FEBS Letters 422 (1998) 94–98 FEBS 19755

Evidence for intrinsic DNA bends within the human *cdc2* promoter

T. Murlidharan Nair*

Department of Biochemistry and Molecular Biology, Kansas University Medical Center, Kansas City, KS 66160, USA

Received 30 October 1997; revised version received 14 December 1997

Abstract Cyclin-dependent protein kinases (Cdks) are key regulatory proteins of the eukaryotic cell cycle. The product of the *cdc2* gene, p34^{cdc2} (cdk1), is the catalytic subunit of a serine/threonine protein kinase that is expressed in S phase and functions in the G2 to M phase transition. Previous studies indicate that the human *cdc2* gene expression is dependent on cell growth, and is transcriptionally regulated in a complex manner involving multiple transcription factors binding to specific sites in the promoter. One possible mechanism by which these transcription factors regulate transcription is that by binding to their cognate sites they induce bends in the DNA helix, thereby allowing their interaction with the basal transcription machinery through protein-protein contacts. Such protein-induced DNA bending is also influenced by intrinsic bends in the regulatory region. Using both theoretical and experimental approaches, the study reports that the human *cdc2* promoter has an intrinsic DNA bend with a broad locus of curvature.

Key words: Intrinsic DNA curvature; *cdc2* promoter

1. Introduction

The conformation of DNA is a function of its nucleotide sequence [1,2]. The existence of such sequence-dependent structural forms was first observed in certain DNA restriction fragments derived from kinetoplast DNA of *Leishmania torentolae* which exhibited anomalous electrophoretic mobility in gel [3]. This sequence dependence of DNA structure which refers to the net (time-averaged) deflection of the helix axis from linearity has since been a subject of intense theoretical and experimental investigation [1–5] and is now an established fact. There are mainly two classes of models that have been proposed to explain the sequence-directed structural deformations in DNA. The wedge model, originally proposed by Trifonov [6–8], is based on the assumption that dinucleotide segments have non-coplanar base pair planes, which gives rise to the formation of wedges between base pair planes, and is particularly significant for the AA sequence. The repetition of wedges in phase with the DNA helix repeat (~10.5 bp) produces macroscopic curvatures which result from additive local effects. This hypothetical wedge forms the basis of one of the two main classes of models that are used to describe DNA curvature, known as nearest-neighbor models. The second major class of models, known as the junction bending model [9,10], ascribes DNA curvature to the distortions at the interface between conformationally distinct forms of DNA (e.g. B-DNA and A-DNA). Both models require the phasing of [A]n tracts, which assures that the curvature of individual bending elements additively produces a large overall bend. DNA curvature is not limited to sequences containing poly-A runs, since a number of DNA fragments whose sequence contains no poly-A tracts are known to be curved [11]. DNA curvature can be conveniently studied by polyacrylamide gel electrophoresis [3,12]. Theoretical investigations of Lerman and Firsh [13] and Zimm and Levene [14] evince the presence of a correlation between DNA curvature and its anomalous migration in polyacrylamide gel. Based on the reptation model [15] they predict that the DNA mobility is directly related to the mean square end to end distance. De Santis et al. [16,17] have theoretically investigated the physical basis of sequence-dependent DNA curvature. They found that the nearest-neighbor differential interaction between base pairs [6] was sufficient to explain the origin of superstructures in DNA.

There have been numerous reports on the biological relevance of DNA curvature and on the importance of the loci of curvature in functionally important regions (e.g. promoters, replication origin) [1,2]. Intrinsic DNA curvature and protein-induced bending is thought to contribute to the activation of transcription by enhancing the interaction between upstream transcriptional activators and the basal transcription machinery [18,19]. Further, functional equivalence between protein-induced DNA bending and intrinsic DNA curvature has been shown in the case of the protein-induced DNA bending locus of the gal promoter [20] and the lac promoter [21] by substituting synthetically curved DNA. Synthetic curved DNA has also been shown to functionally replace bending induced by integration host factor (IHF) or catabolite activator protein (CAP), thus assigning functional significance to curvature per se [21–23]. DNA structural features has also been shown to be important in being recognized by a particular protein [24,25].

