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Poliovirus RNA recombination in cell-free extracts

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ABSTRACT

Poliovirus RNA has been shown to undergo homologous genetic recombination at a high frequency in infected human cells. Recently it has become possible to mimic the entire intracellular replicative cycle of poliovirus replication in cytoplasmic extracts prepared from HeLa cells, resulting in the generation of infectious poliovirions. The mechanism of poliovirus RNA recombination has been shown previously to be coupled to RNA replication, presumably by template switching during the replication of parental RNAs. Experiments were designed to test whether recombinant poliovirus RNA molecules are produced in a cell-free environment. Recombinant molecules generated bear marker sequences that can be detected physically by reverse transcription and PCR. We report here successful detection of poliovirus RNA recombination in a cell-free replication system. The frequency measured for cell-free RNA recombination between two polymorphic marker loci 656 nt apart was between 10^{-2} and 10^{-3} recombinants/genome, a frequency comparable to or slightly higher than that measured for RNA recombination in infected cells.

Keywords: brefeldin A; copy-choice; enviroxime; replicative recombination; reverse transcriptase; RNA-dependent RNA polymerase; template switching

INTRODUCTION

Poliovirus, a positive-strand RNA virus, undergoes homologous recombination at a frequency estimated to be as high as 10^{-1}–10^{-2} recombination events per genome in infected cells (King, 1988; Jarvis & Kirkegaard, 1991; Lai, 1992). The frequency at which recombination occurs has been measured both by selecting for recombinant progeny that show the combined growth characteristics of two different parent viruses (Hirst, 1962; Ledinko, 1965; Cooper, 1968; Kirkegaard & Baltimore, 1986), and by direct physical detection of recombinant RNA molecules in the absence of selection (Jarvis & Kirkegaard, 1992). Poliovirus RNA recombination is coupled to RNA replication: it was shown that nonreplicating RNAs could donate the 5′ end but not the 3′ end of recombinant viruses, and that the 3′ end must be donated by an actively replicating genome. This genetic result was interpreted to mean that RNA recombination in poliovirus occurs by template switching during negative-strand synthesis, in which an RNA replication complex that has initiated on the 3′ end of one parental RNA (donor) switches to a second parental RNA (acceptor) to complete synthesis of the negative strand (Kirkegaard & Baltimore, 1986). According to this model, the preference for template switching during negative-strand synthesis could be explained by the 50-fold higher concentration of acceptor positive strands in infected cells (Novak & Kirkegaard, 1991; Jarvis & Kirkegaard, 1992).

To examine the mechanistic details of copy-choice RNA recombination, such as whether template switching is processive or nonprocessive and what constituents of the replication complex are required, it would be useful to stage the recombination event in vitro. However, reconstituting authentic poliovirus RNA replication outside the confines of a cell posed a formidable challenge. In infected cells, poliovirus replication complexes are found attached to membranous vesicles induced during infection (Bienz et al., 1992). Identification of the different proteins and RNA elements that make up the poliovirus replication complex is ongoing. Studies have implicated various proteolytic precursors of poliovirus proteins, such as 3AB and 3CD (Andino et al., 1990, 1993; Xiang et al., 1995), as con-

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stituents of functional replication complexes. Sam 68, a human protein that associates with Src during mitosis, has been shown to interact physically with the viral polymerase 3D (McBride et al., 1996). A 36-kDa ribosome-associated cellular protein is a component of a ribonucleoprotein complex formed at the 5′ end of the poliovirus genomic RNA. This 5′ RNP is postulated to be required for the synthesis of positive-sense RNA (Andino et al., 1993). The mechanisms of RNA priming, strand displacement, and template recognition remain unknown.

Recently, a cell-free replication system was developed that supports the production of authentic poliovirions (Molla et al., 1991; Barton & Flanegan, 1993; Barton et al., 1996b). When programmed with purified viral RNA, crude cytoplasmic extracts prepared from HeLa cells can perform viral-specific RNA translation, RNA replication, and RNA packaging, giving rise to infectious polioviruses. However, not all aspects of the poliovirus replicative cycle are mimicked in the cell-free replication system. Potent inhibitors of poliovirus replication in mammalian cells, such as brefeldin A (Maynell et al., 1992; Aldabe & Carrasco, 1995) and enviroxime (Heinz & Vance, 1995), were tested in this work and found to cause no significant change in the number of infectious poliovirions produced in these cytoplasmic extracts (Table 1). To test whether this cell-free system would support poliovirus RNA recombination, we employed a replication scheme in which two poliovirus RNAs carrying polymorphic sequences in their 5′ noncoding regions were used to program viral replication reactions. Recombinant RNA molecules generated were then detected physically using reverse transcription and quantitative PCR (Jarvis & Kirkegaard, 1992). We demonstrate here the successful detection of RNA recombination in this cell-free replication system.

TABLE 1. Cell-free production of poliovirions in the presence of antiviral reagents.

Antiviral reagents	Concentrations	Titer (pfu/mL)
None	—	4.5×10^6
Guanidine HCl	0.5 mM	3.1×10^5
Guanidine HCl	1.0 mM	4.5×10^5
Guanidine HCl	2.0 mM	<20
DMSO	2%	2.3×10^5
Enviroxime[a,b]	2 μg/mL	5.0×10^4
Enviroxime[a,b]	100 μg/mL	6.4×10^5
Enviroxime[a,b]	1,000 μg/mL	4.0×10^3
Brefeldin A[a,b]	100 μg/mL	3.2×10^5
Brefeldin A[a,b]	1,000 μg/mL	1.7×10^5

[a] Enviroxime and brefeldin A were solubilized in DMSO. The final concentration of DMSO in each replication reaction was 2%.

[b] Concentrations of antiviral reagents used to inhibit poliovirus replication in mammalian cells: enviroxime (1–10 mg/mL), brefeldin A (2–5 mg/mL).

