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Oncogenesis of Mammary Glands, Skin, and Bones by Polyomavirus Correlates with Viral Persistence and Prolonged Genome Replication Potential

JULIA J. WIRTH, LARRY G. MARTIN,† AND MICHELE M. FLUCK*
Department of Microbiology, Michigan State University, East Lansing, Michigan

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A correlation between polyomavirus-induced oncogenesis and viral persistence on the one hand and/or prolonged genome replication potential on the other was established with respect to their respective organ distributions. Prolonged replication potential is defined as the capacity of a genome to replicate in a given organ from the time of infection up to the onset of oncogenesis. This conclusion was derived following intraperitoneal infection of BALB/c mice with wild-type strain A2. Viral genomes were used as parameters of persistence and replication and were detected by Southern blotting and PCR analysis. The major tumor target organs (mammary gland, skin, and bone), which have not been previously analyzed for persistence, were compared with other, non-tumor-prone organs (kidney, liver, lung, spleen, and salivary gland). A progressive loss of viral genomes was observed in all tissues as a function of time postinfection; however, genomes were shown to persist through 20 weeks postinfection in the mammary glands, skin, and bones to an extent similar to that in the previously described kidneys (D. J. McCance, J. Virol. 39:958–962, 1981; W. P. Rowe, J. W. Hartley, J. D. Estes, and R. J. Huebner, Natl. Cancer Inst. Monogr. 4:189–209, 1960). Thus, tumors arise among organs that sustain a persistent infection, but not all such organs develop tumors (e.g., the kidney). The capacity of organs to support de novo replication at various ages, including the age reached when the first tumors are detected, was also determined using a 3-day infection period for ages between 0 and 7 weeks. For all organs tested, a higher level of genomes was observed in organs of mice infected as neonates than in those infected after the age of 3 weeks. However, marked organ-specific differences were seen in the degree and timing of loss of replication. In particular, viral genome replication, although reduced, was maintained in the mammary glands, skin, and bones of adult animals, in contrast to the kidneys. We conclude that organ-specific oncogenesis correlates with two organ-specific parameters: persistence of viral genomes and prolonged viral genome replication potential. This may reflect a requirement for continued viral genome replication and/or gene expression for tumorigenesis. In turn, these parameters may be linked to the tissue-specific continued capacity for cellular division.

Polyomavirus causes a rapid systemic infection with high-level viremia in neonatal mice (8, 21). The immune response (both antibody and cell mediated) is very efficient and rapidly clears virus and infected cells following immune maturation (i.e., around 7 days of age) (8, 21). However, the virus has been shown to persist long term (14) and to induce tumors in a variety of organs later in life (5). Variables which affect the specific pattern of persistence include the route of infection (7–9), the virus genotype (18), and the genetic background of the mouse (20). These same factors affect the level of viral replication, which ultimately may be important in determining the sites of persistence. With respect to the fate of the viral genome, tumorigenesis can be viewed as a special case of persistence: i.e., the viral genome needs to persist in the infected cells to express the viral transforming protein middle T antigen. Yet, within a given set of parameters of infection (i.e., specific mouse and virus strains and a specific viral dose and route of infection), there is not a good correlation between the sites of persistence identified to date and the targets of oncogenesis. The kidneys and salivary glands have been identified as major targets of replication and persistence (14, 15, 21), while tumors occur in a much wider spectrum of organs (5). In particular, the skin, mammary glands, and bones, which are among the most frequent targets of polyomavirus-induced oncogenesis by various viral strains and in various mouse backgrounds (3, 5, 13, 19), have not been analyzed as sites of viral persistence. On the other hand, a connection has been previously noted between organ specificity in both oncogenesis and viral genome replication (23). In infections of normal neonatal or 6-week-old athymic *nu/nu* BALB/c mice with the A2 strain, the spectrum of organs that can sustain viral genome replication in mice infected as adults is a subset of those that serve as targets in neonates. The mammary glands, skin, and bones sustain viral genome replication in de novo-infected 6-week-old mice, albeit to lower levels than the same organs in neonatal mice. In contrast, the kidneys, liver, and lungs, which support high levels of replication in neonates, support very low levels in adults. The intrinsic capacity of organs to replicate polyomavirus at the adolescent and adult stages may affect persistence and tumorigenesis as well, since tumors develop at these stages. We thus undertook a study of the sites of persistence in BALB/c mice neonatally infected with polyomavirus strain A2. The emphasis was on tumor target organs, and the time frame was extended to the onset of oncogenesis. We report that the mammary glands, skin, and bones are major organs of persistence to a degree and extent equal to those of the previously described kidneys. However, persistence appears to be a necessary but not sufficient condition for oncogenesis in BALB/c mice neonatally infected with wild-type A2.

* Corresponding author. Phone: (517) 353-5014. Fax: (517) 353-8957. E-mail: 21346net@msu.edu.
† Present address: Fred Hutchinson Cancer Research Center, Seattle, WA 98104-2029.

