E1A Represses Wild-Type and F9-Selected Polyomavirus DNA Replication by a Mechanism Not Requiring Depression of Large Tumor Antigen Transcription

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Polyomavirus (Py) DNA replication may be regulated to a low-level replication state in specific target cells in mice as well as in certain undifferentiated murine cell lines, such as embryocarcinoma (EC) cells. To investigate possible mechanisms by which such control may occur, we have examined the effects of E1A on Py DNA replication. Adenovirus E1A proteins repress transcriptional activation of various enhancers, including those of Py, and can stimulate DNA replication in quiescent cells, but E1A effects on Py DNA replication were unknown. We found that constitutive E1A expression in NIH 3T3 cells depressed Py DNA replication very strongly. Two F9 EC cell-selected Py enhancer variants, PyF441 and PyF101, were also examined because undifferentiated EC cells are hypothesized to have an E1A-like activity responsible for the Py restriction, and these variants activate Py DNA replication in *cis* in undifferentiated F9 cells. Both variants were repressed by E1A, indicating that E1A activity in 3T3 cells is not equivalent to undifferentiated F9 cell E1A-like activity. We also examined transient inducible E1A expression in cells supplying Py large tumor antigen (T-Ag). Py DNA replication was again repressed, and the inhibition increased with E1A induction. Analysis of T-Ag mRNA levels indicated that E1A repression of Py DNA replication was not an indirect result of depression of T-Ag transcription. This suggests that E1A may repress Py DNA replication by a more direct mechanism, possibly by blocking enhancer activation of DNA replication in a manner uncoupled with enhancer transcriptional control.

Simian virus 40 (SV40) and polyomavirus (Py) DNA replication are valuable model systems for eukaryotic cellular-DNA replication. They are believed to replicate their circular histone-covered DNA (often referred to as minichromosomes) in a semiconservative bidirectional fashion, with complete dependence on cellular replication proteins plus one viral protein, the large tumor antigen (T-Ag) (14). One major way in which they appear to differ from the mitotic cellular mode of once-per-cell-cycle replication is that these viral origins are runaway replicons which can reinitiate replication repeatedly in one cell cycle, producing thousands of genomic copies per cell (49). The *in vitro* replication systems for SV40 and Py DNA have also been optimized to reinitiate DNA synthesis (for reviews, see references 33 and 57 and references therein). In some circumstances, however, Py replicates in a much slower, more controlled fashion. Following primary Py infection of mice, an episomal persistent-infection state generally occurs in which viral replicons are maintained at low copy levels for months or years in what is presumably a highly regulated replication state (20, 21). How such low-level viral DNA replication is regulated, however, is not known. Similar episomal persistent Py infections can be established in undifferentiated embryonal carcinoma cells (11, 22, 38), but here also little is known of the mechanisms by which such states are achieved.

Most well-studied model systems for DNA replication, including that of virtually all eukaryotic DNA viruses, show that eukaryotic replication origins are associated in *cis* with transcriptional control elements (9, 13). With Py, there appears to be an absolute requirement for an enhancer to activate DNA replication in *cis* (18). Thus it seems likely that

enhancer activation of the Py replication origin may be important in regulation of replication states. Enhancer *cis* regulation of DNA replication is also involved in the tissue-specific replication of Py in many relatively undifferentiated cell types such as undifferentiated embryocarcinoma (EC), neuroblastoma, or Friend leukemic cells (12, 22, 32, 40, 53). Although Py enhancers may restrict both replication and transcription, the enhancer activation of replication works only in *cis*, and cell-specific Py replication is therefore not *trans* activated by the elevated transcription of T-Ag (7, 16, 17, 51).

Enhancer changes that confer *cis*-dependent replication activation have been most studied in undifferentiated EC cells, which are reported to have an E1A-like cellular activity which disappears following differentiation (30). The multifunctional adenovirus E1A proteins are active in transformation and transactivation but also can repress transcription of specific sets of viral and cellular genes (2, 3, 30, 42). This repression is reported to act by inhibiting enhancer activity, both cellular and viral, including that of wild-type Py (4, 50, 56, 59). Because the wild-type Py enhancer's activity is also repressed in undifferentiated F9 EC cells, it is plausible that the E1A-like activity of these cells is responsible for the restriction of wild-type Py, including both early transcription suppression and the repression of wild-type Py DNA replication in *cis*. If so, Py enhancer variants selected in undifferentiated EC cells should also be resistant to E1A repression. The transcriptional portions of this prediction have been tested by two groups. Hen and colleagues examined transcription from a Py enhancer- β -globin reporter construct in a mouse L-cell line constitutively expressing low levels of adenovirus type 2 (Ad2) E1A gene products. They found that the wild-type Py enhancer was repressed about fivefold in the E1A-expressing line but that a point-mutated F9-selected enhancer (from PyECF9.1) was not

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repressed by E1A. These facts were interpreted as evidence that enhancers active in undifferentiated F9 cells are also resistant to E1A repression effects, which is consistent with the view that a cellular repressor in undifferentiated F9 cells blocks the Py enhancer and that this repressor has the same target sequences as the E1A proteins (27). In contrast, Velcich and colleagues found quite different results when they used human HeLa cells to examine E1A effects on transcriptional activity of the Py early and late promoters driven by the Py enhancer. Ad5 (or Ad12) E1A products were transiently expressed by cotransfection of E1A-expressing plasmids, and two EC-cell-selected enhancers (one from PCC4 cells and one from F9 cells) were tested. They found that Py transcription was similarly repressed by E1A with either the wild-type or mutant enhancers (61). The apparent discrepancy between these results may have various explanations, such as different levels of E1A expression, transient versus constitutive E1A expression, the quantities of transfection input reporter plasmid DNA (reported by Hen et al. [27] to be critical for transcriptional repression in their assay system), the different F9-selected variants tested by the two groups, or perhaps the different types and species of cells used. Neither group, however, examined the effects of E1A on Py replication.