RESULTS

Viral production in the presence and absence of antiviral reagents in cell-free extracts

Table 1 shows the effects of guanidine HCl, enviroxime, and brefeldin A on the production of infectious poliovirus in cell-free extracts. Guanidine HCl, which was shown previously to inhibit drastically the generation of infectious poliovirus at 2 mM in these cell-free extracts (Molla et al., 1991; Barton et al., 1995), was used as a positive control for inhibition. Incubation of infected cells with 2 μg/mL of brefeldin A has been shown to cause a reduction in poliovirus titer of 10^4–10^6 (Maynell et al., 1992; Aldabe & Carrasco, 1995). However, even at a concentration of 1,000 μg/mL in the extract, the poliovirus titer remained unchanged compared with the titer obtained by replication in extracts containing 2% DMSO.

Enviroxime at 1 μg/mL when incubated with infected cells causes a 10^3–10^4 reduction in poliovirus titer (Heinz & Vance, 1995). In cell-free extracts, however, only a twofold reduction in poliovirus titer was observed when enviroxime was present at a concentration 100 times higher than that used to inhibit poliovirus production in mammalian cells.

Guanidine HCl, brefeldin A, and enviroxime have all been shown to inhibit the poliovirus replicative cycle at the level of RNA synthesis. These antiviral reagents do not affect translation or protein processing (Molla et al., 1991; Maynell et al., 1992; Heinz & Vance, 1995). The different sensitivity of poliovirus RNA replication to brefeldin A and enviroxime in these cell-free extracts argues that not all features of the poliovirus RNA replication complex are mimicked in these extracts.

Cell-free replication and recombination assay

To test whether RNA recombination can occur in this cell-free replication system, physically marked poliovirus genomes (Fig. 1A) were used to program mixed or individual reactions. These Mahoney type I poliovirus strains, 5NC-104 (Compton et al., 1989) and DNC-65 (Andino et al., 1990), are termed Ab and aB, respectively, in reference to the physical markers they bear (Jarvis & Kirkegaard, 1992). Ab has a 25-base deletion, from nt 632 to 656 (Compton et al., 1989), and aB contains an exchange of nt 10–13 with nt 31–34 (Andino et al., 1990). Aside from the sequence changes in the 5′ noncoding region of the viral genomes, both Ab and aB are wild type in sequence. The primers A, a, B, and b (Fig. 1B) were designed to anneal to the polymorphic marker loci and show very low amounts of priming from their opposite polymorphic locus (Jarvis & Kirkegaard, 1992). By using different sets of primers in PCR reactions, recombination events within the

