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The arrangement of H5 molecules in extended and condensed chicken erythrocyte chromatin

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Chemical cross-linking with dithiobis(succinimidyl propionate) has been used to investigate the relative disposition of neighbouring H5 (H1) molecules in chicken erythrocyte chromatin in the extended (nucleosome filament) and condensed (300 Å filament) states; in this chromatin H5 and H1 are interspersed along the nucleosome filament, rather than segregated into blocks, as shown by the nature of the cross-linked dimers and their relative amounts. Detailed analysis of the cross-linked H5 homopolymers from extended chromatin and condensed nuclear chromatin indicates which domains of H5 are in contact (or close proximity) in the two states. The results suggest a polar, head-to-tail arrangement of H5 molecules along the nucleosome filament. This arrangement persists when chromatin adopts higher-order structure but in the folded state neighbouring basic C-terminal domains, in particular, are more closely juxtaposed than they are in extended chromatin.

Key words: chromatin structure/chemical cross-linking/H5 polymer/H5-H5 contacts

Introduction

Histone H1 is somehow involved in the condensation of the nucleosome filament into the next level of structure, the 300 Å diameter filament (e.g., Thoma *et al.*, 1979; Butler and Thomas, 1980; and references therein). H1 in chromatin can be cross-linked into a homopolymer (poly H1) (e.g., Thomas and Khabaza, 1980) and H1-H1 associations may be important in the formation of chromatin higher-order structure. Specifically, salt-induced H1-helix formation was suggested to be the driving force for formation of a regular superhelix of nucleosomes (or solenoid) on addition of NaCl, the H1 helix lining the central hole of the solenoid (Thoma *et al.*, 1979).

Poly-H1 is not unique to the condensed state but is also generated by cross-linking extended chromatin (Thomas and Khabaza, 1980) indicating that H1 molecules are in close proximity [within 12 Å, or even closer (Ring and Cole, 1983)] along the nucleosome filament as well as in the 300 Å filament. In relation to the possible importance of H1-H1 associations in chromatin folding, the question then arises whether there is a salt-dependent change in the nature of these associations that correlates with chromatin condensation.

We report here an analysis of the cross-linked products from chicken erythrocyte chromatin, chosen for study because of its stable higher-order structure (Bates *et al.*, 1981). This stability is probably largely due to the H1 analogue, H5, which is the major lysine-rich histone in chicken erythrocyte nuclei (Neelin *et al.*, 1964), occurring at ~0.9 molecule per nucleosome on average, together with 0.4 molecule of H1 (Bates and Thomas, 1981). Homopolymers of H5 and of H1 as well as H1/H5 heteropolymers are produced on cross-linking. We have focussed on the H5 homopolymers. For both the extended and condensed states we have defined those regions of neighbouring H5 molecules whose proximity permits cross-linking. We have defined the orientation of H5 molecules along the nucleosome filament and we have identified new juxtapositions favoured in the condensed state.

Results

H5 and H1 are cross-linked into homo- and heteropolymers in both extended and condensed chicken erythrocyte chromatin

Like H1 in rat liver chromatin (Thomas and Khabaza, 1980), H5 and H1 in chicken erythrocyte chromatin are cross-linked into polymers with bifunctional amino-group reagents. These polymers may be extracted with 5% perchloric acid for analysis in SDS-polyacrylamide gels. Figure 1 shows time courses of cross-linking in extended chromatin, $MgCl_2$-condensed chromatin and nuclei with dithiobis(succinimidyl propionate) (Lomant and Fairbanks, 1976). Polymers giving the same general band pattern are obtained in all three cases although marked differences in the recovery of perchloric acid-extractable material are evident. The apparent maximum in the H5(H1) polymer size with increasing time of cross-linking (particularly apparent in Figure 1B), followed by a decrease in the yield of perchloric acid-soluble material, is due to cross-linking of H5(H1) to the core histones giving insoluble products (cf. Thomas and Khabaza, 1980). The possibilities for such cross-linking are evidently greater in $MgCl_2$-condensed chromatin (Figure 1B), and particularly in nuclei (Figure 1C; note the shorter times of cross-linking), than in extended chromatin. Because of competition from cross-linking to the core histones, the kinetics of H5/H5 (H1/H1, etc.) cross-linking in extended and condensed chromatin and nuclei cannot be reliably compared. However, comparison of the polymers produced at early times (e.g., 2 min in Figure 1A and B) when cross-linking to core histones, is negligible, suggests slightly faster cross-linking in $MgCl_2$-condensed than in extended chromatin, possibly due to closer proximity of lysine-containing regions of neighbouring H5(H1) molecules in the higher-order structure.

Under the conditions in Figure 1A, B and C, which were chosen to illustrate the differences between the extents of cross-linking to the core histones in extended and condensed chromatin and nuclei, cross-linked polymers up to $(H5)_5$ etc., were detectable in the gels. Larger polymers could however be generated, e.g., up to $(H5)_9$ etc., in Figure 1D, by serial additions of reagent which presumably offsets reagent hydrolysis.

The cross-linked polymer bands are triplets (particularly evident in Figure 1D where the gel loading is slightly higher) suggesting that each oligomer class consists, in order of decreasing electrophoretic mobility, of H5 homopolymers, H5/H1 heteropolymers and H1 homopolymers, the bands due to the latter being very faint; this is supported by the linearity of semi-log plots of

