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Capillary electrophoresis: separation and quantitation of reverse transcriptase polymerase chain reaction products from polio virus

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Abstract

In the present study reverse transcriptase (RT) polymerase chain reaction (PCR) products were generated from the RNA of polio virus. The products of the RT-PCR were analyzed by slab-gel electrophoresis (SGE) on 4% agarose gels, and capillary electrophoresis (CE). CE separations were performed in a coated capillary containing a linear polyacrylamide. Samples were injected hydrodynamically or electrokinetically. Detection of the RT-PCR products on CE was by UV absorbance at (254 nm) or by laser-induced fluorescence (LIF). While SGE resulted in adequate separation of 163 and 97 base pair RT-PCR products, separation of the 97, 71 and 53 base pair products was minimal. CE separations showed baseline resolution for all the above PCR products. Finally, it was possible to quantitate the amount of RT-PCR product by developing a standard curve showing a linear relationship between the amount of RNA used in the RT-PCR and the amount of product formed in the RT-PCR. These results suggest the greater resolution and enhanced sensitivity observed, together with the ease of quantitation, make CE a powerful alternative to SGE for the separation and quantitation of PCR products.

1. Introduction

The polymerase chain reaction (PCR) [1,2] may be one of the most useful technologies developed in the last ten years for amplification of genomic material. While excelling at amplification, PCR suffers from difficulties in quantitation of the reaction products. One reason for this is the method currently in use for separation of the PCR reaction products, namely slab-gel electrophoresis (SGE). One solution to this difficulty may be to replace SGE with another procedure. Other procedures that have been used include HPLC for the separation of restriction fragments from digests of bacteriophage DNA [3,4] and capillary electrophoresis (CE) [5–8]. Recently, CE has been used successfully for the separation of DNA restriction fragments and PCR products of HIV-1 virus [9], and restriction fragments of the ERBB2 oncogene [10]. In addition, the introduction of laser-induced fluorescence has increased the sensitivity of the detection systems for CE [11]. Given these advances in CE methodology, we have used CE as the method for separation of the PCR products.

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In the present study the RNA from the polio virus was used as the template. The polio virus offered several advantages for evaluation of CE for quantitation of the reaction products. For example, several types of viruses are available, the wild type and three vaccine strains, Sabin 1–3. In addition, primers had been developed for each of these strains to yield PCR products of 163, 97, 71 and 53 bp for the wild type and Sabin 1–3 respectively [12]. In this paper, the RNA from all four viruses was used in a reverse transcriptase-PCR and the reaction products analyzed by both SGE and CE. The results indicated that CE provided better separations and greater sensitivity than SGE. As a result, and as a first step towards quantitation of the PCR products in unknown samples, it was possible to generate a standard curve demonstrating a linear relationship between the amount of template RNA and peak area using the position of the primer peak or a co-injected marker DNA as an internal standard. The results of this study indicate that CE can provide the resolution and sensitivity for quantitation of PCR products.

2. Experimental

2.1. Poliovirus and PCR primer pairs

The wild type poliovirus (PV3/9288/MEX89) was obtained from Dr. Olen Kew (Centers for Disease Control, Atlanta, GA, USA); Orimune (containing the Sabin 1, 2 and 3 strains of vaccine polio virus) was obtained from Lederle Labs (Pearle River, NY, USA). Primer pairs for all polio viruses were a gift from Dr. Kew (CDC, Atlanta, GA, USA).

2.2. Reverse transcriptase and PCR amplification

As polio virus is an RNA virus, cDNA transcripts were prepared from RNA templates using reverse transcriptase (RT). The RT and PCR amplification reactions were performed together in 100 μl reaction mixtures containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 4 m*M* $MgCl_2$, two or more of the PCR primers (20 pmol each), 200 μM each of the dATP, dCTP, dGTP, dTTP (Perkin-Elmer, Norwalk, CT, USA), 10 U placental ribonuclease inhibitor (Boehringer, Mannheim, Germany) and 2.5 U avian myeloblastosis virus (AMV) reverse transcriptase (Life Technologies-Gibco, Bethesda, MD, USA) and 2.5 U DNA polymerase from *Thermus aquaticus* (*Taq*. polymerase; Perkin-Elmer). In most reactions 2–5 μl of template RNA was used. The RT reaction was carried out by incubation of the reaction mixture at 42°C for 30 min and 95°C for 3 min. For the PCR, programmed amplification cycles (denaturation: 94°C, 45 s; annealing: 62°C, 45 s; extension: 72°C, 1 min) were performed in a thermal cycler (Eppendorf, Germany). A typical program of 30 cycles was completed in 4.5 h.