Because replication is *cis* activated by Py enhancer variants in undifferentiated F9 cells, we decided to examine E1A effects on DNA replication of both wild-type and F9-selected Py. This required using a permissive mouse cell line, NIH 3T3, which also avoids some potential cross-species complications. We examined E1A effects from both a constitutively E1A-expressing cell line and from transient expression and at various levels of E1A. We report here that E1A represses but does not eliminate Py DNA replication even in the presence of T-Ag mRNA, thus mimicking the restriction on wild-type Py replication seen in many relatively undifferentiated cell types, including F9 EC cells. However, F9-selected enhancer variants are not significantly resistant to this replication repression, indicating that E1A expression in 3T3 cells does not create conditions equivalent to the undifferentiated F9 EC-cell environment with respect to DNA replication. These results imply a role for E1A in the control of DNA replication, such as suppression of runaway DNA replication.

MATERIALS AND METHODS

Cell lines. The 2G cell line is a derivative of murine 3T3 fibroblasts which constitutively expresses the Ad5 289-amino-acid gene product from an integrated retroviral vector. SVX cells are a matched control line derived from 3T3 cells by the integration of the plain retroviral vector, pZIP-NeoSV(X). Both lines were generously provided by B. Roberts and J. Susan (48). FOP cells are a derivative of murine FM3A mammary cells (generously provided by John Hassell, McGill University, Montreal, Canada). FOP cells were transformed with the Py early region and constitutively express Py T-antigens, allowing high-level replication of Py origin-containing constructs by supplying all the necessary T-Ag in *trans*. CMT-3 cells are a derivative of primate CV-1 cells (generously provided by Yakov Gluzman) and supply the SV40 T-Ag in *trans* from an inducible metallothionein promoter (25). They allow replication of plasmids with SV40 origins either to medium levels or to very high levels when induced with 1 μ M CdSO₄ and 100 μ M ZnCl₂. All cells were grown in Dulbecco modified Eagle medium supplemented

with 5% fetal bovine serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml.

Viral DNA and plasmids. The Py A2 strain was used as the wild type. The PyF441 and PyF101 strains were obtained from Frank Fujimura (22). The Py F441/A2 control strain was constructed with the PyF441 (A3-derived) noncoding region (*Bcl*I to *Bgl*II) placed in the Py A2 coding region (*Bgl*II to *Bcl*II) as described previously (7). Py dID is a deletion of the *Pvu*II d fragment (B enhancer) as described previously (7). For the assays in FOP and CMT-3 cells, Ad2 E1A products were expressed from a cotransfected plasmid, pMSG(sma)E1A(532-A4), which was generously provided by A. Senear (Oncogene, Seattle, Wash.). It was made by inserting the Ad2 fragment from *Hae*III (nucleotide 498) to *Hha*I (nucleotide 1680) into the *Sma*I site of the polylinker of expression vector pMSG from Pharmacia, Inc. This allows controllable levels of expression of E1A products from the inducible mouse mammary tumor virus promoter. Induction was by addition of 100 μ M dexamethasone to the growth medium following transfection. The SV40 replicon used in the CMT-3 cell assays was constructed by placing a 366-bp fragment of SV40 extending from positions 5172 to 300 of the SV40 sequence (containing the origin, the 72-bp repeats, and the 21-bp repeats [6]) into the *Xho*I site of plasmid pIB131 (IBI, Inc.) to generate plasmid pSVOE31. This vector lacks the pBR322 "poison" sequences and replicates to very high levels in CMT-3 cells.

DNA transfections. Before transfections, the DNA corresponding to Py genomes was excised by restriction enzyme digest from cesium chloride-purified plasmid preparations, purified by gel electrophoresis, and recircularized with T4 DNA ligase, as described before (7). DNA was transfected by the DEAE-chloroquine method with slight modifications, as previously described (7, 39). Unless otherwise noted, 2 μ g of DNA was transfected per 100-mm tissue culture plate, with about 5×10^6 cells per plate.

Replication analysis. At 48 h posttransfection, low-molecular-weight DNA was isolated by Hirt extraction (28) and purified by standard methods as described previously (7). Ten-microgram quantities of DNA were digested with *Dpn*I and *Bcl*II to separate input methylated DNA from the newly replicated, unmethylated DNA. *Dpn*I (methylation requiring) digests only input DNA to smaller bands, and *Bcl*II (methylation sensitive) serves to linearize only newly replicated DNA, so that the absence of supercoiled and relaxed circular forms serves as an internal control for complete digestion by both enzymes. The DNA was then subjected to Southern analysis as described previously (7). Briefly, electrophoresis on 1% agarose-TBE (Tris-borate-EDTA) gels was followed by capillary transfer to nylon membranes. Hybridization was to randomly primed (1) ³²P-labeled Py DNA, and exposure was to XAR-5 X-ray film with Cronex Lightning-Plus intensifying screens. For the SV40 replicon, the probe was the plasmid pSVOE31 described above. Quantitation was by scanning exposures in the linear range with an LKB laser densitometer calibrated against the re-constructed copy number controls included on each Southern blot. Each replication assay was done a minimum of two times.