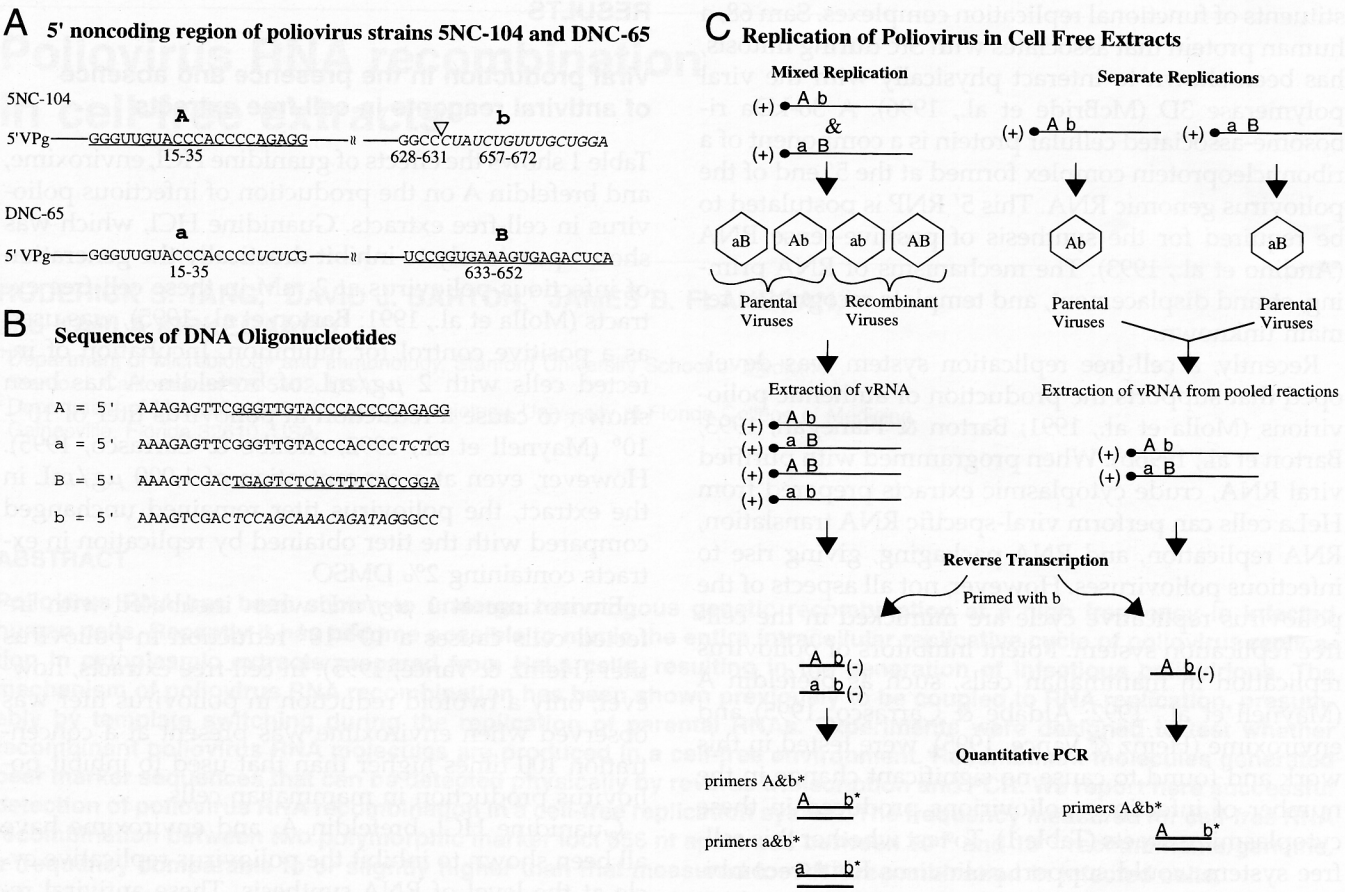

FIGURE 1. Experimental design for the generation and detection of recombinant RNA molecules in cell-free extracts. **A:** Parental poliovirus RNAs that contain polymorphic loci in the 5′ noncoding regions. 5NC-104 (denoted Ab) is a poliovirus strain in which nt 632–656 have been deleted from the region indicated by an inverted triangle. Wild-type sequences from nt 15–35 constitute physical marker "A," mutant sequences from nt 628 on constitute physical marker "b" and are in italics (Compton et al., 1989). DNC-65 (denoted aB) is a poliovirus strain in which nt 10–13 were exchanged with nt 31–34 (Andino et al., 1990). Sequences from 15–35, which contain exchanged nt 31–34 as indicated in italics, constitute physical marker "a". Wild-type sequences from 633–652 constitute physical marker "B". Apart from the sequence changes in the 5′ noncoding region, the remaining sequences of both strains are the same as that of Mahoney type I poliovirus. **B:** Sequences of DNA oligonucleotides for cDNA synthesis and PCR are shown. Sequences encoding sites for restriction endonucleases *Eco*R I and *Sal* I are present at the 5′ ends of oligonucleotides A and a, and B and b, respectively. The 3′ ends of the oligonucleotides contain sequences designed to anneal specifically to the indicated poliovirus sequences. **C:** To assay for the occurrence and frequency of RNA recombination, the two parental viral RNAs carrying polymorphic sequences shown in Figure 1A are used to program replication reactions in HeLa cytoplasmic extracts. Mixed replication reactions contained Ab and aB parental RNAs in a 1:1 ratio. In separate experiments, replication reactions containing only one type of parental RNA are performed. These reactions were mixed before reverse transcription as a control to show that recombinant RNA molecules were not generated during cDNA synthesis or PCR. DNA oligonucleotide b was used to prime reverse transcription reactions from the mixed replication and control reactions. The products of this reaction should be cDNAs with parental sequences Ab and recombinant sequences ab. These cDNAs are amplified by PCR for 35 cycles with the specific DNA primers shown. Primer b is end-labeled with [γ-^{32}P]ATP, as indicated with an asterisk. ——, RNA; ——, DNA.

656-nt interval between locus A/a and locus B/b can be detected (Fig. 1C).

When cell-free extracts are programmed with Ab vRNA, aB vRNA, or both in combination, translation, RNA replication, and RNA packaging should all proceed during the 34 °C incubation (Molla et al., 1991; Barton & Flanegan, 1993). To detect the presence of parental and recombinant RNAs, cDNA synthesis can be primed with oligonucleotide B or b, designed to anneal to viral positive strands. The relative abundance of parental and recombinant cDNAs can then be determined by quantitative PCR (Fig. 1C).

Viral production and RNA accumulation of parents Ab and aB

To test the ability of Ab and aB viral RNAs to program the production of infectious viruses, identical amounts of these viral RNAs were used to program cell-free extracts. In viral infections and transfec-

tions, the phenotypes of Ab and aB have been shown previously to be wild type (Compton et al., 1989; Andino et al., 1990). Figure 2 shows the generation of infectious poliovirions in separate Ab and aB replication reactions and in mixed Ab + aB replication reaction. Surprisingly, replication reactions performed with Ab vRNA consistently gave 10–100-fold more plaque-forming units than replication reactions programmed with aB vRNA. This difference is reproducible with different preparations of HeLa extracts, as well as with different preparations of vRNA used to program the extract, and points to another interesting discrepancy between poliovirus replication in cell-free extracts and in infected cells.

