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Retinoblastoma Protein and Simian Virus 40-Dependent Immortalization of Human Fibroblasts

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Transformation and immortalization of human diploid fibroblasts by simian virus 40 (SV40) is at least a two-stage process, since transformants have a limited lifespan in culture. We have isolated immortalized derivatives (AR5 and HAL) from transformants generated with an origin-defective SV40 genome encoding a heat-labile large T protein (T antigen) and reported that both preimmortal and immortal transformants are continuously dependent on T antigen function for growth as determined by temperature shift experiments. In this study, we demonstrate complex formation between T antigen and the retinoblastoma susceptibility gene product (Rb) at 35°C and observed a reduction in complexes under conditions of loss of T antigen function and growth inhibition at 39°C. Viral oncogenes (polyomavirus large T protein and adenovirus E1A 12S protein) known to bind Rb were introduced into AR5 and HAL cells, both stably by gene transfer and transiently by virus vectors. Such double transformants are still unable to proliferate at 39°C, although complex formation with the newly introduced oncogenes was demonstrated. We suggest that T antigen interacts with other cellular processes in addition to Rb to transform and immortalize human cells in culture. Our finding that p53-T antigen complexes are also temperature dependent in AR5 and HAL cells could provide such an additional mechanism.

Human diploid fibroblasts, like cells from other species, show a limited lifespan in culture. Such cultures initially grow vigorously, followed by a period of decreasing proliferation, growth arrest, and finally, cell death (16). This phenomenon has been termed senescence. Introduction of the simian virus 40 (SV40) *A* gene, encoding the large T protein (T antigen), can overcome senescence and restore proliferation. In most cases, this effect is temporary, resulting only in an extension of lifespan (18, 34). In more limited cases a subpopulation of these SV40-transformed fibroblasts can proliferate indefinitely and become immortal (18, 34, 38). Since immortal cell lines arise rarely even in clonally derived cell populations which uniform express T antigen, we (38) and others (52) have proposed a two-step model for immortalization of human fibroblasts. T antigen is phenotypically involved in both steps, since fibroblasts transformed by an SV40 genome encoding a heat-labile T antigen (*ts*A58 transformants) are temperature dependent for growth both as preimmortal cells (i.e., during the period of extended lifespan) (35, 38) and as immortal cells (38). Wright and co-workers similarly found that immortalized human fibroblasts conditionally transformed by inducible T antigen cease to proliferate when T antigen expression is shut off (52).

Recent studies have demonstrated that SV40 transformation of rodent cells is dependent on interaction of T antigen with cellular proteins, most notably, the retinoblastoma susceptibility gene product (Rb-1) and p53 (25). Mutations in SV40 which decrease binding of T antigen with Rb-1 block transformation in both established (3) and primary (2, 46) rodent cells. Induction of colonies in primary mouse embryo fibroblasts at low cell density and their subsequent propagation ("immortalization") was, however, not affected (2, 46). Inactivation of the Rb-1 protein has been demonstrated to be important in both hereditary retinoblastoma and nonhereditary human tumors (49). A model has evolved which proposes that T antigen and gene products of other DNA tumor viruses, such as adenovirus (50) and human papillomaviruses (8), transform susceptible cells by inactivation of the cellular Rb-1.

We have examined the role of the interaction of T antigen with Rb-1 in temperature-dependent SV40-transformed human fibroblasts (SV*ts*A/HF) to clarify the role of T antigen in immortalization. One might predict that binding ("complex formation") would occur at 35°C (when the cells are proliferating and transformed) but not at 39°C, when the T antigen becomes inactivated. We have indeed observed that complex formation, although low at 35°C, is further reduced at 39°C. Introduction of other viral oncogenes generates formation of appropriate complexes with Rb-1 but does not restore growth at 39°C. These results are interpreted to indicate that T antigen must affect other cellular processes in addition to its interaction with Rb-1 to transform and immortalize human cells in culture.

MATERIALS AND METHODS

Cell lines. The isolation of SV*ts*A/HF-A, a human diploid fibroblast line (HS74) transformed by an origin-defective SV40 genome (*ts*A58) at 35°C, was described previously (38). Three immortalized derivatives have been established: an uncloned cell line established by continuous passage at 35°C (designated immortal SV*ts*A/HF-A), a colony isolated at early passage from SV*ts*A/HF-A (designated AR5), and a colony isolated from immortal SV*ts*A/HF-A at passage 40 (designated HAL). HAL was isolated in conventional medium supplemented with 5×10^{-5} M thioguanine after

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mutagenesis of the population with ethylmethane sulfonate (150 μg/ml); it is defective in the enzyme hypoxanthine phosphoribosyltransferase (HPRT) and unable to grow in hypoxanthine-aminopterin-thymidine (HAT) medium containing 5×10^{-5} M hypoxanthine, 5×10^{-6} M aminopterin, and 1×10^{-5} M thymidine. All SV*ts*A58-transformed cell lines were maintained in culture at 35°C in DF10 medium supplemented with 10% fetal bovine serum under conditions described previously (37). SV/HF-5/39, an immortal derivative of HS74 (33) transformed by an origin-defective SV40 genome, and adenovirus-transformed 293 cells (11) were maintained at 37°C. Cell number was determined with a Royco cell counter as reported previously (33).

