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Analysis of RNA by capillary electrophoresis

Analytical parameters known to be important for the separation of DNA by capillary electrophoresis, including gel polymer concentration, electrical field strength and temperature, were investigated and optimized for the analysis of RNA molecules from 100 to 2000 bases. Denaturation, essential to obtain uniform and identifiable peaks, was accomplished by heating the sample in 80% formamide prior to electrophoresis and the presence of 2–8 M urea in the electrophoresis buffer. Efficient separations were obtained over a wide range of electrical field strengths and temperatures using the gel polymer hydroxypropylmethylcellulose (HPMC) as separation matrix. Low HPMC concentrations (<0.3%) were suited for the separation of high molecular mass RNA (>1000 bases) whereas higher HPMC concentrations were required for optimal separation of low molecular mass RNA. An optimized system was applicable for the separation of the predominating RNA populations (small RNA of 60–300 bases (as a group of unseparated peaks), 18S and 28S rRNA) in total RNA from a human glioma cell line. This is the first systematic investigation of electrophoresis of higher molecular mass RNA in capillaries, and motivates further studies to transfer electrophoresis of RNA to the capillary format.

1 Introduction

Capillary electrophoresis (CE) has been widely used during the last decade in the analysis of single- and double-stranded DNA (ssDNA, dsDNA) [1–3]. The electrophoretic separation, carried out in a gel-filled capillary under high electrical field strength, is in general characterized by short analysis time, high resolution, accurate quantitation and high reproducibility. Applications of CE in DNA analyses include the separation of dsDNA restriction products [2], dsDNA generated by the polymerase chain reaction [4, 5] and ssDNA sequencing products [3, 6, 7]. For more than three decades, gel electrophoresis has been fundamental in the characterization of RNA [8]. The technique is routinely used for the analysis of RNA mass and conformation and in the detection of gene expression (RNA hybridization techniques) [9, 10] and the identification of microorganisms (retroviruses and bacteria) [11, 12]. Recently, data were published describing the separation by CE of low molecular mass RNA (transfer RNA and 5S ribosomal RNA, 70–135 bases) from bacteria [13, 14]. Various system parameters were evaluated for their effect on the separation efficiency. High-resolution separations (1–5 bases) were obtained in 30 min for low molecular mass RNA. To our knowledge, no data have been published on CE of higher molecular mass RNA.

In the present paper we present a systematic study of the electrophoretic separation of RNA in capillaries. Important analysis parameters including gel polymer concentration, electrical field strength, temperature and denaturant are investigated for their effects on the separation of RNA ranging in size from 100 to more than 6000 bases. The results demonstrate the applicability of CE for the analysis of RNA.

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Nosntandard abbreviations: HPMC, hydroxypropylmethylcellulose; **TBE,** Tris-borate-EDTA buffer; **TE,** Tris-EDTA buffer

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2 Materials and methods

2.1 CE instrumentation

CE was performed on a Beckman P/ACE System 2210 equipped with a P/ACE UV Absorbance Detector (Beckman Instruments, Palo Alto, CA, USA). Total capillary length was 27 cm (20 cm effective length). Beckman System Gold software (Version 8.10) was used for system control, data collection and processing. The data collection rate was set to 20 Hz. Electropherograms for preparation of figures were transferred as DIF files to an Apple Macintosh computer and redrawn by Sigma Plot (Version 4.16; Jandel Scientific, Erkrath, Germany).

2.2 Chemicals

Phenylmethylpolysiloxane-coated fused silica capillaries (DB-17, 100 μm ID, 375 μm OD, 0.1 μm film thickness) were purchased from J & W Scientific (Folsom, CA, USA). Membrane filters (0.2 μm pore size) were from Schleicher & Schuell (Dassel, Germany) and MF-Millipore membrane filters (0.025 μm pore size, 13 mm diameter) from Millipore (Bedford, MA, USA). Hydroxypropylmethylcellulose (HPMC, 4000 cP at 25°C, 2% aqueous solution) and other chemicals (analytical or molecular biology grade) were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). Solutions containing HPMC were prepared as described elsewhere [15]. Water, double-distilled and purified on a Milli-Q Plus Water Purification System (Millipore), was used for preparation of all aqueous solutions.

