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Release of Discrete Subunits after Nuclease and Trypsin Digestion of Chromatin

(chromatin electrophoresis/histone complexes/iodination/peptide mapping)

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ABSTRACT **Digestion of chromatin with DNase (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) releases 11–12S nucleoprotein particles. After extensive nuclease digestion, the DNA in these particles consists of a collection of eight discrete DNA fragments. When these nuclease-particles are treated with trypsin (EC 3.4.21.4), only 20 to 30 amino-acid residues are cleaved from histone N-terminals, the histone C-terminal segments being resistant. The resulting 5S nucleoprotein particles have now been shown on acrylamide gels to consist of a series of eight discrete DNA-containing bands. Four of these bands contain C-terminal cleavage fragments from four histones (III, IV, IIb2, and IIb1) tightly bound to them; a fifth contains fragments from only histones III and IV. The remaining three bands contain only DNA. Since these protein-free DNA bands were resistant to nuclease prior to trypsin treatment, they were presumably associated with histone N-terminal segments in the native structure. Trypsin, therefore, appears to split nuclease-particles, releasing two subfractions of DNA—one associated with protein, the other not. The data is compatible with a model in which the majority of DNA in the eukaryotic nucleus is folded into hairpin loops of double-stranded helix, each created by the concerted *cross-linking* action of 6 to 10 histones which interact to form a trypsin-resistant complex composed, for the most part, of all four major histones. These loops may further fold upon themselves to form the "nu" bodies that have been visualized by electron microscopy.**

The DNA in chromatin is digested by staphylococcal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) into eight discrete, limit-digest DNA fragments (1, 2), which range in size between 45 and 145 base pairs. Yet, partial staphylococcal nuclease digestion results in the appearance of transient DNA fragments of 200 base pairs or integer multiples of that size (3, 4). A reasonable interpretation of these findings is that these larger transient segments are generated by a regular arrangement of very accessible, DNase-sensitive sites, while the eight bands of the limit-digest are generated by the further digestion of less accessible but discrete sites within each 200-base-pair repeat. Recent electron microscope data has shown that "bead-like" structures occur at regular intervals of about 200 base pairs along the extended chromosome fiber (5). It is thus tempting to consider that the initial nuclease cut is between these so-called "nu" bodies (5), and that further digestion introduces a limited number of specific cuts within each "nu" body.

Exhaustive treatment of chick erythrocyte chromatin with trypsin (EC 3.4.21.4) results in the complete digestion of histones I and V and the cleavage of only 20 to 30 amino-acid residues from the positively charged N-terminal parts of histones III, IV, IIb2, and (possibly) IIb1 (2). In this communication I describe and analyze a series of discrete sub-chromosomal particles generated after trypsin cleavage of nuclease-treated chromatin. The results indicate that the majority of DNA in the cell behaves as if it were crosslinked by histones.

Abbreviations: ST, produced by treatment with staphylococcal nuclease followed by trypsin; TS, trypsin followed by nuclease.

RESULTS

Sub-Chromosomal Particles Obtained after Trypsin Digestion of Nuclease-Treated Chromatin. Sahasrabuddhe and Van Holde (6) and Noll (4) have shown that treatment of chromatin with staphylococcal nuclease releases as a major product a highly condensed 11–12S sub-chromosomal particle containing both DNA and protein. When this particle is treated with trypsin, it sediments at about 5–6 S (6). I have confirmed these observations. Moreover, the pattern of trypsin digestion of the histones from nuclease-digested chromatin is indistinguishable from that obtained with intact chromatin (2). Thus, with progressive trypsin digestion, histones I and V are first completely digested, followed by the removal of 20 to 30 amino acids from the N-termini of histones III and IV and then by the removal of about 20 to 30 amino acids from histones IIb2 and IIb1. The transition of the 11S nuclease-particle to a 5S species occurs when the N-termini of histones III and IV are cleaved by trypsin. *This indicates that at a minimum the integrity of histones III and IV is required for complete folding of each nuclease-particle.*

