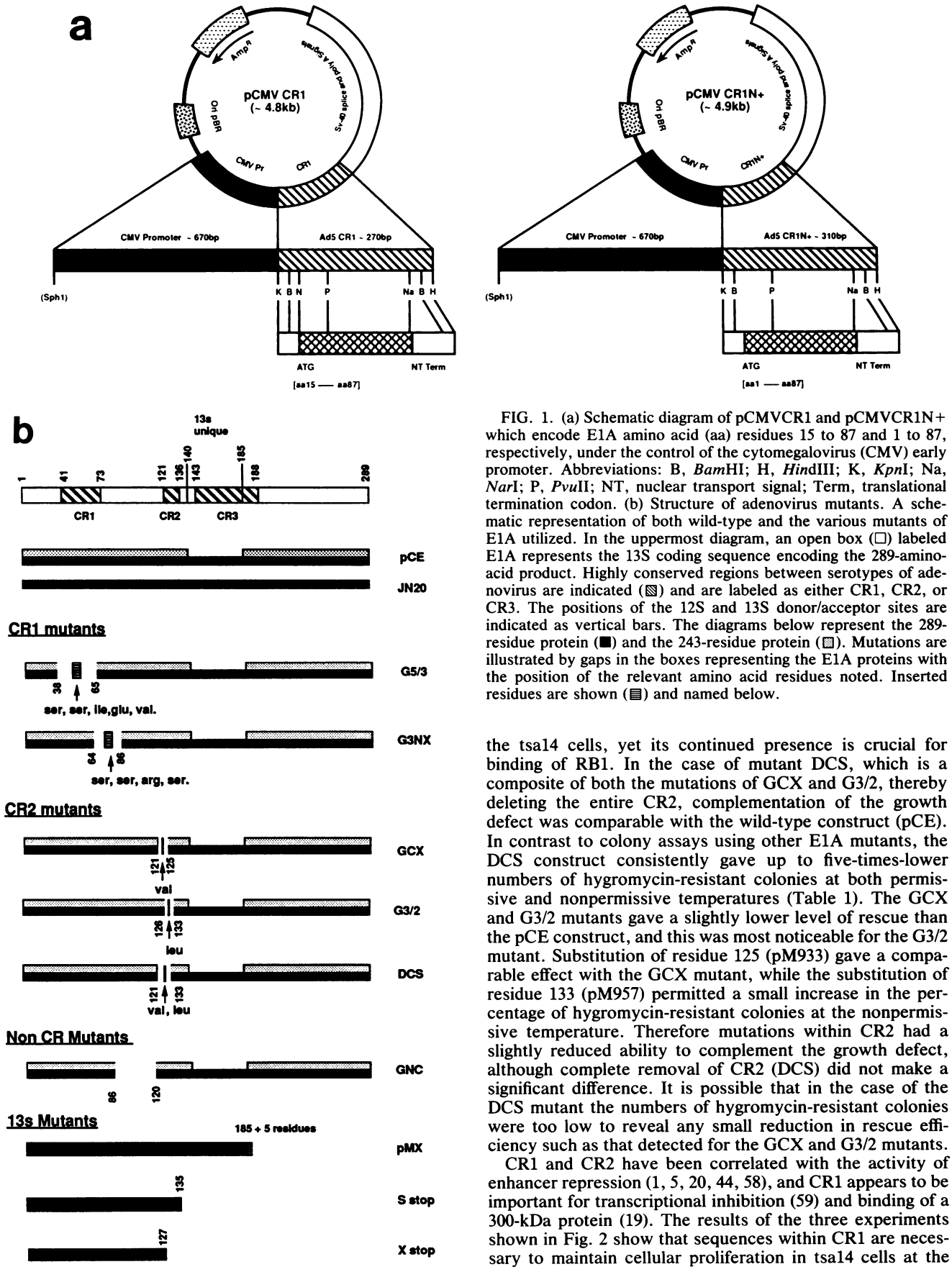

FIG. 1. (a) Schematic diagram of pCMVCR1 and pCMVCR1N+ which encode E1A amino acid (aa) residues 15 to 87 and 1 to 87, respectively, under the control of the cytomegalovirus (CMV) early promoter. Abbreviations: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; Na, *Nar*I; P, *Pvu*II; NT, nuclear transport signal; Term, translational termination codon. (b) Structure of adenovirus mutants. A schematic representation of both wild-type and the various mutants of E1A utilized. In the uppermost diagram, an open box (□) labeled E1A represents the 13S coding sequence encoding the 289-amino-acid product. Highly conserved regions between serotypes of adenovirus are indicated (▨) and are labeled as either CR1, CR2, or CR3. The positions of the 12S and 13S donor/acceptor sites are indicated as vertical bars. The diagrams below represent the 289-residue protein (■) and the 243-residue protein (▩). Mutations are illustrated by gaps in the boxes representing the E1A proteins with the position of the relevant amino acid residues noted. Inserted residues are shown (▤) and named below.

