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COMPLETE NUCLEOTIDE SEQUENCE OF *Pst*I-L FRAGMENT
OF TANAPOX VIRUS GENOMIC DNA

by

Sarah J. Neering

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Sarah J. Neering

COMPLETE NUCLEOTIDE SEQUENCE OF *Pst*I-L FRAGMENT OF TANAPOX VIRUS GENOMIC DNA

Sarah J. Neering, M.S.

Western Michigan University, 1993

Tanapox virus (TPV), of the genus *Yatapoxvirus*, is a human poxvirus that was originally isolated from the Tana River Valley in Kenya in 1962. TPV produces a mild disease characterized by a short febrile illness associated with one or more nodular skin lesions. Although there have been limited studies on TPV, other poxviruses such as vaccinia virus, have been extensively characterized and their genomic DNAs sequenced. In order to examine the molecular features of TPV, we have cloned approximately 17% of the genomic DNA. TPV DNA was digested with *Pst*I restriction endonuclease and cloned into pUC19. The *Pst*I-L fragment, composed of 2,108 base pairs, has been sequenced. Computer analyses show there are several potential open reading frames (ORF) within this fragment. The largest ORF consists of 239 amino acids (27 kDa). None of the ORFs analyzed show a high degree of homology with other poxviruses.

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INTRODUCTION

Tanapox virus (TPV) is the prototype of the *Yatapoxvirus* genus belonging to the family *Poxviridae*. Like vaccinia virus (VV), the most extensively studied poxvirus, TPV contains a large, linear, double stranded DNA genome. The size of TPV genomic DNA is approximately 145 kbp (Knight, Novembre, Brown, Goldsmith & Esposito, 1989). In comparing the genomic size of TPV to VV, which has a genomic size of 192 kbp (Goebel, Johnson, Perkus, Davis, Winslow, & Paoletti, 1990) potentially coding for 200-300 polypeptides (Essani & Dales, 1979), TPV may possibly encode between 150-200 polypeptides. Unlike VV, little is known about the genomic organization of TPV or the molecular mechanisms governing viral gene expression.

TPV was originally isolated in 1962 from human lesions in an outbreak of disease among natives of the Tana River Valley in Kenya (Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971). TPV is believed to be transmitted to man from monkeys via mosquitoes (Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971; Manson-Bahr & Downie, 1973). The same virus gave rise to epidemic infections in macaques monkeys (*Macaca mulatta*) in primate centers in Oregon, Texas and California during 1965-66 (Crandell, Casey & Brumlow, 1969; Hall & McNulty, 1967). Animal handlers at these locations contracted TPV through abrasions or cuts incurred from the monkeys.

TPV produces a mild infection in humans characterized by a short febrile illness lasting for approximately 2 to 4 days. During this period one or more nodular skin lesions appear at the site of entry. The lesion begins as a small papule and develops into a raised circular vesicle that becomes umbilicated (Casey, Woodruff & Butcher, 1967; Crandell, Casey & Brumlow, 1969; Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971; Espana, Brayton & Ruebner, 1971; Hall & McNulty, 1967; Nicholas & McNulty, 1968). The disease is self-limiting and full recovery occurs within 6 to 8 weeks. There have been no reported cases of reinfection (Jezek, Arita, Szczeniowski, Paluku, Ruti & Nakano, 1985).

TPV may provide opportunities to elucidate molecular mechanisms viruses employ to interact with the human immune system, because it is a primate virus which does infect humans. It has been shown in three other poxviruses [cowpox virus (Ray *et al.*, 1992), myxoma virus (Upton, Mossman & McFadden, 1992) and Shope virus (Smith *et al.*, 1990)] that these viruses produce anti-cytokine proteins which may increase virulence by inhibiting host immune cell function. Therefore, the purpose of this study was to further characterize TPV at the DNA sequence level and eventually determine how TPV proteins differ from other well characterized poxvirus polypeptides. In order to do this we took advantage of data previously obtained by Knight *et al.* (1989). These studies described three DNA restriction maps (*Bam*HI, *Mlu*I and *Pst*I). The *Pst*I restriction endonuclease was used in our studies. *Pst*I digests the TPV genome into 15 fragments. These fragments are alphabetized from A to N based on their size, A being the largest fragment and N the smallest.

REVIEW OF LITERATURE

General Characteristics of Tanapox Virus

Tanapox virus (TPV) has been classified in the genus *Yatapoxvirus* of the family *Poxviridae*. TPV, a large enveloped DNA virus, appears oval or brick-shaped under electron microscopy. Thin sectioned virions show a lipoprotein bilayer surrounding a core that appears biconcave with two structures of unknown function, called lateral bodies, within the concavities (Casey, Woodruff & Butcher, 1967; Espana, Brayton, & Ruebner, 1971). The virion contains a non-infectious, linear, double stranded DNA genome of approximately 145,000 base pairs (bp). The terminal ends of the genome are joined by hair-pin loops (Knight, Novembre, Brown, Goldsmith & Esposito, 1989). Like other poxviruses, TPV replicates within the cytoplasm of infected cells (Buller & Palumbo, 1991).

Historical Background

TPV, an endemic, zoonotic virus of equatorial Africa, was originally isolated from human skin biopsy specimens in 1962 (Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971). The disease was first seen in 1957 in a village on the flood plains of the lower reaches of the Tana River in Kenya. In 1962, a more wide spread epidemic

occurred along the middle areas of the Tana River between Garrissa and Garsen (Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971). In 1965 and 1966, animal handlers at primate centers in California, Oregon and Texas contracted the disease through cuts and/or abrasions inflicted while handling infected monkeys or cleaning cages that housed these monkeys (Casey, Woodruff & Butcher, 1967; Crandell, Casey & Brumlow, 1969; Espana, Brayton & Ruebner, 1971; Hall & McNulty, 1967; Nicholas & McNulty, 1968). The virus isolated from these monkeys was shown to be identical to TPV isolated from natives in 1962 (Downie & Espana, 1971). The fact that the same virus was responsible for the outbreak in the primate centers in the United States and in natives of Africa had an important relation to the epidemiology of the disease.

Viral Polypeptides

Knight *et al.* (1989) purified TPV particles by velocity sedimentation in 20-40% (w/w) sucrose gradients followed by a CsCl density gradient. This resulted in generating two viral particle sedimentation bands, one band of aggregated particles that sedimented at 1.25 g/ml in the CsCl gradient and the second nonaggregated band at 1.27 g/ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of each viral band showed approximately 55 proteins ranging in size from 11 kDa to 200 kDa. There were slight differences between the two protein patterns of TPV particles sedimenting at different densities. In addition to common proteins, the slower sedimenting virions (1.25 g/ml) contained 12 proteins that were not present in the faster sedimenting virions. A 30 kDa protein

and a 28 kDa protein were common to both viral patterns, but the 30 kDa protein was markedly decreased in the faster sedimenting virions while the 28 kDa protein was decreased in the slower sedimenting virions. There were two proteins (40 kDa and 29 kDa) unique to the faster sedimenting virions. These differences were most likely due to the fact that the slower sedimenting viral particles were double-enveloped virions while the faster sedimenting particles were single-enveloped virions. The differences in sedimentation densities appeared to be a result of whether the virion was double-enveloped or single-enveloped, whereas the differences in protein patterns could be a result of the second envelope having different proteins than the first envelope.

Viral DNA Analysis

The approximate size of TPV genomic DNA has been previously calculated to be 145 kbp via estimates of DNA digested fragment molecular sizes and field inversion gel electrophoresis. Restriction maps of the TPV genome were established by end-fragment determination and through analysis of overlap cross-hybridization of isolated, individual *Pst*I, *Mlu*I, and *Bam*HI viral DNA fragments (Knight, Novembre, Brown, Goldsmith & Esposito, 1989).

