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Temperature-gradient gel electrophoresis

Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts

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A temperature-gradient gel electrophoresis technique and its application to the study of structural transitions of nucleic acids and protein-nucleic acid complexes are described. The temperature gradient is established in a slab gel by means of a simple ancillary device for a commercial horizontal gel apparatus. The gradient may be freely selected between 10 and 80 °C, and is highly reproducible and linear. In a normal application the biopolymers migrate perpendicular to the temperature gradient so that every individual molecule is at constant temperature throughout electrophoresis. The structural transition of a biopolymer is seen as a continuous band which is retarded or speeded up in the temperature range of the transition. Dissociation processes are mostly irreversible under the conditions of electrophoresis and, therefore, show up as discontinuous transitions from a slow-moving to fast-moving band. As examples the conformational transitions of viroids, double-stranded RNA from reovirus, double-stranded satellite RNA from cucumber mosaic virus and repressor-operator complexes have been studied. It could be shown that by this method dsRNA molecules may be differentiated which differ only in one base-pair, or proteins differing in one amino acid only. As a particular advantage, temperature-gradient gel electrophoresis allows the study of conformational transitions of biopolymers which have not been purified. The biopolymer may either be identified by silver staining as a specific band among many others or, if the study is carried out on nucleic acids, these may be recorded by hybridization with a radioactive probe.

1. Introduction

The study of the structural transitions of biopolymers is of general interest for the understanding of structure-function relationships. For example, helix-coil transitions of double-stranded nucleic acids have been studied in great detail and could be described quantitatively by statistical thermodynamics (for a review, see ref. 1). Our understanding of the stability and cooperativity of nucleic acids is derived mainly from such studies. At a refined level, conclusions about minor variations of helix-coil transitions as being due to mutations and mismatches may be obtained. As another example, the mechanism of protein folding in vitro was the object of many investigations (for a review, see ref. 2). The results helped to broaden understanding of the folding process in the cell after biosynthesis, the mechanism of regulatory conformational changes or, as has become important more recently, the formation of the biologically active structure of bioengineered proteins. Also, the dissociation-association equilibria of protein-nucleic acid complexes may be regarded as a particular type of structural transition.

In experimental studies, conformational transi-

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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tions may be induced by changes in temperature, solvent conditions, ligand concentration and in a few cases, pressure and electric field. Initiation of particular transitions by light, X-ray irradiation, etc., will not be regarded here. So far, the most extensive theoretical treatment has been given for temperature-dependent structural transitions. On a qualitative level, structure denaturation by heat treatment or denaturing solvents is similar, but an accurate relationship has not yet been established [3,4]. In contrast, it has been shown that different double-stranded nucleic acids, the thermal denaturation of which could be described by the same set of parameters, react in a different manner to denaturing solvents [5,6]. Consequently, the most detailed information may be expected from structural transitions if the variable is the temperature.

Another aspect of experiments on structural transitions is illustrated by the method of monitoring structure changes. Spectroscopic methods, hydrodynamic techniques, including chromatography and gel electrophoresis, and monitoring of the changes in chemical reactivity have been applied. Gel electrophoresis and, to some extent, chromatography and centrifugation are different from all other methods in the sense that these techniques are able to fractionate according to size and shape and to monitor structural changes in the same experiment. This means that, in principle, studies on structural transitions may be carried out without purifying the biopolymer of interest. Because of its high power of resolution this advantage seems most apparent in gel electrophoresis. Furthermore, as has been demonstrated by Lerman and co-workers [7] for nucleic acids and Creighton [8] for proteins, the gel electrophoretic properties of biopolymers are very sensitive to structural changes. These authors and others have introduced gel electrophoretic methods in which a concentration gradient of a denaturing solvent is established in a slab gel perpendicular to the electric field; on one side of the gradient the biopolymers migrate as native molecules, on the other as denatured species, and in between the whole transition curve may be recorded. Restrictions exist in the quantitative evaluation and choice of buffer conditions because the presence of a denaturing solvent is a prerequisite.

From these considerations it follows that the combination of gel electrophoresis and temperature as the variable appears still more attractive. It would have all the advantages of gradient gel electrophoresis but circumvents the restriction of a denaturing solvent being present. A temperature-gradient gel electrophoresis method has actually been reported by Thatcher and Hodson [9]. A vertical slab gel was mounted between two thermostatted plates. The authors applied their technique mainly to protein denaturation.

In the present work we have developed a simple technique for establishing temperature-gradient gel electrophoresis. It consists basically of an ancillary device for commercial horizontal gel electrophoresis instruments. It may be utilized by every biochemical laboratory as a routine procedure. In a series of examples we describe its application to nucleic acid structure, mutation analysis, and equilibria of protein-nucleic acid complexes. With a combination of temperature-gradient gel electrophoresis and molecular hybridization it is possible to study conformational transitions of biopolymers in cellular extracts, even if they are present in the extract in minute amounts only.

2. Materials and methods

2.1. Chemicals

Acrylamide was purchased from Serva (Heidelberg, F.R.G.) and bisacrylamide from Bio-Rad (München, F.R.G.). All other chemicals were of reagent grade from commercial sources. Glyoxal was purified over amberlite.

