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STUDIES ON THE *IN VITRO* AND THE *IN VIVO* TRANSCRIPTION OF THE BLUETONGUE VIRUS GENOME

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ABSTRACT

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Bluetongue virus particles, converted to a high density form by the selective removal of two polypeptides from their protein capsids, possess RNA dependent RNA polymerase activity. The enzyme, which can be assayed by its ability to incorporate nucleoside triphosphates into RNA in an *in vitro* system, is dependent on magnesium ions, is stimulated by the presence of manganese ions and shows maximal activity at 28°C. The product of the *in vitro* reaction was isolated and shown to consist of ten single-stranded RNA segments which can be hybridized with double-stranded RNA isolated from purified bluetongue virus (BTV). The hybridization product, when analyzed by means of polyacrylamide gel electrophoresis, is indistinguishable from a hybrid obtained using BTV messenger RNA isolated from infected cells. It is therefore deduced that the BTV genome is fully transcribed both *in vitro* and *in vivo* by an enzyme present in the viral capsid.

INTRODUCTION

Within the group of double-stranded RNA containing viruses, defined by Verwoerd (1970a), the synthesis of viral messenger RNA (mRNA), both *in vivo* and *in vitro,* has so far only been studied extensively in the case of reovirus. A summary of the process, which has been termed transcription because it is strictly analogous to the transcription of the DNA genomes of other organisms into mRNA, can be found in a recent review by Shatkin (1971).

Initially the synthesis of single-stranded RNA (ssRNA) complementary to one of the genome strands was demonstrated in reovirus infected cells. It was then shown by means of molecular hybridization studies that this ssRNA consists of ten discrete segments, each derived from one of the ten genome segments, suggesting a possible role as mRNA (Watanabe, Prevec & Graham, 1967; Bellamy & Joklik, 1967). It has recently been demonstrated that all ten ssRNA segments can be isolated from polysomes derived from infected cells (Ward, Banerjee, La Fiandra & Shatkin, 1972). This substantiates their role as messengers and suggests that all ten segments are being translated, although only eight virus-specific polypeptides have so far been identified (Zweerink, McDowell & Joklik, 1971).

The RNA polymerase, or transcriptase, responsible for the synthesis of mRNA was found to be associated with or part of the viral envelope. In reovirus, which possesses a double capsid, the outer layer must be removed by means of degradation with proteolytic enzymes, by heat treatment or by other chemical means, in order to "activate" the enzyme for *in vitro* synthesis (Borsa & Graham, 1968; Shatkin & Sipe, 1968; Skehel & Joklik, 1969). Similar enzyme activities were also discovered, without prior activation, in cytoplasmic polyhedrosis virus (Lewandowski, Kalmakoff & Tanada, 1969), and in wound tumor virus (Black & Knight, 1970).

In bluetongue virus (BTV) infected cells, a ssRNA is also synthesized during the viral replication cycle (Huismans, 1970). It is not incorporated into the virion, but is associated with ribosomes in the infected cell. It was shown to be heterogeneous with regard to molecular mass and to hybridize uniquely with isolated denatured BTV double-stranded RNA (dsRNA), but not with itself (Verwoerd & Huismans, 1969). These results provided strong evidence for its role as mRNA, and suggested the possibility of a transcription process similar to that found in reovirus replication.

However, previous attempts to demonstrate a RNA polymerase activity in BTV, similar to the transcriptase of reovirus, failed (Verwoerd, 1970b). *In vivo* studies in the presence of inhibitors of protein synthesis indicated the presence of a pre-formed RNA polymerase in the infecting virus, but all the methods used by other workers for various viruses failed to uncover the enzyme activity *in vitro*.

A solution for this problem has now been found and the results presented here describe the method used for activation of the BTV transcriptase, summarize the characteristics of the enzyme and compare the product synthesized *in vitro* with the mRNA produced *in vivo*.

MATERIALS AND METHODS

Virus and cells

Bluetongue virus serotype 10 was used throughout the study. Methods for the production and purification of the virus have been described by Verwoerd (1969). The origin and cultivation of the BHK-21 and L-strain fibroblast cells that were used were described by Howell, Verwoerd & Oellermann (1967).

Density gradient centrifugation

Isopycnic banding in 40 to 50% CsCl density gradients was utilized to simultaneously expose purified bluetongue virus to high salt concentrations and to isolate the high-density particles produced by this treatment, as discussed under Results. Gradients were buffered to pH 7,0 with 0,2 M Tris-HCl, and centrifuged for 16 hours at 200 000 $\times$ *g* in a Spinco SW 50.1 rotor. Rate zonal centrifugation was used to estimate the molecular size of the ssRNA synthesized *in vitro*. Centrifugation was for 4 hours at 55 000 rpm in a Spinco SW 56 rotor, in 10 to 30% sucrose gradients containing 0,1 M NaCl, 0,005 M Tris, 0,5% sodium dodecyl sulphate (SDS) and 0,0005 M EDTA. Labelled ribosomal RNA from L-cells were used as sedimentation markers.