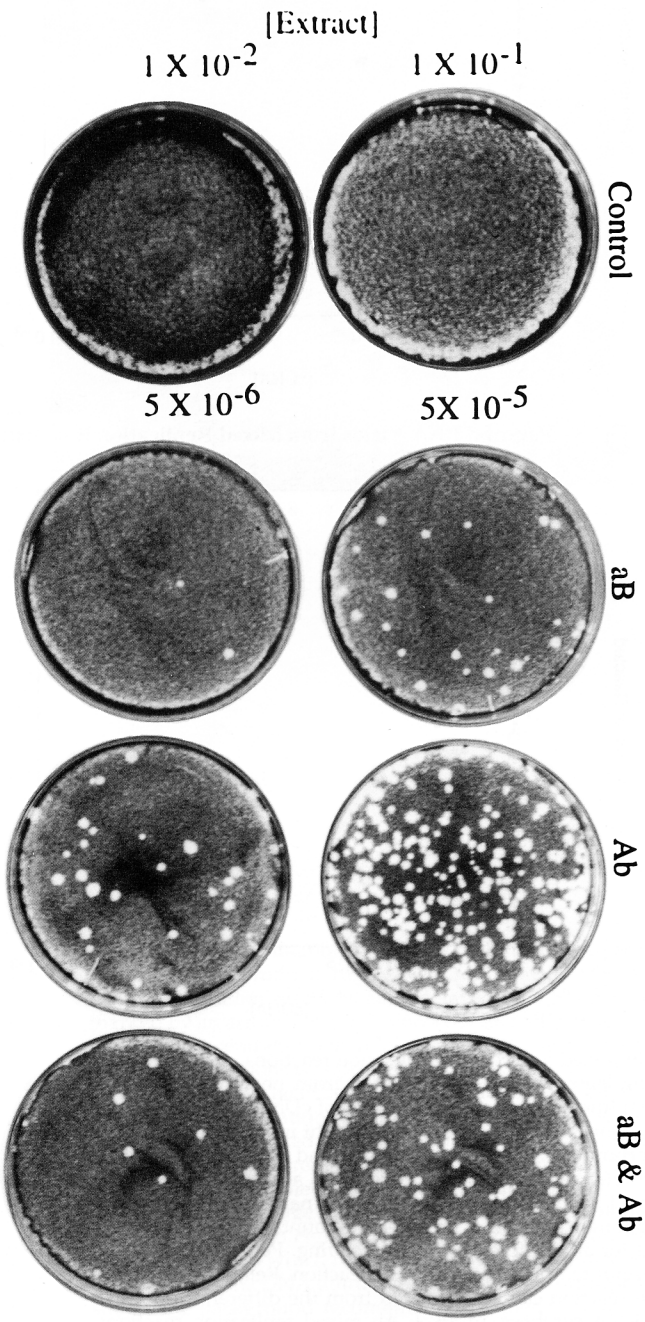

FIGURE 2. Generation of infectious poliovirus with physical markers Ab and aB in cell-free extracts. Plaque assays of ab, Ab, and aB + Ab replication reactions. After replication, reactions were incubated at 34 °C for 18 h, 5% of each reaction was removed and digested with RNase T1 (8 μg/mL) and RNase A (20 μg/mL) at room temperature for 15 min. Serial dilutions and plaque assays were performed as described (Molla et al., 1991; Barton & Flanegan, 1993). Titers for single-parent replication reactions were (1×10^7 pfu/mL) for Ab and (5×10^5 pfu/mL) for aB. The mixed replication reaction gave a titer of 4×10^6 pfu/mL.

To test whether the decreased aB titer reflected drastically lowered yields of aB RNA, we tested the accumulation of aB and Ab RNAs in separate and mixed replication reactions. Because trace amounts of input vRNA used to program the replication reactions can contribute to the parental signals if reverse transcription is performed using positive-sense RNA templates, we decided to probe for the relative abundance of negative-sense parental RNAs, which should originate only from replication reactions initiated in the cell-free extract. RNAs isolated from both control replication reaction, in which separate Ab and aB reactions were pooled after incubation, and a mixed Ab and aB replication reaction were used as templates for reverse transcriptions primed either with DNA oligonucleotide A or a. The first-strand cDNAs were serially diluted 10-fold and amplified by PCR with A and b DNA primers, to detect parent Ab, or with a and B primers, to detect parent aB. The intensity of the expected 656-bp PCR product for both parents are compared in Figure 3A for control replication reaction and in Figure 3C for mixed replication reaction. The PCR signals were quantified as the percentage of primer reacted to eliminate differences in gel loading as a source of error, and plotted as a function of relative cDNA concentration for control replication (Fig. 3B) and for mixed replication reactions (Fig. 3D). Estimates made from these plots of the relative amount of aB cDNA needed to produce the same amount of PCR signal as Ab cDNA indicate that the negative strands of parent Ab is three to five times more abundant than those of parent aB. It is also clear that Ab and aB RNAs can be synthesized either together or separately in the HeLa cell-free extract.

Observation of RNA recombination in cell-free extract

PCR signals from a recombination assay are displayed in Figure 4A. Lanes 1–6 show PCR fragments of the expected size (656 base pairs), indicating the detection of parent Ab in both the mixed Ab and aB parent and control replication reactions. The signals for the Ab parent are comparable in intensity over the range of cDNA dilutions shown. Lanes 7–14 show the signal for recombinant ab from the mixed-parent

