A Rapid Method for the Localization of Lassa Virus in Fixed Tissue Using Labeled Riboprobes Visualized by Enzyme-Linked Immunoassay

by

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ABSTRACT

Lassa fever is a hemorrhagic disease of West Africa affecting as many as 300,000 people per year (Fisher-Hoch, S.P., et al., 1989). Lassa fever is difficult to diagnose because it has a wide range of clinical manifestations and variable severity (McCormic, J.B., et al., 1987a). Lassa virus, the etiologic agent of Lassa fever, is an Arenavirus in the Family Arenaviridae. An assay for the detection of Lassa virus in guinea pig tissue would provide a means for testing the efficacy of potential vaccines as well as contribute to the overall understanding of the pathogenicity of Lassa fever. Conventional immunoassay detection of Lassa virus antigen in formalin fixed, paraffin embedded guinea pig tissue has not been successful in our laboratory. Thus, the aim of this study was to develop and standardize a nonisotopic in situ hybridization assay to detect Lassa virus mRNA in the guinea pig tissue. Digoxigenin labeled riboprobes were transcribed from T7/SP6 multicloning vectors, bearing Lassa virus cDNA inserts. Riboprobes were hybridized to Lassa virus mRNA and tagged with alkaline phosphatase conjugated anti-digoxigenin polyclonal sheep antibody Fab-fragments. Nitroblue tetrazolium and 5-bromo-4-chloro-3-

indoyl phosphate generated a dark blue precipitate in tissues from infected animals but not in uninfected controls. The assay thus provided detection of Lassa virus mRNA with minimal background and clear delineation of cellular detail.

INTRODUCTION

Molecular Organization of Arenaviruses:

Lassa virus, the etiologic agent of Lassa fever, is an Arenavirus in the Family Arenaviridae. An Arenavirus virion consists primarily of a lipid envelope, glycoproteins (GCP), nucleoproteins (NP), RNA genome, and ribosomes. The lipid envelope is obtained from the plasmid membrane of the host cell (Bishop, D.H. 1990). The few glycoproteins projecting outward through the surface of the envelope have a club shaped structure (Murphy, F.A., and S.G. Whitfield 1975). Host ribosomes found within the virion give the grainy appearance for which the virus is named "arena"; meaning sand.

The RNA genome consists of a large segment (L: 2 X 10^6 b.) and a small segment (S: 1 X 10^6 b.) (Vezza, A.C., et al., 1978). Both segments are single stranded, circular, and lack both 3-prime poly A sequences and a 5-prime methylated guanosine cap. The L and S segments also contain different genetic information. The RNA is coated with NP, forming a bead like structure that makes up

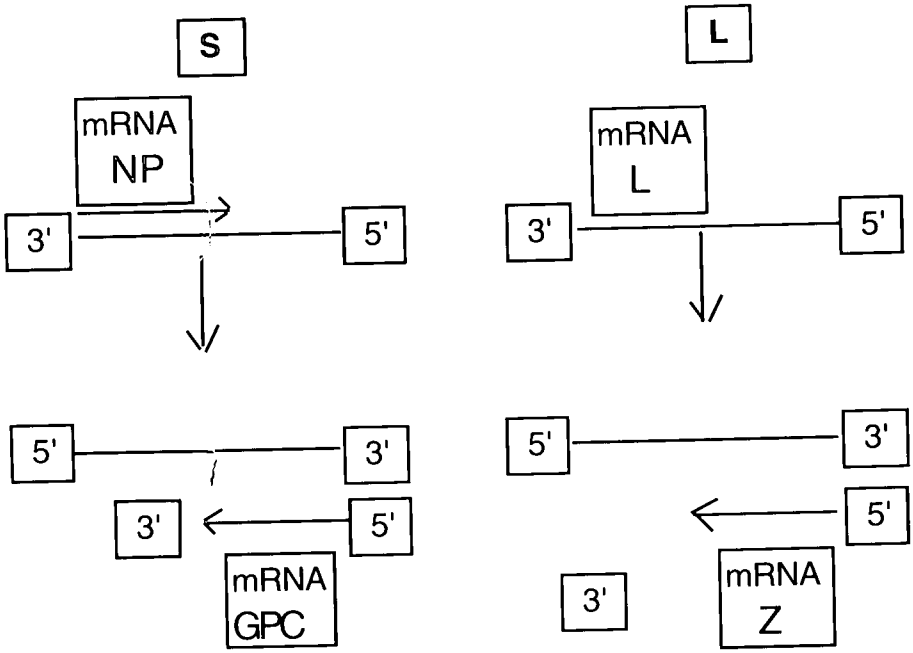
approximately 70% of the protein mass and is referred to as the Ribonucleoprotein (RNP) structure.

Replicative Cycle of Arenaviruses

The process of attachment to the host cell most likely occurs through an interaction between a viral envelope glycoprotein and a cell membrane receptor (Bishop, D.H. 1990). Entry of the ribonucleoprotein (RNP) is thought to occur by membrane fusion or host cell phagocytosis. Once inside, the synthesis of messenger RNA (mRNA) appears to be initiated by a viral specific transcriptase contained within the virion. Host cell ribosomes and a viral envelope are acquired as maturing virions bud though the cell membrane.

Transcription of the viral RNA produces mRNA at or near the 3-prime terminus of the S and L RNA segments (Bishop, D.H. 1990). The mRNA generated is translated into the NP and L proteins. NP and L proteins further stimulate RNA transcription, protein synthesis, and RNA replication. Replication of viral RNA produces both new viral genomic segments and templates for the transcription of GPC and Z mRNA (Figure 1). The transcription of NP and L mRNA from genomic segments and GPC and Z mRNA from templates complementary to the

Figure 1: Arenavirus replication



Replication of viral RNA segments S and L produces both new viral genomic segments and templates for the transcription of GPC and Z mRNA. The transcription of NP and L mRNA from genomic segments and GPC and Z mRNA from templates complementary to the genomic segments is referred to as an ambisense coding strategy.

the genomic segments is referred to as an ambisense coding strategy. Following translation of the GPC and Z mRNA, the genomic segments are incorporated into new virions.

Lassa Fever Epidemiology

The first documented case of Lassa fever was in a missionary working in Nigeria in 1968 (Frame, J.D. 1970). The virus was isolated from the pharynx and pleural fluid of the missionary and subsequently named for the geographic location. Since then, Lassa fever has been found to be endemic in much of western Africa, where persistent outbreaks have been reported in Liberia and Sierra Leone (McCormic, J.B., et al., 1987a). It appears that the Lassa virus is not capable of sustained human to human transmission or else it would have already spread outside of West Africa.

The pool of Lassa virus in endemic areas is maintained by the persistent infection of two species of Mastomys natalensis (Frame, J.D. 1970). These rodents are primarily found in savannah areas, frequenting human dwellings and food stores (McCormick, J.B., et al., 1987a). The virus is spread to humans through direct contact with the urine of infected rodents or from direct contact with infected persons (Holmes, G.P., et al., 1970). One study of village populations

in Sierra Leone found that 5-14% of the noted febrile illnesses were due to infections with Lassa virus. If this study is representative of West Africa, Lassa virus infection causes 100,000-300,000 cases of Lassa fever per year and 5,000 deaths (Fisher-Hoch, S.P., et al., 1989).

Several problems are encountered when attempting to obtain mortality rates for Lassa fever. Differing sensitivities of diagnostic techniques affect the total number of Lassa fever cases reported (Frame, J.D. 1989). The admission of more seriously ill patients to the hospital increases the case fatality rate whereas the admission of less seriously ill patients decreases this rate. Discrepancies in mortality rates could also be due to variations in the quality of the health care received.

Case fatalities are estimated to be 87% for neonates, 50% for patients whose symptoms include hemorrhage, 20% for pregnant women, and an overall 16% for hospitalized patients (Fisher-Hoch, S. P. 1993). The variation in virulence of different viral strains, however, appears to influence mortality rates. Outbreaks in Nigeria in 1969 and 1970 resulted in mortality rates greater than 50%, whereas an outbreak in Liberia in 1972 resulted in a mortality rate

of 36%. Studies using guinea pigs have also shown greatly differing mortality rates for the Macenta and Josiah strains of Lassa virus, suggesting different pathogenic strains of virus.

Pathogenesis of Lassa Fever

The virus is believed to enter the body through cuts or abrasions in the skin, or through the mucosa (Winn, W.C., and D.H. Walker 1975). An incubation period of one to three weeks suggests a silent, primary replication site (Fisher-Hoch, S.P. 1993). Such a site has not yet been located but is thought to be within the reticuloendothelial system. This hypothesis is supported by in vitro studies demonstrating viral ability to replicate in a continuous monocyte cell line (Lewis, R.M., et al., 1988). Interestingly, this ability appears to be enhanced by Lassa-specific antibodies. In vivo models seem to support this evidence as IgG and IgM production by B-cell response early in the infection does not decrease viral titers. High viremia and high titers of Lassa-specific antibodies are often present at the same time in both humans and primates (Fisher-Hoch, S.P., et al., 1987). In humans, the virus may persist in the urine and serum for several months and possibly in occult sites for years.

Little is understood about the pathogenesis of Lassa fever. There is generally a lack of pathological changes of sufficient severity to account for death (McCormic, J.B., et al., 1986). Organ damage is usually mild but gross pathological findings in humans have included: exudative pharyngitis, diffuse encephalopathy, congestion of the viscera, petechiae of the GI tract, pleural effusions, ascites, congested edema of the true and false vocal cords, and nodular kidneys (Winn, W.C., and D.H. Walker 1975). Microscopic pathological findings in humans include interstitial edema of the heart and lungs, tubular and glomerular necroses of the kidneys, atrophy of the white pulp of the spleen, enlarged lymph nodes with depleted germinal centers, petechiae and chronic inflammation of the gastrointestinal mucosa, and hepatocyte necrosis. A substantial macrophage response is also noted but there is little or no infiltration of lymphocytes (Fisher-Hoch, S.P. 1993). Although high viral titers occur in the brain, ovary, pancreas, uterus and placenta, no significant pathological or functional lesions have been observed in these organs.

A pathological indicator to determine the risk of mortality was first looked for in liver tissue because hepatic lesions were the

most severe and consistent pathologic change noted (McCormic, J.B., et al., 1986). Liver damage, however, was not extensive enough to account for death, nor was a correlation found between the degree of necrosis and the duration or severity of illness. Later studies found a correlation between aspartate amino transferase (AST) levels, viremia levels, lymphopenia counts and the severity of the disease (Fisher-Hoch, S.P., et al., 1988).

Animal Models

Monkeys provide the best animal model for the disease (Fisher-Hoch, S.P. 1993). Fever is present five days after inoculation with ensuing anorexia and progressive wasting (Jarhling, P., et al., 1982). Death occurs approximately ten to fifteen days after inoculation from vascular collapse, shock, and hemorrhage. Edema, respiratory distress, encephalopathy and hypovolemic shock are associated with a rapidly rising neutropenia (Fisher-Hoch, S.P., et al., 1988). Endothelial dysfunction from the inability to produce prostacyclin and the loss of platelet function are associated with hemorrhagic phenomena (McCormic, J.B., et al., 1986). Endothelial dysfunction also results in hypovolemic shock as the loss of integrity of the capillary membrane leads to the leakage of fluids and

macromolecules from the extravascular spaces. Histopathological findings are limited to mild hepatic necrosis and pulmonary interstitial pneumonitis.

Diagnosing Lassa Fever

Symptoms at the onset of illness, most often include malaise, high fever, sever headache, weakness, joint and lower back pain (McCormic, J.B., et al., 1987b). Symptoms less often reported are chest and/or epigastric pain, headache, sore throat, and dry cough. Vomiting, diarrhea, and a fever above 40^{0}C are usually present by the fifth day of illness. Illness is usually apparent by the sixth day while most patients begin to recover about the ninth or tenth day of illness.

A minority of the patients go on to develop edema of the head and neck, pleural and pericardial effusions, respiratory distress, encephalopathy, and/or hemorrhagic phenomena (McCormic, J.B., et al., 1987b). Bleeding may occur from old injection sites, as epistaxis, conjunctival hemorrhage, melena, hemoptysis, and hematemesis. Petechiae of the skin and mucosal surfaces has been observed. There are also reports of dizziness, vertigo, and hearing loss in a few of the victims.

Early signs and symptoms of Lassa fever are similar to influenza and the overall clinical course is highly variable (Holmes, G.P., et al., 1970). A fever above 38.3^{0}C as well as the absence of rhinorrhea are used to distinguish Lassa fever from influenza. Rising levels of AST, depressed platelet function, thrombocytopenia and lymphopenia appear to be present only in severe cases (Fisher-Hoch, S.P. et al., 1988). During the first week of illness, detectable levels of antibodies are present in approximately half of the patients (Lunkenheimer, K., et al., 1990).

The most convincing diagnosis is made by virus isolation which requires tissue culture techniques and take several days to perform. Polymerase chain reaction (PCR) has been used to detect Lassa virus in serum and urine specimens, before antibodies were detected. PCR, however, requires high quality reagents, special technical equipment, as well as primer sets able to detect the various viral strains. Detection of viral antigen can be accomplished using standard immunochemistry detection for post mortem diagnosis as a tissue biopsy would not be performed in a patient suspected of having a hemorrhagic disease.

Treatment and Prevention of Lassa Fever

Treatment consists of symptomatic and passive immunization with immune IgG. Prevention of infection follows the Center for Disease Control recommendation for universal precautions to prevent contact with contaminated blood or other body fluids (Holmes, G.P., et al., 1970). Mechanical ventilation, hemodynamic monitoring and intravenous vasopressor therapy available in intensive care units are necessary to treat shock and/or adult respiratory distress syndrome.

The intravenous administration of RNA antiviral Ribavirin has been shown to significantly reduce the mortality of Lassa fever (Huggins, J.W. 1989). A study in Sierra Leone showed viremia levels greater than $10^{3.6}$ $TCID_{50}$ (tissue culture infective dose/ml) at the time of admission, resulted in a mortality rate of 76% in untreated patients and a mortality rate of 32% in those treated with ribavirin. It was also noted that patients treated before the seventh day of illness had an improved chance of survival.

Attempts employing the classic methodologies with Lassa specific antiserum for passive protection in humans as well as killed virus vaccination in rhesus monkeys, have both failed

(Auperin, D.A. 1993). Although the rhesus monkeys elicited a substantial humoral immune response to the major viral structural proteins, no protection against live virus challenge was observed. These results were consistent with the observation that Lassa-specific humoral antibodies are non-neutralizing. Viral clearance, therefore, appears to depend on a cell mediated immune response.

Vaccination with a live attenuated strain of Lassa virus and avirulent cross-reacting strains has met with limited success. It was hoped that such vaccines would stimulate a more effective immune response by presenting viral antigens to the host's immune system in a manner resembling that of the parent virus (Auperin, D.A. 1993). One study showed laboratory animals infected with avirulent viruses that were serologically related to Lassa survived a subsequent challenge with virulent Lassa virus, experiencing only mild, if any, symptoms. The disadvantages of such vaccines include the possibility of a reversion/conversion to virulence, adverse side effects, and the ability of arenaviruses to establish persistent infections as demonstrated in rodents and tissue culture (Kew, O.M., et al., 1981; Arita, I. and F. Fenner 1985).

The ability to produce recombinant viruses capable of expressing foreign genes provides another possible means of preventing Lassa virus infection. Vaccines using recombinant vaccinia viruses expressing the Lassa virus NP or GPC proteins have been investigated in guinea pigs and rhesus monkeys. Inbred strain-13 guinea pigs are uniformly susceptible to Lassa (Jarhling, P.B., et al., 1982). Death occurs in 100% of the animals 11-22 days postinfection, with as few as two plaque forming units (pfu). High titers of virus (10^5-10^7 pfu/ml) can be isolated from the liver, spleen, pancreas, adrenal gland, kidney, lymph node, salivary gland, and lung. Lower mortality rates (70-90%) are noted in outbred, Hartley-strain guinea pigs which are sometimes used because they offer the genetic diversity inherent in outbred populations. Such strains permit a more realistic assessment of vaccine efficacy (Auperin, D.A. 1993). In these animals, vaccination with the vaccinia-expressed NP gene of the Josiah strain of Lassa resulted in a 6% mortality rate. The protection, however, was incomplete as all but one of the animals became viremic and 86% became febrile.

In rhesus monkeys, Lassa virus has been found in all visceral organs examined with mean titers ranging from $10^{6.6}$ pfu/ml in

lymph node tissue to $10^{7.6}$ in liver tissue (Jarhling, P.B., et al., 1982). Monkeys vaccinated with a recombinant vaccinia virus expressing the Lassa virus NP died when challenged whereas those vaccinated with a recombinant vaccinia virus expressing GPC survived (Fisher-Hoch, S.P., et al., 1989). Some of the animals, however, had intermittent episodes of fever for 3 months and virus was detected in the serum at 7-9 days post infection.

Prevention of Lassa fever by vaccination is needed for two reasons. One, Lassa fever affects as many as 300,000 people per year and has a mortality rate of approximately 10% (Fisher-Hoch, S.P., et al., 1989). Secondly, the infection is difficult to diagnose (McCormic, J.B., et al., 1987b). Ongoing Lassa vaccine research at this institute required a means of identifying and localizing Lassa virus in fixed tissue taken from infected guinea pigs. When conventional staining procedures using antibodies to identify and localize Lassa virus antigens in fixed guinea pig tissues were unsuccessful, in situ hybridization (ISH) detection of NP and GPC mRNA in tissues became the assay of choice.

History of In situ hybridization

The hybridization of a nucleic acid probe to its nucleic acid target was first performed in solution and then isolated by equilibrium-density gradient centrifugation (Hall, B.D. and S. Spiegelman 1961). As the procedure was slow, labor intensive, and inaccurate, a simpler solid phase hybridization method was soon developed. It was called the DNA-agar technique and used agar to immobilize denatured DNA (Bolton, E.T. and B.J. McCarthy 1962). DNA was labeled with radioisotopes and hybridized to complementary sequences of DNA within the gel. The gel was washed to remove the unbound, radiolabeled DNA. The bound, radiolabeled DNA was then eluted from the agar with low salt solutions at high temperatures. As the eluted radioactivity was proportional to the amount of hybridized probe, the approach was used for detailed kinetic analysis of DNA annealing reactions.

The ability to detect DNA sequences immobilized on nitrocellulose with radio-labeled probes and to cut the DNA at specific sites, paved the way for more advancements (Nygaard, A.P. and B.D. Hall 1963; Nathans, D. and H.O Smith 1975). Radio-labeled DNA probes were generated from purified mRNA templates following

an incubation with reverse transcriptase (RT) (Weiss, G.B., et al., 1976). Radioactivity was used to quantitate the amount of hybridized probe and estimate the number of genes present. Restriction enzymes cut the DNA into specific fragments that were separated by size with electrophoresis. Using Southern blotting, a specific DNA fragment could be localized by transferring the DNA to a membrane and hybridizing with a radiolabeled probe of complementary sequence (Southern, E.M. 1975). A restriction map could be generated by comparing the sizes of fragments produced from a variety of restriction enzymes (Maniatis, T., et al., 1978).

Molecular cloning generated unlimited quantities of specific DNA fragments for a multitude of uses. The cohesive ends of the DNA fragment were used for insertion into self-replicating elements such as a plasmid (Cohen, S., et al., 1973). Bacteria containing the plasmid were cultured to produce large quantities of the DNA fragment. The plasmid DNA was extracted from the cells and separated from the DNA fragment by restriction endonucleases. The nucleic acid sequence of the DNA fragment was then determined by the chemical or the enzymatic method (Maxam, A. 1977; Sanger, F. 1977).

The first nonradioactive DNA probe was generated by enzymatic polymerization, incorporating biotin labeled deoxyribonucleotide triphosphates (Dale, R.M. and D.C. Ward 1975). The modified nucleotides required a linker arm to ensure access to detection reagents and to avoid interference with hybrid formation. The modification was located at the C-5 position so it would not interfere with hydrogen bonding.

Biotin labelled DNA probes were generated by nick translation (Leary, J.J., et al., 1983). Nick translation used DNA polymerase I to add nucleotide residues to single stranded nicks within a DNA template. The enzyme then moved along the template replacing the strand of DNA with labeled and unlabeled nucleotide residues. Following hybridization, biotin residues are detected by anti-biotin antibodies which are in turn conjugated to enzymes. The addition of a substrate results in the formation of a detectable precipitate such as a colored band on nitrocellulose or as cellular staining (Brigati, D.J., et al., 1983).

Digoxigenin was also used to label nucleic acid probes (Boehringer Mannheim Corp., Indianapolis, IN). Digoxigenin is a hapten not normally found in biological materials as is the

ubiquitous vitamin biotin. Digoxigenin-labeled probes are made by the enzymatic incorporation of digoxigenin-11-UTP (dUTP) into RNA or DNA. Following hybridization and washing, the hybrids are detected by incubating with an anti-digoxigenin alkaline-phosphatase conjugate and colorimetric detection (Holte, H.J., et al., 1988). Digoxigenin-labeled probes were found to produce much lower background staining than biotin labeled probes (Martin, R., et al, 1990).

Restriction enzyme sites and phagemid promotors were placed within plasmid DNA to make cloning vectors (Wolfe, et al., 1987). Foreign DNA could then be placed into the vector next to an RNA promoter. Using the appropriate RNA polymerase, RNA could then be transcribed from the foreign DNA template. Placing the foreign DNA between two different promotors such as SP6 and T7 allowed for the transcription of RNA probes in either orientation.

