THE *IN VITRO* TRANSCRIPTION AND TRANSLATION OF BLUETONGUE VIRUS mRNA

BY

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The world and all that is in it
belong to the LORD;
the earth and all who live on it are his.

Psalm 24 : 1

dedicated to my parents

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SUMMARY

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This investigation was primarily aimed at studying the *in vitro* synthesis of bluetongue virus proteins. Both BTV mRNAs and denatured dsRNAs were used as templates for translation. Double-stranded RNAs could be purified from infected cells, but the only means of obtaining pure BTV mRNAs was to synthesise them *in vitro*. Therefore, some of the factors that influence the *in vitro* transcription reaction were also investigated.

The transcriptase of BTV can be activated *in vitro* by removal of the outer capsid proteins, which results in the conversion of virions to core particles. It was found that the concentration of the core particles has a marked effect on the efficiency of the transcription reaction. At high core concentrations the transcription reaction is severely inhibited, resulting in a complete termination of transcription after a relatively short incubation period. It was found that it is possible to counteract this inhibition by lowering the incubation temperature. Consequently, at high core

concentrations the in vitro transcription reaction is much more efficient at a lower incubation temperature (28°C), than at a higher incubation temperature (37°C). At low core concentrations, this low temperature optimum is not observed. It was furthermore found that the core-mediated inhibition of in vitro transcription is completely reversible. Reactions in which the synthesis of mRNAs is completely terminated at 37°C, can be reactivated by lowering the incubation temperature. Another important finding was that the core-mediated inhibition can be counteracted by including compounds such as sucrose and glycerol in the reaction mixture.

The BTV mRNA species synthesised during the in vitro transcription reaction were analysed by agarose gel electrophoresis. It was found that the 10 different BTV mRNA species are not synthesised in vitro in equal amounts. The results confirm those previously obtained by much more indirect methods.

The purpose of studying in vitro translation of BTV was to identify all the proteins that are specified by BTV mRNA species, and determine the RNA-protein coding assignments of the genome segments.

All 9 BTV proteins previously identified in BTV-infected cells can be synthesised in vitro using BTV mRNAs as templates, a result that confirms that the two non-structural proteins, NS1 and NS2, are indeed virus-specific. Apart from these 9 proteins, three new virus-specific non-structural proteins, NS3A, NS3B and protein C were identified by means of in vitro translation. They were subsequently also detected in infected cells, where they seem to be synthesised only in very small amounts. The relative amounts in which the BTV proteins are synthesised in vitro, differ from that observed in infected cells.

The RNA-protein coding assignments for seven of the ten genome segments, the three medium-sized and the four small-sized segments, were determined by *in vitro* translation of denatured dsRNAs. It correlates with results published for BTV types 1 and 17 respectively. Genome segment 10 codes for both proteins NS3A and NS3B. The peptide maps of these proteins are virtually identical, indicating that they may be translated from overlapping in-phase reading frames. Preliminary results indicate that genome segment 9, which codes for protein 6, also codes for protein C.

It was also investigated whether *in vitro* synthesised NS2 is phosphorylated and has affinity for single-stranded RNA, as has been reported for NS2 synthesised in infected cells. It was found that *in vitro* synthesised NS2 has affinity for ssRNA, similar to NS2 present in infected cells, but no phosphorylation of *in vitro* synthesised NS2 has as yet been observed. On the other hand, it was found that NS2 from infected cells can be phosphorylated *in vitro*. The kinase responsible for phosphorylating NS2 was found to be present in all preparations of *in vivo* synthesised NS2 - even in those purified by means of affinity column chromatography.

OPSOMMING

THE *IN VITRO* TRANSCRIPTION AND TRANSLATION OF BLUETONGUE VIRUS mRNA

DEUR

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Die kern van hierdie studie was 'n ondersoek van die *in vitro* sintese van bloutongvirus (BTV) proteïene. Sowel BTV boodskapper-RNAs as gedenatureerde dubbeldraad-RNAs is as templaat vir die translasiereaksies gebruik. BTV ddRNAs kon uit geïnfekteerde selle geïsoleer kon word, maar suiwer BTV mRNAs kon slegs verkry word deur hulle *in vitro* te sintetiseer. Derhalwe is die faktore wat die *in vitro* transkripsiereaksie beïnvloed ook bestudeer.

Die transkriptase van BTV kan *in vitro* ge-aktiveer word deur die buitedopproteïene van die virus te verwyder. Laasgenoemde prosedure lei tot die omskakeling van virions tot binnedoppartikels. Tydens hierdie ondersoek is gevind dat die konsentrasie van binnedoppartikels 'n groot invloed op die effektiwiteit van die *in vitro* transkripsie-reaksie het. By hoë konsentrasies van binnedoppartikels word die transkripsie-reaksie sterk geïnhibeer, en kom die transkripsieproses na 'n relatief kort inkubasieperiode ten volle tot stilstand. Dit het

egter geblyk dat dit moontlik is om hierdie inhibisie teen te werk deur die inkubasietemperatuur te verlaag. Die in vitro transkripsie-reaksie by hoë binnedoppartikel-konsentrasies is dus baie meer effektief by 'n laer inkubasietemperatuur (28°C), as by 'n hoër inkubasietemperatuur (37°C). By lae binnedoppartikelkonsentrasies is die lae temperatuuroptimum vir in vitro transkripsie nie waargeneem nie. Die binnedoppartikel-bemiddelde-inhibisie blyk volledig omkeerbaar te wees. Dit is moontlik om reaksies waartydens mRNA-sintese ten volle tot stilstand gekom het weer te aktiveer deur bloot die inkubasietemperatuur te verlaag. 'n Ander belangrike bevinding was dat die binnedoppartikel-bemiddelde-inhibisie teëgewerk kan word deur die insluiting van verbindings soos sukrose en gliserol in die reaksiemengsels.

Die BTV-mRNAs wat tydens die in vitro transkripsie-reaksie gesintetiseer is, is deur middel van agarose jel elektroforese ge-analiseer. Daar is vasgestel dat die 10 verskillende BTV mRNAs nie almal in dieselfde hoeveelhede in vitro gesintetiseer word nie. Hierdie bevinding bevestig resultate wat vroëer met heelwat meer indirekte metodes verkry is.

Die doel van die in vitro translasie-studies was om alle proteïene wat deur BTV mRNAs kodeer word, te identifiseer en die koderingsverband tussen die proteïene en genoomsegmente te bepaal.

Al 9 BTV proteïene wat voorheen in geïnfekteerde selle geïdentifiseer is, kon in vitro met BTV mRNAs as templaat gesintetiseer word. Sodoende is bewys dat die 2 nie-strukturele proteïene, NS1 en NS2, wel virus-spesifiek is. Benewens laasgenoemde 9 proteïene, is 3 nuwe virus-spesifieke nie-strukturele proteïene, NS3A, NS3B, en proteïen C in vitro geïdentifiseer. Hierdie proteïene is daarna ook in geïnfekteerde selle opgespoor. Dit blyk dat hulle egter in

uiters klein hoeveelhede *in vivo* gesintetiseer word. Die relatiewe hoeveelhede waarin BTV proteïene *in vitro* gesintetiseer is, het verskil van dié in geïnfekteerde selle.

Die RNA-proteïen koderingsverband vir sewe van die tien genoomsegmente, die drie medium en vier klein genoomsegmente, is deur *in vitro* translasie van gedenatureerde ddRNA segmente bepaal. Dit stem ooreen met gegewens wat vir BTV serotipes 1 en 17 onderskeidelik gepubliseer is. Dit blyk dat genoomsegment 10 vir beide proteïene NS3A en NS3B kodeer. Die peptiedkaarte van hierdie proteïene stem feitlik ooreen en dui daarop dat hulle waarskynlik van oorvleuelende in-fase leesrame getransleer word. Voorlopige resultate toon dat genoomsegment 9, wat die genetiese inligting vir proteïen 6 bevat, ook vir proteïen C kodeer.

'n Verdere oogmerk van die studie was om vas te stel of *in vitro* gesintetiseerde NS2 affiniteit vir enkeldraad-RNA besit, en of dit gefosforileer kan word. Daar is vasgestel dat *in vitro* gesintetiseerde NS2 affiniteit vir enkeldraad-RNA vertoon, netsoos NS2 wat intrasellulêr teenwoordig is, maar dit kon nie *in vitro* gefosforileer word nie. Daarenteen kon proteïen NS2, wat uit geïnfekteerde selle verkry is, wel *in vitro* gefosforileer word. Kinase-aktiwiteit was in alle preparate van *in vivo* gesintetiseerde NS2 teenwoordig, selfs in dié wat met affiniteits-kolom-chromatografie gesuiwer is.

ABBREVIATIONS

A_{260}	- absorbance at 260 nanometer
Adomet	- S-adenosylmethionine
AHSV	- African horsesickness virus
ATP	- adenosine triphosphate
BHK	- baby hamster kidney
BSA	- bovine serum albumin
BTV	- bluetongue virus
β-ME	- beta-mercaptoethanol
cm	- centimeter
cpm	- counts per minute
CPV	- cytoplasmic polyhedrosis virus
°C	- degrees Celsius
ddRNA	- dubbeldraad-ribonukleïensuur
DMSO	- dimethyl sulphoxide
DNA	- deoxyribonucleic acid
ds	- double-stranded
EDTA	- ethylenediaminetetra-acetic acid
EGTA	- ethyleneglycolbis-(aminoethylether)-tetra-acetic acid
EHDV	- epizootic haemorrhagic disease virus
fig	- figure
g	- gram
g	- force of gravity
h	- hour
K	- kilodalton
Km	- Michaelis constant
MAK	- methyl albumin kieselguhr
M	- molar
mA	- milliampere
mg	- milligram
$MgAc_2$	- magnesium acetate
min	- minutes
mM	- millimolar

MMOH	- methylmercuric hydroxide
mmole	- millimole
ml	- milliliter
mRNA	- messenger ribonucleic acid
nm	- nanometer
p.i.	- post infection
PFU	- plague forming units
pTp	- 2'-deoxythymidine-3',5'-diphosphate
PVS	- polyvinylsulfuric acid
RNA	- ribonucleic acid
RNase	- ribonuclease
rpm	- revolutions per minute
S	- Svedberg unit
sec	- seconds
SDS	- sodium dodecyl sulphate
ss	- single-stranded
TCA	- trichloroacetic acid
Tm	- melting temperature
tRNA	- transfer ribonucleic acid
uCi	- microcurie
ug	- microgram
ul	- microliter
UMP	- uridine monophosphate
UTP	- uridine triphosphate
UV	- ultraviolet
VRI	- veterinary research institute
VSV	- vesicular stomatitis virus
WTV	- wound tumor virus

LIST OF BUFFERS

STE-1-buffer - 0,01 M NaCl; 0,01 M Tris-HCl, pH 7,4; 0,001 M EDTA

STE-2-buffer - 0,15 M NaCl; 0,01 M Tris-HCl, pH 7,4; 0,001 M EDTA

TE-buffer - 0,01 M Tris-HCl, pH 7,4

NETS/NP-40/BSA-buffer - 0,15 M NaCl; 0,005 M EDTA; 0,05 M Tris-HCl, pH 7,5; 0,05% NP-40

MTD-buffer - 0,01 M Tris-HCl, pH 8,0; 0,01 M $MgCl_2$; 0,01 M dithiothreitol; 0,2 uCi/ml γ^{32}p-ATP (1 mCi/ml; 5200 Ci/mmole)

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CHAPTER 3

CHAPTER 1

LITERATURE INTRODUCTION

1.1 INTRODUCTION

Viruses were discovered at the turn of the century when it was realised that some diseases were caused by agents smaller than all previously known infectious agents. Among the first diseases attributed to viral infections were tobacco mosaic disease of plants (1892) and foot-and-mouth disease of cattle (1898). In 1906 Theiler established that a virus was the aetiological agent of "Malarial Catarrhal Fever of Sheep" - the disease now known as bluetongue (Theiler, 1906). He subsequently introduced the first vaccine against bluetongue which succeeded in controlling the disease to the extent that it was reported (Du Toit, 1929):

Very little has been published during the last twenty years on bluetongue in sheep. The investigations of Theiler ..... during the early years of the century, cleared up most of the problems in connection with this disease, and enabled veterinary research workers in South Africa to devote their attention to more pressing problems.....Nevertheless cases have been reported each year of unsatisfactory results following the use of bluetongue vaccine. In some cases the vaccine was supposed to cause unduly severe reactions in inoculated sheep; in others it was supposed to produce insufficient immunity, so that such sheep contracted virulent bluetongue when exposed to natural infection.

The problem of insufficient immunity has not yet been entirely overcome, and it is one of the main motivations for the continued research on BTV on a molecular level.

Bluetongue manifests clinically in sheep by a catarrhal

inflammation of the mucous membranes of the digestive and respiratory systems and is associated with degenerative changes in the skeletal muscle. In severe cases the mucous membranes of the mouth, and particularly the tongue, may turn a bluish-purple colour, hence the name bluetongue. The acute disease causes profound emaciation and weakness. Affected animals have a protracted convalescence and diminished productivity. Since there are approximately thirty million sheep in the country, the disease has serious economic implications (Howell & Verwoerd, 1971). Cattle, goats and various species of antelope are also susceptible, but usually do not show any clinical symptoms (Erasmus, 1975; Hourrigan & Klingsporn, 1975).

Viruses are being studied not only because of their importance as disease causing agents, but also because they are particularly suitable models which can be used to study the mechanisms controling replication, transcription and translation of genetic information. This knowledge is fundamental to an understanding of the molecular biology of higher organisms, and is therefore directly applicable to the practice of medicine, veterinary science and the improvement of human welfare in general.

1.2 CLASSIFICATION AND CHARACTERISATION OF BTV VIRIONS

BTV has a genome consisting of 10 double-stranded (ds) RNA species (Verwoerd *et. al.*, 1970) and has consequently been classified as a member of the Reoviridae family. The Reoviridae include six genera: Reovirinae, Rotavirinae, Fijivirinae, Cypovirinae, Phytoreovirinae, and Orbivirinae (Matthews, 1982). Bluetongue virus is the prototype of the orbivirus genus. The name orbivirus was based on the electron microscopic observation of large doughnut-shaped capsomeres (Latin, orbis, meaning ring) seen on the surface of virus particles in negatively stained preparations (Borden *et. al.*, 1971).

Currently orbivirinae consist of 13 serological groups that are distinguished by complement fixation tests. The serotypes within each group are distinguished by serum neutralisation tests (Gorman, 1979). In the bluetongue virus serogroup at least 23 serotypes have been identified to date (Jeggo & Wardley, 1985).

The BTV virion consists of a double layer protein capsid which surrounds the 10 dsRNA genome segments. Seven different structural proteins have been identified - four are major components (P2, P3, P5 and P7), and three are minor components (P1, P4 and P6). Proteins P2 and P5 comprise the outer capsid layer of the virions (Verwoerd et. al., 1972; Martin & Zweerink, 1972).

The synthesis of viral proteins involves two of the basic BTV replication processes, viz., the reaction in which the dsRNA genome segments are transcribed into mRNAs, and the translation of mRNA species into proteins. These aspects will subsequently be discussed in detail, as they are closely related to the aims formulated for this investigation (see 1.7).

1.3 TRANSCRIPTION IN THE REOVIRIDAE

The first virus-associated enzyme discovered, was the DNA-dependent RNA polymerase of vaccinia virus (Kates & McAuslan, 1967). The first RNA-dependent RNA polymerase, that of reovirus, was discovered only a year later (Shatkin & Sipe, 1968; Borsa & Graham, 1968). Some reports claim the existence of RNA-dependent RNA polymerases in plants (Astier-Manifacier & Cornuet, 1971; Duda et. al., 1973; Fraenkel-Conrat, 1983). However, no cellular enzyme capable of transcribing RNA has ever been detected in animal cells. Therefore, all animal viruses that have a dsRNA or a negative strand single-stranded (ss) RNA genome invariably contain a RNA-dependent RNA

polymerase. Since the discovery of the reovirus transcriptase, similar enzyme activities have been detected in several Reoviridae.

In general the transcriptases of the Reoviridae are not active in virions. In vitro activation can be accomplished by various methods that all have the removal of outer capsid polypeptides in common. This treatment of virions results in the formation of particles of higher densities, which are usually referred to as core particles. For example, the reovirus polymerase can be activated by treating the virions with chymotrypsin (Skehel & Joklik, 1969) or heat shock (Borsa & Graham, 1968). These treatments remove the outer capsid proteins μ2, σ1, and σ3. The RNA polymerase of rotavirus becomes active when the outer capsid glycoproteins, 31K and 34K, are removed from fully infectious virions by heat shock or calcium chelation (Cohen, 1977; Cohen et. al., 1979). Three procedures have been described to obtain BTV core particles which have transcriptase activity: CsCl centrifugation at pH 7,0 (Verwoerd et. al., 1972), treatment of virions with chymotrypsin and magnesium (Van Dijk & Huismans, 1980), and isolation of naturally occurring core particles from infected cells (Martin & Zweerink, 1972). All three methods remove the two outer capsid proteins, P2 and P5, from the virion. The chymotrypsin-magnesium treatment has also been used to activate the transcriptases of other orbivirinae such as epizootic haemorrhagic disease virus (EHDV) and African horsesickness virus - AHSV (Van Dijk & Huismans, 1982). Transcriptase activity of Ibaraki virus, another orbivirus, was detected in naturally occurring core particles isolated from infected cells and purified on pH 9,0 CsCl gradients (Namiki et. al., 1983). However, cytoplasmic polyhedrosis virus (CPV) and wound tumor virus (WTV) differ from the above-mentioned Reoviridae in that virions do have transcriptase activity and no activation is required (Lewandowski et. al., 1969; Black & Knight, 1970;

Reddy et. al., 1977). The activity was not diminished when the outer capsid proteins were removed by chymotrypsin treatment, suggesting that these polypeptides were not involved in transcription.

It is not clear why or how modification or removal of the outer capsid shell activates the transcriptase. In the case of reovirus, it has been suggested that these modifications may uncover the central channels of the core spikes through which nucleoside triphosphates may be admitted and transcripts released, or that it may release conformational constraints and thereby permit the polypeptide chains that constitute the enzyme to assume the configuration essential for activity (Joklik, 1974). Yamakawa and co-workers (1982) found evidence supporting the latter postulate. They showed that the polymerase and other RNA-modifying enzymes of reovirus are present in a functional state in intact virions, indicating that chain elongation by the transcriptase is prevented by a structural constraint imposed on the core, which prevents either the advancement of the transcriptase along a stationary genome, or movement of the template RNA through the enzyme catalytic site.

When the optimum reaction conditions for in vitro transcriptase activity of the Reoviridae are compared, some similarities as well as several noticeable differences become apparent. Some of the differences may be related to the different environments in which these viruses replicate (insects, plants and animals).

In vitro transcription of the Reoviridae is dependent on the presence of the divalent cations, notably Mg^{++} and Mn^{++}. Except for Ibaraki virus, which has a pH optimum of pH 7,4, the optimum pH for transcription of the Reoviridae is generally between pH 8,0 and pH 8,5. The optimum temperature for the in vitro transcription reaction of the Reoviridae varies

considerably. The transcriptases of human reovirus (Kapuler, 1970) and rotavirus (Cohen, 1977) function optimally at 47-52°C, while the optimum for WTV, CPV, BTV, EHDV and AHSV varies between 28-32°C. It has been suggested that a low temperature optimum for *in vitro* transcription might be characteristic for viruses which replicate alternatively or exclusively in poikilothermic organisms (Nuss, 1984; Verwoerd *et. al.*, 1972). However, the low temperature optimum for *in vitro* transcription of BTV does not correlate with results obtained *in vivo*, where BTV-infected cell cultures are routinely incubated at 37°C and produce high yields of virus (Huismans, 1979). This particular aspect has not yet been fully investigated.

A feature of the *in vitro* transcription reaction of BTV that has not been described for any of the other Reoviridae, is the inhibition of transcriptase activity which is observed when the core concentration in the transcription reaction mixture is increased (Van Dijk & Huismans, 1980). Vesicular stomatitis virus (VSV) and Sendai virus display a similar endogenous inhibition of *in vitro* transcription with increasing virion concentrations (Perrault & Kingsbury, 1974; Marx *et. al.*, 1974). In both these cases the inhibition could be overcome by including polyanions, such as poly-L-glutamic acid, in the *in vitro* transcription mixtures (Carrol & Wagner, 1978; Stone & Kingsbury, 1973). The factors that influence the core-mediated inhibition of BTV have, however, not yet been identified.

The methyl donor S-adenosylmethionine (Adomet) seems to influence the transcription of the Reoviridae in varying degrees. Adomet has no influence on the transcription by human reovirus (Furuichi *et. al.*, 1975) or WTV (Rhodes *et. al.*, 1977), but it stimulates CPV transcription upto 70 fold, BTV transcription 2 fold (Van Dijk & Huismans, 1980) and Ibaraki virus transcription 15 fold (Namiki *et. al.*, 1983). The original discovery that Adomet increased ssRNA synthesis by

type 1 CPV was thought to indicate a link between transcription and methylation (Furuichi, 1974). However, findings with analogues of Adomet indicated that this interpretation does not hold true. These analogues could not act as methyl donors, but still increased polymerase activity (Mertens & Payne, 1978; Furuichi, 1978; Wertheimer et. al., 1980). It has subsequently been suggested (Mertens & Payne, 1983) that the effects of Adomet, or its analogues, on the transcription of CPV are due to their binding to at least two functionally distinct sites on the CPV particle - the transcription control site and the methyl transfer site(s). It appears that Adomet stimulates the initiation of transcription by a mechanism that lowers the Km for the initiating nucleotide at the promoter site (Furuichi, 1981; Spencer & Garcia, 1984).

In the case of both reovirus (Yamakawa et. al., 1981) and CPV (Furuichi, 1981) it has been observed that RNA polymerases direct multiple abortive initiations during transcription, which result in an accumulation of short oligonucleotides corresponding in nucleotide sequence to the 5' termini of mRNAs. In contrast to prevailing notions in eukaryotic transcription, it has been suggested that chain elongation rather than initiation is the rate limiting stage for CPV and reovirus transcription (Smith & Furuichi, 1982).

Different roles of ATP during in vitro transcription have been reported for the Reoviridae. In vitro transcription of both CPV and rotavirus is inhibited when ATP is replaced by β-γ imido or methylene ATP, which are non-hydrolysable analogues of ATP. In the case of CPV this inhibition can be overcome by pre-incubation with ATP, indicating an involvement of ATP in the initiation process (Wertheimer et. al., 1980). Pre-incubation does, however, not overcome the inhibition in the case of rotavirus and suggests that hydrolysis of ATP is associated with elongation of pre-initiated rotavirus RNA chains (Spencer

& Garcia, 1984).

The mechanism of transcription in the Reoviridae is conservative and asymmetrical. The parental dsRNA genome segments are conserved and remain within the core particles, while the newly synthesised ssRNA transcripts are exported (Joklik, 1974). The *in vitro* synthesised ssRNA transcripts are all of the plus polarity. They do not hybridise with viral mRNAs isolated from infected cells, and direct the synthesis of virus-specific proteins in *in vitro* translation systems. Electron microscopic evidence (Gillies *et. al.*, 1971; Bartlett *et. al.*, 1974) suggests that the reovirus core particle is able to transcribe all 10 genomic dsRNA segments simultaneously, and that the mRNA product is extruded via the 12 hollow spikes of the particle (Luftig *et. al.*, 1972). This confirmed biochemical observations (Skehel & Joklik, 1969; Banerjee & Shatkin, 1970) suggesting the existence of multiple enzymatic sites for the simultaneous transcription of the 10 dsRNA templates.

Several reports on investigations of transcriptional control in the Reoviridae have been published. The genome RNAs of CPV (Smith & Furuichi, 1980) and human reovirus type 3 (Skehel & Joklik, 1969) were reported to be transcribed *in vitro* in molar amounts inversely proportional to their molecular weights. In infected cells, however, reovirus transcription appears to be regulated during the early stages of the multiplication cycle (Watanabe *et. al.*, 1968; Zweerink & Joklik, 1970). Messenger RNA synthesis of bovine rotavirus (Bernstein & Hruska, 1981) appears to have differential rates of transcription for each genome segment both *in vivo* and *in vitro*. Similar results have been obtained in the synthesis of BTV mRNA (Huismans & Verwoerd, 1973). In the case of BTV, it has been found that genome segment 5 is transcribed at double the rate, and genome segment 10 at half the rate that would be anticipated if the 10 different genome segments are individually transcribed at a

constant rate of chain elongation (Verwoerd et. al., 1979). Genome segment 5 of EHDV and genome segment 6 of Ibaraki virus are also transcribed more frequently than expected (Huismans et. al., 1979; Namiki et. al., 1983).

Apart from transcriptase activity, capping enzymes have been detected in reovirus, CPV and WTV (Shatkin, 1976). In the case of rotavirus a capped 5' structure has been found on the plus strands of the genome segments, but the actual enzyme activities have not yet been detected (Imai et. al., 1983). The capping enzymes presumably enable parental virions to initiate the replication process in infected cells without utilising cellular synthetic reactions or inducing new enzyme activities (Joklik, 1981). Studies of the reovirus capping enzymes have been used to analyse initiation of transcription and mRNA capping. It was shown that capping occurs early in the biogenesis of mRNA, probably at the level of initiation (Furuichi et. al., 1976; Darness, 1982). The viral mRNA guanylyltransferase, a key enzyme in the enzymatic steps required for cap synthesis, has been tentatively assigned to the λ2 polypeptide on the basis of its specific, covalent radiolabelling with α^{32}p-GTP (Shatkin et. al., 1983).

Except for the above mentioned-example, no individual enzymatic activities have been assigned to structural polypeptides of the Reoviridae, since the enzyme activities involved in mRNA synthesis and modification are lost upon solubilisation of purified viral particles. Joklik (1981) postulated that the enzymes of the Reoviridae are components of the core shell and that the genes move past transcriptase catalytic sites fixed on the interior side of the core shell. In the case of reovirus this might take place at the base of the spikes in such a way that the dsRNA templates remain within the cores, while transcripts are fed into and through the 12 spikes located on the cores (Gillies et. al., 1971). Apart from transcriptase

activity, no other virus-associated enzymatic activity has as yet been detected in the orbivirinae.

1.4 INFORMATION CONTENT OF THE dsRNA GENOMES OF REOVIRIDAE

A remarkable relationship was observed between the size of the genome segments and polypeptides of reovirus (Zweerink & Joklik, 1970). This was subsequently also found for other dsRNA viruses, including BTV (Verwoerd et. al., 1972; Martin & Zweerink, 1972) and AHSV (Bremer, 1976). These observations formed the basis for the assumption that each genome segment codes for the synthesis of one virus-specific polypeptide. The identification of the primary gene products of a virus and the assignment of biological functions to these proteins is fundamental to understanding the molecular biology of a virus. Both and co-workers (1975) set three criteria for defining the primary gene products of human reovirus: 1) they are present in infected cells after pulse-labelling, 2) they co-electrophorese with cell-free translation products synthesised in response to purified viral mRNA, and 3) they have molecular weights consistent with the coding capacity of the genome segments. These criteria also apply to other Reoviridae such as BTV.

Apart from the seven structural proteins of BTV which have been mentioned previously, Huismans (1979) detected two apparently non-structural proteins, P5A and P6A, in infected cells. These proteins will in future be referred to as NS1 and NS2 respectively. However, it has not been proven unequivocally that these two proteins are indeed virus-specific. If it is assumed that they are viral proteins, then only 9 of the predicted 10 BTV proteins have been accounted for. By means of in vitro translation of BTV mRNAs, and an analysis of the in vitro synthesised proteins, it should be possible to confirm the viral origin of the non-structural proteins, identify any as yet unknown BTV proteins, and determine the protein-RNA

coding assignments of the genome segments.

The two techniques that have been used to determine the information content of the individual genome segments of reovirus, will be outlined briefly. The one technique is based on the slight differences in size that exist between the genome segments and proteins of the three reovirus serotypes. Mustoe and co-workers (1978) produced hybrid viruses by genetic reassortment after pairwise mixed infection. By examining the RNA and protein polyacrylamide gel electrophoresis profiles of the cloned recombinants, they established the protein changes that accompanied specific gene changes and deduced the RNA-protein coding assignments. Kahlon and co-workers (1983) followed a similar approach for BTV serotypes 10 and 11. They were able to determine that the L2 gene of BTV co-segregated with the viral antigens responsible for type specificity, and demonstrated that the L2 gene codes for polypeptide P2.

The other technique involved, was the more direct approach of *in vitro* translation. The 10 species of reovirus mRNA, transcribed by reovirus cores *in vitro*, were isolated individually, hybridised to the various genes to determine from which each was transcribed, and subsequently translated individually in an *in vitro* protein synthesising system. The coding assignments of the four smallest (s-class) reovirus mRNAs were determined using this approach (Levin & Samuel, 1980). However, insufficient quantities of the larger mRNA species were obtained to allow the determination of their protein coding assignments. This urged McCrae and Joklik (1978) to adopt an alternative approach. They isolated the 10 dsRNA genome segments individually and used them successfully as templates in a wheat germ translation system following denaturation with 90% dimethyl sulphoxide. Identification of the proteins was achieved by comparing the electrophoretic mobilities of the translation products with those of authentic

proteins, as well as by Staphylococcus Aureus V8 peptide mapping. The coding assignments obtained by this method corresponded to that obtained by the genetic reassortment procedure. To date, the technique of translating denatured dsRNAs in vitro has been used successfully for reovirus (McCrae & Joklik, 1978), rotavirus (McCrae & McCorquodale, 1982), BTV type 17 (Grubman et. al., 1983), BTV type 1 (Mertens et. al., 1984), infectious pancreatic necrosis virus (Mertens et. al., 1982; Mertens & Dobos, 1982), and Drosophila X virus (Nagy & Dobos, 1984).

1.5 REPLICATION OF THE REOVIRIDAE

It is generally assumed that viral non-structural proteins function during replication. Possible functions which have been suggested for these proteins include the inhibition of cellular metabolism, a role in regulating viral nucleic acid and/or protein synthesis, and involvement in morphogenesis. As has been mentioned, two non-structural proteins have been identified for BTV, namely NS1 and NS2. Protein NS1 is synthesised in large amounts throughout the infection cycle and seems to be the only structural component of the 68 nm tubules found in BTV infected cells (Huismans & Els, 1979). Protein NS2 is a phosphoprotein and has affinity for ssRNA (Huismans & Basson, 1983). However, nothing is known about the functions of either of these proteins.

One of the major unresolved aspects in the replication of the Reoviridae is the mechanism which specifies that each progeny virus particle receives one of each of the 10 different RNA molecules (Joklik, 1981). Reovirus is the only member of the Reoviridae where in vivo replication has been studied extensively. It has been established that morphogenesis of reovirus commences approximately 4 hours after infection. The earliest immature virus particles that were detected contained

virus-coded proteins, including λ1, λ2, μ2, and μ1C (Morgan & Zweerink, 1974), as well as plus-stranded transcripts in RNase-sensitive form (Acs et. al., 1971). Within these particles the plus strands are transcribed once to form minus strands. These minus strands remain associated with the plus strands and represent the progeny dsRNA molecules. Double-stranded RNA never occurs in a free, unencapsidated form within infected cells (Gomatos, 1967; Silverstein et. al., 1970). Therefore, the only way in which genetic information can be passed from parent to progeny is via plus-stranded transcripts (mRNAs). The mechanism that apportions RNA molecules to nascent reovirus progeny particles is efficient, but not infallible, and several examples of reovirus particles with less than 10 genes have been reported (Nonoyama & Graham, 1970; Scheurch et. al., 1974). In the case of WTV, mutants lacking certain genome segments have also been found (Reddy and Black, 1977). Currently all the evidence indicates that assortment of the RNA molecules occurs at the ssRNA stage (Acs et. al., 1971; Sakuma & Watanabe, 1972a; Sakuma & Watanabe, 1972b; Zweerink et. al., 1972).