In the present study, the higher order DNA structure associated with the promoter region of the human *cdc2* gene which encodes a 34 kDa protein [p34^{cdc2}] has been analyzed. The [p34^{cdc2}] is the catalytic subunit of a serine/threonine protein kinase that phosphorylates key substrates that regulate the cell cycle progression. The activity of *cdc2* kinase is controlled post-transcriptionally, and is linked to the growth state of the cells [26–28]. The human *cdc2* promoter has multiple binding sites for transcription factors [29–31], suggesting that the transcriptional regulation of the *cdc2* gene is complex. Further, the human *cdc2* promoter does not contain a classical TATA-like element, but has two TTTGAAA elements located upstream of the transcription start site [29,30]. The human *cdc2* promoter has been examined for the presence of higher order structures using the theoretical models proposed by Trifonov [6,7] and De Santis et al. [16], and experimental methods involving cyclic permutation gel mobility shift assay [23] with a view to understanding the transcriptional regulation of this important member of the eukaryotic cell cycle. The results indicate that the human *cdc2* promoter, the bio-

*Present address: Department of Medicinal Chemistry, 308 Skaggs Hall, University of Utah, Salt Lake City, UT 84112, USA.
Fax: (1) (801) 581-7087. E-mail: nair@alanine.pharm.utah.edu

PII S0014-5793(97)01607-4

logical activities of which have been previously analyzed in detail [29,30,32], has an intrinsic DNA curvature.

2. Materials and methods

2.1. Materials

pCDC-PstI was a generous gift from Bruno Calabretta to R. Padmanabhan [30]. Agarose, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, Tris, boric acid, EDTA and $MgCl_2$ were purchased from Sigma Chemical Co. Ltd. Restriction enzymes, Klenow fragment of DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase were from New England Biolabs. The enzymes were used according to the suppliers' specifications. [γ-^{32}P]ATP (3000 Ci/mmol) and NEF-495 X-ray film were from DuPont NEN. Extraction of DNA fragments from agarose gel was done using QIAEX II gel extraction kit from Qiagen. Unincorporated label was removed using the QIAquick nucleotide removal kit from Qiagen. The CURVATURE program was a generous gift from Trifonov and Shpigelman [33].

2.2. Circular permutation assay

The 645 bp fragment corresponding to the 5′ flanking sequence of the *cdc2* gene from residue −677 (*Nsi*I restriction site) to residue −28 (*Apa*I restriction site) was analyzed by circular permutation assay. This fragment was gel extracted after digesting pCDC2-PstI with *Nsi*I and *Apa*I. It was further treated with Klenow DNA polymerase and self-ligated to generate multimers of the fragment. The ligation product was then digested with different restriction enzymes, each of which cut once within the insert to generate cyclically permutated fragments. The permutated fragments were dephosphorylated by treatment with calf intestinal alkaline phosphatase for 1 h. The reaction mixtures were then heated to 70°C for 15 min and extracted once with phenol and twice with chloroform, and ethanol precipitated. DNAs were subsequently labeled with ^{32}P by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase [34]. The unincorporated label was removed with the QIAquick nucleotide removal kit from Qiagen. These DNA fragments were analyzed on a non-denaturing 6% polyacrylamide gel in 0.5×Tris-borate-ethylenediaminetetraacetic acid buffer (pH 8.3) at a constant voltage of 4–5 V/cm at 4°C, which was pre-run for several hours before applying the sample. After the run the gel was dried and autoradiographed.

2.3. Trajectory plots and curvature map of the cdc2 promoter

The curvature map and the DNA path have been calculated using the CURVATURE program [33], a generous gift from Trifonov and Shpigelman. The program uses the experimentally determined 16 DNA wedge angles [11]. The trajectory plots thus generated provide both the degree of curvature and its orientation.

2.4. Theoretical permutation analysis

The theoretical permutation analysis was done using the De Santis model [16]. The model is based on the theoretical evaluation of local deviations from the standard B-DNA structure of the 16 different dinucleotide steps using conformational energy calculations [16]. The superstructures are predicted by integrating the theoretical deviations. The curvature is represented using a pair of diagrams in which both the modulus and the relative phase calculated from the first nucleotide residue of the curvature vector $C(n, \nu)$. The curvature vector represents in the complex plane the directional change of the double helix axis between sequence number n and $n+\nu$. The curvature dispersion σ^2 for a given DNA fragment is calculated as the second moment of the angular deviation of the local helical axis from the average direction. σ^2 has also been shown to be linearly correlated with gel electrophoretic retardation. Calculating σ^2, by cyclically permutating the sequences, allows the calculation of an analog of the permutation assay [35–37]. The model is described in detail in the original work of De Santis et al. [16,17]. The programs for the model were developed in FORTRAN.