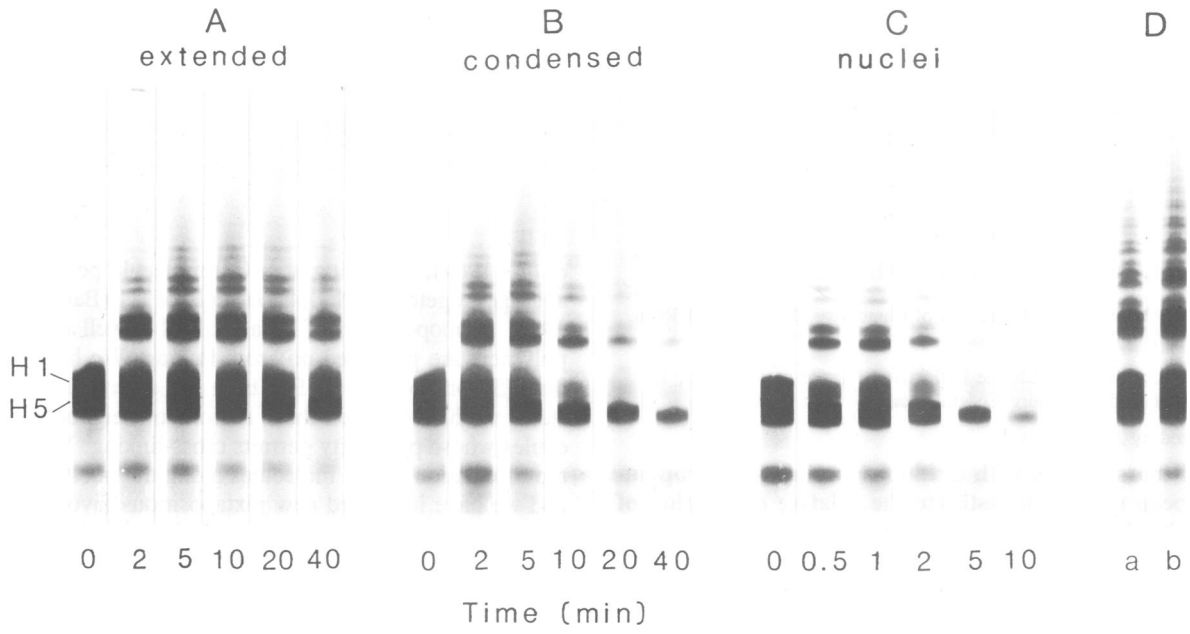

Fig. 1. Cross-linking of H5(H1) in chicken erythrocyte nuclei and extended and condensed chromatin. **(A)** Extended chromatin, **(B)** condensed chromatin and **(C)** nuclei were cross-linked with dithiobis(succinimidyl propionate) (0.5 mg/ml) at 23°C for the times indicated. **(D)** extended chromatin was cross-linked at 23°C by serial additions of reagent (0.2 mg/ml) at 10-min intervals; (a, two additions and b, three additions). H5 and H1 and their cross-linked products were extracted and analysed in 5%-polyacrylamide tube gels. The stain is Coomassie blue.

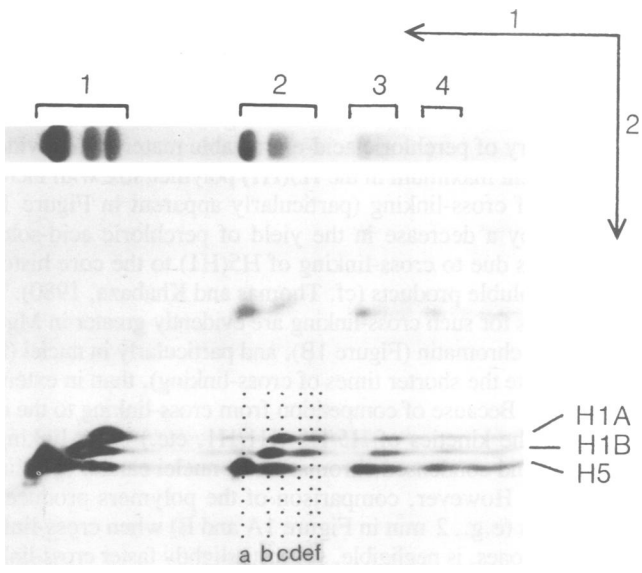

Fig. 2. Analysis of cross-linked dimers of H5 and H1 from chicken erythrocyte nuclei. Nuclei were cross-linked at 23°C with dithiobis(succinimidyl propionate) (0.2 mg/ml) for 10 min at pH 7.5. The H5,H1 were extracted and 15 μg subjected to two-dimensional SDS-polyacrylamide gel analysis. First dimension (no 2-mercaptoethanol), 9% polyacrylamide; second dimension, 18% polyacrylamide; the gel was silver stained. Top, horizontal: a Coomassie blue stained counterpart of the first-dimension gel. Spots lying on the vertical lines arise from components that were initially cross-linked to each other; lines a, b, c, d, e, f therefore indicate the cross-linked dimers $(H5)_2$, H5-H1B, H5-H1A, $(H1B)_2$, H1A-H1B and $(H1A)_2$, respectively.

assumed oligomer mol. wt. versus electrophoretic mobility (not shown). The cross-linked dimers were identified directly by two-dimensional 'diagonal' gel electrophoresis, using the first dimension to resolve the dimers and the second, after thiolysis of the cross-links, to resolve the component histones of the dimers. Figure 2 shows the dimers from condensed nuclear chromatin. The vertically related 'off-diagonal' spots in the second dimension gel arise from the dimers $(H5)_2$, H5-H1B, H5-H1A, $(H1B)_2$, H1A-H1B and $(H1A)_2$ (lines a–f, respectively). The same dimers were obtained from extended chromatin (not shown) and, moreover, from cross-linked dinucleosomes, showing that they genuinely reflect the lysine-rich histone composition of adjacent nucleosomes along the nucleosome filament: in dinucleosomes the possibility of long-range effects is excluded. Evidently H5 and both H1 subtypes, H1A and H1B, are interspersed along the nucleosome filament, rather than segregated into blocks.

The relative proportions of the cross-linked dimers in extended chromatin (where the situation is not appreciably complicated by cross-linking of H1 and H5 to core histones) were determined by densitometry of one-dimensional 9%-polyacrylamide slab gels (not shown), assuming equal staining on a molar basis. The observed proportions $[(H5)_2$:H1-H5:$(H1)_2 = 48:44:8]$ are close to those expected (44.4:44.4:11.1) for a random arrangement of H1 and H5 in a linear array along the nucleosome filament, assuming a 2:1 molar ratio of H5:H1 (Bates and Thomas, 1981) and no differential effect of lysine content [44 lysines in H5 (Briand *et al.*, 1980) and 63 in H1 (Sugarman *et al.*, 1983)]. Similar conclusions about H1 and H5 interspersion were drawn by Pospelov *et al.* (1981) for pigeon erythrocyte nuclei using an approach similar to that described here [although they resolved only $(H5)_2$ homodimers and H1-H5 heterodimers], and by Torres-Martinez and Ruiz-Carrillo (1982) from the H5,H1 content of short oligonucleosomes immunoprecipitated with anti-H5 antibodies.

Nuclei prepared in the usual 'high ionic strength', spermidine/spermine-containing buffer (Hewish and Burgoyne, 1973) and nuclei prepared at low ionic strength (10 mM Tris-HCl) in the presence of 3 mM $MgCl_2$ gave an identical pattern of cross-linked dimers, at least as judged by one-dimensional gel electrophoresis.

