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Chromatin structure of transcriptionally competent and repressed genes

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We have compared transcriptionally competent and repressed genes with respect to their linker histone content and their ability to fold into higher-order structures. Histones were cross-linked covalently to DNA in chicken erythrocyte and oviduct nuclei by UV irradiation, and the DNA that was immunoprecipitated with anti-H1 and (for erythrocytes) anti-H5 antibodies was analysed for particular DNA sequences. None of the sequences investigated was free of H1 (H5). However, in mature erythrocytes the tissue-specific adult β-globin gene (β^A) appears to be partially depleted of H5, and both the β-globin gene and the H5 gene (also tissue-specific), as well as the 'housekeeping' β-actin gene, appear to be partially depleted of H1 relative to inactive genes; in oviduct slight H1-depletion is detected on the ovalbumin gene relative to genes that are inactive in this tissue and the actin gene. Transcriptionally competent erythrocyte chromatin fragments, in contrast to inactive fragments, are unable to self-associate into 'pseudo-higher-order structures'. This is likely to be a consequence of the partial depletion of H5 and/or H1 in active chromatin, resulting in the breakdown of (probably cooperative) interactions between H5 and/or H1 molecules that otherwise mediate the assembly of pseudo-higher-order structures *in vitro* and a stable 30 nm chromatin filament *in vivo*.

Key words: active and inactive genes/chicken erythrocytes/chromatin structure/histones H1 and H5/UV cross-linking

Introduction

Most of the chromatin in the eukaryotic interphase nucleus is present as a 30 nm filament (reviewed by Felsenfeld and McGhee, 1986; Widom, 1989) stabilized by histone H1 or related variants. The chromatin of genes that are transcriptionally competent (although not necessarily active) is distinguished from bulk chromatin by an increased sensitivity to DNase I (Weintraub and Groudine, 1976) which may extend over several kilobases of DNA (Stalder *et al.*, 1980) suggesting structural modification of a whole chromosomal domain. The biochemical basis for this is ill-understood (reviewed by Reeves, 1984; Gross and Garrard, 1987) but probably resides, at least in part, in relaxation of higher levels of chromatin organization as an enabling step before the onset of transcription.

Gene activation through chromatin unfolding might be a consequence of the loss of H1, which is regarded as a general repressor of gene activity (Weintraub, 1984; Schlissel and Brown, 1984; Wolffe, 1989). Even partial loss might well be sufficient to cause unfolding if the folded structure is stabilized by H1–H1 interactions (Thoma *et al.*, 1979; Thomas and Khabaza, 1980), and if interactions between successive H1 molecules in chromatin are cooperative, as they indeed are for H1 bound to DNA (Renz and Day, 1976; Clark and Thomas, 1986, 1988). H5 is an extreme variant of H1 which accumulates during the maturation of avian erythrocytes (Neelin *et al.*, 1964) in parallel with a general shut-down of transcription, and erythrocyte chromatin [which has a combined (H5 + H1) content of 1.3 molecules per nucleosome on average (Bates and Thomas, 1981)] indeed has a more stable higher-order structure than rat liver chromatin with one molecule of H1 per nucleosome (Bates *et al.*, 1981). Repression of transcription by H5 has been demonstrated directly in cell fusion (Appels *et al.*, 1974) and microinjection experiments (Bergman *et al.*, 1988) and on induction of a transfected H5 gene in a non-erythroid cell line (Sun *et al.*, 1989). The distribution of H5 and H1 in chromatin is not known, but various approaches, such as cross-linking (Pospelov *et al.*, 1981; Lennard and Thomas, 1985), immunoelectron microscopy (Mazen *et al.*, 1982) and immunoprecipitation (Torres-Martinez and Ruiz-Carrillo, 1982) have suggested that they are in general interspersed.

To gain further insights into the question of the presence and distribution of H1 and its variants on active and inactive genes, we have investigated the association of the linker histones H5 and H1 with particular DNA sequences in mature chicken erythrocytes, and of H1 with the same sequences in oviduct of laying hens. Although mature erythrocyte nuclei are heterochromatic and largely inert with respect to transcription, the tissue-specific globin genes retain the characteristic DNase I sensitivity of transcriptionally competent chromatin (Weintraub and Groudine, 1976; Stalder *et al.*, 1980), and both the β^A-globin and H5 (also tissue-specific) genes may even still be transcribed (Affolter *et al.*, 1987). Since the inactivation of the erythrocyte nucleus appears to be achieved by the deposition of H5 on the chromatin (Appels and Wells, 1972; Billett and Hindley, 1972; Ruiz-Carrillo *et al.*, 1974), followed by dephosphorylation (Sung, 1977), we have asked whether there is a significant difference in the distribution of H5 (and H1) on expressed and non-expressed genes in the erythrocyte, and particularly whether the β-globin and H5 gene regions are associated with histone H5 or whether they have escaped its deposition.

Histones were fixed at their native binding sites in nuclei by UV cross-linking, and cross-linked protein–DNA fragments were then immunoprecipitated from a restriction digest of the DNA using anti-H5 or anti-H1 antibodies, and analysed for gene content by hybridization to various cloned sequences. This procedure has been used to identify *Drosophila* genes bearing RNA polymerase molecules

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(Gilmour and Lis, 1985), and trout testis genes associated with the non-histone protein HMG-T (Blanco *et al.*, 1985). We find quantitative differences in the cross-linking of the linker histones (most probably due to differences in linker histone content) in active and inactive chromatin, and we have investigated the effect of this on some aspects of chromatin folding. Both sedimentation and gel electrophoresis indicate differences in the folding of transcriptionally competent and repressed chromatin.

Results

Localization of H1 and H5 on particular DNA sequences

Figure 1 outlines the strategy used to investigate the H1 (H5) content of chromatin containing particular DNA sequences in chicken erythrocyte and oviduct nuclei. Briefly, nuclei were irradiated with 254 nm UV light to cross-link proteins to DNA, and the DNA was freed of unbound proteins. After restriction endonuclease digestion (using *Pst*I whose cutting site does not contain -TpT- sequences and would therefore not be impaired by any UV-induced thymidine dimer formation), DNA fragments covalently linked to histones H1 or H5 were immunoprecipitated with H1 or H5 antisera, deproteinized, and analysed for particular DNA sequences.

