Measurement of the differential melting profile of a promoter-containing fragment of T7 DNA by means of a microspectrophotometer

M.A.Grachev and M.P.Perelroyzen

Novosibirsk Institute of Organic Chemistry, Siberian Division of the Academy of Sciences,
Novosibirsk 90, USSR

Received 18 April 1978

ABSTRACT

A double-beam microspectrophotometer with a 5 microliter cell has been used to study denaturation of DNA in an aqueous solution. This instrument enables measurement of high-resolution differential melting profiles simultaneously at several wavelengths with 0.5 to 1 microgram of DNA. Therefore it becomes possible to study nucleic acids which are difficult to obtain in large amounts. The techniques have been employed to measure the differential melting profiles of T7 DNA and of a fragment of this DNA 1000 base pairs long which contains the four early promoters.

INTRODUCTION

High-resolution DNA melting profiles give valuable information on the distribution of sites of decreased and increased thermal stability in bacteriophage genomes (1-4) which may be important for their function. Recently combination of this physical method with chemical modification served a basis for a promising approach to fragmentation of DNA at low-melting sites (5). However, spectrophotometers used for such measurements are not suitable for work with small amounts of nucleic acids.

A few years ago a microspectrophotometer has been designed in this Institute (6). The instrument has been extensively employed since that time as a multiple-wavelength detector for micro-column liquid chromatography (7). The volume of the flow cell of this instrument is about 2 microliters. The optimized optical scheme and the precise electronic measuring unit make it possible to perform accurate measurements with the usual spectrophotometric concentrations of substances in such small volumes.

The present paper reports application of this instrument to measurement of the differential melting profiles of DNA. Reported is the high-

-resolution melting curve of a short fragment of T7 DNA which contains the four early promoters.

MATERIALS AND METHODS

T7⁺ DNA was obtained from T7⁺ bacteriophage (Studier) grown on a pilot plant of this Institute. It was isolated by hot phenol extraction followed by pronase treatment and second phenol extraction, and finally precipitated by ethanol. DNA obtained was homogeneous in gel-electrophoresis in neutral medium and in ultracentrifugation in an alkaline medium.

The promoter-containing fragment was obtained from this DNA as described earlier (8). According to this procedure, a complex was formed between DNA and E.coli RNA-polymerase; the complex was cleaved by restriction nuclease Bsu (cleavage in the middle of the sequence -G-G-C-C-) and the mixture subjected to filtration through nitrocellulose filter. The fragment eluted from the filter was homogeneous in gel-electrophoresis - the total admixture of other fragments did not exceed 10%. In some experiments we used the same fragment subjected to additional purification by gel-electrophoresis on agarose followed by chromatography on hydroxyapatite. The fragment contains according to ref. (8) the left-hand terminus of T7 DNA, the promoters A₁, A₂, A₃ and the minor promoter P_D(9); its length is about 1000 base pairs. It was obtained in this laboratory by E.F.Zaychikov and A.G.Pletnyov.

To measure the melting profiles, the fragment was precipitated by ethanol, washed by 70% ethanol, gently dried to remove ethanol and dissolved in 0.1 SSC (0.015 M NaCl - $1.5 \cdot 10^{-3}$ M sodium citrate pH 7.0) at a concentration 0.2 mg/ml (3 A₂₆₀ units per ml). Freshly prepared solutions were used in all the experiments.

The double-beam microspectrophotometer MSFP-3 was designed and built in this Institute. The optical scheme of this microspectrophotometer invented by S.V.Kuzmin (6, 10) consists of a monochromator and of a special mirror objective. The convex mirror of the objective serves as the means of switching the light beam between the reference and the sample paths. For this purpose the mirror is mounted on an electromagnetic drive activated by the electronic measuring unit of the microspectrophotometer. The mirror turns around a vertical axis to a small angle so that the objective focusses the light

beam alternatively on the reference or the sample cell. The objective is designed in such a way that the beam in the working positions does not touch walls of the cell, and the light spot on the photomultiplier does not move. This scheme ensures practically identical spectral characteristics of the reference and the sample light paths.

The measuring electronic unit of the microspectrophotometer (E.A.Kuper, V.V.Kargaltsev and B.M.Chaldin participated in its design) is a logarithmic analog-to-digital converter with an integration time 0.16 to 20 sec. Accuracy of the measurement of the value $\lg I_0/I$ within the range from 10^{-2} to 2 is 0.1%. The data are recorded in parallel by a flat-bed recorder and by a punch-tape recorder.