RNA analysis. Cytoplasmic RNAs were isolated at 48 h posttransfection by cell lysis with nonionic detergent in the presence of urea as described previously (8). All samples were treated with DNase prior to analysis with Northern (RNA) gels by standard methods (1). Briefly, for Northern analysis, 20- μ g samples were electrophoresed on 1.2% agarose-formaldehyde gels, transferred to nylon membranes,

FIG. 1. Py enhancer-origin region of variant genomes tested for E1A sensitivity. Restriction sites are shown as landmarks, and the alpha and beta replication elements defined by Muller et al. (43) are shown within the A and B enhancers. The locations of the PyF441 point mutation and the PyF101 54-bp tandem duplication (including this mutation) are also indicated. The precise construction of Py dLD was described previously (7). All numbering corresponds to that of GenBank A2 sequence file, release 63.

fixed by UV irradiation, and hybridized to ^{32}P -labeled randomly primed probes specific for Py T-Ag mRNAs. The probe used was the *Hpa*II restriction fragment of Py extending from nucleotide 1490 to 2617.

RESULTS

Because EC cells select for specific enhancer changes in the context of the whole regulatory region, we first examined Py DNA replication in its natural genomic context rather than with a hybrid plasmid replicon. To examine Py replication, a permissive cell line such as murine 3T3 was required. E1A expression was either from a constitutively expressing cell line or by transient expression from a cotransfected plasmid. For constitutive expression, we used E1A-expressing and control 3T3 cell lines which were made and characterized by Bryan Roberts et al. (48). The SVX control line contains only the integrated retrovirus vector pZIP-NeoSV(X) and differs in no apparent way from the parental 3T3 cells. The 2G cells express the Ad5 13S product constitutively (from an integrated pZIP vector), are morphologically altered (more cuboidal), and are presumed to differ from the parental 3T3 cells only in aspects determined by direct or indirect consequences of their E1A expression (48). It was therefore likely that any changes in replication seen in these permissive Py host cells would be due to E1A expression. Following transfections, Hirt extracts were prepared and extensively digested by *Dpn*I (to digest methylated input DNA) and *Bcl*II (to linearize only the unmethylated DNA and thus act as a control), and the newly replicated DNA was visualized by Southern analysis.

Py and F9 EC-cell-selected variant replication with or without E1A. Py strain A2 was used as our wild type (Fig. 1), and genomic DNA replication was analyzed in the SVX (control 3T3) versus the 2G (E1A-expressing 3T3) cells. In repeated transfections, such as the one shown in Fig. 2, Py A2 replication was always much lower in the E1A-expressing 2G cells. Laser densitometry of exposures in the linear range from several transfections show that apparent repression of Py DNA replication was about 100- to 200-fold (see

FIG. 2. Py DNA replication with or without E1A. SVX cells are a control murine 3T3 line, and 2G cells are an E1A-expressing 3T3 line. Replication was assayed at 48 h posttransfection and is represented by the *Dpn*I-resistant, *Bcl*II-linearized, genome-sized bands. Reconstructed copy number controls are included.

also Table 1). Similar transfection efficiencies were presumed (consistent with densitometry of the *Dpn*I-digested input DNA bands, which was very similar for the two cell lines).

We also tested PyF441 and PyF101 (Fig. 1), two well-characterized F9 EC-cell-selected variants which activate both transcription and DNA replication in *cis* in undifferentiated F9 cells (23). The PyF441 phenotypic difference from the wild-type A3 strain is conferred by a single point change in the B enhancer (22). This is the same change studied by Hen et al. (27). PyF101 has a 54-bp tandem duplication including this change (Fig. 1) and is 5 to 10 times more active than PyF441 in undifferentiated F9 cells by a variety of assays (22, 23). As shown in Fig. 2 (and Table 1), PyF441, like wild-type Py, also replicated to much lower levels in the E1A-expressing cells. The F441/A2 control construct, which consists of the F441 noncoding region in an A2 strain coding-region background, was also tested. It behaved identically, showing that the A2 and A3 coding-region differences were not a significant factor in our assay.

When replication only within a given cell type in the same transfection was compared, wild-type Py was always seen to

TABLE 1. Viral DNA replication

Virus	DNA/plate (μg)	Copy no./cell		Repression ^a (fold)
		SVX cells	2G cells	
Py A2	2.0	8,500	50	170
	0.5	2,000	13	150
	0.1	300	3	100
PyF101	2.0	2,000	15	130
	0.5	400	8	50
	0.1	230	1	230
PyF441	2.0	1,600	20	80
Py dLD	2.0	600	13	45

^a Repression here is defined as the apparent relative replication decrease in 2G compared with SVX cells as determined by laser densitometry of *Dpn*I-resistant bands following Southern analysis. The values shown are the average of at least two separate experiments.

FIG. 3. Py A2 versus PyF101 replication with transfection dose response. The indicated quantities of genomic DNA were transfected per 100-mm dish, and replication was assayed as for Fig. 2. Both panels are from the same autoradiograph. Reconstructed copy number controls are included.

replicate better than the F9 variant, PyF441, even in the cells expressing E1A. This result resembles the transcriptional findings of Velcich and Ziff (62) in that an EC-cell-selected genome is apparently not resistant to E1A-mediated repression of replication.

Finally, the entire B enhancer (point mutated or otherwise altered in all F9-selected variants) was totally deleted in Py d1D (Fig. 1). This genome replicated about 10-fold less than wild-type Py in the control cells and was also lower in the E1A-expressing cells (Fig. 2 and quantitation in Table 1), showing that the B enhancer region, which has been suspected as being repressed in F9 cells, is not necessary for E1A repression of replication.

