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# MOLECULAR HYBRIDIZATION STUDIES ON THE RELATIONSHIPS BETWEEN DIFFERENT SEROTYPES OF BLUETONGUE VIRUS AND ON THE DIFFERENCE BETWEEN THE VIRULENT AND ATTENUATED STRAINS OF THE SAME SEROTYPE

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## ABSTRACT

HUISMANS, H. & HOWELL, P. G., 1973. Molecular hybridization studies on the relationships between different serotypes of blue-tongue virus and on the difference between the virulent and attenuated strains of the same serotype. *Onderstepoort J. vet. Res.* 40 (3), 93-104 (1973).

Isolates of $^{3}H$-labelled messenger RNA of a number of different bluetongue virus serotypes were hybridized with saturating amounts of denatured $^{32}P$-labelled double-stranded RNA of different serotypes. These cross-hybridization products were then analysed by polyacrylamide gel electrophoresis. The results indicate relatively large differences between the various serotypes. Only a few of the genome segments in the different serotypes were completely homologous. Each of the cross-hybridization patterns obtained using the genome of Serotype 10 and any one of the other serotypes was unique and characteristic for the strain under investigation. The patterns furthermore clearly indicated different degrees of homology between the genomes of the different serotypes.

The immunological specificity of the serotypes appears to be determined mainly by the second genome segment of the virus while genome segment six could be of secondary importance. These results were supported by a study of the cross-hybridization patterns between different isolates of Serotype 4.

Cross-hybridization experiments between virulent and attenuated strains of the same serotype also indicated small differences. In all the serotypes investigated the process of attenuation involved changes in genome segments two and six. This result would tend to implicate the same genome segments in the determination of both the immunological specificity and the virulence of the virus.


## INTRODUCTION

Strains of bluetongue virus (BTV) from outbreaks of disease amongst naturally infected cattle and sheep in South Africa and other parts of the world have been classified into 16 distinct immunological groups (Howell, 1969). Up to the present time, strains of virus shown to be homologous by serological tests with all but one of the prototypes have been identified in South Africa. The exception is Type 16 which was originally identified in Pakistan in 1959. The majority of these 16 prototype strains have been attenuated by an empirical process of serial passage in eggs and selected passage levels are used for the preparation of a polyvalent vaccine.

The original immunological classification of these strains was based upon the results of serum-virus neutralization tests conducted in tissue culture. However, the recent characterization of BTV as a double-stranded RNA (ds RNA) virus (Verwoerd, 1969) has provided the opportunity of studying the different serological types by a hybridization technique. This technique has been successfully applied to the study of the relationships of many other viruses (Young, Hoyer & Martin, 1968; Dictzschold, Kaaden & Ahl, 1972).

In so far as BTV is concerned it has been previously shown by Verwoerd & Huismans (1969) that the percentage of hybridization between two different BTV serotypes is about 70%. This work has now been extended to include other serotypes so that by analysis of the cross hybridization products, those genome segments of the virus which determine the immunological specificity could be located. This technique has also been applied to study any possible difference between the virulent and the attenuated or egg-adapted strains of the same serotype.

## MATERIALS AND METHODS

### *Virus*

The origin of the particular BTV serotypes used in this study has been described previously (Howell, 1969). Care was taken to use virulent virus that was as close as possible to the original naturally occurring strains. All the egg-attenuated strains were derived from the same original virulent virus with which they are compared in the hybridization experiments.

In addition to these strains three different isolates of Serotype 4 were included. These strains represented the earliest identified strain of the group isolated by Theiler in 1900 (4/Theiler), a more recent strain from a local outbreak in 1958 (4/1314) and a third which was recovered from a natural case of the disease in sheep on the island of Cyprus in 1971 (4/Cyprus).

The freeze-dried, plaque-purified strains were passaged twice in tissue culture for the production of seed virus, prior to the preparation of large quantities of virus in BHK 21 monolayers as described by Verwoerd (1969). The identity and immunological specificity of these aliquots were checked by a plaque inhibition test, conducted according to previously described techniques (Howell, 1969).

### *Cells*

The origin and cultivation of the BHK 21 clone 13 cells and a derivative of Earle's L strain of mouse fibroblasts have been described by Howell, Verwoerd & Oellermann (1967).

### *Isolation of labelled ds RNA*

The isolation of the labelled dsRNA was a modification of the method described by Huismans & Verwoerd (1973). Confluent BHK 21 monolayers grown

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in roller bottles were inoculated with 30 plaque-forming units of BTV per cell. Three hours after infection unadsorbed virus was removed and the cells incubated with phosphate-free Eagle's medium to which $^{32}P$ as orthophosphate was added to a final concentration of between 0,3 and 0,6 $\mu$Ci/ml. The cells were harvested 24 hours after infection and the RNA extracted by the method of Scherrer & Darnell (1962) in the presence of 0,5% sodium dodecyl sulphate (SDS) in 0,01 M sodium acetate pH 5,0.

RNA extraction from the cells was carried out in the presence of the nuclei. It was found that provided the pH was maintained at 5,0, very little DNA was extracted into the waterphase. The total yield of RNA was, however, greatly increased compared to the previous method in which only the cytoplasmic fraction was extracted. Further purification of the dsRNA was undertaken as described by Verwoerd, Louw & Oellermann, (1970). After the final stage of purification on a column of methylated albumin kieselguhr (MAK), the dsRNA was precipitated with ethanol and the precipitate dissolved in and then dialyzed against 0,01 M STE-PVS buffer (0,01 M NaCl, 0,05 M Tris-HCl, 0,001 M EDTA, 0,05% polivinyl sulphate, pH 6,9). The RNA was stored at −20°C at a concentration of 10 $A_{260}$ units/ml.

