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Multiple H4 histone mRNAs of HeLa cells are encoded in different genes

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We have previously reported the presence of at least two distinct mRNAs coding for histone H4 in HeLaS3 cells[1,2]. In this communication we report the resolution of at least seven different histone H4 mRNAs, each of which codes for histone H4. The presence of different mRNAs coding for the same protein could be explained either by the presence of different genes that have diverged without changing the encoded amino acid sequence (there are 20–40 copies of the histone genes per haploid genome in human cells[3]), or by differential processing of the same primary transcript. By hybridizing a series of cloned genomic human H4 histone sequences to the histone H4 mRNAs and digesting with S_1 nuclease, we have shown that different H4 histone mRNA species are protected from nuclease digestion by different H4 histone genes, suggesting that at least three of the HeLa H4 histone mRNAs are the products of distinct genes.

In vitro ^{32}P-labelled 7–11S polysomal RNA from S phase HeLa cells was electrophoresed under denaturing conditions in a 6% (w/v) acrylamide-8.3M urea gel at 55–60 °C (Fig. 1*a*), and the three RNA bands (A, B, and C) in the H4 region were excised from the gel, and the RNA eluted. When each of these H4 histone mRNA fractions was re-electrophoresed on the same type of denaturing gel, single bands were obtained and there was only a small amount of cross-contamination between species (data not shown). These labelled RNA fractions were identified as H4 histone mRNAs by *in vitro* translation[4–6], and from hybridization with a chicken H4 histone gene-containing clone (data not shown). Each of the three RNA fractions yielded several bands when electrophoresed under nondenaturing conditions (Fig. 1*b*). Each of the RNAs directed the translation of H4 histone in a cell-free system (data not shown), and the

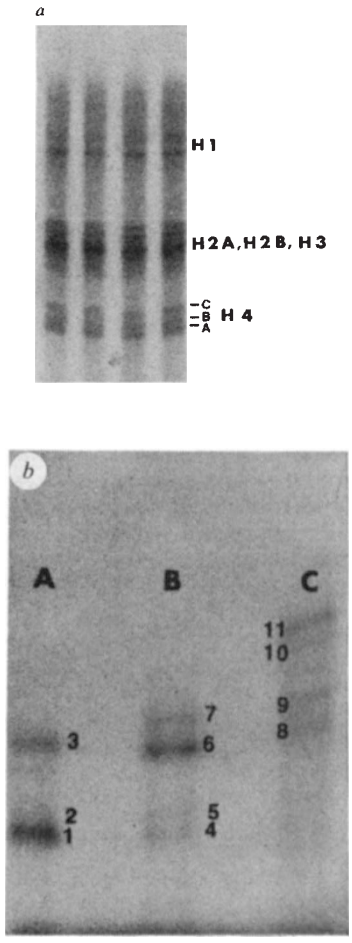

Fig. 1 *a*, HeLa S3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum. Unlabelled total polysomal RNA was isolated from 5×10^9 S phase cells[37,38] synchronized by a single 2 mM thymidine block[39]. ^{32}P-labelled RNA was prepared from one litre of S phase HeLa cells synchronized by two cycles of 2 mM thymidine block[39]. Following release from the second thymidine block the cells were incubated in phosphate-free growth medium buffered with 30 mM HEPES containing 10% dialysed calf serum and 50 mCi of ^{32}P (2×10^6 cells ml^{-1}) at 37 °C for 4.5–5 hours. Cells were collected and lysed in 10 mM Tris-HCl *p*H 7.5, 13 mM $MgCl_2$, 10 mM vanadyl ribonucleoside, prepared as in [40] except that KOH was used instead of NaOH, and centrifuged at 12,000 r.p.m. in a Beckman JA 20 rotor. The supernatant was centrifuged through a cushion of 2 M sucrose containing 10 mM vanadyl ribonucleoside at 40,000 r.p.m. in a Beckman Ti 60 rotor for 2 hours and the pellet was resuspended in 20 mM Tris-HCl (*p*H 7.7), 1% (w/v) SDS and extracted with buffered phenol/chloroform/isoamyl alcohol (25:24:1). 7–11S RNA was isolated on 5–30% sucrose gradients as described[38]. Unlabelled 5–18S RNA was isolated using similar procedures, mixed with ^{32}P-labelled RNA and ethanol precipitated. The pellet was dissolved in 8.3 M urea, 5 mM EDTA (*p*H 8.0), heated to 100 °C and quick cooled before loading on a 6% (w/v) polyacrylamide, 8.3 M urea gel buffered with 50 mM Tris-borate, 1 mM EDTA (TBE)[41]. The gel was run for 7 hours at 20 watts, which gives a surface temperature of 55–60 °C. After autoradiography, the bands labelled A, B, and C were excised and eluted electrophoretically[42]. The sizes of the H4 mRNAs, as determined by electrophoresis in MeHgOH–3% (w/v) agarose gels using *Hpa*II/*Pst*I restriction fragments of ϕX174 DNA as markers, are 364, 394 and 439 bases (R. Rickles, personal communication). *b*, RNA eluted from the gel shown in *a* was ethanol-precipitated and the dried pellet was dissolved in 4 M urea, 2.5 mM EDTA (*p*H 8.0), heated to 100 °C for 2 min and quick cooled on ice. The samples were loaded on a non-denaturing 'strand separation' gel[41] containing 5.9% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 50 mM TBE and 0.2% (w/v) SDS. Electrophoresis was at 15 watts (400 V) for 8 hours.

