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Different Roles for Two Enhancer Domains in the Organ- and Age-Specific Pattern of Polyomavirus Replication in the Mouse

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Viral replication in mice infected with murine polyomavirus strains with novel enhancer rearrangements was analyzed by direct in situ hybridization of whole mouse sections and by hybridization of nucleic acids extracted from a specific set of organs. The enhancer rearrangements included a deletion of the B domain as well as duplications within the A domain. Comparisons between enhancer variants demonstrate that the B domain plays an important role in replication in most organs, in particular in the kidney, at the neonatal stage (days 0 to 7 postbirth). In contrast, the B domain is not required in those organs which can sustain replication in the adult, i.e. mammary gland, skin, and bone (class I organs [J. J. Wirth, A. Amalfitano, R. Gross, M. B. A. Oldstone, and M. M. Fluck, J. Virol. 66:3278–3286, 1992]). Altogether, the results suggest that the B and A domains mediate very different functions in infection of mice, controlling the acute and persistent phases of infection, respectively. A model of mouse infection based on the crucial role of differentially expressed host transcription factors is presented.

Murine polyomavirus infects many mouse tissues of both epithelial and mesenchymal origins (9, 12, 13, 37, 48) and induces tumors in a subset of those (3, 6, 8, 9, 18, 19, 39, 42). Such a pleiotropic pattern of replication and tumor induction suggests that the virus has been under selective pressure to interact with a few ubiquitous factors or pathways present in these tissues (8) and/or with a series of tissue-specific factors. By studying mice infected at the neonatal or adult stage (48), we have recently defined an organ-specific and age-dependent pattern of replication for polyomavirus, which can be summarized as follows. In one group of organs (class II [kidney, liver, and lung]), viral replication is very high after infection of neonatal mice but very low after infection of adults, while in the other group (class I [mammary gland, skin, and bone]), replication is also very efficient in neonates and moderately high in adults.

It is presently not clear what determinants of the virus actually control tissue tropism in mouse infections. It might be anticipated that the enhancer region plays an important role in tropism given its potential to bind a plethora of tissue-specific as well as ubiquitous transcription factors (see Fig. 1). This question has been vigorously investigated with tissue culture systems for which the functions of the enhancer domains have been defined. The enhancer has been defined as a 244-nucleotide (nt) fragment between the *BclI* and *PvuII* sites (nt 5023 to 5267) (10, 11, 41) and subdivided by using a convenient restriction endonuclease site into two major domains, A (nt 5023 to 5130) and B (5131 to 5267). These were shown to have redundant activities (21) and to modulate both early transcription and viral DNA replication (11, 20, 30, 31, 40, 41, 43). The A and B enhancer domains play a pivotal role in controlling host range in tissue culture systems, in particular the ability to replicate in cells at different stages of differentiation (1, 21). Studies at the animal level have begun recently with an analysis of sequences required for replication in mice at the acute phase of neonatal infection (36). Nt 5198-5206 (similar to half of a glucocorticoid response element) as well as the B enhancer domain appear to be required for replication in the kidney at this stage (36).

In the present study, we have investigated the organ-specific patterns of replication of viral strains with novel enhancer rearrangements in neonatal and adult infections. As a starting point, we decided to study biologically relevant mutants, i.e., those viable and stable mutants obtained by biological selection. The mutations we chose arose as a compensation for the absence of the polyomavirus oncogene middle T antigen. In these strains, the middle T antigen-responsive elements, PEA1 and PEA3 (45–47) in the A domain, are duplicated, while the non-middle T antigen-responsive elements are sometimes eliminated (7a). Our results suggest that the major control signals for replication at the neonatal and adult stages lie in two separate regions of the enhancer, the B and A domains, the evolution of which corresponds to the emergence of two different phases of polyomavirus infection, i.e., the acute and persistent phases, respectively.

MATERIALS AND METHODS

Viral strains. The enhancer structures of the variants used in this study are shown in Fig. 1 and have been analyzed by others (7a). These enhancer variants were isolated as a *BclI-BglI* fragment from middle T antigen-defective hr-t strains (2, 14) and used to replace the homologous fragment in wild-type (WT) A2 (WTA2) (12).

Infections. BALB/c mice were used. Pregnant mice were purchased from Harlan Industries and infected as described previously (48). Mice that were less than 24 h old were infected intraperitoneally with approximately 1.2×10^6 PFU in 50 μl of the variants under study, by using WTA2 as a reference. For infections at the adult stage, 6-week-old