We have also further refined the analysis of the replication potential in various organs following de novo infection as a function of age. The results suggest a strong organ-specific correlation between the capacity for viral genome replication in de novo infection at the adult stage and the development of tumors.

MATERIALS AND METHODS

Virus, mice, and infections. Polyomavirus wild-type strain A2 (12) was grown either in primary baby mouse kidney cells or in mouse NIH 3T3 cells. BALB/c mice were purchased in midpregnancy from Harlan Sprague Dawley. For the persistence study, neonatal mice were infected intraperitoneally with 5×10^6 PFU of A2 within 14 h of birth. At various times, three to six mice were sacrificed. Beginning at 4 weeks postinfection, the remaining mice were monitored weekly for the development of palpable tumors. For the age dependence study, litters of mice were infected intraperitoneally with 1×10^6 to 5×10^6 PFU at various ages. For each time point, five mice were sacrificed 3 days later. This experiment was performed twice.

Quantitation of viral genomes. Viral genomes were quantified as described previously (23). Briefly, mice were sacrificed and organs were removed, homogenized, and incubated overnight in a buffer solution containing proteinase K. For the persistence study, organs from three mice were pooled, with the exception of the 20 weeks postinfection group, from which two pools of three mice each were prepared. For the age dependence study, organs from two mice were processed separately in one case and organs from three mice were pooled together in the other. Total DNA was extracted and isolated by standard techniques. Equivalent amounts of total DNA (5 μg) were used as a means of quantitating the level of viral genomes per equivalent constant amount of organ tissue. DNA samples were digested with the *Eco*RI restriction endonuclease, which linearizes the polyomavirus genome. Digested samples were electrophoresed on 0.8% agarose gels; DNA was transferred to Hybond filters and hybridized to ^{32}P-labeled probes for 72 h at 65°C. After being washed, the blots were exposed to Kodak X-ray film or to Amersham Hyperfilm.

Probes. ^{32}P-labeled probes were synthesized by using the Amersham Multiprime labeling kit and protocol, with linearized A2 genomic DNA as the template. The specific activity of the probes was always higher than 10^9 cpm per μg of DNA.

Virus assay. A portion of the organ homogenate was used to assay virus as follows. Homogenates were sonicated for 1.5 min at full power with a sonic oscillator (250 W, 115 V, 10 kc, 60 cycles). Lysates were then incubated for 15 min at 45°C and centrifuged at room temperature at 2,000 rpm for 20 min. Supernatants were removed and stored at −20°C until use. Titers were determined by plaque assay on NIH 3T3 cells.

PCR analysis. Five micrograms of organ DNA, obtained as described above, was subjected to 30 cycles of PCR with a 20-nucleotide sense primer starting at nucleotide 1542 and a 20-nucleotide antisense primer starting at nucleotide 2981. The amplified fragment contained 1,458 base pairs and encompassed the origin, the middle T-encoding region, and a segment of the late region.

RESULTS

Persistent polyomavirus genomes in organs of mice infected as neonates. The levels of polyomavirus genomes in organs of mice infected as neonates were determined at various times postinfection (see Materials and Methods). Groups of three to six mice were sacrificed during the acute phase (3 and 7 days postinfection) and the persistent phase (5, 10, and 20 weeks postinfection), and selected organs were removed and pooled. Total organ DNA was isolated, treated with the *Eco*RI restriction endonuclease (which linearizes the viral genome), and analyzed by Southern blotting and hybridization to a genomic virus probe. As seen in Fig. 1A, during the acute phase of infection, viral genomes were detected in all six organs examined (mammary glands, spleen, skin, bones [ribs], salivary glands, and kidneys). The level of genomes increased greatly in all organs between 3 and 7 days postinfection. As previously described by others (8, 9, 14, 21), in mice infected as neonates, live virus and viral genome levels increase until about 6 to 7 days postinfection, after which time the immune response clears most of the virus and viral genomes from the infected organs within 1 week. This transition defines the beginning of the persistent phase (at about 2 weeks postinfection). Analysis of late persistence showed a further, slower progressive decrease in the level of genomes between 5 and 20 weeks postinfection. By 5 to 10 weeks postinfection, genome levels were low in all organs, and they reached the limit of detection by Southern blotting by 20 weeks postinfection. With long film exposure periods, genomes could be detected through 20 weeks postin-

