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Poliovirus RNA recombination: mechanistic studies in the absence of selection

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Direct and quantitative detection of recombinant RNA molecules by polymerase chain reaction (PCR) provides a novel method for studying recombination in RNA viruses without selection for viable progeny. The parental poliovirus strains used in this study contained polymorphic marker loci ~600 bases apart; both exhibited wild-type growth characteristics. We established conditions under which the amount of PCR product was linearly proportional to the amount of input template, and the reproducibility was high. Recombinant progeny were predominantly homologous and arose at frequencies up to 2×10^{-3}. Recombination events increased in frequency throughout replication, indicating that there is no viral RNA sequestration or inhibition of recombination late in infection as proposed in earlier genetic studies. Previous studies have demonstrated that poliovirus recombination occurs by a copy-choice mechanism in which the viral polymerase switches templates during negative-strand synthesis. Varying the relative amount of input parental virus markedly altered reciprocal recombination frequencies. This, in conjunction with the kinetics data, indicated that acceptor template concentration is a determinant of template switching frequency. Since positive strands greatly outnumber negative strands throughout poliovirus infection, this would explain the bias toward recombination during negative-strand synthesis.

Key words: copy-choice/quantitative PCR/RNA recombination/RNA virus/template switching

Introduction

Genetic recombination facilitates the exchange of genetic information. Thus at certain evolutionary junctures, the ability to recombine may be advantageous or even essential to the survival of a species. RNA viruses have long been known to be adept at rapid evolution through mutation (Holland *et al.*, 1982). The capacity to recombine, however, was initially thought to be confined to a small subset of viruses. In particular, the picornavirus family, including poliovirus, was the first for which recombination was demonstrated (Cooper, 1968).

The list of viruses known to undergo homologous recombination continues to grow. Besides poliovirus, examples among positive-strand viruses include foot-and-mouth disease virus (McCahon and Slade, 1981), mouse hepatitis virus (Lai *et al.*, 1985), brome mosaic virus (Bujarski and Kaesberg, 1986), cowpea chlorotic mottle virus (Allison *et al.*, 1990), turnip crinkle virus (Zhang *et al.*, 1991) and tomato ringspot virus (Rott *et al.*, 1991). Sequencing has revealed cases such as Western equine encephalitis virus (Hahn *et al.*, 1988) and mouse hepatitis virus (Luytjes *et al.*, 1988) in which viruses must have arisen by recombination between distantly related viral progenitors. Although there has yet to be a report of recombination in eukaryotic double-stranded RNA viruses, Mindich *et al.* (1992) have detected recombination in the double-stranded RNA bacteriophage $\phi6$. In addition, despite earlier failed attempts to detect recombination in the single-stranded phage $Q\beta$ (Horiuchi, 1975), recent reports indicate that it, too, recombines although at relatively low frequency (Palasingam and Shaklee, 1992). The subject of RNA recombination has been reviewed extensively (King, 1987, 1988a; Jarvis and Kirkegaard, 1991; Lai, 1992).

Mechanistically, poliovirus RNA recombination differs from the breaking and rejoining pathway commonly seen in DNA recombination. Using defined conditional mutants to inhibit selectively the replication of one parent in a recombinant cross, Kirkegaard and Baltimore (1986) demonstrated that poliovirus recombination occurs by a copy-choice mechanism whereby the viral polymerase switches templates during RNA synthesis. Furthermore, recombinants apparently arose only during negative-strand synthesis, posing an intriguing puzzle as to the constraints governing template switching. Experiments in which the inhibition of replication of one parental virus was followed by superinfection with another virus, also demonstrated that a parental RNA need not be replicating itself to serve as an acceptor template for a recombination event from an actively replicating RNA species. (We will refer to the template on which replication initiates as the 'donor' template, and the template to which the replication complex switches as the 'acceptor' template.) It remains unclear whether the copy-choice recombination mechanism is operative in other RNA viruses. No evidence has been found in any other virus system, however, for the enzyme activities required for breaking and rejoining, leading to the widespread belief that template switching accounts for most, if not all, examples of recombination among RNA viruses (Keese and Symons, 1985; Ahlquist *et al.*, 1987; Hahn *et al.*, 1988; Cascone *et al.*, 1990).

Intertypic poliovirus recombination, in which the parent viruses share 85% homology at the nucleotide level, was observed at a 100-fold lower frequency than intratypic recombination between completely homologous parents (Kirkegaard and Baltimore, 1986). Whether this large difference resulted from a stringent requirement for extensive homology during template switching, or selection against

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intertypic recombinants due to biological constraints is unknown. Analysis of intertypic cross-over sites (Kirkegaard and Baltimore, 1986) revealed no obvious consensus sequence for recombination, though an elevated degree of homology between the donor and acceptor RNAs on the downstream side of the cross-over sites has been noted (King, 1988b). Tolskaya *et al.* (1987) examined intertypic recombination events between more distantly spaced markers and observed a non-random distribution of sites consistent with a consensus structural motif; recombination appeared to happen preferentially in the single-stranded loop regions of putative RNA stem–loop structures.

Several genetic studies of picornavirus recombination have indicated that at least with certain crosses, the recombination frequency measured late in infection showed surprisingly little increase compared with that measured much earlier in infection. Since the bulk of RNA synthesis occurred after the first recombination measurement, this suggested that although some recombination occurred late in infection, the majority of the recombination events must happen very early in the replication cycle (Ledinko, 1963; Cooper, 1968, 1977). This led to speculation about changes in the cellular environment late in infection that might account for the inhibition of late recombination. For example, the membranous vesicles that accumulate in the cytoplasm during poliovirus infection and are physically associated with viral replication complexes (Bienz *et al.*, 1980) might sequester RNA templates and physically block recombination. Alternatively, the structure or composition of the replicative intermediate might undergo substantial changes late in infection.

We were interested in developing a sensitive recombination assay that would enable us to pinpoint the primary determinants of the frequency and strand specificity of template switching, and the kinetics of RNA recombination *in vivo*, while avoiding some of the pitfalls encountered in standard genetic assays. The sensitivity of genetic recombination assays in RNA viruses, for example, has sometimes been limited by complementation between parental strains. Furthermore, the reversion frequency of point mutations is high ($10^{-3}-10^{-4}$) (Holland *et al.*, 1982). When such point mutations are used as recombination markers, the reversion frequency sets a threshold below which recombination cannot be detected.

The assay we have devised involves detection of recombinant RNA molecules in cytoplasmic RNA preparations isolated from co-infected cells using reverse transcriptase-polymerase chain reaction (RT-PCR) methodology (Rappolee *et al.*, 1988; Saiki *et al.*, 1988). Instead of phenotypic traits, the parental markers consist of polymorphic loci to which specific PCR primers can anneal. We have established conditions under which the amount of PCR product is linearly proportional to the amount of input template, and the reproducibility is high. The threshold of detection is $<1\times10^{-6}$. A direct, physical assay of this kind enables us to probe the mechanistic aspects of RNA recombination in greater detail by separating the recombination event itself from the subsequent viability of the recombinant progeny. It also provides the unique opportunity to study the process of recombination very early in the replicative cycle of the virus, before virions are produced.

Results

We have developed a physical assay for recombinant RNA molecules, shown schematically in Figure 1A. Total cytoplasmic RNA was isolated from a co-infection of the two parental viruses 'aB' (DNC-65) and 'Ab' (5NC-104). Both viruses show essentially wild-type growth characteristics and are completely homologous except for the mutations at the marker loci. The assay measures the presence and frequency of the two potential reciprocal recombinant types, 'AB' and 'ab'. Separate cDNA reactions were performed using either the primer specific for the 'b' or the 'B' site. Thus in each cDNA reaction, one parent type and one recombinant type was synthesized, using the entire RNA pool as template. The RNA was then removed by RNase digestion and dilutions of the cDNA were used in a quantitative PCR assay using appropriate primer pairs to amplify either the recombinant or the parental cDNA.