Virus preparations. Mutant adenovirus stocks were obtained as crude lysates and propagated in 293 cells, which contain adenovirus sequences encoding E1A and E1B. They were titered for infectivity by an immunofluorescence assay for adenovirus DNA-binding protein in 293 cells as described previously (39). All these viruses are defective in E1A function and unable to express efficiently other viral genes, including the DNA-binding protein. Virus replication and viral protein synthesis detected by immunofluorescence therefore result from transactivation of the viral gene(s) by the functional E1A protein constitutively expressed in 293 cells.

A replication-defective amphotropic retrovirus vector expressing polyomavirus large T antigen (Py LT) was prepared in PA317 cells (32) in the following manner. The recombinant DNA pZIPNeoPyLT (LT4), provided by P. Jat (19), was transfected into PA317 cells by the calcium phosphate-DNA coprecipitation technique (12). (Py LT is expressed from the long terminal repeat of murine leukemia virus; resistance to neomycin is expressed from the SV40 promoter. An SV40 origin of DNA replication is also present.) Pooled and individual clones selected with the neomycin analog G418 were screened for expression of intact Py LT by the immunoblot technique. Virus was prepared from several producer clones and used to infect HAL cells at 35°C. Infected clones were isolated in medium containing G418 (150 μg/ml) at 35°C and screened for Py LT by immunoblot. One HAL/Py-LT clone, HC1-1, was chosen for detailed study.

DNA preparations. Plasmid DNA was prepared as described previously (22) by standard methods (31). pRNS-1 contains an origin-defective SV40 genome expressing a wild-type T antigen and resistance to neomycin from the long terminal repeat of Rous sarcoma virus (28). The recombinant DNA pAd12S-hprt was constructed in the following manner. The human cDNA encoding HPRT in the Okayama-Berg-derived pCD1 vector (36) was provided by S. Weissman. The 2.5-kb *Aat*II-*Nde*I fragment was cloned directly into the corresponding sites of a plasmid containing adenovirus sequences encoding the 12S E1A gene previously cloned into the *Eco*RI and *Sst*I sites of the polylinker of pUC18, kindly provided by E. Moran (33). The final 6.3-kb construct was verified to be intact by restriction enzyme analysis (40a) and transformation of rat embryo fibroblasts (33a). E1A is expressed from the endogenous E1A promoter; HPRT is expressed from an SV40 promoter. An SV40 origin of DNA replication is also present.

Immunoassays. Cell extracts were prepared from 100-mm dishes which had been seeded at 35°C. In a typical experiment, 1×10^6 to 2×10^6 cells were inoculated and either maintained at 35°C or shifted to 39°C for 3 to 4 days. Extracts were typically prepared directly on the dish at 4°C with 0.5 to 1 ml of lysis solution containing 120 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), and a mixture of protease inhibitors, including phenylmethylsulfonyl fluoride (0.15 mM), leupeptin (0.3 mg/ml), α_2-macroglobulin (0.1 mg/ml), and aprotinin (0.1 TIU). The disrupted cells were harvested by scraping with a rubber policeman and centrifuged in a microfuge for 15 min at 4°C. The supernatant was routinely used directly for further analysis.

Extracts containing approximately 1 to 2 mg of protein were immunoprecipitated by the addition of the appropriate monoclonal antibody and Sepharose-conjugated protein A (38). In the case of Rb-1, an additional incubation with rabbit anti-mouse immunoglobulin G (IgG) was included. The immunoprecipitate was washed five times in buffer containing 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), and the protease inhibitor mixture and dissolved by heating in a boiling-water bath (100°C, 1 min) in 50 μl of buffer containing 6% sodium dodecyl sulfate (SDS), 250 mM NaCl, 2.5 M mercaptoethanol, 20% glycerol, and bromphenol blue. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as previously described (34). Viral and cellular proteins were detected by appropriate monoclonal antibodies and affinity-purified goat anti-mouse IgG conjugated with either horseradish peroxidase or alkaline phosphatase (Boehringer-Mannheim), used according to specifications provided by the supplier. In several cases, the same nitrocellulose filter was assayed for individual proteins by sequential reactions. Extracts were analyzed by use of the following monoclonal antibodies: SV40 T antigen was assayed by PAb 419 (13), Py LT was assayed by F4 (37), adenovirus E1A was assayed by M73 (14), Rb-1 was assayed by C36 (50), and p53 was assayed by PAb 421 (13). PAb 210E8 against the *Escherichia coli* RNA polymerase β subunit (42) was used as a negative control.

Measurement of DNA synthesis. Cells were inoculated at different cell densities (5×10^4 per 35-mm dish or 4×10^5 per 100-mm dish) at 35°C for 24 h prior to temperature shift and/or infection. At selected intervals, [^{3}H]thymidine ([^{3}H]TdR; New England Nuclear; specific activity, 70 Ci/mmol) was added to a final concentration of 10 μCi/ml in 1.5 μM thymidine (in DF10 medium) for 1 to 4 h. The medium was removed, and incorporation of radioactivity into trichloroacetic acid (TCA)-precipitatable material was determined as described previously (5).

Determination of protein. The protein concentration of extracts was determined by the Bradford (Bio-Rad) protein assay according to the microassay procedure (1).