2.3 Nucleic acids

An RNA molecular mass marker was purchased from Promega (Madison, WI, USA; RNA transcripts: 281, 623,

0173-0835/96/0909-1512 $10.00+.25/0

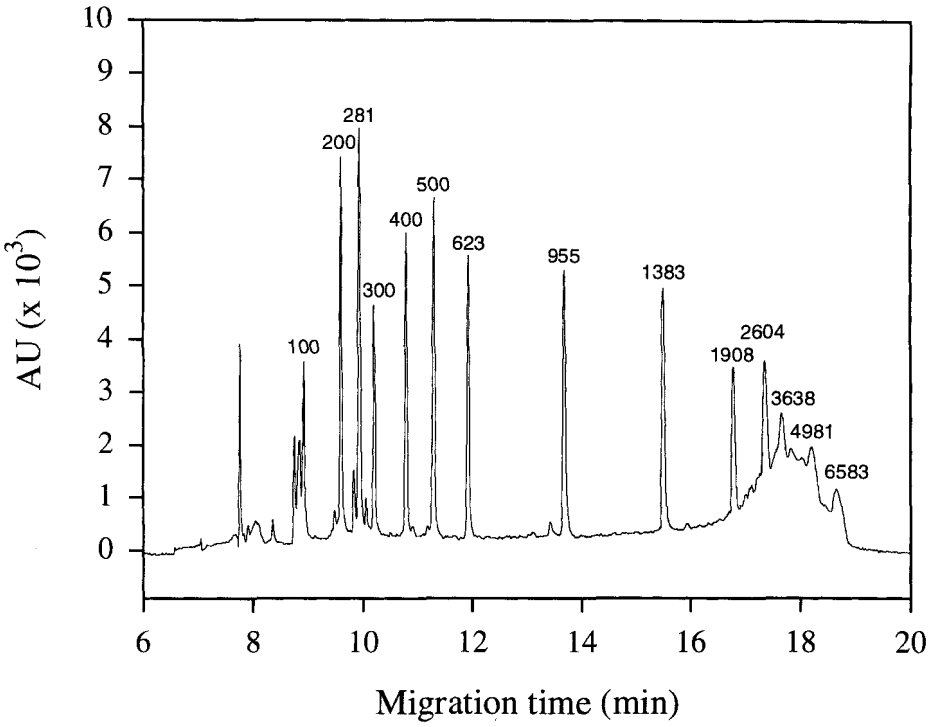

Figure 1. Electropherogram of RNA molecular mass marker. The sample was denatured, injected at 300 V/cm for 10 s and subjected to CE at 200 V/cm in 1 × TBE/8 M urea containing 0.3% HPMC. Other experimental conditions were as described in Section 2.4. AU, arbitrary units.

955, 1383, 1908, 2604, 3638, 4981 and 6583 bases). Another marker was synthesized by *in vitro* transciption of the RNA Century Marker Template Set from Ambion (Austin, TX, USA) using the MAXIScript T7 Kit (Ambion; RNA transcripts: 100, 200, 300, 400 and 500 bases). The RNA markers were dialyzed against 10 mM Tris, pH 8.0, 1 mM EDTA (TE buffer) on an MF-millipore membrane filter (Millipore) for 20 min [16] and mixed to obtain a combined marker with molecular mass distribution from 100 to 6583 bases (RNA molecular mass marker). The DNA Mass Ladder (an equimolar composition of 100, 200, 400, 800, 1200 and 2000 bp DNA fragments) was purchased from Life Technologies (Gaithersburg, MD, USA). Total RNA was extracted from the human glioma cell line GaMg, using Trizol (Life Technologies) [17], dissolved in TE buffer, quantitated by UV spectrophotometry and diluted to the appropriate concentration in formamide [18].