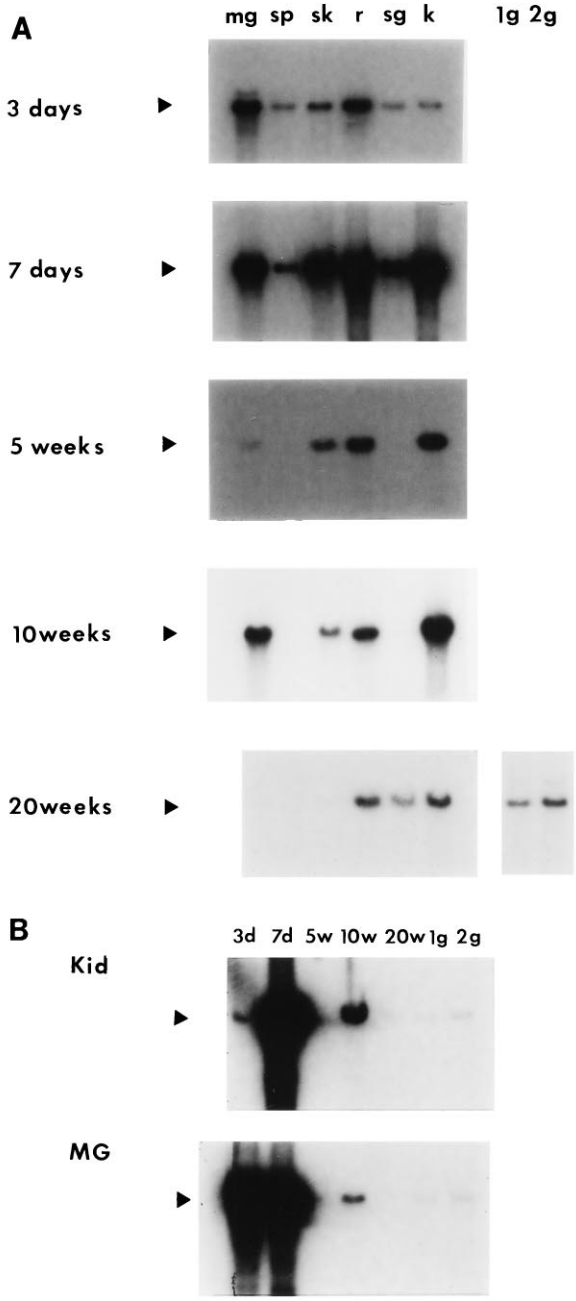

FIG. 1. Polyomavirus genome levels in neonatally infected mice from the acute phase through the persistent stage of infection. Neonatal mice were infected as described in Materials and Methods. At the indicated times, organs from three mice were pooled and processed as described in Materials and Methods. (A) For samples from 3 and 7 days postinfection, Kodak X-ray films were exposed for 2 to 3 days, while samples from 5, 10, and 20 weeks postinfection were exposed for 2 weeks (with Amersham Hyperfilm being used for the 10- and 20-week samples and Kodak X-ray film being used for the 5-week samples). mg, mammary glands; sp, spleen; sk, skin; r, ribs; sg, salivary glands; k, kidney; 1g and 2g, 1 and 2 genomes per cell, respectively. (B) Mammary gland and kidney DNAs from mice sacrificed at the indicated times were electrophoresed on the same gel. d, day; w, week; kid, kidney; MG, mammary glands.

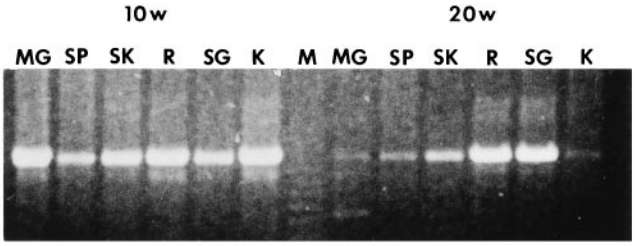

FIG. 2. PCR analysis of DNA from organs of neonatally infected mice sacrificed at 10 and 20 weeks of age. MG, mammary glands; SP, spleen; SK, skin; R, ribs; SG, salivary glands; K, kidney; M, molecular size marker; w, weeks.

fection to an average level of approximately 0.3 to 1 genome/cell in the mammary glands, skin, bones, and kidneys. Persistence in the same organs was also seen in one control and three experimental mice 14 to 17 weeks of age that were used in the pregnancy experiment (see Table 3). Animal-to-animal as well as litter-to-litter variations in the levels of genomes were observed despite good overall reproducibility and consistency as previously discussed (23). For example, viral genomes were detected in the salivary glands in the samples from animals sacrificed at 20 weeks postinfection but not in those sacrificed at 5 and 10 weeks postinfection.

To better compare the number of viral genomes per cell in a given organ as a function of time postinfection, samples from the mammary glands, the major tumor target organ, and the kidneys, previously defined as the major persistence site, were analyzed in a single gel along with the DNA from a cell line containing one integrated genome per cell. As seen in Fig. 1B, high levels of genomes were seen in organs of mice infected for 3 and 7 days, and the level of genomes dropped to the range of one to two copies per cell on average by 20 weeks postinfection. The fact that the level of genomes in the kidney is lower than that in the mammary gland is likely to reflect a delay in reaching the former organ (a secondary replication site), since levels at 7 days are equivalent in both organs.