the tsa14 cells, yet its continued presence is crucial for binding of RB1. In the case of mutant DCS, which is a composite of both the mutations of GCX and G3/2, thereby deleting the entire CR2, complementation of the growth defect was comparable with the wild-type construct (pCE). In contrast to colony assays using other E1A mutants, the DCS construct consistently gave up to five-times-lower numbers of hygromycin-resistant colonies at both permissive and nonpermissive temperatures (Table 1). The GCX and G3/2 mutants gave a slightly lower level of rescue than the pCE construct, and this was most noticeable for the G3/2 mutant. Substitution of residue 125 (pM933) gave a comparable effect with the GCX mutant, while the substitution of residue 133 (pM957) permitted a small increase in the percentage of hygromycin-resistant colonies at the nonpermissive temperature. Therefore mutations within CR2 had a slightly reduced ability to complement the growth defect, although complete removal of CR2 (DCS) did not make a significant difference. It is possible that in the case of the DCS mutant the numbers of hygromycin-resistant colonies were too low to reveal any small reduction in rescue efficiency such as that detected for the GCX and G3/2 mutants.

CR1 and CR2 have been correlated with the activity of enhancer repression (1, 5, 20, 44, 58), and CR1 appears to be important for transcriptional inhibition (59) and binding of a 300-kDa protein (19). The results of the three experiments shown in Fig. 2 show that sequences within CR1 are necessary to maintain cellular proliferation in tsa14 cells at the

TABLE 1. Complementation of the growth effect[a]

Transfected DNA	Mean no. of colonies	
	33°C	39.5°C
pCE	310	272
JN20	271	222
JF12	247	215
G5/3	244	19
G3NX	207	8
GNC	380	327
GCX	308	216
G3/2	302	166
pM933	270	195
pM957	331	258
DCS	79	73
S stop	323	81
X stop	297	214
pMX	220	200
pUC7	301	1
TAg	332	299

[a] The indicated plasmids were transfected into tsa14 cells using hygromycin B as a coselectable marker, as described in Materials and Methods. Transfected tsa14 cells were cultured at either the permissive temperature of 33 or 39.5°C until colonies were clearly visible. At this stage, they were stained with 2% methylene blue in 50% ethanol and counted. Each value is the mean of three independent transfection experiments.

nonpermissive temperature. The removal of sequences in this region severely impaired the ability of E1A to rescue the growth defect of the tsa14 cells at 39.5°C. The percentages of rescued colonies were only 7 and 4%, respectively, for the mutants G5/3 and G3NX compared with the number of hygromycin-resistant colonies obtained at 33°C.

Carboxy-terminal deletions did not significantly affect the ability of E1A proteins to rescue the growth defect. An exception was the mutant bearing the deletion that extended into the CR2 region (X stop) where there was about a threefold reduction in its ability to rescue in comparison to wild-type E1A.

The colony formation assay showed that sequences within CR1 were necessary to complement the growth defect of tsa14 cells at the nonpermissive temperature. However, loss of carboxy-terminal sequences and deletions extending into CR2 (mutant X stop) also compromised the ability to rescue but to a much lesser extent.

Isolation of E1A transfected tsa14 cell lines. Hygromycin-resistant colonies of tsa14 cells were isolated and expanded into cell lines. In the case of E1A CR1 mutant constructs, G3NX and G5/3, colonies were isolated from dishes of cells that had been selected at both the permissive and nonpermissive temperatures. Viable cell lines containing the CR1 mutants were only obtained from colonies that had been selected at the permissive temperature. This showed that the CR1 mutant-transfected colonies that had been scored in the colony formation assay at 39.5°C were not viable. These colonies were morphologically distinct from those colonies at the permissive temperature in that they were composed of cells that displayed prominent stress fibers and were large and flattened, with large nuclei. This flattened morphology is reminiscent of both the parental tsa14 line at the nonpermissive temperature and of embryonic fibroblasts when they undergo senescence in vitro and become growth arrested (24; T. E. W. Riley et al., unpublished data). Ten colonies were isolated from both the G3NX and the G5/3 transfections and screened for the presence of E1A by immunoprecipitation with the PAbM73 anti-E1A monoclonal antibody (18). Four of ten colonies derived from transfection with G5/3 and six of ten colonies derived from transfection with G3NX were found to express E1A. Representative analysis is shown in Fig. 3.

