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Recombination in RNA

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Summary

The aphthovirus genome consists of a single molecule of single-stranded RNA that encodes all the virus-induced proteins. We isolated recombinant aphthoviruses from cells simultaneously infected with temperature-sensitive mutants of two different subtype strains. Analysis of the proteins induced by 16 independently generated recombinants revealed two types of protein pattern, which were consistent with single genetic crossovers on the 5′ side and 3′ side, respectively, of the central P34-coding region. Recombinants invariably inherited all four coat proteins from the same parent, and novel recombinant proteins were not observed. RNAase T1 fingerprints of virus RNA, prepared from representatives of each recombinant type, confirmed the approximate crossover sites that had been deduced from the inheritance of proteins. These fingerprints provide molecular evidence of recombination at the level of RNA and demonstrate the potential of RNA recombination for producing genetic diversity among picornaviruses.

Introduction

Although there is plenty of opportunity within diploid cells for the exchange of genetic information between homologous RNA sequences, it is difficult to test for low levels of recombination among normal messenger RNAs. RNA viruses, however, provide a sensitive system for detecting recombinants. In addition, recombination could have functional or evolutionary importance for RNA viruses, particularly for those with unsegmented genomes. The possibility of genetic recombination in such viruses was first suggested many years ago by Hirst (1962) and Ledinko (1963), who showed that infection of cells with a mixture of inhibitor-sensitive variants of poliovirus resulted in an enhanced yield of resistant progeny that were genetically stable. Similar observations were made with aphthovirus (or foot-and-mouth disease virus) in this laboratory (Pringle, 1965). However, with the exception of retroviruses, which are presumed to recombine as a DNA intermediate, recombination has not been demonstrated in any other family of unsegmented RNA virus despite extensive investigation.

There are two possible explanations for the apparent recombination seen in picornaviruses: either the enhancement in the yield of resistant progeny is due to recombination between mutant genomes or it is due to complementation between them that leads to an increase in the yield of wild-type revertants. Although this question has not, until now, been resolved conclusively, several lines of evidence favor the existence of recombination.

First, recombination frequencies are additive. Linear genetic maps were constructed for both poliovirus (Cooper, 1968; Cooper, 1977) and aphthovirus (Lake et al., 1975; McCahon et al., 1977). Biochemical mapping of mutations has recently given us an insight into the physical basis of the aphthovirus genetic map; loci within the left-hand half, in the middle, and at the right-hand end of the genetic map appear to be correlated with their respective physical locations near the 5′ end (King and Newman, 1980; King et al., 1980), middle (Saunders and King, 1982) and 3′ end of the genome (Lowe et al., 1981; for review, see McCahon, 1981).

Second, we have obtained biochemical evidence of genetic recombination by crossing temperature-sensitive mutants of aphthovirus that possess second-site mutations affecting polypeptide charge. Three different pairwise crosses produced ts^+ viruses with polypeptide markers from both parents (King et al., 1982).

Third, the phenomenon of enhancement is seen even when the parental viruses have defects in the same polypeptide. Two aphthovirus mutants, *ts22* and *ts115*, have been shown, by a variety of chemical and enzymological methods, to encode a temperature-sensitive RNA polymerase (Lowe et al., 1981). Yet infection with a mixture of these mutants resulted in a significantly enhanced yield of ts^+ virus (McCahon and Slade, 1981). Such mutations would be unlikely to complement each other, since the polymerase of picornaviruses functions as a monomeric polypeptide (Flanegan and Baltimore, 1979).

All the work reviewed above was confined to crosses between mutants of the same strain. To assess the evolutionary implications of recombination between picornaviruses, it is important to find out whether genetic information can be exchanged between evolutionarily divergent strains of virus. Recent improvements in the sensitivity of recombination assays (McCahon and Slade, 1981) have opened the way to a study of recombination between viruses having distinct nucleotide sequences. This paper describes a cross between two subtype strains of aphthovirus that has provided the first biochemical demonstration of genetic recombination at the level of RNA.

Results

Parental Mutants

Two aphthovirus strains of serotype O were chosen for studies of genetic recombination: Pacheco of subtype O_1, and V1 of subtype O_6. The former was chosen

because most previous studies had involved crosses between mutants of this strain, and the latter because its RNA was readily distinguishable from that of the former by RNAase T1 fingerprinting, and the approximate genomic locations of the oligonucleotides were known.