Clinical Features of Infection

TPV produces a mild disease in humans characterized by a short febrile illness that lasts for approximately 2 to 4 days. Severe headache, backache and pronounced prostration may occur during this period. One

or more pock-like skin lesions, occurring primarily on exposed areas of the skin (i.e. arms, hands and legs), have been reported (Casey, Woodruff & Butcher, 1967; Crandell, Casey & Brumlow, 1969; Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971; Espana, Brayton & Ruebner, 1971; Hall & McNulty, 1967; Nicholas & McNulty, 1968). The lesion starts as a papule and develops into raised circular vesicle that becomes umbilicated. No pustulation has been recorded and usually only a single lesion occurs. In some cases swelling of regional lymph nodes and inflammation of lymph ducts occurred (McNulty, Lobitz, Hu, Maruffo & Hall, 1968). The lesions spontaneously disappear within 6 to 8 weeks and there have been no reports of reinfection (Jezek, Arita, Szczeniowski, Paluku, Ruti & Nakano, 1985).

Histopathology

Lesions in humans are characterized by epidermal spinoses with extension of the epidermal ridges. Follicular epithelium and epidermal cells show vacuolization within their cytoplasm. The dermal papilla appear thin, elongated, and filled with fluid. Capillaries within the dermal papilla become swollen and congested with red blood cells. The congested capillaries are usually seen at the tips of the papilla which are covered by a layer of necrotic epidermal cells (McNulty, Lobitz, Hu, Maruffo & Hall, 1968).

In early lesions only the upper epidermal layer is affected. As the lesion progresses the dermis also becomes involved. In early stages, where swelling occurs with infiltration of immune cells, the vascular

involvement can be quite extensive with destruction of blood cells that results in fibrosis and blockage of the lumen (McNulty, Lobitz, Hu, Maruffo & Hall, 1968).

Lesions in monkeys, the natural host of TPV, show an increase in the thickness of the epidermis that extends down the hair shafts and into the sebaceous glands forming masses of epidermal cells in the dermis. Swelling of both the cytoplasm and nucleus of cells occurs. Sometimes discrete cytoplasmic eosinophilic inclusions have been seen in all cell layers of the epidermis, follicular cells and basal cells of the sebaceous gland (Hall & McNulty, 1967). In the outer epidermal layer, cells are frequently necrotic with sloughing in some areas. The necrotic crust is composed of keratin, leukocyte discharge and dead unkeratinized epidermal cells. There are a moderate number of inflammatory cells, mostly neutrophils, in necrotic and degenerate regions. Both mononuclear and polymorphonuclear cells are present in perivascular areas of the dermis and subcutaneous tissues (Crandell, Casey & Brumlow, 1969). The mode of transmission of TPV among monkeys has not been identified but direct contact or an insect vector has been suggested. Although death has not been associated with the disease, the extent of secondary infections has determined the clinical course of disease.

Epidemiology

The epidemiology of TPV has not been fully elucidated. Specific characteristics have been deduced from a study conducted in Northern Zaire over a 5 year period (Jezek, Arita, Szczeniowski, Paluku, Ruti &

Nakano, 1985). There appeared to be no discrimination between age, sex and occupation of infected people. The majority of people infected had been immunized against small pox indicating that the small pox vaccine offered no protection against TPV.

TPV infections were more frequent during November through March. This time period coincided with the period that mosquitoes and other blood-sucking insects were active, suggesting mode of transmission could possibly have been arthropod in nature.

The mode of transmission between animals and from animals to man is presently unknown. However, the rapid transmission among monkeys housed together suggests a common source or direct contact between affected and susceptible monkeys. There have been no reported cases indicating that TPV can be transmitted directly from person to person. It has been suggested that TPV can be transmitted from monkeys to humans through arthropod vectors, specifically culicine mosquitoes (Downie, Taylor-Robinson, Caunt, Nelson, Manson-Bahr & Matthews, 1971; Manson-Bahr & Downie, 1973).

Serological Studies

Serologically, TPV has minimal cross-reactivity to only one other pox virus pathogenic to humans, Yaba monkey tumor virus (YMTV) (Downie & Espana, 1972; Nicholas & McNulty, 1968). TPV and YMTV share the same host range and are similar in shape, size and ultrastructure (Casey, Woodruff & Butcher, 1967; Espana, Brayton & Ruebner, 1971). However, lesions produced by these viruses differ in both histological and

gross appearance. YMTV lesions in monkeys and humans are tumor-like and involve mesodermal cells while TPV lesions affect primarily the epidermis.

Monkeys recovering from either TPV or YMTV infections showed partial cross-immunity. Studies with immune sera showed that following absorption each virus possessed common as well as specific antigens (Downie & Espana, 1973).

Analysis of sera collected from natives living in the Tana River Valley in 1971 showed that TPV infections continued to occur in the area since the outbreak in 1962 (Manson-Bahr & Downie, 1973). The examination of repeated serum samples from these natives indicated that antibody to TPV was not detectable after 2 to 3 years. Further serological surveys of the lower Tana River valley in 1976 confirmed the results obtained in 1971 (Axford & Downie, 1979).

MATERIALS AND METHODS

Virus and Cell Lines

TPV was obtained from American Type Culture Collection, Rockville, Maryland, and grown in monolayers of CV-1 monkey kidney cells or Owl monkey kidney (OMK) cells. Both types of cells were maintained in Earle's minimum essential medium (MEM) containing 10% (v/v) heat-inactivated newborn calf serum, 2 mM glutamine and antibiotics (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B). Cells were cultured at 37° in an incubator with 5% CO₂. Cells were infected with 1-2 plaque forming units (pfu) per cell. Adsorption was carried out on a rocking platform at room temperature for one hour. Following adsorption, TPV infected cells were maintained in Earle's MEM containing 2% (v/v) heat-inactivated newborn calf serum, 2 mM glutamine and antibiotics at 35° with 5% CO₂.

TPV for further infections and tests was prepared from cultures of CV-1 or OMK cells in 75 cm² culture flasks. Infected cells were harvested 5-7 days post-infection using a rubber policeman and cells were pelleted by centrifugation at 1500 xg (Sorvall RC-5B refrigerated superspeed centrifuge; Sorvall type GSA rotor at 3000 rpm) for 20 minutes. The pellet was resuspended in sterile deionized water and freeze-thawed three times to release intracellular virus. Large cytoplasmic debris and nuclei were then removed by centrifugation at 3000 xg (SS-34 Sorvall rotor at 5000 rpm). The resulting supernatants were centrifuged at 85,000 xg (Beckman L8-70M

ultracentrifuge; Ti-70.1 fixed angle rotor at 30,000 rpm) for 30 minutes to pellet the virus. The virus pellet was resuspend in 10 mM Tris-HCl, pH 8.0, to a final concentration of 100X and stored at -20°.

Cloning of Tanapox Virus Genomic DNA

Restriction Enzyme Cleavage of DNA

TPV genomic DNA was kindly supplied by Dr. Joseph J. Esposito, Centers for Disease Control, Atlanta, Georgia. *Pst*I restriction endonuclease (Gibco BRL, Gaithersburg, MD) was used to digest, separately, 1µg TPV genomic DNA and 1µg pUC19 under the following conditions: 5 units *Pst*I (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.15% (v/v) TRITON X-100, 50% (v/v) glycerol) per µg DNA, 1X REact 2 buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM NaCl) in a total volume of 10 µl with deionized water. The reactions were incubated at 37° for 12-18 hours. Reactions were terminated by either heat inactivation at 65° for 30 minutes or by addition of 10X stop solution (0.5 M EDTA, 65% sucrose, 0.2% bromophenol blue, 0.2% xylene cyanol).

Dephosphorylation of pUC19 and Ligation of Tanapox *Pst*I Fragments

Dephosphorylation of pUC19 was done using calf intestine phosphatase (CIP) (Boehringer Mannheim, Indianapolis, IN) as suggested by manufacturer. Briefly, 1 µl of CIP (1 µg/ml) was added to 3-5 µg of DNA along with 1 µl of 10X CIP buffer (0.5 M Tris-HCl, pH 9.0, 10 mM MgCl₂, 1 mM ZnCl₂, 10 mM spermidine) and brought up to a total volume of 10 µl

with deionized water. The mixture was incubated at 37° for 15 minutes then at 56° for an additional 15 minutes. Another aliquot of CIP was added and the mixture reincubated as above. The reaction was terminated by heating at 75° for 10 minutes.