Colonies from tsa14 cells transfected with the wild-type

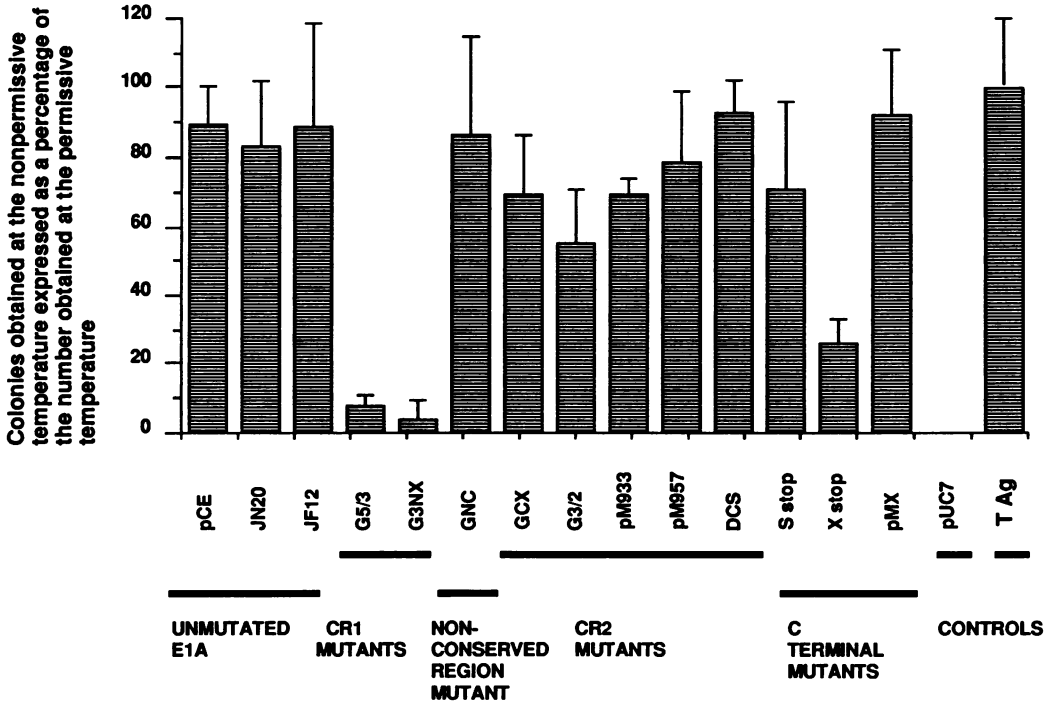

FIG. 2. Growth complementation using the colony formation assay. The results presented here correspond to the ratio of hygromycin-resistant colonies obtained at the nonpermissive temperature as a percentage of those obtained at the permissive temperature calculated from the data in Table 1.

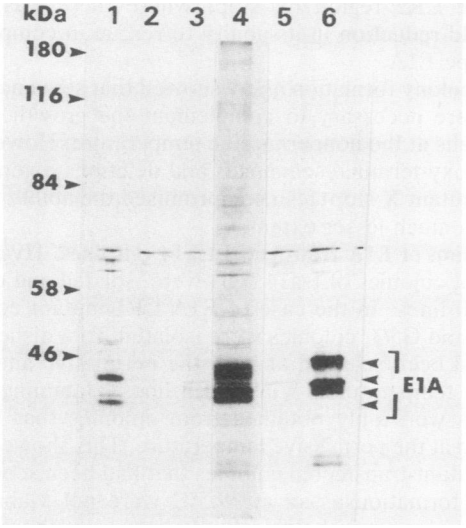

FIG. 3. Immunoprecipitation analysis of E1A proteins carrying CR1 deletions. Lanes: 1, tsa14×5/3.1; 2, tsa14×3/2.3 (non-E1A containing revertant cell line); 3, tsa14×G3NX.2; 4, 293 (E1A-positive control); 5, tsa14; 6, tsa14×pCE.3. Extracts were immunoprecipitated with the PAbM73 anti-E1A mouse monoclonal antibody (17). An equivalent amount of trichloroacetic acid-precipitable counts were immunoprecipitated from each extract. The positions of the wild-type 52-, 50-, 48.5-, and 45-kDa E1A proteins are shown as well as the region of the gel corresponding to the mutated E1A proteins of G5/3 and G3NX. Cell line tsa14×3/2.3 (lane 2) represents a E1A-negative hygromycin-resistant clone. Lane 4 shows a shorter exposure from the same gel.