The use of two separate cDNA reactions, specifically primed by either oligonucleotide 'b' or 'B' at the locus of the 3′ marker, avoids an artifact that can arise when both parental cDNAs are present in the PCR reaction. This is diagrammed in Figure 1B. Frohman and Martin (1990) coined the term PHLOP (polymerase halt-mediated linkage of primers) to describe this phenomenon. In short, if Taq

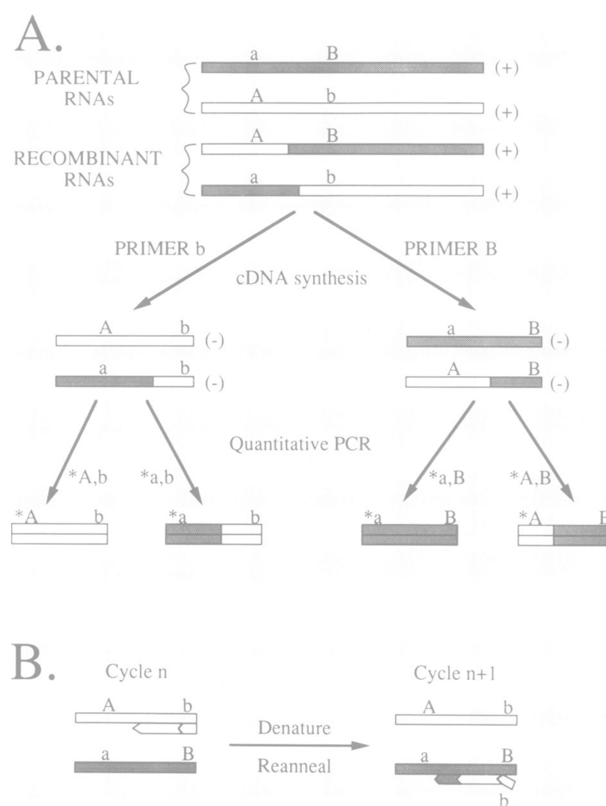

Fig. 1. RNA recombination assay. **(A)** The mutated sequences at each locus are denoted by lower case letters. For example, 'a' is the DNC-65 lesion and 'b' is the 5NC-104 deletion; the corresponding wild-type sequences are indicated as A and B, respectively. Cytoplasmic RNAs from a mixed infection of viruses 'aB' and 'Ab' were used in synthesizing first-strand negative-sense cDNA, primed by either oligonucleotides 'B' or 'b'. These cDNAs then serve as templates for quantitative PCR using oligonucleotide primers specific for either parent or recombinant. **(B)** Formation of PHLOP artifacts (see text).

polymerase terminated elongation before the second marker was reached during a cycle of PCR, the incomplete product could anneal to the other parent template after denaturation and reannealing, thereby creating a recombinant. In the scheme shown in Figure 1A, this problem was avoided by copying only one of the parents in each cDNA reaction. The recombination frequency is then determined by the relative abundance of the 'a' and 'A' markers, with no possibility of recombinational artifacts arising.

Banner and Lai (1991) have reported a somewhat similar assay in which PCR is used to detect recombinants in mouse hepatitis virus. Although they did not attempt to quantify recombination frequency, they did observe an increased amount of PCR product corresponding to recombinant RNAs from co-infections of the two parental viruses compared with that obtained from the two parental RNAs mixed after isolation. Instead, the PCR products were sequenced in order to locate cross-over sites in intertypic recombination events. The PHLOP phenomenon discussed above can unfortunately result in movement of the cross-over site during PCR, rendering this approach difficult to interpret. Zimmer and Gruss (1991) have developed a PCR method to detect DNA recombination in which 10-fold dilutions are used to find the threshold of detection of unlabeled PCR product. Although no further quantification was attempted, they noted the potential for PHLOP artifact formation and succeeded in minimizing it by optimization of the PCR reaction conditions to promote full-length products.

Quantitative PCR: the linear range of the assay

To measure recombination frequency using PCR methodology, it was necessary to establish conditions under which the PCR signal was proportional to the initial template concentration. Figure 2 shows a DNA dose–response curve in which the amount of input template was varied over 10 orders of magnitude. The PCR signal is expressed as the percentage of primers extended into full-length products. We found that the assay was linear between the lower detection limit (~0.1%) and ~2% primer reacted; in this range the signal was directly proportional to the initial template concentration. Above 2%, the slope gradually decreased until a plateau was reached, probably indicating that primer or polymerase concentration replaced template as the limiting factor in the amplification reaction. The dose–response curve is shown for both 20 and 35 cycles of PCR. Not surprisingly, the template concentration corresponding to the linear range is dependent on the number of cycles, and both variables can be adjusted when optimizing the assay for a particular application. This experiment also demonstrated the reproducibility of the assay, shown by duplicate and triplicate points. The average coefficient of variance of triplicate points was 9.8%.

Selecting appropriate viral polymorphic markers

What is the minimal sequence polymorphism in an RNA virus that can be used as a recombination marker? The goal was to design primers to anneal specifically to one parental sequence under PCR conditions while annealing or extending very poorly at the opposite parental locus. Since Taq DNA polymerase lacks 3′–5′ exonuclease activity, even if the primer were to anneal to the opposite polymorphic locus, a mismatch at the 3′ end of the primer might pose an effective barrier to elongation. In Figure 3, the reduction in the PCR signal obtained with a variety of mismatched primers from the signal obtained with a fully base-paired primer is shown. The background from mispairing was more noticeable at the higher number of cycles, presumably because once a mismatched primer has been extended in an early cycle, it presents a perfect priming site in subsequent cycles. Clearly a single G–G mismatch at the primer terminus was not sufficient to provide priming specificity under the temperature conditions chosen. However, single T–T and A–C mismatches may be more efficacious in blocking elongation, according to a report by Kwok *et al.* (1990). Two mismatches at either the first and second or first and third positions from the 3′ end of the oligonucleotide primer, however, greatly reduced the background, and three mismatches at the 3′ end virtually eliminated primer cross-reaction. Four or six internal mismatches (out of a total of 20 bp) also substantially reduced the background, presumably via thermodynamic destabilization of the primer–template. Thus the strategy to find an effective sequence polymorphism is to locate an insertion, deletion or cluster of point mutations and to position the 3′ end of the primer at one of the mutations, thereby taking advantage of both general mismatch destabilization and the impaired ability of Taq polymerase to elongate mispaired 3′ ends.