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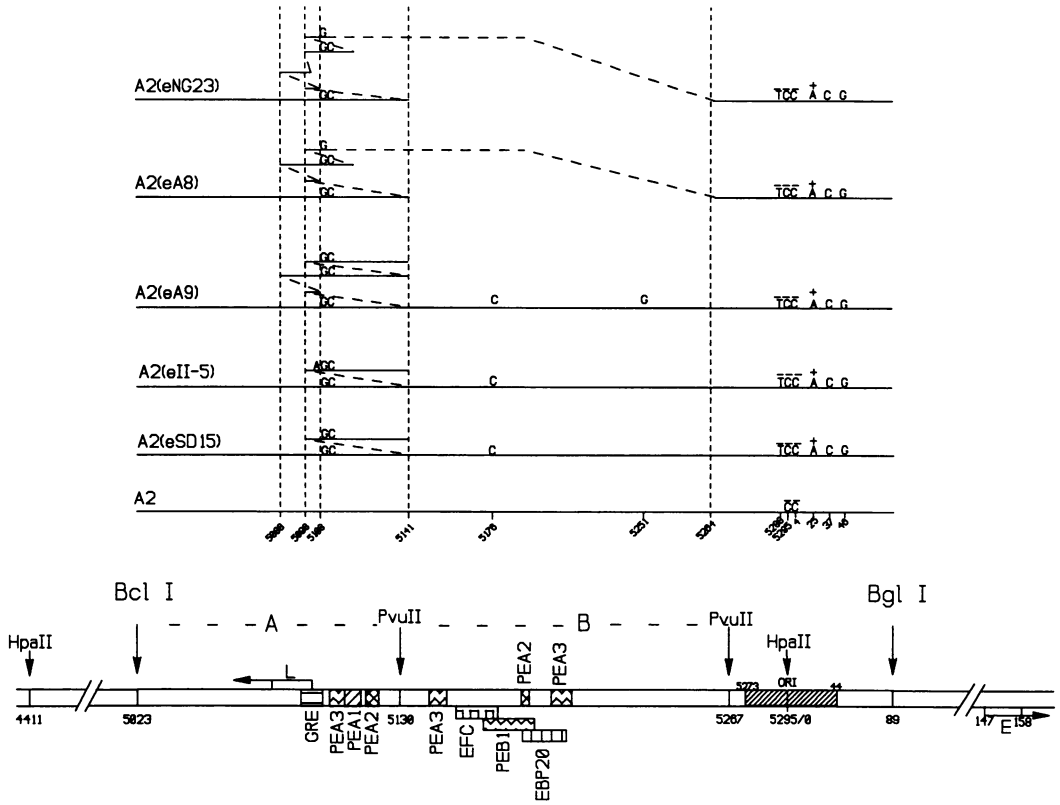

FIG. 1. Physical map of the polyomavirus enhancer and the A2(eX) enhancer variants. The bottom line depicts the enhancer-origin region with the two redundant domains A and B (21, 29, 41, 43), the origin, and the early and late promoters (38). The positions of the following specific transcription factor binding sites are shown: the middle T antigen-responsive (45–47) PEA1 and PEA3 sites (the mouse homolog of AP1 [35] and c-*ets* [45]), and the GRE site (36). Also shown are the following sites in the B domain: PEA3 or EF-1A (5), EF-C (15, 32), PEB-1 (4, 33, 34), EBP20 (16, 22) and PEA2 or PEBP2/3 (23). Nucleotide numbering is that of Salzman (38). The various duplications, deletions, and point mutations of enhancer variants are shown and have been described elsewhere (7a). The end point of the reiterations and deletions is shown on the WTA2 map, as are the positions of point mutations. X^-, one base deletion; X^+, one base addition; X, one base transition or transversion. Note that the C's at positions 5295 and 4 probably represent mistakes in the published WT sequence.

female BALB/c nude (*nu/nu*) animals were used (to bypass the effect of the immune response). These animals were purchased from Life Science and infected subcutaneously similarly to the neonates. All animals were sacrificed at 7 days postinfection for replication studies or at 4 weeks postinfection for persistence studies (neonates only). Cadavers were processed for direct in situ hybridization or organs were removed for extraction of nucleic acids.

In situ hybridization. The protocol for direct in situ hybridization is described elsewhere in detail (48). Briefly, cadavers were embedded in carboxymethylcellulose and frozen. Sagittal sections (40 μm) were collected on tape, fixed, denatured in 95% formamide, and hybridized in high-phosphate buffer with column-purified polyomavirus-specific probe. Sections were washed, stained, and exposed to Kodak X-Omat film with intensifier screens for 24 to 96 h at −70°C. Developed films were directly compared with the stained section to identify the tissues containing virus-specific signals. In some cases, counts which hybridized in a whole section were obtained with a beta scanner (AMBIS).

Organ analysis. The appropriate organs were removed from cadavers immediately following sacrifice and either stored in liquid nitrogen for later processing or placed directly into 1 ml of tissue digestion buffer (100 mM NaCl, 10 mM Tris [pH 8], 0.5% sodium dodecyl sulfate, 0.1 mg of proteinase K per ml). Tissue was homogenized at 30,000 rpm with a Tekmar Tissumizer and digested at 50°C for 12 to 18 h with shaking. Digested tissue was extracted with phenol-chloroform, treated with RNase A (1 μg/ml at 37°C for 1 h), and reextracted first with phenol-chloroform and then with chloroform. Total DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, mixed, and stored overnight at −20°C. DNA was collected by centrifugation at 10,000 rpm in a Sorvall SA-600 rotor and resuspended in 10 mM Tris–1 mM EDTA, and the final DNA concentration was measured by A_{260}. Since total organ DNA was isolated, we assume that comparisons between equivalent amounts of DNA appropriately serve as comparisons between equivalent amounts of organ extracts. Digestions with restriction endonucleases were as described in the figure legends, by using the appropriate reaction conditions.