3. Results and discussion

Sequence-directed structure associated with the *cdc2* promoter has been analyzed using both experimental and theoretical approaches. The nucleotide sequence of the *cdc2* promoter [29,30] region revealed the presence of several 'A' tracts; some of these were separated from each other by approximately 10 bp. Earlier studies had revealed that AA dinucleotide segments have non-coplanar base pair planes and this could lead to distortion of the helix axis [3,8]. The distortions would be significant if the 'A' tracts were separated by one turn of the helix (∼10.5 bp/turn). With a view to understanding the distortion induced in the *cdc2* promoter as a result of its sequence distribution, this region was theoretically analyzed using the models proposed by Trifonov [6] and De Santis et al. [16]. The curvature map of the *cdc2* promoter region as evaluated by the CURVATURE program is reported in Fig. 1. The window used in the calculation of the curvature map was 20. The regions with high curvature correspond to regions which are maximally curved. The curvature profile evaluated using the De Santis model also revealed the presence of regions which are curved. The curvature was evaluated over three turns of the helix, and the curvature dispersion σ^2 calculated as the second moment of the curvature vector is shown in Fig. 2. The curvature dispersion σ^2 retains the overall characteristic of the curvature profile, but in this method the noise caused by fluctuation of the curvature along the sequence is canceled and it allows individuation of sequences with high curvature. A linear correlation between the curvature dispersion σ^2 and gel electrophoretic retardation permits the calculation of an analog of the

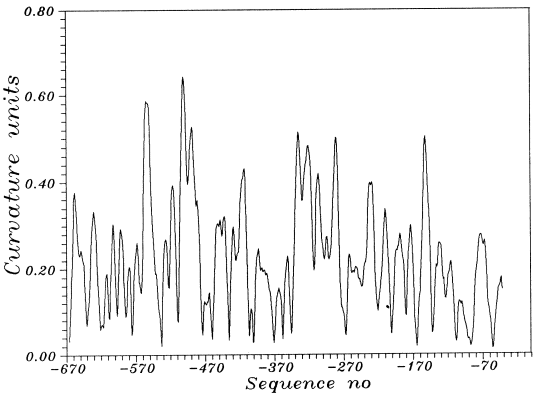

Fig. 1. Curvature map of the human *cdc2* promoter *Nsi*I-*Apa*I fragment expressed in DNA curvature units, which is the average curvature of DNA in the nucleosome core particle, 1/42.8 A.

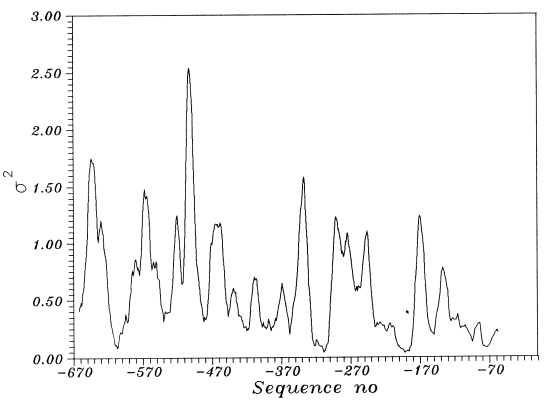

Fig. 2. Curvature dispersion (σ^2) of the *Nsi*I-*Apa*I fragment calculated using the De Santis model [16].

experimental permutation assay, by calculating σ^2 for the cyclically permutated sequences. The simulation results of the theoretical permutation assay are given in Fig. 3. It is important to realize here that only the trend in the mobility behavior versus the sequence is necessary to identify the molecular bend locus.

The analysis of the *cdc2* promoter using the theoretical models revealed that most of the regions corresponding to high curvature lie between the *Nsi*I and *Apa*I restriction sites. Analysis of the region downstream of the *Apa*I site did not reveal the presence of highly curved structures. The molecular bend locus as obtained by the simulation was also found to lie within the *Nsi*I-*Apa*I region.