E1A construct (pCE) and with the CR2 mutants GCX and G3/2 were isolated from cultures at the nonpermissive temperature and expanded into cell lines. Apart from one exception (cell line tsa14×3/2.3 [Fig. 3]), all pCE and CR2 mutant-transfected lines were positive for E1A expression. Cell lines containing pCE, GCX, and G3/2 all displayed a characteristic E1A morphology reminiscent of 293 cells in contrast to the tsa14 cells carrying E1A CR1 mutants which shared the fibroblastic morphology of the parent cell line tsa14 at the permissive temperature. The transformed morphology was not evident in cell line tsa14×3/2.3, which had undetectable levels of E1A (Fig. 3).

To ensure that the tsA58 LT Ag was still thermolabile in the cell lines expressing the various mutants of E1A, they were assayed by indirect immunofluorescence, using the anti-SV40 LT Ag monoclonal antibody PAb412. In all cell lines except tsa14×3/2.3, the tsA58 LT Ag was as thermolabile as in the parental cell line. In contrast, tsa14×3/2.3 cells were found to contain equivalent levels of LT Ag at both temperatures. Representative cell lines derived from transfection with the E1A construct pCE (cell line tsa14×pCE.3), mutant G5/3 (cell line tsa14×5/3.1), mutant G3NX (cell lines tsa14×G3NX.2 and tsa14×G3NX.3), mutant G3/2 (cell line tsa14×3/2.11), and mutant GCX (cell line tsa14×GCX.12) were then chosen for further characterization.

Analysis of the growth of tsa14×E1A cell lines. To further substantiate that cells transfected with E1A constructs with deletions within the CR1 region were unable to complement the growth defect at 39.5°C, colonies from cultures transfected at 33°C were isolated and expanded into cell lines. They were then assayed for growth at both the permissive and nonpermissive temperatures. A total of 10^3 cells were plated on 60-mm-diameter dishes and cultured over a period of 8 days. The cell numbers obtained over this period were compared with those for the parent cell line tsa14, a cell line derived by rescuing tsa14 cells with wild-type SV40 LT Ag (tsa14×SV40TAg) and SV4, a cell line isolated by immortalizing rat embryo fibroblasts with wild-type SV40 LT Ag (24). Cell lines containing wild-type E1A (pCE) and mutations within the CR2 of E1A were also assayed.

As shown previously, we observed a significant difference in the growth potential of tsa14 cells between the permissive and nonpermissive temperatures. There was a sixfold reduction in cell number at 39.5°C over that at 33°C (Fig. 4). In contrast, the SV4 cell line exhibited a fivefold increase in cell number at 39.5°C, indicating that this cell line not only continues proliferating but actually divides faster at the higher temperature. The wild-type SV40 LT rescued line (tsa14×SV40TAg) also proliferates at 39.5°C but not as rapidly as SV4.

The inability of tsa14 cells to grow at the nonpermissive temperature is not overcome in cell lines tsa14×G3NX.3, tsa14×G3NX.2, and tsa14×5/3.1 that contain E1A molecules bearing deletions within the CR1. In accordance with the colony formation assay, these cell lines showed a very limited growth potential at the nonpermissive temperature; none of them gave net growth over the 8-day time period. The tsa14×5/3.1 cell line grew more slowly at 33°C compared with the parental cell line, but the difference between the permissive temperature and the nonpermissive temperature was still approximately 15-fold.

The inability to grow at 39.5°C was overcome in those cell lines that carried the wild-type E1A construct (tsa14×pCE.3) or E1A constructs with deletions in CR2 (tsa14×GCX12 and tsa14×3/2.11 [Fig. 4]). The growth of the tsa14×pCE.3 cell line at the permissive temperature was comparable with that of the parental cell line. After shift up to the higher temperature, a slightly increased growth rate was observed which verified the ability of E1A to complement the temperature-dependent growth defect of this cell line. Even though the E1A-rescued cell lines are not as prolific as SV4 at 39.5°C, they clearly continue to divide just like the wild-type SV40 LT Ag rescued line and do not undergo the morphological changes characteristic of senescent cells. Similarly, cell lines containing E1A with deletions in CR2 also grow at the nonpermissive temperature at a slightly increased rate over that at the permissive temperature. This growth assay therefore substantiated the colony formation assay which showed that mutations within CR2 produced little or no effect upon growth complementation, which however requires sequences within CR1.