RESULTS

Replication patterns of novel enhancer variants. (i) In situ hybridization studies. To study the role of enhancer domains in replication in different organs, in particular with regard to class I and class II organs in neonatal and adult mice, we made use of mutant viruses with novel, biologically selected, stable enhancer rearrangements. These rearrangements arose in hr-t mutant strains which harbor deletions in the

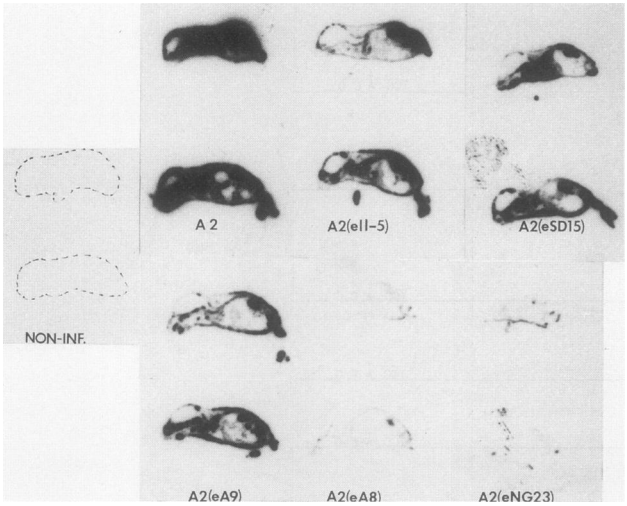

FIG. 2. Replication analysis of enhancer variants in mouse organs by direct in situ hybridization. Neonatal mice were infected as described in Materials and Methods and sacrificed at 7 days postinfection; cadavers were processed for direct in situ hybridization as described in Materials and Methods with a probe corresponding to the whole polyomavirus genome. Sections from two individual mice as well as sections from uninfected mice are shown for each viral strain. Note the complete lack of hybridization to noninfected (NON-INF.) sections.

viral oncogene, middle T antigen (2, 7a, 14). These variants, depicted in Fig. 1, contain reiterations of the middle T antigen-responsive elements (46, 47) within the A enhancer domain, coupled, in two cases, with a 123-bp deletion encompassing the whole B domain. The A domain rearrangements of mutants A8, A9, and NG23 are very similar though not identical. A8 and NG23 share an additional deletion of the B domain. The rearrangements permitted the independent test of both the effect of the A reiterations (comparisons of variant A9 with WTA2) and the effect of the B deletion (comparisons of A8 and NG23 with WTA2). Five such enhancer variants were cloned into the WTA2 background by homologous replacement of the small *Bcl*I-*Bgl*I fragment (Fig. 1). The resulting recombinants were designated A2(eX), where e stands for enhancer and X refers to the viral strain from which the variant enhancer was isolated. These recombinants could not be distinguished from WTA2 in their abilities to grow in NIH 3T3 cells except when in direct competition with WTA2 (7a). In the latter case, mutants with duplications but no deletions replicated to higher levels than the WT, while mutants with a deletion of the B enhancer replicated slightly less well than the WT.

The abilities of these enhancer variants to replicate in neonatal mice were assayed by direct in situ hybridization of whole mouse sections (48), and representative results are shown in Fig. 2. For this purpose, mice were infected within a few hours of birth (as described in Materials and Methods) and sacrificed 7 days postinfection. This time corresponds to the maximal accumulation of viral genomes and shortly precedes viral clearance by the immune system (12, 13). As described elsewhere (48), and in agreement with results of others (12), 7-day infections with the WTA2 resulted in the highest levels of polyomavirus sequences in the kidney, salivary glands, bones, and skin layers; lower levels in the liver, intestines, and lungs; and a notable absence of signal in the brain, muscle, mucosa of the stomach, and bladder. The patterns of replication of viral strains with A domain duplications [A2(eA9), A2(eSD-15), and A2(eII-5)] were similar to that of A2, except for slight decreases in the total amount of sequences hybridizing in each section. In contrast, strains with a deletion of the B domain [A2(eA8) and A2(eNG23)] demonstrated a major deficit in replication compared with WTA2. Quantitation with a beta scanner of the amount of hybridized signal averaged for two sections of independently infected mice showed 15.5- and 13.3-fold overall reductions in A2(eA8)- and A2(eNG23)-infected sections compared with WTA2-infected sections. In contrast, a 2.6-fold reduction was seen with A2(eA9), and smaller effects were seen with simple duplication mutations [A2(eSD15) and A2(eII-5)]. Similar results have been obtained with a minimum of 10 mice each for A2(eSD-15) and A2(eII-5) and up to 16 mice each for WTA2, A2(eA8), A2(eA9), and A2(eNG23) in different experiments. Greater variations from animal to animal were observed with mutants with low replication potentials [A2(eA8) and A2(eNG23)] than with the WT or with mutants with high replication potentials. We conclude from these results that the deletion of the B enhancer domain in strains A2(eA8) and A2(eNG23) results in a major defect in replication in neonatal mouse tissues. In contrast, the effect of a reiteration in the A domain is minor and possibly proportional to the number of reiterations.