RNA probes have several advantages over DNA probes. RNA probes are single stranded so there is no competition from complementary strands. RNA/RNA hybrids are more stable than RNA/DNA hybrids. RNA probes have a fixed length. Excess or non-specifically bound RNA can be removed with RNAse as it degrades

only single stranded RNA; RNA/RNA hybrids and RNA/DNA hybrids were not effected by the enzyme (Simmons, D.H., et al., 1989).

It was the goal of this study to develop an assay that could be used to study disease progression and test the efficacy of potential vaccines. Although PCR is very helpful in determining a diagnosis, it does not allow for light microscopy localization of virus and tissue pathology. Immunohistochemistry is the more commonly used technique for eluding disease progression and pathology within this institute, but it is theoretically inferior to in situ hybridization. The antisera generally recognizes a short sequence of amino acids synthesized by the virus. This antigenic epitope may be altered by fixation and processing, causing loss of immunohistochemical signal. The signal may also be lost if the antigenic epitope is not expressed throughout the period of infection. Thus a nonisotopic in situ hybridization (ISH) assay was developed and standardized for the detection of mRNA from the Josiah strain of Lassa fever virus in formalin fixed tissues. For this assay, digoxigenin-labeled riboprobes were detected using alkaline phosphatase conjugated anti-digoxigenin antibody Fab fragments (Boehringer Mannheim,

Indianapolis, IN), nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

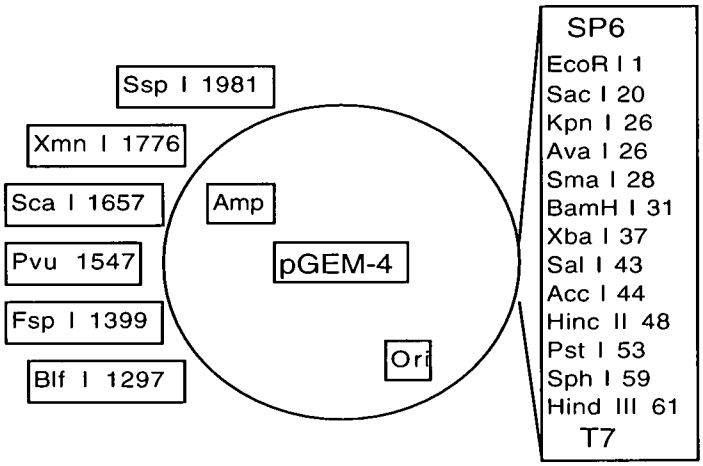
MATERIALS AND METHODS

Generating Lassa cDNA Bearing Plasmids

Luria broth was prepared by dissolving 25 g of Luria broth base (Sigma, St. Louis, MO) into 1 L of dH_2O and autoclaving at 250°C for 20 minutes. The broth was allowed to cool to 50°C before adding 50 mg of ampicillin and storing at 4°C (Sambrook, J., et al., 1989a). Luria agar petri dishes were prepared by dissolving 37 g of Luria agar (Sigma, St. Louis, MO) in 1 L of dH_2O and autoclaving at 250°C for 20 minutes. The agar was allowed to cool to 50°C before adding 50 mg of ampicillin. The petri dishes were covered and the agar allowed to harden prior to storing at 4°C.

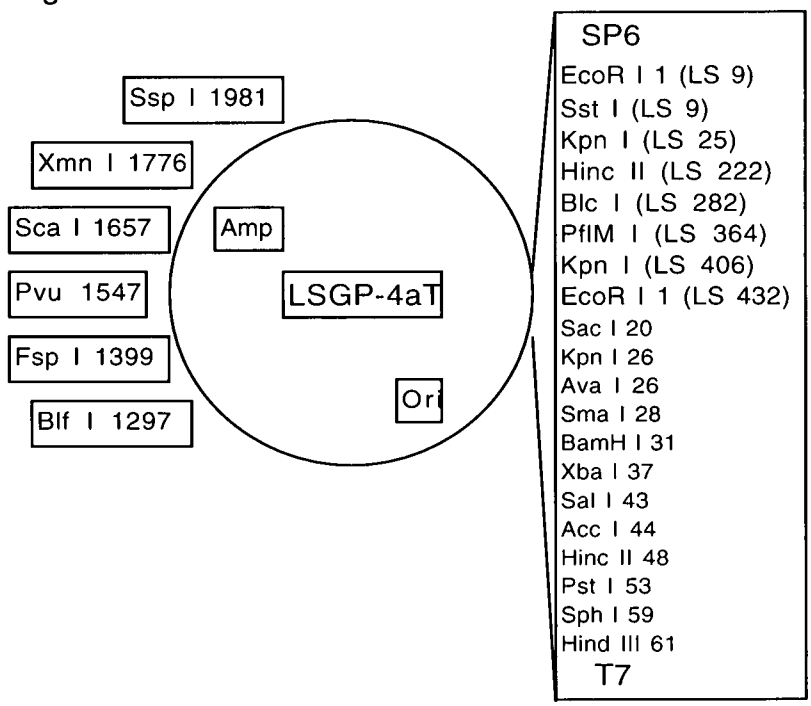
Competent DH5α cells (Gibco BRL, Gaithersburg, MD) were transformed with plasmids bearing cDNA from the Josiah strain of Lassa fever virus (Figures 2-5). The Lassa virus cDNA was generously donated by Dr. David Auperin, of the Pfizer Central Research Department of Molecular Genetics (Auperin, D., et al., 1986). Following manufacturer's instructions, the DH5α cells and polypropylene tubes were chilled on ice. Fifty µl of the cell suspension (1-3 x 10^8 cells/100 µl) were added to each of the

Figure 2: Vector pGEM-4 (Promega, Madison, WI)



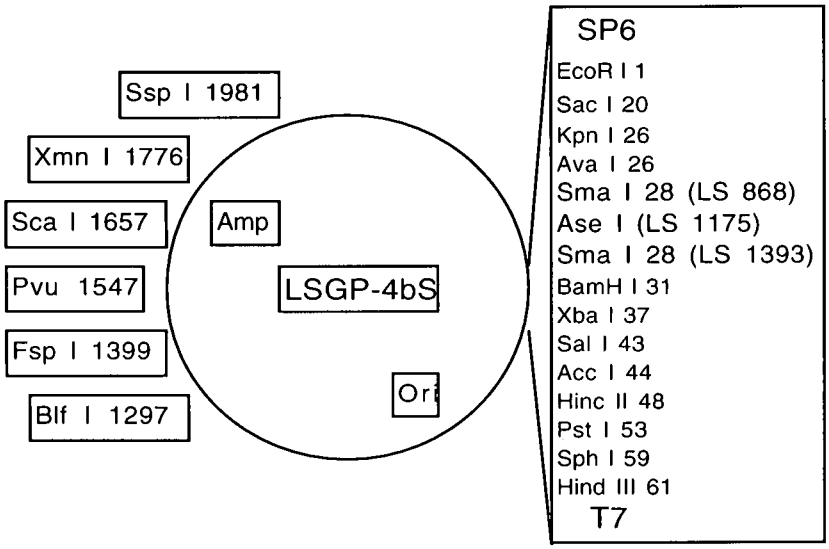
Vector pGEM-4 is shown with restriction sites, multicloning sequence between the Sp6 and T7 promoters, gene for ampicillin resistance, and an origin of replication.

Figure 3: LSGP-4aT



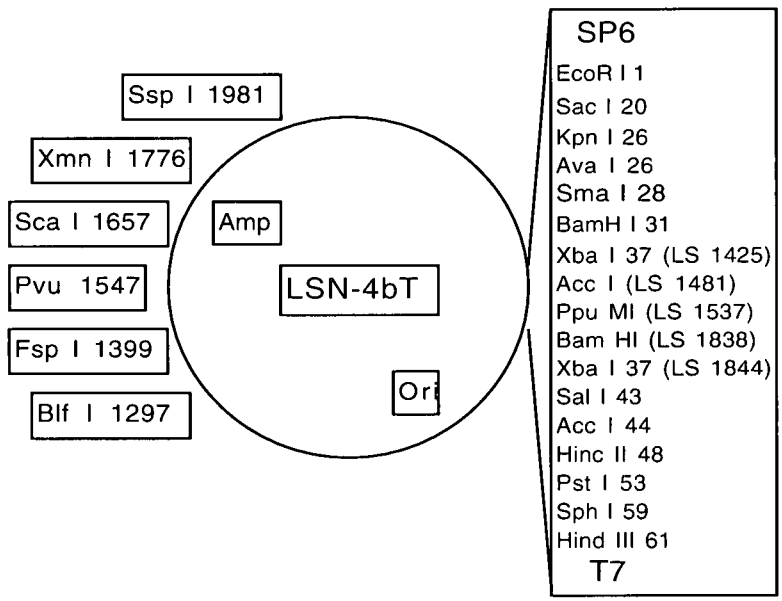
LSGP-4aT contains the Lassa glycoprotein sequence for nucleotide 9 through 432, subcloned into the pGEM-4 multicloning sequence at the EcoR 1 site.

Figure 4: LSGP-4bS



LSGP-4bS contains the Lassa glycoprotein sequence for nucleotides 868 to 392, subcloned into pGEM-4 multicloning sequence at the Sma I site.

Figure 5: LSN-4bT



LSN-4bT contains the Lassa nucleoprotein sequence for nucleotides 1425 to 1838, subcloned into pGEM-4 multicloning sequence at the Xba I site.

chilled tubes, followed by either 1 ng of pGEM-4 (Promega, Madison, WI), 1 ng of a Lassa cDNA bearing plasmid, or 1 µl of dH_2O (control). The cells were incubated on wet ice for 30 minutes, heat shocked at 42°C for 45 seconds, and then rapidly cooled on wet ice for 2 minutes. After cooling, 500 µl of S.O.C. media (Gibco BRL, Gaithersburg, MD) were added and the resulting cell cultures incubated at 37°C for 1 hour. Luria agar plates containing ampicillin were inoculated with 100 µl of the resulting cultures and incubated overnight at 37°C.

Plasmid Isolation

Bacterial colonies from the Luria agar plates were used to generate working stocks of plasmids with Lassa virus cDNA inserts. An isolated colony was transferred to a 250 ml flask containing 50 ml of Luria broth with ampicillin and incubated overnight at 37°C, with shaking. On the following day, 0.4 ml of the resulting culture was set aside until plasmid preps had been performed and it were assured that the cultured cells contained plasmids bearing Lassa virus cDNA inserts. At that time 0.4 ml of the reserved culture were added to 1.6 ml of Luria broth with ampicillin and 2 ml of 20%

glycerol in dH_20 (Vehey, personal communication). After vortexing for 5 seconds, the glycerol-culture mixture was aliquoted into four sterile 1.5 ml polypropylene tubes and stored at -70°C.

Plasmid DNA isolations were a modification of the methodology of J. Sambook, et al., 1989b. Isolations were begun by centrifuging the cell culture at 2,000 x g for 15 minutes and discarding the supernate. The pellet of cells was resuspended by vortexing in 0.5 ml of STE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for approximately 1 minute to inactivate cell wall components thought to interfere with restriction enzyme digestion. Centrifugation was repeated (as before) and the supernate discarded. The cell pellet was then resuspended by votexing in 5 ml of *Solution I* (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCL, pH 8.0) for approximately 1 minute.

Plasmids were isolated from the cell culture by lysing the cells and coagulating the protein. Ten µl of freshly made *Solution II* (0.2 N NaOH, 1% SDS,) were added to the resuspended pellet and gently mixed by inverting the suspension for 5 seconds. After incubating on wet ice for 5 minutes, 7 ml of *Solution III* (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml H_2O)

were added and the lysate vortexed for 10 seconds. Following a 5 minute incubation on wet ice, the mixture was clarified by centrifugation at 3,500 x g for 20 minutes at 4°C. The lysate was filtered through several layers of sterile cheesecloth into a 50 ml conical tube to remove the coagulated protein.

Much of the RNA was removed from the plasmid solution by LiCl precipitation. An equal volume of prechilled (-20°C) isopropanol was added to the filtered plasmid solution and gently mixed by inverting for 2 minutes. After repeating the centrifugation, the supernate was discarded and the pellet resuspended in 2 ml of sterile dH_2O. Two ml of LiCl-MOPS buffer (5 M LiCl, 0.5 M MOPS, pH 8.0) were added and the solution gently mixed by inverting for 1 minute. After a 10 minute incubation on wet ice, the solution was centrifuged as before and resulting supernate transferred to a sterile polypropylene tube.

Plasmid DNA was extracted from the supernate using phenol:water:chloroform (1:1:1) (Applied Biosystems, Foster, CA) and chloroform:isoamyl alcohol (24:1) (Wallace, D.M. 1987). An equal volume of phenol-water-chloroform (Applied Biosystems, Foster, CA) was added to the plasmid solution and vortexed for 1 minute.

After separation at 5,000 x g for 2 minutes, the upper layer was transferred to a new tube and the lower layer discarded. The extraction was then repeated with an equal volume chloroform:isoamyl alcohol (24:1). After transferring the upper layer to a new tube, an equal volume of chilled (-20^{0}C) isopropanol was added and the resulting solution gently mixed by inverting for 5 seconds. The solution was centrifuged at 5,000 x g for 20 minutes at 4^{0}C and the supernate discarded.

Five μg of DNAase-free RNAase (Boehringer Mannheim, Indianapolis, IN) were added to 0.5 ml of 1X TE buffer, pH 7.4 (Digene, Silver Spring, MD) and used to degrade any RNA remaining in the plasmid solution. The pellet was resuspended in the buffer and incubated at 37^{0}C for 15 minutes. The RNAase was then removed by extracting with phenol:water:chloroform (1:1:1) and chloroform:isoamyl alcohol (24:1), as previously explained. Once the upper layer had been transferred to an eppendorf tube, 0.25 ml of 7.5 M ammonium acetate were added and the solution gently mixed by inverting for 1 minute.

To precipitate the plasmid DNA, 0.75 ml of prechilled (-20^{0}C) isopropanol were added and then incubated on dry ice for 15 minutes.

The chilled solution was then centrifuged at 10,000 x g at 4°C for 30 minutes. After discarding the supernate, 1 ml of prechilled, (-20°C) 75% ethanol was gently pipetted over the pellet. The pellet was dried by carefully decanting the liquid and allowing the remaining ethanol to evaporate. The pellet was then resuspended in 50 ul of 1X TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4) and stored at -20°C (Wallace, D.M., 1987).

Plasmid Characterization

The concentration of the plasmid solution was determined by ultraviolet spectrophotometry. The ultraviolet lamp was lit and the wavelength set to 260 nm. A cuvette containing 99 µl of 1X TE buffer was used to calibrate the system. Calibrated pipet tips (BIO 101, La Jolla, CA.) were used to increase the accuracy of the absorbance reading. Accuracy was further verified by adding 1 µl of a known concentration of DNA to the cuvette and recording the absorbence once the reading had stabilized. A reading that did not fluctuate for 10 seconds was considered stable. The method was repeated until acquiring two or three absorbency readings with a difference of 5% or less. The sample DNA was quantitated in the

same manner. Plasmid concentration was determined in nanograms by multiplying the absorbency by a dilution factor of 100 and the absorption coefficient of 50. TE buffer was added as needed to bring the plasmid concentration between 0.5 and 1 μg/μl.

Plasmid length was determined by gel electrophoresis, using the Horizon 11.14 Gel Electrophoresis Apparatus (Gibco BRL, Gaithersburg, MD). The apparatus was assembled following the manufacturer's instructions. A 1% agarose gel was made with 0.25 g of agarose and 25 ml of 1X TBE buffer (50 mM Tris, 50 mM Boric Acid, 1mM EDTA-$Na_2 \cdot 2H_2O$) (Sigma, St. Louis MO). The mixture was dissolved by heating for approximately 2 minutes in a microwave oven set on full power. After cooling, the agarose solution was poured into the mini-gel mold and an eight tooth comb inserted. Once the gel was set, the casting dams were removed and the gel covered with 1X TBE buffer to a depth of approximately 1 mm (Sambrook, J. , et al., 1989c).

To prepare the plasmids for electrophoresis, 1 μl of the plasmid solution was added to 8 μl of water and 1 μl of 10 X Gel Loading Buffer (5 Prime-3 Prime, Boulder, CO). The comb was

carefully removed and the plasmid solution pipetted into a central well. Adjacent wells were loaded in the same manner with Supercoiled DNA Ladder (Gibco BRL, Gaithersburg, MD) and uncut pGEM-4. Once the leads were attached and the power set at 120 volts (1-5 V/cm), the gel was electrophoresed until the loading dye had migrated approximately 3/4 of the length of the gel (Sambrook, J. , et al., 1989c). The leads were then detached and the gel immersed in a 1% solution of ethidium bromide in dH_2O for 5 minutes. Contact with ethidium bromide was minimized by wearing gloves, working under a fume hood and using containers with tightly fitting lids. The ethidium bromide solution was then stored at 4^0C in a brown bottle. After destaining the gel in tap water for approximately 20 minutes, it was viewed on a transilluminator containing a 302 nm UV source and photographed.

To verify the presence of the Lassa cDNA within pGEM-4, the plasmids were digested with restriction enzymes to remove the cDNA inserts. LSGP-4aT was digested with EcoR I, LSGP-4bS was digested with BamH I and EcoR I, and LSN-4bT was digested with Xba I (Figures 2-4). Restriction digestions were performed with 10 ug of DNA, 50 units of enzyme, 3 µl of the appropriate 10X buffer, and

sterile dH_2O, for a total volume of 30 µl. After incubating at 37°C for 2 hours, 3 µl of the restriction digest were electrophoresed on a 1.2% agarose gel. Adjacent wells were loaded with 1 µl of 1kb DNA Ladder (Gibco BRL, Gaithersburg, MD) and 1 µl of ϕX174 RF DNA/Hae III Fragments (Gibco BRL, Gaithersburg, MD). The gel was stained with ethidium bromide, viewed on a transilluminator, and photographed.

Preparation of cDNA Transcription Templates

The pGEM-4 bearing Lassa cDNA inserts were linearized within the multicloning sequence by digestion with restriction enzymes, yielding blunt or 5' overhangs (Figure 2) (Sambrook, J., et al., 1989d). To linearize, 10 µg of plasmid were incubated with 3 µl of the appropriate 10X buffer, in a total volume of 30 µl. Hind III, Sma I, Xba I, Ava I, Sal I, EcoR I and BamH I were available in 10 units/µl concentrations. EcoR I and BamH I were also available in 50 units/µl concentrations. Restriction digestions were performed with 10-30 units of either Hind III, Sma I, Xba I, Ava I, and Sal I, or with 10-100 units of either EcoR I or BamH I. LSGP-4aT was linearized with either Hind III, Sal I, Xba I, or BamH I (Figure 3). LSGP-4bS was

linearized with either BamH I, Ava I or EcoR I (Figure 4). LSN-4bT was linearized with either EcoR I, Ava I, or Sma I (Figure 5). Restriction digests were incubated at 37^{0}C for 2 hours except for Sma I, which was incubated at room temperature.

Linearization of the plasmids was determined by gel electrophoresis and transformation efficiency. Using the previously explained method, 1 μg of the digested plasmid, 1 μg of linearized pGEM-4, or 1 μg of nonlinearized plasmid was loaded into the central wells of a 1% agarose gel. Following electrophoresis, the gel was stained with ethidium bromide, viewed on a transilluminator, and photographed.

The efficiency of the restriction digests was determined by attempting to transform competent cells with the restricted DNA. Following the previously explained method, 50 μl of cell suspension (1-3 x 10^8 cells/100 μl) received either 1 μl of the restriction digest, 1 ng of unlinearized pGEM-4 (positive control), or 1 μl of sterile dH_2O (negative control). After incubating on wet ice for 30 minutes the suspensions were heat shocked at 42^{0}C for 45 seconds, and rapidly cooled on wet ice for 2 minutes. Five hundred μl of SOC

media (Gibco BRL, Gaithersburg MD) were added to each suspension and incubated at 37^{0}C for 1 hour. Luria agar plates containing Ampicillin were inoculated with 100 µl of the resulting cultures and incubated overnight at 37^{0}C. On the following day, the number of colonies on each plate was noted.

Prior to extracting the linearized plasmid, the total volume of the restriction digest was brought to 100 µl by the addition of 70 µl of sterile dH_2O. An equal volume of phenol-water-chloroform was added and the resulting mixture vortexed for 1 minute. After separating the mixture at 5,000 x g for 2 minutes, the top phase was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) added (Wallace, D.M. 1987). The solution was vortexed and separated by centrifugation as before.

Once the top phase had been transferred to an eppendorf tube, the plasmid was precipitated by adding 2/10 volume of 3 M sodium acetate (pH 7.0) and gently mixed by inverting for 5 seconds (Wallace, D.M. 1987). After incubating at room temperature for 3 minutes, 4 volumes of prechilled (-20^{0}C) ethanol were added and the solution vortexed for 5 seconds. Following a 15 minute incubation

on dry ice, the solution was spun at 10,000 x g for 30 minutes at 4^{0}C. The pellet was dried by carefully decanting the liquid and allowing the remaining alcohol to evaporate. The pellet was resuspended in 10 µl of 1X TE, pH 7.4 buffer by vortexing for 5 seconds and incubating at 37^{0}C for 30 minutes. The plasmid was quantitated by ultraviolet spectroscopy, as previously explained, and stored at -20^{0}C.

