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Specific DNA methylation sites in the vicinity of the chicken β-globin genes

METHYLATION of cytosine is the only post-synthetic modification so far detected in the DNA of higher eukaryotes and thus has been made the basis of several proposed mechanisms of gene activity and cellular differentiation[1–3]. All evidence indicates, however, that the overall content of 5-methylcytosine in DNA does not vary significantly between different tissues of the same organism and does not change throughout at least some steps of differentiation[4–6]. As 5-methylcytosine occurs predominantly in the dinucleotide sequence CpG (ref. 7) and as a number of bacterial restriction endonucleases can distinguish whether this sequence is methylated or unmethylated, it is now possible to investigate DNA methylation patterns in the vicinity of single genes[8]. In this report, we use this technique to provide a correlation between DNA methylation and the activity of the chicken β-globin genes.

The restriction endonuclease *Hpa*II cleaves the DNA sequence CCGG (ref. 9) but does not cleave the methylated sequence C(MeC)GG (ref. 10). A second restriction endonuclease, *Msp*I, is able to cleave this same DNA sequence whether it is methylated or not[11]. Thus DNA from any cell or tissue type can be digested with either *Hpa*II or *Msp*I, the cleaved DNA electrophoresed on an agarose gel, transferred to a nitrocellulose filter[12] and hybridised to a gene-specific probe. Any bands seen in the autoradiogram which differ between the two enzyme digests can reasonably be ascribed to differences in methylation of CCGG sites in the vicinity of the specific gene. This kind of experiment, used here to examine the chicken β-globin genes, has recently been used to investigate a single methylation site in the large intervening sequence of the adult rabbit β-globin gene[13].

Figure 1 shows the autoradiogram from an experiment with chicken DNA, using the plasmid pHb1001 as a ^{32}P-labelled hybridisation probe (pHb1001 is a cDNA clone of the adult chicken β-globin gene[14], provided by Dr W. Salser). Figure 1*a* represents the *Hpa*II digest of adult chicken erythrocyte DNA and shows bands at 4.2 and 1.3 kilobase pairs. These fragment sizes should correspond to distances between unmethylated CCGG sites in the DNA regions containing β-globin genes. Figure 1*b* represents the *Msp*I digest of the same adult erythrocyte DNA and shows bands at 2.0 and 1.3 kilobases, as well as a faint but reproducible band at 0.4 kilobases. These fragment sizes should correspond to distances between both methylated and unmethylated CCGG sites in the β-globin gene DNA. The 1.3-kilobase fragment is of similar intensity in both digests.