PyF101 versus PY A2 at various DNA transfection dosages. Because Hen reported that the differential transcriptional repression observed for the PyECF9.1 construct versus that of the wild type depended greatly on assaying at low levels of input-DNA transfection (27) (perhaps because of low E1A levels in their cell line), we decided to test PyF101 at various DNA input levels and compare it with Py A2. The results (shown in Fig. 3 and with the averages from several experiments quantitated in Table 1) show that within our experimental accuracy, there is no significant escape from E1A repression by PyF101 at any of the DNA input levels tested. Although in some experiments PyF101 replicated closer to wild-type levels in the E1A-expressing cells (2G) than in the control cells (SVX), at no time did the F101 mutation confer a replication advantage compared with Py A2 in the presence (or absence) of E1A (see Fig. 3, which represents the experiment with the highest levels of PyF101 replication we observed in the 2G cells). Within the same transfection, wild-type Py always replicated better than PyF101 at each transfection dosage in either cell type. Overall, at our standard transfection amount (2-µg of DNA per 100-mm plate), PyF101 replicated to levels in both cell types roughly equivalent to that of PyF441, and so they showed similar "repression levels" (defined here as replication in 2G compared with that in SVX cells), as did Py A2 (Table 1).

FIG. 4. DNA replication with T-Ags supplied *in trans*. The Py genomic DNA replicon and the SV40 replicon were transfected into the cells, and replication was assayed as described in the text. Variable levels of E1A were supplied by cotransfected inducible plasmids, as described in the text. Note that in panel B, replication of the SV40 replicon was assayed at either induced or uninduced T-Ag levels.

DNA replication with T-Ag supplied *in trans* and E1A transiently expressed to variable levels from an inducible plasmid. To this point we had no evidence whether the E1A repressive effect on DNA replication was direct (i.e., affecting in *cis* enhancer activation of replication) or indirect (i.e., mediated by lowered T-Ag levels via a transcriptional repression effect). We also wanted to check for E1A repression via cotransfected plasmid expression and to test whether variable levels of E1A would produce correspondingly variable levels of replication. To address these questions, we focused on Py replication in a cell which constitutively expresses Py T-Ag. We chose FOP cells, a derivative of murine FM3A cells that constitutively express Py T-Ag, because they are highly permissive for Py DNA replication. To express E1A we obtained a plasmid from A. Senear (Oncogene) which expresses the Ad2 E1A products under the mouse mammary tumor virus promoter control. This promoter is inducible with dexamethasone, allowing the level of E1A to be controlled by an approximately 5- to 10-fold induction with 100 µM dexamethasone, as determined by Western immunoblot analysis (53a). Transfection was with Py A2 DNA, either alone or with the E1A-expressing plasmid pMSG(sma)E1A(532-A4). The cells were incubated for 48 h following transfection either with or without dexamethasone added to the medium to induce higher levels of E1A expression. The results of this experiment are shown in Fig. 4A, with replication represented by the *DpnI*-resistant, *BclI*-linearized bands as before (except that smaller *DpnI*-cut input bands are not shown here). In repeated transfections we saw clear inducible repression of Py replication. Laser densitometry revealed that Py A2 replication was reduced about 6-fold at low E1A levels (uninduced plasmid) and about 30-fold at high E1A levels (with dexamethasone induction). As a control, Py replication with and without dexamethasone was assayed, and we saw no effect by dexamethasone on Py replication in this cell line. Dexamethasone changed Py replication levels only when the E1A expression plasmid was cotransfected (data not shown). These results show that transient E1A expression (as well as constitutive expression in a selected cell line) also effectively represses Py DNA replication. They also show that the level of replication can be regulated by

regulating the level of E1A expression. These results in cells constitutively expressing T-Ag tended to argue against a solely indirect mechanism of replication repression via the lowering T-Ag levels. To further clarify this point, particularly because it has been proposed that newly synthesized T-Ag is most active in DNA replication (14), it was necessary to analyze T-Ag mRNA levels under the conditions of this assay. This issue is dealt with below.

We also examined E1A effects on the closely related virus SV40 under similar assay conditions. This was particularly interesting for several reasons. First, while SV40 has an enhancer also shown to be transcriptionally repressed by E1A, it appears to lack the stringent dependence seen with Py for enhancer region activation of an adjacent replication origin when tested by transfection assays. If E1A directly represses DNA replication activation by the enhancer, then replication repression might not work well with SV40, although a T-Ag depletion mechanism would still be expected to function. Second, as mentioned above, SV40 has been shown to replicate very well in human cells expressing both adenovirus E1A and E1B proteins (26, 37), so it was intriguing to see what might happen in our assay system. To express SV40 T-Ag in *trans*, we used CMT-3 cells, which constitutively express either medium (uninduced) or very high levels of T-Ag when their integrated metallothionein promoter is induced. E1A expression was from the inducible plasmid described above, and we used an SV40 replicon completely dependent on the T-Ag supplied in *trans*. The plasmid, pSVOE31, includes an SV40 fragment (containing the 72-bp repeats [enhancer region], the 21-bp repeats, and the SV40 origin region) cloned into the vector pIBI31 and has been shown to replicate very well in both COS (data not shown) and CMT-3 cells. The experiment whose results are shown in Fig. 4B was very similar to that for Py but was performed at either uninduced (medium) or induced (high) SV40 T-Ag levels, and either zero or high E1A levels are shown here. The higher T-Ag levels boosted replication about 10- to 20-fold, as expected from previous results, but at either T-Ag level, replication was apparently totally unaffected by E1A, in sharp contrast to results with Py. This result may reflect a key difference in regulation of DNA replication between SV40 and Py, as will be discussed below.