2.2. Nucleic acids

Potato spindle tuber viroid (PSTV) was prepared in our laboratory by R. Hecker in crude extracts and in highly purified form as described previously [10,11]. RNA extracts from German hop (*Humulus lupulus*) containing the hop stunt viroid (HSV) were kindly provided by R. Hecker. The manner of preparation was analogous to that of PSTV.

Purified double-stranded satellite RNA 5, WT strain, associated with the cucumber mosaic virus (dsCARNA5) [12] was provided by Dr. J. Kaper (U.S. Department of Agriculture, Beltsville, MD). RNA from reovirus serotype 3 dearing stream [13] was kindly provided by Dr. H. Müller (Justus-Liebig-Universität, Gießen, F.R.G.). A 187 base-pair DNA Tn10-encoded tetracyclin resistance (TET) gene control region from *Escherichia coli* [14] was prepared by Professor W. Hillen and D. Hansen (Institut für Mikrobiologie, Universität Erlangen, F.R.G.).

2.3. Proteins

Two mutants of the Tn10-encoded TET repressor (ref. 15 and unpublished data) were provided by Professor W. Hillen and D. Hansen (Institut für Mikrobiologie, Universität Erlangen).

2.4. Gel electrophoresis

Gels contained 5% acrylamide, 0.12% bis-acrylamide, 0.12% TEMED with or without 8 M urea, respectively, in 'low salt' electrophoresis buffer (8.9 mM Tris, 8.9 mM boric acid, 0.25 mM EDTA) and 0.07% ammonium peroxodisulfate for initiating polymerization.

The electrophoresis procedure as well as the device are described in section 3. Staining of nucleic acid was carried out mostly with silver [16] according to a method modified from that of ref. 17: The gel was shaken slowly twice for 10 min in 10% ethanol, 0.5% acetic acid, then for 15 min in 10 mM $AgNO_3$, followed by three times for 15 s in distilled H_2O. Subsequent treatment was for not more than 15 min in a fresh solution of 375 mM NaOH, 2.3 mM $NaBH_4$, 0.4% HCHO (37%, w/v) and finally for 5 min in 70 mM Na_2CO_3.

For staining nucleic acid with ethidium bromide, gels were incubated in 0.5 μg/ml ethidium bromide for 15 min and washed for 10 min in H_2O. Photographs were taken with a Polaroid SC-70 camera on an ultraviolet transilluminator.

2.5. Blotting, hybridisation and autoradiography

For the specific detection of viroids after electrophoresis of crude RNA extracts, nucleic acids were transferred in an LKB 2005 Transphor electroblotting unit for 2 h with an electric field of 5 V/cm to a Biodyne-A membrane (Pall, GenCove, NY). After heating the membrane for 2 h in a vacuum oven the hybridization procedure described by Melton et al. [18] was followed. The viroid-specific probe was a ^{32}P-labelled SP6 transcript of the plasmid pRH605 using 10 μCi/μl [α-^{32}P]GTP (Hecker, unpublished results). After prehybridization with 10 ml prehybridization solution (250 μg/ml calf thymus DNA) per 100 cm^2 membrane area at 55 °C for 4 h, the membrane was hybridized with 2 ml hybridization solution (cf. ref. 18) per 100 cm^2 membrane area at 55 °C for at least 10 h. Subsequent washing was performed twice in 2 × SSC (SSC: 0.15 M sodium chloride/0.015 M sodium citrate), 0.1% SDS for 10 min at room temperature, in 0.1 × SSC, 0.1% SDS, 50% formamide for 10 min at 50 °C.

An autoradiogram was made with Kodak X-omat S X-ray film and Du Pont Cronex Lightening-plus intensifier, the film being developed after 100 min exposure in Kodak D19 and fixed in Agfa Agefix.

3. Results

3.1. Method of temperature-gradient gel electrophoresis

3.1.1. Instrument

Temperature-gradient gel electrophoresis was carried out in a horizontal gel electrophoresis device, consisting of a commercial electrode buffer tank (LKB Multiphor II; LKB, München, F.R.G.) and a laboratory-built temperature-gradient plate. The gradient plate consists of a copper block of 210 × 190 × 5 mm onto which two brass double channels for cooling (210 × 30 × 30 mm) have been soldered. The construction is illustrated in fig. 1. Fig. 1 also shows the flow of cooling water from two thermostats through the two double channels. The direction of migration during electrophoresis (− to + for nucleic acids) is perpendicular to that of the temperature gradient with a total temperature difference $T_2 - T_1$.

For electric insulation the copper plate was

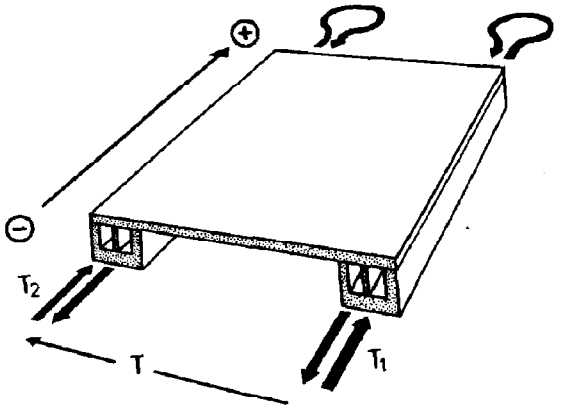

Fig. 1. Temperature-gradient plate for electrophoresis with a gradient of temperature T perpendicular to the direction of migration (− to + for nucleic acids). The copper plate is 210×190×5 mm; each double channel for cooling (210×30×30 mm) is attached to a thermostatting bath of temperature T_1 or T_2, respectively.

covered with a self-adhesive special teflon foil of thickness 80 μm (type 3-15, Von der Brüggen GmbH, Köln, F.R.G.). The insulation withstands voltages greater than 500 V. The foil sticks to the copper plate up to temperatures above 80 °C.