Cell cycle analysis. Cells were harvested by trypsinization, centrifuged at low speed, and suspended in PBS (0.15 M NaCl, 0.01 M $Na_2HPO_4 \cdot NaH_2PO_4$ [pH 7.4]) at a concentration of 10^6 cells per ml. One milliliter was added to 9 ml of 90% cold ethanol and stored at 4°C until analysis. The fixed cells were then sedimented, washed once with and resuspended in PBS (at 10^6 cells per ml), and stained with propidium iodide (50 μg/ml) in the presence of Triton X-100 (1%, wt/vol) and RNase for 20 min at room temperature as described elsewhere (21). Cells were analyzed for DNA content with a System 50H flow cytometer (Ortho Diagnostics Institutes, Westwood, Mass.) interfaced to an Ortho 2150 data analysis system.

RESULTS

Immortalized SV*ts*A/HF-A and its cloned derivatives AR5 and HAL are temperature dependent for growth, as reported previously for AR5 (38) and shown in Fig. 4A for HAL cells. The doubling time at 35°C is typically 24 to 36 h; however, when cultures are shifted to 39°C, only one or no cell

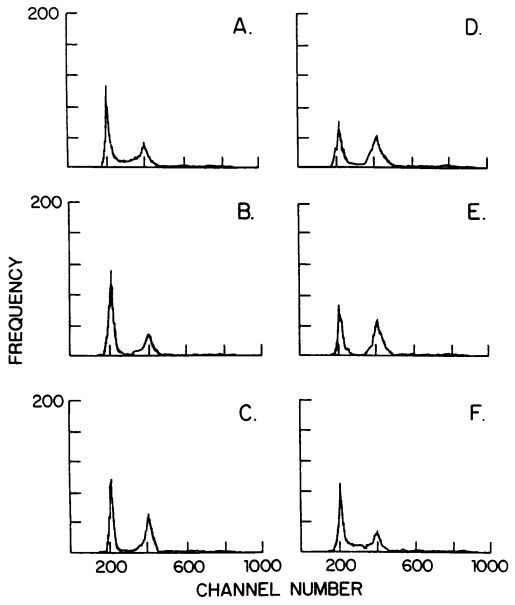

FIG. 1. Cell cycle analysis of SV*ts*A/HF-A. Dishes (100 mm) were seeded with immortal SV*ts*A/HF-A cells (passage 24) and incubated at 35°C until cells were verified to be in logarithmic growth. On day 0, replicate cultures were shifted to 39°C. Cells were harvested at daily intervals and analyzed by flow cytometry as described in Materials and Methods. (A) day 0, 35°C; (B) day 1, 39°C; (C) day 2, 39°C; (D) day 3, 39°C; (E) day 4, 39°C; (F) day 4, 35°C. Channel number on the ordinate represents relative DNA content per cell.

doubling occurs. There is a decrease in DNA synthesis at 39°C. Cell cycle analysis by flow cytometry showed a depletion in the number of cells in S phase (from 26.6% to less than 15.8%) and a relative accumulation of cells in G2 (from 22 to 44%) by 4 days at 39°C (Fig. 1). Similarly, there is a decline in incorporation of [^{3}H]TdR as shown in Fig. 7B. The cells support replication of a plasmid containing an SV40 origin at 35°C but not at 39°C (38). The cells undergo a morphological change following shift to 39°C, assuming a flattened appearance, and remain attached to the dish surface for several days despite gradual loss of viability (data not shown).

We had previously reported that introduction into AR5 of an SV40 genome encoding a wild-type T antigen restored cell growth at 39°C, supporting the interpretation that the inability of these cell lines to proliferate at 39°C was due solely to the inactivation of T antigen function at the elevated temperature. We therefore sought to determine whether complex formation between T antigen and the cellular protein Rb-1 correlated with T function. Preliminary studies involving pulse labeling of cells with [^{35}S]methionine at 35°C, immunoprecipitation of extracts, and PAGE were inconclusive, so we shifted our emphasis to immunoblots. As shown in the immunoblot in Fig. 2, developed with antibodies to T antigen and Rb-1, immunoprecipitation of extracts of HAL cells with the respective monoclonal antibody resulted in precipitation of the appropriate polypeptide (T antigen in lane 1, Rb-1 in lane 2); neither protein was observed with the control antibody. Immunoprecipitation with monoclonal antibody (PAb C36) to Rb-1 resulted in coprecipitation of T antigen at 35°C (lane 2). This represented a small percentage of the intracellular T antigen and was estimated at ≤5% form

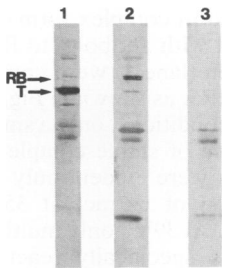

FIG. 2. Rb-T complexes in SV*ts*A58-transformed human cells. Extracts were prepared from SV*ts*A58-transformed HAL cells cultured at 35°C and analyzed for Rb-T complexes by immunoblot as described in Materials and Methods. Immunoprecipitation was performed with PAb 419 to T antigen (lane 1), PAb C36 to Rb-1 (lane 2), and control antibody to *E. coli* RNA polymerase (lane 3). The immunoblot was initially reacted with antibody to T antigen and horseradish peroxidase-conjugated antiglobulin and subsequently with antibody to Rb-1 and alkaline phosphatase-conjugated antiglobulin. Markers are indicated for Rb and T antigen (T).

repeated experiments. A fraction of the Rb-1 also appeared to be coprecipitated with antibody (PAb 419) to T antigen.

