Expression of wild-type and mutant simian virus 40 large tumor antigens in villus-associated enterocytes of transgenic mice

cell cycle regulation/[Val12]KRAS/intestinal neoplasia

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ABSTRACT The four principal gut epithelial cell lineages undergo continuous and rapid renewal during a geographically well-organized migration along the crypt-to-villus axis. The molecules that regulate their proliferation and differentiation programs are largely unknown. The large tumor antigen (TAg) of wild-type (wt) simian virus 40 (SV40) and its mutant derivatives represent tools for describing the contributions of regulators of the cell cycle to the proliferative state of each lineage. Expression of SV40 TAgwt in postmitotic, villus-associated enterocytes of transgenic mice reenters them to reenter the cell cycle without an apparent effect on their state of differentiation. When human KRAS with a Val-12 substitution ([Val12]KRAS) is coexpressed with SV40 TAgwt in villus enterocytes of bitransgenic animals, the two oncoproteins cooperate to produce dedifferentiation (dysplasia). SV40 mutant d1137 expresses a TAg that is unable to complex with p53 but retains N-terminal transforming functions, including the ability to complex pRB, p107, and p500. When SV40 TAgd1137 is expressed in villus enterocytes, they reenter into the cell cycle. However, coexpression of SV40 TAgd1137 and [Val12]KRAS does not produce dysplastic changes. Thus, the N-terminal 121 residues of TAg are sufficient to perturb the proliferative state of the enterocyte but not to produce detectable changes in the state of differentiation when coexpressed with [Val12]KRAS.

The crypt-to-villus axis of the mouse intestine represents a continuous developmental system. A multipotent stem cell, located near the base of each crypt, gives rise to descendants that undergo several amplifying rounds of cell division. Members of this proliferating, transit cell population are allocated to one of four principal lineages. These lineages complete their terminal differentiation programs during a rapid bipolar migration (1). Enterocytes, goblet, and enteroendocrine cells are translocated in vertical coherent bands from each monoclonal crypt to an adjacent villus and are subsequently exfoliated when they reach an extrusion zone located near the villus tip. Paneth cells differentiate as they are translocated to the base of each crypt where they are removed by phagocytosis. Proliferation, commitment, migration-associated differentiation, and exfoliation are completed rapidly—e.g., the turnover time for enterocytes, which compose 90–95% of gut epithelial cells, is ~3 days (2).

The factors that regulate proliferation and differentiation in the gut epithelium are largely unknown. The tumor suppressors p53 and the retinoblastoma susceptibility gene product (pRB) have been implicated in regulating passage through the G1/S checkpoint of the cell cycle. Mice homozygous for a Tprp53 (transformation-related protein p53 gene) null allele have no obvious abnormalities in intestinal morphology (3). The effects of mutational inactivation of Rb-1 on the intestine cannot be determined, since mice that are homozygous for Rb-1 null alleles die before embryonic day 16 (E16)—i.e., prior to cytodifferentiation of the gut epithelium (1, 4, 5). The large tumor antigen (TAg) of simian virus 40 (SV40) binds to and presumably disrupts the tumor suppressor functions of p53 and pRB. Binding to p53 requires two noncontiguous regions, which include residues 350–450 and 532–625. Residues 105–114 are involved in binding to pRB. A third transforming activity, analogous to the p300 binding function of adenovirus E1A, maps near the N terminus of SV40 TAg. SV40 TAg mutants have been identified that are deficient for each of these three functions (6–10). Expression of wild type (wt) and mutant SV40 TAgss in different cell lineages of transgenic mice represents one approach for defining the relative importance of p53, pRB, and related molecules in modulating their proliferation/differentiation programs (6).