*Isolation of labelled single-stranded RNA (ssRNA)*

The method used has been described by Verwoerd & Huismans (1972). Suspension cultures of L cells infected with BTV were labelled with 3 $\mu$Ci/ml $^{3}H$-uridine between 10 and 12 hours after infection. RNA extraction was carried out with the modifications as described above. The RNA was finally dissolved in 0,01 M STE-PVS buffer and stored at −20°C in small 0,1 ml aliquots suitable for a single hybridization experiment.

*Molecular hybridization*

The hybridization procedure has been described in detail by Verwoerd & Huismans, (1972). A saturating amount of $^{32}P$-labelled dsRNA was added to an appropriate amount of $^{3}H$-labelled ssRNA. The mixture was incubated for 5 min at 96°C and then incubated for 30 min at 72°C after the addition of NaCl to a final concentration of 0,3 M NaCl. The product of hybridization was purified by a salt precipitation at 1,25 M LiCl followed by MAK column chromatography. Cold carrier ribosomal RNA was usually added before the salt precipitation in order to enhance the quantitative removal of ssRNA by precipitation.

*Electrophoresis*

Polyacrylamide gel electrophoresis was carried out as described by Huismans & Verwoerd, (1973). The dsRNA hybrids were electrophoresed in 3% polyacrylamide gels in a 20 cm column for 18 hours at 5 V/cm. The gels were stained with a methylene blue solution and after destaining, cut into 0,08 mm slices each of which was dissolved in 10% piperidine and counted.

*Assay of radioactivity*

All determinations of radioactivity were made by scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer with Bray's scintillator solution (Bray, 1960). For dual counting of $^{3}H$ in the presence of $^{32}P$, a correction of 2% was made for any leak-over of $^{32}P$ into the $^{3}H$ channel.

## Results

*Identification of the selected strains of BTV*

The results of cross-neutralization tests between the selected strains of virus and the reference type antisera confirmed their identity. With the exception of types 3 and 16 there was a complete absence of any cross-neutralization. In previously reported tests the difference between these two type strains was considered sufficient to regard them as separate serotypes (Howell, 1969).

The Type 4 strains were identified on the basis of the results of routine screen neutralization tests and no attempt at intratypic differentiation was made.

*Cross hybridization*

The BTV genome consists of 10 dsRNA segments which are transcribed during replication into 10 ssRNA species. These ssRNA species or so-called plus strands hybridize exclusively with the complementary (or minus) strands derived from denatured BTV dsRNA (Huismans & Verwoerd, 1973). In the normal homologous hybridization experiments the $^{3}H$-labelled ssRNA is hybridized with an excess of $^{32}P$-labelled dsRNA from the same serotype. The ten BTV-specific mRNA species are then converted into ten dsRNA segments indistinguishable in electrophoretic mobility from the normal dsRNA genome segments. Cross-hybridization is carried out in a similar manner, the only difference being that the mRNA is hybridized with dsRNA from a different serotype.

In the first series of experiments isolates of $^{3}H$-labelled ssRNA from eight different BTV serotypes were each hybridized with denatured $^{32}P$-labelled dsRNA of Type 10 virus. This strain was arbitrarily chosen because all the previous work on the structure and composition of BTV had been carried out with this virus. The different cross hybridization products were then analysed by gel electrophoretic fractionation. The distribution of $^{32}P$ and $^{3}H$ activity in eight such electrophoretograms is shown in Fig. 1. The following notation applies to the hybrids in both the figures and text. The (+) sign always indicates the strain from which the $^{3}H$-labelled mRNA is derived and the (−) sign that from which the $^{32}P$-labelled complementary strands were derived.

The positions of the ssRNA-derived hybrid segments in the different hybridization patterns are indicated by the peaks of $^{3}H$-activity (dotted lines). The peaks of $^{32}P$-activity (full lines), on the other hand, indicate the position of the normal type 10 dsRNA segments. This is due to the fact that a large excess of denatured $^{32}P$-labelled dsRNA is used for hybridization. Only a small proportion hybridizes with the ssRNA and will therefore co-electrophorese with that of the $^{3}H$-activity. The bulk of the denatured dsRNA strand will recombine to form the normal Type 10 ds RNA segments.

Analysis of the hybridization patterns in Fig. 1 indicates the occurrence of three basic phenomena. Firstly, it can be seen that in a few cases the hybrid segment has exactly the same electrophoretic mobility as the corresponding Type 10 dsRNA segment. This is normally found in homologous hybridization and it indicates that this specific genome segment is probably identical to or at least very similar in the two strains under investigation. The most obvious example is Segment 5 in Fig. 1A, D and E. Secondly, it is frequently observed that the hybrid segment has a

different electrophoretic mobility when compared with the corresponding Type 10 genome segment. These shifts can also be observed visually by staining the RNA bands with methylene blue after gel-electrophoretic fractionation of the hybridization product. This feature is illustrated in Fig. 1 by a shift in the position of the $^{3}H$-activity peak relative to that of the $^{32}P$ peak. In general the migration rate of the hybrid segment seems to be reduced relative to the normal dsRNA segment although the opposite has also been observed.

This reduction in mobility can either be very small as in the case of Segments 1 and 5 in Fig. 1C, or it can be much larger as shown by Segment 5 in Fig. 1B. In most cases it is obvious from which genome segment the hybrid segment is derived. With genome Segments 2 and 3 the situation is more complex. It is obvious that only one of these two genome segments show cross-hybridization, but it is impossible to decide from the evidence in Fig. 1 which one. The changes in electrophoretic mobility are probably due to small regions of mismatched base pairs in the hybrid segments. This would affect the conformation of the molecule and as such also its mobility on polyacrylamide gels. Similar findings have been reported by other workers (Ito & Joklik, 1972).

