Dissecting the Molecular Anatomy of the Nervous System: Analysis of RNA and Protein Expression in Whole Body Sections of Laboratory Animals

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Nucleic acid hybridization and protein blotting procedures have allowed the specific detection of both RNA and protein gene products in whole body sections of a selected host. These procedures permit efficient and reproducible screening of both endogenous and exogenous (viral) gene products, thus facilitating the study of normal differentiation, the localization of virus and the monitoring of viral diseases. Although success with RNA hybridization has thus far been limited to exogenous viral gene expression, the protein immunoblotting procedures have the sensitivity to detect endogenous protein products with high resolution. These combined procedures should prove useful for the study of protein expression in numerous developmental systems.

INTRODUCTION

Specific reagents are now available for tracing normal and aberrant patterns of gene expression. Nucleic acids (genes and messenger RNAs) can be identified in hybridization reactions with labeled probes10 and proteins can be detected with antibodies to predetermined amino acid sequences10 or polyclonal and monoclonal antibodies. These reagents have contributed significantly to the current understanding of gene regulation. Here we present results derived from examining whole body sections of mice — a powerful new approach to the analysis of gene expression in diverse anatomical locations of individual animals. In particular, the high degree of resolution and sensitivity permits detection of proteins in a single continuous plane throughout nervous system tissue.

MATERIALS AND METHODS

Detection of nucleic acids and proteins in whole-body sections

Laboratory animals were anesthetized with ether, exsanguinated, cleared of fur by shaving, embedded in 3.5% carboxy methylcellulose (CMC) and frozen by immersion in dry ice-ethanol. The CMC blocks were placed at -30 °C and maintained at this temperature before sectioning. Since loss of antigenicity was observed with certain endogenous antigens upon extended storage, sectioning was usually performed within 48 h after embedding. The blocks were mounted, trimmed and cut in a LKB cryostat, Model 2258, until the appropriate plane for sectioning was obtained. At that point 40 µm sections were cut, collected on 3M scotch tape (No. 688) and used for either nucleic acid hybridization or protein detection as illustrated in Fig. 1. The procedure followed for nucleic acid hybridization has been recently described12. Briefly, the sections were air-dried, fixed by immersion in freshly prepared 4% paraformaldehyde, washed in phosphate buffered saline (PBS), air-dried and hybridized in Seal-a-Meal bags. Prehybridization was performed at 37 °C for 12 h in 50% deionized formamide, 5 × SSC, 2.5 × Denhardt’s solution with 100 µg/ml1 boiiled, sonicated salmon sperm carrier DNA. Hybridization was performed at 37 °C for 24–72 h, depending on the specific activity.
Fig. 1. A diagram of the procedure used for the molecular detection of nucleic acids or proteins in whole animal body sections. See Materials and Methods for details.

and base pair size of the probe used. $^{32}$P-Labeled nick translated DNA probes of 200–1600 base pairs with specific activities of $1\times 10^6$ cpm/µg of DNA were used. After hybridization, the sections were washed initially with $2\times$ SSC at 37 °C and then with $0.1\times$ SSC at 55 °C, stained with hematoxylin-eosin and exposed at −70 °C using Kodak XAR-5 film and a Cronex lightning fast intensification screen.

To detect proteins, the 40-µm section picked up, as above, on scotch tape, was transferred immediately to Pall Biodyne nylon membrane (0.2 µm, Figs. 1 and 2). The sections were allowed to passively transfer in this manner for 15–30 min. The membrane strips, with the tape still adhered, were then immersed in 70% Laemmli buffer, $(25\ mM\ Tris\-HCl, \ pH\ 8.3,\ 192\ mM\ glycine)$, 30% methanol for 15 min; transferred to 10% acetic acid, 10% methanol for 30 min; and rinsed in deionized water. At this point the tape easily peeled off and excess tissue was removed from strips by carefully scraping with a glass slide. If long-term storage of sections was desired, the strips were air-dried at this stage and stored at −30 °C. Strips have been stored in this manner for several weeks with no decrease in signal. Before treatment, the strips were blocked overnight in Blotto (5% non-fat dry milk, 0.01% antifoam A [Sigma] and 0.001% merthiolate in PBS) to tie up non-specific protein-reactive sites. The strips were then incubated for 2–4 h in appropriate dilution of antibody (usually 1:100) in Blotto, washed 3 times in Blotto, and subsequently reacted with $^{125}$I staphylococcal protein A in Blotto ($5\times 10^5$ cpm/ml) for 1 h. The strips were then washed in Blotto 3 times and then with lithium chloride buffer $(0.5\ M\ LiCl,\ 0.10\ M\ Tris,\ pH\ 8.5,\ 1\%\ nonidet\ p-40)$ two times, 10–15 minutes each. Finally, the strips were rinsed thoroughly with distilled water, dried and subjected to autoradiography using XRP-1 Kodak X-ray film without an intensifying screen or Saran Wrap between the film and the strips. This autoradiographic procedure was found to maximize resolution.