For comparison, Figure 3B shows the specificity of the primers that we used for the poliovirus recombination studies. The only noticeable cross-reaction was seen with

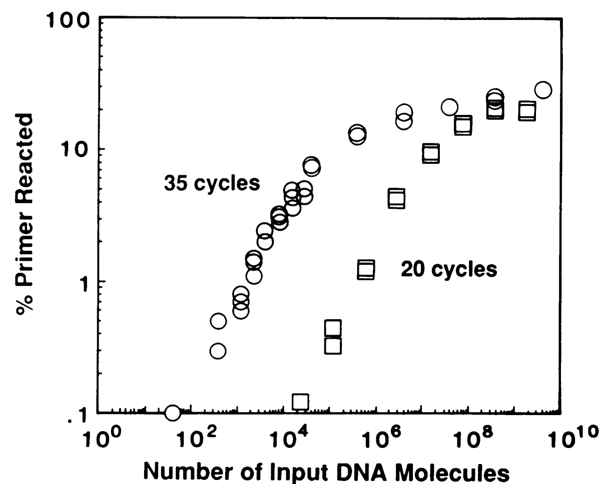

Fig. 2. PCR signal as a function of initial template concentration. PCR reactions were performed with varying amounts of linearized plasmid DNA containing poliovirus cDNA sequences. The target sequence was 1875 nucleotides. The amplification reactions used a temperature profile of 1 min at 94°C, 1 min at 55°C and 3.5 min at 72°C for 20 (□) or 35 cycles (○). Occasionally an inhibition is observed at very high template concentrations. We believe this is due to contaminants in the DNA preparation as it can usually be eliminated by further purification of the DNA. The concentration of Taq polymerase (Materials and methods) used in these studies was optimized to give a high efficiency of amplification while attempting to avoid problems associated with extremely high enzyme concentration, such as the formation of 'primer dimers' (Saiki, 1989). Lower polymerase concentrations result in a lower plateau signal, effectively limiting the linear range of the assay to an unacceptably narrow window. If A is the total amplification following n temperature cycles, then the PCR efficiency, X, is defined as $(1 + X)^n = A$. In the linear range of the assay (~1% primer reacted) the efficiency was ~0.7 for 35 cycles and 0.8 for 20 cycles.

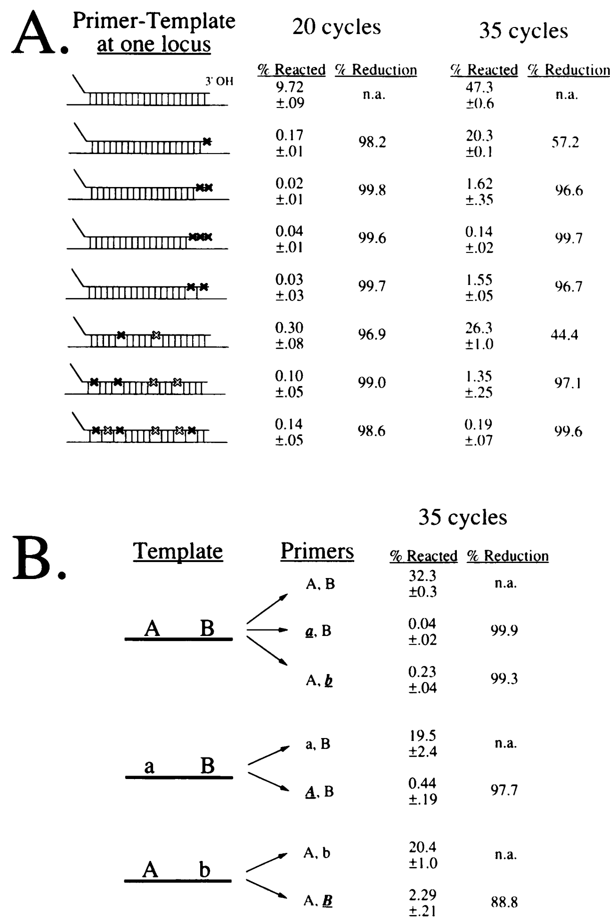

Fig. 3. PCR priming specificity. PCR reactions were performed in the presence of 0.3 ng of a 10 kb linearized plasmid containing poliovirus cDNA sequences. (**A**) Primer 5'ATCCGGTGAAAGTGAGACTC anneals from position 631 to 651 of negative-sense poliovirus and was kept constant throughout the experiment. No reaction was seen when this primer was omitted (not shown). The variable primer nominally anneals to position 2480–2499 of positive-sense poliovirus. The fully base-paired version is 20 nucleotides long; the sequence is 5'TCAAGCATCTGACCTAACCC. (✖) indicates a C→G change (creating a G–G mismatch) and (⌧) indicates an A→T changes (creating a T–T mismatch). The temperature profile was 1 min at 94°C, 1 min at 55°C and 3.5 min at 72°C for 20 or 35 cycles. (**B**) The target sequence was 656 nucleotides (Materials and methods). The temperature profile was 1 min at 94°C, 1 min at 60°C and 2 min at 72°C for 35 cycles. 'AB' indicates the wild-type poliovirus sequence; 'Ab' and 'aB' designate DNC-65 and 5NC-104 mutant poliovirus sequences, respectively. n.a. = not applicable.

primer 'B' annealing to the 'b' allele. Note that the high template concentration in all the experiments shown in Figure 3 exaggerates the background, because the signal from the fully base-paired primer was far above the linear range of the PCR assay (Figure 2). In practice, when the relevant signal was in the linear range of <2% primer reacted, even the degree of cross-reaction of primer 'B' with allele 'b' was insignificant.

Poliovirus recombination is predominantly homologous

The PCR signal was quantified by labeling one of the oligonucleotide PCR primers with ^{32}P at its 5' end, and separating the resulting PCR products by denaturing polyacrylamide gel electrophoresis as shown in Figure 4. The PCR reactions yielded a discrete product of the expected size (656 nucleotides). No such products were seen when control RNA from mock-infected cells was used as a cDNA template (lanes 11 and 12), confirming that cellular RNA sequences cannot produce a product of this size. In addition, when no template was added to the cDNA reactions, no PCR product was seen (lane 13), demonstrating the absence of cross-contamination of the samples. The primer pairs used to detect either recombinants 'AB' or parents 'aB' are indicated, as well as the relative cDNA dilutions. Lanes 1–5 show the background control, in which RNAs from separate parental infections were mixed before cDNA synthesis and carried through the entire assay. Only a very slight background signal was detectable at the highest cDNA concentration (lane 1). In lanes 6–10, cytoplasmic RNA was isolated from a mixed infection in which recombinant viral progeny may have arisen *in vivo*. Here, recombinant-specific primers 'A' and 'B' give a clear PCR signal above background (lanes 6 and 7).

That the PCR product in lanes 6 and 7 was a discrete 656 nucleotide band is consistent with RNA recombination being predominantly homologous. Non-homologous recombination, resulting in deletions or duplications, would have produced different product sizes. Clearly it would be possible to miss such events using this type of assay if they occurred in random positions in the genome and at low frequency. High frequency non-homologous recombination, however, would have resulted in an increase in radioactivity in regions of the gel above and below the 656 nucleotide band compared with that seen when the same degree of amplification was obtained starting with plasmid DNA or parental cDNA. This was not the case, leading us to conclude that non-homologous recombination, if it occurred at all, occurred at a much lower frequency than homologous recombination.

Reciprocal recombination frequencies are equal

Data such as that presented in the autoradiograph in Figure 4 can be quantified by direct scanning on a radioanalytic scanner, giving the percentage of primer that has been extended into full-length products. Figure 5 shows the result of a recombination experiment displayed graphically as percentage primer reacted (i.e. PCR signal) versus relative cDNA concentration. This analysis confirms that the data fall in the linear range of the PCR assay and provides a more accurate frequency determination than would be possible from the autoradiograph. The recombination frequency is given by the shift in the recombinant curve relative to the parent curve. For example, in part A, 2×10^{-4} less cDNA was required to obtain a 1% signal when using the parental primer pair than when using the recombinant primer pair. In this experiment, the background 'recombination frequency' was $<1\times10^{-6}$. Note that each frequency determination derives from ratios of PCR products obtained from a single cDNA reaction: 'AB'/'aB' from cDNA primed by 'B', and 'ab'/'Ab' from cDNA primed by 'b'. The recombination frequency measured was thus internally controlled and independent of the efficiency of cDNA priming and synthesis. However, to measure the true recombination frequency it was necessary to test for potential differences in PCR priming efficiency between primers 'A' and 'a'. In this case, the primers, which are identical in size and GC content, showed no differences when priming efficiency was tested using plasmid DNA of known concentration (not shown), so no corrections were necessary.

