Raman Microspectroscopic Study of Low-pH-Induced Changes in DNA Structure of Polytene Chromosomes

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ABSTRACT: The effects of low-pH treatments on DNA structure in polytene chromosomes of *Chironomus thummi thummi* have been studied by Raman microspectroscopy. Measurements were carried out on chromosomes at low pH and on chromosomes reneutralized after a short exposure to low pH. Protonation of adenine residues and subsequent unpairing of adenine (A) and thymine (T) were found to commence already above pH 3.6 and are completed at pH 2.2. Protonation of the cytosine–guanine base pair also starts above pH 3.6. It leads to an intermediate protonated, most likely Hoogsteen-type, guanine–cytosine base pair. Unpairing of G and C residues starts between pH 2.6 and 2.2 and continues below pH 2.2. Brief treatment of chromosomes at a pH $\leq$ 2.2, i.e., at a pH where both AT and GC base pairs are disrupted, was found to lead to irreversible changes in DNA conformation upon return to neutral pH. These were most pronounced after treatment with 45% acetic acid. More than 10% of the A and T residues was found to have adopted a non-B-DNA conformation. Evidence was found for denaturation of the B-form backbone. The amount of protein extracted from the chromosomes was strongly pH-dependent. Treatment at pH 3.6 did not cause noticeable protein extraction, while treatment with 45% acetic acid extracted more than 50% (by weight) of the chromosomal proteins.

The predominant DNA conformation in chromosomes and in the nucleus of eucaryotic cells is the B-form, the canonical right-handed double helix. Alternative DNA structures and conformations, such as cruciforms (Panayotatos & Wells, 1981; Lilley et al., 1987; Furlong et al., 1989), triplex DNA (Lee et al., 1984; Htun & Dahlberg, 1988; Kohwi & Kohwi-Shigematsu, 1988; Mirkin et al., 1987), Z-DNA (Wang et al., 1979; Klysik et al., 1983; Jovin & Soumpasis, 1987; Hill, 1991), parallel-stranded DNA (Saenger, 1984; Van de Sande et al., 1988; Ramsing & Jovin, 1988; Otto et al., 1991), and (extended stretches of) unpaired bases (Kohwi-Shigematsu et al., 1987; Kohwi-Shigematsu & Kohwi, 1990; Furlong et al., 1989; Kowalski et al., 1988; Kowalski & Eddy, 1989), have sparked much interest. These non-B-DNA structures can be formed under conditions of torsional stress and may play important organizing and/or regulatory roles in vivo (McLean & Wells, 1988; Rahmouni & Wells, 1989; Palecek, 1991). Cytological studies have been carried out with fluorescently labeled antibodies raised against Z-DNA, triplex DNA, and cruciforms in order to assess the presence of such DNA structures in cells and chromosomes. They have indicated the existence of Z-DNA in acid-fixed polytene chromosomes (Nordheim et al, 1981; Arndt-Jovin et al., 1983), metaphase chromosomes (Viegas-Pequignot et al., 1983, 1987; Nordheim et al., 1986; Ueda et al., 1990), and cell nuclei (Lipps et al., 1983; Morgenegg et al., 1983). However, these results do not constitute a straightforward measure of Z-DNA in vivo because of the largely unknown effects of acid fixation. They do show, however, that in all these different forms of chromatin, potential Z-DNA-forming stretches of DNA exist. Unfixed, untreated chromosomes isolated under physiological conditions demonstrate only very weak staining. This may indicate that only small numbers of the potential Z-forming stretches are in the left-handed conformation at any one time or that the Z-conformation is masked by chromatin proteins that block antibody binding. A third possibility is that fixation creates conditions favorable for the induction of additional Z-conformation (Robert-Nicoud et al., 1984; Hill et al., 1984). Fluorescently labeled anti-triplex-DNA antibodies were found to stain fixed mouse and human metaphase chromosomes, interphase nuclei, and polytene chromosomes (Burkholder et al., 1988, 1991). Again, unfixed metaphase chromosomes were only weakly fluorescent.

Because acid fixation of cytological preparations apparently enhances staining with various non-B-DNA antibodies, it is worthwhile to study the effects of a low-pH environment on DNA structure more closely. The enhancement of anti-Z-DNA antibody staining was investigated with polytene chromosomes (Hill et al., 1984; Robert-Nicoud et al., 1984). It was found that fluorescence intensity (i.e., amount of antibody binding) and localization were strongly dependent on the pH employed in the treatment and on the time of exposure. In general, lower pH and longer exposure led to more intense and homogeneous staining. It was concluded that torsional stress, due to negative supercoiling as a result of protein extraction, and (local) strand separation at low-pH facilitate the B to Z transition.

In this work, a confocal Raman microspectrometer (Puppels et al., 1990, 1991a) was employed to investigate the effects of low-pH treatments on DNA structure and DNA–protein ratio of polytene chromosomes of *Chironomus thummi thummi*. Raman spectra provide information about vibra-

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tional energy levels of molecules, which depend on the types of atoms and chemical bonds involved in vibrations. Alterations in molecular structure lead to changes in the Raman spectrum. The technique has been widely applied to study the composition, structure, and interactions of nucleic acids and proteins (see, e.g., Erfurth & Peticolas, 1975; Goodwin & Brahms, 1978; Hayashi et al., 1986; Savoie et al., 1985; Thomas et al., 1977, 1986). Measurements were made of chromosomes at low pH, chromosomes reneutralized after a brief incubation at low pH, and calf thymus DNA at low pH. It is shown that upon protonation GC base pairs adopt a protonated Hoogsteen conformation and that irreversible changes in DNA structure occur, when at low pH both AT and GC base pairs are disrupted.

MATERIALS AND METHODS

Chromosome and DNA Preparations. Unfixed polytene chromosomes were isolated from explanted salivary glands of 4th instar larvae of *C. thummi thummi* according to the micromanipulation technique of Robert-Nicoud (1975). After isolation, they were positioned onto the surface of a microscope slide, to which they readily adhered.

The low-pH treatments were carried out by washing the chromosomes in 50 mM glycine–HCl, 50 mM NaCl buffers of the appropriate pH (3.6, 2.6, 2.2, or 1.8), or 45% acetic acid. Chromosomes were then returned to the neutral pH buffer (Ringer's insect phosphate-buffered saline with added divalent cations, 1 mM $MgCl_2$ + 1 mM $CaCl_2$, pH 7.2) and washed for 20 min before a cover glass was sealed on the microscope slide.

The buffers used for the preparations with chromosomes kept at low pH (3.6, 2.6, 2.2, or 1.8) were the same as used in the short low-pH treatments. Fused silica microscope slides and cover glasses (200-μm thickness) were used. The slides with chromosomes were stored on ice until used in the Raman experiments, which were carried out at room temperature within 24–48 h after preparation of the samples.

