Deoxyribonuclease I Generates Single-Stranded Gaps in Chromatin Deoxyribonucleic Acid†

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ABSTRACT: Production of 10-base multiple DNA ladder fragments during DNase I digestion of chromatin is explained by a model which does not involve site-specific nicking by the DNase I. This model was tested because it explains why 10-base (actually 10.4 base) multiple-related fragments are paradoxically generated by both endonucleolytic (DNase I) and exonucleolytic (exonuclease III) mechanisms. This new model also explains the phenomenon of substantial single-stranded DNA production during DNase I digestion of chromatin. The latter phenomenon has been widely observed but is not explained by previous models. The single-stranded gap model to be presented makes testable predictions. Primarily, these are that DNase I produces single-stranded gaps in chromatin DNA and that the termini of 10-base multiple ladder fragments are separated by single-stranded gaps. Single-stranded gap production by DNase I was confirmed by a number of methods. Sensitivity of ladder band components (from DNase I but not staphylococcal nuclease digests) to S1 nuclease suggested that the ladder fragments themselves may compose a significant portion of these gaps. Separation of ladder fragment termini by single-stranded gaps was verified by demonstrating both resistance to the nick-specific NAD^+-dependent ligase and sensitivity to T4 ligase which can ligate across gaps. Many single-stranded gaps, occurring both individually and in clusters, were observed by electron microscopy using either cytochrome *c* labeling (where the gaps are thinner than duplex) or gene 32 protein labeling (gaps thicker than duplex). Gap sizes were estimated by protecting them with gene 32 protein and digesting away unprotected duplexes. By this method, gap sizes fall into a ladder distribution (from 10 or 20 bases up to 120 bases), which, at least in the region of the shorter sizes, clearly indicates the sizes of single-stranded gaps formed in chromatin by DNase I.

It is generally thought that if we were to understand more about how nucleases digest nucleosomal DNA, we would understand more about the nucleosome itself and the manner in which nucleosomal DNA is packaged. Endonucleases (Noll, 1974; Altenburger et al., 1976; Sollner-Webb & Felsenfeld, 1977; Hewish & Burgoyne, 1973; Whitlock et al., 1977; Axel, 1975) and at least one exonuclease (Riley & Weintraub, 1978) produce from chromatin families of DNA fragments related in size by multiples of 10 bases. Although averaging measurements indicate that the multiple actually averages about 10.4 bases (Lutter, 1979), I will refer to these as 10-base multiples for convenience, and families of such fragments will be referred to as "ladder fragments".

Pancreatic DNase I is an endonuclease which produces a characteristic pattern of 10-base multiple ladder fragments, presumably by making single-stranded nicks at specially accessible "point sites" spaced at 10-base multiples (Noll, 1974). The special accessibility of point sites spaced at 10-base multiples would seem to be an obvious interpretation of DNase I digestion patterns, but it is, however, a hypothesis that has never been challenged. There is, in fact, an interpretation distinct from that of special accessibility of point sites. This new interpretation can account for 10-base multiple ladders and predicts that DNase I produces various sizes of single-stranded gaps in chromatin DNA.

The single-stranded gap model to be presented was developed and tested because it encompasses two phenomena of DNase I digestion not explained by previous models. The first of these is a paradox between the effects on chromatin of two dissimilar nucleases, DNase I and exonuclease III. Both nucleases produce 10-base multiple ladder bands. However, one, DNase I, acts endonucleolytically, while 5′-labeling studies show that the other, exonuclease III, produces ladder bands by 3′ to 5′ exonucleolytic digestion (Riley & Weintraub, 1978). This implies, paradoxically, rapid digestion by the exonuclease through portions of those regions of nucleosomal DNA, which, according to the point-site model for DNase I digestion, are the most protected. I will present the gap model and evidence for it with respect to DNase I and then describe how the effects of DNase I and exonuclease III are reconciled via the gap model (see Discussion).

The second phenomenon of DNase I digestion not previously explained is the fact that a large proportion of the chromatin DNA is rendered single stranded during DNase I digestion (Oliver & Chalkley, 1974). In fact, as will be shown, conversion of duplex to single-stranded DNA appears to be a predominate way in which DNase I digests chromatin.

Predictions of this new interpretation are that DNase I produces single-stranded gaps in chromatin DNA, and that the termini of neighboring ladder fragments in DNase I digested chromatin are actually separated from one another by single-stranded gaps. I will focus primarily on evidence for the production of single-stranded gaps and on the distance separating neighboring ladder fragment termini in DNA from DNase I digested chromatin. Details of the mechanisms involved in single-stranded gap formation will remain largely speculative.

In Figure 1 is presented a schematic model of how 10-base multiple ladder fragments could be generated by DNase I without nicking at specially accessible point sites. Note that the model invokes single-stranded nicking of duplex by DNase I but does not make use of nicking at specially localized point sites. There are many possible variations in the manner in which single-stranded gaps of various sizes could be formed and situated in this illustrative model. More precise models (delineation of which has not as yet been attempted) might take into account different contributions from the nucleosome

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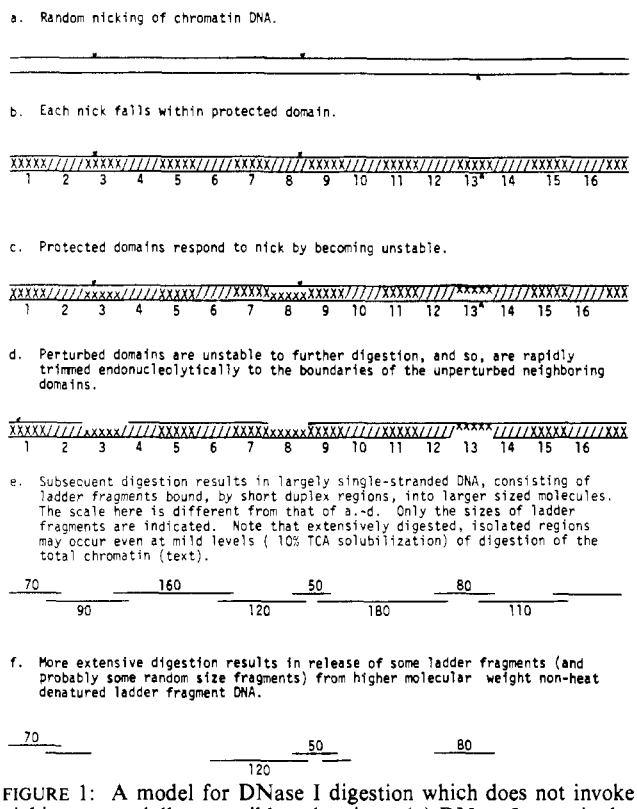

FIGURE 1: A model for DNase I digestion which does not invoke nicking at specially accessible point sites. (a) DNase I puts single-stranded nicks in essentially random locations in the chromatin DNA. (b) Each nick falls somewhere within a protected domain of DNA. Here the protected domains are numbered arbitrarily from left to right. For simplicity, each domain is assumed to cover 10 bases, although domains of any 10-base multiple length are considered possible (see text). Most importantly, each domain abuts directly on its neighbors so that, prior to nicking in step a, all of the phosphodiester bonds are equivalent (or nearly equivalent) in their accessibility to DNase I. (c) In response to nicking, nicked domains become perturbed and unstable (indicated by shift of shading in domains 3, 8, and 13 in the example) to further digestion of the strand which was nicked. (d) The perturbed domains become rapidly digested endonucleolytically along one strand, while other domains remain stable unless new nicks are acquired as in domain no 1. Since protected domains are postulated to be 10 bases or 10-base multiples in length, the single-stranded gaps resulting in d are 10-base multiples in length. Therefore, DNA strands between two fully trimmed gaps on the same strand are members of the 10-base interval-ladder fragment families when seen under denaturing conditions. In the example, a 40-base fragment is generated by perturbation and endonucleolytic trimming of domains 3 and 8. (e) After extensive digestion many of the gaps have extended or run together resulting in primarily single-stranded DNA consisting of ladder fragments, hooked together by short stretches of duplex. This situation, in which the remaining duplex regions are fairly short, will be referred to as a tight cluster of gaps occurring on alternating strands. (f) This represents the apparent situation (see text) of ladder fragment DNA after extensive DNase I digestion (20 to 50% digestion of Cl_3AcOH precipitable counts) of chromatin. Some of the interlocking duplexes may have become shorter releasing some ladder fragments, while other fragments may be shortened or destroyed. The observed loss of intramolecularity for extensive digest ladder fragments (see text) might also be due to random DNase I attack on single-stranded regions of ladder fragments during extensive digestion by DNase I.

core and spacer which could lead to some degree of nonrandomness in the initial nicking. Also not specified is whether protecting groups are shared by the two strands (e.g., becoming committed to the unnicked strand in nick-perturbed domains) or whether each strand has its own set of perturbable domains.

Although I will speculate to some extent on certain mechanistic complications, I have primarily limited what follows to two questions: (1) Does DNase I produce single-stranded gaps in chromatin DNA and (2) more specifically, are ladder fragment termini separated by nicks or by gaps?

Materials and Methods

Pancreatic DNase I and staphylococcal nuclease were from Worthington. T-4 ligase, NAD^+-dependent ligase, and λ-exonuclease were obtained from New England Biolabs. S1 nuclease was obtained from Bethesda Research Laboratories. Gene 32 protein was a generous gift from Bruce Alberts and his colleagues.

Cells and Labeling. Chicken erythroid leukemia (MSB) cells were grown in suspension in RPMI medium (GIBCO) supplemented with 10% fetal calf serum and 1% Penn Strep. Labeling (4 to 24 h, 37 °C) was with [*methyl*-^{3}H]thymidine (50 mCi/mmol, New England Nuclear) used at 25–50 μCi/mL after pelleting actively growing cells and resuspending (10^5 cells/mL) in fresh medium.

Red blood cells from 4- or 14-day-old chick embryos were isolated as described by Riley & Weintraub (1978). Briefly, erythrocytes were collected by vein puncture and washed several times in standard saline–citrate (SSC)[1] (0.14 M NaCl, 0.01 M Tris-HCl (pH 7.4), and 0.015 M sodium citrate). The cells were lysed in reticulocyte standard buffer (RSB) (0.01 M NaCl, 0.005 M $MgCl_2$, and 0.01 M Tris-HCl (pH 7.4), containing 0.5% Nonidet P-40 BDH). The released nuclei were repeatedly washed and pelleted in the same buffer until free of hemoglobin. Nuclei from MSB cells were isolated in the same manner.

DNA Purification. After limited treatment with pancreatic DNase I or staphylococcal nuclease, nuclei in RSB were pelleted and resuspended for two cycles (A_{260} units are not released) to remove enzyme. Digested or undigested nuclear suspensions were made 10 mM in EDTA, 0.1% $NaDodSO_4$, and 0.2 mg/mL proteinase K. Proteinase K digestion was carried on for 1 h at 37 °C. The samples were then extracted repeatedly with phenol–chloroform–isoamyl alcohol ($1{:}1{:}^1/_{24}$) and 3–4 times with chloroform–isoamyl alcohol ($1{:}^1/_{24}$). The aqueous phase was then either made 0.3 M in NaCl and precipitated with 2 volumes of ethanol, or dialyzed overnight against sterile glass-distilled water and lyophilized. Excessive manipulative shear forces were avoided, and samples were aliquoted to avoid multiple cycles of freeze–thawing.

Enzyme Treatments. DNase I digestions were performed on nuclei rinsed for two cycles in RSB without NP40. Nuclei were suspended at 0.3 mg of chromatin DNA/mL and 1 μg/mL (for a 1–2% digest) to 100 μg/mL DNase I was added. Incubation was for 12 to 15 min at 37 °C. The nuclei were kept in suspension by swirling.