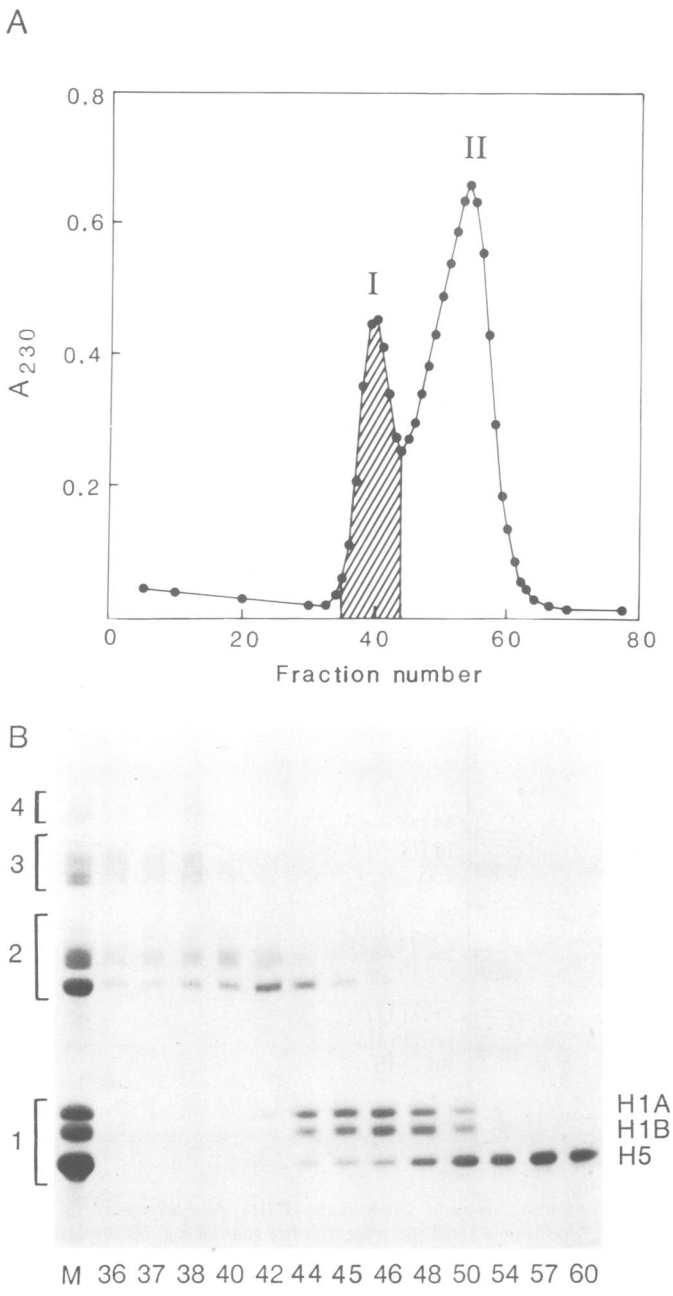

Fig. 3. Isolation of cross-linked oligomers of H5(H1) by gel filtration. **(A)** Chicken erythrocyte nuclei (2000 A_{260} units) were cross-linked at A_{260} = 10 with dithiobis(succinimidyl propionate) (0.2 mg/ml) for 10 min at 23°C. H5 and H1 were extracted, precipitated, redissolved in 10 mM HCl and separated by gel filtration on Sephadex G100; 1 ml fractions were collected. **(B)** SDS-9%-polyacrylamide gel electrophoresis of selected fractions from the Sephadex G100 column. M is the unfractionated mixture.

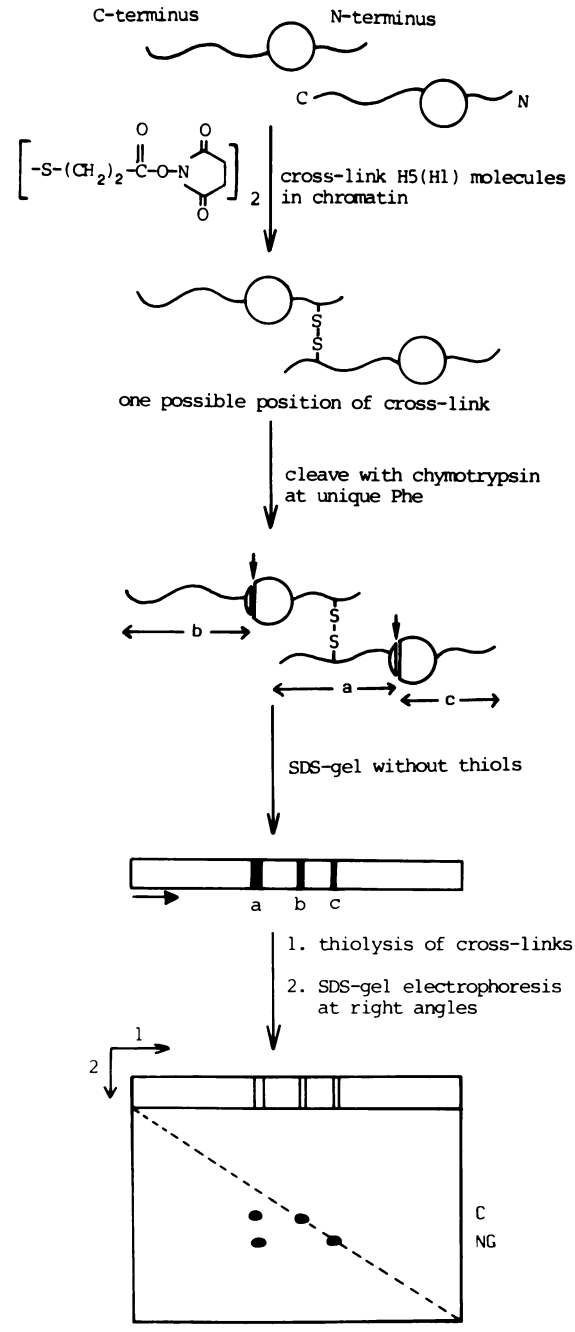

Fig. 4. Strategy for identification of cross-linked domains in H5 (or H1) dimers (see text).

This argues against the possibility that H1 and H5 become randomized between otherwise segregated binding sites during nuclear isolation at elevated ionic strength.

Neighbouring H5(H1) domains in extended and condensed chromatin revealed by dissection of cross-linked products

It is useful to relate the location of the cross-links to the tripartite structure of H5 and H1 (Aviles *et al.*, 1978; Allan *et al.*, 1980), in which a central globular domain (G) of ~80 amino acid residues is flanked by a basic N-terminal region (N) and a longer basic C-terminal region (C). Under certain conditions chymotrypsin will cleave once at the single phenylalanine residue [residue 93 in H5 (Briand *et al.*, 1980) and residue 105 in H1 (Sugarman *et al.*, 1983)] giving two fragments of roughly equal size comprising, respectively, the N-terminal 'half' of the molecule designated NG (i.e., the N-terminal domain, N, together with all but a few residues of the globular domain, G) and the C-terminal 'half' of the molecule, C (Bradbury *et al.*, 1975).