Holmquist showed that at 24 °C in 0.2 N HCl, free DNA and DNA in metaphase chromosomes are depurinated at a rate of ~1 purine/10 000 bases/min (Holmquist, 1979). On the basis of the results of Venner (1964) concerning depurination rates of nucleosides as a function of pH and temperature, it is estimated that for the chromosomes stored on dry ice in the pH 1.8 buffer the depurination rate will be at least 2 orders of magnitude lower. Therefore, total depurination after 48 h with be less than 1%.

Calf thymus DNA (Sigma type I, D-1501; St. Louis, MO) at a concentration of 15 mg/mL was dialyzed against glycine buffers (50 mM glycine, 150 mM NaCl) to obtain DNA samples in the pH range 5–2.5. At pH 2.5, slight aggregation and sedimentation of the DNA were observed.

Experimental. All RS experiments with chromosomes were carried out on the confocal Raman microspectrometer (CRM) mentioned in the introduction and described in detail in Puppels et al. (1991a). The microscope was equipped with a 63× Zeiss Plan Neofluar water immersion objective (NA 1.2, 0.12–0.22). Laser light of 660 nm from a DCM-operated dye laser (Spectra Physics, Model 375 B) was used for excitation. The spectra shown have been processed by means of the software package RAMPAC (De Mul & Greve, 1993). Each measurement on a chromosome was followed by a second (background signal) measurement just next to that chromosome in order to determine the signal contributions from buffer (water and glycine) and substrate (fused silica), which were then subtracted from the chromosome measurement. A slightly sloping broad-band residual background, due to trace amounts of fluorescing impurities, was subtracted from the resulting spectra (method described in detail in Benevides et al., 1984).

Each spectrum is the result of 10 or more measurements (see figure captions) at random positions on chromosomes I, II, and III (excluding telomers), which were averaged in order to minimize effects due to differences in local DNA–protein composition (Puppels et al., 1990, 1993; Puppels, 1991c). The averaged spectra thus obtained showed only minor variations, not of any consequence for the interpretations given below. The wavenumber calibration of the Raman spectra was made on the basis of an indene Raman spectrum recorded with the same instrument setting. Line positions of well-resolved lines are accurate within ±2 cm^{-1}. The spectra were corrected for the wavenumber-dependent detection efficiency of the CRM and for pixel-to-pixel variations in CCD-camera quantum efficiency (Puppels et al., 1991a).

The data shown represent the results of three series of experiments, with independently prepared chromosome samples. The experiments with calf thymus DNA were carried out on a second, largely identical, micro-Raman setup developed in our laboratory, using 514.5-nm laser excitation.

Difference Spectra. Chromosomes constitute an inhomogeneous sample. Therefore, an internal intensity calibration was needed to scale the measured spectra in order to compute difference spectra. The 1094-cm^{-1} line of the DNA backbone PO_2^- symmetric stretching vibration was used for this purpose. Its intensity is not sensitive to DNA denaturation (Erfurth & Peticolas, 1975) or to DNA protonation down to at least pH 2.35 (O'Connor et al., 1981). Protonation of the DNA backbone phosphate groups occurs around pH 1 (Hartmann et al., 1973). In diethyl phosphate, it leads to a decrease in the intensity of the PO_2^- symmetric stretching Raman line (Stangret & Savoie, 1992). However, since no measurements were carried out at pH's lower than 1.8, this will not affect the general validity of the results presented here. The intensity of the 1094-cm^{-1} line is furthermore largely independent of the DNA–protein interactions that occur in nucleosomes and chromatin (Thomas et al., 1977; Hayashi et al., 1986; Savoie et al., 1985) and in DNA–polylysine complexes (Prescott et al., 1976).

Determination of DNA-Protein Ratio. The DNA–protein ratio (weight/weight) of the chromosomes returned to neutral pH after a low-pH treatment and of untreated chromosomes was determined by the method described previously (Puppels et al., 1991b), using the intensities of the 1094-cm^{-1} DNA backbone line and the 1449-cm^{-1} protein line (CH deformations) as internal concentration markers.

RESULTS AND DISCUSSION

Raman microspectroscopic measurements were carried out on *C. thummi thummi* polytene chromosomes at neutral pH (pH 7.2) and at pH 3.6, 2.6, 2.2, and 1.8 and on chromosomes returned to neutral pH after a 10-min incubation at pH 3.6, 2.6, 2.2, and 1.8 or in 45% acetic acid. This choice of pH's was based on the observations (Robert-Nicoud et al., 1984) that treatment of chromosomes at pH > 3 does not result in an enhancement of Z-DNA immunoreactivity and that maximum enhancement was observed with treatments at pH 1.8 or in 45% acetic acid (~pH 1.6), resulting in a homogeneous staining of such chromosomes with fluorescently labeled anti-Z-DNA antibodies.

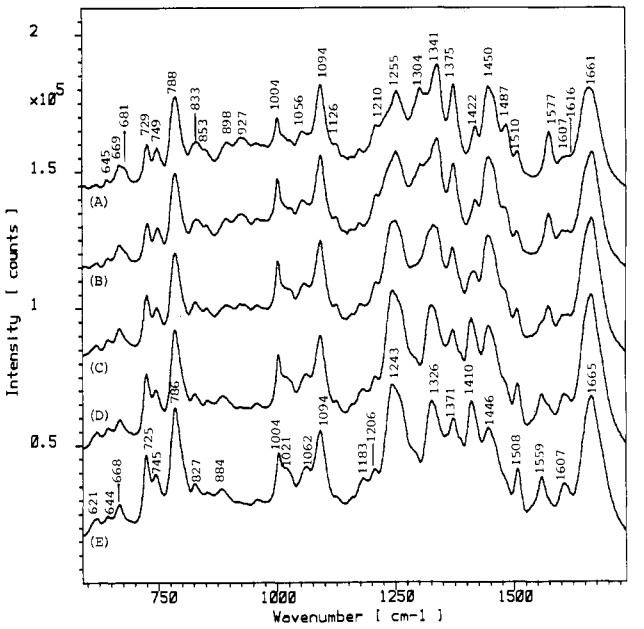


FIGURE 1: Raman spectra of chromosomes at different pH's: (A) neutral pH (pH 7.2), (B) pH 3.6, (C) pH 2.6, (D) pH 2.2, and (E) pH 1.8. For all measurements, the laser power was 14 mW and the measuring time 5 min. Each spectrum is the average of 10 measurements.