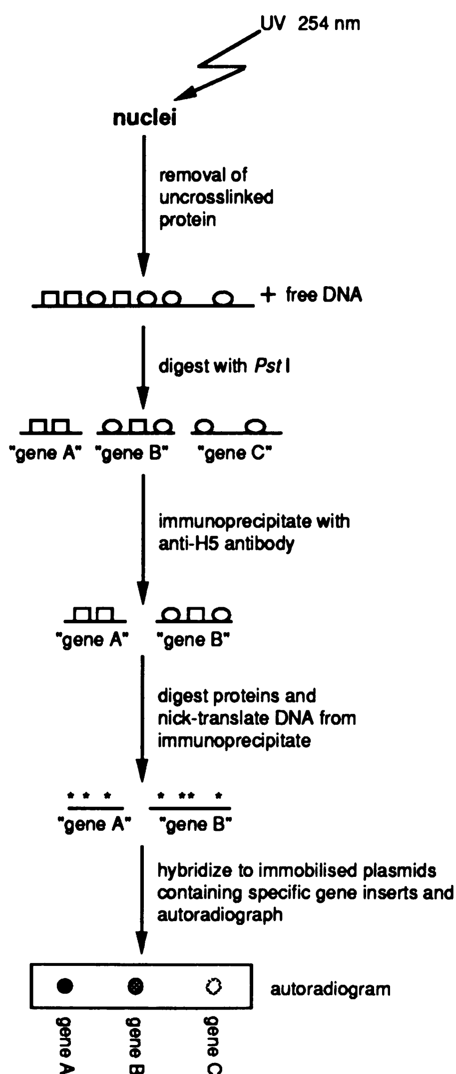

Fig. 1. Strategy for identification of particular proteins cross-linked to particular DNA sequences by UV cross-linking (see text for details). □ and ○ indicate H5 and H1 respectively; an arbitrary distribution is shown in the starting material.

UV-mediated cross-linking of H1, H5 to DNA and its immunodetection. Irradiation was routinely carried out for 10 min on the basis of a time course (Figure 2) up to 30 min, when some H5 degradation became evident. (The H5 was detected by immunoblotting of the proteins liberated by extensive micrococcal nuclease digestion of the cross-linked protein–DNA complexes, rather than by staining, because of the low degree of cross-linking and the large number of proteins cross-linked.) Cross-linking was quantified by probing, with anti-H5 antibody, known amounts of UV-irradiated DNA spotted onto nitrocellulose paper alongside H5 standards (not shown). About 2.5% of the H5 (~5 μg per mg DNA) was cross-linked to the DNA after 10 min irradiation, and 12.5% after 30 min, assuming 0.9 molecule H5 per nucleosome (212 bp DNA) in chicken erythrocyte chromatin (Bates and Thomas, 1981).

Specificity of immunoprecipitation. Figure 3 demonstrates by immunoblotting that the anti-H1 and anti-H5 antisera do not cross-react or recognize the core histones (and also shows that the anti-H5 antibodies recognize both fragments of H5 generated by cleavage roughly in the middle with chymotrypsin whereas the anti-H1 antibodies, which react with both the H1A and H1B subclasses, recognize only the C-terminal fragments of H1). Radioimmunoassay (Figure 4) also showed that the antisera do not cross-react or recognize DNA.

To check the specificity of immunoprecipitation of histone–DNA complexes, restriction-digested cross-linked DNA–protein complexes (and free DNA) were incubated with the appropriate antibody, and fragments with bound antibody were then immobilized on protein A–Sepharose. This was washed successively with buffer containing increasing concentrations of NaCl up to 0.25 M, and the material that remained bound was treated with proteinase K to release the DNA (to be used for hybridization). Analysis of the wash fractions and of the material bound to the protein A–Sepharose by agarose gel electrophoresis showed

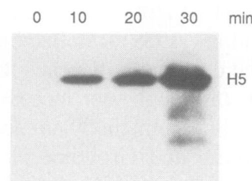

Fig.2. Time course of cross-linking of H5 to DNA by UV irradiation. Nuclei were irradiated for various times, lysed, and the DNA was freed of proteins that were not covalently attached by sedimentation twice through sucrose gradients containing 2.5 M NaCl. Samples containing one A_{260} unit of DNA (cross-linked + noncross-linked) were digested extensively with micrococcal nuclease and subjected to electrophoresis in an SDS–18% polyacrylamide gel. The released proteins were transferred electrophoretically to nitrocellulose and H5 was detected by immunoblotting.

that (i) there was no bound fraction from samples that had not been UV-irradiated; (ii) cross-linked DNA was immunoprecipitated only by the specific antisera and not by preimmune serum; and (iii) the anti-H5 antibody did not generate an immunoprecipitate from oviduct nuclei, which do not contain H5 (data not shown).

Analysis of DNA sequences in the immunofractionated complexes. The immunoprecipitated DNA was radiolabelled and used to challenge a set of pure plasmid DNAs containing the sequences of interest (Figure 5) immobilized on nylon, thus allowing simultaneous screening of several genes. All the plasmids contained inserts of roughly the same size (~2–2.7 kb) and were applied to the support at three loadings (0.05, 0.2 and 0.5 μg) of insert. At each loading they were in excess over the radiolabelled immunoprecipitated sequences so the relative hybridization signals for different plasmids reflect the relative concentrations of the corresponding sequences in the immunoprecipitate. Five chicken genes were examined: the erythrocyte-specific adult β-globin (β^A) and H5 genes; the oviduct-specific ovalbumin gene; β-actin (a housekeeping gene); and a feather keratin gene, inactive in both erythrocytes and oviduct. The cloned sequences that were probed with the immunoprecipitates were genomic in the case of globin (5′ non-coding sequences and the first two exons and introns), H5 and keratin (in both

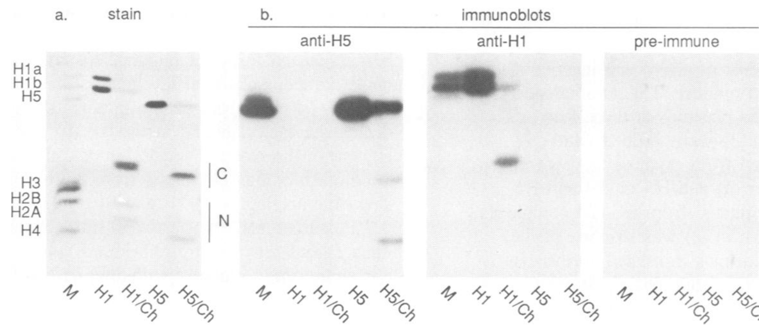

Fig. 3. The specificity of the anti-H1 and anti-H5 antibodies demonstrated by immunoblotting. Chicken erythrocyte histones H1 and H5 and their chymotryptic digests [H1/Ch and H5/Ch arising from cuts at Phe-105 and Phe-93, respectively (Clark *et al.*, 1988)] were analysed in quadruplicate in an SDS–18% polyacrylamide gel. M, 'marker' of total chick histones (in non-stoichiometric amounts). (**a**) One set of samples stained with Coomassie blue; (**b**) identical sets of samples transferred electrophoretically to nitrocellulose, probed with anti-H5, anti-H1 or pre-immune serum, followed by [^{125}I]protein A, and autoradiographed.