In contrast to most genetic assays, this method enables

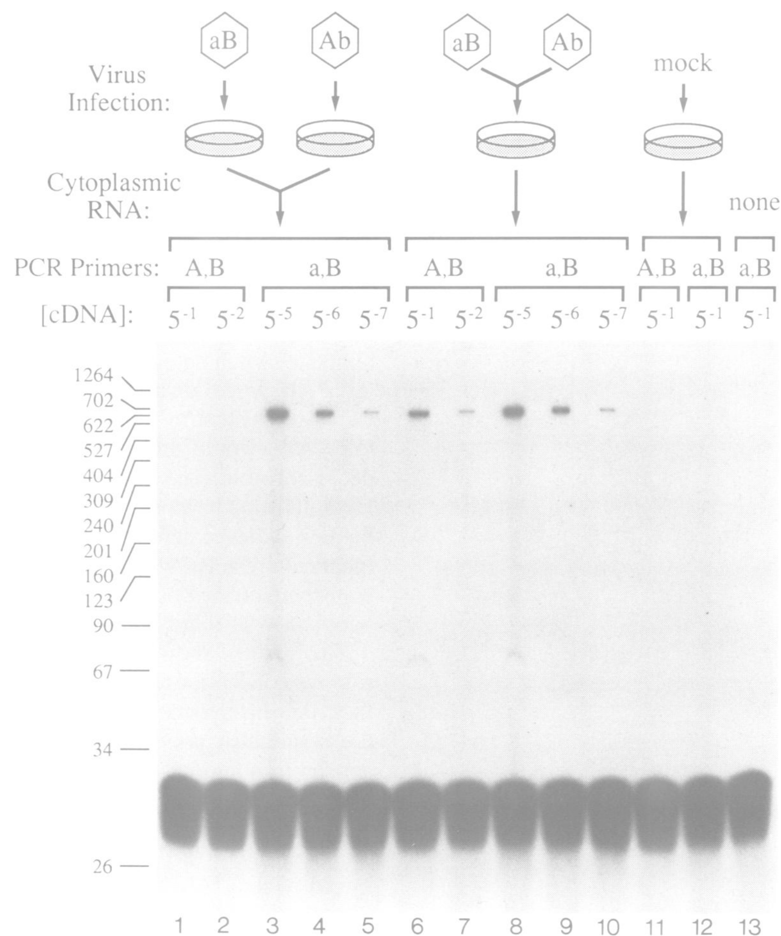

Fig. 4. Denaturing polyacrylamide gel analysis of PCR samples. Cytoplasmic RNA was isolated at 7 h post-infection from a mixed infection (m.o.i. 25 each, 'aB' and 'Ab'), as well as from separate infections (m.o.i. 50 each) and mock-infected cells. All cDNAs were primed by oligonucleotide 'B' and used in serial 5-fold dilutions as templates for 24-cycle PCR reactions containing the indicated primer pairs; 'A' + 'B' oligonucleotide primers were used to detect recombinants and 'a' + 'B' were used to detect parents. Lanes 1–5: RNA from separate 'Ab' and 'aB' infections, mixed prior to cDNA synthesis. Lanes 6–10: RNA from a mixed 'Ab' and 'aB' infection. Lanes 11–12: cellular RNA from a mock infection used as cDNA template. Lane 13: no RNA template present at cDNA step. The >1200 nucleotide band faintly visible above the primary band (lanes 6 and 7) was visible in the background control (lane 1) on longer exposures and also appears when amplifying plasmid DNA containing full-length polio cDNA (not shown). Therefore it probably represents a pseudosite for primer 'B', rather than a non-homologous recombination event.

one simultaneously to assay both reciprocal recombination frequencies. In part A the 'AB'/'aB' frequency was 2×10^{-4}; as shown in part B, the opposite reciprocal recombination frequency 'ab'/'Ab' was also 2×10^{-4}. Since both parental viruses were added at equal m.o.i. and both replicate at the same rate, this result was not unexpected. Altering these parameters might yield a different result, though. For example, since recombination is thought to occur primarily during negative-strand synthesis in poliovirus, one might expect that shutting off negative-strand synthesis of one of the parents via a conditional *cis*-acting mutation would prevent one type of reciprocal recombinant from occurring, while accumulation of the other would be unaffected. Thus, unequal reciprocal recombination frequencies could be diagnostic of differences in the RNA replication of the two parental viruses.

Unequal multiplicity of infection alters reciprocal recombination frequencies

Ledinko (1963) found that the highest poliovirus recombination frequency was obtained when the m.o.i. of each parent was equal. Asymmetric input ratios resulted in lower frequencies, regardless of which parent was in excess. The interpretation of this result was complicated, however, by the fact that the total multiplicity of infection varied as well. Cooper (1968) performed a similar experiment but at constant total m.o.i. In this case, the scatter in the duplicate measurements precluded detection of any significant effect on the recombination frequency. In both cases, the recombination frequency was reported in the standard way as the frequency of obtaining one particular reciprocal recombinant relative to the sum of the parent virus yields. This would tend to under-emphasize the frequency of template switching events in which the donor parent is the minority species in an asymmetric cross. In contrast, we report the frequency of occurrence of one reciprocal recombinant relative to the concentration of one parent only ('AB'/'aB' or 'ab'/'Ab'). Since poliovirus recombination is thought to occur during negative-strand synthesis, the parent in the denominator would be the donor parent; thus our method of reporting recombination frequency reflects the frequency of template switching relative to completion of synthesis on the same template on which replication was initiated.

We addressed the question of input ratio of parental viruses in the experiment shown in Table I. The total m.o.i. was held nearly constant, and the recombination frequency for both reciprocal types was measured at asymmetric and equivalent input m.o.i. ratios. While the recombination frequencies were equal for both reciprocal types at equal m.o.i., this situation was dramatically altered when one parental virus was present in 10-fold excess over the other. When 'Ab' was in excess, the 'AB'/'aB' ratio increased, while the 'ab'/'Ab' ratio decreased. The converse was true when 'aB' was in excess. The results in Table I roughly correlate with a simple statistical probability model. In other words, in an equal m.o.i. cross, each switching event would have a 50% chance of yielding a detectable recombinant, because switching to an acceptor template of the same parental type as the donor would be invisible. When 'Ab' is present in 10-fold excess over 'aB', template switching during negative-strand synthesis initiating on 'aB' would have a 90% probability of generating recombinant 'AB' (i.e. 'AB'/'aB' would go up almost 2-fold relative to the equal m.o.i. case); in the same cross, negative-strand synthesis initiating on 'Ab' would have only a 10% chance of a template switching event producing an 'ab' recombinant (i.e. 'ab'/'Ab' would decrease 5-fold). Conversely, when parent 'aB' was present in 10-fold excess, one would predict that 'ab'/'Ab' would increase 2-fold and 'AB'/'aB' would decrease 5-fold. The examples are given for the case in which recombination happens during negative-strand synthesis since there is evidence that this is the case in poliovirus. However, identical results would be expected if recombination happened during positive-strand synthesis, and the acceptor template was represented in the denominator.

