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Sequencing of antisense DNA analogues by capillary gel electrophoresis with laser-induced fluorescence detection

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Abstract

A method using capillary gel electrophoresis with laser-induced fluorescence detection is described which permits complete sequence determination of antisense DNA analogues of unknown sequence. This method, originally created as a tool to confirm the sequence of antisense oligonucleotides being developed as therapeutic drugs, utilizes data collected under a range of experimental conditions described by the Ogston model as applied to gel electrophoresis. A linear relationship independent of experimental conditions between the relative electrophoretic migration time and the oligonucleotide base number was observed and is shown to be consistent with a simplified version of this model and can be used to facilitate the sequence determination.

1. Introduction

Antisense medicine is still in its infancy, but is maturing rapidly. Several human trials have already begun or are about to begin, and sequencing that determines the target-sense as well as the antisense sequence is the key to successful anti-viral treatment. Antisense compounds under investigation are chemically modified DNA such as phosphorothioates or methylphosphonates and are in general between 15 to 50 bases in length. The chemical modification of phosphodiester DNA is normally performed to inhibit enzymatic activity. For example, phosphorothioates are much more resistant to exonucleases than unmodified DNA (phosphodiesters) [1]. The chemical properties of a modified DNA molecule can be quite different from its phosphodiester counterpart. For example, the pK_a due to charge distribution on phosphorothioates is different than the pK_a of phosphodiesters [2]; phosphorothioates are more hydrophobic than phosphodiesters and therefore exhibit more secondary structure behavior [3]. Consequently, optimization and modification of analytical methods used for phosphodiesters need to be made and protocols need to be updated to reflect this different chemistry [4].

DNA sequence determination is an important structural analysis, especially when information is needed to determine an unknown sequence of a short strand of DNA or DNA analogue or to confirm the specific sequence of a certain antisense drug. At present, two methods are used to prepare phosphodiester DNA fragments for sequencing: the chemical degradation approach of Maxam and Gilbert [5] and the chain-termination method of Sanger et al. [6]. Four separate reactions yield fragments differing in length by only a single nucleotide which terminate at adenosine (A), cytosine (C), guanosine (G) or thymidine (T) residues. These products are gen-

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erally resolved by electrophoresis on a denaturing polyacrylamide gel. The method of product visualization has traditionally been autoradiography, usually ^{32}P or ^{35}S incorporated into the DNA strand; however, fluorescent detection of DNA fragments has been recently introduced. Fluorescent tags are attached either to the primer [7] or to each of the terminating dideoxynucleotides [8]. In either case, detection can be achieved using laser-induced fluorescence (LIF). Automated LIF methods bypass the normal post-electrophoresis manipulations. The fluorescently labeled fragments are detected on-line; the data are collected and sent to a computer via an analog-to-digital converter for processing and analysis.

Many different DNA sequencing protocols originating from the chain termination reaction of Sanger et al. [6] have been developed over the years. Recently, sequencing with LIF detection was used in a variety of protocols including single-dye coding of bases with four different peak heights [9–12], single-dye coding of three bases by peak height ratios plus one base coded by a gap [10,12], two-dye-binary coding of three bases with one base coded by a gap [13], two-dye coding by peak height ratios with two optical channels [14], and four-dye coding with two optical channels [15]. Although all of these methods offer different aspects of flexibility, none of them describe the complete sequencing of a short single-stranded DNA (ssDNA) from the very first to the very last base. Moreover, none of these methods were ever applied in practice for routine sequencing, and sequencing of antisense DNA analogues was not considered.

Successful sequencing depends on the separation step. A problem that is sometimes encountered during sequencing is band compression when different fragments possessing similar electrophoretic migration times are not resolved leading to an ambiguous or incorrect sequence. One cause of this phenomenon is the effect of sequence-specific secondary structure. Denaturing conditions can minimize the effects of secondary structure. Under our experimental conditions, band compression does not appear to be a problem for the short fragments under consideration.

One of the fastest growing areas in separation science today is capillary electrophoresis (CE). The method is similar to high-performance liquid chromatography (HPLC) in its instrumentation and operation, but differs in the principle of separation, CE has high resolving power, and low mass detection limits [16]. As active participants in the introduction and development of oligonucleotide separations by CE, we have utilized capillary columns both in the open-tube mode [17] and the gel-filled mode, also known as capillary gel electrophoresis (CGE) [18]. Separations with a very high resolving power of 30 million theoretical plates per meter have been achieved using CGE [19]. Based on these results, CGE potentially can be utilized in routine DNA sequencing [20]. The method is readily coupled to a variety of detection methods including LIF and mass spectrometry (MS). With the rapid development of matrix-assisted laser desorption ionization (MALDI) MS, the combination of CE and MS promises to be an important analytical tool [21,22].

Enzymatic sequencing of short DNA analogue substrates using MALDI-MS for detection has been documented very recently [23,24]. The method uses exonucleases with phosphodiester DNA as a substrate. The protocol is relatively slow; aliquots are taken every 15 min and directly analyzed by MALDI-MS [25]. When DNA analogues are to be sequenced under these conditions, exonuclease digestion is problematic because the analogues have been designed for their insusceptibility to exonucleases [1]. In any case, the development of a method, by which an antisense DNA analogue sequence can be determined from the very first to the very last base will be a major contribution to the sequencing effort. The method which we now describe is such a method and can be automated, validated, and used for routine sequence determination.

