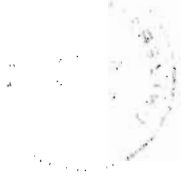
STUDIES ON THE ISOLATION OF THE POLYMERASE GENES FROM THE H1N1 INFLUENZA A VIRUS



by

RICHARD NAIDOO
B.Sc (Hons) (UDW)

Submitted in partial fulfilment of the
requirements for the degree of
Master of Medical Science
in the
Department of Physiology
Faculty of Medicine
University of Natal
Durban

December 1992

PREFACE

The experimental work described in this thesis was carried out in the Department of Physiology, University of Natal Medical School, Durban, from April 1990 to December 1992.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of others it has been duly acknowledged in the text.

RICHARD NAIDOO

ABSTRACT

Vaccines directed against the influenza virus become ineffective due to continuous mutation. An alternative approach might be to control replication at the genomic level by enzymatic methylation of the polymerase genes. Hence in this study, a method to locate and successfully isolate the H1N1 influenza A polymerase genes was investigated.

The virus was cultured in chick embryos via the allantoic route using aseptic techniques. Following incubation, the allantoic fluid was isolated and washed to remove any contaminating blood cells. The allantoic fluid was checked for fungal and bacterial contamination using the blood agar test and the presence of the virus was established by the haemagglutination titration test. Viral particles were pelleted by ultracentrifugation. Electron microscopy verified the morphology and size of these viruses while immunofluorescence studies, using a monoclonal antibody, confirmed the influenza A strain. The ribose test verified the presence of RNA in the samples.

Purified viral pellets were pooled and homogenised in buffer containing guanidine thiocyanate, mercaptoethanol and sarkosyl. The samples were incubated on ice before mechanical disruption of the virus. Viral RNA was isolated from the upper aqueous layer after a standard phenol/chloroform extraction procedure.

RNA was quantified spectrophotometrically and purity assessed initially by the absorbance ratio readings at 260/280 nm. Electrophoresis of the RNA samples was performed together with RNA molecular weight markers on a 1.5% formamide agarose gel.

Five bands were identified and the band containing the polymerase genes was size selected, located and excised. Purification of the polymerase genes from the agarose was achieved by using the BIO 101 RNAid kit. The three isolated polymerase RNAs were reverse transcribed using the Boehringer Mannheim cDNA synthesis kit.

The results indicate that the H1N1 influenza virus was successfully grown and isolated from chick embryos. Absence of contamination and verification of viral presence at different stages of the study were indications that asepsis was successfully achieved. The RNA obtained was sufficient and suitable for cDNA synthesis. This cDNA may now be used for further molecular analysis and subsequent DNA methylation studies. Further, transfection studies may then be performed to determine, if any, the the expression of methylated and unmethylated cDNA.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following people for their contribution to this thesis:

Dr Zainub Khan, who developed the project and guided me throughout the initial stages of the work.

Dr Di Manning, (Head of Biochemistry Division - Physiology Department) for her supervision, invaluable guidance and efficiency, especially with the proof-reading of the thesis.

Dr Kogie Reddi, for her invaluable advice and constructive criticism throughout this project and especially for her willingness to assist whenever problems were encountered.

Dr Peter Olmesdahl (Deputy Head of the Physiology Department) for encouraging me and allowing me time and opportunity to complete this study, above the call of my duties in the department.

Professor Botes, (Head of Department of Biochemistry and Centre for Molecular and Cellular Biology, University of Cape Town) for synthesising the primer used for the complementary DNA synthesis.

Gops Gopaul, Bip Singh and Kinnon Naidoo for their technical assistance in the Virology Department and also for trusting me with the use of their equipment.

Sanjay Maharaj, from Rainbow Chicken Farm Laboratory, for supplying the eggs used in the experiments.

Anita Naicker for her assistance with the electron microscopy.

Seelan Naidoo, for his most useful suggestions during the proof-reading of this thesis.

Sunny Govender for his technical assistance in the Department of Chemical Pathology.

The Research and Travel Committee, Natal University Medical Faculty, for providing the financial support for this study.

Finally, my dear wife Nirmala, for her encouragement and my two little girls (Kimera and Narina) for helping me keep sane most of the time.

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ABBREVIATIONS

5'Me Cyt	5'-methyl cytosine
A,C,G,T,U	adenine, cytosine, guanine,thymine, uracil
Abs	absorbance
AMV	avian myeloblastosis virus
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
CpG	cytosyl-(3-5) guanosine dinucleoside monophosphate
cRNA	complementary DNA
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
g	acceleration due to gravity
GC	gas chromatography
GpC	guanylyl-(3-5) cytidine dinucleoside monophosphate
HA	haemagglutinin
HBc	hepatitis B virus core
HPLC	high pressure liquid chromatography
ml	millilitre
MOPS	4-morpholinpropanesulphonic acid
mRNA	messenger RNA
NA	neuraminidase
NP	nucleoproteins
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMK	primary monkey kidney
poly (A)	polyadenylic acid
PTA	phosphotungstic acid
RNA	ribonucleic acid
RNAse	ribonuclease
RNP	ribonucleoproteins
RT	Room temperature
SDS	sodium dodecyl sulphate

Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
vRNA	viral RNA
µg	micrograms
µl	microlitre

CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

1.1 HISTORICAL BACKGROUND

Influenza is an acute respiratory illness which was described as far back as the middle ages. The virus causing the illness ranks as one of the most contagious infectious agents known, spreading from person to person with great efficiency facilitated by a very short incubation period. As a result, a number of severe pandemics have occurred in the human population, the most severe being that of 1918 in which 20 million people died. A search for the causative agent was initiated as a first step towards controlling the disease.

Shope (1931) first showed that the disease could be transmitted in swine by filtered human mucus, suggesting that the causative agent was a virus. Two years later the first human isolates of the virus were obtained from throat washings (Smith *et al.*, 1933). The first human experiments were carried out by inoculating the virus subcutaneously (Francis and Magill, 1936) and subsequent work resulted in the human influenza virus being successfully cultivated in developing chick embryos for the first time (Burnet, 1936). Some of the methods used by these investigators are still routinely employed in many laboratories for propagation of the virus, especially in laboratory studies requiring large quantities of virus for vaccine production.

An important development was a sensitive method for detecting the presence of the virus. Hirst (1941) showed that red blood cells could be agglutinated by allantoic fluid isolated from chick embryos infected with the virus.

This later proved to be an excellent method for establishing both the presence of the virus and quantitative determination of viral titres (Hirst, 1942). Since the advent of cell culture systems in the 1950's, numerous additional methods for preparing virus stocks have become available.

Although it was known that the virus agglutinated erythrocytes, the structure and mechanism of attachment was not known. Some twenty years later, during the early nineteen sixties, the first electron microscope pictures of the influenza virus were produced (Horne *et al.*, 1960; Choppin *et al.*, 1960), providing valuable information about its morphology and size. The viruses were shown to be spherical structures with spike-like projections on their outer covering. The mode of attachment and penetration into cells were subsequently demonstrated (Dales and Choppin, 1962).

The next major breakthrough by researchers was the estimation of the molecular weight of the RNA genome (Skehel, 1971) followed by the demonstration that the genome consisted of eight individual RNA segments (McGeoch *et al.*, 1976). This information, together with the relatively new techniques of molecular biology at that time, resulted in increased interest in the molecular analysis of the influenza A genome, the surface glycoproteins and the replication of the virus. These aspects are important for understanding the pathogenicity of the virus.

Epidemiological studies have shown that the viruses responsible for the recurring pandemics were antigenically similar. Antigenic similarity of these influenza viruses indicates that the viruses probably undergo mutation after a certain time lapse.

Extensive research has been carried out on the genes coding for the two surface antigens or glycoproteins, named haemagglutinin (HA) (Porter *et al.*, 1979; Wilson *et al.*, 1981; Gething *et al.*, 1981) and neuraminidase (NA) (Colman *et al.*, 1983), in an attempt to understand and control viral antigenicity. However, due to continued mutation of the virus, vaccines prepared against the viral proteins rapidly become ineffective. Mutation of the surface antigens is said to occur by processes referred to as antigenic shifting and antigenic drifting. Antigenic shift refers to major changes to the HA and NA genes when compared

with other strains of influenza virus. This shift is due to replacement of entire gene segments. Antigenic drift occurs as a result of minor changes in the genes coding for the surface glycoprotein within a family of related influenza virus strains. Pandemics and endemics are said to be the result of antigenic shifts and antigenic drifts. The 1957 pandemic which was caused by the H2N2 subtype (Asian influenza) was a direct result of an antigenic shift which replaced the H1N1 subtype, originally isolated in 1933. Similar events have been described for other pandemics. The emergence of these "new" viruses creates a major problem with respect to control of viral spread. It would seem relevant therefore, that understanding the regulation of viral transcription and replication could be an alternative to vaccines for control or interruption of pathogenicity.

1.2 NOMENCLATURE

Influenza viruses can be grouped into three types, A, B and C. This classification is based on the antigenic differences between the nucleoprotein and the matrix proteins.

Influenza A is subdivided into different types based on the system adopted by the World Health Organization Memorandum (Assaad *et al.*, 1980).
The system of nomenclature includes the host of origin (for strains isolated from non-human sources), the geographic origin, strain number and the year of isolation. The antigenic description of the haemagglutinin and the neuraminidase is also included, eg., A/swine/Iowa/15/30 (H1N1). Traditionally the human isolates do not include the host of origin, as in the case of A/Brazil/1/84 (H1N1).

1.3 STRUCTURE OF THE INFLUENZA A VIRUS

1.3.1 General morphology

A complete understanding of the biological properties and the genetic behaviour of the influenza virus is not possible without knowledge of the molecular structure and its mode of replication. The influenza A virus, (Figure 1) is a member of the Orthomyxoviridae.

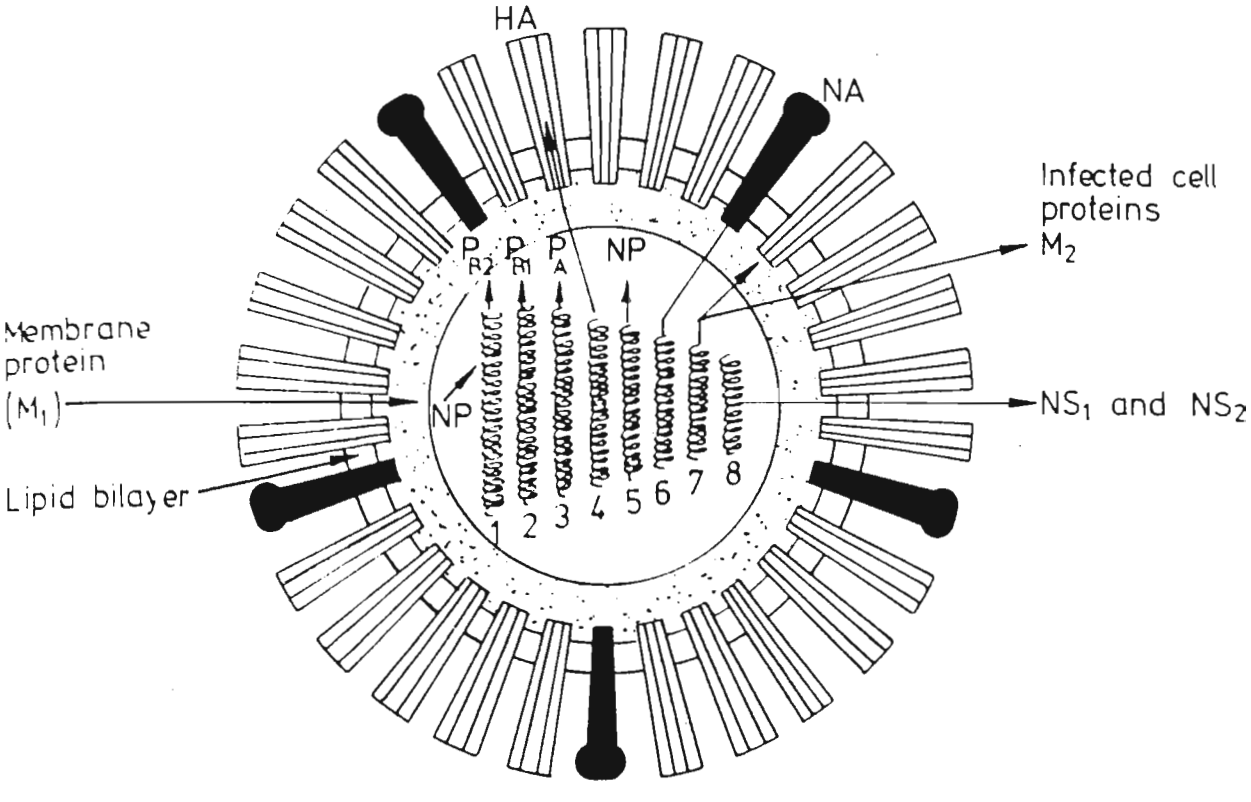

Figure 1. Schematic representation of the influenza A virus showing the composition of the RNA genome and the viral proteins they code for (from Stuart-Harris *et al.*, 1985).

These viruses are roughly spherical in shape, having diameters of 80-120 nm. Electron microscopic studies using negative staining have also demonstrated the presence of filamentous forms of the virus (Choppin *et al.*, 1961). The virus has an outer envelope composed of a lipid bilayer membrane from which spike-like glycoproteins project outwards. There are two types of membrane-associated glycoproteins (Laver and Valentine, 1969): haemagglutinin and neuraminidase. The HAs appear as rod-like spikes having a triangular appearance in cross section and they protrude approximately 12 nm from the lipid bilayer. The NAs have a typical mushroom-like morphology with narrow stalks inserted into the lipid layer (Figure 1).

The HA protein is involved in the attachment of the virus to specific cellular receptors on the host cell surface. It also aids in penetration into the host cell. The NA glycoprotein cleaves neuraminic acid, sialoglycoproteins and glycolipids in the cell surface receptors. It has also been shown to play an important role in the fusion of the virus with the host cell-receptor (Huang *et al.*, 1980).

The membrane or matrix protein (M1) which provides structural integrity to the virus, is the most abundant protein in the viral particle and is situated below the lipid bilayer. It is hydrophobic in nature and is composed of 252 amino acids (molecular weight 28000 daltons). M1 has recently been found to be closely associated with the viral nucleoproteins (NP) (Ruigrok *et al.*, 1989). The nucleoproteins are enclosed within the matrix as are the polymerase proteins (P proteins: PA, PB1 and PB2). The NP and the P proteins are closely associated with the viral genome. The influenza viral genome, housed within the matrix, is biochemically unique in that it is composed of eight separate single-stranded RNA segments (Figure 1) (McGeoch *et al.*, 1976; Inglis *et al.*, 1976; Pons, 1976; Palese, 1977). Chemically, the virus is composed of 60 to 75% protein, 20 to 30% lipid and approximately 7% carbohydrate. The RNA constitutes only 1% of the total weight of the virus.

1.3.2 The genome

The unusual composition of the influenza viral genome and its role in transcription has been well documented in the literature (Richey *et al.*, 1976; Pons, 1976; McGeoch *et al.*, 1976; Palese, 1977). As previously mentioned, eight RNA segments make up the viral genome, varying in length from 890 to 2341 nucleotides (Lamb and Choppin, 1983). Each of the eight RNA segments codes for structural and/or non-structural viral proteins (Figure 1). The three largest RNA segments, 1, 2 and 3, code for the three viral polymerase proteins, PB2, PB1 and PA, respectively. These proteins form a complex with the viral RNA together with the nucleoproteins and participate in viral transcriptase activity (see section 1.5.1).

The surface membrane glycoproteins HA and NA are coded for by RNA segments 4 and 6, respectively (Palese and Schulman, 1976) and segment 5 codes for the ribonucleoprotein or nucleocapsid, a protein associated with the RNA genome. Segment 7 codes for a structural protein M1 and a non-structural protein M2. The latter is produced only in the infected host cell. Segment 8 codes for two non-structural proteins, NS_1 and NS_2, both of which also occur only in the infected cell. It was originally thought that each RNA segment codes for a single protein but it is now known that segments 7 and 8 each code for two proteins by overlapping reading frames (Bredis *et al.*, 1981). The exact function of the NS proteins is not well characterised, however the NS_1 protein plays a role in certain post-transcriptional processes during viral protein synthesis (Hatada *et al.*, 1990).

