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# A COMPARISON OF AN AUSTRALIAN BLUETONGUE VIRUS ISOLATE (CSIRO 19) WITH OTHER BLUETONGUE VIRUS SEROTYPES BY CROSS-HYBRIDIZATION AND CROSS-IMMUNE PRECIPITATION

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ABSTRACT

HUISMANS, H. & BREMER, C. W., 1981. A comparison of an Australian bluetongue virus isolate (CSIRO 19) with other bluetongue virus serotypes by cross-hybridization and cross-immune precipitation. *Onderstepoort Journal of Veterinary Research*, 48, 59–67 (1981).

No major differences in size were observed when both the double-stranded RNA and the polypeptides of the Australian bluetongue virus (BTV) isolate CSIRO 19 (BTV-20) were compared with those of other BTV serotypes such as BTV-10 and BTV-4. Minor capsid polypeptide P6 of both BTV-20 and BTV-4, which electrophoreses as a single band on continuous phosphate buffered gels, is separated into 2 distinct bands on discontinuous glycine-buffered gels. This was not the case with BTV-10.

Cross-immune precipitation of BTV-20 with BTV-10, BTV-17, BTV-4 and BTV-3 indicated strong immunological cross-reaction of the group-specific antigen P7 of the different serotypes. There was also some cross-immune precipitation of the serotype-specific polypeptide P2 of BTV-20 and BTV-4. This result is in agreement with the observed cross neutralization of these 2 viruses.

The main distinction between BTV-20 and the other BTV serotypes was observed in cross-hybridization experiments. The homology between the nucleic acid of BTV-20 and other BTV serotypes was less than 30%, whereas homology normally found between BTV serotypes is at least 70%. The hybridization products of the different BTV serotypes were analysed by electrophoresis and fluorography. Two main hybrid segments were observed in all heterologous hybridizations with BTV-20 as compared with 7 hybrid segments in hybridizations between BTV-4 and BTV-10. In order to determine from which genome segment of BTV-20 these 2 hybrid segments were derived, the hybridizations were carried out with individually purified double-stranded RNA segments. These results indicate that the 2 segments of BTV-20 that show the largest homology to corresponding segments of a heterologous BTV serotype are No. 7 and 10.

*Résumé*

*COMPARAISON D'UN ISOLAT AUSTRALIEN (CSIRO 19) AVEC D'AUTRES SÉROTYPES DU VIRUS PAR HYBRIDATION CROISÉE ET PRÉCIPITATION IMMUNE CROISÉE DU VIRUS DE LA FIÈVRE CATARRHALE DU MOUTON*

*Aucune différence majeure dans la taille ne fut observée quand les ARN et polypeptides du virus de la fièvre catarrhale du mouton (BTV), isolat australien CSIRO 19 (BTV 20) furent comparés avec ceux d'autres sérotypes tels que BTV-10 et BTV-4. Le polypeptide capside mineur P6 de BTV-20 et de BTV-4 qui donne une seule raie en électrophorèse en milieu gélifié et continuellement tamponé de phosphate est séparé en deux raies distinctes en electrophorèse en milieu gelifiè aux tampons de glycine discontinus. Ceci ne fut pas le cas avec BTV-10.*

*Une précipitation immune croisée de BTV-20 avec BTV-10, BTV-17, BTV-4 et BTV-3 indiqua une forte réaction croisée immunologique du groupe de l'antigène spécifique du groupe P7 des différents sérotypes. Il y eut aussi une certaine précipitation immune croisée du polypeptide P2 spécifique de serotype du BTV-20 et BTV-4. Ce résultat est en concordance avec la neutralisation croisée de ces deux virus.*

*La distinction principale entre BTV-20 et les autres serotypes de BTV a été observée dans des expériences d'hybridation croisée. L'homologie entre l'acide nucléique du BTV-20 et les autres serotypes de BTV fut moindre que 30% tandis que l'homologie normalement trouvée entre les sérotypes de BTV est d'au moins 70%. Les produits d'hybridation des différents serotypes de BTV furent analysés par électrophorèse et fluorographie. Deux segments d'hybrides principaux furent observés dans toutes les hybridations hétérologues utilisant BTV-20 en comparaison avec 7 segments hybrides dans des hybridations entre BTV-4 et BTV-10. Afin de déterminer de quel genome segmental du BTV-20 ces deux segments hybrides étaient dérivés, les hybridations furent menées avec des segments d'ARN purifiés individuellement. Ces résultats indiquent que les deux segments de BTV-20 qui montrent la plus grande homologie aux segments correspondants d'un serotype BTV hétérologue sont les numéros 7 et 10.*

## INTRODUCTION

Orbiviruses belong to the family Reoviridae, a group of segmented, double-stranded RNA (dsRNA) -containing viruses. Their biochemical, biological and immunological characteristics have been the subject of a number of reviews (Gorman, 1979; Verwoerd, Huismans & Erasmus, 1979). In recent years a large number of orbiviruses were isolated in Australia. The interest in them stems mainly from the potential hazard which the prototype member of the group, bluetongue virus (BTV) constitutes for the sheep industry. Until recently most of these isolates proved to be serologically distinct from BTV and non-pathogenic. More recently, however, an orbivirus strain CSIRO-19 was isolated in Australia and identified as BTV (St. George, Standfast & Cybinski, 1978). Preliminary serological tests have indicated that, even though the virus shows some cross-neutralization with a few of the other BTV serotypes, it is sufficiently distinct from these to be classified as a new serotype, BTV-20. Some of the serological and biological characteristics of BTV-20 have been described in a separate communication (Erasmus, Pieterse & Boshoff, 1981).