The temperature gradient is linear as may be deduced from the heat equation. This was confirmed experimentally by measuring the temperature over the whole plate with a calibrated thermistor (PT 100 FKG 4304, Heraeus, Hanau, F.R.G.) with an active measuring area of 3×4 mm. Fig. 2 shows a diagram of the temperature gradient. The temperature in the gel was estimated as the mean value of the temperatures on top of the gel and between the gel and teflon foil. Within the limits of experimental error identical gradients were measured, whether determined at the site of the water inlet/outlet, water flow inversion, or in the middle between both positions. By virtue of this feature it is guaranteed that a molecule which migrates in the electric field as shown in fig. 1 is at constant temperature throughout electrophoresis.

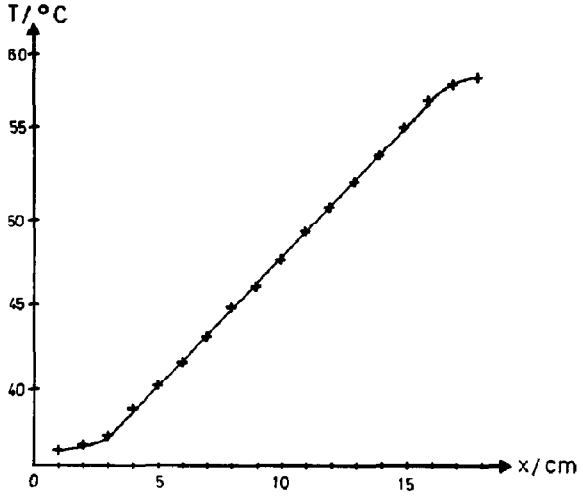

Fig. 2. Temperature vs. position on the copper plate between both cooling channels. Temperature was measured with a thermistor as described in the text. The temperature profile is the same whether measured between the inlets/outlets, reversal of the water stream or at the middle between both positions. $T_1 = 35\,°C$, $T_2 = 60\,°C$.

3.1.2. Handling

Polyacrylamide gels were prepared on gel bond films. For preparation the gel was poured between one glass plate and the gel bond film which was pressed onto a second glass plate; the first glass plate carried the spacers for sealing and for forming the slots.

For electrophoresis with detection of nucleic acid by staining, the hydrophobic side of the gel bond film is in contact with the glass plate, the hydrophilic side being covalently bound to the gel. The spacers were 1 mm thick. The slot for the biopolymers under study was 140 mm (perpendicular to the direction of migration) × 5 mm. On the low- and high-temperature sides in line with the sample slot, two additional slots (10 × 5 mm) for reference samples were established.

For electrophoresis with nucleic acid detection by blotting and molecular hybridization the hydrophobic side of the gel bond film was in contact with the gel, thus preventing covalent binding of gel and film. Because neither capillary forces nor an electric field work through the insulating gel bond film, gels have to be separated from the film after electrophoresis. For easier handling gels were 2 mm thick.

After polymerization on the gel bond film, the gel is laid on the temperature-gradient plate, and the slots are filled with the samples. In the first

step of temperature-gradient gel electrophoresis the biopolymers are allowed to migrate into the gel matrix under conditions of high electrophoretic mobility and uniform temperature. In the experiments of this paper, these were a temperature of 10 °C over the whole gel and an electric field of 6.7 V/cm. The electrical contact of the gel with the electrodes is through electrode contact cloths (strongly soaking rag), which are – except for the contact sides – sealed in polyethylene foil in order to prevent drying out at higher temperature. The assembly of gel, contacts and the different covers (see below) is depicted in fig. 3. This first step is stopped by switching off the voltage, when appropriate dyes indicate that all the biopolymers under study have entered the gel matrix.

In the second step, the gel is immersed in electrode buffer, and a protection film, the hydrophobic side of a 150 × 210 mm gel bond film, is laid on top of the gel. This procedure and the sealing of the electrode contact cloths in polyethylene foil provide good protection against drying out and therefore guarantee a uniform electric current. As may also be seen from fig. 3, the gel is covered furthermore with two 5-mm glass plates for thermal insulation; these allow observation of indicator dyes during the subsequent electrophoresis. After switching on the temperature gradient