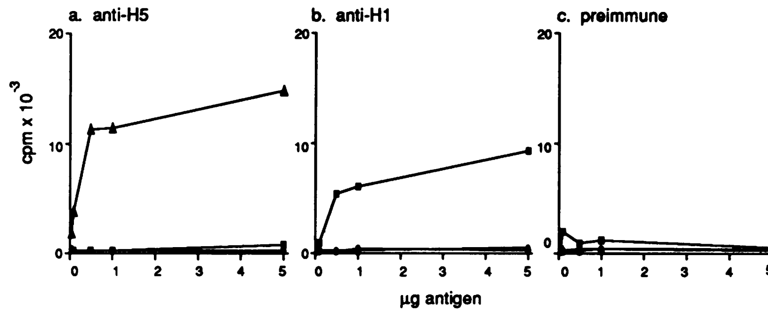

Fig. 4. The specificity of the anti-H1 and anti-H5 antibodies demonstrated by radioimmunoassay. Wells of microtitre plates were coated with (**a**) anti-H5, (**b**) anti-H1 or (**c**) pre-immune serum, then incubated successively with various amounts of antigen (DNA —●—; H1 —■— or H5 —▲—), a 1:300 dilution of the appropriate antiserum or pre-immune serum and [^{125}I]protein A; bound radioactivity was measured by gamma counting.

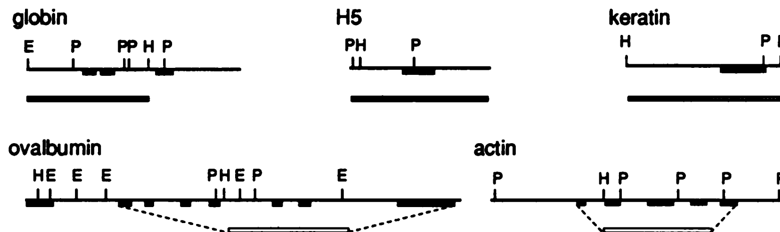

Fig. 5. The five cloned DNA sequences used (as inserts in pBR322) to analyse immunoprecipitates. β-Globin, H5 (as plasmid p2.6H5; Krieg *et al.*, 1983) and keratin (pH 2.7; R.Presland, personal communication; Presland *et al.*, 1989) are genomic sequences (shaded bars below the genomic restriction maps); β-actin (pA1; Cleveland *et al.*, 1980) and ovalbumin (pHha ov; Breathnach *et al.*, 1977) are cDNA sequences (open bars below the genomic restriction maps). Black bars indicate exons. The β-globin fragment (indicated by the shaded bar) was the *Eco*RI–*Hin*dIII 2 kb fragment subcloned from the clone pCAβG1 (Ginder *et al.*, 1979) which contains the 6.2 kb region shown above the bar. All five inserts are in the size range ~2–2.7 kb. E, P and H indicate cleavage sites for *Eco*RI, *Pst*I and *Hin*dIII respectively.

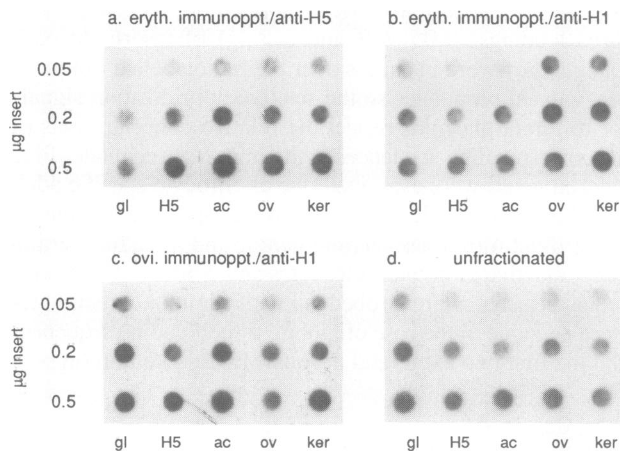

Fig. 6. The relative H5 and H1 contents of active and inactive chromatin in chicken erythrocytes and oviduct. The autoradiograms are of nylon membranes bearing denatured plasmids at three loadings (corresponding to 0.05, 0.2 and 0.5 μg insert), probed with radiolabelled DNA immunoprecipitated from erythrocyte cross-linked histone–DNA complexes using either (**a**) anti-H5 or (**b**) anti-H1 antiserum or with DNA immunoprecipitated from oviduct complexes using anti-H1 (**c**). (**d**) The filter shown in (a) was stripped of the hybridized DNA and rehybridized with nick-translated erythrocyte DNA that had not been fractionated by immunoprecipitation; hybridization to the different plasmids is much more uniform than in (a)–(c). gl, β-globin genomic insert; H5, histone H5 genomic insert; ac, β-actin cDNA insert; ov, ovalbumin cDNA insert; ker, keratin genomic insert. Further details of the sequences are given in Figure 5.

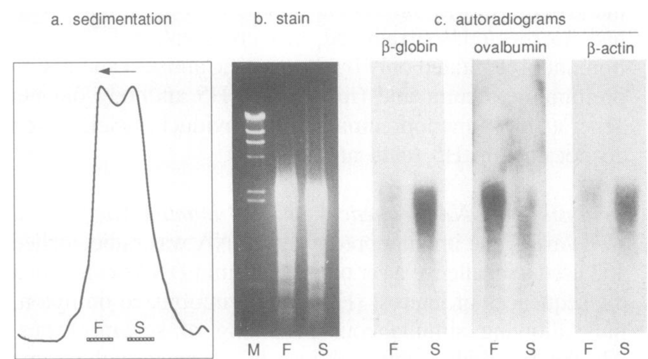

Fig. 7. Active and inactive sequences partition differently between the self-associated and non-associated populations of chicken erythrocyte oligonucleosomes. (**a**) A bimodal distribution of the associated and non-associated forms of oligonucleosomes (average length ~10 nucleosomes, isolated at low ionic strength) upon sedimentation in 5–30% (w/v) sucrose density gradients containing 70 mM NaCl (for details see Materials and methods). (**b**) The active and inactive gene content of the material in the bimodal distribution, determined by Southern blotting. The DNA from the material in the fast and slow-sedimenting peaks (F and S) was analysed in triplicate in a 0.7% agarose gel, transferred to nitrocellulose and three equivalent nitrocellulose strips were hybridized with nick-translated plasmid DNA containing the β-globin, ovalbumin or β-actin gene inserts (Figure 5). Autoradiograms were exposed for 12–16 h.

cases 5′ and 3′ non-coding sequences and the entire coding region), and cDNAs in the case of actin (entire coding sequence) and ovalbumin (all but the first exon) (Figure 5).

Figure 6 shows autoradiograms of filters probed with UV cross-linked DNA immunoprecipitated with anti-H5 and anti-H1 antibodies. Differences in the relative amounts of different DNA sequences in an immunoprecipitate suggest differences in the amount of H1 or H5 associated with them [or, less likely (see Discussion), altered binding of H1 or H5]. Histone H5, whose appearance on the chromatin during erythrocyte maturation is associated with general shut-down of transcription, is present on all the sequences investigated (Figure 6a). However, the chromatin containing the tissue-specific and transcriptionally competent β-globin gene is partially depleted of H5 relative to the inactive ovalbumin and keratin (or the 'housekeeping' β-actin) gene chromatin. Perhaps surprisingly, the tissue-specific H5 gene is not significantly depleted of H5. However, the presence of a 3′-flanking region in the cloned H5 gene sequence but not the globin sequence (Figure 5) might contribute to the apparent difference in H5 content of the chromatin containing the two genes, if both were partly H5-depleted but depletion was confined to their 5′-ends (and coding regions).