The primary aim of this investigation was to establish how closely BTV-20 is related to other BTV serotypes and in particular to BTV-4. Of all the existing BTV serotypes, BTV-4 seems to be the most widespread. The first BTV strain isolated in South Africa by Theiler in 1905 (reviewed Howell, 1963) was serotype 4, and the same serotype has since been isolated, not only in South Africa but also in other countries such as Cyprus, Israel and Turkey. The almost complete nucleic acid homology that was found between dsRNA from the BTV-4 strain isolated in Cyprus in 1971 (4/Cyprus) and dsRNA of the strain isolated in South Africa by Theiler in 1900 (4/Theiler) would

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seem to suggest a close relationship between these different Type-4 strains (Huismans & Howell, 1973). The observation that BTV-20 also cross-reacts with BTV-4 in neutralization tests (Erasmus *et. al*, 1981) suggested the possibility that an equally close nucleic acid homology would be found between BTV-4 and the Australian BTV isolate. The relationship of BTV-20 to other BTV serotypes has important ecological implications for the spread of BTV and could indicate the route of introduction of the virus into Australia.

To investigate this relationship a comparative study was made of the biochemical composition of BTV-20 and some of the other BTV serotypes. Nucleic acid homology was studied by a cross-hybridization technique (Huismans & Howell, 1973), whereas immunological relatedness was studied by cross-immune precipitation (Huismans & Erasmus, 1981).

## MATERIALS AND METHODS

### *Virus and cells*

The serotype BTV-4 was first isolated in 1978 and originated in Turkey. The BTV-10 and BTV-6 strains originated and were isolated in South Africa and the BTV-17 strain in the USA. The CSIRO-19 strain, BTV-20, was isolated in Australia in 1977 and sent for serotyping to the International Reference Centre for BTV at Onderstepoort.

BHK cells were obtained from the American Type Culture Collection, USA and grown in BHK-Eagle's medium supplemented with 5% bovine serum. The different BTV serotypes were all propagated in confluent cultures of BHK cells with serum-free Eagle's medium, using a low passage stock virus as an inoculum (Huismans, 1979) and harvested 48 h after infection. The purification method has been described (Huismans, 1979).

### *Electrophoresis*

*Proteins:* Freeze-dried samples, resuspended in 20 $\mu\ell$ of a solution containing 0,8 M NaCl, 8 M urea, 1,5% sodium dodecyl sulphate (SDS) and 4% mercapto-ethanol, were eletrophoresed for 16 h at 55 V/gel on continuous 7,5% polyacrylamide slab gels in the presence of urea, using a phosphate buffer (Stone, Smith & Joklik, 1974).

Where indicated under Results, an alternative method was used for the electrophoresis of protein samples, using discontinuous gels as described by Laemmli (1970). Electrophoresis was carried out at 4 °C for 16 h at 180 V/gel with a separating gel of 12,5%.

Gels were stained in Coomassie Brilliant Blue G-250 (Anderson, Cawston & Cheeseman, 1974) and destained in 4% acetic acid. They were dried on filter paper under vacuum on a heated gel-drying apparatus.

*RNA*: Double-stranded RNA was electrophoresed on 15 cm long 4% acrylamide slab gels, cross-linked with 0,1% bisacrylamide, using a Loening buffer system (Loening, 1967). Sodium dodecyl sulphate (SDS) was added to a final concentration of 0,1% unless otherwise indicated. Electrophoresis was for 16 h at 80 V at room temperature. Gels were stained in methylene blue and destained in water.

Preparative RNA electrophoresis was carried out as described for the isolation of individual dsRNA segments.

### *Fluorography and autoradiography*

Gels with $^{3}$H-containing labelled components were prepared for fluorography by impregnation with 22,5% w/v PPO in dimethyl sulphoxide as described by Bonner & Laskey (1974). The dried gels were exposed to Kodak DF 96 X-ray film without pre-exposure.

Dried gels with $^{14}$C-containing labelled components were directly exposed to Kodak DF 96 X-ray film.

### *Immune precipitation*

$^{14}$C-labelled, soluble protein extracts of BTV-infected cells were obtained, as described by Huismans (1979). Immune precipitations were carried out by the addition of 0,2 m$\ell$ of the soluble S 100 extracts to 50 $\mu\ell$ of the indicated guinea-pig antiserum. Immune precipitates were analysed by electrophoresis and autoradiography as described (Huismans, 1979).

### *Preparation of BTV messenger RNA (mRNA)*

Four BHK monolayer cultures in Roux flasks were infected with BTV at an input multiplicity of between 20 and 40 plaque-forming units/cell. After 9,5 h at 37 °C Actinomycin D was added to the medium to a final concentration of 0,1 $\mu$g/m$\ell$. After 30 min the medium in each Roux flask was replaced by 15 m$\ell$ of Eagle's medium containing 0,1 $\mu$g/m$\ell$ Actinomycin D and 15 $\mu$Ci/m$\ell$ $^{3}$H-uridine*. The cells were incubated for 2 h at 36 °C on a shaking platform. After harvesting, the RNA was extracted as previously described (Huismans & Verwoerd, 1973). The LiCl precipitation step was repeated 3 times to ensure the quantitative removal of dsRNA. The single-stranded (ss) mRNA was stored in 0,01 M STE-PVS buffer (0,01 M NaCl, 0,01 M Tris-HCl, pH 7,4, 0,001 M EDTA, 0,05% polyvinyl sulphate) in 0,1 m$\ell$ aliquots which contained between 150 000 and 200 000 $^{3}$H cpm each.

### *Isolation of dsRNA*

Unlabelled dsRNA was isolated from infected cells by phenol extraction, LiCl precipitation and purification on a column of methylated albumin on Kieselguhr, as described (Verwoerd, Louw & Oellermann, 1970; Huismans & Verwoerd, 1973).

Labelled dsRNA was obtained from infected BHK monolayers in roller bottles in the same way except that 4 h after infection the incubation medium was replaced with 10 m$\ell$ of Eagle's medium containing 8 $\mu$Ci/m$\ell$ $^{3}$H-uridine. After 48 h incubation the cells were harvested and the dsRNA extracted, as described above.

### *Isolation of individual dsRNA segments*

At least 10 aliquots of 100 $\mu$g of BTV dsRNA were each electrophoresed on 20 cm long, 3% acrylamide gels cross-linked with ethylene diacrylate. Electrophoresis was carried out with TNE buffer (0,02 M NaAc, 0,04 M Tris, pH 7,8, 2 mM EDTA) for 17 h at 6 mA/gel. The gels were stained in 0,01% ethidium bromide, and the 10 different dsRNA segments cut out from the gel. RNA was recovered from the gel pieces by electrophoresis. Traces of acrylamide were removed by phenol extraction or by separation on a column of methylated albumin on Kieselguhr (Verwoerd *et al.*, 1970). Segments that were found to be cross-contami-

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nated with other segments were purified again, as described above. RNA was finally dissolved in 0,01 M STE-PVS at a concentration of 160 μg/mℓ.