(ii) Organ studies. To better quantitate the replication levels observed by in situ hybridization, replication in specific organs was analyzed by extracting total DNA from those organs previously shown to have the highest replication potential (12, 13, 37), i.e., kidney, liver, and lung, or class II organs (48). The DNAs extracted from organs of three to six similarly infected neonatal mice were pooled, the concentrations were normalized, and blot hybridization analysis was carried out (Fig. 3A). Consistent with the results from in situ hybridization (and previous results [12, 13]), infections with the WTA2 strain generated the highest levels of polyomavirus DNA in the kidney, with liver, lung, and spleen exhibiting lower levels (Fig. 3A). Very similar results were obtained with strains A2(eSD-15), A2(eA9), and A2(eII-5) with amounts of polyomavirus DNA equivalent to those for WTA2-infected mice (Fig. 3A). In contrast, a very different pattern was observed with strains carrying a B domain deletion [A2(eA8) and A2(eNG23)] (Fig. 3A). On the basis of densitometric scanning analysis, a decrease in signal in infections with A2(eA8) and A2(eNG23) amounted to at least 30-fold in all four tissues analyzed. Thus, the B deletion profoundly diminished the replication potential in class II organs. Comparisons of the replication of these variants in a larger group of class I and class II organs of neonatally infected mice (pooled from five animals) are shown in Fig. 3B. Hybridized counts were quantitated with a beta scanner, and relative counts are presented in Table 1 and are assumed to approximate relative replication levels. Relative to the replication of WTA2, a small reduction was seen for A2(eA9) in the kidney and the liver. In contrast, a pronounced decrease was observed with A2(eA8) and A2(eNG23) rearrangements in all organs tested. To attempt to separate the effect of the B deletion from that of the A rearrangement(s), the relative replication levels of A2(eA8) and A2(eNG23) versus that of A2(eA9) were computed (Table 1). These results suggest that the B deletion affects replication in all tissues at the time assayed (e.g., in a window between birth and 7 days of age), though replication in the kidney was more affected than replication in bone and in skin.

Replication studies with adult animals. To compare the replication potential in adult animals, which best reveals the

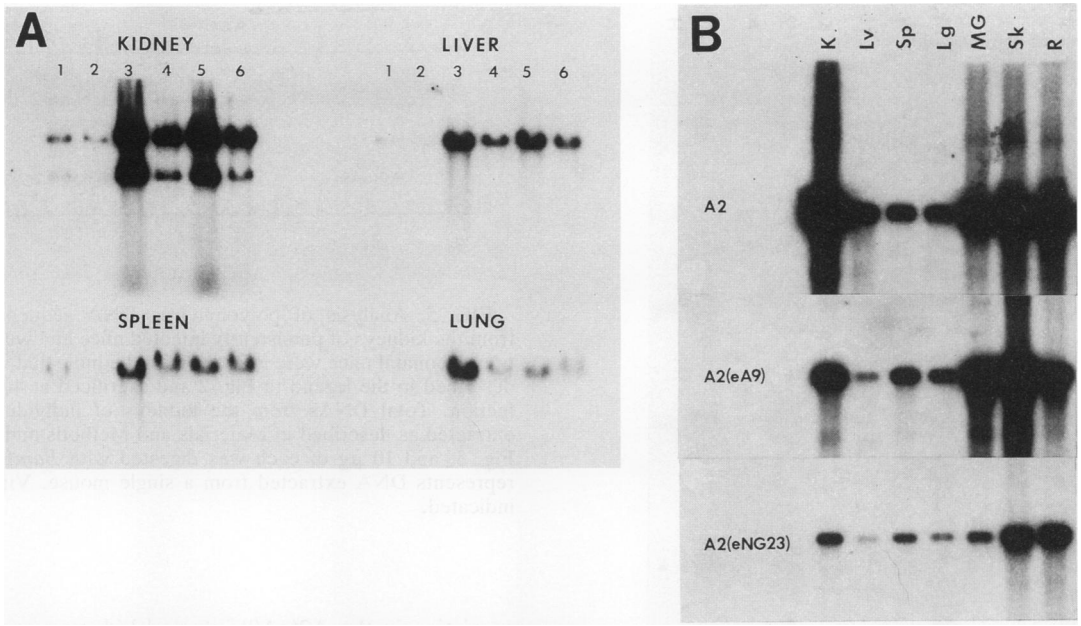

FIG. 3. Analysis of polyomavirus genomes extracted from specific organs of mice infected as neonates. Neonatal mice were infected as described in the legend to Fig. 2 and sacrificed 7 days postinfection. Organs were removed and total DNA was extracted as described in Materials and Methods. Results for pooled organs from five or six animals are shown. Five micrograms of each DNA sample was digested and electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a polyomavirus-specific probe containing the complete viral genome. Panels A and B show results from different experiments. (A) Digestion with *Bam*HI, with a single cleavage site in the polyomavirus genome, thus linearizing the genome. Lanes contain viral strains as follows: 1, A2(eA8); 2, A2(eNG-23); 3, WTA2; 4, A2(eA9); 5, A2(eSD-15); 6, A2(eII-5). The kidney samples yielded partial digestions, with linearized and relaxed circles. (B) Digestion with *Eco*RI, with a single site in the polyomavirus genome. Abbreviations for organs: K, kidney; Lv, liver; Sp, spleen; Lg, lung; MG, mammary gland; Sk, skin; R, rib. Virus strains are designated.