RNA polymerase assay

The optimal incubation mixture was determined by the experiments reported under Results. The mixture contained, unless otherwise stated, per 0,5 ml: 0,5 μmole of each of the four ribonucleoside-triphosphates, 30 μmoles of Tris-HCl (pH 8,2), 4 μmoles of $MgCl_2$, 1 μmole of $MnCl_2$, 1 μmole of phosphoenolpyruvate kinase, 1 μmole of dithiothreitol (DTT) and 200 μg of virus (1 A_{260}-unit). ^{3}H-labelled UTP was added to a final concentration of 2 μCi/μmole. Incubation was for

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2 h at 28°C unless otherwise indicated. The reaction was stopped by the addition of 2 mg of carrier yeast RNA and 1 ml of 10% trichloro-acetic acid (TCA) containing 0,05 M sodium pyrophosphate. After standing for an hour at 4°C the precipitates were collected on Millipore filters, dried and counted.

Isolation of the in vitro *product*

For the large scale production of the transcriptase product, 20 A_{260}-units of BTV were added to 10 ml of the above mixture. Macaloid in a final concentration of 400 μg/ml was also included in the mixture in order to inhibit ribonuclease activity. In most experiments the concentration of the tritiated uridine triphosphate was increased to 5 μCi/μmole. After incubation for 4 to 6 hours at 28°C one tenth volume of 0,2 M sodium acetate (pH 5,0) was added and the mixture deproteinized with phenol. The RNA was precipitated with 2,5 volumes of cold ethanol, and dissolved in 0,01 M STE buffer (0,01 M NaCl, 0,05 M Tris-HCl, 0,001 M EDTA, pH 6,9) containing 0,05% polyvinylsulphate. In order to separate the mRNA from viral dsRNA it was precipitated in 1,0 M LiCl for 16 hours at 4°C, dissolved in STE buffer again and stored at —20°C.

Cell fractionation

Cytoplasmic extracts were prepared by suspending the cells in ice cold hypotonic RSB buffer (0,01 M NaCl, 0,01 M Tris-HCl, 0,005 M $MgCl_2$, pH 7,4) at a concentration of $3,0 \times 10^7$ cells/ml. After 15 min at 4°C the cells were disrupted by 20 strokes with a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 3000 rpm for 3 min. The nuclear pellet was again suspended in a suitable small volume of RSB buffer, homogenized with a further five strokes and the cytoplasmic extract, after removal of the nuclei, combined with that obtained in the first extraction.

Isolation of double-stranded RNA

Double-stranded RNA was isolated from BTV-infected cells as described previously (Verwoerd, Louw & Oellermann, 1970). Cytoplasmic extracts from infected cells were deproteinized by two phenol extractions. Single-stranded RNA was removed by precipitation with 1,0 M NaCl and the remaining mixture of tRNA and dsRNA separated by chromatography on a column of methylated albumin kieselguhr (MAK).

Isolation of labelled single-stranded RNA

A suspension culture of L-cells was infected with purified BTV at an input multiplicity of 30 plaque forming units/cell. Six hours after infection Actinomycin D was added to a final concentration of 0,5 μg/ml in order to inhibit DNA dependent RNA synthesis. The cells were labelled with ^{3}H-uridine (2 μCi/ml) between 10 and 12 hours after infection. After the labelling period the cells were harvested by centrifugation and a cytoplasmic extract prepared as described. The extract was deproteinized by phenol extractions in the presence of SDS and the RNA precipitated with 2,5 volumes of ethanol at —20°C. Single-stranded RNA was separated from dsRNA and tRNA by overnight precipitation with 1,0 M LiCl at 4°C. The ssRNA precipitate was dissolved in 0,01 M STE buffer and precipitated a second time by addition of NaCl to a final concentration of 1,0 M. The second precipitation removed residual traces of dsRNA contaminant in the ssRNA preparation. The ssRNA was again dissolved in 0,01 M STE containing 0,05% PVS and stored at —20°C.