1.3.3 Haemagglutinin glycoprotein

Haemagglutinin, which constitutes twenty five percent of the total viral protein, is one of the most widely studied glycoproteins of the influenza A virus. The HA glycoproteins form a dense radial arrangement around the entire outer surface of the virus. It is so named because of its ability to agglutinate red blood cells. The HA monomer is synthesised as a single polypeptide chain in the infected cell. It is proteolytically cleaved into two polypeptides, designated HA1 and HA2,

having molecular weights of 36000 and 27000 daltons respectively (Ward and Dopheide, 1976). Cleavage of the HA momomer molecule into HA1 and HA2 is a prerequisite for viral infection (Kawaoka and Webster, 1988) (Figure 2). The two polypeptides (HA1 and HA2) are held together by a single disulphide bond (Figure 2). A protease produced by the host cell is responsible for the cleavage of the HA monomer (Orlich *et al.*, 1990). The HA2 subunit is said to trigger fusion with the host cell during viral infection (Stegmann *et al.*, 1991).

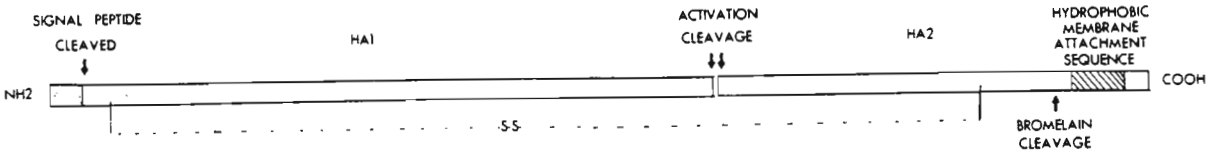

Figure 2. Diagrammatic representation of haemagglutinin polypeptide showing momomers HA1 and HA2 linked together by a disulphide bond (from Murphy and Webster, 1985).

Neutralising antibodies produced against the virus are usually directed at the HA antigen. Most cell-surface glycoproteins and glycolipids have oligosaccharides terminating in N-acetyl neuraminic acid (sialic acid) residues. These sialic acid residues serve as receptor sites to which the influenza A virus binds (Wiley and Skehel, 1987). Detailed work on the three-dimensional structure of the the HA glycoprotein has been carried out by Wiley and Skehel (1987) and Weis *et al.* (1988). These workers proposed the approach that anti-viral drugs could be synthesised to block viral-cell receptor attachment sites and thus prevent viral entry into the host cells. Complementary double-stranded DNA made from RNA coding for the haemagglutinin gene has been extensively studied with respect to antigenic shifting and drifting (Gething *et al.*, 1980; Jou *et al.*, 1980; Sleigh *et al.*, 1980; Lai *et al.*, 1980; Ward and Dopheide, 1981; Gething and Sambrook, 1981). Similarly the HA2 region has been sequenced and studied to further clarify the concept of antigenic shifts and drifts (Threlfall *et al.*, 1980).

1.3.4 Neuraminidases

The neuraminidases constitute five to ten percent of the total viral protein and appear as mushroom-shaped spikes on the surface of the virus (Figure 1). Each spike consists of a central stalk and a tetramer made up of four roughly spherical subunits. The tetramer is centrally attached to the stalk which has a hydrophobic region responsible for anchoring the NA molecule to the viral membrane.

Various functions have been postulated for the NA molecule, including cleavage of the α-ketosidic linkage between the terminal sialic acid and adjacent sugar residues on the host cell. This permits transport of the virus through mucin and destruction of the HA receptor on the host cell, allowing the newly synthesised progeny to leave the infected cell (Palese *et al.*, 1974).

1.3.5 Polymerase proteins

The three largest proteins present in the virus are the polymerase or P proteins designated PB1, PB2 and PA, with molecular weights of 96000, 87000 and 85000 daltons, respectively. The three proteins are not easily distinguished from each other by standard separation techniques because of the very small differences in their molecular weights. Two-dimensional separation using a combination of non-equilibrium pH gradient electrophoresis and polyacrylamide gel electrophoresis has been more successful in isolating these proteins (Horisberger, 1980).

These P proteins are found in association with the nuclear proteins (NP). Together, they are known as the ribonuclear proteins (RNP) and are associated with each of the genomic RNA segments (Scholtissek and Becht, 1971). A recent study using immunogold labelling/electron microscopy clearly demonstrated the P proteins and the NP as part of a helical complex (Murti *et al.*, 1992). Ribonuclear proteins facilitate polymerase activity which is responsible for transcription of the viral RNA (see 1.5).

A relatively early study (Rochovansky, 1976) indicated that synthesis of viral RNA could be stimulated *in vitro* using RNP complexes isolated from the influenza virus, thus demonstrating its role in transcription.

The polymerase proteins have been extensively investigated by Palese and co-workers who have provided valuable information with regard to its structure and function in transcriptional activity.

A successful method to purify the three polymerase proteins and the NP was described by Szewczyk *et al*. (1988). The purified polymerase complex was then renatured with RNA isolated from the influenza virus using thioredoxin. This was an important finding since earlier attempts to solubilise the polymerase were unsuccessful. This study also showed that isolated P proteins are collectively required for transcription. Active polymerase complexes were also isolated from nucleoprotein cores using cesium-chloride gradient centrifugation (Parvin *et al*., 1989). The purified complex of PA, PB1, PB2 and NP was used to study the promoter signals for polymerase activity. Later a unique transfection system was devised, in which recombinant RNA (CAT1RNA) molecules containing the chloramphenicol acetyltransferase (CAT) gene were transfected in cell culture (Luytjes *et al*., 1989). CAT activity was detected in the transfected cells after infection with the influenza virus and it was shown that the 3' and 5' non-coding sequences provided the necessary signal for RNA transcription. Most recently, vRNA was synthesised from synthetic cRNA templates using the purified viral polymerase complex (Li and Palese, 1992). The results indicate that alteration of the 3' and 5' non-coding sequences of the influenza viral RNA, alters expression of the genes.

Although collectively the P proteins are essential for polymerase activity, the individual function of each of these P proteins is not well understood at present. It is thought that PB1 is responsible for initiation and elongation of viral mRNA synthesis. PB2 is involved in catalysing the initiation of transcription by incorporating guanosine residues onto the primer and the recognition of host mRNAs during transcription (Ulmanen *et al*., 1981).

1.4 VIRUS REPLICATION

When the influenza virus infects a cell, a number of events follows which results in the conversion of the metabolically inert virus into the dynamically active intracellular form of the virus. The outcome of this process of infection is multiplication of the virus which ultimately causes the death of the cell. The replication of the virus involves a sequence of specific events, namely: adsorption and entry, maturation and assembly, and finally, budding and release of the newly synthesised progeny viruses.

1.4.1 Viral attachment or adsorption

Successful infection requires the transfer of the viral genome from the external environment to the interior of the host cell. This first stage of the replication cycle is initiated by the binding of the influenza virus to the outer membrane of the host cell. Although very little is known about the detailed molecular interaction between the virus and the host cell, the amino acid sequence of the viral glycoproteins responsible for attachment is of great importance. Sialic acids are known to be essential components of the cell surface receptors and are distributed in cells of many different tissue types. Binding of haemagglutinin, the major glycoprotein found on the surface of the virus, to sialic acid residues on the host cell surface receptors initiates infection. Single amino acid substitutions in the haemagglutinin have been shown to alter receptor binding capacity and have aided in the localisation of the receptor binding sites (Roger *et al.*, 1983). Hence site specific mutation in the cytoplasmic domain of the haemagglutinin molecule results in varying degrees of internalisation of the virus (Lazarovits and Roth, 1988). Once binding to the host cell membrane is achieved, the next stage is the transport of the virus into the host cell.

1.4.2 Viral entry

The mechanism of entry into the host cell is a subject of much controversy. Entry is thought to occur by phagocytosis, direct penetration of the cell membrane or the process of receptor-mediated endocytosis (viropexis). Recently there has been a growing acceptance that viral entry is achieved by the process of receptor-mediated endocytosis (Helenius *et al.*, 1983; Goldstein *et al.*, 1985; Wileman *et al.*, 1985; Lazarovits and Roth, 1988; Marsh and Helenius, 1989; White, 1990).

Receptor mediated endocytosis occurs at specialised sites on the plasma membrane known as "coated pits" (Figure 3). Ultrastructurally these sites are seen as thickenings on the cytoplasmic side of the membrane, due to the presence of a protein called clathrin.

The virus first binds to the receptors present on the host cell surface. Once bound the virus-receptor complex triggers the membrane around the complex to invaginate and form "coated pits". These pits internalise to form coated vesicles, which are often referred to as endosomes or phagosomes. The virus then releases its nucleic acid in order to initiate new viral production. This is achieved by the fusion of the endosome with the cellular lysosome (Huang *et al.*, 1980; Stegmann *et al.*, 1990). The pH within the lysosomal area (pH 5.5) facilitates the fusion of the endosome and the lysosome. Viral RNA is released and rapidly transported to the the host cell nucleus where transcription and replication of the viral RNA occur (see 1.5). The exact mechanism by which the RNA is transported to the nucleus remains obscure. However, a recent study indicates that the membrane proteins and the RNPs dissociate after internalisation into the host cell, thus allowing the RNPs to be transported from the cytoplasm into the nucleus (Zhirnov, 1990; Martin and Helenius, 1991).

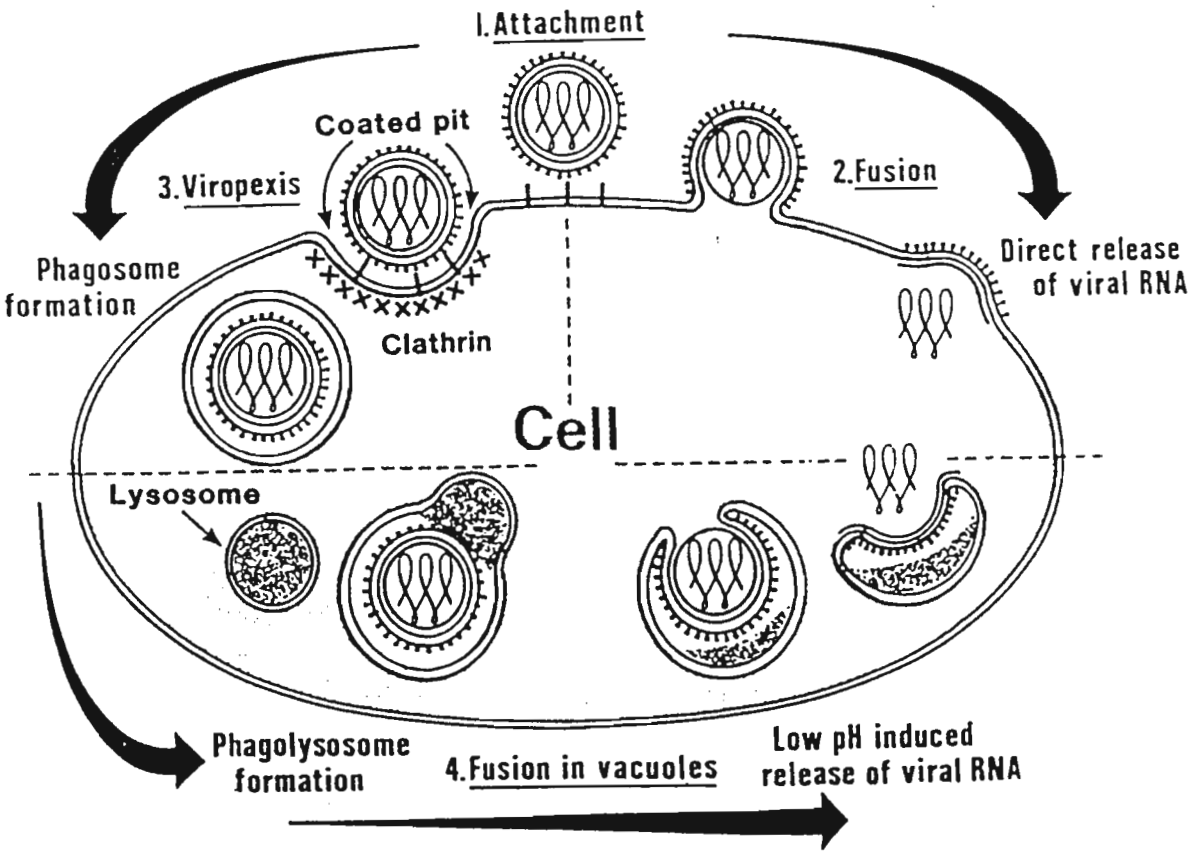

Figure 3. Schematic representation of infection of cells by influenza virus. The sequence of events required for infection are outlined numerically (modified from Stuart-Harris *et al.*, 1985).

1.4.3 Viral maturation and assembly

The process resulting in assembly of the genome and ribonucleoproteins into viral particles is not clearly understood. Viral maturation and assembly are said to occur in the cytoplasm of the host cell. The period between entry of the virus into the host cell and assembly of the components of the progeny virus is known as the "eclipse phase".

Once transcription and replication of the RNA genome (see 1.5) are complete, translation of the messenger RNA (mRNA) into the different viral proteins occurs in the cytoplasm of the host cell. Messenger RNA is translated by the host cell's machinery for synthesising proteins, (ribosomes, transfer RNA, initiation, elongation and termination factors), with only minor modifications being made by the virus, such as cleavage and capping (see 1.5).

During the infective cycle the influenza virus exercises translational control by shutting off host cell protein synthesis and enhancing viral protein synthesis (Lee *et al.*, 1990). The synthesis of the viral proteins is controlled at the transcriptional level. During the early stages of transcription, the only proteins synthesised are PA, PB1, PB2, NP and NS_1. These proteins are referred to as the early proteins. The HA, NA, NS_2 and M proteins are synthesised at a later stage and consequently referred to as the late proteins. The rate of viral protein synthesis is largely dependent on the rate of mRNA synthesis which varies considerably between the eight RNA strands and is said to be under temporal control (Shapiro *et al.*, 1987). Temporal control has was originally shown to exist for mRNAs coding for the NS and NA proteins (Yamanaka *et al.*, 1988), but a recent study by Yamanaka *et al.* (1991) indicates that this is the case for all the mRNAs of the influenza virus. Garfinkel and Katze (1992) have indicated that selective and efficient translation of viral mRNA and not host cell mRNA was due the overall structural configuration of the viral mRNA, which in some way escapes the viral-imposed shutoff of host protein synthesis.

The initial event in the assembly of the progeny virus is the insertion of the membrane glycoproteins HA and NA into the host cell's plasma membrane (Figure 4). Haemagglutinin and NA are thought to reach the membrane via the membranes of the host cell endoplasmic reticulum. Recently, monoclonal antibodies were used to demonstrate the involvement of the endoplasmic reticulum and the golgi apparatus in the assembly of the viral HA molecule (Yewdell *et al.*, 1988). This event is preceded or accompanied by the removal of host proteins from the plasma membrane, followed by alignment of the viral matrix proteins on the internal side of the membrane. The viral RNA is presumed to enter the cytoplasm by diffusion. The exact mechanism by which the progeny virus assumes it final shape is still obscure.

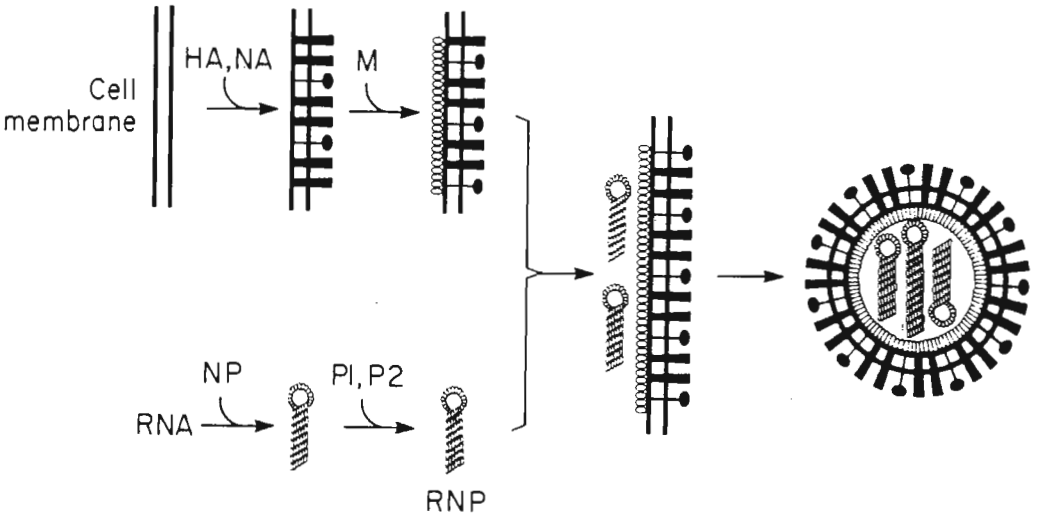

Figure 4. Schematic representation of the stages in influenza virus assembly (from Kilbourne, 1987).