Promoter mapping studies conducted in transgenic mice have shown that nucleotides from −1178 to +28 of the rat gene encoding intestinal fatty acid binding protein (I-FABP) contain cis-acting sequences that restrict foreign gene expression to postmitotic enterocytes distributed along the length of duodenal, jejunal, and ileal villi (11). I-FABP−1178 to −28/reporter transgenes are activated coincident with the initial cytodifferentiation of the gut epithelium (E15/E16), and expression is sustained throughout the first year of life. Studies of I-FABP−1178 to −28/SV40 TAgwt transgenic mice have revealed that production of this viral oncprotein in postmitotic enterocytes results in their reentry into the cell cycle (12, 13). The nature of the cell cycle differs from that observed in crypts where the transgene is silent—i.e., S phase is longer in SV40 TAg-positive enterocytes and there may be a block at the G2/M boundary (12). These findings are consistent with the notion that differentiation affects the accumulation of cell cycle regulators. SV40 TAgwt-induced reentry into the cell cycle causes suppression of CAAT enhancer binding protein α accumulation but has no discernible effects on the state of differentiation of these cells (12–14). Addition of human Val-12-substituted KRAS ([Val12]KRAS) to SV40 TAgwt-producing enterocytes causes marked dysplasia (13). In contrast, I-FABP−1178 to −28/KRASVal12 (mutant KRAS encoding [Val12]KRAS) transgenic mice display no abnormalities in gut epithelial proliferation or differentiation (13). These experiments indicate that one or more SV40 TAg activities are required for reentry of enterocytes into the cell cycle and cooperation with [Val12]KRAS. In this report, we have analyzed enterocytic proliferation and differentiation programs in I-FABP−1178 to −28/SV40 TAgwt transgenic mice and in animals that produce a truncated mutant derivative, SV40 TAgd1137, that lacks the ability to bind p53 but retains its N-terminal transforming function and capacity to bind

Abbreviations: SV40, simian virus 40; TAg, large tumor antigen; wt, wild type; KRASVal12, mutant KRAS encoding [Val12]KRAS; I-FABP and L-FABP, intestinal and liver fatty acid binding proteins; I-FABP−1178 to −28, nucleotides −1178 to +28 of the gene encoding rat I-FABP; pRB, retinoblastoma protein.

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additional pedigrees of bitransgenic mice were examined that coexpress [Val^{12}]KRAS and SV40 TAg^{wt} or SV40 TAg^{1137}. The results have allowed us to genetically separate TAg activities that induce proliferation in villus-associated enterocytes and activities required for cooperation with [Val^{12}]KRAS to induce dysplasia.

MATERIALS AND METHODS

Generation of I-FABP^{-1178} to +28/ SV40 Tag^{1137} Transgenic Mice. A plasmid containing SV40 Tag^{1137} DNA (10) was digested with Bcl I and BstXI, releasing a 2-kb fragment (nucleotides 4759–2770 of SV40 DNA). A plasmid containing I-FABP^{-1178} to +28/ SV40 TAg^{wt} (nucleotides 5235–2533; ref. 13) was also digested with Bcl I and BstXI to excise nucleotides 4759–2770 of the SV40 genome. The 2-kb SV40 Tag^{1137} Bcl I/BstXI fragment was then ligated into the remaining Bcl I/BstXI vector DNA. A 4.2-kb EcoRI/Sal I fragment containing I-FABP^{-1178} to +28/ SV40 Tag^{1137} (Fig. 1A) was used for pronuclear injections into FVB/N zygotes. Surviving eggs were transferred to the oviducts of pseudopregnant Swiss Webster recipients. Live born mice were screened for the presence of transgenes by using the PCR, tail DNA, and the primers described in Fig. 1A. One pedigree, containing the three copies of the transgene/haploid genome, was used for subsequent studies together with one line of FVB/N I-FABP^{-1178} to +28/ SV40 TAg^{wt} transgenic mice (pedigree 48 in ref. 13) and two lines of FVB/N I-FABP^{-1178} to +28/ SV40 Val^{12} mice (pedigrees 33 and 73 in ref. 13).

Labeling Cells in S Phase and Regional Dissection of the Intestine. Mice were maintained in microisolator cages and fed autoclaved Ralston Purina Chow (no. 5010) ad libitum. All animals received an i.p. injection of BrdUrd (120 mg/kg of body weight) and 5-fluoro-2'-deoxyuridine (12 mg/kg) 1.5 hr prior to sacrifice at 5–47 weeks of age. The gastrointestinal tract was removed en bloc and subdivided into stomach (ST), duodenum (DU), proximal and distal jejunum (PJ, DJ), ileum (IL), cecum (CE), and proximal and distal colon (PC, DC) (11). Samples (1 cm) were removed from each segment and placed in Bouin’s solution. The remaining portions of each segment were frozen in liquid N_{2} as were the brain, heart, lung, liver, spleen, pancreas, kidney, and skeletal muscle.