Finally, it was observed in all the serotype cross-hybridizations carried out that some of the genome segments do not hybridize at all. This is best illustrated by Segment 6 of Type 10 which was never found to hybridize with ssRNA from a heterologous serotype. This failure to hybridize is not due to an inherent defect in the mRNA species involved, because full hybridization of all ten mRNA species was obtained with homologous dsRNA. The failure to hybridize probably reflects large differences between the genome segments involved.

The results in Fig. 1 can therefore be summarized as follows. All the cross-hybridization patterns shown in Fig. 1 as well as others not shown, were found to be different from one another. Each pattern is, within limits, characteristic for the strain under investigation. The average number of genome segments of a specific serotype that hybridize with the corresponding Type 10 genome segments is 7 with a maximum of 8 in Type 9 and a minimum of 6 in Types 3, 13 and 16. In all cases observed, Segment 6 and either Segment 2 or 3 did not show cross-hybridization with the equivalent Type 10 genome segments. Segments 1, 4, 5 and 8 always hybridized, although the homology was usually incomplete.

When ssRNA preparations of the different serotypes were hybridized with denatured dsRNA from strains other than Type 10, the hybridization patterns were found to be very similar to those depicted in Fig. 1. One important difference was, however, observed. Two patterns were obtained where cross-hybridization between genome Segments 6 had taken place. These, as well as those of two other cross-hybridizations, are shown in Fig. 2. It can also been seen that again, neither Segment 2 nor 3 shows cross-hybridization with a heterologous serotype.

Subsequently, similar results were obtained with all the cross-hybridizations carried out with other serotypes.

*Cross-hybridization between different BTV isolates of the same serotype*

The three different isolates of BTV Serotype 4 were identified as 4/Theiler, 4/Cyprus and 4/1314. From the widely divergent dates and places of isolation of these strains it could be expected that the genome segments of these strains might reflect some differences. Serologically however, the isolates are considered to be homologous. It was therefore considered expedient to determine whether the cross-hybridization patterns between different Type 4 isolates could be distinguished from those involving different serotypes.

The results of four cross-hybridization experiments carried out are shown in Fig. 3.

It is obvious that the genome segments of the three Type 4 strains are not identical. Not a single genome segment of 4/Theiler gave homologous type hybridization with the genome segments of 4/Cyprus and 4/1314. No cross-hybridization between genome Segment 10 in 4/Theiler and Segment 10 in any one of the others was found. The different isolates nevertheless have one feature in common, namely that both Segments 2 and 3 show cross-hybridization.

It can be seen from Fig. 3 that 4/Cyprus is more closely related to 4/1314 than both are to 4/Theiler. This is not entirely unexpected as the 4/Theiler strain was isolated about 60 to 70 years before the other two strains. Furthermore it was shown that the cross-hybridization pattern 4/Cyprus+: 4/Theiler$^{-}$ is not identical to 4/Theiler+: 4/Cyprus$^{-}$. In the first pattern, hybrid Segment 9 is either absent or has shifted so far towards a lower mobility that it co-electrophoreses with that of Segment 8. In the second cross, however, all three of the hybrid segments are clearly resolved. Similar observations have been made by Ito & Joklik (1972) for reovirus mutants.

*Differences between virulent and avirulent strains*

Although the naturally occurring strains of BTV vary considerably in their virulence, stable attenuated mutant virus populations can be produced in the laboratory by serial passage in eggs. This modification in virulence should be reflected by changes in the virus genome. It was therefore of interest to determine whether cross-hybridization between virulent and avirulent strains of the same serotype would reflect any changes in the virus genome.

For this study cross-hybridization patterns between virulent and attenuated strains of four different serotypes were obtained. The results presented in Fig. 3 indicate that the two reciprocal cross-hybridizations that can be carried out do not necessarily yield identical results. For this reason all the mRNA isolates (plus strands) of the attenuated strains were first hybridized with the minus strands of the virulent type and then the minus strands of the attenuated strains were hybridized to strands of the virulent type. The different cross-hybridization patterns are shown in Fig. 4.

From these results it is apparent that the differences between the virulent and attenuated strains are generally very small. Only two relatively large changes in the migration of the hybrid segments relative to the dsRNA segments were observed, namely Segment 7 in the hybridization 10 virulent$^{+}$: 10 attenuated$^{-}$ and Segment 9 in the hybrid 10 attenuated+: 10 virulent$^{-}$ (Fig. 4 G, H). The other changes are all very small, and involve a shift to either a higher relative mobility as illustrated by Segment 6 in Fig. 4C or to a lower mobility as seen in Segments 2 and 3 in Fig. 4A.

A comparison of Segments 7 and 9 in Fig. 4D and 4H furthermore clearly indicated that the reciprocal order of cross hybridization can result in entirely