Two mechanisms have been postulated to explain the association of sets of ssRNAs during morphogenesis (Joklik, 1974). The one is based on RNA-RNA interactions and the other on RNA-protein interactions. The hypothesis that complementary sequences at the terminals of the genome segments are involved in recognition was ruled out when sequencing of the reovirus genes was undertaken. It was found that all reovirus genome segments have common 5'- and 3'-ends (Antczak et. al., 1982). Similar results have been obtained by the analysis of the terminal sequences of influenza virus (Desselberger et. al., 1980; Robertson, 1979), some rotaviruses (Clarke & McCrae, 1983; Imai et. al., 1983), CPV (Kuchino et. al., 1982), ϕ6 phage (Iba et. al., 1982), as well as six orbiviruses (Mertens & Sangar, 1985; Kuichi et. al., 1983; Rao et. al., 1983), of which four belong

to the BTV serogroup, namely BTV serotypes 1, 10, 11 and 20. It has been suggested that these conserved terminal non-coding regions contain recognition signals for initiation of transcription by the core-associated polymerase, and for initiation of the complementary negative strand synthesis during the formation of the dsRNA genome segments (Imai et. al., 1983; Mertens & Sanger, 1985; Antczak et. al., 1982).

Evidence has, however, been obtained supporting the hypothesis that RNA-protein interactions form the basis for the assembly of the different ssRNAs during morphogenesis. For instance, the non-structural σNS protein of reovirus may function as a condensing protein to bring together the 10 reovirus ssRNA templates in preparation for dsRNA synthesis (Gomatos et. al., 1981). Temperature-shift experiments showed that protein σNS, synthesised early in infected cells, was sufficient to produce high yields of infectious reovirions. Furthermore, small 13-19S reovirus-specific particles were detected that were composed solely of σNS protein and had poly-C-dependent poly-G polymerase activity as well as affinity for ssRNAs (Gomatos et. al., 1980; Gomatos et. al., 1981). The mRNA segments of reovirus bind to these particles at specific regions unique for members of each size class (Stamatos & Gomatos, 1982). Additional evidence supporting the hypothesis that proteins function as condensing agents during morphogenesis of the Reoviridae are reports that two orbivirinae, namely BTV (Huismans & Basson, 1983) and Wallal virus (Gorman et. al., 1983) both have a non-structural protein, called NS2, capable of binding ssRNAs.

1.6 PHOSPHORYLATION

The finding that BTV NS2 protein is a phosphoprotein (Huismans & Basson, 1983; Grubman et. al., 1983), adds another perspective to its possible functions. Phosphorylation-

dephosphorylation has been demonstrated in almost every type of cellular process including, synthesis of cell constituents, metabolic pathways and gene expression (Krebs & Beavo, 1979). Phosphorylated proteins have been detected in many enveloped DNA- and RNA-containing viruses such as vaccinia virus, certain rhabdoviruses, simian virus 40, adenoviruses, some arboviruses, oncoviruses, and avian and mammalian retroviruses (Rosemond & Moss, 1973; Sokol & Clark, 1973; Tan & Sokol, 1972; Russell et. al., 1972; Axelrod, 1978; Lamb & Choppin, 1977; Collett & Ericson, 1978; Levinson et. al., 1978; Collett et. al., 1979; Lai, 1976; Pal et. al., 1975). Foot and mouth disease virus (FMDV) and adenovirus type 2 and 5 appear to be the only non-enveloped viruses thusfar examined which contain a protein kinase (Grubman et. al., 1981; Akusjärvi et. al., 1978; Blair & Russell, 1978). Some oncornavirus transforming gene products (Eckhart et. al., 1979; Hunter & Sefton, 1980; Smith et. al., 1979; Witte et. al., 1980) and early proteins of papovaviruses (Eckhart et. al., 1979; Schaffausen & Benjamin, 1979; Scheidtmann et. al., 1982; Smith et. al., 1979) have autophosphorylating activity at tyrosine residues.

The presence of both virion-associated protein kinases and phosphorylated viral proteins in non-transforming enveloped RNA viruses has lead to speculation regarding the function and origin of the kinase, and the significance of the phosphorylated viral proteins in the life cycle of these viruses. At this stage evidence has been obtained that the degree of phosphorylation of some proteins seems to determine the extent of their binding to viral nucleic acid (Clinton et. al., 1978; Scheidtmann et. al., 1984). Furthermore, phosphorylation has been shown to stimulate viral transcription in the case of influenza virus (Kamata & Watanabe, 1977) and VSV (Kingsford & Emerson, 1980).

BTV NS2 protein is the only phosphoprotein that has as yet been

detected in the Reoviridae. However, it should be emphasised that apart from reovirus, this aspect has not been thoroughly investigated. Nothing is known about the significance of the phosphorylation, or the kinase involved. The above-mentioned evidence tempts one to postulate that the affinity of NS2 for ssRNA might be regulated by a phosphorylation process. By investigating the phosphorylation state of *in vitro* synthesised NS2, it might be possible to come to a better understanding of the functional significance of this phenomenon in BTV replication.

1.7 OBJECTIVES OF THIS STUDY

The review of the literature on BTV and related viruses indicates that a detailed knowledge of the *in vitro* synthesis of BTV proteins is still lacking. A primary objective of this of this investigation was therefore to study this process. In order to achieve this objective, the *in vitro* transcription reaction also had to be investigated, since BTV mRNAs were required for the translational studies.

It was considered of particular importance to investigate factors that influence the efficiency of the *in vitro* transcription reaction, such as core concentration and incubation temperature (Chapter 2).

Knowledge of the *in vitro* translation of BTV mRNAS opens the possibility to obtian further detailed information on BTV as such, and BTV related aspects. The aim of the *in vitro* translation study of BTV was to identify all the proteins that are encoded by the 10 BTV mRNA species, and prove the viral origin of the non-structural proteins, as well as determining the coding assignments of the 10 dsRNA genome segments (Chapters 3 and 4).

The last aspect of the study was to investigate whether *in vitro* synthesised NS2 differed from its *in vivo* synthesised counterpart with respect to phosphorylation and affinity for ssRNA, and to carry out experiments in order to identify the kinase phosphorylating NS2 (Chapter 5).

The significance of the results obtained for each of these objectives is discussed in detail at the end of the relevent chapter. Finally, some concluding remarks (Chapter 6) are presented relating the overall findings of this investigation, as well as suggestions for future investigations.

CHAPTER 2

THE *IN VITRO* TRANSCRIPTION REACTION OF BLUETONGUE VIRUS

2.1 INTRODUCTION

Transcriptase activity of BTV is associated with core particles. The conversion of BTV virions to core particles involves the removal of the two outer capsid proteins, P2 and P5 (Verwoerd *et. al.*, 1972). The resulting core particles have a density of 1,40 g/ml as opposed to the 1,36 g/ml of virions (Van Dijk & Huismans, 1982). Conditions for the *in vitro* activation of the BTV-associated transcriptase and the *in vitro* transcription reaction have been studied (Verwoerd & Huismans, 1972; Van Dijk & Huismans, 1980). It was found that the BTV transcriptase has some characteristics that distinguish it from other Reoviridae. One is the inhibitory effect observed with increasing core concentrations in the reaction mixture. Another is the low temperature optimum of 28°C, which is in contrast to the 47-52°C optimum reported for reovirus (Kapuler *et. al.*, 1970) and rotavirus (Cohen, 1977). Furthermore, this low temperature optimum for *in vitro* transcription seems to be in conflict with *in vivo* conditions where BTV-infected cells are routinely propagated at 37°C and produce high yields of virus (Huismans, 1979). Therefore, the purpose of this part of the investigation was to study the effect of the concentration of core particles and the incubation temperature on the BTV *in vitro* transcription reaction in detail. This included a comparison between transcriptase activity of core particles isolated from BTV-infected cells and of core particles prepared *in vitro* from purified virions.

2.2 MATERIALS AND METHODS

2.2.1 Cells and Media

Two cell lines were used for this investigation, baby hamster kidney cells (BHK-21 cells), and L-strain mouse fibroblasts (LF-cells). Both lines were originally obtained from the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, USA 20825). The cells were grown as monolayers in Roux flasks or roller bottles in modified Eagle's medium supplemented with 5% bovine serum (Verwoerd et. al., 1967).

2.2.2 Virus

The avirulent strain of BTV serotype 10 was used. It was propagated in monolayer BHK-cells using a plaque-purified, low passage stock virus suspension. The methods for the propagation and attenuation of the stock virus suspension have been described (Verwoerd, 1969). Virus was purified according to a modification of the Freon extraction method of Verwoerd (1969) as described by Huismans (1979).

2.2.3 In Vitro Preparation of BTV Core Particles

2.2.3.1 Chymotrypsin-Magnesium Procedure

The chymotrypsin-magnesium procedure used for the preparation of BTV core particles was that described by Van Dijk and Huismans (1980). Purified BTV virions were incubated at 37°C for 60 min at a concentration of 0,6 mg/ml (1 mg of virus taken as approximately 5 A_{260}-units) in a reaction mixture containing 0,6 M $MgCl_2$; 80 ug/ml chymotrypsin; and 0,1 M Tris-HCl, pH 8,0. After incubation, core particles were recovered by centrifugation through a 40% sucrose layer at the bottom of the

centrifuge tube (100000g for 120 min at 4°C). The pellet was resuspended in 2 mM Tris-HCl, pH 8,0 and further purified by CsCl density centrifugation (150000g for 27 h at 4°C), using 0,1 M Tris-HCl, pH 8,0 to buffer the CsCl solution. Core particles banded at a density of 1,40 g/ml. This fraction was collected with a syringe, diluted at least ten fold with 2 mM Tris-HCl, pH 8,0, and concentrated by centrifugation through a 1 ml 40% sucrose layer at the bottom of the tube (190000g for 45 min at 4°C). The core particles were resuspended in 2 mM Tris-HCl, pH 8,0 and kept at 4°C until used. Alternatively, the CsCl bands were not concentrated but, desalted on a prepacked Sephadex G-25M column (Pharmacia Fine Chemicals, PD-10).

2.2.3.2 Magnesium Procedure

The magnesium procedure for the preparation of core particles was developed by Cloete and Huismans*. Purified BTV virions were resuspended in 2 mM Tris-HCl, pH 8,0 at a concentration of 2 mg/ml, followed by the addition of an equal volume of 2 M $MgCl_2$. After 30 min at 4°C, the core particles were collected by centrifugation through a 40% sucrose layer (100000g for 120 min at 4°C). The pellet was resuspended in 2 mM Tris-HCl, pH 8,0 and purified further on CsCl density gradients as described under 2.3.2.1.

2.2.4 Polyacrylamide Gel Electrophoresis

Electrophoresis was performed with a slab gel unit constructed as described by Studier (1973). The dimensions of the gels were 15 cm x 16 cm x 0,1 cm. The electrophoretic procedure was essentially the same as that described by Laemmli (1970). The concentration of acrylamide in the separating gel was 15% and

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* unpublished methodology used in this laboratory

in the stacking gel 5%. The gels were run at 200 Volt for 17 h at 4°C, unless indicated otherwise. Gels were stained in Serva Blue solution (Anderson et. al., 1974) and destained in 4% acetic acid at 45°C. They were dried on Whatman 3MM filter paper under vacuum on a heated gel-drying apparatus.

2.2.5 In Vitro Transcription Reaction

In vitro transcriptase activity was assayed using a standard transcription reaction mixture described by Van Dijk and Huismans (1980). The final concentration of reagents in the transcription mixture was: 1,7 mM each of ATP, GTP and CTP; 100 mM Tris-HCl, pH 8,0; 6 mM $MgCl_2$; 7,5 mM phosphoenol-pyruvate; 0,1 mg/ml pyruvate kinase; 2 mM $MnCl_2$; 0,25 mM S-adenosyl-L-methionine (Adomet); 0,5 mg/ml bentonite; 40 uCi ^{3}H-UTP (specific activity 23 mCi/mmole), and BTV core particles at 0,5 A_{260}-units/ml. This transcription mixture was incubated at 28°C for 6 h unless indicated otherwise.

The time dependent incorporation of ^{3}H-UMP into RNA was determined by analysis of samples taken from the transcription mixture. The samples were spotted onto Whatman 3MM filter paper discs (2 cm in diameter), and the amount of ^{3}H-cpm incorporated into acid-insoluble material was determined according to the method described by Bergmann and Lodish (1979). The discs were washed 3 times for 10 min in cold 5% TCA and once in cold water. The water was removed by a rinse in cold 95% ethanol followed by a rinse in ether. The discs were dried for 5 min in a microwave oven, or for 30 min at 90°C. The radioactivity on each filter was measured in a toluene based scintillation fluid (0,1% w/v 2,5-diphenyloxazol; and 0,03% w/v 2,2'-p-phenylenebis 4-methyl-5-phenyloxazole in toluene) in a Packard Scintillation Counter.

2.2.6 In Vivo Uncoating of BTV Virions to Core Particles

2.2.6.1 Preparation of Suspension Cultures of LF-cells

Mouse LF-cells were grown to confluent monolayers on Roux flasks. The cells were then trypsinised, pooled and concentrated by centrifugation (2000g at room temperature). The cells were rinsed once with Eagle's medium, collected by centrifugation and then resuspended in Eagle's medium to a final concentration of approximately 5 x 10^6 cells/ml.

2.2.6.2 Uncoating of BTV Virions in LF-cell Suspension Cultures

Suspension cultures of LF-cells in Eagle's medium (5x10^6 cells/ml) were infected with purified BTV virions at a concentration of approximately 0,5 mg BTV/1 x 10^8cells. The infected suspension cultures were incubated at 37°C, while being stirred magnetically. At 1 h p.i. the suspension was diluted 2,6 fold with Eagle's medium and incubated another hour at 37°C. At 2 h p.i. the cells were harvested by centrifugation (1500g at 4°C). This cell suspension was used for the preparation of a crude cell-free extract (S10-extract). The cell suspension was resuspended in 0,5% Triton-X100 which was buffered with 20 mM Tris-HCl, pH 8,0 (2,5 ml/10^8 cells), and disrupted by ten strokes in a Dounce homogeniser. Nuclei were removed by centrifugation (2000g for 5 min). The supernatant formed part of the crude cell-free extract. The nuclear sediment was rinsed once more with half the initial volume 0,5% Triton-X100 buffered with 20 mM Tris-HCl, pH 8,0, and centrifuged again. This supernatant was pooled with the previous supernatant, and the combined fraction formed the S10-extract.

Viral particles were recovered from the S10-extract by centrifugation (100000g at 4°C for 120 min) through a layer of

40% sucrose in 2 mM Tris-HCl, pH 8,8. They were then layered on a 4-40% sucrose gradient in 2 mM Tris-HCl, pH 8,8 and centrifuged for 1 h (100000g at 4°C). The gradients were fractionated and the optical density of each fraction was determined at 260 nm.

2.2.7 Preparation of In Vitro Synthesised BTV mRNA

For large scale preparation of BTV mRNA by *in vitro* transcription, at least 40 A_{260}-units of BTV cores were used. Apart from the components mentioned under 2.2.5, the transcription mixture also contained 0,85 mM UTP. Incubation was at 28°C for 16 h. After the incubation period, polyvinylsulfuric acid (PVS) was added to a final concentration of 0,05%. Bentonite and core particles were removed by centrifugation (100000g at 4°C for 90 min). An equal volume of STE-1-buffer (0,01 M NaCl; 0,01 M Tris-HCl, pH 7,4; 0,001 M EDTA), containing 0,05% PVS, was added to the supernatant. The virions were subsequently deproteinized by phenol extraction, followed by two ether extractions. The NaCl concentration of the waterphase was adjusted to 0,15 M and supplemented with two volumes of ethanol. The mixture was left for 16 h at -20°C, which resulted in the precipitation of RNA. The RNA was collected by centrifugation and resuspended in 20 ml STE-1-buffer. Single-stranded RNA was separated from any remaining double-stranded RNA by overnight precipitation at 4°C in the presence of 2 M LiCl. The ssRNA precipitate was collected by centrifugation, resuspended in water and divided in small aliquots which were kept at -70°C until used.

2.2.8 Agarose Gel Electrophoresis

Submarine horizontal agarose gel electrophoresis was performed using 3% low gelling temperature agarose gels (SeaPlaque; FMC). The electrophoresis buffer contained 0,005 M Na-acetate;

0,001 M EDTA and 0,001 M Tris, pH 7.9. After dissolving the agarose, it was autoclaved for 10 min before pouring the gel. Electrophoresis was at 100 mA for 45 min. The gels were stained in electrophoresis buffer containing 1 ug/ml ethidium bromide and visualised by means of ultraviolet light.

2.3 RESULTS

2.3.1 In Vitro Preparation of BTV Core Particles

The BTV-associated transcriptase is activated by removal of the two outer capsid proteins, P2 and P5, from the virion. Recently Cloete and Huismans (manuscript in preparation) developed a method to dissociate these outer capsid proteins from virions in an intact form. To investigate whether this method resulted in the complete dissociation of proteins P2 and P5 and activate the transcriptase, it was compared to the chymotrypsin-magnesium procedure.

Figure 2.1 shows the result obtained when BTV virions were converted to core particles by the methods as described under 2.2.3.

It is evident that both methods are equally suitable for the *in vitro* preparation of core particles. Removal of the two outer capsid proteins of BTV by either method resulted in the unmasking of the viral-associated transcriptase (result not shown).

2.3.2 The Influence of Core Concentration on the In Vitro Transcription Reaction

The optimum temperature for the *in vitro* transcription reaction of BTV has been reported to be 28°C (Verwoerd & Huismans, 1972; Van Dijk & Huismans, 1980). However, preliminary experiments

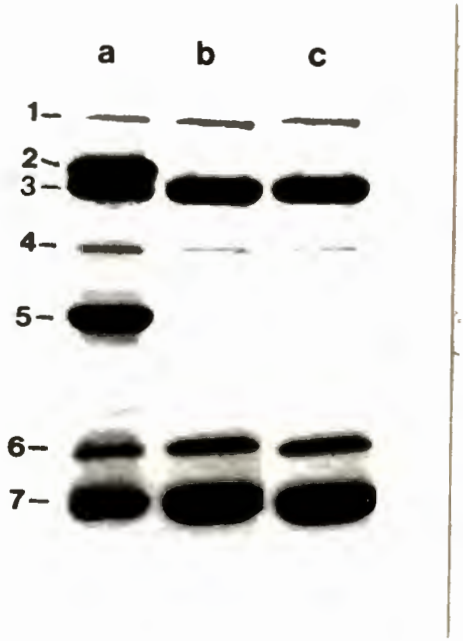

Figure 2.1 Polyacrylamide gel of (a) BTV control, (b) 0,15 A_{260}-units of BTV core particles prepared by the chymotrypsin-magnesium procedure, and (c) 0,15 A_{260}-units of BTV core particles prepared by the magnesium procedure.

indicated that the core concentration in the reaction mixture affected this optimum. To study this, the optimum temperature for the BTV transcriptase was determined at three different core concentrations: 2 A_{260}-units/ml; 0,625 A_{260}-units/ml; and 0,125 A_{260}-units/ml. The BTV cores were prepared as described under 2.2.3.1. Transcriptase activity was assayed at temperatures which varied from 20°C to 47°C by using standard transcription mixtures of 400 ul each from which 25 ul samples were spotted onto filter paper discs every 30 min during a 6 h reaction period. The results obtained are presented in Figure 2.2, and indicate the amount of ^{3}H-cpm incorporated into acid-insoluble material relative to a fixed amount of core particles ($3,125 \times 10^{-3}$ A_{260}-units).

This result shows that the degree to which temperature affects the transcriptase reaction depends greatly on the core concentration. At a core concentration of 0,125 A_{260}-units/ml (Fig.2.2A), there is only a small difference between the reaction rates at 37°C and 28°C, though the reaction appears to proceed best at 31°C. At a core concentration of 0,625 A_{260}-units/ml (Fig.2.2B) the reactions at 28°C and 31°C are almost identical, but there appears to be inhibition of the transcriptase reaction at 37°C. At the highest core concentration, 2 A_{260}-units/ml (Fig.2.2C), the reaction is highly ineffective at 37°C and after 2 h no further synthesis is observed, while at the lower temperatures this inhibitory effect is much less pronounced, and the reaction proceeds somewhat better at 28°C and 24°C than at 31°C.

Similar results were obtained for all the other temperatures investigated. At the highest core concentration (Fig.2.2C) the reaction at 20°C proceeds almost as well as the reaction at 28°C, but transcription only lasted 1 h at 41°C. On the other hand, at the low core concentration of 0,125 A_{260}-units/ml (Fig.2.2A), the reaction rates at 20°C and 41°C were virtually

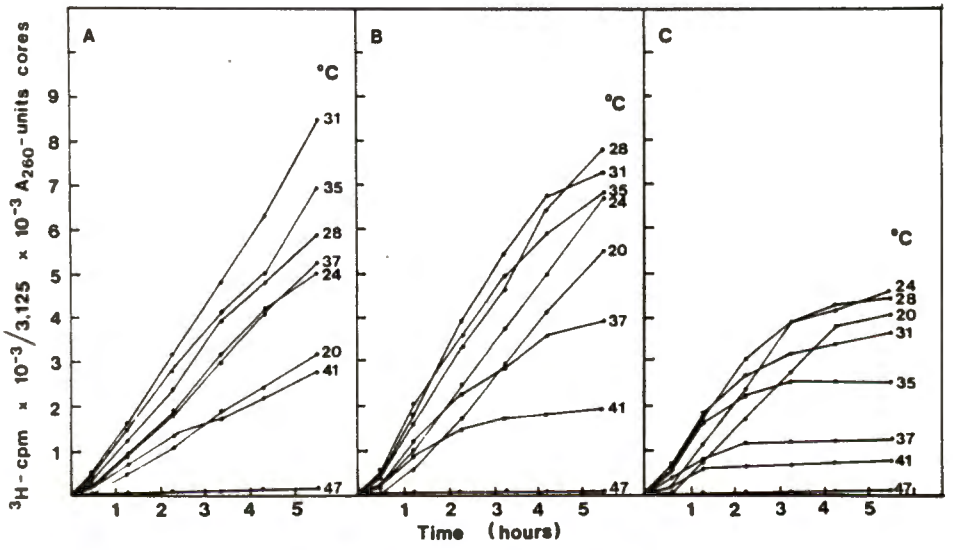

Figure 2.2 Temperature dependence of the <u>in vitro</u> transcription reaction of <u>in vitro</u> prepared BTV core particles, at (A) 0,125 A_{260}-units BTV cores/ml, (B) 0,625 A_{260}-units BTV cores/ml, and (C) 2,000 A_{260}-units BTV cores/ml. The points are the average of two determinations. They represent the ^{3}H-UMP cpm incorporated by 3,125 x 10^{-3} A_{260}-units of BTV core particles, which is the amount of cores present in a 25 ul sample from reaction mixtures in (A).

the same, and transcription continued throughout the experiment.

The results shown in Fig.2.2 indicate that the apparent low temperature preference of the BTV transcriptase is not an inherent characteristic of the enzyme. It seems to be the result of a concentration-dependent inhibitory effect associated with the core particles in the reaction mixture.

2.3.3 Characteristics of the In Vivo Activated BTV Transcriptase

2.3.3.1 In Vivo Preparation of Core Particles

It seems unlikely that factors such as the core-mediated inhibition play a significant role in transcription in infected cells. There is also no evidence for a low temperature requirement for transcription *in vivo*. In fact, radioactive labelling of *in vivo* synthesised BTV mRNA is routinely carried out at 37°C (Huismans, 1970; Huismans & Verwoerd, 1973), and high yields of virus are obtained by propagation of infected cells at 37°C (Huismans, 1979). Therefore, it was of interest to investigate the *in vitro* temperature requirement of transcriptase activity of *in vivo* uncoated virions.

The method for the isolation of BTV cores from an infected suspension culture of L-cells is described under 2.2.6.2*. A 500 ml suspension culture of LF-cells was infected with 12 mg of purified BTV virions. At 2 h p.i., the S10-extract of these cells was prepared and centrifuged at 4°C for 2 h at 100000g. The supernatant was discarded and the pellet was resuspended in 2 mM Tris-HCl, pH 8,8 and layered on a 10-30% sucrose gradient in 2 mM Tris-HCl, pH 8,8. The gradient was centrifuged for 1 h at 100000g (27000 rpm in a Beckman SW 27 rotor) and then

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* the method was communicated by Dr. H. Huismans

fractionated (±2 ml/fraction). The optical density of each fraction was determined at 260 nm and the result is shown in Figure 2.3.

Two optical density peaks were obtained, one with an S value of 550S which correlates with that of virions, and another with an S value of 470S which is that of core particles (Martin & Zweerink, 1972). The standard analysis of the protein content of the particles in the two peaks by means of polyacrylamide gel electrophoresis confirmed that virions were present in the 550S region and core particles in the 470S region (results not shown).

2.3.3.2 In Vitro Transcription of In Vivo Prepared Core Particles

The different fractions of the 470S region of the sucrose gradient of 2.3.3.1, shown in Fig.2.3, was assayed for *in vitro* transcriptase activity both at 28°C and at 37°C, by adding a 200 ul sample of the fractions to 200 ul of double concentrated transcription mixture. At various time intervals, 40 ul samples were taken from the reaction mixtures and analysed for incorporation of ^{3}H-UMP cpm into acid-insoluble material as described under 2.2.5. The results obtained for fraction 11 of Fig.2.3 are shown in Figure 2.4.

Transcriptase activity was observed at both incubation temperatures. The level of activity at 37°C was even slightly higher than that at 28°C. The core concentration for the reaction was approximately 0,2 A_{260}-units/ml. At this concentration all the previous results indicated a higher level of activity at 28°C than at 37°C (compare Fig.2.2). *In vitro* transcriptase activity, similar to that in fraction 11, was detected in all fractions of the 470S region of the sucrose gradient (result not shown).

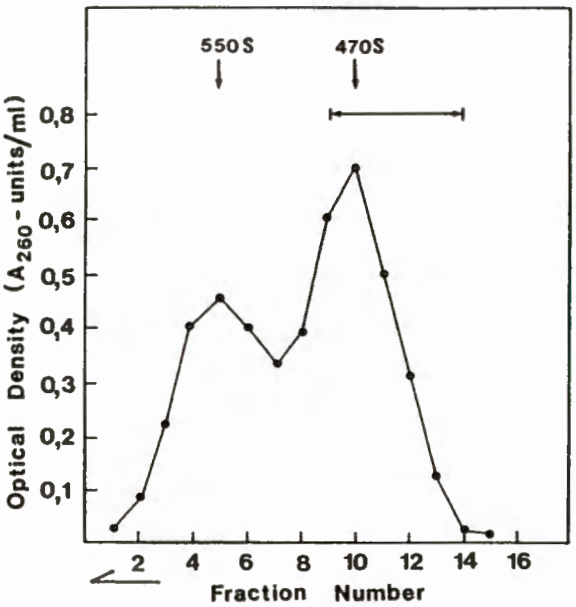


Figure 2.3 Sucrose gradient sedimentation analysis of the pellet of the S10-extract of BTV-infected cells. Sedimentation is from right to left. The position of 550S virus and 470S core particles in control gradients are indicated by arrows. (⟷) indicates the fractions that were pooled for further investigations.

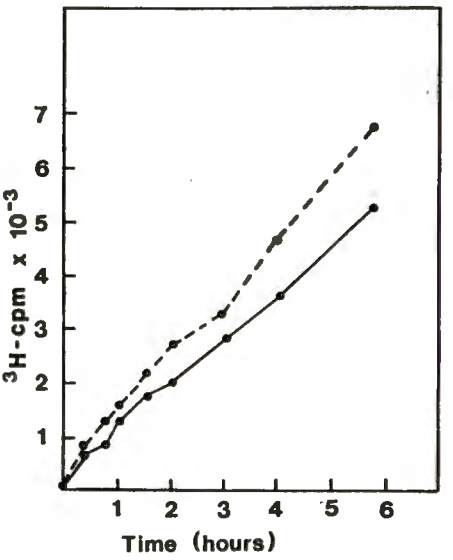


Figure 2.4 In vitro transcription of in vivo prepared BTV core BTV core particles in sucrose gradient fraction 11 at 28°C (•—•), and 37°C (•- - -•).

The fractions of the 470S region of the gradient were subsequently pooled (as indicated in Fig.2.3) and core particles were concentrated by centrifugation and purified on CsCl gradients as described under 2.2.3.1. These core particles were assayed for transcriptase activity in 300 ul reaction mixtures at three different core concentrations: 0,1 A_{260}-units/ml (Fig.2.5A); 0,5 A_{260}-units/ml (Fig.2.5B); and 1,5 A_{260}-units/ml (Fig.2.5C), and five different incubation temperatures between 28°C and 41°C. Transcriptase activity was assayed by taking 25 ul samples every 30 min over a 6 h period and determining the ^{3}H-cpm incorporated into acid-insoluble material as described under 2.2.5. The results obtained are shown in Figure 2.5, and represent the ^{3}H-UMP cpm incorporated by $2,5 \times 10^{-3}$ A_{260}-units of BTV core particles.

The temperature dependence of the in vitro transcription reaction in this experiment differed from that observed in the earlier experiment shown in Fig.2.4, although the same in vivo prepared core particles were used. At all three core concentrations investigated, the in vitro transcription reaction proceeded distinctly better at 28°C than at 37°C. In fact, the results were almost identical to those of in vitro prepared core particles (Fig.2.2). Therefore, it can be concluded that the temperature requirement for in vitro transcription of BTV is the same, irrespective of whether core particles are prepared in vitro or in vivo. One possible explanation for the different temperature dependencies observed in the experiments shown in Fig.2.4 and Fig.2.5 respectively, is that a component of the reaction mixture was responsible for this temperature effect. A possible candidate that might be the cause of this effect, is sucrose. The possible effect of sucrose was therefore subsequently verified.

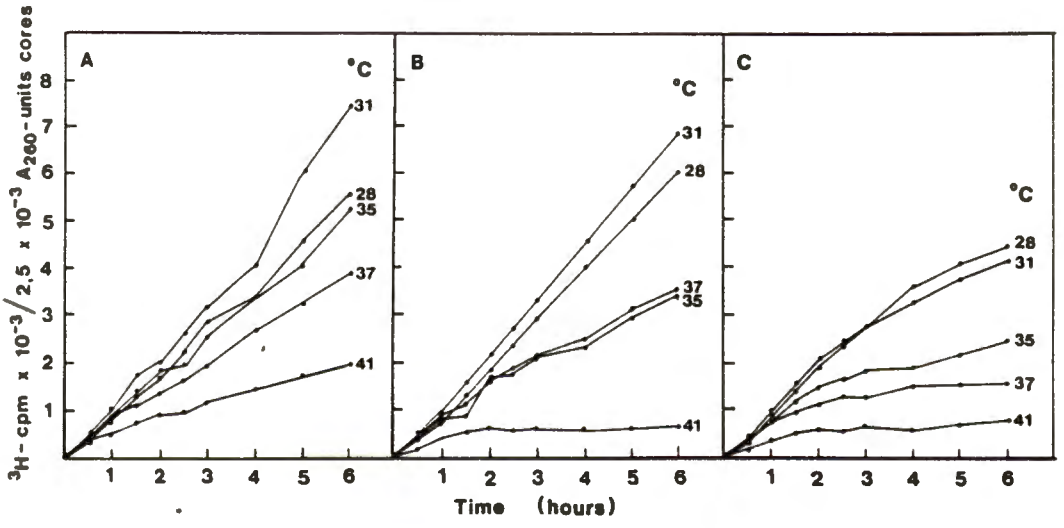


Figure 2.5 The temperature dependence of <u>in vitro</u> transcription of <u>in vivo</u> prepared core particles after CsCl gradient centrifugation at (A) 0,1 A_{260}-units BTV cores/ml, (B) 0,5 A_{260}-units BTV cores/ml, and (C) 1,5 A_{260}-units BTV cores/ml. The points are the average of two determinations. They represent the ^{3}H-UMP cpm incorporated by 2,5 x 10^{-3} A_{260}-units BTV core particles, which is the amount of cores present in a 25 ul sample from reaction mixtures in (A).