Ribonuclease Protection Assays. SV40 TAg^{wt} and SV40 TAg^{1137} mRNAs were detected as 125-nucleotide fragments when total cellular RNA was incubated with a 32P-labeled complementary RNA (cRNA) encompassing nucleotides 5023–4896 of SV40 TAg^{wt} DNA. Human KRAS^{Val^{12}} and mouse c-ras mRNAs were detected as 148 and 54 nucleotide fragments, respectively, after hybridization to a 32P-labeled 199-nucleotide cRNA described in ref. 13. Polyacrylamide gels containing the protected fragments were scanned with a radionuclide imager. The intensity of the signal observed with tissue RNA was compared to the signals generated from known amounts of in vitro transcribed mRNA standards.

Measurement of Steady-State Levels of SV40 TAg^{wt} and SV40 TAg^{1137} Transgenic Mice. SV40 Tag^{1137} transgenic mice were sacrificed at 8 and 47 weeks of age together with comparably aged nontransgenic littermates. The small intestine was removed en bloc and subdivided as described above. Each intestinal segment was frozen in liquid N_{2}, pulverized, and lyophilized. The lyophilized material was resuspended in an equal volume of extraction buffer containing 0.04 M Tris (pH 6.8), 2% 2-mercaptoethanol, 1% SDS, 5% glycerol, aprotinin at 1 μg/ml, leupeptin at 1 μg/ml, N-tosyl-l-phenylalanine chloromethyl ketone at 100 μg/ml, phenylmethylsulfonyl fluoride at 100 μg/ml, pepstatin A at 1 μg/ml, and 1 mM EDTA. The solution was boiled for 5 min, insoluble particulate material was removed by centrifugation for 5 min at 10,000 × g, and the protein
concentration of the resulting supernatant was determined. Total cellular proteins and purified SV40 Tagwt and SV40 Tagw12117 cDNA standards were fractionated by SDS-PAGE and transferred to nitrocellulose filters. Duplicate blots were incubated with the monoclonal antibody PAB419 (15), diluted 1:1000 with blotto (50 mM Tris-HCl, pH 7.5, 100 mM NaCl/5% nonfat dry milk) (1:1000), or with a rabbit anti-rat I-FABP sera (1:1000; ref. 11). Antigen-antibody complexes were visualized with a rabbit anti-mouse IgG sera and 125I-labeled protein A. Blots were scanned to determine the concentration of wt or mutant SV40 Tag and I-FABP in each segment. The concentrations of I-FABP were equivalent in comparably positioned segments of transgenic and normal littermate small intestine (data not shown).

**Immunocytochemical Studies.** Single and multilabel immunocytochemical studies were performed (16) and with the following antiserum: rabbit anti-SV40 Tag (1:1000; from D. Hanahan, University of California, San Francisco), hamster anti-SV40 Tag (1:1000; ref. 12), rabbit anti-I-FABP (1:500; ref. 11), rabbit anti-liver (L)-FABP (1:500; ref. 12), rabbit anti-alkaline phosphatase (1:5000; ref. 12), and goat anti-BrdUrd (1:1000; ref. 13). Antigen-antibody complexes were detected with (i) a donkey anti-rabbit gold-labeled antibody (1:40 dilution; Amersham) and silver staining, (ii) donkey anti-rabbit or rabbit anti-hamster antibodies labeled with fluorescein isothiocyanate (1:100; Jackson Immunoresearch), or (iii) donkey anti-goat antibodies tagged with Cy-3 (1:400; Jackson Immunoresearch).

**Histopathologic Grading System.** Hematoxylin/eosin-stained sections, prepared from the proximal and distal small intestine of transgenic mice and their normal littermates, were graded in a blinded fashion for histopathologic abnormalities: 0 = normal, 1 = minimal to mild dysplasia, 2 = moderate dysplasia, and 3 = severe dysplasia (see ref. 13). The scoring results were subjected to nonparametric statistical analysis using the Van der Waerden test (17).