To further assess this low level of T-Rb complexes and their temperature dependence, we compared the behavior of AR5 cells with that of a temperature-independent SV40 transformant, as shown in Fig. 3. Extracts prepared from SV/HF-5/39, the same normal human fibroblasts transformed by a non-temperature-sensitive SV40 genome which encodes a truncated T antigen (34) and grows well at both 35 and 39°C, demonstrated complexes at both temperatures, as shown in Fig. 3B. Consistent with data obtained for other nonhuman SV40 transformants (29), T antigen preferentially bound to a faster-moving (unphosphorylated) Rb-1 polypeptide (lanes 2 and 5). However, somewhat unexpectedly, when a fivefold-greater amount of the immunoprecipitate was analyzed (lanes 3 and 6), a second (presumably phosphorylated) Rb-1 polypeptide was readily detected. Furthermore, most, if not all, of the unphosphorylated Rb-1 poly-

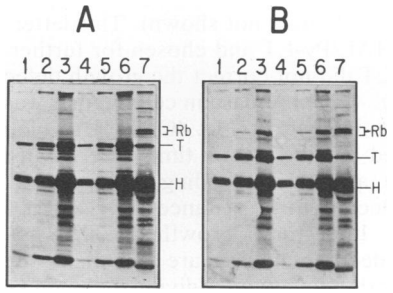

FIG. 3. Rb-T complexes in temperature-sensitive and temperature-independent human cell lines. SV*ts*A58-transformed AR5 cells (A) and SV40-transformed SV/HF-5/39 cells (B) were cultured for 3 days at 35°C (lanes 1, 2, 3, and 7) or 39°C (lanes 4, 5, and 6). Extracts containing equal amounts of protein were immunoprecipitated in all cases, but different amounts of the dissolved precipitate (in 50 μl) were analyzed. Immunoprecipitation was performed with PAb 419 to T antigen (lanes 1 to 6) and PAb C36 to Rb-1 (lane 7). The immunoblot was reacted with both PAb 419 and PAb C36 and developed with alkaline phosphatase-conjugated antiglobulin. Lane 1, 2 μl; lane 2, 8 μl; lane 3, 40 μl; lane 4, 2 μl; lane 5, 8 μl; lane 6, 40 μl; lane 7, 50 μl. Markers are indicated for Rb, T antigen (T), and IgG heavy chains (H).

peptide was involved in complex formation when the same extracts precipitated with antibody to Rb-1 (lane 7) or with antibody to T antigen (lane 3) were compared.

The results with AR5, as shown in Fig. 3A, were strikingly different. Under all conditions, only a small proportion of the Rb-1 was in the form of stable complexes with T antigen. Discrete Rb-1 bands were evident only in the lane with the highest concentration of extract at 35°C (lane 3), and a doublet was evident. At 39°C, only multiple faint bands were seen, and no bands specifically reactive with C36 were observed. (The apparent coprecipitation of T antigen in lane 7 is misleading. In an effort to maximize the likelihood of detection of complexes in the AR5 extracts, the precipitates were not vigorously washed [in contrast to those in other experiments]. Consequently an unusually high level of T antigen was associated with the Sepharose beads. Equivalent amounts of T antigen but no Rb-1 were also seen in extracts incubated with a nonreactive control antibody [data not shown].) These results confirm and extend the prior data with HAL cells. T-Rb complexes were indeed demonstrable in a temperature-dependent manner in the cell lines expressing a heat-labile T function. However, it should be pointed out that variability was noted among different experiments with the temperature-sensitive cell lines and other atypical features were also present.

Introduction of Py LT into HAL cells. In an effort to obtain functional evidence for a role of Rb-1 binding in these temperature-dependent cell lines, we attempted to introduce alternative gene products which could bind Rb-1. As a first choice, we elected to use Py LT, since it is reported to bind Rb-1 at least in cell extracts (6) but does not have many of the other properties of SV40 T antigen (e.g., binding to p53) and might be expected to have limited additional effects on human cells.

We elected to generate a retrovirus suitable for use with human cells by packaging an available construct (pZIP-neoPyLT), which is known to express Py LT, in the amphotropic helper cell line PA317. Multiple colonies were obtained and five individual producer colonies were analyzed; all were positive for Py LT (1 to 1,000 infectious units per ml). All were used to generate G418-resistant HAL colonies at 35°C; three yielded cell lines positive for Py LT by immunoblot, with one of them (HC1-1) producing solely full-length Py LT (data not shown). This latter cell line was designated HAL/Py-LT and chosen for further study.

HAL/Py-LT did not correct the growth defect at 39°C, as shown in Fig. 4. No increase in cell number was observed at 39°C over a 6-day period (Fig. 4C). A HAL clone (HAL/SV-WT) isolated at the same time after transfection with pRNS-1 (28), a plasmid encoding a wild-type T antigen, and similarly selected for resistance to G418 is included for comparison. It restored growth at 39°C as at 35°C, as expected; indeed, the curves are essentially superimposable (Fig. 4B). Both pooled and individual cloned G418-resistant HAL cell lines generated at 35°C with Py LT retrovirus from another producer clone similarly failed to restore growth at 39°C (data not shown).