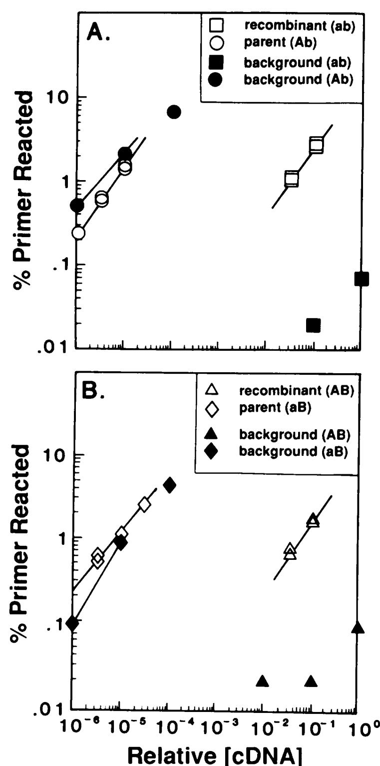

Fig. 5. Determining the reciprocal recombination frequency. RNAs were isolated at 7 h post-infection. The m.o.i. was 5 for each parent in the mixed infection and 10 in the separate infections. Open symbols: mixed infection; closed symbols: background control (separate infections with RNAs mixed prior to cDNA synthesis). Lines show the best fit of points in the linear range. (A) Relative concentrations of recombinant 'ab' and parent 'Ab', determined by dilutions of cDNA primed with 'b'. PCR primer pairs: (□,■) 'a'+'b'; (○,●) 'A'+'b'. (B) Relative concentration 'AB'/'aB', determined by dilutions of cDNA primed with 'B'. PCR primer pairs: (△,▲) 'A'+'B'; (◇, ◆) 'a'+'B'. Recombination frequencies were determined from the cDNA dilution required to superimpose parental and recombinant curves in the linear range of the PCR assay.

The results shown in Table I are consistent with the relative concentration dependent model described above. In fact, the differences between the reciprocal frequencies in the asymmetric crosses compared with the equivalent cross appear in some cases to be even more exaggerated than would be expected from the simple relative concentration model. For example, 'AB'/'aB' drops more than 5-fold in the case where 'aB' is in excess and increases more than 2-fold when 'Ab' is in excess. This may indicate an additional effect of absolute acceptor template concentration on the frequency of template switching during negative-strand synthesis. However, the data are not sufficiently refined to determine the extent to which absolute concentration plays a role. Such an effect would only manifest itself if the two parental templates are not utilized identically as acceptor templates. That is, there must be a slight preference to switch to an acceptor template of the same type as the donor template. The fact that one of the recombination markers consists of a 25-base deletion may introduce a sufficient difference in homology to account for such an effect. Using markers that are farther apart or using a more minimal polymorphism as a marker should eliminate this effect.

The apparent discrepancy between our results and those of Ledinko probably reflects the difference in the way recombination frequencies were calculated, rather than a fundamental difference in the frequency of actual recombination events between the two experiments. We

Table I. Dependence of reciprocal recombination frequencies on multiplicity of infection[a]

aB	Ab	AB/aB ($\times 10^3$)	Relative change[b]	Multiplicity of infection ab/Ab ($\times 10^3$)	Relative change
25	25	0.5 ± 0.1	n.a.[c]	0.6 ± 0.1	n.a.
5	50	1.6 ± 0.5	↑ 2×–5×	0.10 ± 0.05	↓ 3×–14×
50	5	0.02 ± 0.01	↓13×–60×	3.0 ± 1.5	↑ 2×–9×

[a]RNA isolated at 7.7 h post-infection.
[b]Increase or decrease in reciprocal recombination frequency in an asymmetric cross relative to the equal m.o.i. case, given as the range of fold-change based on recombination frequency error.
[c]Not applicable.

conclude that the frequency of recombination events is affected by both the absolute concentration of acceptor templates and the probability of a replication complex on a donor template encountering a template of the opposite parental type (i.e. relative concentration).

Recombination increases throughout RNA replication

We were interested in studying the kinetics of recombination throughout the replication cycle of poliovirus. For the intratypic cross shown in Figure 1, we assume that each of the two recombinant and two parent RNA molecules is equally competent to serve as template for subsequent rounds of replication. It is instructive to consider the predictions for the observed accumulation of recombinant progeny during the course of an infectious cycle for different frequencies of recombination during the cycle. First, if template switching events happen at a constant frequency throughout the course of RNA synthesis, then the fraction of recombinant molecules would increase at a linear rate with respect to RNA accumulation, as shown by the dashed line in Figure 6a. Alternatively, if recombination events were primarily limited to the early rounds of replication, as suggested by the studies of Cooper (1968) and Ledinko (1963), the situation would be better described by the upper (dotted) line in Figure 6a, in which the rate of accumulation of recombinants decreases, and the recombinant fraction plateaus, during RNA replication. Finally, if the frequency of recombination events increases during replication, the increasing rate of accumulation of recombinants would result in an upward sloping curve (solid line).

We addressed the question by isolating RNA at various times post-infection, and measuring the apparent recombination frequency (i.e. recombinant fraction) at those times. The results of one experiment are shown in Figure 6b. To make a meaningful comparison with the theoretical curves in Figure 6a, it was necessary to normalize the x axis to the amount of RNA synthesized (Figure 6c). This was accomplished by quantifying the relative amount of positive-stranded RNA in each sample using a dot-blot hybridization method, as shown in Figure 6d. No recombination was detected above background at the first time point, but controls showed that virtually no new RNA had been synthesized at that time (see legend). Subsequent time points, however, showed that the rate of accumulation of recombinants increased throughout the period of RNA synthesis. This result stood in sharp contrast to the earlier genetic results and seemed to rule out an inhibition of recombination late in infection. Possible reasons for this discrepancy will be addressed in the Discussion.

That the frequency of actual recombination events appeared to increase during replication was again consistent with the hypothesis that RNA concentration itself was a determining factor in template switching frequency. We repeated the time course at two different multiplicities of infection, as shown in Figure 6e. At each time point, the recombination frequency in the low m.o.i. experiment was consistently lower than that of the high m.o.i. experiment. Of course, the entire infection progresses at a faster rate at higher m.o.i., so it was again necessary to normalize to the amount of RNA synthesized at each point (Figure 6f). This caused the curves to superimpose, suggesting a correlation between the absolute RNA concentration and the template switching frequency *in vivo*.

Discussion

We were interested in defining the determinants of template switching during RNA virus replication. Using a physical assay for recombinant RNA molecules, we found that recombinants generated in a poliovirus cross between essentially wild-type parental viruses at an equivalent m.o.i. were predominantly homologous and arose at equal frequencies for both reciprocal types (Figure 5). It is important to recognize that the copy-choice recombination mechanism operative in poliovirus would be expected to yield such a result when both parental templates replicate at the same rate and are equally available to act as acceptor templates in a template switching event. This result does not imply reciprocity at the level of molecular events (that is, both reciprocal types are not necessarily generated simultaneously in a single recombination event) but rather is more simply interpreted as an equal probability of switching in either reciprocal sense. Interestingly, however, we find that infecting with unequal parental m.o.i.s significantly alters the observed reciprocal frequencies in a way that correlates with the mathematical probability of encountering the opposite parental type when switching templates and may indicate a dependence on absolute concentration as well.

The recombination frequency that we have measured for poliovirus mixed infections using a physical assay is $\sim 2 \times 10^{-3}$ per 600 nucleotides when measured very late in infection (Figure 6; note that experiments shown in Table I and Figures 4 and 5 represent recombination frequencies at slightly earlier times in infection). We have also measured a recombination frequency of 6×10^{-3} between markers in the capsid region ~ 1900 nucleotides apart (not shown), indicating a rough proportionality between template switching frequency and distance. Both the frequencies that we measured and the linearity with distance are consistent with previously reported poliovirus intratypic homologous recombination studies (Cooper, 1977; Kirkegaard and Baltimore, 1986). Intertypic poliovirus recombination studies have shown no specific consensus sequences when many different cross-over sites were examined (Kirkegaard and Baltimore, 1986; King, 1988b). Thus, template switching appears to occur at nearly random locations throughout the genome. Although there does not appear to be a consensus sequence for template switching, we cannot rule out the possibility of a common structural motif such as a small hairpin that might promote template switching. It is conceivable, for example, that any combination of structure and/or sequence that serves momentarily to slow the elongation rate of the viral polymerase might provide the kinetic window necessary to switch templates. Evidence for frequently occurring RNA structures that may promote poliovirus recombination have been reported by Tolskaya *et al.* (1987).