This paper describes a genetic cross between temperature-sensitive mutants of the two subtype strains. The O_1 parent, *ts33*, had a temperature-sensitive mutation that was associated with an altered structural polypeptide VP2 (King et al., 1980), encoded near the 5′ end of the genome. For the other parent, a mutant of subtype O_6 with a temperature-sensitive mutation at the 3′ end of the genome was sought. Several spontaneous temperature-sensitive mutants of subtype O_6 were isolated and screened by electrofocusing polypeptides induced in virus-infected cells. One mutant, *ts302*, induced an altered nonstructural polypeptide, P56a, which is encoded at the 3′ end of the genome.

Figure 1 illustrates the electrofocusing patterns produced by the polypeptides of the two wild-type strains and the parental mutants, *ts33* and *ts302*. Conditions of labeling and sample preparation were designed to give simple patterns of stable virus-specific polypeptides, for ease of comparison in one dimension. Polypeptides were identified by two-dimensional electrophoresis as described below. As Figure 1 shows, the O_1 temperature-sensitive mutant differed from the O_1 wild-type only in VP2, and in the precursor of VP2, P38, whereas the O_6 temperature-sensitive mutant differed from the O_6 wild-type only in P56a. The latter alteration was shown to be due to the temperature-sensitive defect by examining the polypeptides of ts^+ revertants of the O_6 parent. Each of six spontaneous revertants demonstrated covariation between the charge change in P56a and the temperature-sensitive defect (data not shown). Significantly, none of these revertants showed any resemblance to the O_1 polypeptide pattern.

Genetic Crosses

Recombinants were isolated by using the sensitive infectious center method (McCahon and Slade, 1981), and the results are shown in Table 1. In addition to the intersubtypic cross (cross 3), each parent was crossed with a temperature-sensitive mutant belonging to the same virus strain. In cross (1), *ts33* was crossed with tsO_3, which has a genetic locus (McCahon et al., 1977) within the region representing the RNA polymerase gene (Lowe et al., 1981). The genetic location of *ts303*, used in cross (2), was not known, but several ts^+ revertants of *ts303* were found to have an electrophoretically altered VP3, suggesting that this mutant had a temperature-sensitive defect of the coat proteins. Thus, in all three crosses in Table 1, parent A was believed to carry a coat protein temperature-sensitive lesion, whereas parent B carried a temperature-sensitive lesion in P56a. In each cross, infection with a mixture of mutants greatly enhanced the proportion of cells yielding ts^+ progeny over that observed from singly infected controls. However, the proportion was not as high when the parents belonged to different subtypes (cross 3) as when isogenic parents were used (cross 1 and 2).

Inheritance of Proteins

Viruses that were ts^+ were cloned at 41°C and were characterized initially by electrofocusing virus-induced polypeptides. We have previously shown this technique to be extremely sensitive to variation even

Table 1. Results of Genetic Crosses

Cross		% of Infected Cells Yielding ts^+ Virus		
Parent A	Parent B	A Only	B Only	A + B
(1) *ts33* (O_1)	tsO_3 (O_1)	0.1	0.3	14.0
(2) *ts303* (O_6)	*ts302* (O_6)	0.8	0.2	19.9
(3) *ts33* (O_1)	*ts302* (O_6)	0.1	0.2	4.4

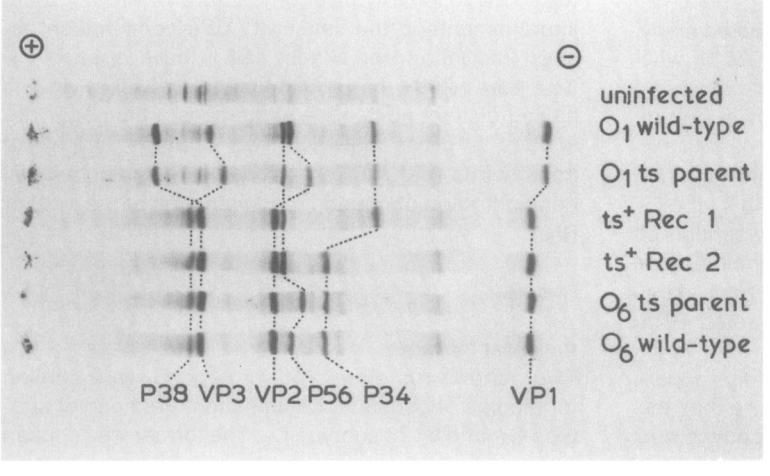


Figure 1. Location of Aphthovirus Mutations and Detection of Recombinants Rec 1 and Rec 2, by Electrofocusing Induced Polypeptides