1.4.4 Viral budding and release

This is the final stage in the reproductive cycle of the virus. The actual budding or evagination process by which the influenza virus particles push out of the host cell plasma membrane is not fully understood. It is thought that once the virus particles are formed they are promptly released. This is accompanied by removal of sialic acid residues from the host cell surface by the viral neuraminidase. Removal of these sialic acid residues prevents readsorption of the progeny virus into the host cell, thus promoting the release of the virus into the surrounding tissue. Detachment of the virus from the membrane is the final step. Viruses are not stored in the cells they infect but are released continuously throughout the infective cycle. Viral replication is terminated by the death of the host cell.

1.5 TRANSCRIPTION AND REPLICATION

Transcription is the synthesis of single-stranded RNA molecules which are complementary copies of either a DNA or RNA template. This RNA, known as messenger RNA (mRNA), is the information-carrier molecule of the cell. Translation is the process whereby this information is used to synthesise the corresponding proteins. The genetic information in mRNA codes (in groups of three bases known as a codon) for specific amino acids. The sequence of these codons determines the sequence of amino acids and hence the specific protein to be synthesised.

Replication is the process by which each of the genomic strands is copied precisely by base pairing with complementary nucleotides. Since the influenza A virus has an RNA genome the processes of transcription and replication are identical.

The influenza A virus has a unique transcriptional mechanism because it requires special viral and host cell interactions. The terminal sequences of each of the eight individual viral RNA segments have become a focus of interest because they seem to have an important role in transcription, translation and replication. The 3' and 5' terminals of all eight RNA segments have common sequences of 12 and 13 nucleotides respectively, suggesting a common function for these termini (Lamb and Choppin, 1983; McCauley and Mahy, 1983; Ishihama and Nagata, 1988). These are referred to as conserved sequences. The 5' common terminal sequence is 5'AGUAGAACAAGG 3' (Skehel and Hay, 1978b). This is followed by a triplet unique to each RNA segment and an oligo(U) tract (uridine tract). In most species the sequence for the RNA segment at the 3' terminal is 3'OH-UCGUUUUCGUCC 5', however there is a single base change in this sequence in RNA segments 1, 2, 3 and 7 in the human strain. The sequence for these segments is 3'OH-UCG**C**UUUCGUCC 5'. The 3' and 5' sequences are flanked by non-coding sequences.

RNA-dependant RNA polymerase, the enzyme also known as transcriptase, is responsible for mediating transcription in the influenza virus (Penhoet *et al.*, 1971; Bishop *et al.*, 1971; Chow and Simpson, 1971). This enzyme recognises

specific sites (promotors) on the RNA template to which it binds before initiating transcription. This transcriptase activity is carried out by the three largest proteins in the virus, PB1, PB2 and PA, (see 1.3.5) as part of the RNA-associated RNP polymerase complex. The associated nucleoprotein forms an important structural component of this complex (Honda *et al.*, 1988). A recent study (Seong and Brownlee, 1992) has conclusively shown that influenza RNA polymerase reconstituted *in vitro*, is responsible for both primary and secondary transcription (1.5.1 and 1.5.2). In addition this study shows that the conserved sequences at the 3' and 5' termini are efficient as promoters of transcription. An investigation by Mukaigawa *et al.* (1991) using temperature-sensitive mutants has shown demonstrated that PB2 plays an important role in viral gene regulation.

Two forms of RNA are synthesised following infection (Figure 5). One form is capped polyadenylated RNA which functions as mRNA. Synthesis of these mRNA's is known as primary transcription and these are incomplete transcripts of the viral RNA (vRNA) (Hay *et al.*, 1977a).

The second form of RNA synthesised, is uncapped non-polyadenylated RNA which is known as cRNA. Synthesis of cRNA is known as secondary transcription. This cRNA is replicated to form the new viral RNA genome (see Figure 5).

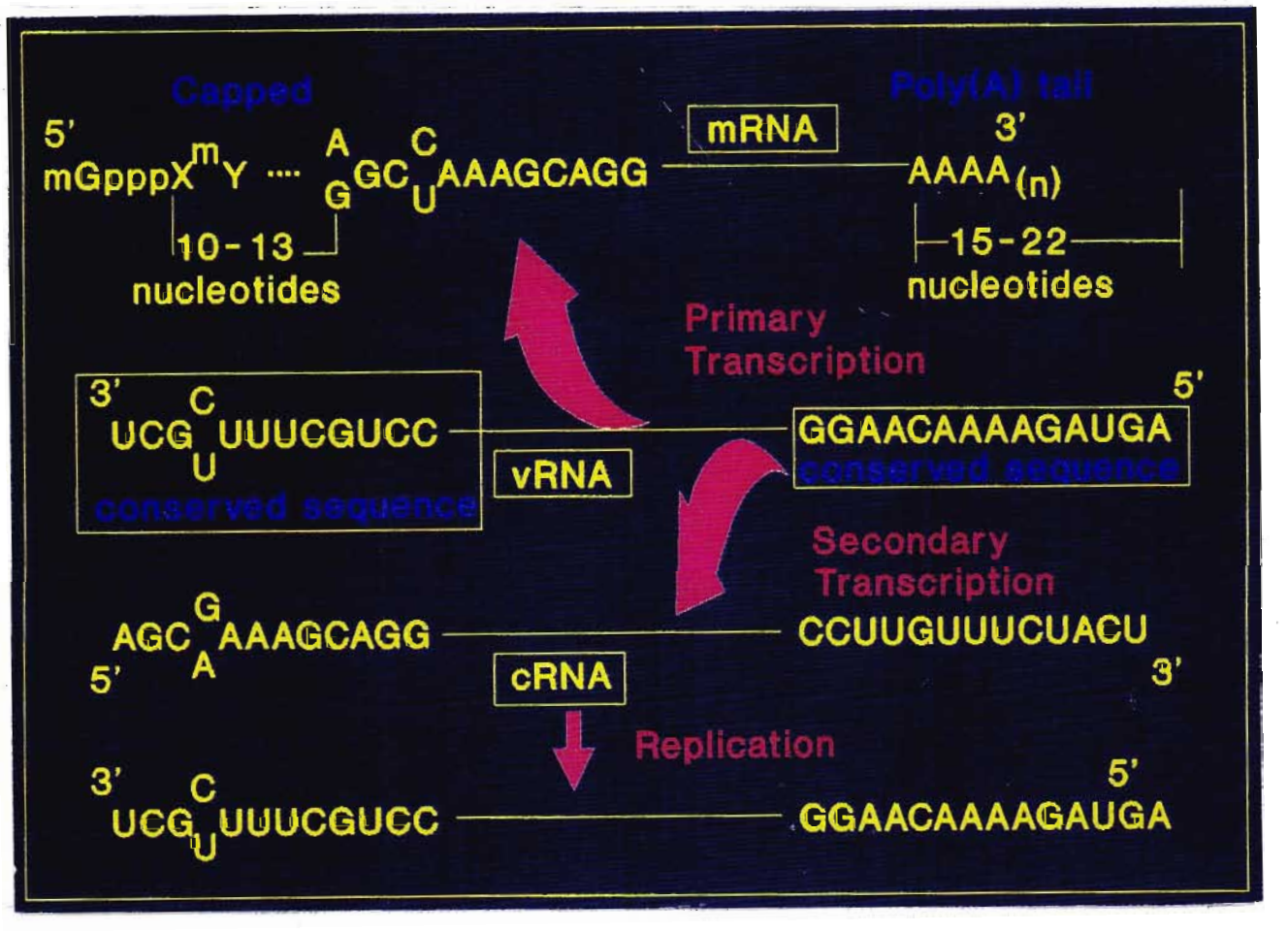

Figure 5. Schematic representation of the synthesis of the two forms of influenza RNA. The conserved sequences are shown at the 5' and 3'-terminal ends in the vRNA and cRNA. Messenger RNA is capped at the 5'-terminal and polyadenylated at the 3'-terminal. Note that secondary transcription to form cRNA, followed by replication of the cRNA will result in an exact copy of the vRNA genome.

1.5.1 Primary transcription

Primary transcription of the viral RNA in the host cell to form mRNA's occurs from all eight strands after infection of a cell. The identification of all eight transcripts of RNA's in the nucleus using pulse labelling suggests that mRNA is synthesised in the nucleus of the infected cell (Herz *et al.*, 1981). These mRNA's are translated into specific viral proteins required for the new progeny virus.

Influenza viruses employ a mode of mRNA synthesis which is different from that of other viruses (Figure 6). The first step is the capture of host cell mRNA's (heterogeneous nuclear RNA). This is then cleaved at specific sites, producing 5' methylated capped RNA (Krug *et al.*, 1976). Capping of the RNA involves the addition of a 7-methylguanosine residue to the 5' end of the mRNA which protects the mRNA from nuclease and phosphatase activity. These methylated capped fragments (m^7GpppNm) are 10 to 13 nucleotides long and are used as primers for viral transcription (Bouloy *et al.*, 1978; Krug *et al.*, 1979; Plotch *et al.*, 1979; Dhar *et al.*, 1980; Beaton and Krug, 1981) (Figure 6). This reaction is catalysed by the PB2 protein (Ulmanen *et al.*, 1981). Utilisation of the host mRNA appears to be unique to the influenza virus. The analysis of the 5' end sequences of the mRNAs confirmed that these sequences are of non-viral origin (Krug *et al.*, 1979). The host cell mRNA's thus act as primers for viral transcription *in vivo*. There is also evidence that the dinucleotides ApG and CpG, when used as primers, can stimulate transcription *in vitro* at specific positions on the viral RNA template (Honda *et al.*, 1986).

Cleavage of the capped RNA primer is followed by the incorporation of guanosine residues onto the 3' terminal end of this primer which initiates transcription. This process requires interaction between the primer and the proteins in the transcriptase complex and is catalysed by the PB1 protein (Ulmanen *et al.*, 1981). Elongation then follows in the presence of the four nucleotide triphosphates which form the building blocks of the new RNA.

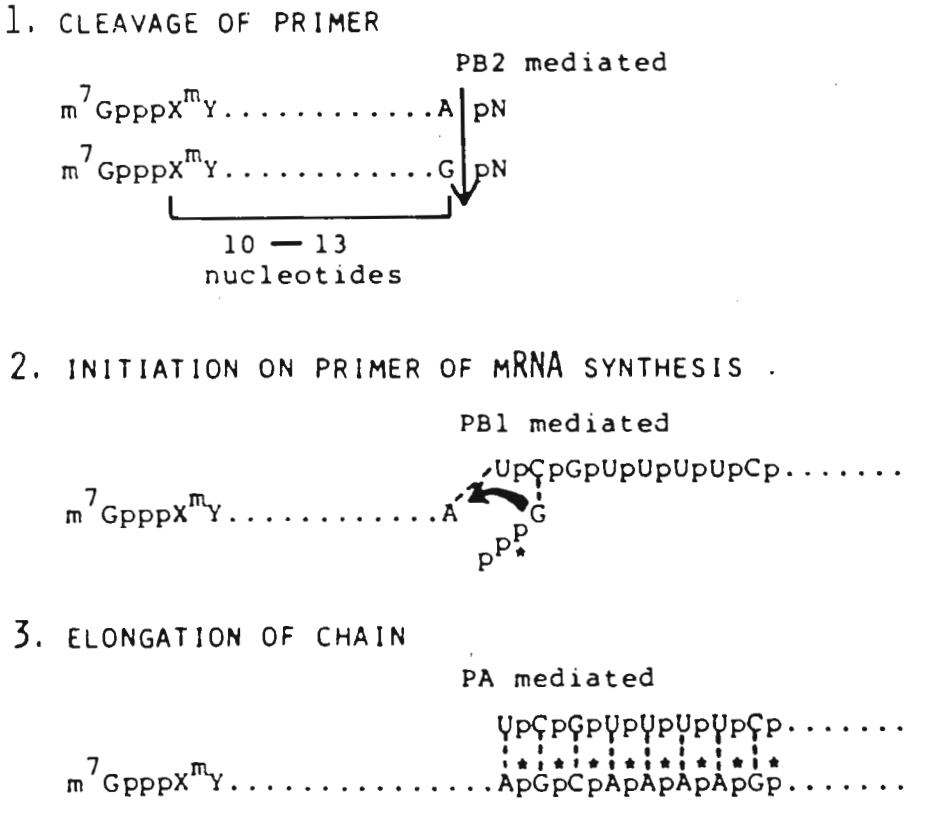

Figure 6. Priming of influenza mRNA transcription using host cell mRNA (from Kilbourne, 1987).

1.5.2 Secondary transcription

Secondary transcription is also thought to occur in the nucleus. The cRNA synthesised is a complete transcript of vRNA and functions as a template for genome replication (Hay *et al.*, 1977b). Following replication, the the new viral RNA is incorporated into newly synthesised progeny viruses. Synthesis of cRNA's is known as secondary transcription. Both classes of RNA synthesis are mediated by the viral RNA-dependent RNA polymerase and the viral nucleoproteins (Shapiro and Krug, 1988).

1.6 METHYLATION

One of the major unanswered questions in biology is the process by which eukaryotic cells control gene expression to give rise to different functional cell types, all of which contain the same genetic information. Cellular differentiation appears to be dependent on the activity or inactivity of certain areas of the genome. Primary control occurs at transcriptional level, but post-transcriptional and post-translational control may also regulate functional activity.

Experimental evidence indicates a link between methylation patterns of genomic DNA or RNA and transcriptional activity (Bird, 1984). Methylated bases have been observed in both prokaryotic and eukaryotic DNA. Methylation occurs in the base adenine at the N^6 position and in cytosine, at the N^4 or N^7 position (Figure 7).

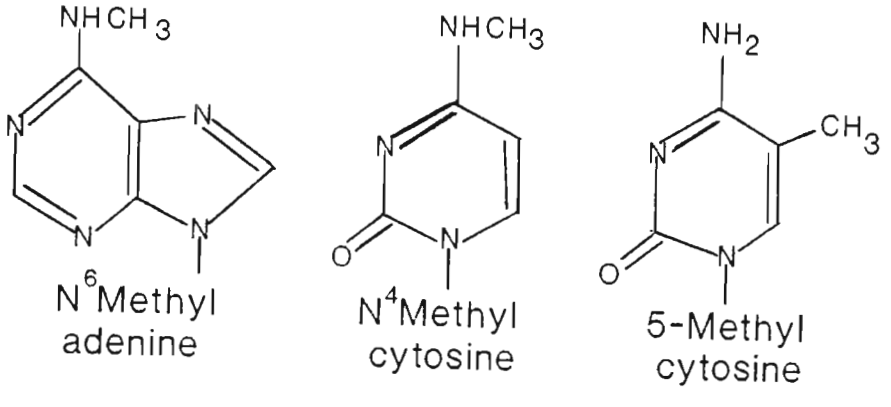

Figure 7. Methylated sites on the bases adenine and cytosine.

Methylation of these bases occurs only after DNA synthesis has occurred. The reaction is catalysed by specific methylase enzymes in the presence of methyl group donors (Figure 8).

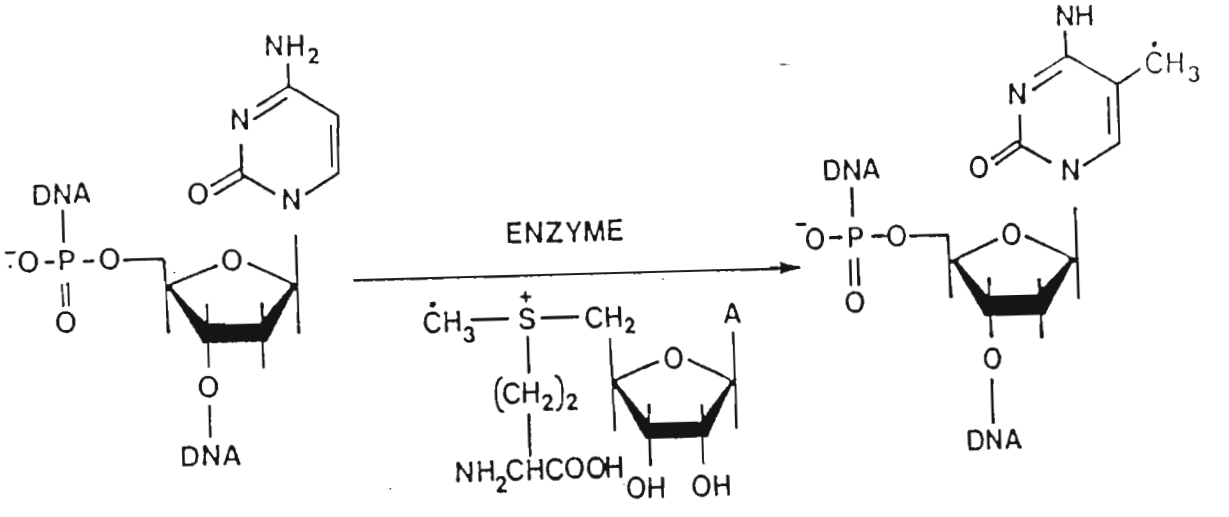

Figure 8. Methylation of cytosine by the transfer of a methyl group to the 5' position of the cytosine residue, using S-Adenosylmethionine as substrate and methylase as the enzyme.