Hybridization

The hybridization procedure used was essentially the same as that described by Verwoerd & Huismans (1969). Labelled ssRNA and unlabelled dsRNA, both in 0,01 M STE buffer, were mixed in a proportion of 2 μg dsRNA for every 1 000 counts per min (cpm) of ssRNA, representing a large excess of dsRNA. The mixture, in tightly stoppered glass tubes, was heated to 96°C in a waterbath. After 5 min at this temperature, the salt concentration was raised to 0,3 M NaCl and the reaction mixture incubated at 72°C for 30 min. The hybridization product was then treated with 4 μg/ml pancreatic ribonuclease for 15 min at 37°C in buffer solution containing 0,3 M NaCl. Prior to electrophoresis the hybridization product was purified from all non-dsRNA material by separation on a MAK column. In some experiments the hybridization products were not analyzed by means of electrophoresis but precipitated with 10% TCA after treatment with ribonuclease. The precipitates were collected on Millipore filters, dried and counted as described below.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out essentially as described by Verwoerd *et al.* (1970). Gels (3%) were prepared in Loening's buffer containing 0,1% SDS, using diacrylate as cross-linking agent. Gel columns were 10 cm long. Single-stranded RNA was heated prior to electrophoresis for 3 min at 90°C and then rapidly cooled in an ice bath before application to the gel column in a volume of 0,05 ml. In the case of ssRNA adequate separation was normally attained after 3 h at 10 V/cm. Separation of dsRNA took 5 h under similar conditions. Gels were cut into 1 mm slices, dissolved in 10% piperidine and counted.

Radio-activity assays

Radio-activity determinations were made in a Packard Tri-Carb liquid scintillation spectrometer. All aqueous samples were counted in Bray's scintillator (Bray, 1960). TCA-precipitates on Millipore filters were counted in a toluene scintillator.

Results

Localization of the viral RNA-dependent RNA polymerase

Bluetongue virions, isolated in the usual way with one or more cycles of rate zonal centrifugation in sucrose density gradients as the final purification step, are completely inactive when tested for the *in vitro* incorporation of radio-active ribonucleotides. This is in contrast to some of the other known diplornaviruses such as wound tumor virus and cytoplasmic polyhedrosis virus (Lewandowski *et al.*, 1969; Black & Knight, 1970). All attempts to "induce" the enzymatic activity, by means of the various methods used by other workers to activate reovirus, were unsuccessful in the case of BTV. These methods all depend on the removal of the outer capsid, a structural feature of reovirus previously shown to be absent in BTV (Els & Verwoerd, 1969).

Recent studies on the polypeptide composition of the BTV protein coat revealed, however, that this virus also possesses two polypeptides which are arranged in a diffuse layer surrounding the capsid proper. This layer, which is present in the virus purified on sucrose gradients and which was shown to be essential for infectivity, can be selectively removed by means of isopycnic centrifugation on CsCl-gradients (Verwoerd, Els, de Villiers & Huismans, 1972). During this process bluetongue virions are converted from a low (1,38) to a high density (1,42), with some intermediate forms also

being seen. The obvious analogy between this loss of two peptides, and the enzymatic removal of a capsid layer consisting of four peptides in reovirus, prompted us to test the "naked" BTV-capsids for RNA polymerase activity.

TABLE 1 Incorporation of ^{3}H-uridine triphosphate by high density bluetongue virus particles in an *in vitro* system

Incubation mixture	Cpm/A_{260} unit of BTV
Complete, no virus	0
Complete with low density BTV (1,38)	5
Complete with high density BTV (1,42)	480
Same minus ATP	12
Same minus GTP	10
Same minus CTP	102
Same minus PEP and P. kinase	385
Same minus Mg^{++}	30
Same minus Mn^{++}	120
Same minus DTT	252
Same with 5-mercaptoethanol replacing DTT	334
Complete plus 0,5 μg/ml Actinomycin D	437

Table 1 illustrates the activity found in these particles and summarizes the general characteristics of the reaction. All four ribonucleoside-triphosphates are necessary, although some incomporation was found in the absence of CTP. The reaction is dependent on the presence of both Mg^{++} and Mn^{++} ions. It is not inhibited by a concentration of 0,5 of μg/ml Actinomycin D, which blocks DNA dependent RNA synthesis completely. Energy in the form of phosphoenolpyruvate and pyruvate kinase stimulate the reaction slightly. Reducing agents DTT and 5-mercaptoethanol have a similar effect.

Optimal conditions for the enzyme reaction

a. *Temperature*

A series of standard incubation mixtures were prepared as described under Methods and incubated at various temperatures as indicated in Fig. 1A. The optimal temperature for maximum incorporation can clearly be seen to be 28°C. At 37°C only about 50% of the maximum activity is obtained.

b. *pH*

The pH of the incubation mixtures was adjusted to the values shown in Fig. 1B, otherwise assays were performed under standard conditions. Maximum incorporation was found in the pH range 8,1 to 8,5. The reaction was relatively insensitive to small changes within this range, but below pH 8,0 the rate decreased sharply. A pH value of 8,2 was selected for our standard conditions.

c. *Mg^{++}-ion concentration*

The dependence of the RNA-polymerase on magnesium ions was determined both in the presence and in the absence of manganese ions. In both cases the reaction was completely dependent on the presence of Mg^{++}. Its optimal concentration, as can be seen in Fig. 2A, is 4 μmoles per 0,5 ml of incubation mixture or 8 μmoles per ml. Higher concentrations were detrimental.