difference between class I and class II organs (48), 6-week-old female immunoincompetent nude (*nu/nu*) BALB/c mice were infected for 7 days and sacrificed. Replication in eight organs was analyzed by blot hybridization, and the results are shown in Fig. 4. Confirming the results presented elsewhere (48), infections with WTA2 yielded no signal in class II adult organs (shown for kidney and liver), while high signal levels were seen in the mammary gland, skin, and bone. Mutants with simple [A2(eII-5)] or complex [A2(eA9)] reiterations of the A enhancer domain showed a pattern similar to that of the WT. In contrast to the results observed in organs of neonates, infections with mutants with a B deletion [shown for A2(eNG23) in Fig. 4A] yielded high levels of signal in adult class I organs. For a better comparison, equal amounts of DNA from the three class I organs from animals infected with A2(eNG23) and WTA2, or A2(eA9) as a control, were compared on the same gel (Fig. 4B). Quantitation obtained with a beta scanner revealed equal hybridization in the mammary gland and skin for WTA2, A2(eA9), and A2(eNG23) and a 2.3-fold reduction in the rib in infection with A2(eNG23) compared with the WT or A2(eA9). Similar results were found in infections of 14-week-old (*nu/nu*) male mice in comparison between WTA2 infections and infections with either of the B deletion mutants [A2(eA8) or A2(eNG23)] (data not shown). We conclude that the rearrangements in strains A2(eA8) and A2(eNG23) profoundly affect the replication potential in neonatal organs, in particular in the kidney, and minimally affect replication in adult class I organs.

TABLE 1. Relative replication levels in neonatally infected organs[a]

Organ	Relative counts of:				
	WT/eA9	WT/eA8	WT/eNG23	eA9/eA8	eA9/eNG23
Kidney	8	117	203	15	26
Liver	7	31	28	4.5	4
Lung	1.3	10	22	7.8	16
Mammary gland	0.8	36	98	43	120
Skin	0.6	11	45	19	77
Rib	1.0	10	33	10	30

[a] Neonatal mice were infected as described in Materials and Methods and sacrificed 7 days later. Organs were removed and processed as described above. Total DNA was isolated, digested with *Eco*RI, electrophoresed, transferred to a nylon membrane, and hybridized to a probe representing the whole polyomavirus genome. The autoradiographs are shown in Fig. 3B. The membranes were scanned with a beta scanner (AMBIS), and, for every organ, the relative level of viral genomes for a given mutant was computed relative to that of the WTA2 or relative to the eA9 mutant, in an attempt to separate the effect of the A reiterations from that of the B deletion.

Persistence studies. Persistence (defined as the infection which remains in the presence of an immune response) in the kidney in long-term infections of neonates was analyzed. Previous studies demonstrated that in neonatally infected mice, the kidney is a major target for polyomavirus persistence (12, 28). The analysis of viral sequences extracted from kidneys of individual mice infected as neonates and sacrificed at 4 weeks postinfection is shown in Fig. 5. In infections with strains WTA2, A2(eSD-15), and A2(eII-5), the level of persisting viral sequences was nearly equivalent. In contrast, infections with strains A2(eA8), A2(eNG23), and A2(eA9) showed a 15-fold decrease compared with the level seen with WTA2. A low level of sequences in long-term