The 5′ flanking region of the *cdc2* gene corresponding to the *Nsi*I (−677) and *Apa*I (−28) restriction sites was further experimentally analyzed following the permutation assay developed by Wu and Crothers [23]. The assay moves the sequence elements in a circularly permutated fashion. The resulting set of DNA fragments possess varying degrees of curvature as a result of the permutated sequences. The magnitude of conformation-induced migration increases with increasing gel concentration. This phenomenon results from the increased frictional coefficient due to decreased pore size [3]. The permutated fragments of the *cdc2* promoter did not show any difference in mobility in a 4% non-denaturing polyacrylamide gel (Fig. 4A). However, these fragments migrated to different degrees in a non-denaturing 6% polyacrylamide gel (Fig. 4B). The mobility of these permutated fragments plotted as a function of the restriction site is shown in Fig. 4C. The maximum and minimum of the resulting curve correspond to molecules having the locus of curvature nearest to their center and end respectively. The permutated fragment generated as a result of *Taq*I digestion migrates with maximum mobility (see lane 3 of Fig. 4B), this corresponds to the fact that the *Taq*I site is at or nearest to the bending locus of the *cdc2* promoter fragment. Digestion at the bending locus results in linear and faster migrating DNA fragments. On the other hand, *Nar*I digestion results in fragments that are maximally retarded (see lane 4 of Fig. 4B). This corresponds to the fact that these fragments have their bending loci in the center. The bending locus as determined experimentally corresponds to the region around the *Taq*I site (−359).

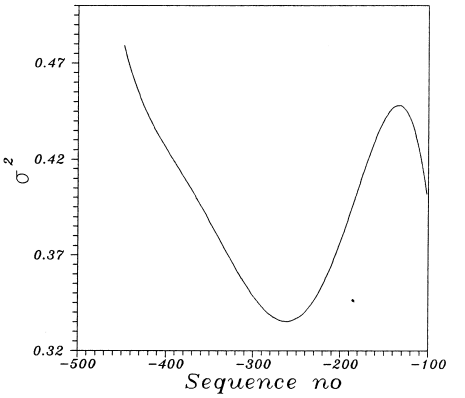

Fig. 3. Curvature dispersion (σ^2) calculated by a cyclic permutation of the sequence. The minimum corresponds to the molecular bend locus.

It is important to note that the permutation approach used in determining the molecular bend locus is an approximate one. The accuracy of the circular permutation approach largely depends on the precision with which the minima and maxima of the curve can be defined experimentally. In other words, in order to determine the putative locus of curvature with a reasonable degree of accuracy, it is important for the fragment of interest to contain enough unique restriction sites which are conveniently distributed throughout the fragment. The presence of other minor loci of curvature within the molecule would also affect the accuracy of the permutation approach [4].