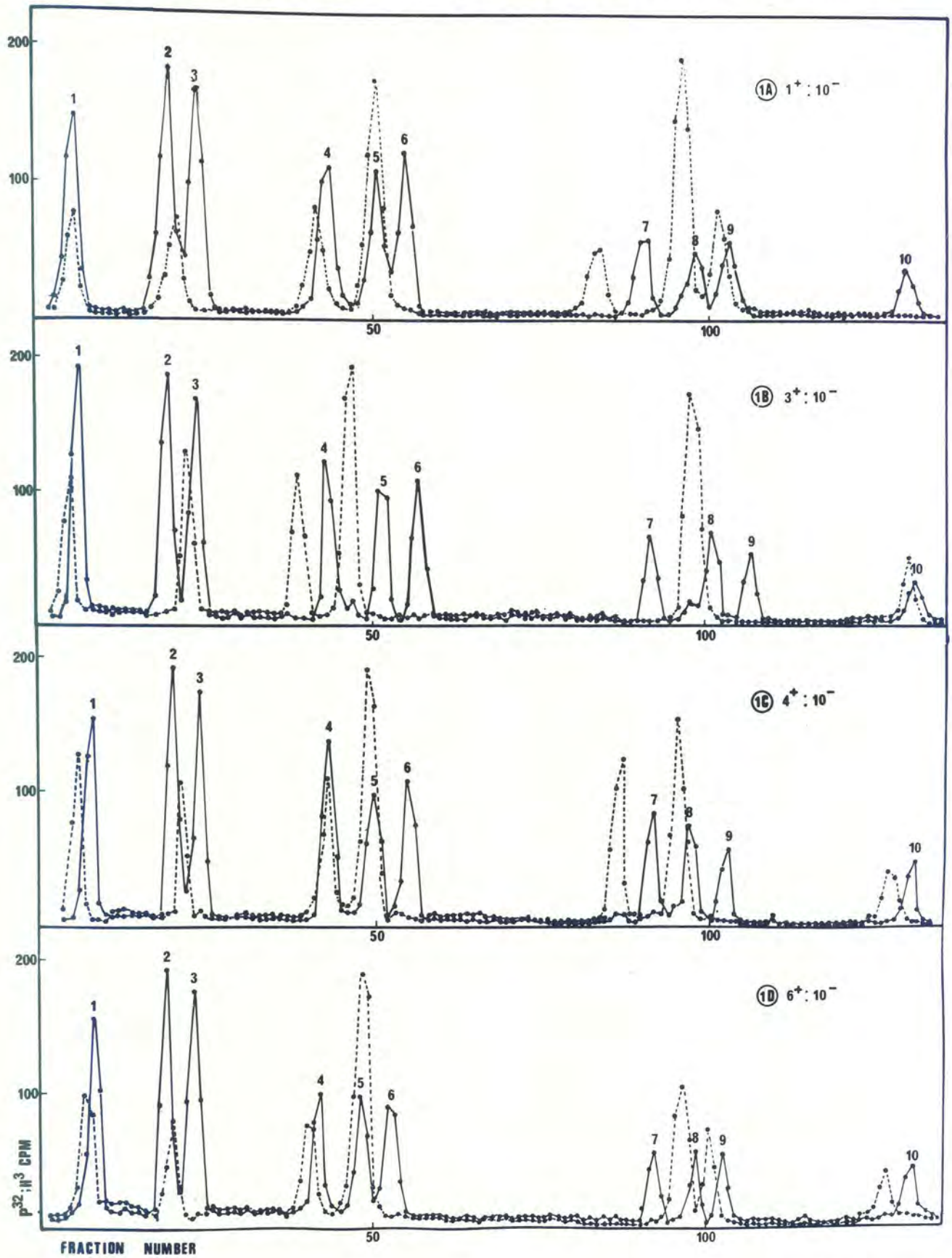
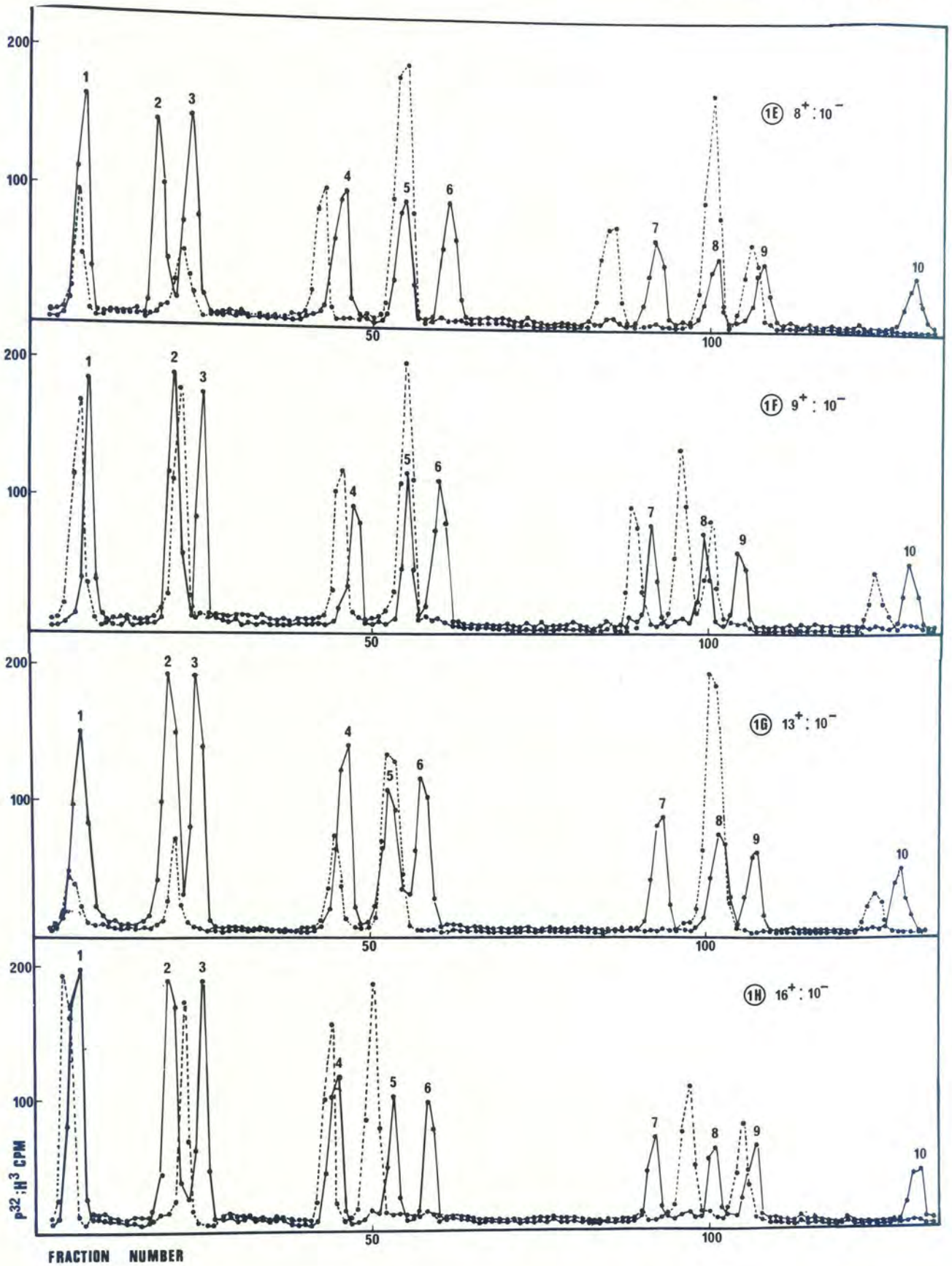