Cross-linked H5 and H1 polymers from nuclei and extended chromatin were separated from residual uncross-linked H5 and H1 by gel filtration (Figure 3), and the entire peak of polymeric material pooled (I in Figure 3A). The strategy for locating cross-links in the N- and C-terminal halves of H5(H1) is outlined in Figure 4 and is similar to that of Nikolaev *et al.* (1981, 1983). The cross-linked products generated with dithiobis(succinimidyl propionate) are digested with chymotrypsin and resolved in an

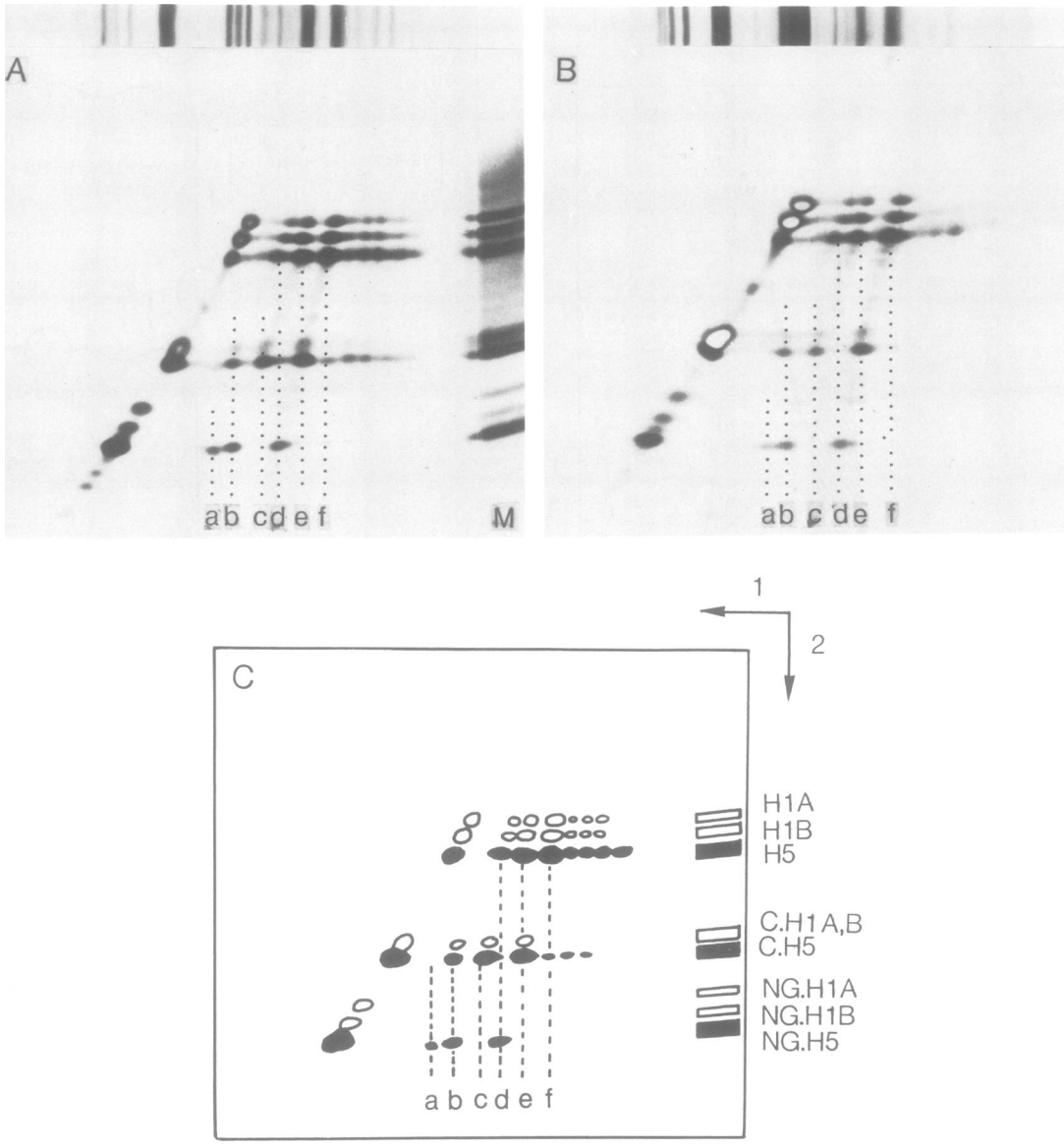

Fig. 5. Analysis of the domains of neighbouring H5 molecules cross-linked in nuclei and extended chromatin. Cross-linked H5(H1) polymers generated by treatment of nuclei or extended chromatin with dithiobis(succinimidyl propionate) at pH 7.5 for 10 min (with the reagent at 0.2 and 0.4 mg/ml, respectively) were isolated by gel filtration, and 14 μg were digested with chymotrypsin for 2 min and analysed by two-dimensional SDS gel electrophoresis (18%-polyacrylamide in both dimensions) as in Figure 4; **(A)** nuclei; **(B)** extended chromatin. The gels were silver stained. Top, horizontal: Coomassie blue stained counterparts of the first-dimension gels. M (applied to the second-dimension gel) is the initial mixture of fragments reduced with 2-mercaptoethanol. The identification of the cross-linked peptides giving rise to spots on vertical lines a – f is given in the text. **(C)** Schematic representation and identification of the peptides in **A**. The solid spots represent peptides that derive from H5; the corresponding H1 peptides migrate just behind them and are particularly visible in the gels for the C-terminal fragments, designated C.H5 and C.H1A,B, respectively, for H5 and H1. (Note that the main differences between H1A and H1B must be in the N-terminal half since two distinct fragments, NG.H1A and NG.H1B, are apparent, whereas the C-terminal fragments, C.H1A and C.H1B, co-migrate.)

SDS-polyacrylamide gel. After thiolysis of cross-links *in situ* the products are resolved in a second-dimension gel. The off-diagonal spots represent products that were initially cross-linked, those lying in a vertical line being cross-linked to each other. Figure 4 shows schematically one of several possibilities, namely cross-linking of two H5(H1) molecules through the N-terminal 'half' of one and the C-terminal 'half' of the other (i.e., NG/C cross-linking).

