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Quantitative Analysis of the Digestion of Yeast Chromatin by Staphylococcal Nuclease†

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ABSTRACT: The DNA in intranuclear yeast chromatin is protected from rapid staphylococcal nuclease degradation so as to yield an oligomeric series of DNA sizes. The course of production and disappearance of the various oligomers agrees quantitatively with a theory of random cleavage by the enzyme at uniformly susceptible sites. The sizes of the oligomers are integral repeats of a basic size, about 160 base pairs, and 80–90% of the yeast genome is involved in this repeating structure. Within this repeat there exists a 140 base pair core of more nuclease-resistant DNA. During the course of digestion, the sizes of the oligomers decrease continuously. The widths of the distribution of DNA sizes increase in the order: monomer (1 × repeat size, half width = 5–7 base pairs) < dimer (2 × repeat size, half width = 30 base pairs) < trimer (3 × repeat size, half width = 40–45 base pairs). The yeast genome thus seems to have variable spacing of the nuclease-resistant cores, to produce the average repeat size of about 160 base pairs. Also, the presence of more than one species of monomer and dimer at certain times of digestion suggests a possible heterogeneity in the subunit structure.

When chromatins from higher eukaryotes are digested by staphylococcal nuclease, the DNA is cleaved into a series of size classes which represent integral multiples of a basic size (cf. Hewish and Burgoyne, 1973), about 200 base pairs (Noll, 1974; Shaw et al., 1976). Approximately half of the DNA in chromatin is ultimately accessible to digestion with staphylococcal nuclease (Clark and Felsenfeld, 1971) and at this limit consists of a distinctive distribution of DNA sizes smaller than the basic repeat size (Axel et al., 1974).

We have previously shown that nuclease digestion of baker's yeast chromatin produces integral DNA size classes as in more complicated eukaryotes, but that the repeat size is smaller (Lohr and Van Holde, 1975). In this study we have followed the time course of nuclease digestion of yeast chromatin using several confluent analyses: accurate sizing of the nuclease-resistant size classes of yeast DNA using PM2 DNA restriction fragments as internal standards; study of the acid solubility to assess susceptibility to nuclease; quantitative analysis of the size classes to assess the amount of the genome involved in the repeating structure; comparison with a random-hit theory of nuclease action. We have also obtained evidence for possible nonuniformity of the repeating structure.

Materials and Methods

Growth of Yeast and Isolation of Nuclei. Baker's yeast, strain Y55, was grown in 1% yeast extract–2% Bactopeptone–2% glucose to a density of about 5×10^7 cells/ml. Cells were harvested, spheroplasts made according to Cabib (1971), and nuclei isolated essentially according to Wintersberger et al. (1973), procedure b, with minor modifications in the HM step. These are: use of 7% Ficoll instead of 5% polyvinylpyrrolidone according to Sajdel-Sulkowska et al. (1974); three "slow" spins at 7000, 6500, and then 6000 rpm in a Sorvall SS34 rotor; a "fast" spin at 20 000*g*, 25 min.

Nuclease Digestion and DNA Isolation. Nuclei were resuspended in 1 M sorbitol, 0.5 mM Ca^{2+}, or 0.05 mM Ca^{2+}, pH 6.3, at a concentration of about 150 μg/ml DNA (about 3×10^9 nuclei/ml). Digestion was done at concentrations of 130 to 180 U/ml staphylococcal nuclease (Worthington Biochemical Corp.) at 37 °C. Samples were removed at designated times and reaction was stopped by addition of cold 8 mM Tris[1]–35 mM Na_2EDTA (pH 6.2) to a final concentration of 11 mM Na_2EDTA. Samples were put immediately on ice and divided into two aliquots for subsequent analysis. (a) An aliquot of each sample was made to 0.3 N in $HClO_4$–0.3 M in NaCl on ice, shaken for 10 min, and centrifuged at 15 000 *g* for 15 min. The pellet was washed once with 0.3 N $HClO_4$–0.3 M NaCl and recentrifuged. The supernatants constituted the acid-soluble and the pellet constituted the acid-precipitable samples, respectively, for DNA analysis. (b) The rest of the digestion sample was made to 100 μg/ml in pancreatic RNase (Schwarz/Mann), 100 U/ml in T_1 RNase (Sigma), and 0.1 U/ml in T_2 RNase (Sigma) and incubated at 37 °C for 15 min. One-tenth volume of 21% Sarkosyl, 1.6 M NaCl, and 50 μl of 10 mg/ml Pronase per ml of sample were added and the samples incubated overnight at 37 °C. DNA was extracted by a modified Marmur procedure (Britten et al., 1974) and pre-

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[1] Abbreviations used: bp, base pairs of DNA; DABA, diaminobenzoic acid dihydrochloride; Hae III, endonuclease R *Hae* III obtained from *Haemophilus aegyptius;* PM2, *Pseudomonas* bacteriophage 2; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

cipitated with 3 volumes of 95% ethanol. The DNA was redissolved in E buffer (40 mM Tris–1 mM Na_2EDTA–20 mM sodium acetate, pH 7.2), RNase treated as above for 15 min at 37 °C and extracted again as above. The purified DNA was dissolved in tenfold diluted E buffer which was 9% in glycerol.