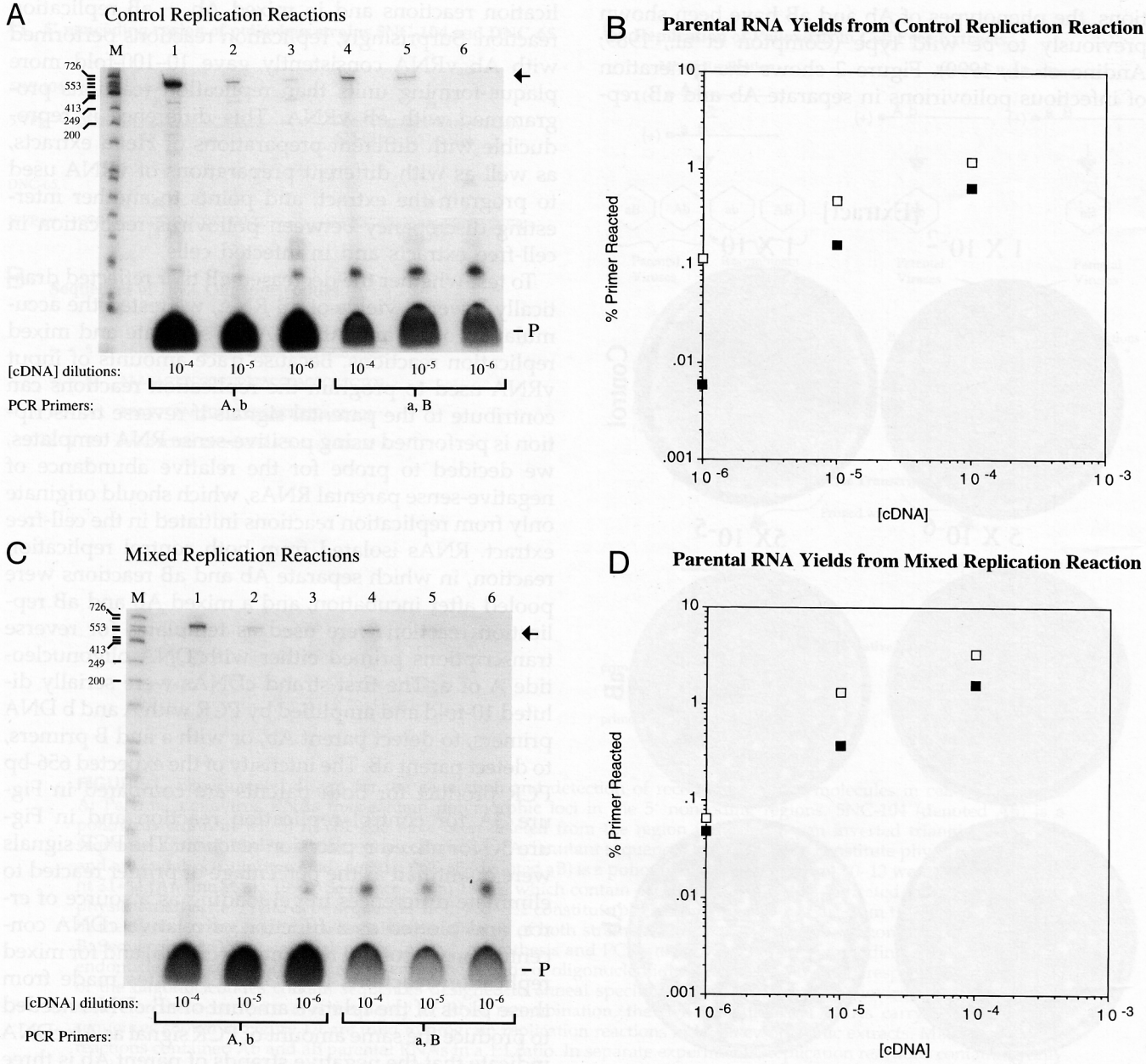

FIGURE 3. Synthesis of RNA that contains markers Ab and aB in mixed and control replication reactions. **A:** PCR signals from cDNA templated with negative-sense parental viral. Complementary DNA templated from negative-sense viral RNAs was made by priming with DNA oligomer A or a. PCR amplification of several dilutions of cDNA were performed to ensure that the amount of PCR product is responsive to cDNA concentration. The arrow at right indicates the position of the expected 656-bp PCR product. The position of the unincorporated ^{32}P-labeled primers B and b are marked with a P. Positions of DNA markers are shown. **B:** Quantitation of the relative abundance of parents aB and Ab in the control replication reaction. The gel shown in Figure 3A was scanned on a Phosphorimager (Molecular Dynamics) to quantify the radioactivity in the 656-bp PCR products and in the unincorporated primers b or B. The amount of PCR product is expressed as the percentage of unincorporated primer to correct for any differences in lane loading. Percentage of incorporated primer was plotted against the cDNA dilution used as input template for each PCR reaction. Relative abundance of parent Ab (□) and parent aB (■) from the control replication reaction can be estimated from the difference in cDNA dilution required to superimpose the two parental curves. **C:** RNA synthesis in aB + Ab mixed replication reactions. Lanes 1–3, parent Ab; lanes 4–6, parent aB. **D:** Quantitation of the relative abundance of parents aB and Ab in mixed replication reactions: Parent Ab (□); parent aB (■).

replication reaction (lanes 7–10) and the control replication reaction (lanes 11–14). The signal for recombinant ab can be seen clearly in the sample in which the parental RNAs were allowed to replicate in the presence of each other, but is reduced drastically in the control reactions. Thus, RNA recombination occurred at a readily detectable frequency in this cell-free extract.