2.4 CE

Electrophoresis buffer was 89 mM Tris-borate, 2 mM EDTA, pH 8.3 (1 × TBE), 8 M urea. Separation buffer was 1 × TBE buffer containing up to 8 M urea and 0.1–1.0% w/v HPMC. All buffers were passed through membrane filters (0.2 μm pore size) and degassed by sonication for 10 min. The capillary was filled with separation buffer by high pressure injection (137 kPa) for 60 s. CE was performed in the reversed polarity mode with the capillary temperature set at 25°C unless otherwise stated. Prior to CE, RNA and DNA samples were dissolved in 80% formamide, heated at 95°C for 3 min to completely denature the nucleic acids, and cooled for 3 min to room temperature. Sample heating and cooling were performed in a programmable thermal cycler. Samples were injected electrokinetically at 300 V/cm for 10 s and separation performed at an electrical field strength of 200 V/cm unless otherwise stated. After each electroseparation, the capillary was flushed with 1 × TBE for 30 s by high pressure. To obtain complete replacement of the high viscosity separation buffers (≥0.7% HPMC), flushing with 1 × TBE and filling with separation buffer was extended to 120 s.

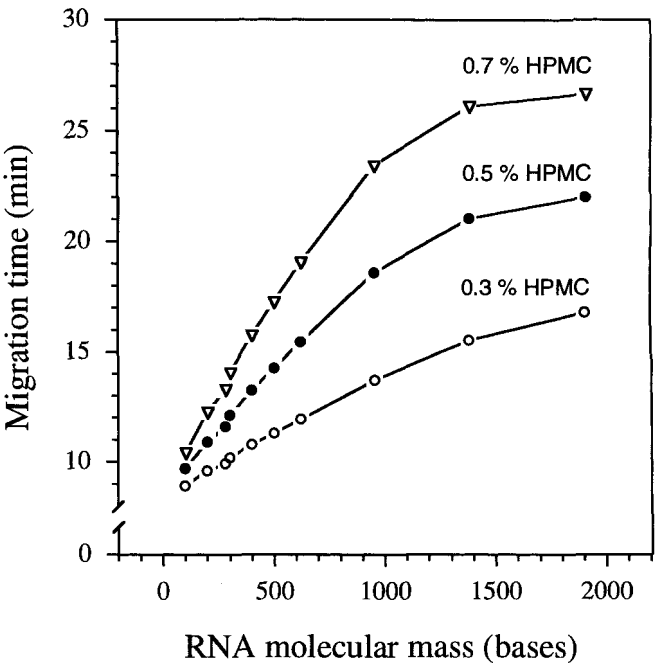

Figure 2. Migration time *versus* RNA molecular mass at three different concentrations of gel polymer. Experimental conditions were as described in the legend to Fig. 1.

3 Results and discussion

The object of this study was to investigate the separation of single-stranded RNA by capillary electrophoresis. As a consequence of the structural similarities of DNA and RNA, we initially focused on physical and chemical parameters known to be major determinants of the electrophoretic separation of DNA in capillaries [19], *i.e.*, electrical field strength, temperature, and gel polymer concentration. The effect of denaturant on the electromigration of RNA was also investigated due to the anomalous migration which can be caused by the formation of inter- and intramolecular (secondary) structures in RNA [8, 20, 21]. Figure 1 illustrates CE of the RNA molecular mass marker (gel polymer concentration, 0.3%). All 14 transcipts were separated in less than 20 min. Peak assignments were confirmed by separate runs of the two components of the RNA marker. High resolution is obtained for RNA molecules up to approximately 1200 bases. At higher RNA molecular masses, resolution drops significantly.

3.1 Migration *versus* molecular mass

In conventional gel electrophoresis, the rate of migration of an RNA molecule is often inversely related to the $\log_{10}$ molecular mass. However, base composition, secondary structure and other factors can affect this relationship [8]. Consequently, analysis under completely denaturing conditions is required for accurate determination of RNA molecular mass. Figure 2 shows a typical plot of migration time *versus* molecular mass for the components in the RNA molecular mass marker (at three different gel polymer concentrations). There is a linear relation between the migration time and the molecular mass from 100 to 500 bases. At higher molecular masses, a deviation from linearity occurs. In CE of ssDNA, a linear relationship has been observed for molecules up to approximately 500 bases [22]. Consequently, RNA and DNA appears to have similar electrophoretic behavior in gel-filled capillaries. In order to compare directly the migration of RNA with ssDNA, we combined the RNA marker and the DNA mass ladder. For subsequent identification of the RNA and DNA components, the two components were denatured and analyzed separately. Figure 3 illustrates a small but significant difference in electrophoretic migration between the RNA and DNA components.

