Detection of DNA and RNA Virus Genomes in Organ Systems of Whole Mice: Patterns of Mouse Organ Infection by Polyomavirus

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A technique which detects viral DNA or RNA in situ in the organ systems of whole mice is described. Frozen thin sections from whole mice were transferred directly to nitrocellulose and hybridized to labeled viral DNA, allowing the detection of viral DNA or RNA. By this procedure, polyomavirus infection of newborn mice inoculated intranasally was followed. We found that the initial inoculum could be detected in the nasal cavity, lungs, and stomach lining after a 5-h absorption period. Primary replication of virus was observed in the nasal cavity, submaxillary gland, and lungs, followed by a systemic phase of infection in which the liver, spleen, kidney, and large colon also became infected. Viral RNA as well as DNA could also be detected as shown by infecting mice intracerebrally with vesicular stomatitis virus. Vesicular stomatitis virus-specific RNA was observed only in the brains of these mice. It is most likely that this technique can be applied to general molecular studies of mice. With this method we should be able to detect all viruses, bacteria, plasmids, and organ-specific transcripts to which a cloned probe exists.

A variety of methods have been used to study the pathogenesis of viral infections in vivo in experimental animal models. Such methods have primarily been directed at locating and quantitating infectious virus and viral antigens. The most quantitative and sensitive of these techniques include (i) virus infectivity titrations from tissue suspensions (assayed by plaquing on susceptible monolayers) (1, 11, 12, 17, 18), (ii) virus antigen localization in tissue sections (assayed by immunofluorescence or immunoperoxidase) (11, 12, 14), and (iii) virus particle localization in tissue sections (assayed by thin-section electron microscopy) (4, 13). Viral nucleic acid can be localized by Southern blot analysis of extracted tissue DNA (6, 10). None of these techniques, however, provide a complete representation of the amount and location of viral genomes within all of the organ systems of the whole animal.

We developed a technique which allows the detection of viral DNA or RNA sequences in situ in the organ systems of whole mice. The use of hybridization of virus-specific nucleic acid probes with frozen sections of whole mice transferred to nitrocellulose has allowed us to quantitate viral genome accumulation in target organs and to combine topological and temporal characteristics of the course of infection. This procedure was designed to compensate for some of the inherent limitations of the previous virus detection assays, since it has been difficult to follow the actual sequence of tissue involvement during viral pathogenesis. The sensitivity of the technique allows detection of initial virus replication sites when viral antigen and infectivity levels are below the limits of detection of the classical virus detection methods. Although immunofluorescence can be applied to whole organs, it is of lower sensitivity than nucleic acid hybridization. Additionally, some viruses appear to be restricted in protein expression during persistence, thereby limiting the general applicability of immunofluorescence, as is the case with persistent lymphocytic choriomeningitis virus infection of mice (14). Virus plaque assays, on the other hand, can only detect infectious virus; during persistent infection virus

may not be infectious, as seen in mice persistently infected with polyomavirus (Py virus) (10).

We applied the whole-mouse hybridization technique to both Py virus (DNA genome), and vesicular stomatitis virus (VSV) (RNA genome) infection of mice. With Py virus infection, the virus inoculum and primary and secondary viral replication was followed. Viral RNA replication in the brain was followed in VSV infection.

MATERIALS AND METHODS

Cells, viruses, and inoculations. The protocol for Py virus propagation on mouse 3T6 cells has been described by Eckhart (3). Cells were grown in the presence of 7% fetal calf serum and nonessential amino acids in Dulbecco medium and maintained in 2% fetal calf serum during virus infection. Titers of ca. 10^{10} PFU/ml were normally obtained. The growth of Indiana serotype VSV on baby hamster kidney (BHK) cells was as described previously (19).

BALB/c mice were inoculated intranasally with 10^8 PFU of Py virus within 24 h of birth. Infection of baby BALB/c mice with VSV was by intracerebral inoculation with 5×10^3 PFU.

Transfer of whole-mouse section to nitrocellulose. Figure 1 shows a schematic representation of a whole-mouse-section transfer to nitrocellulose. Newborn mice were infected with Py virus by intranasal inoculation (Fig. 1, panel 1). Mice were then frozen in O.C.T. (Tissue Tek-II) in a specimen mold over dry ice (Fig. 1, panel 2). For adult mice, the fur, skin, and teeth were removed by dissection before the mice were frozen because these tissues interfered with sectioning. Mice were sectioned to the desired depth at -15 to -20°C . For sectioning, a large sliding sledge microtome (model 860; American Optical) with a 250-mm cryostat blade (C. L. Sturkey, Mfg.) (Fig. 1, panel 3) was used. At the desired plane, a photograph may be taken of the section to be transferred. Untreated nitrocellulose (Schleicher & Schuell Co.) was applied to the top of the mouse-O.C.T. block, followed by layering of 3 to 4 lantern glass plates, prewarmed to 45°C , on the nitrocellulose for ca. 15 to 20 s or until the nitrocellulose became attached to the mouse-