2.3. Slab gel electrophoresis of PCR amplified products

Samples (10 μl) of the PCR reaction mixture were loaded onto agarose gels (9 × 7 cm) 4% NuSieve (FMC Products, Rockland, ME, USA) containing ethidium bromide. After electrophoresis (53 mA and 127 V) for 55 min at room temperature, fluorescent DNA bands were visualized on a transilluminator and photographed.

2.4. Capillary electrophoresis of PCR amplified products

CE was performed on a P/ACE 2200 (Beckman, Fullerton, CA, USA) in the reverse-polarity mode (negative potential at the injection end of the capillary). A capillary (100 μm I.D., 37–47 cm total length, 30–40 cm to the detector, Beckman), coated to reduce electroosmotic flow, was filled with a sieving polymer buffer system containing linear polyacrylamide in a Tris–borate–EDTA (TBE) buffer. This buffer was replaced after each separation. PCR samples were injected hydrodynamically (10 s at 3.45 kPa) or, after desalting by membrane dialysis [13], electrokinetically (10 s at 40 V/cm). In some analyses, the PCR sample and a ϕX174 DNA/*HaeIII* digest (Promega, Madison, WI, USA) were injected sequentially and allowed to

co-migrate in the capillary. The standard digest was not desalted, but diluted to 20 μg/ml in HPLC-grade water prior to injection. Temperature of the capillary cartridge was set at 20°C, and separation of the PCR products was accomplished at a field of 200 V/cm. Detection was on-line, either using the P/ACE standard UV detector at 254 nm, or the P/ACE laser-induced fluorescence (LIF) detector. With the latter, a fluorogenic intercalator, EnhanCE™, was added to the run buffer, and the separation monitored on-column using an argon-ion laser (488 nm line) to excite the resulting DNA–dye complex; fluorescence of the complex occurs at 530 nm. Data was collected at 5 Hz and analyzed using System Gold Software, version 7.12 (Beckman).

3. Results

3.1. Separation of RT-PCR reaction products from wild type and vaccine (Sabin 1) polio virus

In separate reactions, primer pairs were used to generate RT-PCR products from a Mexican strain of wild virus and from the Sabin 1 vaccine strain. When the PCR products were analyzed by gel electrophoresis (SGE) the results shown in Fig. 1 were obtained. Lane 3 shows a band at 163 bp, representing the product from the wild type polio virus RNA, and Lane 4 a band at 97 bp, representing the product from the Sabin 1 RNA [12]. Lane 2 shows a gel of a mixture of both products. In addition, each lane shows a band representing the unreacted primer. While the results shown in lane 2 indicate a clear separation between the two products, attempts to determine the amount of product present on the densitometric scans of each lane were unsuccessful due in part to variations in the density of the background and also from the spreading associated with each band (data not shown).

Samples of these same PCR products were examined using CE and the results of representative electropherograms are shown in Fig. 2. The electropherograms of the reaction product from wild type polio virus RNA (panel A) and the

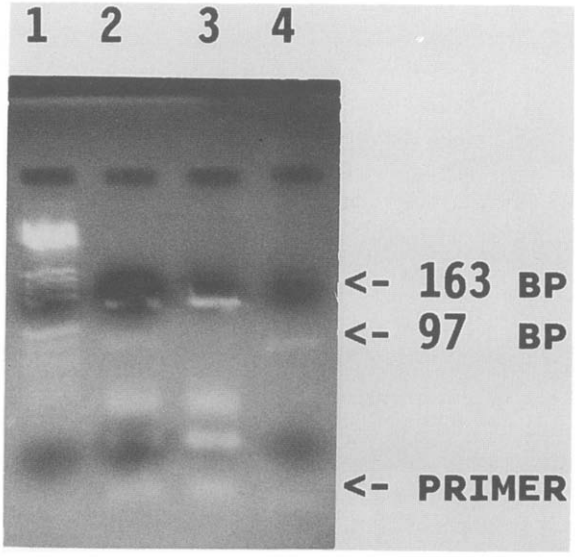

Fig. 1. SGE separation of RT-PCR products. RT-PCR was performed with wild type and Sabin 1 vaccine polio virus. RNA was extracted and RT-PCR performed as described in Experimental. Separations were performed on 4% agarose gels as described in Experimental. Lane 1 = references; lane 2 = mixture of PCR products from wild type and Sabin 1; lane 3 = wild type; lane 4 = Sabin 1.

