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The transcription process of infectious hematopoietic necrosis virus (IHNV), a rhabdovirus of salmonid fish, was studied both in vivo and in vitro. Polyadenylated RNA from IHNV infected-salmon cells was resolved electrophoretically into five bands, one of which was found to contain two mRNA species. The molecular weights and intracellular molar ratios of these bands were determined. Coding assignments were made by hybrid selection and in vitro translation of individual mRNA species using cloned plasmids carrying cDNA to each viral mRNA. Species of mRNA were identified which encode each of the five known virion proteins. In addition, a sixth viral mRNA species which encodes a previously unrecognized non-virion protein was discovered. This protein, designated NV, is the first non-virion protein reported for a

rhabdovirus.

Transcription by the RNA polymerase of IHNV was also examined in vitro. Studies of the optimal reaction conditions revealed that the use of HEPES buffer rather than Tris, and the addition of S-adenosyl methionine to the reactions led to a six-fold increase in enzyme activity. The products of in vitro transcription were found to contain polyadenylated species which co-migrated electrophoretically with IHNV mRNA bands 2, 3, 4, and 5 from IHNV infected-cells. These transcripts were shown to be functional mRNA species by the ability to direct the synthesis of viral proteins in vitro.

The construction of cloned plasmids carrying cDNA to viral mRNA species is described. A set of 21 cloned plasmids was characterized by mRNA blot hybridizations and cross-hybridization studies, which identified subsets of plasmids with cDNA to each of the six viral mRNA species.

The molecular weight of the genome RNA of IHNV was determined to be 3.7×10^{6}. The cDNA clones were used to construct a physical map of the viral genome by heteroduplex analyses. Measurements of R-loops formed between viral genome RNA and cDNA cloned plasmids determined that the gene order on the viral genome is 3' N-M1-M2-G-NV-L 5'.

Molecular Characterization of Infectious Hematopoietic Necrosis Virus Transcription and Genome Organization

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TO RUSTY, OREGON, AND THE PACIFIC OCEAN

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PREFACE

The heteroduplex analyses described in this thesis were done in collaboration with Kevin Ahern in the laboratory of Dr. G. D. Pearson. All project design, sample preparation, and final analyses of results were carried out by myself (G. Kurath). Hybridizations, electron microscopy, measurements of electron micrographs, and photography were done by Kevin Ahern. The composite figures 14 and 15 were prepared by Dr. G. D. Pearson.

MOLECULAR CHARACTERIZATION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS TRANSCRIPTION AND GENOME ORGANIZATION

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus which infects salmon and trout (74). This virus is responsible for increasingly devastating losses of young fish at hatcheries in western North America (32). Although diagnostic methods for detection of IHNV have recently been greatly improved (32), control methods are still limited to avoidance procedures(32). The molecular genetics of the virus have not been studied, and the replication cycle of IHNV in relation to its carrier host is not known.

IHNV is similar to the rhabdovirus prototypes, vesicular stomatitis virus (VSV) and rabies, in that it is a bullet-shaped, enveloped virus with glycoprotein surface projections and a genome of single-stranded, negative sense RNA (4,37,61). The protein structure of IHNV has been reported to resemble that of rabies virus and consists of a viral polymerase (L), a surface glycoprotein (G), two matrix proteins (M1 and M2), and a nucleocapsid protein (N) (37,51,59). Although the proteins of IHNV have been well characterized, the viral nucleic acid species have received little attention. Therefore, in order to further

our understanding of the molecular biology of IHNV in both the carrier state and during lytic infections, it was first necessary to characterize the viral genome and mRNA species.

Studies presented here show the isolation of genome RNA from purified IHNV virions and six viral mRNA species from IHNV infected salmon cells. These molecules were resolved by electrophoresis on denaturing gels and the molecular weights and molar ratios present in infected cells were determined. In addition, the mRNA species were characterized by hybrid selection and *in vitro* translation studies which resulted in the identification of the viral protein coded for by each message. Our results identify mRNA species for all the known viral proteins and, in addition, identify a message for a previously unrecognized viral protein which is present only in the infected cells.This protein has been designated the NV protein due to its non-virion nature.

In order to investigate the transcription process of IHNV in more detail, the activity of the viral polymerase was examined *in vitro*. Reaction conditions previously described for IHNV *in vitro* transcription (60) were tested and modified in order to maximize RNA synthesis. The use of HEPES buffer rather than Tris, and the addition of S-adenosyl methionine to the reactions resulted in a six-fold increase in polymerase activity. The RNA transcripts produced under these optimal conditions included polyadenylated species which co-migrated with IHNV mRNA species 2, 3, 4, and 5.

These transcipts were shown to be complete, functional transcripts by the ability to direct viral protein synthesis in vitro.

The slow growth and relatively low yield of IHNV in tissue culture (21) made it necessary to obtain cloned genetic material for further studies of the molecular biology of IHNV. The construction and characterization of cDNA clones carrying sequences from each of the six mRNA species of IHNV is described. These clones were used in the hybrid selection studies to determine the coding assignments for each mRNA, and in heteroduplex analyses to obtain a physical map of the viral genome.

LITERATURE REVIEW

A. IHNV DISEASE

The disease caused by infectious hematopoietic necrosis virus (IHNV) was first reported in Washington State sockeye salmon in 1953 (86). Since then this virus has caused extensive mortalities in populations of hatchery fish in Washington, Oregon, California, and British Columbia (74). Outbreaks of IHNV have also been reported in Alaska, Idaho, Minnesota, Montana, South Dakota, and Japan (74). The salmonid species which are susceptible to IHNV include sockeye salmon (Oncorhynchus nerka), chinook salmon (Oncorhynchus tschawytscha), rainbow trout (Salmo gairdneri), and steelhead trout (sea-run rainbow trout)(74,101). Although virus can be isolated from adult fish, mortality due to viral infection is generally limited to juveniles (101).

Young fish which are infected with IHNV may show signs including exophthalmia, anemia, distension of the abdomen, petechial hemorrhaging at the base of the fins, and behavioral changes (74,101). Often the first sign of an epizootic in a hatchery population is a rapid onset of mortalities among juvenile fish. Internally, the viscera of infected fish show anemia and hemorrhaging, and there is extensive necrosis of the kidney, spleen, and pancreas (74,102).

The transmission of IHNV has been studied intensively, but is

still not completely understood. The two modes considered to be most significant are vertical transmission via the spawning fluids (3,69,74,99) and horizontal, water-borne transmission (69,86,99). There is evidence that both of these occur, but the question of which, if either, is most responsible for IHNV epizootics at hatcheries is still unanswered. Survivors of IHNV epizootics have been shown to become asymptomatic carriers of the virus (3). These fish appear to be virus-free until the onset of spawning, at which time IHNV can be isolated from the semen, ovarian fluid, and many internal tissues (3). The tissue location and status of the virus during the unexpressed carrier state is not known.

Presently, diagnosis of IHNV involves isolation of the virus from infected fish tissues and replication in cell culture, followed by confirmed identification by neutralizaton with specific anti-IHNV sera (5,52). A more rapid method has been developed recently which conclusively identifies IHNV by its protein pattern on SDS-polyacrylamide gels (52,53).

Although rapid identification of IHNV is essential to diagnosing an outbreak of the virus, control methods currently used are limited to avoidance procedures. Standard practice is destruction of infected fish stock, and quarantine of infected populations in order to prevent the spread of the disease(32). An attenuated vaccine strain of IHNV has been developed and tested (64,92), but it is not available for use at hatcheries due to expense and the risk of reversion to virulence.

The incidence of mortalities due to IHNV at hatcheries in the Pacific northwest of North America has been increasing drastically in recent years. Between 1980 and 1982 the number of juvenile fish lost to IHNV in Columbia River basin hatcheries increased from 150,000 to 5,446,000 (32). This figure does not include the number of eggs which were destroyed due to detection of IHNV in brood stock. This represents a substantial economic loss, as well as a threatening indication of the future.

B. IHN VIRUS

Early studies of the agent which causes infectious hematopoietic necrosis disease indicated that it was an enveloped RNA virus (61,100). The viral genome was subsequently shown to be single-stranded RNA with a sedimentation value of 38-40S (37,61), and electron micrographs revealed the bullet shape morphology of the virions(4,37). Thus, IHNV has been classified as a rhabdovirus.

The protein structure of IHNV has been characterized by several investigators (37,42,51,59). The virion consists of five proteins including the viral polymerase (L, mol.wt. 150,000), a surface glycoprotein (G, mol.wt. 67,000), a nucleocapsid protein (N, mol. wt. 40,500), and two matrix proteins (M1 and M2, mol. wts. 22,500 and 17,000 respectively) (51). This protein composition is very similar to that of rabies virus (18,37,59,88), and differs from vesicular stomatitis virus (VSV) , which has only

one matrix protein but also contains a non-structural protein (NS) associated with the ribonucleoprotein core (58). On the basis of these protein patterns, IHNV has been placed in the Lyssavirus (rabies-like) genera of rhabdoviruses rather than the vesiculovirus (VSV-like) genera (37,58,59).

The mRNA species of IHNV have been described briefly in relation to an in vitro transcription system reported by McAllister and Wagner in 1977 (60). These investigators demonstrated an RNA polymerase activity associated with IHN virions, and reported that the transcripts produced both in vitro and in IHNV infected-cells ranged from 12-17S in size. No individual mRNA species were identified, but the transcripts were shown to be virus-specific by hybridization with the viral genome RNA.

C. OTHER RHABDOVIRUS MODELS

Since IHNV is clearly identified as a member of the rhabdovirus family, it is helpful to briefly review the information which is available regarding the molecular biology and transcription process of other rhabdoviruses.

Several other fish rhabdoviruses have been described in the literature, including pike fry rhabdovirus (PFR), spring viremia of carp virus (SVCV), and the virus of viral hemorrhagic septicemia (VHS) (37,101). Although the biology and pathogenicity of these viruses has been studied, very little molecular or

genetic characterization has been reported. *In vitro* transcription systems have been described in order to demonstrate a viral-associated RNA polymerase activity for each fish virus, but individual transcripts produced *in vitro* were not identified (60,84,85). The *in vitro* transcription system for SVCV has been used extensively in studies of the capping mechanism for viral mRNA synthesis (33,34,35). Although there has been a recent report of the isolation and sequencing of a clone carrying cDNA to the M protein mRNA of SVCV (47), characterization and coding assignments of the mRNA species of SVCV are not available in the literature.

In contrast, the molecular biology and transcription processes of the rhabdovirus prototypes, VSV and rabies, have been studied extensively. The protein structures of these viruses differ as described above, but both are comprised of five proteins (58). The mRNA species associated with VSV and rabies have been isolated from virus infected-cells, and their molecular weights and poly(A) content have been determined (14,39,82). Rabies mRNA species are resolved into five bands by electrophoresis, and VSV mRNA migrates as four bands, the smallest of which contains two unique mRNA species. For both viruses, the proteins encoded by each viral mRNA have been identified by *in vitro* translation, and the mRNA species observed account for all the known viral proteins(14,48,72).

In vitro transcription of rabies virus has been reported, but

the activity of the viral polymerase *in vitro* is extremely low (24,45). In contrast, the VSV polymerase is very active *in vitro*(15,25,68). Many systems for VSV transcription *in vitro* have been used to study the details of the viral transcription process in the absence of host cell influence. Systems have been described in which all of the mRNA species of VSV are produced *in vitro* as functional mRNA (14,25,43,83). Alternatively, systems have been developed which result in aberrant transcription, in order to assess individual mechanisms of "normal" transcription such polyadenylation (83) and replicative RNA synthesis (93). *In vitro* transcription studies with VSV are responsible for the discovery of the "leader" sequence (16,17) , a short 47-48 nucleotide sequence at the exact 3' end of the VSV genome which is transcribed before any of the viral genes and is now known to play a role in inhibition of host cell transcription (30,31).

The genome maps of VSV and rabies have been determined by ultra-violet inactivation studies and are very similar. The order of genes on the genome of VSV is 3' N-NS-M-G-L 5' (2,10), and the rabies genome is 3' N-M1-M2-G-L 5' (23).

Transcription of VSV has been shown to proceed sequentially from the 3' to the 5' end of the genome, with no gene transcribed until the transcription of its 3' neighboring gene is complete (2,10,44). The molar ratios of the mRNA species produced intracellularly indicate an attenuation of transcription at each gene junction (97). This attenuation has also been observed in

in vitro transcription studies (43).

Cloning and primer extension studies with VSV have resulted in the characterization of extensive regions of the viral genome, including the intergenic regions (79). Cloned plasmids carrying cDNA to the mRNA species of VSV have been used to determine the sequences of the entire coding regions of the VSV N, NS, M, and G genes (27,28,80,81). A clone carrying the coding sequence of the G gene of rabies virus has also been reported (6). This sequence information has revealed many interesting features such as the 23 base consensus sequence at the gene junctions on the VSV genome. This sequence contains a putative polyadenylation signal, a dinucleotide spacer, and a sequence common to the 5' ends of all VSV mRNA species (79). This type of information is indispensable in elucidating the signals involved with the complex processes of transcription and replication.

Three main models have been proposed to explain the sequential transcription of the VSV genome. An early model involved the transcription of the entire genome as a large precursor molecule, which was subsequently cleaved to form functional mRNA species by a processing nuclease (11). Although this model has not been disproven, there is little evidence to support it, and an RNA processing system has not been observed. A second model is the single polymerase entry site model, which proposes that RNA polymerase initiates transcription only at the 3' end of the genome, and transcribes the leader sequence and

genes in order from 3' to 5' (11,44). In this model the enzyme remains bound to the template, but pauses at intergenic junctions for termination, release, and possibly polyadenylation of each transcript before proceeding to the next gene. A third model is the multiple entry site model, which suggests that the initiation of transcription for each gene occurs independently, but that elongation and termination of the transcripts occurs sequentially (33,94). Evidence for both the second and third models comes from in vitro transcription studies, but as yet no conclusive data has proven which model is correct.

A complete model of the transcription process of VSV must take into consideration a mechanism by which the polymerase can distinguish genome replication from mRNA transcription. Both of these RNA synthetic processes are carried out by the viral-associated polymerase, but the signals which direct termination or read-through at intergenic junctions are still uncertain. It has been reported that viral N protein synthesis is required for replicative RNA synthesis in vivo (19,70), and in vitro systems which couple translation and replication have been described (19,38,71). It has also been shown that in the presence of certain base analogs replicative RNA synthesis can be demonstrated in vitro without protein synthesis (93).

Mutants of VSV have been isolated which fail to terminate transcription at the leader termination site under standard in vitro transcription conditions (73). It is hypothesized that

these readthrough mutants are defective in the switch which regulates transcription vs. replication. The mapping of this mutation to the N protein has provided support for the hypothesis that the binding of soluble N protein to nascent plus strand leader RNA signals read-through of the leader-N gene junction, and thus stimulates the polymerase to carry out replication of the entire genome rather than transcription of monocistronic mRNA species (54,73).

In summary, although extensive experimentation has provided a great deal of detailed information about the transcription and replication of VSV, there are still several major phenomena which are not completely understood. Nevertheless, the large body of information which has accumulated through studies of VSV is important as a model for the molecular structure and functions of any rhabdovirus.

MATERIALS AND METHODS

VIRUS PROPAGATION

The IHN virus used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte hatchery in central Oregon. Virus was propagated in a chinook salmon embryo cell line (CHSE-214) obtained from J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, Oregon. Salmon cells were grown at 16°C in minimal essential medium (Autopow MEM, Flow Lab.) supplemented with 5% fetal calf serum, 10 mM glutamine, 500 U/ml penicillin, and 500 µg/ml streptomycin. Cell monolayers were infected with IHNV at a multiplicity of infection of 0.001 and incubated at 16°C for seven days. If radioactive labeling of viral genome RNA was desired, (5,6-^{3}H) uridine (New England Nuclear, 30 Ci/mmol) was added to 10 µCi/ml 24 hours post-infection. The supernatant fluid was harvested and clarified of cellular debris by centrifugation at 2400 x g (4000 rpm) in an HS-4 rotor in a Sorvall RC5 centrifuge for ten minutes at 4°C. The clarified supernatant contained 10^{7}-10^{8} $TCID_{50}$ units of IHN virus per ml and was stored at -20°C.

VIRUS PURIFICATION

IHN virions were purified for RNA extraction and protein analyses by banding to homogeneity on discontinuous and continuous sucrose gradients as described (53).

For use in *in vitro* transcription assays, virions were purified by a procedure involving less manipulation in order to preserve polymerase activity. Virus was maintained at 0-4 °C throughout the procedure. IHNV was concentrated from the clarified supernatant by centrifugation at 82,000 x g (24,000 rpm) for 90 minutes at 4 °C in an SW27 rotor in a Beckman L5-65 ultracentrifuge. Virus pellets were resuspended in STE, pH8.3 (0.15 M NaCl, 0.02 M Tris-hydrochloride, 1 mM EDTA) and layered onto pre-formed 5-30% sucrose gradients in STE, pH 8.3. Gradients were centrifuged at 58,000 x g (21,000 rpm) in an SW27 rotor for 30 minutes at 4 °C. Virus bands were collected, diluted in STE, pH 8.3, and concentrated by centrifugation at 82,000 x g (24,000 rpm) for 90 minutes at 4 °C. Pellets of purified virus were resuspended in STE, pH 8.3, distributed into small aliquots, and stored frozen at -70 °C. The protein content of the purified virus preparation was determined by Coomassie Blue G staining (Bio-Rad protein microassay), and ranged from 0.5 to 1.0 mg protein per ml.