Histone H1 is present as ~0.4 molecule per nucleosome on average in mature chicken erythrocytes, which also contain ~0.9 molecule per nucleosome of H5 (Bates and Thomas, 1981), and some nucleosomes therefore contain a second molecule of H1 or H5. Figure 6b shows that although H1, like H5, is present in erythrocytes on all sequences examined, it is partially depleted (to roughly the same extent) on regions that were once active (β-globin, H5, β-actin) relative to the inactive ovalbumin and keratin genes.

Oviduct chromatin contains only H1 [about one copy per nucleosome on average, based on comparison with rat liver chromatin (Bates and Thomas, 1981)] and no H5. In oviduct the active ovalbumin gene is somewhat, although not markedly, depleted of H1 relative to the inactive β-globin, H5 and keratin sequences, and the β-actin gene (Figure 6c).

To check that the observed hybridization differences were not merely a consequence of grossly unequal loadings of the plasmids, the membranes were stripped of hybridized immunoprecipitated DNA and then probed with nick-translated chicken DNA that had not been immuno-fractionated but which had otherwise been isolated from UV-irradiated nuclei as outlined in Figure 1. Figure 6d (which is the reprobed filter from Figure 6a) confirms that all the plasmids were present in very similar amounts. (Slight differences should not matter since the nylon-immobilized sequences were at each loading present in large excess over the calculated maximum concentration of hybridizable sequences in the immunoprecipitates.) The observed hybridization differences with various cloned sequences were therefore due to real differences in the amounts of these sequences immunoprecipitated, and hence differences in the H5 and/or H1 content of the chromatin into which they are packaged.

Differences in sedimentation properties of transcriptionally competent and repressed chromatin

In view of the partial depletion of linker histones (H1 and H5) on the β-globin gene region in erythrocytes relative to inactive genes, we have asked whether this results in differences in chromatin folding. We have taken advantage of the observation (Thomas *et al.*, 1985) that bulk chicken erythrocyte chromatin fragments, ~6–16 nucleosomes long, size-fractionated in sucrose gradients at low ionic strength, fold and partly self-associate when the ionic strength is raised to ~70 mM, forming pseudo-higher-order structures.

The distribution of the β^A-globin, H5, ovalbumin and β-actin gene chromatin within the bimodal distribution of non-associated and self-associated fragments of average

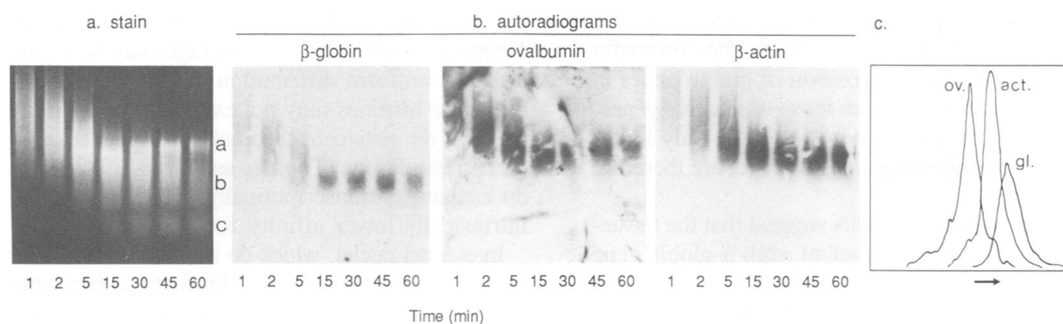

Fig. 8. Active and inactive chromatin partition differently in agarose gels. (**a**) Nuclei were digested with micrococcal nuclease, and the chromatin released by lysis into 0.2 mM Na_2EDTA, pH 7, was analysed in quadruplicate in 1.1% agarose gels containing 20 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA, pH 7.4. The gels were stained with ethidium bromide and blotted onto nitrocellulose; a, b, c refer to the three 'particles' described by Weintraub (1984). (**b**) Autoradiograms of the filters probed with radiolabelled β-globin, ovalbumin or β-actin-containing plasmids (Figure 5). (**c**) Densitometer scans (top to bottom in the direction shown) of the 60 min tracks of the autoradiograms shown in (b) of the β-globin (gl), β-actin (ac) and ovalbumin (ov) blots.

length ~10 nucleosomes (Figure 7a) was investigated. The DNA extracted from the material in the fast (F) and slow (S)-sedimenting peaks was blotted from an agarose gel (Figure 7b) onto nitrocellulose and probed with radiolabelled plasmids containing the gene inserts (Figure 7c). The inactive ovalbumin chromatin partitions with the bulk material that self-associates to form pseudo-higher-order structures; in striking contrast, the β-globin chromatin fractionates almost exclusively with the bulk chromatin fragments that do not self-associate. This material is slightly depleted in H1 and H5, probably due to handling losses (Thomas *et al.*, 1985; and data not shown), and β-globin chromatin probably co-sediments with this population of the bulk fragments because it itself is intrinsically partly deficient in H1 and H5, consistent with the UV cross-linking results described above. The behaviour of the tissue-specific H5 gene is identical with that of the β-globin gene (data not shown).

The β-actin (housekeeping) gene behaves like the β-globin gene rather than the inactive ovalbumin gene (Figure 7c), suggesting that depletion of H1 (see above), even in the absence of obvious differences in the H5 content, is sufficient to influence sedimentation behaviour. This depletion is evidently not confined to the 5′-end of the actin gene and must occur on the coding region (the actin probe is a cDNA).

Differences in the gel-electrophoretic mobility of transcriptionally competent and repressed chromatin

The bimodal sedimentation behaviour of oligonucleosomes and the partitioning of active and inactive sequences described above is reminiscent of the gel-electrophoretic behaviour of chicken erythrocyte chromatin fragments containing active and inactive genes reported by Weintraub (1984). We have therefore examined the gel-electrophoretic properties of the β-globin, ovalbumin and β-actin chromatin using the same DNA probes that were used for the sedimentation analysis. A micrococcal nuclease digest of chicken erythrocyte chromatin subjected to agarose gel electrophoresis (20 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA, pH 7.4) gives a tight aggregate of fragments termed an 'a-particle', a broad band of chromatin fragments separating according to size (the 'b-particle'), and a rapidly migrating 'c-particle' consisting mainly of mononucleosomes (Weintraub, 1984), as shown in Figure 8a. When the chromatin is blotted onto nitrocellulose and the DNA probed (Figure 8b), the ovalbumin gene is found largely in the a-particle and the β-globin gene almost entirely in the b-particle (c.f. Weintraub, 1984). (The c-particle gives no hybridization signal, probably because of poor binding to nitrocellulose of DNA ≤200 bp.) The actin gene chromatin migrates between the a- and b-particle regions of the stained gel, as shown more clearly (Figure 8c) by the relative positions of the ovalbumin, β-globin and β-actin hybridizing bands determined by densitometry of the autoradiograms.