HAL/Py-LT does, however, express a Py LT which binds Rb-1, as shown in Fig. 5. When extracts prepared at 35°C or after a shift to 39°C for 4 days were immunoprecipitated with the anti-Rb antibody C36, a prominent band was observed (lanes 3 and 4) at approximately 100 kDa when the immunoblot was developed with the monoclonal antibody F4, directed against an immunodeterminant in the amino terminus shared by Py LT and the other polyomavirus T proteins. This band corresponded in position with immunoaffinity-purified Py LT prepared from 293 cells (provided by K. Lawlor), which is active in viral DNA synthesis in vitro (4), as shown in lane 7. Conversely, immunoprecipitates prepared from HAL/Py-LT with control antibodies (e.g., the anti-E1A antibody M73) did not contain this band, as shown in lanes 5 and 6. Immunoprecipitation of extracts from HAL cells with the antibody C36 similarly did not result in a band reactive with the antibody to Py LT (data not shown). Southern analysis demonstrated the presence of the expected 4.2-kb *Bam*HI fragment of polyomavirus DNA in HAL/Py-LT, further supporting the conclusion that authentic full-length Py LT was being detected in the immunoblots (data not shown).

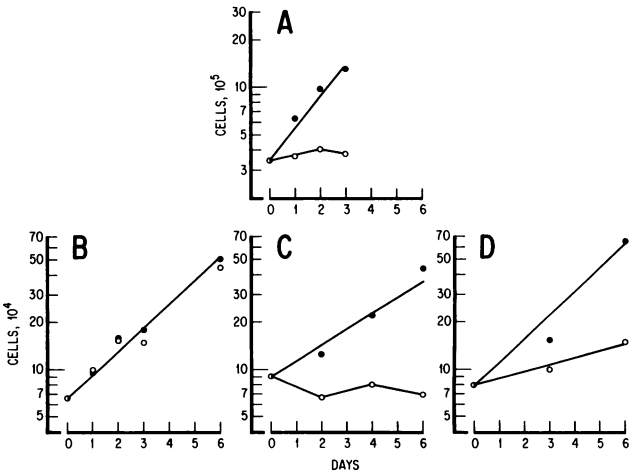

FIG. 4. Growth curves of HAL-derived cell lines. From 5×10^4 to 1×10^5 cells were seeded per 60-mm dish at 35°C and subsequently shifted to 39°C or maintained at 35°C for determination of cell number. Day 0 corresponds to the time at which cultures were shifted to 39°C. (A) HAL cells; (B) HAL/SV-WT; (C) HAL/Py-LT; (D) HAL/Ad 12S. Symbols: ●, 35°C; ○, 39°C.

Introduction of adenovirus 12S E1A into HAL and AR5 cells. Using a strategy similar to that described in the preceding section, we generated HAL cell lines expressing the adenovirus 12S E1A by transfection of HAL cells with calcium phosphate-DNA coprecipitates containing 5 μg of the plasmid pAd12S-hprt per dish. The plasmid construction is described in Materials and Methods. Transfectants were isolated at 35°C in HAT medium by the expression of the *hprt* cDNA in the plasmid. Ten discrete colonies and one

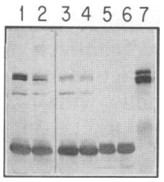

FIG. 5. Rb-Py LT complexes in retrovirus-infected HAL cells. HAL/Py-LT cells were cultured for 4 days at 35°C (lanes 1, 3, and 5) and 39°C (lanes 2, 4, and 6) and analyzed after immunoprecipitation with PAb C36 to Rb-1 (lanes 1 to 4) or control PAb M73 to adenovirus E1A (lanes 5 and 6). Lane 7 contains 200 ng of immunoaffinity-purified Py LT. The immunoblot was separated and reacted with either PAb C36 (lanes 1 and 2) or monoclonal antibody F4 to Py LT (lanes 3 to 7), followed by alkaline phosphatase-conjugated antiglobulin.

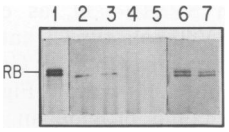

FIG. 6. Rb-E1A complexes in transfected HAL cells. 293 cells (lane 1) or HAL/Ad12S-1 cells cultured for 3 days at 35°C (lanes 2, 4, and 6) or 39°C (lanes 3, 5, and 7) were analyzed after immunoprecipitation with different monoclonal antibodies. The composite immunoblot was reacted with antibody to Rb-1, followed by alkaline phosphatase-conjugated antiglobulin. Lane 1, extract from 293 cells immunoprecipitated with M73 antibody to E1A; lanes 2 and 3, extract immunoprecipitated with M73 antibody to E1A; lanes 4 and 5, extract immunoprecipitated with control antibody; lane 6, residual supernatant from immunoprecipitation in lane 2 reprecipitated with antibody to Rb-1; lane 7, residual supernatant from immunoprecipitation in lane 3 reprecipitated with antibody to Rb-1.