Staphylococcal nuclease was used to obtain mixtures of chromatin monomers and higher multimers for exonuclease III digestion (Riley & Weintraub, 1978) or to obtain submonomer size, double-stranded ladder fragments. To obtain chromatin for exonuclease III digestion, nuclei at 0.3 mg/mL were made 10^{-4} M in $CaCl_2$ and incubated with staphylococcal nuclease at 0.4 μg/mL for 10 min at 37 °C. This yields a chromatin preparation of which over 50% is monomers, and the rest consists of higher multimers. Staphylococcal nuclease was also used (75 to 100 μg/mL, with 0.1 mg/mL chromatin DNA, 15–30 min at 37 °C) to obtain ladder fragments which are over 95% double stranded. These double-stranded ladder

[1] Abbreviations used: SSC, standard saline–citrate (0.14 M NaCl, 0.01 M Tris-HCl, and 0.015 M sodium citrate); RSB, reticulocyte standard buffer (0.01 M NaCl, 0.005 M $MgCl_2$, 0.01 M Tris-HCl, and 0.5% Nonidet P-40); $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

fragments, purified from extensively digested staphylococcal nuclease chromatin, were used to determine the accessibility of small, double-stranded fragments to S1 nuclease and gene 32 protein (see Results).

S1 Nuclease Digestions. S1 nuclease digestions were performed on purified DNA fragments in 30 mM sodium acetate (pH 4.5), 0.15 M NaCl, and 1 mM $ZnSO_4$. DNA concentrations (0.2 mg/mL) in the S1 buffer were constant from experiment to experiment, and S1 nuclease was routinely used at 0.15 unit per μg of DNA. Incubation was at 45 °C for 45 min, and non-S1-treated controls were always incubated under the same conditions.

Ligases. The NAD^+-dependent ligase from uninfected *Escherichia coli* was used in ligation of purified DNAs (at 50 μg/mL in 50 μL). Conditions were as described by Sugino et al. (1977) with 40 mM Tris (7.9), 5 mM $MgCl_2$, 10 mM dithiothreitol, and 60 μM NAD^+. Incubations were at 16 °C for 30 min. Reactions were terminated by addition of EDTA to 10 mM, $NaDodSO_4$ to 0.1%, and 0.2 mg/mL proteinase K (1 h, 37 °C).

T4 ligase (Gellert, 1966; Sugino et al., 1977) was used in ligation reactions with DNA at the same concentration as above for the NAD^+-dependent enzyme. Incubation mixtures consisted of 66 mM Tris (7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.4 mM ATP. Incubation was for 16 h at 4 °C. Reactions were terminated by the EDTA–$NaDodSO_4$–proteinase K treatment described above.

Gene 32 Protein Binding and Protection Experiments. Gene 32 single-stranded DNA binding protein (Alberts et al., 1968; Alberts & Frey, 1970; Curtis & Alberts, 1976) was used under conditions where the protein is specifically bound to single-stranded DNA without appreciable binding or denaturation of native, double-stranded DNA (see, also, Brack et al., 1975). To 0.4 to 0.8 μg of purified DNA (in 40 μL, i.e., 10 to 20 μg of DNA/mL) in 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 10^{-4} M $CaCl_2$ was added 6 to 12 μg (150 to 300 μg/mL) of purified gene 32 protein (stock at 2.4 mg/mL). Binding was allowed to continue for 10 min at either 4 or 37 °C.

Binding was also performed in the absence of divalent cations (substituting 2 mM EDTA for $CaCl_2$ and $MgCl_2$) without detectable effects on the results. DNA not protected by the gene 32 protein was digested in "protection experiments" to small oligonucleotides using from 1 to 100 μg/mL of pancreatic DNase I (as specified in the text) for 15 min at 20 or 37 °C. A technical note is that, although gene 32 very effectively protects single-stranded DNA from DNase I (Huang & Lehman, 1972; Curtis & Alberts, 1976), staphylococcal nuclease cannot be used in a similar way (see legend to Figure 15).

Gene 32 protein was also used to label single-stranded gaps in preparation for electron microscopy as described below.

Preparation of Native DNA with Nicks. Nicked DNAs generally were prepared by two different methods using mild DNase I treatment either of purified, native DNA or of chromatin in the presence of ethidium bromide. To obtain nicked DNA whose denatured profile extends into the region of migration of ladder bands, one can use DNase I at 0.01 μg/mL (high molecular weight DNA at 0.3–0.5 mg/mL) for 15 min at 37 °C. At 5- to 10-fold higher levels of DNase I, much of this "nicked" DNA becomes resistant to ligation (see NAD^+-dependent ligase) probably due to accumulation of random-size gaps (see sections on electron microscopy and gene 32 protein binding).

A more convenient way of mimicking the size distribution of ladder fragment DNA is to digest low concentrations (0.05 to 0.1 mg/mL) of chromatin or nuclei in the presence of 40 μg/mL ethidium bromide, followed by DNA purification. For example, if 10 μg/mL of DNase I is used to obtain ladder fragment DNA from nuclei, the same concentration of DNase I in the presence of ethidium bromide will produce a smear whose size distribution is very similar (except for the presence of bands) to that of the ladder fragment distribution generated without ethidium bromide. However, "nicked" DNA generated in the presence of ethidium bromide is somewhat more resistant to ligases than DNA nicked in the purified form as described above.

DNA, with random-size gaps artificially installed after DNA purification, was prepared by nicking purified DNA as above, followed by treatment with λ-exonuclease to release 10 to 12% of Cl_3AcOH precipitable material. The "gapped" nature of this DNA was verified by electron microscopy and by fairly uniform increased mobility (after exonuclease) of its denatured size distribution.

Electron Microscopy. Cytochrome *c* spreading of purified DNA samples was performed by the method of Davis et al. (1971). Grids were rotary shadowed with 100% platinum at an angle of 6–9° (5 to 8 × 10^{-5} Torr).

Gene 32 protein labeling, which causes an increased diameter of single-stranded DNA over double-stranded DNA, was performed by a modification of the method of Brack et al. (1975). Carbon-coated grids were glow-discharged at a setting of 60 in a Denton DV-502 vacuum evaporator (100 mTorr) for 30 s and used the same day. Gene 32 protein was bound to DNA samples as described above. After 10 min of binding (usually at 37 °C), samples were fixed with 0.02% Ultra Pure glutaraldehyde (Pelco) for 5 min at 37 °C. Samples (10 to 20 μg/mL DNA) were applied by the drop method (30-s contact), after which the samples were stained (30 s) with freshly prepared 5 mM uranyl acetate and shadowed as above. Micrographs were taken at 60 kV on Philips 300 and AEI electron microscopes.

Polyacrylamide Gel Electrophoresis. Methods for preparing polyacrylamide gels for examination of native and denatured components have been described (Riley & Weintraub, 1978).

Results

Single-Stranded Character of DNA from DNase I Digested Chromatin. To test for the production of significant amounts of single-stranded DNA during DNase I digestion of chromatin, nuclei were digested with increasing concentrations of DNase I. The DNA was then isolated and treated with single-strand specific S1 nuclease. Figure 2 shows that at progressively higher levels of DNase I digestion, the DNA becomes progressively more single stranded by the criteria of S1 nuclease sensitivity and thermal hypochromicity. By 50% solubilization of Cl_3AcOH precipitible counts, 75 to 85% of the remaining DNA length is single stranded. It can be seen that at essentially all levels of digestion, a major portion of the Cl_3AcOH precipitible counts solubilized can be accounted for in terms of conversion of duplex to single-stranded material. Although the occurrence of specially accessible point-site nicking cannot be excluded, note that no moderate alteration of the point-site hypothesis explains this generation of single-stranded DNA.

A related experiment is shown in Figure 3 in which DNA from DNase I treated chromatin was run on gels in denatured form as well as after S1 digestion (Materials and Methods) in native form prior to denaturation. The DNA from DNase

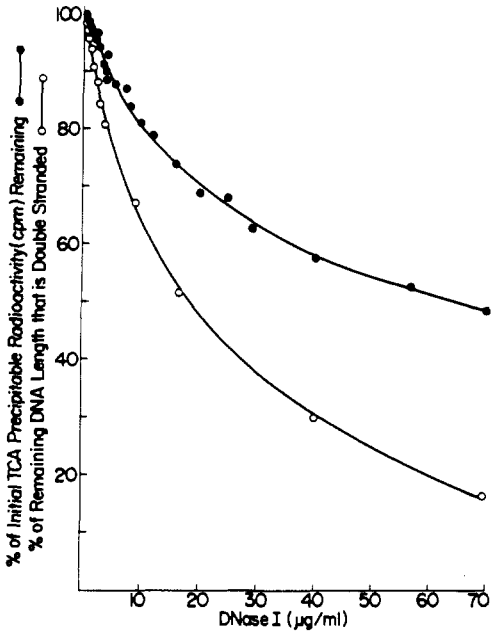

FIGURE 2: Production of single-stranded DNA during DNase I digestion. Multiple aliquots of chromatin (at 0.3 mg of DNA/mL) from MSB nuclei (Materials and Methods) were digested with increasing amounts of pancreatic DNase I. Increasing digestion is expressed as the percentage of Cl_3AcOH-precipitable counts per minute (cpm) remaining (●) and as the percentage of remaining Cl_3AcOH-precipitable cpm that are resistant to S1 nuclease (expressed as the total remaining DNA length that is S1 resistant (○)). When digestion was monitored following thermal hypochromicity of purified DNA from DNase I digested chromatin, results (not shown) were identical with those shown for S1 resistance.

I treated chromatin is seen to be a complex mixture of both single- and double-stranded DNA. The ladder bands themselves are clearly susceptible to S1, even at mild stages of digestion. Ladder band sensitivity to S1 at mild stages of DNase I digestion was not entirely expected. From this, it appears that soon after their formation, the majority of ladder fragments acquire at least partial single-stranded character. However, this result depends on the nondestructiveness of S1 with respect to duplex DNA.

As a control for the S1 treatment, I prepared non-heat-denatured DNA from staphylococcal nuclease chromatin (Materials and Methods) whose monomers had been digested into the core with exonuclease III (Riley & Weintraub, 1978). The purified DNA from exonuclease III digested nucleosome cores has 10 base pair overlapping duplexes with single-stranded tails. For this reason, conversion of the exonuclease III DNA from a smear to a ladder pattern (on nondenaturing gels) serves as an effective routine test for trimming by S1 without destruction of duplex (Figures 3f and g; and see Riley & Weintraub, 1978). More quantitatively, S1 digests, under these conditions, 95% of labeled, control, single-stranded DNA and only 4 to 5% of non-heat-denatured duplex.

Although the gap model of Figure 1 predicts the generation of single-stranded fragments, I did not expect the observed S1 sensitivity of ladder bands themselves at early stages of digestion (Figures 3b and c). That is, neither the specially accessible point-site model or the gap model provides an immediate explanation of the effects of S1. Similar S1 sensitivity has been observed for ladder fragments (which are DNase-I-like in submonomer distribution) produced by a nuclease from *Serratia marcescens* (Pospelov et al., 1979).