Discussion

In view of the likely disruption of higher-order structure in transcriptionally competent chromatin, and the essential role of histone H1 (and in nucleated erythrocytes, H5) in such structures, we have asked whether the chromatin of active regions contains histone H1 (H5), and if so whether there is a full complement. The question has been approached directly by UV cross-linking of histones to DNA, and indirectly through comparison of some physical properties of transcriptionally competent chromatin with those of bulk chromatin and of repressed chromatin in chicken erythrocytes.

The linker histone content of transcriptionally competent and repressed chromatin in chicken erythrocytes and oviduct

Mature chicken erythrocytes contain both H1 and H5 at ~0.4 and 0.9 molecule per nucleosome respectively (Bates and Thomas, 1981) and thus have a higher linker histone content than the 'normal' value of about one molecule per nucleosome. H1 and H5 are not segregated in the chromatin (Pospelov *et al.*, 1981; Mazen *et al.*, 1982; Torres-Martinez and Ruiz-Carrillo, 1982; Lennard and Thomas, 1985), and it is possible that ~30% of the nucleosomes carry a second molecule of H5 or H1 (Nelson *et al.*, 1979; Bates and Thomas, 1981). Despite the general shut-down of transcription that correlates with the accumulation of H5 in erythrocyte chromatin during erythropoiesis (Appels and Wells, 1972; Billett and Hindley, 1972; Ruiz-Carrillo *et al.*, 1974) and the increase in linker histone concentration, the tissue-specific globin genes in mature erythrocytes retain the DNase I sensitivity of transcriptionally competent chromatin (Stalder *et al.*, 1980), they give run-on transcripts in nuclei

supplied with UTP (Gariglio *et al.*, 1981), and they may even be transcribed (Affolter *et al.*, 1987). The chromatin structure and linker histone composition of globin genes in mature erythrocytes, compared with those of inactive genes, and of globin genes in oviduct where H1 is the only linker histone and in which the globin gene is inactive, are therefore of some interest.

First, the UV cross-linking results suggest that the tissue-specific and transcriptionally competent adult β-globin gene in erythrocytes is not free of H5 or H1. Likewise the transcribed ovalbumin gene in oviduct is not H1-free. Similar conclusions with respect to the presence of H1 were reached for active heat-shock genes in *Drosophila* (Nacheva *et al.*, 1989) [after fixation of proteins to DNA using a variation of the chemical cross-linking method used earlier (Karpov *et al.*, 1984), which had led to different conclusions]; and, more recently, using indirect immunofluorescence and immunoelectron microscopy, for active regions in salivary gland polytene chromosomes of *Drosophila* (Hill *et al.*, 1989) and active Balbiani ring genes of *Chironomus* (Ericsson *et al.*, 1990), respectively. The continued presence of at least some H1/H5 on the β-globin gene in erythrocytes and on the ovalbumin gene in oviduct was also inferred from the ionic strength dependence of the sedimentation of nuclease-digested chromatin (Fisher and Felsenfeld, 1986).

Secondly, semi-quantification of the UV cross-linking results reveals differences in the relative immunoprecipitation of DNA sequences containing different genes in erythrocytes as well as of the β-globin gene in erythrocytes and oviduct. The most likely explanation of the differences in cross-linking responsible for this is differences in H5 and/or H1 content. [Subtle differences in the mode of binding of H5 or H1 to, e.g. active and inactive genes seem less likely to be responsible, in view of the apparently low chemical specificity of UV cross-linking with respect to amino acid side chains (Smith, 1969), and the high abundance of lysine which is relatively favourable for cross-linking (Smith, 1969; Schott and Shetlar, 1974) throughout both molecules.] The UV cross-linking results therefore suggest that in erythrocytes the 2 kb β-globin gene fragment, although not free of H1 and H5, is partially depleted of both histones relative to inactive genes (keratin, ovalbumin). The tissue-specific H5 gene and the once-active housekeeping β-actin gene, although not apparently depleted of H5, are both depleted of H1 relative to the permanently repressed genes. The apparent difference in H5 depletion between the H5 and β-globin genes might conceivably reflect differences in transcription rates, although these appear to be similar in mature erythrocytes (Affolter *et al.*, 1987), but it could also be due to the different nature of the probes used [the β-globin probe comprises 5′ non-coding sequences and the first two exons, whereas the H5 probe contains 5′ and 3′ non-coding sequences in roughly the same proportions (Figure 5)], so that differences that were common to the two genes but confined to their 5′-ends (and perhaps coding sequences) would be less evident in the case of the H5 gene. The depletion of histone H1 found for both the β-globin and H5 gene regions is probably not confined solely to their 5′-ends, since the actin gene shows similar depletion which in this case must occur within the coding region of the gene seen by the cDNA probe.

The results taken together suggest that H5 is necessary but not sufficient for complete repression of genes in the erythrocyte nucleus and for chromatin condensation and packing; for this a linker histone stoichiometry in excess of 1 copy per nucleosome on average may be a crucial factor. The non-uniform distribution of H1 and H5 also suggests that these histones may not exchange freely in the nucleus, as they do between chromatin fragments *in vitro* under certain conditions (Thomas and Rees, 1983) or that, if they do exchange, transcriptionally competent regions have an intrinsically lower affinity for H1 and H5.

In oviduct nuclei, which do not contain H5, the H1 content of (inactive) β-globin gene chromatin is the same as that of the inactive keratin or H5 genes, showing that there is no intrinsic tendency of the β-globin DNA sequence to exclude linker histones. The active ovalbumin chromatin is only slightly depleted (by ~20%) of H1 relative to inactive gene chromatin, and no depletion is detected for the housekeeping β-actin gene. However, both the ovalbumin and actin probes were cDNAs and thus any depletion confined to the 5′-end would not be detected.

The main result, therefore, is the difference between the globin gene chromatin and the chromatin of inactive genes in a tissue in which the globin gene is (or was once) expressed and an inactive tissue (oviduct). This points clearly to some depletion of H5 and H1 on the globin gene region in its transcriptionally competent (but minimally active) state in mature erythrocytes, despite a generally high nuclear linker histone content. The depletion (of ~40%) occurs within the region corresponding to the 2 kb probe used here which encompasses 5′ flanking sequences and the first two exons. One nucleosome is known to be missing at the promoter, at a DNase I hypersensitive site where erythroid-specific and non-specific transcription factors are known to bind (Evans *et al.*, 1988; Lewis *et al.*, 1988; and references therein), suggesting that about half the remaining nucleosomes within this stretch of 9–10 also lack H5 and H1. This might be a direct consequence of nucleosomal disruption at the promoter and a local breakdown of cooperative H5/H1 interactions, and/or other signals in the chromatin [e.g. core histone acetylation or the presence of HMG-14 and 17 (Reeves, 1984; Ridsdale and Davie, 1987; and references therein)] which we have not investigated here.