Virus-infected baby hamster kidney cells were labeled with ^{35}S-methionine for 30 min, followed by a 30 min chase with unlabeled methionine, and virus-specific polypeptides were precipitated with antiserum as described previously (King et al., 1981). The origin of electrofocusing is on the left.

among closely related aphthoviruses (King et al., 1981). As Figure 1 shows, large differences between the subtypes were seen in the isoelectric points of all six major polypeptides, and these differences were exploited as markers for studying recombination. Since the O_1 parent was defective in VP2 and the O_6 parent in P56a, ts^+ recombinants would be expected to inherit an O_6 VP2 and an O_1 P56a. Out of 22 ts^+ progeny, 16 possessed these hybrid characteristics. Since each clone was derived from a separate infected cell, these recombinant viruses were all products of independent genetic crossovers.

The recombinants were of two types, termed Rec 1 and Rec 2, and an example of each is shown in Figure 1. Two were of the Rec 1 variety, having inherited all their structural polypeptides VP1, VP2, VP3 and P38, from the O_6 parent, and both nonstructural polypeptides, P34 and P56a, from the O_1 parent. The other 14 recombinants were of the Rec 2 type, in which all six polypeptides except P56a were inherited from the O_6 parent. The remaining six ts^+ progeny were indistinguishable from the O_6 wild-type and were presumably revertants of the O_6 parent, *ts302*. An alternative possibility is that these ts^+ viruses were produced by pairs of genetic crossovers on either side of the O_6 temperature-sensitive mutation.

Rec 1 was the most interesting type of presumptive recombinant, since it appeared to have been generated either by a genetic crossover in the middle of the genome or by a minimum of two separate mutations. A more complete analysis of the induced polypeptides of Rec 1 was therefore undertaken by two-dimensional electrophoresis of whole cytoplasmic extracts prepared from pulse-labeled virus-infected cells, as shown in Figure 2. Under the conditions used, most of the virus-induced polypeptides, precursors as well as products, were labeled. Identification of the larger virus polypeptides in two-dimensional gels has been described by King et al. (1982). The small polypeptides P12, P16, P20a and P20b were identified from their electrophoretic mobilities in the SDS gel dimension. In both subtypes, P20a appeared as two spots differing in isoelectric point.

The two subtypes were compared by running a mixture of polypeptide extracts (O_1 + O_6, Figure 2). Differences between them are seen as pairs of polypeptide spots. The results confirmed the differences seen earlier in one dimension (Figure 1) and revealed further subtype-specific differences in all the other virus-coded polypeptides except for P12, P20b and P88. The absence of a difference in P88, the precursor of the structural proteins, was presumably because differences among its constituent polypeptides cancelled each other out. To improve resolution of the smaller polypeptides, all six analyses of Figure 2 were repeated with a higher concentration of polyacrylamide in the SDS gel (not shown); these gels confirmed the conservation of P12 and P20b. The acidic