FIG. 1 Gel electrophoretic fractionation of the cross hybridization product of the mRNA isolates (+ strands) of different BTV serotypes with complementary strands (— strands) from Type 10. The Type 10 genome segments are numbered in order of decreasing size. The $^{3}$H-labelled mRNA-derived hybrid segments are indicated by - - - · - - - and the normal $^{32}$P-labelled dsRNA segments are indicated by —— · ——. Direction of electrophoretic migration is from left to right.


1E 8+ : 10−
1F 9+ : 10−
1G 13+ : 10−
1H 16+ : 10−
1
2
3
4
5
6
7
8
9
10
200
100
50
100
p32 : H3 CPM
FRACTION NUMBER

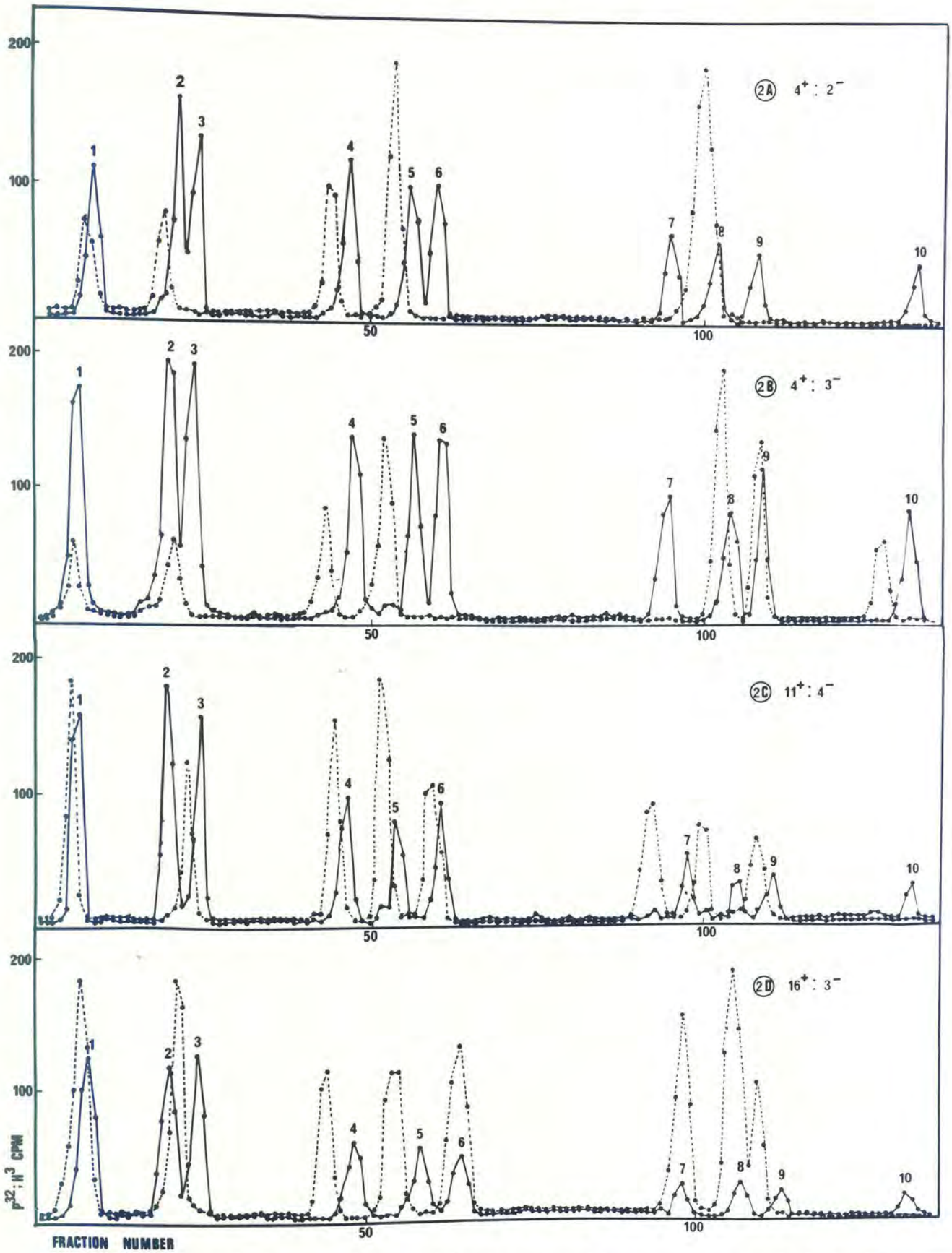



FIG. 2 Gel electrophoretic fractionation of the cross hybridization product of the plus and minus strands of a few different BTV serotypes. The position of the $^{32}$P-labelled dsRNA segments used in the hybridization is indicated by —— · —— and the segments are numbered in order of decreasing size. The $^{3}$H-labelled mRNA-derived hybrid segments are indicated by - - - · - - -. Direction of electrophoretic migration is from left to right.