T-Ag mRNA levels under conditions of E1A repression of Py DNA replication. To more closely examine possible mechanisms of repression of Py DNA replication in FOP cells, we decided to check T-Ag mRNA levels by Northern analysis. Because E1A is believed to exert control over T-Ag levels at the transcriptional level, it is likely that the mRNA levels represent the levels of newly synthesized T-Ag (which is thought to be most active in replication and therefore more relevant than total T-Ag). The transfections were performed exactly as described for Fig. 4. Total cytoplasmic RNA was isolated at 48 h posttransfection under conditions identical to those of the replication assays. In all cases, sharp unstreaked rRNA bands were seen with ethidium bromide staining. These bands serve as markers, indicate little degradation, and confirm that roughly equivalent levels of RNA were loaded (data not shown). (Note that lane 1 had 60 instead of 20 μ g of total RNA loaded to enhance sensitivity.) The three early tumor antigen mRNAs differ in size only slightly, are largely colinear, and were all detected by our probe, as shown in Fig. 5. Lanes 1 and 2 represent the constitutive T-Ag mRNA levels in FOP cells with or without E1A. We found (in repeated analyses) that the FOP cells were producing very low levels of T-Ag mRNA, requiring long exposures

FIG. 5. Py T-Ag mRNA levels in FOP cells. Cellular RNA was assayed for Py T-Ag mRNA by Northern analysis under replication assay conditions as for Fig. 4A. Twenty micrograms of total RNA was loaded per lane except for lane 1, which received 60 μ g. Lanes 1 and 2 show endogenous T-Ag mRNA levels from mock-transfected or E1A plasmid-transfected FOP cells. Lanes 3 through 5 correspond to the three lanes of Fig. 4A. rRNA band positions are shown as markers. The short exposure (above) was for 3 days with one intensifying screen, and the long exposure (below) was for 3 weeks with two screens. st, small tumor antigen; mt, middle tumor antigen; LT-Ag, T-Ag.

to visualize, yet these cells allow the replication of Py origin-containing plasmids (which can make no T-Ag) to very high levels, even with extremely minimized enhancer regions (52). We estimate by both Northern and dot blot analyses (data not shown) that the endogenous levels (with or without E1A plasmid transfection) are at least 10- to 20-fold lower than the levels seen following T-Ag coding plasmid transfection. The fact that our transfection frequency with FOP cells has averaged about 10%, as determined by *in situ* hybridization (data not shown), implies that Py-transfected FOP cells have perhaps 100-fold-greater T-Ag mRNA levels and that therefore the large majority of the T-Ag mRNA in lanes 3 to 5 comes from the Py-transfected fraction of the cells.

Lanes 3, 4, and 5 in Fig. 5 show the levels of T-Ag mRNA in the same three Py replication situations as were used for Fig. 4A. With low E1A (uninduced) expression, the levels of all three Py tumor antigen mRNAs were not repressed but instead were apparently increased two- to threefold in several cases, as estimated by dot blot analysis (data not shown). (This apparent stimulation was unexpected but reproducible, and so it seems that very low levels of E1A may be able to stimulate, while higher levels repress, T-Ag transcription in FOP cells in a previously unreported manner.) Thus the repression of Py DNA replication in this situation cannot be explained by decreased production of T-Ag mRNA. Note also that the levels of the three mRNAs (small tumor antigen, middle tumor antigen, and T-Ag) appear to be regulated coordinately. The level of T-Ag mRNA in Py with high E1A (dexamethasone induced) was reduced by about twofold (also confirmed by dot blot) from

that without E1A. The facts that Py DNA replication with low E1A was already reduced by a factor of about 6 while T-Ag mRNA levels were not depressed and that at high E1A levels Py T-Ag mRNA is reduced only about 2-fold compared with an at least 30-fold repression of DNA replication are strong evidence suggesting that E1A is not depressing Py DNA replication simply by lowering T-Ag transcription. Clearly, at high E1A levels the decrease in T-Ag mRNA might well be contributing to the increasing repression of replication, but this does not alter the body of the conclusion.

DISCUSSION

Although the abilities of E1A gene products to *trans* activate transcription and to repress enhancer-mediated transcription are intensively studied, the effects of E1A on DNA replication have received much less attention. E1A has been reported to stimulate expression of proliferating-cell nuclear antigen (PCNA), a key cellular replication-associated protein, as well as to activate DNA replication and promote mitosis in quiescent cells (42). These effects have been proposed to facilitate adenovirus replication in quiescent human epithelial cells, which are among the sites of normal viral infection (54). Therefore, there appears to be evidence for the participation of E1A in replication control, all apparently in a positive manner. In contrast, we have shown here that in the Py model system, E1A can strongly repress viral DNA replication, or, seen from another viewpoint, perhaps "regulate" or switch Py DNA replication to a low-level state similar to what we have observed in the persistent-infection state in mice. This effect seems clearly not to be mediated solely by the depression of T-Ag transcription. This point suggests a more general mechanism of regulation of DNA replication which may involve transcriptional-element activation of replication directly. Previously we have reported that E1A can suppress a lytic Py infection in tissue culture, leading to persistent infection with episomal maintenance and low-level continuing replication (15). Along with the results presented here, this suggests that E1A does not completely prevent Py DNA replication but may instead prevent enhancer-mediated runaway amplification such as is seen in lytic infection of highly permissive cell types.

In addition to repression of wild-type Py, we have also shown that the F9 EC-cell-selected mutations in PyF441 and PyF101 did not allow replication above the level of wild-type Py in E1A-expressing murine 3T3 cells. This result is similar to the transcriptional results of Velcich et al. (61) in human HeLa cells and in apparent contrast to that of Hen et al. (27) in murine L cells in that F9 EC-cell-selected variants did not escape from E1A-mediated repression. However, our study differs from these in that (i) we examined primarily replication and (ii) we used permissive murine 3T3 cells. The differing results could be due to the numerous differences of the varied cells. For example, it has been observed that the ability of E1A to repress immunoglobulin heavy-chain enhancer-mediated transcription depends greatly on the cell type used (2, 6, 25). It is possible that the effects of E1A depend on the particular cell-specific mix of active transcription (and replication) factors. What is clear is that our results are inconsistent with a simple model, suggested by the results of Hen et al. (27), in which an E1A analog in undifferentiated F9 EC cells restricts Py in a simple repressorlike fashion. There has been an alternative mechanism proposed by which the PyF441 point change may activate

the enhancer by creating a new site for a positively acting factor present in undifferentiated EC cells (31, 34). Activation in differentiating EC cells might then be by the appearance or activation of alpha domain factors PEA1 and PEA2 (35, 41, 45) (with perhaps PEA1 alone as a positively acting factor [64]). Our data so far neither contradict nor strongly support such alternative mechanisms. Regardless of these models, there remains strong and varied evidence for at least some subset of E1A-like activities in undifferentiated EC cells (30, 36, 47, 66), and there is increasing evidence for a relationship between E1A expression and the undifferentiated state (19, 58, 65, 67). Our results showing that E1A can restrict Py DNA replication in a manner similar to that seen in many undifferentiated cells, including EC cells, may also be consistent with the presence of an E1A-like cellular factor.