*Hybridization*

Experiments in which the percentage hybridization between different BTV serotypes was calculated were carried out as follows: Constant amounts of $^3$H-labelled BTV mRNA (3 000–5 000 cpm) were mixed with increasing amounts of unlabelled dsRNA of a homologous or heterologous serotype. The volume was adjusted to 0,2 mℓ with 0,01 M STE-PVS and the mixture heated for 5 min at 100 °C before addition of 20 μℓ of 4 M NaCl. After incubation at 72 °C for 30 min the samples were treated for 15 min with pancreatic RNase at a final concentration of 2 μg/mℓ and precipitated with 10% TCA in the presence of carrier (0,2 mg yeast RNA). Precipitates were collected on nitrocellulose filters, washed extensively with 4% TCA, dried and counted in Toluene scintillation fluid in a Packard Tricarb scintillation counter.

*Analysis of hybrids*

$^3$H-labelled mRNA (about 150 000 cpm) was added to 100 μg of unlabelled dsRNA, diluted to 0,5 mℓ with 0,01 M STE-PVS and heated for 5 min in a stoppered glass tube in a water-bath to 100 °C. After an addition of 40 μℓ 4 M NaCl the mixture was incubated for 60 min at 72 °C. Unhybridized, single-stranded RNA was removed by overnight precipitation in the presence of 2 M LiCl and 200 μg ribosomal carrier RNA. The supernatant of the LiCl precipitation (1 mℓ) was diluted with 6,8 mℓ 0,01 M STE and 2,2 mℓ 96 % ethanol and loaded onto a CF-11 cellulose column equilibrated with 22% ethanol in TSE buffer (0,1 M NaCl, 1 mM EDTA, 0,05 M Tris, pH 6,9) (Franklin, 1966). The unbound material (approximately 10–15% of the total counts in the sample) contained no dsRNA that could be identified by electrophoresis. After a rinse with 75 mℓ of the 22% ethanol containing buffer, the remaining counts were eluted from the column in a single step with TSE buffer containing no ethanol. RNA was precipitated with 2 volumes of ethanol. Precipitates were dissolved in distilled water, lyophilized and stored at —20 °C until analyzed by electrophoresis and fluorography.

Hybridizations with individual dsRNA segments were carried out as described above except that the 100 μg unfractionated dsRNA was replaced with 40–50 μg of a specific individual dsRNA segment. This amount of dsRNA represented a large excess over the amount of the corresponding mRNA available for hydridization.

## RESULTS

*Protein and nucleic acid composition of BTV-20*

$^3$H-labelled dsRNA was isolated from cells infected with BTV-20 and electrophoresed on polyacrylamide slab gels. Labelled dsRNA from serotypes 10 and 4 were used as controls. A fluorogram of the result is shown in Fig. 1.

The fractionation pattern obtained with BTV-20 has the typical size distribution of BTV dsRNA. However, the electrophoretic mobility of the majority of the 10 segments of BTV-20 differs from that of corresponding BTV-4 and BTV-10 segments, indicating that BTV-20 is not identical with either of these 2 strains.

The capsid polypeptides of purified BTV-20 were fractionated on 7,5% continuous polyacrylamide slab gels as described under Materials and Methods. Polypeptides from purified BTV-10 and BTV-4 were co-electrophoresed as controls. The result is shown in Fig. 2A.

The 3 polypeptide patterns are very similar. BTV-20 contains 4 major and 3 minor polypeptide components. There are only minor size differences between corresponding BTV-20, BTV-4 and BTV-10 polypeptide components.

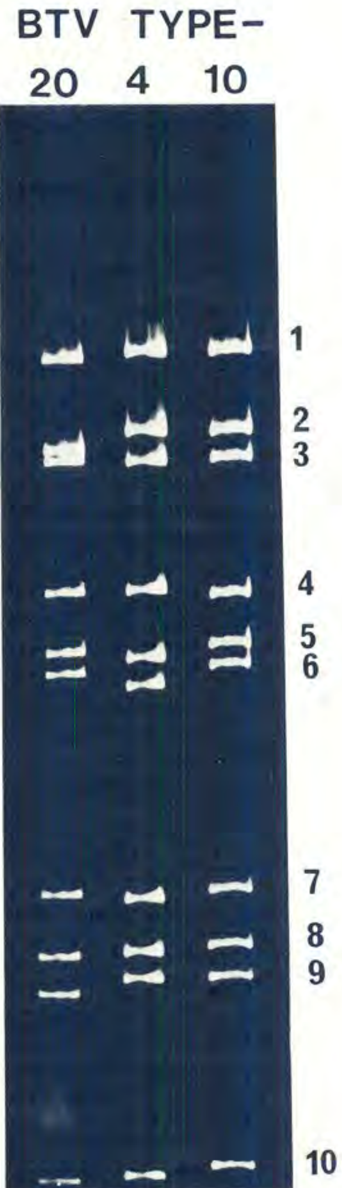


FIG. 1 A fluorogram of the gelelectrophoretic fractionation of $^3$H-labelled double-stranded RNA from BTV-20, BTV-4 and BTV-10

A slightly different result was obtained when the same samples were electrophoresed on a discontinuous stacking gel containing a glycine buffer (Fig. 2B). Under these conditions polypeptide P6 of both BTV-4 and BTV-20 split into 2 distinct bands, giving these viruses a total of 4 major and 4 minor polypeptide components. BTV-10 is different in that P6 always electrophoreses as a single band. Polypeptides P2 and P3 of BTV-20 are not separated on these 12,5% polyacrylamide gels.