PREPARATION OF VIRAL GENOME RNA

Purified virions were suspended in STE (0.1M NaCl, 20 mM Tris buffer, 1 mM EDTA, pH 7.4). Self-digested pronase and sodium

dodecylsulfate (SDS) were added to 1 mg/ml and 1% (v/v) respectively, and the mixture was incubated for 30 minutes at 37° C. RNA was isolated from disrupted virions by two extractions with STE-saturated phenol, followed by one extraction with STE-saturated chloroform:isoamyl alcohol (24:1). The final aqueous phase was made 0.3M with potassium acetate and precipitated overnight with 2.5 volumes of ethanol. RNA was pelleted, resuspended in water, and the quantity of RNA was measured by UV absorbance at 260 nm. One liter of tissue culture fluid yielded 12 to 15 µg of IHNV genome RNA. All solutions (excluding Tris buffers) and glassware used for RNA extraction were treated with diethyl pyrocarbonate (DEP) to inactivate contaminating RNases.

PREPARATION OF INTRACELLULAR RNA

Monolayers of CHSE-214 cells were infected with IHNV at a multiplicity of infection of 10 to establish a synchronous infection, and actinomycin D was added to 0.5 µg/ml to inhibit cellular transcription. Six hours post-infection (5,6 ^{3}H)uridine (New England Nuclear, 30 Ci/mmol) was added to 10 µCi/ml. The infection was allowed to proceed until approximately 25% of the cell monolayer exhibited a cytopathic effect (24 to 28 hours post-infection), at which time the monolayers were rinsed three times with ice-cold Tris buffered saline. A lysing solution

consisting of 0.5% SDS, 250 μg/ml proteinase K, 0.1 M NaCl, 5 mM EDTA, and 30 mM Tris, pH 7.4, was added using four ml per 150 cm^2 flask (95). The flasks were then incubated for 1 hour at 37° C. Cell lysates were then pooled and total nucleic acid was isolated by two extractions with STE saturated phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was extracted with chloroform:isoamyl alcohol (24:1), brought to 0.3M potassium acetate, and precipitated with 2.5 volumes of ethanol.

The total nucleic acid preparation was DNase treated by incubation for one hour at 37 °C in the presence of 0.1 mg/ml proteinase K-treated DNase (96), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM Tris buffer (pH 7.8), and 2mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories, Inc.). Reactions were stopped by the addition of EDTA to 50mM. RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) containing 0.1% 8-hydroxyquinoline until the dye remained yellow, indicating the complete removal of the vanadyl ribonucleoside complexes. The aqueous phase was then brought to 0.3M potassium acetate and precipitated with 2.5 volumes of ethanol. The RNA precipitates were resuspended in water, measured spectrophotometrically for concentration and purity, and stored at -70°C.

Polyadenylated RNA species were selected from the total RNA preparation by column chromatography with oligo dT-cellulose using a modified procedure of Aviv and Leder (8). RNA was

suspended in 1 mM EDTA and heat denatured at 65°C for ten minutes. After cooling on ice, Tris buffer (pH 7.5) and NaCl were added to 10 mM and 0.5 M respectively. Up to 10 mg of RNA was loaded onto an eight ml column of oligo dT-cellulose and washed extensively with binding buffer (10 mM Tris, 0.5 M NaCl, PH 7.5). Bound RNA was eluted by washing with 10 mM Tris (pH 7.5) and fractions containing radioactivity were pooled, brought to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. Precipitated RNA was resuspended in DEP-treated water, quantitated, and stored at -70°C. DEP was used as above to inactivate RNases in solutions and glassware.

ELECTROPHORESIS OF RNA

Glyoxal treatment of RNA and subsequent electrophoresis of the RNA on 1% agarose gels in 10 mM phosphate buffer (pH 7.0) was carried out as described (63) with the exception that the glyoxal (Fluka AG, Buchs, Switzerland) required more extensive de-ionization. Glyoxal was passed twice over each of five ten ml columns of Bio-Rad AG501-X8 mixed-bed resin and stored in small aliquots at -20°C for single use.

Electrophoresis of RNA samples in 5 mM methylmercuric hydroxide (Alfa Products), on 1% agarose gels containing 5 mM methylmercuric hydroxide, was carried out as described (9).

Both types of agarose gels were prepared for fluorography by

soaking for two hours with agitation in liquid autoradiography enhancer (Enhance, New England Nuclear), followed by two hours with agitation in water. Gels were dried between filter paper and cellophane for two hours on a gel dryer (Bio-Rad) and exposed to X-ray film (Kodak X-Omat AR) for 20-48 hours at -70 °C.

CELL-FREE TRANSLATION

Polyadenylated RNA or RNA selected by hybridization to plasmid DNA was translated in vitro in a nuclease-treated rabbit reticulocyte lysate system (Bethesda Research Laboratories, Inc.) as specified by the manufacturer. Reactions were carried out in 15 µl volumes for one hour at 30 °C in the presence of ^{35}S-methionine (New England Nuclear, NEG-009A, 1166.5 Ci/mmol). Newly synthesized proteins were visualized by polyacrylamide gel electrophoresis and fluorography as described below.

ELECTROPHORESIS OF PROTEINS

Proteins were analyzed by polyacrylamide gel electrophoresis on 10% polyacrlyamide gels with a 4.75% stacking gel (49). Samples were boiled for two minutes in SDS denaturing buffer (62.5mM Tris, 10% glycerol (w/v), 5% 2-mercaptoethanol, and 2.3% SDS, pH 6.8) prior to electrophoresis with a constant current of 20 mAmps. The Laemmli gel buffer system was used for gel and

reservoir buffers (49).

Following electrophoresis, gels were fixed for at least one hour in 10% acetic acid, 10% TCA, and 30% methanol. Gels were prepared for fluorography by soaking for one hour with agitation in liquid autoradiographic enhancer (Enhance, New England Nuclear), and then one hour with agitation in water. Gels were dried for one hour between two pieces of cellophane and exposed to X-ray film as above.

DENSITOMETER ANALYSIS OF AUTORADIOGRAMS

Autoradiographs of electrophoretic gel profiles were scanned on a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., model SL-504-XL). Scans were printed on an Apple personal computer using an electrophoresis reporting integrater program. Scans were xeroxed and individual peaks were cut out and weighed. Relative peak weights and mRNA molecular weights were used to calculate the molar ratio of each mRNA band with respect to mRNA band 3, which was given the arbitrary value of 1.0 The equation used was as follows; molar ratio of mRNA X = (peak weight mRNA X/peak weight mRNA 3) x (mol wt mRNA 3/mol wt mRNA X), where mRNA X was any one of the five mRNA bands.

HYBRID SELECTION

Hybrid selection of individual mRNA species was carried out by hybridization of the polyadenylated RNA from IHNV-infected cells to plasmid DNA immobilized on nitrocellulose filters essentially as described (57,77). Plasmids carrying complementary DNA (cDNA) sequences to each of the five translatable mRNA species (table 1), were cleaved with the restriction enzyme Bam Hl or Hind III. Ten µg of each linearized plasmid were heat denatured and spotted onto separate 3mm squares of nitrocellulose filter paper (Schleicher and Schuell, BA 85). The filters were dried overnight and baked at 80°C in vacuo for two hours. Each filter was hybridized with 15 µg of polyadenylated RNA from IHNV-infected CHSE-214 cells for 6 hours at 52 °C under the conditions specified by Maniatis (57). Following hybridization the filters were washed and the RNA released, extracted, and precipitated as described (57). Precipitated RNA was rinsed twice with 70% ethanol and translated *in vitro*.

IN VITRO RNA POLYMERASE ASSAY

The activity of the viral RNA polymerase was assessed in 100 µl *in vitro* transcription reactions containing 15 µl of the purified virus preparation (7.5-15 µg viral protein per reaction). The optimal reaction mixture contained 400 mM HEPES buffer, pH 8.0, 0.05% Triton X-100, 30 mM NH_4Cl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mM each of ATP,CTP, and GTP, and 0.001 mM UTP.

The labeled precursor (5,6-^{3}H)-UTP was included to a final specific activity of 86.7 Ci/mmole. The methyl donor, S-adenosyl methionine (SAM) was included at 0.5 mM unless otherwise indicated. Details of experiments defining these optimal conditions are presented in the results section. Tris-hydrochloride buffered reactions were carried out in 100 µl volumes with 15 µl purified IHNV using the reaction conditions previously described (60). Reactions were incubated for one hour at 18°C unless otherwise indicated. The amount of RNA synthesized was determined by incorporation of ^{3}H-UMP into acid precipitable material.

RECOVERY OF IN VITRO TRANSCRIPTION REACTION PRODUCTS

In vitro transcription reactions were scaled up as necessary and incubated for three hours in order to obtain large quantities of the RNA reaction products for further analyses. Reactions were stopped by the addition of sodium dodecyl sulfate (SDS) to 0.5%. HEPES buffered reactions were diluted with an equal volume of STE, pH 7.4, to avoid volume loss during extractions. HEPES buffered reaction mixtures were extracted twice with an equal volume of phenol equilibrated in STE, pH 7.4, with 200 mM HEPES buffer. The aqueous phase was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1) equilibrated with the same solution. The final aqueous phase was adjusted to 0.3M potassium

acetate and precipitated with 2.5 volumes of ethanol overnight at -20°C. RNA was recovered from Tris buffered reactions in the same way with the exception that the organic solutions were equilibrated in STE, pH 7.4, with no HEPES buffer.

Precipitated RNA was pelleted at 189,000 x g (45,000 rpm) for one hour in an SW 50.1 rotor at 4°C. RNA was resuspended in water and stored at -70°C. Water, solutions (excluding Tris buffers), and glassware used for extractions were treated with diethylpyrocarbonate (Eastman Kodak, Inc.) to avoid RNase contamination (57).

CLONING OF VIRAL mRNA SPECIES

The template for cloning was polyadenylated RNA from IHNV-infected CHSE-214 cells. This RNA was passed twice over an oligo dT-cellulose column to remove all detectable host cell ribosomal RNA. Synthesis of double-stranded complementary DNA (cDNA) was carried out by the procedures of Land et al (50). This procedure includes 4 mM sodium pyrophosphate in the first strand cDNA reaction to prevent the formation of the terminal hairpin loop and eliminate the need for S1 nuclease digestion.

Polyadenylated RNA was reverse transcribed in a 200 µl reaction containing 20 µg RNA template, 2 ug oligo-$dT_{(12-18)}$ primer, 160 units of reverse transcriptase (Life Sciences, Inc.),

20 units placental RNase inhibitor (Enzo Biochem, Inc.), 4 mM sodium pyrophosphate, 100 µg/ml bovine serum albumin, 50 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 2mM dithiothreitol, 0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dTTP, and 0.2 mM ^{32}P-dCTP (specific activity 0.75 Ci/mmol, New England Nuclear). The reaction was incubated for one hour at 42°C and stopped by the addition of EDTA to 25 mM. The RNA template was digested in the presence of 0.4N NaOH for eight hours at room temperature, and the reaction was then neutralized by the addition of HCl to 0.4N. The cDNA was passed over a five ml column of Sephadex G-100, and fractions containing radioactivity incorporated into DNA were pooled, adjusted to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. The amount of ^{32}P-CTP incorporated indicated that 3.9 ug of single-stranded cDNA had been synthesized from 20 µg of template RNA. Products of this first strand cDNA reaction were visualized by alkaline gel electrophoresis (62) and autoradiography.

Homopolymer tails of dC residues were added to the 3' ends of the single-stranded DNA molecules in a 125 µl reaction containing 3.5 µg of cDNA, 120 mM potassium cacodylate, 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 1 mM $CoCl_2$, 10 µM ^{32}P-dCTP (specific activity 5 Ci/mmole, New England Nuclear), and 50 units of terminal deoxynucleotidyl transferase (P-L Biochemicals, Inc.). The reaction was incubated at 37°C for ten minutes, at which time approximately 20 residues had been added

per 3' end. EDTA was added to 10 mM and the reaction was extracted twice with choroform:isoamyl alcohol (24:1). The aqueous phase was adjusted to 0.3 M potassium acetate and precipitated with 2.5 volumes of ethanol.

For synthesis of the second strand of cDNA the dC-tailed single-stranded cDNA was first hybridized with an oligo-$dG_{(12-18)}$ primer (Collaborative Research, Inc.). Hybridization was carried out in a 50 µl mixture which contained 200 ng cDNA, 1.5 µg oligo-$dG_{(12-18)}$, 50 mM Tris-hydrochloride (pH 8.3), 30 mM KCl, and 10 mM $MgCl_2$. This mixture was incubated for five minutes at 68°C, followed by five minutes at 55°C, ten minutes at 50°C, fifteen minutes at 43°C, and cooled on ice (50). The reaction mix was then adjusted to include 10 mM dithiothreitol, 1 mM dATP, 1 mM dTTP, 1 mM dGTP, and 0.2 mM (^{32}P)dCTP (specific activity 0.75 Ci/mmole, New England Nuclear). Twenty-four units of reverse transcriptase (Life Sciences, Inc.) were added and the reaction was incubated at ten minutes for 37°C, followed by six hours at 42°C. The reaction was stopped by adding EDTA to 25 mM, extracted with chloroform:isoamyl alcohol (24:1), adjusted to 0.3M potassium acetate, and precipitated with 2.5 volumes of ethanol.

In order to fill in any nicks, the double-stranded cDNA was incubated for four hours at room temperature in a Klenow enzyme reaction (57) which contained the cDNA, 50 mM Tris-hydrochloride (pH 7.2), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 50 µg/ml bovine serum albumin, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.2 mM

(^{32}P)dTTP (specific activity 5 Ci/mmole, New England Nuclear), and six units of DNA polymerase 1, large fragment (Klenow enzyme, Bethesda Research Laboratories, Inc.). The reaction was stopped by adding 20 mM EDTA, extracted with chloroform:isoamyl alcohol (24:1), and passed over a five ml sephadex G-100 column to remove unincorporated nucleotides. Homopolymer dC tails were added to the double-stranded cDNA molecules in a terminal deoxynucleotidyl transferase reaction as described above.

The plasmid vector, pUC8, was cleaved with the restriction endonuclease Pst 1 (Bethesda Research Laboratories, Inc.) according to the specifications of the enzyme manufacturer, and dG tails of approximately 15 residues were added in a terminal deoxynucleotidyl transferase reaction as described above. The dG-tailed vector and dC-tailed cDNA were annealed at a molar ratio of 1:1 in 10 mM Tris-hydrochloride (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The annealing mixture was heated to 65°C for ten minutes, incubated at 43°C for two hours, and cooled slowly to room temperature. This DNA was used to transform two host strains of E. coli K-12, JM103 (65) and the C600 strain SC181 (7). Transformation was carried out with freshly prepared competent cells by the calcium chloride shock method (56,57). Transformants were plated on LB agar (57) containing 150 µg/ml ampicillin. The transformation efficiencies were 51 and 158 ampicillin-resistant transformants per ng of re-annealed DNA for JM103 and SC181 respectively. The control transformation efficiency was 5 x 10^3

transformants per ng of uncleaved pUC8 for both strains.

PREPARATION OF IHNV GENOME cDNA PROBE

The probe for viral-specific sequences was ^{32}P-labelled cDNA to the IHN viral genome RNA. Probe synthesis was carried out at 42 C for 105 minutes in a 50 µl reaction containing 1 µg of fragmented viral genome RNA, 0.5 µg calf thymus random primer fragments (91), 50 mM Tris-hydrochloride (pH8.3), 40 mM KCl, 8 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, and 0.01 mM (^{32}P)dCTP (specific activity 800 Ci/mmole, New England Nuclear), and 40 units of reverse transcriptase (Life Sciences, Inc.). After synthesis the reaction was adjusted to 0.6N NaOH and incubated at 37°C for 30 minutes. The reaction was then neutralized by adding HCl to 0.6N and Tris-hydrochloride (pH8.1) to 200 mM. This mixture was passed over a five ml column of Sephadex G-50 and fractions containing incorporated radioactivity were pooled, adjusted to 0.3M potassium acetate, and precipitated with 2.5 volumes of ethanol. This probe typically had a specific activity of 2 x 10 7 cpm/µg.

COLONY BLOTS

Transformants were screened for viral sequences by the procedure of Taub and Thompson (90) in which fresh colonies were

replicated onto sterile Whatman 541 filter paper and washed successively in NaOH, lysozyme, proteinase K, and phenol:chloroform:isoamyl alcohol (25:24:1). Filters were hybridized with 2-5 x 10^6 cpm of ^{32}P-labelled cDNA probe (see above) as described (90).

ISOLATION OF PLASMID DNA

Small scale plasmid preparations (1-2 µg) were made by a modification of the alkaline lysis procedure (12). Fresh colonies of each transformant were scraped from plates with a toothpick and dispersed vigorously in 40 µl of a lysing buffer composed of 0.5% sodiom dodecyl sulfate, 50 mM NaOH, 5 mM EDTA, and 2% Ficoll. These mixtures were incubated for 30 minutes at 68°C. Sucrose and bromphenol blue were added to 5% and 0.002% respectively, and the samples were loaded onto a horizontal 0.7% agarose gel in Tris-acetate buffer (0.72M Tris-hydrochloride, 0.1 mM sodium acetate, 20 mM EDTA, pH 7.9). After electrophoresis for 12 to 16 hours at 25 volts the gel was stained with ethidium bromide and examined with an ultraviolet transilluminator (Fotodyne, Inc.).