The methylated base 5' methylcytosine (5'Me Cyt) was first described about 40 years ago and since then it has been demonstrated in a variety of prokaryotic and eukaryotic organisms (Doskocil and Sorm, 1962).

A number of highly sensitive tests (HPLC, GC, mass spectrometry and antibody testing against the 5' Me Cyt) are available to identify and quantify these methylated cytosine bases. These techniques have provided valuable information about the location and distribution of the methyl groups.

Another technique for identification of methylated bases involves the use of restriction enzymes. Restriction enzymes are enzymes isolated from bacteria and are widely used to identify DNA sequences. These enzymes are highly specific in that they are capable of recognising and cutting DNA at unique sequences of four or six nucleotides long.

The methylation site in eukaryotic DNA is almost invariably the 5' end of the cytosine residue. In addition the 5' Me Cyt occurs at specific sequences, usually linked to a guanosine residue on the 3' end (5'CpG 3') (Figure 9) (Ehrlich and Wang, 1981).

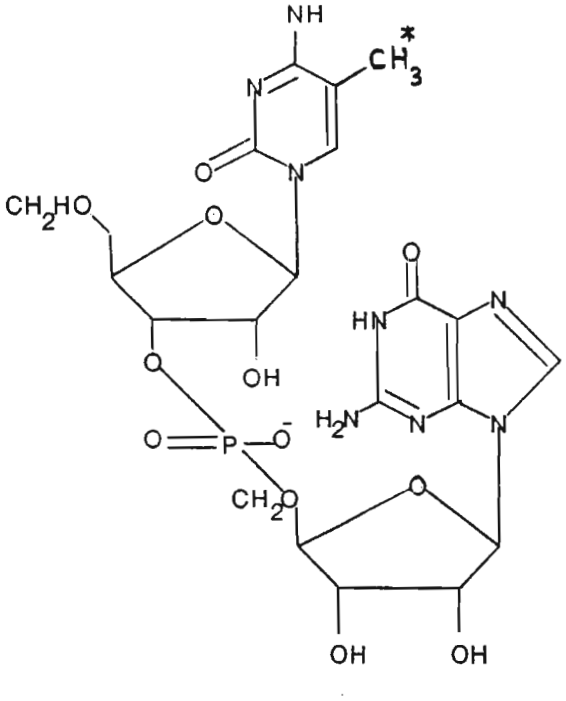

Figure 9. Structure of 5'methyl cytosine linked to guanosine.

Most restriction enzymes do not cleave at restriction sites that have been methylated, but some restriction sites are recognised by more than one restriction enzyme. These restriction enzymes are referred to as isoschizomers (Old and Primrose, 1988). An example of isoschizomers are the restriction enzymes **HpaII** and **MspI**, both of which recognise the four base sequence 5' CCGG 3' and cleave between the inner and outer cytosine residues as shown below.

5'----C/CGG----3'

3'----GGC/C----5'

If the inner C residue within this sequence is methylated then HpaII fails to cut at this site, however MspI activity is not affected (Waalwijk and Flavell, 1978). Hence by using a combination of these enzymes identification of the methylated base is easily achieved.

In addition to identification of methylated sequences, there are methods available that allow specific methylation of DNA sequences. The enzyme HpaII methylase has been used to introduce methyl groups into given DNA sequences at specific bases. The impact of this has been that manipulation of the DNA through methylation has allowed researchers to study functional expression of these gene sequences compared to their unmethylated counterparts.

Investigations into the relationship between gene expression and methylation have shown that the degree of methylation of active regions of the chromosome is considerably lower than that of inactive regions. Critical reviews (Doerfler, 1983; Bird, 1984; Cedar, 1988; Bird, 1992) have described highly methylated regions of DNA to have little or no transcriptional activity (these areas of DNA are said to be transcriptionally silent), whereas unmethylated regions of DNA are found to be transcriptionally active. The impression gained from these studies is that methylation of certain CpG residues results in inactivation of specific genes while demethylation of these residues causes activation of gene expression.

Studies on viral systems grown in cell culture also confirm a link between methylation and gene expression. Herpes virus sequences present in non-producer cells appear to be extensively methylated whereas the viral DNA is not methylated in cells active in viral production (Desrosiers *et al.*, 1979). When a recombinant cell line containing the hepatitis B virus core (HBc) gene was treated with 5'-aza-cytidine (a potent inhibitor of methylation in cytosine), the result was a five-fold increase in the HBc gene transcripts (Korba *et al.*, 1985). This indicates that specific inhibition of methylation results in activation of transcription.

Although methylation has been found to occur in RNA, very little is known about the function of these methyl groups in the RNA strands. Several different enzymes of varying specificities have been reported. Some enzymes are involved in reactions which produce methylated bases in soluble RNA in *E. coli* (Hurwitz *et al.*, 1964), whilst other enzymes specifically methylate the cytosine residue 5' CpG 3' in RNA (Grippo *et al.*, 1968). Methylation of specific bases has also been reported in *E. coli*, in which uracil methylating enzymes catalyse the methylation of uracil to produce thymine (Fleissner and Borek, 1962). The messenger RNA of the Reovirus has also been shown to be methylated during its synthesis (Shatkin, 1974). Introduction of methyl groups onto the cytosine residues in C-G dinucleotides of a retrovirus proviral genomic clone, resulted in reduced activity of this gene (Simon *et al.*, 1983). These studies, however provide very little information on the function of these methylated bases.

With respect to the influenza A virus, a study by Khan *et al.* (1984), showed that methylation of primers resulted in non-recognition of such primers by the influenza A viral polymerase enzyme, and consequently failure of complementary synthesis of the RNA genome. In addition when synthetic polymers (modified to contain 5' Me Cyt) were used as the viral RNA template, the RNA-dependent RNA polymerase failed to carry out complementary synthesis of the RNA genome (Khan *et al.*, 1987). Thus it seems that methylation may contribute to the regulation of transcription and replication of the influenza viral genome.

1.7 FORMULATION OF PRESENT STUDY

The influenza A viral surface glycoproteins have been the focus of extensive research for many years, and have contributed significantly to our understanding of viral structure and the mechanism of host cell entry. However, there has been little progress in attempts to control viral pathogenicity. Recent advances in molecular technology have made it possible to study cellular activity at the genomic level, thus making it possible to probe into mechanisms that control gene transcription, the first step in genomic replication.

From the literature it is apparent that methylation plays a important role in the control of gene expression. However, the mechanism by which methylation brings about such effects is not entirely understood. In addition there is very little information on the degree of methylation of the influenza A viral genome and how this relates to the pathogenecity of the virus. More importantly, the examination of the gene sequence of the influenza polymerase RNAs and possible manipulation thereof, may provide valuable information on the control of influenza viral replication.

The influenza A viral RNA-dependant RNA polymerase is intimately involved in the process of transcription and replication. It would therefore seem useful to investigate the effects of modifying the RNA responsible for the production of the polymerase enzyme by the method of enzymatic methylation. Since the isolated viral RNA cannot be tested in a transfection system, it will be necessary to prepare cDNA copies of the polymerase RNAs which may then be transfected into a suitable vector for expression studies. In order to isolate the RNAs coding for the RNA polymerase, a number of preliminary investigations are necessary.

The aim of the present study was to formulate a method for the successful isolation of the RNA coding for this enzyme from which cDNA could be synthesised. The following experimental procedures were carried out:

1. Allantoic fluid of chick embryos was infected with the influenza A virus, a method commonly used to propagate the virus. The harvested fluid was subjected to extensive tests to verify the presence of the virus and to check for contamination.

2. The virus was isolated and purified by ultracentrifugation and sucrose gradient ultracentrifugation.

3. The presence of the virus was determined by:
    a. Haemagglutination titration
    b. Ribose determination
    c. Electron microscopy
    d. Tissue culture and immunofluorescence

4. The viral RNA genome was isolated and separated by electrophoresis. The polymerase genes were identified using RNA molecular weight markers.

5. The polymerase genes were isolated and purified for use as template for cDNA synthesis.

6. Complementary DNA copies of the polymerase genes were synthesised.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL ASEPTIC TECHNIQUE

Aseptic techniques were employed for all experiments described below, since contamination of any kind would have resulted in incorrect experimental interpretation. One of the major problems encountered when working with RNA is the effect of ribonucleases (RNAses). Virtually all biological cells and tissues, especially human skin, are rich in RNAses. RNA is susceptible to degradation by these ribonucleases which randomly cut the RNA into smaller fragments (Pritchard and Holland, 1985). Further, RNAses are very stable and active enzymes that require no co-factors to function.

A number of precautions were taken to maintain sterility and prevent RNAse activity. Sterile, disposable plastic-ware was used wherever possible. Aluminium foil was fitted over the openings of all glass-ware, before autoclaving at 121°C for 20 minutes. Distilled water was also autoclaved before use. Work surfaces were swabbed with 90% methanol before and after use. Gloves were worn during all experimental procedures. Masks and gowns were worn during operations involving viral inoculation and isolation. All procedures were carried out under the vertical laminar flow hood unless otherwise stated. All chemicals used were of Molecular Biology Grade and were purchased from Merck, Boehringer Mannheim, Promega and Sigma Chemical Corporation.

All disposable equipment was discarded after use into a stainless steel receptacle containing biocide, while glass-ware and re-autoclavable items were soaked in a bucket containing biocide together with detergent, before washing. All materials and apparatus used in the viral inoculation and isolation work, including sub-cellular fractions (eg. pellets and supernatants from centrifugation), were decontaminated by autoclaving before disposing into specialised Biohazard medical waste boxes.

2.2 INFECTION OF CHICK EMBRYOS WITH INFLUENZA A VIRUS

2.2.1 Virus and chick embryos

The mildly pathogenic 1978 Brazilian H1N1 strain influenza A virus was used in this study. The virus was obtained in the freeze-dried form from the the National Institute of Virology, Johannesburg, Transvaal.

Ten day old embryonated chick eggs were obtained from Rainbow Chicken Farms Laboratories (Hammersdale, Natal).

2.2.2 Procedure

Infection of chick embryos with influenza A virus was carried out using the method of Dowdle *et al.* (1979). Immediately prior to inoculation of chick eggs, the freeze-dried virus was reconstituted by suspending it in phosphate-buffered saline (PBS). The eggs were trans-illuminated using an egg candling box to check the viability of the embryos and also to mark the inoculation points. After swabbing the eggs with methanol (90%), 0.2 ml of virus (haemagglutination titre value of 1 : 128 - section 2.4.2) was injected into the allantoic cavity with a 25 gauge syringe needle. The inoculation was carried out on batches of 30 eggs. The inoculation points were then sealed with bees' wax and incubated for 48 hours at 33°C. The eggs were candled daily to check for viability of the embryos. Embryos showing no signs of movement within the first 24 hours were discarded.

2.3 ISOLATION AND PURIFICATION OF THE INFLUENZA A VIRUS FROM THE ALLANTOIC FLUID

2.3.1. Isolation of the allantoic fluid from the embryos

The isolation and purification procedure was adapted from the method of McGeoch and Kitron (1975). After incubation for 48 hours at 33°C, the eggs containing the influenza virus were chilled overnight at 4°C before extraction of the allantoic fluid. Storage at 4°C causes the blood vessels in the embryo to constrict thereby reducing contamination of the allantoic fluid in the event of blood vessels being ruptured during the extraction procedure. Subsequent steps were carried out under the vertical laminar flow hood. The temperature was maintained below 5°C, by working in an ice box. The eggs were swabbed with methanol (90%) before the air sac was exposed. A pair of scissors was used to pierce and cut the egg shell around the air sac. The allantoic fluid was harvested using a sterile Pasteur pipette and pooled into a sterile bottle. Aliquots of 2 ml viral suspension were stored at -20°C for subsequent inoculations, while some was streaked on blood agar plates to check for fungal and bacterial contamination (section 2.4.1). Quantification of the virus was established by the Haemagglutination Titration Test (section 2.4.2). The viral suspension was diluted 10 fold before inoculation into the next batch of eggs. The above procedure was carried out through 6 passages, using 30 eggs for each passage. This was necessary to obtain a large quantity of virus.

2.3.2 Purification of the virus

2.3.2.1 Ultracentrifugation

NTE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA pH 7.6) required for this procedure was prepared as follows:

Sodium chloride (0.58 g), Tris-HCl (0.12 g) and EDTA (0.37 g) were dissolved in 80 ml of sterile water. The pH of the solution was adjusted to 7.6 with 0.1 N HCl and made up to 100 ml with sterile water. The solution was sterilised by autoclaving.

The virus was purified by a series of centrifugation steps. The isolated allantoic fluid was first centrifuged at low speed, (5000 x g) for 15 minutes at 4°C in a Beckman J2-21 centrifuge. The pellet, containing red blood cells and other debris was discarded and the supernatant removed and stored at 4°C. The fluid was then ultracentrifuged at 100 000 x g for 90 minutes at 4°C in a Beckman L8 55 ultracentrifuge, using ultraclear polyallamer tubes in the SW 28 rotor. The viral pellet was carefully resuspended in 0.5 ml of NTE buffer (pH 7.6), transferred to sterile storage tubes and kept at 4°C. Aliquots of this viral sample were used for the ribose estimation (section 2.2.4) and electron microscopy (section 2.4.3).

2.3.2.2 Sucrose gradient ultracentrifugation

In initial experiments the viral pellet obtained after ultracentrifugation (section 2.3.2.1) was purified further using sucrose gradient ultracentrifugation. The viral sample was suspended on 15% to 60% sucrose solution made in NTE buffer (pH 7.6). Ultraclear polyallamer Beckman tubes and the SW 28 rotor were used for this procedure. The use of an automatic Pipetteman facilitated layering the sucrose solutions. The sample was centrifuged in the Beckman L8 55 ultracentrifuge at 60 000 x g for 90 minutes at 4°C. The viral sample was carefully removed and resuspended in 0.5 ml NTE buffer (pH 7.6) before dialysing against NTE buffer (pH 7.6) for 24 hours. Two buffer changes were

made over the same period. The dialysed viral sample was transferred to sterile tubes and stored at 4°C. Subsequent experiments showed that the crude viral pellet gave comparable results to the sample obtained after sucrose gradient ultracentrifugation with regard to the purity. In all subsequent experiments this step was then omitted.

2.4 ANALYSIS OF VIRAL SAMPLE

Since the ultimate aim of the study was the isolation of the polymerase genes from the influenza A virus, it was necessary to verify the presence of the virus in the allantoic fluid and viral RNA. Tests for possible contaminants such as bacteria and fungi were also included.

2.4.1 Inoculation of blood agar plates

It was necessary to inoculate blood agar plates with samples of the extracted allantoic fluid (section 2.3.1) to verify the absence of contamination by bacteria and fungi. Blood agar medium supports growth of many fastidious organisms, including fungi and pathogenic and non-pathogenic bacteria.

Blood agar plates were obtained courtesy of the Microbiology Department, Natal University Medical School. Using a sterile loop, an initial dense inoculum was made on one end of the agar plate. The loop was flamed and a streak was made from the densely inoculated area. The plate was then incubated at 37°C overnight, before checking for any growth in the medium.

2.4.2 Haemagglutination titration test

2.4.2.1 Principle

Influenza viruses have the ability to agglutinate chicken and human O erythrocytes (Murakami *et al.*, 1991). This agglutination property is due to spike-like projections which are found distributed over the entire outer surface of the virus. These glycoproteins, referred to as haemagglutinin, become adsorbed onto the surface of the erythrocytes. As the virus is able to attach to more than one erythrocyte at a time, bridges are formed between the cells, resulting in a lattice-like network causing clumping (agglutination) of the cells. These clumps then settle to the bottom and form a typical haemagglutinating pattern. Where no agglutination occurs, the erythrocytes rapidly sediment to the bottom of the plate, resulting in a typical "button-like" pattern. Button formation was used as the control, since its presence indicated the absence of virus. The agglutination test is a convenient and reliable test to check for the presence of the influenza virus as well as to determine viral concentration.