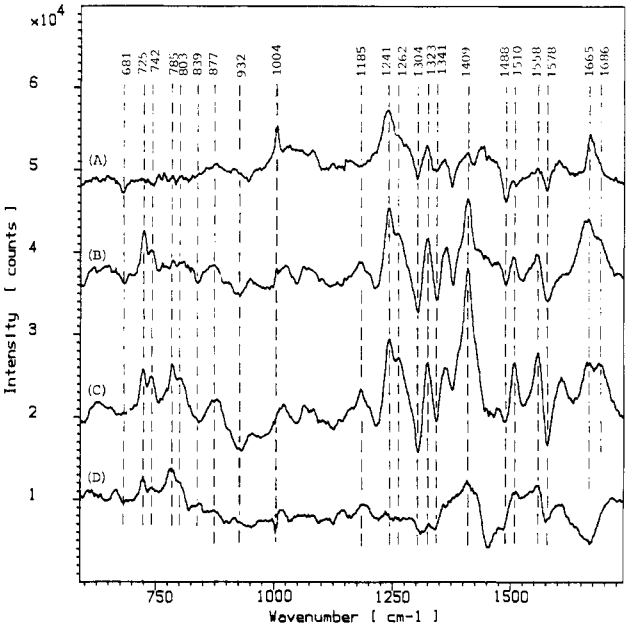


FIGURE 2: Raman difference spectra: (A) pH 3.6 – neutral, (B) pH 2.6 – pH 3.6, (C) pH 2.2 – pH 2.6, and (D) pH 1.8 – pH 2.2. The spectra were scaled on the 1094-cm^{-1} DNA line before subtraction (see Materials and Methods). See Table 1 for line assignments.

Studies on Chromosomes and Calf Thymus DNA at Low pH

In Figure 1, the spectra obtained at neutral pH and in the low-pH region (pH 3.6–1.8) are shown. The events occuring in each pH interval are discussed below and become very clear in the difference spectra in Figure 2. A listing of the spectral changes and Raman line assignments is given in Table 1.

A comparison of the protonation profiles of 5′-CMP, 5′-AMP, and 5′-GMP (O'Connor et al, 1981; based on data of Izatt et al., 1971) shows that in mononucleotides, protonation of cytosine N3 (pK_a = 4.24) and adenine N1 (pK_a = 3.80) commences at a significantly higher pH than protonation of guanine N7 (pK_a = 2.3). The results obtained in mononucleotide studies are not directly transferable to the case of nucleotides in the DNA double helix. In DNA, the N3 of cytosine is hydrogen-bonded to guanine N1, i.e., not freely accessible to a proton as is the case in 5′-CMP. Cytosine protonation in DNA is thought to occur via a mechanism involving a rotation of guanine around the glycosidic bond from an anti to a syn position, leading to an intermediate protonated Hoogsteen GC base pair (Courtois et al., 1968; Zimmer & Triebel, 1969; Kas'yanenko, 1986; Guéron et al., 1990). This "flexibility" of guanine does not depend upon its incorporation in a DNA duplex but has also been documented for guanosine and guanosine monophosphates, which were found to preferentially adopt a syn conformation upon protonation (Guschlbauer et al., 1968: Son et al., 1972). On the other hand, protonation of adenine in an AT base pair was found to induce disruption of the base pair, i.e., no protonated AT base pairs are formed during acid titration of DNA (Hermann & Fredericq, 1977).

Large changes in the Raman spectra of polytene chromosomes were observed upon lowering the pH. These are due to protonation and unstacking of the DNA bases and to changes in DNA backbone conformation. The intensities of the Raman lines of the ring breathing vibrations of guanine (681 cm^{-1}), adenine (729 cm^{-1}), thymine (749 cm^{-1}), and cytosine (784 cm^{-1}) are not sensitive to protonation. These lines, which are markers for the C2′-endo-anti conformation of the nucleotides, typical of B-DNA (Thomas et al., 1986), do respond, however, to unstacking of the bases (O'Connor et al., 1981). The guanine line is hypochromic; its intensity decreases upon unstacking during DNA melting (Erfurth & Peticolas, 1975). The other lines show a hyperchromic behavior, especially, the adenine line shows a large increase in intensity upon unstacking. The base vibrations in the higher wavenumber region (1200–1700 cm^{-1}) are sensitive to both protonation and unstacking (O'Connor et al., 1981).

Chromosomes at pH 3.6. Figure 2A and Table 1 show that at pH 3.6, protonation of GC base pairs had started. Spectral changes were found that are also observed upon protonation of cytosine (shoulder at 1262 cm^{-1}) and guanine in mononucleotides (intensity decrease of the 1488-cm^{-1} line). There was a clear intensity decrease of the guanine 681-cm^{-1} line (further discussed in Chromosomes at pH 2.6). The absence of a line at 785 cm^{-1} indicates that no unstacking of cytosine residues had taken place at this pH.

There were also signs indicating the start of adenine protonation (negative line at 1304 cm^{-1} and positive line at 1323 cm^{-1}) and the unstacking of thymine rings (lines at 1241 and 1665 cm^{-1}). Thymine unstacking indicates that protonation of adenine is accompanied by a disruption of the AT base pair, in agreement with the results of Hermann and Fredericq (1977). The strong increase in the intensity of the phenylalanine (phe) line at 1004 cm^{-1} reflects a change in the chemical microenvironment of this residue, possibly unstacking of phe rings (Thomas et al., 1983).

Chromosomes at pH 2.6. Lowering the pH to 2.6 (Figures 1C and 2B) led to more extensive protonation of adenine (evidence for this are, apart from the features at 1304 and 1323 cm^{-1} mentioned above, also the strong line at 1409 cm^{-1} and the shoulder at 1686 cm^{-1} in Figure 2B; see Table 1). The line at 725 cm^{-1} in Figure 2B shows that unstacking of adenine residues takes place. Thymine unstacking continued (apart from the lines at 1241 and 1665 cm^{-1}, also evident from the

Table 1: Low-pH-Induced Changes in the Raman Spectra of *C. thummi thummi* Polytene Chromosomes and Line Assignments[a]

line position (cm^{-1})	pH 3.6 – neutral	pH 2.6 – pH 3.6	pH 2.2 – pH 2.6	pH 1.8 – pH 2.2	assignments and pH and heat denaturation effects described in the literature (reference)	
					heat denaturation	pH effects
681	↓	↓	–	–	G $\xrightarrow{hd}$ ↓ (1)	G $\xrightarrow{pH}$ ↓ (4)
725	–	↑	↑	↑	A $\xrightarrow{hd}$ ↑ (–5) (1)	A $\xrightarrow{pH}$ ↑ (3)
742	–	↑	↑	↑	T $\xrightarrow{hd}$ (–6) (1)	T $\xrightarrow{pH}$ ↑ (3)
785	–	–	↑	↑	C, DNA:BK $\xrightarrow{hd}$ ↑ (1)	
803	–	–	↑	↑		DNA:BK (tent.) G^+ (3)
830	–	↓	↓	–	DNA:BK $\xrightarrow{hd}$ (–) (1)	DNA:BK $\xrightarrow{pH}$ ↓ (3,4)
877	–	↑	↑	–		DNA:BK (tent.)
934	–	↓	↓	–		p:C–C skeletal modes (denaturation and extraction)
1004	↑	–	–	↓		phenylalanine (see text)
1094		Intensity Calibration Line				DNA:BK PO_2^- sym str (1)