Reagents used for nucleic acid and protein determination

Purified restriction fragments of cloned lymphocytic choriomeningitis virus (LCMV) cDNA, corresponding to the nucleoprotein (NP) coding region of

Transfer 40 µm Whole Body Sections from Tape to Nylon Membrane
Rinse and Fix in Laemnli Buffe/Mehtanol 15 Minutes
Methanol/Acetic Acid, 30 Minutes, Room Temperature
Remove Tape and Excess Tissue from Nylon Membrane
Wash in Water
Block Non-Specific Protein Sites for 16 Hours at Room Temperature
Blocking Solution: 5% Non Fat Dry Milk (Blotto) 0.01% Antifoam A 0.0001 Merthiolate in PBS
React with Specific Antibody Diluted in Blotto, 3 Hours, Room Temperature
Wash in Blotto, 15 Minutes, 3 Times, Room Temperature
React with Second Antibody (if Appropriate) Diluted in Blotto, 1 Hour, Room Temperature
Wash in Blotto, 15 Minutes, 3 Times, Room Temperature
React with $^{125}$I Staph Protein A, 1 Hour, Room Temperature $(5\times 10^5$ to $1\times 10^6$ cpm/ml Diluted in Blotto)
Wash in LICXRP-40, 10 Minutes, 2 Times
Rinse in Water
Air Dry
Exposure Against Kodak XRP-1 Film
Overnight at Room Temperature

Fig. 2. Flow chart detecting proteins in the whole animal body section. See Materials and Methods for details.
Fig. 3. Expression of LCMV RNA and protein in adult 3–4-month-old mice persistently infected with LCMV since birth. Panels 2 and 3 show sections from two persistently infected mice after hybridization with a 32P-labeled specific LCMV cDNA probe obtained from the 5′ end of the LCMV small chromosome (see text and ref. 9 for details). Viral nucleic acid sequences are seen in the brain, salivary gland, brown fat pad, liver, spleen and kidney. Controls were the [32P]LCMV cDNA-labeled probe applied to a section from an uninfected age- and sex-matched mouse and a section from an LCMV persistently infected mouse exposed to an indifferent [32P]HCMV cDNA probe, as shown in panels 1 and 4, respectively. Panels 5–8 record the LCMV proteins detected in whole animal sections. Panels 5 and 6 are sections from an uninfected age- and sex-matched mouse. Panel 5 shows a background of bacteria or auto-antigens in the gut that were absorbed with normal mouse intestine, as panel 6 depicts. Panel 7 and 8 are sections of the same mouse whose viral RNA is depicted in panel 3. In panel 7, antibody to LCMV developed in guinea pigs inoculated with the infectious virus recognized the 3 major viral structural proteins, GP1, GP2 and NP1. In panel 8, rabbit antibody to purified viral NP was used. These two antibodies reacted with viral proteins deposited in the nervous system, salivary gland, lung, liver, spleen and kidney, as detected with 125I-staphylococcal protein A and autoradiography.
Fig. 4. Ten-fold enlargement of the brain section used in Fig. 3. LCMV NP is visible in the lacrimal gland tissue around the orbit (LG), frontal lobe of the cerebral cortex (FC), thalamus (TH), cerebellum (CB), dentate gyrus (DG) and sector CA1 of the hippocampus (HPC/CA1). A section from a 4-month-old persistently infected mouse was incubated with rabbit antibody to LCMV NP followed by \( ^{125} \text{I} \) staphylococcal protein A. This anatomic distribution of LCMV proteins was routinely seen in repeated studies.