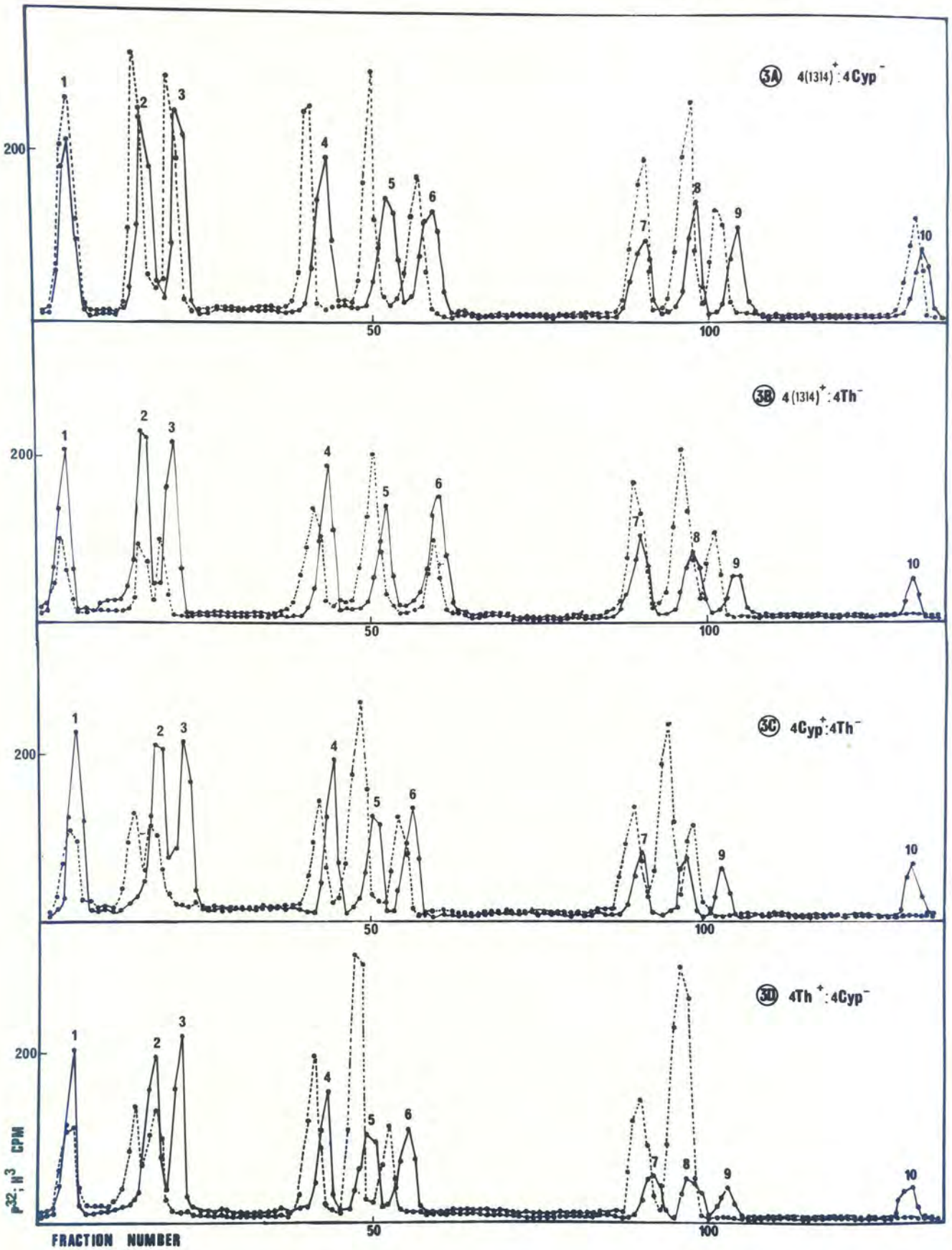



FIG. 3 Gel electrophoretic fractionation of the cross hybridization product of the plus and minus strands of the following Serotype 4 isolates. 4/Theiler; 4/Cyprus; 4/1314. The position of the $^{32}$P-labelled dsRNA segment used in the hybridization is indicated by —— · —— and the different segments are numbered in order of decreasing size. The $^{3}$H-labelled mRNA derived hybrid segments are indicated by - - - · - - -. Direction of electrophoretic migration is from left to right.