I have now extended these observations by analyzing the trypsin digest of nuclease-treated chromatin by electrophoresis through polyacrylamide gels. Chromatin was extensively digested with nuclease and the reaction stopped by adding EDTA to 2 mM. The resulting 11S particles were then treated with increasing concentrations of trypsin and the products were loaded directly onto 6% acrylamide gels (7). After electrophoresis, the DNA was stained with ethidium bromide. Fig. 1 shows the complex pattern obtained by treating the nuclease-particles with increasing concentrations of trypsin. I refer to these sub-chromosomal particles as "ST" particles (for staph followed by trypsin). Before trypsin treatment, the 11S nuclease-particles migrate only marginally into these polyacrylamide gels, probably because they have essentially no net charge. With the removal of histones I and V by trypsin, three discrete slowly migrating, negatively charged bands appear. (When these three bands are run in a second dimension corresponding to a 15% sodium dodecyl sulfate/acrylamide gel, each is seen to contain all histones but I and V in approximately equal proportions.) At high trypsin concentrations, a very stable and reproducible pattern of eight main, sub-chromosomal bands is observed in polyacrylamide gels stained with ethidium bromide. *The same pattern is obtained after trypsin treatment of nuclei that have*

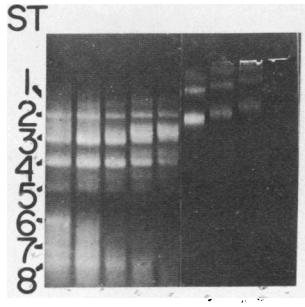

FIG. 1. Sub-chromosomal particles generated by trypsinization of nuclease-digested chromatin. Chromatin (A_{260} = 10) was digested for 30 min at 37° with staphylococcal nuclease in 5 mM Na-phosphate, pH 6.8, 25 μM $CaCl_2$ and the reaction was stopped by the addition of EDTA to 2 mM. Increasing concentrations of trypsin (μg/ml) were added for 30 min at 37° (right to left: 0, 0.5, 1.0, 2.0, 5.0, 25, 50, 100, 300). The sample was then loaded directly on 6% acrylamide gels containing *no* sodium dodecyl sulfate. The gels were stained with 2 μg/ml of ethidium bromide in water and photographed through a red filter. The bands from top to bottom have been defined as "ST" particles (for staph-tryp particles) and are numbered ST-1 to ST-8 in order of increasing mobility. Although it is not mentioned in the *text*, our gels show a faint continuous background of ethidium bromide stained material, particularly in the region above ST-1. This heterogeneous population of DNA molecules may or may not originate from sub-structures similar to the more rigidly defined structures observed as discrete bands, ST-1 through ST-8. Note, moreover, that "ST-3" is clearly heterogeneous. Only ST-1, 2, 3, 4, and 5 stain with Coomassie blue. The fact that the trypsin-resistant histone C-terminal fragments run with the DNA in these gels demonstrates that they are tightly bound, since, being positively charged, they would otherwise migrate in the opposite direction.