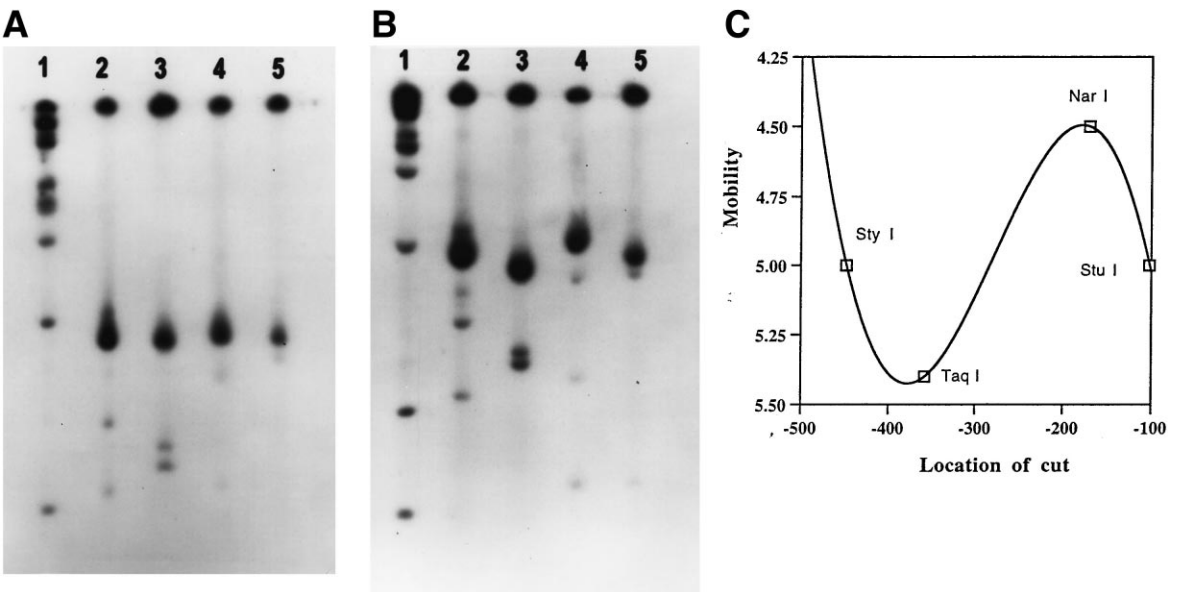

Fig. 4. Circular permutation assay. The circular permutation assay was carried out as described in Section 2. The ligation product of *Nsi*I and *Apa*I, after blunt ending by treatment with *E. coli* Klenow enzyme, was digested with various restriction enzymes, the fragments were end-labeled and analyzed by 6% polyacrylamide gel at 4°C. A: Mobility of the permutated fragments in 4% polyacrylamide gel at 4°C. Lane 1, lambda *Bst*EII marker; 2, *Sty*I; 3, *Taq*I; 4, *Nar*I; 5, *Stu*I. B: Mobility of the permutated fragments in 6% polyacrylamide gel at 4°C. Lane 1, lambda *Bst*EII marker; 2, *Sty*I; 3, *Taq*I; 4, *Nar*I; 5, *Stu*I. C: Plot of the relative mobility vs. the location of cut for the permutated fragment.

Comparing the results of the theoretical permutation with that of the experimental permutation, one finds that the minimum as obtained by the simulation is slightly shifted towards the transcription start point as compared to that obtained experimentally. This shift in the minimum can be explained by analyzing the DNA path of the *cdc2* promoter shown in Fig. 5. The DNA path calculated using the parameters derived by energy minimization [16,17] as well as those derived experimentally [11] reveals the presence of a broad locus of curvature. The curvature locus also seems to be uneven. However, it is interesting to note that the curvature loci as obtained by the theoretical simulations also fall within this broad locus. Further, the theoretical simulations are able to capture the trend in the mobility of the fragment with a reasonable degree of accuracy.

With a view to further understanding the mobility behavior of the permutated fragments, they were further analyzed theoretically. The DNA path shown in Fig. 6 also distinctly demonstrates that *Nar*I is maximally curved as compared to the other permutations. It is also noteworthy that the permutated fragment resulting from *Nar*I digestion has a rather sharp locus of curvature as compared to the other permutations.

The results of our analysis reveal that the *cdc2* promoter is intrinsically curved and has a broad locus of curvature. Recent studies provide evidence that intrinsic DNA curvature is likely to play a role in transcriptional regulation. Cress and Nevins [38] reported that the transcription factor E2F recognition site is intrinsically bent, and binding of free E2F to this site resulted in DNA bending in the opposite orientation to the intrinsic DNA bend. Furthermore, the ability of free E2F to reverse the orientation of the intrinsic DNA bend was abrogated by the retinoblastoma susceptibility gene product, RB, which is a negative regulator of E2F-mediated transcription. Conformational changes in DNA are also known to increase specificity [39] and cooperativity [40] within operator regions. Interaction of factors bound to separate sites on DNA can be regulated by protein-induced DNA bending [41,42]. Further, DNA bending by proteins can select among different potential protein-protein interactions in a region containing multiple factors bound to DNA. This may be achieved

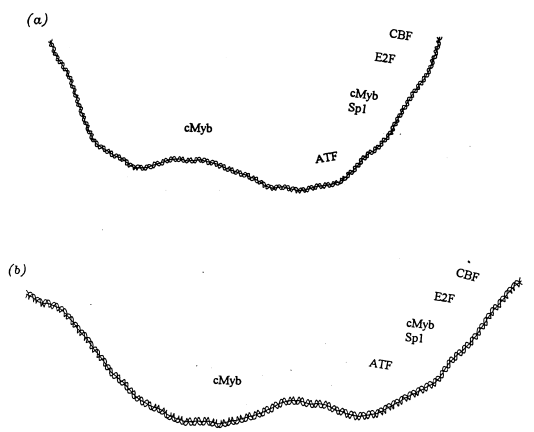

Fig. 5. DNA path of the *cdc* promoter *Nsi*I-*Apa*I fragment calculated (a) using the experimentally determined parameters [11] and (b) using the energy-minimized values of roll, tilt and twist [16]. Also shown in the figure is the approximate location of the binding sites for *cis*-acting factors viz. C-Myb, ATF, Sp1, E2F [29,30] and a CCAAT box binding factor (CBF) [32].