Prevention and Inactivation of RNAase Contamination

Ribonucleases (RNAases) in the dH_2O were inactivated by the addition of diethyl pyrocarbonate (DEPC) to a final concentration of 0.1%, mixing for ten minutes on an oscillating shaker table, incubating at room temperature overnight, and autoclaving at 250^{0}C for 20 minutes (Blumberg, D.D. 1987). The resulting solution was referred to as DEPC-water and used to make all RNAase free solutions. RNA degradation from RNAases in both the transcription and storage solutions was also minimized by the addition of 10^3 units of rRNasin (Promega, Madison, WI) or RNase Inhibitor (Boehringer Mannheim, Indianapolis, IN) and 5 mM DTT (Promega, 1991).

Latex gloves were worn at all times to prevent RNAase contamination of the labware (Blumberg, D.D. 1987). RNAases present on glassware, metal spatulas, and stir bars were inactivated by washing in a 2% solution of Absolve (Dupont, Boston Massachusetts), rinsing with DEPC-water and baking in an oven set at to 200°C for 6 hours. Specific pipettors were designated for RNAase free work, and individually wrapped, sterile, disposable plasticware was used whenever possible. Pipet tips, microcentrifuge tubes and siliconized tubes (PGC Scientific, Gaithersburg, MD) were autoclaved prior to use at 250°C for 15 minutes.

Transcription of DIG-11-UTP Labeled RNA

The Riboprobe Gemini II Core System kit (Promega, Madison, WI), DIG-11-UTP (Boehringer Mannheim Biochemical, Indianapolis, IN), and Lassa cDNA templates were used to transcribe the RNA. The transcription solution included 10 µl of DEPC-water, 8 µl of 5 X transcription buffer, 1 µl of rRNase Inhibitor (40 u/µl), 4 µl of 100 mM dithiothreitol (DTT), 2 µl of 10 mM GTP, 2 µl of 10 mM ATP, 2 µl of 10 mM CTP, and 1 µl of linearized plasmid (1 µg/ul). Three

different ratios of UTP to DIG-UTP were used to vary RNA labeling (Holte, H.J., et al., 1988). One µl of 10 mM DIG-UTP was combined with 1 µl of 10 mM UTP for 1:1 labeling, 0.5 µl of 10 mM DIG-UTP was combined with 1.5 µl of 10 mM UTP for 1:3 labeling, and 1.5 µl of 10 mM DIG-UTP were combined with 0.5 µl of 10 mM UTP for 3:1 labeling. Linearized LSGP-4bS and LSGP-4aT required the addition of 1 µl of SP6 RNA polymerase (40 u/µl) whereas LSN-4bT required 1 µl of T7 RNA polymerase (40 u/µl) for transcription of antisense RNA (Figures 2, 3, & 4). After incubating the transcription solution at 37^{0}C for 1 to 4 hours, the DNA template was degraded by adding 10 units of RNase free, DNase (Boehringer Mannheim, Indianapolis, IN) and incubating at 37^{0}C for 15 minutes.

Instructions included in the Genius RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) were used to precipitate the transcribed RNA. Ten µl of 4 M lithium chloride were added to the transcription solution. The solution was vortexed for 5 seconds and incubated at room temperature for 3 minutes. After adding 750 µl of prechilled ethanol (-20^{0}C), the solution was vortexed for 5 seconds and incubated on dry ice for 30 minutes. The RNA was pelleted by

spinning at 10,000 x g for 30 minutes, at 4°C. The liquid was carefully decanted and the remaining alcohol allowed to evaporate. The DIG-UTP labeled RNA was resuspended in 20 µl of 1X TE buffer, pH 7.4 (Digene, Silver Spring, MD) by vortexing for 30 seconds. Quantitation methodology was the same as for DNA except for an absorption coefficient of 40.

Nomenclature for the labeled RNA refers to the template used for transcription and the percentage of DIG-11-UTP included in the transcription reaction. RNA generated from LSGP-4aT with UTP alone was referred to as LSGP-4aT-0 whereas RNA generated with a 1:3 ratio of DIG-11-UTP to UTP was referred to as LSGP-4aT-25. RNA generated from LSGP-4bS with a 1:1 ratio of DIG-11-UTP to UTP was referred to as LSGP-4bS-50 whereas RNA generated with a 3:1 ratio of DIG-11-UTP to UTP was referred to as LSGP-4bS-75. Control RNA was transcribed from a 400 base segment of the Simian Aids Virus (SIV) glycoprotein gene, subcloned into pGEM-7ZF (courtesy of Dr. Vanessa Hirsch, NIAID, NIH). The control RNA was generated from a 1:1 ratio of DIG-11-UTP to UTP and designated as SIV-50.

Determining the Length of the RNA Transcript

The molecular weight of the DIG-labeled RNA was determined by gel electrophoresis using the Horizon 11.14 Gel Electrophoresis Apparatus (Gibco BRL, Gaithersburg, MD). Two types of agarose gels were used to determine which type would give the best clarification of RNA bands. After assembling the apparatus, 1.8% agarose gels were made with 0.7 g of Seakem ME agarose (FMC, Rockland, ME), 4 ml of 10X Formaldehyde Gel Running Buffer (5 Prime-3 Prime, Boulder, CO), and 35 ml of DEPC-water. The 3% agarose gels were made with 1.2 g of Nusieve 3:1 Agarose (FMC, Rockland, ME), 4 ml of 10X Formaldehyde Gel Running Buffer, and 35 ml of DEPC-water (Rappolee, D., et al., 1989).

The agarose was dissolved by heating in a microwave oven with frequent swirling for approximately 1 minute. Twenty-five ml of the hot agarose solution were mixed with 1.3 ml of 37% deionized formaldehyde with or without 1 µl of ethidium bromide solution (100 µg/ml). The agarose solution was quickly poured into a mini gel mold and an eight tooth comb inserted. Once the gel had set, the comb and gel casting dams were carefully removed. Running buffer was made from a 1:10 dilution of 10X Formaldehyde Gel Running

Buffer with DEPC-water and used to cover the gel to a depth of 1 mm (Sambrook, J., et al., 1989e).

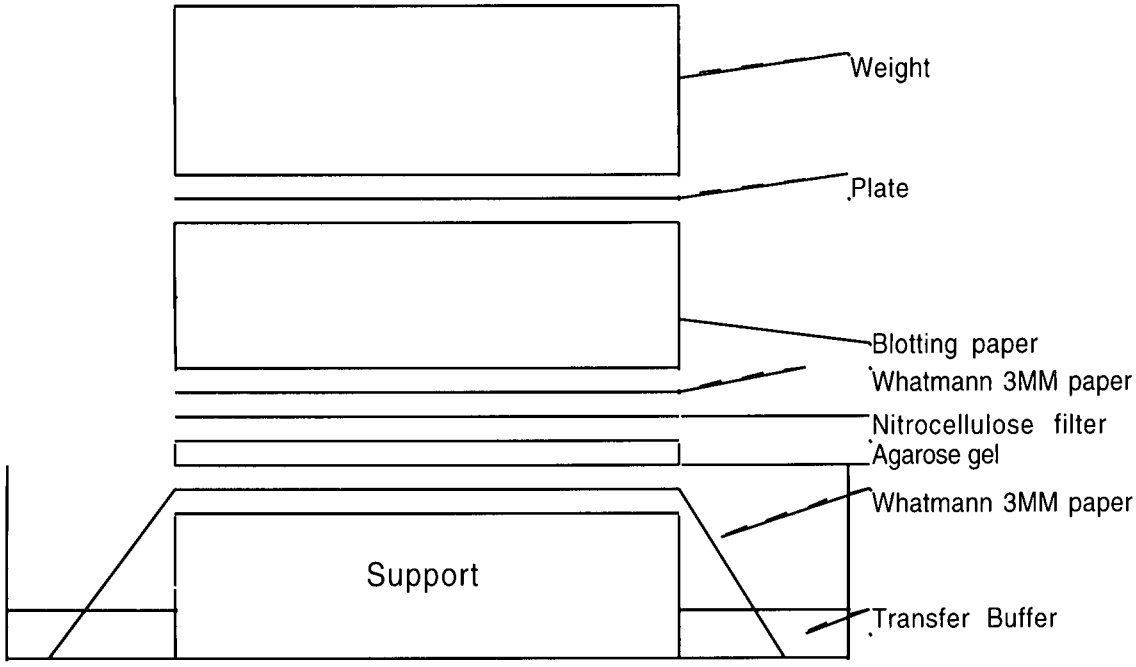
The DIG-labeled RNA and RNA ladders were prepared for electrophoresis. The 2X tracking buffer was made with 500 µl of formamide, 190 µl of 37% formaldehyde, 100 µl of 10X Formaldehyde Gel Running Buffer, 100 µl of 1% bromophenol blue, and 10 µl of DEPC-water. Three to five µl of RNA (~1.5 ug/ul) were added to an equal volume of tracking buffer. One µl of ethidium bromide (100 ug/ul) was added to the solution when not included in the gel. After heating at 65^{0}C for 15 minutes, the DIG-labeled RNA was cooled on ice for 3 minutes and pipetted into a central well (Sambrook, J., et al., 1989e). The 0.16-1.7 Kb RNA Ladder and/or 0.24-9.5 Kb RNA Ladder (Gibco BRL, Gaithersburg, MD) were prepared in the same manner and loaded into the adjacent wells. Electrophoresis was performed at 70 volts and stopped when the dye had migrated 3/4 the length of the gel. Gels containing ethidium bromide were washed in DEPC-water for approximately 1 hour whereas gels loaded with RNA samples containing ethidium bromide were washed in DEPC-water for approximately 10 minutes. Washes were performed at

room temperature on an oscillating shaker table set at 50 rpm. Gels were then viewed on a transilluminator (302 nm) and photographed.

Following gel electrophoresis the DIG-labeled RNA was transferred to a nitrocellulose filter by capillary elution (Sambrook, J., et al., 1989e) (Figure 6). The upper right hand corner of the agarose gel was removed for orientation during succeeding steps. The Blot Transfer Apparatus 11.14 (Gibco BRL, Gaithersburg, MD) was assembled and the buffer tray filled with 20X SSC (3 M NaCl, 0.3 M $C_6H_5Na_3O_7$, pH 7.0). The wicking filter was wetted in 20X SSC and draped over the tray insert such that the ends of the filter reached to the bottom of the reservoir. The agarose gel was centered on the wicking filter in an inverted position (wells down) and a glass rod used to smooth out air bubbles trapped between the wicking filter and nitrocellulose filter.

The nitrocellulose filter (0.45 um) was cut to the same size as the gel and a corner was clipped off for orientation. After wetting in 20X SSC for 10 minutes the nitrocellulose filter was carefully laid over the gel, matching up the clipped corners. A glass rod was

Figure 6: Capillary transfer of RNA transcripts.



Capillary transfer of nucleic acids from agarose gel to nitrocellulose filter. A buffer solution is drawn from the reservoir through the gel and nitrocellulose filter, into the stack of blotting paper. The RNA is deposited on the nitrocellulose filter.

used to smooth air bubbles out from between the wicking filter and nitrocellulose. Very small holes were punched into the nitrocellulose filter overtop the wells of the agarose gel. Two pieces of 3 MM paper were cut to the same size as the gel, and briefly wetted in the 20X SSC. After placing the 3 MM papers over the nitrocellulose filter, the gel was surrounded with plastic wrap and a 5-8 cm stack of blotting papers was placed on top the 3 MM papers. The plastic wrap prevented any contact between the blotting paper and the wicking filter. The transfer was allowed to proceed overnight at room temperature.

On the following day, the Genius Nucleic Acid Detection kit (Boehringer Mannheim, Indianapolis, IN) was used to visualize the riboprobe bands. The blotting paper, 3 MM paper, and nitrocellulose filter were gently peeled off of the agarose gel. After placing the nitrocellulose filter between two pieces of 3 MM paper, it was incubated at 80^{0}C for 1 hour. The filter was then washed in Buffer #1 (100 mM Tris-HCl, 150 mM, pH 7.5) for 3 minutes to remove any debris. All of the washes were performed on an oscillating shaker table set at 50 rpm. Nonspecific binding of the antibody to the filter

was prevented by incubating in Buffer #2 (1% low fat, dry milk in Buffer #1) at room temperature for 30 minutes.

The nitrocellulose filter was then washed in Buffer #1 for 3 minutes to remove excess blocking reagent and prepare for the antibody incubation. The alkaline phosphatase conjugated anti-digoxigenin polyclonal sheep antibody fab fragments (750 U/ml) were diluted 1:5,000 (150 mU/ml) in Buffer #1. The filter was incubated in the antibody solution at 37^{0}C for 30 minutes. Unbound antibody was removed by twice washing in Buffer #1, for 15 minutes each. To prepare the filter for the enzyme-substrate reaction, it was washed in Buffer #3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 3 minutes.

The enzyme-substrate reaction was performed with 45 µl of NBT solution (75 mg/ml of nitroblue tetrazolium salt in 70% dimethylformamide), 35 µl of BCIP solution (50 mg/ml of 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt, in dimethylformamide) and 10 ml of Buffer #3. The nitrocellulose filter was incubated in the dark with the substrate solution for approximately 30 minutes. The enzyme reaction was halted by incubating the filter in Buffer #4 (10 mM Tris-HCl, 1 mM EDTA, pH

8.0) for 1 hour. Once the filter had air dried it was stored at room temperature.

Preparation of the Tissue

Lassa infected, strain 13 guinea pig tissues were generously donated by Dr. Peter Jahrling (Senior Research Scientist, USAMRIID, Frederick, MD). The guinea pigs were given a subcutaneous injection of 650 pfu of the Josiah strain of Lassa virus (Jarhling, P.B., et al., 1982). After sacrificing the animals at 7, 11, and 14 days post inoculation, spleen and lung tissues were collected and fixed in 10% neutral buffered formalin (Columbia Diagnostics, Springfield, VA). The fixative was replaced at weekly intervals over a 30 day period. Tissues were then trimmed into 2-3 mm cross sections and placed in a Tissue Processor (Fisher Scientific, Pittsburgh, PA) for paraffin infiltration and embedment. Tissue sections 4-6 um thick were cut on a microtome, floated on DEPC-water and picked up onto Super Frost slides (Fisher Scientific, Pittsburgh, PA). Slides were placed in an upright position on a slide rack and stored at room temperature when dry.

Guinea pig tissues were prepared for protease digestion by first removing the paraffin and then rehydrating in ethanol. Using an

oscillating shaker table set at 50 rpm, the slides were gently agitated in xyless (Columbia Diagnostics, Springfield, VA) for 10 minutes to remove the paraffin (Boenisch, T., et al., 1989). This step was then repeated using fresh xyless. After sequentially immersing the slides for 3 minutes in 95%, 80%, and 70% ethanol, they were placed in DEPC-water.

Two methods of tissue digestion were compared for the preservation of tissue morphology and the digestion time required for ISH signal detection. One group of tissues was incubated at 37^{0}C in a protease VIII solution (0.05% protease VIII, 0.9 M monobasic sodium phosphate, 0.85 M dibasic sodium phosphate) for 0, 5, 10, 15, and 30 minutes. Another group was incubated at 37^{0}C in a proteinase K solution (0.1 M Tris, 0.05 M EDTA, pH 8.0, 1 ug/ml of proteinase K) for 0, 15, 30, 45, and 60 minutes (Simmons, D.M., et al., 1989). The concentration of proteinase K was then increased to 10 μg/ml and digestion incubations repeated as before. All digestions were followed by a 3 minute wash in DEPC-water, using an oscillating shaker table set at 50 rpms.

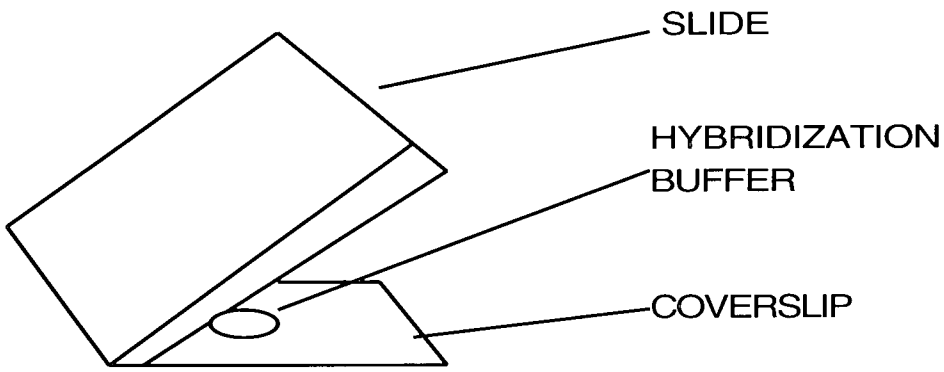
Hybridization and Wash Stringency

Lassa infected spleen and lung tissue sections were prehybridized to block any nonspecific binding of the riboprobe. Prehybridization buffer was made in advance with 40% formamide, 10% dextran sulfate, 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrolidone, 10 mg/ml RNase-free bovine serum albumin), 4X SSC, 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml salmon sperm DNA (Springer, J.E., et al., 1991). The buffer was aliquoted and stored at -70°C until needed.

Fifty µl of the prehybridization buffer were applied to the edge of a 24 x 30 mm coverslip. Slides to be prehybridized were taken from the DEPC-water and shaken to remove any excess liquid. Acting quickly to prevent tissue sections from drying, the slide was gently applied to the edge of the coverslip containing the buffer (Simmons, D.H. et al., 1989) (Figure 7). Once the capillary action had pulled the coverslip up to the slide, it was quickly turned over. The coverslip floated on top of the buffer, providing even coverage.

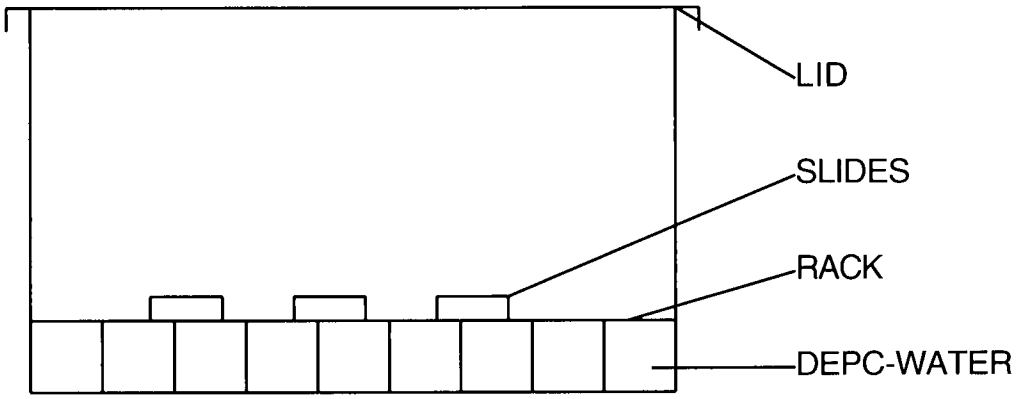
To prevent the tissues from drying out, a humidity chamber was used for slide incubations (Figure 8). The chamber was prepared by adding DEPC-water to below the level of the slide rack. After

Figure 7: Applying the hybridization buffer



The buffer is applied to the coverslip and the slide is gently lowered at an angle across the coverslip, allowing capillary action to pull the coverslip up to the slide.

Figure 8: Humidity chamber



Slides are incubated in a humidity chamber to prevent the tissues from drying.

placing slides on the rack, the lid was applied and the chamber placed in a 60^0 water bath for 1 hour (Angerer, L.M., et al., 1987). Slides were held upright, allowing the coverslips to fall off or gently agitated in DEPC-water until the coverslips fell off. Tissues not prehybridized proceeded directly to the hybridization step.

Hybridization buffer was made by adding Lassa riboprobes to the prehybridization buffer. Each type of riboprobe was used alone or in conjunction with the other two. One ul of each riboprobe was added to 100 ul, 1,000 ul, 10,000 ul, or 100,000 ul of hybridization buffer. The hybridization buffer was vortexed for 5 seconds, incubated at 65^{0}C for 15 minutes, and cooled on wet ice for 5 minutes (Sambrook, J., et al., 1989f). Fifty µl of hybridization buffer were pipetted onto 24 X 30 mm coverslips and applied in the same manner as the prehybridization buffer. Slides were then placed in the humidified chamber and incubated for 1, 2, 3, and 4 hours or overnight. The incubation temperature was varied from 40 to 80^{0}C (Simmons, D.H., et al., 1989).

On the following day, slides were washed in sequentially decreasing concentrations of 20X SSC to remove any unhybridized probe (Simmons, D.H., et al., 1989). The concentrations of SSC used

for cold washes ranged from 1X to 0.01X. Cold washes were performed for 15 minutes on an oscillating shaker table set at 100 rpm. A final 30 minute wash in heated 0.01X SSC was sometimes performed in a shaking waterbath set at 50 rpm. The temperatures used for the heated SSC wash were varied from 50°C to 80°C.