In order to account for S1 sensitive ladder bands by models (i.e., strict forms of the point-site model) that do not involve generation of single-stranded DNA, one has to invoke spontaneous denaturation, during purification of duplex ladder fragments. To obtain an estimate of the possible significance of spontaneous denaturation, DNA from chromatin, digested to various extents with DNase I, was run in both heat-denatured and non-heat-denatured forms (Figures 4 and 5). Although spontaneous denaturation may play a role after extensive DNase digestion (15 to 50% Cl_3AcOH solubilization, Figures 4c and d; discussed later), spontaneous denaturation appears insignificant for mild digests, since no material migrates in the region of ladder fragments unless the purified mild digest DNA is heat denatured (Figures 4a,b and 5a,b).

As a further control for the specificity of S1, it was found that under the same conditions (i.e., using the same S1 nuclease and DNA concentrations as in Figure 3) the same batch of S1 nuclease reproducibly fails to digest known double-stranded ladder fragments, purified from staphylococcal nuclease digested chromatin (Materials and Methods). Double-stranded staphylococcal nuclease ladder fragments as small as 30 base pairs are not detectably digested (not shown). For these reasons, spontaneous denaturation most probably does not explain the effects of S1 on bands from mild DNase I digests. One may conclude that at the level of chromatin digestion by DNase I, the majority of ladder fragments become single stranded in character but, at least for mild digests, are bound by terminal duplexes into larger size, non-heat-denatured molecules.

The primary difficulty for the gap model in explaining these observations is that, although S1-sensitive DNA is predicted, stages of partial digestion as shown in step d of Figure 1 should lead, after S1 treatment, to preservation or even reinforcement of some of the ladder bands. In fact, I reproducibly observe only very subtle banding in the smears left by S1 treatment. However, the gap model can be reconciled with this observation if it is assumed that gaps occur either individually or in rapidly formed tight clusters on alternating strands, as in Figure 1e. I have no explanation for this apparent tight clustering of gaps at early stages of DNase I digestion, but gap clustering is a complication also apparent in other experiments below.

The experiment in Figure 3 is also complicated by the fact that, although the derivation of ladder fragments upon denaturation of longer, native material is obvious at the lower levels of DNase I digestion, it becomes less obvious at higher levels (Figure 4). The origin of this mechanistic complication is probably trivial, as may be more apparent in the following experiments.

Ligases Indicate That Ladder Fragment Termini Are Separated by Gaps. (a) The NAD$^+$-Dependent Ligase. NAD$^+$-dependent ligase from *E. coli* has been shown to be fairly specific for closely juxtaposed 5′-phosphate and 3′-hydroxyl termini (Gellert, 1966; Zimmerman et al., 1967). I attempted to ligate non-heat-denatured DNA from mildly DNase I digested chromatin in order to determine if ladder fragment termini, bound into higher molecular weight DNA, are actually closely juxtaposed as in the case of a single-stranded nick or more distantly removed as in the case of a gap.

The NAD$^+$-dependent DNA ligase was found to ligate various nicked control DNAs, while non-heat-denatured DNA purified from DNase I treated chromatin was resistant to ligation as shown in Figure 5. Selective ligation of smears of fragments from nicked DNA was also seen when nicked and non-heat-denatured ladder fragment DNAs were mixed (Figure 6). The resistance of ladder fragments to ligation by the NAD$^+$-dependent ligase suggests that the ladder fragment termini are separated from one another by single-

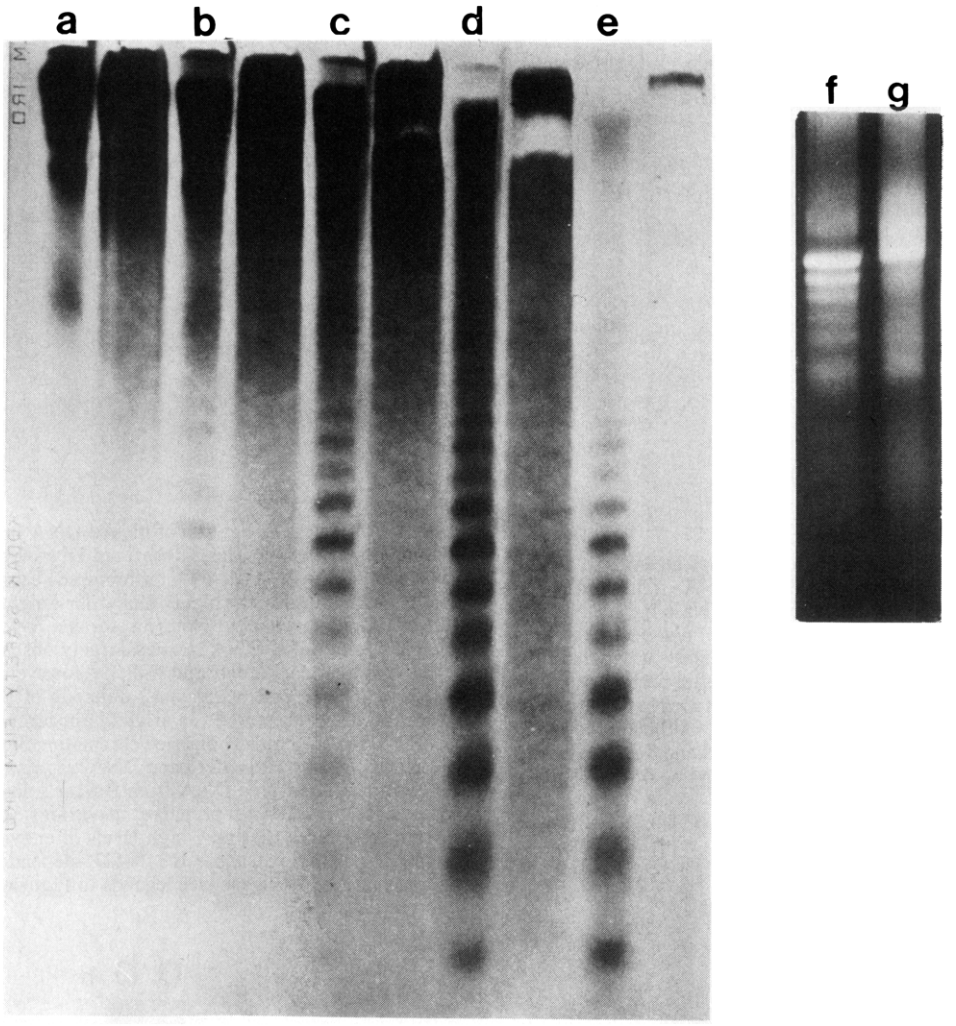

FIGURE 3: S1 nuclease sensitivity of DNase I digestion products. Nuclear chromatin was digested with increasing amounts of DNase I. The DNA was then purified without heat denaturation and duplicate aliquots were incubated with or without S1 nuclease (Materials and Methods). The DNA was then heat denatured and run on denaturing gels. Each S1-treated sample appears to the right of the corresponding non-S1-treated samples which are lettered. S1 nuclease levels were adjusted to digest all but 4 to 5% of control single-stranded DNA, while leaving 94% of control double-stranded DNA intact: (a) 1 μg/mL DNase I in the initial chromatin digest; (b) 3 μg/mL; (c) 10 μg/mL; (d) 30 μg/mL; (e) 60 μg/mL. (f) Precise trimming of single-stranded tails by S1 nuclease. DNA from chromatin digested into the monomer core by exonuclease III contains 10-base repeats which are obvious only if the DNA is either denatured or treated with S1 nuclease (Riley & Weintraub, 1978). At the enzyme to DNA ratios used in a–e, S1 nuclease accomplishes this trimming (f, g). Shown is purified DNA from exonuclease III digested chromatin without (g) and with (f) S1 treatment in non-heat-denatured form.

stranded gaps, which are relatively insensitive to ligation by the NAD^+-dependent ligase (Gefter et al., 1967).

Because the bulk of ladder fragments from mild (about 10% Cl_3AcOH solubilized) digests are reproducibly both S1 sensitive and partially duplexed with other fragments prior to heat denaturation (Figures 3 and 4), I interpret mild DNase I digestion as development, in isolated regions of chromatin, of well-digested, tight clusters of gaps involving the ladder fragments as shown in Figure 1e. Clearly, there are other interpretations but they are limited to variations only in: (1) the length of the interlocking duplexes, which I have made short (i.e., less than 20 to 25 base pairs) to account for the low level of discrete banding after S1 (and see Discussion); (2) the proportion of random size fragments which might be interspersed with ladder fragments; and (3) the number of ladder fragments that occupy the same non-heat-denatured molecule. Presumably, this number could be as low as one if each ladder fragment tended to be associated with one or more large, random-sized fragment to account for the heat denaturation dependent shift in mobility of mild-digest ladder bands.

The evidence shifted in favor of the situation depicted in Figure 1e when the effects of T4 ligase were studied.

(*b*) *T4 DNA Ligase.* Although the two ligases and their properties have often been interchanged and confused, numerous studies indicate prominent differences in substrate preference. Briefly reviewed, the NAD^+-dependent ligase from uninfected *E. coli* has a much more stringent requirement for closely juxtaposed termini, as in the case of hydrogen-bonded, cohesive ends or internal, single-stranded nicks (Gellert, 1966; Zimmerman et al., 1967). T4 ligase, on the other hand, has a much lessened requirement for juxtaposition of the ends, as it will ligate non-hydrogen-bonded cohesive ends, blunt ends (Sgaramella, 1972; Sugino et al., 1977), and cohesive ends with base mismatch (Sgaramella et al., 1970), and it will also ligate across single-stranded gaps (Cozzarelli et al., 1967). Gap ligation presumably occurs by looping out the single-stranded region between substrate termini, although a mechanism involving chain slippage (Olivera & Lehman, 1967) is also possible. I find that at moderate enzyme levels T4 ligase will indeed ligate DNA with gaps (installed after purification by nicking with DNase I, followed by λ-exonuclease treatment;

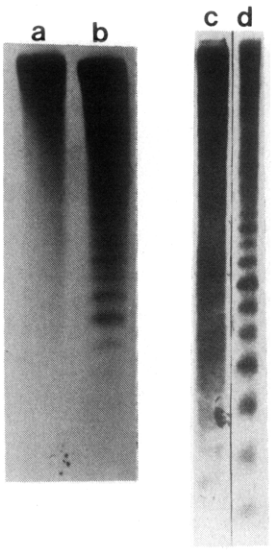

FIGURE 4: Effects of heat denaturation on purified DNA from DNase I digested chromatin. (a, b) Mild-digest DNA. Chromatin was digested to 10% Cl_3AcOH solubilization with DNase I; the DNA was purified and run (Materials and Methods) in (a) non-heat-denatured form and (b) heat-denatured form. For mild digests, the bulk of the ladder band material reproducibly appears only after heat denaturation of substantially larger size material. (c, d) Extensive digest DNA. This DNA was 25% digested in chromatin form. Although the ladder bands become clarified (c is non-heat-denatured and d is denatured), extensive digest DNA, upon denaturation, shows no obvious shift of ladder band material from larger size pieces.