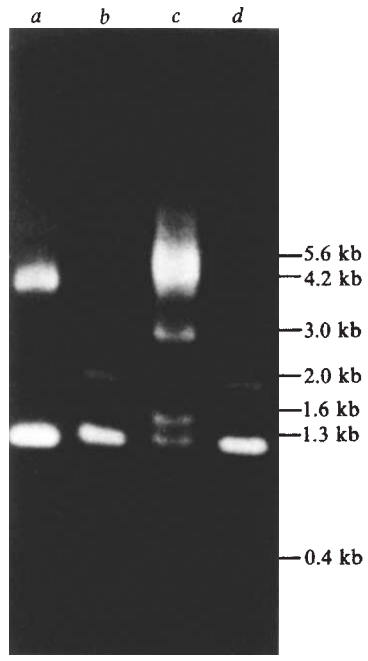

Fig. 1 DNA was isolated from chicken tissues by extensive digestion with proteinase K (Merck) in the presence of 0.2% SDS with or without previous isolation of nuclei. This was followed by at least three extractions with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), three extractions with two volumes of chloroform–isoamyl–alcohol (24:1), and three ethanol precipitations. In a typical digestion protocol, 200 units of restriction enzyme were added to 100 μg DNA in a final buffer of 6 mM KCl, 10 mM Tris-HCl (*p*H 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 μg ml^{-1} autoclaved gelatin. (The enzyme *Msp*I was obtained from New England Biolabs. The enzyme *Hpa*II was obtained from either New England Biolabs, Bethesda Research Laboratories, or Boehringer–Mannheim; all gave identical results. It was verified that, in the digestion conditions used, both *Hpa*II and *Msp*I gave identical cleavage patterns with the plasmid pBR322.) The reaction was incubated at 37 °C for 2 h, a further 200 units of enzyme were added and incubation was continued for an additional 2 h at 37 °C. Identical results were obtained if this second incubation was continued for 10–15 h. The completion of digestion was monitored by adding a small amount of bacteriophage λ DNA to an aliquot of either the first or the second incubation mixture and verifying by electrophoresis that the required cleavage products were present. After digestion, the DNA was repurified by repeating the proteinase K digestion and organic extraction steps described above, and 25–50 μg electrophoresed on each slot of a 1% agarose slab, 6 mm thick, for 2 h at 200 V. DNA was then transferred to Schleicher and Schuell nitrocellulose filters by the method of Southern[12], the filters hybridised to ^{32}P-labelled nick-translated pHb1001 for 24 h at 70 °C in 6×SSC, washed in 3×SSC, and finally washed for 30 min in 0.1×SSC. Filters were exposed to Kodak XR5 X-ray film for 24–96 h at −80 °C, using two Dupont Cronex intensifying screens. A typical autoradiogram is shown above and described in more detail in the text. *a*, Adult erythrocyte DNA digested with *Hpa*II; *b*, adult erythrocyte DNA digested with *Msp*I; *c*, oviduct DNA digested with *Hpa*II; *d*, oviduct DNA digested with *Msp*I. Band sizes were calibrated relative to a *Hin*dIII digest of λ and a *Hae*III digest of ΦX174 DNA, run in an adjacent gel slot, and are probably accurate to 5%. Identical *Hpa*II digestion patterns were obtained with DNA from erythrocytes or reticulocytes isolated from several individual adult roosters and chickens, of both White Rock and Leghorn breeds. *Hpa*II digestion of a mixture of erythrocyte and oviduct DNA yielded a pattern which was the expected sum of the patterns of slots *a* and *c* above. A digest of the same DNA with a mixture of *Hpa*II and *Msp*I yielded only the *Msp*I digestion pattern. The *Msp*I digestion pattern of all DNA samples investigated was identical to that in slots *b* and *d* above. Digestion with several restriction endonucleases which do not have CG as part of their recognition site gave the same digestion patterns in different tissues (see also ref. 16).

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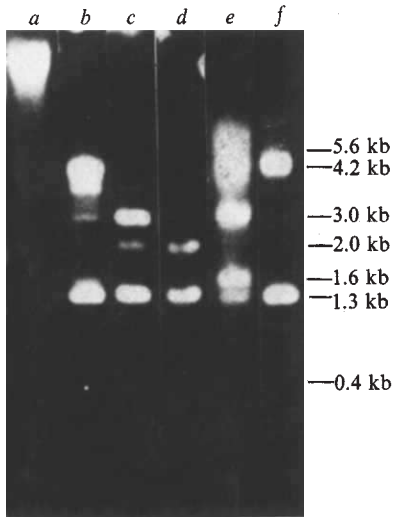

Fig. 2 *a–d*, Partial digestion of adult erytnrocyte DNA with the restriction endonuclease *Msp*I; *a*, no enzyme control; *b*, 2 units; *c*, 12 units; *d*, 100 units of enzyme. All incubations were for 2 h at 37 °C with 50 μg DNA and using the buffer conditions described in Fig. 1. *e*, Complete *Hpa*II digest of erythrocyte DNA from 5-d-old embryos. For comparison, slot *f* is a complete *Hpa*II digest of adult erythrocyte DNA.

However, the *Hpa*II digest (*a*) lacks both the 0.4 and 2.0 kilobase fragments of the *Msp*I digest as well as any other bands which could correspond to a combination of the *Msp*I bands. This suggests that the CCGG sites at the ends of the 1.3-kilobase fragment are completely (>90%) unmethylated in adult chicken erythrocyte DNA and furthermore, that at least two other specific sites are completely methylated. DNA isolated from the circulating reticulocytes of phenylhydrazine-induced anaemic adult chickens gives digestion patterns which are essentially identical to those of adult erythrocyte DNA (data not shown). These results are in contrast to those for the rabbit β-globin gene[13], where in both marrow and spleen cells of anaemic rabbits, only partial methylation of a site in the large intervening sequence was observed; this difference could be due to the different positions of the cleavage sites within the gene sequence or, as the authors have pointed out[13], due to a heterogeneous cell population.