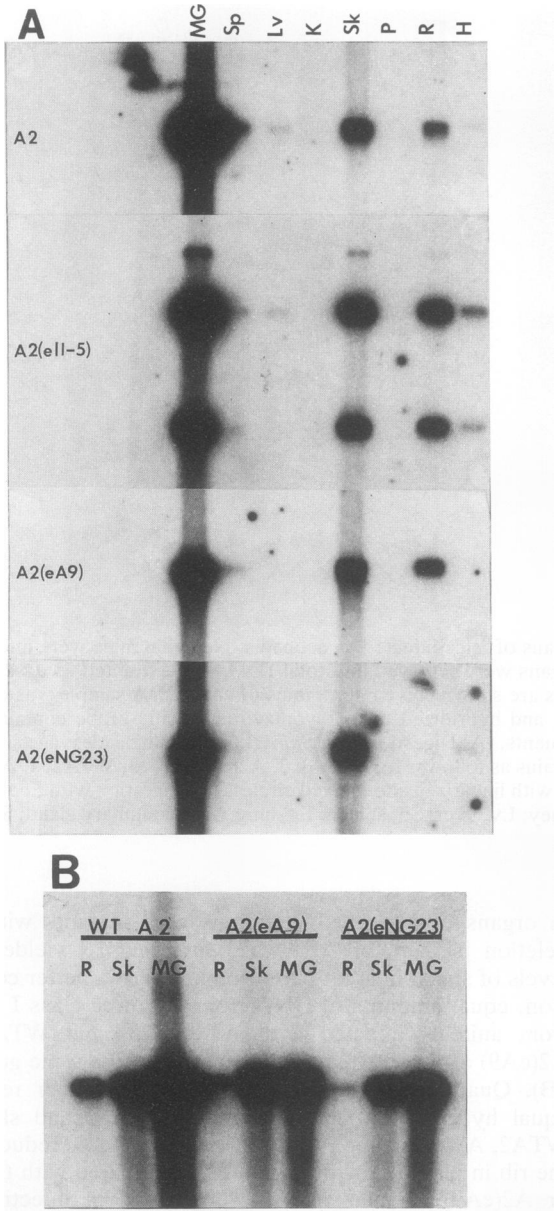

FIG. 4. Analysis of polyomavirus DNA extracted from specific organs of mice infected as adults. Six-week-old female BALB/c nude (*nu/nu*) mice were infected as described in Materials and Methods. Animals were sacrificed 7 days postinfection. Organs were processed and total DNA was isolated as described in the legend to Fig. 3. Results for DNAs from pooled organs from three animals are shown. Digestions were with *Eco*RI. Note that the A2(eII-5) strain contains an additional *Eco*RI site located in the enhancer domain. Abbreviations for organs: MG, mammary gland; Sp, spleen; Lv, liver; K, kidney; Sk, skin; P, pancreas; R, rib; H, heart. Virus strains are shown. (A) Comparisons of four viral strains analyzed in separate electrophoreses. (B) For a more accurate comparison, specific mutants were analyzed in a single electrophoresis.

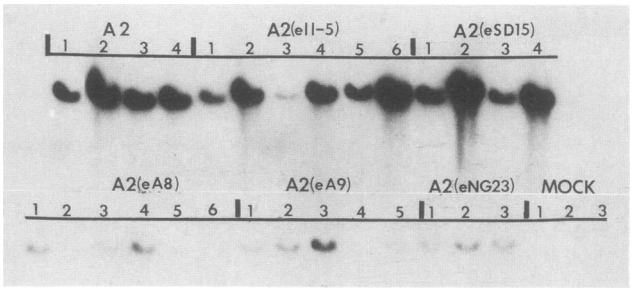

FIG. 5. Analysis of polyomavirus DNA sequences extracted from the kidneys of persistently infected mice at 4 weeks postinfection. Neonatal mice were infected with the indicated viral strains as described in the legend to Fig. 2 and sacrificed at 4 weeks postinfection. Total DNAs from the kidneys of individual mice were extracted as described in Materials and Methods and the legend to Fig. 3, and 10 μg of each was digested with *Bam*HI. Each lane represents DNA extracted from a single mouse. Viral strains are indicated.

kidney infection was the result expected for A2(eA8) and A2(eNG23), since in these infections the levels of viral genomes is already very low before the onset of the immune response and viral clearance. Thus, the low signal level in long-term infections with these strains should not be characterized as a persistence problem. The low level of signal persisting in the A2(eA9)-infected kidney was unexpected, given that a near-WT level of genomes (40%) was observed at 7 days postinfection (Fig. 2 and 3). Since this mutant also replicates to near-WT levels in adult mice, this persistence defect is likely to be due to the accumulated effect of smaller viral bursts over a long period. Whether the reiterations or a point mutation (such as G at nt 5251, which, however, does not affect a known transcription factor binding site) is responsible for the change in the persistence pattern remains to be determined. A negative effect by high repeat copy number of enhancer domains has been documented previously for simian virus 40 (24).

To analyze whether infections of mice selected for new genetic variants, the viral genomes recovered from infected organs were compared with those of the original stocks by restriction endonuclease analysis. *Msp*I was chosen since digestion with this enzyme gives rise to a fragment (no. 3) which contains the enhancer region (Fig. 1) (17) and easily reveals the most common mutations of interest in the enhancer region, i.e., duplications and/or deletions (1). The analysis of the genomes recovered from kidney 4 weeks postinfection is shown in Fig. 6 and demonstrates that a single species of fragment no. 3 of the correct size was recovered for each strain. All *Msp*I fragments were found to be stable throughout the course of this long-term infection. Similar results were obtained in analyses of WTA2, A2(eA8), A2(eA9), and A2(eNG23) sequences recovered from the kidney, liver, lung, mammary gland, skin, and bone in 7-day infections of neonates; the mammary gland, skin, and bone in 7-day infections of adults; and the kidney in 4-week infections of neonates (data not shown). Selection of new deletion or reiteration variants was not observed in any of the infections analyzed.