We have presented kinetic data showing that the frequency of recombination events increases throughout the replication cycle of the virus. This contradicts the results of Ledinko (1963) and Cooper (1968) who both measured only a 2-fold increase in recombinant fraction between a point early and a point late in infection. This discrepancy may simply reflect the paucity of time points in the genetic studies or may be the result of genetic interactions between the mutants used. The data in Figure 6 show that the rate of accumulation of recombinants increases most sharply between 7 and 9 h post-

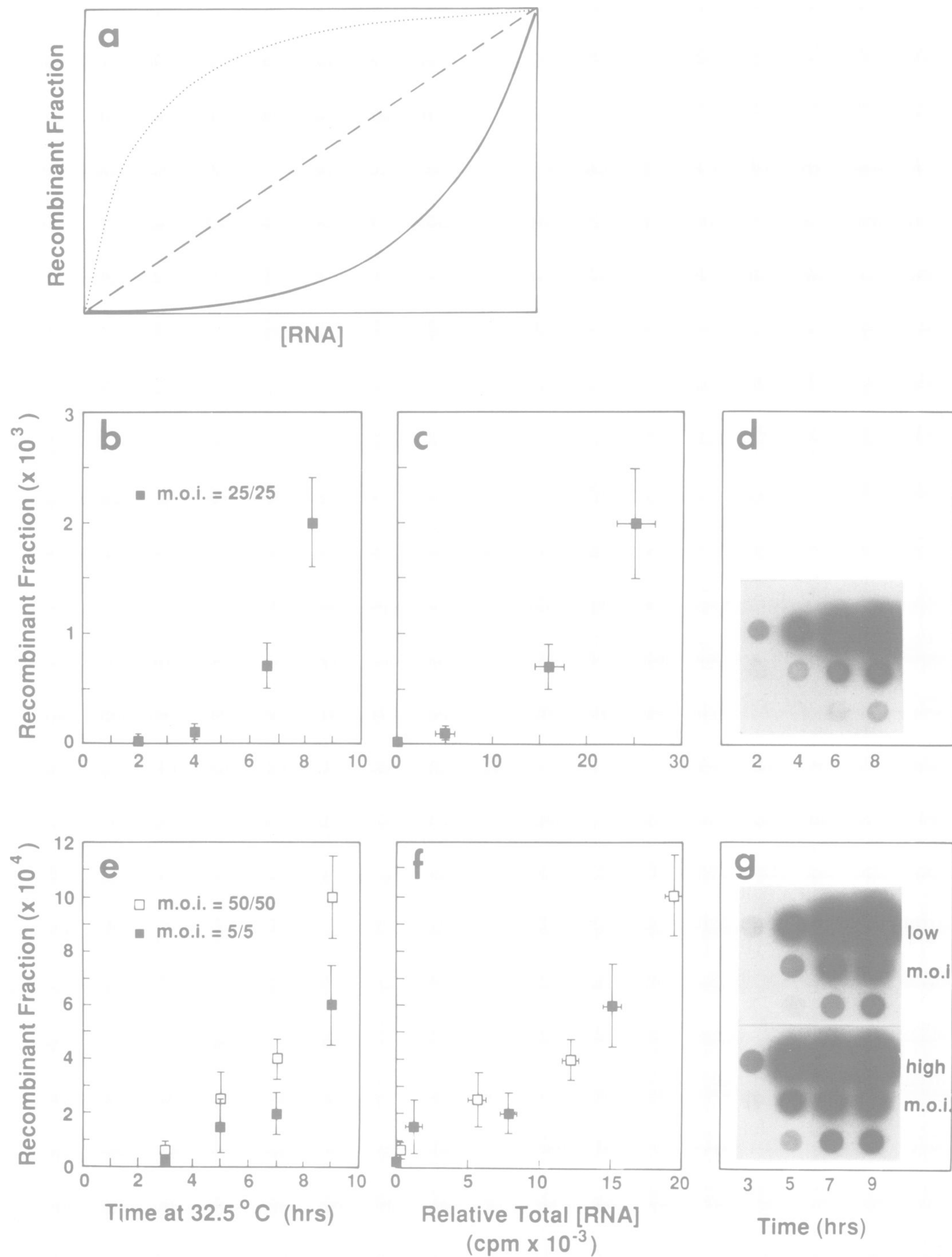

Fig. 6. RNA recombination kinetics during poliovirus infection. (**a**) Theoretical curves depicting the accumulation of recombinants as a function of RNA concentration given. (······), decreasing rate of template switching during RNA synthesis in a single-cycle poliovirus infection; (— — —), constant rate of template switching; (——), increasing rate of template switching. (**b**) The recombination frequency was measured at various times post-infection (32.5°C) in mixed infections at an m.o.i. of 25 for each parent. Each frequency determination was derived from 15–25 PCR reactions, analyzed as shown in Figure 5. *y*-error bars were estimated from the correlation coefficients of the least-squares fit of the parent and recombinant data. (**c**) Data from (b) plotted as a function of the amount of newly synthesized positive-sense RNA at each point as determined by dot-blot analysis. *x*-error bars show the c.p.m. range on duplicate blots. (**d**) Dot-blots of RNA samples from 2, 4, 6.5 and 8 h. Serial 10-fold dilutions (top to bottom) confirm conditions of probe excess. Parallel infections performed in the presence of guanidine, a potent inhibitor of polio replication indicated the amount of input RNA remaining at the early times points (not shown). Very little new RNA had been synthesized at 2 h, while by 4 h >90% of the RNA was newly synthesized. (**e**) Recombinant fraction plotted as a function of time for mixed infections at m.o.i. of 5 each (■) or 50 each (□) for the parental viruses. (*f*) Data in (e) plotted versus relative RNA concentration. (**g**) Dot-blots of RNA samples from 3, 5, 7 and 9 h (in serial 10-fold dilutions). Top: m.o.i. of 5 each parent. Bottom: m.o.i. of 50 each. Comparison with infections in the presence of guanidine showed that the 3 h time point for the high m.o.i. infection contained 90% new RNA, while the 3 h time point for the low m.o.i. infection contained ~50% new RNA. The recombination frequencies in this figure are from measurement of 'ab'/'Ab'. Virtually identical results were obtained for reciprocal type 'AB'/'aB' (not shown).

infection (at 32.5°C). Thus the timing of the final points in the time course is critical in observing this increase. The discrepancy may also be the result of selection. For example, some structural feature of the recombinational intermediate late in infection may block efficient packaging. In any case, our results clearly indicate that there is no RNA sequestration or other inhibition of the recombination event itself late in infection. The data in Figure 6 are not sufficiently refined, however, to allow us to distinguish between a model in which the frequency of recombination events increases steadily throughout replication (perhaps in response to RNA concentration as we have proposed) and one in which the curve is biphasic (i.e. the replication environment suddenly becomes more recombinagenic late in infection).

A more detailed understanding of RNA virus recombination may require a more complete understanding of the structure of the replicative intermediate and the environment in which RNA replication takes place *in vivo*. Although the detailed cell biology of poliovirus infection is unclear, it appears that poliovirus replication occurs in association with membranous vesicles (Bienz *et al.*, 1980, 1987; Guinea and Carrasco, 1990). In addition, the replicative intermediate *in vivo* has a highly single-stranded character, with multiple positive strands being made from a single negative-strand template (Bishop *et al.*, 1969; Richards *et al.*, 1984). The single-stranded regions of the nascent strands could facilitate template alignments required for homologous strand switching. The full complement of viral and cellular proteins required to establish and maintain this replicative structure is as yet unknown.