2.3.4 Effect of Sucrose on the In Vitro Transcription Reaction

In order to investigate the effect of sucrose on transcriptase activity, BTV virions were uncoated *in vitro* by treatment with chymotrypsin and magnesium as described under 2.2.3.1. The effect of sucrose on the *in vitro* transcription reaction of these core particles was studied by including different concentrations of sucrose in identical, separate 400 ul transcription reaction mixtures. Three different incubation temperatures (28°C, 37°C and 41°C) and two core concentrations (1,5 A_{260}-units/ml and 0,25 A_{260}-units/ml) were investigated. The reaction was assayed by spotting 25 ul samples of the reaction mixtures on filter paper disks over a 6 h period. The incorporation of ^{3}H-UMP cpm into acid-insoluble material was determined as described under 2.2.5. The results are shown in Figure 2.6.

At the lower core concentration sucrose had no effect on the reaction at 28°C (Fig.2.6A), but it stimulated the reaction at both 37°C and 41°C (Fig.2.6B, Fig.2.6C). At the high core concentration the presence of sucrose stimulated the transcription reaction at all three incubation temperatures (Fig.2.6D, 2.6E, and 2.6F). Higher concentrations of sucrose resulted in higher levels of stimulation. Sucrose only stimulated the transcription reaction under conditions where inhibition was evident (Fig.2.6B, 2.6C, 2.6, 2.6E, and 2.6F). Sucrose had no effect on the transcription reaction at low core concentrations and low incubation temperatures, where the reaction rate was constant over the 6 h period (Fig.2.6A).

The stimulatory effect on the *in vitro* transcription reaction did not appear to be unique to sucrose, since it could also be demonstrated with glycerol (results not shown).

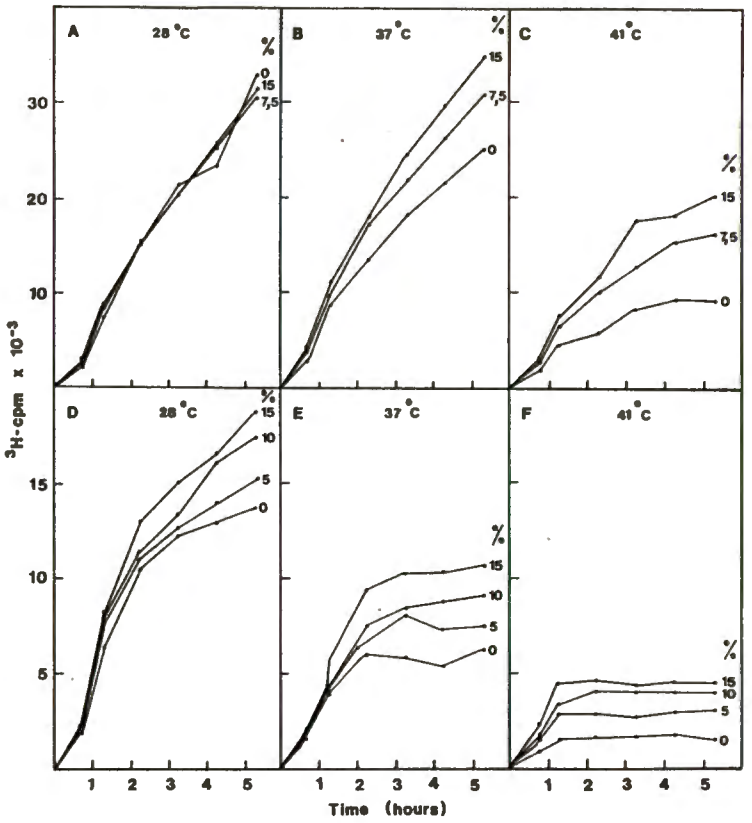


Figure 2.6 The effect of varying amounts of sucrose (%) on the *in vitro* transcription reaction of *in vitro* prepared BTV core particles.

(A), (B), (C) : 0,25 A_{260}-units BTV cores/ml

(D), (E), (F) : 1,50 A_{260}-units BTV cores/ml

2.3.5 The Effect of a Temperature-Shift on In Vitro Transcription

There are at least two possible explanations for the inhibition of *in vitro* transcription observed at high core concentrations. One is that core particles are very unstable under these conditions and disintegrate. The other is that high core concentrations might result in a temporary inactivation of polymerase activity at higher temperatures. In the latter case the inhibition should be reversible. To investigate this, a temperature-shift experiment was carried out: A 2,4 ml transcription mixture with a core concentration of 0,56 A_{260}-units/ml was prepared as described under 2.2.5. One half of this mixture (1,2 ml) was incubated at 28°C, while the other half was incubated at 37°C. After 165 min a 300 ul portion of the reaction mixture at 28°C was removed and incubated further at 37°C. Similarly, 300 ul of the reaction mixture at 37°C was removed and incubated further at 28°C. The remainder of the mixtures were left at the original temperatures. An hour later, 225 min after the start of the reaction, the procedure was repeated. Throughout the experiment 30 ul samples from the different reaction mixtures were removed at the time intervals indicated in Fig.2.7, spotted on Whatmann 3MM filter paper discs and analysed for the incorporation of ^{3}H-UMP cpm into acid-insoluble material. The results obtained are shown in Figure 2.7.

During the first 30 min of incubation the rate of transcription at 37°C was the same as that at 28°C. At 90 min the reaction at 37°C was already severely inhibited, and from 165 min no further incorporation of ^{3}H-UMP was detected. However, the reaction at 28°C continued at its original rate for at least another 4 h. When the incubation temperature of the reaction at 28°C was increased to 37°C, the rate of transcription declined immediately until it terminated completely about 1 h after the shift. On the other hand, a

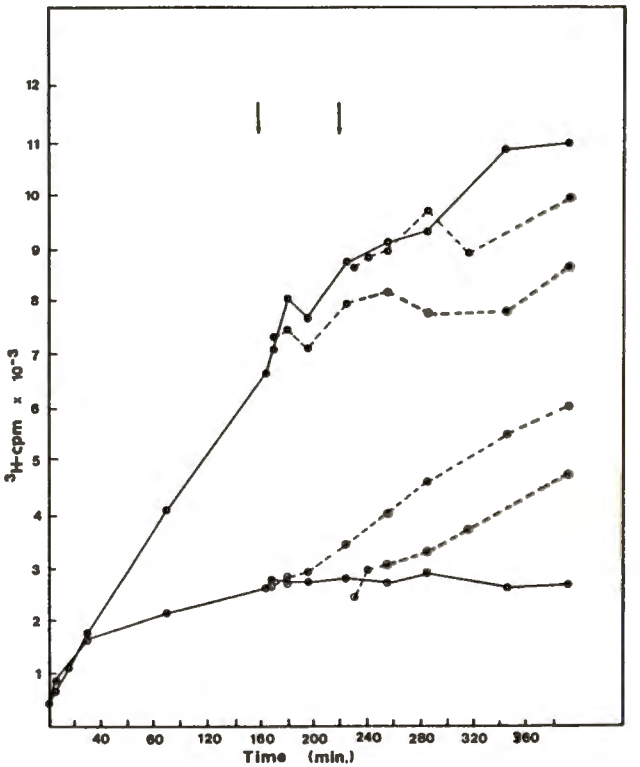


Figure 2.7 The effect of a temperature-shift on the in vitro transcription reaction. (●—●) reaction at 28°C; (●—●) reaction at 37°C; (●---●) reaction upshifted to 37°C; (●-●) reaction downshifted to 28°C. The arrows indicate when the temperature shifts occurred.

decrease in incubation temperature from 37°C to 28°C at a stage when transcription had already completely terminated, resulted in a marked reactivation and increase in the reaction rate.

These findings indicate that the core-mediated inhibition is reversible and therefore not due to unstability or disintegration of BTV core particles under transcription reaction conditions.

2.3.6 Analysis of the Product of In Vitro Transcription

Sucrose gradient analysis and hybridisation are methods which have been used to analyse the BTV mRNA species synthesised during the *in vitro* transcription reaction (Van Dijk & Huismans, 1980; Van Dijk, M.Sc. Thesis, 1980). The S value and size distribution of the newly synthesised mRNA species are obtained on sucrose gradients, but no resolution between individual mRNA species or size classes are possible. The hybridisation procedure converts the ^{3}H-labelled single-stranded mRNAs to ^{3}H-labelled dsRNAs which are readily separated on polyacrylamide gels (Huismans & Verwoerd, 1973), and indicates the relative amounts in which the BTV mRNA species are synthesised. Gel electrophoresis of the mRNA species is, however, the most direct way of analysing the ssRNA product of the transcription reaction. Previous attempts using polyacrylamide gels were not satisfactory, basically due to poor reproducibility and the fact that relatively large amounts of material was required to visualise bands after electrophoresis.

In this investigation the *in vitro* synthesised BTV mRNA species were separated on a 3% low gelling temperature agarose gel, as described under 2.2.8. The result is shown in Figure 2.8. To quantify the result, the negative of Fig.2.8 was scanned (Shimadzu Dual-Wavelenght Thin-Layer Chromato Scanner - Model

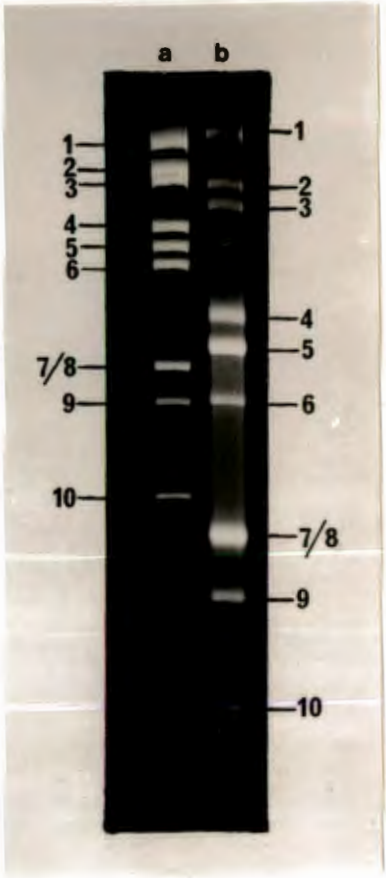


Figure 2.8 3% low gelling temperature agarose gel electrophoresis of: (a) 3 ug BTV dsRNA, and (b) 5 ug BTV mRNA. The gel was stained with ethidium bromide and visualised with ultraviolet light.

CS930). The result is shown in Table 2.1.

The result clearly shows that 5 ug of BTV mRNA was sufficient for agarose gel electrophoresis. This method of separation results in an excellent resolution of the BTV mRNA species. Except for BTV mRNA species 7 and 8, which co-migrated under these conditions, all other species were resolved. There was a marked difference in the relative amounts in which the three mRNA size classes were transcribed. Judged by the intensity of the ethidium bromide staining and the result of the scan, it is clear that the three l-class mRNAs were present in approximately equal amounts relative to one another, but were less abundant than the m- and s-class mRNAs. In the m-class, mRNA-5 was synthesised in an excess compared to the smaller mRNA-6. In fact, it was the most abundantly synthesised of all ten mRNA species. As far as the s-class mRNAs are concerned, mRNA-10 was synthesised markedly less than the somewhat larger mRNA-9. However, as was the case with polyacrylamide gel electrophoresis, the reproducibility of the resolution on agarose gels varied from gel to gel, and many gels could not be used for preparative purposes. Furthermore, the relative migration positions of the mRNA species compared with those of the dsRNA species also varied between gels. An example of this is evident by comparing Fig.2.8 and Fig.4.1, which were run under similar electrophoretic conditions.

Table 2.1 The Relative Amount (%) and Molar Ratio (%) in which the Individual mRNA Species were Synthethised by *In Vitro* Transcription

mRNA Species	Molecular Weight ($\times 10^6$)	Relative Amount (%)	Molar Ratio[a] (%)
1	2,50	7.2	3,03
2	1,99	6.7	3,54
3	1,82	5.9	3,41
4	1,31	14.2	11,41
5	1,16	19.5	17,70
6	1,08	12.6	12,28
7	0,60	23.7	21,89
8	0,54		
9	0,50	6.2	13,06
10	0,03	3.9	13,68

a) calculated by dividing the relative amount (%) by the molecular weight and normalising to a percentage

- molecular weights used were those of Verwoerd *et. al.* (1972)

2.4 DISCUSSION

In this chapter three different methods to convert BTV virions to core particles were used. The chymotrypsin-magnesium and magnesium-pH procedures (2.2.3 and 2.3.1) are both *in vitro* methods. The third method is an *in vivo* procedure using L-cell suspension cultures (2.2.6 and 2.3.3) to convert parental BTV virions to core particles. All three methods unmasked viral-associated transcriptase activity.

The results presented in this chapter illustrate that the core concentration significantly influences the *in vitro* transcription reaction. At low core concentrations the rate of the reaction remains constant for several hours. This is reflected by a linear incorporation of ^{3}H-UMP into acid-insoluble material as a function of time. At higher core concentrations a significant inhibitory effect is observed causing a decline in the reaction rate, until eventually no further incorporation of ^{3}H-UMP can be detected. Depending upon the reaction conditions, the inhibition could come into effect from as early as 15 min to as late as several hours after the start of the reaction. At lower incubation temperatures the inhibition was significantly less than at higher incubation temperatures (Fig.2.2, and Fig.2.5). Evidence was found indicating that the inhibition is of a temporary nature and should be ascribed to specific reaction conditions. The inhibition could be counteracted and even reversed under certain circumstances.

A temperature-shift experiment (Fig.2.7) demonstrated the possibility of reactivating transcriptase activity of a fully inhibited reaction by lowering the incubation temperature of the reaction. The reverse also applied: increasing the incubation temperature resulted in an immediate decrease in

transcriptase activity. The finding that the inhibition could be reversed, ruled out the explanation that it is due to instability and disintegration of core particles under the reaction conditions.

Furthermore, the inhibition of *in vitro* transcription could be reduced by including sucrose or glycerol in the reaction mixture. The characteristics that these compounds have in common are that they both contain multiple hydroxyl groups, and that they have a high viscosity. The stimulatory effect was observed irrespective of the incubation temperature, but only applied to reaction conditions where inhibition of transcripition was evident. Sucrose did not cause additional stimulation under conditions where the reaction rate was not suppressed (Fig.2.6), suggesting that it merely counteracted the inhibition and did not stimulate transcription as such. Polyanions, which could overcome a similar inhibition of VSV and Sendai virus transcriptase (Carrol & Wagner, 1978; Stone & Kingsbury, 1973), had no effect on the inhibition of BTV transcription (data not shown).

There are several alternatives to be considered in attempting to explain the effect of temperature and sucrose on the *in vitro* transcription reaction of BTV.

Serwer and co-workers (1983) found that dextrans and some smaller related compounds, such as sucrose and sorbitol, significantly increased the efficiency of infectious particle formation during the *in vitro* assembly of bacteriophage T7 from procapsids and DNA. Dextrans also inhibited elevated temperature-induced emptying of DNA from bacteriophages. The authors stated that their findings indicated that the stimulation of assembly is partly caused by the stabilisation of packaged DNA in capsids and that protection at elevated temperatures was accompanied by inhibition of the emptying of

DNA from capsids. However, this explanation is not directly applicable in the case BTV, since the reversibility of the inhibition of transcription argues against leakage of RNA as the cause of the inhibition.

On the other hand, polyhydroxylic compounds such as glycerol and sucrose are widely used as stabilising agents for the maintenance of the biological activity of macromolecules (Tanford et. al., 1962; Frigon & Lee, 1972). Glycerol is known to prevent protein denaturation of cell and enzyme preparations during freezing and thawing (Fransler & Loeb, 1974; Valeri, 1975). Back and co-workers (1979) also obtained results indicating that sucrose and other sugars often prevent the thermal denaturation of proteins. The report by Van der Walt (1980) demonstrating that the presence of sucrose was necessary to preserve infectivity of BTV during freeze-drying, seems to fit in with these observations.

A thermodynamic analysis of the aqueous sucrose system by Lee and Timasheff (1981) suggested that the stabilising effect of sucrose on proteins is due to the preferential hydration of proteins in this medium, which can be related to the increase in free energy of cavity formation induced by addition of sucrose to water. Arakawa and Timasheff (1982) concluded that the cohesive force of sugars, which increases the surface tension of water, is a very important factor governing the preferential interaction of proteins with solvent components in aqueous sugar systems, and is responsible for the stabilisation of proteins.

In the case of BTV it is, therefore, quite possible that stabilisation of the core proteins, and in particular the transcriptase itself, could contribute to counteracting the core-mediated inhibitory effect.

Several reports in the literature demonstrate that glycerol and other polyhydroxylic compounds may act to stimulate RNA polymerase activity in reconstituted eukaryotic transcriptive systems. Buss and Stalter (1978) found a marked increase in transcriptive activity of crude or partially purified rat thymic RNA polymerase II on calf thymus DNA in the presence of glycerol, ethylene glycol, glucose or sucrose. Similar to what has been found for BTV, the stimulation of transcription was both concentration and temperature dependent. Furthermore, Travers (1974) reported that glycerol and dimethyl sulphoxide stimulated RNA synthesis in vitro. The extent of stimulation was dependent upon the nature of the DNA template and resulted mainly from an increase in initiation. These findings support the hypothesis that compounds such as sucrose may alter both the DNA template, by lowering the Tm for melting, and the RNA polymerase, by changing the structure of the promoter, in such a way that its recognition parameters were changed. This may lead to stimulation of transcription by facilitating the association of these two compounds to form an active transcriptive complex.

It is possible that a similar explanation accounts for the effect of sucrose on the in vitro transcription reaction of BTV. However, the experimental evidence presented in this study was not sufficient to establish whether elongation or re-initiation, or both, of in vitro transcription are affected by the core-mediated inhibition.

The above-mentioned characteristics regarding the effect of incubation temperature and the concentration of core particles, applied to the in vitro transcription reaction of in vitro as well as in vivo prepared core particles. Therefore, it does not seem as if any inherent differences exist between the in vitro and in vivo activated BTV transcriptases. Consequently, the apparent conflicting temperature requirements of the in vitro

and *in vivo* transcription reactions should be ascribed to the different environments in which these reactions take place. This phenomenon is not unique to BTV, since it has been established that for infectious pancreatic necrosis virus, a bi-segmented dsRNA virus (Mertens *et. al.*, 1982), there is also no correlation between the optimum temperature for *in vitro* transcription (30°C) and the optimum temperature for intracellular IPNV replication (18-20°C).

The phenomenon that BTV core particles are extremely insoluble and virtually always precipitate, might be a contributing factor to the inhibition of BTV transcription. On a few occasions preparations were obtained in which the core particles remained in solution. These results were not properly quantified, but some evidence has been found that in such cases the transcription reaction was much less inhibited (data not shown). However, despite the fact that reovirus core particles also precipitate (flocculate) quite extensively, no concentration-mediated inhibition has been reported. The concentration of reovirus cores during *in vitro* transcription is usually between 5 and 10 fold higher than that used for BTV under optimum conditions. Furthermore, the *in vitro* transcription reaction of reovirus proceeds at much higher rates and produces far higher yields of *in vitro* synthesised mRNA than BTV (Skehel & Joklik, 1969; Joklik, 1972; Schuerch *et. al.*, 1975).

Reovirus cores have 12 hollow projections called spikes (Luftig *et. al.*, 1972) of which protein λ2 seems to be the sole component (Bartlett *et. al.*, 1974). Removal of the spikes was accompanied by a complete loss of transcriptase activity. It has been suggested that these spikes might be the site of synthesis and extrusion of ssRNA. One speculation was that removal of these spikes prevented the newly synthesised ssRNAs to be extruded, and that this accounts for the inhibition of

transcription.

Since precipitates of BTV cores are much finer than that of reovirus cores, it could be postulated that precipitation causes such severe aggregation in the case of BTV, that it becomes physically impossible for any reagents to enter the core particles or for newly synthesised ssRNAs to be extruded - a situation similar to the loss of reovirus spikes. Should this be the case, the effect of sucrose on the transcription reaction could be explained by postulating that it merely acts by counteracting precipitation of core particles due to its high viscosity. However, it remains difficult to explain how core particles which are aggregated to such an extent at 37°C that no mRNA synthesis is possible, can almost immediately be reactivated by a decrease in incubation temperature. In the case of reovirus, indications have been obtained suggesting that conformational changes of core particles are involved in activation and maintenance of in vitro transcription (Kapuler, 1970; Yamakawa et. et., 1982; Powell et. al., 1984). Therefore, it might well be that conformational restraints in the core particles cause the core-mediated inhibition of BTV in vitro transcription.

The resolution of the different BTV mRNA species obtained on agarose gels (Fig.2.8) was comparable to that obtained for reovirus mRNAs on composite polyacrylamide-agarose-urea gels (Schuerch et. al., 1975; Levin & Samuel, 1980). The individual mRNA species were synthesised in vitro in amounts similar to that observed by Huismans and Verwoerd (1973), who reported that BTV genome segment 5 was transcribed at double, and genome segment 10 at half the frequency expected if all the genome segments are transcribed individulally at a constant elongation rate. Genome segment 5 of EHDV (Huismans et. al., 1979) and genome segment 6 of Ibaraki virus (Namiki et. al., 1983) are also synthesised in excessive amounts compared to the other

genome segments. It has been suggested that these findings reflect some form of regulation of transcription in the orbivirinae. As is the case with BTV, the l-class mRNAs of Ibaraki virus are also synthesised in distinctly smaller amounts than the m- and s-class mRNAs. The significance of this phenomenon is not clear yet.

In summary, the results obtained in this part of the investigation contribute towards a better understanding of the factors involved in determining the optimum conditions for the in vitro transcription reaction of BTV. The core-mediated inhibition seems to be the factor that dominates the reaction. It can be counteracted by using lower incubation temperatures and including compounds such as sucrose and glycerol in the reaction mixture. A practical application of these results is that it has now become possible to determine optimum reaction conditions for specific circumstances, thus ensuring the highest possible yield of BTV mRNA.

In the next chapter, experiments will be presented regarding in vitro translation of these in vitro synthesised BTV mRNAs. These experiments should indicate the translational activity of the mRNAs, and thus reflect the fidelity of the in vitro transcription reaction.

CHAPTER 3

IN VITRO TRANSLATION OF BLUETONGUE VIRUS mRNA

3.1 INTRODUCTION

The genetic information of BTV is contained in 10 dsRNA genome segments (Verwoerd et. al., 1970). It has been postulated that each genome segment codes for the synthesis of a single viral protein (Verwoerd et. al., 1972). However, only 9 of the predicted 10 virus-specific proteins have as yet been identified in infected cells - 7 of which are structural, while 2 are non-structural components of the virus (Huismans, 1979). Apart from their presence in infected cells there is, however, no evidence that the latter two proteins are virus-specific and the possibility exists that they are virus-induced cellular proteins. Furthermore, none of the 9 proteins identified so far has a molecular weight of 16K corresponding to the coding capacity calculated for the smallest genome segment (Verwoerd et. al., 1972). A study of the in vitro translation of BTV mRNA was undertaken with the purpose of:

1) confirming the fidelity of the in vitro transcription reaction,
2) identifying all the proteins that are synthesised in vitro from BTV mRNA,
3) determining whether any additional BTV proteins are synthesised in vitro apart from those already identified in infected cells,
4) establishing whether such additional proteins, if any, can be detected in infected cells, and
5) obtaining unequivocal evidence as to the viral origin of the non-structural proteins.

Several cell-free systems have been developed for the in vitro

translation of exogenous mRNAs. They include the E. coli (Ofengand & Haselkorn, 1962), wheat germ (Marcus et. al., 1974), Xenopus laevis oocytes (Gurdon et. al., 1971), Krebs II ascites (Mohier et. al., 1975), and rabbit reticulocyte lysate (Pelham & Jackson, 1976) systems. It was, however, not an objective of this study to compare these systems for the translation of BTV mRNAs. The rabbit reticulocyte lysate system was therefore chosen for this study, basically because it is known to translate large exogenous mRNAs with high efficiency, its general acceptance as a translation system of high fidelity, and finally because it can be considered as a homologous system in view of its eukaryotic origin and the genetic message studied.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of In Vitro Synthesised BTV mRNA

The procedure has been described under 2.2.7.

3.2.2 In Vitro Translation

3.2.2.1 Preparation of Rabbit Reticulocyte Lysate System

Lysates were prepared from rabbits which had been made anaemic by injection of acetylphenylhydrazine as described by Ranu and London (1979). Lysates were stored under liquid nitrogen in 1 ml aliquots.

3.2.2.2 mRNA-Dependent Reticulocyte Lysate System

Lysates were treated with micrococcal nuclease to destroy endogenous mRNAs according to the procedure initially described by Pelham and Jackson (1976), as modified by Neeleman and Van Vloten-Doting (1978). Immediately upon thawing the lysate was

made 25 uM in haemin, 1mM in $CaCl_2$ and 75 U/ml in micrococcal nuclease. It was mixed thoroughly and incubated at 20°C for 15 min. After incubation EGTA, (neutralised with KOH) and pTp (Skup & Millward, 1977) were added to final concentrations of 2 mM and 0,12 mM respectively. The lysate was divided into small aliquots and immediately frozen in and stored under liquid nitrogen until used.

3.2.2.3 Conditions for In Vitro Translation

The in vitro translation procedure that was followed was that described by Pelham and Jackson (1976) and Ranu and London (1979). The translation mixture contained: 2 mM $MgAc_2$; 20 mM Hepes pH 7,5; amino acid mixture containing 19 unlabelled amino acids (lacking L-methionine) each at a concentration of 0,1 mM; 50 ug/ml creatine phosphokinase; 10 mM creatine phosphate; 2 mM dithiothreitol; 160 mM KCl; 40% (v/v) nuclease treated lysate; 20 ug/ml tRNA; 2 ul ^{35}S-methionine (1000 Ci/mmole; ± 12-14 mCi/ml); and 50 ug/ml mRNA. Incubation was done at 30°C for 60 min unless indicated otherwise. At the indicated intervals 5 ul samples were spotted onto Whatman 3MM filter paper discs (2 cm in diameter). The incorporation of ^{35}S-methionine into acid-insoluble material was determined as described under 2.2.5, with the exception that the discs were heated to 95°C for 15 min in 10% TCA before being washed, and acetone was used instead of ether.

3.2.3 Polyacrylamide Gel Electrophoresis

Gels for analytical purposes were identical to those described under 2.2.4. Gels used for preparative purposes were similar, except that the well-forming comb was replaced with a comb having a single well 10 cm in length.

3.2.4 Fluorography

After electrophoresis gels were fixed, destained and fluorographed according to the method of Bonner and Laskey (1974). The gels were then dried and exposed to Cronex MRF31 X-ray film at -70°C.

3.2.5 ^{35}S-Methionine Labelling of BTV Proteins in Infected BHK-21 Cells

Confluent monolayers of BHK-21 cells were grown in 25 cm^2 Falcon flasks or Roux flasks as described under 2.2.1. At various times after infection (see text) the cells were washed with Eagle's medium, inoculated with approximately 5 BTV PFU/cell and incubated at 31°C until the start of the labelling period. The medium was then removed and the cells were washed twice with methionine-free Eagle's medium. Methionine-free Eagle's medium containing 15 uCi/ml ^{35}S-methionine (±12 mCi/ml) was then added to the cells (3 ml/Falcon flask, and 10 ml/Roux flask respectively). The cells were incubated at 37°C for 3 h on a rocker platform. At the end of the labelling period, glass beads were used to harvest the cells. The cells were concentrated by centrifugation at 1500g for 15 min at 4°C, resuspended in STE-1-buffer as indicated and kept at 4°C (usually 4 ml/10^8 cells).

3.2.6 Preparation of Sub-Cellular Fractions from Infected Cells

Soluble (S100) and particulate (P100) fractions were prepared from infected cells resuspended in STE-1-buffer as described under 3.2.5. This is basically according to the method described by Huismans (1979). The only difference was that Triton-X100 was used instead of NP-40. Triton-X100 was added to cells resuspended in STE-1-buffer (±5 X 10^7/ml) to a final

concentration of 0,5%. The cells were disrupted with 5 strokes of a tight fitting Dounce homogeniser. The nuclei were removed by centrifugation at 1500g for 5 min at 4°C and washed once with STE-1-buffer containing 0,5% Triton-X100. Pooled supernatants were referred to as the S10-extract. Soluble (S100) and particulate (P100) fractions were prepared from this S10-extract by centrifugation for 2 h at 190000g at 4°C (45000 rpm in a SW 50.1 rotor) through a 2 ml layer of 40% sucrose. The supernatant (S100) was removed carefully, to avoid contamination with material in the interphase fraction, divided into small aliquots and kept at -20°C. The pellet (P100) was resuspended in STE-1-buffer (20% of the original volume of the S10-extract) and also kept at -20°C.

3.2.7 Preparation of BTV Antiserum

Antiserum against P100 fractions of BTV-infected BHK-cells were prepared as follows: A P100 fraction of BTV-infected cells (1×10^8 cells) was prepared as described above. A 50 ul volume of the preparation was mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly into a rabbit. After 7 days an identical booster injection was given. Two weeks later a second booster, without any adjuvant was administered. A week after the last booster injection the animal was bled and serum prepared. Sheep serum, prepared against purified BTV, was obtained from Mr. N.T. van der Walt, VRI, Onderstepoort. Sheep were inoculated with 100 ug purified BTV together with Freund's adjuvant. A booster injection of 100 ug without any adjuvant was given after 7 days. Three weeks after the booster injection serum was prepared.

3.2.8 Immuneprecipitation

The procedure used was the one described by Harris and co-workers (1981). Volumes of 15 ul translation mixture were

increased to 100 ul by adding 85 ul NETS/NP-40/BSA-buffer (0,15 M NaCl; 0,005 M EDTA; 0,05 M Tris-HCl, pH 7,5; 0,05% NP40). To these, 25 ul rabbit serum, raised against BTV P100 fraction, or 25 ul sheep serum, raised against purified BTV, was added. Precipitation was at 4°C for 16 h. Precipitates were collected by centrifugation (4°C for 10 min at 3000g), washed once with STE-1-buffer, and analysed by gel electrophoresis.