Figure 5 shows the analysis of the cross-linked products from nuclei in the presence of $MgCl_2$ (A) and extended chromatin (B) at similar low ionic strengths (10 – 15 mM). Cross-linked products that migrate close to H5(H1) in the first dimension, but which give off-diagonal peptides in the second dimension, represent two half-molecules cross-linked together. The components of such cross-linked products from H5 lie on the vertical lines labelled a, b and c in Figures 5A, B (for clarity the corresponding weaker H1 peptides are ignored). They have the same electrophoretic mobility as purified, characterised NG and C fragments of H5 (see Materials and methods) designated NG.H5 and C.H5 and arise from NG/NG, NG/C and C/C cross-linking, respectively. The other major off-diagonal peptides (lines d – f) arise, respectively (see Figure 5C), from H5/NG.H5, H5/C.H5 and $(H5)_2$, all products of incomplete cleavage by chymotrypsin. (Note that despite their very similar molecular masses, the very basic C fragment migrates much more slowly than the NG fragment, presumably because of the much higher intrinsic positive charge

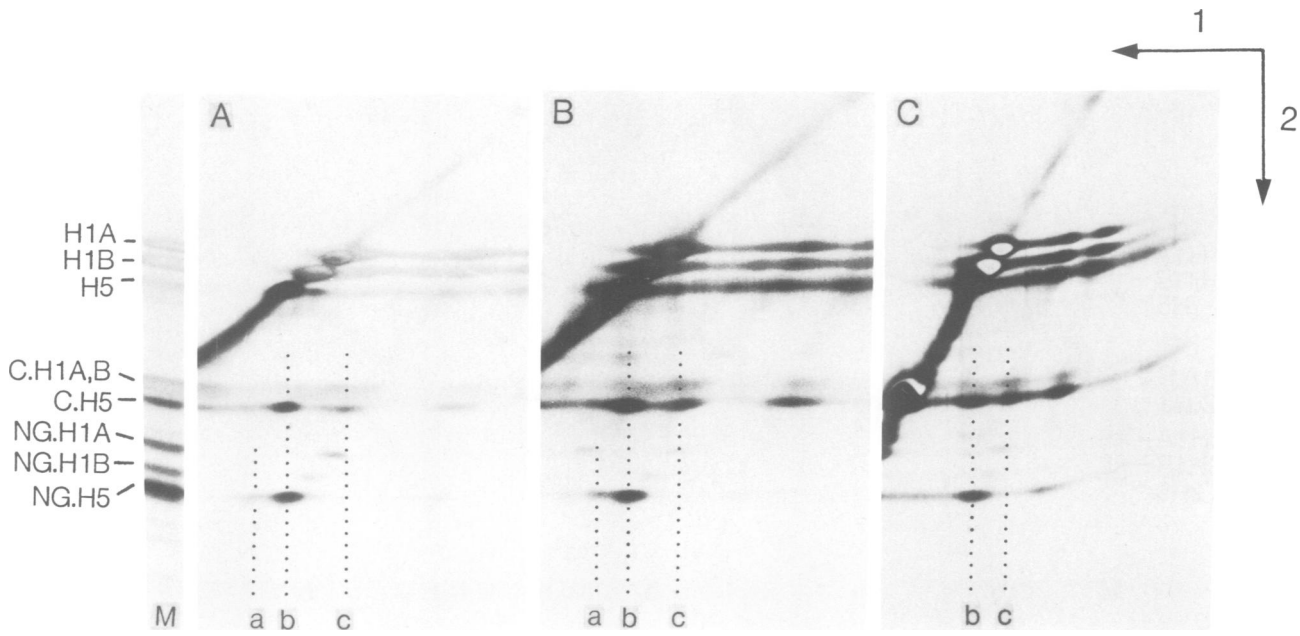

Fig. 6. Analysis of the domains of H5(H1) cross-linked in di-, tri- and tetranucleosomes. H5 and H1 were extracted from **(A)** dinucleosomes, **(B)** trinucleosomes, **(C)** tetranucleosomes, after cross-linking at A_{260} ~2 with dithiobis(succinimidyl propionate) (three treatments of 0.2 mg/ml for 10 min each at 23°C) under the low ionic strength conditions for extended chromatin, and analysed without gel filtration. The procedure was otherwise as in the legend to Figure 5, except that a total of 40 μg of material was used for each two-dimensional analysis. Only the regions of interest of the gels are shown for clarity. Vertical lines a, b, c are as in Figure 5; they indicate the products of NG/NG, NG/C and C/C cross-linking, respectively, for H5 molecules. The large amount of material on the diagonal is due to digestion products of monomeric H5 and H1 which were still present since the gel filtration step had been omitted (see text). M (applied to the second-dimension gel) is the initial mixture of chymotryptic fragments corresponding to **(A)**, reduced with 2-mercaptoethanol.

on C; NG and C also do not silver-stain equally on a molar basis, making quantification of their relative amounts difficult.)

Although the two-dimensional patterns resulting from cross-linked extended chromatin (Figure 5B) and condensed nuclear chromatin (Figure 5A) are broadly similar, there are distinct and reproducible differences. In particular, in extended chromatin the spots lying on the vertical lines a and c are weaker relative to those on line b, indicating less NG/NG and C/C cross-linking relative to NG/C cross-linking. This suggests that the transition from extended to condensed chromatin results in (or may be caused by) a bringing together of C-terminal domains of H5(H1), as well as of N-terminal domains.

In a simple model that would account for a predominance of NG/C cross-linking in extended chromatin, H5 molecules on successive nucleosomes along the nucleosome filament are arranged in a polar, head-to-tail manner represented as NG-C NG-C NG-C, etc. In its simplest form, however, such an arrangement, which had already been noted as a possibility (Hayashi *et al.*, 1978), would not predict NG/NG or C/C cross-linking. That this is observed might be a consequence of the inherent flexibility of the nucleosome filament, resulting in random collisions between distant parts. In order to preclude such effects at a distance, short oligonucleosomes were examined, as described in the next section.

Neighbouring H5(H1) domains in short oligonucleosomes

Cross-linking between the two H5(H1) molecules in a dinucleosome should simply reflect the relative orientation of the two molecules, with no possibility of effects at a distance. Figure 6A shows the two-dimensional analysis of the H5(H1) molecules cross-linked and cleaved as described above for H5(H1) from nuclei and extended chromatin. The analysis in this case was carried out on the total H5(H1) fraction after cross-linking, because of the losses incurred in isolating cross-linked dimers on the necessary (small) scale from dinucleosomes. Figure 6A shows that the predominant cross-linking in dinucleosomes is NG/C, consistent with a polar arrangement of H5 molecules along the nucleosome filament. C/C cross-linking, which is also detectable, is substantially less than NG/C cross-linking, as shown by comparison of the intensities of the C-spots in the two cases: equimolar amounts of C/C and NG/C cross-linked products would give C-spots in the ratio 2:1. The two-dimensional pattern of cross-linked products was insensitive to the extent of the initial nuclease digestion used to prepare dinucleosomes from nuclei (not shown), arguing against the possibility that the arrangement of H5 molecules in dinucleosomes may not be representative of that along the entire nucleosome filament, e.g., because of selective cleavage of certain classes of linker.