Detection of the Digoxigenin Labeled Transcript

Blocking buffer was used to prevent nonspecific binding of the anti-DIG antibody to the tissue. The blocking buffer was made in advance with 2% Teleostean Gelatin (Sigma, St. Louis, MO), 0.5 M Tris-HCl (pH 8.0) 0.2% BSA, 0.1% Triton X-100, and 0.02 M glycine. Tissue sections were covered with 50 µl of the blocking buffer and placed in the humidity chamber. Slides were incubated at 4°C for 30 minutes. Unused blocking buffer was stored at 4°C.

The Genius Nucleic Acid Detection kit (Boehringer Mannheim, Indianapolis, IN) was used to visualize the riboprobes. Tissues were covered with 50 µl of Buffer #1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 3 minutes. Triton X-100 (BIO-RAD, Richmond, CA) was added to Buffer #1 at concentrations of 0%, 0.25%, 0.50%, or 1.0% (v/v) and vigorously vortexed to dissolve. The alkaline phosphatase conjugated anti-digoxigenin polyclonal sheep antibody fab fragments

(anti-DIG) (750 U/ml) were diluted in the Buffer #1/Triton X-100 solution. The antibody dilutions ranged from 1:500 to 1:10,000. Tissue sections were covered with 100 µl of the diluted antibody solution. Slides were placed in the humidity chamber and incubated at 4°C overnight or at 37°C for 1-4 hours.

Unbound antibody was removed prior to developing the ISH signal. Slides were washed in Buffer #1 for 15 minutes on an oscillation shaker table set at 100 rpm. Next, slides were incubated at room temperature in Buffer #3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 minutes and the color-substrate solution was prepared with 45 µl NBT, 35 µl of BCIP and 10 ml of Buffer #3. After returning slides to the humidity chamber, tissue sections were covered with the color-substrate solution and incubated in the dark. Incubation temperatures were set at 24°C, or 37°C and the incubation times were varied from 15 minutes to 2 hours.

The enzyme-substrate reaction was halted by immersing the slides in Buffer #4 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 15 minutes. Slides were then rinsed with dH_20 and counterstained with nuclear fast red (Digene, Silver Spring, MD) for 5 minutes. Tissues

were dehydrated by sequential immersion in 70, 90, and 100% ethanol for 1 minute each. After clearing in xyless, the slides were mounted with permount, viewed by light microscopy, and photographed.

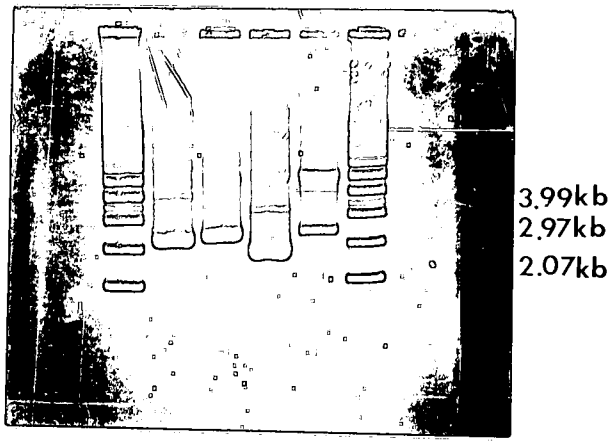
RESULTS

Characterization of Lassa cDNA Plasmids

Plasmids bearing Lassa cDNA inserts were generated by transforming competent cells, culturing those cells, and isolating the resulting plasmids as described in Materials and Methods. Gel electrophoresis confirmed the presence of the Lassa cDNA within pGEM-4. Plasmids were separated by electrophoresis on 1% agarose gels and compared to Supercoiled DNA Ladder (Gibco BRL, Gaithersburg, MD) (Figure 9). The migration of LSGP-4bS (3.396 Kb), LSGP-4aT (3.294 Kb), and LSN-4bT (3.290 Kb) was noted between the 2.97 and 3.99 Kb markers. The migration of pGem-4 (2.871 Kb) was noted between the 2.07 and 2.97 Kb markers.

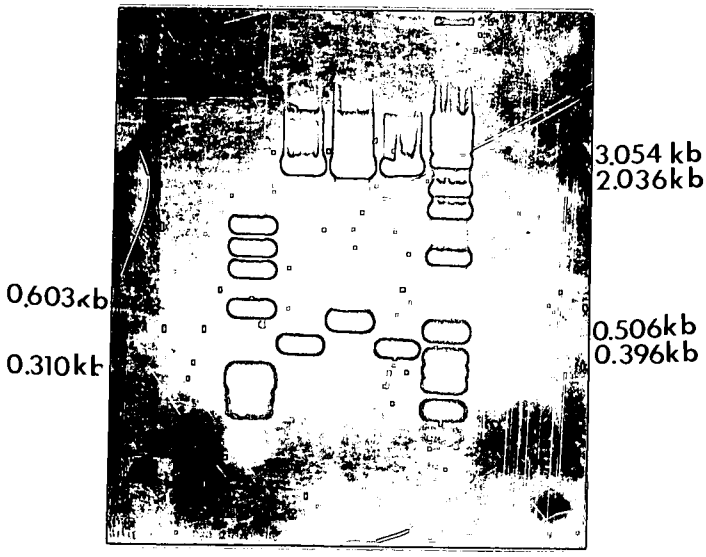
Further confirmation came from digesting isolated plasmids with restriction enzymes to remove the Lassa cDNA inserts (Figure 10). The migration of the resulting bands was compared to the ϕX174 RF DNA Hae III fragments (Gibco BRL, Gaithersburg, MD) and the 1 Kb DNA Ladder (Gibco BRL, Gaithersburg, MD). All three lanes placed pGEM-4 between the 3.054 Kb and 2.054 Kb markers of the 1 Kb DNA Ladder (Gibco BRL, Gaithersburg, MD). This corresponded well with the expected pGEM-4 size of 2.871 Kb. The digestion of

Figure 9: Lassa cDNA bearing plasmids



The 0.9% agarose gel shows 1 Kb Supercoiled DNA Ladder (Gibco BRL, Gaithersburg, MD) in lanes 1 and 6, LSGP-4aT (3.294 Kb) in lane 2, LSGP-4bS (3.396 Kb) in lane 3, pGEM-4 (2.871 Kb) in lane 4, and LSN-4bT (3.290 Kb) in lane 5.

Figure 10: Restriction digest of Lassa cDNA bearing plasmids.



The 0.9% agarose gel shows ϕX174 RF DNA/Hae III Fragments (Gibco BRL, Gaithersburg, MD) in lane 1, LSGP-4aT cut with EcoR I in lane 2, LSGP-4bS cut with EcoR I and BamH I in lane 3, LSN-4bT cut with Xba I in lane 4, and 1 kb DNA (Gibco BRL, Gaithersburg, MD) ladder in lane 5. The bands of pGEM-4 are noted at 3.0 Kb marker and the Lassa cDNA bands are noted at between the 0.396 Kb and 0.603 Kb markers.

LSGP-4aT and LSN-4bT resulted in bands between the 0.396 Kb and 0.506 Kb marker. This correlated well with the expected LSGP-4aT size of 0.423 Kb and the LSN-4bT size of 0.419 Kb. The digestion of LSGP-4bS resulted in a band just above the 0.506 Kb marker which correlated well with the expected LSGP-4bS size of 0.562 Kb.

Completion of the restriction digest was necessary to generate an isolated band of riboprobe. Restriction enzymes having unique sites within the multicloning region and giving rise to either a blunt cut or a 5′ overhang were compared for digestion efficiency. Enzymes giving rise to a 3′ overhang were excluded because the 3′ OH group can serve as a start site for RNA transcription (Promega , 1991). To avoid nonspecific cutting, digestion incubations were limited to 2 hours (Sambrook, J., et al., 1989c). Restriction enzymes contain 50% glycerol to inhibit their activity. To prevent glycerol inhibition of the restriction digestion, the enzyme contribution was kept under 0.1 volume of the final reaction mixture.

Unique restriction sites for Hind III, Sma I, Xba I, Ava I, Sal I, EcoR I, and BamH I located within the pGEM-4 multicloning region were used to linearize the Lassa cDNA bearing plasmids (Figure 2). Samples of restriction digests were separated by electrophoresis on

agarose gels, stained with ethidium bromide and viewed with a transilluminator. More than one band of DNA was generated by digests performed with 20 units of either Hind III, Sma I, Xba I, Ava I, Sal I, EcoR I, or BamH. The increased migration of some of the bands indicated that uncut DNA was present. One band of DNA was generated by digests performed with 100 units of EcoR I or BamH I. The migration of the band indicated that it was linearized DNA.

Competent cells were transformed with linearized plasmid to determine the completeness of the digest. Complete digestion was necessary to prevent transcription from continuing past the cDNA along the pGEM-4 sequence. Digests performed with 10 units of BamH I or EcoR I generated approximately 50 colonies per 100 μl of SOC. Increasing the enzyme concentration to 50 units and repeating the transformation generated no bacterial colonies, indicating that circular DNA was not present. In the controls, competent cells transformed with pGEM-4 generated many colonies whereas cells subjected to the transformation reaction without added plasmid did not give rise to any colonies.

Generating DIG-11-UTP Labeled RNA

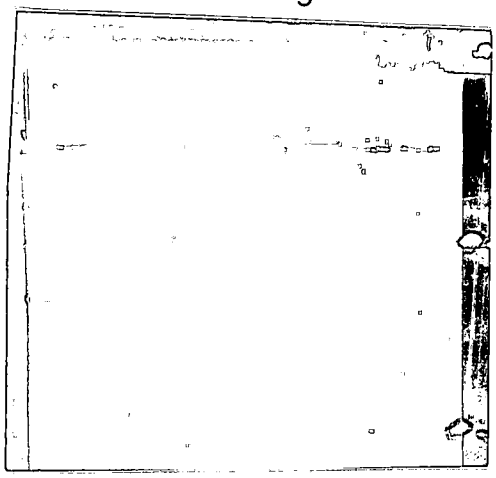
Transcription reactions carried out with the Riboprobe Gemini II Core System kit (Promega, Madison, WI), pGEM-4 bearing Lassa cDNA templates, and DIG-11-UTP generated very consistent quantities of RNA, as noted by ultraviolet spectroscopy. A 1:1 ratio of DIG-11-UTP to UTP in the transcription solution and an incubation period of 1 hour generated approximately 30 μg of RNA per reaction. The quantity of RNA generated did not change when the incubation time was increased to 2 hours, nor when a different Lassa virus cDNA template or RNA polymerase was used. Transcription reactions carried out for 1 hour using either UTP alone, a 1:3 or a 3:1 ratio of DIG-11-UTP to UTP generated the same quantity of RNA, as determined by ultraviolet spectroscopy.

Visualization of RNA transcripts and RNA ladders on formaldehyde-agarose gels was improved by adding ethidium bromide directly to the RNA, and by using Nusieve 3:1 Agarose (FMC, Rockland, ME). Agarose gels containing ethidium bromide required approximately 5 μl of RNA transcribed with a 1:1 ratio of DIG-11-UTP to UTP, for visualization. The background fluorescence of ethidium bromide made it difficult to obtain a satisfactory

photograph (Figure 11). The fluorescence could be decreased by washing the gels in DEPC-water for 1 or more hours but the RNA band also became more diffused. The addition of 1 µl of 100 µg/ml of ethidium bromide to the RNA sample increased the contrast and resulted in a better photograph (Figure 12). Using this method, only 3 µl of riboprobe-50 were needed for visualization. Tighter bands of RNA were also noted when 3% Nusieve was used in place of Seakem ME agarose (Figure 12).

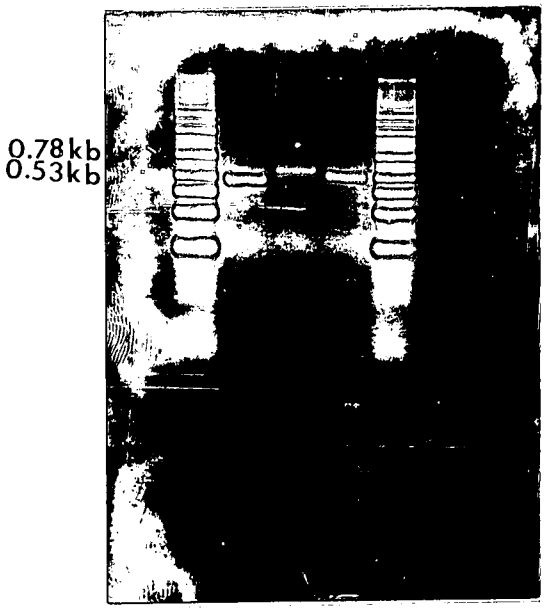
Transcription of linearized LSGP-4aT and LSN-4bT gave rise to RNA fragments of approximately 0.45 Kb in length, whereas transcription of linearized LSGP-4bS gave rise to fragments of approximately 0.55 Kb in length. RNA generated from LSGP-4aT or LSN-4bT without the incorporation of DIG-11-UTP was visualized between the 0.40 Kb and the 0.53 Kb markers whereas RNA generated from LSGP-4bS without the incorporation of DIG-11-UTP was visualized at the 0.53 Kb marker (Figure 13). As the incorporation of DIG-11-UTP increased, migration of the RNA fragments decreased and the visability of the resulting bands decreased (Figure 14). The control RNA, SIV-50 was also visable on formaldehyde-agarose gels between the 0.40 Kb and the 0.53 Kb markers.

Figure 11: Seakem ME RNA gel



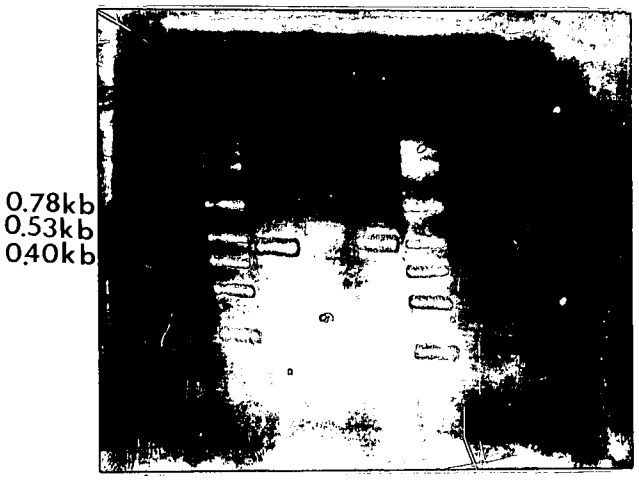
Seakem ME formaldehyde-agarose gel containing ethidium bromide.

Figure 12: Nusieve RNA gel



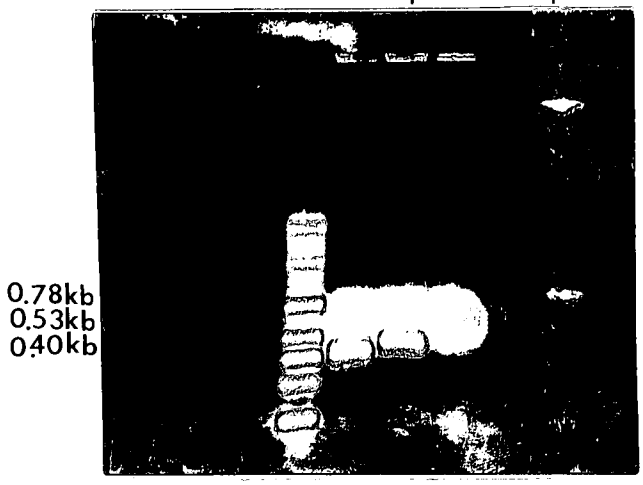
Nusieve formaldehyde-agarose gel showing 0.16-1.77 Kb RNA Ladder in lanes 1 and 5, LSGP-4aT-50 in lane 2, LSGP-4bS-50 in lane 3, and LSN-4bT-50 in lane 4.

Figure 13: Lassa RNA transcripts



Formaldehyde-agarose gel showing 0.16-1.77 Kb RNA Ladder (Gibco BRL, Gaithersburg, MD) in lane 1 and lane 5, LSGP-4aT-0 in lane 2, LSN-4bT-0 in lane 3, and LSGP-4bS-0 in lane 4.

Figure 14: Lassa RNA transcripts incorporated with Digoxigenin.



Formaldehyde-agarose gel showing 0.16-1.77 Kb RNA Ladder (Gibco BRL, Gaithersburg, MD) in lane 1, LSGP-4aT-0 in lane 2, LSGP-4aT-50 in lane 3, and LSGP-4aT-75 in lane 4.

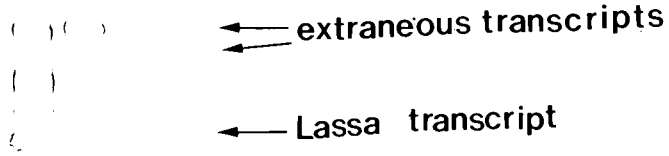
DIG labeled RNA transcripts were transferred from the agarose gels to nitrocellulose filters by capillary elution and visualized with the Genius Nucleic Acid Detection kit (Boehringer Mannheim, Indianapolis, IN). Extraneous RNA bands that were not detected on the ethidium stained agarose gels were detected on the nitrocellulose filters. Plasmids linearized with less than 50 units of restriction enzyme generated extraneous RNA bands (Figure 15). Increasing the restriction enzyme to 50 units or greater resulted in a single RNA band (Figure 16).

Preparation of the Tissue

Tissue sections were well adhered to the slides as there was no loss of sections during the ISH procedures. Methods used for tissue fixation and processing preserved both the tissue morphology and Lassa mRNA. Lassa virus mRNA detected within Lassa infected tissues could be eliminated by treating tissues with RNAase, prior to hybridization.

Tissue digestion methods were varied to optimize the hybridization signal, decrease incubation time, and preserve tissue morphology. No hybridization signal was detected in tissues incubated in the digestion buffer without either Proteinase K or

Figure 15: Extraneous RNA transcripts on nitrocellulose filter.



Extraneous RNA transcripts generated from plasmids linearized with 10 units/μg of restriction enzyme. Transcripts were transferred from a formaldehyde-agarose gel and visualized on a nitrocellulose filter with the Genius Nucleic Acid Detection kit (Boehringer Mannheim, Indianapolis, IN).

Figure 16: Lassa RNA transcripts on nitrocellulose filter.

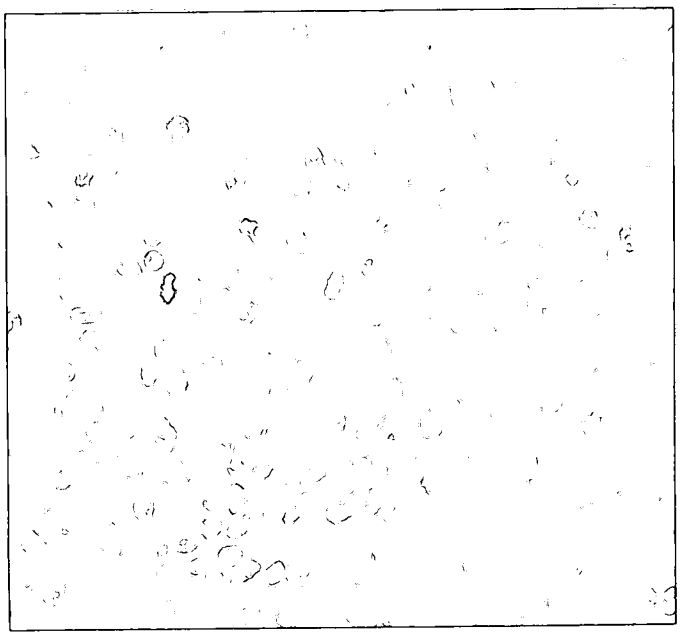
Single band of RNA transcript generated from plasmids linearized with 50 units of restriction enzyme. Transcripts were transferred from a formaldehyde-agarose gel and visualized on a nitrocellulose filter with the Genius Nucleic Acid Detection kit (Boehringer Mannheim, Indianapolis, IN).

Protease VIII (Figure 17). Tissues required digestion incubations of at least 60 minutes to generate ISH signal when 1 μg/ml of Proteinase K was used. Increasing the concentration to 10 μg/ml generated a weak ISH signal after a 30 minute digestion. Optimal ISH signal required a 60 minute digestion. Protease VIII required an incubation of only 15 minutes for optimal signal detection (Figure 18). Digestions of less than 15 minutes generated weaker ISH signal whereas increasing the digestion to 30 minutes or more resulted in a loss of tissue morphology. Therefore, the 15 minute incubation with Protease VIII was selected for routine tissue digestions.

