

RESEARCH NOTE

FAILURE TO DEMONSTRATE *IN VITRO* AS OPPOSED TO *IN VIVO* TRANSCRIPTION OF THE BLUETONGUE VIRUS GENOME

D. W. VERWOERD, Veterinary Research Institute, Onderstepoort

The bluetongue virus (BTV) possesses a segmented, double-stranded ribonucleic acid (RNA) genome, a characteristic it shares with other members of the Diplornavirus group (Verwoerd, Louw & Oellermann, 1970; Verwoerd, 1970). These include reovirus, African horsesickness virus (AHSV), wound tumor virus (WTV), rice dwarf virus (RDV), cytoplasmic polyhedrosis virus (CPV) and possibly a number of other viruses of which the double-stranded nature of the RNA has not yet been proved.

With a genome of this nature, the elucidation of the enzymatic mechanisms concerned in both replication and expression of the double-stranded RNA, poses a problem. Attempts to demonstrate the enzyme activity responsible for the latter process are described in this paper.

Thus far it has been demonstrated unequivocally only in the case of reovirus and RDV that the double-stranded viral RNA is inactive as a template for *in vitro* protein synthesis (Shatkin, 1965; Miura & Muto, 1965). It has been shown, however, that in bluetongue virus-infected cells a single-stranded RNA is synthesized, similar to the messenger RNA in reovirus-infected cells (Huisman, 1970). A large proportion of this RNA is associated with polyribosomes, thus suggesting a messenger function. Furthermore, the single-stranded RNA can be fractionated by means of polyacrylamide gel-electrophoresis into fragments equivalent to the segments of the genome and in hybridization studies it was shown to be completely homologous to the double-stranded RNA. However, it does not show any self-hybridization, indicating that it is a copy of