Cross-linking between NG and C regions of adjacent H5(H1) molecules was, as expected of a polar arrangement, also favoured in trinucleosomes and tetranucleosomes (Figure 6B, C, vertical line b). Surprisingly, however, in these cases C/C cross-linking (vertical line c) was appreciably stronger relative to NG/C cross-linking than in dinucleosomes. One possibility is that there is heterogeneity of H5-H5 contacts along the nucleosome filament, in a regular or irregular manner; this would be hard to determine. Alternatively, the arrangement of H5(H1) molecules is indeed regular and polar, but cross-linking of C-terminal regions is possible, and occurs with increasing ease as the oligomer length increases. This might be due to conformations taken up by the oligomers in solution that are not truly extended (even 'low' ionic strength is 10 – 15 mM); or perhaps the C-terminal 'tails' of the H5(H1) molecules on the terminal nucleosomes are relatively free because the terminal linkers were 'trimmed' during the initial

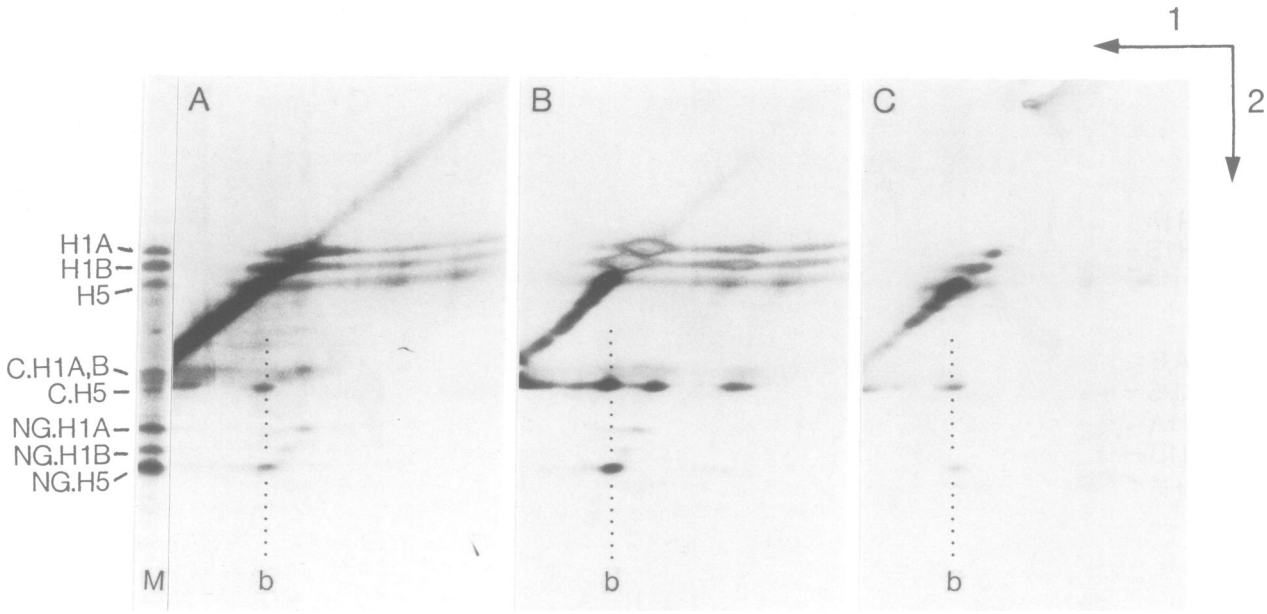

Fig. 7. Estimation of intramolecular H5 cross-linking in mononucleosomes **(A)** and short oligonucleosomes **(B,C)**. **(A)** Mononucleosomes (A_{260} = 1) at low ionic strength were treated with dithiobis(succinimidyl propionate) (three additions of 0.2 mg/ml at 10-min intervals) and the H5 and H1 extracted. A sample (35 μg) was digested with chymotrypsin and analysed by two-dimensional gel electrophoresis as in the legend to Figure 6. The weak products on the vertical dotted line b (designation as in Figures 5 and 6) indicate intramolecular NG/C cross-linking. M (applied to the second-dimension gel) is the initial mixture of chymotryptic fragments, reduced with 2-mercaptoethanol. **(B,C)** Pooled short oligomers of chromatin (di-, tri- and tetranucleosomes) were cross-linked as described in **A** and the total H5(H1) was extracted, dissolved in 10 mM HCl, 20 mM NaCl and fractionated on a column of Sephadex G100 Superfine (1.6 × 52 cm). The monomeric and polymeric material after gel filtration (cf. Figure 3A) were digested with chymotrypsin and analysed (15 μg and 40 μg, respectively) by two-dimensional SDS gel electrophoresis. **(B)** Cross-linked polymers; **(C)** material remaining monomeric after treatment with cross-linking reagent. The relative loadings in **B** and **C** were chosen to give roughly equal amounts of H5 (since the H5:H1 ratio is greater in monomer than in polymer).

oligonucleosome preparation by micrococcal nuclease digestion. In all three cases (di-, tri- and tetranucleosomes) NG/NG cross-linking (vertical line a) was at best barely detectable.

What is the contribution of intramolecular cross-linking to the observed NG/C cross-linking?

The analysis of neighbouring domains described here is concerned with cross-linked fragments, obtained by chymotryptic digestion of cross-linked H5 dimers, whose combined molecular mass is similar to that of intact H5. Where dimers have been separated from monomers (and other products) after cross-linking (e.g., in the case of nuclei and chromatin, Figure 5A, B), the interpretation of the results is unambiguous. However, if residual monomers are present in the analysis of cross-links, which is the case when the gel filtration purification of the cross-linked dimers is omitted (e.g., for small oligonucleosomes; Figure 6), the possibility arises that the monomers contain intramolecular NG/C cross-links and continue to migrate with the molecular mass of monomers after chymotryptic digestion. Analysis of the residual monomeric H5 that eluted from the gel filtration column during isolation of cross-linked H5 dimers from nuclei (Figure 3, peak II) revealed no NG/C intramolecular cross-linking in this case (not shown). However it seemed possible that, in short oligonucleosomes, trimming of terminal linkers might lead to unconstrained H5(H1) tails, perhaps increasing the likelihood of intramolecular cross-linking.