line position (cm^{-1})	pH 3.6 – neutral	pH 2.6 – pH 3.6	pH 2.2 – pH 2.6	pH 1.8 – pH 2.2	assignments and pH and heat denaturation effects described in the literature (reference)	
					heat denaturation	pH effects
1185	–	↑	↑	↑	T $\xrightarrow{hd}$ ↑ (1)	G^+ (2)
1241[b]	↑	↑	↑	–	T $\xrightarrow{hd}$ ↑ (1)	(see text)
1262	↑	↑	↑	–	C^+ $\xrightarrow{hd}$ ↑ (7)	C^+ (2)
1304	↓	↓	↓	–	A,C (2) $\xrightarrow{hd}$ ↑ (1)	A,C $\xrightarrow{pH}$ ↓ (3,4)
1323	↑	↑	↑	–		A^+ (2,3,4)
1341	↓	↓	↓	–	A (2) $\xrightarrow{hd}$ = (1)	A $\xrightarrow{pH}$ ↓ (3,4)
1409	–	↑	↑	↑		A^+ (2)
1488	↓	↓	↓	↓		G,A $\xrightarrow{pH}$ ↓ (2,3,4)
1510	–	↑	↑	↑		A $\xrightarrow{pH}$ ↑ (2,3)
1558	–	↑	↑	↑		A^+,G^+ (2)
1578	↓	↓	↓	↓		G,A (5,6) $\xrightarrow{pH}$ ↓ (3,4)
1665	↑	↑	↑		T (C=O) $\xrightarrow{hd}$ ↑ (1)	T (C=O) $\xrightarrow{pH}$ ↓ (3)
				↓		p:amide I $\xrightarrow{pH}$ ↓ (extraction)
1686	–	↑	↑	–		A^+ (2)

[a] Legend: (1) Erfurth & Peticolas, 1975; (2) Lord & Thomas, 1967; (3) O'Connor et al., 1981; (4) Zama et al., 1978; (5) Prescott et al., 1984; (6) Thomas et al., 1977; (7) Chou & Thomas, 1977. Abbreviations: (+) line specific for protonated nucleotide, (hd) heat denaturation, (pH) lowering of pH, (BK) backbone, (p) protein, (↑) intensity increase, (↓) intensity decrease, ((–*x*)) *x*-cm^{-1} downshift, (=) no change, (sym) symmetric, (str) stretching, (tent.) tentative. [b] Assignment of the 1241-cm^{-1} line in Figure 2. The large increase in intensity of a composite band, centered around 1250 cm^{-1} upon lowering of the pH (Figure 1), was also observed in Raman experiments on DNA (O'Connor et al., 1981) and nucleosome cores (Zama et al., 1978). In those reports, it was attributed to protonation of cytosine. Figure 2 shows, however, that the intensity increased mainly due to a band positioned at 1241 cm^{-1}. In our opinion, this is indicative of the unstacking of T rings. The following arguments support this view: (A) Neither in cytosine, cytidine, or 5′-CMP at pH 1 (Lord & Thomas, 1967) or pH 1.8 (O'Connor et al., 1981) is a line found at ~1241 cm^{-1}. (B) It is clear from the spectra in Figures 1 and 2 and from Table 1 that protonation of A and the intensity increase of the 1241-cm^{-1} line occur simultaneously. It is known that protonation of A results in local stand separation of the DNA double helix (Zimmer & Triebel, 1969; Hermann & Fredericq, 1977) and that T rings do not show significant stacking in single stranded poly(dT) (Riley et al., 1966). Thermal denaturation of DNA (implying unstacking of T rings) leads to an increase of the intensity of the 1241-cm^{-1} T line (Erfurth & Peticolas, 1975). This implies that an increase in the intensity of the T line at 1241 cm^{-1} is expected when protonation of A occurs. (C) The 1241-cm^{-1} line is also present in the difference spectra of low-pH-treated chromosomes returned to neutral pH and untreated chromosomes (shown in Figure 4) where it can, of course, not be due to protonated C.

line at 742 cm^{-1} in Figure 2B), as did the protonation of the GC base pair. From the absence of a line at 785 cm^{-1} follows that still no unstacking of cytosine residues had occurred. The 681-cm^{-1} guanine line has almost completely disappeared in Figure 1C.

Further information is obtained from the band centered around ~830 cm^{-1}, which is ascribed to an antisymmetric PO_2 stretching mode of the B-form DNA backbone, i.e., with the furanose rings in a C2′-endo conformation (Prescott et al., 1984). The negative line at 839 cm^{-1} in Figure 2B, the

high-frequency side of this band where signal contributions from adenine and thymine are found (Benevides et al., 1991), indicates that changes in DNA backbone structure occur first at the AT sites.

At the same time, a broad positive band, centered around 877 cm^{-1}, appears. This line was also found in Raman spectra of calf thymus DNA at low pH (O'Connor et al., 1981). There, it was suggested that this could be indicative of the formation of C-type DNA (Goodwin & Brahms, 1978; Thomas et al., 1986) at low pH. This would be enabled by an overall decrease in the charge of the DNA molecule, due to protonation, permitting closer approach of the phosphates. However, as described below, the line at 877 cm^{-1} presisted in the spectra of reneutralized chromosomes. Tajmir-Riahi et al. (1988) ascribed a line at 875 cm^{-1} in spectra of calf thymus DNA in the presence of Cu(II) to a back bone mode of denatured DNA. Benevides et al. (1991) showed a line at 875 cm^{-1} to be correlated with thermal denaturation of the B-form backbone. Therefore, the appearance of the line at 877 cm^{-1} in our spectra, concommittant with the loss of intensity of the B-form backbone marker band, is interpreted as a sign of acid-induced loss of ordered DNA backbone structure.

Experiments with Calf Thymus DNA. The chromosome experiments were supplemented with experiments carried out with calf thymus DNA. Spectra were recorded of DNA at neutral and acidic pH's (4, 3.7, 3.4, 3.1, 2.8, and 2.5). This was done because these spectra are not complicated by changes in protein-signal contributions occurring at the same time as changes in DNA-signal contributions (as is the case with the chromosome spectra). They, moreover, shed light on differences in protonation characteristics between free DNA and chromosomal DNA.