Following dephosphorylation, *Pst*I digested pUC19 and TPV DNA were purified, to remove the enzyme, using Geneclean (Promega, Madison, WI) following manufacturers instructions. Briefly, 3 volumes of 6M NaI was added to the digested DNA along with 5 µl of Glassmilk®. The solution was vortexed and put on ice for 5 minutes with occasional mixing. The suspension was briefly centrifuged, pelleted and washed 3 times with NEW® wash solution. The final pellet was resuspended in deionized water equal to volume of pellet. The mixture was incubated at 45-55° for 2-3 minutes, centrifuged and the supernatant containing eluted DNA was collected.

The heat inactivated mixture containing *Pst*I digested fragments of TPV DNA were ligated into pUC19 as described in Maniatis (Maniatis, Fritsch & Sambrook, 1989). Briefly, 2 µl of 1 unit/µl T4 DNA ligase (Gibco BRL, Gaithsburg, MD), 1 µl of 10 mg/ml bovine serum albumin (BSA), 1 µl of 5X ligase buffer [0.25 M Tris-HCl, pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG-8000] were added to a 1:5 mixture of pUC19-TPV DNA. The reaction was incubated at 12° for 12-14 hours. The reaction was terminated by heating at 75° for 10 minutes.

Transformation

Ligated TPV DNA was transformed into commercially available DH5 α -MCR competent cells (Gibco BRL, Gaithersburg, MD) by adding 5 μ l of ligation mixture to 50 μ l of competent cells and incubating at 4° for 30 minutes. The mixture was then heated at 42° for 45 seconds and returned to 4° for 2 minutes. Nine hundred microliters of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added to the chilled mixture and incubated at 37° in a shaker at 200 rpm for 1.5 hours. The suspension was then plated out in triplicate on 2X YT (1.0 % yeast extract, 1.6 % tryptone, 85 mM NaCl, 1% agar) plates containing 50 μ g/ml ampicillin using 20 μ l, 50 μ l and 100 μ l of the suspension. Plates were incubated at 37° for 18-24 hours. Three hundred ampicillin resistant colonies were isolated.

Purification of Plasmid DNA

Plasmid DNA was extracted using the Magic Mini Prep DNA Purification System (Promega, Madison, WI) following manufacturer's instructions. Briefly, bacterial cultures were grown in 2X YT medium containing 50 μ g/ml ampicillin for 12-16 hours. Cells were pelleted by centrifugation at 2000 xg (Damon/IEC PR-6000 centrifuge at 3000 rpm) for 5 minutes and resuspended in cell resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μ g/ml RNase A). The cells were lysed in a solution of 0.2 M NaOH and 1% SDS, neutralized with 2.55 M KC₂H₃O₂, pH 4.8, and pelleted by centrifugation at 16,000 xg (Brinkman 5415C eppendorf centrifuge at 14,000 rpm) for 5 minutes. The supernatant was

mixed with a resin and applied to a mini-column. The plasmid DNA was eluted from the column by addition of deionized water preheated to 65°.

Analysis of Plasmid DNA by Electrophoresis and Southern Blotting

Plasmid DNA was digested using *Pst*I enzyme as described above. The reactions were terminated by addition of 10X stop solution. The samples were loaded onto a 0.7% agarose gel made with 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) containing 1 µg/ml ethidium bromide. The gel was electrophoresed at 50 V for 18-24 hours. Electrophoresis was in 1X TBE solution, pH 8.0. After electrophoresis the gel was placed under a UV light source and photographed through a red filter with polaroid type 665 film (Polaroid Corporation, Cambridge, MA). The gel was then denatured by submerging it in 0.5 M NaOH, 1.5 M NaCl at room temperature for 1 hour with gentle shaking. The gel was then neutralized in 1.0 M Tris-HCl, pH 7.4, 1.5 M NaCl for 1 hour and the denatured DNA fragments transferred to a Biotrans nylon membrane (ICN, Irvine, CA) using standard methods as described in Maniatis *et al.* (1989). The membrane was rinsed in 6x SSC (0.9 M NaCl, 99 mM Na₃C₆H₅O₇ · 2H₂O, pH 7.0) for 5 minutes, baked at 80° for 2 hours in a vacuum oven and then placed in a plastic bag.

Hybridization and Autoradiography

The probe used for hybridization was obtained by excision of the *Pst*I-L fragment (2.1 kbp) from a 0.7% low melting point agarose gel. For

each gram of agarose, 3 ml of deionized water was added. The mixture was heated to 100° for 3-5 minutes and then aliquoted into 35 µl portions. The DNA was labeled using the multiprime DNA labeling system (Amersham, Arlington Heights, IL) following manufacturer's instructions. Briefly, the DNA was denatured by heating at 100° for 5 minutes, cooled to 37° and the following ingredients were added: 2 µl of BSA (10 mg/ml), 10 µl of Oligo-labeling buffer minus cytosine, 5 µl (30 µCi) ³²P d-CTP (400 Ci/mmol) and 2 µl Klenow (10 units/ml). The mixture was incubated at room temperature for 12-18 hours.

Nylon membranes following transfer were prehybridized in 5-10 ml of hybridizing solution (HS) containing 50% deionized formamide, 1 M NaHPO₄, pH 7.2, 5 M NaCl, 250 mM EDTA, 10 mg/ml denatured salmon sperm DNA and 7% SDS (w/v) at 42° for 12-24 hours. The HS was then replaced with 5-10 ml of fresh HS to which heat denatured ³²P-labeled probe of the *Pst*I-L fragment had been added. The nylon membrane was incubated at 42° for 24 hours and then washed three times, 30 minutes each, with 6x SSC, 0.5% SDS at 62°. The filter was then exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 3-4 days.

DNA Sequencing

Both strands of the 2.1 kbp *Pst*I-L fragment were sequenced using the dideoxynucleotide chain termination-based Sequenase Version 2.0 sequencing kit (United States Biochemical Corporation, Cleveland, OH) following manufacturer's instructions. Briefly, 1 µg of plasmid DNA containing the *Pst*I-L fragment was denatured by adding 1 µl of 2 N

NaOH and deionized water to a final volume of 5 μ l and placed on ice for 5 minutes. To this 1 μ l of primer (200 ng/ml), 1.5 μ l 3 M $\text{NaC}_2\text{H}_3\text{O}_2$ and 28 μ l ethanol (100%) were added and the mixture was put on dry ice/ethanol for 20 minutes. The DNA/primer were pelleted and washed once with 60 μ l of 70% ethanol and vacuum dried. The pellet was resuspend in 5 μ l annealing buffer (1X), heated to 65° for 2 minutes and slowly cooled to room temperature. To the annealed template-primer the following were added: 1 μ l DTT (0.1 M), 2 μ l labeling nucleotide mix (1.5 μ M dGTP, 1.5 μ M dCTP, 1.5 μ M dTTP), 1 μ l Sequenase® enzyme and 1 μ l (10 μ Ci) ^{32}P d-ATP (400 Ci/mmol). Reactions were terminated by adding the reaction mixture, in equal volumes, to each of 4 dideoxynucleotide termination mixtures and incubated at 37° for 3-5 minutes. The reaction mixture was further terminated by the addition of 4 μ l stop solution. The reaction mixtures were heated to 80-90° for 5 minutes prior to loading and run on a 8% acrylamide gel (acrylamide:bis-acrylamide ratio, 19:1) in 1X TBE, at 40 Watts for 2.5 hours. The gel was exposed to Kodak X-Omat film for 3-5 days. Sequencing of the complete L fragment was done by walking along the insert using 20-22 base primers.