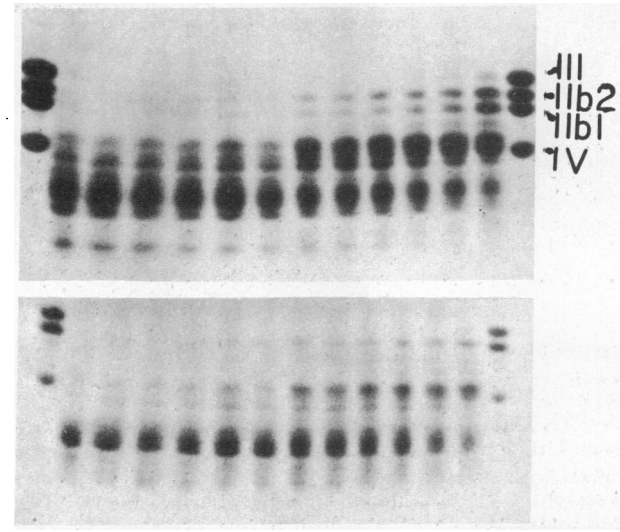

FIG. 2. Preservation of [^{35}S] methionine in histones during tryptic digestion of chromatin. Four day erythroblasts were labeled *in vitro* with [^{35}S]methionine as previously described (2). Chromatin was isolated and digested with increasing concentrations of trypsin for 30 min at 37°. One volume of 1% sodium dodecyl sulfate, 2% 2-mercaptoethanol was added and 20 μl aliquots were loaded directly onto 15% dodecyl sulfate/acrylamide gels (2). The upper panel shows the stained gel. The lower panel shows an autoradiograph of the same gel. Note that IIb1 contains no methionine. The differences in density between corresponding areas of the stained gel and areas of the autoradiograph probably reflect positions where IIb1 limit-digest histones are present. The concentrations of trypsin (μg/ml) from right to left are: 0, 5, 10, 25, 50, 75, 100, 200, 250, 300, 400, 500, 0. The small amount of radioactivity migrating near the tracking dye is digested very early and probably comes from non-histone proteins. The amount of methionine in III and IIb2 is about the same, but twice that in IV, indicating the fact that IV has only 1 methionine and III and IIb2 each contain two methionine residues. No methionine is detected in histone I by this procedure.

been digested with nuclease. Of these eight DNA-containing bands, the top five stain with Coomassie blue, indicating that they also contain protein, whereas the bottom three bands do not. Thus, *both protein-associated and protein-free DNA fragments are generated by treating 11S nuclease-particles with trypsin.* This finding has been confirmed by banding these ST particles on CsCl equilibrium gradients after irreversible fixation with formaldehyde (unpublished observations). The three protein-free DNA particles (ST-6, ST-7, and ST-8) are released after different extents of trypsin treatment; ST-8 appears first, followed by ST-7, and finally by ST-6.

Origin of the Protein-Free ST Particles. The results presented here show that when nuclease-treated particles are digested with trypsin, discrete DNA-containing particles are obtained on acrylamide gels. Some of these ST particles contain histone and some do not. Since trypsin cleaves associated chromosomal histones at their N-termini, leaving the C-terminal sections intact (2), the simplest explanation for the generation of protein-free DNA fragments among the ST particles is that they were formerly associated with the N-termini of the digested histones, while the protein-containing ST particles remain associated with histone C-termini (see below). (After removal of histones I and V by limited trypsin treatment or with 0.5 M NaCl, the sensitivity of the depleted chromatin to nuclease is unchanged. Moreover, all of the DNA-containing bands seen in polyacrylamide gel electrophoresis for both the nuclease limit-digest and the ST particles are preserved; in addition, reconstitution of chromatin in the absence of histone I and V results in the same ST pattern as shown in Fig. 1. Since the generation of the ST pattern is, therefore, independent of the presence of histone I and V, the cleavage of these histones is unlikely to be the source of the protein-free, ST-DNA.)

An alternate explanation for the generation of protein-free ST particles is that, after trypsin treatment, histone C-terminal fragments are released from *some* nuclease-particles, leaving protein-free DNA fragments behind. We have looked for such released polypeptides by reversing the polarity used for gel electrophoresis of the ST particles. (Being positively charged, these protein fragments would migrate in the opposite direction from ST particles.) No large molecular weight histone fragments were observed to migrate into the gel or remain at the origin when the polarity of the electrophoresis was reversed. Consequently, the large histone fragments are probably not released from nuclease-particles, consistent with previous sedimentation data (2, 6, 8). We have also tested the possibility that the protein-free ST fragments are generated from a sub-population of histones that are completely digested by trypsin. In order to estimate the percentage of histones that are completely digested by trypsin, erythroblasts were labeled with [^{35}S]methionine. Chromatin was isolated, digested with increasing concentrations of trypsin, and loaded

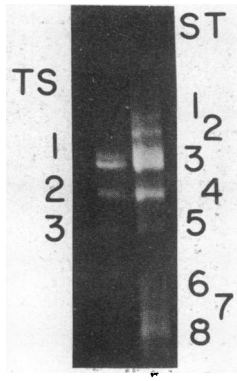

FIG. 3. Correspondence between TS particles and ST particles. To generate TS particles, chromatin (A_{260} = 20) was digested with trypsin (300 μg/ml; 30 min; 37°), the reaction was stopped with trypsin inhibitor, and the sensitive DNA was digested with nuclease as described in the legend to Fig. 1. ST particles were generated as described in the legend to Fig. 1. The ST and TS particles were loaded without fixation onto 6% acrylamide gels and run for 1.5 hr at 150 V, in a slab apparatus. The gels were then stained with ethidium bromide. Staining of the TS particles with Coomassie blue showed that all particles contained protein. Note that as ST-3 is heterogeneous, so is TS-1.