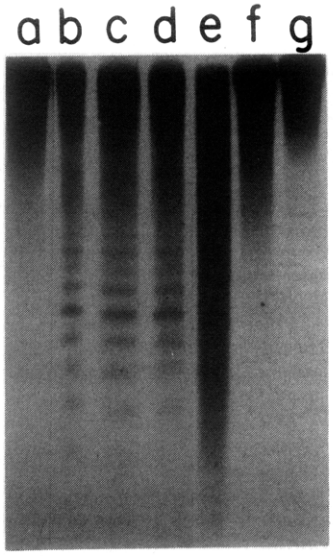

FIGURE 5: NAD^+-dependent DNA ligase ligates nicked control DNA but not non-heat-denatured DNA from DNase I digested chromatin: (a) non-heat-denatured DNA from chromatin about 8% Cl_3AcOH solubilized by DNase I; (b) a heat-denatured aliquot of the sample shown in a; (c) an aliquot of the sample shown in a was treated with 0.5 unit of NAD^+-dependent DNA ligase at 16 °C for 0.5 h (Materials and Methods) prior to heat denaturation; (d) treatment with 2.5 units of NAD^+-dependent DNA ligase; (e) DNA randomly nicked in nondenatured, purified form by DNase I (Materials and Methods) and then run in denatured form; (f) an aliquot of the sample in e treated with 0.5 unit of NAD^+-dependent ligase prior to denaturation; (g) an aliquot of e treated with 2.5 units of NAD^+-dependent ligase prior to denaturation.

see Materials and Methods), while, for the NAD^+ ligase, gap ligation is inefficient (about 30 to 40% maximal) and requires extremely high levels of enzyme (see legend to Figure 6).

Like the NAD^+-dependent enzyme, T4 ligase prefers nicked control DNA to non-heat-denatured ladder fragment DNA.

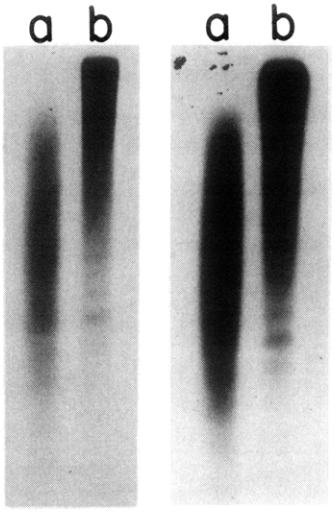

FIGURE 6: Selective ligation of nicked DNA in mixtures. A preparation of purified, non-heat-denatured DNA from DNase I digested chromatin (7 to 10% Cl_3AcOH solubilized) became randomly nicked in its duplex (mostly the higher molecular weight material; see Figure 3) by an unknown activity during storage. Upon denaturation, this partially degraded DNA showed largely obscured ladder bands at all exposures (e.g., a, left and right). However, treatment (lanes b) with NAD^+-dependent ligase (2 units/μg of DNA) prior to denaturation largely restored the original appearence (denatured form) of this DNA from mildly digested chromatin. Similar selective ligation of nicked DNA over ladder band DNA was also observed in mixtures of non-heat-denatured DNA from DNase I digested chromatin and nicked control DNAs prepared in various ways (Materials and Methods). Note that very high levels of enzyme (irrelevant to the comparisons above) cause the NAD^+-dependent ligase to behave somewhat like T4 ligase (see legends to Figures 7 and 8) in ligating across gaps.

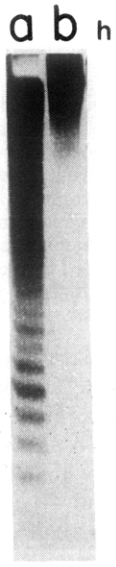

FIGURE 7: T4 ligase. Unlike the NAD^+-dependent ligase, moderately high levels of T4 ligase will ligate mild-digest ladder fragment DNA: (a) no ligase prior to heat denaturation; (b) 4 units of T4 DNA ligase/μg of DNA prior to heat denaturation. However, T4 ligase will not ligate ladder fragment DNA from extensive digests (see Figure 8).

However, unlike the NAD^+-dependent ligase, T4 ligase will ligate all of the ladder fragment DNA from mild digests at moderate enzyme levels (Figure 7). More importantly, T4 ligase, which is competent in ligating across gaps, is strikingly incapable of ligating ladder fragments from extensive digests (Figure 8) except for some inefficient ligation occurring at extreme concentrations (enzyme excess).

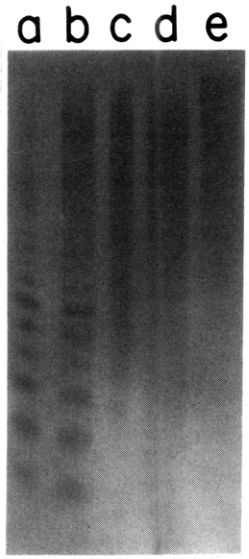

FIGURE 8: Non-heat-denatured ladder fragment DNA was purified from an "extensive" DNase I digest (30% Cl_3AcOH solubilized). High levels of T4 ligase failed to ligate ladder bands from extensive digests [(a) no ligase; (b) 10 units of T4 ligase/μg of DNA], while DNA with random-size single-stranded gaps prepared using DNase I and λ-exonuclease; (Materials and Methods) was ligated at moderately high levels [(c) no ligase; (d) 1 unit of T4 ligase/μg of DNA; (e) 5 units of ligase/μg of DNA]. Note: extremely high levels of enzyme (approaching 100 units/μg of DNA) cause some ligation by T4 ligase of both denatured mild-digest, ladder fragment DNA and non-heat-denatured ladder fragment DNA from extensive digests.

The result of interest is that the T4 DNA ligase detects a clear change in the properties of non-heat-denatured, ladder fragment DNA comparing mild and extensive digests. A clue to this difference between mild and extensive digests is given by the fact that T4 ligase will not ligate unassociated single-stranded termini, free in solution (Sgaramella et al., 1970; D. Riley, unpublished observations). Thus, the most apparent interpretation for the reproducible ligatabilities of these various DNAs is what was already suspected from the effects of heat denaturation on ladder fragment DNA patterns (Figure 4). That is, 5′-phosphate and 3′-hydroxyl termini, which are potential substrates for T4 ligase, are much more closely associated (intramolecular) in mild digest, non-heat-denatured DNA than in the DNA from chromatin more extensively digested by DNase I where many termini have apparently become unassociated. For mild-digest, ladder fragment DNA, one can mimick this loss of intramolecularity (and corresponding insensitivity to T4 ligase) by simply heat denaturing the DNA prior to ligase treatment. However, the results of Figure 4 suggest that extensive-digest, ladder fragment DNA, which is insensitive to T4 ligase, is not completely denatured prior to heat but rather looks more like the illustration in Figure 1f. The fact that non-heat-denatured ladder fragments from mild digests are ligatable by T4 ligase strongly suggests that a minimum of three fragments, presumably mostly ladder fragments, remain associated per non-heat-denatured DNA molecule from mild DNase I digests. This was the basis for preferring the arrangement shown in Figure 1e over other interpretations discussed above (see section on NAD^+-dependent ligase).

The results with T4 ligase also suggest that, since substrate termini are available intramolecularly in mild-digest DNA, spontaneous dissociation of some termini cannot account for the resistance of mild-digest, ladder fragment DNA to ligation by the NAD^+-dependent ligase. If spontaneous dissociation were significant in the mild-digest, non-heat-denatured, ladder fragment DNA, this DNA should have been strongly resistant to both ligases and, also, should not show a strong heat-denaturation-dependent shift in size.

The change in ladder fragment properties, comparing ladder fragments from mildly and extensively digested chromatin, could represent fragment production by fundamentally different mechanisms operative on different classes of chromatin. However, as discussed below, several observations suggest that the changes in ladder fragment properties are not due to different mechanisms but to the mechanistic complication referred to as "gap clustering".

Single-Stranded Gaps Detected by Electron Microscopy. Using either cytochrome *c* or gene 32 protein labeling methods (Materials and Methods), it was possible to observe, in non-heat-denatured DNA from DNase I digested chromatin, many clearly defined single-stranded gaps ranging in size from 100 bases to about 2 kilobases (kb) (Figure 9a). Careful inspection also reveals clusters of even smaller gaps at least down to about 40 bases ± 20 (Figures 9 and 11). Individual small gaps, not apparently associated with clusters, were also observed (Figures 9 and 11).

(*a*) *Long Single-Stranded Gaps* (*Nominally 0.4 to 2 kb*). Non-heat-denatured DNA from mildly DNase I treated chromatin shows a variety of sizable, single-stranded gaps not observed in DNA from undigested nuclei (Figures 9 and 10). Many of these longer gaps appear to be uninterrupted by duplex, and, it is believed, they could actually be uninterrupted, since, for mild digests, there is high molecular weight DNA observed on denaturing gels after selective protection by gene 32 protein (Figure 16, arrow). Moreover, the smoothly labeled long gaps and high molecular weight, denatured material seen on gels disappear if the original chromatin is slightly more extensively digested (i.e., more than 10%) with DNase I. Because of their appearance in mild DNase I digests and disappearnce at slightly higher levels of digestion, we speculate that the long gaps may in some way represent intermediates in the digestion of "active" stretches which are readily attacked by DNase I (Weintraub & Groudine, 1976). However, this tenative assignment requires much further investigation. Also unclear is whether these larger gaps are formed by a mechanism consistent with the model of Figure 1, which has no inherent restriction (except for the 10-base multiple) on the sizes of gaps formed, or whether some other mechanism, such as biased or polar protection of one strand, in some regions of chromatin, is involved.

(*b*) *Short Single-Stranded Gaps.* In addition to the long gaps described above, I also observed short gaps from about 40 bases (the lower detection limit, by electron microscopy) to 500 bases in length. These short gaps are present individually (Figures 9, 11, and 12) but also in clusters which accumulate at the higher levels of digestion. Clusters of short single-stranded gaps are found internally (Figures 11a and b), but often (depending on the extent of DNase I digestion) the most extensive clusters are found at the ends of non-heat-denatured, ladder fragment DNA molecules (Figures 11c and d). The frequent end location of small gap clusters suggests that these clusters may be sites sensitive not only to breakage but to spontaneous dissociation. As the level of DNase I digestion is increased, the gap clusters become even more single stranded in character (Figure 12; i.e., the gene 32 protein labeling becomes smoother) and, unlike earlier stages of digestion, non-heat-denatured DNA molecules found at higher stages of digestion have fairly common (about 40% at 12 to 15% digestion) gene 32 protein labeling of the end stretches (Figure 12). Although the clusters found at higher stages of

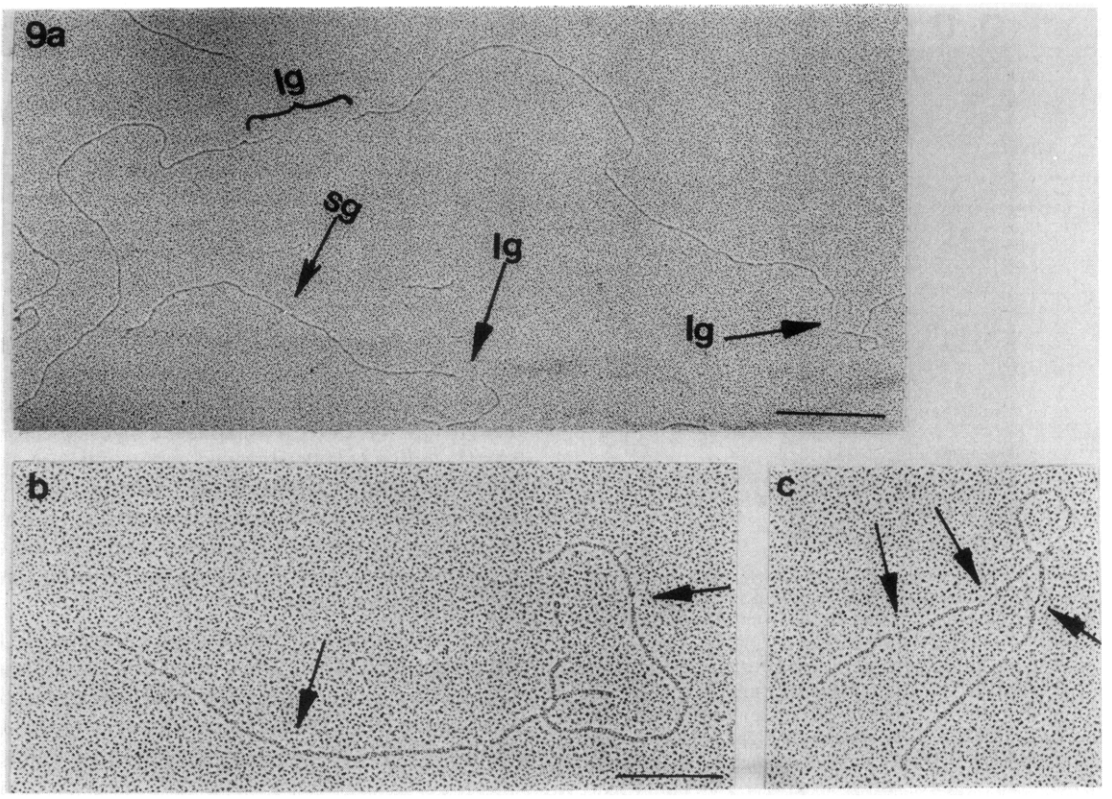

FIGURE 9: (a) Cytochrome *c* spreading of non-heat-denatured DNA from chromatin about 5% digested (Cl_3AcOH solubilized) with DNase I: large single-stranded gaps (lg); small single-stranded gaps (sg). The bracketed large gap in the upper left is approximately 2.2 kb in length (Materials and Methods). The small gap in a is about 70 ± 20 bases in length (scale bar = 0.3 μM). (b) Higher magnification of some fragments from an 8% digest. The small gap (arrow) on the left is approximately 60 ± 20 kb in length, while the one on the right is about 40 ± 20 bases, which approaches the detection limit. (b and c) Scale bar = 0.12 μM.