3.2.9 Peptide Mapping

The procedure was essentially that of Cleveland and co-workers (1977) as modified by Burge and Huang (1979). ^{35}S-Labelled proteins were located on the dried gels by means of autoradiography and excised. After removal of the filter paper backing, the rectangular protein-containing fragments (0,5 cm x 0,3 cm) were inserted in the sample wells of a slab gel. As far as possible, proteins containing similar amounts of radioactivity (judged by the intensity of the bands on autoradiograms) were used for comparative purposes. The stacking gel part of these gels was 4 cm in length. The gel fragments were overlaid with 20% glycerol in stacking gel buffer. A 10 ul volume 10% glycerol in stacking gel buffer, containing varying amounts of Staphylococcus Aureus V8 protease, as indicated in the text, was layered over the 20% glycerol buffer. The current was switched on immediately and the voltage was adjusted to 150 Volt. When the tracking dye had moved 3,5 cm into the stacking gel, the current was turned off for 30 min to allow digestion. After the 30 min digestion period the current was turned on again, the voltage was adjusted to 200, volt and electrophoresis was continued until the tracking dye had moved to the bottom of the resolving gel (about 6 h). The peptides were visualised by means of autoradiography.

3.3 RESULTS

3.3.1 Determination of Reaction Conditions for In Vitro Translation of BTV mRNA

Rabbit reticulocyte lysate was prepared and treated with micrococcal nuclease as has been described under 3.2.2. The optimum KCl and $MgAc_2$ concentrations for the translation of BTV mRNA were determined using 30 ul translation mixtures: the $MgAc_2$ concentration was varied at a constant KCl concentration of 160 mM, and the KCl concentration was varied at a $MgAc_2$ concentration of 2 mM. At 0, 15, 30 and 60 min after the start of the reaction 5 ul samples were taken and the amount of ^{35}S-methionine incorporated into acid-insoluble material was determined as described under 3.2.2.3. The results are shown in Figure 3.1.

The optimum $MgAc_2$-concentration was found to be 2 mM at a KCl concentration of 160 mM (Fig.3.1A). The optimum concentration for KCl varied between 160 mM and 240 mM depending upon the incubation period (Fig.3.1B).

Similar experiments were conducted to determine the optimum concentration for haemin. The addition of haemin did not affect the reaction (result not shown).

The reaction conditions for *in vitro* translation of BTV mRNA (2 mM $MgAc_2$; 160 mM KCl; and 10 uM haemin) were selected to favour initiation and maintain a constant reaction rate for a reasonable incubation period. Haemin was included routinely as a precautionary measure to avoid activation of the heme-regulated translational inhibitor (Hunt *et. al.*, 1972). Using these conditions the linear incorporation of ^{35}S-methionine generally lasted at least for 30 min.

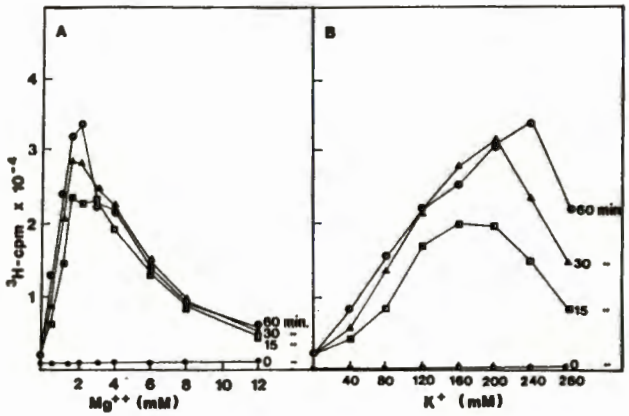


Figure 3.1 Determination of tha optimum concentration of (A) $MgCl_2$ and (B) K^+-acetate for <u>in vitro</u> translation of BTV BTV mRNA after (■—■) 15 min; (▲—▲) 30 min, and (●—●) 60 min of incubation.

3.3.2 In Vitro Translation of BTV mRNA

To determine which proteins were synthesised in vitro from BTV mRNA, two separate 25 ul translation mixtures were compiled as described under 3.2.2.3. One of these served as a control and no exogenous mRNA was added. BTV mRNA was added to the other to a final concentration of 50 ug/ml. Both mixtures were incubated at 30°C for 60 min before a 7,5 ul sample of each was analysed by gel electrophoresis and autoradiography, as described under 3.2.4. The result is shown in Figure 3.2.

^{35}S-Labelled purified BTV served as a control to locate the 7 structural proteins on the gel, while the ^{35}S-labelled P100 sample from infected cells indicated the position of the two non-structural proteins, NS1 and NS2. Proteins which co-migrated electrophoretically with the 7 structural proteins (P1-P7) as well as the two non-structural proteins (NS1, and NS2) of BTV were synthesised in vitro from BTV mRNA (lane c). No endogenous protein synthesis was detected in this experiment (lane d), but an endogenous protein co-migrating with NS2 was sometimes observed.

3.3.3 Kinetics of the In Vitro Translation of BTV mRNA

The kinetics of in vitro protein synthesis of BTV was studied using a 500 ul translation mixture as described under 3.2.2.3. At the indicated time intervals two 15 ul samples were taken: one was immuneprecipitated with rabbit serum raised against the particulate fraction of BTV-infected cells, while the other was immuneprecipited with a sheep serum against purified BTV (3.2.7). The two sera were selected in order to conduct the immuneprecipitation with as wide a range as possible of BTV antibodies. BTV does not replicate in rabbits, but it does in sheep. Subsequent experiments showed that immuneprecipitation was unnecessary, since precipitation was largely non-specific. The

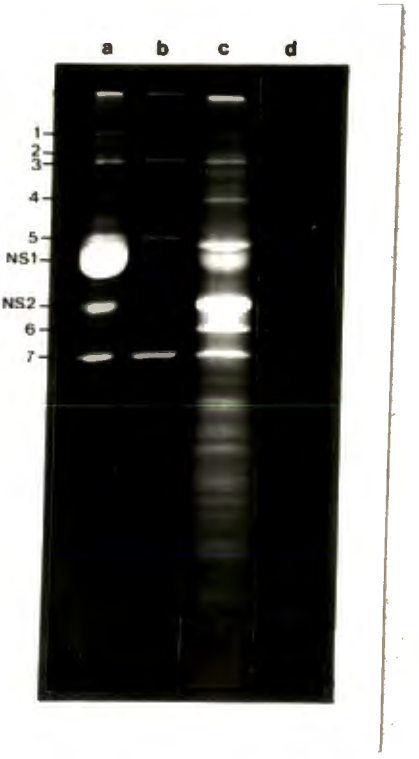


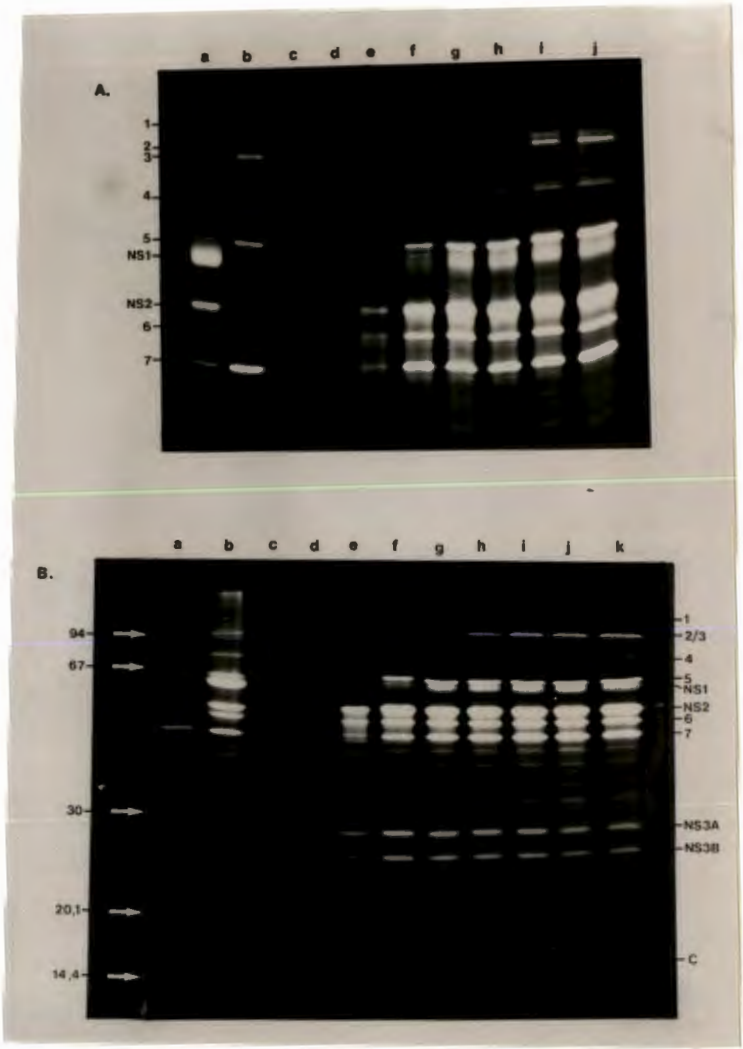
Figure 3.2 Autoradiogram of the polyacrylamide gel electrophoretic analysis of: (a) P100 control, (b) BTV control, (c) in vitro synthesised proteins from BTV mRNA, and (d) in vitro synthesised endogenous proteins.

translation mixtures could therefore just as well have been analysed directly. The immuneprecipitates were analysed by electrophoresis and fluorography (as described under 3.2.4). The gel containing the sheep serum immuneprecipitates was electrophoresed for 16 h at 100 Volt, which is considerably shorter than usual, while the one with the rabbit serum immuneprecipitates was electrophoresed as described under 2.2.4. The results are shown in Figure 3.3. Commercially available low molecular weight markers (Pharmacia) were co-electrophoresed on the gel shown in Fig.3.3B.

Fig.3.3 indicates the time required to synthesise complete copies of the different BTV proteins. In general, the proteins were completed in order of increasing size. The one exception was NS1, which seemed to consist of a number of different polypeptides (Fig.3.3A lane f), and was only completed after P5. Proteins which co-migrated electrophoretically with BTV proteins P7, P6 and NS2 were synthesised within 10 min. After 15 min, synthesis of protein P5 was completed. NS1 and P4 could be detected after 20 min, while P3 and P2 were only detected after at least 25 min of incubation. No synthesis of P1 was detected in this experiment. However, as seen from the results in Fig.3.2 (lane c), P1 could also be synthesised <u>in vitro</u>.

In addition to the 9 above-mentioned proteins, two other proteins (NS3A and NS3B) were synthesised <u>in vitro</u> in significant amounts (Fig.3.3B). They were detected after 10 min of incubation. Their molecular weights, determined from their electrophoretic mobilities relative to that of the low molecular weight markers (Fig.3.3B), were found to be 25K and 28K respectively.

Figure 3.3 Kinetics of the *in vitro* synthesis of BTV proteins analysed by polyacrylamide gel electrophoresis and autoradiography after immuneprecipitation with (A) rabbit serum, and (B) sheep serum. Samples were taken after (c) 0, (d) 5, (e) 10, (f) 15, (g) 20, (h) 25, (i) 30, (j) 35, and (k) 45 min of incubation. (a) and (b) are P100 and BTV controls respectively. The arrows at the left of the figure in B indicate the position of molecular weight markers ($x10^{-3}$).

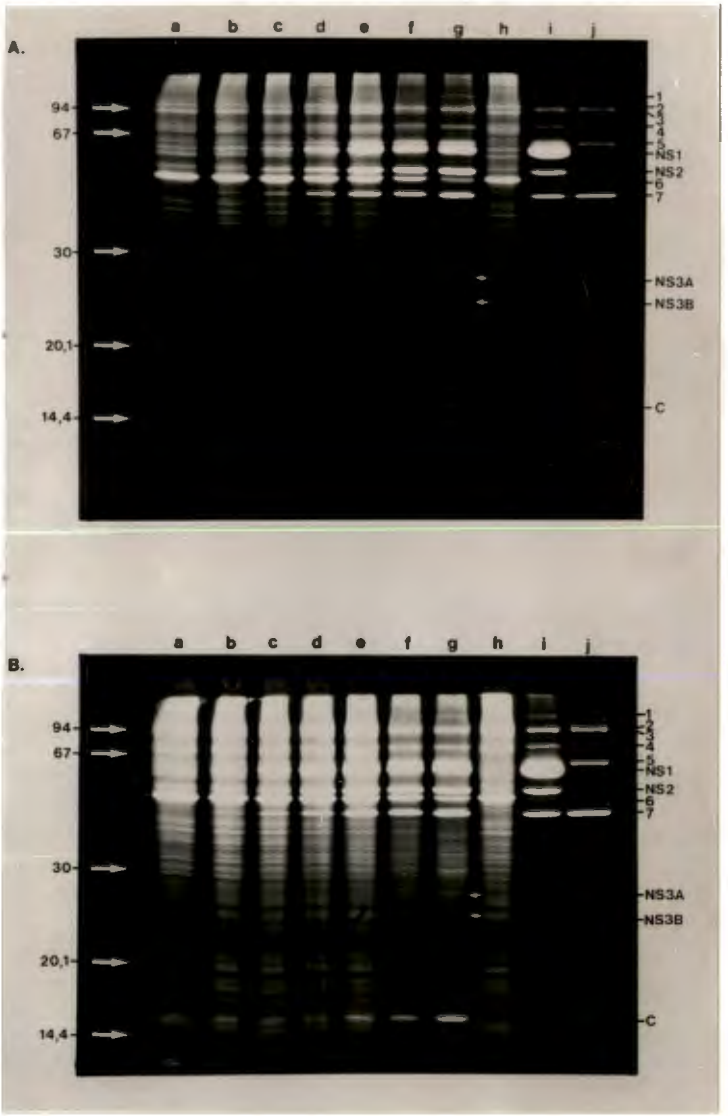

A.
a b c d e f g h i j
1
2
3
4
5
NS1
NS2
6
7
B.
a b c d e f g h i j k
94
67
30
20,1
14,4
1
2/3
4
5
NS1
NS2
6
7
NS3A
NS3B
C

3.3.4 Analysis of Protein Synthesis in BTV-Infected Cells

The detection of proteins NS3A and NS3B described above, raised the question as to whether they are unique to *in vitro* translation, or could also be found under *in vivo* conditions. To investigate this, seven 25 cm^2 Falcon flasks each containing a confluent monolayer of BHK-cells were prepared and infected with BTV. Protein synthesis was monitored by consecutive ^{35}S-methionine labelling (described under 3.2.5) of each of the flasks for a different 3 h interval during the 1 to 21 h p.i. period. After the labelling period, the cells were harvested, concentrated by centrifugation and resuspended in STE-1-buffer at a concentration of 2,5 x 10^7 cells/ml. The 7 cell suspensions were sonicated (MSE sonicator; 1,4 inch tip; amplitude 12; 60 sec), and a sample representing approximately 5 x 10^5 cells of each was analysed by gel electrophoresis and fluorography as described under 3.2.4. Commercially available low molecular weight markers (Pharmacia) were co-electrophoresed. The result is shown in Figure 3.4.

As has been described (Huismans, 1979), the first virus-specified protein synthesis could be detected as early as 4-7 h p.i. (protein P7 in Fig.3.4A lane b). By 10 h p.i. protein synthesis was predominantly virus-specified and BTV proteins P1, P2, P3, P4, P5, NS1, NS2, P6 and P7 (Fig.3.4A lanes d, e, f, and g) were readily distinguishable among the background of cellular proteins (Fig.3.4A lane f). No proteins corresponding in molecular weight to NS3A and NS3B could initially be detected in the low molecular weight region of the gel. However, when the exposure period for autoradiography was doubled (Fig.3.4B), two weakly labelled low molecular weight proteins similar in size to NS3A and NS3B were detected in the 19-21 h p.i. labelling period (Fig.3.4B lane g). They could not be detected during the early stages of infection (Fig.3.4B lanes a-f) and were not synthesised in uninfected cells

Figure 3.4 Kinetics of protein synthesis in BTV-infected cells analysed by polyacrylamide gel electrophoresis and autoradiography. Samples were taken after different 3 hour labelling periods: (a) 1-4 h, (b) 4-7 h, (c) 7-10 h, (d) 10-13 h, (e) 13-16 h, (f) 16-19 h, and (g) 19-21 h p.i. (A) 20 h exposure to X-ray film, and (B) 40 h exposure to X-ray film. (h) mock-infected cells labelled 16-19 h p.i., (i) P100 control, and (j) BTV control. The arrows at the left of the figure indicate the position of the molecular weight markers ($x10^{-3}$).


A.
a b c d e f g h i j
94
67
30
20,1
14,4
NS1
NS2
NS3A
NS3B
C
B.
a b c d e f g h i j
94
67
30
20,1
14,4
NS1
NS2
NS3A
NS3B
C

(Fig.3.4B lane h).

Furthermore, a protein with a molecular weight of approximately 16K, designated C, was synthesised in infected cells simultaneous with the virus-specified proteins that have been mentioned (Fig.3.4A+B lanes b-f). This protein was also synthesised *in vitro* from BTV mRNAs (Fig.3.3 and Fig.3.6). However, the synthesis of protein C varied considerably between *in vitro* translation experiments. This is illustrated by the difference between Fig.3.3, where it could hardly be detected, and Fig.3.6, where it is clearly visible. Protein C was not found in uninfected cells (Fig.3.4 lane h).

To determine whether NS3A, NS3B and protein C were present in a soluble or particulate form in infected cells, one Roux flask containing a confluent monolayer of BHK-cells was infected with BTV at a high multiplicity of infection (40 PFU/cell). Proteins were labelled with ^{35}S-methionine at 12 h p.i. At 16 h p.i. S100 and P100 fractions were prepared as described under 3.2.6. Samples (50 ul) were taken from these S100 and P100 fractions and analysed by gel electrophoresis and autoradiography. The result is shown in Figure 3.5.

Proteins of similar molecular weight as NS3A and NS3B were detected in the soluble (S100) fraction of infected cells (lane c), a protein with the same molecular weight as protein C was present in both the S100 and P100 fractions (lanes b and c).

3.3.5 Peptide Map Analysis of the Non-Structural Proteins of BTV

Co-electrophoresis of proteins is not sufficient evidence that they are identical (Mertens *et. al.*, 1984). Much more conclusive evidence can be obtained by comparison of specific peptide maps prepared for the proteins under discussion. The experimental procedure used for peptide mapping is described

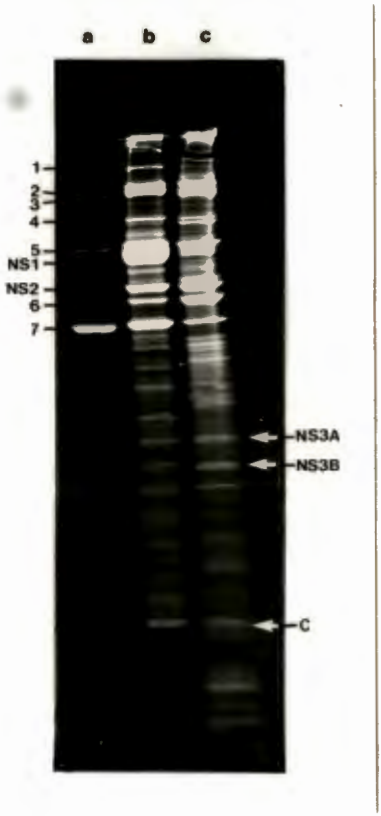


Figure 3.5 Sub-cellular localisation of the newly identified proteins in BTV-infected cells by means of polyacrylamide gel electrophoresis and autoradiography. (a) BTV control, (b) P100 fraction of infected cells, and (c) S100 fraction of infected cells.

under 3.2.9. The technique involves the digestion of proteins with Staphylococcus Aureus V8 protease - an enzyme that cleaves peptide bonds at the C-terminal side of aspartic and glutamic acid residues, resulting in the formation of a limited number of peptides for each protein - and comparing the sets of peptide fragments derived from them by gel electrophoresis, followed by autoradiography.

The proteins which were used for peptide mapping were obtained as follows: ^{35}S-labelled in vivo synthesised NS1 and NS2 were excised from gels after preparative electrophoresis (3.2.3) of a ^{35}S-P100 fraction (prepared as described under 3.2.5 and 3.2.6) from approximately 2 x 10^7 BTV infected cells (Figure 3.6A). ^{35}S-labelled in vivo synthesised NS3A , NS3B and C were similarly excised from preparative gels after electrophoresis of ^{35}S-labelled BTV infected cell-material (3.2.5) from 5 x 10^6 cells (Figure 3.6B). ^{35}S-labelled in vitro synthesised NS1, NS2, NS3A, NS3B and C were all obtained by in vitro translation of BTV mRNA in a 150 ul translation mixture as described under 3.2.2.3. The translation mixture was immuneprecipitated (3.2.7) and subjected to preparative gel electrophoresis (Figure 3.6C).

The peptide maps obtained for proteins NS1 and NS2 are shown in Figure 3.7A and Figure 3.7B respectively.

A characteristic peptide pattern was obtained for each protein. Both the partial and fully digested peptide patterns of in vivo synthesised NS1 were virtually identical to that of in vitro synthesised NS1 (Fig.3.7A). The same applied to the results obtained for NS2 (Fig.3.7B).

A similar experiment was also conducted with protein C. The result is shown in Figure 3.8.

Figure 3.6 Autoradiogram of preparative polyacrylamide gel electrophoresis of (A) P100 fraction of BTV-infected cells, (B) total protein content of BTV-infected cells, and (C) immuneprecipitates of *in vitro* synthesised BTV proteins.

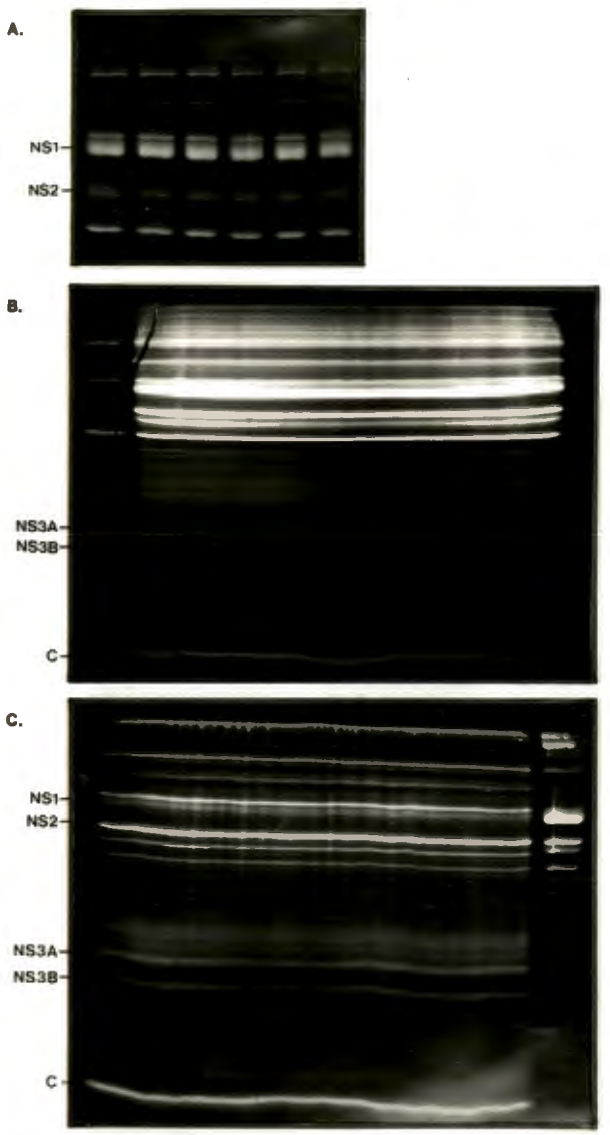
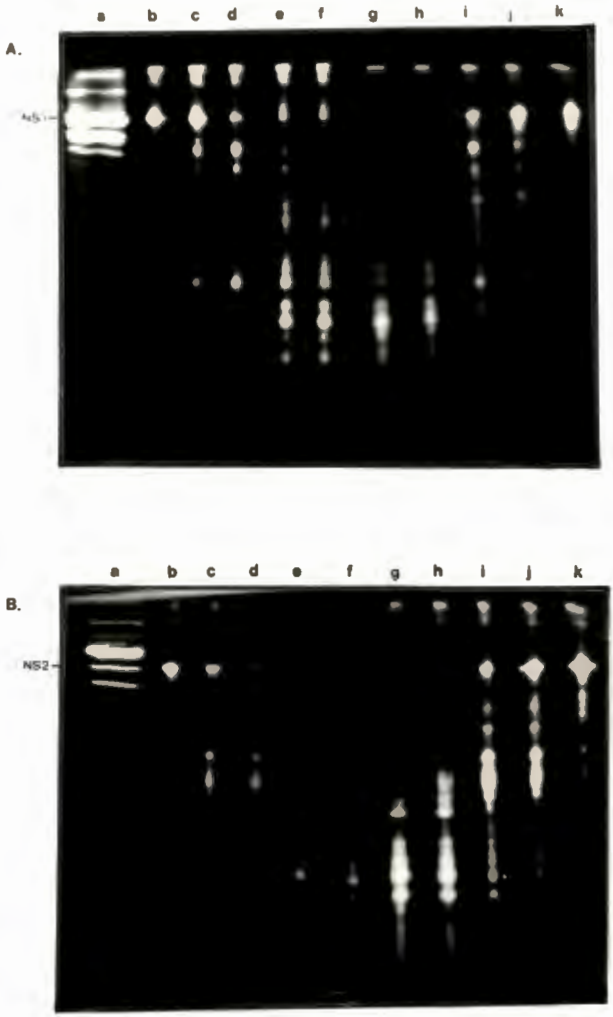

A.
NS1
NS2
B.
NS3A
NS3B
C
C.
NS1
NS2
NS3A
NS3B
C

Figure 3.7 Protease V8 digestion patterns of *in vivo* (lanes b to f) and *in vitro* (lanes g to k) synthesised proteins NS1 (A), and NS2 (B), as analysed by polyacrylamide gel electrophoresis and autoradiography. Amounts of protease V8 used for digestion were (b and k) 0 ng, (c and j) 5 ng, (d and i) 25 ng, (e and h) 500 ng, and (f and g) 2500 ng. (a) P100 control.


a b c d e f g h i j k
A.
NS1
a b c d e f g h i j k
B.
NS2

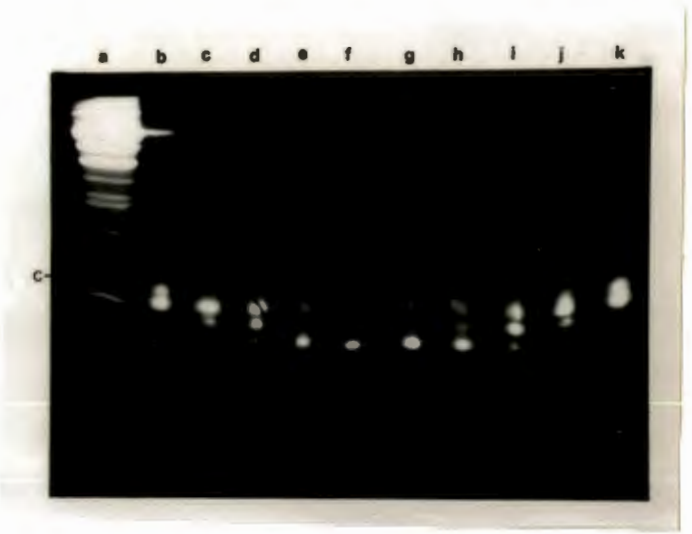


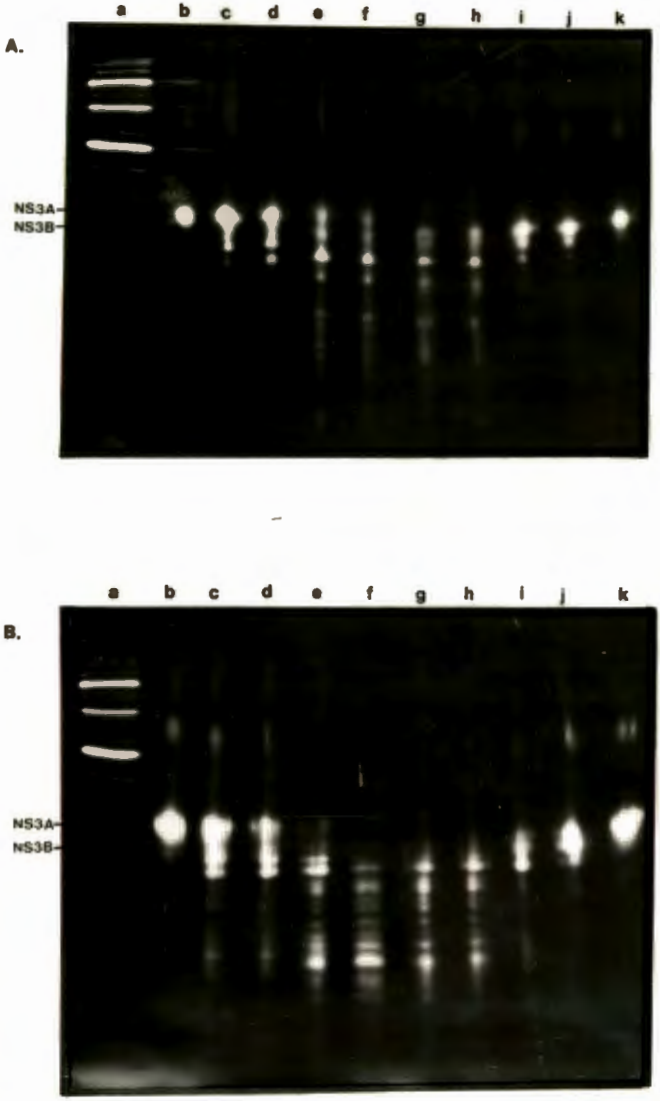
Figure 3.8 Protease V8 digestion patterns of <u>in vivo</u> (lanes b to f), and <u>in vitro</u> (lanes g to k) synthesised protein C by means of polyacrylamide gel electrophoresis and autoradiography. Amounts of protease V8 used for digestion: (b and k) 0 ng, (c and j) 5 ng, (d and i) 25 ng, (e and h) 500 ng, and (f and g) 2500 ng. (a) P100 control.

The peptide patterns of *in vivo* and *in vitro* synthesised protein C were virtually identical. In all cases (Fig.3.7, 3.8, and 3.9; lanes b and k) some digestion of proteins occurred in wells that did not receive any protease. This should be attributed to diffusion of the protease during the 30 min digestion period when electrophoresis was interrupted.

During the study indications were found (see 4.3.5) that NS3A and NS3B could be related. In order to investigate this, the peptide patterns of *in vivo* synthesised NS3A and NS3B were compared with one another. The same was done with *in vitro* synthesised NS3A and NS3B. The results are shown in Figure 3.9.

Except for the initial difference in molecular weight, the peptide map of NS3A was almost identical to that of NS3B. This applied to both the *in vitro* (Fig.3.9B) and *in vivo* (Fig.3.9A) synthesised proteins. Furthermore, comparison of the peptide maps of the *in vivo* and *in vitro* synthesised NS3A (Fig.3.9A lanes b to f and Fig.3.9B lanes b to f) showed a remarkable resemblance. The same applies to NS3B (Fig.3.9A lanes g to k and Fig.3.9B lanes g to k).

Figure 3.9 Protease V8 digestion pattern of: (A) *in vivo* synthesised NS3A (lanes b to f) and NS3B (lanes g to k), and (B) *in vitro* synthesised proteins NS3A (lanes b to f) and NS3B (lanes g to k), as analysed by polyacrylamide gel electrophoresis and autoradiography. Amounts of protease V8 used for digestion: (b and k) 0 ng, (c and j) 5 ng, (d and i) 25 ng, (e and h) 500 ng, and (f and g) 2500 ng. (a) BTV control.


a b c d e f g h i j k
A.
NS3A
NS3B
a b c d e f g h i j k
B.
NS3A
NS3B

3.4 DISCUSSION

In this chapter the *in vitro* translation of BTV mRNA was investigated. The viral origin of the non-structural proteins NS1 and NS2 was established, and a possible translation product of genome segment 10 was identified.