directly onto 15% sodium dodecyl sulfate/acrylamide gels (2). Fig. 2 shows the stained pattern of histones and histone cleavage products as well as an autoradiograph of the same gel. The amount of radioactivity associated with the undigested histone controls (III, IIb2, and IV, since I and IIb1 have no methionine) was determined by eluting the protein from the gel and comparing the radioactivity to the amount associated with the high-molecular-weight trypsin-resistant cleavage products. (Material that ran within 2 cm of the tracking dye was not included.) From three separate experiments done in duplicate, the amount of radioactivity lost from histone was no greater than 9 ± 4%. Since methionine is located only in the C-terminal part of three major histones, complete degradation of a significant sub-population of histones appears very unlikely.

Are Protein Rearrangements Induced by the Nuclease Treatment? In order to test the possibility that proteins rearrange by sliding during the nuclease digestion, the enzyme treatments were reversed, i.e., chromatin was first treated with maximal level of trypsin and then with nuclease (generating trypsin-staph particles, denoted as "TS"). Fig. 3 demonstrates that to a great extent the same particles are obtained and thus that symmetry is conserved. As would be expected, the free DNA defined by ST-6, 7, and 8 is absent from the T-S pattern. Moreover, TS-1, 2, and 3 correspond to ST-3, 4, and 5, respectively. It, therefore, seems likely that these particles are present in the native chromatin, and not induced by histone rearrangements during nuclease digestion, since the histone C-terminals would be unlikely to rearrange identically to whole histones.

However, bands corresponding to ST-1 and 2 are missing from the TS particles. Since they stain with Coomassie blue and, therefore, contain protein, ST-1 and 2 would be expected to be present when the enzyme treatments are reversed. These particles represent about 10% of the total DNA. Some type of rearrangement could be occurring here; however, I think that this is unlikely for the following reason: When ST-1 and 2 are eluted from a gel with 5 mM Na-phosphate, pH 6.8, and then digested again with nuclease in the presence

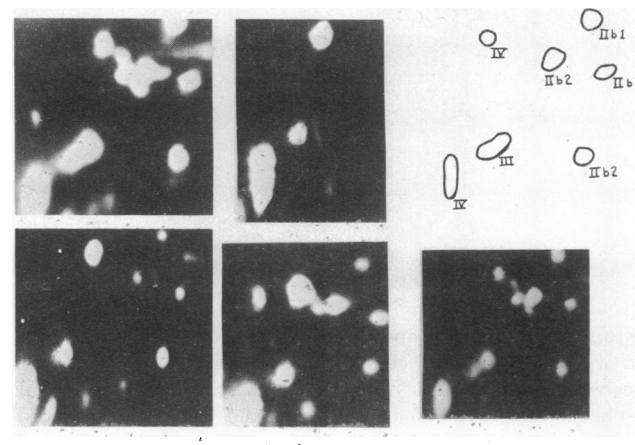

FIG. 4. Tryptic fingerprints of the iodinatable peptides in ST particles. Iodination with ^{125}I and the mapping procedures have been described previously (2). pH 3.5 electrophoresis was from left to right. Chromatography was from top to bottom. The proteins from ST-1–5 were eluted from the chromatin gels in the presence of 0.1% sodium dodecyl sulfate. The top frames (left to right) represent (*1*) the map of whole histone, (*2*) ST-5, and (*3*) a representation of the whole histone map with the individual peptides identified according to their parental molecules. The bottom frames show the maps for (*1*) ST-4, (*2*) ST-3, and (*3*) ST-1 (left to right). Additional experiments have shown that ST-1 and ST-2 have maps that are identical and that the polypeptides in TS-1 correspond to those in ST-3; those in TS-2, to those in ST-4; and those in TS-3, to those in ST-5. The intensity of each peptide in these maps depends on its particular reactivity with ^{125}I and is *not* directly related to its molar concentration. The protein to DNA ratio in each "ST" particle was estimated with a labeling procedure. Erythroblasts were labeled with [^{14}C]thymidine and [^{3}H]leucine and the $^{14}C/^{3}H$ ratio was determined in chromatin and in the various ST particles eluted from the gel. For those particles that contain protein, the ratio was not significantly different from that obtained from total chromatin, indicating that the particles corresponding to ST-1, 2, 3, 4, and 5 (and TS-1, 2, and 3) have a DNA to protein ratio of about 0.9.