digestion begin to resemble the large gaps described above, they are distinguishable in being more interrupted or "rougher" in appearance, in their increase in number at higher levels of digestion, and in their frequent end location. These observations suggest that short duplex regions, which interrupt clusters of gaps, become shorter during the digestion to the extent that they become sites of spontaneous dissociation. In both mild and extensive digests, fragments smaller than about 0.5 to 1 μm (2 to 4 kb) typically appear to be more single stranded (with an intervening duplex occasionally obvious, Figure 12) than double stranded in character. Beyond these observations, I have not attempted to quantitate the various kinds of gaps and gap clusters by electron microscopy. Such quantitation would probably be unrealistic, since not only are some of the gaps and clusters transitory, but the chromatin DNA is clearly not digested uniformly by the DNase I.

Effects on Purified DNA. Native DNA, purified from undigested nuclei, is smooth in appearance (double-strand diameter) with only a very occasional small gap. However, if MSB nuclei are incubated at 37 °C (Materials and Methods), the purified DNA contains both long and short gaps suggesting the presence of an endogenous gap forming nuclease. When digested in purified form, native DNA from unincubated nuclei shows a variety of small gaps, some grouped into loosely formed clusters, but no tight clusters or long gaps. Since DNA, mildly digested in purified form with DNase I, is relatively easy to ligate by NAD^+-dependent ligase (Figure 5), one assumes that single-stranded nicks predominate over gaps when the DNA is digested in purified form.

Gap Sizes Determined by Gene 32 Protein Protection. Gene 32 protein has selective affinity for single-stranded DNA and, under appropriate conditions, does not denature or bind appreciably to native, double-stranded DNA (Alberts et al., 1968; Curtis & Alberts, 1976; Alberts & Frey, 1970; Brack et al., 1975). Gene 32 protein has also been shown to protect single-stranded DNA from digestion by nucleases (Huang & Lehman, 1972; Curtis & Alberts, 1976). If gene 32 protein specificity and protection from nucleases were assumed to be ideally precise, one should be able to bind gene 32 protein to purified, non-heat-denatured DNA from DNase I digested chromatin and obtain an accurate picture of possible gaps and their size distribution by then digesting away the unprotected duplex regions.

Before presenting the results of such an experiment, consider predictions made by the model of Figure 1. Since a given 10-base, multiple ladder fragment is formed as a result of destruction of 10-base multiple domains on either side, an absolute requirement of the model is that the gap sizes will have precisely the same 10-base multiple characteristic as the ladder fragments produced. However, other than the 10-base multiple property, the model does not predict whether a large variety of 10-base multiple gap sizes are possible or how frequencies of various gap sizes are related to ladder fragment size frequencies.

The results of binding gene 32 protein to non-heat-denatured DNA from DNase I digested chromatin, followed by digestion of unprotected DNA, are shown in Figures 13, 16, and 17. By looking at various exposures, I found three components in this "protection" pattern. The first component is a heavy smear from 10 or less than 10 bases, up to about 60 bases. At low exposures, one observes somewhat diffuse bands at 40, 30, and 20 bases (Figure 13, lane b). These values are assigned according to mobility identical with the original ladder fragments run next to the protected samples. The third component consists of ladder-size protected fragments seen only in heavy exposures, from 50 bases to about 120 bases. The relative

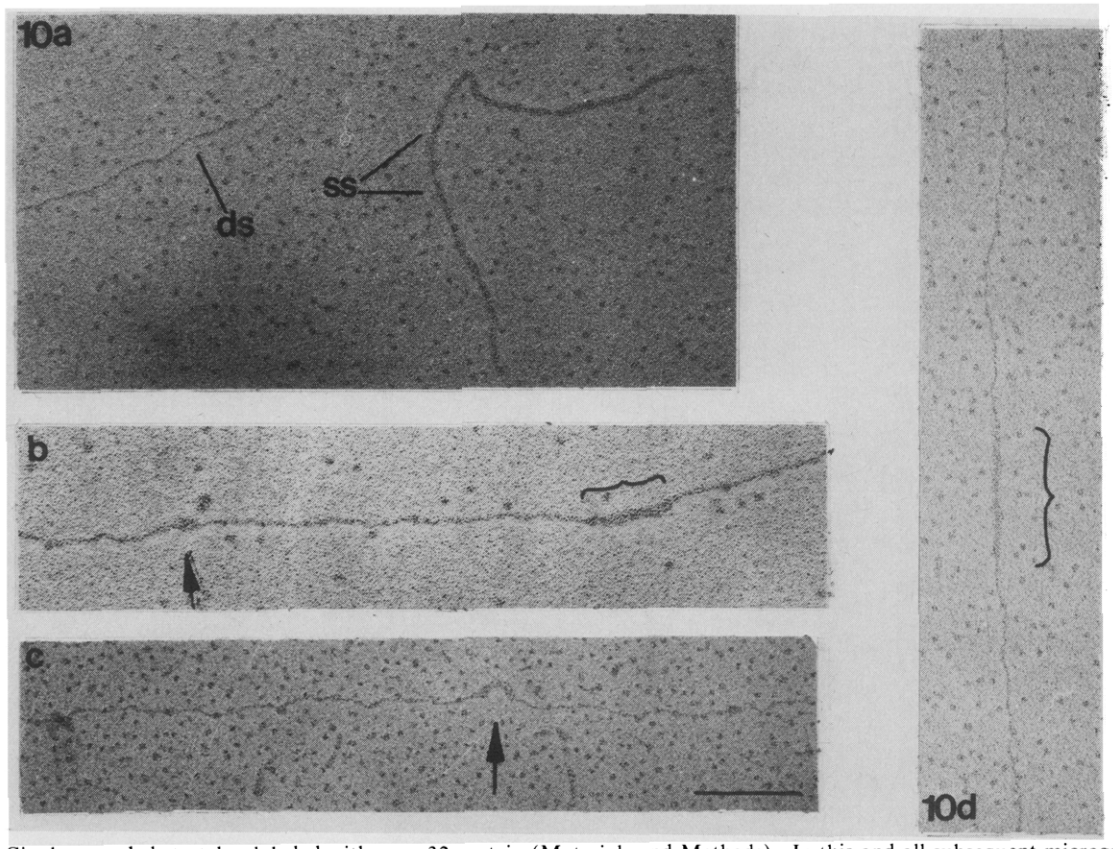

FIGURE 10: Single-stranded stretches labeled with gene 32 protein (Materials and Methods). In this and all subsequent micrographs, gene 32 protein binding causes an increased diameter of single-stranded regions compared to double-stranded regions. (a) A mixture of native and denatured DNA; ds, double-stranded; ss, single-stranded; (b, c, and d) non-heat-denatured DNA from chromatin about 5% Cl_3AcOH solubilized by DNase I. Shown are various long, single-stranded gaps (brackets or arrow) ranging from about 400 bases (c) to 1 kb (d). Note that the thinner, double-stranded regions vary somewhat in diameter, possibly reflecting binding of gene 32 protein to very small (e.g., less than 40 bases) indistinguishable gaps. Scale bar for a–d = 0.18 μM.

contributions of these various components depend on the conditions under which the experiment is done, as described below.

The Smear. It was found that native DNA, digested after purification with DNase I in the presence of gene 32 protein, also resulted in a heavy smear, but in this case no bands were present. Since DNA, purified from mildly digested (5 to 10% Cl_3AcOH solubilization) chromatin, is mostly (90 to 95%) native duplex, it appeared that the heavy smears were generated artifactually by DNase I digestion, during the protection experiment, of large stretches of native duplex. That is, although for native, purified DNA, DNase I puts in ligatable nicks (see ligation), some small gaps were also observed (see section on electron microscopy). Since the gene 32 protein was always in 15-fold excess during the protection experiments, these random-size gaps may become protected as well, generating the smear.

This suspicion was verified when it was found that adding an excess of carrier single-stranded DNA, to bind unassociated, excess gene 32 protein, led to a substantial reduction in the smear compared to the bands (Figure 17). Carrier addition was after gene 32 protein binding but before DNase I digestion of unprotected DNA. Although in this experiment all of the bands become weaker (no longer in excess, some of the gene 32 protein dissociates), it is clear that the smear is substantially more reduced than the bands (compare Figures 13, far-right lane, and 17b).

Without adding carrier, gene 32 protein protects about 15 to 20% either of native duplex DNA or of non-heat-denatured, ladder fragment DNA from chromatin which was about 5% digested by DNase I. With single-stranded carrier, only 2 to 3% of native duplex is protected, and protected is about 4% of non-heat-denatured, ladder fragment DNA. These values help describe the effects of carrier but are not considered as an accurate measurement of the amount of DNA in gaps at a given level of digestion, since the intensity (but not the basic appearance) of the protection patterns varies widely depending on the concentration of gene 32 protein, nuclease, and carrier used in the protection experiments.

Bands Seen at 20, 30, and 40 Bases. As seen in Figure 15, the gene 32 protein appears to protect all of the original ladder bands when these are first heat denatured, and has some tendency to artifactually protect duplexes 20 bases and smaller (Figure 14). However, gene 32 protein is seen to have no tendency to protect duplexes 30 bases and larger. Thus, except for the smear effect and uncertainty in the size range 20 bases and smaller, gene 32 protein specificity is appropriate to the question of gap size distribution. One concludes from the protection experiments, as for the S1 experiments, that ladder fragments in non-heat-denatured, ladder fragment DNA exist in partially denatured form. The term "partially" is used because, for ladder fragments from mildly digested chromatin, heat denaturation is always required before ladder fragments are observed (Figures 4a,b and 5a,b).

The disruption of duplexes 20 bases and smaller suggests caution in interpreting size distributions, since it is possible that the gene 32 protein, after binding to some gaps, may dissociate short terminal duplex regions (although these would have to be smaller than 20 to 25 bases), converting some clustered gaps into prematurely dissociated ladder fragments which become protected.