## RESULTS

**SV40 Tagw12117 Causes Reentry of Villus-Associated Enterocytes into the Cell Cycle.** SV40 mutant d11137 carries a 31-bp deletion in the viral early region and as a result expresses a truncated Tag cDNA consisting of the initial N-terminal 121 amino acids of Tagwt plus 11 missense residues (ref. 7; see Fig. 1A). The mutant Tag retains the ability to complex cellular PRB and p107 but has lost the p53 binding domain as well as all known replicative functions. d11137 can transform some established cell lines (9) and induce chordoid plexus tumors in transgenic mice (6). Transformation of other established cell lines (9) or induction of B- and T-cell lymphomas in transgenic mice (6, 18) requires the p53 binding domain.

The two pedigrees of I-FABPw1178 to +28/SV40 Tag transgenic mice used for this study were selected because the steady-state levels of their SV40 Tagwt and SV40 Tagw11173 mRNAs were similar along the duodenal-to-colonic axis at comparable ages (Fig. 1, B, C, and E). Although immunocytochemical surveys using several polyclonal antibodies indicated that SV40 Tagwt is readily detectable in villus-associated enterocytes distributed from the base to the tips of duodenal, jejunal, and ileal villi, none of these antibodies was able to detect SV40 Tagw11173, even under a wide variety of fixation conditions (data not shown). Therefore, Western blots were prepared containing total protein extracted from the duodenum, jejunum, and ileum of 8- and 47-week-old transgenic animals plus similarly aged normal littermates. When probed with a well-characterized monoclonal antibody directed against Tag (PAB419; ref. 14), proteins of the expected mass were visualized. The SV40 Tagwt transgenics but not their normal littermates expressed a 90-kDa immunoreactive protein with a distribution along the duodenal-to-ileal axis that paralleled that of the corresponding mRNA (e.g., compare lanes 5 and 8 in Fig. 2 with Fig. 1C). I-FABPw1178 to +28/SV40 Tagw12117 transgenic mice expressed a 15-kDa (but not a 90-kDa) immunoreactive protein that also showed similar regional variations in concentration along the cephalaocolical axis of their small intestine (lanes 6 and 7 in Fig. 2). In any one segment of gut, the steady-state molar concentration of the mutant protein was 2-10 times higher than the wt protein (e.g., Fig. 2). The steady-state concentrations of wt and mutant Tag mRNA and protein within a given segment of gut did not change appreciably during the 10-month period surveyed (data not shown). Evidence for expression of the mutant viral oncprotein in villus-associated enterocytes was also provided by the observation that members of this cell lineage reenter the cell cycle (Fig. 3 A-C). The distribution of BrdUrd-positive, S-phase cells follows the expression domain of I-FABP/SV40 Tagw12117 along the cephalaocolical axis of the gut—i.e., the number of enterocytes in S phase is greater in duodenal and jejunal than in ileal villi. The number of S-phase enterocytes overlying villi located at comparable positions of the duodenal-to-colonic axis is similar in 10- to 12-week-old I-FABPw1178 to +28/SV40 Tagwt and I-FABPw1178 to +28/SV40 Tagw12117 mice (n = 7-10). In each pedigree, the number of villus-associated cells in S phase exceeds the number of cells in M phase by several fold. SV40 Tagw12117- or SV40 Tagwt-induced reentry into the cell cycle is not associated with any detectable change in crypt-villus architecture, in the morphologic characteristics of enterocytes, or in the patterns of expression of several markers of the enterocyte differentiation program (e.g., Fig. 3B), even when mice are monitored for a period of 6-7 months. These results indicate that post-mitotic, villus-associated enterocytes are capable of reentering the cell cycle and that activities contained in the N-terminal 121 residues of SV40 Tag are able to initiate their release from cell cycle arrest. SV40 Tagw12117 Is Unable to Cooperate with [Val12]K Ras To Produce Dedifferentiation of Villus-Associated Enterocytes. Production of [Val12]K Ras in villus-associated enterocytes has no demonstrable effect on proliferation or differentiation (13). I-FABPw1178 to +28/SV40 Tagwt transgenic mice were mated to members of two pedigrees of I-FABPw1178 to +28/K RasVal12 mice. The steady-state levels and distribution of K RasVal12 mRNA along the duodenal-to-colonic axis are similar in members of these two K RasVal12 lines (13) (Fig. 4E). The SV40 Tagwt X K RasVal12 transgenic mice exhibited marked dysplasia of their villus-associated enterocytes (Fig. 3F and H). Distortions in crypt-villus architecture were
FIG. 3. Analysis of the proliferation and differentiation programs of villus-associated enterocytes in transgenic mice containing one or two I-FABP$^{\text{1178 to +28}}$/oncogene DNAs. (A–E) Sections from the proximal jejunum of 10- to 12-week-old mice stained with goat anti-BrdUrd and rabbit anti-L-FABP sera. Antigen-antibody complexes were detected with Cy-3-labeled donkey anti-goat and fluorescein-labeled donkey anti-rabbit sera. (A) A normal FVB/N mouse. Proliferating BrdUrd-positive cells (orange-red) are confined to the crypt. L-FABP is first detectable in postmitotic enterocytes (green) as they exit the crypt. (B) An I-FABP$^{1178}$ to $^{+28}$/SV40 TAg$^{wt}$ transgenic mouse. Villus-associated, L-FABP-positive enterocytes have reentered the cell cycle (arrows). (C) An I-FABP$^{1178}$ to $^{+28}$/SV40 TAg$^{d'1137}$ transgenic mouse. Enterocytes are able to reenter the cell cycle. (D and E) A SV40 TAg$^{wt}$ × KRAS$^{Val12}$ bitransgenic mouse (D) and a SV40 TAg$^{d'1137}$ × KRAS$^{Val12}$ bitransgenic animal (E) both exhibit proliferative abnormalities in their proximal jejunal villus enterocytic populations. (F) Hematoxylin/eosin-stained section of proximal jejunum prepared from a normal 12-week-old FVB/N mouse. No M-phase cells are evident in villus-associated epithelial cells. (G) Proximal jejunum from a young adult SV40 TAg$^{wt}$ × KRAS$^{Val12}$ transgenic mouse stained with anti-L-FABP and anti-BrdUrd sera. Villus architecture is distorted. This is shown by polyploid lesions as well as branched villi. Numerous enterocytes in S phase are evident. (H) Hematoxylin/eosin-stained section of proximal jejunum from a SV40 TAg$^{wt}$ × KRAS$^{Val12}$ bitransgenic mouse showing proliferative and dysplastic changes in villus-associated enterocytes. These changes include increases in the nuclear-to-cytoplasmic ratio, nuclear hyperchromatism, nuclear atypia, and M-phase cells (arrow). (I) Proximal jejunum of a SV40 TAg$^{d'1137}$ × KRAS$^{Val12}$ bitransgenic mouse showing the lack of dysplastic changes in enterocytes. However, proliferative abnormalities are present—e.g., a cell in M phase is indicated by the arrow. (Bar = 25 μm.)
readily apparent and include polyloid structures as well as bifurcated and trifurcated villi (Fig. 3G). Analysis of seven, 5- to 28-week-old mice indicated that these changes are evident in the duodenum, jejunum, and ileum at the earliest time-point surveyed, that they persist, and that they do not progress to adenomas or adenocarcinomas.