A transcriptionally competent state of chromatin regions containing tissue-specific genes such as the β-globin gene in erythrocytes and the ovalbumin gene in oviduct thus appears to be achieved with only partial depletion of the histones (H5, H1) that stabilize higher-order structure. This also appears to be true of actively transcribed heat-shock genes in *Drosophila* (Nacheva *et al.*, 1989), of other genes in puffed regions of *Drosophila* polytene chromosomes (Hill *et al.*, 1989), and of the active Balbiani ring genes in *Chironomus* polytene chromosomes (Ericsson *et al.*, 1990). If the interactions between H1 (H5) molecules in chromatin, like those between the histones bound to free DNA (Clark and Thomas, 1986, 1988; and references therein), are indeed cooperative, as they are generally assumed to be (for example, Weintraub, 1985), then partial depletion might be sufficient to disrupt interactions over entire chromatin domains and result in a looser (although not necessarily completely unfolded) higher-order structure, in principle competent to support transcription. In active Balbiani rings, unfolded nucleosomes co-exist with 30 nm filaments which re-form when successive RNA polymerase molecules are relatively far apart, indicating that chromatin is in a dynamic state that is transiently perturbed by passage of a polymerase (Ericsson *et al.*, 1990).

Physical properties of active and inactive genes in chicken erythrocytes

Perhaps not surprisingly, the partial depletion of both H5 and H1 from chromatin containing the β-globin sequence, and of H1 from the H5 gene-containing and actin gene-containing chromatin in erythrocytes, results in discernible differences in certain folding-dependent physical properties between chromatin containing these sequences and bulk or inactive chromatin. Active and inactive genes partition differently within the bimodal population of previously size-fractionated chicken erythrocyte chromatin fragments generated by sedimentation at ~70 mM ionic strength (Thomas *et al.*, 1985). The β-globin, H5 and actin genes segregate almost entirely with the slowly sedimenting fraction and the inactive ovalbumin gene very largely with the more rapidly sedimenting (self-associated) fraction. These results are entirely compatible with the relative linker histone contents deduced from the UV cross-linking experiments. Sedimentation of the globin gene, H5 gene and actin gene chromatin with the fraction of the bulk chromatin that has lost up to ~20% of its histones H1 and H5 [possibly to other chromatin fragments (Thomas *et al.*, 1985)] suggests that these genes are packaged in chromatin structures that may themselves be intrinsically partly depleted of histones H1 and/or H5, and thus unable to form fully folded structures as a prerequisite for self-association by stacking. This association is probably mediated by H5−H5 interactions resembling the interactions that occur between turns of higher-order structure in an intact chromatin filament (Thomas *et al.*, 1985), and which are believed to be responsible for the more stable higher-order structure of chicken erythrocyte chromatin than, for example, rat liver chromatin, suggested by hydrodynamic measurements (Bates *et al.*, 1981). The bulk chromatin in the slowly sedimenting peak of the bimodal distribution appears from electron microscopy of fixed samples to be folded into ~30 nm diameter structures (Thomas *et al.*, 1985); however, the small proportion of globin gene chromatin may or may not be in this form, since the resolution in the gradients is probably not sufficient to distinguish between folded (30 nm diameter) and unfolded (10 nm diameter) short chromatin fragments in the slowly sedimenting material.

The inactive ovalbumin gene fractionates bimodally, as expected from the above explanation, but is substantially enriched in the self-associated fraction which contains a full complement of H5 and H1, consistent with the UV cross-linking results and suggesting a fully folded higher-order structure for the repressed ovalbumin gene. That the β-actin and H5 genes fractionate like the β-globin gene, suggests that the partial depletion of H1 on the actin and H5 genes in erythrocytes detected by UV cross-linking, even in the absence of any detectable or significant depletion of H5, is enough to destabilize the higher-order structure sufficiently to disrupt the interactions within it; this is reflected here in the breakdown of self-association between chromatin fragments. Thus both the tissue-specific β-globin and H5 genes in the mature erythrocyte which are expressed late in erythropoiesis, and the housekeeping β-actin gene whose transcription probably ceased earlier (Hinssen *et al.*, 1987), presumably accompanying the deposition of histone H5, appear to retain distinct characteristics that suggest looser folding than for inactive (e.g. ovalbumin) genes. In the case of actin at least (cDNA probe), the slower sedimentation is not due to structural disruption confined to the 5′-end of the gene and must reflect some degree of chromatin unfolding over the coding region.

These conclusions based on the sedimentation behaviour of size-fractionated chromatin at 70 mM ionic strength are entirely in accord with those deduced from the electrophoretic behaviour of a micrococcal nuclease digest of chicken erythrocyte chromatin. The inactive ovalbumin gene partitions with the fragments of the bulk population that self-associate, probably again through linker histone (e.g. H5−H5) interactions, whereas the β-globin gene partitions with the bulk fragments that do not, presumably because of disrupted linker histone interactions (cf. Weintraub, 1984). Migration of the actin chromatin between the ovalbumin and globin chromatin suggests that weak self-association might persist between actin chromatin fragments during gel electrophoresis that do not survive sedimentation in sucrose gradients. The overall state of chromatin condensation of the housekeeping β-actin gene in erythrocytes may thus be intermediate between that of the condensed, repressed ovalbumin gene and the more relaxed, expressed β-globin gene, possibly reflecting the relative transcription rates. Alternatively, the apparent differences between β-actin and β-globin may arise from additional structural changes at the 5′-end of the β-globin gene over and above those presumably shared by both genes on their coding regions.

Other studies of the folding of β-globin chromatin in chicken erythrocytes

Others have studied the sedimentation behaviour of β-globin chromatin fragments relative to bulk or inactive fragments of the same size in digests of chicken erythrocyte nuclei. Kimura *et al.* (1983) attributed the slightly slower sedimentation in 80 mM NaCl of β-globin chromatin produced by *Eco*RI digestion, than of inactive gene fragments of about the same size, to some loss of H5/H1 with consequent relaxation of the higher-order structure; similar conclusions were drawn for micrococcal nuclease digested chromatin (Fisher and Felsenfeld, 1986). However, the slow sedimentation of the *Eco*RI-generated chromatin fragments was subsequently attributed to the presence of nuclease hypersensitive sites flanking the β-globin gene, rather than to loss of H5/H1 *per se*, since the shorter fragments that did not include hypersensitive sites sedimented with bulk fragments of the same size (Caplan *et al.*, 1987). It was concluded from density measurements that, apart from local disruptions at the hypersensitive sites, probably resulting in the loss of one octamer in each case, the chromatin had its full complement of histones, including H5 and H1, and therefore presumably adopted the 30 nm filament structure characteristic of the bulk chromatin between the disjunctions at the hypersensitive sites (Caplan *et al.*, 1987). However, hypersensitive sites might well not persist in micrococcal nuclease-generated fragments although sedimentation differences between active and inactive genes were still apparent (Fisher and Felsenfeld, 1986). Taken together, all these studies, and the results reported in this paper, thus suggest a less tightly folded structure for active than for inactive chromatin, and although hypersensitive sites probably do constitute conspicuous sites of disruption, and may initiate it, a more general relaxation of the structure also seems likely.