Hybridization And Wash Stringency

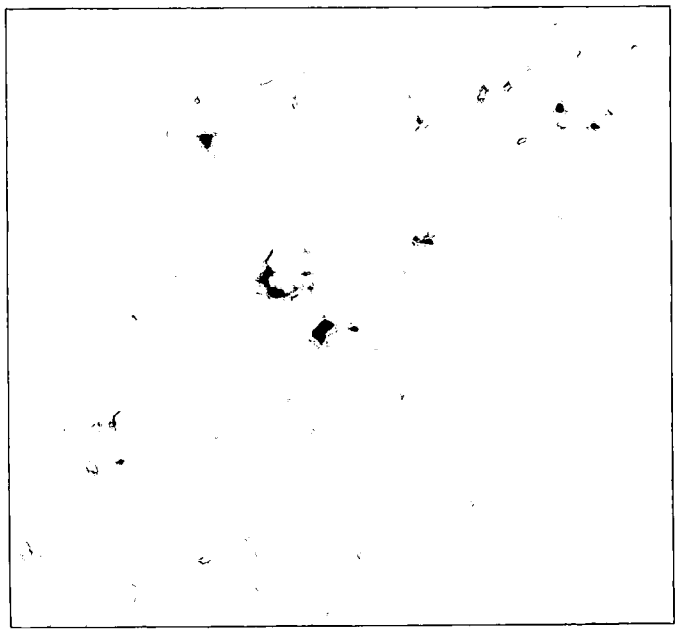
The temperature of the hybridization incubation was varied to optimize the Lassa mRNA detection. ISH using transcripts from plasmids bearing Lassa nucleoprotein or glycoprotein cDNA inserts detected Lassa virus mRNA within Lassa infected tissue sections but not within uninfected sections (Figures 19 and 20). All of the infected spleen sections hybridized overnight at 40, 50, 60, 70, or 80^{0}C, and washed for 15 minutes each in 1 X SSC and 0.1X SSC, generated ISH signal. The 70^{0}C and 80^{0}C incubations also resulted in a loss of tissue morphology. The hybridization signal generated by

Figure 17: Spleen section that was not digested prior to hybridization.



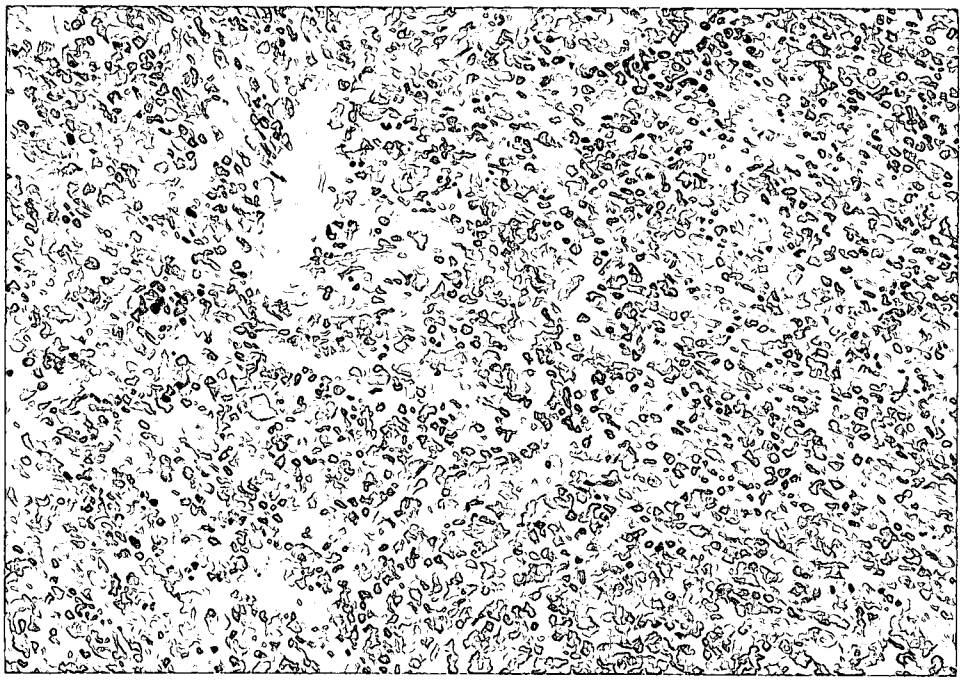
40X magnification of spleen section from Lassa virus infected guinea pig incubated in digestion buffer without Proteinase K or Protease VIII. ISH employed riboprobes LSGP-4aT-50, LSGP-4bS-50 and LSN-4bT-50. ISH signal as a blue precipitate from NBT and BICP was not noted. Counterstained with Nuclear Fast Red.

Figure 18: Spleen section that was digested with Protease VIII prior to hybridization.



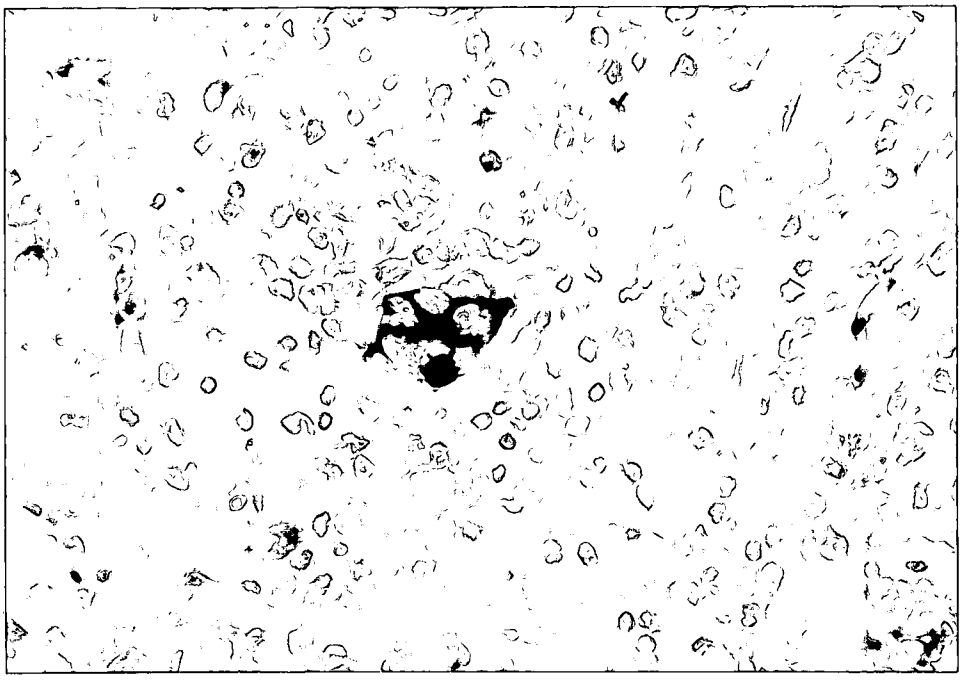
40X magnification of spleen section from Lassa virus infected guinea pig, incubated in 0.05% Protease VIII digestion buffer for 15 minutes. ISH employed riboprobes LSGP-4aT-50, LSGP-4bS-50 and LSN-4bT-50. ISH signal was noted as a blue precipitate from NBT and BICP. Counterstained with Nuclear Fast Red.

Figure 19: Spleen section of an uninfected guinea pig following the ISH assay



20X magnification of spleen tissue taken from an uninfected guinea pig. ISH employed riboprobes LSGP-4aT-50, LSGP-4bS-50 and LSN-4bT-50. No blue precipitate from NBT and BICP was noted. Counterstained with Nuclear Fast Red.

Figure 20: Spleen section from a Lassa virus infected guinea pig following the ISH assay



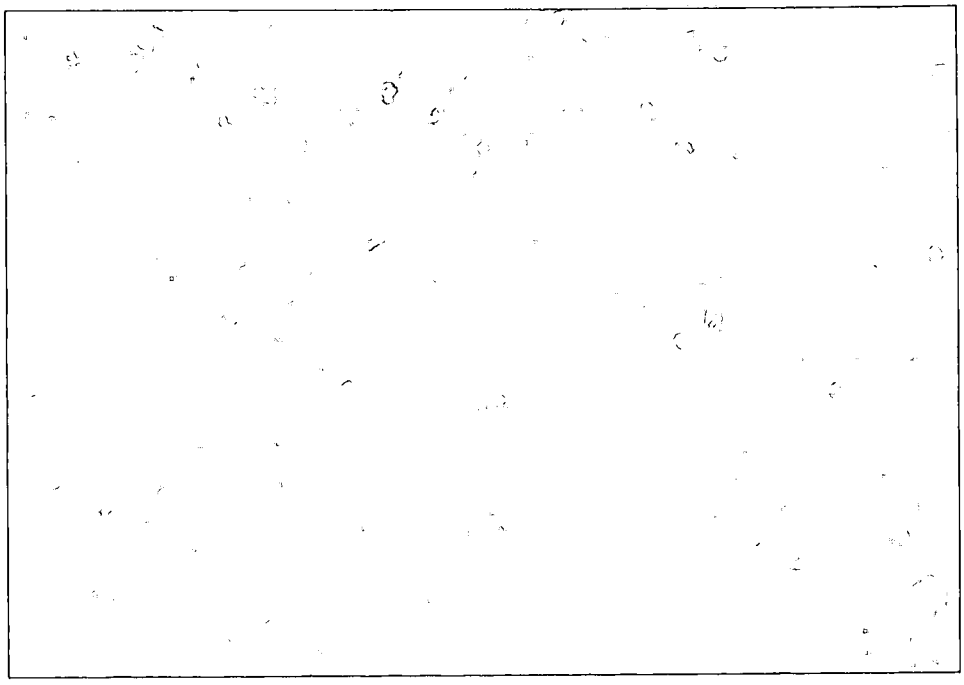
40X magnification of spleen tissue taken from a Lassa virus infected guinea pig. ISH employed riboprobes LSGP-4aT-50, LSGP-4bS-50 and LSN-4bT-50. Blue precipitate from NBT and BICP was noted over specific cells. Counterstained with Nuclear Fast Red.

the 40°C incubation showed more nuclear and connective tissue staining. Hybridizing at 60-70°C generated stronger hybridization signal than at 50°C and without any nuclear staining.

The length of the hybridization period was varied to determine the minimal time needed for optimal detection of Lassa mRNA. Hybridization incubations were performed at 60°C for 1, 2, 3, 4 hours or overnight. No hybridization signal was detected when slides were incubated for 1 hour (Figure 21). Hybridization signal was difficult to detect in slides incubated for 2 hours. Incubating slides for 3 hours generated hybridization signal equal to that of tissues incubated overnight (Figure 22).

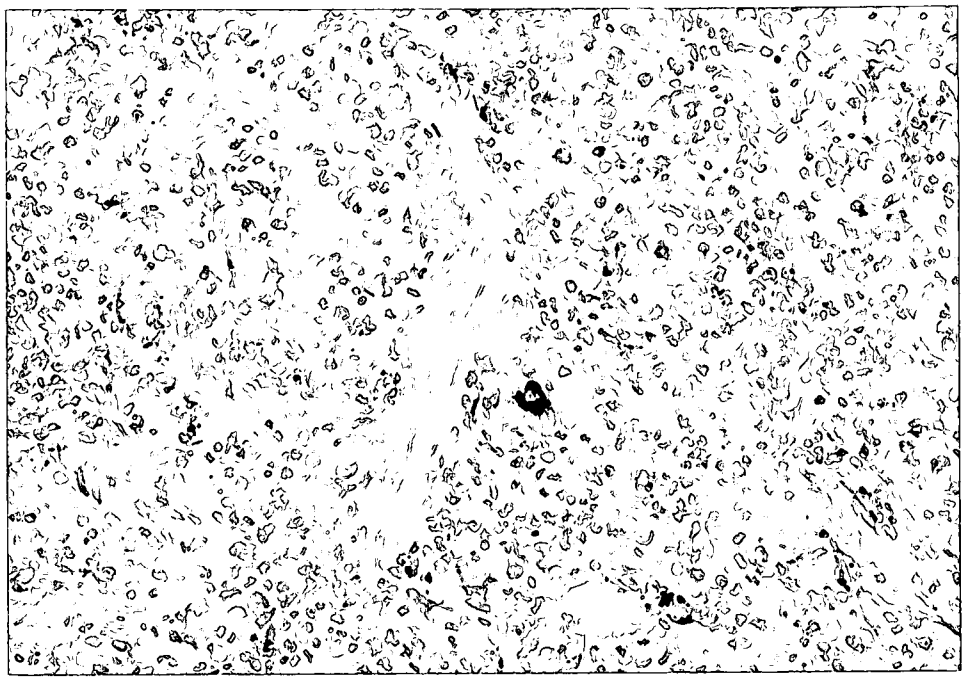
The temperature of the hybridization was varied to determine its effect on the ISH signal. Slides were hybridized for 3 hours at 40, 50, 60, or 70°C and washed for 15 minutes each in 1X SSC, 0.1X SSC, and 0.01X SSC. The strongest, most consistent ISH signal was noted in tissues hybridized at 60-70°C (Figure 22). Tissues hybridized at 70°C, however, sometimes showed a loss of morphology so a hybridization temperature of 60°C was selected for routine use.

Figure 21: Lassa infected spleen section hybridized for 1 hour.



40X magnification of spleen section from Lassa virus infected guinea pig tissue, hybridized for 1 hour at 60^{0}C with LSGP-4aT-50, LSGP-4bS-50, and LSN-4aT-50. No blue precipitate from NBT and BCIP was noted. Counterstained with Nuclear Fast Red.

Figure 22: Lassa infected spleen section hybridized for 3 hours.



20X magnification of spleen section from Lassa virus infected guinea pig tissue. Tissue was hybridized for 3 hour at 60^{0}C with LSGP-4aT-50, LSGP-4bS-50, and LSN-4aT-50. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

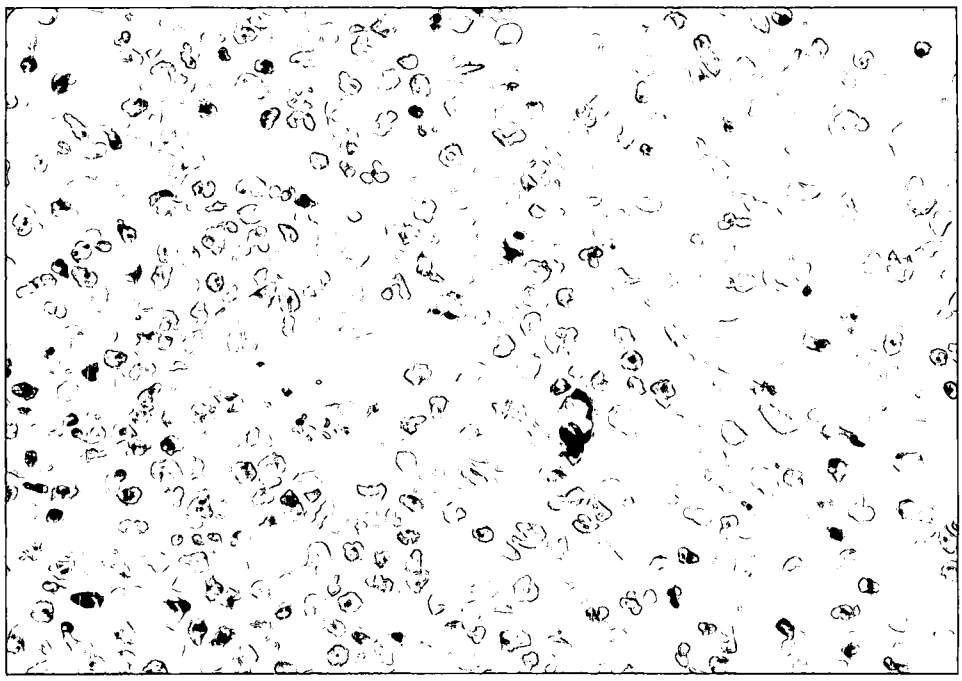
The primary difficulty with this assay was the inability to quantitate the riboprobe. Absorbency readings by ultra violet spectroscopy did not correlate with the strength of the riboprobe bands detected by agarose gel electrophoresis or the Genius Nucleic Acid Detection kit. Neither phenol chloroform extraction nor purification over a sephadyl column generated a detectable riboprobe band by gel electrophoresis. Dot blot quantification using the Genius Nucleic Acid Detection kit was not a possibility, as a control riboprobe labeled in the same manner was not available. To minimize the problem of quantification, we first diluted the precipitated riboprobe into a 20 µl volume of TE and then did a series of serial dilutions in hybridization buffer.

To determine the optimal concentration of DIG labeled RNA needed, serial dilutions of the RNA transcripts were made in hybridization buffer. One µl each of LSGP-4aT-50 (~1.5 µg/µl), LSGP-4bS-50 (~1.5 µg/µl), and LSN-4bT-50 (~1.5 µg/µl) were diluted into 100 µl, 1,000 µl, 10,000 µl, and 100,000 µl of hybridization buffer. The strongest, most consistent ISH signal was generated

with the 1:100 and 1:1,000 dilution (Figure 23). A very weak ISH was generated with the 1 to 10,000 dilution and no signal was generated with the 1 to 100,000 dilution of labeled transcripts (Figure 24). The assay was then repeated using each RNA transcript alone. The hybridization signal was weaker but still present at the same dilutions (Figures 25 and 26). The 1 to 1,000 dilution of all three transcripts was selected for routine use.

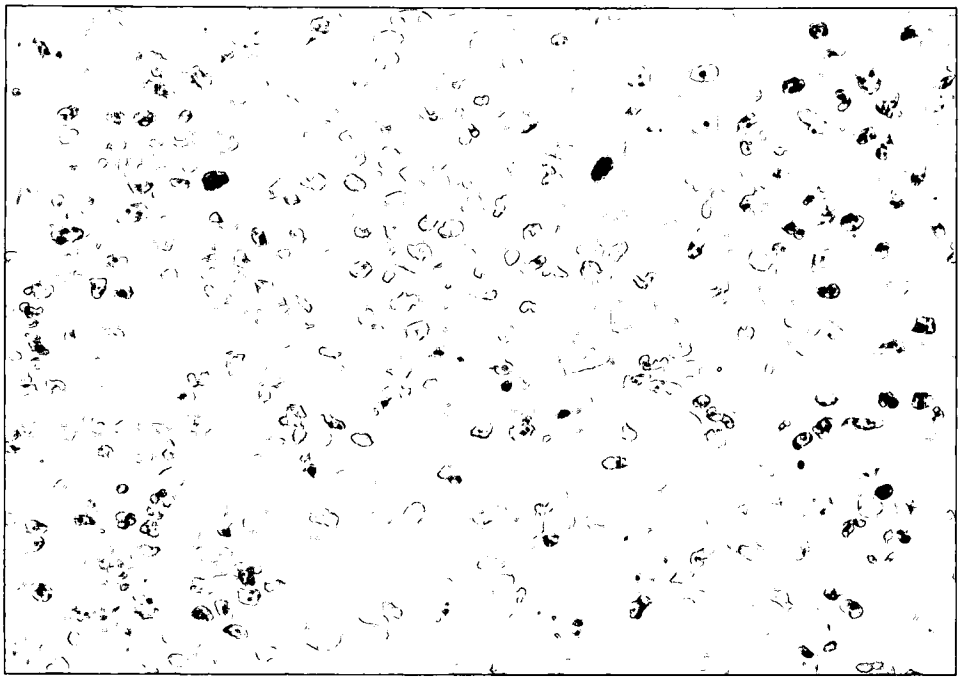
The optimal labeling was determined by repeating the assay with a cocktail of LSGP-4aT-75 (~1.5 μg/μl), LSGP-4bS-75 (~1.5 μg/μl), and LSN-4bT-75 (~1.5 μg/μl) or with a cocktail of LSGP-4aT-25 (~1.5 μg/μl), LSGP-4bS-25 (~1.5 μg/μl), and LSN-4bT-25 (~1.5 μg/μl). Hybridizing with a 1 to 10,000 μl dilution of either cocktail did not generate any ISH signal. Hybridizing with a 1 to 1,000 μl dilution of either cocktail generated a weak signal (Figures 27 and 28). The LSGP-4aT-50, LSGP-4bS-50, and LSGP-4bT-50 were selected as the optimal concentrations as the ISH signal could be detected at greater dilutions than for either of the above RNA transcripts. Slides were sequentially washed in decreasing concentrations of 20X SSC to determine the stringency needed to

Figure 23: Lassa virus infected spleen section hybridized with a 1 to 1,000 dilution of Lassa RNA transcript.



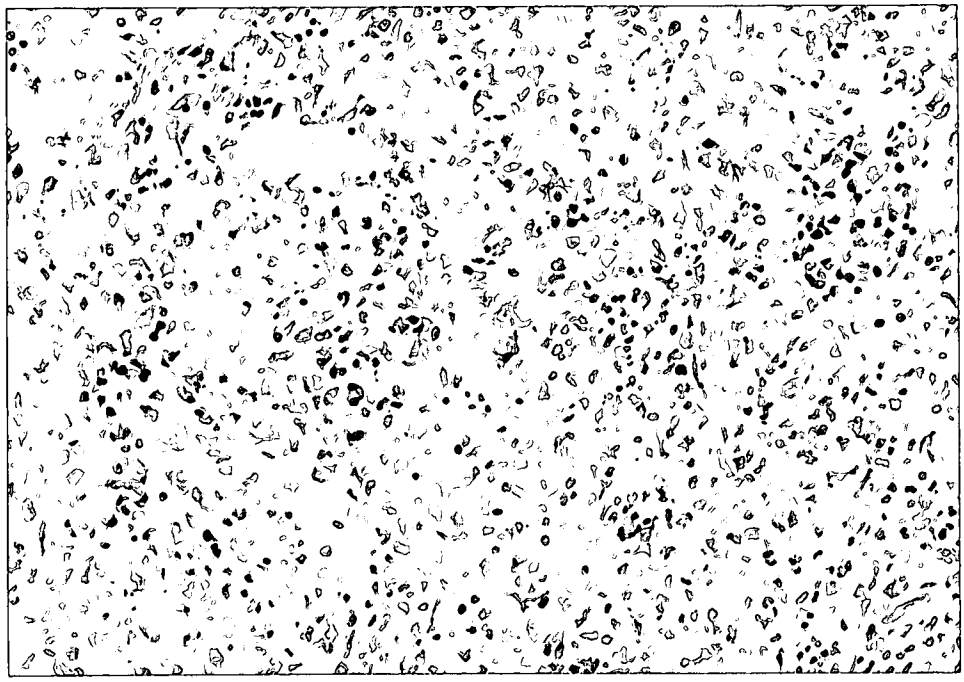
40X magnification of a spleen section from a Lassa virus infected guinea pig. Tissue was hybridized with a 1 to 1,000 dilution of LSGP-4aT-50, LSGP-4bS-50, and LSN-4aT-50. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 24: Lassa virus infected spleen section hybridized with a 1 to 10,000 dilution of Lassa RNA transcript.