2.4.2.2 Procedure

The method used was that of Dowdle *et al.* (1979). Phosphate-buffered saline pH (7.6) (50 µl) was added to each of the 12 wells (from left to right) of a round-bottom haemagglutination microtitration plate (Figure 11). This was repeated in 3 rows (from top to bottom). Serial dilutions of 50 µl of viral fluid were added in duplicate to the first well in each of the first two rows. The third row had no viral fluid and this served as the control. Fifty microlitres of washed human O erythrocytes (0.4% in PBS) was added to all the wells. The titration plate was gently agitated and incubated for 1 hour at 4°C before checking for agglutination.

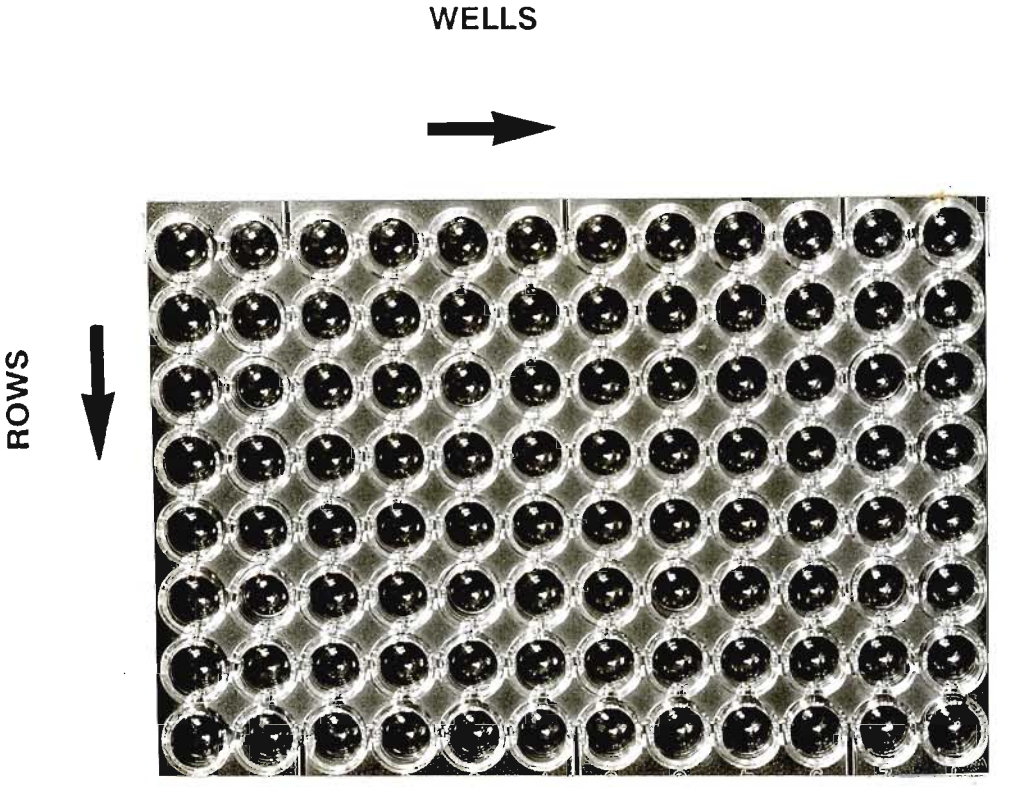

Figure 10. Haemagglutination titration plate showing wells, from left to right and rows, from top to bottom.

2.4.3 Electron microscopy

Electron microscopy is an important diagnostic tool used for identifying viruses (Doane and Anderson, 1987) and likely contaminants (bacteria and fungi). The shape and size of these organisms are the main features used for identification. Both negative and positive staining techniques were used to identify the virus.

2.4.3.1 Principle

Negative staining is a rapid method for detecting viruses (Doane and Anderson, 1987). Metal salts such as phosphotungstic acid (PTA) when mixed with the specimen and air-dried on the specimen grids, form an electron-dense matrix around the more electron-translucent virus particles. This process enhances the contrast and makes it possible to visualise the specimen. Positive staining uses conventional processing methods where the virus is fixed, embedded and cut.

2.4.3.2 Positive staining for virus identification

The procedure adopted for processing of the virus was the routinely used double fixation method in Karnovsky's fixative (Karnovsky, 1965).

Some of viral pellet obtained from ultracentrifugation (2.3.2) was placed in an Eppendorf tube and microfuged to collect the viral material into a tight pellet. The processing schedule is outlined in Appendix A.

2.4.4 Ribose estimation

2.4.4.1 Principle

Since the influenza A virus is an RNA virus the ribose estimation test is a good indication of the presence of RNA in the isolated sample. The test is based on a colour reaction produced as a result of the reaction between a pentose sugar and concentrated HCl. This reaction forms furfurals which condense with orcinol in the presence of ferric ions to give a blue-green colour (Bruckner, 1955).

2.4.4.2 Reageants

Ribose standard solution (30 µg/ml) D-Ribose (50 mg) was dissolved in 500 ml of distilled water. This solution was stored at 4°C after use.

Orcinol solution Orcinol (0.6 g) was dissolved in 10 ml of 90% ethanol.

Ferric chloride (0.5% in concentrated HCl) $FeCl_3.6H_2O$ (0.25 g) was dissolved in 500 ml concentrated HCl.

2.4.4.3 Procedure

D-ribose (0-1 ml aliquots of the 30 mg/ml standard solution) was diluted to a final volume of 1 ml with distilled water to give the required ribose concentration (0-30 µg/ml). Ferric chloride (2 ml) was added to the D-ribose standard

solutions and the viral sample. Orcinol (0.14 ml) was subsequently added and the mixture was vortexed before incubating in a boiling water bath for 20 minutes. The mixture was cooled and the absorbance at 665 nm was read against a blank of distilled water. A standard ribose curve was obtained and the ribose content in the viral sample was calculated from the standard curve.

2.4.5 Immunofluorescence

Immunofluorescence was used to confirm that the isolate was the influenza A virus (Dowdle *et al.*, 1979) because of possible contamination from influenza B virus. Vervet Primary Monkey Kidney cells (PMK cells) were infected with the allantoic fluid isolated from 10 day old chick embryos and tested with anti-mouse IgG fluorescein conjugated anti-influenza A and B monoclonal antibodies. These antibodies were made against the HA and NA surface glycoproteins of the influenza A virus.

2.4.5.1 Infection of Vervet Monkey Kidney cells with the virus.

Vervet Primary Monkey Kidney cells were obtained courtesy of the Virology Department, Natal University Medical School. These cells were infected with allantoic fluid isolated from chick embryos (section 2.3.1).

The cells were grown at 37°C as rolling culture tubes containing 5 ml of Minimum Eagles Medium (MEM). These tubes also contained a glass cover slip. The allantoic fluid isolated from chick embryos infected with influenza virus was filter-sterilised (Millex - GS 0.22 μm Millipore filter) before inoculating the culture medium (200 μl per 5 ml culture). Culture tubes were incubated at 37°C for 48 hours.

2.4.5.2 Preparation of cells for Immunofluorescence

Preparation of the cells for immunofluorescence was carried out according to the method described in the kit supplied with the antibody (Gull Laboratories, Inc. SLC, UT, USA). The cells from the inside of the culture tube were carefully scraped off with a sterile Pasteur pipette. These cells together with the growth medium were transferred to a Bijou bottle and discarded. Approximately 3 to 4 ml of cold acetone was added to the culture tube containing the cover-slip. The tube was shaken gently until there was a turbid appearance before discarding the contents. A second incubation with 4 ml acetone was then performed for 10 minutes at 4°C. The cover-slip was removed and fixed onto a glass slide with the cells facing upwards. A diamond knife was used to split the cover-slip in two. Two drops of anti-influenza A conjugate (monoclonal antibody against influenza A) was added onto the one half of the cover-slip and anti-influenza B was added to the other half of the cover-slip. These slides were incubated for 30 minutes at 37°C, in the dark before viewing with a fluorescence microscope (40X objective in the dark). The slides were photographed with 64 ASA Ectochrome slide colour film using a shutter speed of 2 minutes.

2.5 ISOLATION OF RNA

It was particularly important to obtain a good quality RNA preparation since this ultimately determines the quality and yield of the polymerase genes as well as the cDNA synthesised from it. Many methods are currently used to isolate RNA, but in this study the method of Chirwin *et al.* (1979) was chosen because of its proven ability to restrict ribonuclease activity. This method uses guanidinium thiocyanate which is a strong detergent and ß-mercaptoethanol which is a reductant. The combination of these two reagents breaks down protein disulphide bonds which are essential for ribonuclease activity.

2.5.1 Reagents

Homogenising solution [4 M Guanidinium thiocyanate, 0.025 M Sodium citrate, 0.5% sarcosyl, 0.1 N ß-mercaptoethanol]. Guanidinium thiocyanate (250 g), was dissolved in the original bottle using 293 ml of sterile water. 17.6 ml of 0.75 M sodium citrate pH 7.0 (previously prepared and autoclaved) and 26.4 ml 10% sarcosyl (previously heated to 65°C to dissolve) were added to this solution. This stock solution was stored for up to three months at room temperature. Immediately before use, 0.36 ml ß-mercaptoethanol was added to 50 ml of the stock solution in a fume hood.

Phenol (water saturated). Phenol (100 g) (Sigma), was dissolved in its bottle by heating at 65°C in a water bath. Sterile water was added up to the neck of the bottle before shaking thoroughly. Most of the water was removed and this solution was stored at 4°C for one month.

Sodium Acetate (2 M pH 4). Sodium acetate (16.4 g) was dissolved in 80 ml of sterile water and glacial acetic acid was used to adjust to pH 4, before making up to 100 ml with sterile water. This solution was sterilised by autoclaving.

Chloroform/isoamyl alcohol. Chloroform (49 ml) was mixed with isoamyl alcohol (1 ml) in a fume hood and stored at room temperature.

Ethanol. Absolute ethanol was diluted to 75% with sterile water and stored at -20°C.

2.5.2 RNA extraction from viral pellet

The viral pellet obtained from ultracentrifugation (2.3.2) was resuspended in NTE buffer (2 ml). The resuspended pellets were pooled in a polypropylene tube and an equal volume of homogenising solution (pre-cooled on ice for 5 minutes) was added to the tube. This was then transferred to a "Dounce Type" glass homogeniser and gently homogenised. The homogenate was then transferred to a sterile polypropylene tube. Sodium acetate (pH 4, 0.1 ml of a 2 M solution), 1 ml water saturated phenol and 0.2 ml chloroform/isoamyl alcohol (49:1) were added per ml of homogenate present. The above solutions were added sequentially with gentle mixing after each addition. The final mixture was shaken vigorously for at least 10 seconds and incubated on ice for 15 minutes. The emulsion was centrifuged for 20 minutes at 10 000 x g at 4°C in the Beckman J2-21 centrifuge. The upper aqueous phase was very carefully removed with a sterile Pasteur pipette and transferred to a new polypropylene tube. Care was taken not to disturb the protein precipitate at the interface of the upper and lower phases. The sample was re-extracted using the above procedure. The clear upper aqueous phase was saved for RNA precipitation.

2.5.3 RNA precipitation

The aqueous phase obtained from the second extraction step was transferred to a new tube and mixed with 2 volumes of cold (-20°C) absolute ethanol. The mixture was placed in the deep freeze (-20°C) overnight to allow the RNA to precipitate. Following centrifugation at 10 000 x g for 20 minutes at 4°C, the supernatant was discarded. The pellet was washed using 400 µl of 75% ethanol (-20°C) and then centrifuged for 2 minutes. The pellet was then air dried. The RNA pellet was resuspended in 100 µl of sterile water and the RNA concentration was estimated as described in section 2.5.4 before storing at -70°C.

2.5.4 Assay for concentration and purity of RNA

The RNA concentration was estimated using ultraviolet spectrophotometry (Davis *et al.*, 1986) in a Milton Roy Spectronic 3000 Array Spectrophotometer. The RNA sample (4 µl) was mixed with 996 µl of sterile water to give a dilution of 1:250. This was then transferred to an acid-washed quartz cuvette. The spectrophotometer was blanked with sterile water before the absorbance was read between wavelengths 200 nm and 300 nm. A crude estimation of the sample purity was determined from the ratio of the absorbance at 260 nm to the absorbance at 280 nm. The concentration was determined by reading at 260 nm where an estimate of 40 µg/ml is equal to an absorbance of 1.

2.6 FORMAMIDE/AGAROSE GEL ELECTROPHORESIS OF RNA

The formamide/agarose gel electrophoresis was previously used for separation of eukaryotic single-stranded RNA (Lehrach *et al.*, 1977) and this procedure was adopted for separating the influenza genomic RNA.

2.6.1 Reagents

MOPS Running Buffer (10x) [0.2 M MOPS, 0.01 M EDTA, 0.05 M Sodium Acetate pH 7]. MOPS (8.36 g), EDTA (0.74 g) and Sodium Acetate (0.82 g) were dissolved in 150 ml of sterile water. The solution was then adjusted to pH 7 using 5 M NaOH and then made up to 200 ml with sterile water.

Agarose gel (1.5%). Agarose (1.5 g) was dissolved in 72.1 ml of sterile water and 10 ml of running buffer (10x) in a covered 200 ml conical flask (baked). This mixture was heated in the microwave for 2 minutes before cooling to 45°C. Formaldehyde (17.9 ml) was added to the cooled mixture. The gel was then poured into the casting tray and the combs were positioned 10 mm from one end of the gel. The gel was allowed to set for 30 to 60 minutes in the fume hood.

Sample Buffer. Running buffer (200 µl of 10x solution), 1 ml deionised formamide and 356 µl formaldehyde (37%) were freshly prepared before each electrophoretic run.

Dye solution. [7.5% Ficoll 400, 0.1% bromophenol blue and ethidium bromide (1 mg/ml)].

Sample Preparation. RNA (4 µl), equivalent to a concentration of 40 µg was heated for 5 minutes at 65°C before 8 µl sample buffer, 4 µl dye and 1 µl ethidium bromide (10 µg/ml) were sequentially added.

RNA marker. The RNA marker (5 µl) was treated exactly as the sample above.

Tank buffer. Running buffer (10x, 150 ml) was diluted to 1.5 L with sterile water.

2.6.2 Procedure

The electrophoretic tank was placed on a level surface and filled with 1.5 L running buffer. The comb used to form the wells was carefully removed and the gel tray was submerged into the electrophoretic tank. The samples and the RNA markers (3911 to 363 base pairs) were carefully loaded into the wells using a micropipette. Electrophoresis was carried out at 20 V for 17 hours.

2.6.3 Photography of the gel

Following electrophoresis the gel was placed on the Camag ultra-violet transilluminator and viewed at long wavelength (300 nm). Polariod 667 film was use to photograph the gel.

2.7 ISOLATION OF RNA FROM FORMAMIDE/AGAROSE GEL

The appropriate RNA band (2300 base pairs) (Lamb and Choppin, 1983) was identified by correlating the position of the band with the molecular weight markers. A sterile scalpel blade was used to excise the RNA band from the gel. The gel slices were transferred into labelled, pre-weighed Eppendorf tubes and extracted using the BIO 101 RNAid kit which was developed using methods described by Chirgwin *et al*. (1979) and Chomozynski and Sacchi (1987). The weight of each gel slice was determined and 3 volumes of RNA binding salt (pH 5) were added to each tube before extraction was performed as outlined in Appendix B. Following extraction the RNA concentration was determined as previously described (section 2.5.4)

2.8 COMPLEMENTARY DNA (cDNA) SYNTHESIS OF THE POLYMERASE GENES OF INFLUENZA A VIRUS

2.8.1 Introduction

Complementary DNA (cDNA) synthesis was carried out using the cDNA synthesis kit purchased from Boehringer Mannheim which is essentially the method of Gubler and Hoffmann (1983). The kit allows for the transcription of 25 μg of RNA into cDNA, using avian myeloblastosis virus (AMV) reverse transcriptase. Since total RNA and not mRNA was isolated from the virus the 3' end primer had to be synthesised. The 3' end of the polymerase genes has a 12-nucleotide sequence that is identical in all influenza A viruses. A 12-nucleotide primer complementary to the 3' conserved sequence of the polymerase genes was synthesised and used for cDNA synthesis. The primer (3'GGACGAAAACGA 5') was synthesised by Professor Botes using a Beckman DNA synthesiser at The Centre for Molecular and Cellular Biology, University of Cape Town.

2.8.2 Reagents

4 µl buffer 1
1 µl RNAse inhibitor
2 µl (10 mmol) deoxynucleotide mixture [(dATP, dCTP, dGTP and dTTP).
9 µl redistilled water
2 µl AMV reverse transcriptase
40 µl buffer 11
1 µl RNAse H
34 µl redistilled water
5 µl *E. coli* DNA polymerase 1
4 µl T_4 DNA polymerase
The reagents listed above were supplied with the cDNA synthesis kit.