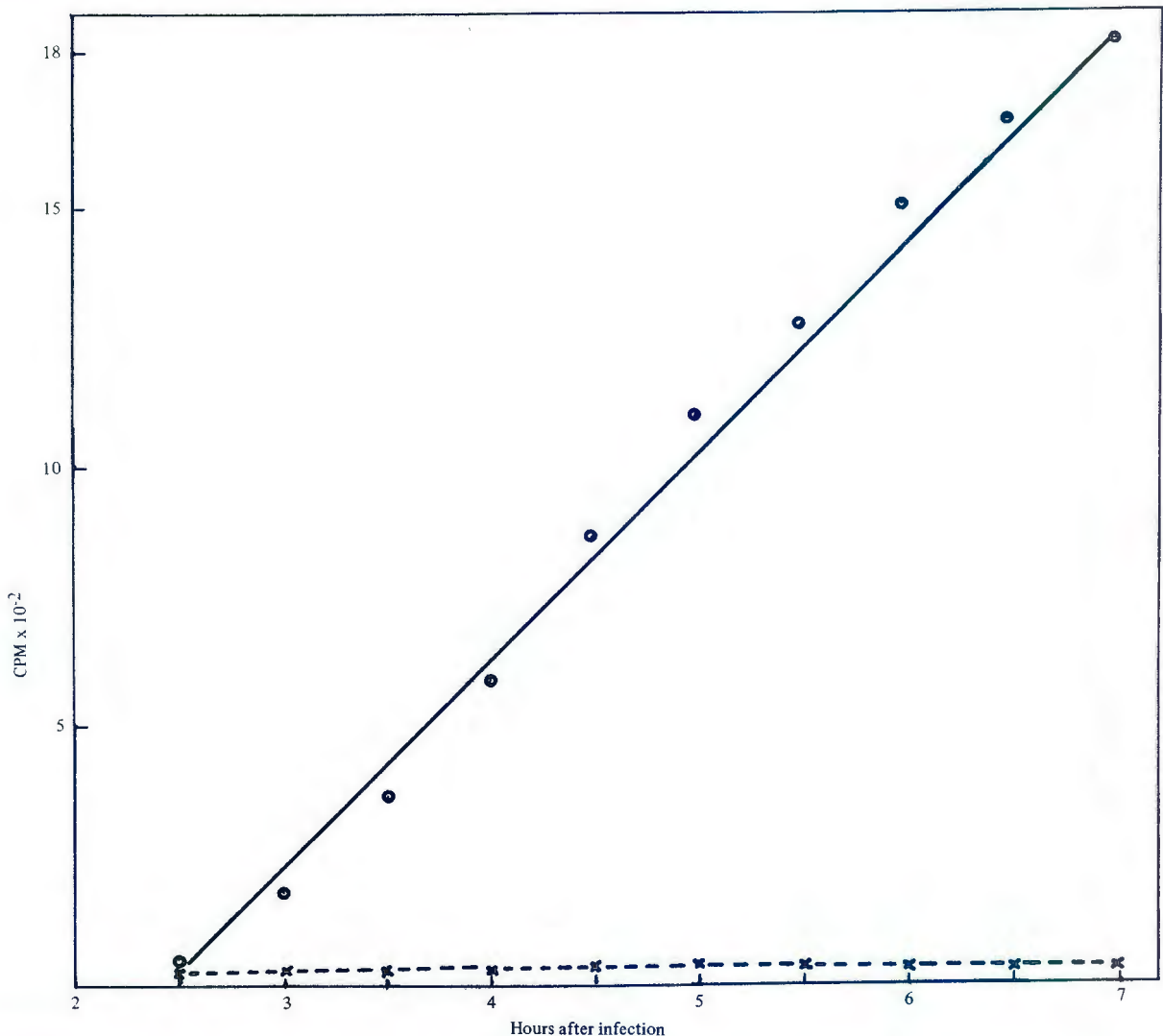


FIG. 1 Incorporation of ¹⁴C-uridine (0.02 μ Ci/ml) by BTV-infected BHK-cells in the presence of 2 μ g/ml actinomycin D and 20 μ g/ml cycloheximide (○—○ infected cells, x····x uninfected control cells).

Received 2 December 1970

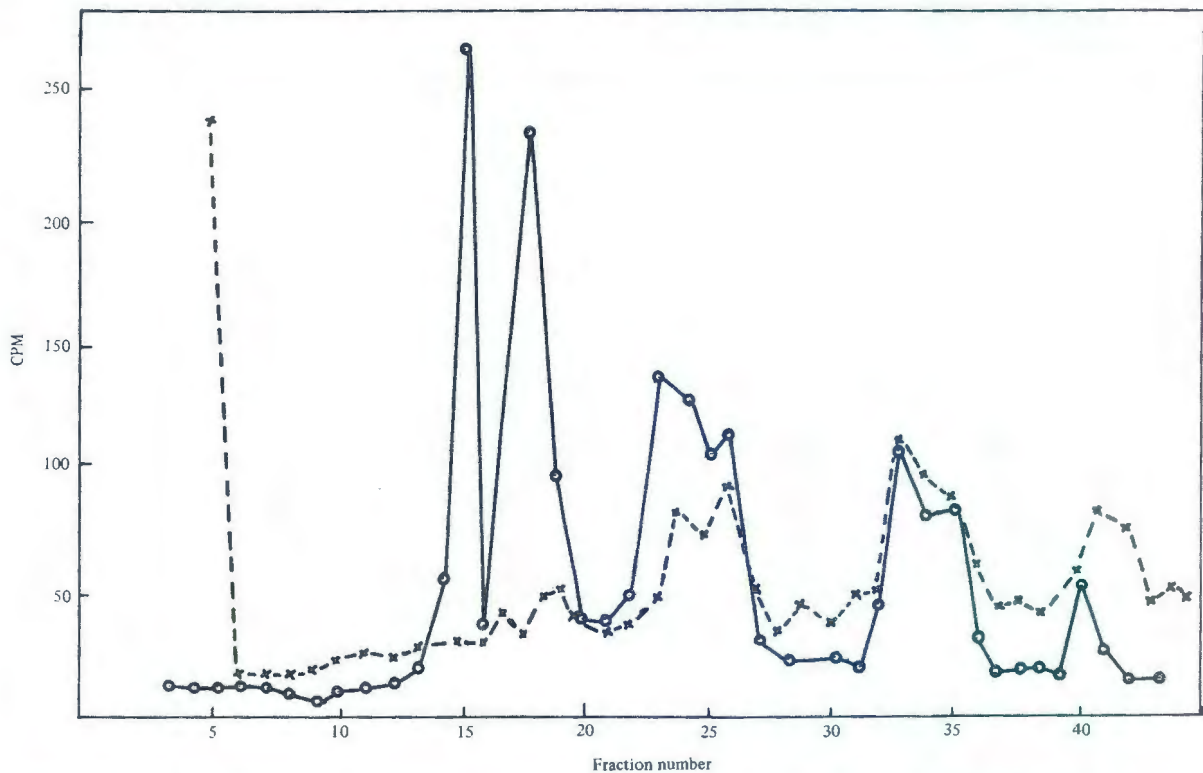


Fig. 2 Comparison of the electrophoretic patterns obtained on polyacrylamide gels with the hybrid product (x····x) and with labelled double stranded BTV-RNA (O—O).

only one of the strands of the double-stranded RNA. From these observations it has been concluded that the genome of the bluetongue virus is transcribed into a single-stranded RNA which functions as a messenger in the synthesis of viral-specific proteins (Huismans, 1970).