Large scale isolations of plasmid DNA were carried out by the boiling method of Holmes and Quigley (40). Transformants were grown at 37°C in 500 ml of LB broth (57) containing 150 µg/ml ampicillin. When the cultures reached an OD_{540} of 1.0, chloramphenicol was added to 150 µg/ml and incubation at 37°C was

continued for 20-24 hours to amplify the plasmids. Plasmid DNA was isolated from lysed cells as described (40) and banded on CsCl-ethidium bromide gradients for 15-18 hours at 300,000 x g (68,000 rpm) using a VTi 80 rotor in a Beckman L8-70 ultracentrifuge. Plasmid bands were collected by side-puncture and extracted several times with CsCl-saturated butanol to remove the ethidium bromide. Two volumes of Tris-hydrochloride (pH8.0) with 1 mM EDTA were added to the aqueous phase, which was then adjusted to 0.3M potassium acetate and precipitated with 2.5 volumes of ethanol.

DETERMINATION OF CLONED VIRAL SEQUENCE SIZE

The restriction endonuclease Pst 1 (Bethesda Research Laboratories, Inc.) was used to cleave 300 ng of each purified plasmid under the conditions specified by the enzyme manufacturer. The released cloned insert DNA was separated from the plasmid vector DNA by electrophoresis on a vertical 7.5% acrylamide gel with a 3.75% acrylamide stacking gel. The Laemmli gel system (49) was used with the exception that sodium dodecyl sulfate was omitted from all buffers. Electrophoresis was carried out at 30 mAmps through the stacking gel and 50 mAmps through the lower gel. Following electrophoresis the gel was stained with ethidium bromide and photographed using an ultraviolet transilluminator (Fotodyne, Inc.) and Polaroid type 47 film. The sizes of the

cloned inserts were determined by comparison with Hinf I and Hae III pBR322 restriction fragment size standards.

PREPARATION OF 32P-NICK TRANSLATED PROBES

Nick translation of cloned plasmids was carried out as described (57,78) in 10 µl reactions containing 200 ng plasmid DNA, 50 mM Tris-hydrochloride (pH7.2), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 50 µg/ml bovine serum albumin, 20 uM dATP, 20 uM dGTP, 20 uM dTTP, 2 uM (^{32}P)dCTP (specific activity 1 Ci/mmol, New England Nuclear), 2 ng/ml DNase I (DPFF, Worthington Biochemical Corp.), and 1-1.5 units of DNA polymerase I (Bethesda Research Laboratories, Inc.). After incubation for one hour at 16 °C reactions were stopped by the addition of 20 mM EDTA, diluted to 50 µl in 10 mM Tris-hydrochloride (pH 8.0) with 1 mM EDTA, and passed over a 5 ml Sephadex G-50 column. Fractions with radioactivity incorporated into DNA were pooled, adjusted to 0.3M potassium acetate, and precipitated with 2.5 volumes of ethanol. Nick translated probes had a specific activity of 2-5 x 10^7 cpm per µg.

DNA BLOT HYBRIDIZATIONS

Cloned plasmids were cleaved with the restriction endonuclease Pst I (Bethesda Research Laboratories, Inc.) under

the conditions specified by the enzyme manufacturer and the cloned inserts were separated from vector DNA by electrophoresis on 0.7% agarose gels in Tris-acetate buffer (see above). The DNA was alkaline denatured, transferred to a nitrocellulose membrane by the Southern blot method (89), and baked onto the filter for two hours at 80 °C in vacuo. Hybridization of the blots was as described (57,89) with the exception that both pre-hybridization and hybridization were carried out in 50% formamide at 42°C. Each hybridization included 2-10 x 10^6 cpm of ^{32}P-labelled cDNA probe or nick translated probe. Following hybridization, blots were washed as described (57) and exposed to Kodak X-Omat AR X-ray film with a DuPont Lightening Plus intensifying screen at -70 °C.

mRNA BLOT HYBRIDIZATION

Hybridization of ^{32}P-labelled nick translated probes to blots of viral mRNA were carried out following the procedures of P. Thomas (95). Polyadenylated RNA from IHNV-infected cells was resolved into five bands by glyoxal gel electrophoresis and transferred to a nitrocellulose membrane as described (95). The blot was baked for two hours in vacuo at 80°C and cut into strips, each containing one gel lane with 2 µg of RNA. The strips were boiled for five minutes in 20 mM Tris-hydrochloride (pH 8.0) to remove the glyoxal adducts, and hybridized individually as described (95) with 5-10 x 10^6 cpm of ^{32}P-nick translated probe.

Following hybridization blots were washed as described (95) and exposed to X-ray film as above.

RESULTS

A. CHARACTERIZATION OF mRNA FROM IHNV-INFECTED CELLS.

mRNA MOLECULAR WEIGHTS

Isolation of the viral mRNA species was carried out by infecting CHSE-214 cells with IHNV in the presence of actinomycin D and tritiated uridine. Polyadenylated RNA from IHNV-infected cells was resolved by both glyoxal and methylmercury gel electrophoresis into six species of RNA (Figure 1). The largest band of RNA co-migrated with purified IHNV genome RNA and was presumed to be genome length positive- or negative-sense molecules involved in the replication process. When purified IHNV genome RNA was fractionated with oligo-dT cellulose, less than 2% of the RNA bound to the column. Therefore, the presence of this genome-length RNA in the polyadenylated RNA preparations does not indicate a true poly-A tail, but perhaps some internal short poly-A sequences, or carry-over of genome template molecules hybridized to polyadenylated mRNA molecules during oligo-dT cellulose chromatography.

The five sub-genomic polyadenylated RNA bands were designated IHNV mRNA 1 through 5, from largest to smallest, as indicated in Figure 1. In all preparations mRNA bands 2, 3, 4, and 5 were relatively abundant, while mRNA 1 was present in minor, more variable quantities. The marker RNA species (VSV genome,

Figure 1. Denaturing gel analyses of IHNV mRNA from infected cells.

Polyadenylated RNA from IHNV-infected cells was analyzed by electrophoresis under denaturing conditions. A) Glyoxal gel electrophoresis, (a) ribosomal RNA markers, 18S and 28S, (b) IHNV genomic RNA extracted from purified virions, (c) VSV genomic RNA marker, (d) polyadenylated RNA from IHNV-infected cells. B) Methylmercury gel electrophoresis, (a) VSV genomic RNA marker, (b) IHNV genomic RNA from purified virions, (c) polyadenylated RNA from IHNV-infected cells, (d) ribosomal RNA markers, 18S and 28S. The number designations for the major species of IHNV-infected cell RNA are shown for each gel.

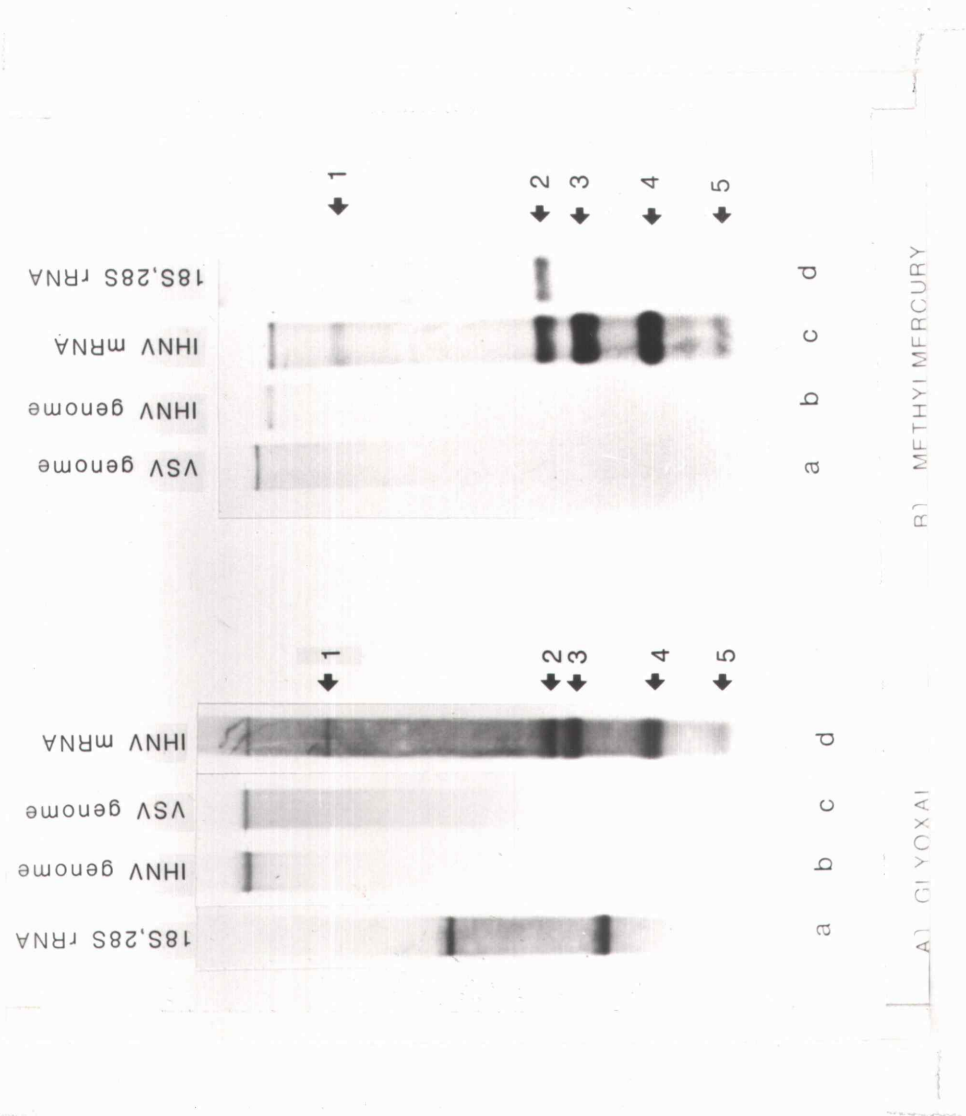
A) GLYOXAL
B) METHYLMERCURY
a b c d
1
2
3
4
5
18S,28S rRNA
IHNV mRNA
IHNV genome
VSV genome

Figure 1.

3.82 x 10^6; 28S ribosomal RNA, 1.7 x 10^6; 18S ribosomal RNA, 6.5 x 10^5) (66,76) established a reproducibly linear plot of log molecular weight vs mobility for each glyoxal gel (Figure 2). This was used to determine the following values for the molecular weights of the IHNV mRNA species: mRNA 1, 2.26±0.035 x 10^6; mRNA 2, 5.63±0.08 x 10^5; mRNA 3, 4.84±0.07 x 10^5; mRNA 4, 3.0±0.05 x 10^5; mRNA 5, 1.95±0.03 x 10^5. These values were calculated as the average of 8-10 separate determinations including four different preparations of polyadenylated RNA.

Although the methylmercury gels provided excellent resolution of the RNA species, in our hands the log molecular weight vs mobility plots were not linear over the size range of the marker RNA species. Therefore these gels were not used to determine the molecular weights of unknown RNA species.

mRNA MOLAR RATIOS

The molar ratios of each mRNA species produced intracellularly during IHNV infection were determined by densitometer analysis of the autoradiograms of several glyoxal gel profiles (Figure 3). The areas under each peak and the molecular weights of the mRNA species were used to compute the following molar ratios normalized to 1 mole of mRNA 3: mRNA 1, 0.02±0.01; mRNA 2, 0.49±0.03; mRNA 3, 1.00; mRNA 4, 2.52±0.40; mRNA 5, 0.41±0.14.

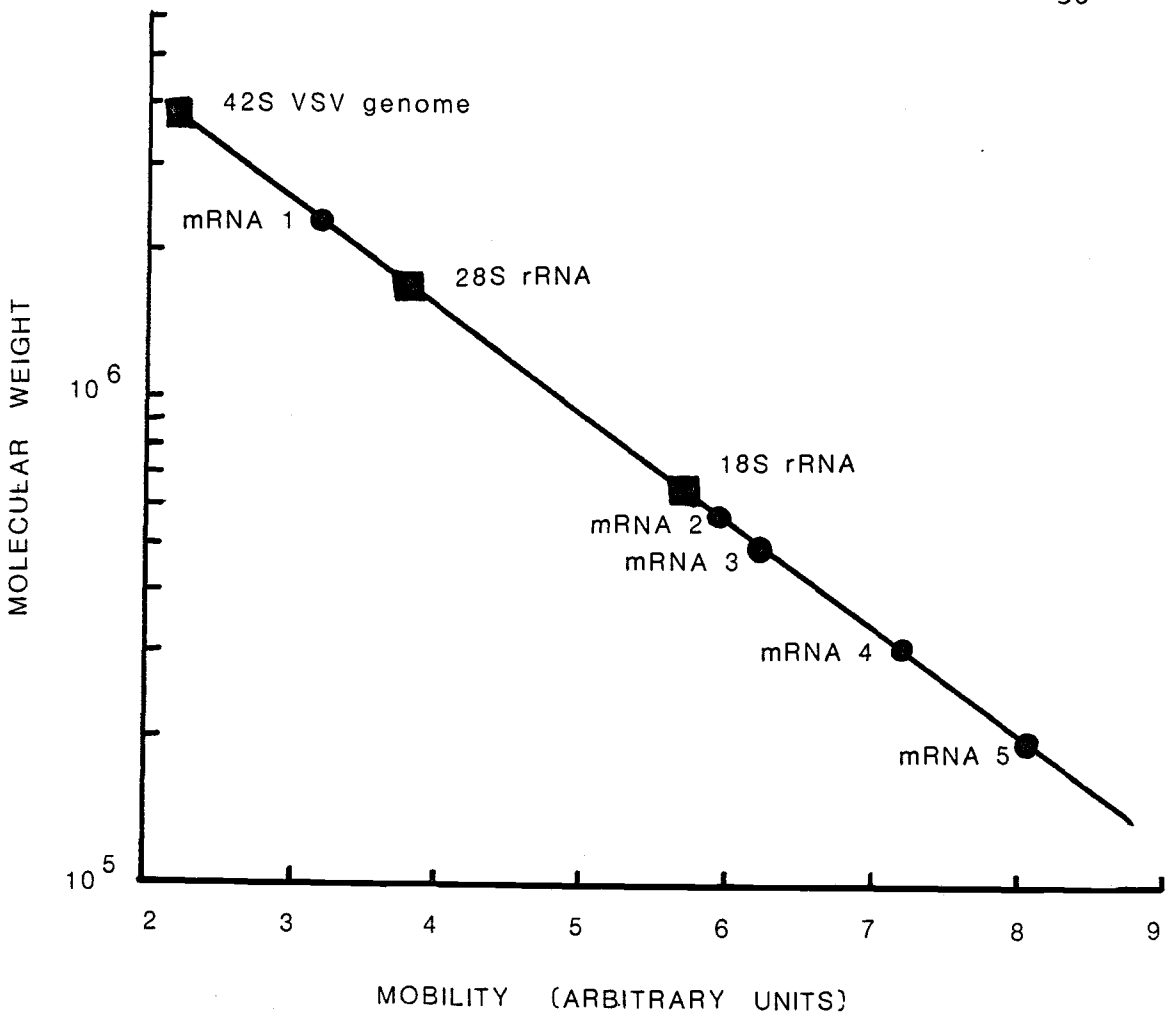

Figure 2. Log molecular weight vs. mobility plot for RNA resolved by glyoxal gel electrophoresis. Marker RNA species (■) were used to define the linear relationship which was then used to determine molecular weights for unknown RNA species (●).

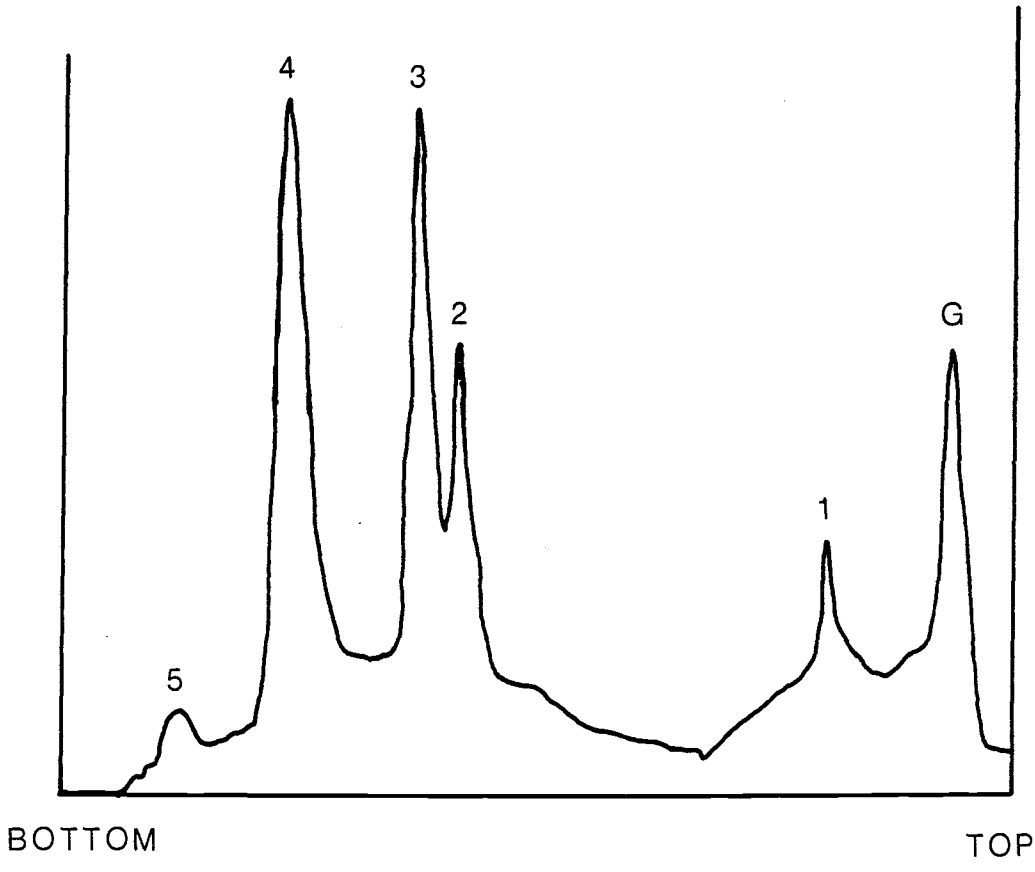

Figure 3. Densitometer scan of the glyoxal gel profile of IHNV mRNA from infected cells. Gel profiles such as shown in Figure 1A were analyzed with a soft laser scanning densitometer and results were printed by a personal computer using an electrophoresis integration program. The mRNA band number corresponding to each peak is indicated at the top, and the letter 'G' indicates IHNV genome length RNA.