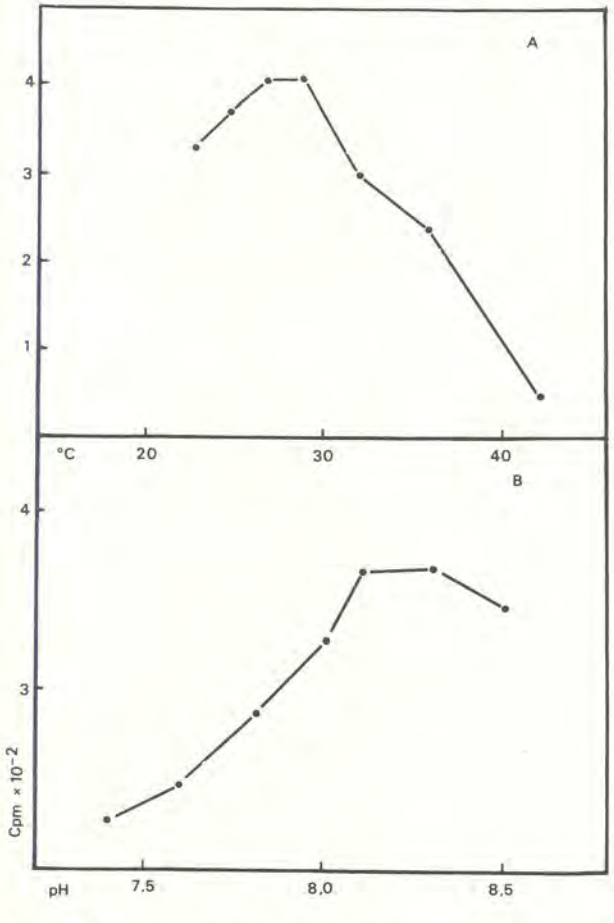

FIG. 1 The influence of variations in temperature (1A) and pH (1B) on the activity of BTV RNA-polymerase in incorporating ^{3}H-uridine triphosphate into RNA in an *in vitro* system

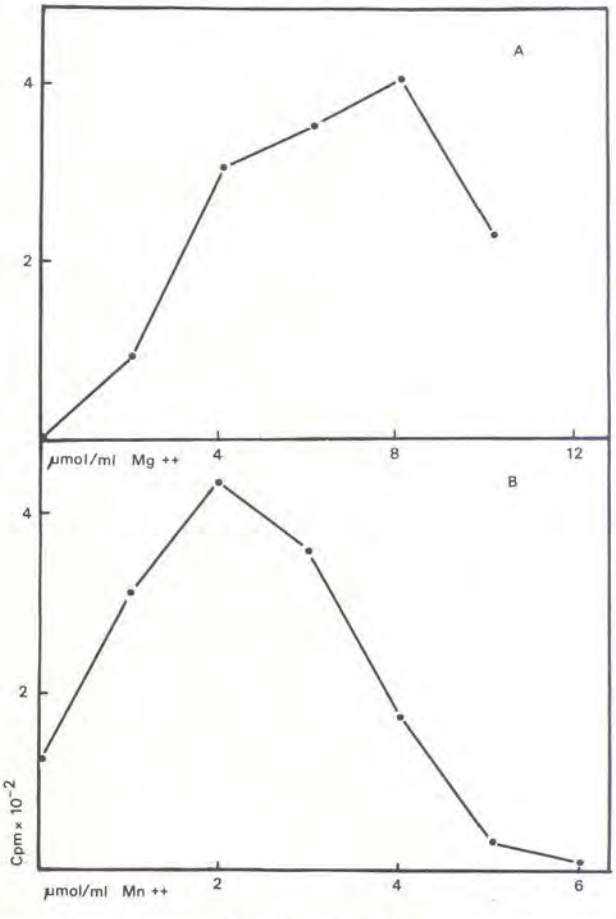

FIG. 2 The influence of Mg^{++} and Mn^{++} concentration on the incorporation of ^{3}H-UTP into RNA by BTV RNA-polymerase

d. *Mn^{++}-ion concentration*

Fig. 2B illustrates the effect of Mn^{++} concentration on the incorporation of ^{3}H-UTP into TCA-precipitable RNA. Although not as dependent on manganese as on magnesium, the former ion has a stimulatory action at a concentration of 2,0 μmol/ml. Higher concentrations again inhibit the reaction.

e. *Substrate concentration and the rate of the reaction*

From an incubation mixture containing ten times the amount of the standard assay mixture, aliquots of 0,5 ml were taken in duplicate at hourly intervals and precipitated and counted as described under Methods. The values obtained were used to plot the reaction rate in Fig. 3. It can be seen that the rate is linear for the first 3 hours at 28°C but then declines. The addition of all four nucleoside-triphosphates at half concentration after 6,5 hours increased the rate to its original value. This indicates that the concentration of the nucleotides is the rate-limiting factor of the reaction under our conditions. If the addition of substrate is continued, the reaction was shown to proceed at an approximately linear rate for a total of at least 12 to 14 hours.