product from the Sabin 1 RNA (panel B) showed a single main peak, representing 163 bp and 97 bp products respectively. As a test of the separation capacity of the CE system a mixture of these two PCR products was prepared and a sample of the mixture injected for analysis. Panel C shows a representative electropherogram of the mixture. Two peaks, identifiable by comparison to the peaks in panels A and B, as the 163 and 97 bp products, were observed and clearly separable by this procedure.

While the results shown in Fig. 2 were obtained with pressure injection of the sample, a separation, similar to that shown in Fig. 2 was achieved using electrokinetic injection (data not shown). Although some enhancement of sensitivity was obtained with the electrokinetic injection method, due to sample focussing, the necessity for first having to desalt the sample made this manner of injection too time consuming for routine use. Also, in addition to the LIF detection method used to obtain the electropherogram in Fig. 2, UV detection, at 254 nm,

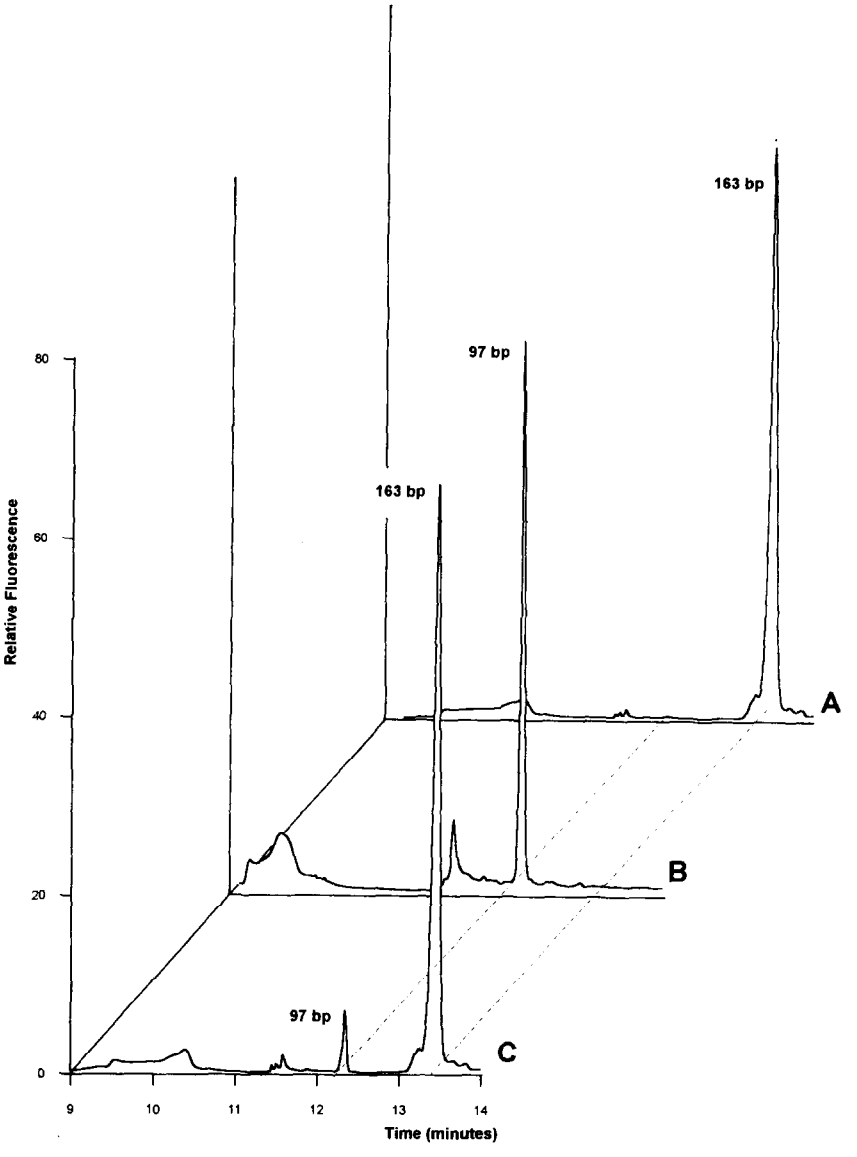

Fig. 2. CE separation of RT-PCR products from wild type and Sabin 1 RNA. Separations were performed on a column of 37 cm × 100 μm I.D. (effective length of 30 cm) as described in Experimental. EnhanCE was added to the TBE buffer at an effective final concentration of 3 μg/ml. (A) wild type, (B) Sabin 1, (C) mixture.