For a more sensitive, qualitative analysis, the presence of viral sequences from organs of mice sacrificed at 10 and 20 weeks postinfection was examined by PCR amplification. By using polyomavirus-specific primers (see Materials and Methods), viral sequences could be amplified in all the organs tested, including those in which the signal was very low or negative by Southern blotting (e.g., the spleen and salivary glands at 10 weeks and the mammary glands, spleen, and skin at 20 weeks in Fig. 1) (Fig. 2). Although these results are not quantitative, they show that viral genomes persisted in all organs tested through 20 weeks postinfection. These results are summarized in Table 1.

Age dependence of polyomavirus replication during limited de novo infections. To examine the possible relationship between the levels of viral persistence in selected organs of mice at different times postinfection (i.e., at different ages) and the capacities of these organs to support viral replication at the time of the assay for persistence, mice were infected at various ages, from the neonatal through the young adult stage. Previously, we compared the viral replication capacities at two ages only (neonatal and 6-week-old mice) by studying infection of normal neonatal mice and that of athymic nude adult mice (23). In the present experiment, 10 ages were studied and all mice were normal. To avoid the antagonistic effects of the antiviral immune response expected in mice infected beyond 10 days of age, an infection period of 3 days was chosen, after which the mice were sacrificed. A short infection period also has the advantage of avoiding multiple cycles of infection, which might mask differential de novo replication rates in different organs at the same time or in one given organ at different ages. On the other hand, a short infection cycle may lead to incomplete dissemination and hence absence of or partial replication in distant targets. In each of two separate experiments, 9 or 10 groups of normal mice ranging in age from 0 to 48 days were infected. All mice within the same group (two to five mice) originated from the same litter. After the animals were sacrificed 3 days later, specific organs from mice of a given group were analyzed separately (Fig. 3A) or pooled together in the other experiment (Fig. 3B). The emphasis of the analysis was on the set of organs chosen for the persistence study, i.e., the mammary glands, skin, bones [ribs], kidneys, spleen, and salivary glands. For a more quantitative comparison of signal levels in the mammary glands and kidneys, the blots from the experiment shown in Fig. 3A were scanned, and the counts are shown in Table 2. Variations between different animals under otherwise identical conditions within a single experiment were observed. Such variations are more obvious when the overall level of genomes is low (23), as is the case for a short infection and for persistence. Variations are inherent in such complex biological systems. Even in inbred mice there are many observable variations, including the gestation time (which may affect many factors, including the maturity of the immune system) and the size of mice within a litter. A certain amount of subjective interpretation has been used. Our bias is as follows. In general, variations can be placed into three categories: (i) variations between animals involving a single organ, (ii) variations between animals from a single litter involving all organs, and (iii) variations between animals from different litters. The first category of variations

TABLE 1. Polyomavirus signal in organs of neonatally infected mice

Time postinfection[a]	Method of analysis	Signal in:[b]					
		Mammary glands	Spleen	Skin	Ribs	Salivary glands	Kidneys
3 days	Southern	+++	+	++	+++	+	+
7 days	Southern	++++	++	++++	+++++	+++	+++++
5 weeks	Southern	+	0	++	+++	0	+++
10 weeks	Southern	++	0	+	++	0	+++
	PCR	+	+	+	+	+	+
20 weeks	Southern	±	0	0	+	+	+
	PCR	+	+	+	+	+	+

[a] Mice, three per time point, were sacrificed at 3 days, 7 days, 5 weeks, 10 weeks, and 20 weeks postinfection.
[b] For Southern blots, relative signal intensities of films, ranging from 0 (no signal) to +++++ (very strong signal), after 2 weeks of film exposure or longer are indicated. The results of PCR analysis are indicated as follows: +, signal detected; 0, no signal.