2. Experimental

2.1. Chemicals and reagents

Ultra-pure Tris base, urea, acrylamide and EDTA were purchased from Schwartz/Mann Biotech (Cleveland, OH, USA). Ammonium

persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA, USA). Boric acid was obtained from Sigma (St. Louis, MO, USA). All phosphorothioate oligomers were synthesized in the laboratory, desalted, lyophilized, and reconstituted in sterile water for injection (Lyphomed, a division of Fujisawa USA, Deerfield, IL, USA). The fluorescently tagged primers were obtained from Applied Biosystems (Foster City, CA, USA).

2.2. CE apparatus

The CE apparatus with UV and LIF detection and the preparation of gel-filled capillaries for the separation of DNA molecules have been described previously [20]. A 30 kV, 500 μA direct-current high-voltage power supply (Model ER/DM; Glassman, Whitehouse Station, NJ, USA) was used to generate the potential across the capillary. UV detection of phosphorothioates at 270 nm was accomplished with a Spectra 100 (Spectra-Physics, San Jose, CA, USA). For LIF detection an argon ion laser (Model 543 100BS; Omnichrom, Chino, CA USA) was employed. All CE runs were performed at room temperature. The data were acquired and stored on an AcerPower 486/33 computer (Acer American, San Jose, CA, USA) through an analog-to-digital converter (Model 970; PE Nelson, Cupertino, CA, USA).

2.3. Gel-filled capillaries

Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm, an outer diameter of 375 μm, an effective length of 10–15 cm, and a total length of 30–60 cm was treated with (methylacryloxypropyl)trimethoxysilane (Petrarch Systems, Bristol, PA, USA) and then filled with a de-gassed solution of polymerizing acrylamide in aqueous media with formamide [1–3 × TBE buffer (1 × TBE buffer = 0.1 *M* Tris–borate, 2 m*M* EDTA), pH 8.3 containing 6–8 *M* urea]. Polymerization was achieved by adding ammonium persulfate solution and TEMED.

2.4. Sequencing method

A method was developed to determine the sequence of a short strand of DNA or DNA analogue. This method consists of 6 steps: (1) synthesis of auxiliary DNA (auxiliary DNA phosphorylated at the 5′ end was purchased from New England Biolabs, Beverly, MA, USA), (2) ligation of the auxiliary DNA to the DNA for which the sequence has to be determined by either "bridge" ligation or "blunt" ligation, (3) primer annealing (the primer is tagged with a fluorescent label), (4) sequencing using Sequenase 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH, USA), (5) separation of the sequencing mixture (by CGE–LIF) and (6) sequence determination.

3. Results and discussion

As mentioned earlier, antisense DNA molecules under investigation are chemically modified analogues which may possess quite different chemical properties compared to their phosphodiester counterparts and are often modified to inhibit enzymatic activity. The outcome of any pre-sequencing enzymatic preparation of these compounds is not apparent. Consequently, several different approaches were investigated.

3.1. "Bridge" ligation using T_4 DNA ligase

In developing our sequencing method, we began by using T_4 DNA ligation as the pre-sequence enzymatic preparation step. If the Sanger approach is used to sequence a short ssDNA, some base sequence information is lost at the 3′ end. This loss of information is primer-size dependent and normally 15–17 bases, i.e., sequence information will only be available right after the primer. Because we want to determine the sequence from the very first base, base 1, to the very last base, we need to protect the first base at the 3′ end by ligating a known sequence of single-stranded oligonucleotide which can hybridize to the primer. This segment of oligonucleotide is defined as the auxiliary DNA. The auxiliary DNA is composed of two identified

sequence regions. One region from the 3′ end consists of 17 bases complementary to the sequence of the primer, in our case the M13mp18(−21) primer, followed by a signaling region of nine T bases (schematically shown in Fig. 1). The function of the signaling region is to denote the beginning of the reading sequence. Base 1 of the oligomer to be sequenced is located right after the auxiliary DNA. A 12-mer bridge DNA is also used in the ligation reaction mixture to support both the antisense and the auxiliary DNA and to facilitate the ligation reaction. This 12-mer bridge consists of two regions of six bases, one complementary to the last six bases of the auxiliary DNA at the 5′ end and the other to the first six bases of the antisense DNA to be sequenced. This imposes a limitation since the first six bases from 3′ end of the antisense oligomer must be known prior to using this procedure. This limitation can be overcome using a different ligation approach as will be described later.