Whatever the sequence of events, β-globin (and actin) gene-containing chromatin fragments are evidently unable to fold sufficiently regularly and tightly to self-associate into

'pseudo-higher-order structures', as indicated here by both sedimentation and gel electrophoresis. This probably reflects the general situation for transcriptionally competent genes *in vivo*, in which the first, enabling, step in gene activation (namely, relaxation of higher-order structure) has been achieved. This state is probably reflected in, and may account for, the characteristic enhanced DNase I sensitivity of transcriptionally competent genes (Weintraub and Groudine, 1976; Smith *et al.*, 1984;), but it is likely that the slight depletion in H5 and H1 is only one of a number of modifications that results in enhanced DNase I sensitivity over whole chromatin domains.

Materials and methods

Isolation of nuclei

Chicken erythrocyte nuclei were isolated essentially as described by Bates *et al.* (1981) and modified by Thomas and Rees (1983), except that for all UV cross-linking experiments, 10 mM Tris−HCl, pH 7.5, 3 mM $MgCl_2$, 0.34 M sucrose (TMS) buffer (Lennard and Thomas, 1985) was used for nuclear isolation instead of Buffer A −0.34 M sucrose (Hewish and Burgoyne, 1973) in order to minimize possible exchange of histones H1 and H5 (Thomas and Rees, 1983).

Chicken oviduct nuclei were isolated from the magnum portion of the oviducts of laying hens killed by cervical dislocation (Fritton *et al.*, 1983) and washed twice in TMS buffer.

UV cross-linking of nuclei; isolation and restriction digestion of UV cross-linked protein−DNA complexes

20 ml of nuclei at A_{260} = 20 in a 10 cm plastic Petri dish placed 10 cm from a 254 nm light source [Chromato-Vue C-61 transilluminator (Ultra-Violet Products, Inc.) with the filter removed] were irradiated for up to 30 min. The intensity at 10 cm was 7.5×10^{-3} W/cm^2 as measured by a UV photovoltaic intensity meter (Blak-Ray J225). The nuclei were collected by centrifugation and lysed in 2−3 ml of 0.2 mM Na_2EDTA, pH 7. An equal volume of 5 M NaCl was added, and the total lysate was loaded onto a 12 ml linear (20−45%, w/v) sucrose gradient containing 2.5 M NaCl, 1 mM Na_2EDTA, 10 mM Tris−HCl, pH 8, 0.25 mM PMSF which was centrifuged at 4°C in a Beckman SW40 rotor at 38 000 r.p.m. for 18 h. The pellet (cross-linked DNA−protein complex and free DNA) was resuspended in a small volume of 10 mM Tris−HCl, 1 mM Na_2EDTA, pH 8.0, dialysed against the same buffer, recentrifuged through a sucrose gradient containing 2.5 M NaCl and redialysed. The DNA, free of proteins that were not covalently attached as a result of UV cross-linking, was then digested to completion with *Pst*I (Amersham), using 1−2 U enzyme for 12−14 h in the recommended digestion buffer.

Immunoprecipitation and nick translation of cross-linked H5(H1)−DNA complexes

100 μg of *Pst*I-digested DNA in ~300 μl of digestion buffer was dialysed overnight into 50 mM Tris−HCl, pH 7.5, 5 mM Na_2EDTA, 150 mM NaCl and then cleared by centrifugation for 30 s at 30 000 r.p.m. in a microfuge. 10 μl of anti-H5 or anti-H1 antiserum was added and the sample was mixed on an end-over-end rotator overnight at 4°C. 30 mg (10% w/v; 50% v/v) of protein A−Sepharose (Pharmacia) in the same buffer was then added and the mixture was incubated at room temperature for 4 h. After dilution of the slurry with 10 ml of the same buffer containing 0.5% NP40 the Sepharose−protein A was collected by brief centrifugation and washed successively with 10 ml each of 50 mM Tris−HCl, pH 7.5, 5 mM Na_2EDTA containing first 0.2 M and then 0.25 M NaCl. The bound immunoprecipitated DNA was then released by digestion with proteinase K for 4−12 h at 42°C, purified by phenol extraction followed by chloroform−isoamyl alcohol extraction, and then ethanol precipitated in the presence of 0.3 M sodium acetate and resuspended in distilled water (100 μl).

The DNA was radiolabelled by nick translation with [α-^{32}P]dCTP (Rigby *et al.*, 1977) and freed from unincorporated nucleotides by passage through a Sephadex G-50 'spun column' (Maniatis *et al.*, 1982). Specific activities of $3 \times 10^7 - 10^8$ c.p.m./μg were routinely obtained, with an average DNA size of ~1000 bp (determined by agarose gel electrophoresis).

Analysis of the immunoprecipitated DNA with immobilized plasmids containing specific gene sequences

The various plasmids containing the sequence inserts of interest were denatured with 0.5 M NaOH−1.5 M NaCl for 10 min at 37°C followed by 1 min in a boiling water bath. They were then applied, in a final volume of 100 μl, at three loadings (0.05, 0.2 and 0.5 μg insert) to a nylon membrane (Hybond-N, Amersham) wetted with 20 × SSPE (Maniatis *et al.*, 1982) in a dot-blotting apparatus (BioRad) with a flow rate of ~100 μl/min. The membrane was then placed (DNA side up) for 5 min on a sheet of Whatman 3MM paper saturated with 0.5 M NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris−HCl, pH 7.0, 1.5 M NaCl for 30 s, dried and finally UV-irradiated (302 nm) for 2 min to fix the DNA. Prehybridization was carried out for 1 h in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 200 μg/ml sonicated salmon sperm DNA (Maniatis *et al.*, 1982), and hybridization overnight at 62−63°C in the same buffer containing 20 ng nick-translated immunoprecipitated DNA per ml solution. The membrane was then washed at the same temperature twice for 15 min each in 2 × SSPE, twice for 15 min each in 2 × SSPE containing 0.1% SDS, and then covered with Saran wrap and exposed wet to pre-flashed Fuji RX X-ray film with an intensifying screen at −70°C. Membranes to be reused were stripped of hybridized DNA by immersion for 30 min in 0.1% SDS which had been brought to the boil.

Sedimentation at ~70 mM ionic strength of chromatin fractionated at low ionic strength and analysis of the bimodal sedimentation profile

Soluble chromatin was prepared by micrococcal nuclease digestion of chicken erythrocyte nuclei and size-fractionated in 5−30% (w/v) linear sucrose gradients containing 10 mM Tris−HCl, pH 7.0, 1 mM Na_2EDTA, 0.25 mM PMSF (Butler and Thomas, 1980). Fractions containing fragments of average size ~10 nucleosomes (cf. Thomas *et al.*, 1985) were dialysed against 5 mM triethanolamine−HCl, pH 7.5, 1 mM Na_2EDTA, 0.5 mM PMSF and then 1 M NaCl was added to a final concentration of 70 mM. After 1 h at 4°C the samples were centrifuged through 17 ml linear sucrose gradients (5−30%, w/v) containing 70 mM NaCl in a Beckman SW28 rotor at 4°C for 16 h at 16 000 r.p.m.