Purification of Primers

Primers were obtained from National Biosciences, Plymouth, MN. The primers were purified using a BioGel P4 (Bio-Rad Laboratories, Richmond, CA) column. One gram of BioGel P4 was suspended in 50 ml of 10 mM Tris-HCl, pH 7.4, and left at room temperature for 1 hour. The

mixture was then poured into an Econo-column (Bio-Rad Laboratories, Richmond, CA) and buffer (10 mM Tris-HCl, pH 7.4) was run through for 1 hour before loading primers. The column dimensions were 14 cm x 1 cm.

Primers were resuspended in 100 μ l of buffer (10 mM Tris-HCl, pH 7.4) and loaded onto the column. The flow rate of buffer through the column was 2 ml per minute. The absorbance of the DNA eluting off the column was monitored on an LC detector (Gilson, Middleton, WI). Fractions were collected and samples were analyzed on a Gilford Response spectrophotometer (Gilford Instrument Lab, Inc., Boston, MA) at a wavelength of 260 nm. Primers were used at a concentration of 200 ng/ml.

Data Analysis

Sequence analysis and data bank searches were done on a VAX 2000 computer using the IntelliGenetics Suite version 5.35 software (IntelliGenetics, Mountain View, CA). Data bases searched were University of Geneva protein data bank and Protein Identification Resource data bank.

RESULTS

Cloning of the Tanapox Virus Genome

DNA restriction maps of TPV DNA have previously been published (Knight, Novembre, Brown, Goldsmith & Esposito, 1989). Using this information, we chose the restriction endonuclease *Pst*I because it produced manageable size DNA fragments for cloning into pUC19.

TPV genomic DNA was digested with *Pst*I restriction endonuclease and the resulting DNA fragments were 'shot-gun' cloned into pUC19. The plasmids were then transformed into competent DH5 α MCR cells as described in Materials and Methods. A total of 300 ampicillin resistant colonies were selected and purified plasmids obtained were restriction digested with *Pst*I to determine which fragment of TPV DNA they contained. Table 1 lists the clones and the *Pst*I fragment they contain. Of the 300 colonies screened only 116 contained a *Pst*I TPV DNA fragment. Figure 1 shows 6 of the 15 possible *Pst*I TPV DNA fragments that were successfully cloned. Approximately 17% of the TPV genome has been cloned. Lanes A and B are ϕ X 174-*Hae*III digested and λ -*Hind*III digested markers, respectively. Lane C contains *Pst*I digested TPV genomic DNA and Lane D is *Pst*I digested pUC19. Lanes E through K show the TPV DNA fragments obtained from cloning.

We then selected the 2.1 kbp L fragment (clone #36) to sequence and analyze due to its relatively short length and proximity to the end of the genome. The L fragment is towards the 5' end of the TPV genome and it

Table 1

Identification of Clones Containing *Pst*I
TPV Genomic DNA Fragments

TPV DNA Fragment and MW (kbp)	Clone Number
F Fragment (9.0 kbp)	20, 45, 139
H Fragment (6.6 kbp)	45, 66, 67, 119, 385, 489
J Fragment (5.0 kbp)	9, 44, 62, 72, 78, 388, 389, 393, 447, 460, 469, 470, 495, 501
L Fragment (2.1 kbp)	7, 10, 16, 22, 24, 28, 32, 34, 36, 42, 48, 85, 92, 101, 102, 109, 113, 121, 130, 134, 300, 310, 311, 321, 324, 330, 334, 336, 341, 357, 364, 365, 374, 378, 382, 402, 442, 448, 455, 461, 468, 478, 481, 493, 496, 505
M Fragment (1.4 kbp)	3, 26, 55, 96, 111, 125, 145, 309, 316, 318, 323, 340, 344, 350, 366, 370, 380, 392, 403, 412, 423, 439, 452, 463, 476, 487, 490, 498
N Fragment (1.1 kbp)	4, 53, 93, 94, 144, 301, 325, 326, 337, 342, 346, 363, 401, 409, 445, 462, 471, 497, 506

has been previously reported that poxvirus genomes (e.g. vaccinia virus) are organized with essential genes clustered in the middle whereas virulence markers that govern pathogenesis tend to map towards the ends of the genome (Traktman, 1990). Some of these virulence markers are secreted proteins that enhance the ability of the virus to propagate in its natural host.

Before sequencing the 2.1 kbp fragment, we did a Southern blot to verify that the fragment we cloned into pUC 19 was in fact the TPV *Pst*I-L

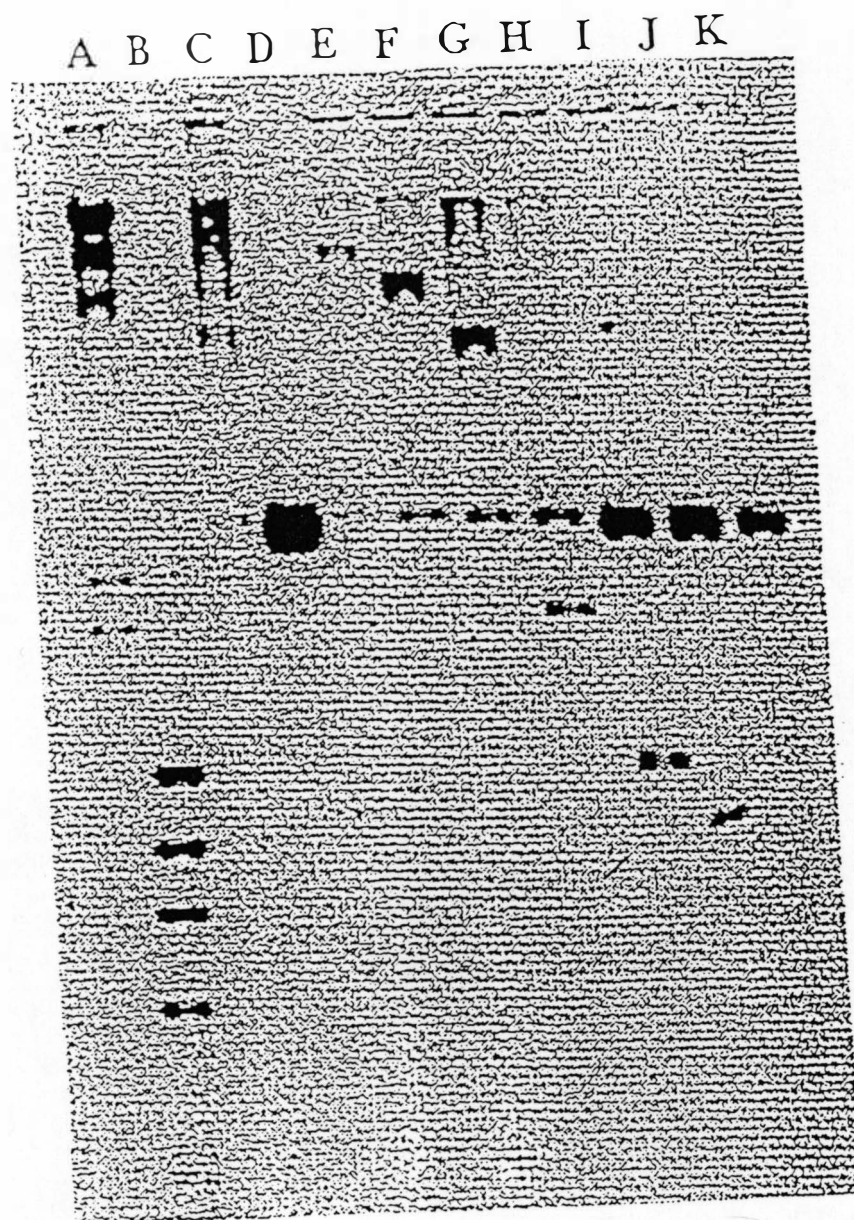


Figure 1. *Pst*I Restriction Digest of Cloned TPV Genomic DNA Fragments. Purified plasmid DNA was digested with *Pst*I restriction endonuclease. Lanes A and B are λ and ϕ X174 DNA markers digested with *Hind*III and *Hae*III, respectively. Lane C is TPV genomic DNA digested with *Pst*I. Lane D is pUC19 digested with *Pst*I and Lanes E through K are plasmid DNA containing TPV DNA fragments restricted with *Pst*I endonuclease.

fragment reported by Knight *et al.* (1989). Figure 2 shows the Southern blot of the gel shown in Figure 1 using the cloned 2.1 kbp fragment as the probe. Lane H is the 2.1 kbp *Pst*I-L fragment which corresponds to the *Pst*I-L fragment of TPV genomic DNA in Lane C.