E1A proteins with deletions within CR2 do not coprecipitate a 105-kDa polypeptide. Mutants of E1A carrying deletions of CR2 were assayed for their ability to coprecipitate polypeptides implicated in the transforming activity of E1A. Whole-cell lysates of the cell lines tsa14×pCE.1, tsa14×GCX.12, and tsa14×3/2.11 were immunprecipitated with the PAbM73 anti-E1A monoclonal antibody (18). The results in Fig. 5 show that the wild-type E1A coprecipitated polypeptides of 105 kDa (a doublet) and approximately 300 kDa in the rat-derived tsa14 cells.The association of a 105- and 300-kDa polypeptide with E1A has been well documented (19, 65). The 105-kDa doublet band has been shown to consist of both 105- and 107-kDa polypeptides (11, 64). It has also been demonstrated to be the product of the RB1 gene and requires CR2 of E1A for complex formation (11, 64).

Immunoprecipitation of extracts prepared from the E1A CR2 mutant cell lines show that while the 300-kDa polypep-

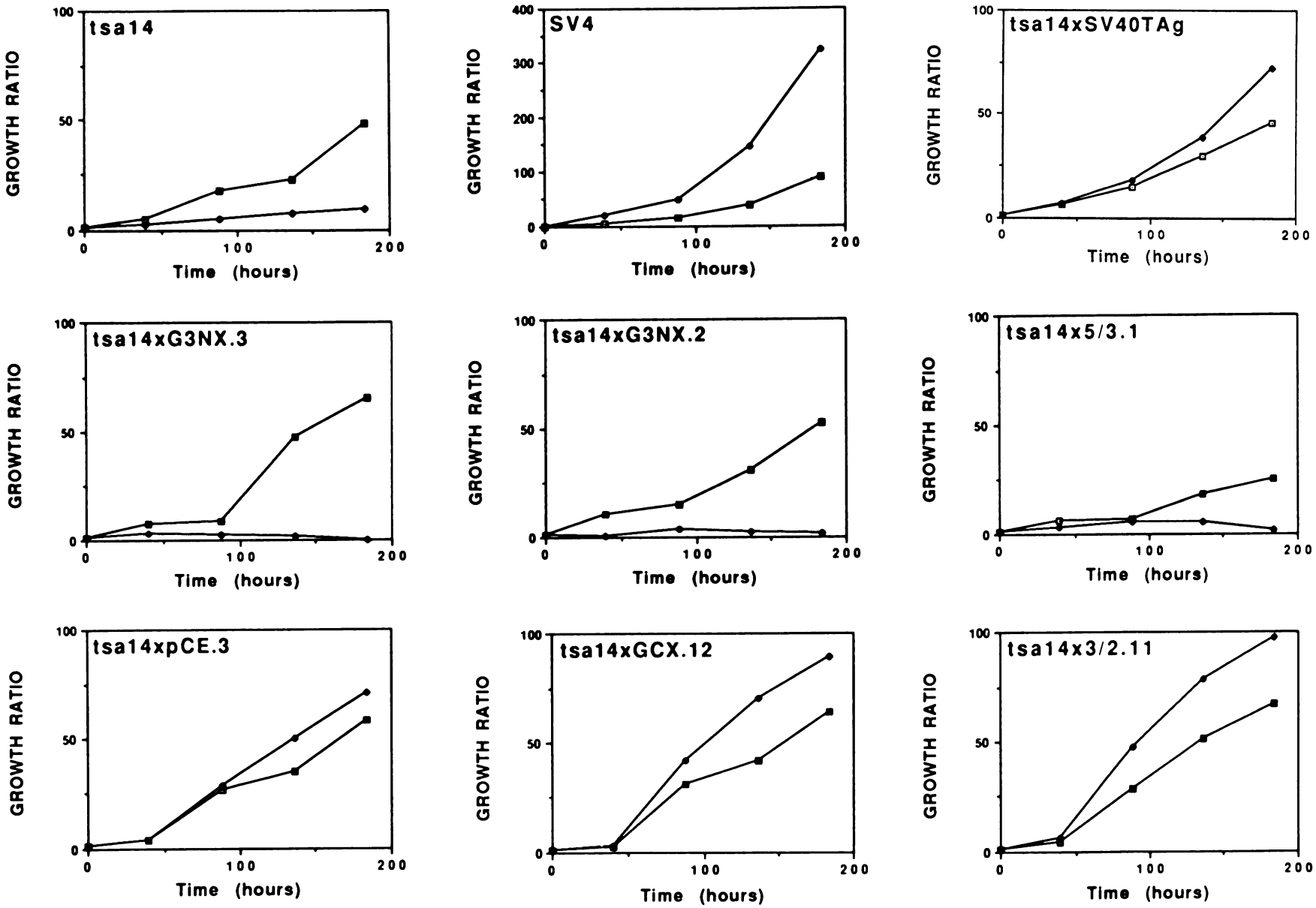

FIG. 4. Quantitative cell growth analysis. The indicated cell lines were analyzed for their growth capability when cultured at either 33 (□) or 39.5°C (◆). A total of 10^3 cells were plated on 60-mm-diameter dishes in triplicate and cultured for no less than 172 h. Growth ratio corresponds to the ratio of the number of cells at the time of sampling to the number of cells plated.