Finally, we have observed that mice which were infected neonatally with viruses which replicate to high levels [WTA2, A2(SD-15), A2(II-5), and A2(A9)] were more apt to display a previously described runting syndrome (8, 42) than mice infected with A2(eA8) or A2(eNG23) or noninfected mice. Possibly, the high levels of replication in the neonatal kidney tissue damages the tubules (25), allowing accumulation of toxic metabolites in the blood and causing a generalized failure to thrive.

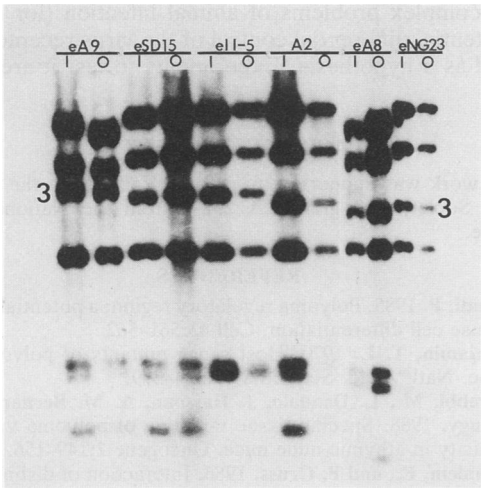

FIG. 6. *Msp*I size analysis of viral DNA isolated from persistently infected kidneys. Mice were infected and handled as described in the legend to Fig. 5. Kidney DNAs from equivalently infected mice were pooled and digested with *Msp*I. Electrophoresis was done in a 2% agarose gel and hybridization conditions were as described in the legends to in Fig. 3 to 5. Five to 10 μg of DNA from pooled samples of A2-, A2(eSD-15)-, and A2(eII-5)-infected kidneys were digested, and 40 to 80 μg from A2(eA8), A2(eA9), and A2(eNG23) samples were used to generate images of comparable intensity. Band distortions due to the high DNA concentrations are visible. To compare the genomes recovered from the infection (output [O]) to that used to infect (input [I]), 200 pg of input viral DNA was mixed with 10 to 80 μg of calf thymus DNA, digested, and run side by side with the recovered (output) DNA. The various *Msp*I fragments are marked (the enhancer rearrangements are within fragment no. 3). Viral strains are indicated.

DISCUSSION

In these experiments, we have made use of naturally selected variants with enhancer rearrangements to begin to analyze the factors involved in the recently reported age dependence and organ specificity of polyomavirus replication in mice (48). Our results show that reiterations within the A enhancer domain had mild, if any, effects, while the deletion of the B domain had a profound negative effect on viral replication during the acute phase of infection in neonatally infected mice. A 10- to 200-fold reduction in replication was seen in all tested organs of neonatally infected mice, the most severe reduction taking place in the kidney. In contrast, replication of B deletion mutants was not markedly affected in three organs during infection of adult mice (class I organs [mammary gland, skin, and bone]). Thus, the B deletion had a stage-specific and organ-restricted effect. Three types of differences between the WT and B deletion mutants (Fig. 1) suggest three possible causes for the replication defect.

(i) There are a number of single base pair additions, substitutions, and deletions in the A2(eA8) and A2(eNG23) viral sequences between the *Pvu*II site at nt 5265 and the *Bgl*I site, on either side of the *ori* core sequences marking the initiation of DNA replication. The facts that other viruses, such as A2(eSD-15) and A2(eII-5), also have these changes and are able to replicate to levels equal to those of WTA2 suggest that these changes are not detrimental to viral replication at least in short-term infections.

(ii) The multiple duplications of sequences around nt 5088 to 5141 found in A2(eA8) and A2(eNG23) may interfere with viral replication, possibly by increasing interactions with negatively acting factors, by creating a new binding site for a negative factor, by preventing synergy between some factors, or by other *cis*- or *trans*-acting effects as previously documented for other similar situations (24). However, the fact that A2(eA9) replicates to levels almost comparable to those of WTA2, with an enhancer region containing duplications nearly equivalent to those found in A2(eA8) and A2(eNG23), argues against this possibility.

(iii) Thus, by elimination, the 123-bp deletion in the B domains of both the A2(eA8) and A2(eNG23) strains appears to be responsible for the loss of replication in neonatal tissues.

A role for the B enhancer domain in kidney replication in neonatal infections has also been demonstrated in a recent study from Villareal's laboratory (36), as has a role for a GRE-like half sequence between nt 5097 and 5105. However, in that case, the loss of B enhancer function could be replaced by duplication of the A enhancer. This is clearly not the case in our study, and the reason for this discrepancy is not clear at the present time. Possibly, a binding site for a positive factor was created in the A duplication studied by the Villareal group (as has been observed in other cases in enhancer duplications of other polyomaviruses [7, 26]). Alternatively, the nucleotide changes or the duplication joints in the reiterated A sequences of strains A2(eNG23), A2(eA8), and A2(eA9) (Fig. 1) may prevent a compensatory effect for the lack of B sequences. Whatever the case may be, in our experiments, two different constructs with triplication of the crucial A domain sequences (the PEA1, PEA2, and PEA3 binding sites as well as the GRE-like element) could not overcome the effect of the B deletion in direct comparisons with two different pairs of strains, in which pair members differed almost exclusively by the B deletion [A2(eA9) compared with A2(eA8) and A2(eA9) compared with A2(eNG23)]. This strongly suggests that a major determinant for replication in neonatal organs lies in the B element. Whether any control site for neonatal replication lies in the A domain in addition to the previously documented GRE sequences (36) remains to be studied.