Figure 3A shows the spectral changes in the interval 640–860 cm^{-1} upon lowering of the pH from 7 to 3.1. The measurements confirm the results of the experiments with chromosomes; the guanine 681-cm^{-1} line disappears upon lowering of pH, whereas the intensity of the 785-cm^{-1} cytosine line remains unchanged. The intensity of the DNA backbone C2′-endo marker band centered at 830 cm^{-1} decreases only slightly. Further lowering of pH to 2.8 led to complete disappearance of this band (i.e., complete loss of B-DNA backbone structure) and unstacking of cytosine residues, as is evident from the intensity increase at 785 cm^{-1} (Figure 3B). At pH 2.5, no further changes of importance occurred. This means that in free DNA, the acid-induced denaturation occurs at higher pH than in chromosomal DNA and that it is confined to a much narrower pH interval. A similar finding was reported by Zama et al. (1978) in a study of nucleosome core particles. The DNA spectra also reveal a spectral feature that is not discernible in the chromosome spectra, namely an initial increase in intensity at 669 cm^{-1}, concommittant with the intensity decrease of the 681-cm^{-1} line. This intensity increase was most pronounced at pH 3.1 (Figure 3A) and disappeared at pH 2.8 (Figure 3B), together with the B-DNA backbone marker at 830 cm^{-1}. This indicates that the line is due to a guanine vibration in a protonated GC base pair, which disappears upon disruption of the GC base pair at lower pH (see below). A line at 670 cm^{-1} is expected for C2′-endo-syn guanine (Benevides et al., 1984). A guanine line at 664 cm^{-1} is often used as a marker for the C3′-endo-anti conformation, found in A-DNA, but in that case, a clear shift of part of the intensity in the C2′-endo marker band to a lower wavenumber (~807 cm^{-1}) should have been observed (Thomas et al., 1986). Apparently, in the chromosome spectra (which also contain protein-signal contributions), the occurrence of

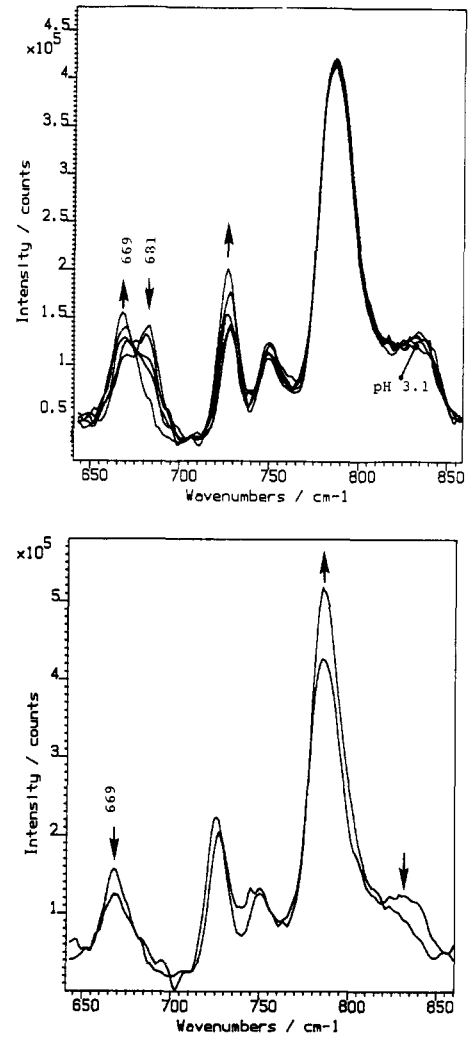


FIGURE 3: (Top) Raman spectra of calf thymus DNA obtained at pH 7, 4, 3.7, 3.4, and 3.1. The arrows indicate intensity increase (↑) or decrease (↓) upon lowering of pH. Buffer-signal contributions have been subtracted. The spectra were scaled to have equal intensity in the 1094-cm^{-1} line. See Table 1 and text for line assignments. (Bottom) Raman spectra of calf thymus DNA obtained at pH 3.1 and 2.8. The arrows indicate intensity increase (↑) or decrease (↓) upon lowering of the pH from 3.1 to 2.8. Other details under (A).

the guanine C2′-endo-syn marker line is masked by other spectral changes, together appearing as a "broad-band" rise in intensity in the spectral interval 625–670 cm^{-1}. This could easily happen because the intensity increase at 669 cm^{-1} is only about half as large as the intensity decrease at 681 cm^{-1} (Figure 3A). Moreover, the chromosomal DNA contains only ~30% GC base pairs (Puppels et al., 1991d) against ~41% GC base pairs in calf thymus DNA. In a recent communication, Duguid et al. (1993) reported the results of a Raman spectroscopic study of DNA–divalent (transition and alkaline) metal complexes. Some of the spectral features that were found were quite similar to those discussed here, but a different interpretation was given. Metal binding to the N7 of guanine was found to lead to a sharp decrease in the intensity of the 1489-cm^{-1} line and a shift of the guanine 681-cm^{-1} line to 665 cm^{-1}. This was explained in terms of a reduction in π-electron density within the imidazole ring as a consequence of metal complexation, resulting in a downshift of the guanine ring breathing mode. However, the decrease in intensity of the 681-cm^{-1} line in the DNA–metal complexes correlates strongly with a decrease in intensity of the B-DNA backbone marker at 830 cm^{-1}, whereas these are clearly unrelated effects in the

work presented here (Figures 1–3). This shows that DNA protonation and metal complexation of DNA have different structural consequences in spite of the fact that the resulting spectral changes are partly similar.

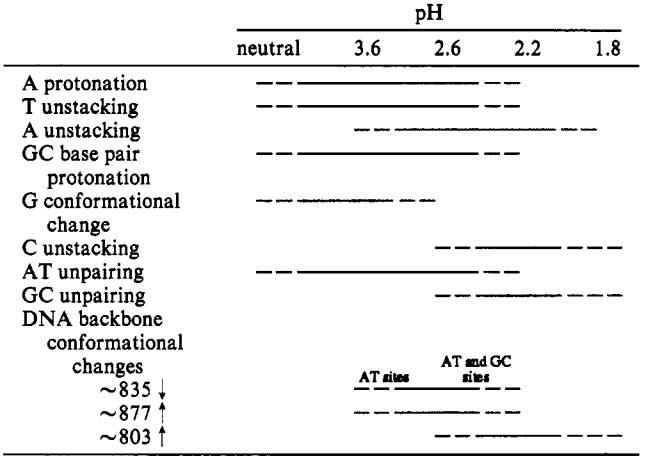
Protonated Hoogsteen GC Base Pairs. The Raman data on chromosomes and calf thymus DNA provide evidence for the reversible formation of protonated GC base pairs under acidic conditions. The pH range in which this occurs and the conformational transition of guanine from C_2'-endo-anti to C_2'-endo-syn are in agreement with NMR, optical rotatory dispersion (ORD), and spectrophotometric pH–titration studies of DNA (Guéron et al., 1990; Courtois et al., 1968; Zimmer & Triebel, 1969). In those studies, evidence was found indicating that the protonated GC base pair is a Hoogsteen base pair, in which the guanine conformational change enables hydrogen bonding of the N7 of guanine and N3 of cytosine. Pulleyblank et al. (1985) showed that protonated Hoogsteen GC base pairs, with the dG residues in a syn conformation, do not give rise to any serious stereochemical impediments.