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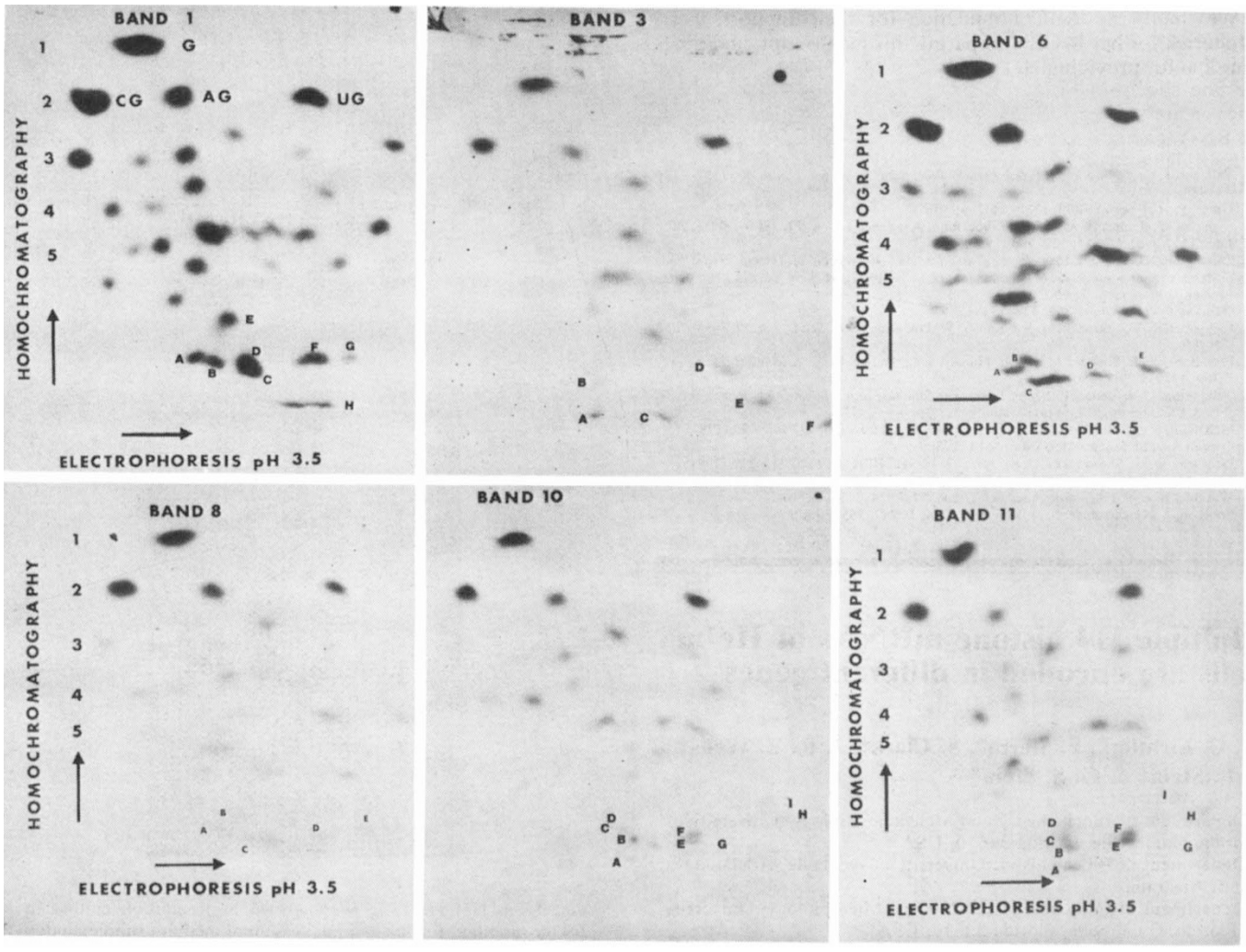