In Fig. 2 the electropherograms before and after ligation are shown, using CGE with UV detection. We observe in Fig. 2a the fast-migrating 12-mer bridge DNA followed by the phosphorothioate DNA analogue (GEM) to be sequenced; the last migrating peak is the 32-mer auxiliary DNA. The mixture is incubated for 30 min. at 37°C after the addition of T_4 DNA ligase and ATP and is then injected into the capillary for analysis by CGE. The ligation product of the GEM 25-mer to be sequenced and the auxiliary DNA, a 32-mer, is a 57-mer which appears as an additional peak shown in Fig. 2b. This 57-mer is then isolated and subjected to chain-termination reactions. As indicated previously, to design the appropriate helper bridge and use this procedure, the sequence of the six bases at the 3′ end of GEM or any ssDNA molecule to be sequenced must be known; however, if the six bases at the 3′ end of the ssDNA analogue to be sequenced are unknown, a bridge DNA cannot be designed and used in the ligation reaction. This disadvantage limits the technique to sequence confirmation instead of a general sequencing method.

Another problem encountered using the bridge ligation approach was an incomplete sequencing reaction. Since the bridge oligonucleotide has a complementary sequence to a site on the ligated product, it can interfere with the enzymatic sequencing reaction. Under the experimental conditions, the bridge oligonucleotide can rehybridize to the complementary site on the DNA to be sequenced right after $(T)_9$ from the 3′ end, and consequently, the sequencing reaction will be stopped before completion. Al-

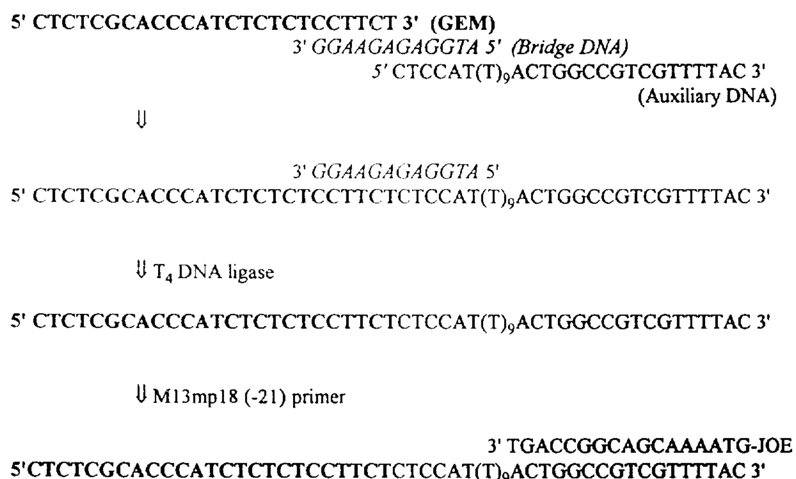

Fig. 1. Schematic representation of the "bridge" ligation protocol.