DNA primer (3' to 5') GGACGAAAACGA (Purchased from University of Cape Town)

0.2 M EDTA pH 7.2. EDTA (1.48 g) was dissolved in 15 ml of sterile water and adjusted to pH 7.2. The solution was made to 20 ml with sterile water.

10% (w/v) N-Lauryl Sarcosine. N-Lauryl Sarcosine (10 g) was dissolved in 100 ml of sterile water.

Phenol (water saturated), 2 M sodium acetate pH 4 and chloroform/isoamyl alcohol were prepared as described in section 2.5.1

2.8.3 Procedure

All reagents obtained with the kit were stored at -20°C. These reagents were thawed and mixed well before microfuging to collect the solutions at the bottom

of the tubes. All these solutions together with the primer were kept on ice throughout the experiment. The polymerase RNA isolated from the virus was aliquoted, precipitated and air dried. The following solutions were sequentially added to the tube containing the RNA. They were mixed throughly between additions.

1. 4 µl buffer 1
2. 1 µl RNAse inhibitor
3. 2 µl deoxynucleotide mixture
4. 2 µl primer
5. 9 µl redistilled water
6. 2 µl of AMV reverse transcriptase

The total reaction mixture (20 µl) was once more mixed on ice and incubated at 42°C for 60 minutes. The mixture was then put on ice and the components for synthesis of the second strand were pipetted into the same tube as follows;

1. 40 µl of buffer 11
2. 1 µl RNAse H
3. 34 µl redistilled water
4. 5 µl *E. coli* DNA polymerase 1

The total reaction mixture (100 ml), was mixed well and microfuged for 10 seconds before incubating at 12°C for 60 minutes, 22°C for 60 minutes and 65°C for 10 minutes. T4 DNA polymerase (4 µl) was added to the mixture and centrifuged in a microfuge for 10 seconds before incubating at 37°C for 10 minutes. The reaction was stopped by the addition of 10 µl EDTA solution and 2 µl sarkosyl solution.

The cDNA was extracted with phenol/chloroform and precipitated twice with ethanol. This procedure was carried out as described in 2.5.2. Concentration of the cDNA was determined using UV spectroscopy as previously described for RNA (section 2.5.4). The concentration was determined by reading the absorbance at 260 nm where an estimate of 50 µg/ml is equal to an absorbance reading of 1.

CHAPTER 3

RESULTS

3.1 VIRAL INOCULATION

The H1N1 influenza A virus was successfully cultured in 10 day old chick embryos. The allantoic fluid isolated from these eggs had a straw coloured appearance. Fluid having a bloody appearance due to ruptured blood vessels was discarded. Thirty eggs produced a yield of 250 ml of allantoic fluid. The average number of eggs discarded due to trauma was five per batch of thirty. This yield was consistent throughout all the experiments.

3.2 ANALYSIS OF ALLANTOIC FLUID AND VIRAL PELLET

The allantoic fluid which was ultracentrifuged as described (section 2.3.2) and the resultant pellet were used for further analysis.

3.2.1 Blood agar test

The allantoic fluid obtained from infected embryos and plated on blood agar showed no growth after 48 hrs. It was clear from this result that the allantoic fluid was free of both fungal and bacterial contamination.

3.2.2 Haemagglutination titration test

Allantoic fluid was serially diluted with PBS (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048) and human O erythrocytes before incubating at 4°C for 30 min. The results of the haemagglutination titration test are shown in Figure 11. Rows A, B and C showed sedimentation ("button" formation) of the red cells in wells with sample dilutions of 1:256 and above. The agglutinated cells were shown in all samples up to dilution 1:128, which indicated a haemagglutination titre of 1:128. The control (Row D) showed no agglutination and in all wells in this row the erythrocytes settled to the bottom of the wells resulting in typical "button" formation.

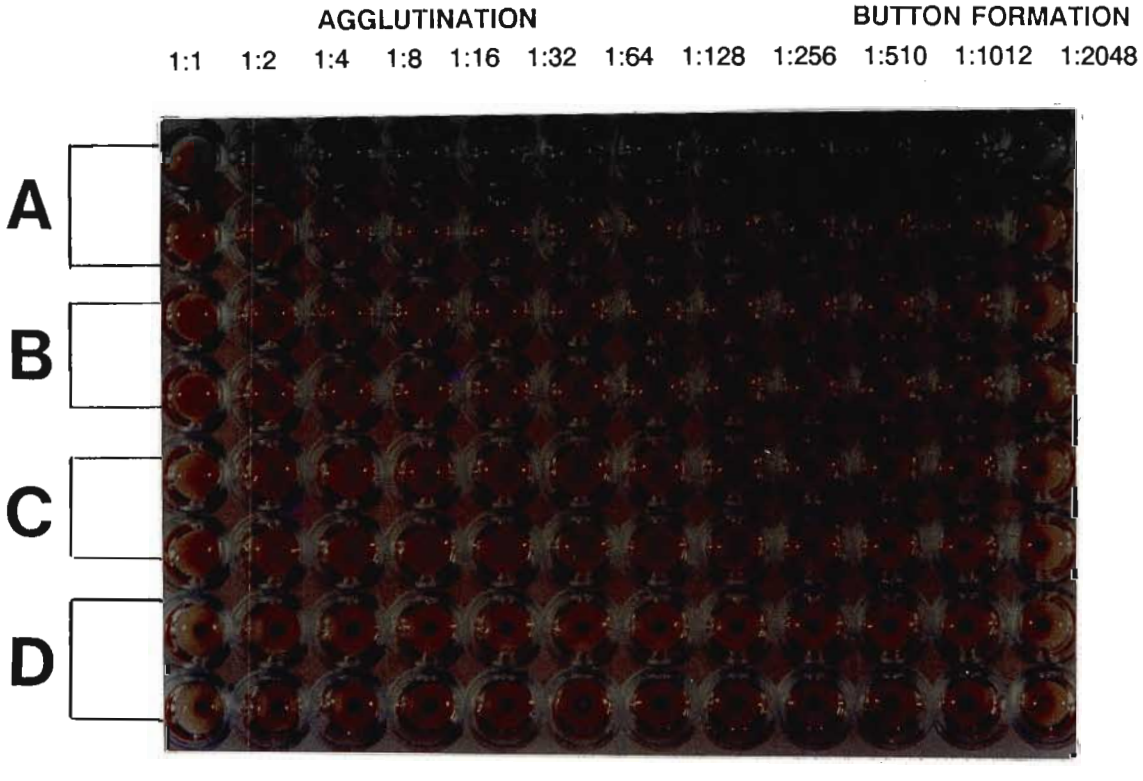

Figure 11. Haemagglutination titration test of allantoic fluid infected with influenza A virus. Rows A, B and C represent serial dilutions of viral samples obtained from infected chick allantoic fluid. Row D, which contained only PBS and erythrocytes served as the control. Absence of agglutination was observed in sample dilutions of 1:256 and above (haemagglutination titre 1:128).

3.2.3 Electron microscopy

The results of electron microscopy are illustrated in the electron micrographs (Figures 12a and 12b). The virus was observed as a spherically shaped structure. The diameter of the viruses ranged from 80 to 110 nm and the size of the surface glycoproteins varied from 10 to 13 nm in thickness. The nucleic acid core ranged in size from 40 to 60 nm in diameter. A very prominent lipid bilayer was present (labelled L in Figure 12a) and the nucleic acid core was clearly visible within the lipid bilayer (labelled N in Figures 12b). The surface glycoproteins appeared as projecting particles uniformly distributed on the lipid bilayer membrane (labelled G in Figure 12a). Positive staining also demonstrated the surface glycoproteins as a "fuzzy coat" (labelled F in Figure 12b).

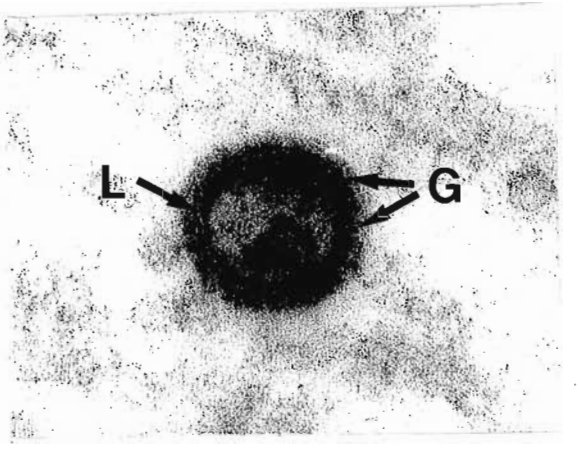

Figure 12a. Electron micrograph of a positively stained Brazilian H1N1 influenza A virus obtained from infected chick embryos. The lipid bilayer (L) is very prominent together with the surface glycoproteins (G) which are uniformly arranged around the lipid bilayer X 240 000.

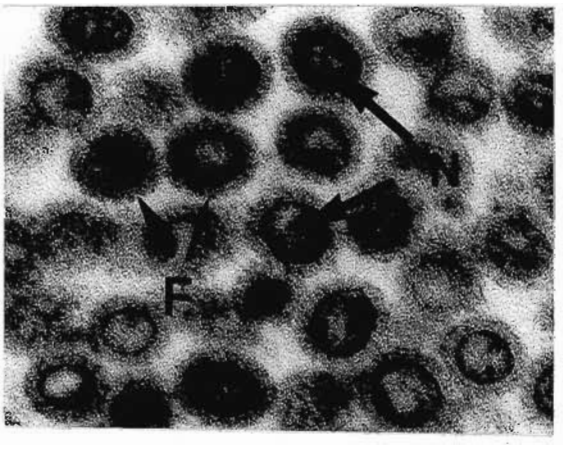

Figure 12b. Positively stained viruses which are predominantly spherical in shape. Note the uniformly distributed, radially arranged surface glycoproteins which have a "fuzzy coat" (F) appearance. The nucleic acid cores are arrowed (N) X 136 000.

3.2.4 Ribose estimation test

The ribose test was carried out to establish the presence of RNA in the sample. Figure 14 shows a typical ribose standard curve obtained using different dilutions of D-ribose in the ribose test. From the graph the average concentration in the five experiments performed was 15 µg/ml of sample.

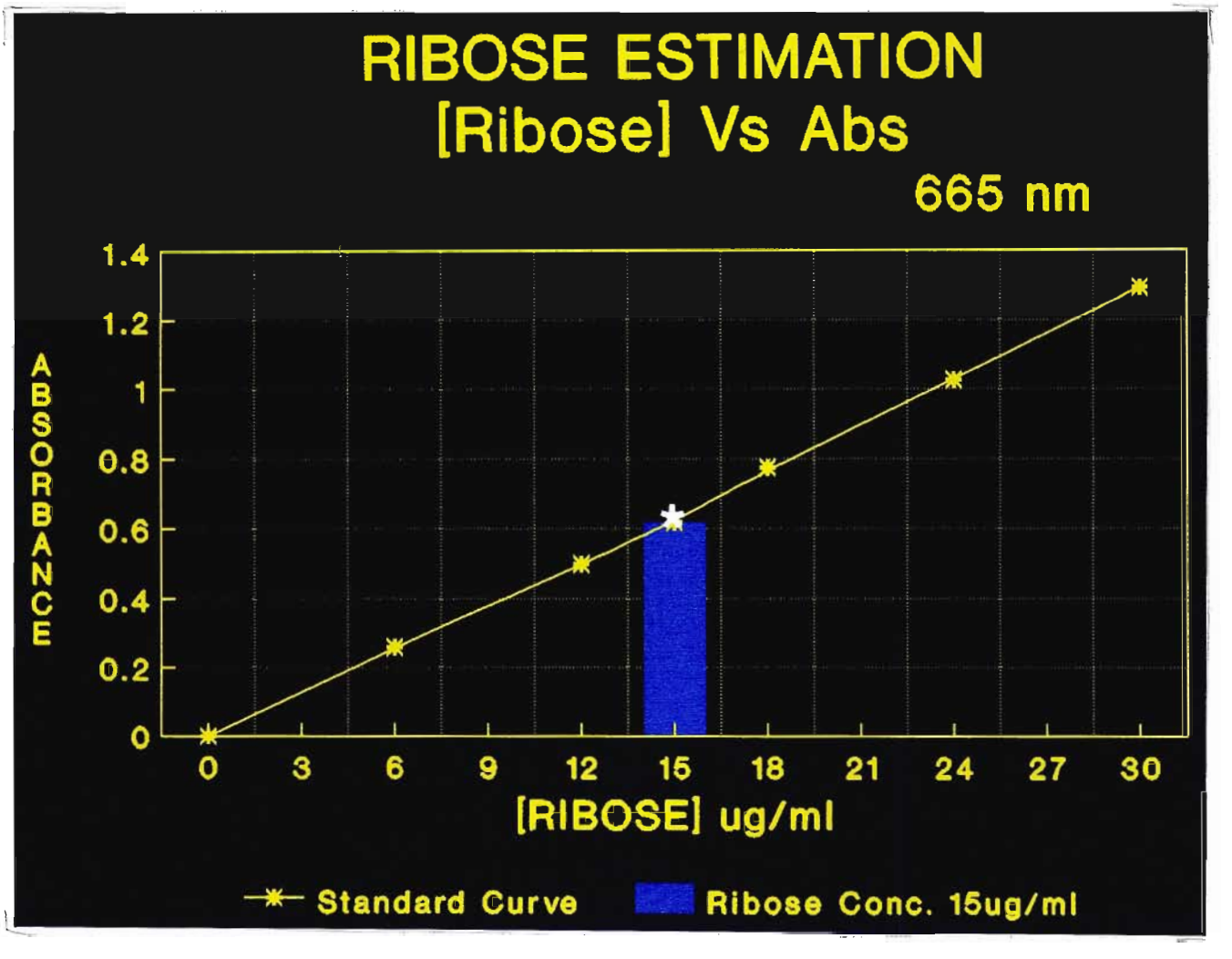

Figure 13. Standard ribose curve obtained using ribose concentrations ranging from 0-30 µg/ml. The ribose concentration was read off the standard curve. Average concentration for five experiments was 15 µg/ml sample.

3.2.5 Immunofluorescence

Immunofluorescence was carried out to verify that the virus used in this study was an influenza A strain and not strain B. The photomicrograph, (Figure 14a) depicts the results obtained after reacting the infected cells in culture with anti-influenza A immunofluorescent antibody. Immunofluorescence was seen as distinct yellow/green areas throughout the Primary Monkey Kidney cells (PMK cells). The PMK cells have a morphology typical of cells in culture, with a very distinct centrally situated nucleus. Varying degrees of binding (detected as fluorescence) were noted throughout the cell surface and cytoplasm (shown by the arrows). The control which was treated with anti-influenza B immunofluorescent antibody showed no indication of fluorescence (Figure 14b).

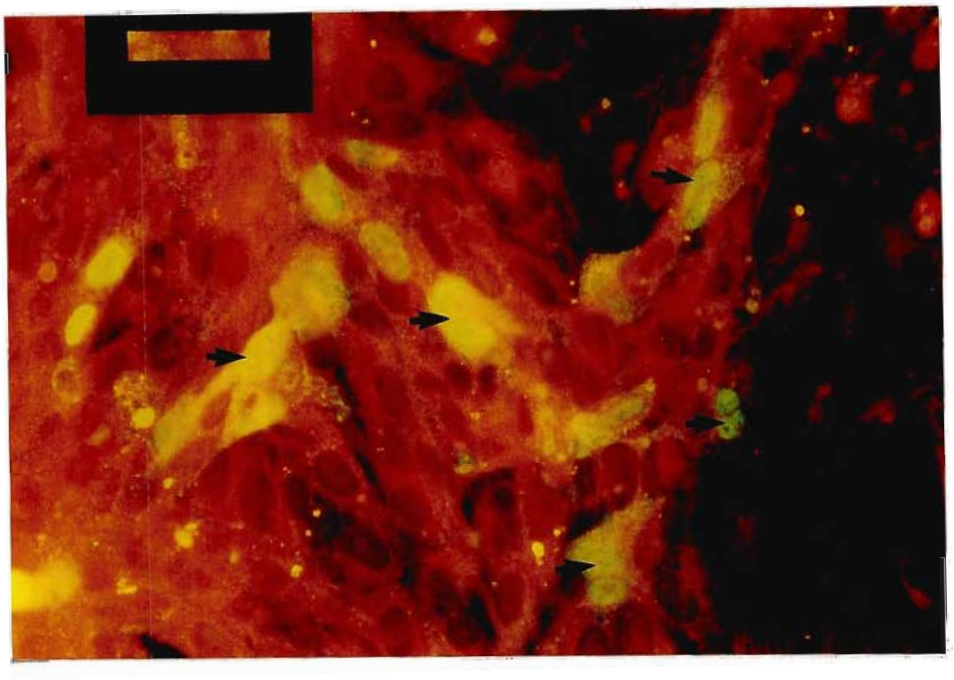

Figure 14a. Primary Monkey Kidney Cells (PMK cells) stained with anti-influenza A monoclonal antibody after infection with H1N1 influenza A virus for 48 hrs at 37°C. Immunofluorescence shown by the yellow/green areas (arrowed) X1480.