3.2 Gel polymer concentration

A number of linear gel polymer matrices have been used for the separation of DNA in CE [23–25]. Among these, the cellulose derivative HPMC has shown several favorable properties, including ease of preparation and low viscosity [23]. The concentration of HPMC in the separation buffer determines the optimal molecular mass range of the separation, and high concentrations favor the resolution of small fragments. We investigated the effect of varying the HPMC concentration (0.1%–1.0%) in the separation buffer on the migration time (Fig. 4A), plate number (Fig. 4B) and resolution (Fig. 4C) of the components in the RNA molecular mass marker. Migration

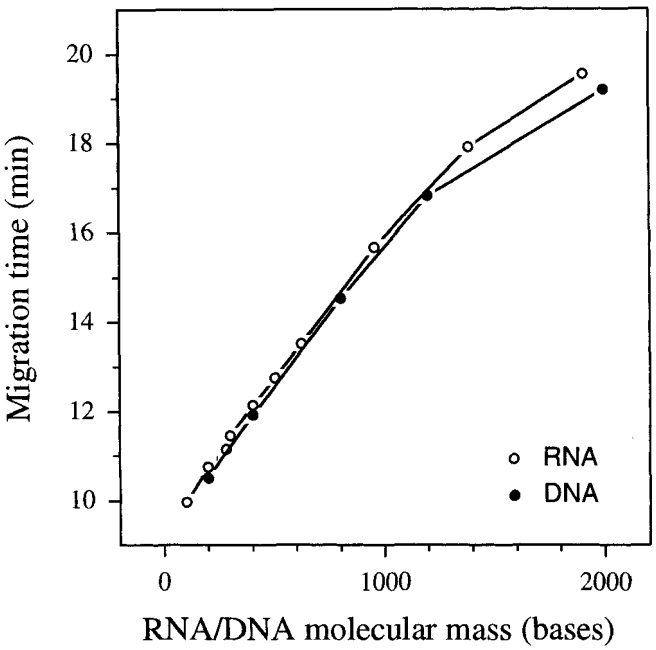

Figure 3. Comparison of migration of RNA and single-stranded DNA. The combined molecular mass RNA marker and the DNA mass ladder were mixed, denatured by preincubation at 95°C for 3 min in presence of 80% formamide and subjected to electrophoreis in a separation buffer containing 8 M urea and 0.3% HPMC. Experimental details are given in the text. Migration time is plotted *versus* molecular mass for the RNA and DNA molecules.

time is linearly related to gel polymer concentration for each of the RNA species up to about 0.6% HPMC. Plate numbers for RNA molecules smaller than 1000 bases increase up to a polymer concentration of 0.2% and subsequently decrease. For the larger RNA molecules, plate numbers decrease as a function of gel polymer concentration over the whole concentration range. Resolution increases with HPMC concentration up to 0.7%. Consequently, maximum resolution of RNA for a given molecular mass range may be obtained by careful optimization of this analysis parameter.

3.3 Electrical field strength

Figure 5A illustrates the decline in migration times for RNA molecules up to approximately 2000 bases as a function of increasing electrical field strength. Except for the 1383-bases RNA transcript, the plate number is highest at low electrical field strengths (Fig. 5B). Resolution is essentially constant for the three RNA doublets up to about 200 V/cm, and subsequently declines (Fig. 5C). At higher electrical field strengths (>300 V/cm), resolution for the RNA doublets shows a marked decline (approximately by a factor of two), indicating that lower electrical field strengths are preferable for the optimal separation of higher molecular mass RNA.

3.4 Temperature

Temperature has been found to influence total analysis time and system efficiency in capillary gel electrophoresis of DNA [19]. The effects are mainly mediated by a decrease in separation buffer viscosity. Figure 6 illustrates a similar relation for CE of RNA. The migra