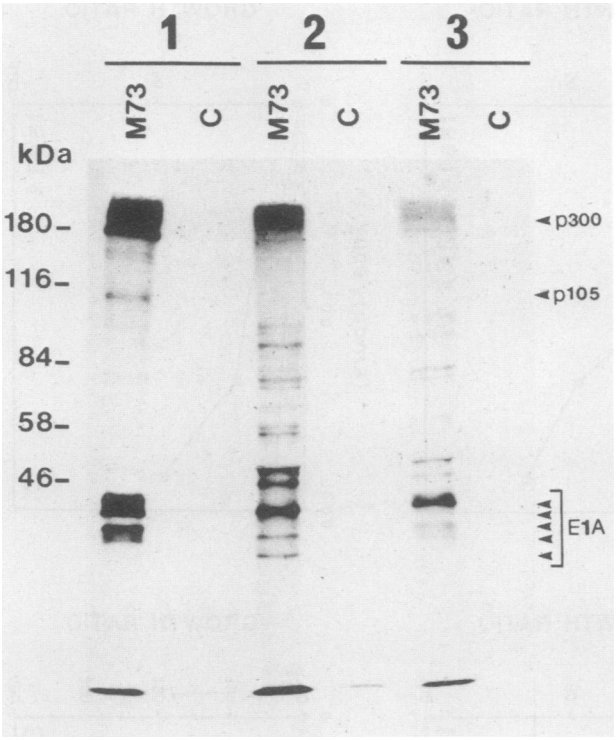

FIG. 5. Immunoprecipitation analysis for coprecipitating polypeptides in the E1A proteins containing CR2 deletions. Lanes 1, 2, and 3 correspond to extracts from cell lines tsa14×pCE.3, tsa14×GCX.12, and tsa14×3/2.11, respectively. Extracts were either immunoprecipitated with the PAbM73 anti-E1A monoclonal antibody (17) or with a control monoclonal antibody F4/pA5 (45).

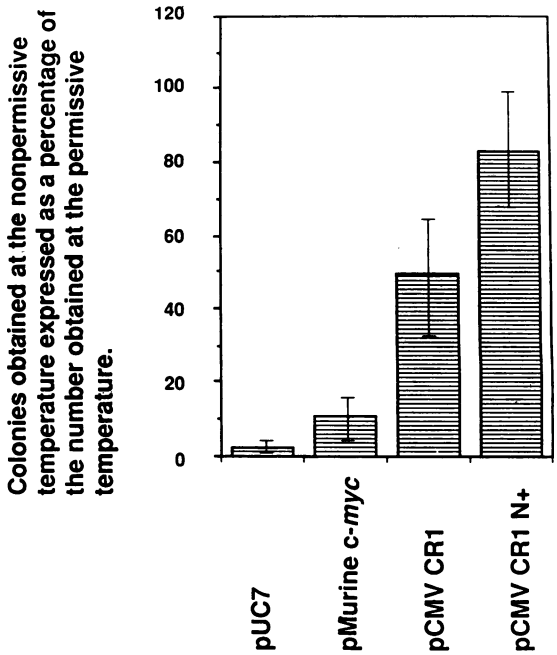

FIG. 6. Growth complementation of *tsa*14 cells using the cytomegalovirus CR1 constructs in comparison with pUC7 negative control and pMurine c-*myc*. Values represented here are the mean of three independent transfection experiments.

tide was precipitated, the 105-kDa polypeptides were not detectable. In NRK cells, the mutant G3/2 has similarly been shown not to complex the 105-kDa polypeptide (59).

In addition to the two documented E1A-associated polypeptides, we also observed a band that migrated in front of the 300-kDa band and which was peculiar to the wild-type E1A in this analysis. This band was not observed in the normal serum controls shown in Fig. 5, even though it had previously been observed in normal serum controls for other immunoprecipitation experiments, as well as in immunoprecipitation analyses of tsa14 cells that did not contain E1A. This suggested that this protein was not associated with E1A but was an artifact of the procedure. In accordance with our observations, van Dam et al. (59) have observed a similar sized band as an artifact in control antibody immunoprecipitations.