Fig. 2 Two-dimensional T_1 ribonuclease fingerprint analysis of H4 histone mRNAs. ^{32}P-labelled RNAs extracted from a gel similar to that shown in Fig. 1*b* were digested with T_1 ribonuclease and analysed according to the method of Volkaert *et al.*[7]. Briefly, T_1 ribonuclease-digested RNAs were electrophoresed on cellulose acetate strips at *p*H 3.5 in 7 M urea, transferred to PEI-cellulose thin layer chromatography plates, and developed with homochromatography mix B[7] at 70 °C. The fractionated oligonucleotides were detected by autoradiography and the labelled spots were eluted, subjected to ribonuclease A digestion, and analysed according to Barrell[8].

amount of H4 histone synthesized correlated with the amount of radioactivity present in the RNA bands. When the RNAs in each of the 11 bands in Fig. 1*b* were eluted from the gel, denatured by heating at 100 °C in 4M urea and re-electrophoresed in another nondenaturing gel, each migrated as a single species with the same relative mobility as in the original gel (data not shown). These results suggest that the multiple H4 mRNA bands seen in the nondenaturing gel system represent several different H4 histone mRNA species, rather than conformational isomers of a single species.

To examine the extent of differences among the various H4 histone mRNAs, we analysed the oligonucleotides generated by T_1 ribonuclease digestion of histone H4 mRNA bands A1, A3, B6, C8, C10 and C11 from Fig. 1*b* using the two-dimensional fingerprint technique of Volkaert *et al.*[7] (Fig. 2). The largest oligonucleotides indicated in each panel of Fig. 2 were further analysed by ribonuclease A digestion[8] (data not shown). The results of this analysis establish that the histone H4 RNAs A1, A3 and B6 are completely distinct species, with only one of the large T_1 oligonucleotides shared by A1 and B6. C8 was identical to B6 and probably represents cross-contamination of H4C by H4B. C10 and C11 shared seven of nine T_1 oligonucleotides, and were both different from A1, A3 and B6.

We have isolated several λ Charon 4A recombinant clones, containing human histone gene sequences[9] (designated λHHG). The seven clones thus far characterized fall into three distinct types of arrangements, based on their restriction site patterns and the location of protein coding regions. We have used these clones in hybridisation experiments to elucidate the origins of the multiple histone H4 mRNAs. Clones representing each of the three classes were used: λHHG 41 represents one class, λHHG 39 a second class, and λHHG 6 and λHHG 17 a third class.

The assignment of histone H4 mRNAs to their respective genomic coding sequences was based on resistance of the mRNA-DNA hybrids to S_1 nuclease, which under the conditions used in our studies will hydrolyse a single mismatched base pair[10]. Our experiments involved annealing mixtures of electrophoretically purified ^{32}P-labelled H4 histone mRNAs with the histone gene-containing recombinant phage DNA in 80% formamide, and digesting with S_1 nuclease according to the procedure of Berk and Sharp[11] as modified by Haegeman *et al.*[12]. The RNA species which remain S_1 nuclease resistant are those which are complementary to the DNA over their full length. To identify the RNA species that were protected from S_1 nuclease digestion by the cloned DNA, the S_1 digested