IN VITRO TRANSLATION OF mRNA

In order to confirm the viral mRNA character of the polyadenylated RNA preparations, they were tested for the ability to direct viral protein synthesis *in vitro*. Figure 4 shows a polyacrylamide gel of the proteins synthesized in an mRNA-dependent cell-free translation reaction primed with 1 ug of the polyadenylated RNA from IHNV-infected cells. Proteins were synthesized which co-migrated with IHNV standard proteins N, M1, and M2. A fourth protein was synthesized which migrated faster than the G protein and had a molecular weight of 56,000. This corresponds with the molecular weight of the unglycosylated G protein, G_0 (the molecular weight of G_0 is 55,000±3000, Hsu, personal communication). The viral L protein was never observed in polyacrylamide gels of the *in vitro* translation products. A fifth protein with a molecular weight of 12,000 was also synthesized *in vitro*. Although this protein does not correspond to any of the proteins in purified virions, there is a protein of this size induced in relatively high quantities in IHNV-infected cells (Figure 4). We have designated this the NV protein due to its non-virion nature.

Figure 4. Electrophoretic analysis of proteins translated *in vitro* fron IHNV mRNA produced in infected cells.

Polyadenylated RNA from IHNV-infected cells was used to prime an *in vitro* translation system, and the ^{35}S-labelled proteins produced were analyzed by SDS-polyacrylamide gel electrophoresis along with IHNV protein standards. (a) proteins from uninfected CHSE-214 cells, (b) proteins from IHNV-infected cells, (c) proteins from purified IHNV virions, (d) proteins translated *in vitro* from IHNV mRNA from infected cells. The nomenclature of the IHNV proteins is indicated on the right.

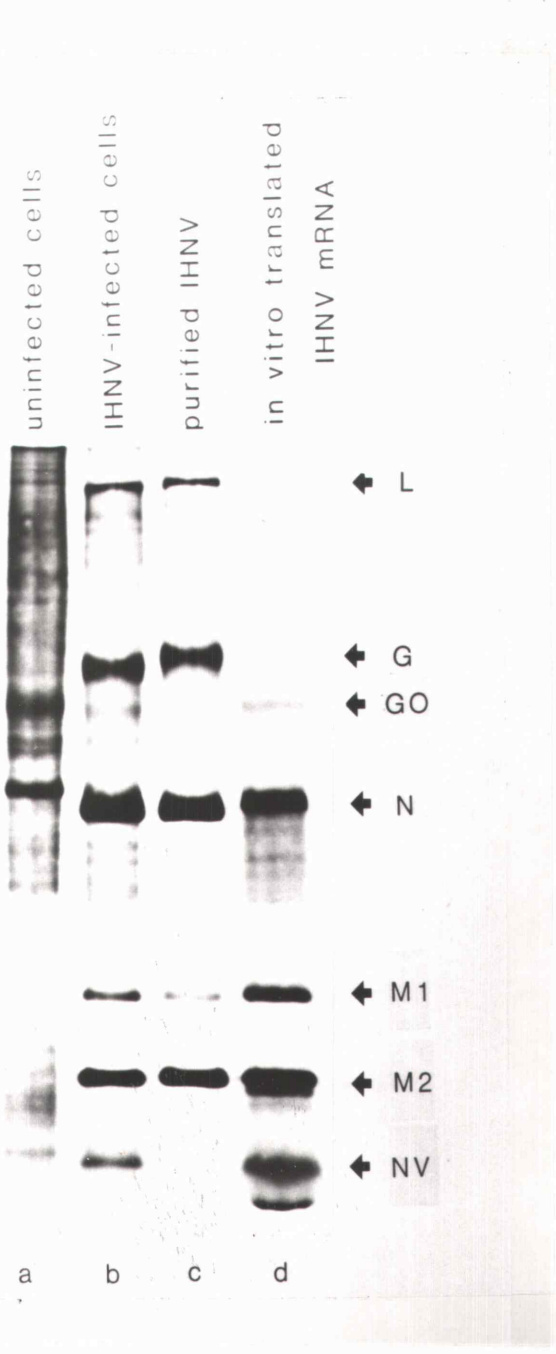
uninfected cells
IHNV-infected cells
purified IHNV
in vitro translated IHNV mRNA
L
G
GO
N
M1
M2
NV
a
b
c
d

Figure 4.

mRNA CODING ASSIGNMENTS

It was necessary to determine which viral mRNA encoded each of the viral proteins. This was done by hybrid selection of individual mRNA species from the mixture using cloned plasmids carrying partial cDNA sequences from each viral mRNA. The construction and characterization of these clones is described in section C of these results. In vitro translation of the mRNA selected by hybridization with each plasmid then identified the protein encoded by that mRNA.

In this way the protein coding assignments were made for all the mRNA species which could be translated in vitro. Examples for each protein are shown in Figure 5, and a summary of the hybrid selections carried out is presented in Table 1. The plasmid which carried cDNA to mRNA band 2 hybrid selected mRNA which translated into the G_0 protein. Two plasmids which carried cDNA to band 3 both selected the mRNA which coded for the N protein. Of four plasmids which carried cDNA to mRNA band 4, three selected an mRNA which coded for the M2 protein, and one selected the mRNA for the M1 protein. Three plasmids which carried cDNA to mRNA band 5 all hybrid selected mRNA which translated into the small molecular weight NV protein.

Thus we conclude that mRNA 2 encodes the G protein, mRNA 3 encodes N, mRNA 4 contains two co-migrating mRNA species which encode M1 and M2, and mRNA 5 encodes the NV protein.

Figure 5. Electrophoretic analysis of proteins translated *in vitro* from individual IHNV mRNA species after hybrid selection.

Protein was translated from individual mRNA species selected from polyadenylated RNA from IHNV infected cells by hybridization with the following cDNA plasmids; (a) pM1163, (b) pM219, (c) pN144, (d) pG480, (e) pNV58. The mRNA band selected by each plasmid is indicated at the top of each lane. Lane (f) is a marker lane of IHNV proteins translated *in vitro* from the total mRNA preparation.

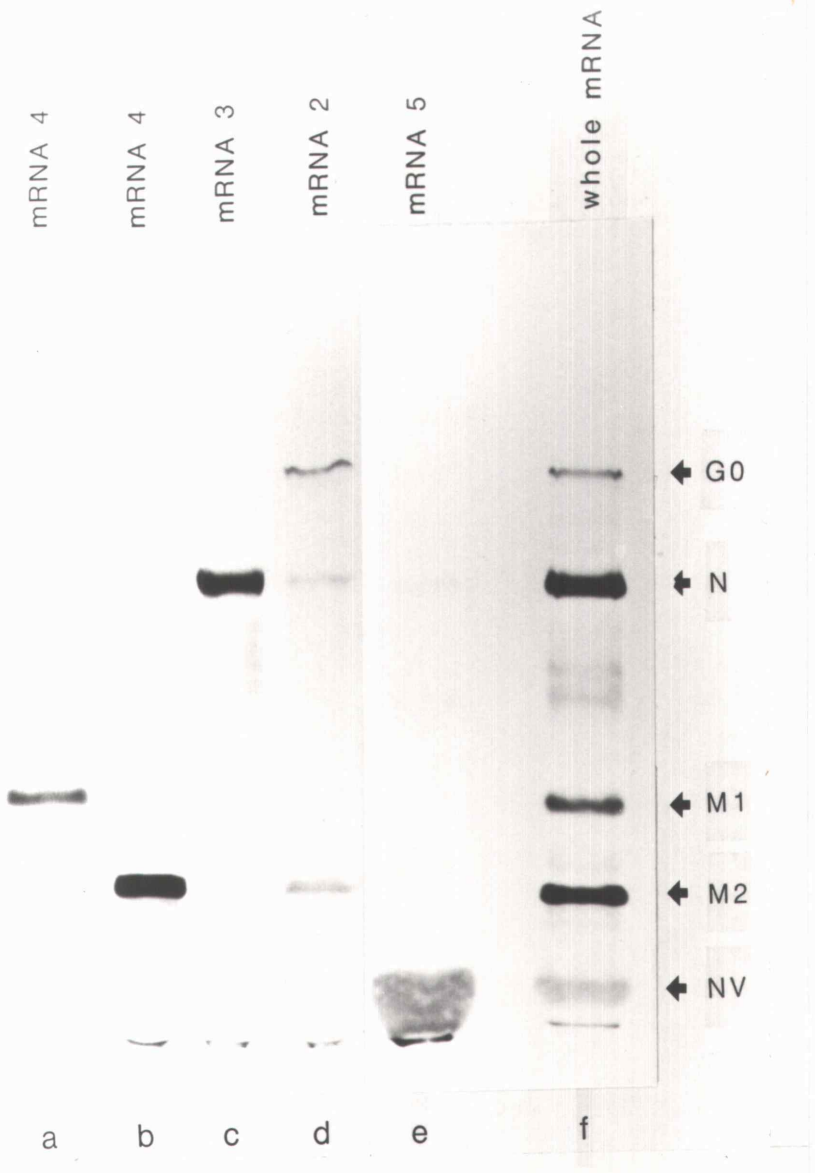

Figure 5.

Table 1. Summary of hybrid selection results.

plasmid	mRNA band* selected	protein encoded
pG480	2	G_0
pN421	3	N
pN419	3	N
pM1163	4	M1
pM219	4	M2
pM2132	4	M2
pM2173	4	M2
pNV58	5	NV
pNV137	5	NV
pNV711	5	NV

* Determined by mRNA blot hybridizations, see figure 13 and table 2.

B. IHNV TRANSCRIPTION *IN VITRO*

OPTIMAL CONDITIONS

The results of several studies defining optimal conditions for RNA transcription *in vitro* by the RNA polymerase of IHN virus are summarized in Figure 6. Sets of reactions were carried out in which only one reaction component was varied and the amount of tritiated UTP incorporated into TCA precipitable material in one hour was measured. In this way the concentration of each reaction component which stimulated the maximum amount of RNA synthesis was determined.

An investigation of the buffer conditions showed that HEPES buffer stimulated three-fold more RNA synthesis than Tris buffer (Figure 6a). The optimum concentration of HEPES buffer was 400 mM, while the optimum for Tris buffer was 50-100 mM. The pH of the HEPES buffer which allowed for maximum RNA synthesis was 8.0 (Figure 6b).

Detergent lysis of the virus particles was required for transcription, and maximum activity occurred in the presence of 0.5% Triton X-100 (Figure 6c). Three non-ionic detergents (Triton X-100, Triton N-101, and NP40) were tested and found to stimulate comparable levels of polymerase activity (data not shown).

Figure 6. Determination of the optimal reaction conditions for *in vitro* activity of the IHNV polymerase.

Sets of *in vitro* transcription reactions were carried out in which one component of the reaction was varied in order to determine the conditions which stimulated maximum RNA synthesis. The reaction components tested were (a) buffer, (b) pH, (c) lysing conditions, (d) reducing agent, (e) monovalent cation, the upper line is a set of reactions in 400 mM HEPES and the lower line is reactions in 100 mM HEPES, (f) divalent cation, (g) temperature, (h) S-adenosyl methionine, (note log scale).

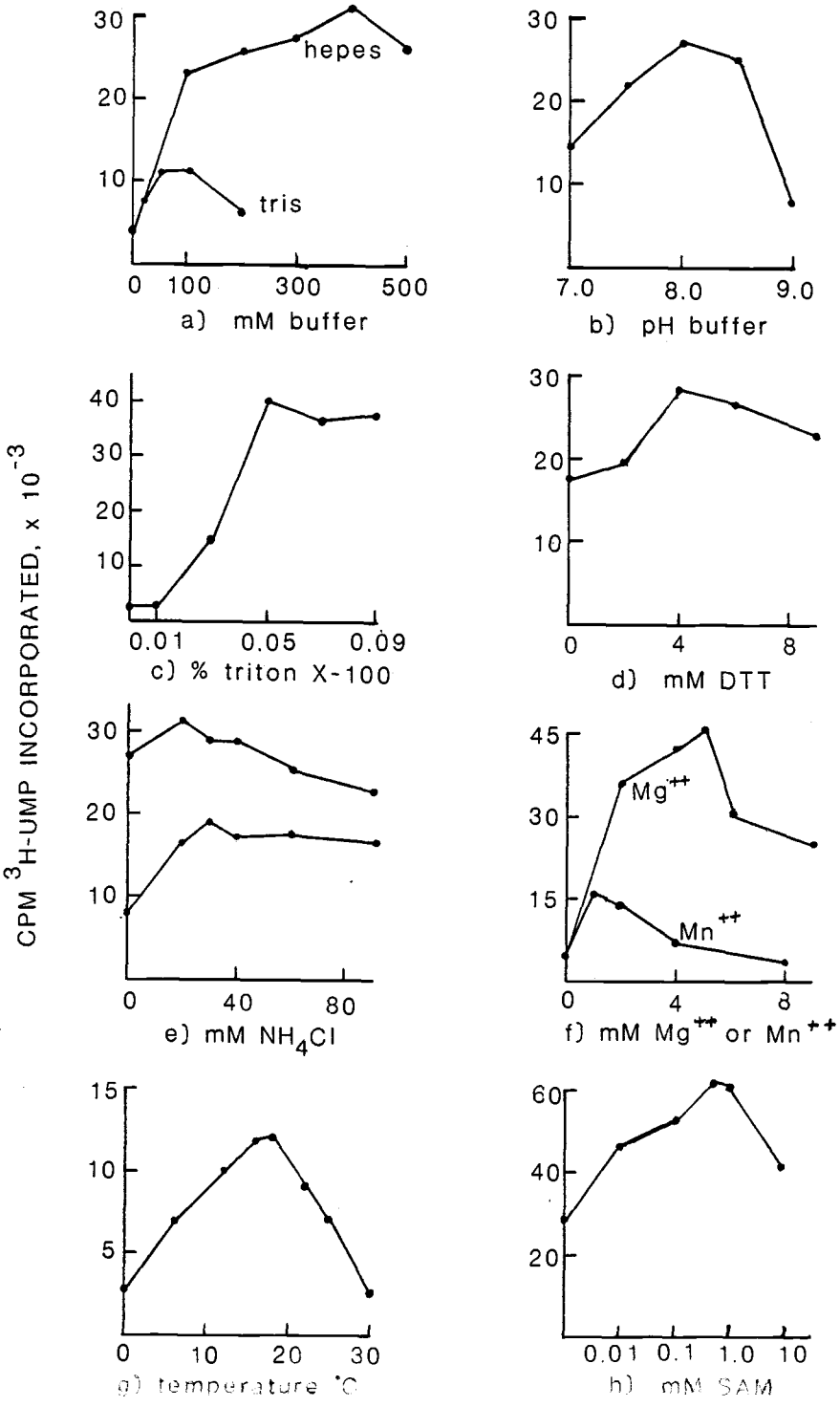
CPM ^{3}H-UMP INCORPORATED, x 10^{-3}
hepes
tris
a) mM buffer
b) pH buffer
c) % triton X-100
d) mM DTT
e) mM NH_4Cl
Mg^{++}
Mn^{++}
f) mM Mg^{++} or Mn^{++}
g) temperature °C
h) mM SAM

Figure 6.

Tests of the requirement for a reducing agent showed that the optimum concentration of dithiothreitol was 4 mM (figure 6d), while reactions which included 2-mercaptoethanol showed significantly less activity (data not shown).

The results of experiments defining the cation requirements for the IHNV polymerase are shown in Figure 6e and 6f. The salts of three monovalent cations, KCl, NaCl, and NH_4Cl, were tested at concentrations ranging from 0-100 mM. While all three gave comparable patterns of activity, the presence of 30 mM NH_4Cl in the reaction mixture consistently resulted in the highest enzyme activity. In the presence of 400 mM HEPES the polymerase requirement for a monovalent cation was not critical, and NH_4Cl concentrations greater than 60 mM were actually inhibitory. However, if the HEPES concentration was lowered to 100 mM the addition of NH_4Cl did stimulate enzyme activity significantly (Figure 6e). Thus, the monovalent cation requirement can be at least partially fulfilled by high concentrations of HEPES buffer.

The viral polymerase did show a definite requirement for a divalent cation, with a sharp optimum of activity at 5 mM $MgCl_2$ (Figure 6f). Manganese was not able to replace magnesium in HEPES buffered reactions. At best, RNA synthesis in the presence of manganese was 30% of the activity with magnesium.