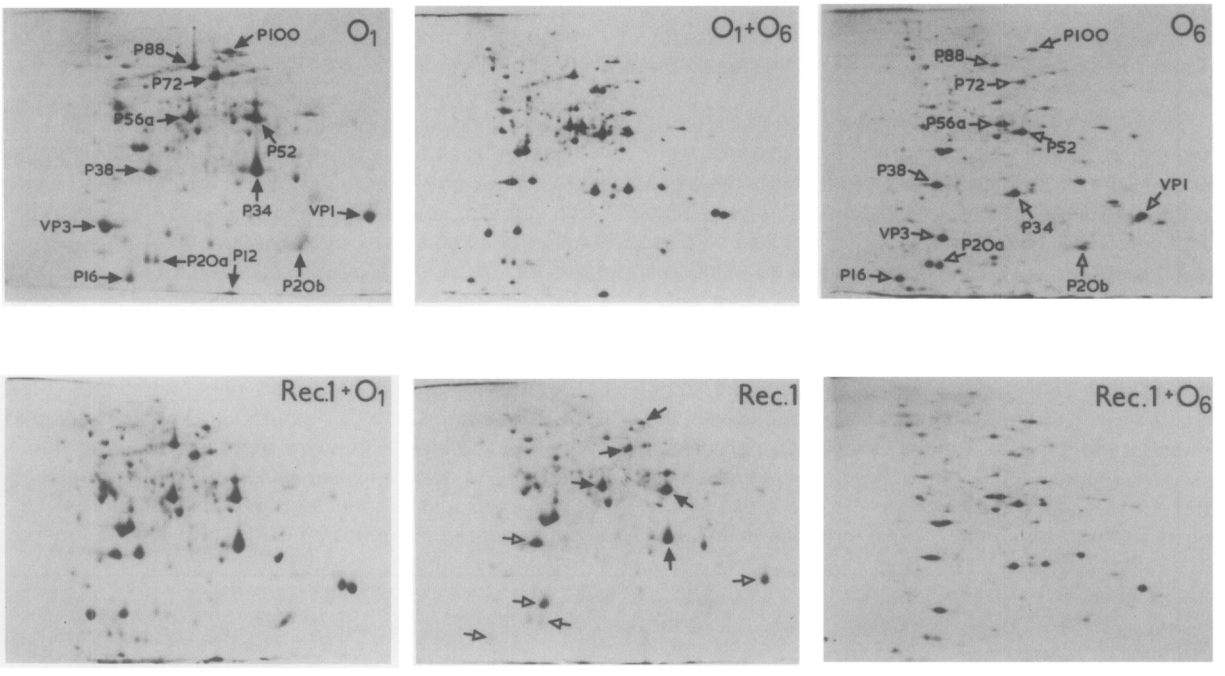


Figure 2. Detailed Comparison of Polypeptides Induced by a Type 1 ts^+ Recombinant (Rec 1) with Those of Its Parental Temperature-Sensitive Viruses (O_1 and O_6) Using Two-Dimensional Electrophoresis

Infected cells were labeled with ^{35}S-methionine for 10 min, and cytoplasmic extracts were subjected to electrofocusing (left to right), followed by electrophoresis in 10% SDS–polyacrylamide gels in the vertical dimension as described by O'Farrell (1975). The parental origins of polypeptides in the Rec 1 preparation are indicated by: subtype O_1 (→); subtype O_6 (⇢).

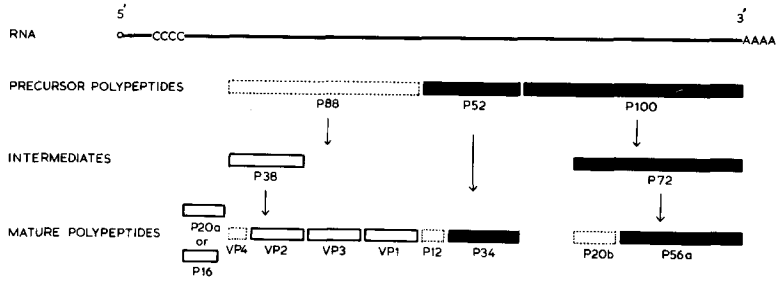


Figure 3. Biochemical Map of Polypeptides Encoded by a Type 1 *ts*$^+$ Recombinant (Rec 1)

Assignments of parental origin are based on the data of Figures 1 and 2 and are indicated by: subtype O_1 (■); subtype O_6 (□); indistinguishable ([⋮]).

polypeptide VP4 does not enter these electrofocusing gels, but the isoelectric point of this polypeptide is known to be highly conserved (King et al., 1981). Differences among the other polypeptides were predominantly in the electrofocusing (horizontal) dimension. Thus the polypeptides of the two subtype strains differed greatly in isoelectric point but not in size.

The subtype origins of Rec 1 polypeptides were determined by running mixtures of Rec 1 and parental extracts. As can be seen from the Rec 1 + O_1 mixture, polypeptides P16, P20a, VP1, VP3 and P38 of the recombinant differed from their O_1 counterparts in exactly the same way as the two parents differed from each other, showing that these five polypeptides (indicated by open arrows on the Rec 1 gel) were inherited from the O_6 parent. In contrast, the same mixture gave rise to only single polypeptide spots for the other nonconserved polypeptides, P34, P52, P56a, P72 and P100, showing that these were inherited from the O_1 parent (solid arrows on the Rec 1 gel). As expected, the situation was reversed in the Rec 1 + O_6 mixture—the two viruses were indistinguishable in the former group of polypeptides, but were different in the latter.