of 25 μM $CaCl_2$, they are completely absent when rerun on a second acrylamide gel, whereas undigested ST-1 and 2 run true. As controls, the eluted ST-3, 4, and 5 remain resistant to nuclease when similarly re-exposed to nuclease. Moreover, exposure of unfractionated ST particles to nuclease yields a pattern identical to that obtained from TS particles. Thus, the DNA associated with ST-1 and 2 is sensitive to nuclease after, but not before, trypsin treatment. The proteins associated with these fragments could thus be either a unique subset of histones or histones present in a unique conformation. This will be discussed later.

Protein and DNA Composition of the ST and TS Particles. In order to identify the large molecular weight histone fragments associated with these particles, individual TS and ST bands were eluted from the gel, precipitated with trichloroacetic acid, iodinated with ^{125}I, digested with trypsin, and mapped as previously described (2) (Fig. 4). This procedure can be used to identify each histone on a single two-dimensional peptide map. The results, in conjunction with similar data from [^{35}S]methionine histone peptides, shows clearly that ST-1, 2, 3, and 4 each contain all histones except I and V, while ST-5 contains only the C-terminal portion of histones III and IV.

TABLE 1. *Correspondence between the DNA band, the ST particles, and the TS particles*

DNA	Length in base pairs	ST	TS	Histone binding site
1	145	1		NH_2 and COOH
2	130	2		NH_2 and COOH
3	110	—		NH_2
4	95	3	1	COOH
5	80	6		NH_2
6	68	4	2	COOH
7	55	7, 5	3	NH_2/COOH
8	48	8		NH_2

A DNA band is thought to be protected by histone N-terminals if it becomes sensitive to nuclease after trypsin treatment (2) or if it is released as free DNA after trypsinization of the 11S nuclease particles (Fig. 1). A DNA band is thought to contain histone C-terminals if it is protected from nuclease after trypsin treatment or if it is associated with limit-digest histone fragments in the ST or TS particles. DNA-1 and 2 are thought to contain histone C-terminals because they are associated with these fragments in ST-1 and 2. They are also thought to contain histone N-terminals since they are sensitive to nuclease after N-terminals are released by trypsin. A distinct ST particle corresponding to DNA-3 is not invariably observed. In about 30% of the analyses, a free DNA fragment corresponding to DNA-3 is observed on ST gels. No obvious explanation for this variability is apparent except that the EDTA may not fully inactivate the nuclease. In any event, DNA-3 is listed as being associated with histone N-terminals because it is digested after trypsinization of chromatin (2). Since DNA-7 is released as free DNA (as ST-7) and is also present in protein associated DNA (as ST-5), it is likely that there are two classes of fragments that migrate as DNA-7, one class binds histone N-terminals and the other binds histone C-terminals. Particular *pairs* of C-terminal and N-terminal binding sites combine to yield DNA molecular weights of about 150 base pairs. Thus, the sums of DNA-3 and 7, 4 and 8, and 5 and 6 add to 165, 143, and 148 base pairs, respectively. This is quite close to the 170 base pairs Noll (4) obtains for partial nuclease digestion and is consistent with the idea that extensive nuclease digestion splits each 170 base pair DNA segment into two small segments.

Assuming that each ST fragment is homogeneous, I conclude that, in general, histones III, IV, IIb2, and IIb1 are *all* located *as a group* over extremely short distances of DNA (45 to 145 base pairs) in the nuclease particles. This is consistent with the fact that isolated histones can interact with each other in a very defined and often complex manner (9–12).