Sequencing of the 2.1 kbp *Pst*I-L Fragment

Sequencing of the *Pst*I-L fragment was accomplished by the dideoxynucleotide chain termination method (Sanger, Nicklen & Coulson, 1977). The complete sequence of the *Pst*I-L fragment has been obtained. The entire sequence contains 2,108 base pairs. Over 75% of the sequence (bp 431 to 2108) was obtained from both strands, the remaining 25% (bp 1 to 430) from one. Figure 3 shows the complete nucleotide sequence of the *Pst*I-L fragment. The fragment reads in the 5' to 3' direction. Bases are in groups of ten and the unconfirmed region is underlined. Up primer sites are in bold and down are in italics. The names of the primers are above the sequence.

Analysis of the *Pst*I-L Fragment

The complete sequence of the *Pst*I-L fragment was analyzed for possible open reading frames (ORFs) using IntelliGenetics software. The sequence was translated in each of the six possible frames to locate potential ORFs. There are 37 possible ORFs located within the *Pst*I-L fragment and 1 ORF partially contained within the fragment that extends toward the 3' end of the genome. In order to simplify our analysis, we only analyzed ORFs that were 100 base pairs or more in length. Given this

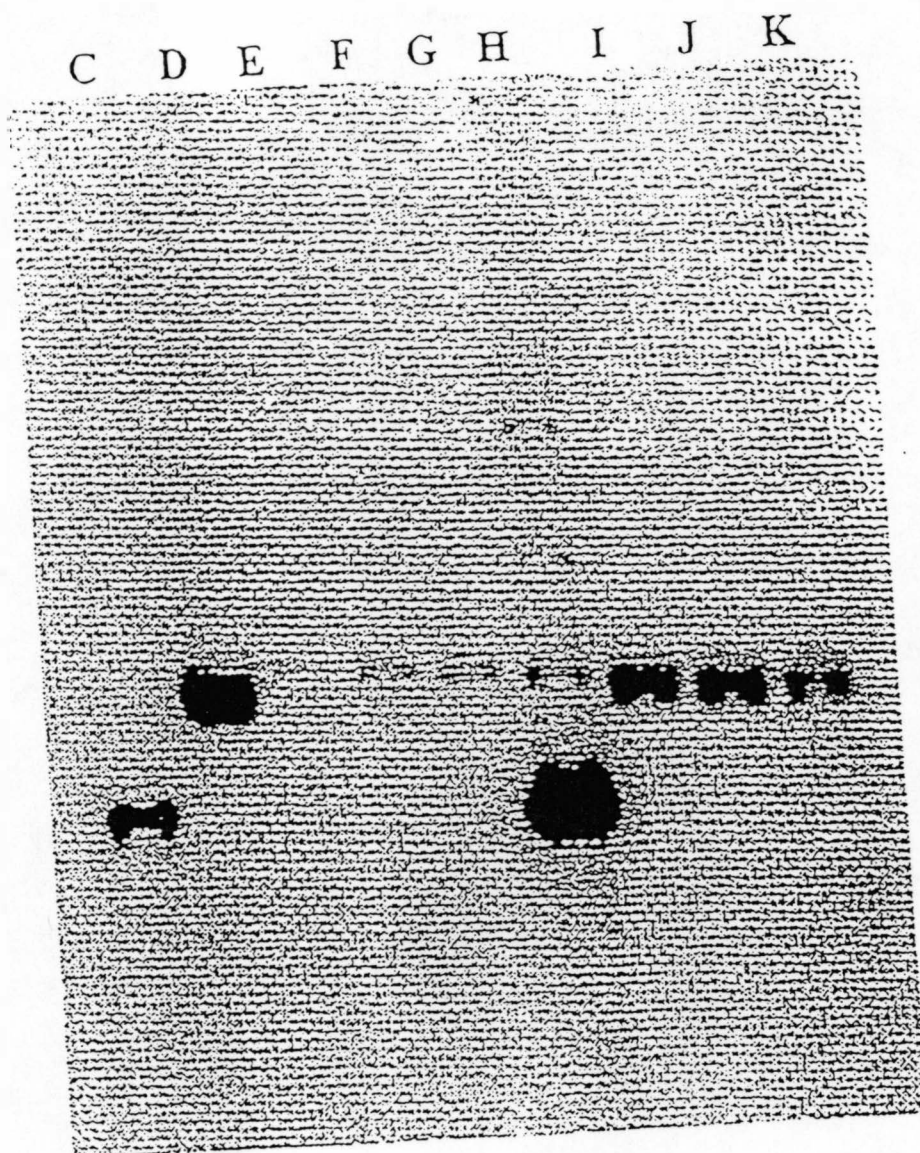


Figure 2. Southern Blot Analysis of TPV Cloned DNA.

A Southern blot was done on the gel shown in Figure 1 using the 2.1 kbp DNA fragment insert in pUC19 as a probe to verify that it was of TPV genomic origin. Lane C contains *Pst*I digested TPV genomic DNA. Lane D is pUC19 DNA digested with *Pst*I. Lanes E through K are plasmids containing TPV DNA fragments. Lane H shows the plasmid that contains the 2.1 kbp *Pst*I-L fragment of TPV.

10
CTGCAGCTTT TTGCTATCAA GGTTATGTGT TTAACATCAA
 50
GTGCCAAAAA AAAAATTAAT AATGTTAAAA TACCAATAAT
 90
AGTATTACAT GGAATAAACG ATGTTATATG CGATGTAAAA
 130 TP-1 UP
TGGTCAAAAT ATATAATTAA GAGCGTTGGA AGCTACGACA
 170
GAACAATAAA ATTATACAAA GGTGCAAATC ATGACCTGCA
 210
TAGAGAAGTT GAAGATATTA GAGATACTGT ATTTAGCGAT
 250
ATTAAAGTAT GGCTAATAAA TAGATCTAAA GTTAGTTACT
 290
ATGATGTCTT AATATAAAAG TAAAAAATAA ACGCAACATC
 330
TTTTAGACAT GAATAATCAC AAAAGTAAGT TGGCGTTTTG
 370 TP-2 UP
TTACGCGTTT CCAACTGTCTG GAACCGTAAC AAAAGGAATT
 410
GTCACAGTTA AAGATGCAGA CTTTACAGTA TTTTACCAG

Figure 3. DNA Sequence of the *Pst*I-L Fragment of TPV.

The 2.1 kbp *Pst*I-L fragment of TPV was sequenced using the dideoxynucleotide chain termination method as described in Materials and Methods. The sequence of the *Pst*I-L fragment is presented in the 5' to 3' direction. The region not sequenced in both directions is underlined. Up primer sites are in bold and down primer sites are in italics with the name of primer above the sequence.