was used as well (data not shown). When the results obtained with the two methods were compared the LIF detection produced a >100-fold increase in sensitivity. In addition, these reaction mixtures contained other components, such as the dNTPs, which appeared on the electropherogram when UV detection methods are used. In contrast, because the LIF detection method uses a fluorogenic compound that only intercalates the oligonucleotides, the dNTPs were not displayed on the electropherogram with LIF detection.

3.2. Separation of the 97, 71 and 53 bp poliovirus RT-PCR products

To compare the resolving capacity of the SGE and CE methods further, 97, 71, and 53 bp RT-PCR products were generated in a single reaction from the Sabin 1, Sabin 2, and Sabin 3 vaccine virus RNA. When a sample of the RT-PCR mixture was analyzed by gel electrophoresis (SGE) the results shown in Fig. 3, lane 2 were obtained. As shown, three bands were visible representing the reaction product of amplification of Sabin 1, 2 and 3 polio virus RNA respectively. As before, attempts to determine the amount of each product by scanning the lane were difficult as the spreading of each band made it impossible to obtain base line separation between each band.

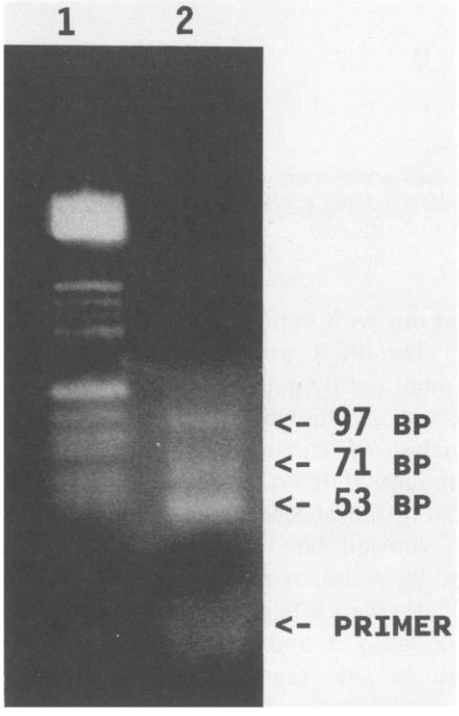

Fig. 3. SGE separation of RT-PCR products from Sabin 1, 2 and 3 RNA. RNA was extracted and RT-PCR performed as described in Experimental. Separations were performed on 4% agarose gels as described in Experimental. Lane 1 = references; lane 2 = mixture of RT-PCR products from Sabin 1, 2, and 3.

In contrast, when a sample of this same reaction mixture was analyzed by CE, the electropherogram (Fig. 4 panel D) showed three peaks, representing the RT-PCR products of each of the three viruses, with base-line separation between each. For confirmation of the identification of each peak, an RT-PCR was performed with RNA from only one virus and Fig. 4A, B and C shows the relative migration position for each of the products. From a comparison of the migration times of each of the products in Fig. 4A–C it was possible to confirm the identify of each of the components on the electropherogram shown in Fig. 4D.

3.3. Quantitation of the RT-PCR product

In an electropherogram, the area under a peak for a particular DNA fragment can be correlated to the quantity of that fragment. However, as the peak area of the fragment is related to its residence time in the detector, slower migrating (large) fragments will remain in the detector window longer than a faster migrating (small) fragment. Thus, the peak area may not be representative of the quantity of DNA passing through the detector. In addition, as DNA fragments from restriction digests and the PCR typically are contained in a high-salt matrix, their mobility will vary depending on sample salt concentration. To account for these variations and obtain quantitation of DNA fragments requires the use of an internal standard to normalize analyte velocity and correct for differences in fragment mobility. Candidates for internal standards include the primer or primer–dimer peaks, as both components are already present in the PCR mixture, or a co-injected standard DNA peak, such as those found in a ϕX174 DNA *Hae*III digest. Attempts to use any of these fragments as the internal standard required their separation from one another and any PCR product, a pre-condition not easily met when the size of the PCR product is below 60 bp. To explore the capacity for separation by CE of the primer, the primer–dimer, the 53 bp product, and any co-injected standards, a RT-PCR was carried out with the Sabin 3 viral RNA. A