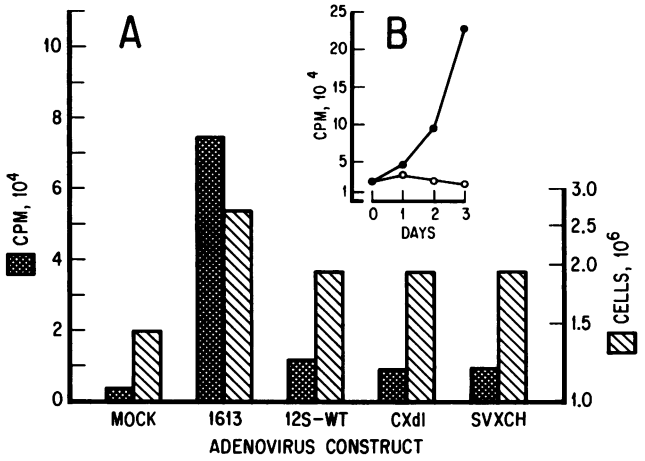

FIG. 7. (A) Virus-infected AR5 cells. Cells (4×10^5) were plated into 100-mm dishes at 35°C, mock-infected or infected with virus at an estimated multiplicity of infection of 5 to 10 at 35°C for 1 h, and shifted to 39°C for 3 days, at which time the cultures were incubated with [^{3}H]TdR for 2 h. TCA-precipitable counts were determined as described in Materials and Methods. Replicate cultures were used for determination of cell number. Virus preparations were Ad/SV1613, Ad/12S-WT, Ad/CXdl, and Ad/SVXCH. (B) Uninfected AR5 cells. Cells were plated as in panel A, shifted to 39°C (at time zero) or maintained at 35°C, and incubated with [^{3}H]TdR for 3 h at daily intervals for TCA-precipitable counts. Symbols: ●, 35°C; ○, 39°C. The two experiments in panels A and B were performed separately.

culture containing multiple pooled colonies were analyzed for E1A polypeptides by immunoblot with M73 antibody; four clones were positive (data not shown).

None of the clones were able to restore growth to HAL cells at 39°C, as shown for one of them (HAL/Ad 12S-1) in Fig. 4D. However, extracts of HAL/Ad 12S-1 immunoprecipitated with antibody (M73) to adenovirus E1A resulted in the coprecipitation of Rb-1, as demonstrated in the immunoblot shown in Fig. 6. Similar levels were evident from cells grown at 35°C or shifted to 39°C for 3 days, but most Rb-1 appeared not to be complexed with E1A at either temperature (compare lanes 2 and 3 with lanes 6 and 7). These results raised the possibility that the failure to restore cell growth at 39°C was due to persistently elevated free unphosphorylated Rb-1 resulting from insufficient E1A. We therefore considered an alternative strategy for testing adenovirus 12S sequences.

Deletion mutants of adenovirus E1A (e.g., *dl*312) replicate poorly and have reduced cytopathic effects when human diploid cells are infected at a low multiplicity of infection (e.g., 1 to 10 PFU per cell) but not high virus input (50 to 100 PFU per cell) (43). Moran (33) has prepared a series of adenovirus mutants in which either wild-type 12S sequences or 12S mutant sequences were substituted for the intact E1A region. With such mutants, it might be possible to develop a transient infection assay compatible with limited viral gene expression and transient cell growth, even though the SV40-transformed human fibroblasts are expected to be permissive for wild-type adenovirus replication. Studies were performed initially with both AR5 and HAL cells; however, the results were more encouraging with AR5, and it was thus chosen for further experimentation.

Since we have previously found that wild-type T antigen restored growth of AR5 cells at 39°C, we elected to initially use an adenovirus type 5 (Ad5) recombinant virus, Ad/SV1613, constructed by Van Doren and Gluzman (47). In brief, the early region encoding Ad5 E1A and E1B had been deleted (bp 455 to 3330) and an origin-defective SV40 genome encoding a wild-type SV40 early region had been introduced. This virus efficiently transforms the human diploid fibroblast line HS74 (47), which is the parent line of AR5. After a series of preliminary experiments, the following protocol was established. AR5 cells were seeded at 35°C. On the next day, they were infected with Ad/SV1613, refed, and shifted to 39°C or maintained at 35°C for 3 to 4 days. Alternatively, cultures were reincubated at 35°C for 24 h after infection prior to being shifted to 39°C. Over the course of such an experiment, there was a severalfold increase in DNA synthesis in the infected cells at 39°C, in marked contrast to inhibition of DNA synthesis in uninfected cells shifted to 39°C. Indeed, the level of [^{3}H]TdR incorporation in Ad/SV1613-infected cells approached that obtained in uninfected cultures maintained at 35°C, indicating an increase not only in DNA synthesis but in cell division as well.

We therefore assessed the ability of Ad/12S-E1A to similarly restore DNA synthesis and cell growth, as shown in Fig. 7A. All samples were pulse labeled with [^{3}H]TdR on the third day after the shift to 39°C. Whereas Ad/SV1613 resulted in a severalfold stimulation in DNA synthesis and at least a twofold increase in cell number, Ad/12S-WT had only a minimal effect, a twofold increase in [^{3}H]TdR incorporation and a 50% increase in cell number. Moreover, this effect did not correlate with the expected behavior in Rb-1 binding by E1A, since a deletion mutant (Ad/E1A-CXdl), in which domain 2 has been removed, which markedly reduces Rb-1 binding by E1A, behaved similarly to wild-type 12S. Another construct (Ad/E1A-SVXCH) in which the Rb-1 binding domain from SV40 has been substituted for domain 2 in 12S E1A, also showed no beneficial effect. The absence of growth-stimulatory effect by the adenovirus mutants did not appear to be due to viral cytopathic effect, since parallel cultures maintained at 35°C after infection showed only 50% or less inhibition of DNA synthesis compared with uninfected cultures and similar results were observed over several concentrations of virus. As would be expected, increasing the virus multiplicity 5- to 10-fold did result in complete cytopathic effect.