The DNA and protein contents of fractions across the bimodal sedimentation profile (Thomas *et al.*, 1985) were analysed in 0.7% horizontal agarose gels containing 40 mM Tris, 2 mM Na_2EDTA, 24 mM acetic acid (Loening, 1967) and ethidium bromide (0.5 μg/ml), and in SDS−18% polyacrylamide gels (Thomas and Kornberg, 1978), respectively.

The gene content of the DNA was analysed by Southern blotting (Southern, 1975; Maniatis *et al.*, 1982). The agarose gel was first soaked in 0.25 M HCl for 1−3 min to nick the DNA, then in 0.5 M NaOH, 1.5 M NaCl for 2 × 15 min to denature it, and finally neutralized in 0.5 M Tris−HCl, pH 7.0, 1.5 M NaCl. The DNA was then transferred by diffusion from the gel to nitrocellulose (BA85, Schleicher and Schuell) for 12−15 h at room temperature. The filter was air-dried, baked at 80°C for 2 h, prehybridized for 1 h at 65°C in a solution containing 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 10% dextran sulphate, 200 μg/ml sonicated salmon sperm DNA (100 μl solution/cm^2 filter) and hybridized for 18 h at 65°C with the appropriate heat denatured nick-translated radiolabelled plasmid (specific radioactivity 10^8 c.p.m./μg) at a concentration of 10 ng/ml. The filters were washed twice for 15 min with 2 × SSPE, 0.1% SDS at room temperature, then with 0.2 × SSPE, 0.1% SDS at 55°C, and exposed to X-ray film as above. They were kept moist (covered with clingfilm) so that they could be stripped (see above) and reprobed.

Fractionation of chromatin in nucleoprotein gels and analysis of DNA

Nuclei (A_{260} = 100) in 0.34 M sucrose-buffer A (Hewish and Burgoyne, 1973) were digested with micrococcal nuclease (Sigma) essentially as described by Noll *et al.* (1975), using 17 U enzyme for various times at 25°C and lysed into 0.2 mM Na_2EDTA, pH 7.0 [1 unit (U) of enzyme activity releases 1 A_{260} unit of oligonucleotide/min from DNA at 37°C and pH 8.8.] The soluble chromatin obtained after centrifugation to remove nuclear debris was diluted to a concentration of 1 μg/ml (A_{260} ~0.01) in 10 mM Tris−HCl, pH 7.5, 1 mM Na_2EDTA and 10 μg was loaded onto a 1.1% agarose gel containing 20 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA, pH 7.4 (Weintraub, 1984) and ethidium bromide (0.25 μg/ml). The gel was run at 70 V for 2−3 h and the nucleoprotein complexes visualized by UV transillumination. Southern blotting of the DNA onto nitrocellulose was carried out directly from the nucleoprotein gel, exactly as described for DNA in agarose gels in the previous section.

Antisera, radioimmunoassay and immunoblotting

Antibodies against H1 and H5 from chicken erythrocyte nuclei (Clark and Thomas, 1986), were raised in New Zealand white rabbits, by repeated (monthly intervals) multiple intradermal injections of H1 or H5 in Freund's complete adjuvant. Antisera were stored in aliquots at −20°C. Their specificity was checked by immunoblotting and radioimmunoassay.

For radioimmunoassay (Johnstone and Thorpe, 1982) the wells of a microtitre plate were incubated overnight at room temperature with 50 μl of antiserum at 0.2 mg/ml in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4). They were then washed repeatedly with PBS containing 2% (v/v) newborn calf serum (NBCS; Gibco), incubated with H1, H5 or DNA diluted in PBS-NBCS overnight at room temperature, washed again several times in PBS-NBCS and finally incubated for 2 h at room temperature with the original antibody at a 1:300 dilution in the same buffer. After washing in PBS-NBCS the wells were further incubated for 2 h at room temperature with [^{125}I]protein A (1 μCi/ml; Amersham International) in the same buffer, then washed, dried and counted in a gamma counter.

Proteins for immunoblotting were separated in an SDS−18% polyacrylamide gel and transferred to nitrocellulose (BA85 Schleicher and Schuell) in a Transblot apparatus (BioRad) for 2−4 h at 250 mA in 25 mM Tris base, 190 mM glycine, 20% methanol, 0.1% SDS (Burnette, 1981). The nitrocellulose was blocked overnight in 'blocking solution' [5% non-fat dried milk (Cadbury's Marvel) in 20 mM potassium phosphate, pH 7.2, 0.15 M NaCl (Johnson *et al.*, 1984)] at 4°C, then incubated for 2 h at room temperature with the appropriate antibody diluted with blocking solution (1:200), rinsed and exposed to [^{125}I]protein A (0.5 μCi/ml in blocking solution) for 45 min at room temperature. It was washed three or four times for 15 min each with 100 ml of blocking solution, rinsed in water, air-dried and exposed overnight to preflashed Fuji RX film at −70°C with an intensifying screen.

Plasmids and subcloning

Plasmids (see legend to Figure 5), all pBR322-derived, were isolated by standard procedures (Maniatis *et al.*, 1982), from *Escherichia coli* TG1 cells (genotype K12, Δ(*lac-pro*), *supE*, *thi*, *hsd* D5/F′, *tra*D36, *pro*A$^+$B$^+$, *lac*I$_q$, *lacZ*$^-$, ΔM15) by a modified alkaline lysis method following amplification. They were further purified in sucrose gradients to remove chromosomal DNA and RNA; up to 500 μg of DNA were loaded onto a 12 ml linear sucrose gradient (15−40% w/v) containing 10 mM Tris−HCl, pH 8.0, 1 mM Na_2EDTA, and centrifugation was carried out at 32 000 r.p.m. in a Beckman SW40 rotor for 18 h at 4°C. Gradients were fractionated by pumping from the bottom, and the plasmid DNA was precipitated from pooled fractions with 0.3 M sodium acetate and 3 vol. absolute ethanol.

The original β-globin plasmid (pCAβG1; Ginder *et al.*, 1979) contained a 6.2 kb insert (including repetitive DNA sequences) which was subcloned before use. *Hin*dIII (Amersham) released a fragment containing a 2 kb *Eco*RI−*Hin*dIII segment of the chicken adult β-globin gene (the 5′ non-coding sequence and the first two exons; Figure 5) and 50 bp from the pBR322 vector, which was eluted electrophoretically from a 1% agarose gel on to DE81 paper (NA45, Schleicher and Schuell), retrieved as recommended by the manufacturer, and cloned into the *Hin*dIII site of pBR322 (Maniatis *et al.*, 1982).

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