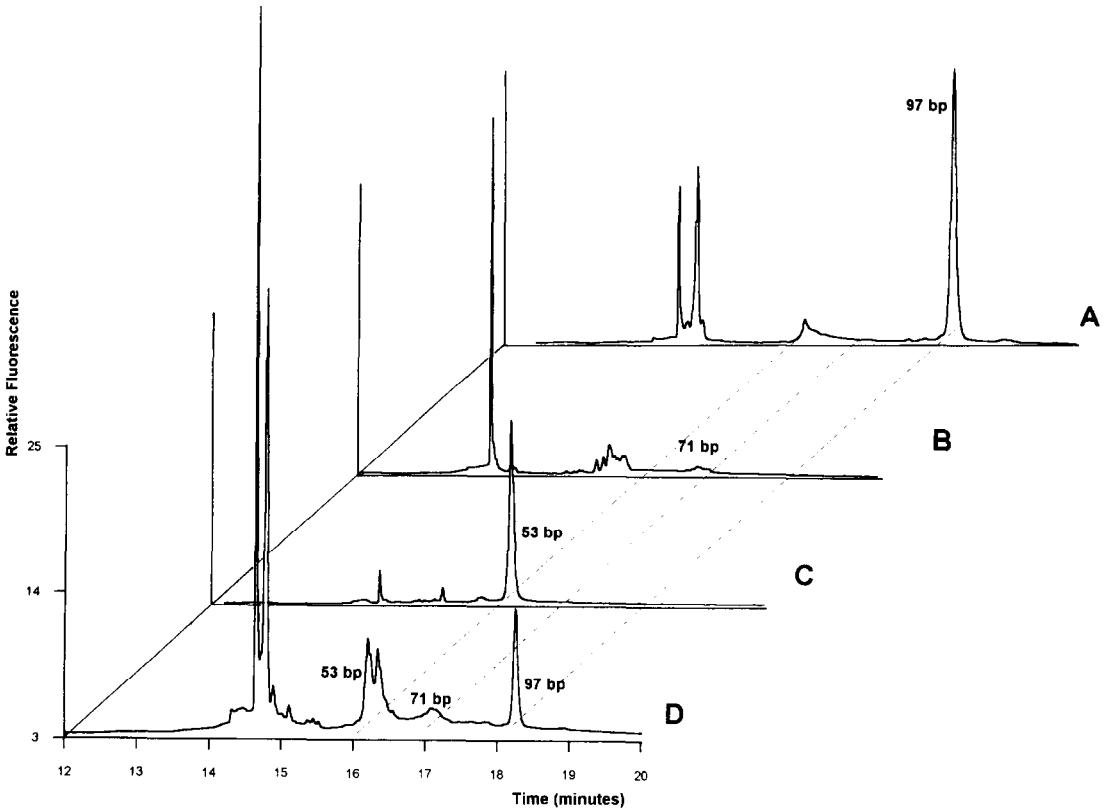

Fig. 4. CE separation of RT-PCR products from Sabin 1, 2 and 3 RNA. Separations were performed on a 47 cm total length capillary, under conditions as described in Fig. 2. (A) Sabin 1, (B) Sabin 2, (C) Sabin 3, (D) mixture of Sabin 1, 2, and 3.

sample reaction product was analyzed by CE and Fig. 5 shows a representative electropherogram. Several clearly resolved peaks were visible. In this case the main peak of interest, Sabin 3, was identified using the relative migration times of the primer peak and the 72 bp fragment of the ϕX174 DNA/*Hae*III digest as references. The success of this separation indicated that either the primer, the primer–dimer, or co-injected DNA fragments $\geq$72 bp could be used as internal standards.