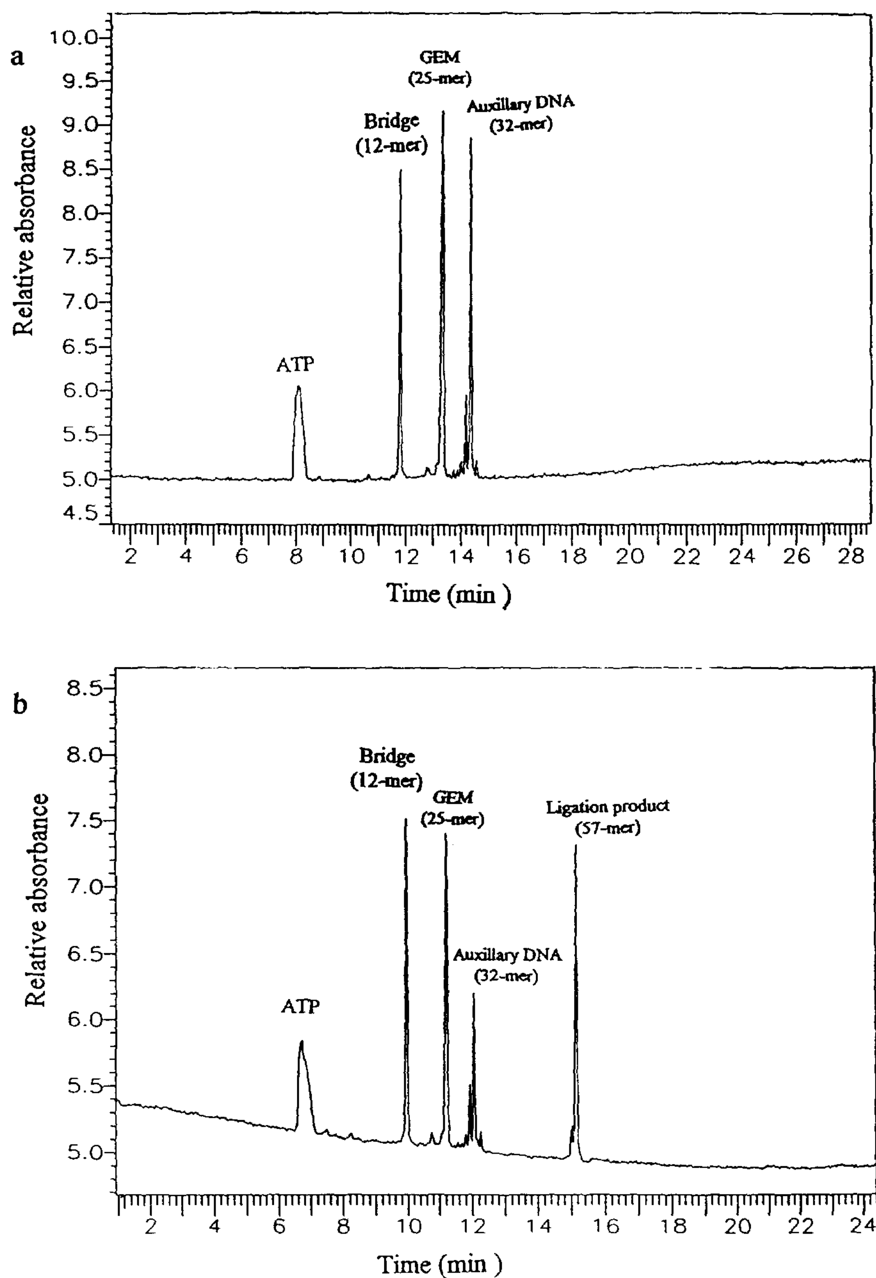

Fig. 2. UV electropherograms of the T_4 DNA ligase reaction mixture. (a) Prior to ligation. Migration order of the detected peaks: ATP, bridge DNA (12-mer), GEM (25-mer) and auxiliary DNA (32-mer). Running buffer was 1 × TBE, and the gel was 9% T polyacrylamide, 7 *M* urea. The applied electric field was 300 V/cm. (For more details, see Experimental section). (b) After the addition of T_4 DNA ligase. The slowest migrating peak is the ligation product (57-mer).

though this interference can be prevented by slab gel purification to remove the bridge DNA, the procedure is cumbersome. We have, therefore, utilized T_4 RNA ligase enzyme, which under the appropriate conditions can ligate any two unknown sequences of ssDNA [26]. Because a bridge DNA is not needed for ligation, this "blunt" ligation procedure overcomes the limitations of the bridge ligation approach.

3.2. "Blunt" ligation using T_4 RNA ligase

Experimental results in Fig. 3a show the products of the T_4 RNA ligase reaction. Because the 3′ end on the auxiliary DNA is unprotected, the enzyme forces the ligation process to proceed in cycles, and several cycles are observed. This undesirable phenomenon, which complicates the ligation procedure, can be prevented simply by having a dideoxy group or an amino group at the 3′ end of the auxiliary DNA. Results of the ligation reaction with the auxiliary DNA protected by an amino group at the 3′ end are shown in Fig. 3b. These results demonstrate that only one ligation cycle was obtained when the 3′ end of the auxiliary DNA was protected, and as with the T_4 DNA ligase procedure, ATP, GEM 25-mer, the 32-mer auxiliary DNA, and the 57-mer ligation product can be observed.

3.3. Antisense DNA analogue sequence determination

Our goal in pursuing this research was to develop an automated ssDNA sequencer for routine antisense analysis. With this in mind, we next turned to develop a working strategy to examine enzymatic sequencing of antisense DNA analogues using the T_4 RNA ligase procedure described in the previous section. Good modeling of the electrophoretic migration of the sequencing fragments is essential for automated data processing; therefore, we focused our attention on developing a better understanding of the migration behavior of the sequencing fragments under our experimental conditions.

We and others [27] have observed experimentally that over a narrow range of molecular size, a linear relationship between time and base number can be established. This relationship, which we have used to simplify the sequencing step, will be shown to be consistent with a simplified version of the Ogston model. We begin by noting that the range of experimental conditions we have selected are such that the electrophoretic mobility of the probe molecule falls within the so-called Ogston regime (see [28]). In other words, the probe molecules are small compared to the matrix mesh size and are unentangled by the polymer matrix [29]. By invoking the Ogston model, we can develop a simplified expression relating the number of bases to the migration time. We define a migration time t_n that is relative to the migration time of two internal standards, A and B. Thus,

$$t_n = \frac{t - t_B}{t_A - t_B} \quad (1)$$

where t is the migration time of the probe molecule and t_A and t_B are the migration times of standards A and B, respectively. Standards A and B are oligonucleotides of the same chemical nature as the probe molecule and possess base numbers, N_A and N_B, respectively, that bracket the probe's base number N ($N_A > N > N_B$). The selection of internal standards is facilitated by our sequencing method; one standard is the primer (N_{17}) and the other is an extra fragment created by Sequenase 2.0 terminal transferase activity after the end of the sequence (e.g., N_{58}).

Starting with the Ogston model, we can relate this relative time to the probe's base number. Ogston derived the distribution of spaces in a random network of fibers available to a spherical object [30]. This distribution was related to the electrophoretic mobility by Rodbard and Chrambach [31] and can be written in the following simplified form [32]:

$$\mu = \mu_0 \exp(-\alpha\lambda CN) \quad (2)$$

where μ is the electrophoretic mobility, μ_0 is the electrophoretic mobility in free solution, α and λ are constants, C is the matrix concentration and N is the base number. This simplified form assumes that the probe chain's radius of gyration