Some parameters of the *in vitro* translation of BTV mRNA were determined (Fig.3.1) and found to be comparable to that which has been reported for a number of viral mRNAs including rotavirus (Cohen & Dobos, 1979; McCrae & McCorquodale, 1982) and turnip yellow mosaic virus (Benicourt *et. al.*, 1978). The optimum concentration for KCl, however, varied at different stages of the translation reaction. This is in agreement with the finding of Mathews and Osborn (1974) who showed that the optimum concentration of KCl for elongation in *in vitro* translation systems was higher than that for initiation.

The *in vitro* synthesised BTV mRNAs were translationally active, thus proving their structural integrity. Proteins co-migrating on gels with the 7 structural proteins of BTV were synthesised *in vitro* from BTV mRNAs. In addition, the two non-structural proteins NS1 and NS2 previously described by Huismans (1979) were also synthesised *in vitro* (Fig.3.2). The peptide maps of *in vitro* synthesised NS1 and NS2 were similar to that of their respective counterparts in infected cells (Fig.3.7). Therefore, it can be concluded that NS1 and NS2 are indeed virus-specified proteins.

The *in vitro* synthesis of the large BTV proteins appeared to be considerably less efficient than that of the medium- and small-sized proteins (Fig.3.2; Fig.3.3). This could be due to inefficient elongation, which has also been found to be responsible for poor synthesis of the large reovirus proteins

in the wheat germ system (McCrae & Joklik, 1978). The efficiency of the in vitro translation of BTV mRNAs seemed to be different from what has been observed in vivo. The intensity of the bands on the autoradiograms indicated that NS1, which is usually synthesised in excessive amounts relative to all the other BTV proteins in infected cells (Huismans, 1979), was synthesised rather poorly in vitro when compared to the other medium- and small-size proteins. The result is especially surprising in view of the finding that mRNA segment 5, postulated to encode this protein, was transcribed in vitro in excess compared to the other mRNAs (Fig.2.8). On the other hand, NS2 and P6 were synthesised in larger relative amounts in vitro compared to the in vivo situation (compare Fig.3.2 and Fig.3.3 to Fig.3.4 and 3.6B). Mertens and co-workers (1984) performed in vitro translation experiments on BTV type 1, using denatured dsRNAs as template, and also speculated about the possibility that a regulatory mechanism controls the expression of genome segments 1 and 10. Huismans (1979) found that all that BTV proteins, with the exception of P1, were synthesised in infected cells with a relative frequency that did not differ significantly from that of the corresponding mRNA species. Therefore, more detailed studies of the translation efficiencies of the BTV mRNAs are needed to verify the results.

In contrast to BTV, neither the in vivo nor the in vitro translation of reovirus proteins was directly related to to the relative abundance of corresponding mRNA species (Joklik, 1974; Both et. al., 1975), and indications of translational control have been reported (Joklik, 1981). In this regard Levin and Samuel (1980) found that differences in translational frequencies between the four s-class reovirus mRNAs that exist in vivo, also existed in vitro. These four reovirus mRNAs were translated in wheat germ protein synthesising systems in vitro with efficiencies that differed by as much as sevenfold. It has been postulated that structural characteristics of the mRNAs

are the basis of their differential translational efficiencies.

In general, the *in vitro* synthesis of BTV proteins was completed in order of increasing size. The small-sized proteins were synthesised within 10 min, the medium-sized proteins required about 20 min, while the large proteins were only completed after at least 25 min of incubation (Fig.3.3). Protein NS1 was, however, the exception. It was completed only after P5, which is a slightly larger protein. It appeared as if several polypeptides accumulated in the same molecular weight region of the gel as protein NS1 during *in vitro* translation (Fig.3.3A, lane f). Similar observations have been made regarding *in vivo* synthesised NS1 (H. Huismans, unpublished results). The significance of this phenomenon is not known. Similar to what has been observed for encephalomyocarditis virus (Shih & Shih, 1981), the rate of peptide chain elongation for BTV also varied for different preparations of reticulocyte lysates. Collins and co-workers (1982a) calculated the maximum rate of elongation of individual Sindbis virus polypeptide chains in the rabbit reticulocyte lysate system to be in the order of 100 amino acids per minute. This is at least twice that estimated to have been obtained during the present study.

In addition to the 9 previously described BTV-specific proteins, 3 new virus-specified proteins (NS3A, NS3B, and C) with molecular weights 28K, 25K and 16K respectively, were detected by *in vitro* translation. These proteins were subsequently also detected in infected cells (Fig. 3.4). The molecular weight of protein C (±16K) corresponded to the coding capacity calculated (Verwoerd *et. al.*, 1972) for the smallest of the 10 dsRNA genome segments. Protein C was present both in a soluble (S100) and particulate (P100) form in infected cells (Fig.3.5). It was detected as early as 4-7 h p.i., which is the stage when viral protein synthesis becomes evident in infected cells. Peptide mapping confirmed the viral origin of protein C.

On the other hand, NS3A and NS3B could only be detected in infected cells at a late stage in the infection cycle (19-21 h p.i.). This may well be due to the fact that they are synthesised in very small amounts (Fig.3.4), and are therefore only detectable under conditions where cellular protein synthesis is markedly reduced. It does not necessarily indicate that they are only expressed late in the infection cycle. Proteins NS3A and NS3B were present in a soluble (S100) form in infected cells (Fig.3.5). The peptide maps of NS3A and NS3B were virtually identical, indicating that they have the same amino acid sequence. This was found to apply to both the *in vitro* and *in vivo* synthesised proteins (Fig.3.9). Furthermore, the peptide maps of NS3A and NS3B showed remarkable resemblance. Unfortunately, not enough material was available at the time to verify this resemblence by analysis on a single gel, but the conclusion that they are virus-specific can, nevertheless, be justified. The main reason why NS3A, NS3B and C were not previously detected, is probably because they are all synthesised in very small amounts compared to the other viral proteins.

The presence of two proteins, such as NS3A and NS3B, which have similar peptide maps, but markedly differ in molecular weight, can be explained in several ways:

(i) post-translational modifications such as proteolytic cleavage, glycosylation or phosphorylation,
(ii) premature termination of protein synthesis, or
(iii) secondary initiation of translation downstream from the first AUG codon in-phase with the first.

In the case of rotavirus (Ericson *et. al.*, 1982), pulse-chase experiments and tunicamycin blockage of glycosylation together with immunoprecipitation and peptide mapping showed that a 35,5K polypeptide was the precursor to the 38K structural glycoprotein, and that glycoproteins 23K and 29K disappeared

concomitant with the build-up of an unglycosylated 20K polypeptide. Futhermore, pulse-labelling experiments and labelling with sugar precursors suggest that modifications to the rotavirus 38K and 29K proteins occurred very rapidly, and most probably co-translationally rather than post-translationally. A precursor-product relationship has also been reported for reovirus. The μ2 protein, which is required for the formation of the spikes, is a cleavage product of structural protein μ1 (Zweerink & Joklik, 1970). No product-precursor relationship between any of the BTV proteins is apparent from experimental data thusfar obtained. This does, however, not exclude such a possibility.

The explanation that premature termination of translation or secondary initiation on BTV mRNAs accounts for the presence of protein NS3B, can also not be ruled out. Experimental evidence such as the amino acid sequences of the proteins, as well as the nucleotide sequences of the genome segments from which the mRNA(s) are derived, will be required to investigate these possibilities. Furthermore, the determination of the coding assignments of the individual genome segments are necessary to identify the genome segments encoding NS3A and NS3B, and to determine whether they are primary translation products encoded by a single genome segment. It will also be required to verify whether protein C is encoded by the smallest genome segment.

Two other reports on the *in vitro* translation of BTV have been published. The one concerns BTV type 1 (Mertens *et. al*, 1984), and the other BTV type 17 (Grubman *et. al*., 1983). However, instead of BTV mRNAs which were used in the experiments described in this chapter, both above-mentioned groups used denatured dsRNAs as templates. The primary purpose of their investigations was to determine the protein coding assignments of the genome segments. Mertens and co-workers (1984) detected two proteins, 8 and 8a, encoded by genome segment 10, which

could resemble NS3A and NS3B respectively. Their results will be discussed in more detail in the next chapter.

CHAPTER 4

CODING ASSIGNMENTS OF BLUETONGUE VIRUS 10A dsRNA GENOME SEGMENTS

4.1 INTRODUCTION

The experiments presented in this chapter were carried out to determine the nucleic acid-protein coding assignment of BTV type 10. In vitro translation of individual mRNAs is one way to achieve this. However, as has been found for reovirus, it is technically difficult to prepare sufficiently large amounts of all 10 individual BTV mRNA species. The dsRNA genome segments of BTV, similar to reovirus (Schuerch et. al., 1975; McCrae & Joklik 1978), migrate as very tight bands when electrophoresed in polyacrylamide gels (Huismans & Verwoerd, 1973) and can readily be isolated in bulk. McCrae and Joklik (1978) developed a technique using dimethyl sulphoxide (DMSO) to physically separate the two strands of the genome segments, which allows the positive strand to be translated in vitro. This technique has been used successfully in the rotavirus, BTV serotype 17 and Drosophila X virus systems (McCrae & McCorquodale, 1982; Grubman et. al., 1983; Smith et. al., 1980). Recently Mertens and co-workers (1984) obtained the coding assignments of BTV 1 using methylmercuric hydroxide (MMOH) as denaturing agent. The denaturing ability of MMOH is due to reactions of MMOH with nucleic acids involving NH bonds essential for Watson-Crick base pairs (Gruenwedel & Davidson, 1966). It reacts reversibly with the nucleotides and can be fully removed by reaction with a suitable strong complexing agent leaving the primary structure of the polynucleotide unaffected (Bailey & Davidson, 1976). Experiments will be presented which were conducted to investigate the denaturation of BTV dsRNA genome segments by DMSO, as well as MMOH, in order to determine the coding assignments of the genome segments. The information thus

obtained was used to elucidate speculation as to whether protein C is the product of the smallest genome segment, and whether NS3A and NS3B are primary gene products from a single genome segment.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of BTV dsRNA

BHK-21 monolayer cells on Roux flasks or roller bottles were inoculated with BTV (1-10 PFU/cell). To be able to monitor the extraction, at least 5 roller bottles were inoculated with 20 PFU/cell of BTV and labelled with ^{3}H-uridine as described by Huismans and Verwoerd (1973). After incubation at 37°C for 48 h, the labelled and unlabelled cells were harvested together and dsRNA was purified as described by Verwoerd and co-workers (1970). The extraction procedure basically involved two phenol extractions, followed by two ether extractions and ethanol precipitation. The dsRNA was subsequently further purified by LiCl precipitation and affinity chromatography on a methylated albumin kieselghur (MAK) column (Verwoerd & Huismans, 1969). The final dsRNA precipitate was dissolved in water and kept at -20°C until used.

4.2.2 Fractionation of Individual dsRNA Species

^{3}H-Labelled dsRNA was prepared as described under 4.2.1 , and fractionated on 4% or 8% preparative slab gels (15 cm x 18 cm x 0,3 cm) as indicated, using the buffer system of Loening (1967) to which 0,01% SDS was added . The 4% gels were run at 70 mA, 100 volt for 16 h, while the 8% gels were run at 70 mA, 100 volt for 42 h. Following electrophoresis dsRNA bands were visualised either by staining with ethidium bromide (1 ug/ml in electrophoresis buffer), or by UV-imaging (Clarke et. al., 1982). Approximately 0,5 mg of BTV dsRNA was fractionated per

gel. The RNA bands were excised from wet gels. After adding 20 ml of STE-1-buffer, the individual bands (usually pooled from 4 gels) were homogenised for 30 sec with an Ultra Turrax homogeniser. The dsRNA was eluted by overnight diffusion at room temperature in a shaker waterbath. Acrylamide residues were removed by centrifugation (5000g for 30 min at 4°C). The NaCl concentration of the supernatant was adjusted to 0,1 M before dsRNA was ethanol precipitated. The precipitate was collected by centrifugation and purified on a MAK-column (4.2.1). No gradient was applied, instead the RNA was loaded in 0,2 M NaCl and eluted with 1,6 M NaCl buffered with 0,05 M Tris-HCl, pH 6,7. A freshly prepared column was used for each individual dsRNA segment. The ^{3}H-dsRNA containing fractions were pooled. RNA was ethanol precipitated and washed successively with 70% and 100% ethanol before being dissolved in water and stored at -20°C until used.

4.2.3 Agarose Gel Electrophoresis

The dimensions of the gels used were 10 cm x 6,5 cm x 0,5 cm.

The following agarose was used:

For 1% gels : Agarose, Ultra Pure DNA Grade from Biorad

For 3% gels : SeaPlague, Low Gelling Temperature Agarose from FMC Corporation

The gels were prepared as described by Maniatis and co-workers (1982). The electrophoresis buffer contained: 0,04 M Tris-HCl, pH 7,0; 0,005 M NaAc; 0,001 M EDTA. Electrophoresis was at 150 mA until the bromophenol blue tracking dye reached the bottom of the gel. The gels were stained with ethidium bromide (1 ug/ml in electrophoresis buffer) and photographed over a UV-light transmittor. Polaroid 665 film was used.

4.3 RESULTS

4.3.1 Denaturation of BTV 10A dsRNA Species

The presence of small amounts of dsRNA is known to inhibit *in vitro* translation reactions (Ehernfeld & Hunt, 1971; Hunter *et. al.*, 1975; Content *et. al.*, 1978). Since dsRNAs have to be denatured in order to render the positive strand available for translation, it is important to ensure complete denaturation of dsRNAs prior to using them as templates for *in vitro* translation. Both DMSO and MMOH can be used as denaturing agents for dsRNAs. Therefore, an experiment was conducted to investigate and compare the denaturation of BTV dsRNA by DMSO and MMOH. Two separate 50 ug amounts of BTV dsRNA (prepared as described under 4.2.1) were precipitated with ethanol, and then freeze-dried. One precipitate was resuspended in 25 ul 10 mM MMOH and left at room temperature for 60 min. The other was resuspended in 2,5 ul water, nine volumes (22,5 ul) of DMSO was added and the mixture was incubated at 50°C for 60 min. Samples (2 ug) of each denaturation reaction were then analysed by electrophoresis both on a 3% low gelling temperature agarose gel and a 1% agarose gel as described under 4.2.3. The result is shown in Figure 4.1.

It can be concluded that DMSO and MMOH denatured BTV dsRNA equally well. No undenatured dsRNA could be detected. Resolution of individual ssRNA species on the 3% gel (Fig.4.1A) was superior to that on the 1% gel (Fig.4.1B). The additional bands present in the denatured samples (lanes c and d) were due to the presence of negative strand ssRNA species, which have electrophoretic characteristics that differ from their corresponding positive strand mRNA species. Unfortunately, as has been mentioned previously (2.3.6), resolution on the 3% low gelling temperature agarose gels was not always reproducible, and for routine analysis of dsRNA denaturation the 1% gels were

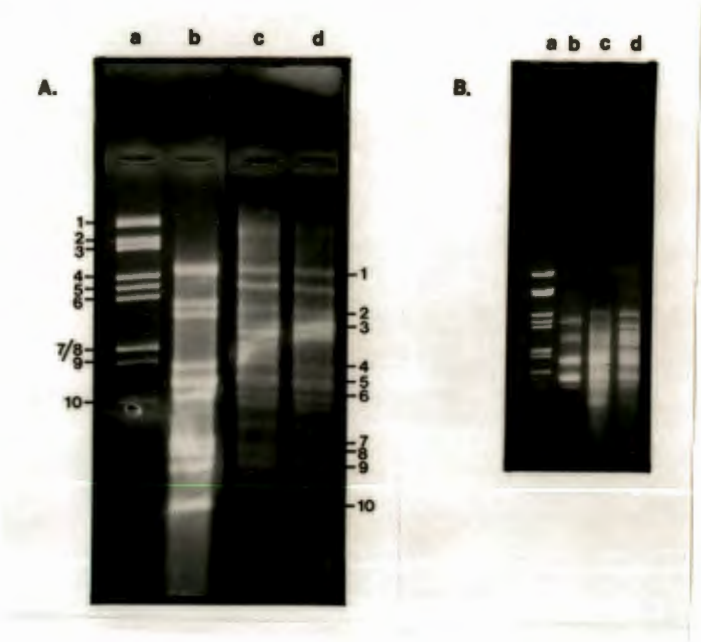


Figure 4.1 Denaturation of BTV dsRNA analysed on (A) 3% and (B) 1% agarose gels.. (a) BTV dsRNA control, (b) BTV mRNA control, (c) DMSO denatured BTV dsRNA, and (d) MMOH denatured BTV dsRNA. The positions of the dsRNA segments are indicated at the left, and those of the mRNA species at the right respectively of figure A.

used.

4.3.2. Translation of Unfractionated BTV dsRNA Species

In vitro translation was conducted using both the DMSO and MMOH denatured BTV dsRNA preparations. Three 50 ul translation mixtures were compiled as described under 3.2.2.3. BTV mRNA was included in the first mixture, while DMSO and MMOH denatured BTV dsRNA was included in the second and third mixtures respectively at a final concentration of 64 ug/ml. After incubation at 30°C for 75 min, a 6 ul sample of each mixture was analysed directly by gel electrophoresis and autoradiography as described previously (3.2.3 and 3.2.4). The result is shown in Figure 4.2.

In the translation control (lane a) all the BTV proteins were synthesised from BTV mRNA. The medium- and small-sized BTV proteins were synthesised *in vitro* equally well from both preparations of denatured dsRNA (lanes b and c). Protein NS2 migrated as a double band. This phenomenon was also observed in Fig.3.6A for *in vivo* synthesised NS2. However, no synthesis of the three largest proteins (P1, P2 and P3) could be detected.

4.3.3 Preparation of Individual dsRNA Species

BTV dsRNA was extracted from BTV-infected cells and fractionated on 4% preparative polyacrylamide gels as described under 4.2.1 and 4.2.2. After staining with ethidium bromide the individual dsRNA species were located over a UV-light transmittor. A typical preparative scale gel is shown in Figure 4.3.

The 10 individual dsRNA bands were excised. The dsRNA was eluted by diffusion into STE-1-buffer, purified as described under 4.2.2, and analysed on a 1% agarose gel such as the one

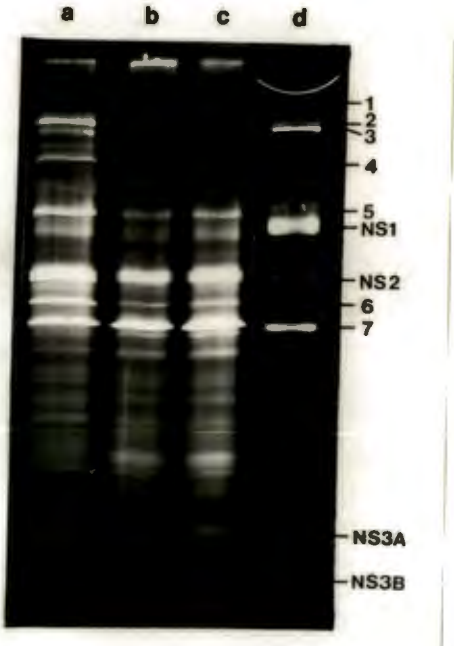


Figure 4.2 In vitro translation of denatured BTV dsRNAs as analysed by polyacrylamide gel electrophoresis and autoradiography. (a) proteins synthesised from BTV mRNA, (b) proteins synthesised from DMSO denatured BTV dsRNA, (c) proteins synthesised from MMOH denatured BTV dsRNA, and (d) P100 control.

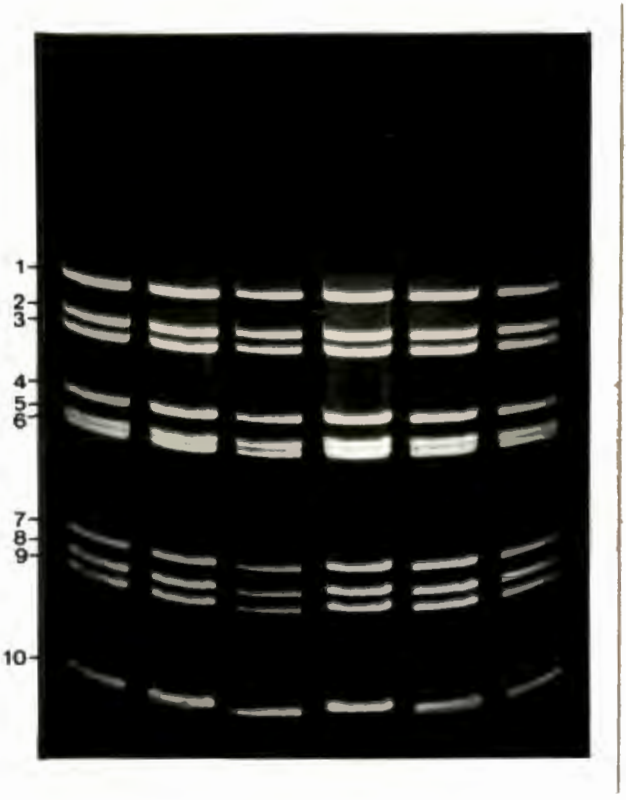


Figure 4.3 Preparative 4% polyacrylamide gel of BTV dsRNA visualised with ethidium bromide.

shown in Figure 4.4.

Genome segments 1, 7 and 10 appeared to be pure. Varying degrees of cross-contamination from neighbouring segments were detected in the other 7 segments. Subsequently these preparations of individual dsRNA segments were each re-run on a separate polyacrylamide gel and extracted as described above, which virtually always eliminated cross-contamination.

Ethidium bromide staining, combined with the relatively long periods of UV-exposure (several minutes) required to excise individual dsRNA bands from gels, is known to cause damage to nucleic acids. To avoid this, the UV-imaging technique developed by Clarke and co-workers (1982) was introduced. It requires only a few seconds of UV-exposure. Figure 4.5 shows the result that was obtained using this method to visualise dsRNA on an 8% polyacrylamide gel.

All the dsRNA genome segments were clearly visible. Although 8% gels were much easier to handle than 4% gels, they had the disadvantage that genome segments 8 and 9 co-migrated and that much longer electrophoresis periods were required to obtain satisfactory resolution.

4.3.4 Denaturation of Individual dsRNA Species

Prior to *in vitro* translation, 4 ug of each individual genome segment was ethanol precipitated. The precipitates were washed with 70% ethanol, freeze-dried and each was resuspended in 8 ul 10 mM MMOH. After 60 min at room temperature, 0,5 ug of each segment was analysed on a 1% agarose gel. The result is shown in Figure 4.6.

Denaturation of all 10 genome segments was observed. The two bands detected after denaturation of each individual dsRNA

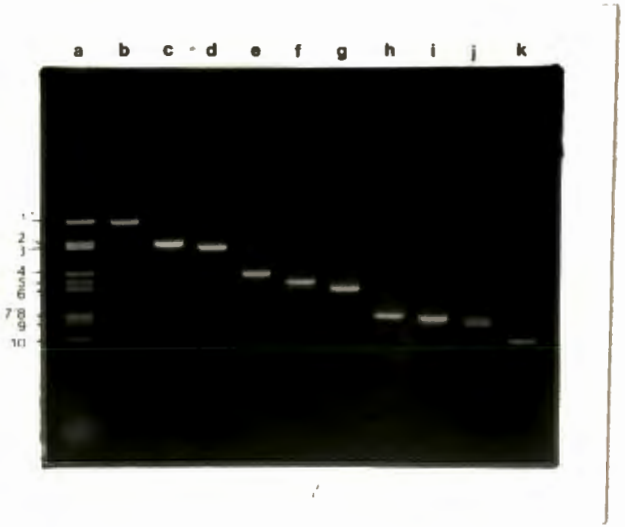


Figure 4.4 Agarose gel electrophoresis of individual BTV dsRNA genome segments. (a) BTV dsRNA control, (lanes b to k) individual dsRNA genome preparations 1 to 10 respectively.

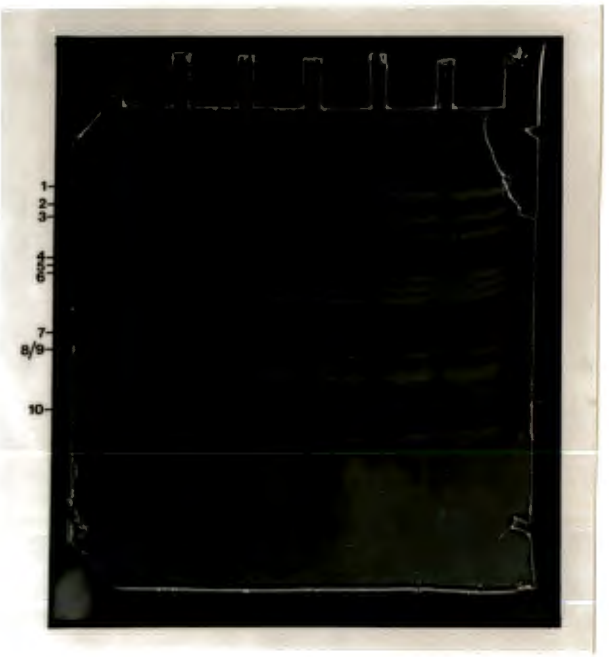


Figure 4.5 Preparative 8% polyacrylamide gel of BTV dsRNA visualised by means of UV-imaging.

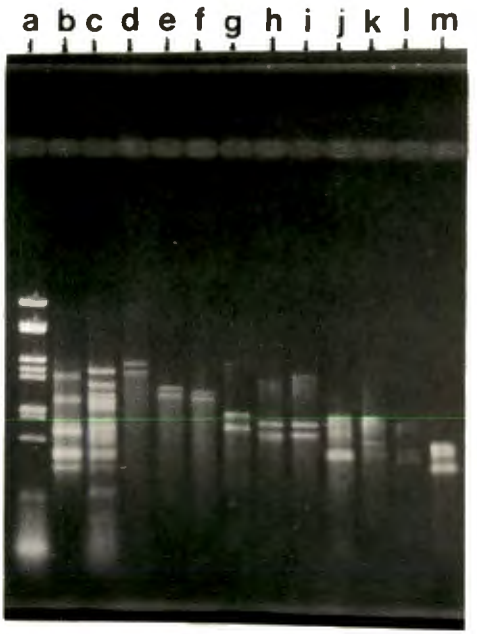


Figure 4.6 Agarose gel electrophoretic analysis of MMOH denaturated BTV dsRNA genome segments.(a) 3 ug undenatured BTV dsRNA control, (b) 5 ug BTV mRNA control, (c) 3 ug unfractionated denatured BTV dsRNAs, and (d to m) 0.5 ug each of denatured dsRNA segments 1 to 10 respectively.

genome segment represented the positive and negative ssRNA species of the genome segments. There was some evidence of either renaturation or incomplete denaturation with the small genome segments.

4.3.5 Translation of Individual Species of Denatured dsRNA

The denatured individual dsRNA genome segments of 4.3.4 were translated *in vitro* in 30 ul reaction mixtures, as described under 4.3.2. Initially the translation products were identified by gel electrophoresis. The results obtained are shown in Figure 4.7 and Figure 4.8.

Translation of the 3 denatured medium-sized genome segments 4, 5 and 6 produced proteins co-migrating respectively with P4, P5 and NS1 (Fig.4.7). Translation of the 4 denatured small genome segments 7, 8, 9 and 10 resulted in the synthesis of proteins co-migrating respectively with proteins P7, NS2, P6 and NS3A and NS3B (Fig.4.8). Although translation was obtained with the 3 denatured large genome segments, it was extremely inefficient and no full size proteins were detected to allow the determination of their respective coding assignments (results not shown). Some cross-contamination between genome segments 8 and 9 remained, even after two rounds of gel electrophoresis. It was, however, possible to deduce the respective coding assignments. Two proteins which had molecular weights identical to NS3A and NS3B were synthesised from genome segment 10. They were usually synthesised in equimolar amounts. However, occasionally the relative amounts in which NS3A and NS3B were synthesised varied and NS3A was synthesised in larger amounts than NS3B (see Fig.4.8 lane f). None of the genome segments seemed to have protein C as a primary gene product, and there was no evidence that protein C was encoded by genome segment 10. However, in one experiment, depicted in Figure 4.9, similar to that described above, there was some indication of a protein

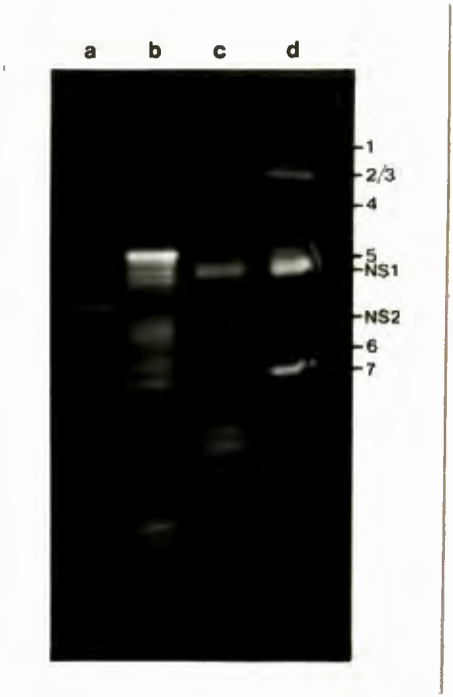


Figure 4.7 Autoradiogram of the polyacrylamide gel electrophoretic analysis of proteins synthesised <u>in vitro</u> from denatured dsRNAs of the medium size-group:
(a) dsRNA segment 4, (b) dsRNA segment 5, and (c) dsRNA segment 6. (d) P100 control.

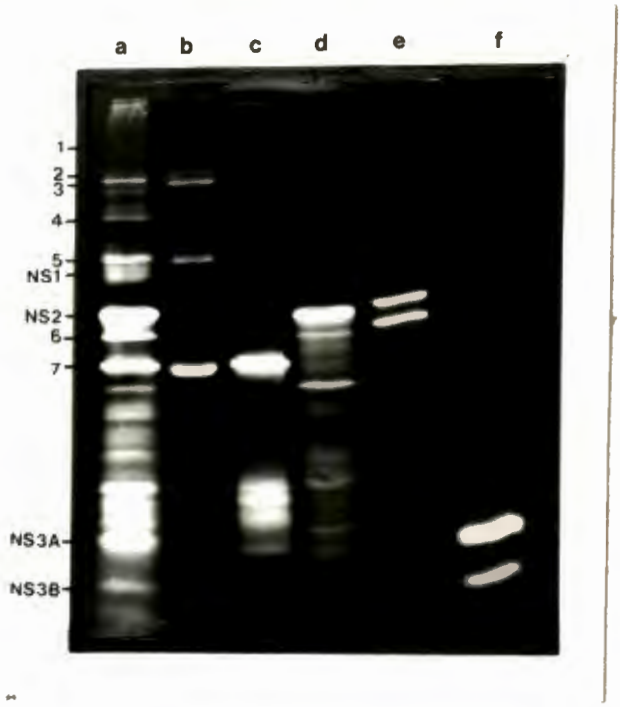


Figure 4.8 Autoradiogram of the polyacrylamide gel electrophoretic analysis of proteins synthesised <u>in vitro</u> from denatured dsRNAs of the small size-group:
(c) dsRNA segment 7, (d) dsRNA segment 8, (e) dsRNA segment 9, and (f) dsRNA segment 10. (a) proteins translated from BTV mRNAs, and (b) BTV control.