The extent of intramolecular cross-linking in short olignucleosomes was investigated in two ways. On the one hand, mononucleosomes – as an extreme case – were treated with cross-linking reagent, and the extracted H5(H1) digested with chymotrypsin and analysed by the usual two-dimensional procedure (Figure 7A). Some intramolecular NG/C cross-linking was apparent (vertical line b), but far less than that observed in dinucleosomes, etc. (Figure 6). In a second approach, the monomeric and the polymeric H5(H1) after cross-linking of pooled di-, tri- and tetranucleosomes were separated by gel filtration and analysed in parallel (Figure 7B and C). It was clear that the majority of NG/C cross-links in H5(H1) dimers (Figure 7B, vertical line b) were likely to be intermolecular rather than intramolecular, since little NG/C cross-linking was found in the monomer (Figure 7C, vertical line b) where intramolecular cross-linking is the only possibility. Thus, since NG/C is the major mode of cross-linking between neighbouring H5 molecules in short oligonucleosomes, and at ionic strengths at which they ought to be in a relatively extended conformation, we conclude that H5 molecules in chicken erythrocyte chromatin are probably arranged along the nucleosome filament in a polar, head-to-tail manner.

Discussion

The cross-linking studies described here show that H5 and H1 molecules in chicken erythrocyte chromatin exist in linear arrays along the nucleosome filament, close enough to be cross-linked with dithiobis(succinimidyl propionate). The length of the arrays is not easy to determine from the length of the cross-linked polymers since these fail to reach a true maximum due to competing cross-linking to the core histones (cf. Thomas and Khabaza, 1980), which is more apparent in condensed than in extended chromatin. The nature of the cross-linked dimers and their relative amounts in extended chromatin correlate well with those expected for a generally random interspersion of nucleosomes along the nucleosome filament, each having one H5 or H1. However, since H5 and H1 together add up to 1.3 molecules/nucleosome in

chicken erythrocyte nuclei and chromatin (0.9 molecule of H5 and 0.4 molecule of H1 per nucleosome on average, Bates and Thomas, 1981), up to about a half of the nucleosomes might contain H1 as well as H5 (cf. Nelson *et al.*, 1979; Bates and Thomas, 1981); alternatively, but perhaps less likely, some nucleosomes might bind two H5 and others two H1 molecules. All that can be said with certainty is that H5 and H1 are not segregated in blocks in chicken erythrocyte chromatin.

The major part of this study was concerned with elucidation of the contacts between H5 molecules in chicken erythrocyte chromatin in the extended state (the nucleosome filament) and in the condensed state (the solenoid) with the aim of identifying contacts that might be unique to the higher-order structure. First, detailed dissection of cross-linked H5 dimers from extended chromatin and dinucleosomes revealed a polarity in the contacts between successive H5 molecules along the nucleosome filament, which appear to be arranged head-to-tail or NG-C NG-C NG-C, etc., where NG and C each represent about half of the molecule.

Secondly, when the nucleosome filament is folded into a higher-order structure under condensing conditions, the folding is such that the C-terminal regions on different H5 molecules are now closer together. This would indeed account for the slightly increased rate of cross-linking in condensed chromatin (Figure 1 and see text). NG regions are also detectably closer together in the condensed state. Less prominent cross-linking between these regions than between the C-regions may simply reflect the relative lysine contents of NG and C ($\sim$1:3); if cross-linking were determined (solely) by lysine content then C/C, NG/C and NG/NG cross-linked products would occur in the ratio 9:6:1.

The central globular domain (G) of H1(H5) appears to seal the two turns of DNA around the octamer (Allan *et al.*, 1980), but the location of the flanking N- and C-terminal basic regions is less well defined. A likely possibility is that one or both bind to the linker DNA. If the linkers are then brought together in the central hole of the solenoid which is generated by folding the nucleosome filament (Finch and Klug, 1976; Thoma *et al.*, 1979; Widom and Klug, 1985), as has been suggested (Pearson *et al.*, 1983), and if, e.g., the H5 C-terminal regions remain bound to them, the observed increased C/C cross-linking in condensed chromatin would indeed be expected. However, other juxtapositions of C-terminal regions in the solenoid can also be envisaged.

The nature of the NG/NG cross-linking that occurs to a rather greater extent in the condensed than in the extended state is at present incompletely defined. Our method of analysis, in which the H5 molecule is cleaved only once, between the NG and C regions, does not distinguish between G/G, N/N and G/N cross-linking. Tryptic excision of the N- and C-terminal tails of H5 after cross-linking indicates some G/G cross-linking (A.C.Lennard and J.O.Thomas, unpublished observations) but the extent to which this contributes to the overall NG/NG cross-linking is as yet unclear. We can therefore say nothing from these results about interactions between the globular regions of H5 as the means of providing the necessary specificity for folding of the nucleosome filament into a solenoid (cf. the H1-helix of Thoma *et al.*, 1979), although such a mechanism is both attractive and plausible.

Previous studies of the relative orientation of H1 molecules in chromatin

While this work was in progress, Nikolaev *et al.* (1981, 1983) reported studies of the arrangement of H1 molecules in calf thymus chromatin using the reagent methyl 4-mercaptobutyrimidate in a two-stage cross-linking procedure. Cross-linking was found between all combinations of the N- and C-terminal 'halves' of H1 generated by chymotryptic cleavage, both in nuclei and in extended chromatin, and moreover in the presence of 8 M urea; however, short oligonucleosomes (e.g., dinucleosomes) were not examined, nor was the contribution of intramolecular NG/C cross-linking directly determined. Although the relative amounts of C/C and NG/C (or in their terminology N/C) cross-linking is hard to assess from the studies of Nikolaev *et al.* (1981, 1983) because of poor resolution in the relevant region of the two-dimensional gels, there appears to be considerably less NG/C cross-linking, relative to C/C, than we observe, especially in extended chromatin (Figure 1A of Nikolaev *et al.*, 1983). Whether this has anything to do with the use of a different cross-linking reagent or a somewhat higher pH (7.9 rather than 7.5), or with the use of chromatin from a different source is not clear; the latter seems unlikely since we obtained the same results for H1 in rat liver chromatin (not shown) as described here for H5 in chicken erythrocyte chromatin. The pronounced formation of C-C cross-links in 8 M urea (Nikolaev *et al.*, 1983) is not easily reconciled with our conclusions that C/C cross-linking is promoted by chromatin condensation; however, the relevance of structures in 8 M urea, which denatures the histones but tolerates electrostatic interactions with DNA, is unclear.