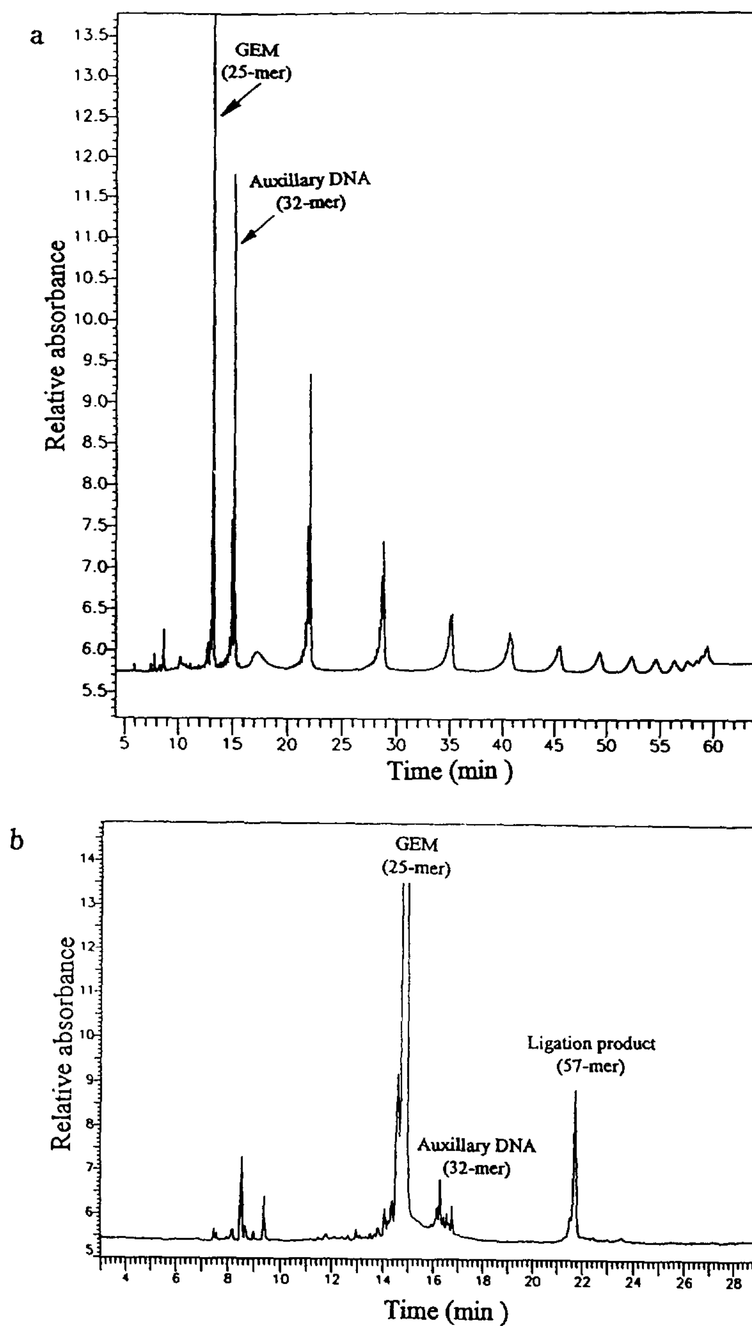

Fig. 3. UV electropherograms of T_4 RNA ligation products. (a) Several extended ligation cycles are observed. Auxiliary DNA (32-mer) with 5′ phosphate and 3′-OH was used. All other conditions as in Fig. 2. (b) Auxiliary DNA (32-mer) with 5′ phosphate and 3′ amino protection was used. No extended ligation cycles are observed.

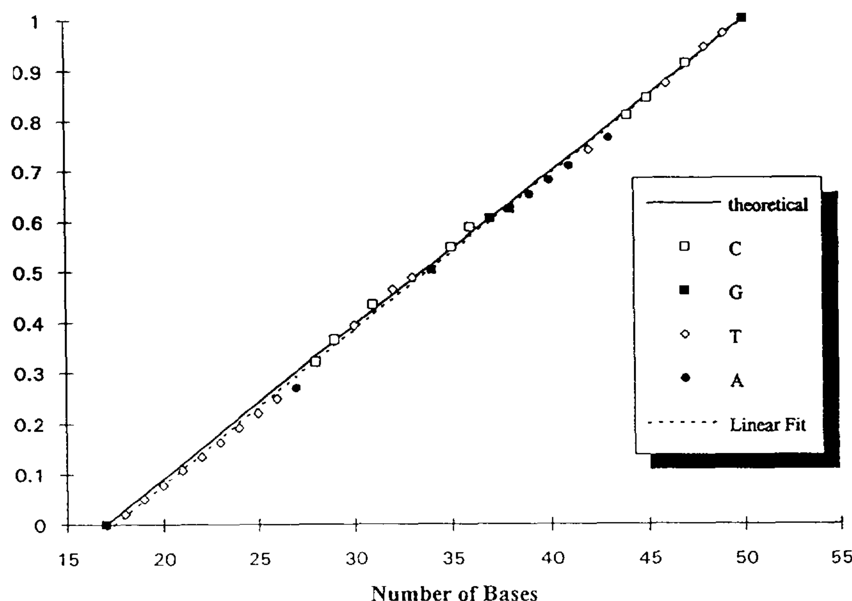

Fig. 4. Relative time (y-axis) plotted as a function of base number. Oligonucleotide standards: $N_A = 50$; $N_B = 17$. Capillary gel electrophoresis: 11% T linear polyacrylamide; 6.5 M urea, 45% formamide, 2 × TBE buffer; 400 V/cm for each sequencing reaction. Dotted line represents a linear fit with $R^2 = 0.9988$. Solid line represents the right-hand side of Eq. 6.

is much larger than the polymer matrix strand radius and that the probe chain's persistence length is small compared to its contour length (i.e., the chain should be highly flexible). For ssDNA under denaturing conditions in a polyacrylamide matrix, these assumptions appear reasonable.