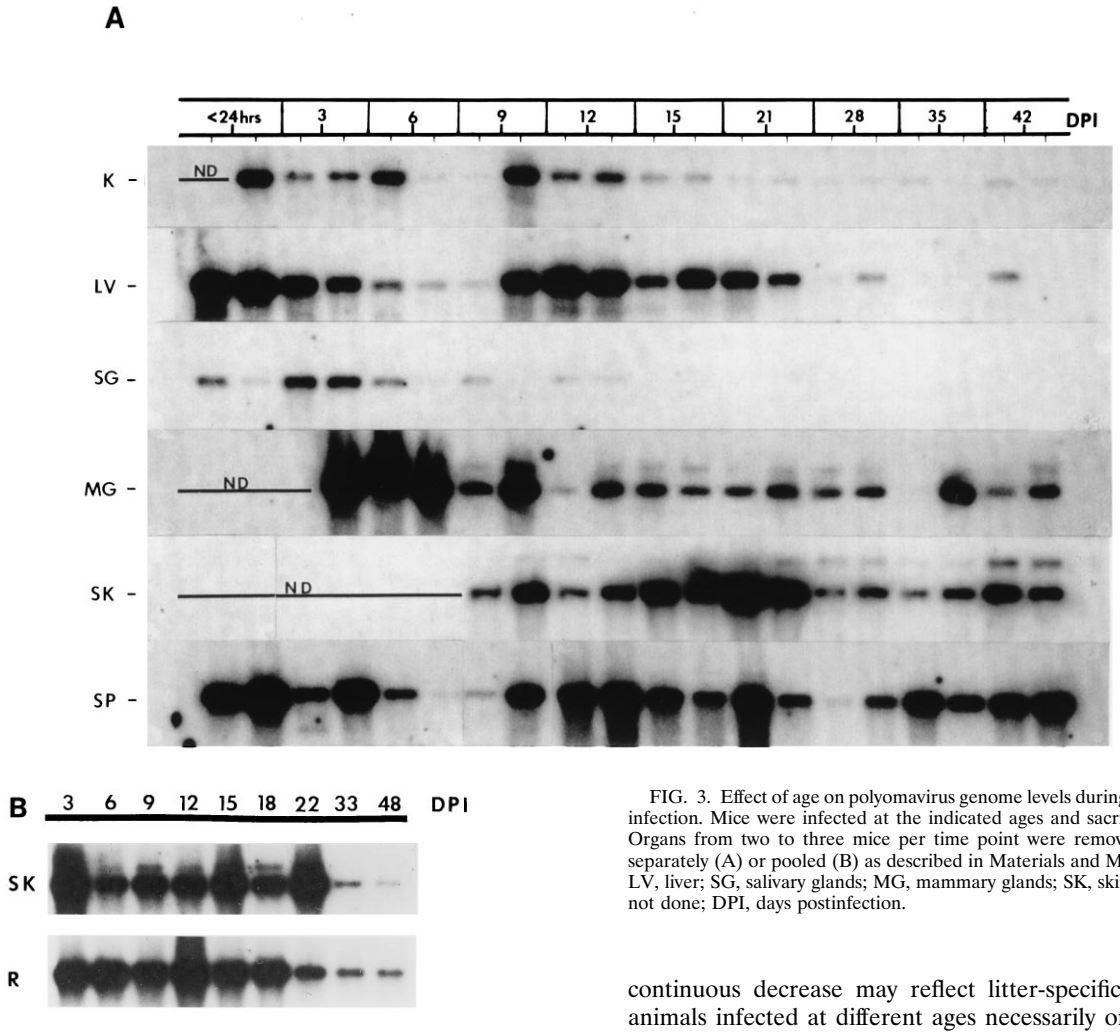

FIG. 3. Effect of age on polyomavirus genome levels during a limited de novo infection. Mice were infected at the indicated ages and sacrificed 3 days later. Organs from two to three mice per time point were removed and processed separately (A) or pooled (B) as described in Materials and Methods. K, kidney; LV, liver; SG, salivary glands; MG, mammary glands; SK, skin; SP, spleen; ND, not done; DPI, days postinfection.

may be due to focal areas of replication. This is very clear in the skin when replication is analyzed by in situ hybridization (see reference 23 for an example). In the second case, the infection dose may be responsible for differences in signals among all tissues of a given animal. The infection inoculum can leak out. We watch for this situation and usually reinsert the inoculum. In addition, there are possible biological variations among animals of a single litter; e.g., often there is a runt. Finally, litter-to-litter variations are only obvious when animals are kept in separate cages. Interestingly, we have found very clear differences for litter-specific effects on oncogenesis (23a). In the present experiments, the levels of viral genomes in organs from mice from the same litter infected at the same age were satisfactorily similar with two noticeable exceptions, i.e., two mice infected at 6 weeks of age and two infected at 9 weeks of age (Fig. 3A). These might be examples of accidental variations in the infection dose. Results from two separate experiments also showed good concordance (Fig. 3A and B).

The major results of this analysis are as follows. First, overall, in all organs tested except the spleen, a decrease in the 3-day accumulation of genomes was seen as a function of age at time of infection. As previously noted (23), accumulation of genomes in the spleen follows a different pattern, with no apparent decrease in de novo replication as a function of age. For the other organs shown, some slight deviations from a continuous decrease may reflect litter-specific effects, since animals infected at different ages necessarily originated from different litters. Second, sharper transition points were apparent, and they occurred at different ages in different organs: an early breakpoint between 12 and 15 days of age was detected in the kidneys, the liver, the skin, and the bones. Beyond 3 weeks of age, the only organs in which the viral genome was replicated to substantial levels were the mammary glands, the skin, and the bones (in addition to the spleen as noted above), confirming our previous results (23).