In order to quantitate the amount of product in a PCR reaction performed with an unknown amount of template, a standard curve can be generated showing a linear relationship between peak area and known amounts of template. To develop such a standard curve, RT-PCRs were carried out with various amounts of the Sabin 3 RNA. The PCR products from each reaction were analyzed by gel electrophoresis (SGE) and the results are shown in Fig. 6. Lanes 2–5 show the analysis of reactions carried out with increasing amount of RNA. Lane 2 showed no band, suggesting the absence of any product. Lanes 3 and 4 showed bands of about equal intensity suggesting a similar amount of product in each. Lane 5 showed a band slightly less intense than that in lanes 3 and 4 suggesting less product formed in this reaction. Densitometric scans (data not shown) of these lanes did not alter what was inferred about product levels from visual inspection of the gel.

When the RT-PCR reaction products from each reaction were examined by CE the electro-

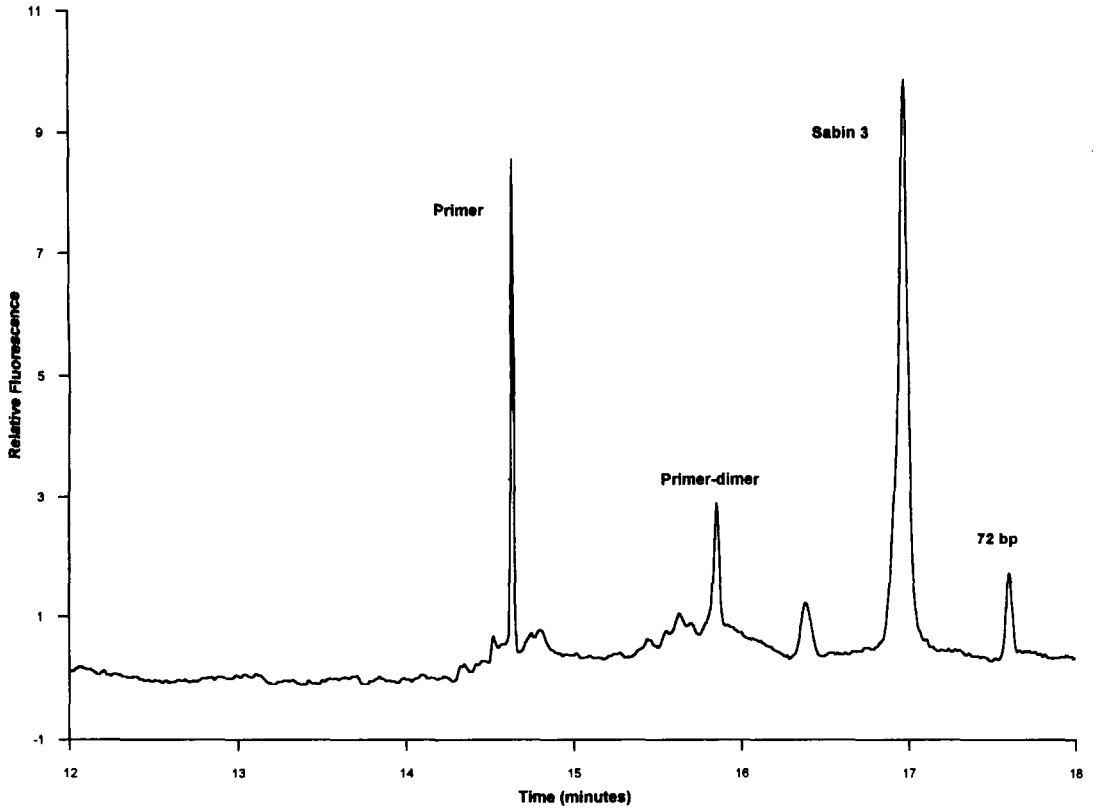

Fig. 5. CE separation of RT-PCR products from Sabin 3 RNA. RNA was extracted and RT-PCR performed as described in Fig. 1. The sample of ϕX174 restriction digest was prepared in water at final concentration of 20 μg/ml. For this separation, the RT-PCR product was injected first (a 10-s injection), and immediately followed by a second injection of the ϕX174 sample.