Figure 1*c* shows the *Hpa*II digest of oviduct DNA. The observed band pattern is not only different from the *Msp*I digest of the same DNA (Fig. 1*d*) but also different from the *Hpa*II digest of erythrocyte DNA (Fig. 1*a*). There are now prominent bands at 4.2, 3.0, 1.6 and 1.3 kilobases (as well as several weaker but poorly resolved bands at higher molecular weight). As Fig. 2*b* and *c* show, all these bands appear as partial digestion products of adult erythrocyte DNA by the enzyme *Msp*I. This suggests that, in the oviduct, CCGG sequences in the vicinity of the β-globin genes are only partially methylated. Nevertheless, the absence of the 2.0-kilobase band suggests that at least one such sequence is completely methylated. As shown in Fig. 2*e*, DNA isolated from the circulating red blood cells of 5-d-old embryos (in which the adult β-globin gene is not being expressed[14,15]) or from adult brain (data not shown) gives digestion patterns essentially identical to that of oviduct DNA.

The legend to Fig. 1 summarises control experiments which show that the above results are not due to partial digestions, DNA isolation methods, enzyme inhibitors, DNA sequence rearrangements or individual differences between chickens. Figure 2*a* also provides an important control, showing that undigested DNA is still transferred to the nitrocellulose filter for hybridisation; thus, any high molecular weight bands would have been detected.

One current difficulty in assigning the observed bands to their positions within the gene sequence arises from the fact that the adult chicken β-globin gene in pHb1001 shares considerable sequence homology with at least one of the embryonic β-globin genes[16], and both sequences can show up on the filter hybridisations. However, our preliminary results using a genomic clone (G.D.G., W. I. Wood and G. Felsenfeld, unpublished) indicate that the prominent 1.3-kilobase fragment shown in Figs 1 and 2 is derived from the adult β-globin gene sequence; moreover one end of this fragment lies within about 30 bases of the 5′-end of the coding sequence[14].

It is undoubtedly premature to relate DNA methylation patterns to a specific biological activity. It does seem clear, however, that the observed tissue differences do not solely reflect different rates of cell division, as both adult and embryonic red cells have ceased division and yet their DNA shows different methylation patterns. Moreover, brain, a non-proliferating tissue, shows the same pattern as the proliferating oviduct. On the other hand, at least some correlation can be made between these methylation patterns and globin gene activity. In cells which are expressing or have expressed the adult β-globin gene (adult reticulocytes and erythrocytes) the CCGG sites near the ends of the gene sequence (that is at the ends of the 1.3-kilobase fragment) seem to be completely unmethylated. In cells which are not expressing this gene (oviduct, brain and embryonic red blood cells) these sites can be at least partially methylated. The determination of how these specific methylation events occur during cellular differentiation must await a more detailed description of their exact positions in the gene sequence. Nevertheless, we believe these results to be the first correlation between site-specific DNA methylation and eukaryotic gene expression.

We thank Dr Gary Felsenfeld for advice and encouragement, and Dr W. Salser for providing an unpublished characterisation of the plasmid pHb1001.

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Received 12 March; accepted 31 May 1979.

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Long regions of single-stranded DNA in human cells

WHEN DNA is isolated from actively growing animal cells by a variety of standard procedures, 1–2% of the total DNA is recovered in single-stranded form[1–5]. Pulse labelling experiments have established that the large majority of the single-stranded DNA is not newly synthesised[3,5]. However, a minor part of this DNA fraction consists of short fragments of newly replicated DNA released by branch migration during isolation[6,7]. Studies with synchronised human cells have shown that single-stranded DNA is mainly found during the period of DNA synthesis[3,4]. In agreement with this, a specific antiserum against denatured DNA used for immunofluorescence has revealed the presence of single-stranded DNA in nuclei of a human lymphoid

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