Recombinants encoding the CR1 of E1A are able to complement tsa14 cells at the nonpermissive temperature. The colony formation assay and the growth analysis of the tsa14 cells containing E1A molecules with mutations in CR1 provided direct evidence that sequences within CR1 of E1A were necessary to maintain cellular proliferation in tsa14 cells at the nonpermissive temperature. To fully explore this hypothesis, we determined whether constructs bearing the CR1 region alone were able to provide complementation of the growth defect.

By colony formation, we assayed two CR1-containing expression constructs that were driven off a cytomegalovirus promoter and which differed only in whether or not they possessed the amino-terminal sequences of E1A (pCMVCR1N+ and pCMVCR1, respectively, in Fig. 1a). The percentage rescue of tsa14 cells at the nonpermissive temperature by pCMVCR1 and pCMVCR1N+ was compared with that of an expression construct of murine c-*myc* (pBS.cM1.smaRC.SVo) and a control plasmid (pUC7) (Fig. 6). The pMurine c-*myc* (pBS.cM1.smaRC.SVo) contains the wild-type genomic murine c-*myc* 6.5-kb *Sma*I-to-*Kpn*I fragment including 422 bases of 5′ sequence. In rat fibroblasts, this construct has previously been shown to express full-length transcripts derived from both P1 and P2 promoters (T. E. W. Riley, Ph.D. thesis, Cambridge University, Cambridge, 1987). In accordance with previous data, the murine c-*myc* was unable to complement the tsa14 cells at 39.5°C, as there were only 12% hygromycin-resistant colonies at the higher temperature (24, 62). Five clones were isolated to determine whether they were viable; none of them expanded. These cells were able to exclude trypan blue and exhibited the large flattened morphology characteristic of growth-arrested tsa14 cells.

In support of the results obtained from the CR1 deletion mutants, the two constructs containing the CR1 of E1A both showed partial complementation of the growth defect. Recombinant pCMVCR1 had only a limited ability to maintain cellular proliferation, whereas pCMVCR1N+ was able to achieve a rescue value of 80%. This suggests that in addition to CR1, sequences from within the amino-terminal portion of E1A are also probably required for maintaining cellular proliferation.

Colonies isolated from transfection with either CMVCR1 recombinant were unable to proliferate extensively at 39.5°C. This may have been due to an inherent instability of the oligopeptides derived from the constructs or due to an inability of cells expressing these constructs to grow under conditions of limiting dilution. The colonies isolated from the pCMVCR1N+ transfections were able to undergo a few rounds of division in contrast to the pCMVCR1 transfectants

which showed very limited proliferative potential. Nevertheless, the effect of rescue with the pCMVCR1N+ construct was pronounced compared with pMurine c-*myc* and pUC7 control transfections, supporting the earlier results that there is a requirement for CR1 sequences and also for certain amino-terminal sequences.

DISCUSSION

We have assayed various adenovirus mutants to define regions of the E1A protein that are necessary for complementation of the temperature-sensitive growth defect of the cell line tsa14. This cell line was derived by immortalization of rat embryo fibroblasts with the thermolabile tsA58 LT Ag and proliferates indefinitely at the permissive temperature but rapidly growth arrests upon shift up to the nonpermissive temperature in either the G_1 or G_2 phase of the cell cycle. Previously it has been shown that the loss of growth potential can be readily complemented by the exogeneous introduction of wild-type SV40 LT Ag, adenovirus E1A 12S, and HPV16 E7 (24, 62). These observations suggested to us that this temperature-sensitive assay system would provide an excellent strategy for defining those regions of E1A necessary for complementation of the growth defect. We have demonstrated that complementation activity maps to amino-terminal and CR1 sequences but surprisingly not to CR2 sequences. These results raise the intriguing possibility that while the loss of RB function may be involved in initiation of immortalization, maintenance of the immortalized phenotype is mediated via an additional activity which is dependent upon CR1 and amino-terminal sequences.

Consistent with earlier findings, the 12S and 13S E1A molecules expressed either singularly (constructs JF12 and JN20, respectively) or together (construct pCE) provided full complementation. Furthermore, the deletion of sequences that are not conserved between the various adenovirus serotypes from residues 86 to 120 did not compromise the ability of E1A to rescue tsa14 cells at the nonpermissive temperature (31, 60). The fact that the "13S unique region" and the nonconserved sequences between CR1 and CR2 were not required for complementation of the SV40 LT Ag is in accord with the findings of Schneider et al. (52), who showed that the GNC mutant which lacks the nonconserved sequences still retained the properties of enhancer repression, establishment, co-operativity with Ha-*ras*, and transactivation and that transactivation was dependent upon the 13S unique region.