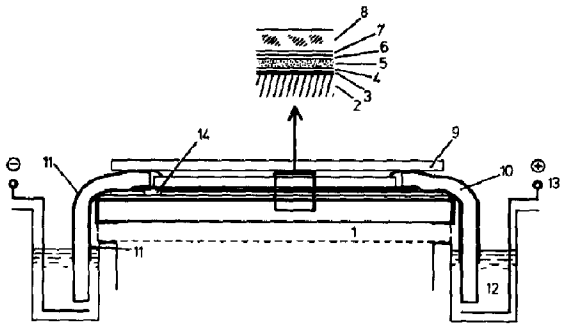

Fig. 3. Arrangement of the components of temperature-gradient gel electrophoresis under working conditions. (1) Double channel for cooling, (2) copper plate, (3) teflon foil for electric insulation, (4) gel bond film, (5) gel, (6) moisture film between gel and protection film, (7) protection film, (8) glass plate on the protection film, (9) glass plate for pressing the electrode contact cloths, (10) electrode contact cloths, (11) polyethylene foil around the electrode contact cloths, (12) electrode buffer tank, (13) electrode, (14) gel slot.

the biopolymers are allowed to assume the equilibrium conformation at each temperature of the gradient in the absence of the electric field. The time necessary for total equilibration depends upon the molecules studied. In the examples described in section 3.2, 15 min were used as a compromise between as much equilibration as possible and as little diffusion as possible.

The third step is the actual electrophoretic analysis of the biopolymers in the presence of the temperature gradient. The electric field applied was between 15 and 35 V/cm in different experiments. After the end of electrophoresis the protection devices are removed from the gel and detection of the analysed biopolymers is carried out either by staining or by hybridization as described above.

3.1.3. Blotting and hybridization

When a specific nucleic acid in the temperature-gradient gels was detected by hybridization, ^{32}P-labelled complementary nucleic acid was applied. In order to transfer the nucleic acid onto the nylon membrane by a Northern blot, potential secondary and tertiary structures must be dissociated. The denaturation can of course only be carried out after temperature-gradient gel electrophoresis. This was done by chemical modification with glyoxal. The entire gel was incubated for 2 min in 10% ethanol, 0.5% acetic acid at room temperature for fixation of the nucleic acids and subsequently incubated in 6% glyoxal, 10% sodium phosphate (pH 6.5) at 50 °C for 15 min. Transfer was carried out as described in section 2. The membrane was baked for 2 h in the vacuum oven to remove glyoxal. The nucleic acid bound to the membrane could be detected subsequently by hybridization as described in section 2.

3.2. Examples of application

3.2.1. Viroids

Viroids are infectious agents of several plant diseases which consist merely of a protein-free, circular, single-stranded RNA of about 350 nucleotides (for a review, see ref. 6a). Temperature-gradient gel electrophoresis of purified PSTV shows a conformational transition from a rod-like

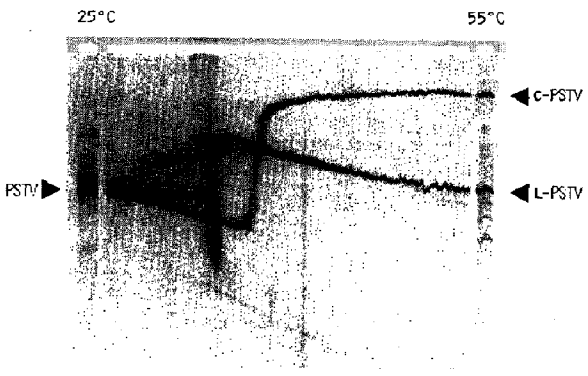

Fig. 4. Temperature-gradient gel electrophoresis of purified viroid PSTV (85 ng in the sample slot and 4 ng in each reference slot). The position of native PSTV (▸) and those of linear (L) and circular (c) PSTV in the denatured state (◂) are indicated. $T_1 = 25\,°C$, $T_2 = 55\,°C$. Electrophoresis in the presence of the temperature gradient was carried out at 500 V for 90 min; buffer, 17.8 mM TBE; gel, silver stained.

to an open-circle structure. This transition is seen as a drastic reduction in mobility to less than a third (fig. 4). The midpoint temperature and the half-width of the transition are in accordance with previous results obtained from ultraviolet absorption melting curves [6,19]. In addition to the circular viroids several transition curves of linear viroids, which are derived from the circular molecules by single nicks, are observed in fig. 4. Their lower T_m values are also in accordance with optical data [20]. Whereas in optical melting only the superimposition of all transition curves could be measured, transition curves of single molecular species have been determined by the electrophoretic technique. It may be concluded from this analysis that different positions of the nicks yield different thermal stabilities of the linear molecules. As only distinct curves appear, one has to assume that the nicks are not randomly distributed but restricted to very few sites.

3.2.2. dsRNA from reovirus

The genome of reovirus consists of 10 dsRNA segments [13]. The melting curve with utraviolet absorption recording yielded three main transitions which could not be attributed to the single segments [5]. The temperature-gradient analysis, the details of which are described by Meyer [21], is shown in fig. 5. In contrast to optical melting curves, it allows one to observe the conformational transitions of single RNA segments.