When the same experiment was conducted at 37°C the reaction rate was seen to be about half of that measured at 28°C. This reduced rate persisted from the onset of the reaction. This proved that the increased incorporation at 28°C was not due to increased stability of the enzyme at low temperatures.

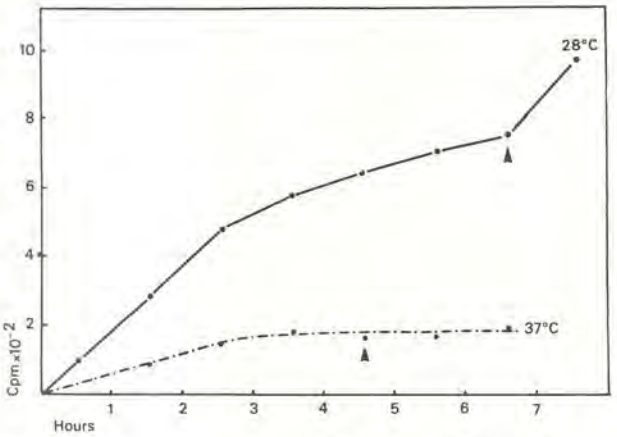

FIG. 3 The rate of incorporation of ^{3}H-UTP into RNA by the BTV RNA-polymerase in an *in vitro* system, at 28°C and 37°C. The time at which the four nucleoside triphosphates at half concentration are added is indicated by arrows

Molecular size of the reaction product

In order to characterize the product formed during the *in vitro* reaction, the volume of the incubation mixture was scaled up to 10 ml, using 20 A_{260}-units of high density BTV as a source of the RNA polymerase. To prevent degradation of the product by contaminating nucleases, Macaloid was added to the mixture at a concentration of 400 μg/ml. At this concentration it was found to have no inhibitory effect on the reaction. After incubation for 4 to 6 hours at 28°C the product was isolated by means of phenol extraction and subjected to rate zonal sedimentation analysis on a sucrose-SDS gradient as described under Methods.

The result is illustrated in Fig. 4. Most of the incorporated ^{3}H-uridine is recovered as high molecular mass material in the gradient, with a heterogeneous sedimentation constant distribution between 12 S and 22 S. This is very similar to the pattern obtained with mRNA synthesized *in vivo* and isolated from polysomes derived from BTV-infected cells (Huismans, 1970). Treatment of the product with pancreatic ribonuclease at a concentration of 4 μg/ml for 30 minutes at 37°C prior to sedimentation analysis leads to a complete degradation, indicating that it is single-stranded in nature (Fig. 4).

In both its molecular size distribution and its single-strandedness, the *in vitro* product was therefore indistinguishable from BTV mRNA.

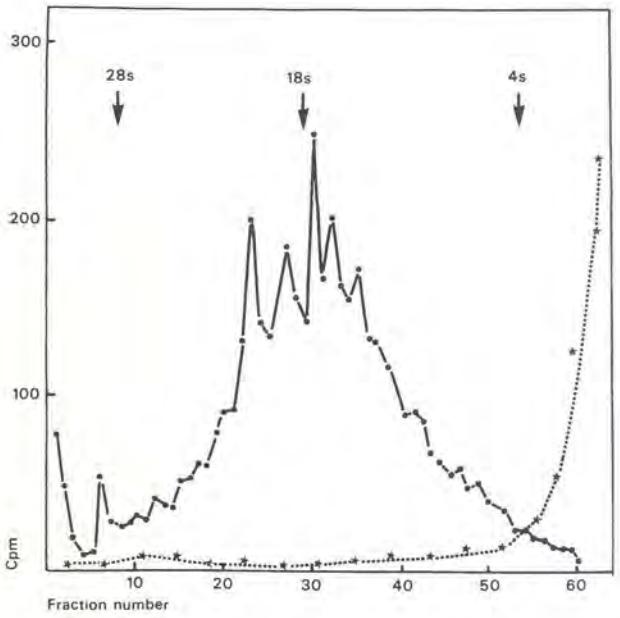

FIG. 4 Sedimentation analysis on sucrose-SDS gradients of the *in vitro* product of BTV RNA-polymerase. Sedimentation was from right to left. The positions of markers are indicated by arrows; gradients were centrifuged for 4 hours at 300 000 $\times g$. The full line represents an untreated sample, the dotted line a sample treated with ribonuclease

Hybridization analysis of BTV messenger RNA synthesized in vitro *and* in vivo

In order to prove that the *in vitro* product of the BTV RNA-polymerase is homologous to the viral double-stranded RNA genome, molecular hybridization of the two RNA types was carried out. Specific hybridization has previously been found between *in vivo* synthesized BTV - mRNA and the genome RNA (Verwoerd & Huismans, 1969; Huismans, 1970). It has not been proved unequivocally, however, that this mRNA represents a mixture of the transcription products of all ten genome segments. The present hybridization experiments were therefore extended to an analysis of the hybridization products by means of polyacrylamide gel electrophoresis.