We have shown that complementation of the growth defect was not significantly affected by the removal of exon 2 sequences since the mutant pMX was able to rescue. However further deletion of the carboxy terminus to residue 135 (mutant S stop) and to residue 127 (mutant X stop) caused a reduction in the complementation activity. The respective reduction in rescue for the mutants S stop and X stop was 20 and 60% that for mutant pMX. Hence while the removal of carboxy-terminal sequences contributed to the reduced rescue, the additional deletion of sequences within CR2 appeared to have a more pronounced effect. Thus, there was either an intrinsic requirement for CR2 sequences in order to rescue the tsa14 cells or mutations within this region were effecting a conformational change that perturbed the remainder of the protein. The increased effect of these deletions was reminiscent of the effects observed upon Ha-*ras* cooperativity (52).

Ha-*ras* cooperativity has a clear requirement for CR2 sequences (52) but in the complementation assay CR2 sequences were not essential for rescue of the growth defect. This was surprising as CR2 has been implicated as having a possible role in cellular proliferation on the basis of its ability to associate with the retinoblastoma susceptibility gene product RB1 (11, 63) and its necessity for immortalization (10, 34, 39, 41, 52, 65). Certainly the loss of the RB1 gene or an inability to synthesize its product has been correlated with cellular proliferation (14, 23, 32, 33) and in human cells infected or transformed by adenovirus or SV40, the complexing of RB1 by the viral transforming proteins is postulated to deprive the cell of a negative governor of cellular proliferation (8, 36, 63, 64). However, our results clearly show that CR2 mutants of E1A are able to maintain cellular proliferation in cells that would otherwise be growth arrested. Immunoprecipitation analysis confirmed that the CR2 mutants did not complex the 105- to 107-kDa polypeptides. The tsa14 complementation assay is therefore clearly different from BRK immortalization (52) wherein CR2 sequences are necessary for immortalization of primary cells. The activities of initiation of immortalization and maintenance of the immortalized phenotype are therefore distinguished by this assay.

Complexing of the RB1 protein may be important in allowing initiation of virus-mediated immortalization, but other interactions appear to be necessary for maintenance of immortalization. Clearly, our results show that any such putative interaction impinges upon CR1 sequences, which if deleted prevent E1A from rescuing the growth defect. It has been reported that in the case of the HPV16 E7 molecule certain mutations within the CR2 homologous region (CR2-hr) abrogate complementation of tsa14 cells at 39.5°C (6). HPV16 E7 possesses a region 5′ and contiguous to the CR2-hr that bears a limited sequence homology to the CR1 region of E1A (9) and which may have a similar role to the CR1 of E1A. Therefore the results of Vousden's group may be reconciled with our own by virtue of this juxtaposition of the CR1 homologous region (CR1-hr) to CR2-hr such that mutations in the latter may effect conformational changes elsewhere in the molecule. A similar phenomenon may have occurred in this report with respect to the X stop mutant. The requirement for CR1 was emphasized by the ability of the construct bearing only CR1 sequences to yield colonies at the nonpermissive temperature. The capability to rescue by the CR1 sequence alone could be further augmented by the presence of the amino-terminal sequences of E1A, thereby suggesting their requirement in complementation of the growth defect. Interestingly, the activity of maintenance of proliferation assayed here is consistent with previous studies that have shown the N-terminal sequences to be necessary for transformation (52, 53, 56). While both CR1 and CR2 appear necessary for the complexing of RB1, it has been shown that both the N-terminal region and CR1 of E1A are required for binding of the 300-kDa polypeptide. It is therefore possible that interaction of E1A with the 300-kDa polypeptide is necessary for complementation in the tsa14 cells. A specific role for the 300-kDa polypeptide has not yet been ascribed.

It is tempting to speculate whether the 300-kDa polypeptide mediates the activity of maintenance of proliferation in the conditionally immortalized tsa14 cells. Candidate functions for the 300-kDa protein include both transcriptional repression of growth factor-inducible genes (59) and most intriguingly the regulation of proliferating-cell nuclear antigen gene expression and DNA synthesis (56, 66) which also map to CR1 sequences. These latter two properties may underpin the characteristic G_1 and G_2 cell cycle arrest of

tsa14 cells that occurs at the nonpermissive temperature (24). The possible involvement of the 300-kDa polypeptide in the cell cycle control of tsa14 cells is currently under investigation.

ACKNOWLEDGMENTS

We are grateful to Ed Harlow, Elizabeth Moran, Phil Branton, and members of our laboratories for helpful discussions. We would also like to thank Dan Dumont for technical advice, Dominique Davidson for the F4/pA5 antibody, Robin Brown for pBS+hygro, and Paris Ataliotis for photography.

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