Several lines of evidence suggest that RNA recombination frequency in poliovirus-infected cells may be a direct function of acceptor RNA template concentration. First, the observed increase in recombination frequency during the infectious cycle could result from the concomitant increase in intracellular RNA concentration (Figure 6). Since the recombination frequency is determined as a ratio between recombinant RNAs and donor parental RNA molecules, the increased concentration of donor parental RNAs should not affect the calculated recombination frequency. Therefore, any dependence on absolute intracellular viral RNA concentration must be a dependence on acceptor parental template RNA concentration.

In further support of the RNA concentration dependent model, increasing the m.o.i. of both parental viruses resulted in a higher recombinant fraction at any given time post-infection (Figure 6e). High m.o.i. infections progress at an accelerated rate compared with low m.o.i. infections. However, the data at low and high m.o.i. could be roughly normalized to the stage of infection by measuring the amount of RNA synthesized as a function of time (Figure 6f). The two data sets superimposed upon normalization, again suggesting a correlation between the concentration of the RNA and the frequency of template switching. Of course, one cannot rule out the influence of some other factor in the cellular environment that changes in parallel with RNA concentration as the infection progresses.

A third line of evidence suggesting a dependence of RNA recombination frequency on absolute RNA concentration is the frequency of formation of different reciprocal recombinants when the ratios of input parental viruses are altered (Table I). Compared with the expected effect of relative concentration alone, a 10-fold asymmetry in the amount of input parental virus has the effect of exaggerating the difference between the two reciprocal recombination frequencies. Specifically, the reciprocal recombinant whose 5′ end derives from the more abundant parent shows a >2-fold increase in frequency and the recombinant whose 5′ end derives from the less abundant parent shows a >5-fold decrease in frequency. This is consistent with a dependence on absolute concentration for template switching during negative-strand synthesis.

Finally, an intriguing consequence of the concentration dependent model is that it provides an explanation for the anomalous bias toward recombination during negative-strand synthesis. Positive strands outnumber negative strands by about 50:1 throughout poliovirus infection (Novak and Kirkegaard, 1991). Thus if a limiting factor in the template switching process is the concentration of the acceptor template, replication complexes making negative strands would have a much higher concentration of available acceptor templates (positive strands) than would replication complexes making positive strands.

The assay we have described represents a valuable tool for future RNA recombination studies. For example, the RNA concentration dependence model will be tested by measuring the strand specificity of recombination in poliovirus mutants showing altered positive-to-negative strand ratios. In addition, we can test whether selection plays a role in limiting the frequency of intertypic recombination. Finally, we would like to take advantage of both the sensitivity and the absence of selection in the assay to test for recombination in other viruses in which recombination has not previously been observed. In so doing we hope to learn whether or not the ability to recombine is a ubiquitous property of all RNA viruses.

Materials and methods

Cells and viruses

HeLa cells were grown in Petri dishes in DME (Dulbecco's modified Eagle's) medium supplemented with 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Penstrep; GIBCO-BRL). Poliovirus stocks were derived from single plaque isolates resulting from transfection of either infectious cDNA (Racaniello and Baltimore, 1981a) or infectious *in vitro* transcribed RNA (van der Werf *et al.*, 1986; Sarnow, 1989). Parent strain 'Ab' was 5NC-104, a 25 base deletion from nucleotides 632–656 (Compton *et al.*, 1989) and strain 'aB' was DNC-65, an exchange of nucleotides 10–13 with nucleotides 31–34 in an RNA stem–loop structure, a gift from Raul Andino (Andino *et al.*, 1990). 5NC-104 and DNC-65 are both derivatives of Mahoney type 1 poliovirus and are phenotypically wild-type.

Virus infection

Unless otherwise stated, all recombinant crosses were between 5NC-104 and DNC-65. Four infections were required for each recombination experiment: mixed infection of both parental types at equal multiplicity of infection (m.o.i. × as indicated), single infections of each parental type (m.o.i. 2×), and mock infection (no virus). Monolayers of 60–80% confluent HeLa cells ($\sim 8\times10^6$ cells per 150 mm Petri dish) were washed twice with 10 ml PBS^+ (phosphate buffered saline containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$). Plates were inoculated with the appropriate combination of viruses in a total volume of 1.5 ml PBS^+, and the virus was allowed to adsorb for 30 min at 32.5°C. The plates were then washed with 20 ml PBS^+ to remove unbound virus, followed by the addition of 25 ml of DME supplemented with 10% fetal calf serum and 1% Penstrep. The plates were incubated at 32.5°C for the stated length of time; time zero indicates the time at which the medium was added. These studies were performed at 32.5°C because the infections progress somewhat slower than at 37°C and this facilitated RNA isolation at frequent time intervals. Experiments performed at 37°C showed no significant difference in recombination frequency (not shown).

Total cytoplasmic RNA preparation

Cells were harvested by washing with 25 ml ice-cold RSB (10 mM Tris−Cl pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$), scraping and pelleting for 5 min at 200 *g* at 4°C. The supernatant was removed and the cells were lysed by resuspending the pellet in 0.9 ml cold RSB + 1% Nonidet P-40 + 5 mM ribonucleoside vanadyl complex (VRC; New England Biolabs). The nuclei were pelleted for 10 min at 2000 *g* at 4°C. The supernatant was transferred to a tube containing 50 μl of 10% SDS and 0.7 ml phenol was added. 0.9 ml of the aqueous phase was removed and ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 30 mM to dissociate the VRC. The samples were divided into aliquots, ethanol precipitated twice and stored under ethanol at −20°C. The typical yield of total cytoplasmic RNA was about 10−20 pg per cell.

Oligonucleotide synthesis and purification

Oligonucleotide primers were synthesized as described (Gauss *et al.*, 1987). The nine nucleotides at the 5′ ends contain restriction sites; the 3′ ends contain poliovirus sequences (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981b). Primers were designed to anneal with high specificity to polymorphic loci in the parent virus genome. Particular care was taken to avoid sequences in which the 3′ end of the primer would be likely to anneal to the opposite parental sequence or to pseudosites elsewhere in the poliovirus genome. The oligonucleotides were purified by polyacrylamide gel electrophoresis and stored in TE (10 mM Tris−Cl pH 7.5, 0.1 mM EDTA) buffer at −20°C.

'A' = 5′ AAAGAATTCGGGTTGTACCCACCCCAGAGG, complementary to negative-sense 5NC-104 from nucleotides 15 to 34.

'a' = 5′ AAAGAATTCGGGTTGTACCCACCCCTCTCG, complementary to negative-sense DNC-65 from nucleotides 15 to 34.

'B' = 5′ AAAGTCGACTGAGTCTCACTTTCACCGGA, complementary to positive-sense DNC-65 from nucleotides 633 to 652.

'b' = 5′ AAAGTCGACTCCAGCAAACAGATAGGGCC, complementary to positive sense 5NC-104 3′ of nucleotide 627.

cDNA synthesis

First-strand cDNAs were synthesized by incubating 50 μg total cytoplasmic RNA from infected cells with 10 nM of the appropriate oligonucleotide primer molecules, 'B' or 'b', in a 50 μl reaction volume containing 50 mM Tris−Cl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 40 U RNasin (Promega), 1.5 mM deoxynucleotide triphosphates, and 40−200 U Superscript (RNase H^-) Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories) for 1 h at 47°C. The reaction was initiated by adding prewarmed template (15 μl in TE) to prewarmed enzyme/primer mix (35 μl). As discussed below, it was necessary to optimize the amount of reverse transcriptase for each batch of enzyme to maintain priming specificity at the cDNA step. The reaction mixtures were then adjusted to 4 M urea and incubated for 1 h at 60°C with 1 U of DNase-free RNase (Boehringer Mannheim). The samples were diluted to 500 μl in TE, placed in Centricon-100 microfiltration unit (Amicon) and centrifuged at 900 *g* for 15 min in a Beckman JA-20 fixed angle rotor, followed by a second dilution to 1 ml in TE and centrifugation for 35 min. The molecular weight cut-off of the filter allows nucleotides, primers and small pieces of RNA to pass through, while retaining cDNA larger than ~300 nucleotides. The remaining volume (~50 μl) was stored at −70°C.