However, this possible deviation of the protection pattern

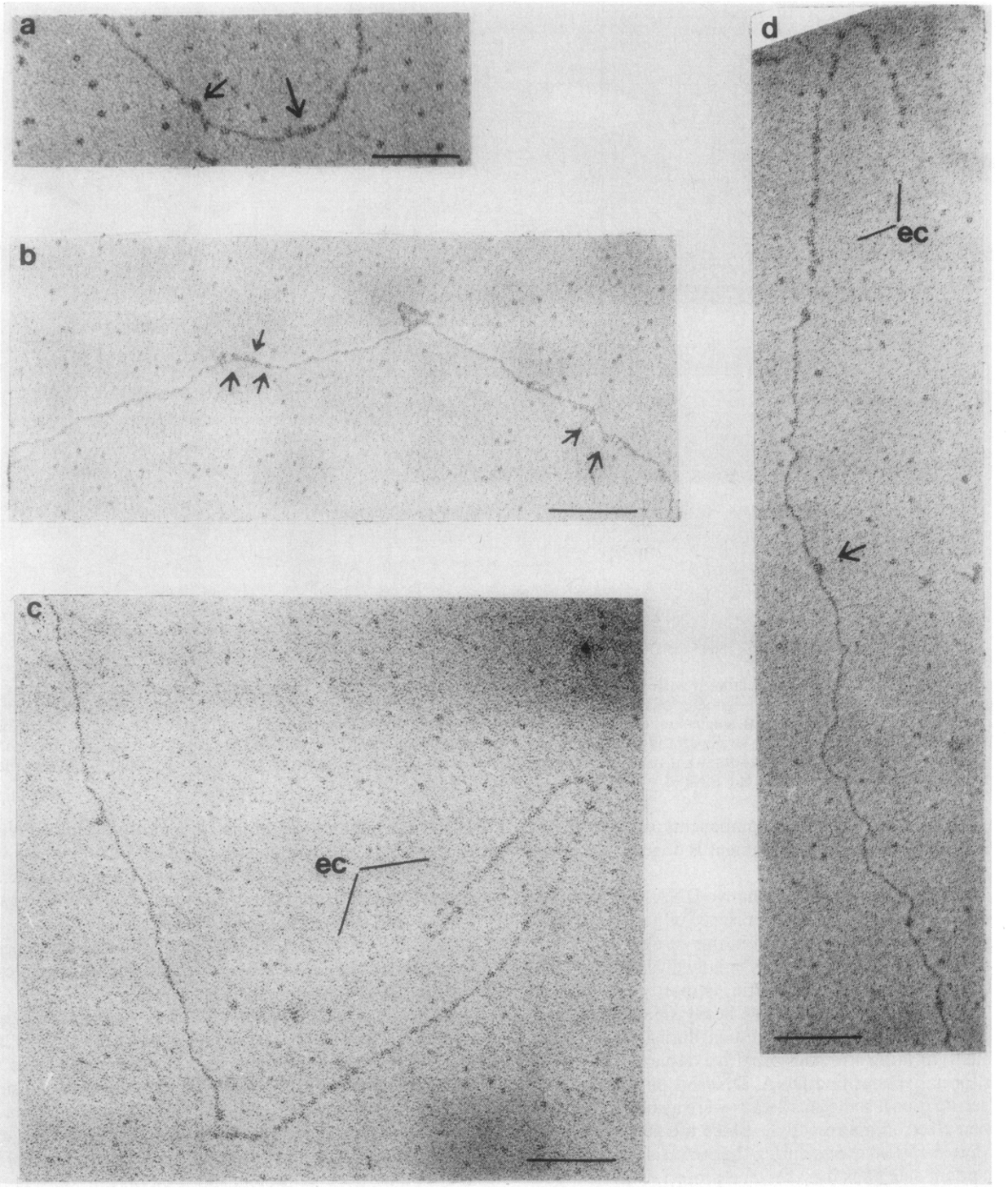

FIGURE 11: Small gaps and gap clusters visualized by gene 32 protein labeling. Although it is relatively easy to find individual small gaps (a and other arrows in b–d) down to about 40 bases, by far the majority of them occur in clusters either internally (b) or at the ends (c, d) of DNA fragments from a mild (8% Cl_3AcOH solubilized) digest. In 8% digests, approximately 10 to 12% of the fragments show end clusters (ec), while 15% digests have nearly 40% end clustering (subsequent figure). Scale bar for a = 0.12 μM. Scale bar for b, c, and d = 0.3 μM.

from the actual gap size distribution appears only in need of consideration for gaps larger than 40 bases, since the gene 32 protein protection experiment generates more material at 20, 30, and 40 bases than was originally present in the ladder fragment population (study carefully both exposures and the legend of Figures 13a and b). That is, at least these smaller 10-base multiple bands of the protection pattern appear to accurately represent the sizes of gaps formed by DNase I digestion of chromatin.

Note that material migrating in the position expected for a 10-base protected fragment (gap) is much reduced in intensity (Figure 13) compared to the bands at 20, 30, and 40 bases. Although 10-base gaps were assumed for simplicity in the illustrative model, they are actually the least amenable to analysis by gene 32 protein protection, since one or two molecules of gene 32 protein that could be accommodated by a 10-base stretch are expected to have far less affinity (nearly 1000-fold less; Kelly et al., 1976) for the DNA than is the case for cooperative binding of several gene 32 protein molecules to longer stretches (Alberts & Frey, 1970; Jensen et al., 1976; Carrol et al., 1972, 1975; Kelly et al., 1976). Thus, the very faint banding below 20 bases may be largely underrepresented.

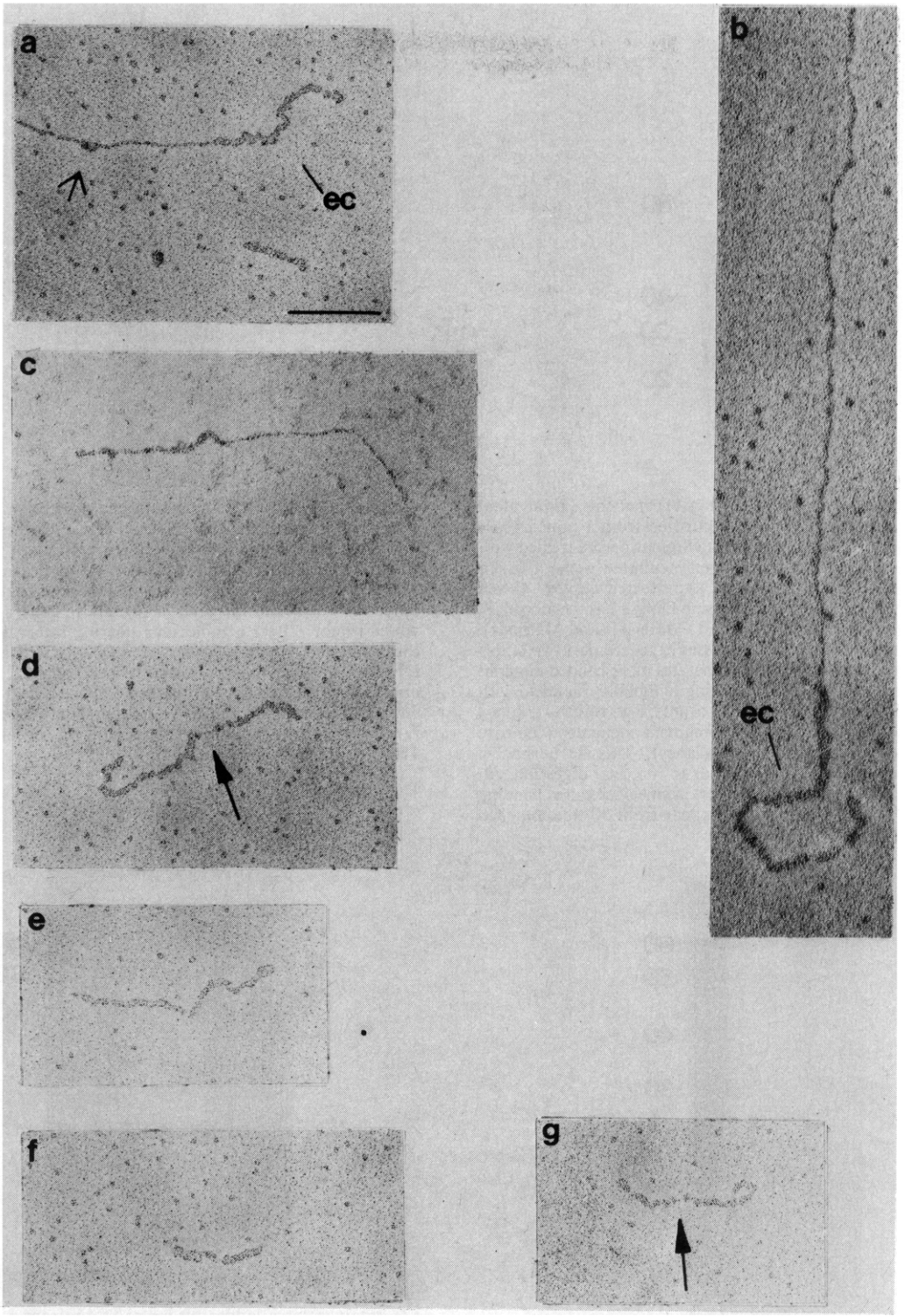

FIGURE 12: Gene 32 protein labeling of non-heat-denatured DNA from 15% Cl_3AcOH-solubilized chromatin. Note that, compared with milder digests (Figure 11), the end clusters (ec in a and b) have become shorter and are somewhat more smoothly labeled. Although may variations were observed, fragments shorter than about 3.5 kb (d–g) typically appeared more single stranded than double stranded in character. These fragments are interpreted to be parts of end clusters which have been attacked by DNase I at either the double-stranded or single-stranded component of Figure 1e (see Discussion). Occasionally, double-stranded interruptions were obvious within clusters (arrow) (d, g). Scale bar = 0.18 μM.

Also, I cannot distinguish (and have not specified in the model; see legend to Figure 1b) whether larger gaps (e.g., 40 bases) are formed because of large independent domains or by fusion of 10-base gaps.

10-Base Multiple Bands from 50 to 120 Bases. Although the intensity of the entire pattern varies, the presence of 10-base multiples (from 50 to 120 bases, as well as the smaller multiples) in protection experiments is remarkably independent of the gene 32 protein to DNA ratio, the presence or absence of carrier, the salt concentration up to 0.3 M NaCl, and the amount of nuclease used (from 1 to 100 μg/mL) to digest unprotected DNA. It also makes little difference whether the gene 32 protein is bound at 4 or 37 °C or whether unprotected DNA is digested away at 20 or 37 °C. The relative proportions of the various bands are also invariant, with the following exceptions: (1) DNA from chromatin very

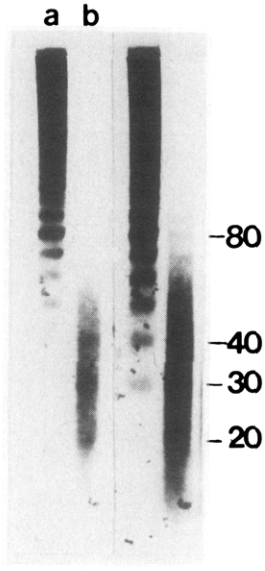

FIGURE 13: Gene 32 protein protection experiment. Equivalent aliquots of non-heat-denatured DNA purified from a mild DNase I digest (10% Cl_3AcOH solubilization) of chromatin were treated with gene 32 protein. The samples were then incubated without (a) or with (b) 25 μg/mL DNase I to digest unprotected duplex. (Note the reasons in Figures 15 and 16 for choosing DNase I as the secondary nuclease.) Samples were then processed (Materials and Methods), denatured, and run on urea gels. The lesser exposure (left two lanes) reveals that the protection experiment generates more banded material at 20, 30, and 40 bases (assigned according to mobility identical with ladder bands observed in the higher exposures, e.g., right two lanes) than was present originally in the ladder fragment population (compare a and b in the lighter exposure left two lanes). This is true since a and b represent equivalent aliquots prior to the final digestion. In twofold higher exposures (right two lanes), a smear obscures banding at 20, 30, and 40 bases, while bands appear from 50 to about 120 bases.