Protonation of GC base pairs led to the loss of intensity of the 1488-cm^{-1} line, which obtains most of its intensity from a guanine vibration (ν_{11} ring stretching; Nishimura et al., 1986). This vibration is known to be very sensitive to any ligation or complexation involving the guanine N7. When, e.g., protonation (Lord & Thomas, 1967), methylation (Goodwin et al., 1979), or binding of poly-L-arginine or non-histone proteins (Mansy et al., 1976; Goodwin & Brahms, 1978; Goodwin et al., 1979) takes place, the intensity of this line is strongly reduced. In crystal studies of guanine mononucleotides, it was observed to shift to lower relative wavenumbers upon binding of a proton or divalent metal ions to N7 (Nishimura et al., 1986). In the light of the anti to syn transition of guanine and the literature referred to above, the most likely interpretation for the loss of intensity of the 1488-cm^{-1} line in the present case is that it is caused by a hydrogen bond between the guanine N7 and the cytosine N3 in a Hoogsteen GC base pair.

The formation of protonated GC base pairs is reversible (see Figure 5A,B and the discussion of the spectra of reneutralized chromosomes below), which makes clear that they exist in equilibrium wtih the usual Watson–Crick GC base pairs. This means that protonated Hoogsteen GC base pairs may exist as a minority species in chromosomal DNA under physiological conditions. A calculation of the amount of protonated GC base pairs under physiological conditions, based on the data presented here, can only be made with great caution, since the equilibrium conditions may be influenced by, e.g., DNA–protein interactions, which may have been irreversibly changed at low pH. Keeping this in mind, it appears that under physiological conditions at pH 7, about 0.01% of the GC base pairs may be in the protonated Hoogsteen conformation because the midpoint of conversion of Watson–Crick to protonated GC base pairs lies around pH 3 (judging from the intensity of the 681-cm^{-1} line).

Chromosomes at pH 2.2. At pH 2.2 (Figures 1D and 2C), further protonation and unstacking of A residues occurred as well as further unstacking of T residues. As evidenced by the line at 785 cm^{-1} in Figure 2C, the unstacking of C residues had initiated. This signifies the disruption of the protonated GC base pair most likely caused by further protonation. The line at 1262 cm^{-1} in Figure 2C could be due to this protonation, although it may also derive part of its intensity from the unstacking of the cytosine rings (Chou & Thomas, 1977). Adjacent to the 785–cm^{-1} line a new line is found at 803 cm^{-1} in Figure 2C. Like the band at 877 cm^{-1}, it is probably indicative of changes in DNA backbone structure. A line around 807 cm^{-1} is diagnostic of C3'-endo conformation (Prescott et al., 1984), found in A-DNA. However, Chou and Thomas (1977) found a line around 800 cm^{-1} in Raman spectra of heat-denatured poly(rC) and poly(rC)·poly(rC+) and in the spectrum of poly(rC+). Therefore, the line at 803 cm^{-1} in our difference spectra is probably indicative of a disordered DNA backbone, arising when not only AT but also GC base pairs are disrupted. As shown in Figure 5, it remains present upon return to neutral pH.

Table 2: Overview of the Changes in DNA Structure Occurring in Separate pH Intervals[a]



[a] Legend: (– – —) starts in this interval, (—) continues in this interval, (— – –) completed in this interval.

Chromosomes at pH 1.8. The absence of lines at 1262 and 1409 cm^{-1} in Figure 2D, the difference spectrum pH 1.8–2.2, shows that in this pH interval no significant further protonation of adenine or cytosine occurred, but their unstacking continued as evidenced by the lines at 725 and 785 cm^{-1}. The negative intensities around 1450 and 1668 cm^{-1} and the negative line at 1004 cm^{-1} are due to loss of protein, as is confirmed by the results obtained with chromosomes returned to neutral pH (see Figure 5). Further change in or loss of DNA backbone structure may again be indicated by the line at 803 cm^{-1}. Table 2 summarizes the changes in chromosomal DNA structure that occur upon lowering the pH from neutral to 1.8.

Studies on Chromosomes Reneutralized after a Brief Treatment at Low pH

Figure 4 shows Raman spectra of untreated chromosomes (A) and of chromosomes treated at different acid pH's and returned to neutral pH (B–F). The most obvious change observed in these spectra is the intensity decrease of the 1450-cm^{-1} line, belonging to protein CH_2 and CH_3 vibrational modes (Tu, 1982), due to protein extraction. Using the intensity of this line as a measure of protein concentration and the 1094-cm^{-1} line as a measure of DNA concentration, the DNA–protein ratio in the chromosomes was determined (method described in Puppels (1991b)). Table 3 shows how this ratio depends on the pH of the buffer used in the acid treatment.

In Figure 5, Raman difference spectra are displayed that document the irreversible changes in the chromosomes caused by acid treatments at pH 3.6, 2.6, 2.2, and 1.8 and with 45% acetic acid. Treatment at pH 3.6 did not give rise to any noticeable irreversible changes in the polytene chromosomes, as seen in Figure 5A. The negative bands centered at ~1450

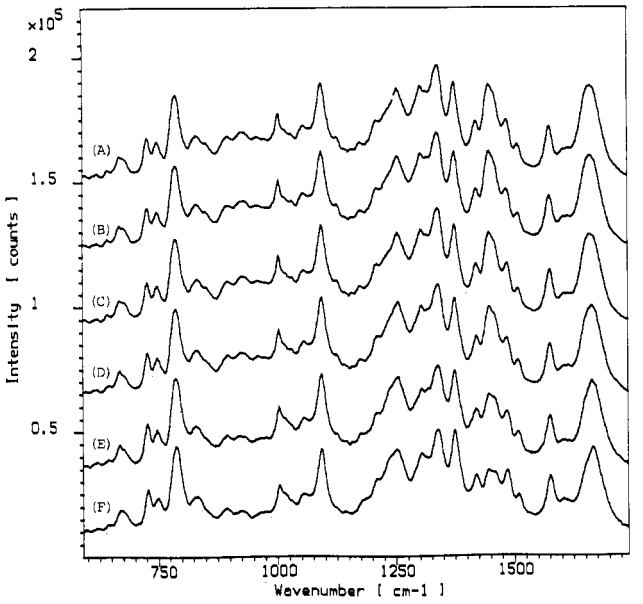


FIGURE 4: Raman spectra of polytene chromosomes at neutral pH after a 10-min incubation at low pH: (A) untreated chromosomes, (B) chromosomes treated at pH 3.6, (C) chromosomes treated at pH 2.6, (D) chromosomes treated at pH 2.2, (E) chromosomes treated at pH 1.8, and (F) chromosomes treated with 45% acetic acid. Experimental conditions: laser power, 15 mW (660 nm); measuring time per measurement, 5 min (B, C, D, and E) or 10 min (A and F); number of measurements, 10 (B, C, D, and E) or 20 (A and F). The spectra shown were averaged and scaled to have equal intensity in the 1094-cm^{-1} line. Intensity scale is for (B). Differences in intensity before scaling were small (±10%).