Ring and Cole (1983) analysed H1-H1 cross-links formed in nuclei with two cross-linking reagents of different lengths, and although cross-linking between amino-terminal regions was not detected, strong cross-linking was found between the carboxy-terminal regions and between the amino-terminal and carboxy-terminal portions of neighbouring H1 molecules, in agreement with our results and those of Nikolaev *et al.* (1981).

Materials and methods

Isolation of nuclei and chromatin, and extraction of H1 and H5

Chicken erythrocyte nuclei were isolated, and chromatin prepared by micrococcal nuclease digestion and fractionated in sucrose gradients as described previously (Thomas and Rees, 1983).

H1 and H5 were extracted from long chromatin or from pelleted nuclei with 5% (v/v) perchloric acid (Johns, 1964), precipitated with 25% (w/v) trichloroacetic acid and washed with acetone/HCl and acetone as already described (Thomas and Khabaza, 1980). The mixture was applied to a column of carboxymethylcellulose (Whatman CM52) in 10 mM Na phosphate, 0.25 mM PMSF, pH 7.0, and H1 and H5 were eluted with a linear gradient (0.3 – 0.75 M NaCl in the same buffer), after first washing the column with buffer containing 0.3 M NaCl. H1 eluted before H5, as indicated by A_{230} and SDS-polyacrylamide gel electrophoresis of column fractions. Pooled fractions were dialysed against 10 mM HCl and stored at −20°C.

Isolation and identification of the chymotryptic cleavage products of H1 and H5

H1 and H5, isolated as described above, were digested with chymotrypsin (1:500 w/w) in 50 mM Tris-HCl, pH 6.5, at 37°C. After $\sim$2 min (exact time determined by SDS gel electrophoresis) the H1 and H5 had been extensively digested and there was minimal further degradation of the N-terminal fragment. The products were precipitated with 25% (w/v) trichloroacetic acid, washed as described above, dissolved in 10 mM Na phosphate, 0.25 mM PMSF, pH 7.0 and applied to a carboxymethylcellulose (Whatman CM52) column in the same buffer. The N- and C-terminal 'halves' were eluted, in that order, with a gradient of 0.1 – 1.0 M NaCl. The effluent was monitored by absorbance at 230 nm and by SDS gel electrophoresis. The material in the pooled fractions was precipitated with 25% (w/v) trichloroacetic acid, redissolved in 10 mM HCl and gel filtered through Sephadex G100 (2 × 58 cm) in 10 mM HCl (4 ml/h). Fractions selected on the basis of SDS gel electrophoresis were stored at −20°C and characterized by amino acid analysis and limited amino acid sequence determination.

Generation, isolation and fractionation of cross-linked H1 and H5

Nuclei were twice pelleted and resuspended in 0.34 M sucrose, 5 mM triethanolamine/HCl, 3 mM $MgCl_2$, 0.25 mM PMSF, pH 7.5, at a final A_{260} $\sim$6. Long chromatin was diluted from a concentrated solution (A_{260} >50) in 0.2 mM Na_2EDTA (Noll *et al.*, 1975) into 5 mM triethanolamine/HCl, 0.25 mM PMSF

containing either 0.2 mM Na_2EDTA (for extended chromatin) or 0.3 mM $MgCl_2$ (condensed chromatin) to give a final $A_{260} \sim 5$. Short oligonucleosomes were dialysed into the buffer used for extended chromatin. Cross-linking was carried out at 23°C by addition of dithiobis(succinimidyl propionate) (Pierce) from a concentrated solution (50 mg/ml) in dimethylformamide to a final concentration, and for times specified in the relevant figure legends. For short oligomers (mono- to tetranucleosomes), serial additions were made to increase the extent of cross-linking. The reaction was terminated with 25 mM glycine buffered to pH 8 with triethanolamine, and residual monomeric and cross-linked H1 and H5 were extracted with 5% (v/v) perchloric acid and precipitated with trichloroacetic acid as described above.

Cross-linked and monomeric material were separated by gel filtration through a column of Sephadex G100 (2 × 58 cm) in 10 mM HCl, pumped at 4 ml/h. The absorbance was monitored at 230 nm and the fractions analysed by SDS-polyacrylamide gel electrophoresis. Cross-linked polymers (dimers and larger) and monomeric H1 and H5 emerged as two peaks (see Figure 3). They were precipitated with trichloroacetic acid and washed as above.

Chymotryptic cleavage of cross-linked H5(H1)

The cross-linked H5(H1) mixture was dissolved in 50 mM Tris-HCl pH 6.5 at 0.2 mg/ml (based on A_{230} = 1.85 for 1 mg/ml H1 or H5; Camerini-Otero *et al.*, 1976) and digested at 37°C with α-chymotrypsin (EC 3.4.21.1; Sigma, 3 × crystallised, type 1-5) at a ratio of 1:500 (w/w) enzyme:histone. Digestion times suitable for the purpose in hand (see figure legends) were determined by monitoring the course of digestion by electrophoresis in SDS 18%-polyacrylamide gels (2 mM PMSF was used to stop the reaction at different times). Digestion times were chosen such that the N-terminal fragment (NG), comprising the N-terminal 'half' of H5(H1), was not appreciably degraded further. The samples were then subjected to two-dimensional ('diagonal') gel electrophoresis to determine the composition of the cross-linked products (see below).

SDS-polyacrylamide gel electrophoresis

Electrophoresis in 5% polyacrylamide tube gels and 18% polyacrylamide slab gels was as described previously (Thomas and Kornberg, 1978); 2-mercaptoethanol was omitted from the 'sample buffer' for cross-linked samples. The procedure for two-dimensional (diagonal) gels for cross-linked material was as described (Thomas and Kornberg, 1978) except that the reduction step was carried out at pH 8.8 rather than 6.8, and the gel strip then re-equilibrated at pH 6.8 before electrophoresis in the second dimension. 9% (w/v) polyacrylamide slab gels containing 0.24% (w/v) bisacrylamide were otherwise prepared and run as for the 18% polyacrylamide gels. Gels were either fixed and stained with Coomassie brilliant blue R-250 (Thomas and Kornberg, 1978), or silver stained by the method of Wray *et al.* (1981) as indicated in the figure legends.

Miscellaneous procedures

Limited N-terminal amino acid sequence determination was carried out by the DABITC method (Chang *et al.*, 1978). Amino acid analysis was carried out using an LKB 4400 amino acid analyser, after hydrolysis in 6 M HCl-0.1% (w/v) phenol (Sanger and Thompson, 1963) for 24 h *in vacuo* at 105°C.

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