Temperature studies revealed a fairly sharp optimum of activity at 16-18°C (Figure 6g).

The addition of the methylating agent, S-adenosyl methionine

(SAM), to the transcription reactions resulted in a doubling of the amount of RNA synthesis (Figure 6h). The minimum concentration of SAM capable of stimulating the viral polymerase was approximately 10 uM, and maximum activity was observed in the presence of 0.2-0.5 mM SAM.

KINETICS

Kinetics of the RNA polymerase activity under the optimal conditions defined above were examined by determining the amount of tritiated UTP incorporated into RNA after various lengths of incubation (figure 7). The rate of RNA synthesis was linear for the first three hours of incubation, after which the amount of RNA remained essentially constant.

CHARACTERIZATION OF REACTION PRODUCTS

In order to assess the polyadenylation of RNA transcribed in vitro, the tritiated products of in vitro reactions were chromatographed on an oligo dT-cellulose column. For each RNA preparation, 10-30% of the radioactivity was retained by the column, indicating a significant proportion of molecules with polyadenylate tracts.

RNA synthesized in vitro, under the optimal conditions defined above, was denatured by glyoxal treatment and examined by agarose

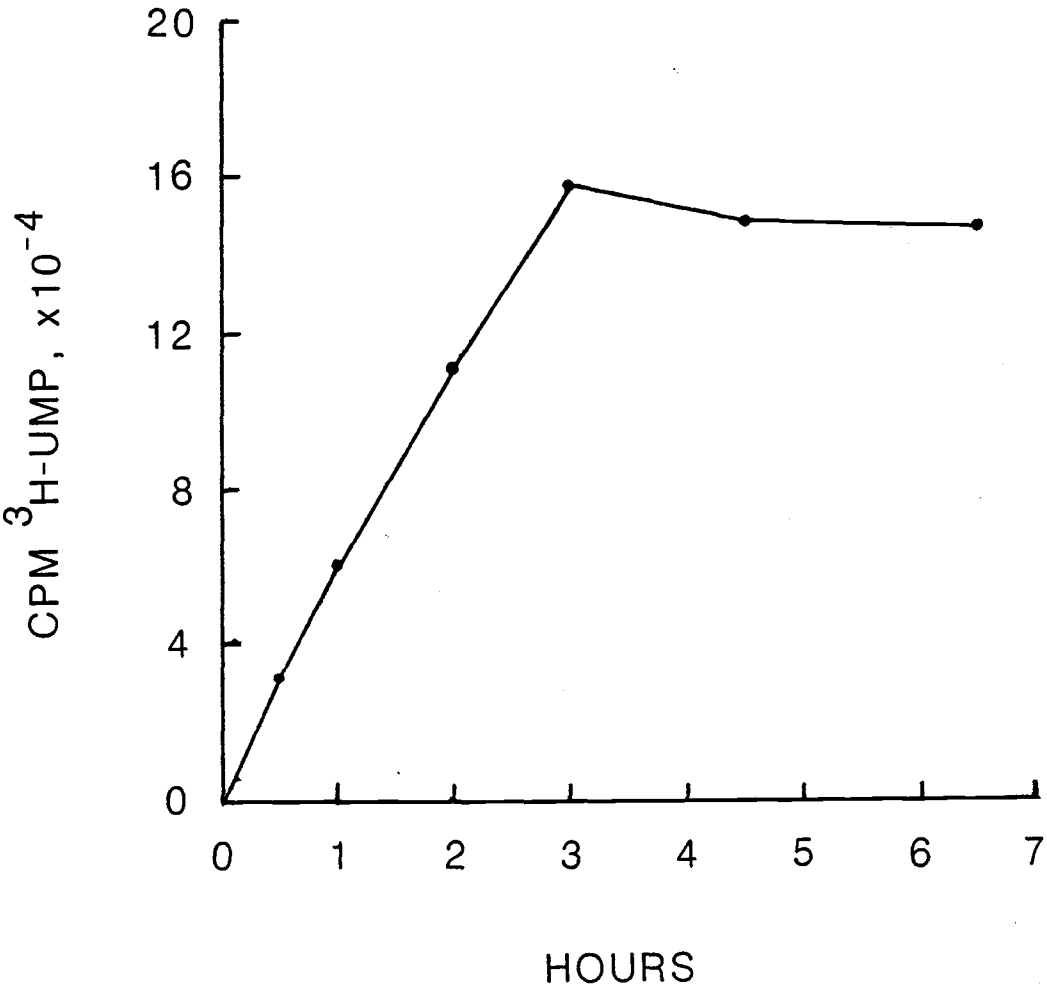

Figure 7. Kinetics of IHNV transcription *in vitro*. IHNV *in vitro* transcription reactions using the optimal conditions described in the text (Figure 6, Table 8) were terminated at different times and the amount of ^{3}H-UMP incorporated into TCA precipitable material was determined.

Figure 8. Denaturing gel analysis of *in vitro* transcripts.

Products of *in vitro* transcription reactions were recovered and resolved by glyoxal gel electrophoresis. (a) ribosomal RNA markers, 18S and 28S (the top band is cellular DNA), (b) polyadenylated RNA from IHNV-infected cells, (c) whole products of *in vitro* transcription in HEPES buffer with SAM, (d) polyadenylated RNA selected from *in vitro* transcription products, (e) non-polyadenylated RNA from *in vitro* transcription products. Lanes (g) and (h) are a long exposure of lanes (c) and (d), included to show mRNA band 2. Arrows indicate *in vitro* transcription products which co-migrate with IHNV mRNA bands 2, 3, 4, and 5.

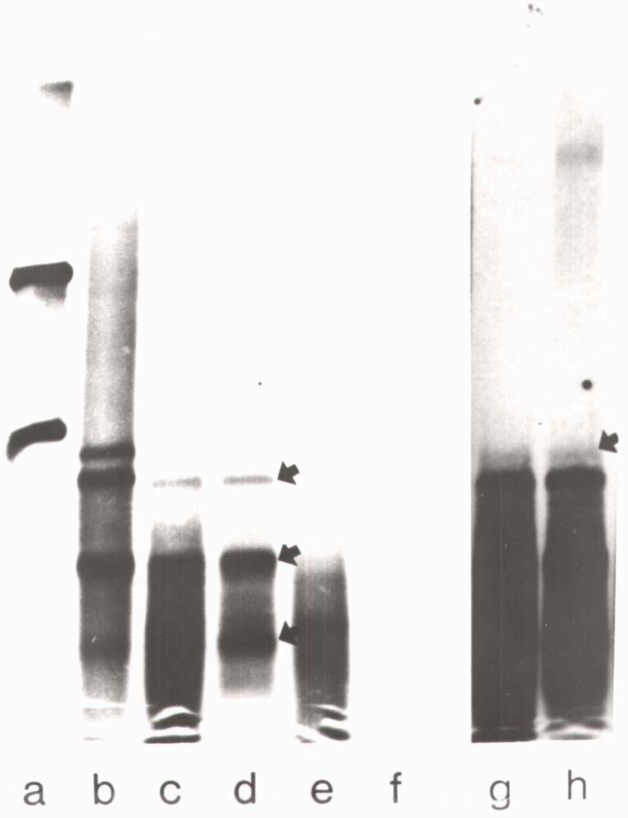

Figure 8.

gel electrophoresis (Figure 8). The reaction products ranged in size from approximately 4.0×10^4 to 5.6×10^5, and contained three major discreet species which co-migrated with IHNV mRNA species 3, 4, and 5 from IHNV-infected salmon cells. There was also a minor species which co-migrated with IHNV mRNA 2.

Polyadenylated RNA selected from the *in vitro* reaction products was comprised mainly of these four distinct species (Figure 8). The visability of the bands co-migrating with mRNA 4 and mRNA 5 was enhanced by poly(A) selection due to the removal of most of the heterogeneous background material including essentially all molecules of molecular weight less than 1.4×10^5. This poly(A) minus material is shown in lane (e) of Figure 8, and is presumed to be incomplete or degraded transcripts.

In order to determine whether the poly(A) RNA transcribed *in vitro* was functional mRNA, it was used to prime a rabbit reticulocyte *in vitro* translation system. The ^{35}S-methionine labelled proteins synthesized *in vitro* are shown in Figure 9. Although there is a background of heterogeneous material, a protein co-migrating with the viral N protein is clearly visible and there are faint bands which co-migrate with the M1 and M2. Thus, the *in vitro* transcription system is capable of producing functional mRNA molecules. The lack of visible G and NV proteins is probably due to the small quantity of mRNA template translated *in vitro*, and the relatively dark background may be due to the tritium labelled template RNA.

Figure 9. Electrophoretic analysis of proteins translated *in vitro* from RNA transcribed *in vitro*.

(a) Negative control showing proteins translated *in vitro* with no addition of exogenous RNA, (b) proteins translated *in vitro* from approximately 0.5 μg of *in vitro* transcription reaction products, (c) proteins translated *in vitro* from polyadenylated RNA from IHNV-infected cells, (d) IHNV proteins from infected cells, (e) IHNV proteins from purified virions.

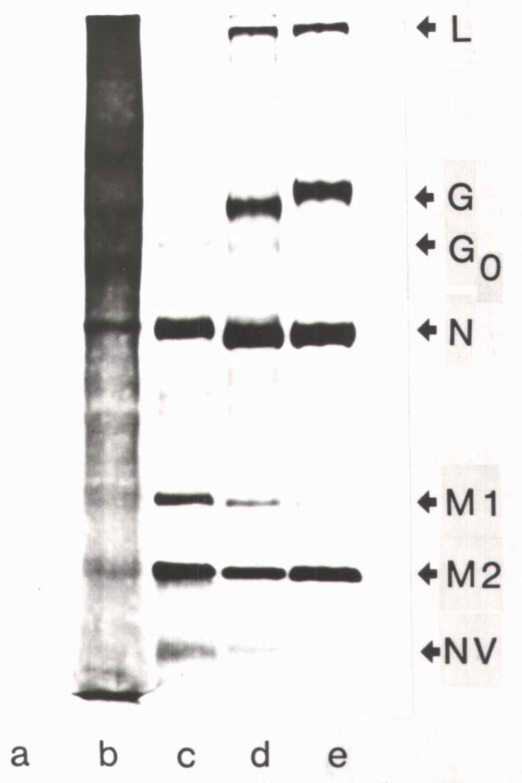

Figure 9.

C. CLONING OF VIRAL mRNA SPECIES

PREPARATION OF CLONED PLASMIDS CARRYING VIRAL cDNA

The mRNA species of IHN virus were isolated from IHNV-infected salmon cells and used as a template for the preparation of cDNA clones carrying IHN viral mRNA sequences. Briefly, polyadenylated RNA from IHNV-infected cells was copied into cDNA using reverse transcriptase and an oligo-$dT_{(12-18)}$ primer. Analysis of the products of the first strand cDNA reaction by alkaline gel electrophoresis showed four distinct species corresponding in size to full length copies of the mRNA bands 2, 3, 4, and 5 (Figure 10A). This single-stranded cDNA was copied into double-stranded molecules (Figure 10B), poly-C tailed with terminal transferase, and annealed into the Pst 1 site of the plasmid vector pUC8. Two E. coli K-12 host strains, JM103 and SC181 (C600) were transformed and approximately 800 ampicillin resistant transformants were isolated from each.

Transformants were screened by colony blot hybridizations using ^{32}P-cDNA to purified viral genome RNA as the probe (Figure 11). Colony blotted DNA of approximately 90% of the transformants of both host strains hybridized to the probe, indicating the presence of viral genetic material. Negative controls transformed with pUC8 showed no hybridization with the probe.

Small scale plasmid preparations (1-2 µg) showed that approximately 10% of the transformants had relatively large

Figure 10. Electrophoretic analysis of cDNA prepared by reverse transcription of mRNA from IHNV-infected cells.

A) Single-stranded cDNA product of the first reverse transcriptase reaction resolved by alkaline gel electrophoresis (62). Sizes of the major reaction products are indicated on the left in kilobases, and the sizes of marker DNA bands are on the right. (a) single-stranded cDNA, (b) lambda marker DNA cut with Hind III.

B) Agarose gel electrophoresis (non-denaturing) of the reaction products at each stage of the preparation of double stranded cDNA. (a) Lambda marker DNA cut with Hind III, (b) single-stranded cDNA after the first reverse transcriptase reaction, (c) single-stranded cDNA after dC-tailing (this lane was loaded with insufficient counts for comparison with the other samples) (d) double-stranded cDNA after the second reverse transcriptase reaction, (e) double-stranded cDNA after Klenow reaction to fill in nicks or gaps.

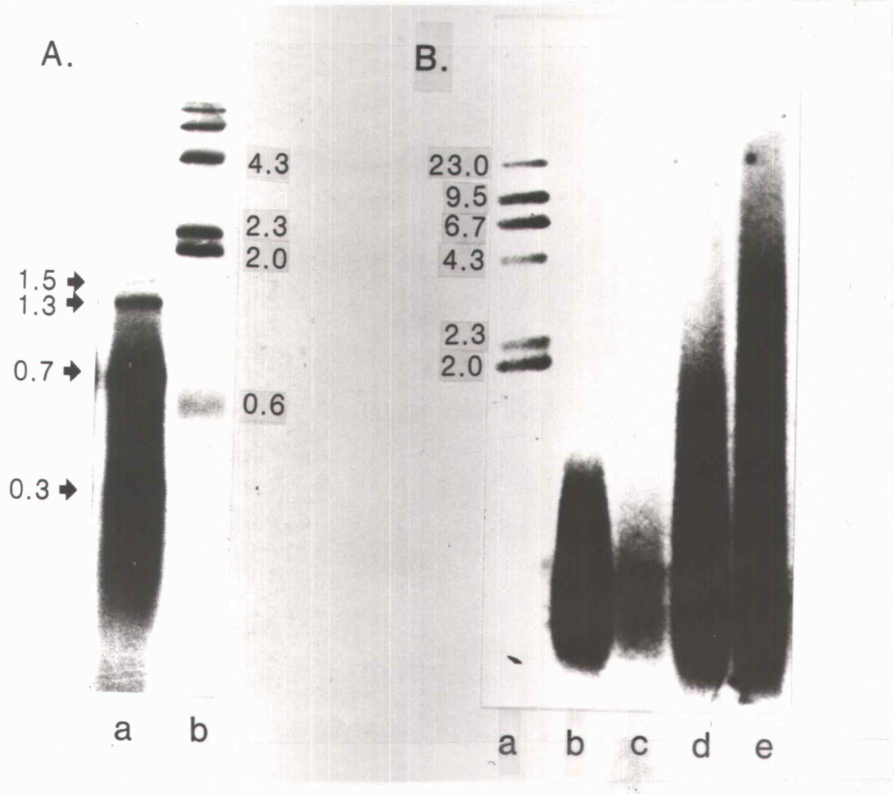

Figure 10.

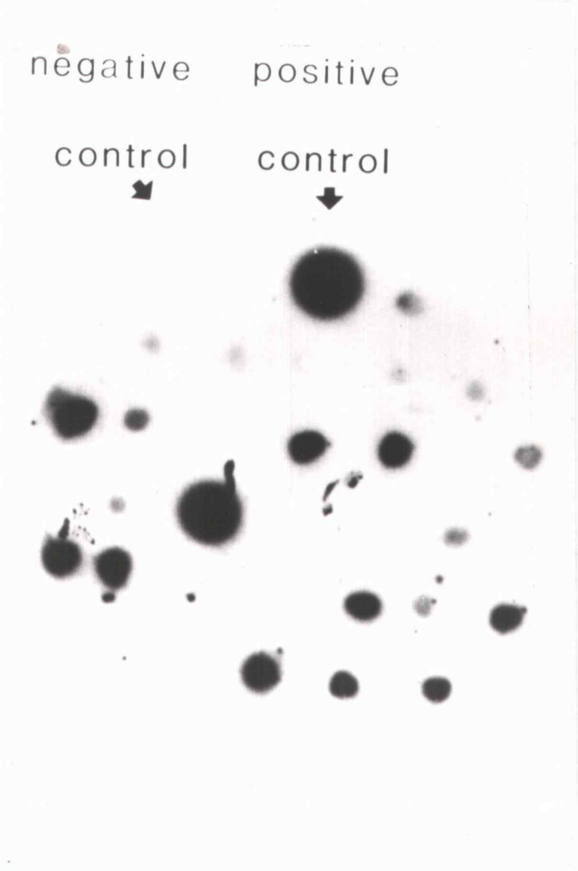

Figure 11. Colony blot analysis of transformants for the presence of viral-specific sequences.

Colonies of transformants transferred to filter paper were lysed, and DNA prints were prepared by the method of Taub and Thompson (90). Filters were hybridized with a ^{32}P-labelled probe of cDNA to IHNV genomic RNA, and autoradiographed. The positive control was 0.5 µg of viral genomic RNA, and the negative control was a transformant which carried pUC8 with no insert.

plasmids. The cDNA inserts of plasmids purified from 21 of these transformants were released by Pst 1 cleavage and determined by electrophoresis to range in size from 155 to 640 base pairs (Table 2). Southern blot analyses of Pst 1 cleaved plasmids showed that the insert DNA hybridized strongly with the viral genome cDNA probe, re-confirming that the cloned sequences were virus-specific (Figure 12).

mRNA BLOT ANALYSES

Characterization of the cloned plasmids required that we identify the specific viral mRNA species which was complementary to each cDNA insert. This was carried out by probing nitrocellulose blots of the mRNA electrophoretic pattern with ^{32}P-labelled probes made by nick translation of the 21 purified plasmids . Examples of plasmids which hybridized specifically to each of the five mRNA bands are shown in Figure 13.