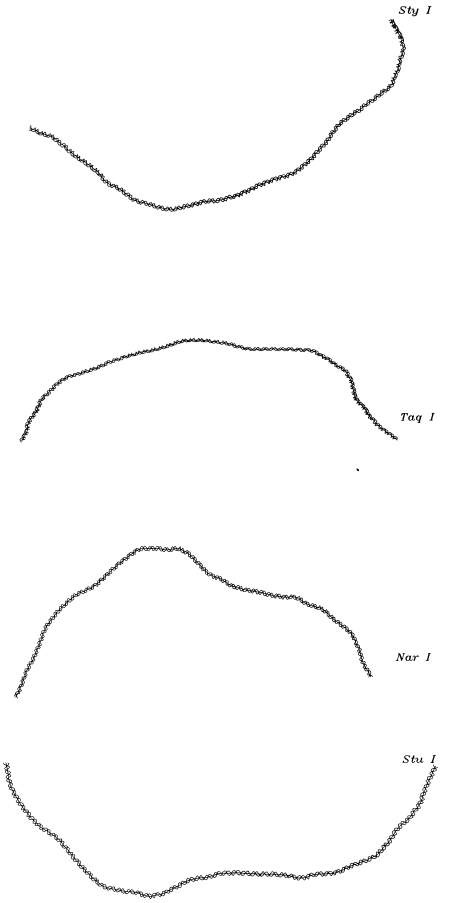

Fig. 6. DNA path of the permutated fragments of the *cdc2* promoter calculated using the experimentally determined parameters and plotted on the maximum curvature plane [11].

by changing the orientation and magnitude of the bend [43]. These results, implicating the role of DNA structure in transcription regulation, suggest that the intrinsically curved region of the *cdc2* promoter may also be functionally important. Moreover, the *cdc2* promoter harbors the binding sites for numerous *cis*-acting factors, viz. C-Myb, ATF, Sp1, E2F [29,30] as well as a CCAAT box binding factor [32]. Earlier analysis also revealed that deletion of sequences upstream of the *Taq*I site (−359) increased the promoter activity, while deletions between the *Nar*I and *Apa*I sites (−171 and −28) decreased promoter activity [32]. Recent evidence from in vivo DNase footprinting analysis of the human *cdc2* promoter indicates that there are 11 sites that were protected by protein binding along the *cdc2* promoter in quiescent fibroblasts and at different periods after serum stimulation. These results suggest that the transcriptional regulation of *cdc2* gene expression is complex [31]. The results reveal an interesting possibility that this broad locus of curvature associated with the *cdc2* promoter may serve as a binding pocket for these transcription factors and help in selective interaction with the basal transcription machinery by changing the degree and orientation of the bend.

Acknowledgements: I thank Prof. E.N. Trifonov for his comments and suggestions on the work. I also thank Prof. R. Padmanabhan for all the support in doing the work. Comments of the referees on the previous version of the manuscript are greatly acknowledged. This

work is supported by the National Science Foundation Grant MCB-9507034.