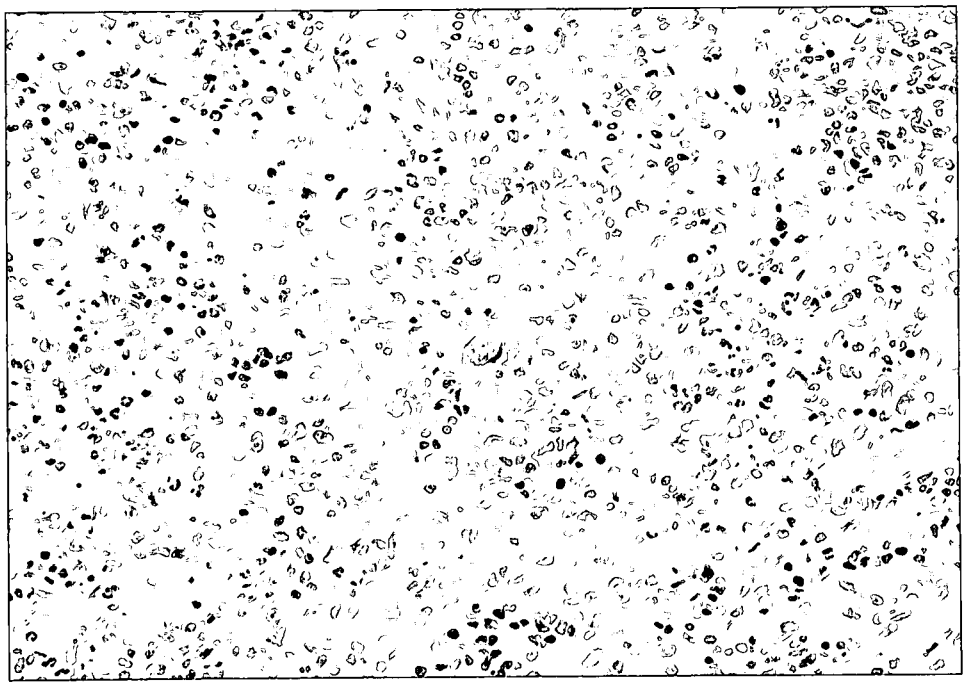
40X magnification of spleen section from Lassa virus infected guinea pig tissue. Tissue was hybridized with 1 to 10,000 dilution of LSGP-4aT-50, LSGP-4bS-50, and LSN-4aT-50. Faint blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 25: Lassa virus infected spleen section hybridized with 1 to 1,000 dilution of LSGP-4aT.



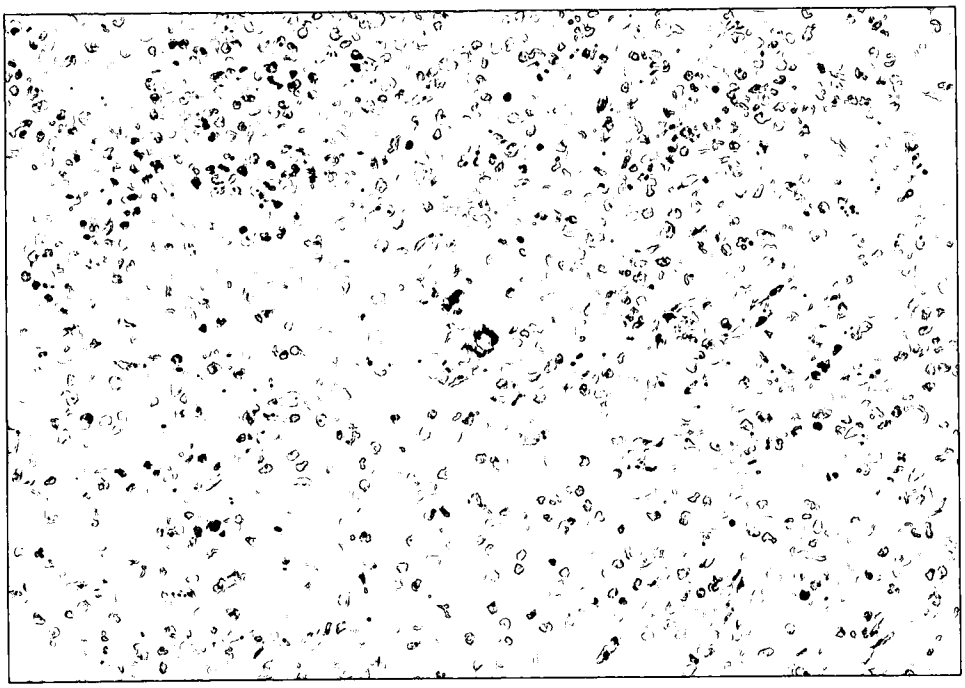
20X magnification of spleen section from Lassa virus infected guinea pig. Tissue was hybridized with a 1 to 1,000 dilution of LSGP-4aT-50. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 26: Lassa virus infected spleen section hybridized with a 1 to 10,000 dilution of LSGP-4aT-50.



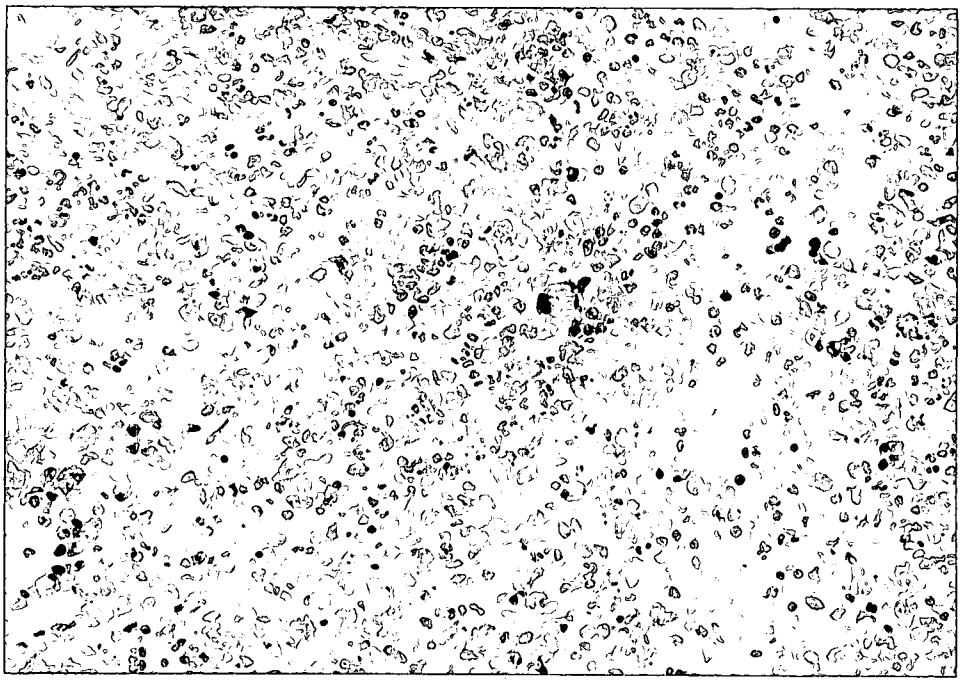
20X magnification of spleen section from Lassa virus infected guinea pig tissue. Tissue was hybridized with a 1 to 10,000 dilution LSGP-4aT-50. Faint blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 27: Lassa virus infected spleen section hybridized with LSGP-4aT-75, LSGP-4bS-75, and LSN-4bT-75.



20X magnification of spleen section from Lassa virus infected guinea pig. Tissue was hybridized with a 1 to 1,000 dilution of LSGP-4aT-75, LSGP-4bS-75, and LSN-4bT-75. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 28: Lassa virus infected spleen section hybridized with LSGP-4aT- 25, LSGP-4bS-25, and LSN-4bT-25.



20X magnification of spleen section from Lassa virus infected spleen section. Tissue was hybridized with a 1 to 1,000 dilution LSGP-4aT-25, LSGP-4bS-25, and LSN-4bT-25. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

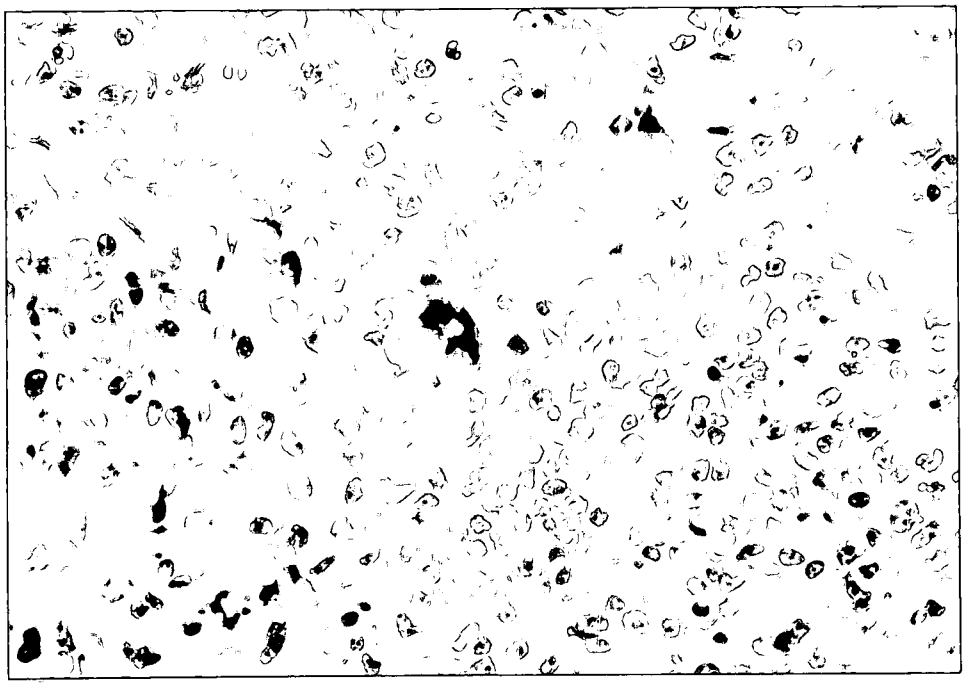
eliminate ISH signal. Increasing the cold wash stringency from 1X SSC, to 0.1X SSC, and to 0.01X SSC did not appear to affect the signal (Figures 29 and 30). ISH signal was eliminated by washing with 0.01X SSC at 64^{0}C for 30 minutes and greatly lessened by washing at 63^{0}C for 30 minutes (Figure 31). The ISH signal did not appear to be affected by washing at temperatures of 60^{0}C or less (Figure 32). Therefore, stringency washes were performed for 15 minutes in 0.01X SSC.

To minimize the time required for the assay the necessity of the prehybridization step was examined. Prehybridized tissue did not generate less ISH signal than nonprehybridized tissue (Figure 33). Nor did there appear to be greater nonspecific signal in the tissue not prehybridized (Figure 34). Therefore, the prehybridization step was no longer used.

Verification of the In situ Hybridization Signal

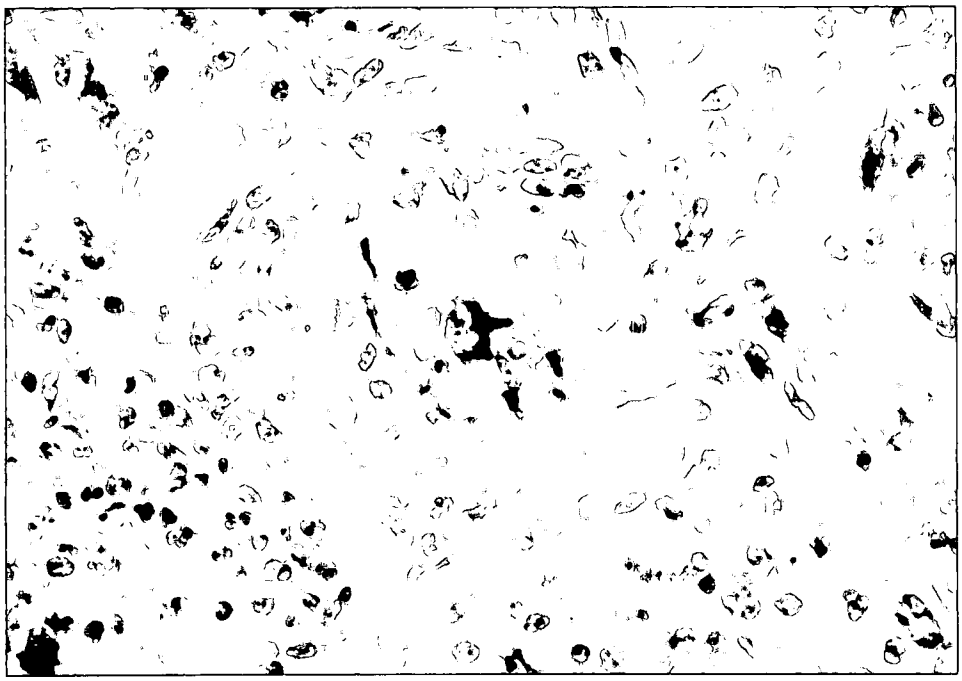
Several controls were used to verify the ISH signal. All controls were hybridized for 3 hours at 60^{0}C and washed for 15 minutes in 0.01X SSC. Uninfected guinea pig tissues and SIV infected monkey tissues did not generate ISH signal when hybridized with Lassa RNA transcripts (Figures 35 and 36). Lassa virus

Figure 29: Lassa virus infected spleen section washed in 1X SSC.



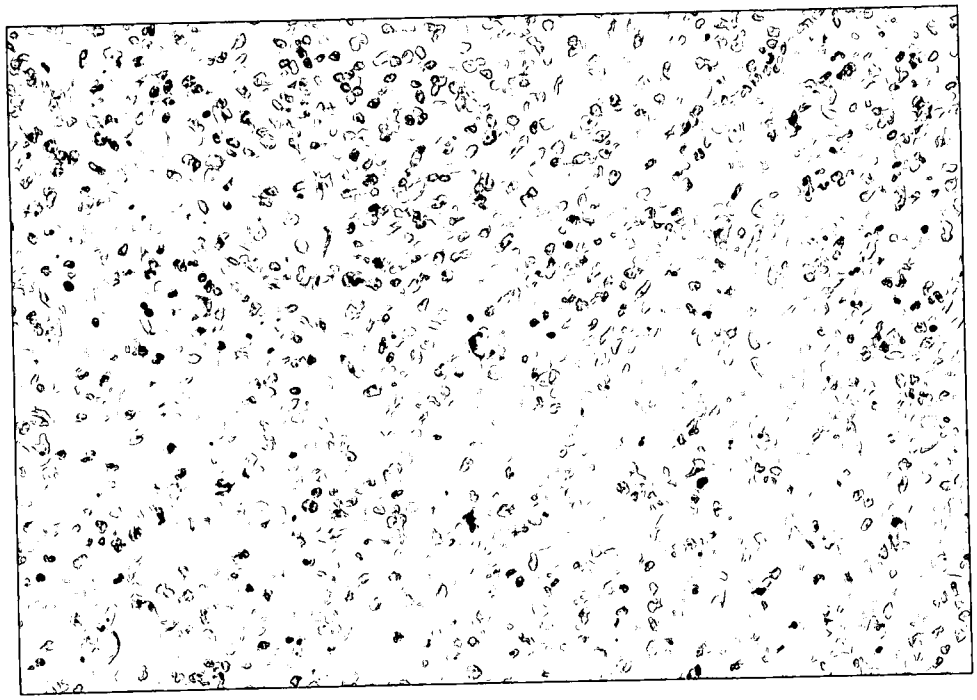
40X magnification of spleen section from Lassa virus infected guinea pig. Tissue was washed for 15 minutes at room temperature in 1X SSC. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 30: Lassa virus infected spleen section washed in 0.01X SSC.



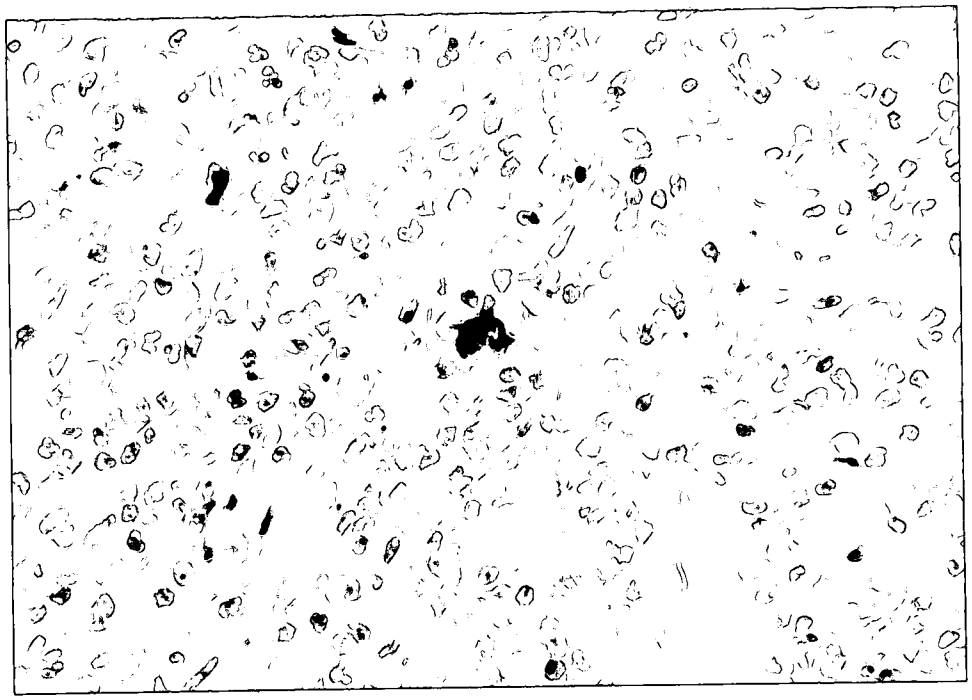
40X magnification of spleen section from Lassa virus infected guinea pig. Tissue was washed at room temperature for 15 minutes each in 1X SSC, 0.1X SSC, and 0.01X SSC. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 31: Lassa virus infected spleen section washed at 63°C.



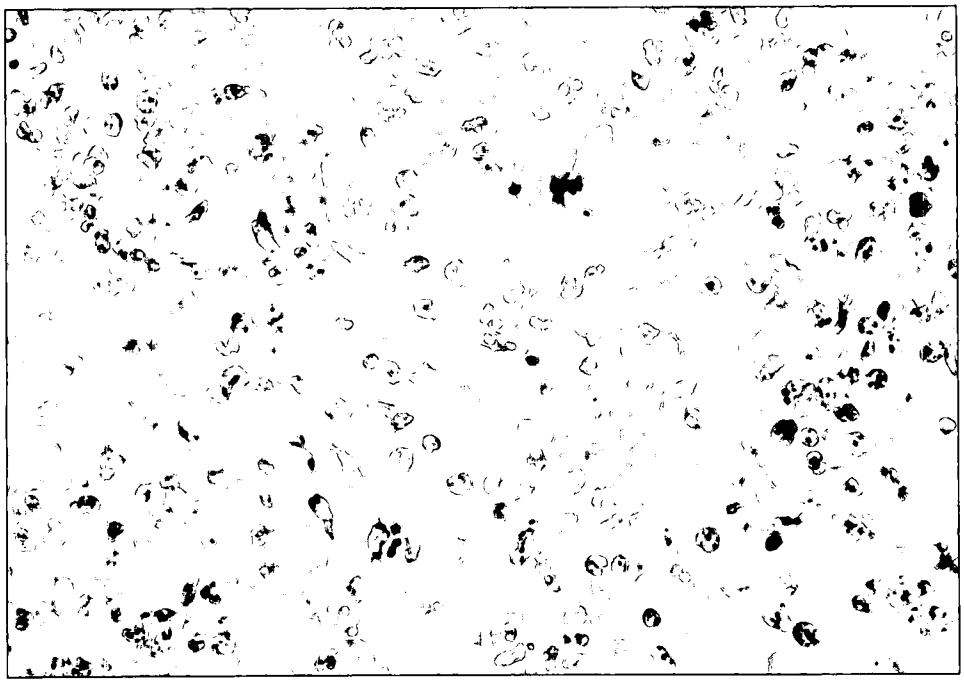
20X magnification of spleen section from Lassa virus infected guinea pig. Tissue was washed at 63°C, in 0.01X SSC for 30 minutes. Faint blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 32: Lassa virus infected spleen section washed at 60°C.