The amount of the *in vitro* product available for hybridization was limited, although relatively high specific activities could be obtained. It was therefore necessary to determine once more the optimal relative concentration of the dsRNA and ssRNA during hybridization. To ensure maximum hybridization efficiency the effects of salt concentration and length of incubation time were also investigated.

(*a*) *Double-stranded RNA concentration*

A constant amount of labelled ssRNA containing 1 000 TCA precipitable counts/min was mixed with increasing amounts of unlabelled dsRNA. The total volume of the hybridization mixture was adjusted to 0,05 ml by addition of 0,01 M STE buffer. The solutions were then heated for 5 min at 96°C and incubated at 72°C after addition of NaCl to a final concentration of 0,3 M NaCl. After an incubation period of 30 min the products were treated with RNase and precipitated with TCA as described under Methods. The percentage hybridization was obtained by calculating the percentage of the total ssRNA counts recovered as TCA-precipitable counts in the RNase resistant hybridization product. The results are shown in Fig. 5A.

If dsRNA is omitted from the hybridization mixture, less than 0,1% of ssRNA can be recovered as RNase resistant material. An increase in the amount of dsRNA results in a corresponding increase in percentage hybridization until maximum hybridization is reached. The minimum amount of dsRNA required for optimum hybridization is in the order of 2 μg dsRNA/1000 cpm single-stranded RNA.

(*b*) *Salt concentration*

The Tm value for the thermal denaturation of BTV-RNA depends on the ionic strength of the solution (Verwoerd *et al.*, 1970). It can therefore be assumed that ionic strength will have a similar effect on the renaturation of complementary single-stranded RNA molecules.

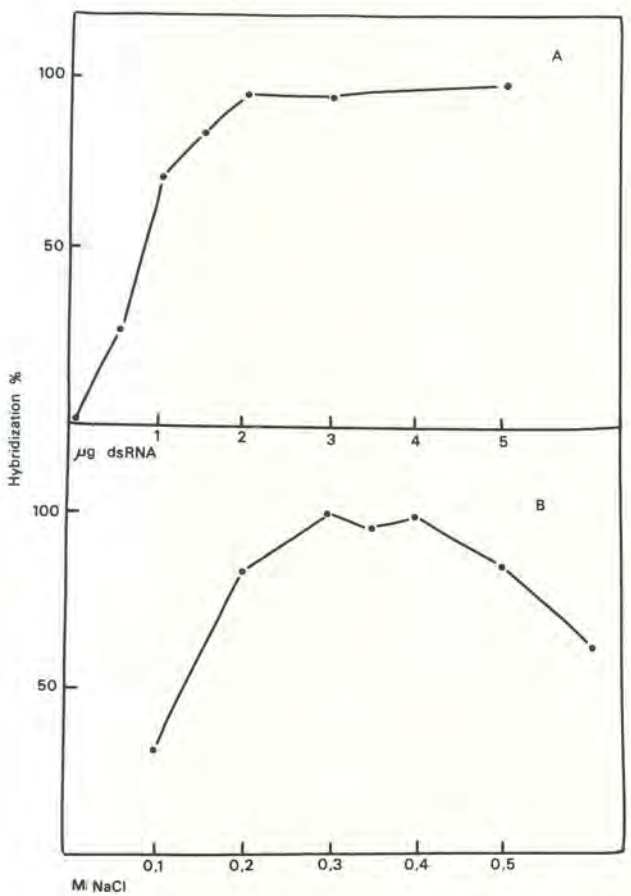

FIG. 5 The effect of double-stranded RNA concentration (5A) and sodium chloride concentration (5B) on the hybridization efficiency of a labelled bluetongue virus messenger RNA sample containing 1000 cpm

In order to investigate this effect and determine the optimal salt concentration, a series of hybridization studies at different salt concentrations was carried out. The optimum relative RNA concentrations as determined in the previous experiment were used. The results are shown in Fig. 5B. At salt concentrations lower than 0,1 M the hybridization is still incomplete after 30 min.

There is an optimal salt concentration between 0,3 and 0,4 M NaCl. The efficiency of hybridization under these conditions is approximately 100%.

(*c*) *Rate of renaturation*

The minimal incubation time required for quantitative hybridization under optimal conditions was also determined. A series of identical hybridization mixtures was set up. These mixtures were then incubated at 72°C for various lengths of time from 5 to 90 min. The percentage hybridization was then calculated in each case. The result is shown in Fig. 6. Hybridization is extremely rapid. After only 5 min approximately 80% renaturation has taken place. After 15 min quantitative hybridization (100%) has been obtained.