(Continued on next page)

450
AGTTTGGGTT AGAAGCATT ATTGTAACT ATTTTCAGT

490
AAACGCAAAT ATAGCAGAAA AATTATCAAA AGAACTATCT

530
GGAAAAACCA TTAATGTACA AGTAATTAGA ACAGATAAAT

570 TP7-DOWN
TAAAAGGATA TGTGATGTT CGACACATAA CATGATATTA

610 TP-3 UP
TAATATAATA TGTCATGTCA GTAATGCCGA TTTTTTATAT

650
AGAGAGTGGT ATACAAGATT ATTTAATTA CCCTTTACAT

690
AGCACTATTT ACTTCTTAAA AGACGAAAAA AAGGTAAAGT

730
ATTTATTAGA TCATGGATAT GATATAAATC AAAAAGCAAA

770 TP6-DOWN
TAATTCTTTA ACTCCGCTTC ATTATGCAGT TTAACTAAC

810
AATGTTGAAA TAGTTAAATT ATTAATATCA AAAGGTGTTA

850 TP-4 UP
ATGTTAATGC TACTGATAGA TACGGATGTT CTCCTCTATA

890
TTATTATATT ATGACTAAAA AGGAAAATTA TGAAATTATA

930
AAACTATTGT TGGACAACGG TGCAGATGTA AATGCTGTTT

970
CTAGCATACA GGAAAATGTA TTACATGCAT TTACAGAATA

(Continued on next page)

1010
CGGATGTAAG AACACCGAGG TTTTAAAAAC AATTATTAAA

1050 TP5-DOWN
AAAACCTACA ACATAAACTC AAAAAATAAG TGGGGTAAAA

1090
CTCCGTAAAA TTTTGCTGTA GAAAAAGATA ATATGAATAT

1130 TP-5 UP
AATAAAAAATA TTGTTACATT ATGGAGCTAA TCCTTTTACA

1170
CTTTCAAATA ACATGGATAC ACTAATGCAT TGTTTTTTAA

1210
ACAACATAAA TTTGTTTAAA AAAGTAAAAC TGTTATTAGA

1250
TATTGGTTTA GATCCAAATA GTAAAAATAT AGACGGTGAT

1290
TCTCCATTAC ATAAGATATG TTCAGTAAAT CCAAGTTTAT

1330 TP-4 DOWN
AGACAGTCAG TTTATTAGTT AGTAAAGGAG CAAATGTAAA

1370
CTCGTTAAAC AATGATAAAA ATACACCTCT TCATGTGTAT

1410
ATGTATGAAT ATCCAGACAA ATTTTGCAAA AGCGTGTTTG

1450 TP-6 UP
TTTTTTTGCT CAAAAGGGA GCTAACATAC ATATAAACAA

1490
TAAATATAAT AAACAACCGT TTAATATTTT ATCTTGTAAT

1530 TP-3 DOWN
AAAGAAATAA CTATTGATTT AATTGAATTG TTTATTAAAA

(Continued on next page)

1570 TP-3A DOWN
AAAATGTACA TGTGAATAAT AAAAACGTGT ACGGATATTC

1610
ACCTATTCAT AATTTTCAA ACAATCCAAA CATTGACATT

1650
GTAAAAAGAT GGCTGGATTA CGGAGCAAAT CCTAACGACA

1690
AAACCGTTAA TGGAGTAACC CCAGTGCATA TAAGTGCAAA

1730 TP-7 UP
AAATAAAAAC ACAAATGTTT TTAAGTTAAT AGTAAGTAAT

TP2-DOWN 1770
GGAGGTGATG TTAATGCTGT AGATCAATAT GGGAATACTC

1810
CATTGCACGA ATCTGTAGTT AATGAAAATA ATTTTAAAAT

1850
ATTTGCTTAG TTAGGAGTT AAAGATGTAC CAAACAAACG

1890 TP-1 DOWN
GGGAGAAACT GCTCTTTTAA AAGCAGTAAG ACATGACAAA

1930
TTAAATTCTG TTAAGTTTTT GTTACAACAA CAAAATAATT

1970
TTTTAAATTA TGTAACATAAT GATGGAAACA CATGTATTTC

2010
AGAATGCTTT GATACCTTCA ATGAAGCAAT TTTTAACGAA

2050
CTTATAATTA ACAGACCTAA TATTTCTACC ATGATCATTT

2090 2108
CGTTAAACAA AGTTAACAAA TACTGCAG - 3'

criteria, there are 8 possible ORFs contained within the *Pst*I-L fragment and 1 ORF extending towards the 3' end of the genome.

Figure 4 shows the ORFs and the respective sizes of the putative polypeptides. The numbering of the ORFs was according to Rosel *et al.* (1986), using the letter (L) to designate the *Pst* fragment in which they originated and then numbering successively from left to right. The reading frame direction is indicated by adding a L (left) or R (right) after the numbering.

Computer analysis enabled us to compare the amino acid sequences of the putative polypeptides with the University of Geneva and Protein Identification Resources libraries. The FASTP program of Lipman and Pearson (Lipman & Pearson, 1985) was used to analyze the data. Table 2 shows a list of the ORFs, their molecular weights (MW) and homologies to other known proteins. The term homology is loosely defined as the number of amino acids that match between the two proteins when they are aligned. The program may introduce gaps within the amino acid sequence to help align the proteins. The ORFs may also only be homologous to a small portion of a larger protein.

The amino acid comparison revealed that most of the ORFs within the *Pst*I-L fragment have some degree of homology to proteins that function in cellular growth and development. ORFs *Pst*I-L5L and -L6R show homology to insulin-like growth factor of rat (Shimatsu & Rotwein, 1987) and transforming growth factor β -5 of frog, respectively (Kondaiah *et al.*, 1990). The largest ORF (27 kDa) *Pst*-L4R shows homology to the M1 protein of VV (30% in 40 amino acids), which is involved in drug