The monochromator of the instrument is equipped by a programming unit which makes it possible to change the wavelength in the course of experiment according to a cyclic program (240, 250, 260, 270, 280 and 290 nm); time of the measurement at each wavelength may be selected between 1.2 and 20 sec. Reproducibility of the wavelength setting is better than 0.1 nm.

The cell of the instrument modified for thermal studies is shown in Fig.1.

Fig.1. Scheme of the cell of the microspectrophotometer. Sizes are given in millimeters.

The Teflon gasket A with cylindrical channels is pressed by a stainless-steel holder (not shown in the Figure) between two quartz windows B. The channels of the cell are completely filled, respectively, by 0.1 SSC and by a solution of DNA in the same buffer. To remove air bubbles, the cell is subjected to brief centrifugation at $1000 \times g$, and the channels are tightly closed by silicone rubber stoppers C. After this, an O-shaped spring is put around the cell to press down the stoppers, and the cell together with this spring is put into the instrument inside a nest in a brass thermostating jacket. This jacket contains a wide circular channel through which water is pumped from a thermostat. The jacket with the cell is covered from outside by heat-insulating plastic with conical holes for the light beam. The thickness of the cover is 8 mm. The thermostating jacket has a groove around the cell nest, into which glued is the hot contact of a copper-constantan thermocouple. The cold contact of the thermocouple is placed into a Dewar flask with ice.

The E.M.F. of the thermocouple is measured by means of an integrating digital voltmeter equipped by a punch-tape recorder. A modified 10-liter U-10 (GDR) ultrathermostat is used to feed the cell jacket with hot water. The thermostat itself and the tubes are thoroughly insulated by a thick layer of foamed polystyrene.

Before the experiment water in the ultrathermostat is heated to 60° . The subsequent increase of the temperature at a rate $0.1^{\circ}/\text{min}$ is performed by means of a heater fed by constant voltage (power about 100 W). Within the necessary interval, growth of the temperature is close to linear. Such a way of heating completely eliminates false peaks of the derivative caused by changes of heating rate due to changes of heater power, which would take place if any kind of regulation were employed.

Treatment of the multiple-wavelength absorbance record was performed by means of Minsk-32 computer using the system CHROM-6 written in FORTRAN-IV. This system, intended initially for the treatment of chromatographic and spectral data in liquid chromatography, yields the optical densities D_{λ} (λ - wavelength) and the absorbance ratio D_{λ}/D_{260} at equal time intervals. To calculate accurately the absorbance ratio from the data obtained with the cyclic six-wavelength program, it is necessary at first to interpolate the values of optical densities within the cycles to given moments - moments of the measurements at the

last wavelength of the cycle; at the same moments temperature was recorded. Interpolation is performed by calculating the values at these abscissae of cubic polynomes fitting consecutively four values of optical density at given wavelength, from the first one to the fourth one, from the second one to the fifth one, and so on.

To calculate differential curves, the file of interpolated values of optical densities at necessary wavelengths is at first condensed by substitution of each five consecutive values of optical density at given wavelength by their mean value. After this calculated are the derivatives of cubic polynomes best fitting the condensed values from the first to the seventh, the second to the eighth, etc. The wavelength was changed once per 1.2 sec; therefore the step of differentiation after data treatment appears to be equal to ca. 0.1°. The data treatment procedure described above gives satisfactory results, but it is not believed to be optimal, and we are intending to modify it in the future.

RESULTS AND DISCUSSION

Fig.2 shows the differential melting profile of DNA of T7⁺ phage in

Fig.2. Differential melting profile of T7⁺ DNA in 0.1 SSC. o - values of the derivatives of the best-fitting polynomes (see text).

0.1 SSC at 260 nm. Qualitatively and quantitatively this profile does not differ considerably from those described by other workers (2, 4). This result demonstrates firstly that the techniques described are in fact suitable for such experiments, and secondly that increase in the concentration of DNA by a factor of 3-5 compared with the usual one does not lead to any considerable changes. The practical consumption of DNA in a single experiment is 0.5 to 1 microgram. The only technical difficulty is rapid evaporation of small volumes before filling the cell which may increase the ionic strength and thus shift the differential profile to higher temperatures. Therefore one must be somewhat pre-cautious in comparison of different profiles in an absolute temperature scale.