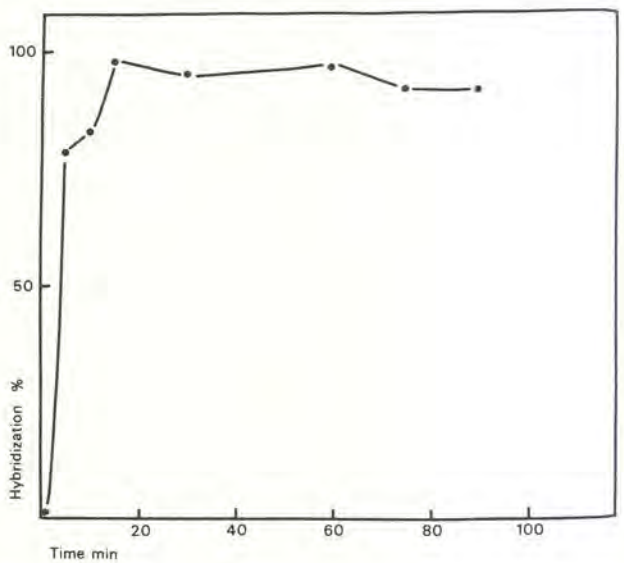

FIG. 6 Renaturation rate of bluetongue virus messenger RNA with its complementary strand from denatured double-stranded BTV RNA under optimal conditions

Polyacrylamide gel electrophoresis of the hybridization products

Labelled BTV mRNA of both *in vivo* and *in vitro* origin was hybridized with unlabelled BTV dsRNA under optimal conditions. The hybrids were treated with RNase and then prior to electrophoresis purified by MAK column chromatography as described above. In Fig. 7 typical electropherograms of the hybrids are shown together with that of labelled dsRNA which is included as a reference. The components are numbered from 1 to 10 in order of decreasing size. It is apparent that both hybrids contain at least 10 different molecular mass components. These components are resistant to RNase treatment and have electrophoretic mobilities identical to those of normal dsRNA segments. The isolated mRNA therefore consists of at least ten different lengths of ssRNA. Each one of these hybridizes with a specific denatured BTV dsRNA segment of equal length to form a dsRNA hybrid molecule. Complementary strands to all ten dsRNA genome segments

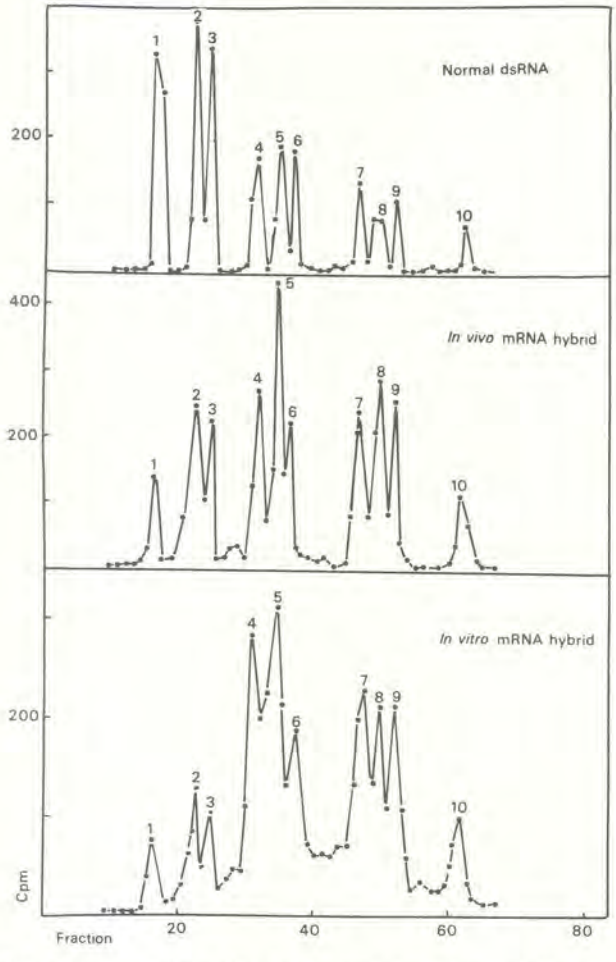

FIG. 7 A comparison of the gelelectrophoretic fractionation pattern of normal double-stranded BTV RNA and those of the hybridization products of BTV mRNA synthesized *in vivo* and *in vitro*. The direction of migration is from left to right

are therefore synthesized. The BTV genome is thus fully transcribed both in the *in vivo* and in the *in vitro* system described in this paper.