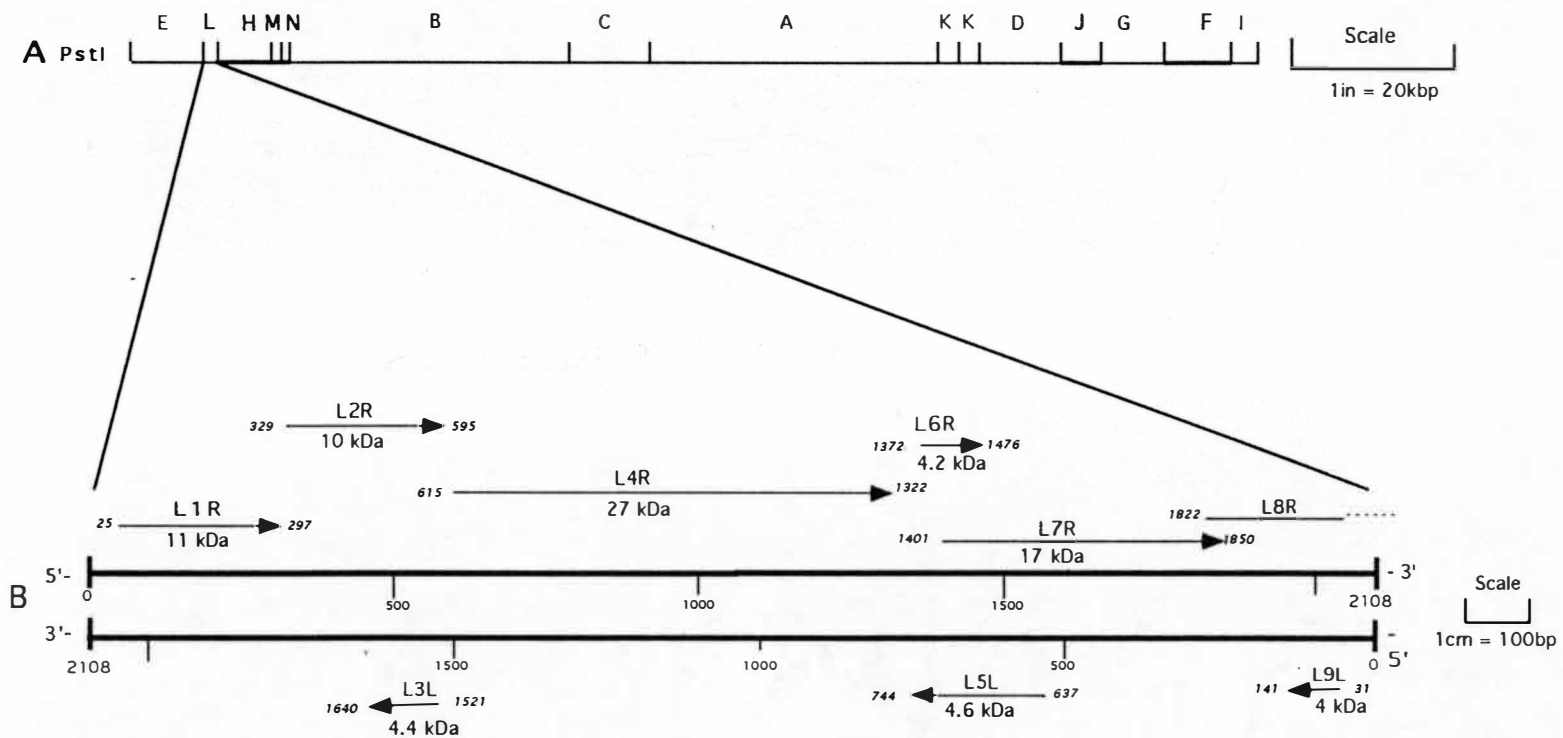


Figure 4. *Pst*I Restriction Map of TPV Genomic DNA and Location of the Potential ORFs on the *Pst*I L Fragment.

(A) *Pst*I restriction map of the TPV genome: fragments are designated A to N according to their size. Bold letters indicate fragments that have been cloned. (B) Positions of the potential ORFs and their molecular weights with respect to the 5', 3' sequence of the *Pst* I-L fragment. Italic numbers indicate base pair ORF starts and stops. Dotted line extending from ORF L8R indicates that it extends into the *Pst* I-H fragment.

resistance to α -amanitin (Tamin, Villarreal, Weinrich & Hruby, 1988), and also to human erythrocyte ankyrin (27%) which contains repeated structures that have homology to tissue differentiation and cell-cycle control proteins (Lux, John & Bennett, 1990).

Table 2

Amino Acid Comparison of the Putative Polypeptides
to Protein Data Bases

ORF	MW (kDa)	Percent Homology
L1R	11	30% to K4 protein of VV 25% to A42R protein of VV
L2R	10	27% to Glutamine receptor of human, mouse and rat 25% to ATP-dependent proteinase Ia in <i>E. coli</i>
L3L	4.4	31% to alpha amino butyric acid receptor of rat 27% to DNA binding protein of human cytomegalovirus 27% to RNA directed DNA polymerase in <i>E. coli</i>
L4R	27	31% to <i>Bam</i> HI ORF13 protein of fowlpox virus 30% to M1 protein of VV 27% to human erythrocyte ankyrin 25% to B4R protein of VV 24% to <i>Hind</i> III C protein of VV 23% to host range protein of cowpox virus

Table 2-Continued

ORF	MW (kbp)	Percent Homology
L5L	4.6	35% to UL10 protein of human cytomegalovirus 30% to Insulin-like growth factor IA precursor of rat 27% to hypothetical protein of VV (mRNA capping enzyme)
L6R	4.2	31% to human rhinovirus 14 genome polyprotein 25% to transforming growth factor beta-5 precursor of frog
L7R	17	26% to M1 protein of VV 26% to human erythrocyte ankyrin 25% to B4R protein of VV
L8R	-	35% to host range protein of cowpox virus 34% to 27.4 kDa <i>Hind</i> III C protein of VV 26% to F8L protein of VV 25% to human brain ankyrin
L9L	4	35% to serotonin receptor of rat 35% to transcriptional factor Oct-1 in frog 33% to serotonin receptor of human

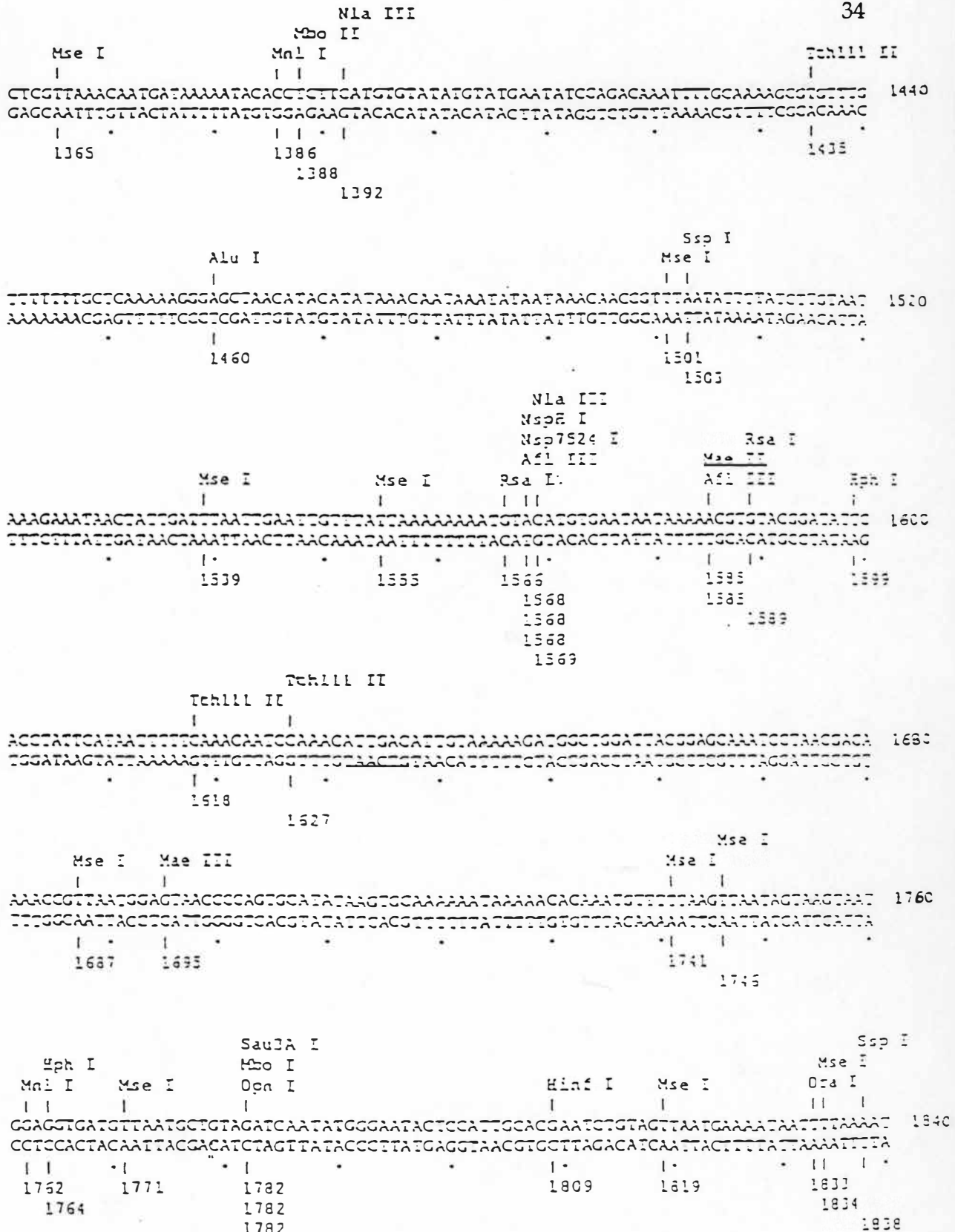
In order to further characterize the *Pst*I-L fragment, computer assisted restriction analysis of the L fragment was done. Figure 5 shows other restriction endonuclease sites within the *Pst*I-L fragment. There are 157 restriction sites within the *Pst*I-L fragment. The unique restriction endonucleases (i.e. endonucleases that cut at one site within the L fragment) are underlined in the following figure.