We found that SV40 DNA replication in primate cells, unlike that of Py, was not affected by E1A, an interesting but puzzling result consistent with the observed strong replication of SV40 in E1A-expressing human 293 cells (26, 37) and in extracts from these cells (33, 57). This result might reflect basic differences in replication control strategy between these two closely related viruses. It is known that SV40 T-Ag, unlike Py T-Ag, binds p53 and that this binding competes with binding to DNA polymerase alpha-primase complex (5, 24). This suggests an intriguing point for possibly divergent control mechanisms. Alternatively, the effects of E1A may be complicated by the crossing of species lines. It is possible that murine adenovirus E1A may have quite different effects on Py replication, and this should be tested in the future.

What is the target for E1A-mediated repression of enhancer transcriptional activation? Recent reports of others who used genetic deletion analysis within the insulin gene enhancer show that the insulin enhancer seems to have no simple subenhancer target for E1A repression (55). This is consistent with a very recent report which examined E1A repression of enhancer activity and found that E1A represses every one of a wide variety of single and multimeric enhancers, suggesting that E1A repression may extend to all enhancer *trans* activators, perhaps by interfering with some unknown crucial mechanism which all enhancers must utilize (50). This contrasts with the earlier finding by Hen et al. (27) that the point-mutated enhancer derived from PyECF9.1 was E1A resistant (see Introduction). It has also been reported that E1A can *trans* activate an enhancerlike region of the E2A gene (29). Perhaps transactivation effects (which usually seem to act on promoter elements) can predominate over repressive effects in specific cellular environments. If E1A truly represses enhancers in a very general way, then future clarification of this mechanism is likely to be involved with the fundamental mechanisms by which enhancers function. Our results, involving replication repression, also seem inconsistent with any simple enhancer changes conferring resistance to E1A. We observed that neither F9-selected changes nor a deletion of the entire B enhancer (the site of almost all F9 EC-cell-selected changes) allow escape from E1A repression of Py replication. This appears to fit with our previously reported inability to select for E1A-resistant mutants of Py (15) in a fashion analogous to that used for Py selection in undifferentiated EC cells (38). We have yet to identify an enhancer which is resistant to E1A repression, but it would be logical to test multimers of E2F or ATF (activating transcription factor)-binding sites, which are identified targets for E1A *trans* activation (44) and which

therefore are perhaps among the best candidates to mediate E1A-resistant replication of Py, if this is possible.

How might E1A affect Py DNA replication? Three observations may suggest that E1A repression of DNA replication acts at the level by which transcription elements themselves activate Py DNA replication. These observations are the necessity of the Py origin core to have an adjacent full or partial enhancer region to activate DNA replication (63); the ability of E1A to inhibit activation of transcription by many enhancers, including that of Py (4, 60, 62); and the observed activation by variant enhancers of Py DNA replication in *cis* in specific cell types such as EC cells with their E1A-like activity (see Introduction). The activity of the enhancer is paramount for the control of DNA replication of Py, and E1A interaction with this control seems probable. Interestingly, the *in vitro* replication of naked Py DNA shows no enhancer dependence (46), unlike the situation *in vivo*. This may imply that chromatin involvement is essential for normal enhancer-mediated regulation of Py replication. With SV40, it has recently been shown that *in vitro* DNA replication can be made dependent on an adjacent NF1-binding site plus the addition of purified NF1 and replication proteins, mimicking the *in vivo* situation (10). This requires prior coassembly of chromatin onto the DNA along with NF1. These results suggest that transcription factor-mediated control of replication may be involved with chromatin assembly and the setting up of stable structures favoring replication. Whether E1A affects Py DNA replication *in vitro* has yet to be determined, but it is likely that accurate assessment will require the refinement of currently described Py *in vitro* replication systems so that they exhibit enhancer dependence *in vitro* as Py does *in vivo*.