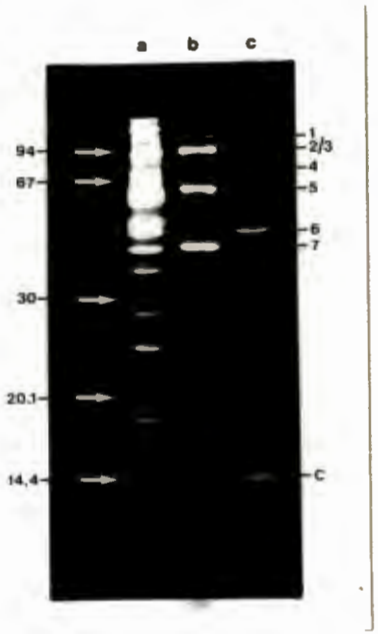


Figure 4.9 Autoradiogram of the polyacrylamide gel electrophoretic analysis of (a) P100 fraction of BTV infected cells, (b) BTV control, and (c) the <u>in vitro</u> translation of denatured dsRNA segment 9. The arrows at the left of the figure indicate the position of molecular weight markers ($x10^{-3}$).

with a molecular weight similar to protein C among the low molecular weight proteins synthesised from genome segment 9 (Fig.4.9 lane c).

4.3.6 Peptide Mapping of Translation Products from Denatured Genome Segments

The coding assignments for the group-specific (P7) and non-structural proteins (NS1, NS2, NS3A and NS3B) of BTV were confirmed by peptide mapping. In the case of the other proteins, insufficient material was available to conduct similar experiments.

The peptide pattern of the protein product of denatured dsRNA genome segment 7 was compared to both that of P7 synthesised *in vitro* from unfractionated BTV mRNAs (Figure 4.10A), and P7 obtained from BTV virions (Figure 4.10B).

The peptide map of the protein synthesised from denatured genome segment 7 was similar to that of P7, which confirms unequivocally that the group-specific antigen, P7 (Huismans & Erasmus, 1981), is encoded by genome segment 7.

The RNA-coding assignments for non-structural proteins NS1 and NS2 were confirmed by comparing the peptide maps of fully digested samples of the proteins *in vitro* translated from BTV dsRNA genome segments 6 and 8 respectively, to proteins NS1 and NS2 present in the P100 fraction of BTV-infected cells (3.2.6). The results are shown in Figure 4.11 and Figure 4.12 respectively.

The respective peptide maps were virtually identical, indicating that BTV dsRNA genome segment 6 codes for protein NS1, and genome segment 8 for protein NS2.

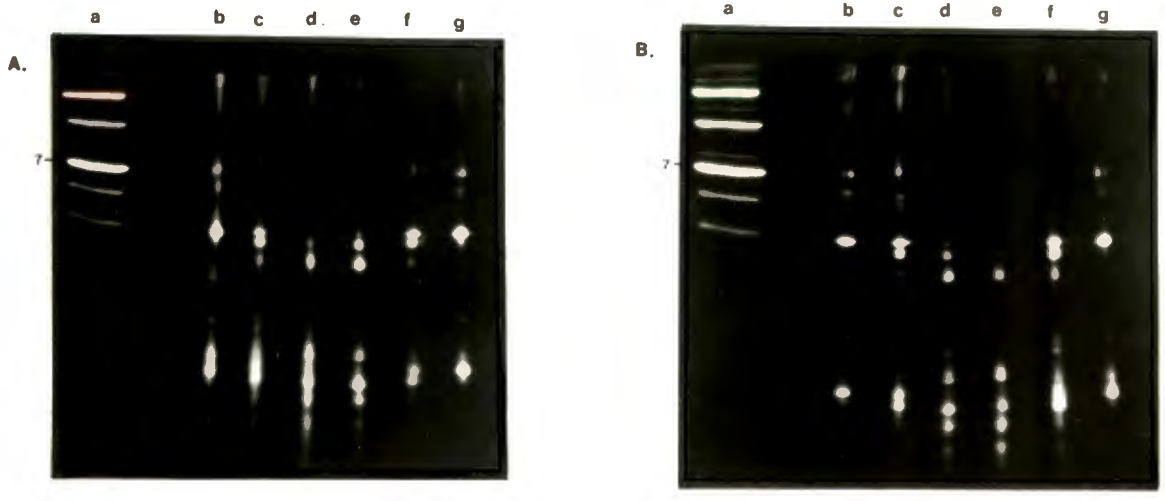


Figure 4.10 Autoradiogram of the polyacrylamide gel electrophoretic comparison of the protease V8 digestion patterns of protein P7: (A) synthesised from BTV mRNAs (lanes b,c and d) with that of the protein synthesised from denatured dsRNA segment 7 (lanes e,f and g), and (B) from purified BTV (lanes b,c and d) with that of the protein synthesised from dsRNA segment 7 (lanes e,f and g). Amounts of protease V8 used were: (b and g) 10 ng, (c and f) 500 ng, and (d and e) 2500 ng. (a) BTV control.

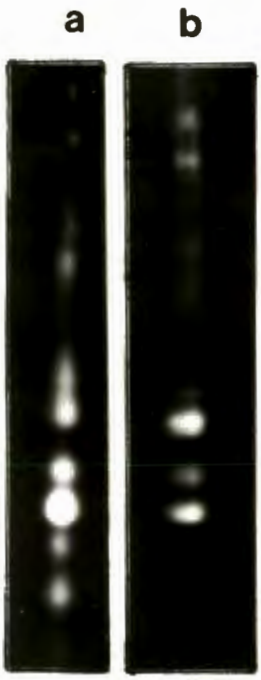


Figure 4.11 Autoradiogram of the polyacrylamide gel electrophoretic comparison of the 2500 ng protease V8 digestion pattern of (a) protein NS1 from the P100 fraction of infected cells with (b) the protein synthesised <u>in vitro</u> from dsRNA segment 6.

Figure 4.12 Autoradiogram of the polyacrylamide gel electrophoretic comparison of the 2500 ng protease V8 digestion pattern of (a) protein NS2 from the P100 fraction of infected cells with (b) the protein synthesised *in vitro* from dsRNA segment 8.

The peptide maps of proteins NS3A and NS3B, which were synthesised *in vitro* from denatured dsRNA genome segment 10, were compared to one another. The result is shown in Figure 4.13.

The peptide maps of NS3A and NS3B were almost identical, except for the initial difference in molecular weight. This result is similar to that obtained with NS3A and NS3B from BTV-infected cells as well as from *in vitro* translation of unfractionated BTV mRNAs, although the digestion was not as complete (compare Fig.3.8).

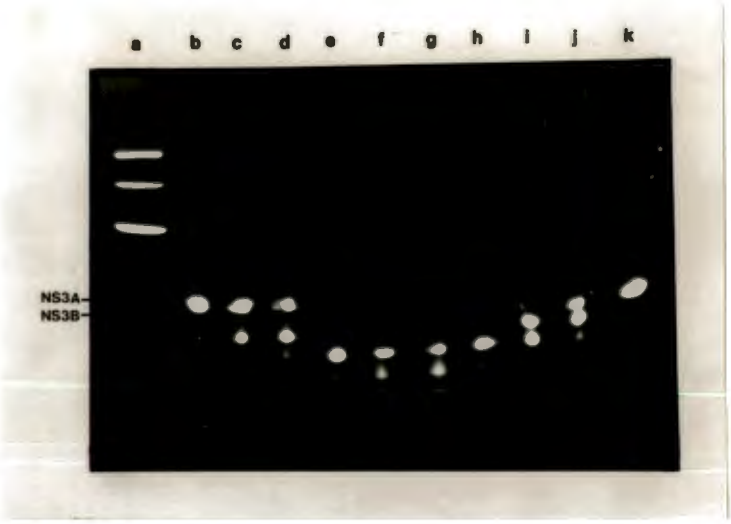


Figure 4.13 Autoradiogram of the polyacrylamide gel electrophoretic comparison of the protease V8 digestion pattern of: in vitro synthesised NS3A (lanes b, c, d, e and f) with in vitro synthesised NS3B (lanes g, h, i, j and k). Amounts of protease used were: (b and k) 0 ng, (c and j) 5 ng, (d and i) 25 ng, (e and h) 500 ng, (f and g) 2500 ng. (a) BTV control.

4.4 DISCUSSION

In this chapter experiments were presented from which the coding assignments for the medium and small-sized genome segments could be determined. The protein product(s) of the smallest dsRNA genome segment were also identified.

It was possible to evaluate the denaturation of BTV dsRNA genome segments on agarose gels. Both DMSO and MMOH were found to denature BTV dsRNAs equally well (Fig.4.1). The positive (+) and negative (-) ssRNA species of the genome segments were separated by electrophoresis, in agreement with results reported for denatured BTV dsRNA genome segments on polyacrylamide gels (Kuichi *et. al.*, 1983). The UV-imaging procedure (Clarke *et. al.*, 1982) was investigated (Fig 4.5), and preferred to the more generally used ethidium bromide staining method for locating dsRNA on preparative polyacrylamide gels since it avoided extended periods of UV-exposure.

No difference was observed between *in vitro* translation products of DMSO and MMOH denatured dsRNAs (Fig.4.2). This confirms the results of Grubman and co-workers (1983), except for the fact that they reported a greater stimulation of protein synthesis with MMOH as opposed to DMSO denatured dsRNAs, which was not observed in the present study. Mertens and co-workers (1984) also used MMOH as denaturing agent for BTV dsRNAs. *In vitro* translation of denatured dsRNA genome segments resulted in the synthesis of the medium- and small-sized BTV proteins. Protein NS1 was again synthesised in amounts considerably less than in infected cells, as has been observed in 3.3.3. However, contrary to the results of Mertens and co-workers (1984), translation with the 3 large denatured genome segments was very inefficient. Similar problems were

encountered during *in vitro* translation of individual denatured genome segments. All the BTV proteins , including the large proteins P1, P2 and P3 were synthesised in control experiments from BTV mRNAs. Therefore, the failure to synthesise the large proteins has to be ascribed to a problem with the dsRNAs. It could be due to trace amounts of undenatured dsRNAs, or rehibridisation of dsRNA during the translation reaction, both these possibilities would result in a dsRNA-mediated inhibition of translation (Content *et. al.*, 1978).

During translation of each individual denatured genome segment, varying amounts of low molecular weight polypeptides were synthesised (Fig.4.8; Fig.4.9). Other investigators also observed this phenomenon (McCrae & McCorquodale, 1982; Mertens *et. al.*, 1984). It was suggested (Grubman *et. al.*, 1983) that it might be the result of degradation of the dsRNA segments. However, it is difficult to explain the characteristic set of proteins for the different genome segments by random degradation. It is also possible that the plus strands of the various BTV dsRNA genome segments have multiple initiation and termination codons, in-phase as well as out-phase with respect to the main reading frame. The rather large number of these small proteins (Fig.4.7 and Fig.4.8) would, however, argue against such an explanation. A more likely explanation for this phenomenon is premature termination of translation at specific places on the plus strands of the genome segments, or incomplete translation. These various postulates will probably only be confirmed once the nucleotide sequences of the different genome segments have been determined.

The protein-coding assignments for the three medium and four small dsRNA genome segments of BTV 10A were determined. A schematic summary of the results obtained, is presented in Fig.4.14. Genome segments 4, 5, 6, 7, 8 and 9 coded for proteins P4, P5, NS1, P7, NS2 and P6 respectively. *In vitro*

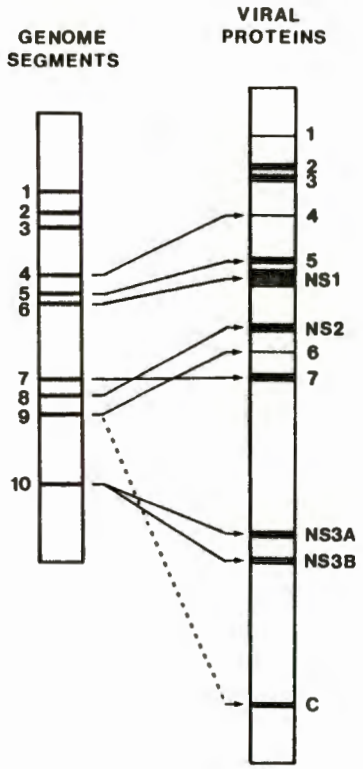


Figure 4.14 Schematic presentation of the protein coding assignments determined for BTV type 10 dsRNA genome segments.

translation of genome segment 10 resulted in the synthesis of two proteins, NS3A and NS3B, with molecular weights of 25K and 28K respectively. The protein-coding assignments that were obtained in this investigation for the genome segments of BTV type 10 correspond to that of BTV type 1 (Mertens et. al., 1984), as well as to that of BTV type 17 (Grubman et. al., 1983). Proteins NS3A and NS3B are, however, much larger than the 16K coding capacity of genome segment 10 that was calculated by Verwoerd and co-workers (1972). Mertens and co-workers (1984) found that genome segment 10 of BTV serotype 1 also coded for two proteins with almost identical peptide maps. The molecular weights of these two proteins were reported to be 15-20K. Since Mertens and co-workers (1984) gave no indication as to how they determined these molecular weights, it is assumed that they used the molecular weights of BTV proteins reported by Verwoerd and co-workers (1972) as markers for their calculations. When these values were used to determine the molecular weights of NS3A and NS3B, values of 18K and 14K are obtained which are very similar to those of Mertens and co-workers (1984). Therefore, it is concluded that the 15-20K proteins of BTV type 1 are the equivalents of NS3A and NS3B in BTV type 10A. Unfortunately their peptide patterns could not be compared to those obtained during this investigation, since they used a different serotype in their study, as well as a different protease, viz. chymotrypsin, for digestion.

Proteins with different molecular weights that are encoded by the same gene and have virtually identical peptide maps have been found in several other viruses. One example is Sendai virus where a non-structural protein, C, is coded for by the same mRNA as the P protein (Giorgi et. al., 1983). This non-structural protein exists in two electrophoretic forms (C and C') which have similar peptide maps and molecular weights of 22K and 24K respectively. No product-precursor relationship

could be detected to account for their presence (Lamb & Choppin, 1978). It was postulated that C' is initiated at a second AUG while C is initiated at a third or fourth AUG. This would be consistent with the difference in electrophoretic mobility between C and C', and it would also be in agreement with the finding that the ratio of C and C' can vary considerably from one *in vitro* translation to another. Such variation was also occasionally observed for BTV NS3A and NS3B proteins. In the case of Sendai virus it was found that none of the four AUG codons implicated in initiation of protein C were in the most favoured context according to the initiation model proposed by Kozak (1981). It appears, therefore, as if the Sendai virus P/C gene mRNA is functionally tricistronic, using one sequence to code for 3 proteins, 2 of which (C and C') are in-phase in the same open reading frame.

Two other paramyxoviruses, New Castle disease virus (Collins *et. al.*, 1982) and mumps virus (Herrler & Compans, 1982), also have pairs of non-structural proteins (36K and 33K; 28K and 19K respectively) in which the peptide map of the one closely resembles that of the other. Furthermore, 2 polypeptides of Herpes simplex virus (43K and 39K) are also translated from a single mRNA by 2 in-phase initiation codons (Marsden *et. al.*, 1983).

The presence of BTV non-structural proteins NS3A and NS3B could, therefore, equally well be the result of two in-phase overlapping open reading frames on genome segment 10. Unfortunately, no sequence data is as yet available to confirm this postulate.

No evidence was found that the 16K BTV protein C was synthesised as the main product of any of the individual genome segments. However, preliminary results indicate that one of the low molecular weight proteins synthesised from genome segment

9, which codes for protein P6, has a molecular weight similar to that of protein C (Fig.4.9). Unfortunately not enough material could be obtained at the time to confirm its identity by peptide mapping. Additional experimental analysis is therefore required to verify this, but should this indeed prove to be protein C, its existence might well be explained by an overlapping reading frame on genome segment 9. Depending upon whether its peptide map corresponds to that of P6 or not , an in-phase or an out-phase reading frame with respect to that of P6 would be involved. The results presented by Grubman and co-workers (1983), which suggested that genome segments 9 and 10 coded for a number of proteins, are therefore in agreement with the observations made during this study.

There are several reports of cases where eukaryotic mRNAs are read in two overlapping reading frames and produce different proteins. They include single transcripts specified by adeno-, myxo-, paramyxo-, reo- and rotavirus genes (Bos et. al., 1981; Briedis et. al., 1981; Briedis et. al. , 1982 ; Shaw et. al., 1983; Cashdollar et. al., 1985; Richardson et. al., 1984).

In the case of reovirus, Cashdollar and co-workers (1985) reported that the s1 genes of the 3 reovirus serotypes all possess two out-phase open reading frames. The first starts with a "weak" initiation codon and can code for a protein of the size expected for the σ1 protein. The other reading frame starts with a "strong" initiation codon about 70 residues downstream from the 5'-terminus and extends for only approximately 120 codons. Ernst and Shatkin (1985) have shown that purified s1 mRNA of reovirus directed the synthesis of a previously unrecognised 14K protein in addition to the σ1 protein. The molecular weight of this protein was in agreement with that predicted by Cashdollar and co-workers (1985).

As far as BTV is concerned, further experiments have to be

conducted to determine the coding assignments of the three large dsRNA genome segments of BTV type 10. The nucleotide sequences of the genome segments are required before the postulate that NS3A, NS3B and C are translated from overlapping reading frames can be confirmed.

CHAPTER 5

FURTHER CHARACTERISATION OF *IN VITRO* SYNTHESISED BTV NON-STRUCTURAL NS2 PROTEIN

5.1 INTRODUCTION

It has been shown (Fig.3.7B, and Fig.4.2) that the peptide maps obtained after *Staph. Aureus* V8 digestion of BTV NS2 protein are similar for the *in vivo* and *in vitro* synthesised products. This strongly indicates identity between them as far as their primary structures are concerned. The purpose of this part of the investigation was to determine whether the identity between them also extends to functional characteristics, and post-translational modifications. In this regard, it is known that *in vivo* synthesised BTV NS2 is phosphorylated, and that it has affinity for ssRNA (Huismans & Basson, 1983; Grubman *et. al.*, 1983). Therefore, it was to be investigated whether *in vitro* synthesised NS2 also has these characteristics.

The poly-(U)-Sepharose affinity column chromatography procedure described by Huismans and Basson (1983), would be suitable to determine whether *in vitro* synthesised NS2 has affinity for ssRNA. No *in vitro* phosphorylation results have as yet been described for BTV. One approach to investigate whether *in vitro* synthesised NS2 can be phosphorylated, would be to include γ^{32}P-ATP in the *in vitro* translation mixture. An alternative approach would be to investigate whether NS2 can be phosphorylated by methods such as the immuneprecipitation procedure described by Collett and Erikson (1978), and Witte and co-workers (1980).

Finally, it was to be investigated whether indications regarding the nature and origin of the kinase involved, could

be obtained.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of ^{35}S-Methionine Labelled S100 and P100 Fractions from BTV-Infected Cells

A roller flask with a monolayer of approximately 7 x 10^8 BHK-21 cells was infected with BTV and pulse-labelled between 11 and 14 h p.i. at 37°C with 25 ml of methionine-free Eagle's medium containing 10 uCi/ml ^{35}S-methionine as described under 3.2.5. S100 and P100 fractions were prepared as described under 3.2.6.

5.2.2 Poly-(U)-Sepharose 4B Affinity Chromatography*

Poly-(U)-Sepharose 4B was packed in a Pasteur pipet to a height of 4 cm. The column was equilibrated with STE-2-buffer containing 0,01 M β-ME. Before loading onto the column, the NaCl concentration of the samples to be analysed was reduced to 0,15 M with STE-1-buffer. Samples were loaded at a rate of 0,1 ml/min. The column was washed with STE-2-buffer containing 0,01 M β-ME until no more unbound material eluted. This was monitored by optical density and/or radioactivity as indicated. The bound material was eluted from the column at the same rate, with 20 ml of a linear gradient of 0,15 M to 1,15 M NaCl in TE-buffer containing 0,01 M β-ME. The NaCl concentration at which material eluted from the column was determined from the refractive index. Peak fractions were pooled as indicated in the text.

The proteins present in the pooled fractions were precipitated

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* I would like to thank Miss A.R. Bauskin for her co-operation in the poly-(U)-Sepharose column chromatography.

by adding SDS to a final concentration of 0,5% and KCl to 0,3 M. After 30 min at 4°C the precipitate was collected by centrifugation and characterised by gel electrophoresis.

5.2.3 In Vitro Phosphorylation Assay

In vitro kinase activity was assayed using the MTD-buffer (0,01 M Tris-HCl, pH 8,0; 0,01 M $MgCl_2$; 0,01 M dithiothreitol and 0,2 uCi/ml γ^{32}p-ATP (1 mCi/ml; 5200 Ci/mmole) described by Moyer and Summers (1974). The final assay mixture was usually obtained by mixing equal amounts of sample and double concentrated MTD-buffer. The mixtures were incubated at 37°C for 30 min. Incorporation of ^{32}p into proteins was detected by polyacrylamide gel electrophoresis and autoradiography.

5.2.4 Simultaneous Detection of ^{35}S- and ^{32}p-Labelled Proteins on Gels

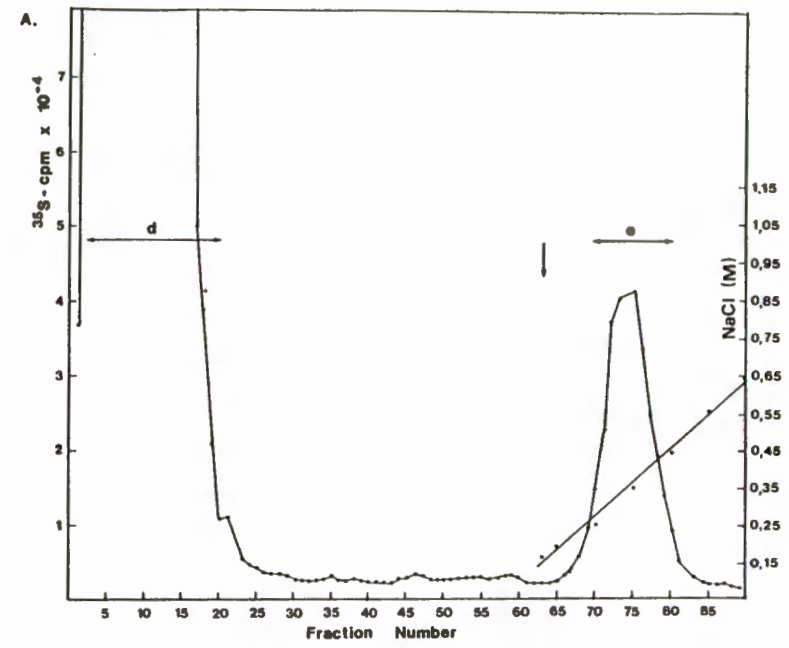
Phosphoproteins, labelled with both ^{35}S and ^{32}p, were characterised by a direct autoradiographic detection method which makes it possible to distinguish between ^{32}p- and ^{35}S-labelled components (Cooper & Burgess 1982). Polyacrylamide gels were dried on Whatmann 3MM filter paper, as described under 2.2.4. Two X-ray films were exposed simultaneously at -80°C: The one was placed directly onto the dried gel, thus recording the ^{32}p- and ^{35}S-label simultaneously. The other was placed on the filter paper side of the gel and was further shielded by placing a 0,015 mm thick sheet of aluminium foil between the filter paper backing of the gel and the X-ray film. This film only recorded the ^{32}p-label.

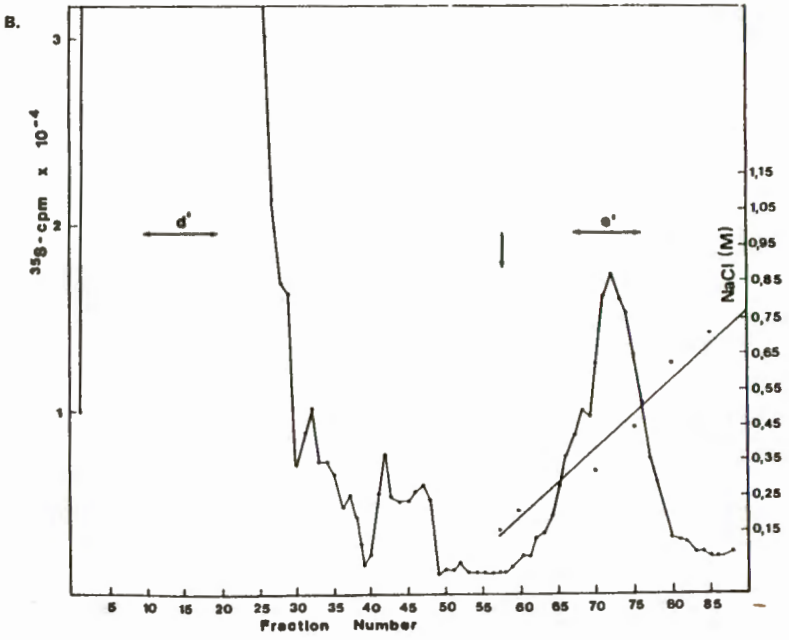
5.3 RESULTS

5.3.1 Poly-(U)-Sepharose 4B Affinity Purification of In Vitro and In Vivo Synthesised BTV Proteins

The first aspect to be investigated was whether *in vitro* synthesised NS2 had affinity for ssRNA similar to that which was reported for NS2 synthesised in infected cells. The approach followed was to compare the affinity of *in vitro* and *in vivo* synthesised NS2 for poly-(U)-Sepharose. A 25 ug quantity of unfractionated BTV mRNA was translated *in vitro* in a 500 ul translation mixture as described under 3.2.2.3. After 50 min, a 5 ul sample of the reaction mixture was spotted onto a filter paper disc to determine the incorporation of ^{35}S-methionine into acid-insoluble material. The NaCl concentration of the remaining material was raised to 1 M in order to dissociate any of the NS2 that could be present in a complexed form. The mixture was kept on ice for 10 min, and after dilution with STE-1-buffer to a final NaCl concentration of 0,15 M, it was loaded on a poly-(U)-Sepharose 4B column (5.2.2). Fractions of approximately 400 ul each were collected. After extensive washing of the column with STE-2-buffer, the bound material was eluted with a NaCl gradient as described under 5.2.2. The salt-gradient was determined from the refractive index of the fractions. Fractions of the unbound and bound peaks were pooled as indicated. Proteins were precipitated from 200 ul of the respective pools by means of SDS/KCl as described, and analysed by gel electrophoresis and autoradiography. Similarly, ^{35}S-labelled S100 material from 1,3 x 10^8 BTV infected cells (prepared as described under 5.2.1) was also subjected to poly-(U)-Sepharose 4B column chromatography and analysed as described above. The results are shown in Figure 5.1.

About 10% of the TCA-precipitable ^{35}S-labelled material in the


A.
^{35}S - cpm x 10^{-4}
d
e
NaCl (M)
Fraction Number


B.
^{35}S - cpm x 10^{-4}
d'
e'
NaCl (M)
Fraction Number

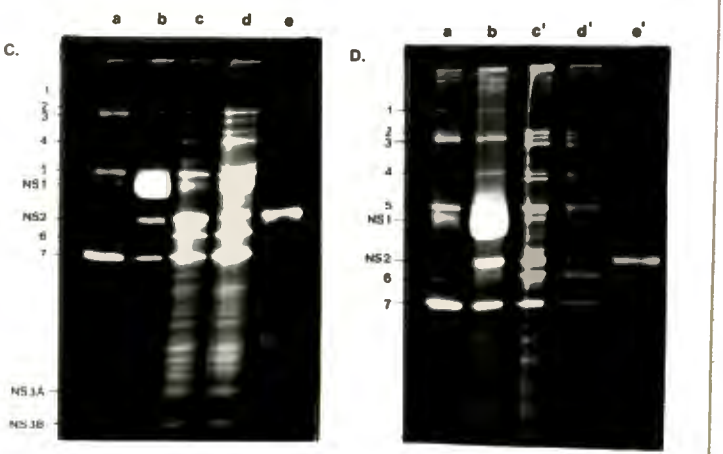


Figure 5.1 Poly-(U)-Sepharose 4B affinity purification of *in vitro* and *in vivo* synthesised BTV proteins. Radioactivity profiles of column fractions from *in vitro* (A), and *in vivo* (B) synthesised BTV proteins. Autoradiograms of gel electrophoretic analysis of column fractions from *in vitro* (C) and *in vivo* (D) synthesised proteins. (a) BTV control, (b) P100 control, (c) *in vitro* synthesised proteins before column chromatography, (d) *in vitro* synthesised proteins in unbound fractions 2 to 20, (e) *in vitro* synthesised proteins in bound fractions 69 to 80, (c') S100 before column chromatography, (d') *in vivo* synthesised proteins in unbound fractions 10 to 20, (e') *in vivo* synthesised proteins in bound fractions 67 to 76. The arrows indicate the fractions where the gradients started.

in vitro translation mixture bound to poly-(U)-Sepharose, and eluted at approximately 0,4 M NaCl. Gel electrophoresis showed that all the BTV proteins were present in the unbound fractions (Fig.5.1C, lane d). However, NS2 was present in considerably reduced quantities, and it was the only viral protein that bound to poly-(U)-Sepharose (Fig.5.1C, lane e). Essentially the same result was obtained for NS2 present in the soluble fraction (S100) of infected cells (Fig.5.1D). Therefore, it can be concluded that *in vitro* synthesised BTV NS2 has affinity for poly-(U)-Sepharose, similar to NS2 synthesised in infected cells. Although the result was not quantified, it seems as if relatively more unbound NS2 was present in the preparation of *in vitro* (Fig.5.1C, lanes d and e) than *in vivo* synthesised NS2 (Fig.5.1D, lanes d' and e').

5.3.2 Phosphorylation

5.3.2.1 In Vitro Phosphorylation of NS2

The next aspect to be investigated was to determine whether NS2 becomes phosphorylated during the *in vitro* translation reaction. To study this, BTV mRNA was translated *in vitro* in a standard reaction mixture as described under 3.2.2.3 which contained approximately 0,1 uCi/ul γ^{32}p-ATP. The proteins in the reaction mixture were analysed by gel electrophoresis and autoradiography as described under 5.2.4. None of the ^{35}S-labelled proteins synthesised during the reaction were phosphorylated. A few of the endogenous proteins in the translation mixture did, however, become phosphorylated, indicating the presence of kinase activity, even though no phosphorylation of any of the viral proteins could be detected (data not shown).

This result caused speculation as to whether the lack of phosphorylation of NS2 was due to the translation reaction

conditions being unsuitable for phosphorylation, or to the absence of the kinase that phosphorylates NS2 *in vivo*. The possibility that the actual amount of NS2 synthesised *in vitro* was so small that the detection procedure was not sensitive enough, also has to be considered. To answer some of these questions, it was decided to investigate whether the *in vitro* phosphorylation procedure for the avian sarcoma virus src gene product, described by Collett and Erikson (1978), could be applied to BTV. The procedure basically involves immuneprecipitation of the viral proteins and resuspension in a phosphorylation mixture.