The relative distribution of radio-activity in the dsRNA pattern in Fig. 7A is directly proportional to the molecular masses of the segments. This distribution reflects the equimolar amounts in which the segments are present in the viral genome. In both hybridization products the relative distribution of radio-activity is obviously different (Fig. 7B & C). This difference could be due to imperfect hybridization, but could also indicate some selective control mechanism in the transcription process. This aspect is at present being investigated further.

DISCUSSION

Although the presence of RNA-polymerase activity in the bluetongue virion was suggested by *in vivo* studies using inhibitors of protein synthesis in virus-infected cells, previous attempts to demonstrate the enzyme in *in vitro* studies have failed (Verwoerd, 1970b). This demonstration has now been successful, using bluetongue virions which have been exposed to high salt concentrations under controlled conditions, a process which was shown to remove a diffuse protein layer consisting of two polypeptides from the virus (Verwoerd *et al.*, 1972). This "activation" of the enzyme by removal of a protein layer is similar to the equivalent process in reovirus, although the structure and stability of the outer coats are different in the two viruses.

The dissociation of certain proteins from a nucleoprotein particle after exposure to CsCl has also been found in the case of ribosomes, and is a widely used method for the study of ribosomal structure (Itoh, Otaka & Osawa, 1968).

As far as the requirements of the respective enzymes are concerned the difference in the effect of manganese ions can be mentioned. Only one worker reported a dependence on Mn^{++} for the reovirus enzyme (Gomatos, 1970). In our hands, this ion had no influence on the reovirus reaction, but caused a distinct increase in ^{3}H-UTP incorporation in the BTV *in vitro* system.

The most pronounced difference between enzymes of the two viruses was found in the optimal temperature for the transcription reaction. The reovirus enzyme has been shown to operate optimally at temperatures between 47 and 52°C (Kapuler, 1970). In the case of BTV, the reaction rate is maximal at 28°C. A possible explanation for this difference can be found in the fact that BTV is an insect-transmitted virus, and has been shown to multiply actively in the tissues of *Culicoides,* its insect host (Du Toit, 1944). Reovirus, in contrast, replicates only in mammalian hosts. An interesting implication of this difference is that BTV should probably be regarded as primarily an insect virus with the vertebrate as its secondary host. It could also explain why a four to tenfold higher yield of reovirus, compared with BTV, is consistently obtained at 37°C in mammalian cell cultures (Verwoerd, unpubilished result).

The demonstration of an RNA polymerase in the BTV particle was extended to showing that the product is single-stranded and identical in its size distribution to BTV mRNA synthesized *in vivo*. Little doubt therefore remains that the enzyme is a true transcriptase, transcribing the BTV-genome *in vitro* into mRNA.

To confirm this relationship, hybridization experiments with double-stranded BTV-RNA have been carried out. The hybridization product was found to consist of ten dsRNA fragments with a molecular mass distribution similar to that of normal dsRNA. This result can be regarded as proof that the full BTV genome is transcribed.

It has been shown by Verwoerd *et al.* (1972) that an excellent correlation is found between the molecular masses of the polypeptide components of BTV on the one hand and the sizes of the genome segment on the other. This observation strongly suggests a direct coding relationship between the genome segments and the virus polypeptides. However, only seven polypeptides are found in the BTV capsid. The possibility that the other three "genes" might be completely repressed has not been ruled out by the demonstration that full transcription of the BTV genome occurs. Therefore some of the mRNA species are either not translated, or they are translated into proteins which are not incorporated into the BTV capsid.

It can be seen in Fig. 7 that the different fragments in the hybridization product are not present in equimolar amounts, as is the case with the genome RNA segments. The question arises as to whether this molar proportion is an accurate reflection of the molar ratio in which the mRNA species are synthesized. The answer to this question depends mainly on the efficiency of the hybridization process. A number of experiments were carried out in which the optimal hybridization conditions were determined. Under these conditions a 100% conversion of ssRNA to the dsRNA hybrid

form can be effected. Furthermore, this conversion is highly specific, as demonstrated by the fact that less than 1% hybridization occurs when BTV mRNA is hybridized with denatured reovirus dsRNA (Verwoerd & Huismans, 1969). However, Watanabe, Millward & Graham (1968) mentioned that re-annealing of the high molecular mass RNA strands of reovirus was a less efficient process than that of the smaller dsRNA segments. In the case of BTV a similar effect was observed. It was observed (Huismans, unpublished results) that if native dsRNA was denatured and then allowed to re-anneal under the normal hybridization conditions, the recovery of the larger dsRNA segments was lower than when native dsRNA was analyzed directly on polyacrylamide gel.

Therefore, no deductions can be made with regard to the actual molar proportion in which mRNA species are synthesized. Experiments are, however, under way in which the hybridization experiments are carried out in such a way that a more accurate estimation of the molar proportion of mRNA species can be obtained.

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