the genomic S RNA segment\(^{12,23} \), were labeled in vitro by nick translation in the presence of \( d[\text{P}] \text{dCTP} \)deoxyribonucleotide triphosphates\(^{15} \). Probes were boiled and immediately chilled on ice before addition to the hybridization reactions\(^{22} \). A double-stranded cDNA probe to detect rabies virus glycoprotein gene and mRNA, ca. 1650 bp in length\(^{5} \), was a gift from Dr. W. Wunner, Wistar Institute, Philadelphia. Rabies virus strain CSV was a gift from Dr. H. Koprowski of the Wistar Institute, Philadelphia. Antibodies used to detect myelin basic protein and glial fibrillary acid protein were purchased from Dako Corporation, Santa Barbara. Antibodies to the nucleoprotein of LCMV\(^{7} \) and the glycoprotein of rabies virus were made by rabbits inoculated with purified viral proteins. Rabbit antibody to mouse prolactin\(^{14,20} \) was a gift from Y. Sinha, Whittier Institute for Diabetes and Endocrinology, Scripps Memorial Hospital, La Jolla, and selected amino acid sequences of Thy1.2\(^{1} \) from R. Lerner, Department of Molecular Biology, The Research Institute of Scripps Clinic, La Jolla. Antibody to mouse growth hormone provided by Y. Sinha was made in monkeys\(^{14,20} \). Guinea pig antibody to the 3 major LCMV polypeptides (glycoprotein [GP] 1, GP2 and NP) was raised by inoculation of infectious virus\(^{5} \). Rabbit antibody to guinea pig 7S immunoglobulin G (IgG) and to human IgG and sheep antibody to rabbit IgG were raised as described\(^{12,13} \). The procedures for chemical purification and purity testing of the various immunogens, animal inoculations and immunochemical analysis of the various antibodies for purity have been detailed\(^{1,3,4,12,13,14,20} \).
RESULTS

Specificity and sensitivity of unearthing gene expression in whole body sections

Detection of exogenous viral nucleic acid sequences and proteins in the nervous system

The first series of experiments established the specificity, sensitivity and resolution of the whole body hybridization procedure. To perform such studies it was necessary to introduce into host animals an exogenous agent that would accumulate in several body tissues including those of the nervous system. For this purpose we selected a persistent viral infection, LCMV, in its natural host, the mouse. Newborn mice of BALB/WEHI and SWR/J strains were infected at birth with 60 plaque-forming units (PFU) of LCMV Armstrong strain. This virus is non-cytopathic per se in that it does not cause cell lysis in cultured cells or in the intact animal (see review ref. 5).

However, LCMV persists throughout the animal’s life-span. Such 2- to 5-month-old persistently infected animals were sacrificed and prepared for in situ nucleic acid hybridization and protein studies by using whole body sectioning (Fig. 1). Fig. 3 represents the results from over 50 such persistently infected mice; viral nucleic acid sequences are shown in two individual mice, panels 2 and 3, after accumulating in multiple tissues throughout their bodies. Although the topographical localization in several organs was possible with the in situ technique, the resolution with the 35S labeled probe did not allow detection of specific structures within an organ system.

Use of guinea pig antibody to all 3 viral structural components (GP1, GP2, NP) and rabbit monospecific antibody directed to only one viral component, the NP, (panel 8) detected protein in the section pictured in panel 3. As one sees in panels 7 and 8 of Fig. 3, the resolution resulting from this method was adequate to outline several organ structures. Extraction of viral nucleic acid from a 40-um section and its subsequent quantitation by dot blot analysis indicated that the whole body in situ nucleic acid hybridization technique was about 10-fold less sensitive than the dot blot procedure. Analysis of protein sensitivity with a reconstitution technique whereby varying amounts of purified viral protein diluted in 50 μl of PBS are spotted onto a nylon membrane and carried through several fixations, antibody reactions, and washing procedures indicated that the lower level of detection was 10–40 ng of viral antigen.
These reactions were specific in that neither the viral nucleic acid probes nor the protein detection systems indicated comparable materials in uninfected mice (Fig. 3, panels 1, 5 and 6), and LCMV genetic materials and proteins did not react to nucleic acid or protein probes directed towards different viruses. That is, $^{32}$P-labeled nucleic acid probes directed against either human cytomegalovirus (HCMV) or rabies virus failed to label whole body sections from mice persistently infected with LCMV, and $^{35}$P-labeled LCMV nucleic acid probes did not stain whole body sections from mice acutely infected with rabies virus. The specificities were identical in protein blots of whole animal sections from mice either infected persistently with LCMV or acutely with rabies virus after using the appropriate monoclonal or polyclonal antibodies. Fig. 3, panel 5, demonstrates non-specific binding in the protein detection system to structures in the gut. These are, presumably, in part, bacteria being recognized by antibodies to bacteria found in the rabbit antiserum used and perhaps rabbit auto-antibody to gut antigens. The removal of such non-specific staining by absorption with tissue from a normal intestinal track is shown in Fig. 3, panel 6.

To test this protein detection system further, we enlarged 5- to 10-fold the involved area in the central nervous system. Analysis indicated that the expression of LCMV viral proteins was clearly seen in discrete tracts and topographical sites in the central nervous system. Fig. 4 shows LCMV NP in the cerebellum, cerebral cortex, thalamus, hypothalamus and tissues surrounding the orbit of the eye. The LCMV NP also localized to the dentate and hippocampal gyri. Further, LCMV antigen was observed in hippocampus CA1 but not CA4 or CA3 (Fig. 4). These findings were identical when repeated and were con-

Fig. 6. Ten-fold enlargement of the brain section shown in Fig. 5, panel B. The viral protein signal in the brain of the rabies virus infected mouse is heavy and diffuse with distribution to areas of the anterior olfactory nucleus (AON), hypothalamus (HY), brainstem (BS), cerebellum (CB), occipital cortex (OC), corpus callosum (CC) and frontal cortex (FC). Similar diffuse distribution was noted in 5 other test mice with acute rabies virus infection.
firmed by immunochemical studies at the light and
electron microscopic levels\textsuperscript{11,16}.