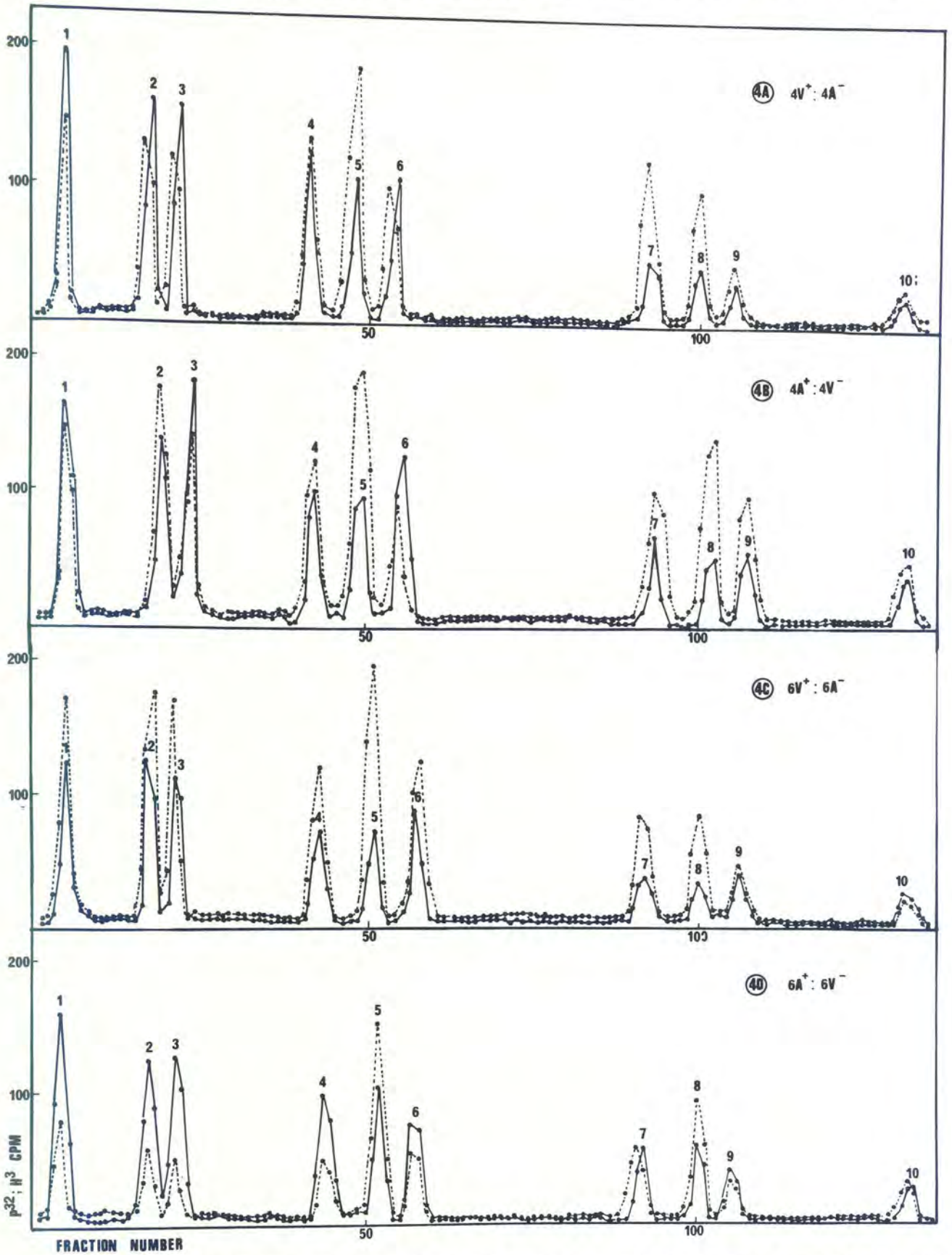
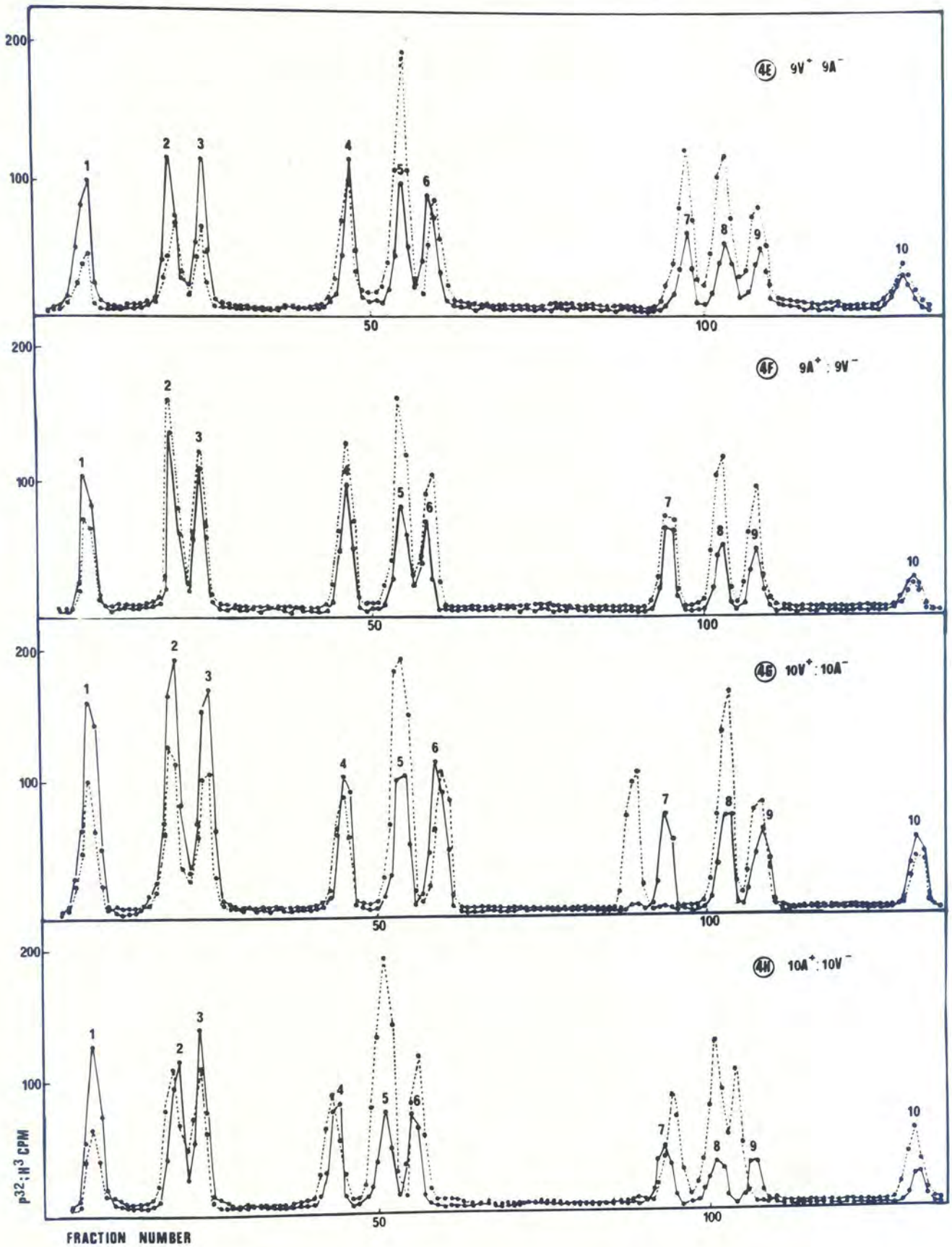



FIG. 4 Gel electrophoretic fractionation of the cross hybridization product between the plus and minus strands of the virulent (V) and attenuated (A) strands of the same serotype. Both reciprocal cross hybridizations are shown. The position of the $^{32}$P-labelled dsRNA segments used in the hybridization is indicated by —— · —— and the different segments are numbered in order of decreasing size. The $^{3}$H-labelled mRNA derived hybrid segments are indicated by - - - · - - -. Direction of electrophoretic migration is from left to right.