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REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
- Berk, A. J. 1986. Adenovirus promoters and E1A transactivation. *Annu. Rev. Genet.* 20:45-79.
- Berk, A. J. 1986. Functions of adenovirus E1a. *Cancer Surv.* 5:367-387.
- Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature (London)* 312:608-612.
- Braithwaite, A. W., H. W. Sturzbecher, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins. 1987. Mouse p53 inhibits SV40 origin-dependent DNA replication. *Nature (London)* 329:458-460.
- Campbell, B. A., and L. P. Villarreal. 1985. Host species specificity of polyomavirus DNA replication is not altered by simian virus 40 72-base-pair repeats. *Mol. Cell. Biol.* 5:1534-1537.
- Campbell, B. A., and L. P. Villarreal. 1986. Lymphoid and other tissue-specific phenotypes of polyomavirus enhancer recombinants: positive and negative combinational effects on enhancer specificity and activity. *Mol. Cell. Biol.* 6:2068-2079.
- Campos, R., and L. P. Villarreal. 1982. An SV40 deletion mutant accumulates transcripts in a paranuclear extract. *Virology* 119:1-11.
- Challberg, M. D., and T. J. Kelly. 1989. Animal virus DNA replication. *Annu. Rev. Biochem.* 58:671-717.
- Cheng, L., and T. J. Kelly. 1989. Transcriptional activator nuclear factor I stimulates the replication of SV40 minichromosomes *in vivo* and *in vitro*. *Cell* 59:541-551.
- Dandolo, L., and D. Blangy. 1984. T-antigen-independent replication of polyomavirus DNA in murine embryonal carcinoma cells. *Mol. Cell. Biol.* 4:317-323.
- Delli Bovi, P., V. De Simone, R. Giordano, and P. Amati. 1984. Polyomavirus growth and persistence in Friend erythroleukemic cells. *J. Virol.* 49:566-571.
- DePamphilis, M. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. *Cell* 52:635-638.
- DePamphilis, M., and M. K. Bradley. 1986. Replication of SV40 and polyoma virus chromosomes, p. 99-246. *In* N. Salzman (ed.), *The Papovaviridae: the polyomaviruses*. Plenum Press, New York.
- DePolo, N. J., and L. P. Villarreal. 1989. E1A suppresses wild-type and F9-selected polyomavirus DNA replication and can lead to maintenance of episomal DNA, p. 239-254. *In* L. P. Villarreal (ed.), *Common mechanisms of transformation by small DNA tumor viruses*. American Society for Microbiology, Washington D.C.
- De Simone, V., and P. Amati. 1987. Replicative *cis*-advantage of polyomavirus regulatory region mutants in different murine cell lines. *J. Virol.* 61:1615-1620.
- De Simone, V., G. La Mantia, L. Lania, and P. Amati. 1985. Polyomavirus mutation that confers a cell-specific *cis* advantage for viral DNA replication. *Mol. Cell. Biol.* 5:2142-2146.
- de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen. 1984. Polyoma virus DNA replication requires an enhancer. *Nature (London)* 312:242-246.
- Dooley, T. P., M. Miranda, N. C. Jones, and M. L. Depamphilis. 1989. Transactivation of the adenovirus E1a promoter in the absence of adenovirus E1A protein is restricted to mouse oocytes and preimplantation embryos. *Development* 107:945-956.
- Dubensky, T. W., F. A. Murphy, and L. P. Villarreal. 1984. Detection of DNA and RNA virus genomes in organ systems of whole mice: patterns of mouse organ infection by polyomavirus. *J. Virol.* 50:779-783.
- Dubensky, T. W., and L. P. Villarreal. 1984. The primary site of replication alters the eventual site of persistent infection by polyomavirus in mice. *J. Virol.* 50:541-546.
- Fujimura, F. K., P. L. Deininger, T. Friedmann, and E. Linney. 1981. Mutation near the polyoma DNA replication origin permits productive infection of F9 embryonal carcinoma cells. *Cell* 23:809-814.
- Fujimura, F. K., and E. Linney. 1982. Polyoma mutants that productively infect F9 embryonal carcinoma cells do not rescue wild-type polyoma in F9 cells. *Proc. Natl. Acad. Sci. USA* 79:1479-1483.
- Gannon, J. V., and D. P. Lane. 1987. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature (London)* 329:456-458.
- Gerard, R. D., and Y. Gluzman. 1985. New host cell system for regulated simian virus 40 DNA replication. *Mol. Cell. Biol.* 5:3231-3240.
- Grass, D. S., D. Read, E. D. Lewis, and J. L. Manley. 1987. Cell- and promoter-specific activation of transcription by DNA replication. *Genes Dev.* 1:1065-1074.
- Hen, R., E. Borrelli, C. Fromental, P. Sassone Corsi, and P. Chambon. 1986. A mutated polyoma virus enhancer which is active in undifferentiated embryonal carcinoma cells is not repressed by adenovirus-2 E1A products. *Nature (London)* 321:249-251.