pherograms shown in Fig. 7A–D were obtained. Each electropherogram showed three peaks identified as the primer, the primer–dimer, and the 53 bp Sabin 3 RNA reaction product. A visual inspection of panel A, revealed that a product was in fact formed in this reaction although this product was not detected on the SGE (Fig. 6, lane 2). Again, in contrast to what was observed on the gel, with increasing amounts of RNA, the height and area of the peaks representing the reaction products from reactions two and three increased. Finally, consistent with the results of the gel analysis (Fig. 6, lane 5) the electropherogram in panel D showed a peak height slightly less than that in panel C. To develop a standard curve, the area under each product peak was determined, and corrected for transit time through the detector, using as an internal standard either the primer or a co-injected DNA fragment of known size. When these corrected areas were plotted as a function of the amount of RNA used in the PCR reaction, a linear relationship between these two parameters was observed for the first three data points while inclusion of the fourth point causes the result to become non-linear (Fig. 7, inset).

4. Discussion

The purpose of this study was to evaluate CE for the separation of PCR reaction products and

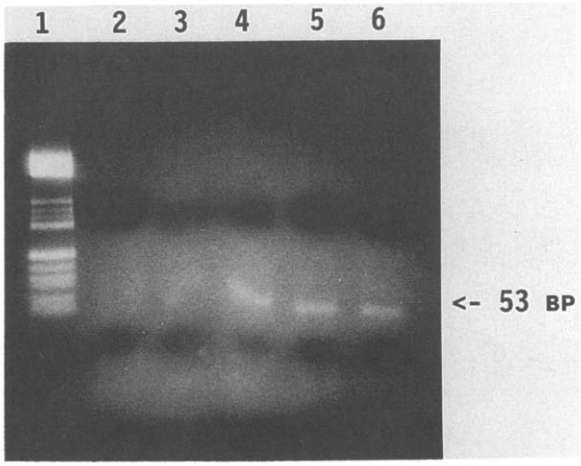

Fig. 6. SGE separation of RT-PCR products from Sabin 3 RNA. RNA was extracted and RT-PCR performed as described in Fig. 1 except that increasing amounts of RNA were used as template in the RT-PCR. All separations performed on 4% agarose gel as described in Experimental. Lane 1 = references, lanes 2–5 = products obtained from RT-PCR with increasing amounts of template; lane 6 = duplicate of lane 5.

to quantitate the amount of PCR product formed during the reaction. To evaluate CE, RT-PCR products were formed in reactions with polio virus RNA and the products were analyzed by both SGE and CE.

The results of this study indicate that the linear polyacrylamide used for the separation was able to resolve the reaction products below 100 bp. Of additional interest was the separation of the 53 bp product from the primer and primer–dimer. This separation allowed for the use of these as migration time standards for quantitation.

The results of this study also showed that CE could be performed with a minimum of sample preparation, for example desalting was not found to be necessary as samples could be injected hydrodynamically. When compared to UV, the use of LIF detection proved to have advantages. For example, whereas with UV the dNTPs were displayed on the electropherogram, this was not the case with LIF. In addition, with the LIF detection system the run buffer was formulated to contain a "dye", EnhanCE, which specifically interacts with DNA (and RNA). This dye acts as a mono-intercalator, inserting itself between every two base pairs of DNA. Intercalation changes the molecular length, conformation, and charge on the DNA molecules, resulting in a change in electrophoretic behavior. Moreover, this DNA–dye complex fluoresces when excited by the 488 nm line of an argon-ion laser, whereas the dye alone, as well as non-DNA sample components, will not. In all, this results in improved base-pair resolution between closely migrating fragments, as well as >100-fold enhanced sensitivity when compared to UV detection. In addition, studies performed with a similar dye [14] suggests that the intercalator used in the present studies would not be base-composition specific and therefore would be suitable for any DNA fragment.

While the problems associated with developing a quantitative PCR procedure have been discussed by others [15], in this study the focus was on dealing with problems associated with the use of CE for separation, namely a variation in migration time. As part of this study the primer peak (and co-injected standard peak) were used to correct peak area for this variation. The corrected peak area for a DNA fragment can be compared to a standard curve to determine absolute amount, that is, DNA output following RT-PCR. Alternatively, RT-PCR can be performed using two sets of primers, one set to amplify the desired target, the other set to amplify a second region as an internal control or "housekeeping" function. Here the amount of the target is quantified relative to that of the internal control [16]. Finally, competitive techniques, based on the relative amounts of products produced from a target and competitive template, may be attractive for clinical samples [15,17] since the absolute amount of product derived from the PCR may not always relate to the amount of target (DNA or RNA) present. With either technique, some procedure is needed to correct peak areas for variations in mobility through the detector. The linearity of the standard curve suggests that the use of internal migration time standards, either through the presence of PCR components such as primer, or co-injection of known-size DNA, is necessary to