The image displays a DNA sequence with various restriction enzyme sites marked above it. The sequence is as follows:
 TTTAGACATGAATAATCAGAAAAGTAAGTGGGCTTTGGTACGGCTTTCCAACTGTGGAGCCCTAACAAAAGCAAT
 AAAATCTGTACTTATTTAGTGTTTTCATTCACCCGCAAAACAAATGGGCAAAAGCTTGACAGCTTTGGCATTTGCTTTCTTAA
 CAGTGTCAATTTCTACGCTCTGAAAATGTCATAAAAAATGGTCTCAAAACCCCAATCTTCGTAATTAACAATTGATAAAAAAGTCA
 AAACCCAAATATAGCAGAAAAATTATCAAAAGAACT. ATCTGGAAAAACCTAATGTACAGTATATTAGAACAGATAAAT
 TTTGGCTTTATATCGTCTTTTAAATAGTTTTCTTGATAGACCTTTTTTGGTAAATTACATGTTCAATATCTTGTCTATTCA
 TAAAAGGATATGTTGATGTTTCGACACATAACATGATATTATAATATAATATGTCAATGTCAGTAATGCCGATTTTATAT
 ATTTTCTTATACAACCTACAAGCTGTGTATTGTACTATAATATTATATTATACAGTACAGTCATTACGGCTAAAAAATATA
 AGAGAGTGGTATACAGATTATTTTAATTACCCCTTACATAGCACTATTTTACTCTTAAAAGACGAAAAAAGGTAAAGT
 TCTCTCACCATATGTTCTAATAAAATTAAATGGGAAATGTATCGTGATAAATGAAGAATTTTCTGCTTTTTCATTTC
 ATTATTAGATCATGGATATGATATAAATCAAAAAGCAAAATAATCTTTAACTCCGCTTCATTATCCAGTTTAACTAAC
 TAAAATATCTAGTACCTATACTATAATTAGTTTTTCTGTTATTAAGAAATTCAGGCGAAGTAATACGTCAAAATTCATTG

Restriction enzyme sites marked above the sequence include:
 Nla III, Bst I, Mlu I, Afl III, Mae III, Nla IV, Hpa I, Mse I, Hinc II, PflM I, SfaH I, Xba I, Aag I, Sau3A I, Dpn I, Mse I.

The sequence is divided into segments with the following lengths indicated on the right side:
 400, 480, 560, 640, 720, 800.

(Continued on next page)



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DISCUSSION

In order to further to characterize TPV on a DNA sequence level, we cloned approximately 17% of the TPV DNA genome into pUC19. Table 1 lists the clones and the TPV DNA fragment insert. Out of the 300 ampicillin-resistant colonies selected, only 116 contained TPV DNA fragments. Many of the clones contained the same fragment (e.g. 46 of the 116 clones contained the *Pst*I-L fragment). Overall, only 6 of the 15 possible *Pst*I TPV DNA fragments were successfully cloned (Figure 1). The two end terminal fragments of the TPV genome could not be cloned using the 'shot gun' method due to the terminal ends being joined by hair-pin loops. Some fragments were not successfully cloned into pUC19 most likely because of their size. One exception is the *Pst*I-K fragment (2.7 kbp). This fragment co-migrates with *Pst*I digested pUC19. The *Pst*I-K fragment may have been successfully cloned (clones 5, 58, 84, 100, 131, 335, 343, 347, 383, 384, and 444), but could not be detected by gel electrophoresis.

Traktman *et al.* (1990) have previously reported that in VV the essential genes are clustered in the middle of the genome whereas virulence markers, which play a role in pathogenesis, are located near the termini. Some of these virulence markers are thought to be proteins that mimic host cell immune functions thereby increasing the viruses survival in the host. With these concepts in mind, the 2.1 kbp *Pst*I-L cloned fragment was chosen for sequencing due to its relatively short length and proximity to the end of the TPV genome. Before sequencing, a

Southern blot was done in order to verify that the cloned fragment was of TPV genomic origin (Figure 2).

The complete *Pst*I-L DNA fragment (clone #36) from TPV was sequenced (Figure 3). The predicted amino acid sequence and putative ORFs were analyzed using IntelliGenetics software. Potentially, there are 37 complete ORFs and 1 partial ORF within this fragment. Further analysis of the 9 largest ORFs are shown in Figure 4 and listed in Table 2.

Analysis of the DNA sequence and proposed protein coding regions required assumptions which may generate possible sources of error. One assumption was there would not be any proteins within the L fragment that were less than 4 kDa. The decision to establish probable amino acid comparison of only the 9 largest ORFs (MW \geq 4 kDa) was based on the understanding that most VV proteins have molecular weights greater than 7 kDa (Goebel, Johnson, Perkus, Davis, Winslow & Paoletti, 1990). This decision may be an underestimate, but only further mRNA analysis from TPV-infected cells will determine if this is true.

In order to minimize DNA sequence error, a minimum of three gels were done on a specific region in order to verify the sequence. There were problems with secondary structures in certain regions of the fragment. In order to sequence through these areas we tried two different approaches. One was to use the *Taq* DNA polymerase in the sequencing reactions. This polymerase is stable at high temperatures. This allowed the reactions to be done at higher temperatures thereby relaxing the secondary structure. All attempts with this procedure failed. We then used different primers further upstream of the region of interest. This

procedure was successful in all cases except for the last 430 base pairs. This region of the *Pst*I-L fragment has been verified in one direction, multiple times.

Comparison of the ORF amino acid sequences revealed that most of the putative ORFs within the *Pst*I-L fragment show a certain degree of homology to proteins which function in cellular growth and development. The largest ORF *Pst*-L4R (27 kDa) shows a relatively high degree of homology to human erythrocyte ankryin (27%) which contains repeated structures that have homology to tissue differentiation and cell-cycle control proteins (Lux, John & Bennett, 1990). It also shows homology to the M1 protein of VV (30% in 40 amino acids), which is involved in drug resistance to α -amanitin (Tamin, Villarreal, Weinrich & Hruby, 1988). Similarly, ORF *Pst*-L7R (17 kDa) also shows homology to the M1 protein of VV (26%) and to human erythrocyte ankryin (26%). Given that these two ORFs are translated in the same frame suggests that they may be one continuous ORF. This would be possible if there was a base pair substitution at base number 1322, thereby changing the termination site of ORF *Pst*-L4R. Further sequence analysis of this region showed this is not the case.

Another interesting ORF is *Pst*-L9L which shows a high degree of homology to the serotonin receptor of rat and human. This ORF also has a high percentage of hydrophobic amino acids (54% of the 37 amino acids) suggesting that it may be a membrane bound protein. Two other ORFs that are of interest; *Pst*-L5L and *Pst*-L6R, have homology to growth factor proteins. ORF *Pst*-L5L shows 30% homology to insulin-like rat growth

factor IA which is structurally and functionally related to insulin but has a much higher growth-promoting activity (Shimatsu & Rotwein, 1987). ORF *Pst*-L6R shows a 25% homology to transforming growth factor β -5 of frog (Kondaiah et al., 1990).

Some of the ORFs show amino acid homology to other poxviruses. ORF *Pst*-L4R has 31% homology to *Bam*HI ORF 13 of fowlpox virus (Tomley, Binns, Campbell & Boursnell, 1988). ORFs *Pst*-L4R and *Pst*-L7R both have 25% homology to B4R protein of VV which is related to the tumor necrosis factor receptor family (Howard, Chen & Smith, 1991). ORF *Pst*-L8R has a 35% homology to a host range protein of cowpox virus. This protein enables cowpox virus to multiply in Chinese hamster ovary cells (Spehner, Gillard, Drillien & Kirn, 1988).

Restriction analysis of the *Pst*I-L fragment using the IntelliGenetics software is shown in Figure 5. This data will be helpful to further subclone the *Pst*I-L fragment for future DNA and RNA analyses.

Ultimately, the goal of this type of research is to identify gene products. We have only begun, in this study, to characterize TPV at the DNA level. Although no significant degree of homology to other known virulence marker proteins were found within the *Pst*I-L fragment, further analysis of other TPV *Pst*I fragments may show otherwise. Studying these types of proteins will help in understanding viral pathogenesis and host cell immune response to infection.

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