samples were divided and half of each sample was electrophoresed in a denaturing gel containing urea, and half in a non-denaturing gel. RNA bands eluted from a gel similar to the one shown in Fig. 1*a* were electrophoresed in adjacent lanes as markers. An 8.3 M urea—6% (w/v) acrylamide gel of the RNA species which are protected from S_1 nuclease digestion by hybridization with λHHG 17, λHHG 39 or λHHG 41, along with marker histone mRNAs H4A, H4B and H4C, is shown in Fig. 3*a*. λHHG 17 protects RNAs comigrating with two H4 histone RNA species, H4A and H4B, which is consistent with other evidence that this clone contains two H4 histone genes[9]. λHHG 39 protects one RNA that comigrates with H4B, as does λHHG 41. The results of fractionation of the S_1 nuclease resistant RNA samples in a nondenaturing gel are shown in Fig. 3*b*. λHHG 17 protects two H4 mRNAs (lane 2), one a subspecies of H4A (band A3, Fig. 1*b*), the other a minor species of H4B (band B7, Fig. 1*b*). The independent isolate λHHG 6, apparently identical to λHHG 17, also protects subspecies H4A3 and H4B7; Fig. 3*b* lanes 4 and 6 shows that the two *Eco*RI fragments of λHHG 6 each protect a different subspecies. The H4 histone mRNAs complementary to λHHG 39 (lane 1) and λHHG 41 (lane 8) both comigrate with the major H4B subspecies, H4B6 (see Fig. 1*b*). In the case of λHHG 41, we have additionally been able to show that the H4 histone mRNA protected from S_1 nuclease digestion has a two-dimensional T_1 ribonuclease fingerprint identical to that of the H4B6 mRNA (not shown).

The results presented here suggest that there are at least three types of human H4 histone genes, each encoding a different H4 histone mRNA. Two of the types of H4 histone genes are each present in the genomic clones λHHG 6 and λHHG 17. Although λHHG 39 and λHHG 41 both appear to protect the H4B6 mRNA, it is possible that there may be minor variations in the H4 genes of these two clones. Although λHHG 17 and λHHG 41 reproducibly protected a large fraction of their complementary H4 mRNA species, λHHG 39 always yielded a weak mRNA band, suggesting that λHHG 39 may be complementary to a less abundant H4 mRNA, which co-migrates with H4B6 in both the denaturing and non-denaturing gel systems used here. Although we have distinguished at least seven different H4 histone mRNA species, we have been able to assign only three of them to the corresponding genes. These results imply that there may be four or more types of human H4 histone genes in addition to those we have characterized.

Several examples of multiple mRNAs coding for similar or identical proteins have been described. Some, such as mouse dihydrofolate reductase[13] and α-amylase[14] mRNAs are produced from a single gene by variable transcription or RNA processing. Others, including the actin mRNAs of humans[15,16] and *Dictyostelium*[17,18] are encoded in multiple, non-identical gene copies.

Histone genes and histone mRNAs have been most extensively studied in sea urchins. Sea urchins have two major classes