The DNA fragments associated with the individual ST and TS particles were purified and their electrophoretic mobilities were compared (Fig. 5) to the eight purified DNA fragments obtained from the staph nuclease limit-digest of chromatin (1, 2). Table 1 is a summary of the correspondence between the various DNA bands, the various ST bands, and the TS bands. Each ST particle contains only *one* size class of DNA molecules. I have also indicated in Table 1 whether the band behaves as if it contains histone C-terminals or N-terminals, or both.

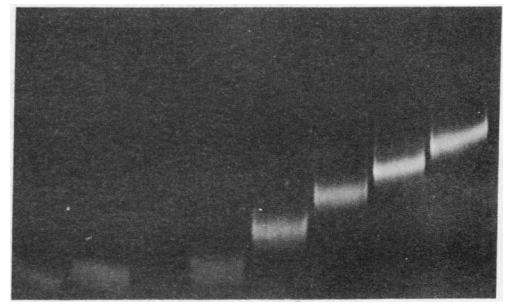

FIG. 5. The DNA components associated with individual ST particles. Individual ST particles were eluted from gels, and the DNA was isolated as previously described (2), and rerun on 6% acrylamide gels. Mobilities were compared to those obtained for the DNA fragments obtained from a total nuclease digest of chromatin. The results for ST and TS fragments are given in Table 1. From right to left are the DNA components associated with ST-1, 2, 3, 4, 5, 6, 7, and 8. The DNA associated with ST-6 is poorly visualized. Longer exposures indicate that it corresponds to DNA-5. Conditions for electrophoresis were exactly the same as in Fig. 1 and approximate mobilities can, therefore, be compared.

DISCUSSION

A Model for Chromosome Packaging. How might the ST particles arise? In deciding on a mechanism, several additional facts should be kept in mind. The 11.2S nuclease-particles generated by *partial* enzyme treatment of nuclei contain a DNA fragment of 170 to 200 base pairs (4) and have a protein to DNA ratio of 1.3, corresponding to about 10 histones per particle. More extensive nuclease digestion yields a particle that sediments at about the same rate, but contains a collection of discrete DNA molecules of about 100 base pairs (6). The latter particles have a slightly higher protein to DNA ratio, indicating that slightly more DNA is digested, even though the sedimentation rate hardly changes. Yet, the size of the associated DNA fragments is about half, suggesting to me that the nuclease-particles obtained from extensive digestion contain *two* DNA molecules per particle rather than one.

These findings as well as our own data are explained by the model shown in the upper left of Fig. 6. In this model, chromosomal DNA is seen as a series of loops which are held in place by the *crosslinking* (2) action of *groups* of histones, their N-termini arranged predominantly along one side and their C-termini along the other side of the loop. There are two kinds of nuclease-sensitive open regions, one at the apex of each loop and another at the base. Complete digestion of this proposed structure by nuclease would result in the production of several types of particles distinguished by different lengths of DNA and possibly by different ratios or conformations of histones. Trypsin treatment of these particles, which digests the basic N-terminal histone "arms" (perhaps "elbow" might be a better description) (2), would release both discrete protein-free DNA fragments and protein-associated DNA fragments, as we have observed.

If this scheme is correct, an important conservation of symmetry must be observed. When the enzyme treatments are reversed, generating tryp-staph particles, gel electrophoresis of the products should show ST bands 1 to 5 (but not the protein-free DNA bands, ST-6–8) (Fig. 3). The data shows that six of the eight ST particles behave as expected. ST-1 and 2 clearly do not.

The finding that the proteins associated with ST-1 and 2 are basically similar to those associated with ST-3 and 4 raises a major question about why, as noted above, the former (but not the latter pair) are sensitive to nuclease after, but not before trypsin treatment. Although clearly not the only