The decrease in replication potential with age also fits well with age-dependent survival of infected immunoincompetent nude mice. While severe symptoms and death before 1 month

TABLE 2. Polyomavirus genomic sequences in the mammary glands and kidneys of mice infected at ages 1 to 45 days[a]

Organ	Polyomavirus genomic sequences in mice infected at age (days):									
	<1	3	6	9	12	15	18	22	33	45
Mammary gland	ND[b]	17,231	9,695[c]	4,574	1,006	953	979	747	1,295	929
Kidney	2,517[c]	527	807	1,358	744	203	73	31	33	93

[a] Counts (from Fig. 3A) were obtained with an AMBIS Data Analyzer. Unless otherwise indicated, values represent the averages of counts from two organs analyzed separately.
[b] ND, not determined.
[c] Only one organ was analyzed.

of age result when these mice are infected at birth (23), apparently symptomless infection and survival are observed for mice infected at 3 weeks of age (19), and 80% survival is observed in mice infected at 10 or 14 days of age (23b). Thus, 3-day infection of mice between 3 and 48 days of age resulted in high (infection before 3 weeks) or moderate (infection beyond 3 weeks of age) levels of accumulated genomes in the skin, bones, and mammary glands. This suggests that factors required for replicating the viral genome are present in these organs through 48 days of age. In contrast, these factors appear to be missing in the kidney beyond 2 weeks of age, as also suggested by others (2). As discussed previously (23), negative results are harder to interpret, since factors other than the intrinsic capacity to replicate the genome can affect the level of genomes observed in a given organ. This is particularly true in the case of the very short infection period used here, which may be insufficient for substantial secondary replication at distant sites (e.g., the kidneys, liver, and lungs). In contrast, a long infection period in athymic nude mice accentuates the differential in replication between the mammary glands and the kidneys in mice infected at the adult stage, in support of the above hypothesis of an organ-specific differential replication potential at the adult stage (23).

The level of genomes that accumulates in a 3-day de novo infection is higher at all times than the level present in organs of persistently infected mice at the same time. This suggests that the immune response is constantly eliminating infected cells in persistently infected mice.

It should be noted that the relatively high level of genomes seen in the bones, skin, and spleen is in sharp contrast with the negative results reported for the same organs in CBA mice infected for 2 days at 4 to 6 weeks of age with the same viral strain and through the same route of infection (4) and also analyzed by PCR amplification. This may be due to a variety of factors: (i) host differences, (ii) the increase in the infection period from 2 to 3 days in the present experiment, (iii) differences in the infection dose (these cannot be easily compared since different virus assays were used—5×10^6 PFU was used in the present experiment, and 10 to 25 hemagglutination units [or probably 2×10^8 to 5×10^8 physical particles or 2.5×10^6 PFU] was used for the CBA mice), and (iv) differences in detection threshholds.

Assays for live virus were also performed on the same samples (Fig. 4). Although the variations in virus levels were significant, the same general trend was found for live virus as was observed for the level of genomes. The highest levels of virus were found in the skin and bone at all times, with a decrease in mice infected past 3 weeks of age.

Effect of pregnancy on polyomavirus reactivation. The observation of viral persistence in the mammary glands raised the possibility that polyomavirus may be transmitted in the milk. This possibility was also suggested by the fact that progesterone markedly increases the yield of virus in mammary gland cells in tissue culture (18a). Viral reactivation has been previously reported in the kidneys of persistently infected mice during pregnancy, as determined by induction of cytopathology in primary mouse embryo fibroblast cultures (16). As another measure of persistence, we sought to determine whether pregnancy reactivates viral replication in various organs. To this end, 12-week-old female mice, infected as neonates, were mated and then sacrificed at different stages of pregnancy or after delivery of the pups. As a control, one female was sacrificed at the time of mating (Table 3). Very low levels of polyomavirus genomes, similar to those seen in mice sacrificed at 10 to 20 weeks postinfection, were detected in the mammary glands, skin, ribs, and kidneys of this mouse. Analyses of individual organs from two mice and their fetuses sacrificed at midpregnancy, two mice and their pups sacrificed on the day of delivery, and three mice and their pups sacrificed 7 days postdelivery revealed very low or undetectable levels of signal. The results are summarized in Table 3. Virus assays performed at each stage of pregnancy or delivery were negative (data not shown). Thus, in the present conditions, pregnancy did not lead to detectable reactivation of polyomavirus replication. These results vary somewhat from those previously reported by McCance and Mims (16). This may be related in part to the age of the mouse at the time of pregnancy and hence to the level of remaining persistent genomes at that time. The probability of reactivation decreased as a function of age, and hence time postinfection, from 100% in mice mated at 6 weeks of age to 33% in mice mated at 12 weeks and 0% in mice mated at 23 weeks. In the present experiment, mice were mated at 12 weeks postinfection. Furthermore, the experiments of McCance and Mims relied on further extensive amplification of the virus assayed by immunofluorescence for large T antigen in tissue explants after many days of incubation. This manipulation also involves tissue injury and regeneration, which in itself can induce reactivation (2).