The question which immediately arises is where the enzyme responsible for the transcription process is situated. It is unlikely that such an enzyme would be present in the cell, where RNA-dependent RNA synthesis does not normally occur. The information for such an enzyme could exist in the viral genome, but this information would also have to be transcribed before the enzyme could be synthesized in the virus-infected cell, unless a limited portion of the genome could act directly as messenger in the translation process. Although the latter possibility cannot be excluded completely, it does not seem very probable. The most likely explanation is the presence of such an enzyme in the virus particle itself.

In fact, an enzyme of this nature, provisionally named transcriptase, has been found to be present in reovirus (Borsa & Graham, 1968; Shatkin & Sipe, 1968; Skehel & Joklik, 1969; Levin, Mendelsohn, Schonberg, Klett, Silverstein, Kapuler & Acs, 1970), in CPV (Lewandowski, Kalmakoff & Tanada, 1969) and in WTV (Black & Knight, 1970). In the case of reovirus, demonstration of *in vitro* enzyme activity is dependent on prior treatment of the virions with chymotrypsin, in order to remove the outer protein capsid. For the other viruses, where an outer capsid is absent, this "activation" was not found to be necessary.

However, all attempts to demonstrate *in vitro* RNA synthesis with purified BTV as a source of enzyme were unsuccessful. Using the standard incubation mixture (Skehel & Joklik, 1969) and reovirus, purified in exactly the same way as BTV (Verwoerd, 1969), as a positive control, no incorporation of ^{14}C -uridine was

found with BTV over a wide range of conditions. These included treatment with chymotrypsin at concentrations ranging from zero to 500 $\mu\text{g}/\text{ml}$, pH values between 6.0 and 9.0, temperatures of incubation between 25° and 40°C, ionic concentrations between 0.01M and 0.2M and the addition of various ions, e.g. Mn^{++} , NH_4^{++} and K^+ . Alternative procedures were also employed in an attempt to "activate" the enzyme, such as treatment of the virus with trypsin, pronase, Nonidet P-40, Triton N-100, sodium dodecyl sulphate, urea, alkaline treatment according to Lewandowski *et al.* (1969), heat-shock according to Borsa & Graham (1968), ultrasonification and preincubation with cytoplasmic and nuclear extracts. In view of the relative instability of BTV in comparison with reovirus, either one per cent bovine serum albumin or four per cent sucrose (Howell, Verwoerd & Oellermann, 1967) was also added in an attempt to stabilize the virus during incubation.

No appreciable incorporation of ^{14}C -uridine in the presence of BTV was found in any of these experiments, whereas active synthesis of RNA was present in the reovirus controls throughout. As a result of the failure to detect *in vitro* transcription with BTV, an attempt was made to demonstrate *in vivo* activity. The approach was to prevent the synthesis of any virus-induced enzyme in infected cells by the inhibition of all protein synthesis with high concentrations of cycloheximide. In order to detect virus-specific RNA synthesis, the formation of cellular RNA was concomitantly inhibited by means of actinomycin D. Under these conditions, incorporation of labelled uridine was regarded as viral-specific RNA synthesis mediated by a viral enzyme.

The result of such an experiment is shown in Fig. 1. A suspension culture of baby hamster kidney (BHK) cells was pre-incubated for one hour with actinomycin D at a concentration of 2 $\mu\text{g}/\text{ml}$ and cycloheximide at a concentration of 20 $\mu\text{g}/\text{ml}$ (Watanabe, Kudo & Graham,

1967). Half of the culture was then infected with purified BTV at an input-multiplicity of 20 virions per cell. Two hours later, ^{14}C -uridine was added to both the infected and the non-infected control cultures at a concentration of $0.02 \mu\text{Ci/ml}$. Duplicate samples of 2×10^6 cells were taken at 30 minute intervals from $2\frac{1}{2}$ to 7 hours after infection and prepared for scintillation counting as previously described (Verwoerd & Huismans, 1969). The remaining cells were collected by centrifugation and single-stranded RNA isolated by means of phenol extraction and salt-precipitation according to procedures described elsewhere (Bellamy, Shapiro, August & Joklik, 1967). Hybridization of the isolated product with unlabelled double-stranded BTV-RNA and analysis of the hybrid by means of polyacrylamide gel electrophoresis was carried out as described by Huismans (1970).