References

[1] Trifonov, E.N. (1985) CRC Crit. Rev. Biochem. 19, 89–106.
[2] Hagerman, P.J. (1990) Annu. Rev. Biochem. 59, 755–781.
[3] Marini, J.C., Levene, S.D., Crothers, D.M. and Englund, P.T. (1982) Proc. Natl. Acad. Sci. USA 83, 862–866.
[4] Hagerman, P.J. (1992) Biochim. Biophys. Acta 1131, 125–132.
[5] Nair, T.M., Madhusudan, K., Nagaraja, V., Kulkarni, B.D., Majumdar, H.K. and Singh, R. (1994) FEBS Lett. 351, 321–324.
[6] Trifonov, E.N. (1980) Nucleic Acids Res. 8, 4041–4053.
[7] Ulanovsky, L.E. and Trifonov, E.N. (1987) Nature 320, 501–506.
[8] Trifonov, E.N. and Sussman L, J. (1980) Proc. Natl. Acad. Sci. USA 75, 3815–3820.
[9] Koo, H.-S., Wu, H.-M. and Crothers, D.M. (1986) Nature 320, 501–506.
[10] Levene, S.D. and Crothers, D.M. (1986) J. Biomol. Struct. Dynam. 1, 429–435.
[11] Bolshoy, A., McNamara, P., Harrington, R.E. and Trifonov, E.N. (1991) Proc. Natl. Acad. Sci. USA 88, 2312–2316.
[12] Harrington, R.E. (1993) Electrophoresis 14, 732–746.
[13] Lerman, L.S. and Frisch, H.L. (1986) Biopolymers 21, 995–997.
[14] Zimm, B.H. and Levene, S.D. (1992) Q. Rev. Biophys. 25, 171–204.
[15] De Gennes, P.G. (1971) J. Chem. Phys. 55, 572–578.
[16] De Santis, P., Palleschi, A., Savino, M. and Scipioni, A. (1988) Biophys. Chem. 32, 305–317.
[17] De Santis, P., Fua, M., Savino, M., Anselmi, C. and Bocchinfuso, G. (1996) J. Phys. Chem. 100, 9968–9976.
[18] Lamb, N.J., Fernandez, A., Wartim, A., Labbe, J.C. and Cavadore, J.C. (1990) Cell 60, 151–165.
[19] van der Vliet, P.C. and Verrijzer, C.P. (1993) BioEssays 15, 25–32.
[20] Bracco, L., Kotlarz, D., Kolb, A., Diekmann, S. and Buc, H. (1989) EMBO J. 8, 4289–4296.
[21] Gartenberg, M.R. and Crothers, D.M. (1991) J. Mol. Biol. 219, 217–230.
[22] Goodman, S.D. and Nash, A.H. (1989) Nature 341, 251–254.
[23] Wu, H.M. and Crothers, D.M. (1984) Nature 308, 509–513.
[24] Nair, T.M., Kulkarni, B.D. and Nagaraja, V. (1995) Biophys. Chem. 53, 241–245.
[25] Werner, M.H., Gronenborn, A.M. and Clore, G.M. (1996) Science 271, 778–784.
[26] Simanis, V. and Nurse, P. (1986) Cell 45, 261–268.
[27] Morgan, D.O. (1995) Nature 374, 131–134.
[28] Pines, J. (1995) Semin. Cancer Biol. 6, 63–67.
[29] Dalton, S. (1992) EMBO J. 11, 1797–1804.
[30] Ku, D.-H., Wen, S.C., Engelhard, A., Nicolaides, N.C., Lipson, K.E., Marino, T.A. and Calabretta, B. (1993) J. Biol. Chem. 268, 2255–2259.
[31] Tommasi, S. and Pfeifer, G.P. (1995) Mol. Cell. Biol. 15, 6901–6913.
[32] Chen, H., Campisi, J. and Padmanabhan, R. (1996) J. Biol. Chem. 271, 13959–13967.
[33] Shpigelman, E.S., Trifonov, E.N. and Bolshoy, A. (1994) CABIOS 9, 435–440.
[34] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[35] Nair, T.M., Madhusudan, K., Nagaraja, V., Kulkarni, B.D., Majumdar, H.K. and Singh, R. (1996) Electrophoresis 17, 633–641.
[36] Boffelli, D., De Santis, P., Palleschi, A., Risuleo, G. and Savino, M. (1992) FEBS Lett. 300, 175–178.
[37] De Santis, P., Palleschi, A., Savino, M. and Scipioni, A. (1990) Biochemistry 29, 9269–9273.
[38] Cress, W.D. and Nevins, J.R. (1996) Mol. Cell. Biol. 16, 2119–2127.
[39] Hard, T. and Lundback, T. (1996) Biophys. Chem. 62, 121–139.
[40] Merabet, E. and Ackers, G.K. (1995) Biochemistry 34, 8554–8563.
[41] Moitoso de Vargas, L., Kim, S. and Landy, A. (1989) Science 244, 1457–1461.
[42] Kim, S., Moitoso de Vargas, L., Nunes-Duby, S.E. and Landy, A. (1990) Cell 63, 773–781.
[43] Moitoso de Vargas, L. and Landy, A. (1991) Proc. Natl. Acad. Sci. USA 88, 588–592.