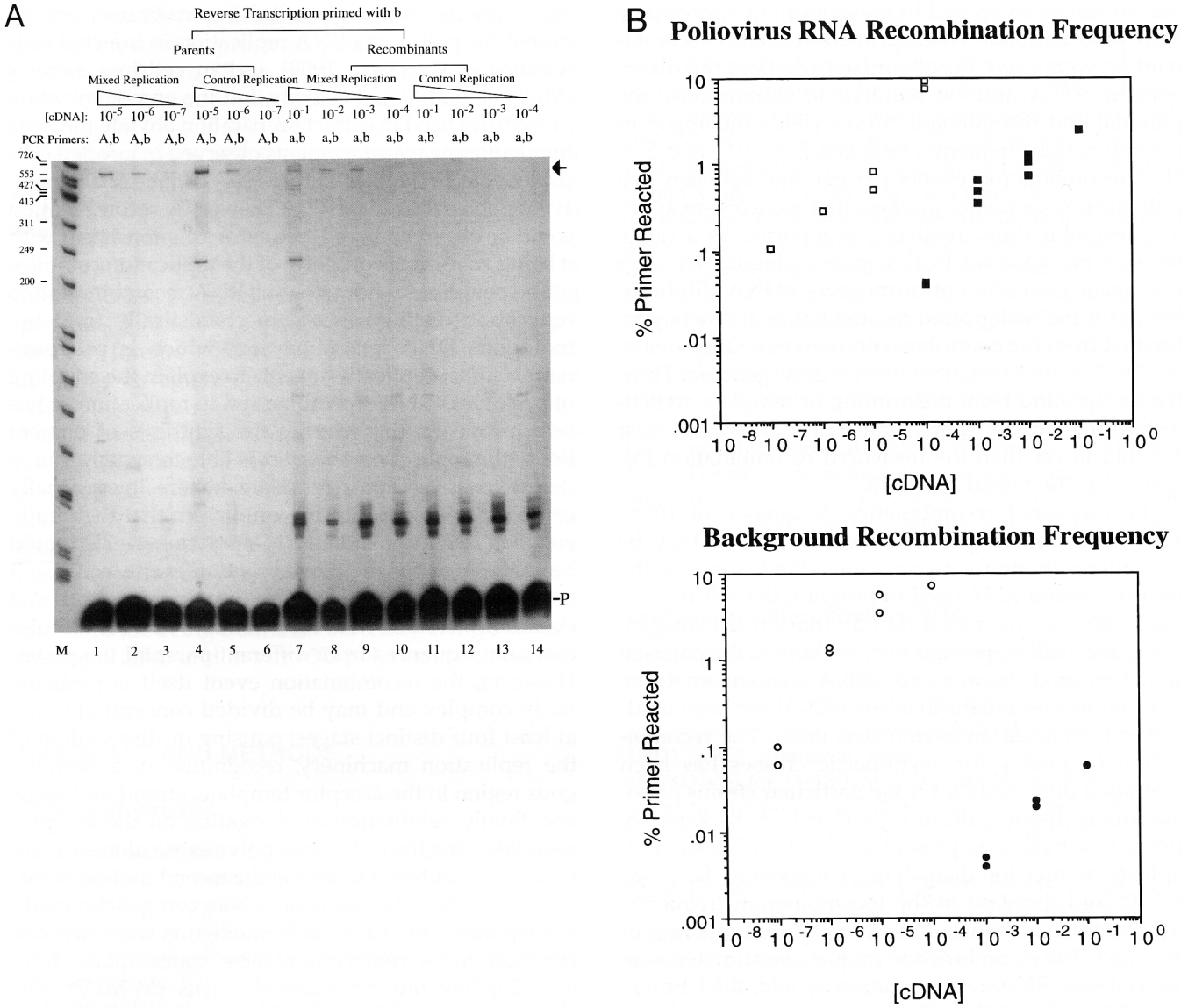

FIGURE 4. Determination of RNA recombination frequency by quantitation of PCR signal from recombinant and parental RNAs. **A:** PCR products from several dilutions of cDNAs made from positive strands present in mixed Ab and aB and control replication reactions are displayed on a 5% denaturing polyacrylamide gel. The expected 656-bp PCR product is marked by an arrow at right. Unincorporated ^{32}P labeled primer b is marked with a P. The PCR signal is linearly responsive to cDNA concentration when less than 2% of the primer is utilized (Jarvis & Kirkegaard, 1992). cDNA synthesis in mixed and control reactions was primed with deoxyoligonucleotide b. Lanes 1–6 show the PCR signals of parent Ab in mixed and control reactions. The PCR signals of putative recombinant ab are shown in lanes 7–14. Lanes 1–3 and 7–10 are signals from mixed parent replication reactions. Lanes 4–6 and 11–14 are signals from control replication reactions in which parent Ab and aB were replicated separately and mixed for cDNA synthesis. The mobilities of DNA markers are shown at left. **B:** Quantitation of apparent recombination frequency in mixed and control reactions. The gel shown in A was scanned on a Phosphorimager (Molecular Dynamics) to quantify the radioactivity in the 656-bp PCR products and in the unincorporated primer. The percentage of primer incorporated was plotted against cDNA dilutions used as input template for each PCR reaction as shown in Figure 3. The top graph shows the parental signal Ab (□) and the recombinant signal ab (■) from the mixed reaction, in which Ab and aB RNAs programmed the same reaction. The recombination frequency is estimated from the difference in cDNA dilution required to superimpose the parental curve and the recombinant curve. The bottom graph shows the background recombination frequency from the control replication reaction in which Ab and aB RNAs programmed separate reactions. Parental signal Ab (○); recombinant signal ab (●).

Frequency of recombination in cell-free extracts

The PCR signals were quantified as the percentage of primer reacted and plotted in Figure 4B as a function of relative cDNA concentration. It is clear that 1,000-fold higher concentration of recombinant ab was present in the mixed than in the control reactions. To determine the recombination frequencies, the amount of 656-bp PCR product made with primers to detect par-

ent Ab can be compared to the amount of appropriate PCR product made when primers to detect recombinant ab were used. For the mixed reaction, the difference in cDNA dilution required to superimpose the parental and recombinant curves yields the apparent recombination frequency, between 2×10^{-3} and 2×10^{-2} recombination events per genome between two polymorphic sequence markers that were 656-nt apart. The recombination frequency is reported as a range because the parental PCR signals in Figure 4A were not linear over the entire range of cDNA dilutions. Similarly, the background recombination frequency estimated from the control reactions gives a range of 1×10^{-6} to 3×10^{-6} recombination events/genome. Thus, the background from mispriming or template switching during reverse transcriptase or PCR was at least 10^3-fold lower than the measured recombination frequency in the mixed reactions.

The measured recombination frequency of 10^{-2}–10^{-3} recombinants per genome over 656 nt may be an underestimate for two reasons. First, some of the positive-strand RNA used to program the cell-free extract, although degraded rapidly (Barton & Flanegan, 1993), may still be present to contribute to the parental signal. Second, because aB vRNA was shown to be 3–5-fold less abundant than Ab vRNA, we have inadvertently enacted an asymmetric cross. The recombination frequency for asymmetric crosses has been examined previously for these particular strains of poliovirus in tissue culture cells (Jarvis & Kirkegaard, 1992). When Ab was present at a 10-fold higher multiplicity of infection than aB in a coinfection, there was a 3–14-fold decrease in the recombination frequency relative to coinfections done at equal multiplicities of infection. The recombination frequency estimated here for cell-free RNA recombination would then be expected to be lower than that of an analogous cross that was symmetric.

DISCUSSION

The ability to mimic all intracellular steps of the poliovirus replicative cycle in cell-free extracts was a major breakthrough (Molla et al., 1991) that should allow the biochemical analysis of viral translation, protein processing, negative-strand and positive-strand synthesis, and viral packaging in vitro (Molla et al., 1991; Barton & Flanegan, 1993; Barton et al., 1995, 1996a, 1996b). However, not all intracellular features of the poliovirus replicative cycle are reflected in these extracts. Specifically, brefeldin A and enviroxime, two compounds that inhibit viral RNA synthesis in infected cells (Maynell et al., 1992; Aldabe & Carrasco, 1995; Heinz & Vance, 1995), have no comparable effect on viral production in cell-free extracts (Table 1). We suggest that the enzymology, but not the cell biology, of the poliovirus infectious cycle is reproduced in cell-free extracts. Although cellular membranes are required for poliovirus RNA replication in infected cells (Guinea & Carrasco, 1990) and in cell-free extracts (Molla et al., 1993), it is likely that the homogenization procedures used in extract preparation obviate the need for membrane rearrangements observed in infected cells (Dales et al., 1965; Bienz et al., 1987; Aldabe & Carrasco, 1995; Schlegel et al., 1996). That RNA recombination could be observed in cell-free extracts is consistent with it being an intrinsic property of the replication complex.

The coupling of homologous RNA recombination to replication distinguishes it mechanistically from homologous DNA recombination, which is predominantly a post-replicative event. To explain the coupling of poliovirus RNA recombination to replication, it has been proposed that, during the synthesis of nascent RNA, the replication complex is able to switch from a donor to an acceptor template, where it eventually completes its synthesis. It would seem that the virally encoded RNA-dependent RNA polymerase 3D would be at the heart of the recombination event, because it is responsible for the polymerization reaction that eventually creates new recombinant RNA molecules carrying sequences from different parental templates. However, the recombination event itself is probably fairly complex and may be divided conceptually into at least four distinct stages; pausing or dissociation of the replication machinery, recognition of a homologous region in the acceptor template, strand exchange, and finally, reinitiation of elongation on the acceptor template. Can the poliovirus polymerase alone accomplish all the different phases of the recombination event?