Mature aphthovirus polypeptides are generated by cleavage of precursor polypeptides, which are synthesized from a single messenger RNA that has the same sense as genomic RNA, as shown in Figure 3. The location of P12 and P20b on the biochemical map (A. M. Q. King and K. Saunders, unpublished work) will be described elsewhere; positions of the other polypeptides are as reported by Sangar (1979). Figure 3 also summarizes our knowledge of the parental origins of the Rec 1 polypeptides. It is clear that this recombinant virus inherited the polypeptides encoded in the 5′ half of the genome from its subtype O_6 parent, but inherited those in the 3′ half of the genome from its O_1 parent, consistent with a single genetic crossover in the middle of the genome. The inheritance of P16 and P20a is of interest, since they have no analog among other picornaviruses. Our results, which show that these nonstructural polypeptides are linked genetically to the coat proteins, agree with a 5′-coding position determined biochemically (Sangar et al., 1980).

Inheritance of T1 Oligonucleotides

The provisional identification of recombinants on the basis of induced polypeptides was confirmed by RNA fingerprinting. Virion RNA from a representative of each of the recombinant types (Rec 1 and Rec 2) was compared with the parental RNAs by two-dimensional electrophoresis of RNAase T1 digests. A mixture of parental RNAs, O_1 + O_6, was also analyzed, and all five fingerprints are reproduced in Figure 4A. The complexity of the mixture showed that there were many differences between the RNAase T1-resistant oligonucleotides of these two subtype strains. The diagrammatic version in Figure 4B indicates 22 oligonucleotides unique to the O_1 component of the mixture, and at least 19 oligonucleotides unique to the O_6 component. The RNA fingerprint of the O_6 mutant in Figure 4A is virtually identical to that of the wild-type O_6 strain previously published by Harris et al. (1980); all but one of the 19 O_6-specific oligonucleotides correspond to spots on the published wild-type pattern, and these are identified in Figure 4B with use of the same numbering system. (The exception is a spot, or group of spots, at the bottom left of the diagram that appears more complex than in the version previously published.)

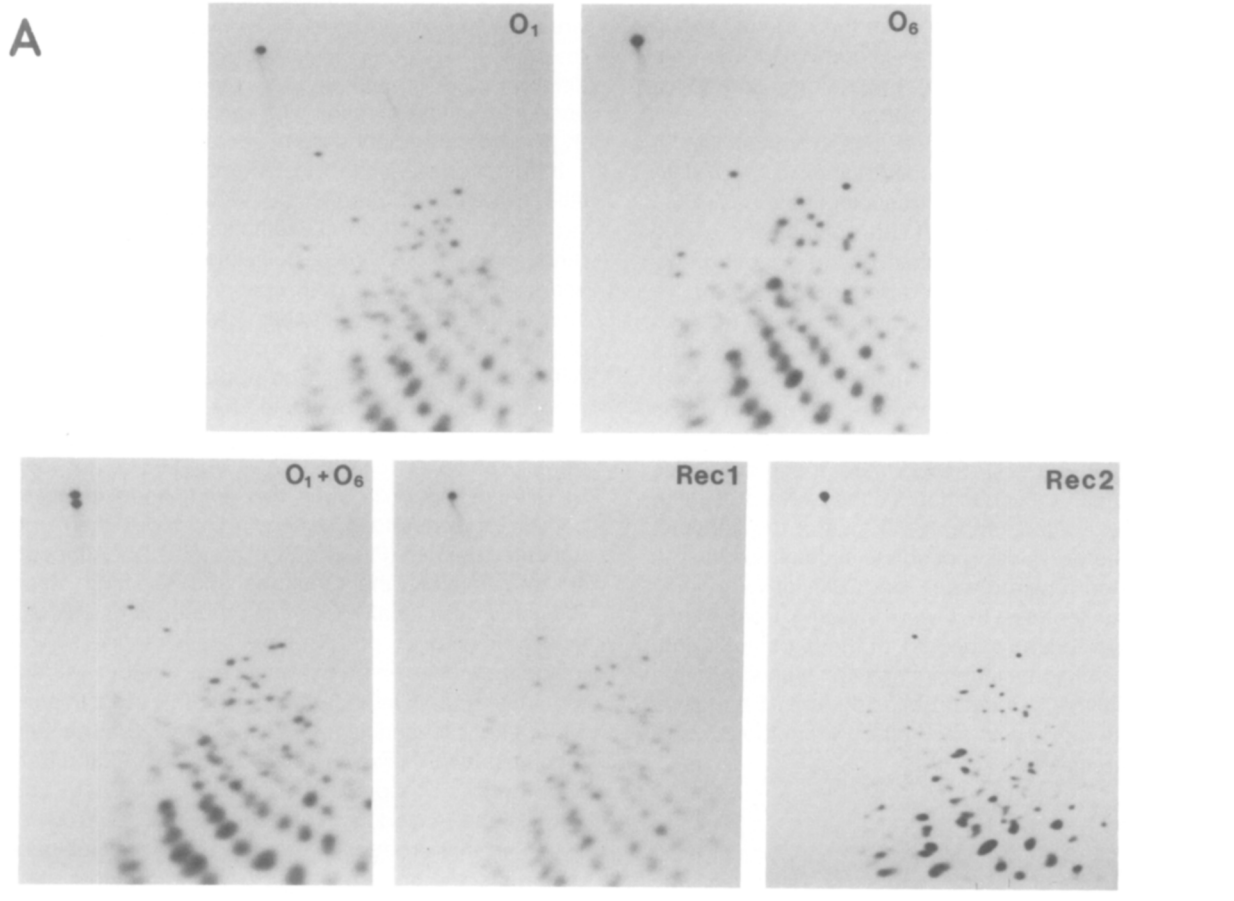
The fingerprint of Rec 1 RNA resembled neither parent, although all the Rec 1 oligonucleotides were present in the O_1 + O_6 mixture. Of the 22 O_1-specific oligonucleotides, nine were represented in the Rec 1 pattern, 11 were missing and the parental origins of the two largest oligonucleotides, poly(C) and poly(A), could not be assigned. Similarly, of the 18 numbered