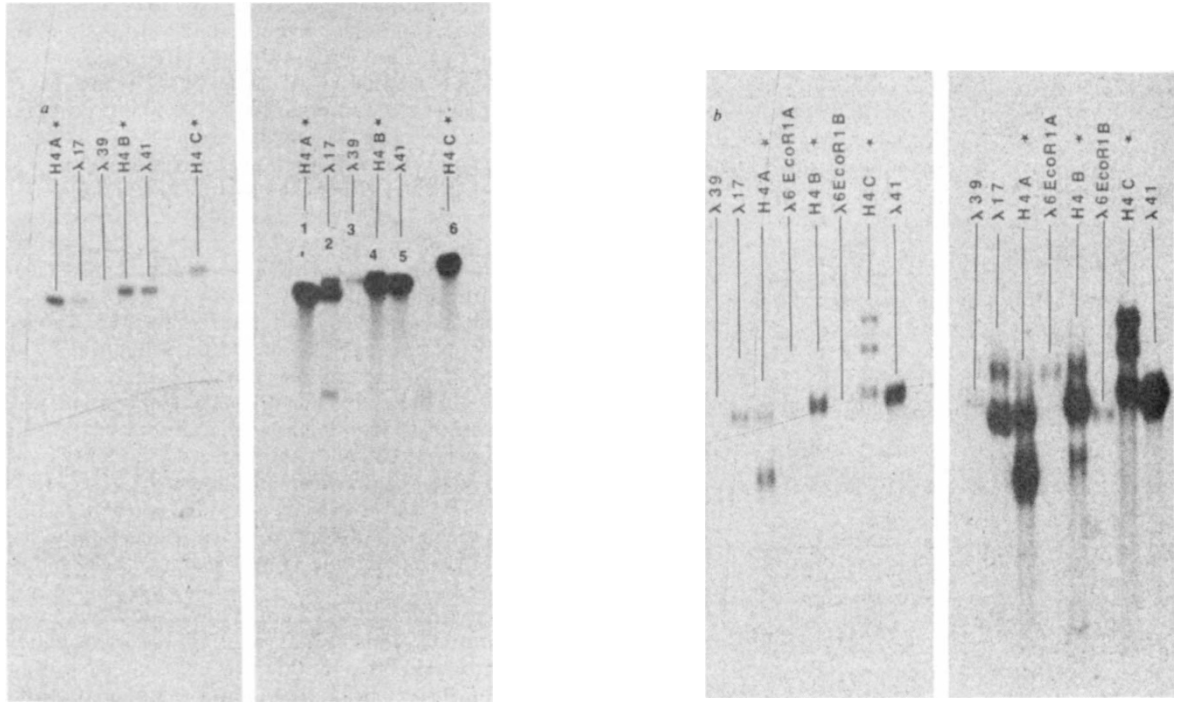

Fig. 3 Identification of H4 histone mRNA species which are S_1 nuclease resistant after hybridization to histone DNA clones. 10–20 μg of λHHG recombinant DNAs were mixed with 500–2,000 c.p.m. of ^{32}P-labelled total H4 histone mRNA isolated from a gel similar to the one shown in Fig. 1 and precipitated with ethanol in a 1.5 ml microfuge tube. The pellet was drained thoroughly and dissolved in 20 μl redistilled but not deionized formamide (Bethesda Research Laboratories). 4 μl of 2 M NaCl, 0.4 M PIPES (*p*H 6.4), 5 mM EDTA were added, and the tube was placed at 80 °C for 5 min. Hybridization was at 50 °C for 5 hours. After hybridization, 225 μl of ice cold 0.25 M NaCl, 25 mM Na acetate (*p*H 4.4), 0.45 mM $ZnSO_4$ were added and the tube was mixed thoroughly and immediately placed on ice. 110 units of S_1 nuclease (Sigma) in 5 μl were then added, and the sample incubated at 37 °C for 30 min, 20 μl of 10% (w/v) SDS, 20 μl 0.2 M EDTA (*p*H 8.0), 20 μg tRNA, 200 μl of phenol, and 200 μl chloroform/isoamyl alcohol (24 : 1) were added, and the mixture was vortexed, centrifuged, and the supernatant was ethanol precipitated. The dried pellet was dissolved in 8.3 M urea, 5 mM EDTA, heated to 100 °C for 2 min and electrophoresed on an acrylamide gel. *a*, S_1 nuclease resistant samples electrophoresed in an 8.3 M urea gel. Lane 1, H4A histone mRNA. Lane 2, λHHG 17 protected RNA. Lane 3, λHHG 39 protected RNA. Lane 4, H4B histone mRNA. Lane 5, λHHG 41 protected RNA. Lane 6, H4C histone mRNA. Light (left panel) and dark (right panel) exposures of the autoradiograms are shown. *b*, S_1 nuclease resistant samples electrophoresed in a nondenaturing gel. Lane 1, λHHG 39 protected RNA. Lane 2, λHHG 17 protected RNA. Lane 3, H4A histone mRNA. Lane 4, λHHG 6 *Eco*RI fragment A protected RNA. Lane 5, H4B histone mRNA. Lane 6, λHHG 6 *Eco*RI fragment B protected RNA. Lane 7, H4C histone mRNA. Lane 8, λHHG 41 protected RNA. Light (left panel) and dark (right panel) exposures of the autoradiograms are shown.

of H4 histone mRNAs, which are preferentially expressed at different stages of development[19,20]. The early H4 mRNAs of *Lytechinus pictus* have three very similar forms[21], the genes for which are repeated several hundred times and are arranged in tandemly repeated clusters[19,20]. Although they encode exactly the same protein the late and early H4 mRNAs are very divergent[22–27]. The late sea urchin histone genes are repeated 25–50-fold[22] but they have only recently been cloned[28] and details of their organization have not yet been published.