For comparison, we next studied the localization
and expression of a rabies virus, microbial agent
known to infect the nervous system. Five-day-old
mice were inoculated intracerebrally with $3 \times 10^4$
PFU of rabies virus, CVS strain. Mice were sacrificed
4–5 days after inoculation, one day before they die of
acute rabies virus infection. Unlike persistent LCMV
infection in which viral proteins infected multiple tis-
sues throughout the body (Fig. 3), rabies virus nu-
cleic acid sequences and protein expression, detected
with a rabies virus-specific probe, were restricted
mostly to the central nervous system (Fig. 5). The en-
larged photo in Fig. 6 demonstrates the enormous in-
volve of most of the nervous system during
acute rabies virus infection and the virus' topo-
graphic localization within the gray matter of the cerebel-
lum. These results were observed in repeated experi-
ments with suckling mice, as represented in Fig. 6 or
with adult (4–6-week-old) mice of the BALB/WE-
HI or SWR/J strains. The rapid spread and diffuse
distribution of rabies virus proteins into neuronal
networks is currently under study (Bloom and
Koprowski, and Lipkin et al., unpublished observa-
tions 1985).

Detection of endogenous proteins in the nervous sys-
tem

We next tracked several hormones and proteins
expressed in the nervous system, with the results
shown in Fig. 7. Specific antibodies directed against
prolactin or growth hormone localized within the pi-
tuitary gland but to no other sites throughout the
body (Fig. 7, panel A). However, antibodies directed
against either myelin basic protein or glial fibrillary
acid protein deposited exclusively in the nervous system. Involved structures in the nervous system are clear in panels B and C of Fig. 7. In contrast to myelin basic protein and glial fibrillary acid protein, two endogenous antigens whose expression is restricted to the nervous system, the theta isoantigen was evident in structures of the brain, spinal cord and thymus (Fig. 7, panel D) as well as other lymphoid organs.

DISCUSSION

Here we show the feasibility and potential of using whole animal sections to study the distribution of expressed genes by in situ nucleic acid hybridization and protein blotting. After introducing neurotropic infectious agents into mice, we recorded the expression and distribution of these exogenous genes and their products in the nervous system. We found that viral RNA and proteins were clearly identifiable while anatomic integrity was maintained. The high degree of resolution using the protein blotting procedure enabled the detection of viral proteins to discrete nervous system tracts and gyri. Hence, this procedure may be useful in charting the tropisms of viruses to specific anatomic locations within the nervous system. Further, it may suggest correlations between neurotropic viruses and neurotransmitters associated with specific nuclear tracts within the nervous system. Elsewhere we reported in situ hybridization analysis of tissues in persistently infected pregnant mice and their infected embryos. We have also used strand specific probes from SP6 vectors that increased the sensitivity of the technique by enhancing the specific activity of the probes and distinguished between RNA species of opposite polarity (genomic sense and complementary sense) (Southern et al., in preparation). Now we demonstrate that proteins can also be detected by applying specific antibodies to whole body sections. Hence, one can monitor gene expression and protein synthesis in the whole animal throughout the course of infection and during development.

In addition, we detected normal host gene products in whole body sections. By focussing on gene expression, we located the hormones prolactin and growth hormone exclusively in the pituitary gland as well as the endogenous proteins myelin basic protein and glial fibrillary acid protein in the nervous system. The limitation of the procedure was the availability of antibodies and an antigen concentration of 10–40 ng. Since there is increasing evidence that several of the neurotransmitters or neuropeptide hormones may be produced in areas other than the nervous system, this technique allows rapid screening of all physiologic sites. The advantage of the procedure reported here is that simultaneous sampling of all tissues in an animal is possible by sectioning at various planes of the body.

The availability of libraries of brain-specific cDNA probes and antibodies permits one to follow gene expression and protein synthesis during development. Similarly, genetic disorders and viral infections can be examined in the whole animal. For example, it will be of interest to study myelin basic protein transcription and protein expression with genetic and environmental models of demyelination and remyelination. The molecular analysis of whole body sections is greatly complemented by focal in situ hybridization procedures that provide higher resolution within limited areas. By employing both techniques, it is now possible to detect expression of endogenous and exogenous genes localized in the nervous system and elsewhere while maintaining suitable morphology for anatomical study.

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