From the definition of electrophoretic mobility, we can write

$$\mu = \frac{\nu}{E} = \frac{x}{tE} \tag{3}$$

where ν is the average velocity, E is the externally applied electric field strength and x is the distance traveled in time t. Using Eq. 2 and solving for t, we obtain

$$t = \left(\frac{x}{E\mu_0}\right) \cdot \exp\,(\alpha\lambda CN) \tag{4}$$

Substituting Eq. 4 into Eq. 1 and rearranging, we obtain

$$t_n = \frac{\exp\,[\alpha\lambda C(N - N_B)] - 1}{\exp\,[\alpha\lambda C(N_A - N_B)] - 1} \tag{5}$$

Using a Taylor series expansion and neglecting higher order terms, we can linearize the expression to

$$t_n = \frac{t - t_B}{t_A - t_B} = \frac{N - N_B}{N_A - N_B} \tag{6}$$

If we plot data obtained by CGE–LIF, we indeed observe this linear relationship as is illustrated in Fig. 4. A linear fit of this data closely matches the theoretical value (the right-hand side of Eq. 6) as shown in the figure. Although the data used in the figure are taken from different runs performed under identical conditions, we note that the relationship between N and t is independent of experimental parameters such as matrix concentration and electric field strength. We should be able, therefore, to use the results from several different runs under very different conditions to obtain the relative times. We have observed this independence for different electric field strengths from 200 to 400 V/cm as well as different gel concentrations ranging from 6 to 14% T[1] linear polyacrylamide and effective lengths from 6 to 15

[1] T = (g acrylamide + g N,N′-methylenebisacrylamide)/100 ml solution

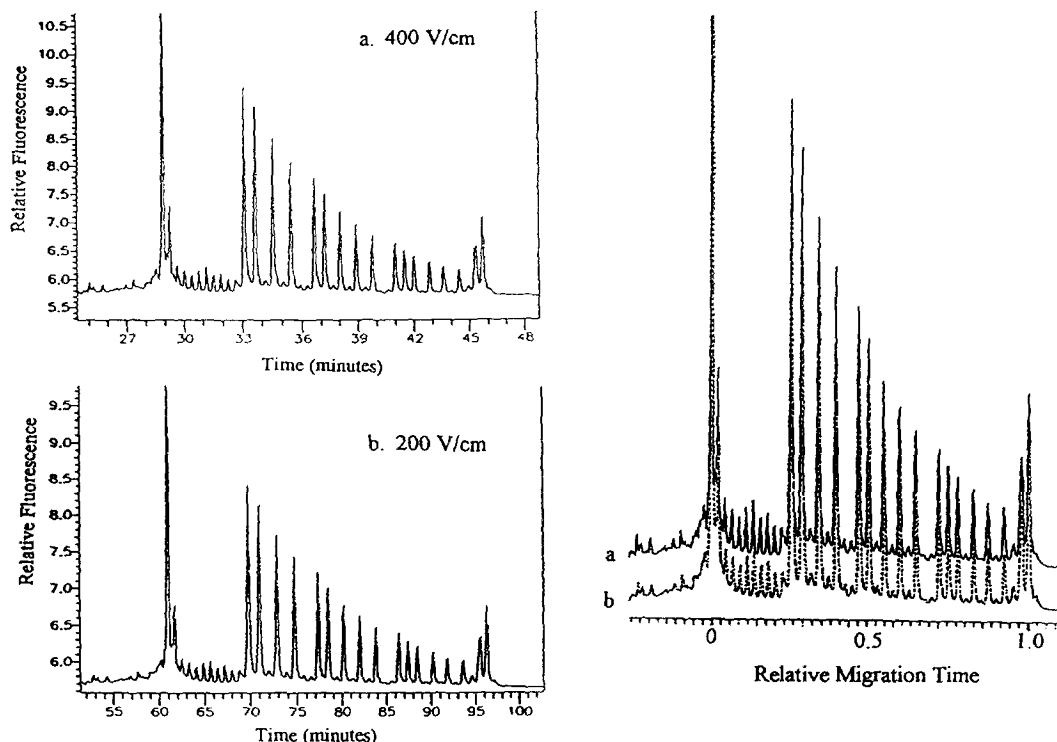

Fig. 5. Effect of electric field strength on the analysis of the ddG terminated sequencing reaction. (a) 400 V/cm, (b) 200 V/cm. Conditions: 13% T polyacrylamide, 2 × TBE buffer, 6 *M* urea, 40% formamide, effective length 12 cm.