Table 3: Changes in Chromosomal DNA–Protein Ratio (weight/weight) Induced by Low-pH Treatment

	DNA:protein	
treatment	absolute (±19%)	normalized (±9%)
no	0.7	1
pH 3.6	0.7	1
pH 2.6	0.8	1.1
pH 2.2	0.9	1.3
pH 1.8	1.3	1.8
45% acetic acid	1.7	2.3

and ~1655 cm^{-1} (protein amide I region (Tu, 1982)) in Figure 5B show that treatment at pH 2.6 caused a slight loss of protein, as was already made clear in Table 3. Treatments at pH 2.2 and 1.8 and with 45% acetic acid, apart from increasing loss of protein, caused irreversible changes in the overall DNA structure. It appears from spectra in Figure 5C–E that the extent but not the nature of these changes depended on the pH used in the treatments. Table 4 lists the most evident spectral changes and their tentative assignments and interpretations. The lines at 730 (A), 1185 (T), and 1240 (T) cm^{-1} and the shoulder at 1670 (T) cm^{-1} in spectrum 5E are most likely due to a hyperchromic effect indicating an altered stacking geometry or unstacking of adenine and thymine residues. From a comparison of spectrum 5E with published difference spectra of B-DNA – A-DNA and B-DNA – Z-DNA (Thomas et al., 1986; Prescott et al., 1984) follows that such conformational transitions cannot account for the increased intensity of the above mentioned adenine and thymine vibrational modes in the spectra of the low-pH treated chromosomes.

We have made an estimate of the amount of adenine and thymine residues that is affected by or involved in the DNA structural changes. This estimate was based upon a comparison of the increase in the intensities of the adenine (730

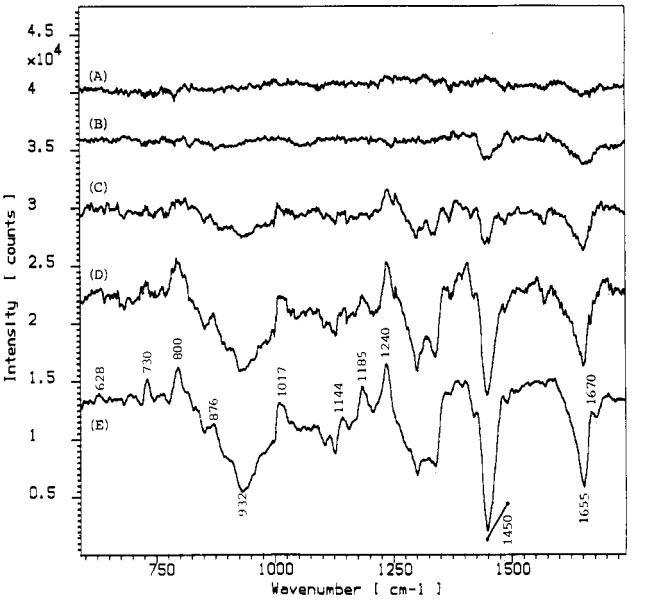


FIGURE 5: Raman difference spectra showing the irreversible changes in chromosomal composition and DNA conformation. The spectra of Figure 4 were used: (A) chromosome treated at pH 3.6 – untreated chromosomes (4B – 4A), (B) chromosomes treated at pH 2.6 – untreated chromosomes (4C – 4A), (C) chromosomes treated at pH 2.2 – untreated chromosomes (4D – 4A), (D) chromosomes treated at pH 1.8 – untreated chromosomes (4E – 4A), and (E) chromosomes treated with 45% acetic acid – untreated chromosomes (4F – 4A).

Table 4: Listing of DNA Structural Changes in Polytene Chromosomes Caused by Low-pH Treatment

spectral change (cm^{-1})	compositional or structural change
732 ↑[a]	A, unstacking
800 ↑	BDA:BK
875 ↑	DNA:BK denaturation
900–1000 ↓[b]	protein extraction
1017 ↑	DNA:BK
1144 ↑	DNA:BK
1185 ↑	T, unstacking
1239 ↑	T, unstacking
1300–1340 ↓	protein extraction
1450 ↓	protein extraction
1655 ↓	protein extraction
1670 ↑ (sh)[c]	T, unstacking

[a] (↑) intensity increase. [a] (↓) intensity decrease. [c] (sh) shoulder in Figure 5E.

cm^{-1}) and thymine (1240 cm^{-1}) lines in the chromosome spectra with the increase in intensity of these lines in calf thymus DNA spectra upon heat denaturation (Erfurth & Peticolas, 1975). In the chromosome spectra, this increase is about 15% for the adenine line (in Figure 3A, peak intensity is ~12 000 counts; in Figure 4E, peak intensity is ~1500–2000 counts). For the thymine line, only a lower limit for the percentual intensity increase could be determined due to overlapping protein-signal contributions. The intensity at 1240 cm^{-1} with respect to an imaginary base line drawn between the intersections of the spectrum with the ordinates was taken as the intensity of the thymine line. The result of ~35 000 counts is an overestimation of course. In spectrum 4E, the difference of the intensities at 1240 and 1205 cm^{-1} was taken as the thymine-line intensity (~4000 counts). The DNA spectra of Erfurth and Peticolas (1975) show intensity increases upon melting of about 100% for the 729-cm^{-1} adenine line and of 100–200% of the 1240-cm^{-1} thymine line. It follows that the changes in DNA structure involved at least 10% and probably a significantly higher percentage of the adenine and thymine residues.

As already argued in the section on low-pH measurements, the positive contributions at 800 and 876 cm^{-1} are most likely due to alterations in DNA backbone structure. This interpretation is supported by the appearance of lines at 1017 and 1144 cm^{-1} assigned to vibrational modes of the DNA backbone. According to Savoie et al. (1985), these DNA modes are suppressed upon nucleosome formation. Their presence in spectra 5C–E could therefore signify the disruption of nucleosome structure, caused by protein extraction and denaturation, at low pH. It is noted, however, that the results of a Raman study of DNA–protein interactions in nucleosome core particles by Hayashi et al. (1986) do not corroborate the conclusions of Savoie et al. (1985) about the suppression of the intensity of the 1017- and 1144-cm^{-1} lines upon nucleosome formation.