The result of a hybridization experiment is shown in Fig. 2. The pattern obtained was identical to that obtained by Huismans for a hybrid of messenger RNA and BTV-RNA. These results taken together clearly indicate that a virus-specific single-stranded RNA, capable of hybridizing with the viral genome, is synthesized in BTV-infected BHK cells at a linear rate for at least 7 hours after infection, under conditions where synthesis of cellular RNA and protein is inhibited.

Although the possibility of some residual protein synthesis sufficient for the formation of a small amount of virus-induced enzyme cannot be excluded, the RNA synthesis seems to be dependent on a pre-existing, actinomycin D-resistant, double-stranded RNA-dependent RNA polymerase. The fact that no RNA synthesis could be detected in non-infected control cultures and the improbability of such an enzyme being present in normal cells, clearly indicates the presence of an enzyme, similar to transcriptase described for reovirus, in the bluetongue virus particle.

The complete failure to demonstrate the activity of such an enzyme *in vitro* cannot be explained at present. It could be a reflection of the structural differences between the viruses, or could be related to the relative instability of purified BTV. Conceivably some cellular activity might be necessary to "uncover" or to "activate" the enzyme. These possibilities are being explored further.

REFERENCES

- BELLAMY, A. R., SHAPIRO, L., AUGUST, J. T. & JOKLIK, W. K., 1967. Studies on Reovirus RNA. I. Characterization of Reovirus Genome RNA. *J. molec. Biol.*, **29**, 1-17.
- BLACK, D. R. & KNIGHT, C. A., 1970. Ribonucleic acid transcriptase activity in purified wound tumor virus. *J. Virol.*, **6**, 194-198.
- BORSA, J. & GRAHAM, A. F., 1968. Reovirus: RNA polymerase activity in purified virions. *Biochem. Biophys. Res. Commun.*, **33**, 895-901.
- HOWELL, P. G., VERWOERD, D. W. & OELLERMANN, R. A., 1967. Plaque formation by bluetongue virus. *Onderstepoort J. vet. Res.*, **34**, 317-332.
- HUISMANS, H., 1970. Macromolecular synthesis in bluetongue virus infected cells. I. Virus-specific ribonucleic acid synthesis. *Onderstepoort J. vet. Res.*, (This journal).
- LEVIN, D. H., MENDELSON, N., SCHONBERG, M., KLETT, H., SILVERSTEIN, S., KAPULER, A. M. & ACS, G., 1970. Properties of RNA transcriptase in reovirus subviral particles. *Proc. natn. Acad. Sci.*, **66**, 890-897.
- LEWANDOWSKI, L. J., KALMAKOFF, J. & TANADA, Y., 1969. Characterization of a ribonucleic acid polymerase activity associated with purified cytoplasmic polyhedrosis virus of the silkworm *Bombyx mori*. *J. Virol.*, **4**, 857-865.
- MIURA, K. I. & MUTO, A., 1965. Lack of messenger RNA activity of a double-stranded RNA. *Biochem. Biophys. Acta.*, **108**, 707-709.
- SHATKIN, A. J., 1965. Inactivity of purified reovirus RNA as a template for *E. coli* polymerase *in vitro*. *Proc. natn. Acad. Sci.*, **54**, 1721-1728.
- SHATKIN, A. J. & SIPE, J. D., 1968. RNA polymerase activity in purified reoviruses. *Proc. natn. Acad. Sci.*, **61**, 1462-1469.
- SKEHEL, J. J. & JOKLIK, W. J., 1969. Studies on the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. *Virology*, **39**, 822-831.
- VERWOERD, D. W., 1969. Purification and characterization of bluetongue virus. *Virology*, **38**, 203-212.
- VERWOERD, D. W. & HUISMANS, H., 1969. On the relationship between bluetongue, African horsesickness and reovirus: hybridization studies. *Onderstepoort J. vet. Res.*, **36**, 175-180.
- VERWOERD, D. W., 1970. Diplornaviruses: A newly recognised group of double-stranded RNA viruses. *Progr. med. Virol.*, **12**, 192-210.
- VERWOERD, D. W., LOUW, H. & OELLERMANN, R. A., 1970. Characterization of bluetongue virus ribonucleic acid. *J. Virol.*, **5**, 1-7.
- WATANABE, Y., KUDO, H. & GRAHAM, A. F., 1967. Selective inhibition of reovirus RNA synthesis by cycloheximide. *J. Virol.*, **1**, 36-44.