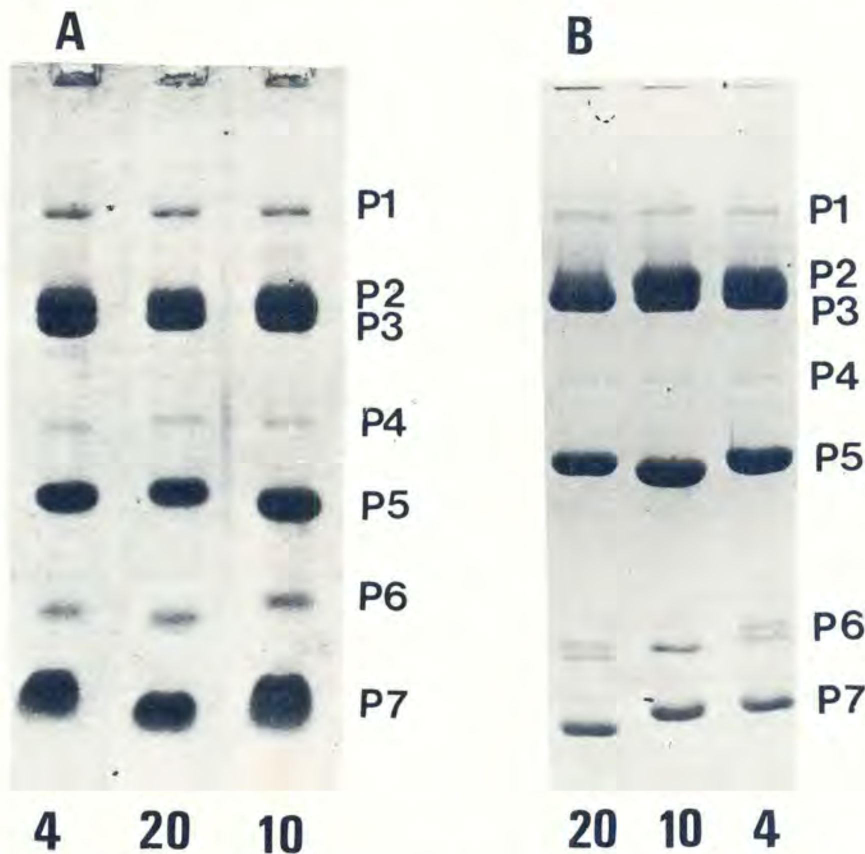


FIG. 2A The gelelectrophoretic fractionation of the capsid polypeptides of BTV-4, BTV-20 and BTV-10 on continuous polyacrylamide slab gels with a $PO_4^{=}$ buffer system and in the presence of urea.

FIG. 2B Gelelectrophoretic fractionation of the same capsid polypeptides as in 2A but on a discontinuous stacking gel with a glycine buffer system.

*Cross immune precipitation of BTV-*20 *and other serotype strains*

The immunological relatedness of the BTV-20 polypeptides to those of other serotype strains was investigated by cross immune precipitation (Huismans & Erasmus, 1981). The precipitations were carried out as described under Materials and Methods, using guinea-pig immune sera agains BTV serotypes, 20, 17, 10 and 4 with respectively $^{14}C$-labelled virus polypeptides from BTV serotypes 20, 17, 10, 6 and 4. These serotypes were selected for the following reasons: Serotypes 20, 17 and 4, because they cross-react in neutralization tests (Erasmus *et al.*, 1981); serotype 10 because evidence obtained with cross-immune precipitation indicated a relatedness to BTV-4 (Huismans & Erasmus, 1981) and serotype 6 because it was considered to be a typical non-cross-reacting strain which, with regard to the precipitation of P2, reacts very much like any one of the other BTV serotypes in cross-immune precipitation experiments. An analysis of the immune precipitates was carried out by electrophoresis and autoradiography. The result is shown in Fig. 3.

Most of the homologous immune precipitates (Fig. 3A lane a and Fig. 3B lane c) show precipitation of polypeptides P2, P3, P6A and P7. Polypeptide P6A is a non-capsid polypeptide (Huismans, 1979), whereas P3 and P7 are both major polypeptide components of the BTV core particle. The immune precipitation of P2, a polypeptide of the outer capsid layer (Verwoerd, Els, De Villiers & Huismans, 1972), is seroptye specific (Huismans & Erasmus, 1981). This is also illustrated in Fig. 3 by the fact that P2 from BTV-6 is not precipitated by heterologous serum (lane d in Fig. 3A, 3C & 3D). In the case of the other 4 strains, there is much more cross-immune precipitation of P2. This is seen in the precipitation of P2 from BTV-10 by both serum against BTV-20 (Fig. 3A, lane c) and BTV-4 (Fig. 3C, lane c). These differences in P2 immune precipitation are schematically summarized in Fig. 4.

In this diagram an arrow between 2 serotypes indicates immune precipitation of polypeptide P2 in a reaction between the soluble polypeptides from one serotype (indicated by the head of the arrow) and immune serum from another (tail of the arrow). It must be pointed out that in this representation no attempt was made to quantify the degree of cross-immune precipitation of P2 in the different reactions, even though this varied to a considerable degree. Cross-reaction was considered positive unless P2 was present in no more than trace amounts relative to that of the homologous control.

The most striking result from Fig. 4 is the close immunological relatedness of BTV-20 and BTV-4. They are the only 2 strains that cross-react in both ways in the immune precipitation reactions. Furthermore, serum from BTV-20 and BTV-4 both precipitate P2 from BTV-10, whereas P2 from BTV-4 and BTV-20 are both precipitated by BTV-17 immune serum.