28. Hirt, B. J. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
29. Imperiale, M. J., R. P. Hart, and J. R. Nevins. 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninucleated and E1a-induced transcription. *Proc. Natl. Acad. Sci. USA* **82**:381-385.
30. Imperiale, M. J., H. T. Kao, J. R. Feldman, J. R. Nevins, and S. Strickland. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1a-like activity. *Mol. Cell. Biol.* **4**:867-874.
31. Jones, N. C., P. W. Rigby, and B. Ziff. 1988. Trans-acting protein factors and the regulation of eukaryotic transcriptions: lessons from studies on DNA tumor viruses. *Genes Dev.* **2**:267-281.
32. Katinka, M., M. Vasseur, N. Montreau, M. Yaniv, and D. Blangy. 1981. Polyoma DNA sequences involved in control of gene expression in murine embryonal carcinoma cells. *Nature (London)* **290**:720-723.
33. Kelly, T. J., P. J. Rosenfeld, R. J. Wides, E. A. O'Neill, J. J. Li, and M. S. Wold. 1987. Replication of adenovirus and SV40 chromosomes in vitro. *Philos. Trans. R. Soc. London (Biol.)* **317**:429-438.
34. Kovessdi, I., M. Satake, K. Furukawa, R. Reichel, Y. Ito, and J. R. Nevins. 1987. A factor discriminating between the wild-type and a mutant polyomavirus enhancer. *Nature (London)* **328**:87-89.
35. Kryszke, M. H., J. Piette, and M. Yaniv. 1987. Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. *Nature (London)* **328**:254-256.
36. La Thangue, N. B., and P. W. Rigby. 1987. An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* **49**:507-513.
37. Lebkowski, J. S., S. Clancy, and M. P. Calos. 1985. Simian virus 40 replication in adeno-transformed human cells antagonizes gene expression. *Nature (London)* **317**:169-171.
38. Levine, A. J. 1982. The natural host range restriction of SV40 and polyoma viruses in embryonal carcinoma cells. *Curr. Top. Microbiol. Immunol.* **101**:1-30.
39. Luthman, H., and G. Magnusson. 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**:1295-1308.
40. Malone, R., C. Passananti, V. De Simone, P. Delli Bovi, G. Augusti Tocco, and P. Amati. 1985. Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication. *EMBO J.* **4**:3215-3221.
41. Martin, M. E., J. Piette, M. Yaniv, W. J. Tang, and W. R. Folk. 1988. Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins. *Proc. Natl. Acad. Sci. USA* **85**:5839-5843.
42. Moran, E., and M. B. Mathews. 1987. Multiple functional domains in the adenovirus E1A gene. *Cell* **48**:177-178.
43. Muller, W. J., D. Dufort, and J. A. Hassel. 1988. Multiple subelements within the polyoma enhancer function synergistically to activate DNA replication. *Mol. Cell. Biol.* **8**:5000-5015.
44. Pei, R., and A. J. Berk. 1989. Multiple transcription factor binding sites mediate adenovirus E1A transactivation. *J. Virol.* **63**:3499-3506.
45. Piette, J., and M. Yaniv. 1987. Two different factors bind to the alpha-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. *EMBO J.* **6**:1331-1337.
46. Prives, C., Y. Murakami, F. G. Kern, W. Folk, C. Basilico, and J. Hurwitz. 1987. DNA sequence requirements for replication of polyomavirus DNA in vivo and in vitro. *Mol. Cell. Biol.* **7**:3694-3704.
47. Reichel, R., I. Kovessdi, and J. R. Nevins. 1987. Developmental control of a promoter-specific factor that is also regulated by the E1A gene product. *Cell* **48**:501-506.
48. Roberts, B. R., J. S. Miller, D. Kimelman, C. L. Cepko, I. R. Lemischka, and R. C. Mulligan. 1985. Individual adenovirus type 5 early region 1A gene products elicit distinct alterations of cellular morphology and gene expression. *J. Virol.* **56**:404-413.
49. Roberts, J. M., and H. Weintraub. 1986. Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. *Cell* **46**:741-752.
50. Rochette-Egly, C., C. Fromental, and P. Chambon. 1990. General repression of enhancer activity by the adenovirus-2 E1A proteins. *Genes Dev.* **4**:137-150.
51. Rochford, R., B. A. Campbell, and L. P. Villarreal. 1987. A pancreas specificity results from the combination of polyomavirus and Moloney murine leukemia virus enhancer. *Proc. Natl. Acad. Sci. USA* **84**:449-453.
52. Rochford, R., C. T. Davis, K. K. Yoshimoto, and L. P. Villarreal. 1990. Minimal subenhancer requirements for high-level polyomavirus DNA replication: a cell-specific synergy of PEA3 and PEA1 sites. *Mol. Cell. Biol.* **10**:4996-5001.
53. Sekikawa, K., and A. Levine. 1981. Isolation and characterization of polyoma host range mutants that replicate in nullipotent embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* **78**:1100-1104.
- 53a. Senear, A. Personal communication.
54. Spindler, K. R., C. Y. Eng, and A. J. Berk. 1985. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. *J. Virol.* **53**:742-750.
55. Stein, R. W., and J. Whelan. 1989. Insulin gene enhancer activity is inhibited by adenovirus 5 E1a gene products. *Mol. Cell. Biol.* **9**:4531-4534.
56. Stein, R. W., and E. B. Ziff. 1987. Repression of insulin gene expression by adenovirus type 5 E1a proteins. *Mol. Cell. Biol.* **7**:1164-1170.
57. Stillman, B. 1989. Initiation of eukaryotic DNA replication in vitro. *Annu. Rev. Cell Biol.* **5**:197-245.
58. Suemori, H., S. Hashimoto, and N. Nakatsuji. 1988. Presence of the adenovirus E1A-like activity in preimplantation stage mouse embryos. *Mol. Cell. Biol.* **8**:3553-3555.
59. Timmers, H. T., H. van Dam, G. J. Pronk, J. L. Bos, and A. J. van der Eb. 1989. Adenovirus E1A represses transcription of the cellular JE gene. *J. Virol.* **63**:1470-1473.
60. van Dam, H., R. Offringa, A. M. Smits, J. L. Bos, N. C. Jones, and A. J. van der Eb. 1989. The repression of the growth factor-inducible genes JE, c-myc and stromelysin by adenovirus E1A is mediated by conserved region 1. *Oncogene* **4**:1207-1212.
61. Velich, A., F. G. Kern, C. Basilico, and E. B. Ziff. 1986. Adenovirus E1a proteins repress expression from polyomavirus early and late promoters. *Mol. Cell. Biol.* **6**:4019-4025.
62. Velich, A., and E. Ziff. 1985. Adenovirus E1a proteins repress transcription from the SV40 early promoter. *Cell* **40**:705-716.
63. Veldman, G., S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. *Mol. Cell. Biol.* **5**:649-658.
64. Wasylyk, B., J. L. Imler, B. Chatton, C. Schatz, and C. Wasylyk. 1988. Negative and positive factors determine the activity of the polyoma virus enhancer alpha domain in undifferentiated and differentiated cell types. *Proc. Natl. Acad. Sci. USA* **85**:7952-7956.
65. Webster, K. A., G. E. Muscat, and L. Kedes. 1988. Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. *Nature (London)* **332**:553-557.
66. White, R. J., D. Stott, and P. W. Rigby. 1989. Regulation of RNA polymerase III transcription in response to F9 embryonal carcinoma stem cell differentiation. *Cell* **59**:1081-1092.
67. Young, K. S., R. Weigel, S. Hiebert, and J. R. Nevins. 1989. Adenovirus E1A-mediated negative control of genes activated during F9 differentiation. *Mol. Cell. Biol.* **9**:3109-3113.