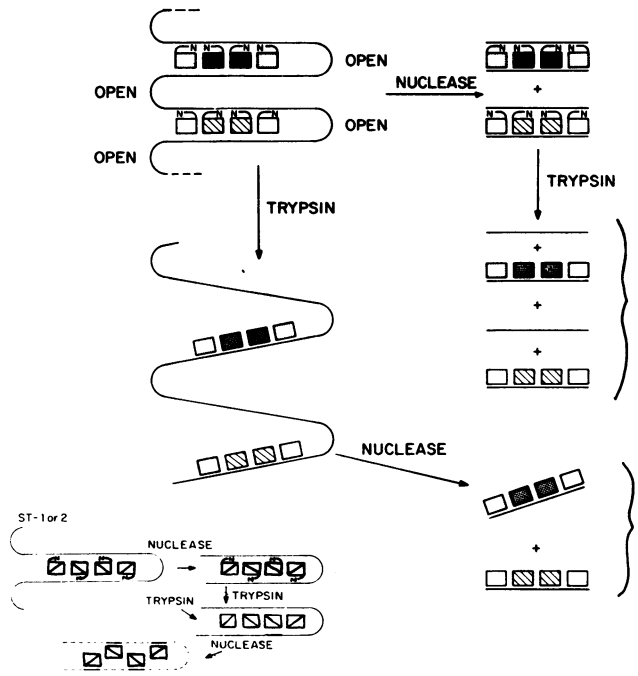

FIG. 6. A possible explanation for the generation of ST particles (upper left). The DNA (upper left) is seen to consist of a series of loops (corresponding to "nu" bodies) (5) which are held intact by groups of histones (rectangles). (The different patterns within the rectangles do not necessarily reflect different histones, but rather those particular conformations or combinations of histone groups that determine the multiple sites of staph nuclease digestion.) N-terminals are aligned along one arm of the loop and C-terminals along the other. Exhaustive digestion of this structure with nuclease (upper right) results in a number of defined particles, which sediment at 11–12 S and are thought to be characterized by two DNA fragments held together by histone bridges. When these 11S nuclease-particles are treated with trypsin (converting most of the chromosomal material to 5 S), the histone N-terminals are removed and protein-free DNA is released as well as a complex of specific DNA fragments and histone C-terminals (which interact in such a way as to preserve their resistance to further trypsin or nuclease treatment). If, however, the structure in the upper left is treated first with trypsin (lower left) and then treated with nuclease (lower right), no protein-free fragments should appear; however, the complexes of histone C-terminals and specific DNA fragments should persist. The model (upper left) only shows a two-dimensional projection of a structure that can explain the data. It is likely that the histones in each loop will fold into a more globular type of configuration as found for nuclease-particles by Sahasrabuddhe and Van Holde (6) and, in doing so, fold the DNA into an even more tightly packed conformation. The number or types of histones within a given loop may vary, but in general the data is consistent with from 6 to 10 histones per loop (4, 6).

Inset: A possible explanation for the sensitivity of ST-1 and 2 to nuclease after, but not before, trypsin treatment. Instead of a contiguous arrangement of histone N-terminals along one arm of the loop, the N-terminals are thought to be arranged in an antiparallel arrangement. ST-1 and 2 correspond to DNA-1 and 2, respectively (see Table 1). The DNA in the loop is sensitive to nuclease after, but not before trypsin treatment.

explanation, one possibility is that ST-1 and 2 originate from nuclease-particles in which the histones are in a different conformation. An example is shown in the inset to Fig. 6, where histone N-terminals are *not* all aligned along one side of a DNA loop, but instead alternate in an antiparallel arrangement.

The Complexity of Chromosome Sub-Structures. The qualitative finding (Fig. 4) that the same histones are found in different types of structures (as reflected by the different electrophoretic mobilities of the structures after nuclease and trypsin treatment) offers a further explanation for the conservation of histone sequences during evolution, since the extent to which these sequences can diverge is clearly more restricted if they must form *several* different types of substructures than if they form only one.

In summary, these experiments lead to the conclusion that histone–histone and histone–DNA interactions are complex, with several different packing patterns present in the interphase chromosome. Our particular model for chromatin structure, proposed in Fig. 6, is consistent with the general view of chromatin structure recently elaborated by others (13–15). However, it extends these models both in indicating how histones might fold the nuclease-particles by cross-linking the DNA, and in accommodating the fact that histone aggregates, as revealed in the nuclease-particles, are clearly heterogeneous. How this complexity is generated and how it is used remains to be explained.

Note Added in Proof. Histones can be successfully reconstituted to the small DNA fragments generated by exhaustive nuclease digestion of chromatin (F. Van Lente and H. Weintraub, manuscript in preparation). The resulting particles are resistant to further nuclease digestion and upon treatment with trypsin yield a pattern identical to that shown in Fig. 1.

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