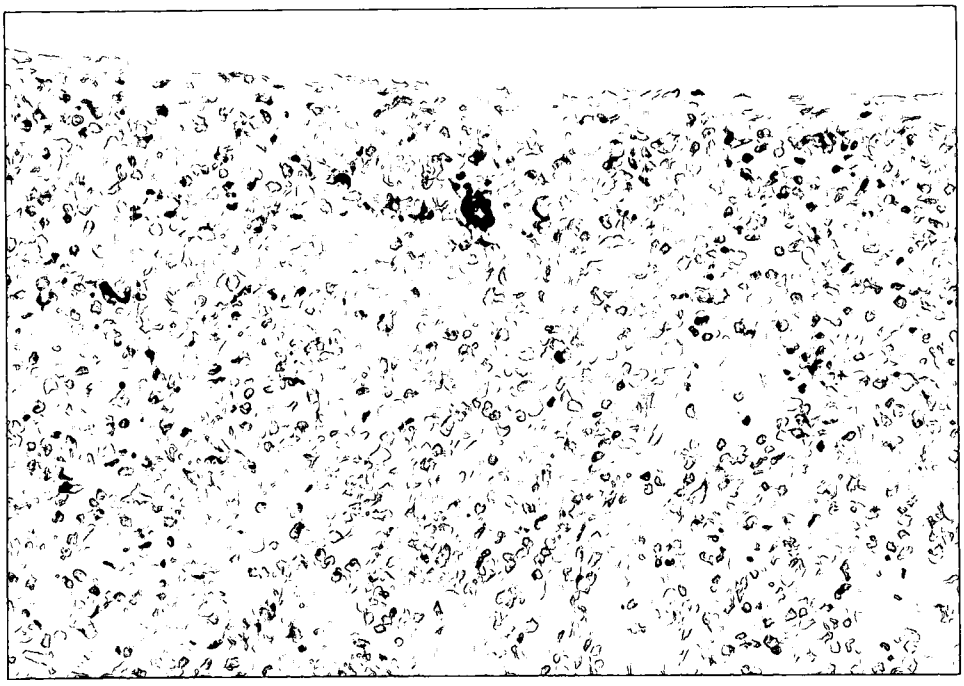
40X magnification of spleen section from Lassa virus infected guinea pig. Tissue was washed at 60°C in 0.01X SSC for 30 minutes. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 33: Lassa virus infected spleen section that received the prehybridization step.



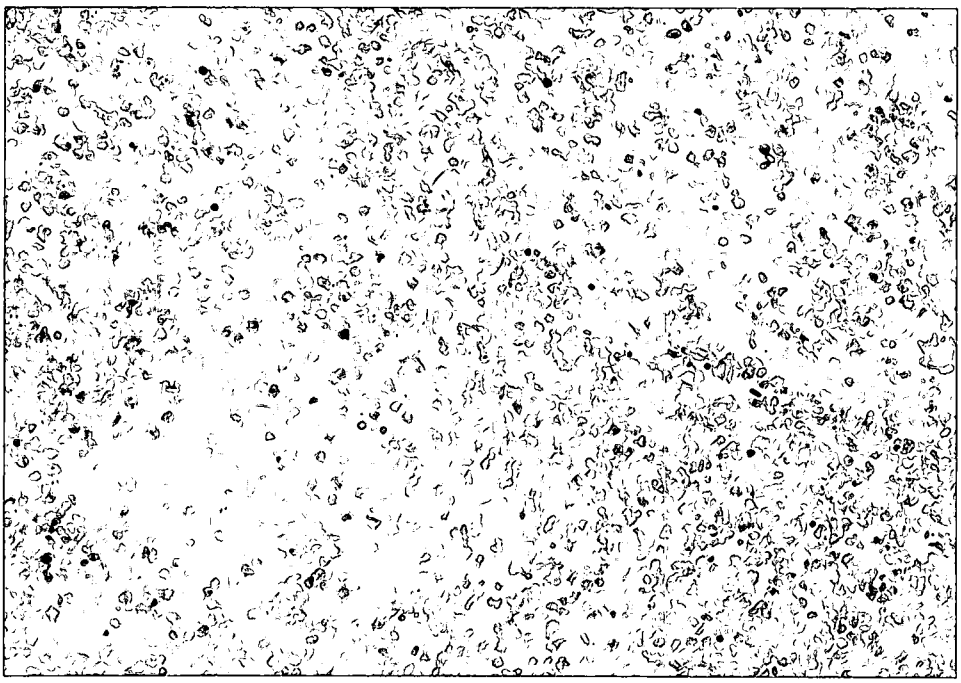
40X magnification of spleen section taken from Lassa virus infected guinea pig. Tissue received the prehybridization step. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 34: Lassa virus infected spleen section that did not receive the prehybridization step.



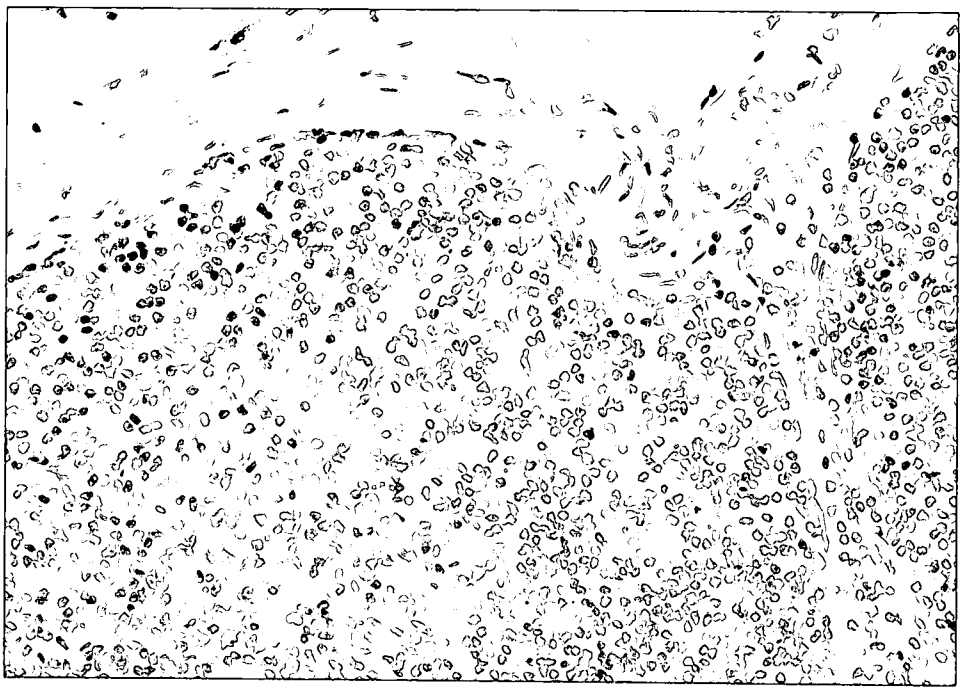
20X magnification of spleen section taken from Lassa virus infected guinea pig. Tissue did not receive the prehybridization step. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 35: Uninfected tissue section hybridized with Lassa virus RNA transcripts



20X magnification of spleen section taken from uninfected guinea pig. Tissue was hybridized with Lassa virus RNA transcripts. Blue precipitate from NBT and BCIP was not noted. Counterstained with Nuclear Fast Red.

Figure 36: SIV infected tissue section hybridized with Lassa virus RNA transcripts



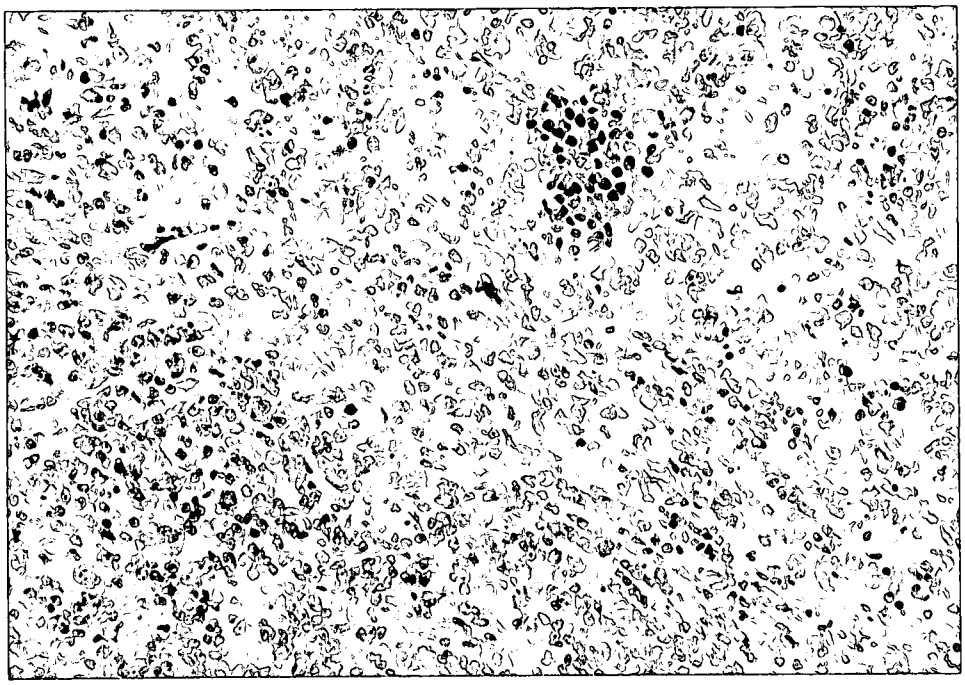
20X magnification of spleen section taken from SIV infected monkey. Tissue was hybridized with Lassa virus RNA transcripts. Blue precipitate from NBT and BCIP was not noted. Counterstained with Nuclear Fast Red.

infected tissues incubated with hybridization buffer not containing the Lassa riboprobes did not generate ISH signal. Lassa virus infected tissue hybridized with the SIV RNA transcripts did not generate ISH signal (Figure 37). SIV infected tissue did generate ISH signal when hybridized with the SIV transcripts (Figure 38). Lassa virus infected guinea pig lung tissues and monkey liver tissues generated ISH signal when hybridized with Lassa RNA transcripts (Figures 39 and 40).

Detection of the Digoxigenin Labeled Transcripts

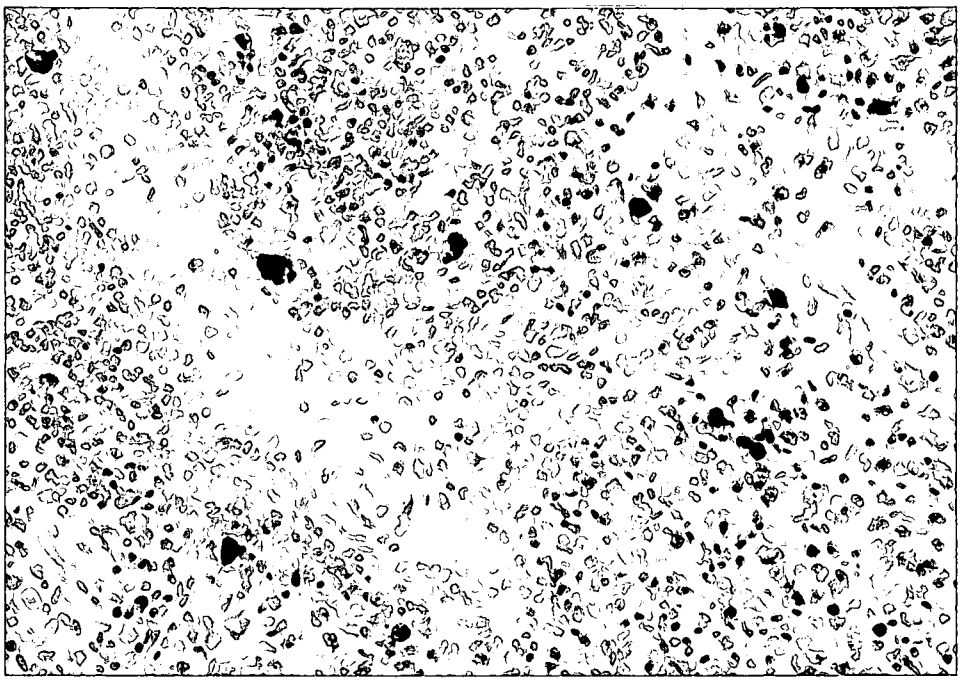
The antibody incubation period and the antibody concentration were varied to optimize signal detection. Tissues were covered with a 1:1,000 dilution of anti-DIG antibody (alkaline phosphatase conjugated anti-digoxigenin polyclonal sheep antibody fab-fragments) and incubated at 37^{0}C for 1, 2, 3, 4 hours and at 4^{0}C overnight. Slides incubated for 1 hour showed no less signal detection than those incubated for longer time periods (Figures 41 and 42). The anti-DIG antibody solution was serially diluted from 1:500 to 1:10,000 and applied to the tissues for 1 hour at 37^{0}C. The ISH signal did not appear to weaken until the antibody was diluted to greater than 1:5,000 (Figures 43 and 44).

Figure 37: Lassa virus infected tissue section hybridized with SIV RNA transcripts



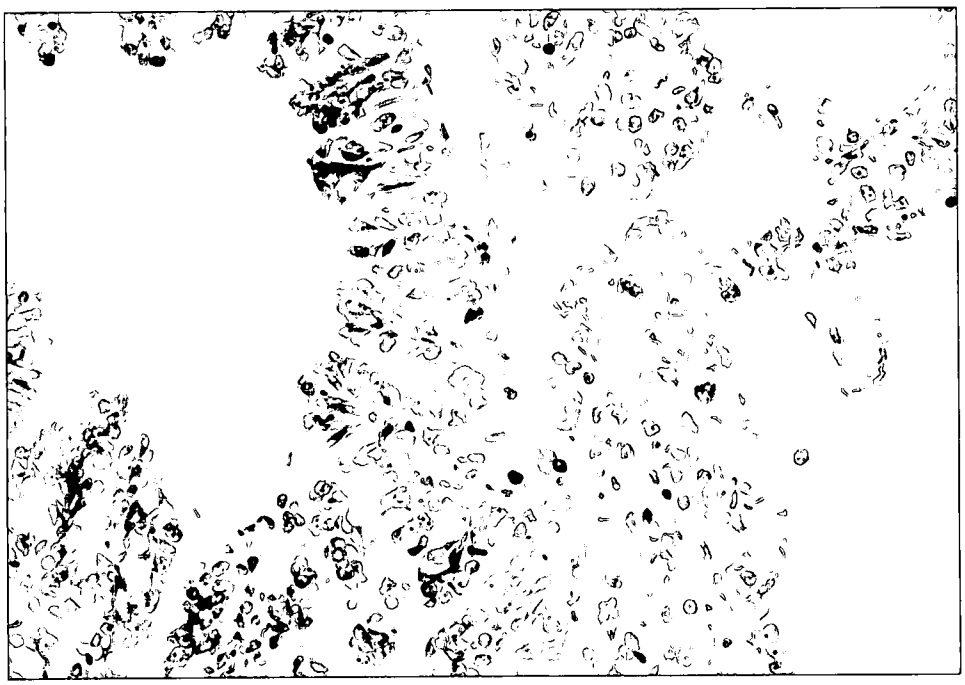
20X magnification of spleen section taken from Lassa virus infected guinea pig. Tissue was hybridized with SIV RNA transcripts. Blue precipitate from NBT and BCIP was not noted. Counterstained with Nuclear Fast Red.

Figure 38: SIV infected monkey section hybridized with SIV RNA transcripts



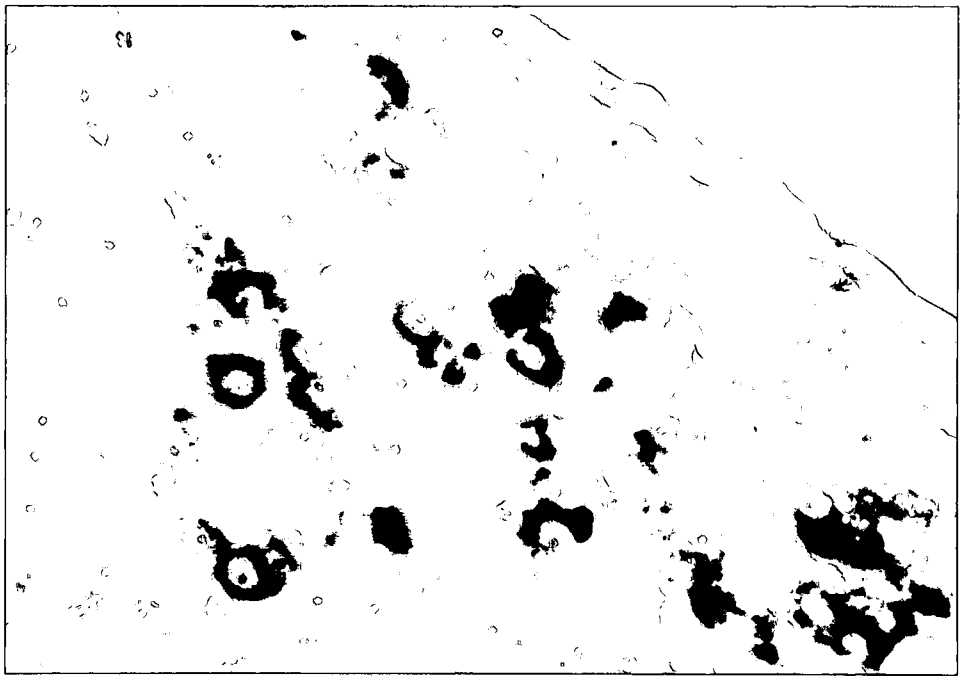
20X magnification of spleen section taken from an SIV infected monkey. Tissue was hybridized with SIV RNA transcripts. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 39: Lassa virus infected lung section hybridized with Lassa virus RNA transcripts.



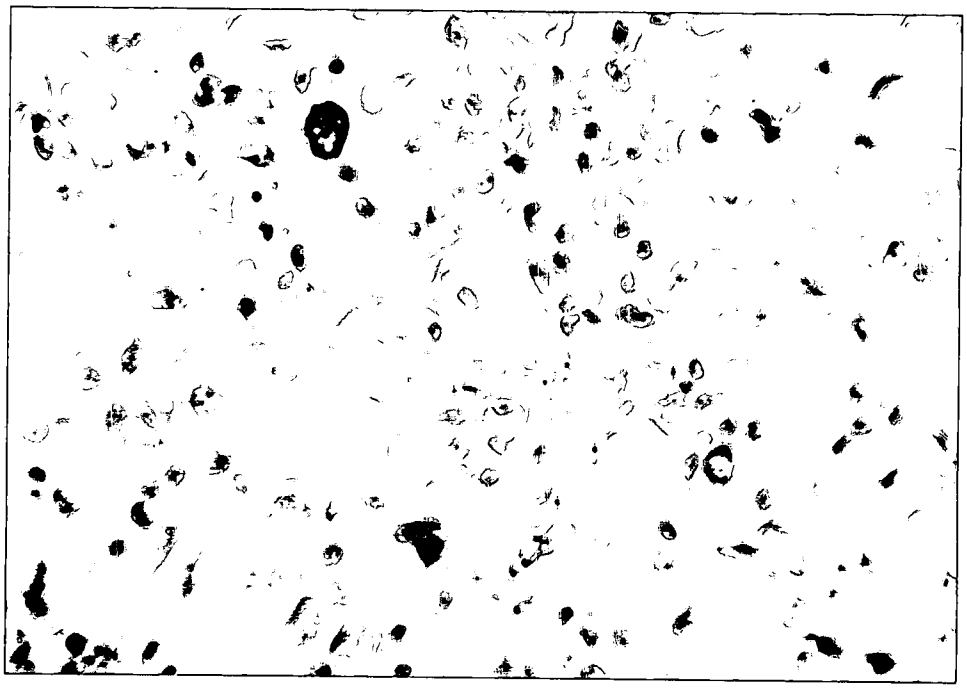
20X magnification of lung section from a Lassa virus infected guinea pig. Tissue was hybridized with Lassa virus RNA transcripts. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 40: Lassa virus infected liver section hybridized with Lassa virus RNA transcripts.



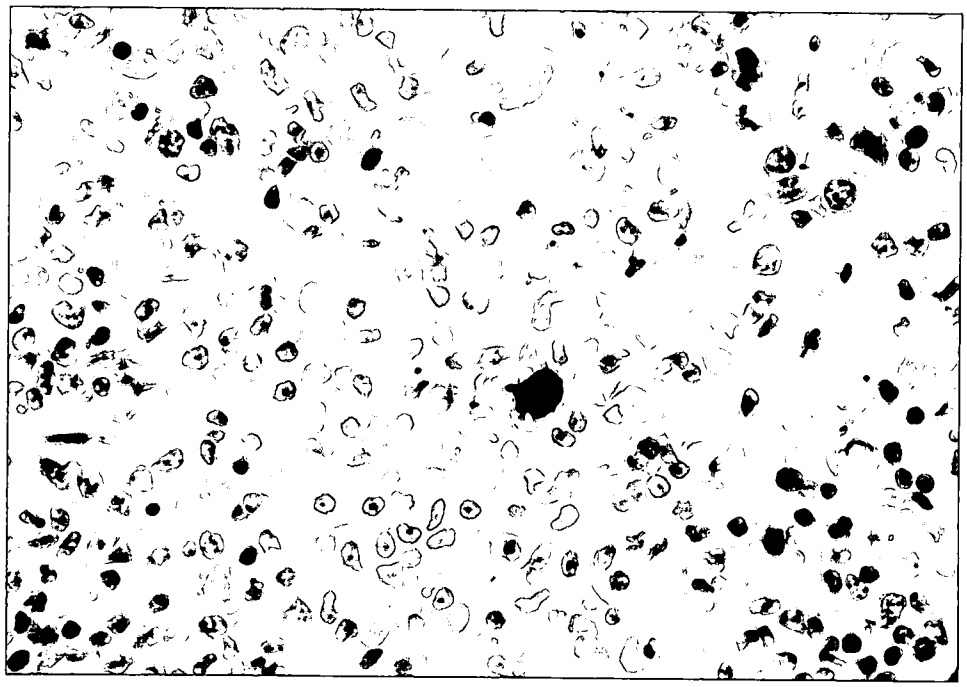
40X magnification of liver section from Lassa virus infected monkey. Tissue was hybridized with Lassa virus RNA transcripts. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 41: Lassa virus infected tissue incubated for 1 hour with an anti-DIG antibody.