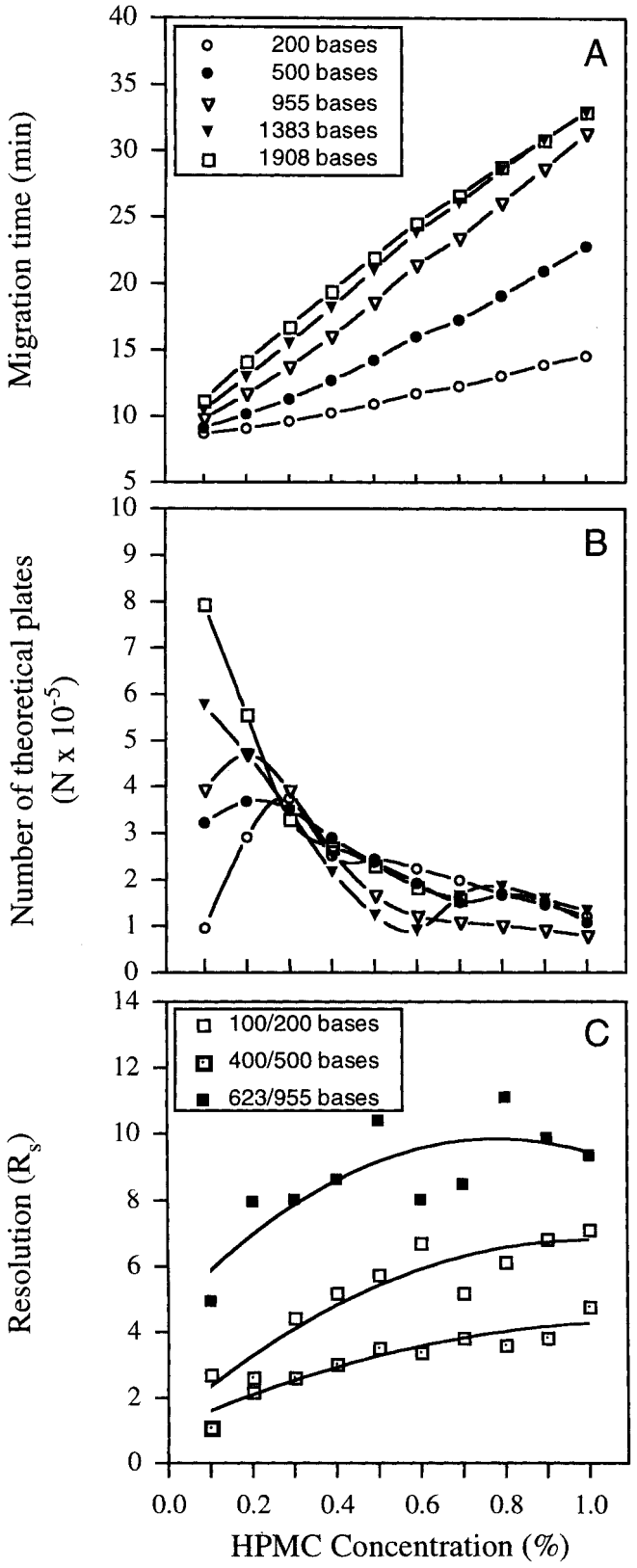

Figure 4. Migration time, plate numbers and resolution of components of the RNA molecular mass marker as a functin of gel polymer concentration in separation buffer. The sample was subjected to electrophoresis in the separation buffers containing increasing concentrations of HPMC. Other experimental conditions were as described in the legend to Fig. 1. Data were obtained from an automatic sequence of single analyses. Resolution, R_s, was calculated using the following expression: $R_s = \Delta x/4\sigma$, where Δx is the distance between the peak centers and 4σ is the mean of the two standard deviations of the peaks. The lines drawn in *C* represents a second-order polynomial regression line through the data.