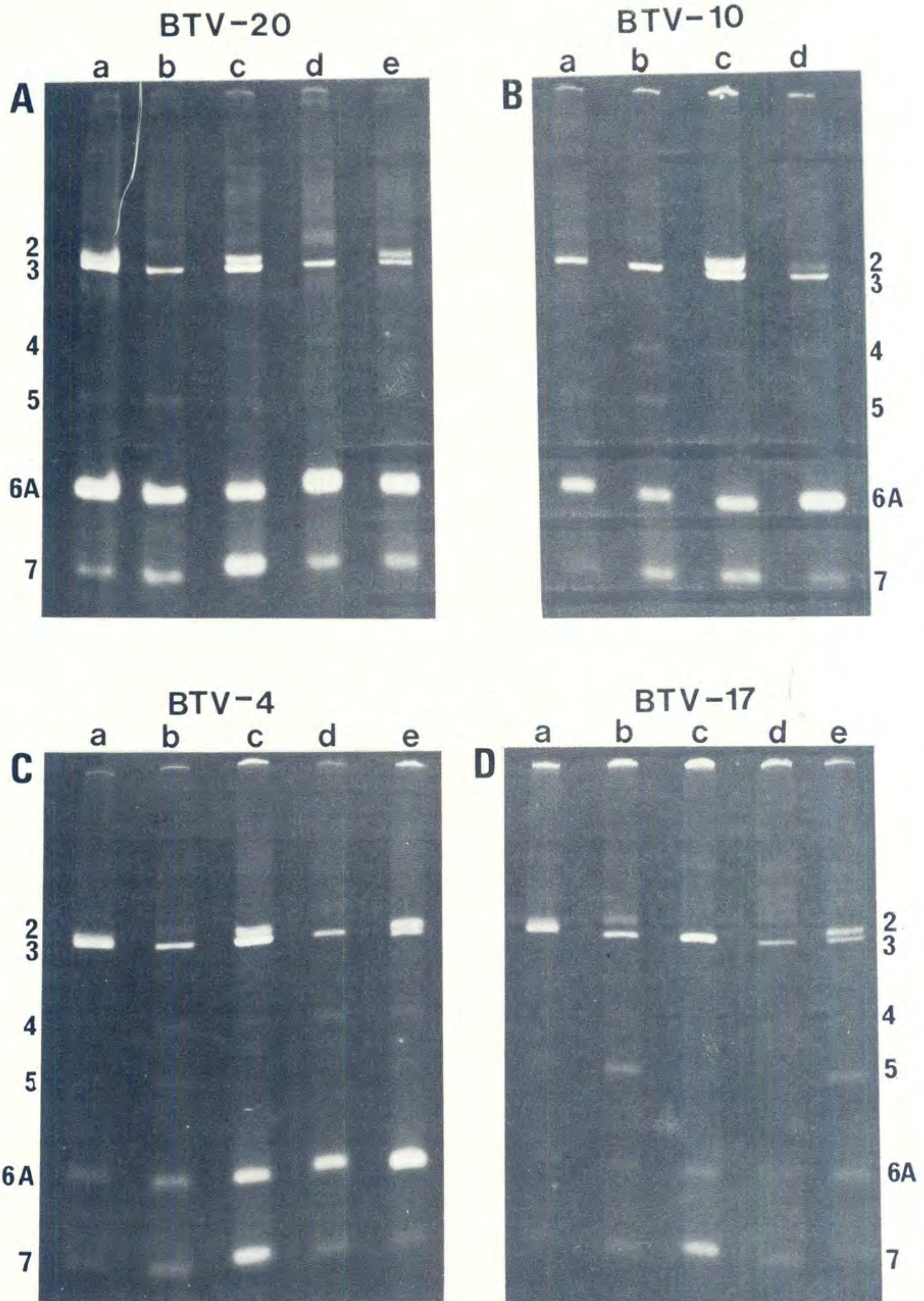


FIG. 3 Autoradiograms of the gelelectrophoretic fractionation of immune precipitates obtained in the reaction of $^{14}C$-labelled polypeptides from cells infected with (a) BTV-20, (b) BTV-17, (c) BTV-10, (d) BTV-6 and (e) BTV-4 with guinea-pig sera prepared against BTV-20 (Fig. 3A), BTV-10 (Fig. 3B), BTV-4 (Fig. 3C) and BTV-17 (Fig. 3D)

*Cross hybridization of BTV-*20 *and other serotype strains*

Hybridization between 2 heterologous serotypes was measured as the % of mRNA converted to an RNase resistant form relative to that of the homologous control. Fig. 5 shows the % hybridization of BTV-4 mRNA with increasing amounts of BTV-20, BTV-10 and BTV-4 dsRNA. In all 3 cases the maximum level of hybridization was attained after addition of approximately 10 μg of dsRNA.

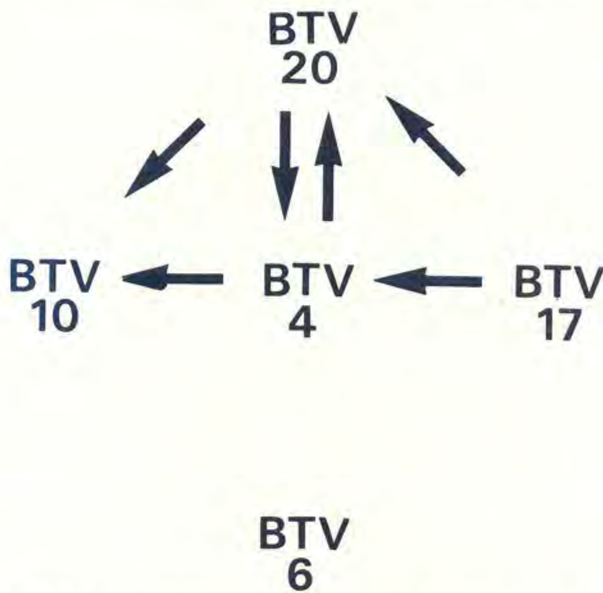


FIG. 4 A schematic representation of the cross-immune precipitation of polypeptide P2 between different serotypes of BTV

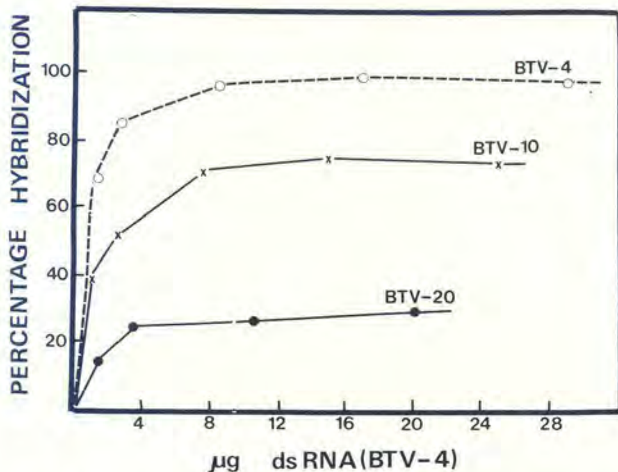


FIG. 5 Hybridization of $^{3}$H-labelled mRNA from BTV-4 (- - - o - - -), BTV-10 (——x——) and BTV-20 (——•——) with increasing amounts of de-natured unlabelled dsRNA from BTV-4

Similar experiments were carried out with mRNA from BTV-10 and BTV-20.