SV40 TAgG1117 transgenic mice were mated to members of the two KrasVal12 pedigrees to determine amino acids 1–121 of SV40 TAg contained the elements needed to produce cooperation with [Val12]KRAS. Bitransgenic mice were sacrificed at 5–7 and 10–12 weeks of age. The distributions and steady-state concentrations of SV40 TAgG1117 and KrasVal12 mRNAs were identical to those observed in comparably aged, single-transgene-containing mice and similar to SV40 TAg and KrasVal12 transgensics (Fig. 1 C–E). Despite these similarities in mRNA levels and the 2- to 10-fold higher steady-state levels of TAgG1117 versus TAg in small intestinal segments, the proliferative changes observed in SV40 TAgG1117 × KrasVal12 bitransgenic mice (Fig. 3E) were less prominent than in comparably aged SV40 TAg × KrasVal12 animals (Fig. 3D)—i.e., there were generally fewer enterocytes in S phase in duodenal, jejunal, or ileal villi. Moreover, the enterocyte dysplasia and distortions in crypt-villus architecture noted in seven 5- to 28-week-old SV40 TAgG1117 × KrasVal12 mice (Fig. 3G and H) were not evident in any of the six 5- to 12-week-old SV40 TAgG1117 / KrasVal12 mice examined (Fig. 3J). This was true when either pedigree of I-FABP/KrasVal12 mice was used to generate bitransgenic animals. These differences in enterocyte differentiation between SV40 TAg × KrasVal12 and SV40 TAgG1117/KrasVal12 transgenic mice were also reflected in the histopathologic scores of their proximal small intestine (1.9 ± 0.4 versus 0.7 ± 0.5, respectively; P = 0.005). No significant differences in histopathologic scores were noted in the distal half of their small intestine, where levels of transgene expression were lower (1.3 ± 0.5 versus 0.7 ± 0.5). Together, these findings indicate that the N-terminal 121 residues of SV40 TAg contain elements that can lead to disruption of the cell cycle arrest in villus-associated enterocytes but do not contain the necessary activities required to support the dysplasia induced by coexpression of intact TAg and [Val12]KRAS.