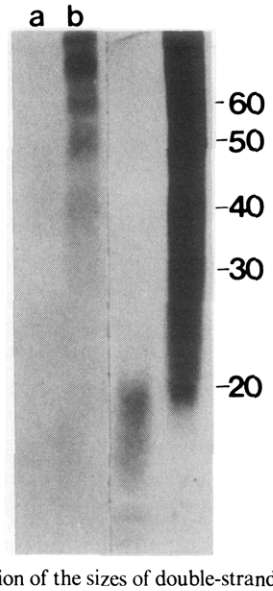

FIGURE 14: Estimation of the sizes of double-stranded fragments which might be protected by gene 32 protein. A double-stranded ladder was obtained using DNA purified from an extensive staphylococcal nuclease digest of chromatin (Materials and Methods). As in other protection experiments, gene 32 protein was bound, and unprotected DNA digested (left lane in each exposure) with DNase I (25 μg/mL). In the lighter exposure (left two lanes) it is seen that double-stranded fragments 40 base pairs and longer are not detectably protected by the gene 32 protein. In the heavy exposure (right two lanes) it is seen that, while there is actually very little protection above 20 to 25 base pairs, material at 20 base pairs and shorter is protected (left lane). (a) 25 μg/mL DNase I; (b) no DNase I.

mildly digested, to the extent that none of the original ladder bands are yet observed, shows protection experiment bands

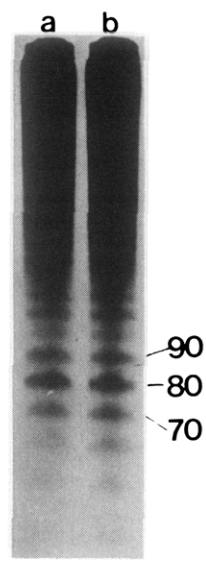

FIGURE 15: Fidelity of single-stranded protection. Purified ladder fragment DNA from a mild DNase I digest was first heat denatured and then submitted to the protection experiment. In this case, gene 32 protein reproducibly protects [(a) no nuclease; (b) 25 μg/mL DNase I)] the entire pattern from DNase I digestion. Also, no tendency was found for gene 32 protein to generate its own repeat which might reflect a molecular binding interval for the protein. Surprisingly, although single-stranded DNA is strongly protected from DNase I by gene 32 protein, staphylococcal nuclease is apparently oblivious to the presence of the protein, since heat-denatured, gene 32-bound ladder fragment DNA is completely digested over a short range (0.4 to 2 μg/mL) of low level enzyme concentrations (Figure 16).

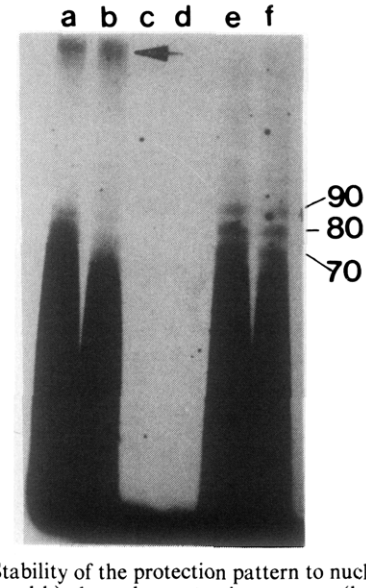

FIGURE 16: Stability of the protection pattern to nucleases. The left two lanes (a and b) show the protection pattern (legend to Figure 13) obtained from ladder fragment DNA from a very mild digest (about 2 to 3% Cl_3AcOH solubilized). The arrow indicates high molecular weight, protected material which is absent in protection patterns from slightly more extensive digests (e.g., chromatin 10% Cl_3AcOH solubilized, e and f). During this protection experiment, a and e were digested with 5 μg/mL DNase I, while b and f were digested with 50 μg/mL DNase I. The patterns are essentially formed at 1 μg/mL and are stable up to 100 μg/mL, except for a gradual weakening of the basic pattern which also occurrs if the gene 32 protein is not in excess. Lanes c and d show the results of staphylococcal nuclease digestion, used in a protection experiment at only fivefold higher levels than that required to obtain detectable Cl_3AcOH solubilization of equivalent amounts of unprotected duplex.

at 20, 30, and 40 bases but no higher multiples. This observation clearly shows that at least the 10-base multiples, 40 bases and smaller, are generated de novo during the protection

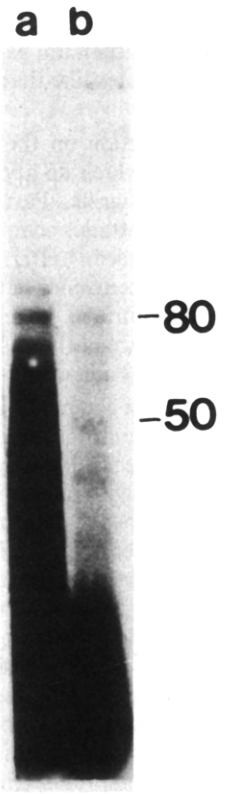

FIGURE 17: The effects of single-stranded carrier. It was found that high molecular weight, duplex DNA prepared without DNase I yielded no bands in protection experiments but did yield a smear, similar to that observed in previous experiments, between about 10 and 60 bases (not shown). To prevent binding to random size gaps, possibly formed during the protection experiment (text), excess unlabeled single-stranded DNA was added after gene 32 protein was bound to non-heat-denatured ladder fragment DNA. Subsequent DNase I digestion (now in the absence of excess unbound gene 32 protein) resulted in a substantial reduction in the smear relative to the bands [(a) no carrier; (b) 0.5 mg/mL single-stranded DNA carrier].

experiments. (2) DNA from chromatin between 1 and 5% Cl_3AcOH solubilized shows, in protection experiments, protected, high molecular weight DNA which barely enters the gels and which diminishes as one uses DNA from chromatin approaching 10% digested (Figure 16). This material possibly represents the long gaps found by electron microscopy.

It was suggested previously that for multiples greater than 40 or 50 bases, it is possible that gene 32 protein might generate protected fragments slightly larger than the actual gap sizes by dissociating short terminal duplexes of ladder fragments. That this might in some cases be true is suggested by the observation of a prominent 80-base band in both the protection experiment ladders and the original ladders (Figures 13, 16, and 17). However, it appears that the enhancement of the 80-base band is much more subtle in the protection experiment ladders than in the original ladders (Figures 13–18; compare bands at 70, 80, and 90 bases).

The protection experiment ladders also show independence from the original ladders in that ladder bands present in the original ladders above 150 bases (Figure 18; these are usually visible in brief exposures) were completely absent from protection ladders under all conditions and exposures tested.

The differences between the two types of ladders mitigate trivial explanations for the protection experiment bands between 50 and 120 bases, although the possibility of some artifactual contributions to the apparent gap size distribution above 50 bases cannot be excluded. It appears that either original ladder fragments above 150 bases (see, also, Lohr et al., 1977) are less susceptible to dissociation at their termini than fragments around 80 bases or, alternatively, gaps may be limited, with exceptions already noted, to subnucleosomal length. At present, precise answers to these questions are obscured by complications believed to be related to gap clustering (see Discussion).

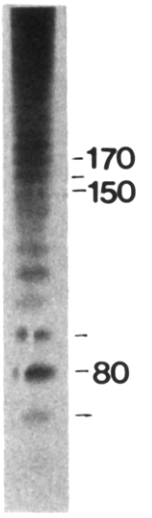

FIGURE 18: Ladder fragment bands above 150 bases. As digestion of chromatin proceeds, ladder multiples above 150 bases appear prior to multiples 120 and below. In most mild digests (5 to 10% Cl_3AcOH solubilized) appropriate exposures show that the higher multiples are also nearly as intense as the lower multiples.

Discussion

It was observed that a prominent characteristic of DNase I digestion of chromatin is the conversion of duplex DNA to single-stranded DNA. Consistent with this observation, others have presented evidence for the generation of single-stranded DNA during DNase I digestion (Oliver & Chalkley, 1974) and for single-strand specific interactions between histone octamers and chromatin DNA (Palter et al., 1979; Mirzabekov et al., 1978). Also, a nuclease from *Serratia marcescens* produces a distinctive but DNase-I-like (in the submonomer fragments) pattern, whose ladder bands have single-stranded character prior to heat denaturation, as judged by S1 sensitivity and hydroxylapatite chromatography (Pospelov et al., 1979).

While the specially accessible point-size hypothesis offers no obvious explanation for the large-scale conversion of duplex to single strandedness, the gap model of Figure 1 explains both this phenomenon and the production of ladder bands themselves. Ligase experiments strongly indicate that in non-heat-denatured DNA from DNase I treated chromatin, neighboring ladder fragment termini are intramolecular but separated by single-stranded gaps. Furthermore, experiments involving gene 32 protein showed that approximate 10-base interval fragments are generated from DNA purified from DNase I digested chromatin, in experiments designed to protect single-stranded gaps. Faithful protection of gaps by gene 32 protein was more clearly free from artifact for gaps 20, 30, and 40 bases in length than for larger multiples, although circumstantial evidence suggests gap sizes fall in a ladder type of pattern from 10 or 20 bases up to 120 bases. Many small gaps consistent in size with those of the proposed model were observed by electron microscopy using either cytochrome *c* or gene 32 protein labeling (Figures 9b, 9c, and 11).

Substantially larger gaps were also protected by gene 32 protein and observed by electron microscopy. With regard to these larger gaps, note that long stretches of single-stranded

DNA have been observed in total DNA from undigested nuclei (Bjursell et al., 1979). However, the "long gaps" of the present report (0.5 to 2 kb—smaller than those observed by Bjursell et al.) were observed only after digestion of the nuclei, suggesting that the frequency of naturally occurring single-stranded stretches is low enough, at least in these cells, so as not to be confused with gaps produced by DNase I.

Other Evidence. Another way of stating the fundamental differences between the specially accessible point-site hypothesis and domain perturbation is that in the latter, 10-base or 10-base-multiple stretches are the unit of rapid digestion, while for the point-site model, point sites are assumed to be the unit of rapid digestion. Although the gap model was presented with regard to DNase I action, it is important to emphasize that the model actually refers to a property of chromatin, in that the 25-fold protection conferred on stretches of DNA in chromatin is perturbed in domain-like fashion in response to nicking the DNA. The most obvious indication of this property of chromatin actually comes not from DNase I, but from exonuclease III (Riley & Weintraub, 1978).