The maximum levels of hybridization that were obtained are summarized in Table 1. Results shown are the average of at least 3 experiments.

TABLE 1 Percentage cross-hybridization between $^{3}$H-labelled mRNA of 3 different BTV serotypes and de-natured dsRNA of the homologous or heterologous serotype strains. Heterologous hybridizations are expressed as a percentage of that of the homologous control

| mRNA from: | De-natured dsRNA from: | | |
|---|---|---|---|
| | BTV-4 | BTV-10 | BTV-20 |
| BTV-4 | 100 | 77 | 22 |
| BTV-10 | 71 | 100 | 22 |
| BTV-20 | 30 | 19 | 100 |

Serotypes 10 and 4 show approximately 71–77% homology, which agrees with the previously described values for the homology between different BTV serotypes (Verwoerd & Huismans, 1969; Huismans & Howell, 1973). The homology between BTV-20 and BTV-4 is in the order of 20–30% and that between BTV-20 and BTV-10 about 19–22%. In a few additional experiments with BTV-17 (results not shown) it was found that its homology to BTV-20 was approximately 25% as against a 70% homology between BTV-17 and the other serotypes.

*Analysis of the hybridization product*

The same cross-hybridizations as indicated in Table 1 were carried out, using the larger amounts of material and the reaction conditions described under Materials and Methods. Hybrids were analysed by electrophoresis and fluorography. The results are shown in Fig. 6.

The homologous hybridization products, shown in lanes a, d and g, confirm that all 3 mRNA preparations contained 10 distinct mRNA species that could each be hybridized with the complementary strand of a denatured homologous dsRNA segment. The heterologous hybridization products between BTV-10 and BTV-4 (lanes b and c) confirm the extensive homology between these serotypes, Only 3 of the 10 genome segments (2, 6 and 10) do not hybridize, whereas the others hybridize with about the same efficiency as in the homologous controls. The only exception is that the heterologous hybrid segments are usually slightly reduced in electrophoretic mobility as compared to that of the corresponding homologous hybrid segments. For example, segment 7 in lane b (a heterologous hybrid of BTV-4 mRNA and BTV-10 dsRNA) electrophoresed slower than segment 7 in the homologous control (lane a). It is diffcult to judge the extent of these reductions from Fig. 6 and they are best demonstrated by using a double-labelling technique (Huismans & Howell, 1973).

In the heterologous hybrids with BTV-20 (lanes e, f, h and i) only 2 labelled segments called x and y were observed. Their electrophoretic mobility depended on the particular cross-hybridization carried out. For example, the x and y segments obtained in the hybridization of BTV-10 mRNA with BTV-20 dsRNA (lane e) had a slightly different electrophoretic mobility from those obtained when BTV-20 mRNA was hybridized with BTV-10 dsRNA (lane f).

The hybridization experiment described above was repeated at least 5 times. The x and y segments were always observed, but in a few experiments an additional faint band was observed in the region of segment 6 and on prolonged exposure trace amounts of even slower moving segments were sometimes observed. The relative amounts of these segments when compared to homologous hybridizations (lanes a, d and g) or to heterologous hybridizations not involving BTV-20 (lanes b and c) were so small, however, that it is difficult to judge their significance. It is quite clear however, that only a few segments of BTV-20 are capable of hybridizing with corresponding segments of a heterologous serotype. In order to identify these segments the hybridizations were carried out with individually purified dsRNA segments.

*Hybridizations with single dsRNA segments*

The purification of the 10 dsRNA segments of BTV-4 is described under Materials and Methods. Electrophoretic analysis of the purified segments indicated no detectable cross-contamination and no