4E 9V+ 9A−
4F 9A+ : 9V−
4G 10V+ : 10A−
4H 10A+ : 10V−
200
100
50
100
1
2
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4
5
6
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8
9
10
p32 : N3 CPM
FRACTION NUMBER

different cross hybridization patterns. A number of of other similar examples can also be found in Fig. 4.

In summary, incomplete cross-hybridization of genome Segments 2 and 6 is indicated for each serotype in at least one of the two reciprocal cross-hybridizations carried out. Genome Segment 7 also frequently shows incomplete cross-hybridization although this was not found with Serotype 9. Incomplete cross-hybridization is also indicated by a few other genome segments, but less frequently. A similar pattern of cross-hybridization was shown by two other serotypes (3 and 12), subsequently investigated.

## Discussion

A large number of molecular hybridizations between the RNA's of different serotypes of BTV was carried out. The objective was twofold. Firstly, to determine whether hybridization studies could provide any information on the degree of relatedness between the 16 different BTV serotypes and secondly, to locate those genome segments of the virus which determine the immunological specificity.

It was found that the cross-hybridization patterns do indicate different degrees of relatedness between different serotypes. This is illustrated in Fig. 2A, B and C in which the hybridization patterns of Serotype 4 with Types 2, 3 and 11 respectively, are shown. In the hybridization of $4^+$: $2^-$ only five of the segments possess homology. In the hybrid $4^+$: $3^-$ there are two more and in $11^+$: $4^-$ a total of eight segments show cross-hybridization. Consequently it can be deduced that Serotype 4 is more closely related to Serotype 11 than to Serotypes 3 and 2 in that order. Similarly the extent to which Serotype 10 is related to the other serotypes can be determined from Fig. 1. It must be pointed out, however, that this relationship refers to the degree of homology between the total genomes of the strains and it does not necessarily refer to any degree of immunological relatedness.

In order to locate the genome segment(s) that are the determinants of the immunological specificity it was important to establish which of them failed to hybridize in most or all of the cross-hybridizations carried out between the different serotypes. It is obvious from Fig. 1 and 2 that there is only one possible candidate, namely either Segment 2 or 3. Although it has not been rigorously proved for all the strains it is most likely the second genome segment that fails to hybridize. In almost all cases of incomplete hybridization the gel electrophoretic mobilities of the hybrid segments are lower than those of the normal dsRNA segments. If this is also true of the hybrid segment that lies between the second and third dsRNA segments in Fig. 1A to 1H this component could only be derived from Segment 3. This conclusion was confirmed in experiments in which the individual dsRNA genome segments of Type 10 were isolated and used for hybridization (Huismans, unpublished results). In these experiments Segment 3 but not Segment 2 showed cross-hybridization.

It has been shown that both the second and third genome segments of BTV code for major polypeptides in the BTV protein coat (Verwoerd, Els, De Villiers & Huismans, 1972; Martin, Zweerink & Joklik, 1972). The polypeptide that is coded for by the third genome segment is part of the nucleocapsid protein while the polypeptide coded for by the second genome segment is one of the two major polypeptides in the diffuse protein layer surrounding the capsid. This diffuse protein layer can be removed by selective treatment in CsCl gradients at different pH values (Verwoerd *et al.*, 1972). The first polypeptide removed by this treatment is the one coded for by the second genome segment. This polypeptide therefore constitutes or is part of the surface protein of the virus coat and as such very important in determining the serological specificity. This also points to the second genome segment as being implicated in the immunological specificity of the BTV serotypes.

Genome Segment 6 failed to show cross hybridization in all but a few isolated cases. According to Verwoerd *et al.* (1972), this segment also codes for one of the polypeptides in the protein layer surrounding the capsid. These two results suggest that Segment 6 could also be of some importance as a determinant of immunological specificity. In this respect the cross-hybridization pattern $16^+$: $3^-$ was of interest. The result obtained (Fig. 2D) illustrates the only observed case of homologous cross hybridization between genome Segments 6 of two serotypes. This result could perhaps explain why Serotypes 16 and 3 are the only two strains that give some measure of cross-neutralization.

Strains which are serologically indistinguishable do not necessarily have identical number 2 and number 6 genome segments. This is shown by the cross-hybridization between different strains of Serotype 4 (Fig. 3). Complete homologous cross-hybridization of these segments was never observed. However, the small differences that obviously do exist between these genome segments are not sufficient to affect the immunological specificity of the strain.

The cross-hybridization patterns of the virulent and avirulent strains of the same serotype also indicate differences between some of the corresponding genome segments. The differences are, however, very small and very often only shown in one of the two reciprocal cross hybridizations carried out between the virulent and avirulent strains. It appears that the only two genome segments that invariably undergo a change during attenuation are Segments 2 and 6. These segments, as already mentioned, code for the two major polypeptides in the outer protein layer of the virus.

This result would therefore suggest that the same polypeptides involved in the determination of the immunological specificity of the virus are also involved in determining the virulence of the virus. If the virulence of a particular virus strain is associated with the ability of the virus to attach to susceptible cells, the same polypeptides could be implicated in both the immunizing activity and the virulence of the virus as has been shown with foot-and-mouth disease virus by Wild, Burroughs & Brown (1969).

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