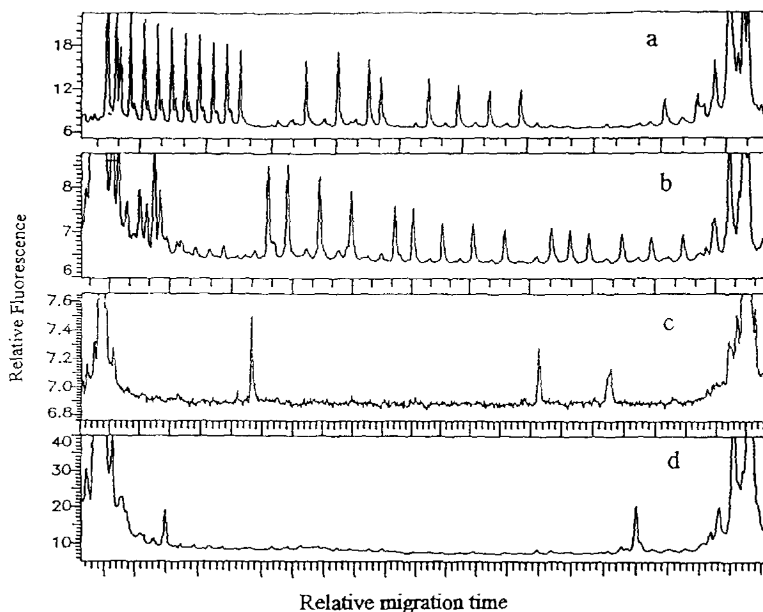

Fig. 6. LIF electropherogram of sequencing fragments from T_4 DNA ligation product (57-mer) (see Experimental section for details). Running buffer is 2 × TBE and the gel contained 14% T polyacrylamide, 7 *M* urea, 54% formamide. (a) ddA, (b) ddG, (c) ddT, (d) ddC terminated sequencing reaction. All other conditions as in Fig. 2.

cm. For example, the effect of electric field strength is shown in Fig. 5. Although the actual migration times differ by about a factor of two, when the relative migration times are used, the two runs can be readily superimposed.

By performing CGE–LIF on separate sequencing reactions for each base using the same fluorescent label to avoid potential band compression, we are able with the aid of a simple computer program to deduce the correct sequence for short oligomers using the relative time. Care should be taken in using Eq. 6 because it is related to the Ogston model only in a very limited context. Although the Ogston model may have limited applicability in a strict sense to flexible macromolecules [33], it is nevertheless a useful starting point, and we have found that the linear relationship of Eq. 6 is sufficient for the purpose of sequencing short oligomers.

Eq. 6 gives us a useful expression for t_n which is only fragment-size dependent. This expression is, of course, a conditional statement since it holds only under Ogston limitations. The best way to validate this relationship is to test this new model under experimental conditions. The ligated product (a 57-mer) was subjected to enzymatic chain termination reaction for four different bases independently (i.e., A, G, C and T). Each of the four bases ran separately as

Table 1
Relative migration time obtained from the data of Fig. 6

Fragment length (number of bases)		Relative migration time, $t_n = (t - t_B)/(t_A - t_B)$; base				Reading sequence
		A	T	C	G	
Real	GEM					
						3′
33	1		0.370			T
34	2			0.391		C
35	3		0.421			T
36	4		0.441			T
37	5			0.460		C
38	6			0.485		C
39	7		0.512			T
40	8			0.537		C
41	9		0.570			T
42	10			0.586		C
43	11		0.620			T
44	12			0.636		C
45	13		0.671			T
46	14	0.680				A
47	15			0.707		C
48	16			0.735		C
49	17			0.763		C
50	18	0.786				A
51	19			0.812		C
52	20				0.828	G
53	21			0.856		C
54	22		0.896			T
55	23			0.904		C
56	24		0.948			T
57	25			0.953		C
						5′

t = Migration time of sequencing fragment; t_B = migration time of primer; t_A = migration time of the last peak.