The reverse transcriptase concentration was crucial in avoiding the production of PHLOP products (see Results) and minimizing primer cross-reaction; if too much reverse transcriptase was used, priming specificity was lost. In fact, under some conditions, a significant amount of viral cDNA could be made even in the absence of exogenous primer, probably as a result of priming at nicks in the viral RNA downstream of the 'B/b' locus, or of priming by cellular RNAs. By lowering the enzyme concentration, however, it was possible to achieve conditions under which only perfectly annealed primers were extended. As a control, cytoplasmic RNA from separate infections of each of the parent viruses was mixed prior to the cDNA synthesis step and the appearance of any apparent recombinants was monitored. Table II shows the apparent recombination background from cDNA reactions performed using varying amounts of two commercially available reverse transcriptase preparations. Clearly, from the standpoint of lowering the background, it was desirable to keep the enzyme concentration low. Although this resulted in lowered yield of cDNA, even the lowest yield shown in Table II provided ample signal for the PCR assay. In subsequent experiments we used a derivative of M-MLV reverse transcriptase that lacks the RNase H domain. This enzyme is stable at 45−50°C; the higher reaction temperature was found to increase priming specificity. Furthermore, literature reports indicate that RNase H activity may promote strand switching of reverse transcriptase *in vitro* and we hoped to minimize this additional potential source of background (Luo and Taylor, 1990).

An alternate protocol is currently being developed in this laboratory (J.Pata, unpublished results). Using biotinylated cDNA primers, first-strand cDNA products can be immobilized on streptavidin beads. Specifically primed second-strand cDNA can then be synthesized by a thermostable DNA polymerase, eluted from the beads and used for quantitative PCR analysis. This approach circumvents the problem of non-primer dependent first-strand cDNA priming at high reverse transcriptase concentrations, and also would be applicable as a DNA recombination assay.

Quantitative PCR

One of the PCR primers was end-labeled to a specific activity of ~1 μCi/pmol with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). Labeled primers were drop-dialyzed (Berger and Kimmel, 1987) on Millipore VSWP 0.025 μm filters against TE to remove unincorporated [γ-^{32}P]ATP and kinase buffer components. PCR reactions contained 50 mM potassium glutamate, 20 mM potassium HEPES pH 8.4, 3.3 mM $MgCl_2$, 0.1 mg/ml acetylated BSA (US Biochemicals), 0.5 mM deoxynucleotide triphosphates (Pharmacia), 0.5 μM [3′OH] cold primer 'B' or 'b', 0.45 μM cold primer 'A' or 'a', 0.05 μM radioactively labeled primer 'A' or 'a', 2 U Amplitaq DNA polymerase (Perkin Elmer-Cetus) and varying amounts of template DNA (either linearized plasmid DNA or cDNA as indicated). Reproducibility was enhanced by creating a master mix containing the enzyme and buffer components, which was then aliquoted into four mixes containing the four different combinations of primers ('A'+'B', 'A'+'b', 'a'+'B' and 'a'+'b'). 15 μl aliquots of these primer/enzyme mixes were then transferred to 0.5 ml tubes on ice. Template dilutions (5 μl) were added on ice, followed by 25 μl light mineral oil (Sigma). Unless otherwise indicated, a temperature profile of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C was used for 20−35 cycles in a programmable thermal cycler (MJ Research). Following the reaction, the samples were

Table II. Varying reverse transcriptase concentration affects background recombination frequency

M-MLV reverse transcriptase[a]			AMV reverse transcriptase[b]		
Units[c]	Background frequency[d]	Relative yield (%)[e]	Units	Background frequency	Relative yield (%)
40	$<1\times10^{-6}$	0.5	3.6	5×10^{-5}	15
400	1×10^{-5}	5	36	5×10^{-3}	25
2000	3×10^{-4}	10	180	1×10^{-2}	100

[a]RNase H^+; incubated 1 h at 37°C.

[b]Avian myeloblastosis virus reverse transcriptase; incubated 1 h at 47°C in the same buffer as MLV reverse transcriptase except 50 mM KCl and 0.1 mg/ml bovine serum albumin (BSA). Note that the difference between AMV and M-MLV reverse transcriptase may be indicative of a difference in enzyme activity, rather than any intrinsic difference in ability to elongate a mismatched primer-template.

[c]Units of enzyme in a 50 μl reaction.

[d]Indicates the apparent recombination frequency detected by the PCR recombination assay when parental RNAs from separate infections were mixed prior to cDNA synthesis.

[e]Approximate yield of full-length cDNA, normalized to highest amount obtained (18 ng from 50 μg of total cytoplasmic RNA). This amount corresponds to at least 75% conversion of available poliovirus positive strands (of both parental types).

stored at 4°C. Samples were analyzed by electrophoresis on 5% polyacrylamide−7.5 M urea gels. 5 μl from each PCR reaction plus 25 μl formamide loading buffer (96% formamide, 10 mM EDTA) were heated for 1 min at 95°C and quick-chilled on ice prior to loading. Gels were either dried on Whatman DE-81 paper or fixed in 7% acetic acid and dried on Whatman 3MM paper. Gels were scanned directly on an AMBIS Systems radioanalytic scanner to quantify the radioactivity. Size markers for the gels were λ *Bst*EII and pBR322 *Msp*I digests (New England Biolabs).

Quantitative PCR methods for determining the absolute amount of RNA using an internal standard have been recently reported (Wang *et al.*,1989, for example). This strategy is valuable for ascertaining the absolute concentration of mRNA species in a narrow concentration range (about two orders of magnitude). In contrast, our assay is particularly designed to measure the concentration of a low frequency species (the recombinant RNA) relative to a much more abundant species (the parent RNA), covering a range of up to six orders of magnitude.

Quantifying of RNA by dot-blot hybridization

A radiolabeled negative-sense poliovirus RNA probe (from 6016 to 5601) was made by *in vitro* transcription in the presence of [α-^{32}P]UTP. To 15 μl of each cytoplasmic RNA sample in TE were added 9 μl 20×SSC (1×SSC = 0.15 M NaCl, 0.015 M trisodium citrate) and 6 μl 37% formaldehyde; the mixtures were heated at 60°C for 15 min. Serial 10-fold dilutions of the RNA into 15×SSC were performed in microtiter dishes (VWR). 80 μl of each dilution were applied to BA85 0.45 μm nitrocellulose filters (pre-wetted with 15×SSC) in a dot Minifold (Schleicher and Schuell) with gentle suction, followed by washing with an equal volume of 15×SSC. The filters were then baked for 90 min at 80°C. Prehybridization was performed by rocking overnight at 68°C in the presence of 6×SSC, 2×Denhardt's reagent (Maniatis *et al.*, 1982) and 0.1% SDS in a sealed plastic bag. The radioactive RNA probe was then added, followed by rocking overnight at 68°C. The filters were then washed for 20 min at 25°C in 1×SSC and 0.1% SDS, followed by three 20 min washes at 55°C in 0.2×SSC and 0.1% SDS. Radioactivity in each dot was quantified on an Ambis Systems radioanalytic scanner and by autoradiography.

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