Immunoblot analysis of AR5 cells infected with the Ad/12S-WT virus demonstrated E1A-Rb complexes at 39°C, as shown in Fig. 8. Extracts prepared from infected cells were immunoprecipitated with antibody to E1A and analyzed for

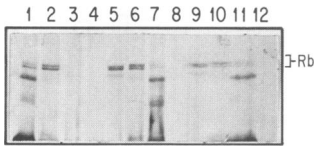

FIG. 8. Rb-E1A complexes in virus-infected AR5. Cells (2×10^6) were seeded in 100-mm dishes and infected as described in the legend to Fig. 7; mock-infected (lanes 1 to 4), Ad/12S-WT (lanes 5 to 8), and Ad/SVXCH (lanes 9 to 12). Extracts were prepared after 3 days at 39°C and immunoprecipitated with monoclonal antibodies for T antigen (lanes 1, 7, and 11), Rb-1 (lanes 2, 6, and 10), E1A (lanes 3, 5, and 9), or control antibody for RNA polymerase (lanes 4, 8, and 12). Immunoblots were developed with antibody to Rb-1 and alkaline phosphatase-conjugated antiglobulin.

the presence of Rb-1. As shown in lane 5, both forms of Rb-1 were detected, as expected. Moreover, the amount of Rb is similar to that immunoprecipitated by antibody to Rb-1 (compare lanes 5 and 6), suggesting that virtually all the Rb-1 is complexed to E1A under the conditions of the experiment. Similar results were obtained with cells infected with Ad/E1A-SVXCH (as also shown in Fig. 8); no Rb was detected in immunoprecipitates obtained from cells infected with Ad/E1A-CXdl (data not shown).

p53-T antigen complexes in HAL cells. SV40 large T antigen has been demonstrated to bind to cellular proteins in addition to Rb-1, namely, p53 (24, 27) and a series of polypeptides designated p120/p107 by different laboratories (7). Mutations in p53 have been associated with human tumors (9). We therefore assessed whether there was a defect in p53-T complexes in HAL and AR5 cells. As shown in Fig. 9A, complex formation was readily demonstrable in HAL cells at 35°C when extracts were immunoprecipitated with antibody to p53 and the immunoblots were analyzed with antibodies to p53 and T antigen. At 39°C, the level of p53 was reduced and no complexes were evident. The reduced level of p53 is consistent with a defect in intracellular complexes at 39°C, since wild-type p53 has a short half-life and complex formation with T has been reported to stabilize p53 (40). Similar results were obtained with AR5 cells (data not shown). Complex formation was not temperature dependent in SV/HF-5/39, as expected (Fig. 9B). Introduction of an SV40 wild-type T antigen into HAL cells (HAL/SV-WT) restored growth at 39°C (Fig. 4) and resulted in p53-T antigen complex formation (Fig. 9C). This defect in complexes at 39°C could provide an explanation for the failure of Py LT and adenovirus to restore growth at 39°C, since it is well established that these polypeptides do not interact with p53 (25).

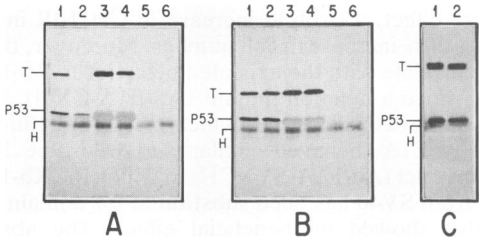

FIG. 9. p53-T complexes in human cell lines. SV*ts*A58-transformed HAL cells (A), SV40-transformed SV/HF-5/39 cells (B), and SV40-transformed HAL cells (HAL/SV-WT) (C) cultured for 3 days at 35°C (lanes 1, 3, and 5) or 39°C (lanes 2, 4, and 6) were analyzed after immunoprecipitation with different monoclonal antibodies as described in the legend to Fig. 2. Immunoprecipitation was performed with PAb 421 to p53 (lanes 1 and 2), PAb 419 to T antigen (lanes 3 and 4), and control antibody to *E. coli* RNA polymerase (lanes 5 and 6). The immunoblots were reacted with PAb 421 and PAb 419 and developed with alkaline phosphatase-conjugated antiglobulin. Immunoblots A and B were performed together and separately from C. Markers are indicated for T antigen (T), p53, and IgG heavy chains (H).

DISCUSSION

Multiple observations have emphasized the importance of the retinoblastoma susceptibility gene product Rb-1 in the regulation of cell proliferation of normal and tumor cells. Several diverse tumors in addition to hereditary retinoblastoma show mutations resulting in gene inactivation (49). Alteration in the state of phosphorylation has been found to correlate with entry of cells into S phase (30). Agents such as transforming growth factor β which inhibit cell proliferation also inhibit phosphorylation of Rb-1 (23). Stein and coworkers have recently reported that senescent human fibroblasts are defective in phosphorylation of Rb-1 (44). Since SV40 T antigen has been shown by several criteria to interact with Rb-1 and phenotypically mimic inactivation of Rb-1, similar to that proposed for phosphorylation, it would appear that the role of T antigen in transformation and immortalization of human fibroblasts could be explained by its effects on Rb-1.