The method was first investigated using *in vivo* synthesised BTV NS2 as substrate. The following experiment was conducted: S100 and P100 fractions were prepared from approximately 3 x 10^8 BTV-infected and uninfected cells respectively. The proteins in a sample of the S100 fractions, representing material from 1.8 x 10^7 cells, were concentrated by immuneprecipitation as described under 5.2.6. These immuneprecipitates were each resuspended in separate 150 ul phosphorylation mixtures containing γ^{32}p-ATP. Samples of the uninfected and infected P100 fractions, representing material from 4,8 x 10^7 cells, were concentrated by centrifugation and also resuspended in 150 ul phosphorylation mixtures containing γ^{32}p-ATP. All four reaction mixtures were incubated at 37°C for 30 min as described under 5.2.3. After the incubation period, a 20 ul sample of each mixture was analysed by gel electrophoresis and autoradiography. The result is shown in Figure 5.2.

In all the samples multiple cellular proteins were phosphorylated *in vitro* (lanes a,b,c,d). NS2 was the only additional phosphoprotein detected in the infected as opposed to the uninfected samples. This applied both to the S100 (lane a vs b) and the P100 (lane c vs d) fractions, indicating that the kinase phosphorylating NS2 was active under these

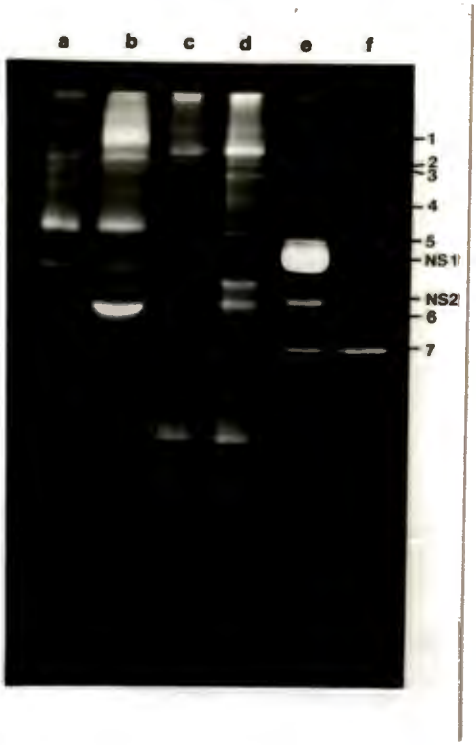


Figure 5.2 *In vitro* phosphorylation of P100 fractions (c and d), and immuneprecipitates of S100 fractions (a and b) of uninfected (a and c), and BTV-infected (b and d) cells determined by polyacrylamide gel electrophoresis and autoradiography. (e) P100 control, and (f) BTV control. Samples in lanes a,b,c and d were labelled with ^{32}P only. Samples in lanes e and f were labelled with ^{35}S only.

experimental conditions. None of the other viral proteins were phosphorylated *in vitro*. Their presence in infected S100 and P100 fractions were confirmed in a control experiment where proteins were labelled using ^{35}S-methionine (data not shown).

Having demonstrated that *in vivo* synthesised NS2 can be phosphorylated *in vitro*, it needed to be investigated whether the same applied to *in vitro* synthesised NS2. The following experiment was therefore carried out. 2,5 ug of unfractionated BTV mRNAs was translated *in vitro* in a 50 ul reaction mixture (3.2.2.3). Endogenous translation in an identical 50 ul reaction mixture was performed as a control. After translation, the proteins were concentrated from the respective translation mixtures by immuneprecipitation with sheep serum raised against BTV as described under 3.2.7, resuspended in separate 70 ul phosphorylation mixtures and assayed for *in vitro* phosphorylation as described under 5.2.3. Both at the start (0 min) and at the end (30 min) of the reaction, a 15 ul sample was taken and added directly to 20 ul of electrophoresis sample buffer (0,0625 M Tris pH 6,5; 2% SDS; 10% glycerol; 5% β-ME; 0,001% bromophenol blue). These samples were analysed by gel electrophoresis and autoradiography under the conditions that distinguished between ^{35}S- and ^{32}P-label described under 5.2.4. The results obtained are shown in Figure 5.3.

The autoradiogram recording both the ^{35}S- and ^{32}P-labelled components indicates that most of the BTV proteins synthesised *in vitro*, including NS2, were present in the immuneprecipitate (lane g), and that no *in vitro* synthesised proteins were present in the immuneprecipitate of the endogenous control (lane e). The autoradiogram recording only the ^{32}P-labelled components revealed that several proteins were phosphorylated in the immunecomplexes (lanes b and d), but no phosphorylation of NS2 could be detected (lane d). Exactly the same proteins were phosphorylated in the immuneprecipitates of the endogenous

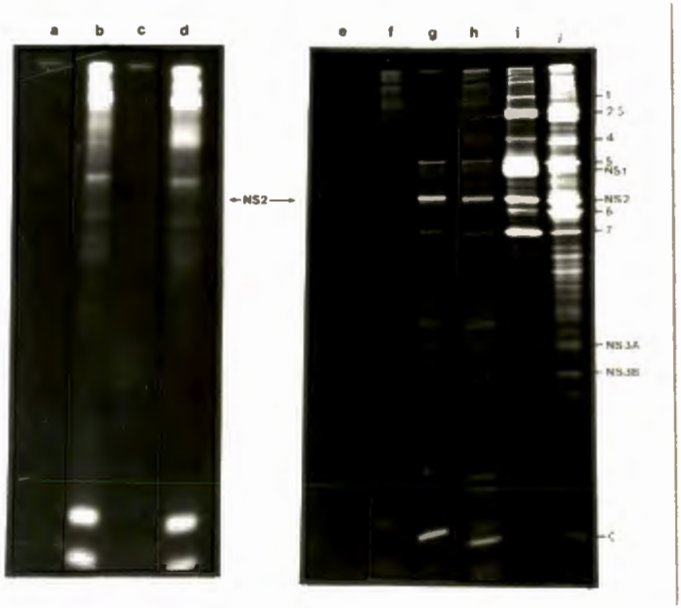


Figure 5.3 In vitro phosphorylation of immuneprecipitates of endogenous (lanes a,b,e and f) and BTV (lanes c,d,g and h) prpteins after in vitro translation as analysed by polyacrylamide gel electrophoresis and autoradiography. Samples were taken after 0 min (a and c), and 30 min (b and d). (a to d) represent the ^{32}P image of the gel, and (e to h) shows the simultaneous ^{32}P and ^{35}S image. (i) ^{35}S-P100 control, and (j) ^{35}S in vitro synthesised BTV control.

control (lane b), and in the in vitro synthesised BTV proteins (lane d), indicating that they are either immunoglobulins, or endogenous proteins of the reticulocyte lysates that co-precipitated. The reason why the strong ^{32}P-label present in lanes b and d were not equally strong in lanes f and h, is because the X-ray film which recorded the ^{32}P-label was overexposed in attempting to detect even very weakly labelled NS2.

5.3.2.2 In Vitro Phosphorylation of In Vitro Synthesised NS2 by Cellular Kinases

The result that in vivo synthesised NS2 could be phosphorylated in vitro, while no phosphorylation of in vitro synthesised NS2 was detected, could indicate that a cellular kinase, which is present in BHK-cells and absent in reticulocyte lysates, is involved. Therefore, the possibility that the kinase is a soluble cellular protein was investigated. Firstly, 2,5 ug of BTV mRNA was translated in vitro as described under 3.2.2.3. As a control an endogenous translation was also performed. Thereafter, the in vitro synthesised BTV and endogenous proteins were immuneprecipitated with a rabbit serum as described under 3.2.8, and resuspended in 100 ul double concentrated MTD-buffer. Two 50 ul samples of a S100 fraction, prepared from uninfected BHK-cells as described under 3.2.6, were each diluted to 100 ul and added to the above-mentioned preparations of in vitro synthesised BTV and endogenous proteins respectively. In vitro phosphorylation was conducted as described in 5.3.2. As an additional control, kinase activity in a 50 ul sample of the uninfected S100 preparation was also assayed in a 200 ul phosphorylation mixture. 20 ul samples of the various reaction mixtures were analysed by gel electrophoresis and autoradiography as described under 5.2.4. The results are shown in Figure 5.4.

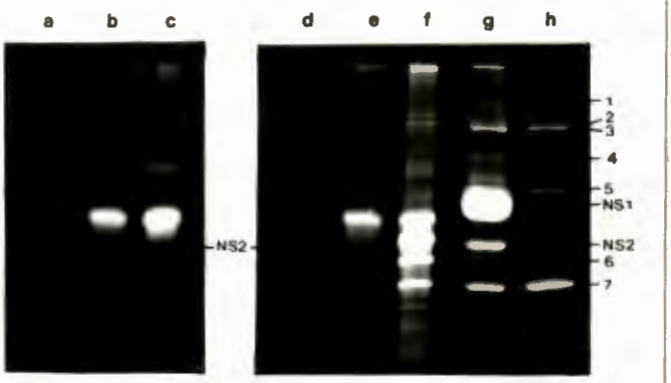


Figure 5.4 Effect of S100 fraction from uninfected cells on the in vitro phosphorylation of the immuneprecipitates of in vitro synthesised endogenous (lanes b and e) and BTV (lanes c and f) proteins, analysed by polyacrylamide gel electrophoresis and autoradiography. (lanes a and d) in vitro phosphorylation control of uninfected S100, (g) ^{35}S P100 control, (h) ^{35}S BTV control. (lanes a to c) represent the ^{32}P image of the gel, and (lanes d to h) represent the simultaneous ^{32}P and ^{35}S image.

The autoradiogram, recording both the ^{35}S- and ^{3}P-labelled components, indicated that *in vitro* synthesised NS2 was present in the immuneprecipitate which was used as substrate for the *in vitro* phosphorylation reaction (lane f), and that no *in vitro* synthesised proteins were present in the immuneprecipitate of the endogenous control (lane d). The autoradiogram recording the ^{32}P-labelled components showed that the only proteins that were phosphorylated were endogenous reticulocyte lysate proteins that co-precipitated and soluble cellular proteins (lanes a and b). No phosphorylation of *in vitro* synthesised NS2 was detected (lane c).

5.3.2.3 *In Vitro Phosphorylation of Poly-(U)-Sepharose Purified NS2*

An obvious advantage in studies concerning the kinase associated with phosphorylation of NS2, would be to have pure preparations of both *in vivo* and *in vitro* synthesised NS2 available for use as substrate. Purification of *in vitro* synthesised NS2 would have the additional benefit of removing possible contaminating agents that could have inhibited the *in vitro* phosphorylation reaction in the previous experiments. Poly-(U)-Sepharose affinity column chromatography seemed to be a particularly suitable method for the purification of NS2. Therefore, a ^{35}S-labelled S100 fraction from BTV infected cells, as well as ^{35}S-labelled *in vitro* synthesised BTV proteins were prepared, and subjected to poly-(U)-Sepharose column chromatography, using similar amounts of material and conditions as those described under 5.3.1. Instead of concentrating NS2 by means of KCl/SDS precipitation, NS2 was concentrated from the total pools of the bound fractions by immuneprecipitation with rabbit serum as described under 3.2.8. The immuneprecipitates were dissolved in 50 ul H_2O and assayed for kinase activity as described under 5.2.3. 20 ul samples were taken from the phosphorylation reaction mixtures both at

the beginning (0 min) and at the end (30 min) of the reaction, and were analysed by gel electrophoresis and autoradiography as described under 5.2.4. The result obtained is shown in Figure 5.5.

Similar to results obtained in 5.3.1, NS2 was the only viral protein that bound to the column (lanes b and e). Surprisingly, the affinity column purified preparation of *in vivo* synthesised NS2 still contained kinase activity which phosphorylated NS2 (lane d). Although kinase activity was also detected in the purified *in vitro* NS2 preparation, NS2 itself was not phosphorylated (lane g). Therefore, it appears as if the kinase active in the *in vitro* NS2 preparation is a serum protein that co-precipitates with NS2, while phosphorylation of *in vivo* synthesised NS2 could be due to autophosphorylation. If the latter hypothesis is correct, it has to be postulated that the inability to detect *in vitro* phosphorylation of *in vitro* synthesised NS2 indicates that the kinase still needs to be activated after *in vitro* translation.

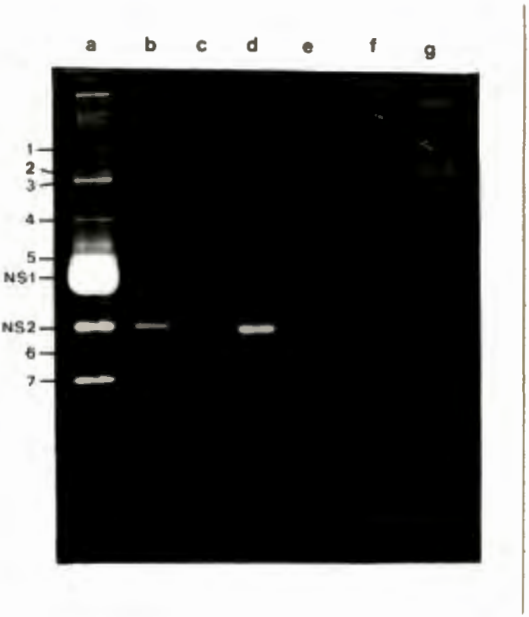


Figure 5.5 Autoradiogram showing in vitro phosphorylation of immuneprecipitated in vivo and in vitro poly-(U)-Sepharose purified NS2 as analysed by gel electrophoresis. (a) ^{35}S P100 control, (b) ^{35}S image of immuneprecipitated in vivo NS2, (c) ^{32}P image at 0 min of phosphorylation of in vivo NS2, (d) ^{32}P image after 30 min of phosphorylation of in vivo NS2, (e) ^{35}S image of immuneprecipitated in vitro NS2, (f) ^{32}P image at 0 min of phosphorylation of in vitro NS2, (g) ^{32}P image after 30 min of phosphorylation of in vitro NS2.

5.4 DISCUSSION

The experiments described in this chapter were carried out to compare *in vitro* synthesised NS2 to its *in vivo* synthesised counterpart with regard to affinity for ssRNA and phosphorylation.

Huismans and Basson (1983) reported that NS2, obtained from the particulate fraction of infected cells by a salt-wash procedure, had affinity for ssRNA. In the present investigation it was found that NS2, which occurs in the soluble (S100) fraction of infected cells, also binds to poly-(U)-Sepharose, as did *in vitro* synthesised NS2 (5.3.1). However, both the *in vivo* and *in vitro* preparations of BTV proteins contained some NS2 that did not bind to poly-(U)-Sephrose. In the case of *in vivo* synthesised NS2, there is the possibility that it could be due to saturation of the column. However, since the latter argument certainly does not apply to the *in vitro* synthesised proteins, it seems justified to conclude that NS2 exists in two forms - one that has affinity for ssRNA, and another that has no affinity for ssRNA. Although the result was not quantified, it appeared as if *in vitro* synthesised preparations of NS2 contained relatively more NS2 which did not have affinity for poly-(U)-Sepharose than preparations of NS2 obtained from S100 fractions of infected cells. The significance of this observation requires further investigation. It could, for instance, reflect differences in the phosphorylation state of NS2. An experiment that should give an indication whether this is the case, would be to label NS2 in infected cells with both ^{35}S-methionine and ^{32}P and compare the $^{35}S/^{32}P$ ratio of NS2 that does not bind to ssRNA to that which does bind. Although protein NS2 was the only virus-specific protein with affinity for poly-(U)-Sepharose, in some experiments several other proteins, mostly smaller in size than NS2, were also detected in the fractions that did bind to poly-

(U)-Sepharose. They may either be incomplete or degradation products of protein NS2 itself, or else represent reticulocyte proteins of the lysate preparation, or be cellular proteins which have affinity for ssRNA. The origin of these proteins should be established, especially in view of the result which will be discussed later concerning the identification of the kinase responsible for the phosphorylation of NS2.

Two other non-structural viral proteins with affinity for ssRNA have already been found in the Reoviridae. The one is protein NS2 from Wallal virus, another member of the orbivirus subgroup (Gorman et. al., 1983). However, apart from mentioning that Wallal virus NS2 has affinity for ssRNA, no other information has as yet been published in this regard. The other example is the σNS protein of reovirus. The affinity of σNS for ssRNA has been extensively studied. Whereas significant amounts of BTV NS2 is present in the S100 fraction of infected cells, σNS was not detected in similar fractions of reovirus infected cells, but was shown to be associated with ssRNA in 40-60S complexes in the cytoplasm of such cells (Huismans & Joklik, 1976). Furthermore, Gomatos and co-workers (1980; 1981) established that σNS is the sole component of small 13-19S reovirus-specific particles which can bind different ssRNA species and also has a poly-(C) dependent poly-(G) polymerase activity. It has been postulated that the affinity of reovirus σNS for ssRNA might indicate that it functions during morphogenesis as a condensing agent to select a set of the 10 plus-sense ssRNA templates in preparation for dsRNA synthesis (Gomatos et. al., 1981). Although not nearly as much information regarding the replication process of BTV is as yet available, it is tempting to speculate that NS2 could have a similar function.

However, a major difference between BTV NS2 and reovirus σNS is that BTV NS2 is a phosphoprotein. The functional significance of this phenomenon is as yet unknown.

In the present investigation it was found that BTV NS2, which occurs in the soluble as well as particulate fractions of infected cells, could be phosphorylated *in vitro* (Fig.5.2). Since it has been established that NS2 is phosphorylated in infected cells (Huismans & Basson, 1983; Grubman *et. al.*, 1983), the result either implies that hyperphosphorylation occurred, or that ^{32}P-labelling took place through phosphate exchange due to a dynamic phosphorylation-dephosphorylation reaction. It was, however, not possible to demonstrate phosphorylation of *in vitro* synthesised NS2 in any of the experiments carried out during the course of this investigation, or to determine whether NS2 is phosphorylated or not during the *in vitro* translation reaction itself. The results cannot be taken as conclusive evidence that no phosphorylation of *in vitro* synthesised NS2 occurred, since the actual amounts of *in vitro* synthesised NS2 that were used during the experiments were very small when compared to that of *in vivo* synthesised NS2. Furthermore, it is possible that the autoradiographic procedure was not sensitive enough to detect extremely small amounts of ^{32}P. The results should therefore be confirmed by using much larger quantities of *in vitro* synthesised NS2, and increasing the amount of $\gamma^{32}P$-ATP in the reaction mixture. It should also be kept in mind that the reaction conditions for phosphorylation were not optimised, and they should also be investigated.

The presence of kinase activity, which phosphorylated *in vivo* synthesised NS2 in poly-(U)-Sepharose purified preparations, could indicate that NS2 might be involved in an autophosphorylation reaction, since no other viral proteins were detected in such preparations. Should this be the case, another possible explanation for the observation that *in vitro* synthesised NS2 was not phosphorylated, could be that the viral kinase (NS2) was not present in an activated state. This possibility also needs further investigation. Kinase activity

phosphorylating *in vivo* synthesised NS2 was also detected in the material of S100 fractions that did not bind to poly-(U)-Sepharose (data not shown). Therefore, whatever the factor(s) are that determine the ability of NS2 to bind to ssRNA, they do not seem to be involved in the activation of kinase activity.

In the literature several examples have been described where a balance of phosphorylation-dephosphorylation appears to be a fundamental mode of control in the viral infectious process (Kamata & Watanabe, 1977; Smith *et. al.*, 1979; Mellon & Emerson, 1978). In particular, in the case of murine type C virus p12 protein (Privalsky & Penhoet, 1977), vesicular stomatitis virus NS protein (Clinton *et. al.*, 1978), and SV 40 large T antigen (Scheidtmann *et. al.*, 1984), the degree of phosphorylation seems to determine the extent of binding to viral nucleic acid: the more highly phosphorylated the protein, the lesser the extent of binding. Furthermore, phosphorylation of influenza nucleocapsid protein stimulates viral transcription (Kamata & Watanabe, 1977). A similar protein kinase requirement for *in vitro* activation of VSV transcriptase has been demonstrated (Witt & Summers, 1980). Different phosphorylated NS species appear to regulate the level of *in vitro* transcription of VSV (Kingsford & Emerson, 1980). The kinase responsible for the phosphorylation in VSV has as yet to be identified (Sinacore & Lucas-Lenard, 1982).

The experiments presented in this study on the phosphorylation of BTV NS2 protein and its affinity for ssRNA should be regarded as preliminary studies. An immediate aim of future studies would be to determine whether or not NS2 is phosphorylated during the *in vitro* translation reaction, and to investigate whether the ability of *in vivo* synthesised NS2 to bind to ssRNA is affected by hyperphosphorylation *in vitro*.

CHAPTER 6

CONCLUDING REMARKS

This investigation involved a study of two of the fundamental processes in the replication of BTV, namely the transcrption and translation reactions. The results that were obtained, have been discussed in detail in the relevant chapters. In this conclusion the main results of this investigation that contribute to a better understanding of the molecular biology of BTV will be mentioned briefly, and some suggestions for future research in this field will be made.

The most significant finding regarding *in vitro* transcription, is the characterisation of the factors that affect the efficiency of the reaction. The concentration of core particles in the reaction mixture appears to be the major factor involved. The efficiency of the *in vitro* transcription reaction declines rapidly with increasing core concentrations. This decline is, however, reversible, and can also be counteracted in several ways, such as by including the polyhydroxylic compounds, sucrose or glycerol in the reaction mixture, or by using a relatively low incubation temperature. These results explain the low temperature optimum observed for *in vitro* transcription of BTV, which has previously been postulated to be a unique characteristic of the virus. It can therefore now be stated that the latter hypothesis is incorrect. Similar explanations probably also apply to the low temperature optimum for transcriptase activity which has been observed for other orbiviruses such as EHDV, and AHSV. The reason why high concentrations of core particles affect the efficiency of the *in vitro* transcription reaction has not as yet been identified. Further investigations should be undertaken to study the effect of core concentration in the reaction mixture on the

conformational requirements of BTV core particles during transcription.

Another important contribution of this investigation is the finding that excellent separation of BTV mRNA species can be obtained by means of agarose gel electrophoresis. It is the first time that separation of the individual BTV mRNA species has been achieved. The results confirmed previous observations that the BTV mRNA species are not synthesised in equal amounts, and therefore lends further support to the hypothesis that BTV transcription might be subjected to regulation. Studies aimed at analysing characteristics of individual mRNA species can now be undertaken.

Several contributions regarding the synthesis of BTV proteins have been made during this investigation. The results represent the first report on *in vitro* translation of *in vitro* synthesised mRNAs for the orbivirinae. The findings on the non-structural proteins are of particular importance. The result that the non-structural proteins NS1 and NS2 could be synthesised *in vitro* using *in vitro* synthesised BTV mRNAs as templates, confirmed their viral origin. Three previously unknown non-structural proteins, namely NS3A, NS3B and C were also identified by *in vitro* translation. They were subsequently identified in infected cells. The finding that proteins NS3A, NS3B, and C are synthesised *in vivo* in extremely small amounts compared to the other viral proteins explains why they were not previously detected.

Another major contribution is the determination of the protein coding assignments of the medium- and small-sized genome segments. It revealed that proteins NS3A and NS3B are both encoded by the smallest of the ten genome segments, for which previously no protein product had been identified. The finding that the peptide maps of proteins NS3A and NS3B are very

similar, could mean that they are synthesised from two overlapping in-phase reading frames. Another genome segment that appears to have two overlapping reading frames is segment 9. It seems to encode both proteins P6 and C. The coding assignments obtained in this investigation for BTV type 10, are similar to those reported for BTV type 1 and 17 respectively.

Future studies on BTV proteins could benefit greatly by the use of recombinant DNA technology. The nucleotide sequences of the various genome segments will become available, and should confirm the observations regarding overlapping reading frames. Genetic manipulation of cloned genome segments, and their subsequent re-introduction into virions should be of considerable use in the identification of the functions of the various proteins.

The finding that it is possible to phosphorylate *in vivo* synthesised BTV NS2 *in vitro*, is also important. It is the first report on *in vitro* phosphorylation in the Reoviridae. The finding that kinase activity remained associated with NS2 after affinity column purification, contributes considerably to the eventual identification of the enzyme, since it rules out any of the other viral proteins as candidates for the kinase, and strongly suggests the possibility of autophosphorylation. The ultimate aim of future investigations in this field would be to determine the functional significance of the phosphorylation, and identify the kinase involved. Initial steps in achieving these objectives might be by investigating alternative ways of separating NS2 from its associated kinase, investigating a possible correlation between phosphorylation of NS2 and its affinity for ssRNA, studying the effect of phosphorylated and unphosphorylated forms of NS2 on *in vitro* transcription and translation, and also using recombinant DNA technology to genetically manipulate NS2.

Parts of the in vitro transcription results have been published.

VAN DIJK, A.A. and H. HUISMANS. 1982. The effect of temperature on the in vitro transcriptase reaction of bluetongue virus, epizootic heamorrhagic disease virus and African horsesickness virus. Onderstepoort J. Vet. Res. 49 : 227-232.

Parts of the results are included in the following papers.

VAN DIJK, A.A. and H. HUISMANS. The effect of sucrose and glycerol on the in vitro transcription reaction of bluetongue virus. Manuscript in preparation.

VAN DIJK, A.A. and H. HUISMANS. In vitro synthesis of bluetongue virus proteins. Manuscript in preparation.

HUISMANS, H.; VAN DIJK, A.A. and H.J. ELS. Uncoating of parental bluetongue virus to core and sub-core particles in infected L-cells. Submitted for publication in Virology.

HUISMANS, H.; BAUSKIN, A.R. and A.A. VAN DIJK. Isolation of a phosphorylated non-structural protein of bluetongue virus with affinity for single-stranded RNA. Submitted for publication in J. of Virology.

Parts of the results have been presented as papers at scientific meetings.

HUISMANS, H.; VAN DIJK, A.A. and D.W. VERWOERD. In vivo uncoating of bluetongue virus.
Fifth International Congress of Virology, Strasbourg, 1981. Workshop 47.

VAN DIJK, A.A. Translational studies with bluetongue virus genes. 23rd Annual Congress of the South African Society of Pathologists, Pretoria, 1983. Paper V20.

VAN DIJK, A.A. and A.R. BAUSKIN. Characteristics of a phosphorylated non-structural protein of bluetongue virus. Third Congress of the South African Society for Microbiology, 1984, Cape Town.

REFERENCES

ACS, G.; KLETT, H.; SCHONBERG, M.; CRISTMAN, J.; LEVIN, D.H., and S.C. SIVERSTEIN. 1971. Mechanism of reovirus double-stranded ribonucleic acid synthesis in vivo and in vitro. J. Virol. 8 : 684-689.

AKUSJÄRVI, G.; PHILIPSON, L. and U. PETTERSON. 1978. A protein kinase associated with adenovirus type 2. Virology 87 : 276-286.

ANDERSON, M.; CAUSTON, T. and G.C. CHEESEMAN. 1974. Molecular weight estimates of milk-fat-globule membrane protein-sodium sulphate complexes by electrophoresis in gradient acrylamide gels. Biochem. J. 139 : 653-660.

ANTCZAK, J.B.; CHMELO, R.; PICKUP, D.J. and W.K. JOKLIK. 1982. Sequences at both termini of the 10 genes of reovirus serotype 3 (strain Dearing). Virology 121 : 307-319.

ARAKAWA, T. and S.N. TIMASHEFF. 1982. Stabilization of protein structure by sugars. Biochemistry 21 : 7193-7201.

ASTIER-MANIFACIER, S and P. CORNUET. 1971. RNA-dependent RNA polymerase in Chinese cabbage. Biochim. Biophys. Acta 232 : 484-493.

AXELROD, N. 1978. Phosphoproteins of adenovirus 2. Virology 87 : 366-383.

BACK, J.F.; OAKENFULL, D. and M-B. SMITH. 1979. Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry 18 : 5191-5196.

BAILEY, J.M. and N. DAVIDSON. 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70 : 75-80.

BANERJEE, A.K. and A.J. SHATKIN. 1970. Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase. J. Virol. 6 : 1-11.

BARTLETT, N.M.; GILLIES, S.C.; BULLIVANT, S. and A.R. BELLAMY. 1974. Electron microscopy study of reovirus reaction cores. J. Virol. 14 : 315-326.

BENICOURT, C.; PERE, J-P. and HAENNE. 1978. Translation of TYMV RNA into high molecular weight proteins. Febs. Lett. 86 : 268-272.

BERGMANN, J.E. and H.F. LODISH. 1979. Translation of capped and uncapped vesicular stomatitis virus and reovirus mRNAs. J. Biol. Chem. 254 : 459-468.

BERNSTEIN, J. and J. HRUSKA. 1981. Characterization of RNA polymerase products of Nebraska calf diarrhea virus and SA 11 rotavirus. J. Virol. 37 : 1071-1074.

BLACK, D.R. and C.A. KNIGHT. 1970. Ribonucleic acid transcriptase activity in purified wound tumor virus. J. Virol. 6 : 194-198.

BLAIR, G.E. and W.C. RUSSELL. 1978. Identification of a protein kinase activity associated with human adenovirus. Virology 86 : 157-166.

BONNER, W.W. and R.A.LASKEY. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46 : 83-88.

BORDEN, E.C.; SHOPE, R.E. and F.A. MURPHY. 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus - a new taxonomic group. Physicochemical and serological studies. J. Gen. Virol. 13 : 261-271.

BORSA, J. and A.F. GRAHAM. 1968. Reovirus RNA polymerase activity in purified virions. Biochem. Biophys. Res. Comm. 33 : 895-901.

BOS, J.L.; POLDER, L.J.; BERNARDS, R.; SCHRIER, P.I.; VAN DEN ELSEN, P.J.; VAN DER EB, A.J. and H. VAN ORMOND. 1981. The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell 27 : 121-131.

BOTH, G.W.,; LAVI, S. and A.J. SHATKIN. 1975. Synthesis of all the gene products of reovirus genome in vivo and in vitro Cell 4 : 173-180.

BREMER, C.W. 1976. A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. Onderstepoort J. Vet. Res. 43 : 193-200.

BRIEDIS, D.J.; LAMB, R.A. and P.W. CHOPPIN. 1981. Influenza B virus RNA segment 8 codes for two non-structural proteins. Virology 112 : 417-425.

BRIEDIS, D.J. and R.A. LAMB. 1982. Influenza B virus genome : Sequences and structural organization of RNA segment 8 and the mRNAs coding for the NS_1 and NS_2 proteins. J. Virol. 42 : 186-193.

BURGE, B.W. and A.S. HUANG. 1979. Conserved polypeptides in the proteins of vesicular stomatitis virus. Virology 95 : 445-453.

BUSS, W.C. and K. STALTER. 1978. Stimulation of eukaryotic transcription by glycerol and polyhydroxylic compounds. Biochemistry 17 : 4825-4832.

CARROLL, A.R. and R.R. WAGNER. 1978. Reversal by certain polyanions of an endogenous inhibitor of vesicular stomatitis virus associated transcriptase. J. Biol. Chem. 253 : 3361-3363.

CASHDOLLAR, L.W.; CHMELO, R.A.; WIENER, J.R. and W.K. JOKLIK. 1985. Sequences of the S1 genes of the three serotypes of reovirus. Proc. Natl. Acad. Sci. USA 82 : 24-28.

CLARKE, I.N. and M.A. McCRAE. 1983. The molecular biology of rotaviruses. VI. RNA species-specific terminal conservation in rotaviruses. J. Gen. Virol. 64 : 1877-1884.

CLARKE, P.; LIN, H-C. and G. WILCOX. 1982. Ultraviolet imaging : A simple method for detecting nucleic acid in preparative gels. Anal. Biochem. 124 : 88-91.