The histone genes of *Drosophila*[29] and *Notophthalmus*[30] are similar to the early sea urchin genes in their highly repetitive nature and ordered structure, while the histone genes of *Xenopus*[31–33], chicken[34,35] and human[9,36] are more analogous to the late sea urchin genes in being reiterated 10–50-fold and in not being highly ordered. It is not known however if higher eukaryotes have classes of histone genes that are functionally equivalent to the early and late histone genes of sea urchins. The ability to resolve a separate H4 histone mRNA for each human H4 histone gene provides us with a unique opportunity to address this question. Because H4 histone proteins do not have variant forms, the study of variant H4 mRNAs is the most direct method for detecting the expression of different H4 histone genes. Variant histone H4 mRNAs have not been detected in organisms other than sea urchins and humans.

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A model for base overlap in RNA

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Duplexed RNA in the solid state conforms to the A-family of structures with 3′-*endo* sugars and bases arranged outside the helix axis in a spiral staircase arrangement[1–4]. Recent experiments[5–11] suggest that parameters of RNA and DNA helices depend on the sequence of bases in the chain. Furthermore, the average conformation of these flexible molecules may differ between the solution and solid states. The NMR chemical shifts of the base protons in ten oligo-RNA duplexes[5,12–18] have been determined. We have now adjusted the helix winding angle, *W*, between adjacent base pairs (see Fig. 1*a*) to fit these data. There is extensive overlap of the hydrophobic surfaces of the bases at the proposed angles. These overlaps depend on the arrangement of purines and pyrimidines along the chain and offer a mechanism for recognition of specific sequences by enzymes.

The analysis forbids ranges in W shown as bars in Fig. 2. Figure 2*a* indicates that a purine next to a purine on the same strand favours $W = 50° \pm 5°$, while Fig. 2*c* shows that a 5′Py–Pu3′ arrangement leads to $W = 45° \pm 10°$ except for the C-G : C-G stacking interaction, which is likely to prefer $W \leq 35°$. Figure 2*b* illustrates that the current data do not restrict W as tightly for 5′Pu–Py3′ orientations; note the large blank areas. However, $W = 20° \pm 5°$ is satisfactory in all cases. The internal consistency of the analysis is remarkable. None of the proposed winding angles is the same as the 30° value for A′-RNA (RNA-12) derived from X-ray diffraction of RNA fibres[1].

Base-stacking, rather than hydrogen-bonding, confers stability to nucleic acid helices. Note the extensive contact between the hydrophobic surfaces of the bases at the proposed winding angles (see Fig. 1*d–f*). This is a compelling molecular explanation of our observations.

Simple rules of base overlap lead naturally to ideas regarding the enzymatic recognition of specific sequences by their shape. RNA and DNA sequences should be analysed for regularities in Pu–Pu, Pu–Py, Py–Pu, or nearest-neighbour arrangement. For example, splice junctions in unprocessed messenger RNA, control regions of genes and repeated sequences in DNA may contain such regularities. The unusual C-G : C-G orientation may provide a special signal as it is represented in eukaryotic mRNA much less often than predicted on a random basis[19].

The NMR spectrometer views an average conformation of the nucleic acid. The microsecond- to nanosecond-scale events known to occur in the motion of duplexed DNA[20] might correspond to oscillations in base overlap, the hydrophobic surfaces of the bases sliding over one another.

The base protons of an isolated mononucleotide are shielded by an amount, $\Delta\delta$, when the monomer is incorporated into a duplexed oligomer. The $\Delta\delta$ values of the three or four protons on each base pair are fitted by adjusting the winding angles on either side of that pair, and are estimated from isoshielding contours including ring-current and local atomic anisotropies[21]. Figure 3 illustrates the process for several protons in duplexed $\overset{1}{A}\overset{2}{A}\overset{3}{G}\overset{4}{C}UU$. The first four proton 'maps' exclude certain combinations of winding angles W_{12} and W_{23}, that is, the solid regions where theory and experiment disagree by more that 0.25 parts per million (p.p.m.). The four maps are then correlated in Fig. 3*e*, the solid areas occupying most of this map. The process is repeated for the next base pair, yielding a new correlation map in Fig. 3*f* relating W_{23} and W_{34}. Figure 3*e* and

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