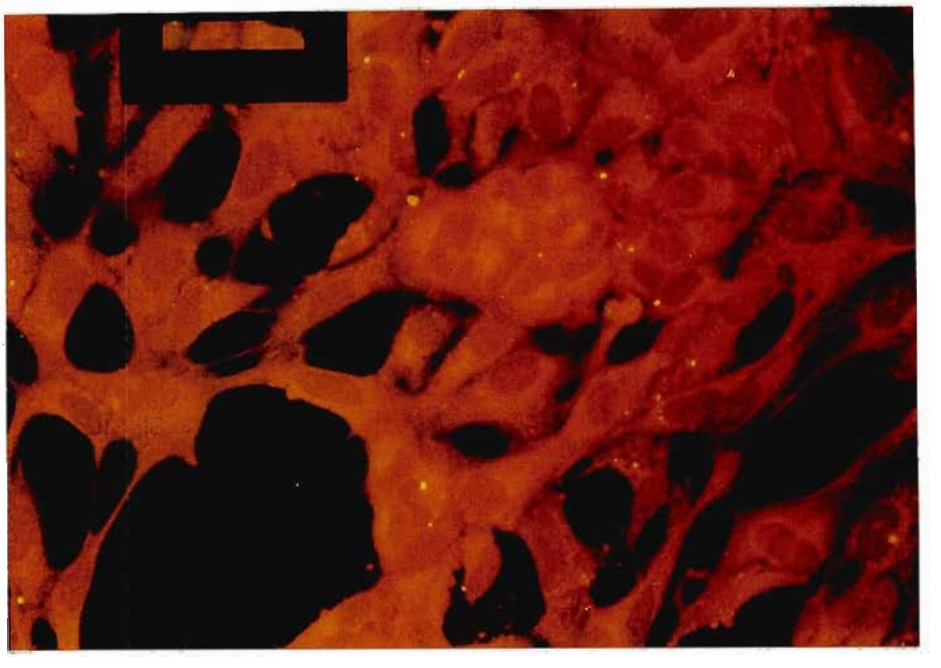

Figure 14b. Primary Monkey Kidney Cells (PMK cells) stained with anti-influenza B monoclonal antibody after infection with H1N1 influenza A virus for 48 hrs at 37°C. Immunofluorescence was not observed in these cell X1480.

3.3 ISOLATION OF RNA

The concentration of RNA isolated from the virus was determined by ultraviolet spectral analysis. Figure 15 shows a plot of wavelength against absorbance (Abs). An absorbance (Abs_{260}) of 1 represents a concentration of 40 µg/ml. A sample calculation is outlined below. The following absorbances were obtained from a typical sample; (Abs_{260}; - 0.175 and Abs_{280} - 0.097) (Figure 15). In this sample the ratio of the Abs_{260}/Abs_{280} was 1.809. Any sample with a ratio < 1.5 would normally be discarded. This was not necessary for any of the experiments performed (see Table 1).

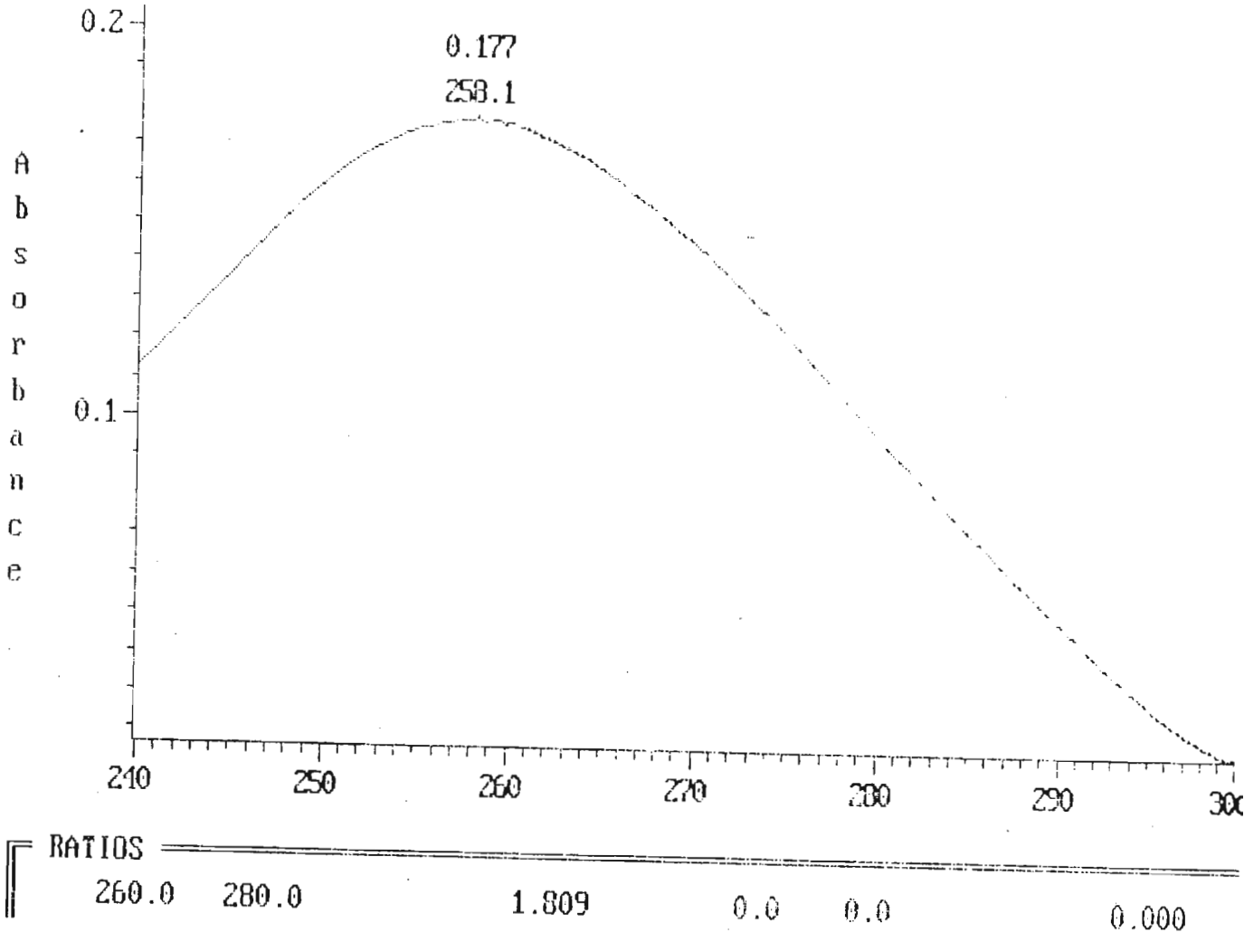

Figure 15. Ultraviolet absorbance spectrogram of total genomic RNA of sample A. Abs_{260} obtained from the spectrogram was 0.175 nm and Abs_{280} was 0.097 nm. The Abs_{260}/Abs_{280} was 1.809. Concentration of RNA estimated at 1.75 µg/µl.

The RNA concentration for sample A was determined as follows;

Sample was diluted 1:250 as follows;
(4 µl sample + 996 µl water)

Abs_{260} = 0.175
therefore Abs_{260} x dilution factor is
0.175 x 250 = 43.75

Abs_{260} of 1.0 represents 40 µg/ml
RNA content of sample = 43.75 x 40 = 1750 µg/ml
therefore RNA content/µl = 1.75 µg/µl

total volume of sample : 100ml
therefore total influenza RNA content was 175 µg/100 µl.
This figure was obtained by pooling sample material from 25 eggs.

TABLE 1: Absorbance values at 260 nm and 280 nm for 6 RNA samples. The ratio Abs_{260}/Abs_{280} are also shown.

Abs_{260}	Abs_{280}	Ratio-Abs_{260}/Abs_{280}
0.159	0.096	1.67
0.163	0.091	1.79
0.179	0.101	1.77
0.197	0.115	1.71
0.198	0.120	1.64
0.166	0.092	1.80

3.4 FORMAMIDE/AGAROSE GEL ELECTROPHORESIS OF RNA

The total genomic RNA was separated on a 1.5% formaldehyde agarose gel using a RNA molecular weight markers to locate the polymerase genes (Figure 16). Five RNA bands were located. The polymerase band, which was most distinct, was size selected using the molecular weight markers. The polymerase genes which are 2300 base pairs (arrowed) were located between RNA molecular weight markers 2800 base pairs and 1800 base pairs.

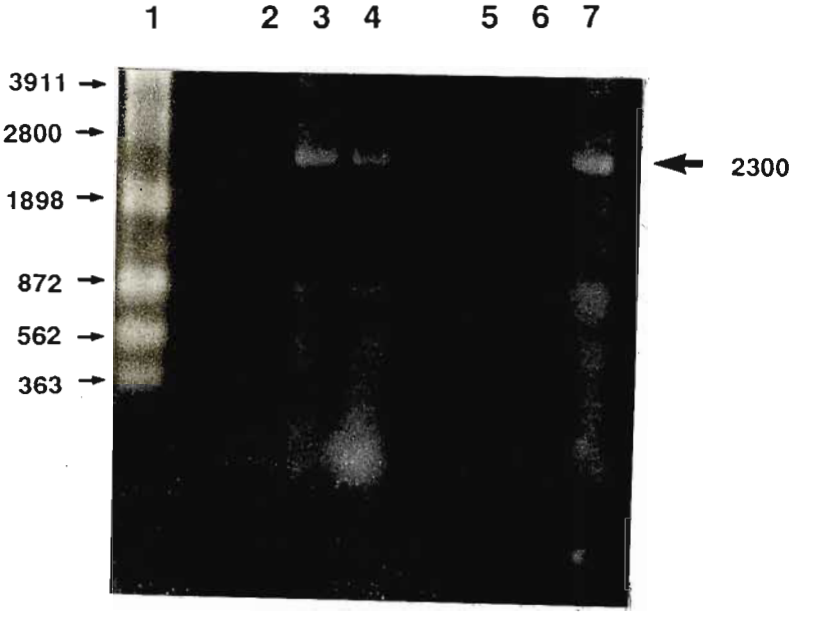

Figure 16. Electrophoresis of Brazilian H1N1 influenza A viral RNA isolated and purified from infected chick embryos. Electrophoresis was performed for 17 hrs at 20 V. Lane 1 contains the RNA molecular weight markers. Lanes 2 to 7 contained influenza viral RNA isolated from different preparations.

3.5 ISOLATION OF POLYMERASE RNA FROM AGAROSE/FORMAMIDE GEL

The polymerase band (2300 base pairs) was identified from the corresponding RNA molecular weight marker. The RNA concentration of the polymerase band was determined by ultraviolet absorbance spectral analysis. Average recovery of RNA following purification from agarose gel was approximately 80% Using the calculation outlined below, the amount of RNA purified was 41 μg.

Sample was diluted 1:250 as follows:
(4 μl sample + 996 μl water)

Abs_{260} = 0.041
therefore Abs_{260} x dilution factor is
0.041x 250 = 10.25

Abs_{260} of 1.0 represents 40 μg/ml
RNA content of sample = 10.25 x 40 = 410 μg/ml
therefore RNA content/μl = 0.41 μg/μl

total volume of sample : 100ml
therefore total influenza RNA content was 41 μg/100 μl.

3.6 COMPLEMENTARY DNA SYNTHESIS OF THE POLYMERASE GENES

The three isolated RNA strands coding for the polymerase genes were reverse transcribed using the cDNA synthesis kit from Boehringer Mannheim and a primer synthesized at the University of Cape Town. 2 μg RNA was used for the synthesis. The product was analysed by ultraviolet spectroscopy. The amount of cDNA synthesised was 1.6μg.

CHAPTER 4

DISCUSSION

The influenza virus has been the subject of intense research since it was isolated in 1933. It has fascinated virologists, microbiologists and molecular biologists because of its ability to evade the immune system. The disease has been aptly referred to as "an unchanging disease due to a changing virus" (Stuart-Harris and Schild, 1976).

Despite extensive research on the genes coding for the two surface antigens HA and NA (Gething *et al.*, 1980; Jou *et al.*, 1980; Sleigh *et al.*, 1980; Lai *et al.*, 1980; Ward and Dopheide, 1981; Gething and Sambrook, 1981) with the aim of controlling antigenic drifts and shifts in the influenza virus, man is still plagued by annual epidemics with enormous health and economic impact. More recently attention has been focused on the molecular mechanisms involved in replication and transcription. With the recent advances in gene manipulation and biotechnology, it is hoped that the isolation of the polymerase genes, and subsequent *in vitro* manipulation by methylation, would provide useful information on possible control mechanisms in gene transcription in the influenza A virus.

A major drawback in molecular research is the enormous cost incurred during such studies. The present study was therefore carefully planned to keep costs at a minimum. It was necessary to avoid contamination of any kind, therefore the aseptic techniques described in Chapter 2 were stringently adhered to during all experimental procedures. This was easily achieved and none of the experiments performed as described showed any signs of contamination.

A clear indication of the absence of contaminating bacteria and fungi in the allantoic fluid was demonstrated in the blood agar test. This was important since the isolated genomic RNA needed to be specific for the influenza A virus.

Blood agar was the medium used since it supports the growth of these contaminants. Subsequent electron microscopy showed the presence of the viruses only, and the absence of bacteria and fungi in the electron micrographs verified this result.

In order to validate the methods used it was necessary to verify the presence of the virus or the RNA genome at each step of the study.

4.1 ISOLATION OF ALLANTOIC FLUID

The straw-coloured appearance of the allantoic fluid is indicative of the absence of contaminating chick embryo blood. The volume of fluid obtained (250 ml/batch of 30 eggs) was the approximate volume obtained at the Rainbow Chicken Farm Laboratory during their routine viral propagation work (Mr S Maharaj, personal communication).

4.2 HAEMAGGLUTINATION TITRATION TEST

The presence of the virus in the allantoic fluid was clearly demonstrated by the haemagglutination titration test. This was an easy test to perform and the results were obtained within one hour. In addition, the test has been adapted to estimate viral titre by using serial dilutions of sample material, a step that was necessary for subsequent inoculations and viral propagation. The use of human O erythrocytes for the agglutination test ensured reliable, reproducible results. It has been shown that human O erythrocytes are completely agglutinated, as opposed to chicken erythrocytes which are commonly used and have been shown to produce incomplete agglutination (Murakami *el al*., 1991).

4.3 ELECTRON MICROSCOPY

The results of electron microscopy, using positive staining, clearly showed the presence of the virus obtained from the isolated allantoic fluid. The predominantly spherical forms of the virus and the size (80-110 nm in diameter) compare favourably with the findings by Choppin *et al.* (1960) who found the size to range from 80-120 nm. However unlike the findings of Choppin *et al.* (1961), no filamentous forms of the virus were found in the samples of the present study. This is consistent with most laboratory-adapted strains of influenza A viruses of which H1N1 is an example. Internal components of the virus (RNP), most recently shown by Murti *et al.* (1992) were not observed in the present study. It is accepted that partial digestion of the virus is required for identification of the RNP. This step was avoided to ensure the integrity of the virus for RNA isolation.

The distribution of the surface glycoproteins was also similar to previous observations (Horne *et al.*, 1960; Stuart-Harris *et al.*, 1985; Doane and Anderson, 1987; Betts and Gordon Douglas, 1990). The size of these glycoproteins (8-10 nm in thickness) showed some similarity to those observed for other strains of influenza viruses. Doane and Anderson, (1987) found the size to range from 8-10 nm.

Although the virus used in the present study was obtained freeze-dried, morphologically these viruses showed no difference when compared to the freshly isolated counterparts using negative and positive staining procedures (Naidoo *et al.*, 1991). However, the freeze-dried forms always had lower haemagglutination titres. In freeze-dried preparations of influenza there is clumping of the virus resulting in lower titre (Prof J W Moodie, personal communication). Freeze-dried viruses propagated through three or four successive passages, showed an increase in titre value which was similar to that of the fresh virus.

4.4 RIBOSE ESTIMATION

The presence of RNA was confirmed by the ribose estimation test. Similar results were obtained by Khan (1982), using the identical procedure. Since the influenza A is an RNA virus, this result further confirmed the conclusions reached from the previous viral analysis experiments. Although the ribose test was carried out, it was clear from the haemagglutination titration test and electron microscopy that the influenza virus was present in the isolated sample. Hence, for further experiments involving isolation of viral RNA samples, this step may be omitted as a cost effective measure.