Fig.3 shows the differential melting profile of a 1000 base pairs-long fragment of T7⁺ DNA isolated according to ref. (8). This fragment contains the four early promoters for RNA-polymerase of *E. coli* (9). The profile consists of four well-resolved peaks whose positions are in a correlation with the stepwise change of the absorbance ratio D_{280}/D_{260} . It is seen also that the profile at 280 nm follows that at 260 nm.

Comparison of these profiles with that of the differential melting of a fragment of ϕ X 174 DNA (11) suggests that the low-temperature peaks reflect the presence of internal low-melting regions rather than being due to mere terminal effects.

Fig.3. Differential melting curve of a fragment of T7⁺ DNA ; the fragment is 1000 base pairs long, it contains four early promoters.

—○—○— dD_{260}/dT ; - - - dD_{280}/dT ; ●●● D_{280}/D_{260}

Assuming that the complete profile corresponds to melting of 1000 base pairs, areas of the peaks show that the first peak corresponds to transition of 80, the second - of 50, the third - of 200, and the fourth - of 670 base pairs. It is interesting that the first two peaks have areas which contradict with the theory (12) giving the dependence between the peak width and the size of the melting region. The widths of these peaks are such as if cooperative transition involved 150 and 120 base pairs, respectively, rather than 80 and 50 pairs. In order to rule out the possibility that these peaks are due to impurities, the fragment was subjected to additional purification by gel-electrophoresis followed by chromatography on hydroxyapatite. Complete coincidence of the differential melting profile of the additionally purified fragment (Fig. 4) with that of the starting one (Fig. 3) strongly suggests that the low-temperature peaks are not due to impurity.

However, detailed discussion of these effects, as well as of the additional information delivered by spectral evidence, is beyond the task of the present communication. The main result of this stage of our studies is the fact that application of the microspectrophotometer to differential melting makes it possible to investigate nucleic acids which are difficult to obtain in large amounts.

It is interesting that the above evidence on the presence of low-melting regions in the promoter-containing fragment is in line with the

Fig.4. The same as in Fig.3, but the fragment was additionally purified by gel-electrophoresis on agarose followed by chromatography on hydroxyapatite and ethanol precipitation.

finding of Aleksandrov and Cherny (13) who demonstrated by means of electron microscopy that the lowest-melting region of the denaturation map of T7 DNA overlaps with the site of the early promoters.

ACKNOWLEDGEMENTS

Our thanks are due to S.V.Kuzmin, V.G.Nekipelov, Yu.A.Bolvanov who participated in construction of the equipment, to Dr. Yu.L.Lyubchenko for his interest to these studies and for the critical discussions, and to Drs. E.F.Zaychikov and A.G.Pletnyov for the preparation of the promoter-containing fragment of T7 DNA.

REFERENCES

- 1 Yabuki,S., Gotoh,O., and Wada,A. (1975) Biochim. biophys. Acta **395**, 258-273
- 2 Akiyama,C., Gotoh,O., and Wada,A. (1977) Biopolymers **16**, 427-435
- 3 Vizard,D.L., and Ansevin,A.T. (1976) Biochemistry **15**, 741-750
- 4 Lazurkin,Yu.S. (1977) Molekularnaya biologia **11**, 1311-1324
- 5 Pavlov,V.M., Lyubchenko,Yu.L., Borovik,A.S., and Lazurkin,Yu.S. (1977) Nucleic Acids Research **4**, 4053-4062
- 6 Kuzmin,S.V. (1973) in Ultramicroanalysis of Nucleic Acids, Knorre, D.G., and Venkstern,T.V., Eds., pp. 95-103, Nauka, Moscow
- 7 Grachev M.A. ibid., pp. 104-121
- 8 Grachev,M.A., Zaychikov,E.F., Kravchenko,V.V., and Pletnyov,A.G. (1978) Dokl. Akad. Nauk SSSR **239**, 475-479
- 9 Stahl,S.J., and Chamberlin,M.J. (1977) J. Mol. Biol. **112**, 577-601
- 10 Kuzmin,S.V. (1971) U.S. Patent No. 3 749 497
- 11 Wada,A., Tachibana,H., Ueno,S., Husimi,Y., and Machida,Y. (1977) Nature **269**, 352-353
- 12 Lyubchenko,Yu.L., Trifonov,E.N., Lazurkin,Yu.S., and Frank-Kamenetskii,M.D. (1971) Molekularnaya biologia **5**, 772-779
- 13 Aleksandrov,A.A., and Cherny,D.I. (1976) Dokl. Akad. Nauk SSSR **231**, 471-473