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FIG. 1. Schematic representation of the whole-mouse hybridization technique and transfer of the mouse section to nitrocellulose. Panel 1 shows intranasal infection. Panel 2 shows the mouse cast into frozen O.C.T. Panel 3 shows the sectioning of the mouse to the desired plane. Panel 4 shows the attachment of the nitrocellulose to the face of the section. Panel 5 shows the cutting of the nitrocellulose that adhered to the section.

O.C.T. block (Fig. 1, panel 4). It is important that the glass plates be kept on the mouse-O.C.T. block for a minimum time to preserve the integrity of the section. The block was then cooled, the microtome blade was lowered, and a 40- μ m section was cut. The nitrocellulose with the adhered mouse section was then peeled off from the microtome blade (Fig. 1, panel 5).

Whole-mouse-section hybridization. For detection of Py virus DNA, sections were fixed to the nitrocellulose by blotting the nitrocellulose (section side up) onto 1.0 M NaOH with 1.0 M NaCl-soaked 3MM paper (Whatman, Inc.) as described previously (21). The alkali was then neutralized with 1.0 M Tris (pH 7.5)–1.0 M NaCl, air dried, and baked for 2 h in a vacuum at 80°C. Both prehybridization and hybridization was for 24 h at 45°C in 50% formamide–5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% dextran sulfate–0.3% sodium dodecyl sulfate–2 $\times$ Denhardt (2) (1 $\times$ Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone)–200 μ g of calf thymus DNA per ml. The hybridization solution in addition included 10^6 cpm (per mouse section) of 32 P-labeled, nick-translated (15), genomic-length Py virus cloned into pBR322 at the *Bam*HI site with a minimum specific activity of 10^8 cpm/ μ g of DNA. Washing was done four times in 2 $\times$ SSC (pH 7.5) with 0.1% sodium dodecyl sulfate for 5 min at room temperature, followed by two times in 0.1 $\times$ SSC–0.1% sodium dodecyl sulfate for 30 min at 50°C. Dried mouse blots were exposed to Kodak XAR-5 film at room temperature for the times indicated in the figure legends.

To detect viral RNA, sections were fixed to the nitrocellulose by blotting the nitrocellulose (section side up) onto 3MM paper soaked with an RNA fixing-denaturing solution. This solution is a modification of a previously described method (5, 8) and consists of 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 10 mM sodium acetate, 1 mM EDTA, and 1% Nonidet P-40. The sections were then blotted onto 3 $\times$ SSC-soaked filter paper, air dried, baked, hybridized, washed, and exposed as described above. The probe was 32 P-labeled, nick-translated VSV glycoprotein G cDNA cloned into pBR325 at the *Eco*RI site and was kindly provided to us by John Rose (Salk Institute).

Southern blot analysis of tissue DNA. Total tissue DNA

was extracted with modifications of techniques described previously (10) from the organs and at the times indicated (see Fig. 4). Nuclei were first isolated from homogenized tissue in reticulocyte standard buffer (0.25 M sucrose, 25 mM NaCl, 5 mM MgCl₂, 10 mM Tris [pH 7.5]) with 0.2% Nonidet P-40. Nuclei were then treated for 1 h each with RNase at a concentration of 10 μ g/ml at 37°C and then proteinase K at a concentration of 100 μ g/ml at 50°C. This treatment was followed by extraction with phenol-chloroform (1:1, water saturated). The aqueous phase was dialyzed extensively against 10 mM Tris (pH 7.5)–1 mM EDTA (pH 7.5) buffer and concentrated within the dialysis tubing by being covered with dry polyethylene glycol (Baker's 8000). Approximately 10 μ g of DNA was digested with *Bam*HI (which cuts Py virus DNA once) according to the conditions of the supplier, run on a horizontal 1% agarose gel, and then blotted to nitrocellulose as described previously (20). Hybridization and washings were also as described above. Py virus probe (10^6 cpm per lane) was used for hybridization. Dried filters were exposed to Kodak XAR-5 film at –70°C for 24 h in the presence of a Du Pont Lighting-Plus intensifying screen.