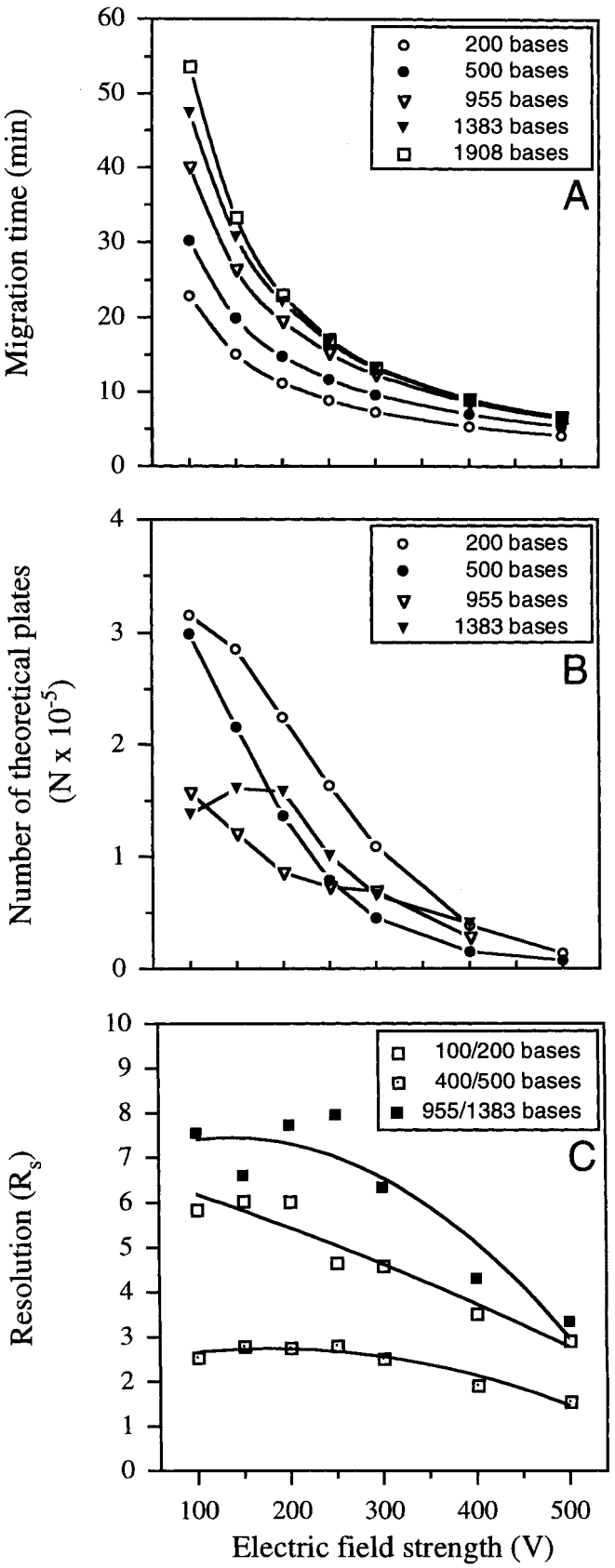

Figure 5. Migration time, plate numbers and resolution of components of the RNA molecular mass marker as a function of electrical field strength. The sample was subjected to electrophoresis under increasing electrical field strengths. Other experimental conditions were as described in the legend to Fig. 1. Data were obtained from an automatic sequence of single analyses. Resolutions, R_s, was calculated as described in the legend to Fig. 4. The lines drawn in *C* represents a second-order polynomial regression line through the data.

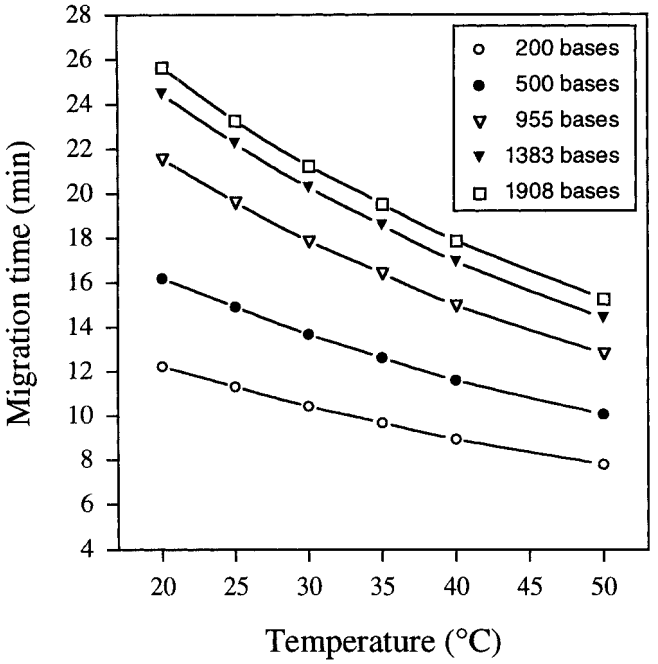

Figure 6. Migration time of components in the RNA molecular mass marker as a function of electrophoresis temperature. The sample was subjected to electrophoresis at increasing temperatures from 20°C to 50°C. Other experimental conditions were as described in the legend to Fig. 1.