**DISCUSSION**

One simple interpretation of the data presented in this study is that both SV40 TAgG1117 and the truncation mutant TAgG1117 allow postmitotic, villus-associated enterocytes to reenter the cell cycle because of their ability to interact with pRB, p107, and/or p130 and that TAg-mediated inactivation of p53 is not necessary to overcome their normal cell cycle arrest. In addition, the greater proliferative response observed when [Val12]KRAS is added to SV40 TAgG1117-positive animals to SV40 TAgG1117-positive enterocytes suggests that the role of p53 as a regulator of the cell cycle may be limited to members of this lineage, which contain aberrant signal transduction pathways. However, this interpretation remains quite speculative. For example, our transgenic mouse experiments cannot exclude the possibility that SV40 TAg activities other than p53 complex formation(s) are responsible for the dysplastic state of the molecule are responsible for mediating cooperative effects with [Val12]KRAS, including both the proliferative and dysplastic responses of enterocytes. This latter hypothesis will have to be tested by comparing the intestinal epithelium of I-FABP/KrasVal12 mice that are Trp53−/−, −/+; and +/+ (3).

Analyses of regulation at the G1/s boundary in mammalian cells have been conducted in cultured cell lines. Little is known about the effects of differentiation of various mouse cell lines in vivo on the levels of regulators of the G1/s boundary. The enterocyte’s rapid progression from proliferation to cell cycle arrest and subsequent differentiation during a geographically well-organized (and perpetual) migration makes it an attractive model for exploring this question. Moreover, the normally quiescent villus-associated enterocyte is a good target for gain-of-function experiments. For example, E2F is a transcription factor that appears to regulate a number of genes that are activated in late G1, when cells are about to reenter S phase. Phosphorylation of pRB is associated with release of E2F from the pRB–E2F complex. SV40 TAg-mediated disruption of E2F interaction with pRB could account for the observed reentry of enterocytes into S phase (19). The potential roles of pRB and E2F in affecting the state of enterocytic proliferation along the crypt-to-villus axis could be tested by introducing SV40 TAg mutants that lack the pRB binding domain but retain p33 binding and trans-activation functions (6, 8) and/or by examining mice that contain an I-FABP11178 to +28/E2F transgene. The assays for proliferative effects in enterocytes are straightforward as demonstrated in this report.

The gut epithelium of I-FABP11178 to +28/SV40 TAg × KrasVal12 bitransgenic mice provides a useful assay system for other types of experiments. [Val12]KRAS-induced dysplasia can be exploited to evaluate the functional significance of polyisoprenylation of c-ras by noting the effects of inhibitors of farnesyl-protein transferase or other drugs that may block [Val12]KRAS-mediated effects or inactivate [Val12]KRAS itself. Since it only takes 2–3 days for enterocytes to complete their migration from the base to the apical extrusion zone of a villus, administration of such agents could modulate [Val12]KRAS-induced dysplasia after only a few days of treatment. The inability of SV40 TAg/[Val12]KRAS-producing, dysplastic enterocytes to undergo clonal expansion and progress to frank neoplasia is likely due to their rapid exfoliation (13). Thus, these mice may also provide a sensitive assay system for identifying gene products that affect cellular translocation rates along the crypt-to-villus axis.

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