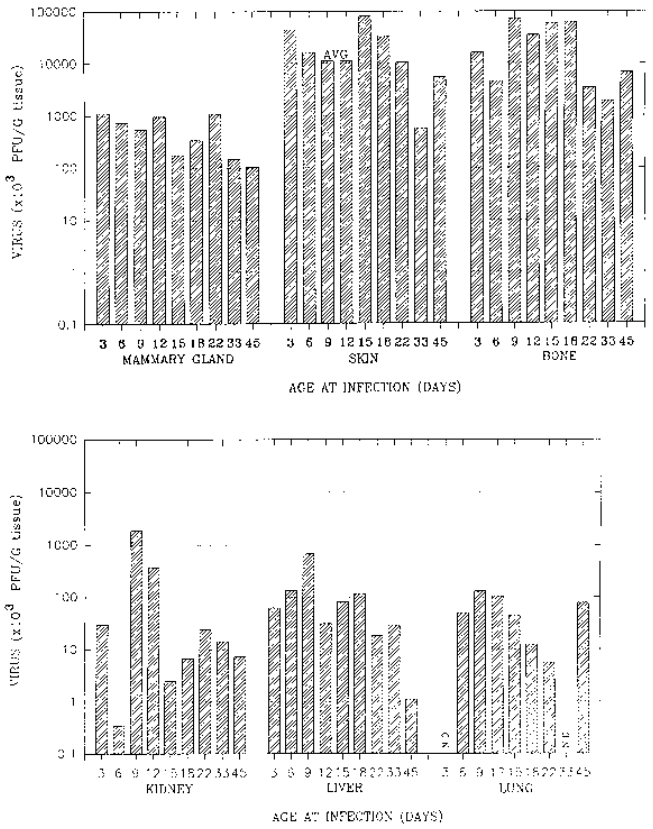

FIG. 4. Effect of age on the production of live polyomavirus in organs of mice infected at different ages. Mice were infected and sacrificed, and organs were processed for the recovery of live virus as described in Materials and Methods. Organ lysates of one-half of the litter (two to three mice per time point) were analyzed for live virus by plaque assays with NIH 3T3 cells. Data are given as PFU per gram of tissue. AVG., average; N.D., not determined.

DISCUSSION

In this report we have analyzed the level and organ distribution of persistent polyomavirus genomes following neonatal

TABLE 3. Polyomavirus signal in organs, fetuses, or pups of mice

Mouse and status	Age at sacrifice (wk)	Signal in[a]:						
		Mammary glands	Spleen	Skin	Ribs	Salivary glands	Kidneys	Fetus or pup
Prepregnant mouse	14	+	+	+	+	+	+	ND[b]
Midpregnant mouse 1	14	+	0	±	0	0	+	ND
Midpregnant mouse 2	17	+	0	+	++++	0	0	0
Mother, 1 day postpartum	17	+	0	+	+	0	++	0
Mother mouse 1, 7 days postpartum	16	0	0	0	0	0	0	ND
Mother mouse 3, 7 days postpartum	16	0	0	0	0	0	0	ND
Mother mouse 2, 7 days postpartum	16	+	0	+	++	0	+	0

[a] Relative signal intensities, ranging from 0 (no signal) to ++++ (strong signal), are indicated.
[b] ND, not determined.

infection of BALB/c mice with an emphasis on a crucial group of tissues—the major tumor target tissues (i.e., mammary gland, skin, and bone)—which had not been analyzed previously. The fate of the virus in these organs was compared to that in the kidney. The kidney was chosen as a reference, since it has been considered to be the major target of polyomavirus replication and persistence to date (14, 21). While all organs examined (mammary glands, skin, bones, kidneys, salivary glands, and spleen) showed moderate to high levels of accumulated genomes during the acute phase (3 and 7 days postinfection), these levels had dropped significantly by the time the persistent phase occurred (followed from 5 weeks postinfection) and continued to decrease during the time course of the experiment. By both Southern blotting and PCR analysis, viral genomes were detected through 20 weeks in all organs tested: skin, bones, mammary glands, kidneys, salivary glands, and spleen. By that time, overt tumors had developed in the mammary glands of female mice and the bones of male mice (23a). Thus, the pattern of persistence in the tumor target organs up to the onset of oncogenesis was found to be very similar to that previously described for the kidney.

Early reports on polyomavirus persistence after infection of normal neonatal mice showed that the kidneys harbored live virus up to 35 days postinfection (14), while virus could be detected in the salivary glands and thymus of outbred mice for up to 120 days (21). Since those reports were issued, it has become clear that a variety of factors, including the strain of virus (18), route of inoculation (7–9), and mouse strain (20), affect which organs become persistently infected and at what level. A high tumor-inducing strain of polyomavirus, PTA, when inoculated subcutaneously into neonatal mice of the highly susceptible mouse strain D10/Bida), led to the persistence of live virus to high titers in the kidney through 50 days (10). High levels of persistent virus in this case may result from the absence in the D10/Bida mice of a specific cytotoxic T-cell clone presumed to be responsible for the killing of polyomavirus-infected cells (11). The viral strain used in the present study, A2, was also shown to persist in the kidneys of intraperitoneally infected neonatal BALB/c or unidentified mice for various lengths of time in some (9) but not other (6) studies. While intraperitoneal injection favored persistence in the kidneys, intranasal inoculation resulted in persistence in the lungs (9). Thus, even when the same virus, mouse strains, and route of inoculation are used, variations in the sites of persistence have been described. The present study establishes that within each given animal unintegrated polyomavirus genomes persist in the tumor target organs at least to the same extent as in the kidneys up to the onset of oncogenesis. This finding suggests that persistence may be necessary for oncogenesis. Since the kidney is not a site of oncogenesis in the case of BALB/c mice neonatally infected with A2, the results suggest that persistence may be necessary but not sufficient for oncogenesis.