3.2.3. Double-stranded satellite RNA from cucumber mosaic virus in perpendicular and parallel gradients

Some strains of cucumber mosaic virus contain in addition to the four genomic RNAs satellite RNA, the so-called cucumber mosaic virus-associated RNA5 (CARNA5) [12]. In the infected cell CARNA5 is produced as a double-stranded form in large quantities. Different variants of CARNA5 are able to suppress disease symptoms or to increase their severeness. In fig. 6a the gradient analysis of dsCARNA5, variant WT, is shown. Six different RNA species not previously analysed (a–f) are detectable. They all have nearly the same size of about 335 nucleotides. As was outlined in detail by Tien Po et al. [22] and Steger et al. [23], the drastic retardation between 40 and 46 °C is induced by partial dissociation of the duplex structure from the ends, and the abrupt increase in mobility above 52 °C is due to complete strand separation. From a series of experiments on different variants it could be shown that variants with

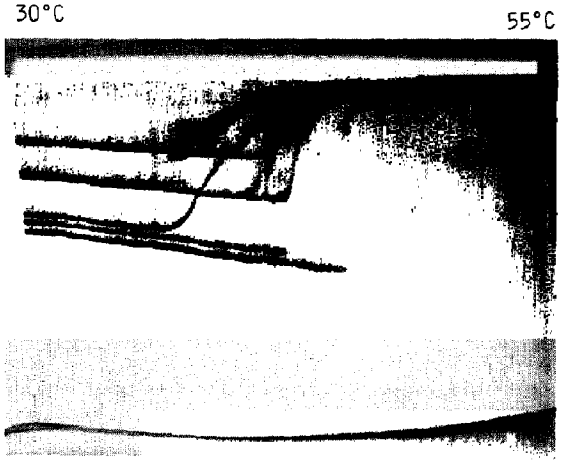

Fig. 5. Temperature-gradient gel electrophoresis of 8.0 µg of the genome of reovirus, consisting of 10 dsRNA segments. $T_1 = 30\,°C$, $T_2 = 55\,°C$. Electrophoresis in the presence of the temperature gradient was carried out at 200 V for 4 h and 40 min; buffer, 8.9 mM TBE and 8 M urea; gel, silver stained.

only one base-pair difference could be separated [23]. Furthermore, a correlation between the temperature of the retardation transition and severeness of the symptoms induced by the virus containing CARNA5 was derived.

The temperature gradient may also be applied parallel to the electric field. In this case different samples may be laid into small slots as in a multi-sample analysis of a normal slab gel (cf. fig. 6b). All molecules run from low to high temperature. They are drastically retarded when they reach the characteristic temperatures of their retardation transitions. If the gel electrophoresis is stopped shortly after that point of time, the position of the bands is characteristic for the temperature of the retardation transition. In fig. 6b different variants of CARNA5 have been analyzed on one parallel temperature-gradient gel electrophoretic run. It can be seen that some samples are homogeneous and are indicative of only one satellite, whereas others contain several components.

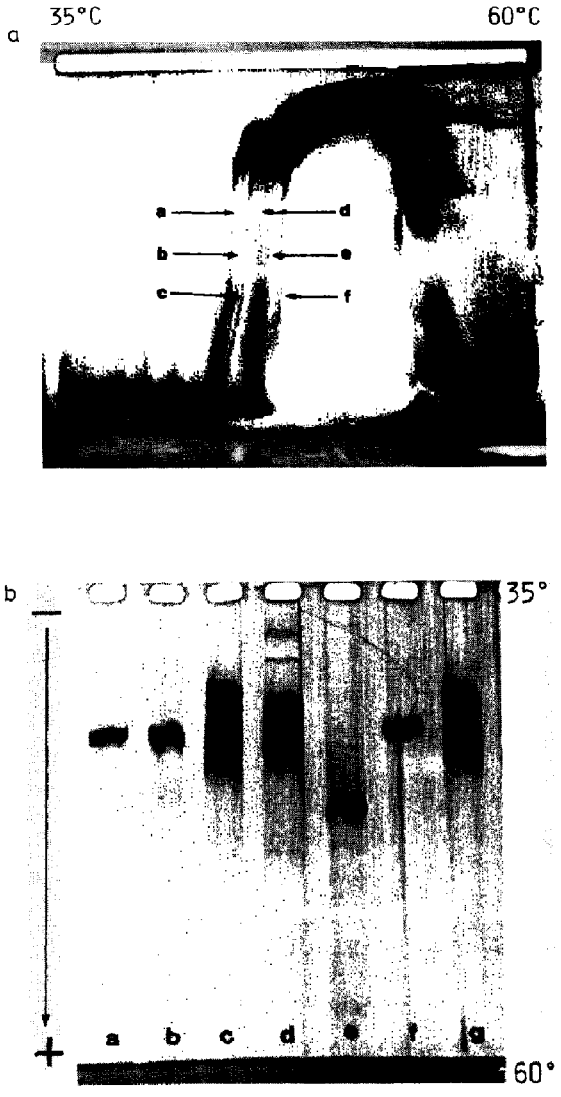

Fig. 6. (a) Temperature-gradient gel electrophoresis with the gradient perpendicular to the electric field of 1.1 μg dsCARNA5. $T_1 = 35°C$, $T_2 = 60°C$. Electrophoresis in the presence of the temperature gradient was carried out at 500 V for 2 h; buffer, 8.9 mM TBE and 8 M urea; gel, silver stained. The very broad and smeared bands in the region of low mobility are possibly an artefact from overloading. (b) Temperature-gradient gel electrophoresis with the gradient parallel to the electric field. The slots contained the following dsCARNA5 variants: clone 42-dsCARNA5 (a), 1-dsCARNA5 (b), WT-dsCARNA5 (c), S52-dsCARNA5 (d), dsRNA5 associated with the peanut stunt virus (e), Valencia 24-dsCARNA5 (f), WT-dsCARNA5 (g). Details of the variants are described by Tien Po et al. [22]. $T_1 = 35°C$, $T_2 = 60°C$. Electrophoresis in the presence of the temperature gradient was carried out at 300 V for 75 min; buffer, 8.9 mM TBE and 8 M urea; gel, silver stained.