Identification of the single mRNA band which hybridized with each plasmid DNA probe was made by comparison of the blot autoradiogram with a marker lane of all five mRNA bands. However, the proximity of mRNA bands 2 and 3 made it difficult to distinguish hybridization to mRNA band 2 with certainty. Therefore, a double hybridization was carried out with two probes together, pG480 and pN144 (Figure 13g). The presence of two bands of hybridization confirmed that cDNA clones with sequences from both mRNA bands 2 and 3 had been isolated. A summary of these

Table 2. Viral insert size and mRNA specificity for cloned plasmids.

Cloned plasmid	insert size, bp	mRNA specificity *	viral gene **
pL232	640	1	L
pL262	640	1	L
pG480	440	2	G
pN512	370	3	N
pN933	320	3	N
pN421	350	3	N
pN154	405	3	N
pN156	400	3	N
pN419	460	3	N
pN125	450	3	N
pN144	450	3	N
pM1163	425	4	M1
pM1420	155	4	M1
pM219	505	4	M2
pM2173	265	4	M2
pM211	510	4	M2
pM2112	515	4	M2
pM2132	510	4	M2
pNV58	420	5	NV
pNV137	395	5	NV
pNV711	220	5	NV

*Determined by mRNA blot hybridizations, figure 13.

**Determined by hybrid selection and in vitro translation, table 1, figure 5.

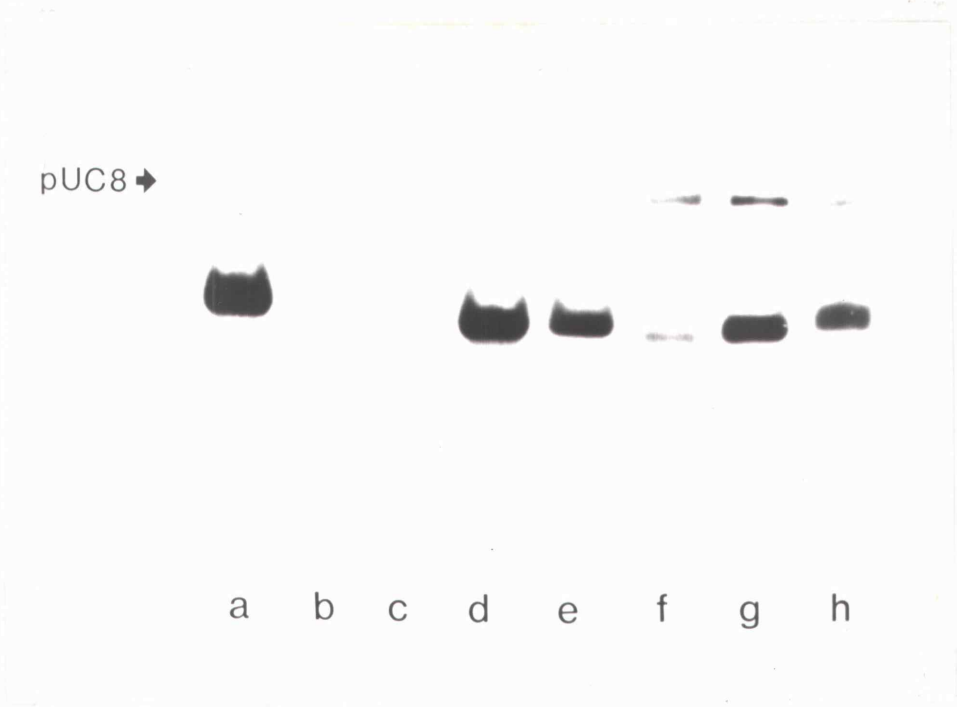

Figure 12. DNA blot hybridization of cloned plasmid inserts with a viral-specific probe.

Cloned plasmids were cleaved with Pst I, resolved by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with a ^{32}P-labelled probe of cDNA to IHNV genomic RNA. An autoradiogram of the hybridized blot is shown, and the distance of migration of the pUC8 DNA is marked. Lane (a) pM219, (b) p15, (c) pN425, (d) pN421, (e) pNV137, (f) pN933, (g) pN512, (h) pNV58. Note the lack of hybridization in lanes (b) and (c), indicating that these plasmids carried insert DNA which was not viral cDNA.

Figure 13. mRNA blot hybridization analyses of cloned plasmids.

Blots of IHN viral mRNA were hybridized individually with ^{32}P-labelled probes of nick-translated cloned plasmids. The plasmids used to make each probe are shown at the top of each lane. Lane (a) is an autoradiogram of the ^{3}H-IHNV mRNA electrophoretic profile identical to those used to prepare blots for hybridization. Lanes (b) through (g) are autoradiograms of blots after hybridization with the following probes : (b) pNV58, (c) pM219, (d) pN144, (e) pG480, (f) pL262, (g) pG480 and pN144.

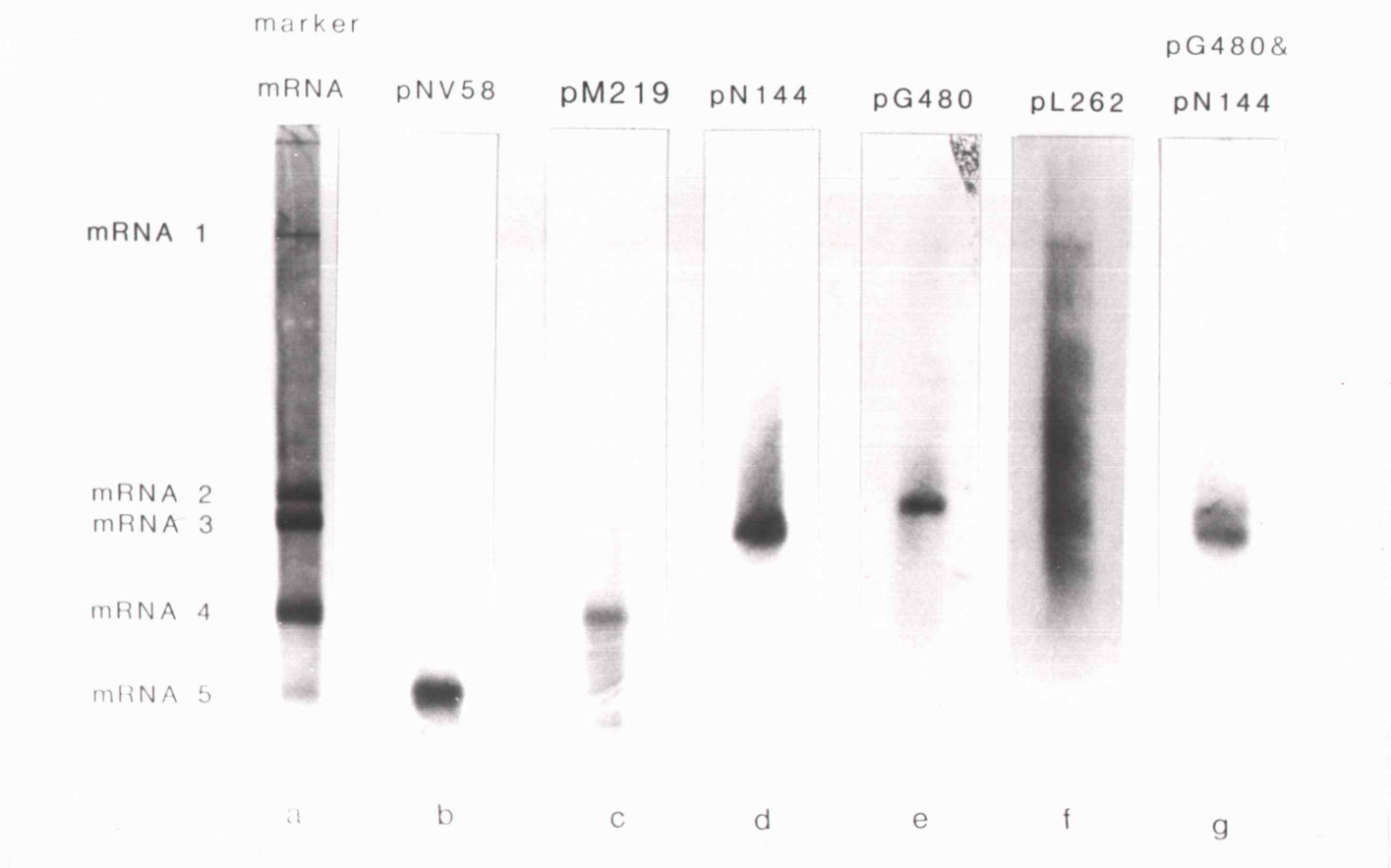
marker
mRNA
pNV58
pM219
pN144
pG480
pL262
pG480&
pN144
mRNA 1
mRNA 2
mRNA 3
mRNA 4
mRNA 5
a
b
c
d
e
f
g

Figure 13.

studies is included in Table 2. Of the 21 plasmids tested, two hybridized to mRNA 1, one to mRNA 2, eight to mRNA 3, seven to mRNA 4, and three to mRNA 5.

CROSS-HYBRIDIZATION STUDIES

It was of interest to examine the extent of cross-hybridization between the cDNA inserts of these cloned plasmids. This was done by hybridizing blots of Pst1 restricted plasmid DNA with probes made by nick translation of other cloned plasmids. As was expected, there was strong hybridization of every probe to the linear pUC8 DNA. However, hybridization to the released insert DNA was dependent on the specific cDNA insert of each probe. Results of all cross-hybridizations carried out are compiled in Table 3. In no case was there hybridization between inserts which had been previously identified as carrying cDNA to to different mRNA bands.

Within plasmid sets that hybridized to the same mRNA band there was considerable homology. Salient results are summarized in Table 4. The inserts of the three plasmids which carried cDNA to mRNA band 5 all showed specific cross-hybridization. In like manner, the inserts of the two mRNA band 1 plasmids hybridized to each other. Of the eight plasmids with cDNA to mRNA band 3, seven of the inserts did cross-hybridize, and one insert, pN419, did not. Hybridization selection studies have shown that both pN419 and the other mRNA band 3 plasmids were complementary to the mRNA

Table 3. Cross-hybridization results for all cloned plasmids.

blot \ probe	pL232	pL262	pG480	pN512	pN933	pN421	pN154	pN156	pN419	pN125	pN144	pM1163	pM1420	pM219	pM2173	pM211	pM2112	pM2132	pNV58	pNV137	pNV711
pL232	+	+	-	-		-			-			-					-	-	-		-
pL262	+	+	-	-		-			-			-					-	-	-		-
pG480			+			-						-					-	-	-		
pN512					+	+			-												
pN933					+	+			-												
pN421					+	+			-												
pN154		-			+	+			-			-					-	-			-
pN156					+				-												
pN419			-		-	-			+			-					-	-	-		
pN125					+	+			-								-	-			
pN144			-		+	+			-			-					-	-	-		
pM1163			-									+	+					-	-		
pM1420			-									+	+					-	-		
pM219			-									-	-		+			+	-		
pM2173												-	-		+			+			
pM211		-		-		-						-	-		+		+	+			-
pM2112		-		-		-						-	-		+		+	+			-
pM2132		-	-	-		-						-	-		+		+	+	-		-
pNV58	-		-						-			-							+		
pNV137	-		-						-			-							+		
pNV711	-		-						-										+		

Table 4. Cross-hybridization results within sets of plasmids with cDNA to the same viral mRNA species.

A. mRNA band 5 plasmids:

	pNV58
pNV58	+
pNV137	+
pNV711	+

B. mRNA band 1 plasmids:

	pL262
pL262	+
pL232	+

C. mRNA band 3 plasmids:

	pN144	pN419
pN512	+	-
pN933	+	-
pN421	+	-
pN154	+	-
pN156	+	-
pN125	+	-
pN144	+	-
pN419	-	+

D. mRNA band 4 plasmids:

	pM219	pM1163
pM219	+	-
pM2173	+	-
pM211	+	-
pM2112	+	-
pM2132	+	-
pM1163	-	+
pM1420	-	+

for the viral N protein.

Analysis of the seven plasmids which hybridized to mRNA band 4 indicated that there were two mutually exclusive subsets of homologous cDNA plasmids. (Table 4d). Hybrid selection studies revealed that mRNA band 4 was comprised of two distinct co-migrating mRNA species which encode the viral M1 and M2 proteins. A subset of five cross-hybridizing mRNA 4 plasmids carried cDNA to the mRNA for M2, and a subset of two mRNA 4 plasmids carried cDNA to the mRNA for M1 (Tables 2 and 4).

D. CHARACTERIZATION OF VIRAL GENOME

GENOME MOLECULAR WEIGHT

The genome of IHN virus has been estimated by sucrose gradient sedimentation to be 38-40S (37). To obtain a more exact size estimate, the genome RNA was extracted from purified virions and analyzed by denaturing gel electrophoresis. This RNA migrated as a single high molecular weight band on both glyoxal and methylmercury gels (Figure 1) indicating that it was intact, genome-length RNA. Although glyoxal and methylmercury gels are both reported to fully denature RNA (9,63), the IHNV genome RNA ran differently in the two systems when compared with VSV genome standard RNA (the VSV genome has a molecular weight of $3.82 \pm 0.14 \times 10^6$)(76). Glyoxal treated IHNV genome RNA migrated just slightly faster than glyoxal treated VSV genome RNA, indicating a molecular

weight of 3.7±0.05 x 10^6 (Figure 2). This corresponds to a genome length of approximately 10,900 ribonucleotides. On methylmercury gels the same IHNV genome RNA migrated significantly faster than the VSV genome, indicating a smaller molecular weight of approximately 3.5-3.6 x 10^6. While this discrepancy should be kept in mind, I consider the glyoxal gel size estimate to be more accurate due to the consistent linearity of the log molecular weight vs. mobility plots for glyoxal treated RNA marker species.

PHYSICAL MAPPING OF THE VIRAL GENOME

The construction of cDNA clones which carried sequences from each of the six viral genes made it possible to determine the physical order of the genes on the viral genome by heteroduplex mapping with the viral genome RNA.

The first set of heteroduplexes were prepared by annealing viral genome RNA with a single cloned plasmid carrying cDNA to each viral gene. Plasmids pL262, pG480, pN144, pM1163, pM219, and pNV58 were used to locate genome sequences of the L, G, N, M1, M2, and NV genes respectively. In addition, plasmid pN419 was included since it failed to cross-hybridize with the other plasmids carrying cDNA from mRNA 4 (Table 4C). Examples of the R-loops observed are shown in Figure 14. Measurements of 15 to 20 R-loops formed with each plasmid were used to locate the region of hybridization along the viral genome. The percent of the full length genome RNA on both sides of each R-loop was determined and

Figure 14. Single R-loop heteroduplexes.

IHNV genome RNA was hybridized with cloned plasmids and visualized by electron microscopy to determine the location of each cloned viral sequence on the genome. Interpretive drawings of each heteroduplex are shown below each photomicrograph, where the thin line represents IHNV RNA and the thick line is double-stranded plasmid DNA. The following plasmids were hybridized; (A,B) pN419, (C,D) pN144, (E,F) pM1163, (G,H) pM219, (I,J) pG480, (K,L) pNV58, (M,N) pL262. The bar in panel B represents 1000 base pairs.

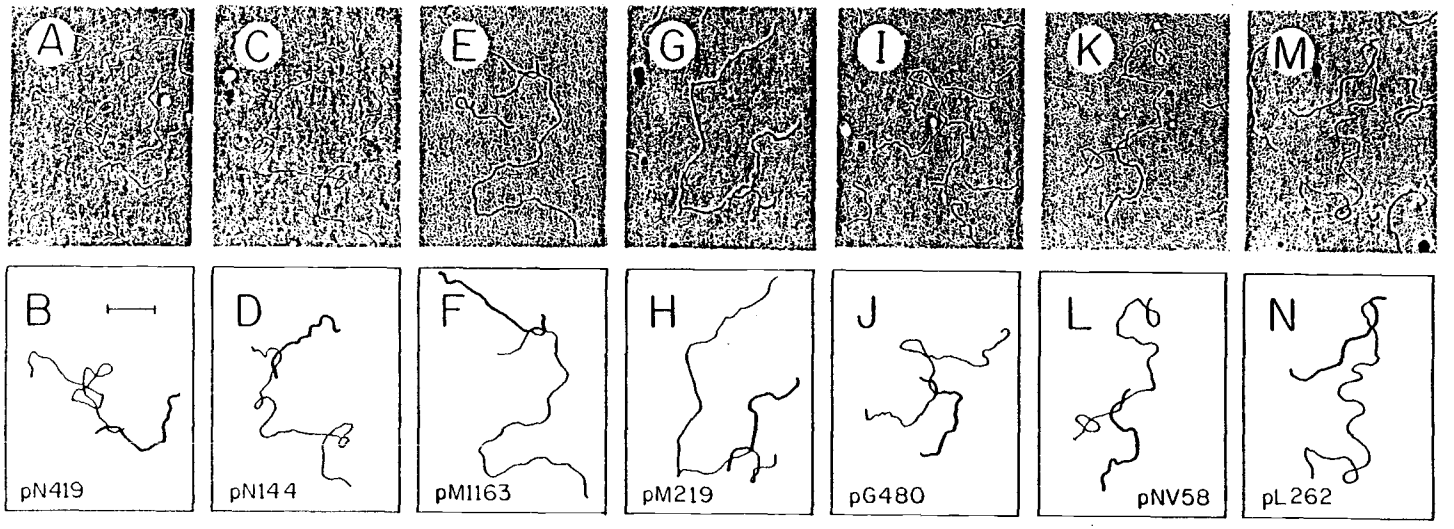
A
C
E
G
I
K
M
B
D
F
H
J
L
N
pN419
pN144
pM1163
pM219
pG480
pNV58
pL262

Figure 14.

converted to nucleotide values, assuming 10,900 bases as the full length of the genome (table 5).