Immunoprecipitation and immunoblots were used to demonstrate that T antigen formed stable complexes with Rb-1 in SV40-transformed human fibroblasts (SV/HF-5/39) as in other systems. Much lower levels of complexes were demonstrable with fibroblasts transformed by an SV40 genome encoding a mutant *ts*A58 T antigen at the temperature permissive for T function. The reduced level was not due to dissociation of complexes in the immunoprecipitates during the wash procedure (data not shown). However, additional complexes could also be present intracellularly but more labile to extraction. Indeed, *ts*A58 T antigen has been observed to form more labile complexes with cellular proteins (e.g., DNA polymerase α) at the permissive temperature in transformed rodent cells (10). Further evidence supporting the functional importance of Rb-T interactions in human cells was the reduced ability to detect complexes between Rb and *ts*A T antigen when extracts were prepared from cells incubated at 39°C for several days. Under these conditions, there was a decrease in cell DNA synthesis and a depletion of S-phase cells, consistent with the proposed models for the inhibitory effects of active Rb-1. The accumulation of AR5 cells in G2 phase would suggest an additional role for Rb-1 and/or other cellular proteins in entry into mitosis (30).

In view of the virtual absence of T-Rb complexes in AR5 or HAL cells at the restrictive temperature, we investigated whether restoration of Rb binding by other viral oncogenes would correct the growth defect. Two different oncogenes were evaluated, Py LT and adenovirus E1A. In both cases stable transformants were generated at 35°C and complexes were demonstrated at both 35 and 39°C. However, little or no effect on cell proliferation at 39°C was observed, in contrast to HAL cells into which a wild-type T antigen had been introduced. Although constructs were chosen to minimize the possible multiple effects of these viral oncogenes, prolonged expression could have diverse effects on cell proliferation. This possibility might particularly be a factor for HAL/Ad 12S. Indeed, we observed that the expression of

E1A polypeptides was unstable. The level of immunodetectable E1A was markedly reduced when HAL/12S-1 cells were recovered after frozen storage, and it decreased upon further passage.

One interpretation is that stable coexpression of T antigen and the adenovirus sequences is not compatible. For example, even though the 12S construct does not retain the transactivation function associated with E1A, it does encode other possible regulatory functions, including repression of the SV40 enhancer (48). Consequently, we used an alternative strategy to attempt to transiently correct the defect at 39°C by using adenovirus mutants with limited viral gene expression. However, the AR5 cells still did not grow at 39°C when E1A 12S was introduced, in contrast to the stimulation in DNA synthesis and cell proliferation observed when a wild-type T antigen (Ad/SV1613) was introduced. In this case as well, E1A-Rb complexes were demonstrable at 39°C. Interestingly, a slight stimulation of DNA synthesis was observed; however, this did not appear to be dependent on Rb-1 binding, since another adenovirus construct (Ad/CXdl) gave similar results even though it is defective in Rb binding and transformation (33). Recent data have demonstrated the importance to induction of cell DNA synthesis of the binding of E1A to a 300-kDa cellular protein; that function is dependent on domain 1 of E1A and is preserved in all three constructs examined (17, 45).

It would appear, therefore, that the effects of T antigen on transformation and immortalization cannot be explained solely by its interaction with unphosphorylated Rb-1. Several alternative explanations are possible. First, AR5 and HAL cells could have aberrant interactions with Rb-1 which induce abnormal growth effects. Supporting this model, T binding to other polypeptides besides unphosphorylated Rb-1 have been observed, as in Fig. 3. On the other hand, this observation is not peculiar to the SV*ts*A58-transformed cell lines (Fig. 3B), and introduction of a wild-type T antigen into AR5 or HAL cells restores cell growth at 39°C. It is also well known that adenovirus E1A binds phosphorylated as well as unphosphorylated Rb-1; the significance of this difference from T antigen is unknown at present. Second, it is possible that binding of Py LT is not sufficient for functional inactivation of Rb-1 in a heterologous system. There are several examples of other systems in which complex formation is necessary but not sufficient for function, and no data are available on the effects of Py LT on Rb-1 in human cells. On the other hand, this possibility cannot apply to the 12S product of E1A, for which ample data have demonstrated a functional role in both rodent and human cells. Third, T is likely to require interaction with additional cellular proteins to exert its full effect on cell proliferation. Incubation of the SV*ts*A58-transformed cells at 39°C would result in loss of more than just the effect of T on Rb function. Even though we restored this effect of T, the other interactions might not have been restored by Py LT or E1A. A likely candidate would be the cellular polypeptide p53, which has antiproliferative properties similar to (but distinct from) those reported for Rb-1, including inactivation through mutation in human tumors (9). Indeed, we have found that T-p53 complexes can be readily detected in AR5 and HAL cells at 35°C but not at 39°C. It must, however, be borne in mind that E1A has been shown to bind multiple other cellular polypeptides (15, 53), and it is possible that T antigen does as well, so the final explanation might be more complicated. For example, Jat and coworkers have described an immortalized rodent cell line, *ts*a14, obtained by transformation of tertiary rat embryo fibroblasts with a *ts*A58 SV40 large T antigen, which shows temperature-dependent growth. Introduction of Py LT did not restore growth at 39°C, but 12S E1A did (20), in contrast to our results. Further analysis of E1A sequences have indicated that domain 1 (CR1) rather than domain 2, containing the Rb-1 binding site, was critical (41). These results support the finding that Rb-1 binding is not the determinant in immortalization of rodent cells (2, 46). However, the results also suggest that mechanisms involving p53-T complex (25) or p107-T complex (51) formation are unlikely to be critical to restoring functions initially provided by T antigen in *ts*a14. It must be noted that the specific relationship between immortalization of rodent and human cells remains unclear (26, 34).

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