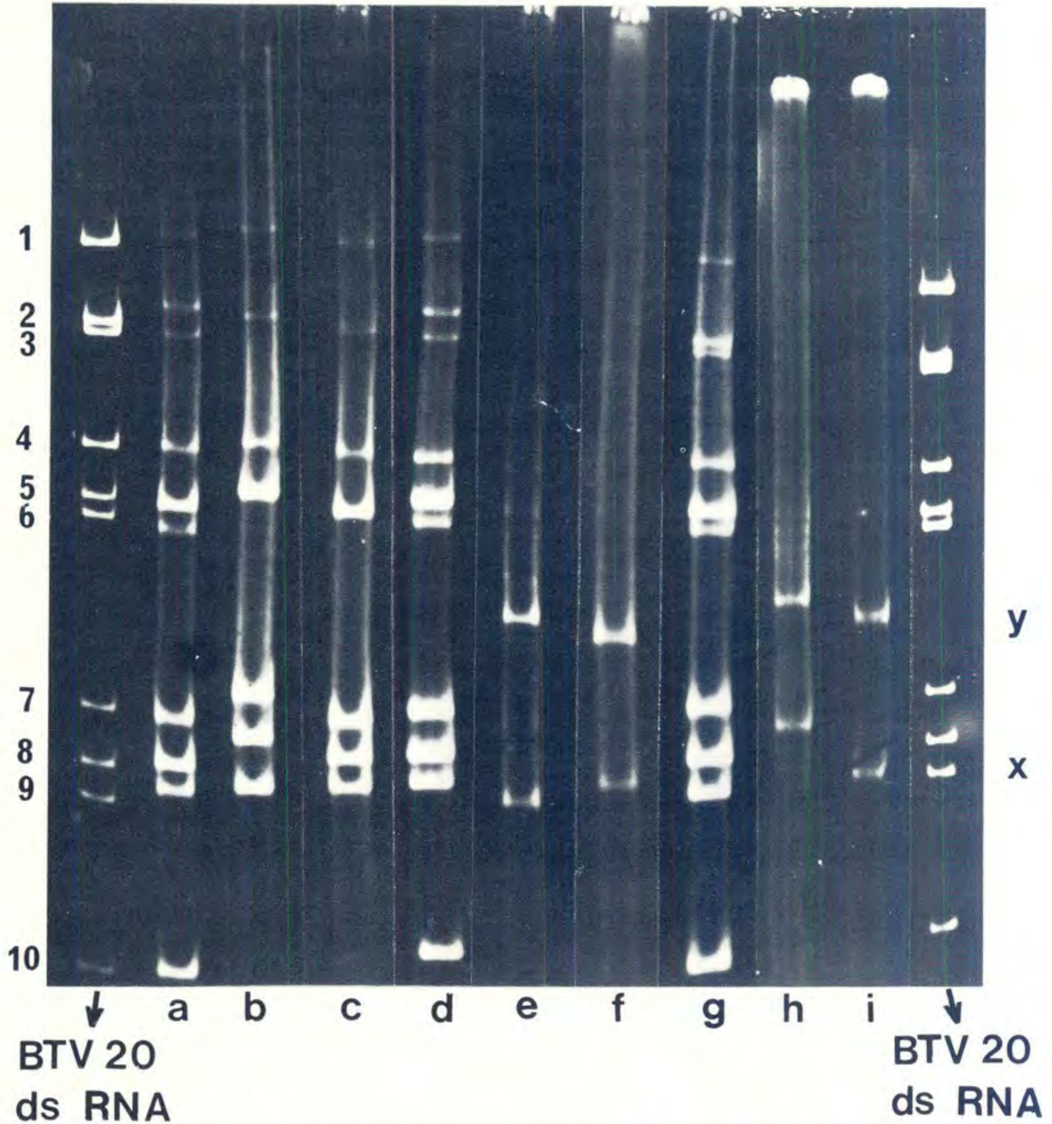


FIG. 6 Fluorogram of the gelelectrophoretic fractionation of the hybrids obtained in the hybridization of $^{3}$H-labelled mRNA from BTV-4 with dsRNA from (a) BTV-4, (b) BTV-10 and (i) BTV-20. Hybrids from $^{3}$H-labelled mRNA from BTV-10 with dsRNA from (c) BTV-4, (d) BTV-10 and (e) BTV-20. Hybrids from $^{3}$H-labelled mRNA from BTV-20 with dsRNA from (f) BTV-10, (g) BTV-20 and (h) BTV-4. The $^{3}$H-labelled BTV-20 control on the left was run on the same gel slab as samples a, b, c, d, e, f and g. The BTV-20 control on the right was run together with samples h and i on a separate gel

breakdown (results not shown). The electrophoretic mobility of the individual segments was also unchanged, provided SDS was present in the electrophoresis buffer. In the absence of SDS small anomalies in the electrophoresis of fractionated and unfractionated dsRNA were seen. This discrepancy remains unexplained, therefore all subsequent electrophoretic separations of single dsRNA segments or their corresponding hybrids were carried out in the presence of SDS.

The integrity of the individual BTV-4 dsRNA segments was further confirmed by hybridizing each segment with type 4 mRNA as described under Materials and Methods. The resulting hybrid segments were indistinguishable (result not shown) from those, obtained in the homologous hybridization with unfractionated RNA (Fig. 6, lane a).

Hybrids of the individual BTV-4 dsRNA segments with BTV-20 mRNA are shown in Fig. 7.

Hybrid segments x and y, previously found in cross-hybridization of unfractionated BTV-4 dsRNA and BTV-20 mRNA (Fig. 6, lane h), are also observed in Fig. 7. Hybrid segment x is derived from cross-hybridization of dsRNA segment 10 and y from segment 7. Hybrid segment z, of which only trace amounts were occasionally detected in the results described in Fig. 6, is derived from segment 8. The most striking aspect of these results is the very large reductions in the electrophoretic mobility of the hybrid segments relative to that of the normal dsRNA segments from which they are derived.

Trace amounts of hybrids derived from segments 6, 5, 4 and 3 were also seen. Again these hybrids electrophoresed much more slowly than the corresponding