Recently, template switching between genetic markers separated by 900 nt of homologous sequence was reported for a retroviral reverse transcriptase from the Moloney murine leukemia virus (M-MLV). The frequency of recombination for a symmetric cross performed in vitro with purified M-MLV reverse transcriptase in the presence of two different RNA templates was approximately 1×10^{-3} recombinants/RNA template. This frequency fell to background levels of 7.7×10^{-5} recombinants/RNA template when a mutant of M-MLV reverse transcriptase, which lacked RNase H activity, was used for cDNA synthesis (Negroni et al., 1995). A requirement for RNase H activity in promoting internal strand transfer has also been reported for the human immunodeficiency virus reverse transcriptase (Stefano et al., 1992, 1994). These studies indicate that retroviral polymerases alone are sufficient for promoting strand switching between regions of homology in the parental RNA templates. However, degradation of the donor RNA template appears to be an essential step in this reaction and is likely to be responsible for the recognition and alignment of homologous sequences during retroviral homologous recombination. Although we do not know whether highly purified poliovirus RNA-

dependent RNA polymerase can switch templates during processive primer-dependent polymerization reactions in vitro (Pata et al., 1995), this precedent from reverse transcriptases that lack RNase H activity suggests that it will not.

No ribonuclease activities have been associated with purified poliovirus polymerase or the replication complex. Yet, the poliovirus RNA replication complex is able to recombine homologously at a frequency that is comparable to that for retroviral polymerase, both in infected cells and in cell-free extracts. Many intriguing questions continue to surround poliovirus RNA recombination, foremost of which is the mechanism by which homologous regions on different templates are aligned before template switching can occur. Does 3D polymerase merely form covalent linkages between ribonucleotides, or are there conformations it can assume by interactions with other viral or cellular proteins that allow it to recognize and bring homologous regions in different RNA templates together? Of course, these functions may not be performed by 3D polymerase, but by other viral or host proteins directly. These questions are being examined currently using both cell-free replication and in vitro 3D polymerase elongation assays.

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). Suspension cultures were grown in spinner culture in Eagle's MEM supplemented with 7% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate at 37 °C. For the preparation of HeLa cytoplasmic extracts, S3 HeLa cells were obtained from ATCC and grown in suspension in Eagle's MEM supplemented with 5% calf serum, 2% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were split 1:2 daily when they reached a density of $\sim 4 \times 10^5$ cells/mL. On the day before harvesting cells for cytoplasmic extract preparation, cells were pelleted and resuspended in fresh medium. Poliovirus 5NC-104 (Ab) contains a 25-base deletion from 632 to 656 (Compton et al., 1989). DNC-65 (aB) is a poliovirus mutant in which nt 10–13 were exchanged with nt 31–34 (Andino et al., 1990). Aside from these changes in their 5′ noncoding regions, both viruses contain sequences derived from Mahoney type 1 poliovirus. They have both been shown previously to be wild type phenotypically (Compton et al., 1989; Andino et al., 1990).

Preparation of HeLa cytoplasmic extracts

Extracts were prepared according to published protocols (Molla et al., 1991; Barton & Flanegan, 1993; Barton et al., 1996b). Briefly, 5×10^8–1×10^9 S3 HeLa cells were pelleted and washed with isotonic buffer (35 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES] adjusted to a pH of 7.4 with 10 M KOH, 146 mM NaCl, and 11 mM glucose). The total volume of wash used was equivalent to the volume of medium used to grow the cells. The cell pellet was resuspended in 1.5 volume of hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $Mg(CH_3CO_2)_2$, and 1 mM dithiothreitol [DTT]) and allowed to swell on ice for 10 min. Cells were lysed on ice in a prechilled 15-mL capacity Wheaton-33 Dounce homogenizer using a tight-fitting pestle. Lysis was usually complete after 25 strokes. Lysates were adjusted with 0.1 volume of a buffer consisting of 0.2 M HEPES, pH 7.4, 1.2 M KCH_3CO_2, 40 mM $Mg(CH_3CO_2)_2$, 50 mM DTT, and pelleted at $2{,}200 \times g$ in a Sorvall H-1000B rotor at 4 °C for 10 min to remove nuclei. The supernatant was pelleted again at $13{,}000 \times g$ in a Beckman JA-20 rotor to remove other cellular debris. After centrifugation, the supernatant was adjusted to 1 mM $CaCl_2$ and digested with 75 Units/mL of micrococcal nuclease (Pharmacia) for 15 min at 20 °C. The micrococcal nuclease was inactivated by the addition of EGTA to a final concentration of 2 mM. Lysates were repelleted at $13{,}000 \times g$, aliquoted at 4 °C, and stored at −80 °C.

Preparation of poliovirus RNA

HeLa cells were infected with high-titer stocks of Ab and aB poliovirus in suspension cultures. Infections were performed at multiplicity of infection of 15–20. Infected cells were lysed and cytoplasmic extracts were prepared (Barton & Flanegan, 1993). CsCl gradients were allowed to equilibrate at $160{,}000 \times g$ for 15–18 h at 22 °C. Banded viruses were collected in polypropylene ultrabottles (Nalgene) and diluted 10-fold with 0.5% SDS buffer [10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5% SDS]. The diluted virus samples were then pelleted by centrifugation at $160{,}000 \times g$ at 22 °C for 2 h. Pellets containing viruses were resuspended in approximately 1 mL of 0.5% SDS buffer and extracted six times with 25:24:1 phenol:chloroform:iso-amyl alcohol followed by three extractions with 24:1 chloroform:iso-amyl alcohol. Ratios of solvents are vol:vol. The samples were precipitated with 68 mM sodium acetate and 3 volumes of ethanol. Precipitated vRNAs were washed at least once with 100 μL of 70% ice cold ethanol. vRNAs were resuspended in deionized distilled water and assessed for purity and integrity by spectrophotometric scans and agarose gel electrophoresis. The amount of vRNA was quantified by its absorbance at 260 nm and stored in sodium acetate and ethanol at −80 °C.