3.2.4. Repressor-operator complexes

Complexes of the repressor and its operator have been formed by incubation of the purified components in a buffer suitable for electrophoretic analysis. As known from other experiments [14], two repressor proteins bind to one molecule of nucleic acid. The degree of complex formation and its dependence upon the temperature were analyzed by temperature-gradient gel electrophoresis. In fig. 7 the analysis of operator DNA (187 base-pairs) alone (fig. 7a) and complexes with two mutants of the repressor (fig. 7b and c) is shown. The DNA moiety was stained by ethidium bromide. In these experiments the temperature gradient increases from right to left.

The operator alone shows in its double-stranded structure a continuous increase in mobility up to an abrupt decrease at 68°C, which represents complete denaturation with strand separation [27].

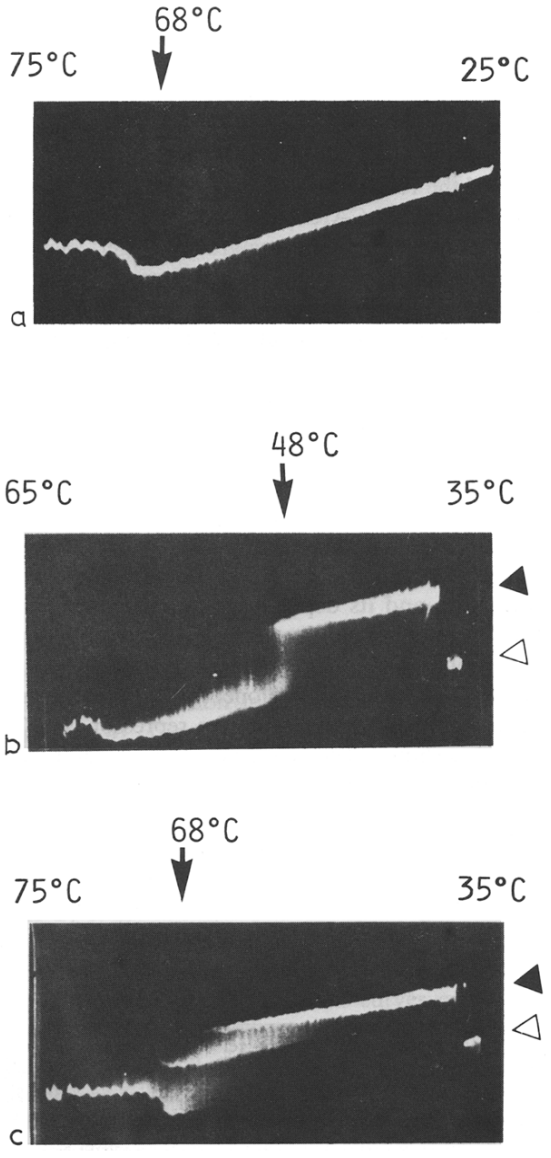

Fig. 7. Temperature-gradient gel electrophoresis of 6.5 μg of the 187 base-pair DNA Tn10-encoded tetracycline resistance gene control region alone (a) and in a complex with 12 μg of two different mutants of the tetracycline repressor (b, c). Electrophoresis in the presence of the temperature gradient was carried out at 250 V for 2 h and 30 min; buffer, 89 mM TBE; gels, stained with ethidium bromide. The arrowheads indicate the bands of protein-nucleic acid complex (◀) and free nucleic acid (◁). Reference slots contain free nucleic acid. Temperature gradient is from right to left. Temperature limits; $T_1 = 75\,°C$, $T_2 = 25\,°C$ (a); $T_1 = 65\,°C$, $T_2 = 35\,°C$ (b); $T_1 = 75\,°C$, $T_2 = 35\,°C$ (c).

If the repressor protein is added, the band of the protein-nucleic acid complex (◀) becomes clearly visible in addition to the band of free nucleic acid (◁). Discontinuous transitions from the complex to free nucleic acid occur at temperatures specific for the two repressor mutants under study. During the dissociation process both repressor proteins may dossociate via a one-step (fig. 7b) or two-step mechanism (fig. 7c). Comparison of the two mutants shows that a single amino acid change yields a marked difference in the capability of forming a complex with the operator. The systematic studies will be published by Hansen and Hillen (manuscript in preparation).

3.2.5. Viroids in crude extracts

These examples have been chosen to demonstrate that transition curves of particular nucleic acids may be recorded even if they are present as a minor component of complex mixtures and amount to no more than 0.1% of all nucleic acids.