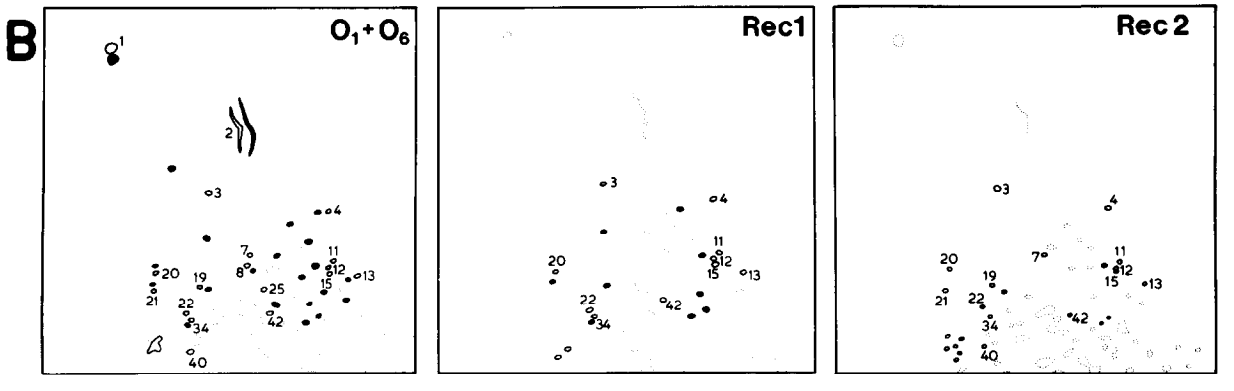
Figure 4. RNAase T1-Resistant Oligonucleotides of the Various Virus RNAs

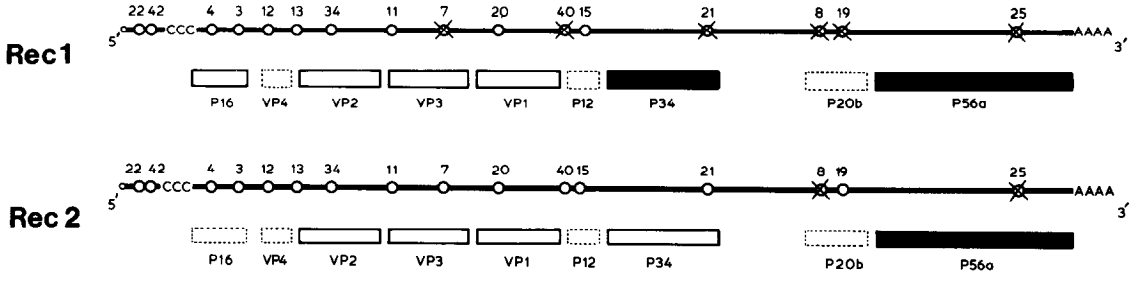
O_1: *ts33* of subtype O_1. O_6: *ts302* of subtype O_6. Rec 1: *ts*$^+$ recombinant type 1. Rec 2: *ts*$^+$ recombinant type 2.