The potential contribution of prolonged viral genome replication to persistence was examined by testing the capacity of organs for de novo replication in mice infected for a 3-day period at various ages. The infection period used was too short to result in an antiviral immune response, and it reduced the number of replication cycles. Viral genomes were detected in the mammary glands, skin, bones, kidneys, spleen, and liver in mice infected at all ages up to 45 days. There was considerable variation in the pattern of the accumulated genomes in these organs. However, two clear patterns emerged. First, an overall decrease in the level of genomes was observed in all organs as a function of age at time of infection. Second, the levels of genomes in the mammary glands, skin, and bones of animals infected beyond 3 weeks of age were much higher than those found in the kidneys. Thus, the results imply that the factors required for polyomavirus genome replication are present in the tumor target organs through 48 days of age. Therefore, prolonged replication of the genome may contribute to persistence in these organs. In contrast, persistence of the viral genome in the kidney may be due mostly to survival of the genome or perhaps continued import of sequences synthesized elsewhere and transported by the blood or by components of the immune response. In addition, the continued ability to replicate the genome up to the onset of oncogenesis—termed prolonged replication potential—parallels the tumor-inducing capacity of the virus. These results also correlate with data on the state of the viral genome in tumors; even in the D10/Bida mouse strain, in which kidney sarcomas are observed following infection of neonatal mice with either the PTA or A2 strain, a difference in the state of genomes can be observed (22). In the case of mammary gland and hair follicle tumors, high levels of unintegrated genomes were found, in contrast to renal sarcomas, which contained little or no free DNA and, hence, mostly integrated genomes (22).

It should be noted that we have not strictly shown that persistent genomes are undergoing replication at all times prior to oncogenesis. This conclusion is inferred from the genome replication pattern observed during de novo infections. Two unlikely circumstances would invalidate this assumption: genomes might persist in a state in which they can't replicate (e.g., heterochromatin-like state) and/or only in cells other than those in which replication can take place. The tumors that do arise following persistent infections contain genome levels that are higher than those seen during the persistent phase, implying that persistent genomes can engage in replication and persist in or migrate to cells in which there is potential for viral replication. Reactivation of replication of persistent genomes is also observed following cellular injury (2). During the per-

sistent phase, there must be a dynamic balance between prolonged replication of persistent genomes and elimination of infected cells and virus by the immune system.

The observation that polyomavirus persists and is capable of continued replication in the skin, bones, and mammary glands increases our knowledge of the host range of this virus and provides new insights into the oncogenic process. Heretofore, the limited number of organs reported to support a persistent infection made it theoretically difficult to explain the constellation of polyomavirus-induced tumors. Requirements for both persistence and prolonged genome replication for polyomavirus-induced oncogenesis can be understood in terms of known aspects of the life cycle of the virus. A requirement for persistence is likely to reflect the requirement for the continued presence of the middle T antigen viral oncogene for oncogenesis. Continued genome replication may be a means of ensuring persistence; indeed, with the exception noted above (22), in tumors analyzed to date, the viral genome is present in an unintegrated state (3, 13, 19) even in tumors originating in the presence of the immune response (e.g., tumors observed in the present study [23a]). Alternatively or additionally, a requirement for continued replication may stem from a requirement for high-level expression of the viral oncogene and may be a direct consequence of the well-documented link between viral replication and viral gene expression. Interestingly, at this stage of infection, the viral genome appears to be maintained as an episome, with a steady-state genome copy number (23).

As previously noted, it is interesting that the tumor targets consist of tissues that are still undergoing growth at the adult stage. Nonreplicating cells may be a poor host, especially in infection of animals in which it is unlikely that the multiplicity of infection is even high enough to induce a capsid-mediated mitogenic signal (24). With regard to what specific host factors control the tissue-specific potential for viral replication, little is known at present. At least in some instances, a connection with transcription factors important for viral gene expression has been implicated (1, 17). Clearly, cells in which higher levels of the viral oncogene are expressed may become preferentially transformed to the neoplastic state.

Interestingly, factors that have been shown to affect the organ-specific oncogenic outcome, such as sex, ovarian hormone status, and age at time of infection (13, 19), may also affect the growth state of the target cells. However, while persistence and prolonged genome replication potential may be common requirements for most polyomavirus-induced tumors, it is clear that other factors (e.g., immunological responsiveness) may also greatly contribute to the oncogenic outcome.

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