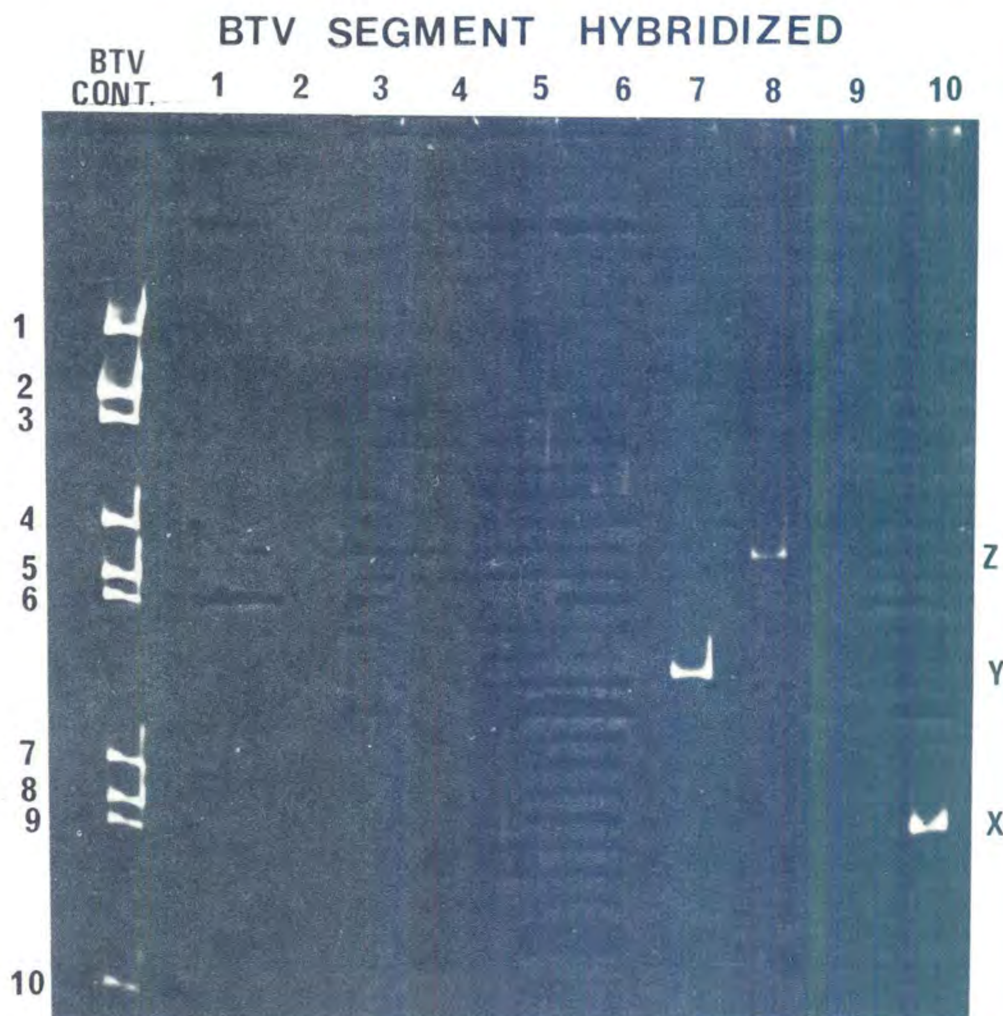


FIG. 7 A fluorogram of the gelelectrophoretic fractionation of the hybrids obtained in the hybridization of $^3$H-labelled BTV-20 mRNA with individual double-stranded RNA segments (1–10) of BTV-4

normal dsRNA segments. It must also be pointed out that these trace amounts represent only a very small fraction of the total amount of mRNA that is converted to dsRNA in case of homologous hybridization. At best the cross-hybridization of segments such as 6, 5, 4 and 3 is extremely inefficient and probably only occurs in the presence of a very large excess of dsRNA such as was the case in the experiment shown in Fig. 7.

## Discussion

The nucleic acid of BTV-20 consists of 10 dsRNA segments with a typical BTV-like size distribution. The only distinctive characteristic of the dsRNA segments is that the electrophoretic mobilities of most of the 10 segments differ from those of corresponding BTV-4 or BTV-10 segments. In the majority of other BTV serotypes the differences in electrophoretic mobilities of corresponding genome segments are usually confined to 2 or 3 segments only (Huismans, unpublished observation).

The size distribution of the 7 capsid polypeptides of BTV-20 are also typical of that of BTV. An interesting observation, however, is that on discontinuous gels polypeptide P6 of BTV-20 is separated into 2 distinct bands. The same result was found with BTV-4 polypeptides and it may be a common characteristic of many more BTV serotypes. The 2 P6 polypeptides could represent different phosphorylated or glycosylated versions of the same protein. Such modifications are known to affect electrophoretic mobility (Dietzschold, Cox & Schneider, 1979). No phosphorylated or glycosylated capsid polypeptides of BTV have been identified as yet, but non-capsid polypeptide P6A of BTV-10 is known to be phosphorylated (Huismans, unpublished result).

One of the aims of this investigation was to determine how closely BTV-20 is related to existing BTV serotypes such as BTV-4 and BTV-10. Cross-immune precipitation experiments demonstrated common antigenic determinants on the P2 polypeptides of BTV-4 and BTV-20. P2 has been shown by Huismans & Erasmus (1981) to determine serotype specificity. BTV-20 and BTV-4 also show a certain amount of cross-neutralization (Erasmus *et al.*, 1981). Cross-immune precipitation of P2 alone, however, is not enough to indicate cross-neutralization. This is demonstrated by the fact that P2 from BTV-10 was immune precipitated by immune serum from BTV-4 or BTV-20, even though there is no evidence of cross-neutralization between those strains. It is possible that cross-neutralization between 2 sero-types is only found between strains that have several antigenic determinants in common, whereas cross-immune precipitation of P2 might only require one such determinant.

In view of the close immunological relatedness of BTV-20 and BTV-4, the hybridization results were surprising. The observed homology of 20–30% between BTV-20 and BTV-4 was found to be much less than the 70% homology between BTV-4 and BTV-10. It was also found that heterologous hybridization products of BTV-20 contained no more than 2 or 3 stable hybrid segments as opposed to the 7 out of a possible 10 found in hybrids of BTV-10 and BTV-4.

Both segments 7 and 10 of BTV-4 dsRNA could be hybridized to BTV-20 mRNA. It is tempting to speculate that segment 7 codes for the group specific antigen, P7 (Huismans & Erasmus, 1981). One would expect a considerable degree of homology amongst genome segments of different BTV serotypes coding for such an antigen. According to Verwoerd *et al.*, (1972), P7 could be coded for by any one of segments 7, 8 or 9. Of these, segment 9 shows the least homology and genome segment 8 very little. The significance of the homology between segments 10 of BTV-4 and BTV-20 is unclear. Genome segment 10 is too small to code for any one of the known capsid or non-capsid polypeptides (Verwoerd *et al.*, 1972; Huismans, 1979).

One striking characteristic of the hybrid segments formed during the hybridization of BTV-20 with BTV-4 is the very large reduction in electrophoretic mobility of these segments when compared to that of the normal dsRNA segments from which they are derived. Such reductions in electrophoretic mobility were also seen in heterologous hybrids not involving BTV-20, but these reductions are relatively small. In the case of BTV-20 it probably indicates fairly large regions of mismatched base pairs. This could give rise to one or more ssRNA loops in the dsRNA hybrid segment and cause the reduction in electrophoretic mobility. It is possible that large ssRNA loops might even prevent the hybrids from entering the gel, leading to a false interpretation of no homology. The existence of ssRNA regions in the dsRNA hybrids was also indicated by the fact that RNase treatment of BTV-20 heterologous hybrids degraded the hybrids to much smaller fragments (Huismans, unpublished results). Hybrids of BTV-4 and BTV-10 were much less sensitive to RNase treatment.

In summary, the hybridization results indicate that BTV-4 and BTV-20 are neither identical nor even very closely related. The striking immunological relatedness is therefore no indication of a general homology between all genome segments but can be explained by a very specific homology between genome segments coding for the group specific antigen (probably segment 7) and common antigenic determinants on the serotype specific antigen P2.

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