(A) RNAase T1 fingerprints.

(B) (Top) Tracings of three of the fingerprints shown in (A). Filled symbols: O_1-specific oligonucleotides. Empty symbols: O_6 oligonucleotides. Dotted lines: indistinguishable. O_6 oligonucleotides are numbered according to Harris et al. (1980). (Bottom) Biochemical maps of recombinant RNAs, showing locations of O_6 oligonucleotides reported by Harris et al. (1980) and the parental origins of the induced proteins. (X) Missing O_6 oligonucleotides.


A
O1
O6
O1 + O6
Rec1
Rec2


B
O1 + O6
Rec1
Rec 2


Rec1
Rec 2
5'
3'
CCC
AAAA
P16
VP4
VP2
VP3
VP1
P12
P34
P20b
P56a

O_6 oligonucleotides, ten were present in the Rec 1 RNA and six were missing. Thus the oligonucleotide composition shown in Figure 4B confirmed that both subtype strains contributed genetic information to Rec 1 in approximately equal amounts.

Rec 2 RNA also contained a recombinant set of oligonucleotides, which included four from the O_1 RNA and 14 of the numbered oligonucleotides in O_6 RNA (Figure 4B). This confirmed the conclusions of the polypeptide analysis that most of the genetic information of Rec 2 was inherited from the O_6 parent.

The locations of the oligonucleotides in O_6 RNA, as reported by Harris et al. (1980), are shown in Figure 4B, (bottom). Although their method of mapping oligonucleotides was rather imprecise, the data of Harris et al. confirm very clearly that the recombinants inherited the 5′ end of O_6 RNA, but not the 3′ end. Thus all ten of the O_6 oligonucleotides that were present in Rec 1 RNA are located in the 5′ half of the genome, whereas the six missing oligonucleotides are heavily concentrated towards the 3′ end. Similarly, the only two oligonucleotides that were missing from Rec 2 RNA are located, as expected, at the extreme 3′ end. When these oligonucleotide maps are aligned with the biochemical maps of the proteins (Figure 4B), the two sets of data agree with each other within the limits of mapping precision.

Discussion

The inheritance of oligonucleotides by Rec 1 and Rec 2 indicates that each recombinant genome was generated by either a single genetic crossover or, conceivably, several crossovers close together. It will not be possible to decide between these alternatives until the oligonucleotides have been located more precisely in the parental nucleotide sequences. However, our results leave little room for doubt that recombination between RNA molecules really can occur, for several reasons. First, the aphthovirus genome consists of a single molecule of single-stranded RNA, and most of the fingerprints in this study were obtained from full-length fractions of virus RNA. Second, the RNA is replicated in the cytoplasm with no requirement for DNA synthesis. Third, the hybrid oligonucleotide patterns of Rec 1 and Rec 2 showed no evidence of virus mixtures. Finally, the same genetic crossovers were inferred independently from the inheritance of virus-coded proteins and were as predicted from the locations of the parental temperature-sensitive lesions.

By using the infectious center method, up to one third of cells infected with a mixture of temperature-sensitive viruses have yielded ts^+ progeny (McCahon and Slade, 1981). Since most ts^+ progeny of the intersubtypic cross proved to be genuine recombinants, RNA recombination appears to be a normal concomitant of picornavirus replication. It should be emphasized that this cross is far from unique. We are currently exploiting recombination between these two virus strains for the purpose of mapping guanidine-resistance mutations. Thus far, crosses between six different pairs of mutants have been carried out and each has yielded viruses with recombinant genomes (K. Saunders, unpublished observations).