A weak positive line is observed at 628 cm^{-1} in spectrum 5E, accompanied by a weak negative line at 681 cm^{-1}. This could be indicative of the presence of Z-DNA in the low-pH-treated chromosomes, since the total added intensity of the guanine lines at 625 cm^{-1} (Z-DNA) and 681 cm^{-1} (B-DNA) remains constant during the B to Z transition (Benevides & Thomas, 1983). However, these spectral features are only very weak, and verification of the assignment of the 628-cm^{-1} line to Z-DNA in other parts of the spectrum is hampered by the fact that these are dominated by changes due to protein extraction. Therefore, a definite statement about the presence (or the amount) of Z-DNA in the acetic-acid-treated chromosomes cannot be made on the basis of the results presented here but will have to await the results of future work (see Concluding Remarks).

The immunochemical studies on polytene chromosomes conducted by Hill et al. (1984) and Robert-Nicoud et al. (1984) demonstrated that low-pH or acetic acid treatments place the chromosomal DNA under a topological stress. This is due to the extraction of histones, which implies a disruption of the nucleosomal organization of the DNA. Torsional stress was found to be a necessary but not sufficient condition to enhance Z-DNA immunoreactivity. DNA "melting" at low pH or high temperature was also required. It was observed that increasing Z-DNA immunoreactivity paralleled the lowering of pH from 2.5 to 2.0 (or 45% acetic acid), whereas at pH 3, no Z-DNA immunoreactivity was elicited (Robert-Nicoud et al., 1984).

Our results are in perfect accordance with these findings in that they show that treatments with buffers of a pH $\geq$ 2.6 do not lead to noticeable changes in DNA conformation, in contrast to treatments below this pH. As evidenced by Figure 5, a treatment at pH 2.2 and, to a larger extent, treatments at pH 1.8 or with 45% acetic acid affect the chromosomal DNA conformation. These involve the DNA backbone and the stacking geometry of the adenine and thymine residues. Irreversible changes in DNA structure therefore take place when the pH of the treatment is low enough to cause not only AT unpairing but also GC unpairing (see Table 2).

In Raman spectroscopic studies of the effects of torsional stress on DNA structure in circular DNA's, it was found that adenine, thymine, and backbone vibrational modes are affected. Hayashi et al. (1985) noted changes around 819 cm^{-1} (assigned to backbone O–P–O stretching) as well as a decrease in the intensity of the 728-cm^{-1} adenine line in spectra of pFb100 under conditions of negative supercoiling (superhelical density between –0.03 and –0.1). No changes were found in guanine or cytosine vibrations. This led them to conclude that local deformation of DNA due to supercoiling takes place at AT base pairs and not at GC base pairs. Brahms et al. (1989), comparing supercoiled and relaxed pBR322, also found evidence (an increase in the intensity of vibrational modes at 810 and 818 cm^{-1}) for an enhanced tendency of bases to adopt non-B-type C3′-exo and/or O4′-endo conformations instead of C2′-endo (predominant in B-DNA) due to torsional stress. Christens-Barry et al. (1989) studied supercoiled and nicked ColE1 DNA. They also concluded accommodation of supercoiling to take place mainly in AT base pairs and backbone moieties, based on a decrease of the intensities of the thymine line at 755 cm^{-1} and the DNA backbone C–O line at 1060 cm^{-1}. Also, evidence for a C3′-endo (A-type) sugar pucker was found. No changes in the Raman lines of guanine and cytosine were found. Although the above mentioned studies are based on different spectroscopic features, they all suggest that torsional stress affects the DNA structure predominantly at the AT base pairs. This is in agreement with our findings. The fact that the features in our spectra leading to this conclusion are again different from those in the studies mentioned above could be due to the fact that, in the chromosomes we studied, optimum conditions were present for accommodation of the torsional stress (i.e., unpairing and unstacking of bases at low pH) while this was not the case in the plasmid DNA studies. This means that the observed spectral changes are probably due to new DNA structures not present in the plasmid DNA. Possible candidates are, apart from Z-DNA, triplex DNA, cruciforms, and AT-rich base-unpaired regions (see, e.g., Frank-Kamenetskii, 1990; Palecek, 1991).

CONCLUDING REMARKS

The results shown above exemplify the unique possibilities of Raman microspectroscopy for studying biomolecular structure in situ. The Raman spectroscopic data on *C. thummi thummi* polytene chromosomes obtained in this study (a) document in detail the changes in DNA structure elicited by low-pH treatment, (b) show that protein extraction and DNA denaturation at low pH cause large scale irreversible changes in the DNA conformation upon return to neutral pH, and (c) provide evidence for the presence of protonated Hoogsteen GC base pairs in chromosomal DNA in equilibrium with Watson–Crick GC base pairs.

It was found that at low pH (1.8 and therefore undoubtedly in 45% acetic acid too), the chromosomal DNA is largely (and maybe completely) denatured and that the bases are unstacked. This is, of course, an ideal situation for a "restructuring" of the DNA, upon return to neutral pH, in a manner that relieves torsional stress, caused by nucleosome disruption. Other effects, not directly related to torsional stress, may also be involved. Because GC unpairing occurs at a lower pH than AT unpairing, it is likely that GC base pairs are formed more quickly than AT base pairs upon return to neutral pH. This may lead to a pairing of "wrong" guanines and cytosines (i.e., guanines and cytosines not originally paired), other than in the above mentioned conformations, giving rise to predominantly unpaired adenine and thymine residues. (Erfurth and Peticolas (1975) also found, in their Raman study of DNA melting, that the process of DNA denaturation is not completely reversible when in the experiments the temperature was raised above the melting temperature.) This would not necessarily be limited to wrong pairing within the original double helix. Because of the fact that in polytene chromosomes many identical DNA strands are close together, it could also involve base pairing between strands of neighboring double helices. This could even constitute a "fixation" mechanism.

The data once more and very clearly show that immunological and chemical assays carried out with acid-fixed cytological preparations do not allow conclusions with respect to the biological significance of the results but rather only underline the polymorphic nature of DNA structure and the complex equilibrium between extrinsic (e.g., pH, ions, or protein binding) and intrinsic (sequence, torsional stress) factors in determining the predominant conformation.

Future Raman studies will aim to obtain a better insight into which of the changes are due to torsional stress and to better understand the role of DNA denaturation in accommodating this stress. Spectra of low-pH-treated chromosomes will be compared with those of low-pH chromosomes that have subsequently been relaxed by, e.g., topoisomerase. This will also be a way to obtain more reliable information concerning the presence of Z-DNA in the low-pH-treated chromosomes because the Raman difference spectra of spectra of low-pH-treated chromosomes and low-pH-*and*-subsequently-topoisomerase-treated chromosomes will not be dominated by large differences due to protein extraction, as is the case in the spectra of Figure 4. Studies are furthermore required to elucidate whether the equilibrium between protonated and nonprotonated GC base pairs can be influenced by, e.g., DNA-protein interactions and/or the presence of different counterions. In this way, it should be possible to obtain more information about the possible significance of these protonated base pairs in cellular processes.

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