4.5 IMMUNOFLUORESCENCE

The H1N1 strain used in this study was confirmed by immunofluorescence using a specific anti-influenza A monoclonal antibody. The areas of fluorescence seen presumably represent complexes formed between the surface glycoproteins present on the virus and the anti-influenza A antibody. Absence of fluorescence in the control, which was incubated with anti-influenza B monoclonal antibodies, helps to confirm that the influenza virus particles seen in the electron microscopy studies and responsible for the haemagglutination test, were in fact influenza A and not the B virus.

The above experiments confirmed the successful isolation of the specific virus and provided the necessary material of suitable quality for isolation and purification of the RNA genome.

4.6 RNA ISOLATION

Although the RNA isolation procedure was previously used in eukaryotic systems (Chirgwin *et al.*, 1979), this procedure proved to be very effective for isolation of viral RNA. This was demonstrated by the spectrogram of the isolated genomic RNA which shows a strong peak only at absorbance 260 nm, indicative

of the presence of nucleic acids (RNA). This value was also used to calculate the concentration of the RNA. Since proteins have a very strong absorbance at around 280 nm, the low absorbance at 280 nm indicated the absence of protein in the sample. The overall purity of the RNA can be estimated from the Abs_{260}/Abs_{280} ratio. Any value between 1.6 and 2.0 usually indicates RNA free of contamination (Davis *et al.*, 1986). Using the extraction method described, this ratio was above 1.6 in all samples, proving that the choice of the method was adequate for isolation of the H1N1 genomic RNA. The RNA was accepted to be of sufficiently high quality to continue with the subsequent electrophoresis step.

4.7 ELECTROPHORESIS

Separation of the influenza A RNA genome was previously performed by polyacrylamide gel electrophoresis (PAGE) using radioactive labels to identify the bands (McGeoch *et al.*, 1976; Pons, 1976; Inglis *et al.*, 1976). PAGE, using radioactive labelling is an extremely sensitive technique and has been useful in identifying the various RNA strands of the viral genome. However, this technique does not allow for further investigation or manipulation of the RNA strands, such as methylation, and expression studies. Since the present study required retention of the RNA it was necessary to use an alternative method. The formamide agarose gel electrophoresis has been successfully used previously for isolation of eukaryotic RNA of sufficient sensitivity for cDNA synthesis (Lehrach *et al.*, 1977). The present study demonstrates clearly that this method is also useful for the isolation of viral RNA.

The nucleotide lengths of the three RNA strands coding for the polymerase genes are almost identical in size (PB2-2341, PB1-2341 and PA-2233), and these co-migrated in the formamide gel as an intense band. Since the amount of fluorescence is proportional to the concentration of the RNA (Sambrook *et al.*, 1989), the other less concentrated bands were not as distinct as the polymerase band. The concentration of the RNA was optimized in order to easily distinguish and identify the various RNA bands using formamide electrophoresis. It was therefore necessary to use 40 μg of total RNA in each sample well.

4.8 PURIFICATION OF POLYMERASE RNA

The isolation and purification of the RNA from the agarose gel was easily achieved by the RNAid kit. This provided a quick and practical method, and was certainly less tedious when compared with the conventional method described by Maniatis *et al.*, (1982). Recovery of RNA following purification was 80% and provided sufficient substrate for cDNA synthesis. The successful use of the RNAid kit was also most recently demonstrated by Gentsch *et al.* (1992), for isolation of rotavirus RNA and by Clerch and Massaro (1992), for isolation of mRNA from rat lung tissue.

4.9 COMPLEMENTARY DNA SYNTHESIS

Since the isolated RNA cannot be used for transfection studies, cDNA copies of the polymerase genes will be required. There are various techniques currently available that allow for *in vitro* manipulation of DNA, most notably amplification of DNA by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988). PCR is a powerful *in vitro* technique that has revolutionised various aspects of molecular biology. A full description of the potential uses of PCR is beyond the scope of this study.

As mentioned previously, the influenza virus has a segmented RNA genome. This was the basis for the reverse transcription of the polymerase genes isolated from H1N1. The enzyme, AMV reverse transcriptase catalyses the synthesis of DNA from the viral RNA (vRNA) strand in a 5' to 3' direction (Old and Primrose, 1988). Therefore, in order to ensure specificity of the reverse transcription, the primer used was a synthetic oligomer complementary to the 3'-terminus of the polymerase vRNA. An identical synthetic primer has been used previously for cDNA synthesis (Huddleston and Brownlee, 1982) as well as for PCR (Rajakumar *et al.*, 1990).

The quantity of cDNA synthesised is sufficient substrate for specific amplification using PCR. However, should a need for a greater quantity of cDNA arise, reverse transcription in multiplication would provide the required yield.

4.10 CONCLUSION

The aim of the present study was to isolate the polymerase genes of the H1N1 influenza A virus. This was successfully completed using the methods outlined. These methods have proved practical and reliable. In this particular study each step was carefully validated to minimise the destruction of the viral genome. The isolated cDNA can now be amplified using PCR, if necessary.

Since the nucleotide sequences of both the 3' and 5'-terminals are conserved, both these sequences can be synthesised for use in the PCR reaction. The amplified DNA would provide the necessary starting material for all subsequent analyses.

The cDNA would be enzymatically methylated using the Hpa II methylase. This enzyme selectively adds a methyl group at the N^6 position of the inner cytosine residue in the sequence 5'CCGG 3'. In a control study unmethylated cDNA would be used. Both unmethylated and methylated cDNA would then be incorporated into a suitable vector in a tissue culture based system. The polymerase genes would then be assayed for by means of Northern blotting.

It is hoped that these studies on the methylation of the cDNA may then be applied to developing a method for methylating the influenza viral genome.

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APPENDIX A

Positive staining procedure

1. Fix virus in Karnovsky's fixative for 1 hour.

2. Wash twice for 10 minutes in 0.2 M cacodylate buffer at 4°C.

3. Stain with osmiun tetroxide for 1 hour 4°C.

4. Wash twice in cacodylate buffer for 10 minutes at 4°C.

5. Dehydrate in ascending grades of ethanol: 70% - 30 minutes, 90% - 30 minutes and absolute (twice for 30 minutes).

6. Infiltrate in propylene oxide for 30 minutes.

7. Infiltrate in propylene oxide/araldite resin 50:50 for 30 minutes.

8. Infiltrate in araldite at 50°C (3 x 1 hour).

9. Embed in Beem capsules and polymerise at 60°C for 48 hours.

10. Stain in 1% uranyl acetate in 50% ethanol solution and Reynolds lead citrate.

APPENDIX B

Isolation of RNA from formamide/agarose gel

1. Transfer gel slices into pre-weighed Eppendorf tubes and label.

2. Determine mass of gel slices and add 3 volumes of RNA binding salt (pH 5).

3. Incubate at 37°C for 10 minutes to melt the agarose. Vortex to ficilitate dissolution of gel.

4. Add 10 of RNAMatrix, mix well to allow RNA to adsorb to matrix for 10 minutes at RT.

5. Centrifuge for 1 minutes at maximum speed in a microfuge to pellet the RNA/RNAMatrix complex.

6. Remove supernatant to new tube and save for possible re-adsorption. Spin briefly to collect remaining liquid at the bottom of the tube. Remove all traces of liquid with a small bore pipette tip.

7. Repeat step 4 to wash pellet and remove any traces of agarose and formaldehyde. Mix throughly with a pipette tip and spin for 1 minutes. Remove supernatant and spin again to remove traces of liquid with a small bore pipette.

8. Resuspend pellet in 500 µl RNAWASH solution by mixing with a pippette tip. Spin for 1 minutes and remove supernatant.

9. Repeat previous washing step (2 times). Re-spin and remove liquid traces of liquid as described in step 7.

10. Resuspend pellet completely in 50 µl RNAse-free (autoclaved water) by mixing with pipette tip.

11. Elute RNA by incubating at 80°C for 10 minutes. Spin for 1 minutes at maximum speed and remove supernatant containing RNA to a sterile Eppendorf tube.

12. Repeat elution step to recover additional RNA.

13. Heat RNA to 80°C for 10 minutes to further dissociate residual formaldehyde. Cool to RT and place on ice immediately.

14. Label and store RNA at -70°C .

APPENDIX C

Elektronmikroskopievereniging van Suidelike Afrika — KAAPSTAD (1991)

A COMPARATIVE STUDY OF THE MORPHOLOGY OF FREEZE-DRIED AND FRESHLY ISOLATED INFLUENZA A VIRUSES

** *Richard Naidoo,* ** *Thajasvarie Naicker and* * *Zainub Khan*

** Department of Physiology, Natal University Medical School
* Department of Biochemistry, University of Durban-Westville

It is known that viruses which have been freeze-dried and stored at -70°C can be reconstituted after many years in sterile water or isotonic saline and remain viable. Whether freeze-drying affects the morphology of the virus is not known and hence, in this study, the morphology of freeze-dried and freshly isolated Brazilian H1N1 influenza A virus was compared using positive and negative staining methods by TEM.

Fresh viral samples were obtained from the National Institute of Virology. The sample (0.2 ml.of 1:10 dilution) was introduced in 10 day old chick embryos via the allantoic route and incubated at 33°C for 48 hrs. Following incubation, the allantoic fluid was aspirated with sterile pasteur pipettes after the air space was exposed. The presence of the virus in the allantoic fluid was established by haemagglutination titration; fungal and bacterial sterility of the samples were confirmed by negative growth after plating on blood agar. A series of ultracentrifugation steps ensured proper washing of the fluid. Isolation of these fresh viruses was achieved by sucrose density gradient ultracentrifugation, following dialysis of the layered isolates against NTE buffer (0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA, pH 7.6). Pelleting of the virus was obtained by centrifugation at 10 000 Xg for 15 minutes. The sample was fixed in 0.25% glutaraldehyde and 1% osmium tetroxide and prepared for TEM using conventional techniques. Samples of H1N1 obtained by the identical procedure adopted above were freeze-dried under vacuum and stored at -70°C since 1982. This was now reconstituted in sterile water and pelleted in an Eppendorf tube. Processing was identical to that adopted for the fresh virus. Negatively stained preparations of both fresh and freeze-dried viruses were also made for TEM (1).

No gross morphological differences were noted under TEM. The virus was found to be roughly spherical in shape as was demonstrated previously (2). In both fresh and freeze-dried samples the surface glycoproteins were found to be uniformly distributed around the lipid bilayer (Fig. 1 and 2) and gave the appearance of a "fuzzy coat" (Fig. 3) in the negatively stained samples. The lipid bilayer was found to be intact in all instances. The diameter of the viruses was found to range from 80 to 110 nm. The size of the surface glycoproteins was between 10 and 13 nm while the diameter of the nucleic acid cores (Fig. 2 and 4) was between 40 and 60nm.

The results show that freeze-drying has no visible effect on the morphology of the H1N1 influenza A virus as demonstrated using negative and positive staining procedures by TEM.

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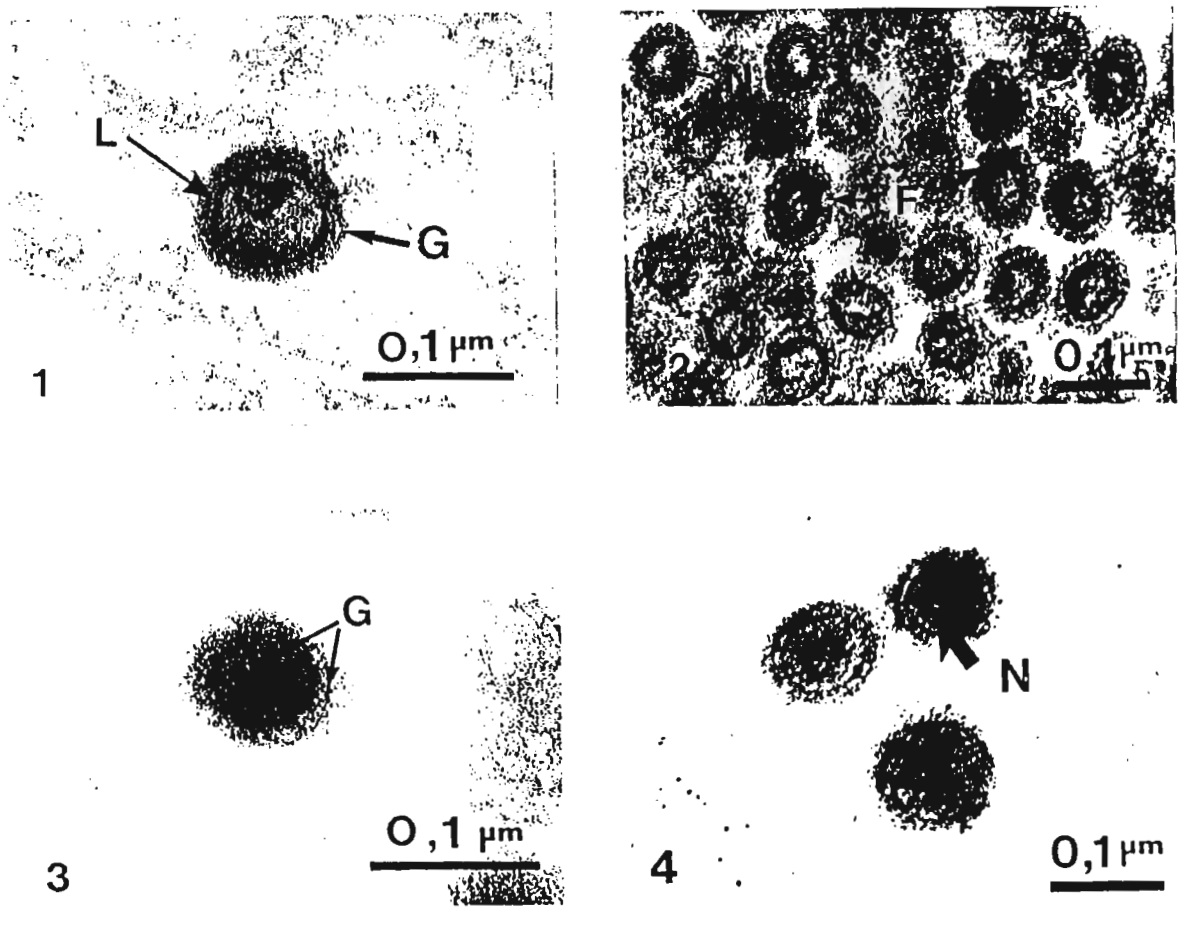

Fig. 1 Positively stained fresh virus; lipid bilayer membrane (L) and surface glycoproteins (G).

Fig. 2 Positively stained freeze-dried virus; note the surface glycoproteins appear as a "Fuzzy coat" (F) and the nucleic acid core (N).

Fig. 3 Negatively stained fresh virus; uniform distribution of outer surface glycoproteins (G).

Fig. 4 Negatively stained freeze-dried virus; nucleic acid core (N).

ISOLATION OF THE POLYMERASE GENE FROM H1N1 INFLUENZA A VIRUS USING A FORMAMIDE DENATURING GEL

Richard Naidoo, **Zainub Khan and *Kogie Reddi

Department of Physiology and *Chemical Pathology, University of Natal Medical School; **University of Miami Medical School.

In this study, we investigated a possible method to successfully locate and isolate the polymerase gene of the influenza A virus. Isolation of the polymerase gene is necessary to investigate the effects of enzymatic methylation on the polymerase gene transcriptase activity.

H1N1 influenza A virus was cultured in 10 day old chick embryos via the allantoic route and incubated at 33^{O}C for 48 hrs. Following incubation, the allantoic fluid was isolated and washed to remove large proteineous debris and any blood cells. The presence of the virus was established by haemagglutination titration test and electron microscopy. Viral particles were pelleted by ultracentrifugation of the allantoic fluid at 100 000 xg. Purified viral pellets were pooled and homogenized in buffer containing guanidine thiocyanate, mercaptoethanol and sarkosyl. The samples were incubated on ice for 10 min before mechanical disruption of the virus with a "Dounce-Type" homogenizer. Viral RNA was isolated from the upper aqueous layer after a standard phenol/chloroform extraction procedure. RNA was quantitated spectrophotometrically and purity assessed initially by ratio readings at 260/280. Electrophoresis of the RNA samples was performed together with a RNA marker at 20v for 17 hrs on a 1.5% formamide agarose gel.

Five bands were identified and the band containing the polymerase gene was size selected, located and excised. Purification of the polymerase gene from the agarose was achieved by using the BIO 101 RNAid kit.

The above is procedure previously used for separation of eukaryotic RNA and was adapted for the isolation of viral RNA. The RNA obtained is of sufficient quality for reverse transcription and subsequent DNA methylation studies.