Is the process limited to picornaviruses? There is little evidence of recombination among other RNA viruses, although several examples of sequence rearrangements in RNA have been discovered in recent years. These include both specific rearrangements, such as the splicing of nuclear RNA and the deletion of genomic sequences from coronavirus messenger RNAs (Lai et al., 1981), and apparently nonspecific rearrangements, as exemplified by the RNA of defective interfering (DI) particles. The latter can be extremely complex (Lehtovaara et al., 1981), and one DI RNA of influenza virus has even been found to consist of sequences derived from more than one genome segment (Fields and Winter, 1982). Although splicing involves the breakage and rejoining of an RNA chain, explanations for the production of DI RNAs usually invoke a "promiscuous" polymerase, which jumps from one region of template to another without terminating replication (Lazzarini et al., 1981). In principle, either mechanism might be involved in genetic recombination. However, there is an essential difference between recombination as described here and any of the molecular rearrangements referred to above, in that production of a functional recombinant requires some mechanism for bringing together separate RNA molecules in a precise manner. The appropriate juxtaposition of homologous sequences would occur whenever RNA molecules from two different viruses become hybridized to a single complementary strand. This situation could arise in various ways, for example, by a negative strand of RNA hybridizing to virion RNAs derived from both parents, or, alternatively, by hybridizing to a nascent positive strand in the replicative intermediate. If splicing enzymes could act on such structures, the former would give rise to a recombinant positive strand, whereas the latter would give rise to a recombinant negative (template) strand. However, all models of this type require the existence of free single-stranded RNA of negative sense, for which there is as yet no evidence in picornaviruses.

The existence of a genetic recombination map implies that crossovers can occur at many different places in the aphthovirus genome; indeed, the assumption behind recombination mapping is that crossovers are randomly located. In contrast, only a limited variety of recombinants was produced by crossing two different subtype strains, despite the fact that the parental temperature-sensitive mutations were at opposite ends of the genome. This suggests that recombination between subtypes is restricted to limited regions of the genome, a limitation reflected in the much

lower recombination frequency from this cross compared with the result obtained with isogenic parents. More work will be needed to define the nature of these constraints, although it may be significant that the only crossovers that did occur were in regions coding for the conserved polypeptides, P12 and P20b. The fact that all the polypeptides of the 16 recombinants resembled one or other of the parental electrophoretic types suggests that there may be functional constraints on the generation of recombinant polypeptides with novel biochemical properties.

What purpose does RNA recombination serve? One possibility is that recombination acts as an RNA repair mechanism by reconstructing complete genomes from overlapping fragments. The rescue of genetic markers from ultraviolet-inactivated viruses (Pringle, 1965) and the surprisingly low dependence of recombination frequency on multiplicity of infection (McCahon and Slade, 1981) give some credence to this idea. Our finding that genetic information can be exchanged between different virus subtypes raises a second possibility—that recombination is a natural source of genetic diversity among aphthoviruses. Thus recombination could play the same evolutionary role for unsegmented RNA viruses that gene reassortment appears to for segmented viruses such as myxoviruses (Scholtissek et al., 1978) and reoviruses (Sugiyama et al., 1981).

Experimental Procedures

Viruses

The origin of the wild-type aphthovirus strain, O_1 Pacheco, and its chemically induced mutant, *ts33*, have been described by Lake et al. (1975). The wild-type strain O_6V1 was provided by D. J. Rowlands and was cloned at 41°C before use. The other temperature-sensitive mutants were spontaneous and were isolated by the method of McCahon et al. (1977).

Electrofocusing

Electrofocusing was performed in gels containing pH 3.5–10 Ampholine ampholytes as described by King et al. (1982).

RNA Fingerprints

^{32}P-labeled virus was grown in baby hamster kidney monolayers in phosphate-free Earle's saline containing 0.01 M Tris–HCl (pH 7.6), 1 μg/ml actinomycin D and 200 μCi/ml ^{32}P-orthophosphate. Virus was purified as described by King and Newman (1980), except that the sucrose gradient was in TNE buffer (0.15 NaCl, 0.05 M Tris–HCl and 5 mM EDTA, pH 7.4) instead of phosphate buffer. The RNA was extracted with a 1:1 mixture of phenol and chloroform. For all the viruses except Rec 2, full-length 35S RNA was isolated by centrifugation on a 5%–25% sucrose gradient in TNE buffer containing 0.1% SDS. Purified RNA was digested with RNAse T1 and subjected to two-dimensional electrophoresis as described by Harris et al. (1980).

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