In fig. 8 the temperature-gradient analysis of a pre-purified RNA extract from leaves of diseased German hop is depicted. PSTV was added as a reference. In addition to the transition curve of

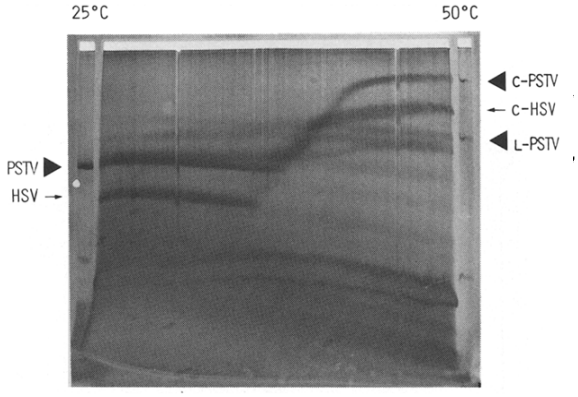

Fig. 8. Temperature-gradient gel electrophoresis of a mixture of 620 ng of purified PSTV and 280 μg of an RNA extract from leaves of diseased German hop. The positions of native PSTV (▶), HSV (→) and of linear (L) and circular (c) viroid in the denatured state (◀, PSTV; ←, HSV) are indicated. Reference slots contained 30 ng of purified PSTV. $T_1 = 25\,°C$, $T_2 = 50\,°C$. Electrophoresis in the presence of the temperature gradient was carried out at 350 V for 100 min; buffer, 8.9 mM TBE; gel, silver stained.

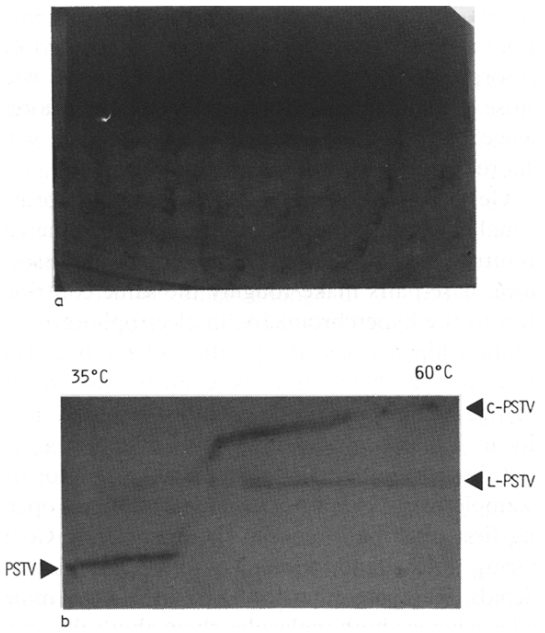

Fig. 9. Temperature-gradient gel electrophoresis of 1.2 mg nucleic acid from a cellular extract of PSTV-infected tomato plants in 8.9 mM TBE. $T_1 = 35\,^{\circ}C$, $T_2 = 60\,^{\circ}C$. Electrophoresis in the presence of the temperature gradient was carried out at 200 V for 4 h and 5 min. (a) Silver-stained gel after electroblotting. (b) Specific detection of PSTV by molecular hybridization with a ^{32}P-labelled SP6 transcript of pRH605. The position of native PSTV (▶) and those of linear (L) and circular (c) PSTV in the denatured state (◀) are indicated.

PSTV and many other curves, a second transition curve showing the characteristics of a viroid molecule with somewhat lower molecular weight is seen. It could be concluded that the disease was due to a viroid infection, the so-called hop stunt viroid (HSV).

Fig. 9 depicts temperature-gradient gel electrophoresis of a crude extract of PSTV-infected tomato plants. Viroid could not be detected by silver staining of the gel after electrophoresis because it is hidden by the dominant staining of all other components. Fig. 9a shows such a silver-stained gel from which, however, part of the nucleic acids had already been transferred to a membrane. If the nucleic acids on the membrane are hybridized to a viroid-specific probe, as described above, viroids may be detected specifically. In fig. 9b the transition curve of PSTV is detected to a reasonably good resolution and contrast, although PSTV is present with less than 0.1% of all cellular RNA. The transition curves agree with that obtained with purified PSTV.

4. Discussion

In this paper we try to emphasize that temperature-gradient gel electrophoresis may be applied to analyse quite different problems in biochemistry and molecular biology. The analysis of CARNA5 variants with expression of different symptoms has been described as one of the examples here; the details are reported elsewhere [22,23]. Similarly, manuscripts on the systematic studies on viroids (Hecker et al.) and on the repressor-operator interaction (Hansen et al.) are in preparation. Here, we concentrate on some general aspects of the method.

4.1. Instrumental and methodical aspects

The temperature-gradient gel electrophoresis as described in this work may be handled like a routine procedure. Therefore, several points are of interest. The device for the gradient has been constructed as an accessory to a commercial horizontal gel electrophoresis. It can be easily changed to fit to other instruments. All procedures have been described in section 3.1.2 in such detail that it is simple to establish this technique in every biochemical laboratory. The different steps have been optimized in order to prevent drying out and to establish a temperature gradient during electrophoresis, which is linear, reproducible and selectable within wide limits. According to our experience, these criteria are best fulfilled by horizontal electrophoresis where the temperature equilibration is only from the lower side. The very steep but sharp transition curves, for example, in fig. 6a (WT dsCARNA5), demonstrate that the temperature is exactly the same on the lower and upper sides of the gel and that molecules from every starting position run at constant temperature throughout electrophoresis.