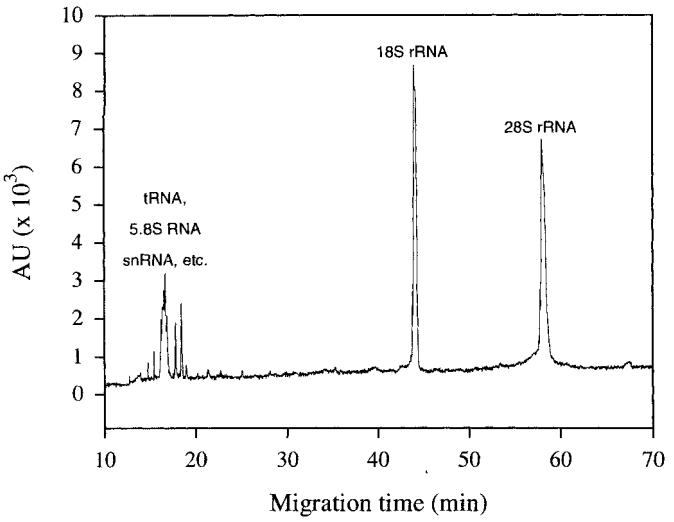

Figure 7. CE analysis of total RNA isolated from human glioma cells. The sample was denatured by preincubation at 95°C for 3 min in the presence of 80% formamide, injected at 300 V/cm for 10 s, followed by electrophoresis at 100 V/cm in 1 × TBE/8 M urea containing 0.5% HPMC. Other experimental conditions were as described in Section 2.4. AU, arbitrary units.

tion times of the RNA molecules decrease as a function of temperature between 20–50°C. Plate numbers and resolution for the RNA species remain essentially the same over the whole temperature range (data not shown). Consequently, high temperature confers an increasing denaturing effect and shorter migration times without a significant loss in efficiency.

3.5 Denaturant

In order to prevent anomalous electrophoretic migration of RNA resulting from the formation of inter- and intramolecular (secondary) structures, samples were denatured prior to electrophoresis and electrophoresis was carried out under denaturing conditions. In our experiments, the samples were dissolved in 80% formamide, heated at 95°C for 3 min, cooled to room temperature and injected. Urea (8 M) was added to the separation buffer as denaturant. The CE instrument was cleaned frequently to prevent current error resulting from precipitation of urea at the cathode. In a series of experiments, the urea concentration of the separation buffer was gradually reduced from 8 M to 0 M. This resulted in a decrease in migration times for all RNA molecules of the marker (data not shown). Plate numbers of all the RNA molecules remained essentially the same for urea concentrations from 8 M to 2 M (data not shown). In the absence of urea, we observed anomalous migration and marked reduction in efficiency, especially for RNA of higher molecular mass. The electropherogram also displayed a more complex profile, and the separate RNA components were not readily identified. The phenomenon probably reflects the formation of secondary structures in RNA.

3.6 Analysis of human total RNA

We demonstrated the applicability of CE for the separation of RNA from biological samples by analyzing total RNA extracted from the human glioma cell line, GaMg. The RNA sample was denatured and subjected to electrophoresis under the same conditions as for the RNA molecular mass marker (Fig. 1). The resulting electropherogram displays three main RNA populations (Fig. 7). The first group of peaks comprises small RNA species (tRNA, 5.8S rRNA, snRNA, *etc.*, 60–300 bases in length), the second peak represents 18S rRNA (1868 bases) and the third peak 28S rRNA (5025 bases).

4 Concluding remarks

The present paper represents the first systematic study on the separation of RNA by CE. On the basis of experimences with CE of DNA, an analytical system was developed and parameters including gel polymer concentration, electrical field strength, temperature and denaturant were investigated for their effect on total analysis time, system efficiency and resolution. Efficient separations of RNA molecules ranging from 100 to more than 6000 bases were achieved within minutes through optimization of gel polymer concentration and electrical field strength. The results demonstrate the applicability of CE as an analytical technique for the analysis of RNA and should motivate further analytical work with the intent to transfer RNA analyses from the slab gel to the capillary format.

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