The inserts of the N gene plasmids, pN144 and pN419, both hybridized within the terminal 10% of the complete genome length. The L gene plasmid, pL262, hybridized within the terminal 7% of the genome length. Since the L and N insert sequences did not cross-hybridize (table 3), these clones must hybridize to opposite ends of the genome. Thus, the genome was oriented with respect to N-ward and L-ward ends.

These data alone were not sufficient to determine the gene order because the two ends of the genome were indistinguishable in the electron micrographs of single R-loops. Therefore, in order to determine the relative positions of the viral genes, double R-loop reactions were carried out in which pairs of cloned plasmids were annealed simultaneously to genome RNA. Examples of genome RNA with two R-loops are shown in Figure 15 and appropriate measurements are given in Table 6. The distances between pairs of R-loops formed between pN144 and pM1163, pM219, pG480, or pNV58 indicate that the N, M1, M2, G, and NV genes are clustered, in that order, within the N-ward half of the genome. The long distance between double R-loops formed with pL262 and pM1163 indicated that the L gene insert did hybridize to the end of the genome distal from the other five genes. Thus the order of the genes from the N-ward to the L-ward ends of the genome was concluded to be N-M1-M2-G-NV-L.

Table 5. Position of hybridization for sigle R-loop heteroduplexes.

plasmid	short end*	loop*	long end*	N-ward** nucleotide	L-ward** nucleotide
pN419	---	490 ± 50	10,410 ± 500	--	490 ± 50
pN144	960 ± 110	440 ± 40	9500 ± 450	960 ± 110	1400 ± 150
pM1163	1520 ± 220	410 ± 60	8970 ± 390	1520 ± 220	1930 ± 285
pM219	2020 ± 250	530 ± 60	8350 ± 470	2020 ± 250	2550 ± 310
pG480	4180 ± 190	400 ± 40	6320 ± 240	4180 ± 190	4580 ± 230
pNV58	4430 ± 240	450 ± 40	6020 ± 270	4430 ± 240	4880 ± 280
pL262	---	710 ± 70	10,190 ± 500	10,190 ± 500	10,900 ± 570

*in nucleotides, assuming 10,900 nucleotides as complete length of genome

** nucleotide position of R-loop boundaries from 0 (N-ward end) to 10,900 (L-ward end).

Figure 15. Double R-loop heteroduplexes.

IHNV genome RNA was hybridized with pairs of cloned plasmids and visualized by electron microscopy to determine the length of the gap between the two positions of hybridization. Interpretive drawings of the double R-loop heteroduplexes are shown beneath each photomicrograph, where the thin line represents IHNV RNA and the thick line is double-stranded plasmid DNA. The following pairs of plasmids were used; (A,B) pN144 and pM1163, (C,D) pN144 and pM219, (E,F) pN144 and pG480, (G,H) pN144 and pNV58, (I,J) pM1163 and pL262, (K,L) pM219 and pG480, (M,N) pM219 and pNV58. The bar in panel B represents 1000 base pairs.

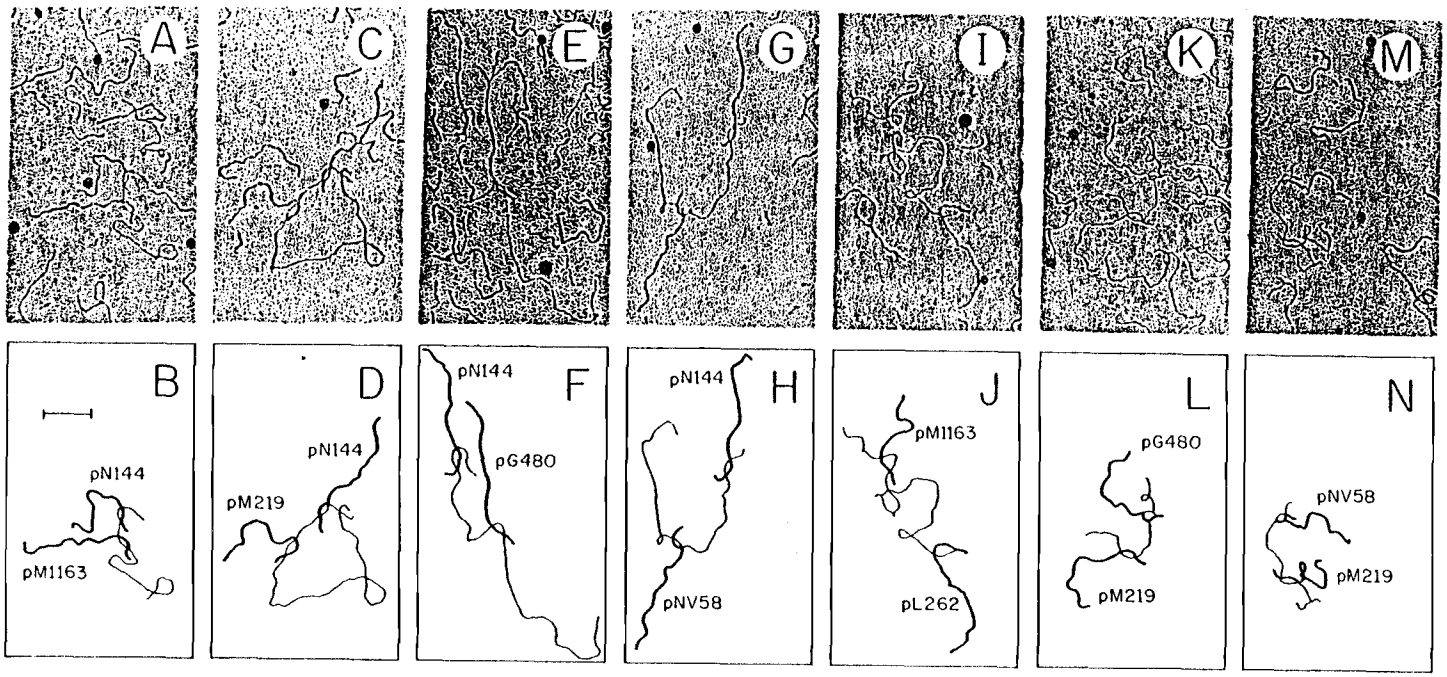
A
C
E
G
I
K
M
B
pN144
pM1163
D
pN144
pM219
F
pN144
pG480
H
pN144
pNV58
J
pM1163
pL262
L
pG480
pM219
N
pNV58
pM219

Figure 15.

Table 6. Gap measurements for double R-loop heteroduplexes.

Plasmids	gap*
pN144 and pM1163	440 ± 30
pN144 and pM219	1140 ± 110
pN144 and pG480	2710 ± 410
pN144 and pNV58	3260 ± 400
pM1163 and pL262	7630
pM219 and pG480	1470 ± 70
pM219 and pNV58	2160 ± 100

* in number of nucleotides between adjacent boundaries of the two R-loops.

DISCUSSION

A. CHARACTERIZATION OF mRNA FROM IHNV-INFECTED CELLS

The mRNA species of IHN virus have been isolated and characterized as to molecular weight, molar ratios, and coding assignments. The results are summarized in Table 7. The molecular weights of the six IHNV mRNA species were used to estimate their coding capacities, after subtracting the molecular weight of a 100 residue poly(A) tail. Our estimate of 100 residues was based on data for the poly(A) tails of VSV mRNA (64-125 residues)(82) and rabies virus mRNA (100-250 residues)(39). Table 7 shows the estimated coding capacity of each IHNV mRNA, along with the actual size of the protein encoded. In each case the size of the mRNA species is more than sufficient to code for its assigned protein product. The sum of the molecular weights of the six mRNA coding sequences (excluding 100 residue poly(A) tails) is 3.9×10^6. Thus, these six genes account for 105% of the IHNV genome length, which is 3.7×10^6 .

The molar ratios of the viral mRNA species produced intracellularly during infection are also shown in Table 7. The ratios for mRNA species 2, 3 and 4 were very consistent between different preparations of polyadenylated RNA, while those for mRNA species 1 and 5 were more variable. The molar ratio calculated

Table 7. Characteristics of IHNV mRNA species.

IHNV mRNA	molecular weight	molar ratio	estimated coding capacity*	protein encoded	protein** molecular weight
1	2.26×10^6	0.02	240,000	L	150,000
2	5.63×10^5	0.49	57,000	G_0	55,000
3	4.48×10^5	1.0	48,500	N	40,500
4	3.00×10^5	2.52	28,700	M1	22,500
				M2	17,000
5	1.95×10^5	0.41	17,400	NV	12,000

*calculated assuming a poly(A) tail of 100 residues (39,82)

** (51)

for mRNA 1 is most likely lower than the actual value due to the increased probability of degradation for such a large mRNA. Since the M1 and M2 mRNA species co-migrate in the gel, their individual ratios cannot be distinguished. However, the combined ratio of 2.52 indicates that one or both of these mRNA species is present in molar quantities greater than the N mRNA, which was given the arbitrary molar ratio of 1.0.

The gene order on the IHNV genome has been determined by heteroduplex mapping to be 3' N-M1-M2-G-NV-L 5'. With the exception of the NV gene, this is identical to the genetic maps of VSV (3' N-NS-M-G-L 5')(2,10) and rabies virus (3' N-M1-M2-G-L 5')(23). In VSV, transcription has been shown to proceed sequentially from the 3' to the 5' end of the genome, with no gene transcribed until the transcription of its 3' neighboring gene is complete (2,10,44). In addition, studies with VSV have shown an attenuation of transcription at each gene junction, resulting in a decrease in the molar quantities of mRNA transcribed from each gene from the 3' to the 5' end of the genome (43,97). If IHNV transcription also proceeds from the 3' to 5' along the genome, then the increase in the molar ratio of transcription from the N to the M1 and/or M2 genes suggests a lack of the attenuation phenomenon. However, 5' to the M2 gene sequential attenuation may occur, since the G, NV, and L mRNA species respectively are found in decreasing molar quantities (Table 7)

Although five bands of mRNA were resolved by electrophoresis

(figure 1), there was not a one-to-one correspondence between these mRNA bands and the five known viral proteins. Instead it was found that the mRNA species for two viral proteins co-migrated in mRNA band 4, and mRNA 5 encoded a previously undescribed viral protein.

The viral polymerase protein, L, was never observed as an in vitro translation product, presumably due to its large size (mol. wt. 150,000)(51) and the difficulty of translating large mRNA species in in vitro systems. However, there is evidence to indicate that mRNA 1 encodes the L protein, even though this could not be proven directly by hybrid selection and in vitro translation. First, cloning data confirm that mRNA 1 is of viral origin by the use of plasmids carrying cDNA to mRNA 1. These cDNA inserts were shown to be viral sequences by hybridization with a cDNA probe to the viral genome, and by heteroduplex formation with the viral genome. Second, the large molecular weight of the L protein (150,000) means that its mRNA must have a minimum molecular weight of 1.39×10^6. Thus, mRNA 1 is the only viral mRNA of sufficient size to encode the L protein (Table 7), in addition to being the only mRNA not accounted for by other viral proteins (Table 1).

Coding assignments for the four smaller mRNA bands were made by hybrid selection of individual mRNA species, followed by in vitro translation (Fig 4, Table 1). IHNV mRNA bands 2 and 3 encoded the viral G_0 and N proteins respectively. Hybrid

selection with different plasmids carrying cDNA to mRNA band 4 showed the synthesis of the M1 or M2 protein, but not both. Thus, it was proven that mRNA band 4 contained two co-migrating mRNA species which code for the virion M1 and M2 proteins. This is analogous to the situation in VSV, in which the fourth mRNA band includes both the NS and M mRNA species (14,82).

All five of the known virion proteins were accounted for by mRNA species 1 through 4, and the identity of mRNA 5 remained to be determined. Proof that mRNA 5 was of viral origin came again from the cloning data. Three plasmids were isolated which carried cDNA to mRNA 5. The cDNA inserts of these plasmids hybridized to a cDNA probe to the viral genome, and also formed heteroduplexes with the viral genome. Thus, mRNA 5 is coded for by the IHNV genome, and is not a cellular mRNA.

Hybrid selection using the plasmids with cDNA to mRNA 5 resulted in the synthesis of a protein of molecular weight 12,000 . Electrophoretic profiles of purified IHN virions labelled with ^{35}S-methionine confirmed that this protein is not present in mature virions (Figure 4). However, IHNV-infected cell proteins labelled with ^{35}S-methionine did show the induction of substantial amounts of a 12,000 molecular weight protein . Since the function of this protein remains to be determined, it was designated the NV protein, due to its non-virion nature. The NV protein translated from hybrid selected mRNA 5 appeared on gels as a more diffuse band compared to the intact band produced during in

in vitro translation of the whole polyadenylated RNA preparation (Figures 4 and 5). This may be due to some alteration or preferential degradation of this small mRNA during the manipulations of hybrid selection.

Hybrid selected preparations of individual mRNA species were sometimes contaminated with trace amounts of mRNA for other proteins, as shown by the faint N and M2 protein bands in lanes a and d of Figure 5. This may be due to some regions of homology between these mRNA species, or simply to the high relative abundance of the N and M2 mRNA species. Nevertheless, in each case a single viral protein was reproducibly the major species translated from hybrid selected mRNA, resulting in conclusive coding assignments which are summarized in Table 1.

The NV protein is the first non-virion viral protein reported for a rhabdovirus. Viral proteins synthesized in infected cells but not assembled into mature virions have been described for positive-strand RNA viruses such as picornaviruses and togaviruses, in which the function(s) proposed for the non-virion proteins is generally related to an RNA replicase activity (55). However, IHNV, as all rhabdoviruses, carries a viral polymerase in the virion. This polymerase is capable of carrying out the transcription of viral mRNA species in *in vitro* reactions containing lysed purified virions (60, this study). Since these reactions would not contain any NV protein, it appears that NV is not necessary for viral transcription. It may be involved with

viral replication or influence host cell processes, both of which occur in the infected cells.

With the exception of the NV protein, IHNV resembles VSV and rabies virus very closely in its proteins, mRNA species, genome size, and genome organization. Thus, one is lead to question if there might be a non-virion protein analogous to the IHNV NV protein associated with VSV and/or rabies. The small molecular weight of the NV protein (12,000) and its mRNA (1.95×10^5, approx 570 bases) means that the coding sequence on the genome would be only 370-470 bases, depending on the length of the NV mRNA poly(A) tail (39,82). Classical genetics with VSV mutants indicate the possible existence of six complementation groups in both the Indiana and New Jersey serotypes (22,75). This suggests that there are six viral genes rather than five. However, cloning studies with VSV have lead to detailed characterization of extensive regions of the genome, and no unidentified gene sequence has been reported (27,28,79,80,81). In addition, the original characterizations of the mRNA species of VSV and rabies virus did not show any mRNA species which were not accounted for by the virion proteins (14,72,82).

If there is a non-virion protein analogous to the IHNV NV in VSV and other rhabdoviruses, then this protein may have a general role in the rhabdovirus replication cycle. Alternatively, if VSV and rabies virus have no analog to the NV protein, it may be that the role of this protein is somehow specific to replication in the

fish cells of its host, or to replication at colder temperatures. The optimum temperature for IHNV replication is 16°C and the virus is inactivated at 20°C (74). It would be of interest to see if a protein analogous to the NV protein is coded for by other piscine rhabdoviruses such as spring viremia of carp virus, pike fry rhabdovirus, or viral hemorrhagic septicemia virus (74,101).

B. IN VITRO TRANSCRIPTION OF IHNV

Optimal conditions for RNA transcription *in vitro* by the virion-associated polymerase of IHNV have been determined, and are summarized in Table 8. With the exception of the buffer and the temperature optima, the requirements of the IHNV polymerase are similar to those of rabies (45), VSV (1,13,68), and other fish rhabdoviruses (60,84,85). In comparison with the previous report of Tris buffered IHNV polymerase activity (60), our results agree as to the temperature optimum, but differ in that manganese was not able to replace magnesium with equal efficiency. As suggested (60), the temperature optimum of 16-18°C for the IHNV polymerase correlates with both the optimum temperature for virus replication in tissue culture and the natural environmental temperature of its poikilotherm host.

Many systems described for the *in vitro* transcription of various viruses have included 50-100 mM Tris as the reaction buffer (1,13,45,60,68,83,84,85). However, Miller and Stone

Table 8. Optimal conditions for *in vitro* transcription of IHNV.

Reaction condition	Optimal value
buffer	400 mM HEPES
pH	8.0
detergent	0.05% Triton X-100
monovalent cation	30 mM NH_4Cl
divalent cation	5 mM $MgCl_2$
reducing agent	4 mM DTT
ribonucleotides	0.5 mM
temperature	18 °C
S-adenosyl methionine	0.5 mM

reported that the use of HEPES buffer rather than Tris for *in vitro* transcription of Newcastle Disease virus resulted in increased RNA synthesis and longer transcripts (67). Similarly, results presented here show that the activity of the IHNV polymerase is increased three-fold by the use of HEPES buffer (Figure 1a).