As outlined in section 1, curves similar to those

reported here had been obtained with gels containing a gradient of a denaturing solvent such as urea or formamide. One may see two advantages of the temperature gradient. First, the results may easily be correlated to thermodynamic calculations because the temperature is the variable in theory and experiments. As mentioned above, the calculations do not hold for solvent denaturation as well. The absence of organic solvents is particularly important if protein-nucleic acid complexes are studied as in the example of fig. 7. Second, the gradient is established completely by an instrumental device where the gel matrices are the same as in normal gel electrophoresis. This has the advantage that the composition of the gel and the buffer conditions in the gel may also be chosen without any restriction compared to normal gel electrophoresis. Furthermore, it is more convenient experimentally to use normal gels instead of pouring gradient gels.

It should be mentioned that a temperature-gradient gel electrophoresis method has been reported already by Thatcher and Hodson [9]. However, their device was quite different from ours; as the main difference the temperature gradient was established by two metal plates contacting both glass plates of a vertical gel. The authors studied mainly the thermal denaturation of proteins by this method [24]. Systematic studies on nucleic acids, which is the main emphasis in this work, had not been reported before.

4.2. New experimental possibilities

The examples listed in section 3 show that temperature-gradient gel electrophoresis may be applied to resolve quite different questions. In some cases, similar results have also been obtained by other methods. For example, the properties of conformational transitions of purified viroids have been studied previously by using a number of physico-chemical techniques [6]. Also the properties of conformational transitions of other isolated nucleic acids and proteins have been investigated previously with other techniques.

Gradient gel electrophoresis, however, bears the advantage that amounts of only 100 ng or less are needed because of the very sensitive staining techniques. In this respect, this method is evidently more sensitive than those based on ultraviolet absorption. The sensitivity is comparable with those obtained by techniques based on fluorescence detection, e.g., hydrodynamic studies with fluorescence detection [25].

Gel electrophoresis is sensitive to conformational changes in a characteristic manner. Whereas in ultraviolet absorption melting curves all dissociating base-pairs make roughly the same contribution to the hyperchromicity, in electrophoresis the relationship between the position of the base-pair in a double helix and its contribution to the change in electrophoretic mobility is highly nonlinear. It has been described by Lerman et al. [7] and discussed in detail by Steger et al. [23] for the example of dsCARNA5 that the base-pairs opening first contribute most to the retardation. Comparing linear and circular viroids, the circular viroids are more retarded than the linear molecules whereas both molecules show about the same hyperchromicity. Strand dissociation, which is detected as an increase in mobility, may also be clearly differentiated from partial denaturation, normally seen as retardation. In ultraviolet absorption, however, both effects have the same sign. In summary, the melting curves in temperature-gradient gel electrophoresis are sensitive to particular details of the structure, such as the structure of the ends, circularity and strand dissociation – for proteins similar characteristics may be determined – whereas ultraviolet absorption melting curves record the state of all parts of the molecule in a similar manner.

Most evidently, gradient gel electrophoresis is advantageous over other techniques, if conformational transitions of biopolymers which are not available as highly purified samples have to be studied. Because electrophoresis is basically a separation technique, the structural transitions of several components present in one sample may be studied in the same experiment. This is seen from the examples where different viroids and cellular RNAs are present (figs. 4 and 8), or when the natural mixture of the 10 RNA segments from reovirus (fig. 5) or the satellite RNA consisting of six different variants (fig. 6) are investigated. These studies could not be carried out at all using ultra-

violet absorption melting curves. Having very similar components in the same sample, it allows one to determine minor differences in thermal stability due to the high relative accuracy of the midpoint temperatures. The differences in T_m values of the different variants of CARNA5 are about 1° C and can be determined with an accuracy of about 0.1° C (cf. fig. 6). Consequently, exchange of a single AU to GC base-pair or vice versa may be detected by the small change in T_m value. This effect has been studied in detail by Steger et al. [23] on CARNA5 variants.

Temperature-gradient gel electrophoresis may even be applied to crude extracts, in which the nucleic acid of interest is present only as a minor component and not visible in gel electrophoresis after silver staining (cf. fig. 9). It may be made visible, however, after blotting and hybridization with a specific probe. This combination of temperature-gradient gel electrophoresis and molecular hybridization offers the novel and unique opportunity of studying thermodynamics of nucleic acids which cannot be purified. It is clearly a step towards in vivo thermodynamics because nucleic acids may be studied in their cellular extracts. If it were possible to avoid denaturing agents such as phenol or others during the preparation of the cellular extract, the intracellular structure of a nucleic acid could be studied for the first time. In principle, similar possibilities open up for the study of proteins, if they can be stained after blotting by specific antibodies.

The examples in the present work show a number, but clearly not all, of the new experimental possibilities. For example, we have studied base-pair exchanges in satellite RNAs which occur naturally in the double-stranded form. Similar studies could be performed on single-stranded RNA, if they were to be transformed into double strands by reannealing with complementary DNA or RNA strands. If the complementary strand were cut out or transcribed, respectively, from a cloned sequence, deviations of the sequence under study from the cloned sequence would lead to a mismatch in the reannealed double strand and could be detected in gradient electrophoresis. Such experiments applying a urea gradient have actually been carried out with influenza virus RNA and interpreted on a qualitative level [26]. Because of the outstanding range of silver staining, from picograms to micrograms, one may hope to detect mutants of low frequency in a natural mixture of sequences.

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