RESULTS

Whole-mouse hybridization of Py virus-infected mice. Py virus was administered intranasally to newborn mice within 24 h of birth. The location of viral DNA was determined by thin-section hybridization after a 5-h absorption period and also at 3 days postinfection (dpi). The results of this analysis are shown in Fig. 2. Uninfected control thin sections showed no background hybridization (data not shown), and nonspecific hybridization with the infected mouse sections was not seen. After a 5-h absorption period, the virus inoculum was easily detected in the nasal cavity, lung, and stomach lining. Primary replication of virus is shown in the 3-dpi panels (Fig. 2). At this time an increase in signal of ca. 10-fold above that of the inoculum was seen in the lung, indicating primary viral replication. No primary virus replication occurred in the

FIG. 2. Whole-mouse hybridization of newborn mice infected intranasally with Py virus. Mice, after a 5-h absorption period (A) or at 3 dpi (B), were transferred to nitrocellulose and hybridized as described in the text. Results at (a) 14 and (b) 48 h of exposure of the autoradiogram. The broken line represents the perimeter of the mouse section.

stomach or gut. The absorbed virus detected after 5 h was lost by 3 dpi. The strong signals detected in the submaxillary gland and nasal cavity at this time also indicate additional sites of primary viral replication. No adsorbed virus inocula was detected in the submaxillary gland, and the signal in the nasal cavity at 3 dpi was clearly increased over the 5-h-absorption-period section. Viral DNA was also detected in the trachea and was seen leading from the nasal area to the lung at 3 dpi in the 48-h-exposure panel (Fig. 2). It is clear that even though the virus inoculum gained access to both the alimentary canal and the respiratory tract, primary replication of virus occurred only in the respiratory tract and associated tissues of the oral cavity.

Systemic infection was seen by 6 dpi and is shown in Fig. 3. The liver, kidney, spleen, and large colon and rectum became infected, in addition to those organs involved at the primary phase of viral replication. Infected splenic tissue was not included in the sections shown. Organs were identified by inspection of the various section planes. The 24-h-

exposure autoradiograms reveal a predominance of viral DNA in the lung and liver, indicating that viral replication is most active in these tissues at this time. In addition, the uniform consistency of the signal in the lung and liver suggests that Py virus can replicate throughout these tissues. A 10-fold increase from 3 to 6 dpi in the amount of viral DNA found in the associated tissues of upper respiratory tract tissues (nasal cavity, submaxillary gland, and trachea) also indicates viral replication in this area. The kidney and large colon and rectum also showed ongoing viral replication at 6 dpi. The signal from the kidney at 6 dpi (more clearly shown in Fig. 3, right panel), did not appear throughout the organ, suggesting foci of viral DNA; localization of Py virus within the kidney has been observed by others (9).

Reconstruction experiments in which 10 pg of Py virus DNA was spotted onto nitrocellulose in a 20-mm² area and hybridized under conditions identical to those used in whole-mouse hybridization gave a peak optical density at 570 nm of 0.30 absorbance units above background autoradiographic

FIG. 3. Whole-mouse hybridization at 6 dpi. The photographs at the top of the figure are the actual faces of the sections used for hybridization before sectioning. Section is (left panel) proximal to the left side of mouse below the spleen and (right panel) distal to the left side of mouse. The autoradiographs directly beneath the photographs were exposed for 24 h. The autoradiographs at the bottom of the figure were exposed for 72 h. Organs were labeled as follows: N, nasal-oral cavity; S, submaxillary area; Lu/i, lung and liver; K, kidney; C, large colon; Lu, lung; and Li, liver.

signal after a 96-h exposure period. This is equivalent to 0.5 pg of DNA per mm².

Southern blot quantitation of Py virus DNA. The quantitation of viral DNA at 3 and 6 dpi, as detected by blot analysis, is shown in Fig. 4. These times encompass the primary and secondary phases of virus infection. A reconstructed control track was included, and autoradiograms were exposed for various times. The lane containing results for undigested lung at 6 dpi shows that Py virus exists autonomously as free super-coiled DNA. From densitometer readings of these data, we estimated the average copy number of Py virus DNA to be 10 copies per cell at 3 dpi and 1,000 copies per cell at 6 dpi in the lung and 10 copies per cell in the kidney at 6 dpi.

Detection of an RNA virus by whole-mouse hybridization. Since the whole-mouse hybridization method was successful in detecting the presence of and replication from a DNA virus genome, the ability of this method to detect replication of an RNA virus was then tested. Newborn mice were infected intracerebrally with 5×10^3 PFU of Indiana serotype VSV and sectioned at 24 h postinfection. The nitrocellulose sections were hybridized to ³²P-labeled cloned cDNA of the VSV glycoprotein gene. Figure 5 shows the results of this experiment. The sections were first fixed under various conditions to determine which procedure gave the strongest intensity of signal after hybridization. We then tested whether formaldehyde fixation or formamide denaturing or both were needed to detect RNA. From Fig. 5, it is apparent that only Nonidet P-40 is necessary; denaturing with formaldehyde or formamide or both did not enhance RNA detection. In fact, the signal in the section treated with Nonidet P-40 alone was at least twice the intensity of the sections treated with additional RNA-denaturing reagents. The uninfected controls showed no background hybridization; in addition, the signal could be eliminated by RNase treatment of the section before hybridization (data not shown), further establishing that RNA was detected.