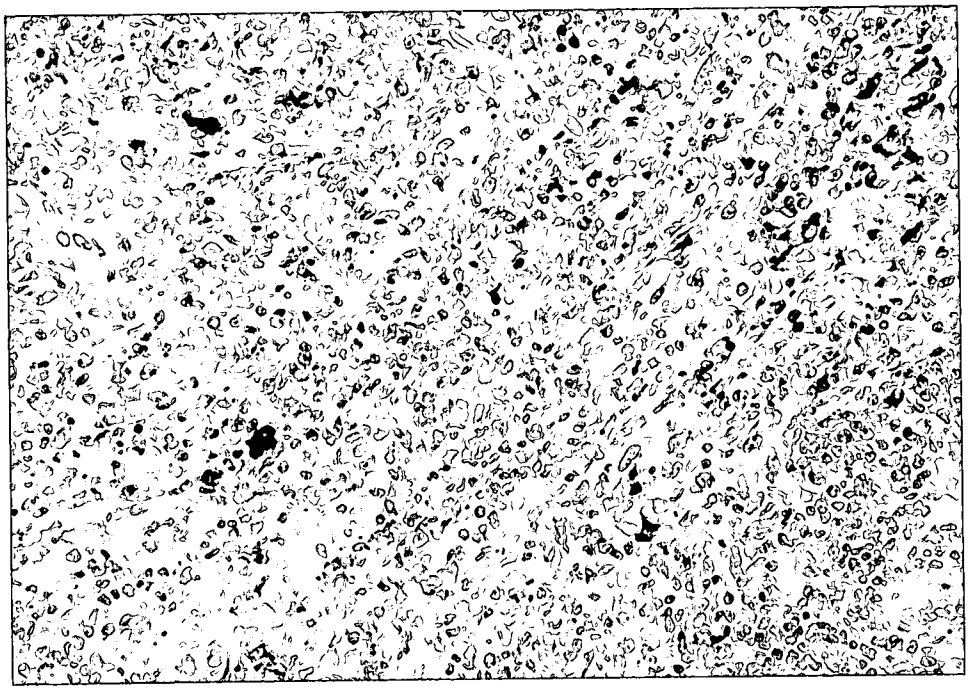
40X magnification of spleen section from a Lassa virus infected guinea pig. Tissue was incubated for 1 hour at 37^{0}C with a 1:1000 dilution of anti-DIG antibody. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 42: Lassa virus infected tissue incubated for 4 hours with anti-DIG antibody.



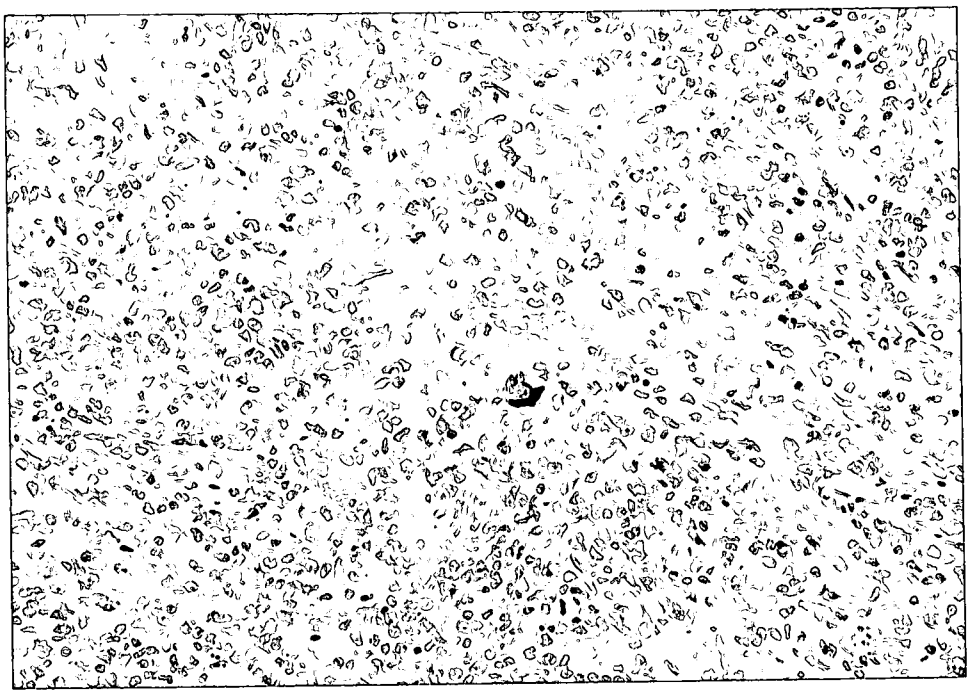
40X magnification of spleen section from a Lassa virus infected guinea pig. Tissue was incubated for 4 hours at 37^{0}C with a 1:1,000 dilution of anti-DIG antibody. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 43: Lassa virus infected tissue section incubated with a 1:5,000 dilution of anti-DIG antibody.



20X magnification of spleen section from a Lassa virus infected guinea pig. Tissue was incubated for 1 hour at 37^{0}C with a 1:5,000 dilution of anti-DIG antibody. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Figure 44: Lassa virus infected tissue section incubated with a 1:10,000 dilution of antibody.



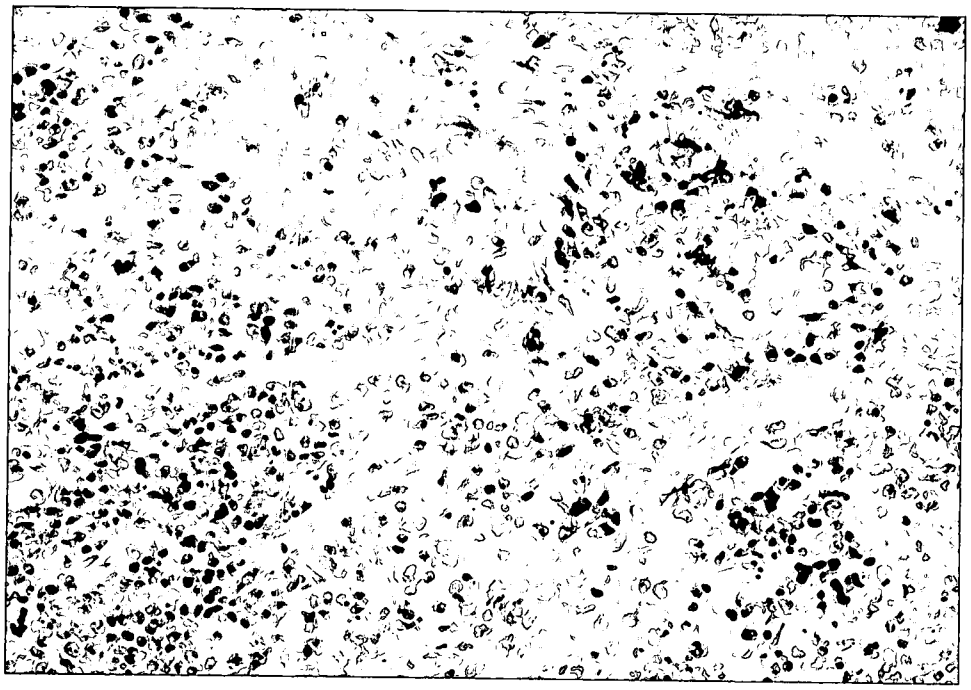
20X magnification of spleen section taken from Lassa virus infected guinea pig. Tissue was incubated for 1 hour at 37^{0}C with a 1:10,000 dilution of anti-DIG antibody. Blue precipitate from NBT and BCIP was noted over specific cells. Counterstained with Nuclear Fast Red.

Strong background staining was noted along the connective tissue, and weak background staining was generally noted throughout the tissue. Antibody blocking steps to decrease background staining were explored. The group of slides incubated with Buffer #1 generated a faint blue precipitate throughout the tissue whereas slides incubated with blocking buffer were much cleaner (Figures 45 and 46). The formation of blue precipitate along the connective tissue was prevented by adding 0.5% Triton X100 (BIO-RAD, Richmond, CA) to the blocking buffer (Springer, J.E., et al., 1991) (Figures 47 and 48).

Incubation time and temperature in the substrate solution were varied to optimize the production of the blue precipitate (ISH signal). Slides were first incubated at room temperature for 30 to 90 minutes. Incubations beyond 60 minutes often resulted in blue crystals that could not be washed off the tissue. The maximum ISH signal required approximately 50 minutes. Increasing the incubation temperature to 37^{0}C generated maximum ISH signal in 30 minutes.

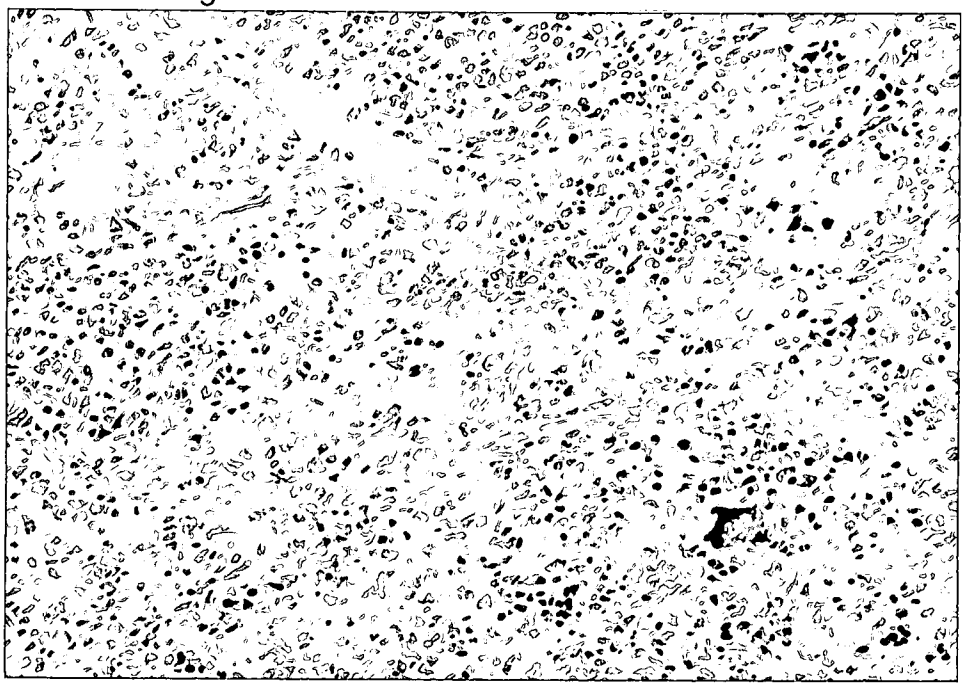
The distribution of the ISH signal within a specific tissue was consistent between different Lassa infected animals of the same species. In guinea pig spleen sections, the signal was usually a

Figure 45: Lassa virus infected tissue section that was not treated with blocking buffer.



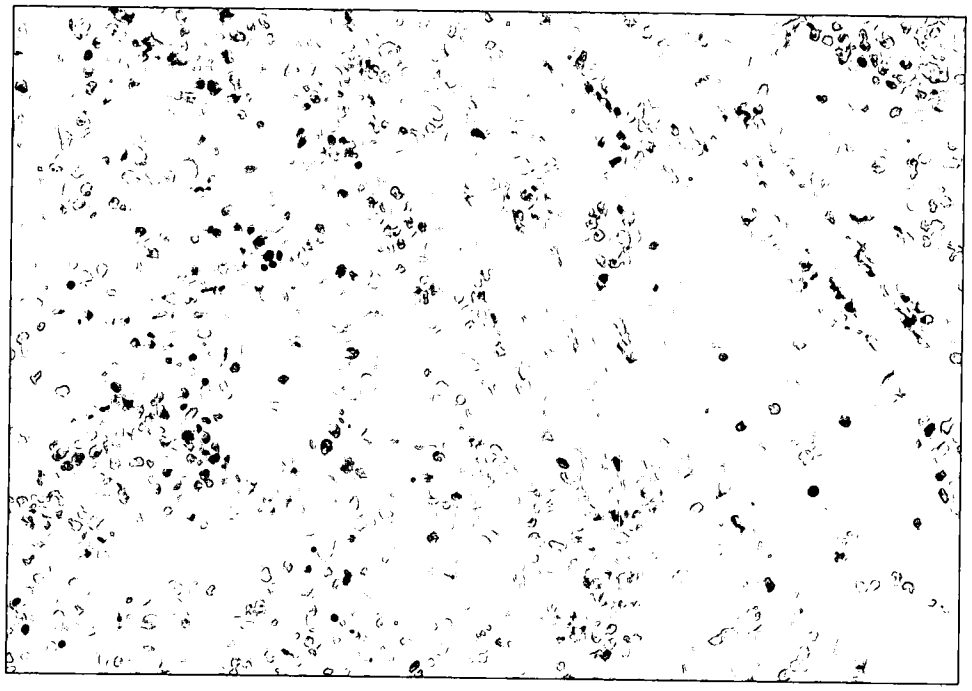
20X magnification of spleen section from a Lassa virus infected guinea pig. Tissue was not treated with blocking buffer prior to the antibody incubation. Blue precipitate from NBT and BCIP was not specific to cells. Counterstained with Nuclear Fast Red.

Figure 46: Lassa virus infected tissue section that was treated with blocking buffer



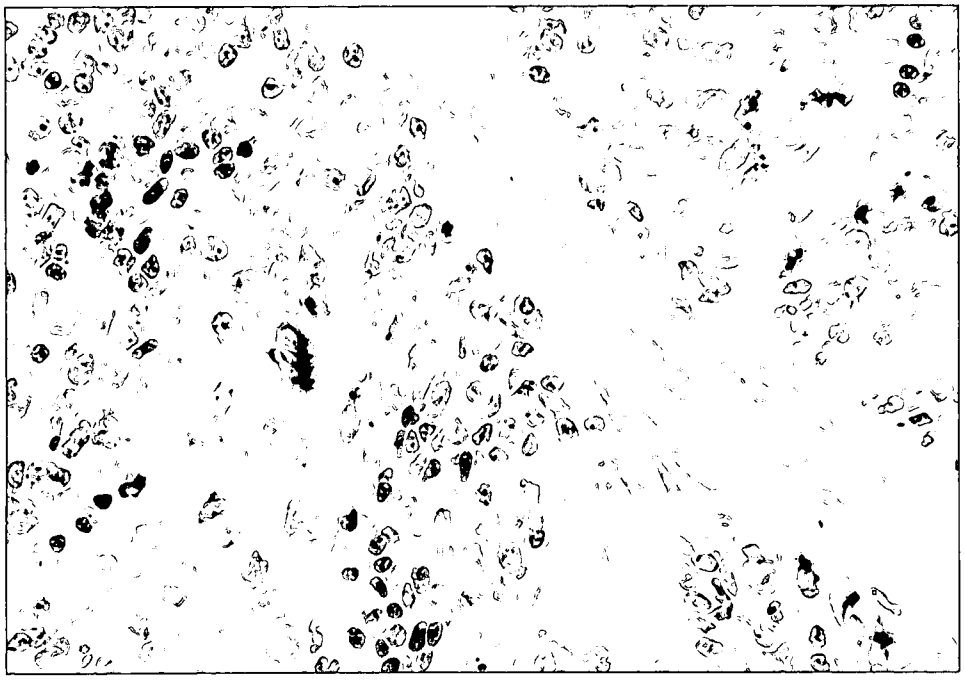
20X magnification of spleen section from a Lassa virus infected guinea pig. Tissue was treated with blocking buffer prior to the antibody incubation. Blue precipitate from NBT and BCIP was noted over specific cells.

Figure 47: Lassa virus infected tissue incubated with an antibody solution that did not contain Triton X-100.



20X magnification of spleen tissue from Lassa virus infected guinea pig. Antibody solution did not contain Triton X-100. Blue precipitate was noted over connective tissue. Counterstained with Nuclear Fast Red.

Figure 48: Lassa virus infected tissue incubated with an antibody solution containing Triton X-100.



40X magnification of spleen tissue from a Lassa virus infected guinea pig. Tissue was incubated with an antibody solution containing 0.5% Triton X-100. Blue precipitate was noted over specific cells. Counterstained with Nuclear Fast Red.

small blue spot just outside the nuclear membrane (Figure 22). Sometimes, the blue precipitate would cover the cytoplasm and completely surround the nuclear membrane (Figure 22). In guinea pig lung sections, the ISH signal was noted in the alveoli tissue and in the bronchiloar epithelium (Figure 39). Again, the blue precipitate was usually found in the cytoplasm and along the nuclear membrane. In monkey liver sections the blue precipitate would often fill the cytoplasm, completely surrounding the nucleus (Figure 40).

DISCUSSION

This thesis describes and characterizes the development of a non-isotopic in situ hybridization assay for Lassa fever RNA. The assay is rapid, efficient and acute, as compared to antibody-based immunocytochemical methods previously used to detect Lassa virus infection at this institute. In situ detection of virus RNA, if equally sensitive to the immunologically based assay, has the advantage of detecting the genomic RNA pre-requisite to virus protein synthesis, on which antibody assays are dependent. Specificity of in situ hybridization depends on a probe complementary to viral RNA and/or transcripts of virus RNA. These riboprobes were synthesized in vitro to specific regions of viral RNA. RNA/RNA hybrids are more sensitive and subject to greater stringency allowing specificity for detection of target sequences herein, Lassa fever viral RNA.

Optimal conditions for in situ hybridization included (a) formalin-fixed tissue stored 30 days, while eliminating infectivity, allows for detection with riboprobes; (b) morphology integrity maintained through formalin-fixation to observe cellular types in sections of tissues; (c) treatment of tissues with protease VIII gave better penetration of the riboprobe than protease K; (d) digoxigenin

labelling of the probe provided sensitive detection, allowing histological reading in a fast and safe bioassay.

Lassa virus genomic organization is ambisense strategy which means 5'-3' transcript synthesis occurs early after infection of the 3'-5' template of the segments of the virus genome. The synthesis of the full length complementary strand of viral RNA allows for transcription of the region not transcribed in early subunits, leading to mRNA for other later proteins (Figure 1).

Subcloning of regions 868-1393 which encodes late GPC protein in plasmids under T7 promoter response was then used to generate Dig-11-UTP labeled riboprobes (Figures 3 and 4). Subcloning region 1425-1825 in plasmids under Sp6 promotor response and labelling with Dig-11-UTP provided riboprobes to the genome region encoding NP (Figure 5). Several methods to quantify the riboprobes were tested (see Results) and a terminal titration method was selected for probe quantification.

The ambisense strategy of Lassa virus RNA precludes the synthesis of a negative strand which is generally of the same sense as the mRNA to prevent detection of viral RNA and mRNA simultaneously. However, given that cRNA templates are generated

in order to transcribe some mRNAs and the genome as template produces mRNA also, there is no region where the probe would be considered negative because it would bind to either the genomic or antigenomic RNA (Figure 1).

The T_m for Lassa riboprobes calculated via the formula (see Materials and Methods) (Wahl, G.M. et al.,1987) for 500 nucleotides was 72^{0}C in 40% formamide. Maximum stringency was at T_m - 10^{0}C = 62^{0}C and was the calculated condition for the in situ hybridization assay to detect Lassa RNA. However, results showed that hybridizing at 60^{0}C provided stable hybridization and preservation of tissue morphology, whereas 62^{0}C, while more stringent for detection of RNA-RNA hybrids, also resulted in a loss of some cellular morphology. Thus 60^{0}C was consistently used for viral RNA detection.

The relationship between antibody concentration, time, and temperature was used to define the optimal immunological detection (Boenisch, T., et al., 1989). As documented in the Results section, the optimal conditions for the detection of in situ hybridization were 1 hr incubation at 37^{0}C, 1:5,000 antibody concentration with 1% Triton X-100, anti-dig antibody Fab fragments and a blocking

solution containing teleosteam gelatin, bovine serum albumin, Triton X-100, and glycine. The production of a specific dark-blue precipitate by NBT and the counterstain of Nuclear Fast Red gave specific localized hybrid detection and accurate cellular morphology for tissues examined.

Lassa fever, a segmented RNA virus presents a diagnostic challenge since virus presence proceeds antibody levels needed for immunological detection. Immunocytochemical detection requires time consuming antibody production and the handling of biologically infectious material. Thus the synthesis of in vitro generated riboprobes and in situ hybridization provides a new and reliable sensitive detection assay for diagnosis and evaluation of vaccine efficaces. In conclusion, the thesis demonstrates an improved in situ hybridization assay that is rapid, safe, and simpler to perform than the existing methods.

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