The action of HEPES buffer on the virion polymerase may be related to its better buffering capacity in the pH range of 6.9-7.9 (87). In addition, the stimulatory effect of HEPES may be due to side effects described by Good et.al.(29). These investigators demonstrated that in enzyme systems which are relatively indifferent to pH, HEPES is markedly superior to Tris in sustaining enzyme activity. The mechanism involved in this stimulation is not known.

Alternatively, Tris buffer may somehow inhibit polymerase activity. Tris has a primary aliphatic amine which may be inhibitory to some enzymes (29). There has been a suggestion that Tris may partially inhibit RNA transcription *in vitro* by acting as a phosphate acceptor (H. O. Stone, personal communication). For VSV, the NS protein is a phosphoprotein which appears to play a regulatory role in viral polymerase activity (20). Although there are several phosphorylated forms of NS in VSV, only the more highly phosphorylated NS protein is active in transcription (41,46). In IHNV both N and M1 are phosphoproteins (59). It is possible that Tris inhibits RNA transcription by affecting the

phosphorylation of a regulatory protein(s) in IHNV.

The presence of SAM results in increased RNA transcription *in vitro* for some viral polymerases. Cytoplasmic polyhedrosis virus requires 0.5-1.0 mM SAM for any significant polymerase activity *in vitro* (26). The same concentration of SAM stimulated a doubling of *in vitro* transcription activity for the salmonid rhabdovirus SVCV (85). In contrast, only slight increases in RNA synthesis were observed for VSV and PFR when SAM was added (84,85).

Presumably, SAM acts as a methyl donor for the capping of mRNA and could exert a stimulatory effect on transcription if required methylating compounds were limiting in the reaction. Rose et. al. have reported that in the presence of SAM and a cytoplasmic cell extract the VSV polymerase transcribes normal mRNA species *in vitro* (83). However, if methylation is prevented by including S-adenosyl homocysteine (SAH) in the reaction rather than SAM, the transcripts have long heterogeneous poly(A) tails of 200-2000 residues. This suggests that methylation is somehow involved with proper polyadenylation of *in vitro* transcripts. However, Gupta and Roy have reported that both SAM and SAH stimulate transcription of SVCV without resulting in this aberrant polyadenylation (35).

A two-fold increase in RNA synthesis was observed upon the addition of 0.5 mM SAM to IHNV *in vitro* transcription reactions (Figure 1,h). There was no apparent difference in the RNA species synthesized in the presence or absence of SAM (data not shown).

Therefore, SAM stimulated the rate or initiation of transcription without altering the sizes or composition of the RNA transcripts.

The previous system described for IHNV transcription in vitro reported the activity of the polymerase to be 1040 pmol UMP incorporated/mg viral protein/hour (60). The polymerase activity obtained using the modified reaction conditions described here was 6,400 pmol UMP incorporated/mg viral protein/hour. This is largely due to the use of HEPES buffer and the addition of SAM to the reactions. In comparison with the polymerases of other rhabdoviruses, this increased activity is higher than that reported for rabies virus (140 pmol GMP incorporated/mg viral protein/hour)(45), but still substantially lower than that of VSV IND. (21,000 pmol UMP incorporated/mg viral protein/hour) (15,25,68).

The IHNV RNA synthesized in vitro under optimal conditions includes species with molecular weights of 5.6×10^5, 4.8×10^5, 3.0×10^5, and 2.0×10^5. These species co-migrated with the IHNV mRNA species 2, 3, 4,and 5, which encode the viral G, N, M1 and M2, and NV proteins respectively. IHNV mRNA 1, which encodes the L protein, has a molecular weight of 2.3×10^6. An RNA transcript of this size was never observed in in vitro reaction products. The gene order on the IHNV genome is (3')(N-M1-M2-G-NV-L)(5'). With the exception of the NV gene, this is identical to the order of the corresponding genes on the rabies and VSV genomes (2,10,23). If transcription of IHNV proceeds sequentially from

the 3' end of the genome, as is the case for VSV (2,10,44), then polarity effects and the large size of the L transcript could explain the absence of mRNA 1 from the *in vitro* transcription products.

In vitro translation of the polyadenylated RNA transcribed *in vitro* resulted in the synthesis of the viral N protein and trace amounts of M1 and M2. These are the viral proteins for which mRNA species are most abundant in the *in vitro* transcription products (mRNA bands 3 and 4). The absence of detectable G and NV proteins is probably due to the small quantity of *in vitro* transcribed RNA (approximately 0.5 ug) used to prime the system. Nevertheless, the synthesis of intact viral proteins *in vitro* shows that this *in vitro* transcription system is capable of producing functional viral mRNA. Therefore, this system is useful for both the production of viral nucleic acid and study of the process of transcription in a controlled system which is not influenced by the host cell.

C. CLONES CARRYING cDNA TO VIRAL mRNA SPECIES

The construction and characterization of cDNA clones carrying sequences from each of the six mRNA species of IHNV have been presented. The strategy for constructing the clones involved the use of an oligo-dT primer, which would hybridize to the 3'

poly-adenylated tail of viral mRNA and prime cDNA synthesis in the 5' direction. Theoretically all the cloned plasmids should contain the intact 3' terminal sequence of the corresponding viral mRNA and various lengths of sequence in the 5' direction. Although alkaline gel analysis of the first strand cDNA products indicated the synthesis of complete copies of mRNA bands 2, 3, 4, and 5, there were no full length sequences found upon analysis of the cloned plasmids. This probably occurred as a result of the lack of size selection prior to annealing, and the preferential annealing of shorter double stranded cDNA molecules into the vector. Thus, the clones described carry partial sequences of each viral mRNA and should include the 3' terminal sequences.

The plasmids from a set of 21 transformants were characterized by hybridization to blots of viral mRNA, and the specific mRNA of origin was determined for each cloned sequence (Table 2). The number of clones carrying cDNA to each mRNA band generally reflected the relative abundance of that mRNA in the total mRNA preparation used as the template for cloning. Thus, the majority of the clones carried cDNA to mRNA bands 3 and 4, which had the highest molar ratios of the mRNA species in the total preparation (Table 7).

Cross-hybridization studies showed that the majority of cloned sequences which hybridized to the same mRNA band also hybridized with each other (Table 4). This indicated that the same or significantly overlapping regions of the mRNA sequence had

been cloned in most cases, as would be expected if each contained the 3' mRNA terminus. The only exception to this was plasmid pN419, which hybridized to mRNA band 3, but did not hybridize with any of the other seven clones exhibiting hybridization to mRNA band 3 (Table 4). R-loop mapping with these plasmids showed that pN419 hybridized to a region covering approximately 490 bases at one end of the genome (Table 5). One of the other seven cross-hybridizing mRNA band 3 plasmids, pN144, hybridized at approximately 960 to 1400 bases from the genome end (Table 5). Since the entire length of mRNA 3 is estimated to be 1420 bases (mol. wt. 4.84×10^5, Table 7), these hybridization measurements indicate that these two plasmids contain non-overlapping sequences from the two ends of the N gene. If, as the cloning strategy predicts, the majority of the clones carry the 3' sequences of the mRNA, then pN144, representing the seven cross-hybridizing plasmids, contains the 3' sequences of mRNA 3. The insert of pN419 would then contain the 5' mRNA band 3 sequences. This could conceivably have originated from a full length single-stranded cDNA molecule which, upon 5' priming, carried out incomplete second strand synthesis. An analogous clone containing 5' sequences of the N mRNA has been reported for VSV (81).

D. CHARACTERIZATION OF THE VIRAL GENOME

The molecular weight of IHNV genome RNA was determined by glyoxal gel electrophoresis to be $3.7 \pm 0.05 \times 10^6$. This is consistent with the known genome sizes for other rhabdoviruses, which are all approximately 4×10^6 (55,58).

There is evidence to suggest that the discrepancy between the genome size estimates from glyoxal gels and methyl mercury gels is due to a high degree of secondary structure in the IHNV genome. Initial attempts to spread IHNV genome RNA for electron microscopy using conditions which spread VSV genomic RNA (36) resulted in molecules with an abundance of tightly coiled loops and stem structures. After testing a variety of procedures, the disruption of this secondary structure was finally accomplished by spreading the IHNV genome RNA from 80 % formamide with 4.0M urea to 50% formamide. These conditions are much stronger than those used to spread the VSV genome.

It is possible to assess whether glyoxal treatment of RNA has resulted in complete denaturation by the difference in mobility between native and glyoxal treated ribosomal marker RNA (63). In all gels used for size determinations, denaturation was complete. However, one instance was observed in which the glyoxal treatment failed to totally denature the RNA samples, as judged by a much less dramatic shift in the mobility of the marker rRNA. Upon electrophoresis of these samples, the IHNV genome RNA migrated significantly faster than the the VSV genome, as it does on methylmercury gels. Together with the more variable, non-linear

nature of the log molecular weight vs mobility plots for methylmercury gels, these facts indicate that the conditions of methylmercury gel electrophoresis are not as totally denaturing as glyoxal treatment. The difference of mobilities for the two genome RNA species on the different gel systems can then be explained if the IHNV genome has more secondary structure than the VSV genome, and requires the stronger conditions of glyoxal treatment for complete denaturation.

The gene order on the IHNV genome was determined by double R-loop mapping to be N-M1-M2-G-NV-L. This is shown diagramatically in Figure 16. With the exception of the NV gene, this order is identical to that of the analogous genes of VSV (3') N-NS-M-G-L (5')(2,10) and rabies (3') N-M1-M2-G-L (5')(23). Thus, although no 3'-5' orientation was determined directly for the IHNV genome, we assume that the N-ward end is 3', since an exact inversion of gene order for this rhabdovirus is highly unlikely. In addition, assuming that the cDNA clones contain the 3' mRNA sequences (with the exception of pN419), then the position of each R-loop agrees with the N3'-L5' orientation (Figure 16). In particular, the hybridization of the pL262 insert to the extreme terminus of the genome, rather than in the middle, implies that the L terminus is the 3' end of the mRNA sequence, and thus the 5' end of the gene and genome.

Examination of the heteroduplexes between pN419 and the viral genome revealed a very short length of unhybridized terminal

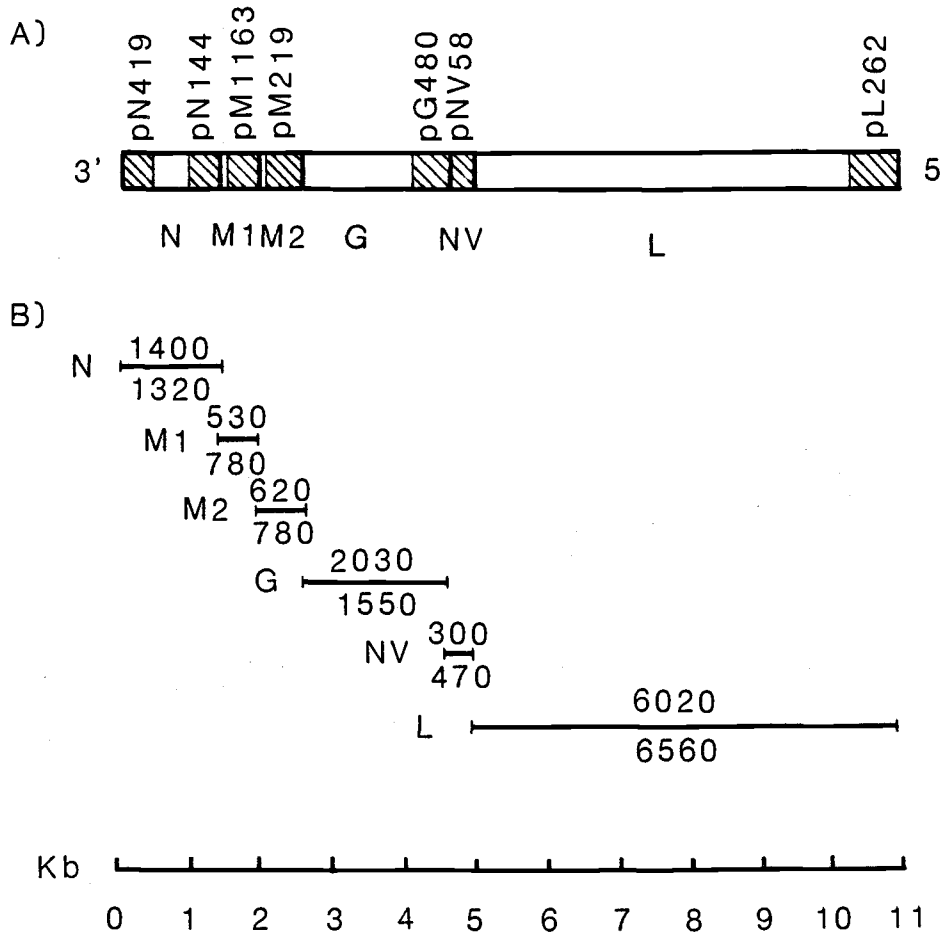

Figure 16. Physical map of the IHNV genome.

A) Physical map showing the order of genes on the genome (delineated by heavy vertical lines) and locations of cloned sequences (shaded areas) as determined from R-loop measurements (Tables 5 and 6).

B) Horizontal lines represent individual genes. Numerical values (in nucleotides) above each line are gene sizes estimated from R-loop measurements, and values below each line are the corresponding mRNA sizes (Table 7).

genome RNA (Figure 3A,B). This may be indicative of a short terminal leader sequence next to the N gene, as in VSV (16,17).

The size of each gene was estimated from the R-loop measurements by assuming that the 5' boundaries of the R-loops of pN144, pM1163, pM219, pG480, pNV58, and pL262 corresponded to the 5' ends of the N, M1, M2, G, NV, and L genes respectively. These gene sizes are shown in Figure 16, and agree quite well with the known sizes of the corresponding mRNA species (Table 7).

Since the NV gene of IHNV has not been reported to exist in any other rhabdovirus, its position on the genome between the G and L genes was of great interest. Measurements of the pG480 and pNV58 R-loops showed that these sequences were very close, and raised the possibility that they might overlap (Table 5). In order to obtain more accurate measurements, double R-loops of each of these plasmids with pM219, the most proximal gene, were carried out (Table 6). The gap measurements between M2 and G, and M2 and NV hybridizations showed a better separation of the G and NV R-loops, indicating that they do not overlap but are adjacent. This is supported by the fact that the insert sequences of pG480 and pNV58 do not cross-hybridize. The cDNA insert of pNV58 is 420 base pairs in length (Table 2), and thus represents a minimum of 73% of the mRNA sequence (575 bases, mol. wt. 1.95×10^5). If one excludes a 100 residue poly-A tail from the mRNA length, then this insert could contain as much as 89% of the mRNA coding sequence. This explains the proximity of the R-loop measurements between the

pNV58 insert and the pG480 insert from the adjacent G gene. The possibility that the NV and G gene sequences overlap is also excluded by the mRNA blot hybridizations and hybrid selection results. If the NV and G genes overlapped, one would expect mRNA blot hybridization of pNV58 to both mRNA bands 2 and 5, and hybrid selection by pNV58 of mRNA for both the NV and G proteins. No such results were seen.

However, the types of data which prove that the NV and G genes do not overlap can not be used to conclusively rule out the possibility that the NV sequence overlaps, or is contained within, the 3' end of the L gene. Due to the size of the L gene (Figure 16) one would not expect hybridization between cDNA sequences from its opposite ends. Hybridization to mRNA band 1 on blots was inconsistent and difficult to detect. The quantity of mRNA 1 in various mRNA preparations was extremely small and variable, and its large size (mol. wt. 2.26×10^6, 6.56 kb) may have resulted in a lower efficiency of blotting transfer. In addition, the increased problem of degradation of such a large RNA was evident in the high background of lower molecular weight hybridization on blots probed with pL232 (Figure 13d). This inconsistency of blot hybridizations to mRNA 1 and the failure of mRNA 1 to translate <u>in vitro</u> mean that the lack of double hybridization on mRNA blots and double selection during hybrid selection may be due to technical difficulties specific to mRNA 1.

The most straight-forward genetic arrangement is that the NV

gene is a separate gene, located between the G and L genes. However, while there is no evidence to indicate that the NV gene sequence does overlap the L sequence, this is an alternative genetic arrangement which has not been ruled out.

SUMMARY

The studies described here involve many interrelated aspects of the molecular genetics and transcription of IHNV. The characterization of the mRNA species produced in vivo correlates well with the protein structure of the virus, and is re-confirmed by comparison with the products of transcription *in vitro*. The sizes of the six viral transcripts together account for the entire length of the viral genome, indicating that all the viral genes have been identified. Data from the heteroduplex mapping studies reconfirms the existence of six viral genes, and indicates gene sizes compatible with the molecular weights of the mRNA species.

Combined with the recent detailed studies of the protein structure and protein synthesis of IHNV (42,51,53), this information provides a much more complete picture of the molecular biology of IHNV than was available previously. This is significant for future studies of IHNV itself, and also as a model of a non-mammalian rhabdovirus, for comparison with the well studied mammalian rhabdovirus prototypes.

Many characteristics of IHNV clearly place it in the rhabdovirus family. The genome size and gene order described here reaffirm this classification, despite the existence of the NV gene in IHNV. Future studies of the function of the NV protein and its occurrence in other rhabdoviruses may provide some clues to its evolutionary origin.

The clones carrying cDNA to each of the viral mRNA species comprise an extremely useful tool for studying many aspects of the molecular biology of IHNV. These clones have already been used to determine the coding assignments for the viral mRNA species and the gene order on the viral genome. Future uses include the development of a viral vaccine, use as probes to investigate the life cycle of IHNV during the carrier state of its host, and more detailed analyses of the replication of the virus.

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