These results suggest that a cellular factor (or factors) is interacting with sequences between nt 5141 and 5265 of the polyomavirus genome and that this factor facilitates viral replication in these organs at that developmental stage. The nucleotides within 5141 to 5265, deleted in A2(eNG23) and A2(eA8), define one of two major functionally redundant enhancer domains; these sequences are designated as B (as shown in Fig. 1) (11) or further divided into two subdomains: B plus C (43) or beta core plus beta auxillary (30). As depicted in Fig. 1, a number of factors binding to the B domain which saturate the origin-distal half have been described. These include PEA3, also reported as EF-1A, (two binding sites between nt 5144 and 5152 and 5203 and 5152) (5), EF-C (nt 5157 to 5177) (15, 32), PEB-1 (nt 5170 to 5195) (4, 33, 34), EBP20 (nt 5189 to 5210) (16, 22), and PEA2 or PEBP2/3 (nt 5191 to 5198) (23). Of these, it is probably safe to eliminate PEA3 (EF-1A), since this binding site is present in the A domain (34, 40) and is duplicated or triplicated in the eA8 and eNG23 rearrangements. Another unlikely candidate is EBP20, a member of the C/EBP family, since these factors are usually activated concomitantly with cellular differentiation. Indeed, the prototype factor, EBP20, was isolated from adult liver (16). A case can also be made against PEA2, or PEBP2/3, since this factor is presumed to have an inhibiting activity in undifferentiated cells (44); however, recent reports suggest that it is controlled in a complex

manner (23), and it is certainly premature to totally eliminate it at the present time. Thus, this reasoning leaves two factors, PEB-1 and EF-C, present in murine tissue culture cells and so far defined only by their ability to bind the polyomavirus B domain, and possibly PEA2, or PEBP2/3. Other candidates are also possible since, for example, the origin-proximal half of the B domain has yet to be characterized and may also contain important elements; two stretches homologous to the BPV enhancer core can be seen. Whether the same (a single or few) neonatal factor(s) controls replication in all tissues at the neonatal stage or whether multiple tissue-specific factors are involved awaits further dissection of the B domain. As discussed elsewhere (48), it appears that the ability to replicate in the kidney is restricted to an area of the kidney in which tubules still undergo growth and differentiation postnatally. The timing of gene expression controlled by this factor(s) will also be interesting to pursue. In recent experiments, we have shown that the ability to replicate in the kidney drops somewhat abruptly between 12 and 15 days postbirth (26a); this pattern of replication may be correlated to the extinction of a fetal or neonatal factor, such as the B-binding factor candidate. Since overlapping sequences in the polyomavirus enhancer have a *cis*-acting dual role in both viral transcription and DNA replication (11, 30, 31, 40, 43), this factor(s) may regulate early viral transcription, leading to reduced levels of large T antigen and hence reduced DNA replication, the factor may directly control viral DNA replication, or both.

Of great interest is the fact that the B deletion does not noticeably alter replication in the adult mammary gland, skin, and bone (i.e., adult class I organs [48]). Thus, we assume that the major control of replication for the class I adult organs must reside outside of the B domain and hence, by deduction, in the A domain. The A domain has been extensively studied by using tissue culture, and the site for factor binding in this domain are PEA1, PEA2, and PEA3 (27, 35, 40, 45, 46). Of these, PEA1 (the mouse homolog of AP1 [35]) and PEA3 (the mouse homolog of c-*ets* [46]) are particularly interesting, since these are middle T antigen-responsive elements (45–47).

In summary, the results presented above and elsewhere (48) suggest that the neonatal and adult phases of murine polyomavirus replication are controlled in a distinct manner, in part by making use of differentially expressed host transcription factors. A simple model of the infection of mice compatible with the data available at the present time emerges. An acute infection is observed in neonates, accompanied by viremia and very high levels of replication in most organs. This phase appears to be overcome not only by the onset of the immune response to the virus but also by the dependence of replication, mediated by the B enhancer domain, on a ubiquitous transcription factor (or a set of tissue-specific factors) which is expressed at the neonatal stage but whose expression or activity is turned off before adulthood. However, the virus has also evolved with an independent ability to replicate at the adult stage (albeit at more moderate levels); the adult replication potential presumably contributes to the ability of the virus to persist following neonatal infection. Control of the adult replication phase is mediated by the A enhancer domain and makes use of transcription factors which are present (expressed or activated) at the adult stage in a restricted group of organs (class I). Since these factor candidates are responsive to the viral oncogene, middle T antigen, this model proposes that one of the major roles for middle T antigen in the infection is to mediate persistence. This overly simplified model ignores many complex problems of animal infection (for example, the potential differential control of the virus receptor) and is offered as a hypothesis. Experiments to test it are in progress.

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