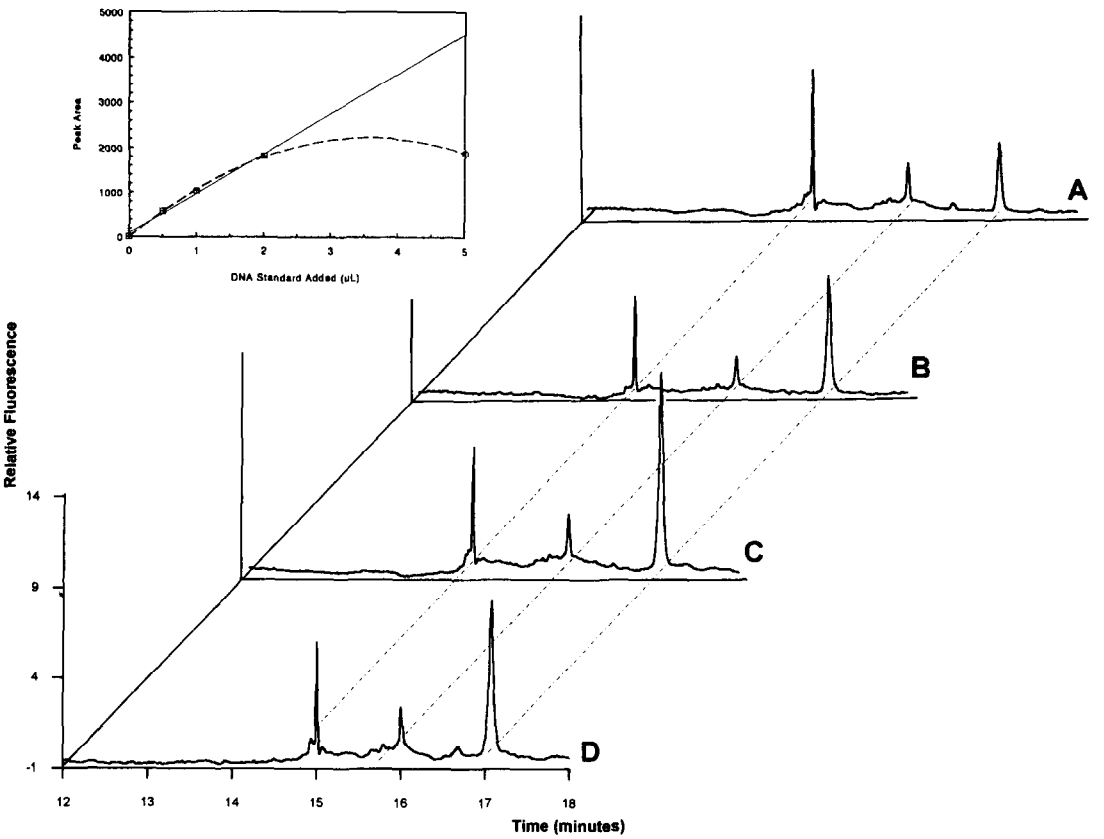

Fig. 7. CE separation of RT-PCR products from Sabin 3. Samples were generated in reactions described in Fig. 6. Separations were performed on a 47-cm column (effective length 40 cm) in TBE buffer containing 3 μg/ml (final concentration) of EnhanCE. At this concentration the dye was found to be in excess but not enough to quench. It should be noted that when these electropherograms were monitored by UV absorbance, no peaks were detected indicating that the amount of RT-PCR product was below the lower limit of detection by this method. (A) Sample from lane 2 in Fig. 6, (B) sample from lane 3 in Fig. 6, (C) sample from lane 4 in Fig. 6, (D) sample from lane 5 in Fig. 6. The inset shows data obtained from the area of the 53 bp product peak corrected for variations in migration time and detector residence time using the primer peak as an internal standard.

accurately quantify PCR products. Taken together, these results suggest that the greater resolution and enhanced sensitivity observed, together with the ease of quantitation, make CE a powerful alternative to SGE for the separation and quantitation of PCR products.

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