It has been shown that exonuclease III, without contaminating endonuclease activity, generated 10-base multiple fragment ladders from nucleosome monomers and dimers by rapidly digesting 10-base or 10-base multiple stretches of DNA. In terms of the specially accessible point-site model for DNase I action, the behavior of exonuclease III represents a substantial paradox. One has to propose that for some reason exonuclease III digests most rapidly through those stretches or parts of those stretches of DNA which, according to the specially accessible point-site model, are the most protected. I now prefer the domain perturbation model in which 10-base multiple fragment ladders produced by dissimilar nucleases, DNase I and exonuclease III, are explained without paradox. In contrast to the case of DNase I, domain perturbation is very easy to grasp in terms of exonuclease III. The exonuclease simply has the most difficulty perturbing or entering a domain, but, once entered, the perturbed domain is easily digested. These conclusions also appear consistent with NMR studies (Feigon & Kearns, 1979; Kallenbach et al., 1978; Klevan et al., 1979) which indicate no observable variation in phosphodiester bond angles in chromatin, the latter being indistinguishable from the bond angles in free DNA.

It should be noted that the gap model and the site-specific point-site model may not be as mutually exclusive as this presentation has so far suggested. That is, at least some degree of nonrandomness in the initial nicking by DNase I is certainly possible. However, the gap model in the extreme form presented in Figure 1 appears sufficient to explain the bulk of the data (including end-labeling studies, below), while strict forms of the point-site model require modification to account for the generation of single-stranded DNA.

Gap Clustering. Both, changes in sensitivity to T4 ligase, comparing DNA from mildly and more extensively DNase I digested chromatin, and the clustering of gaps seen by electron microscopy suggested that DNase I digestion is characterized by rapid accumulation of well-digested, isolated stretches of chromatin DNA (Figure 1e) as opposed to accumulation of partially digested stretches (Figure 1d) more uniformly throughout the chromatin. This conclusion was also supported by marked S1 sensitivity, prior to heat denaturation, of ladder bands from mild digests. It should be noted that some degree of faint banding is usually visible after S1 and seems to be somewhat more prominent in DNA from selected sources (e.g., 14-day-old chick red blood cells, not shown, and frozen duck erythrocytes; Sollner-Webb & Felsenfeld, 1977). The occurrence of some S1-resistant ladder band material suggests that, for some stretches of chromatin, gap clustering may not be important and intermediates like that shown in Figure 1d accumulate.

Already there is information on the properties of some discrete duplex fragments which do appear below 100 base pairs in extensive DNase I digests. Particularly, the stagger between the ends of the two strands comprising these resistant duplexes is fairly well established (Lutter, 1979; Sollner-Webb & Felsenfeld, 1977). The occurrence of resistant duplexes in subnucleosomal size distributions is another complication (possibly related to the absence of the clustering complication in some areas of chromatin) not dealt with in detail here.

Clearly, the single-stranded gap model is also oversimplified to the extent that it only accounts for the generation of 10-base multiple fragments and not their individual intensities. Presumably, slightly differing environments in various regions of the nucleosome core and spacer determine which 10-base multiples are the most frequent, and, in fact, 5′-end-labeling experiments have already defined the location of various ladder multiples with respect to the 5′ ends of the core (Whitlock et al., 1977). However, it seems premature to speculate on the actual reasons for the various band intensities, since, as shown by the occurrence of gap clustering, much about the ladder patterns produced by DNase I is governed by presently uncharacterized properties of the chromatin. Although the data require imposition of tight gap clustering on the model of Figure 1, mechanisms responsible for clustering are completely unelucidated. One can at present only clarify observations of what happens to the ladder fragment DNA: (1) After mild DNase I digestion, ladder fragments are single stranded in character but bound by terminal duplexes into higher molecular weight, non-heat-denatured DNA. (2) This appears to be a rapidly formed situation that is metastable to digestion, since ladder fragments, apparently nonassociated at their termini, accumulate in more extensive digests.

To further illustrate the diversity of possible variation in the simplified model of Figure 1, it should be noted that, although gap clustering was clearly suggested in the studies by electron microscopy, I have no evidence that the clustering observed in mild digests is sufficiently "tight" (i.e., the interlocking duplexes sufficiently short) to account for the low level of discrete banding observed after S1 (Figure 3). I point this out because there is an interesting alternative explanation. That is, possibly DNase I "sees" no definite phasing between the 10-base multiple determinants (or domains) on the opposing strands in chromatin DNA. Possibly the apparent phasing observed, by S1 treatment of DNA from exonuclease III treated chromatin (Riley & Weintraub, 1978) or by polymerase filling-in experiments (Sollner-Webb & Felsenfeld, 1977; Lutter, 1979), is induced in the chromatin by the pressure of enzyme digestion. Alternatively, phasing inherent in the chromatin and detected by other nucleases and polymerase may for some reason be disrupted or randomized by DNase I, although some discrete banding after S1 suggests that exceptions would exist. In any case, the complexity of, and variations in, interpretations of nuclease digestions increase greatly when one attacks the assumption that protecting groups are immobile.

Clearly, it is only the final steps (Figures 1e and f) in the presented model that are directly supported by the data presented. Since the proposed intermediates (Figures 1a–d) were not readily demonstrated by these methods (although electron microscopy observations were consistent with Figure 1d), there is at least one fundamental alternative to the domain

perturbation model. This is a static model where gaps are predetermined in the chromatin by relatively immobile protecting groups protecting domains on alternating strands. The primary reason for choosing the perturbation model is that the static model seems inconsistent with the results of exonuclease III digestion, where larger ladder bands precede smaller ones (Riley & Weintraub, 1978) in sequential fashion as the level of enzyme is increased.

I have also not specified in detail the mechanism by which ladder fragment properties change, comparing mild and extensive digests. Already mentioned was the possibility of shortening of interlocking duplexes, which are presumably short enough in mild digests to be attacked by S1. An even simpler interpretation is that, after extensive digestion, DNase I (which prefers duplex but will also attack single-stranded DNA; Matsuda & Ogoshi, 1966) attacks the single-stranded components of Figure 1e. That is, since in Figure 3 each channel represents equivalent amounts of DNA prior to digestion, some of the ladder fragments destroyed after extensive digestion (compare intensities of Figures 3c, d, and e) may have bridged the termini between some of the ladder fragments which remain. In any case, there is no reason at present to assume that ladder fragments in mild and more extensive digests are formed by different mechanisms.

Biological Significance. A distinction that should be made, however, is that the ladder fragment production by DNase I is a certain property only for bulk nucleosomal chromatin. Thus, it is unclear as to what contribution, if any, "active" stretches of chromatin might make to the mild-digest ladder patterns.

With regard to bulk, inactive chromatin, the domain perturbation model could have some interesting implications. It has been found that, at least in some cases, nucleosomes appear to occupy sites very close to the passage of replication forks (McKnight & Miller, 1977). One interpretation is that the histones defining the nucleosome never leave the DNA during the passage of DNA polymerase. Possibly, division of the DNA into independent 10-base multiple, perturbable domains plays some role in the complex process of DNA replication in eukaryotes (Sheinin & Humbert, 1978).

It is also worth considering that if a single-stranded nick is capable of perturbing an entire domain of nucleotides, possibly such domains are also sensitive to other variations such as a modified base or a mismatched base pair. With regard to mismatched base pairs, eukaryotic cells exhibit fidelity in replication far greater than the fidelity of polymerase copying in vitro (Loeb et al., 1977). Perhaps domain perturbation plays a role in magnifying errors made by the polymerase, so that these can be repaired by enzymes of generalized specificity.

It is particularly intriguing that a modified base might perturb an entire domain of some 10-base multiple length. A speculative mechanism is that modification of a particular base, in a given stretch of inactive chromatin, might be sufficient to render a conformational change in an entire domain to the "active" configuration.

Certainly, the actual role of the nucleosome in various processes of replication and regulation will soon be apprehended as more of its properties become clarified.

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Distribution within Chromatin of Deoxyribonucleic Acid Repair Synthesis Occurring at Different Times after Ultraviolet Radiation†

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ABSTRACT: We have compared the initial distribution and subsequent redistribution within chromatin of nucleotides incorporated during the early ("rapid") phase and the late ("slow") phase of UV-induced DNA repair synthesis. As has been observed for the early repair phase, most or all of the nucleotides incorporated during the late repair phase are initially staphylococcal nuclease and DNase I "sensitive" (i.e., rapidly digested). This initial enhanced sensitivity is accompanied by both an underrepresentation of these nucleotides in the 145-165 base pair (core) DNA produced by staphylococcal nuclease digestion and an absence of these nucleotides in the ~10-base repeat pattern produced by DNase I digestion. Furthermore, nucleotides incorporated at late times after damage are involved in nucleosome rearrangement as reported previously for repair synthesis occurring at early times [Smerdon, M. J., & Lieberman, M. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4238-4241]. The kinetics of redistribution, however, appear to be more rapid than those observed for early times. Following redistribution the average nucleosome repeat length of DNA containing repair-incorporated nucleotides is the same as that of bulk DNA regardless of the time after damage that repair occurs; also, many of these nucleotides coelectrophorese with the ~10-base repeat fragments generated by DNase I. These results yield a new interpretation of our previous studies [Smerdon, M. J., Tlsty, T. D., & Lieberman, M. W. (1978) *Biochemistry 17*, 2377-2386] on the distribution of nucleotides incorporated at long times after UV irradiation.

Our understanding of DNA repair in the chromatin of mammalian cells has grown rapidly over the last few years [for recent reviews of chromatin structure, see Kornberg (1977), Chambon (1977), and Felsenfeld (1978)]. When nucleotides are incorporated by DNA repair synthesis occurring immediately after damage by either UV[1] (Cleaver, 1977; Smerdon et al., 1978, 1979; Smerdon & Lieberman, 1978a,b; Williams & Friedberg, 1979) or chemicals (Bodell, 1977; Tlsty & Lieberman, 1978; Bodell & Banerjee, 1979; Oleson et al., 1979), a majority of these nucleotides are located in DNA which is initially staphylococcal nuclease sensitive (i.e., rapidly digested). This enhanced sensitivity is accompanied by an underrepresentation of repair-incorporated nucleotides in the DNA of intact nucleosome cores (i.e., 145-165-bp DNA protected from rapid digestion by staphylococcal nuclease) (Smerdon et al., 1978, 1979; Smerdon & Lieberman, 1978a; Tlsty & Lieberman, 1978; Oleson et al., 1979). These results have been interpreted as evidence that DNA repair synthesis occurring immediately after damage is located mainly in linker DNA (Cleaver, 1977; Smerdon et al., 1978; Bodell & Banerjee, 1979); however, an alternative explanation is that repair synthesis is associated with a perturbation of the nucleosome structure such that some (or all) of the core DNA becomes nuclease sensitive (Lieberman et al., 1979; Oleson et al., 1979).

Two different laboratories have reported that during the first few hours after UV irradiation the distribution of repair-incorporated nucleotides becomes more uniform as the labeling times are increased (Cleaver, 1977; Smerdon & Lieberman, 1978b). Two possible explanations for these results have been proposed: (1) following repair synthesis nucleosome rearrangement occurs, resulting in a more uniform distribution of repair-incorporated nucleotides and/or (2) nuclease-sensitive regions of chromatin are repaired more rapidly than nuclease-resistant regions. Clearly, these two possibilities are not mutually exclusive. In the report by Cleaver (1977), pulse-chase experiments failed to show a change in the distribution of repair-incorporated nucleotides during the chase period. Thus, he concluded that the second possibility was more likely. Pulse-chase experiments performed in our laboratory, however, indicate that repair synthesis occurring immediately after damage by either UV (Smerdon & Lieberman, 1978a; Smerdon et al., 1979), 2-(acetylacetoxyamino)fluorene (Tlsty & Lieberman, 1978), or 7-(bromomethyl)benz[*a*]anthracene (Oleson et al., 1979) is followed by a rapid and

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[1] Abbreviations used: UV, ultraviolet; dThd, thymidine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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