CLEVELAND, E-W.; FISCHER, S.G.; KIRSCHNER, M.W. and U.K. LAEMMLI. 1977. Peptide mapping by limited proteolysis in sodium dodecylsulphate and analysis by gel electrophoresis. J. Biol. Chem. 252 : 1102-1106.

CLINTON, G.M.; BURGE, B. and A.S. HUANG. 1978. Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. J. Virol. 27 : 340-346.

COHEN, J. 1977. Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. 36 : 395-402.

COHEN, J. and P. DOBOS. 1979. Cell-free transcription and translation of rotavirus RNA. Biochem. Biophys. Res. Comm. 88 : 791-796.

COHEN, J.; LaPORTE, J.; CHARPILIENNE, H. and R. SCHERRER. 1979. Activation of rotavirus RNA polymerase by calcium chelation. Arch. Virol. 60 : 177-186.

COOPER, P.C. and A.W. BURGESS. 1982. Simultaneous detection of ^{35}S- ^{32}P-labeled proteins on electrophoretic gels. Anal. Biochem. 126 : 301-305.

COLLETT, M.S. and R.L. ERIKSON. 1978. Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. USA 75 : 2021-2024.

COLLETT, M.S.; ERIKSON, E. and R.L. ERIKSON. 1979. Structural analysis of the avian sarcoma virus transforming protein : sites of phosphorylation. J. Virol. 29 : 770-781.

COLLINS, P.L.; FULLER, F.J.; MARCUS, P.I.; HIGHTOWER, L.E. and L.A. BALL. 1982a. Synthesis and processing of Sindbis virus non-structural proteins in vitro. Virology 118 : 363-379.

COLLINS, P.L.; WERTZ, G.W.; BALL, L.A. and L.E. HIGHTOWER. 1982. Coding assignments of the five smaller mRNAs of New Castle disease virus. J. Virol. 43 : 1024-1031.

CONTENT, J.; LEBLEU, B. and E. CLERCQ. 1978. Differential effects of various double-stranded RNAs on protein synthesis in rabbit reticulocyte lysates. Biochemistry 17 : 88-94.

DARNESS, J.E. Jr. 1982. Variety in the level of gene control in eukaryotic cells. Nature 297 : 365-371.

DESSELBERGER, U.; RACANIELLO, V.R.; ZAZRA, J.J. and P. PALESE. 1980. The 3' and 5' terminal sequences of Influenza A , B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 8 : 315-328.

DUDA, C.T., ZAITLIN, M. and A. SIEGEL. 1973. In vitro synthesis of double-stranded RNA by an enzyme system isolated from tobacco leaves. Biochim. Biophys. Acta 319 : 62-71.

DU TOIT, P.J. 1929. The nature and duration of the immunity against bluetongue in sheep. 15 th Annual Report of the Director of Veterinary Services, Union of South Africa: 69-78.

ECKHART, W.; HUTCHINSON, M.A. and T. HUNTER. 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell 18 : 925-933.

EHRENFELD, E. and T. HUNT. 1971. Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates. Proc. Natl. Acad. Sci. USA 68 : 1075-1078.

ERASMUS, B.J. 1975. Bluetongue in sheep and goats. Australian Vet. J. 51 : 165-170.

ERICSON, B.L.; GRAHAM, D.V.; MASON, B.B. and M.K. ESTES. 1982. Identification, synthesis and modification of simian rotavirus SA 11 polypeptides in infected cells. J. Virol. 42 : 825-839.

ERNST, H. and A.J. SHATKIN. 1985. Reovirus heamagglutinin mRNA codes for two polypeptides in overlapping reading frames. Proc. Natl. Acad. Sci. USA 82 : 48-52.

FRAENKEL-CONRAT, H. 1983. RNA-dependent RNA polymerases of plants. Proc. Natl. Acad. Sci. USA 80 : 422-424.

FRANSLER, 8.S. and L.A.LOEB. 1974. Sea urchin nuclear DNA polymerase. In: Colowick, S.P. and O. Nathan, eds. Methods in Enzymol. XXIX Part E Academic Press. pp. 53-70.

FRIGON, R.P. and J.C. LEE. 1972. The stabilization of calf-brain microtubule protein by sucrose. Arch. Biochem. Biophys. 153 : 587-589.

FURUICHI, Y. 1974. Methylation-coupled transcription by virus-associated transcriptase of cytoplasmic polyhedrosis virus containing double-stranded RNA. Nucleic Acid Res. 1 : 809-822.

FURUICHI, Y. 1978. Pre-transcriptional capping in the biosynthesis of cytoplasmic polyhedrosis virus mRNA. Proc. Natl. Acad. Sci. USA 75 : 1086-1090.

FURUICHI, Y. 1981. Allosteric stimulatory effect of S-adenosyl-methionine on the RNA polymerase in cytoplasmic polyhedrosis virus : A model for the possitive control of eukaryotic transcription. J. Biol. Chem. 256 : 483-493.

FURUICHI, Y.; MORGAN, M.; MUTHUKRISHNAN, S. and A.J.SHATKIN. 1975. Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: $m^7G(5')ppp(5')G_mpCp-$. Proc. Natl. Acad. Sci. USA 72 : 362-366.

FURUICHI, Y.; MUTHUKRISHNAN, S.; TOMASZ, J. and A.J. SHATKIN. 1976. Mechanism of formation of reovirus mRNA 5'-terminal blocked and methylated sequence , m^7GpppG^mpC. J. Biol. Chem. 251 : 5043-5053.

GIORGI, C.; BLUMBERG, G.M. and D. KOLAKOFSKY. 1983. Sendai virus contains overlapping genes expressed from a single mRNA. Cell 35 : 829-836.

GILLIES, S.; BULLIVANT, S. and A.R. BELLAMY. 1971. Viral RNA polymerases : electron microscopy of reovirus reaction cores. Science 174 : 694-696.

GOMATOS, P.J. 1967. RNA synthesis in reovirus infected L929 mouse fibroblasts. Proc. Natl. Acad. Sci. USA 58 : 1798-1804.

GOMATOS, P.J.; PRAKASH, O. and N.M. STOMATOS. 1981. Small reovirus particles composed soley of sigma NS with specificity for binding different nucleic acids. J. Virol. 39 : 115-124.

GOMATOS, P.J.; STAMATOS, N.M. and N.H. SARKAR. 1980. Small reovirus-specific particle with polycytidylate-dependent RNA polymerase activity. J. Virol. 36 : 556-565.

GORMAN, B.M. 1979. Variation in orbiviruses. J. Gen. Virol. 44 : 1-15.

GORMAN, B.M.; TAYLOR, J. and P.J. WALKER. 1983. Orbiviruses. In : The Reoviridae. Joklik, W.K., ed. Plenum Press, New York. pp. 287-375.

GRUBMAN, M.J.; APPLETON, J.A. and G.J. LETCHWORTH III. 1983. Identification of bluetongue virus type 17 genome segments coding for polypeptides associated with virus neutralization and intergroup reactivity. Virology 131 : 355-366.

GRUBMAN, M.J.; BAXT, B.; LA TORRE, J.L.and H.L. BACHRACH. 1981. Identification of a protein kinase activity in purified foot-and-mouth-disease virus. J. Virol. 39 : 455-462.

GRUENWEDEL, D.W. and N. DAVIDSON. 1966. Complexing and denaturation of DNA by methylmercuric hydroxide. J. Mol. Biol. 21 : 129-144.

GURDON, J.B.; LANE, C.D. and H.R. WOODLAND. 1971. Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature 233 : 177-182.

HARRIS, T.J.R.; BROWN, F. and D.V. SANGAR. 1981. Differential precipitation of foot and mouth disease virus proteins made in vivo and in vitro by hyperimmune and virus particle guinea pig antisera. Virology 112 : 91-98.

HERRLER, G. and R.W. COMPANS. 1982. Synthesis of mumps virus polypeptides in infected Vero cells. Virology 119 : 430-438.

HOURRIGAN, J.L. and A.L. KLINGSPORN. 1975. Bluetongue : The disaese in cattle. Australian Vet. J. 51 : 170-174.

HOWELL, P.G. and D.W. VERWOERD. 1971. Bluetongue virus. In : Gard, S.; Hallower, C. and K.F. Meyer, eds. Virology Monographs. Springer Verlag, New York. pp. 35-74.

HUISMANS, H. 1970. Macromolecular synthesis in bluetongue virus infected cells. I. Virus specific nucleic acid synthesis. Onderstepoort J. Vet. Res. 37 : 191-198.

HUISMANS, H. 1979. Protein synthesis in bluetongue virus infected cells. Virology 92 : 385-396.

HUISMANS, H.; BREMER, C.W. and T.L. BARBER. 1979. The nucleic acid and proteins of epizootic haemorrhagic disease virus. Onderstepoort J. Vet. Res. 46 : 95-104.

HUISMANS, H. and H.M. BASSON. 1983. The characterization of a phosphorylated non-structural polypeptide of bluetongue virus with affinity for RNA. In : Compans, R.W. and D.H.L. Bishop, eds. Double-Stranded RNA Viruses. Elsevier Biomedical. New York. pp. 173-181.

HUISMANS, H. and H.J. ELS. 1979. Characterization of the tubules associated with the replication of three different orbiviruses. Virology 92 : 397-406.

HUISMANS, H. and B.J. ERASMUS. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. Onderstepoort J. Vet. Res. 48 : 51-58.

HUISMANS, H. and W.K. JOKLIK. 1976. Reovirus-coded polypeptides in infected cells : Isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA respectively. Virology 70 : 411-424.

HUISMANS, H. and D.W. VERWOERD. 1973. Control of transcription during expression of the bluetongue virus genome. Virology 52 : 81-88.

HUNT, T.; VANDERHOFF, G. and I.M. LONDON. 1972. Control of globin synthesis: The role of heme. J. Mol. Biol. 66 : 471-481.

HUNTER, T.; HUNT, T.; JACKSON, R.J. and H.D. ROBERTSON. 1975. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. J. Biol. Chem. 250 : 409-417.

HUNTER, T. and B.M. SEFTON. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA 77 : 1311-1315.

IBA, H.; WATANABE, T.; EMORI, Y. and Y. OKADA. 1982. Three double-stranded RNA genome segments of bacteriophage O6 have homologous terminal sequences. Febs. Lett. 141 : 111-115.

IMAI, M.; AKATANI, K.; IKEGAMI, N. and Y. FURUICHI. 1983. Capped and conserved terminal structures in human rotavirus genome double-stranded RNA segments. J. Virol. 47 : 125-136.

JEGGO, M.H. and R.C. WARDLEY. 1985. Bluetongue vaccine : cells and/or antibodies. Vaccine 3 : 57-58.

JOKLIK, W.K. 1972. Studies on the effect of chymotrypsin on reovirions. Virology 49 : 700-715.

JOKLIK, W.K. 1974. Reproduction of Reoviridae. In : Fraenkel-Conrat, H. and R.R. Wagner, eds. Comprehensive Virology. Plenum Press. pp.231-334.

JOKLIK, W.K. 1981. Structure and function of the reovirus genome. Microbiol. Reviews 45 : 483-501.

KAHLON, J.; SUGIYAMA, K. and P. ROY. 1983. Molecular basis of bluetongue virus neutralization. J. Virol. 48 : 627-632.

KAMATA, T. and Y. WATANABE. 1977. Role for nucleocapsid protein phosphorylation in the transcription of influenza virus genome. Nature (London) 267 : 460-462.

KAPULER, A.M. 1970. An extraordinary temperature dependence of the reovirus transcriptase. Biochemistry 9 : 4453-4457.

KAPULER, A.M.; MENDELSOHN, N.; KLETT, H. and G. ACS. 1970. Four base-specific 5'-triphosphatases in the subviral core of reovirus. Nature 225 : 1209-1211.

KATES, J.R. and B.R. McAUSLAN. 1967. Messenger RNA synthesis by a "coated" viral genome. Proc. Natl. Acad. Sci. USA 57 : 314-320.

KINGSFORD, L. and S.U. EMERSON. 1980. Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. J. Virol. 33 : 1097-1105.

KOZAK, M. 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acid Res. 9 : 5223-5252.

KREBS, E.G. and J.A. BEAVO. 1979. Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48 : 923-959.

KUCHINO, Y.; NISHIMURA, S.; SMITH, R.E. and Y. FURUICHI. 1982. Homologous terminal sequences in the double-stranded RNA genome segments of cytoplasmic polyhedrosis virus of the silkworm Bombyx mori. J. Virol. 44 : 538-543.

KUICHI, A.; RAO, C.D. and P. ROY. 1983. Analysis of bluetongue viral RNA sequences. In : Compans, R.W. and D.H.L.Bishop, eds. Double-Stranded RNA Viruses. Elsevier Biomedical, New York. pp. 55-64.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of head bacteriophage T4. Nature 227 : 680-685.

LAI, M. 1976. Phosphoproteins of Rous sarcoma viruses. Virology 74 : 287-301.

LAMB, R.A. and P.W. CHOPPIN. 1977. The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. Virology 81 : 382-397.

LAMB, R.A. and P.W. CHOPPIN. 1978. Determination by peptide mapping of the unique polypeptides in Sendai virions and infected cells. Virology 84 : 469-478.

LEE, J.C. and S.N. TIMASHEFF. 1981. The stabilization of proteins by sucrose. J.Biol. Chem. 256 : 7193-7201.

LEVIN, K.H. and C.E. SAMUEL. 1980. Biosynthesis of reovirus-specified polypeptides. Virol. 106 : 1-13.

LEVINSON, A.D.; OPPERMANN, H.; LEVINTOW, L.; VARMUS, H.E. and J.B. BISHOP. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15 : 561-572.

LEWANDOWSKI, L.J.; KALMAKOFF, J. and Y. TANADA. 1969. Characterization of a ribonucleic acid polymerase activity associated with purified cytoplasmic polyhedrosis virus of the silkworm Bombyx mori. J. Virol. 4 : 857-865.

LOENING, U.E. 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide gel electrophoresis. Biochem. J. 102 : 251-257.

LUFTIG, R.B.; KILHAM, S.S.; HAY, A.J.; ZWEERINK, H.J. and W.K. JOKLIK. 1972. An ultrastructural study of virions and cores of reovirus type 3. Virology 48 : 170-181.

MANIATIS, T.; FRITSCH, E.F. and J. SAMBROOK. 1982. Molecular cloning : A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York.

MARCUS, A.; EFRON, D. and D.P. WEEKS. 1974. The wheat embryo cell-free system. In : Colowick, S.P. and O. Nathan, eds. Methods in Enzymology XXX. Academic Press. pp. 749-754.

MARSDEN, H.S.; HAARR, L. and C.M. PRESTON. 1983. Processing of herpes simplex virus proteins and evidence that translation of thymidine kinase mRNA is initiated at three separate AUG codons. J. Virol. 46 : 434-445.

MARTIN, S.A. and H.J. ZWEERINK. 1972. Isolation and characterization of two types of bluetongue virus particles. Virology 50 : 495-506.

MARX, P.A.; PORTNER, A. and D.W. KINGSBURY. 1974. Sendai virion transcriptase complex : Polypeptide composition and inhibition by virion envelope proteins. J. Virol. 13 : 107-113.

MATHEWS, M.V. and M. OSBORNE. 1974. The rate of polypeptide enlongation in a cell-free system from Krebs II ascites cells. Biochem. Biophys. Acta 340 : 147-152.

MATTHEWS, R.E.F. 1982. Classification and nomenclature of viruses. Intervirol. 17 : 1-199.

McCRAE, M.A. and W.K. JOKLIK. 1978. The nature of the polypeptide encoded by each of the double-stranded RNA segments of reovirus type 3. Virology 89 : 578-593.

McCRAE, M.A. and J.G. McCORQUODALE. 1982. The molecular biology of rotaviruses. II. Identification of the protein assignments of calf rotavirus genome RNA species. Virology 117 : 435-443.

MELLON, M.G. and S.U. EMERSON. 1978. Rebinding of transcriptase components (L and NS proteins) to the nucleocapsid template of vesicular stomatitis virus. J. Virol. 27 : 560-567.

MERTENS, P.P.C.; BROWN, F. and D.V. SANGAR. 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. Virology 135 : 207-217.

MERTENS, P.P.C. and P. DOBOS. 1982. Messenger RNA of infectious pancreatic necrosis virus is polycistronic. Nature 297 : 243-246.

MERTENS, P.P.C.; JAMIESON, P.B. and P. DOBOS. 1982. In vitro RNA synthesis by infectious pancreatic necrosis virus-associated RNA polymerase. J. Gen. Virol. 59 :47-56.

MERTENS, P.P.C. and C.C.PAYNE. 1978. S-adenosyl-L-homocysteine as a stimulator of viral RNA synthesis by two distinct cytoplasmic polyhedrosis viruses. J. Virol. 26 : 832-835.

MERTENS, P.P.C. and C.C. PAYNE. 1983. The effect of S-adenosylmethionine (Adomet) and its analogues on the control of transcription and translation in vitro of the mRNA products of two cytoplasmic polyhedrosis viruses. Virology 131 : 18-29.

MERTENS, P.P.C. and D.V. SANGAR. 1985. Analysis of the terminal sequences of the genome segments of four orbiviruses. Virology 140 : 55-67.

MOHIER, E.; HIRTH, L.; LE MEUR, M-A. and P. GERLINGER. 1975. Translation of alfalfa mosaic virus RNAs in mammalian cell-free systems. Virology 68 : 349-359.

MORGAN, E.M. and H.J. ZWEERINK. 1974. Reovirus morphogenesis: Core-like particles in cells infected at 39°C with wilde-type reovirus and temperature-sensitive mutants of groups B and G. Virology 59 : 556-565.

MOYER, S.A. and D.F. SUMMERS. 1974. Phosphorylation of vesicular stomatitis virus in vivo and in vitro. J. Virol. 13 : 455-465.

MUSTOE, T.A.; RAMIG, R.F.; SHARPE, A.H. and B.N. FIELDS. 1978. Genetics of reovirus identification of the dsRNA segments encoding the polypeptides of the u and s size classes. Virology 89 : 594-604.

NAGY, E. and P.DOBOS. 1984. Coding assignments of Drosophila X virus genome segments : In vitro translation of native and denatured virion dsRNA. Virology 137 : 58-66.

NAMIKI, M.; SUZUKI, Y.; NAKAGAWA, S.; YOSHIOKA, I. and Y. SAITO. 1983. Characterization of ribonucleic acid transcriptase in Ibarakivirus core particles. J. Gen. Virol. 64 : 1397-1400.

NEELEMAN, L. and L. VAN VLOTEN-DOTING. 1978. Excess of micrococcal nuclease may harm the exogenous mRNA-dependent rabbit reticulocyte cell-free system. Febs. Lett. 95 : 103-106.

NONOYAMA, M. and A.F. GRAHAM. 1970. Appearance of defective virions in clones of reovirus. J. Virol. 6 : 693-694.

NUSS, D.L. 1984. Molecular biology of wound tumor virus. In : Lauffer, M.A. and K. Maramorosch, eds. Advances in Virus Research. Academic Press Inc. pp.57-93.

OFENGAND, J. and R. HASELKORN. 1962. Viral RNA dependent incorporation of amino acids into protein by cell-free extracts of E. coli. Biochem. Biophys. Res. Comm. 6: 469-474.

PAL, B.K.; McALLISTER, R.M.; GARDNER, M.B. and P. ROY-BURMAN. 1975. Comparative studies on the structural phosphoproteins of mammalian type C viruses. J. Virol. 16 : 123-131.

PELHAM, H.R.B. and R.J. JACKSON. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67 : 247-256.

PERRAULT, J. and D.W. KINGSBURY. 1974. Inhibitor of vesicular stomatitis virus transcriptase in purified virions. Nature 248 : 45-47.

POWELL, K.F.H.; HARVEY, J.D. and A.R. BELLAMY. 1984. Reovirus RNA transcriptase: Evidence for a conformational change during activation of the core particle. Virology 137 : 1-8.

PRIVALSKY, M.L. and E.E. PENHOET. 1977. Phosphorylated protein component present in influenza virions. J. Virol. 24 : 401-405.

RANU, R.S. and I.M. LONDON. 1979. Regulation of protein synthesis in rabbit reticulocyte lysates : preparation of efficient protein synthesis lysates and the purification and characterization of the heme-regulated translational inhibitory protein kinase. In : Colowick, S.P. and N.O. Kaplan, eds. Methods in Enzymology. LX. Acad. Press pp.459-484.

RAO, C.D.; KUICHI, A. and P.ROY. 1983. Homologous terminal sequences of the genome double-stranded RNAs of bluetongue virus. J. Virol. 46 : 378-383.

REDDY, D.V.R. and L.M. BLACK. 1977. Isolation and replication of mutant populations of wound tumor virions lacking certain genome segments. Virology 80 : 336-346.

REDDY, D.V.R.; RHODES, D.P.; LESNAW, J.A.; MacLEOD, R.; BANERJEE, A.K. and L.M. BLACK. 1977. In vitro transcription of wound tumor virus RNA by virion-associated RNA transcriptase. Virology 80 : 356-361.

RHODES, D.P.; REDDY, D.V.R.; MacLEOD, R.; BLACK, L.M. and A.K. BANERJEE. 1977. In vitro synthesis of RNA containing 5'-terminal structure $^{7}mG(5')ppp(5')Ap^{m}$ by purified wound tumor virus. Virology 76 : 554-559.

RICHARDSON, M.A.; IWAMOTO, A.; IKEGAMI, N.; NOMOTO, A. and Y. FURUICHI. 1984. Nucleotide sequence of the gene encoding the serotype-specific antigen of human (Wa) rotavirus : Comparison with the homologous genes from simian SA 11 and UK bovine rotaviruses. J. Virol. 51 : 860-862.

ROBERTSON, J.S. 1979. 5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res. 6 : 3745-3756.

ROSEMOND, H. and B. MOSS. 1973. Phosphoprotein component of vaccinia virions. J. Virol. 11 : 961-970.

RUSSELL, W.C.; SKEHEL, J.J.; MACHADO, R. and H.G. PEREIRA. 1972. Phosphorylated polypeptides in adenovirus infected cells. Virology 50 : 931-970.

SAKUMA, S. and Y. WATANABE. 1972a. Reovirus replicase-directed synthesis of double-stranded ribonucleic acid. J. Virol. 10 : 628-638.

SAKUMA, S. and Y. WATANABE. 1972b. Incorporation of in vitro synthesized reovirus double-stranded ribonucliec acid into virus core-like particles. J. Virol. 10 : 943-950.

SCHAFFAUSEN, B.S. and T.L. BENJAMIN. 1979. Phosphorylation of polyoma T antigens. Cell 18 : 935-946.

SCHEIDTMANN, K-H.; ECHLE, B. and G. WALTER. 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. J. Virol. 44 : 116-133.

SCHEIDTMANN, K-H.; HARDUNG, M.; ECHLE, B. and G. WALTER. 1984. DNA-binding activity of simian virus 40 large T antigen correlates with a distinct phosphorylation state. J. Virol. 50 : 1-12.

SCHUERCH, A.R.; MUTSUHISA, T. and W.K.JOKLIK. 1974. Temperature-sensitive mutants of reovirus. VI. Mutant ts447 and ts556 particles that lack either one or two genome RNA segments. Intervirol. 3 : 36-46.

SCHUERCH, A.R.; MITCHELL, W.R. and W.K. JOKLIK. 1975. Isolation of intact individual species of single- and double-stranded RNA after fractionation by polyacrylamide gel electrophoresis. Anal. Biochem. 65 : 331-345.

SERWER, P.; MASKER, W.E. and J.L. ALLEN. 1983. Stability and in vitro DNA packaging of bacteriophages. Effects of dextrans, sugars and polyols. J. Virol. 45 : 665-671.

SHATKIN, A.J. 1976. Capping of eukaryotic mRNAs. Cell 9 : 645-653.

SHATKIN, A.J.; FURUICHI, Y.; LaFIANDRA, A.J. and M. YAMAKAWA. 1983. Initiation of mRNA synthesis and 5'-terminal modification of reovirus transcripts. In : Compans, R.W. and D.H.L. Bishop, eds. Double-stranded RNA viruses. Elsevier Biomedical. pp. 45-54.

SHATKIN, A.J. and J.D. SIPE. 1968. RNA polymerase activity in purified reovirus. Proc. Natl. Acad. Sci. USA 61 : 1462-1469.

SHAW, M.W.; CHOPPIN, P.W. and R.A. LAMB. 1983. A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. Proc. Natl. Acad. Sci. USA 80 : 4879-4883.

SHIH, C-Y. T. and D.S. SHIH. 1981. Cleavage of the capsid protein precursors of encephalomyocarditis virus in rabbit reticulocte lysates. 1981. J. Virol. 40 : 942-945.

SILVERSTEIN, S.C.; LEVIN, D.H. SCHONBERG, M. and G. ACS. 1970. The reovirus replicative cycle: Conservation of parental RNA and protein. Proc. Natl. Acad. Sci. USA 67 : 275-280.

SINACORE, M.S. and J. LUCAS-LENARD. 1982. The effect of the vesicular stomatitis virus-associated protein kinase on viral mRNA transcription in vitro. Virology 121 : 404-413.

SKEHEL, J.J. and W.K. JOKLIK. 1969. Studies on the in vitro transcription of reovirus RNA catalized by reovirus cores. Virology 39 : 822-831.

SKUP, D. and S. MILLWARD. 1977. Highly efficient translation of messenger RNA in cell-free extracts prepared from L-cells. Nucleic Acid Res. 4 : 3581-3587.

SMITH, A.E; SMITH, R.; GRIFFIN, G. and M. FRIED. 1979. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell 18 : 915-924.

SMITH, M.L.; LAZDINS, I. and I.H. HOLMES. 1980. Coding assignments of double-stranded RNA segments of SA 11 rotavirus established by in vitro translation. J. Virol. 33 : 976-982.

SMITH, R.E. and Y. FURUICHI. 1980. Gene mapping of cytoplasmic polyhedrosis virus of silkworm by the full-length mRNA prepared under optimized conditions of transcription in vitro. Virology 103 : 279-290.

SMITH, R.E. and Y. FURUICHI. 1982. The double-stranded RNA genome segments of cytoplasmic polyhedrosis virus are independently transcribed. J. Virol. 41 : 326-329.

SOKOL, F. and H.F. CLARKE. 1973. Phosphoproteins, structural components of rhabdovirus. Virology 52 : 246-263.

SPENCER, E. and B.I. GARCIA. 1984. Effect of S-adenosylmethionine on human rotavirus RNA synthesis. J. Virol. 52 : 188-197.

STAMATOS, N.M. and P.J. GOMATOS. 1982. Binding to selected regions of reovirus mRNAs by a non-structural reovirus protein. Proc. Natl. Acad. Sci. USA 79 : 3457-3461.

STONE, H.O. and O.W. KINGSBURY. 1973. Stimulation of Sendai virion transcriptase by polyanions. J. Virol. 11 : 243-249.

STUDIER, W.F. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79 : 237-248.

TAN, K.B. and F. SOKOL. 1972. Structural proteins of simian virus 40 : phosphoproteins. J. Virol. 10 : 985-994.

TANFORD, C.; BUCKLEY, C.E.; DE, P.K. and E.P. LIVELY. 1962. Effect of ethylene glycol on the conformation of γ-globulin and β-lactoglobulin. J. Biol. Chem. 237 : 1168-1171.

THEILER, A. 1906. Bluetongue in sheep. Ann. Rep. Dir. Agric. Transvaal for 1904-1905 : 110-121.

TRAVERS, A. 1974. On the nature of DNA promotor conformations. The effects of glycerol and dimethylsulphoxide. Eur. J. Biochem. : 47 : 435-441.

VALERI, C.R. 1975. Simplification of the methods for adding and removing glycerol during freeze preservation of human red blood cells with the high or low glycerol methods : biochemical modification prior to freezing. Transfusion 15 : 195-218.

VAN DER WALT, N.T. 1980. A heamagglutination and heamagglutination inhibition test for bluetongue virus. Onderstepoort J. Vet. Res. 47 : 113-117.

VAN DIJK, A.A. 1980. Die in vitro aktivering van transkripsie-aktiwiteit by bloutongvirus. M. Sc. Thesis. University of Potchefstroom for Christian Higher Education.

VAN DIJK, A.A. and H. HUISMANS. 1980. The in vitro activation and further characterization of the bluetongue virus-associated transcriptase. Virology 104 : 347-356.

VAN DIJK, A.A. and H. HUISMANS. 1982. The effect of temperature on the in vitro transcriptase reaction of bluetongue virus, epizootic heamorrhagic disease virus and African horsesickness virus. Onderstepoort J. Vet. Res. 49 : 227-232.

VERWOERD, D.W. 1969. Purification and characterization of bluetongue virus. Virology 38 : 203-212.

VERWOERD, D.W.; ELS, H.J.; DE VILLIERS, E-M. and H. HUISMANS. 1972. Structure of the bluetongue virus capsid. J. Virol. 10 : 783-794.

VERWOERD, D.W. and H. HUISMANS. 1969. On the relationship between bluetongue, African horsesickness and reovirus: hybridization studies. Onderstepoort J. Vet. Res. 36 : 175-180.

VERWOERD, D.W. and H. HUISMANS. 1972. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. Onderstepoort J. Vet. Res. 39 : 185-192.

VERWOERD, D.W.; HUISMANS, H. and B.J. ERASMUS. 1979. Orbiviruses. In : Fraenkel-Conrat, H. and R.R. WAGNER, eds. Comprehensive Virology 14. Plenum Press, New York. pp.285-345.

VERWOERD, D.W.; LOUW, H. and R.A. OELLERMANN. 1970. Characterization of bluetongue virus ribonucleic acid. J. Virol. 5 : 1-7.

VERWOERD, D.W.; OELLERMANN, R.A.; BROEKMAN, J. and K.E. WEISS. 1967. The serological relationship of South African bovine enterovirus strains (Ecbo SA-I and II) and the growth characteristics in cell culture of the prototype strain (Ecbo SA-I). Onderstepoort J. Vet. Res. 34 : 41-52.

WATANABE, Y.; MILLWARD, S. and A.F. GRAHAM. 1968. Regulation of transcriptase of the reovirus genome. J. Mol. Biol. 36 : 107-123.

WERTHEIMER, H.M.; CHEN, S.Y.; BORCHARDT, R.T. and Y. FURUICHI. 1980. S-adenosylmethionine and its analogues : structural features correlated with synthesis and methylation of mRNAs of cytoplasmic polyhedrosis virus. J. Biol. Chem. 255 : 5924-5930.

WITT, D.J. and D.F. SUMMERS. 1980. Relationship between virion-associated kinase-effected phosphorylation and transcription activity of vesicular stomatitis virus. Virology 107 : 34-49.

WITTE, O.N.; DASGUPTA, A. and D. BALTIMORE. 1980. Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283 : 826-831.

YAMAKAWA, M.; FURUICHI, Y.; NAKASHIMA, K.; LaFIANDRA, A.J. and A.J. SHATKIN. 1981. Excess synthesis of viral mRNA 5'-terminal oligonucleotides by reovirus transcriptase. J. Biol. Chem. 256 : 6507-6514.

YAMAKAWA, M.; FURUICHI, Y. and A.J. SHATKIN. 1982. Reovirus transcriptase and capping enzymes are active in intact virions. Virology 118 : 157-168.

ZWEERINK, H.J.; ITO, Y. and J. MATSUHISA. 1972. Synthesis of reovirus double-stranded RNA within virion-like particles. Virology 50 : 349-358.

ZWEERINK, H.J and W.K. JOKLIK. 1970. Studies on the intracellular synthesis of reovirus-specified proteins. Virology 41 : 501-518.