RNA replication in HeLa cytoplasmic extracts

Replication reactions containing 1.25 μg vRNA, 25 μL HeLa extracts, 5 μL reaction buffer [1 mM ATP, 0.2 mM GTP, CTP, and UTP, 120 mM $K(CH_3CO_2)$, 2.75 mM $Mg(CH_3CO_2)_2$, 35 mM HEPES, pH 7.4, 25 mM creatine phosphate, and 100 μg of creatine phosphokinase] were adjusted to a final volume of 50 μL with deionized distilled water. Reactions were incubated at 34 °C for 15–20 h. At least three different reactions are required for each recombination assay. In the mixed replication experiments, 0.625 μg of Ab and 0.625 μg of aB vRNAs were used to program the HeLa extracts. In the separate replication experiments, 1.25 μg of Ab and 1.25 μg

of aB were used to program separate replication reactions. To generate sufficient virus for the recombination assays, 10 50-μL reactions containing the mixed parents were pooled at the end of incubation. Control replication reactions consisted of five tubes of 50-μL replication reactions programmed with Ab vRNA and 5 tubes of 50-μL replication reaction programmed with aB vRNA. These separate parent replication reactions were pooled at the end of incubations. Note that the final concentration of input RNAs for both the pooled mixed parent and the pooled control reactions are the same: 0.025 μg/μL of Ab and 0.025 μg/μL of aB.

RNA replication in HeLa cytoplasmic extracts containing antiviral reagents

To assess the effect of brefeldin A and enviroxime on the production of infectious poliovirions in the cell-free system, 1.25 μg of wild-type poliovirus vRNA was used to program 50-μL replication reactions in the presence and absence of antiviral reagents. Brefeldin A (Epicentre Technologies) was solubilized in DMSO to concentrations of either 50 mg/mL or 5 mg/mL. Enviroxime dissolved in DMSO to a concentration of 50 mg/mL was kindly provided by B.A. Heinz (Lilly Research Laboratories, Indianapolis, Indiana). Guanidine HCl (Sigma) was solubilized in deionized distilled water to a final concentration of 100 mM. The antiviral reagents were mixed with cytoplasmic extracts prior to the addition of viral vRNA. Reactions were incubated at 34 °C for 15 h, treated with RNases, and used to infect HeLa monolayers. Dilutions of identical antiviral stocks were inhibitory to poliovirus production in infected cells, as expected (data not shown) (Maynell et al., 1992; Aldabe & Carrasco, 1995; Heinz & Vance, 1995).

Preparation of cytoplasmic RNA

Pooled replication reactions were diluted by the addition of 5 volumes of 0.5% SDS buffer and extracted six times with 25:24:1 phenol:chloroform:iso-amyl alcohol followed by three extractions 24:1 of chloroform: iso-amyl alcohol. RNA samples were treated and stored in the same manner described above for viral RNAs.

Reverse transcription and PCR

Reverse transcription and PCR were performed according to published protocols (Jarvis & Kirkegaard, 1992). Briefly, 10–20 μg of RNA from replication reactions were used as templates for cDNA synthesis. To detect positive strands, reverse transcription reactions were primed with 0.1 μM of oligonucleotide B or b (Fig. 1B) and consisted of: 50 mM Tris-HCL, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 40 U RNasin (Promega), 1.5 mM deoxynucleotide triphosphates, and 100 U Superscript (RNase H^-) Moloney murine leukemia reverse transcriptase (GIBCO-BRL). The template and reaction mix were pre-warmed in separate tubes at 45 °C for 10 min. They were then mixed, allowed to incubate at 45 °C for 1 h, diluted with deionized distilled water, and concentrated with Centricon-100 ultrafiltration units (Amicon), which also remove nucleotides and nucleic acid fragments shorter than 300 nt. To detect negative strands, RNAs were incubated with 15 mM CH_3HgOH (Alfa Æsar) for 15 min at room temperature. CH_3HgOH was neutralized before reverse transcription by incubating the RNAs with 190 mM 2-Mercapto-ethanol (Sigma) at room temperature for 2 min. Reverse transcription primed with 0.1 μM of oligonucleotide A or a were performed using the conditions described above for the detection of positive strands. Ten-fold serial dilutions of the products of the reverse transcription reactions were used to template PCR amplifications.

PCR reactions contained the following components: 50 mM potassium glutamate, 20 mM potassium HEPES, pH 8.4, 3.3 mM $MgCl_2$, 0.1 mg/mL acetylated BSA, 0.5 mM deoxynucleotides, 0.45 μM cold primer B or b, 0.5 μM cold primer A or a, 0.05 mM radiolabeled B or b, 2 Units Amplitaq DNA polymerase (Perkin Elmer-Cetus), and different dilutions of cDNA. Amplification of the cDNAs used 35 cycles with the following thermal profile: 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. The products of PCR amplification were displayed on 5% polyacrylamide–7.5 M urea gels. Twelve percent of each PCR reaction was loaded with an equal volume of dye containing formamide and EDTA. The gels were dried on Whatman DE-81 paper to prevent loss of unreacted primer (Jarvis & Kirkegaard, 1992). Dried gels were exposed to X-ray films and phosphorimager screens to quantify the radioactivity. PCR signals generated in this way are linearly responsive to template DNA concentration when 2% or less of the primer is incorporated (Jarvis & Kirkegaard, 1992).

An artifact called polymerase halt-mediated linkage of primers (PHLOP) can be produced by PCR reactions and lead to the generation of false parents and recombinants. This is brought about when elongation is stalled prematurely before the second primer binding site. When this halted product is melted, it can reanneal to the other parental template, thereby generating a PCR-mediated recombinant molecule (Frohman & Martin, 1990). To avoid this artifact, only primer B or b was used to generate cDNAs. For example, when b is used in isolation (Fig. 1C), cDNAs to only the Ab parent and ab recombinant can be generated. Then, quantitative PCR is used to determine the frequency of the A and a alleles in this population. PHLOP may change the crossover location between the A/a and the b allele, but will not change the relative frequency of A and a alleles.

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