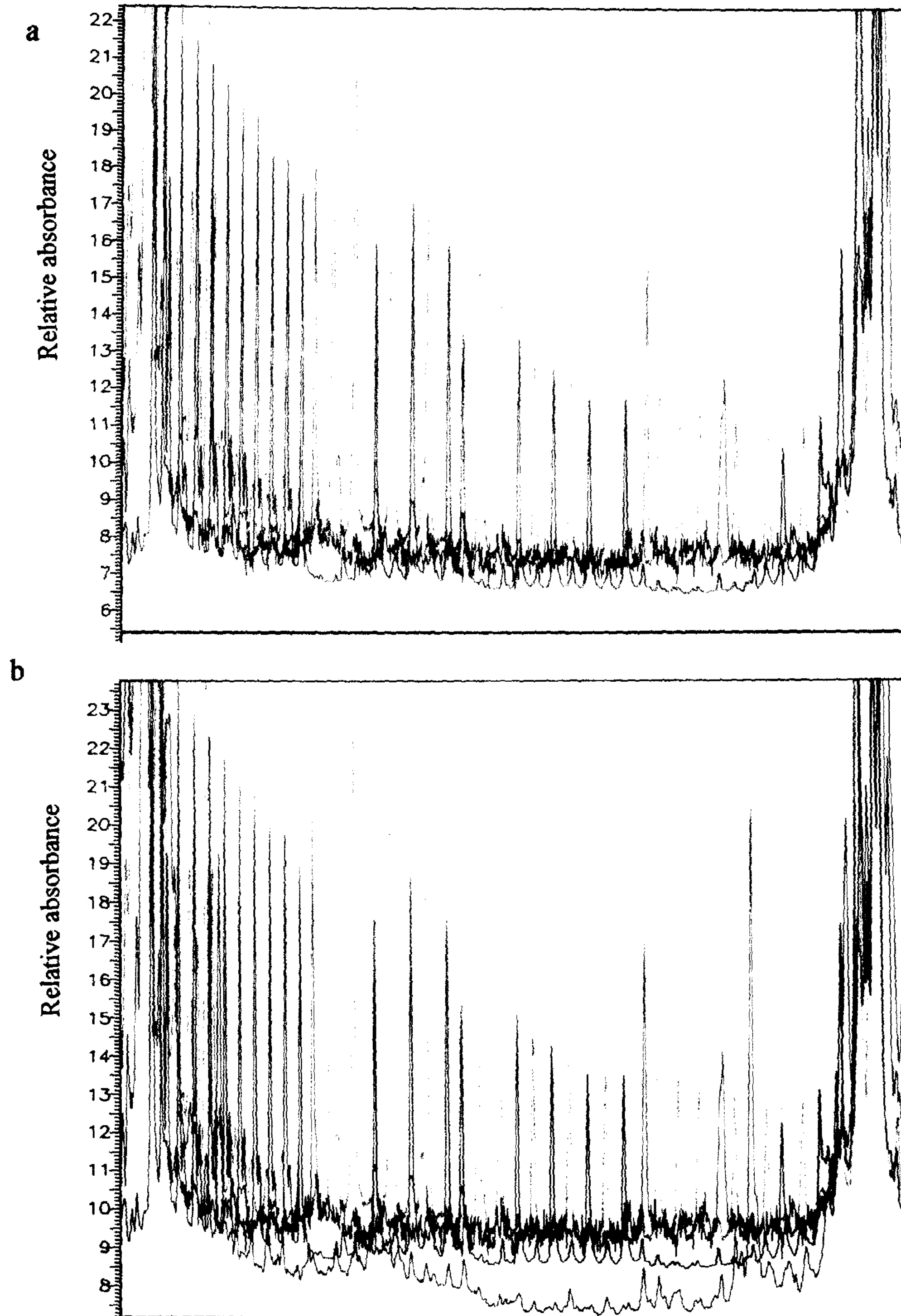

Fig. 7. Computer overlay of LIF electropherograms. (a) a, b and c electropherograms from Fig. 6 using two point re-size alignment (see text for details). Red = A bases; blue = C bases; purple = T bases. (b) a, b, c and d electropherograms from Fig. 6. Red = A bases; blue = C bases; purple = T bases; magenta = G bases. All other conditions as in Fig. 6.

illustrated in Fig. 6. The absolute migration times of the primer (N_{17}) for the A, G, T and C reactions were disperse but have been aligned in this figure as were the times for the latest migrating fragment N_{58}. The term t_n in Eq. 6 was calculated individually for each of the detected fragments between 17 and 58 bases in length. The obtained values of t_n were then rearranged in order from low to high according to the occurrence in the four different runs for the four individual bases shown in Fig. 6. The results are summarized in Table 1. Fragment 33 corresponds to fragment 1 on the GEM molecule. As indicated in this table, the sequence of GEM is determined in the right-most column from the 3′ to 5′ end. A linear relationship ($R^2 = 0.999$) is observed between the relative migration time and the base number of GEM (25-mer) for this data. Computer software capable of facilitating the sequence determining step and performing data processing was designed in the laboratory. The output of this software is in a format where the determined sequence is displayed. The software is interfaced with commercial software, Turbochrom III by PE Nelson (Cupertino, CA, USA) which can manipulate the obtained data and plot the final electropherogram of the sequencing as shown in Fig. 7.

4. Conclusions

Sequencing of antisense ssDNA analogues (phosphorothioates) using CGE interfaced with LIF is presented for the first time. As expected and published by us [4] and others [28,34], several times in the past, DNA fragments up to 100 bases in length or even longer can obey the Ogston model. Within the limitations of this model, a mathematical expression is derived and successfully used for computer-assisted antisense DNA analogue sequence determination. The strength of this expression is that t_n as defined in Eq. 6 for the relative migration of sequencing fragments is related only to the fragment length N as expressed in terms of base number. Moreover, this expression to a large extent is independent of the experimental conditions given that all sequencing fragments are separated. This latter issue was never a problem, since CGE can separate sequencing fragments with very high efficiency and resolution, as has been previously demonstrated by several laboratories [12,17,35,36]. The fact that column variance can be eliminated allows the use of high-concentration linear polyacrylamide (over 9%) and cross-linked gel columns with a fixed matrix and very high resolving power for short sequencing fragments. Under our experimental conditions we have not observed band compression for these short fragments. Finally, the sequencing method that was presented is a single-dye LIF detection approach. It is not, by any means, meant to limit the scope of application since any method described in the introduction can also be applied.

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