DISCUSSION

Both RNA and DNA virus genomes can be detected by this whole-mouse hybridization procedure, making it appli-

cable to the detection of all virus infections of mice. Since the actual sequence of appearance of viral nucleic acid can be followed, this technique is a valuable tool for studying viral pathogenesis. The question of how a virus gains access to its host can be directly answered by allowing mice to naturally acquire an infection and monitoring them for viral nucleic acid by whole-mouse hybridization. With respect to our Py virus-mouse system, it is generally accepted that virus entry occurs via a respiratory rather than an alimentary route, since a successful infection can be established with a lower amount of virus in the respiratory tract (17, 18). However, because Py virus is somewhat resistant to the adverse conditions present in the gut, it was not clear whether primary replication could also be occurring in the alimentary canal as with adenovirus (16). It is apparent that although the inoculum is absorbed in both the alimentary and respiratory tracts, primary viral replication occurs only in the respiratory tract and associated tissues, thereby establishing the portal of virus entry.

Both the temporal and the tissue pattern of systemic viral infection can also be determined by the whole-mouse blot. In Py virus-infected mice, the upper respiratory tract, lung, liver, kidney, and large colon all show viral DNA during the systemic phase of infection (6 dpi). At this time viral replication predominates in the lung and liver, showing quantitatively in which organs virus replication is most successful. In addition, this method also detects focal virus replication occurring within the kidney.

The probable portals of virus exit from the host mouse were also apparent. The large colon and rectum transiently show a large amount of viral DNA, implying that virus may be shed into the feces. The lungs also contained high levels of viral DNA, implying that this previously unrecognized site is a probable portal of virus exit. It is also clear that viral DNA is present in the kidney, and this result is in agreement with previous reports that Py virus is shed into the urine (13, 14). Our method, therefore, is clearly able to identify the route of viral entry and the primary and secondary organs of virus replication. The blot hybridization data with total extracted tissue DNA (Fig. 4) confirm the results seen in the mouse blots, although the mouse blots provide a

FIG. 4. Southern blot hybridization with extracted tissue DNA from Py virus-infected mice at 3 and 6 dpi. Newborn mice were infected as described in the text. The Py virus marker lanes are labeled M. Lung^a was not digested; all other tracks were digested with *Bam*HI. Exposure was for 24 h.

FIG. 5. Whole-mouse hybridization of VSV-infected mice. New-born mice were infected intracerebrally with VSV as described in the text. The composition of the fixing-denaturing solution was as follows: A, 1% Nonidet P-40 (NP40); B, 1% NP40-7% formaldehyde; C, 1% NP40-50% formamide; and D, 1% NP40-50% formamide-7% formaldehyde. Exposure time was for 12 h.

clear topographical view of the actual movement of the virus.

The sensitivity of this method has been estimated by several approaches. Reconstruction experiments with a ^{32}P -labeled probe with a minimum specific activity of 10^8 cpm/ μg of DNA indicated that 1 to 10 copies of viral DNA per cell can be detected in a 1-mm^2 area. This is equivalent to ca. 0.1 pg of viral DNA in ca. 10^4 cells. By Southern blot hybridization with total extracted tissue DNA, we have determined that at 3 dpi 10 copies per cell of Py virus DNA are present in the lung. At 6 dpi, 1,000 copies per cell are present in the lung, and 10 copies per cell are present in the kidney (2a). These levels of DNA were easily detected by the mouse blot hybridization and establish that our method can indeed detect viral DNA at 10 copies per cell.

In addition, it is known that viral DNA can be detected in the absence of detectable plaque-forming ability (10). Recently, we have successfully detected Py virus DNA in persistently infected kidneys by our method (T. W. Dubensky and L. P. Villarreal, manuscript in preparation).

It is probable that this technique can be used for mapping viral transcription from a DNA genome. When RNA is to be detected, the nucleic acid is not denatured and the sections can be digested with DNase, thereby minimizing the hybridization from the double-stranded DNA template. It should be possible, therefore, to map the levels of gene expression of Py virus in any tissue during infection. It also seems likely that the mapping of organ-specific expression of various host cellular genes should be possible with this technique, making it generally useful for molecular studies of gene expression in whole mice. An additional application could be to characterize bacterial and viral flora. This would be especially useful for identifying sequences such as bacterial plasmids which may be involved in pathogenesis.

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