Analytical Methods for Analysis of DNA. The acid-soluble DNA samples from above (a) were adjusted to 0.5 N in $HClO_4$ and hydrolyzed at 70 °C for 15 min; this hydrolysate is designated the S fraction. The acid-precipitable DNA samples from a were hydrolyzed in 0.5 N $HClO_4$ at 70 °C for 20 min. The samples were spun at 15 000*g* for 15 min, the supernatant was removed, and the pellet was reextracted with 0.5 N $HClO_4$ for 15 min at 70 °C and spun again at 15 000 *g* for 15 min. The two supernatants were combined and constituted the acid-insoluble, P, fractions. DNA was measured by the modified diphenylamine reaction (Burton, 1968) or by a version of a fluorescence assay (Kissane and Robins, 1958) in which 0.1 ml of decolorized 2 M diaminobenzoic acid dihydrochloride in distilled H_2O was mixed with 0.2 ml of hydrolyzed DNA and incubated at room temperature for 60 min. The reaction mixture was diluted with 0.9 ml of 0.6 N $HClO_4$ and samples, standards (acid-hydrolyzed calf-thymus DNA), and blanks (digestion buffer + nuclease, treated as samples above) were read in an Aminco-Bowman fluorimeter at λ_{exc} 405 nm and λ_{em} 520 nm. Emission and excitation spectra were similar to spectra of DNA standards.

Gel Electrophoresis and Staining. DNA samples (aliquots (b) from above) were electrophoresed on 3, 4.5, or 6% polyacrylamide tube gels, according to Loening (1967), at 5 mA/gel for about 6, 8, or 10 h, stained 1 h in 0.5 μg/ml ethidium bromide and photographed under "blacklight" illumination on Polaroid Type 55 film. Analysis of gels involved one of three procedures. (1) Gels were scanned directly at 265 nm in a Gilford gel scanner, then stained in 40 μg/ml toluidine blue (MCB) for 6–8 h, destained in H_2O, and scanned at 546 nm. (2) Gels were scanned at 265 nm, then stained for 10–12 h in 0.005% Stains-all (Kodak) in 1:1 H_2O–formamide (pH 7.3), destained in the light in H_2O until the background cleared, and then scanned at 600 nm. (3) The scans at 265 nm were omitted and the gels were stained in toluidine blue or Stains-all as above. We find that the three techniques mentioned above (scanning at 265 nm, staining with toluidine blue, and scanning at 546 nm, or staining with Stains-all and scanning at 600 nm) give very comparable results. We prefer using Stains-all, because it is faster and more sensitive than toluidine blue. Both toluidine blue and Stains-all give linear responses to DNA concentration (maximum deviation from average values 7–8%), within the concentration range used here, as determined from the areas of the PM2 DNA restriction fragments run at various loadings on 3% polyacrylamide gels and from the relative areas for the fragments on a single gel (Kovacic, Lohr, and Van Holde, in preparation).

For analysis of single-strand DNA, 4.5% polyacrylamide–formamide (denaturing) gels were made according to Staynov et al. (1972) and Boedtker et al. (1973). Gels were run for 14–16 h at 6 V/cm in 0.02 M phosphate buffer and stained in ethidium bromide and Stains-all as described above.

In order to provide an accurate measure of the sizes of the double-strand or single-strand yeast DNA fragments produced by the staphylococcal nuclease digestion, *Hae* III restriction endonuclease fragments of PM2 DNA were used as markers. The preparation and calibration of these fragments has been described (Van Holde et al., 1975). We have found it necessary to recalibrate our original sizes (Noll, 1976; H. Zachau, private communication) against several series of SV40 and λ restriction endonuclease DNA fragments. The sizes in this paper reflect this recalibration and differ somewhat from those we used previously (cf. Figure 4). Details of the recalibration will be discussed elsewhere (Kovacic and Van Holde, 1977).

Results

Acid Solubility as a Function of Time. The action of staphylococcal nuclease on chromatin may be followed by monitoring the production of acid-soluble deoxyribonucleotides (cf. Clark and Felsenfeld, 1971). Because yeast is rich in RNA and because staphylococcal nuclease has RNase activity, the action of the enzyme on yeast chromatin cannot be followed by measuring the absorbance at 260 nm. Furthermore, we found that some material in the acid-soluble samples inhibits color development in the diphenylamine reaction for DNA. This interference has been previously noted (Munro and Fleck, 1954). However, the highly DNA-specific fluorimetric reaction (Kissane and Robins, 1958) with 3,5-diaminobenzoic acid dihydrochloride (DABA) is free from this interference.

Figure 1 shows the kinetics of the digestion of intranuclear yeast chromatin by staphylococcal nuclease. The nuclease renders increasing percentages of the DNA acid soluble as digestion continues. There is no evidence of a plateau but these experiments were not intended to analyze the long-time or limit digestion behavior of yeast chromatin. Even in experiments carried to very long times or using very high nuclease concentrations we do not observe as much acid-soluble DNA as in higher eukaryotes (Clark and Felsenfeld, 1971). Although such experiments will be discussed in detail elsewhere (Lohr et al., in press), we can say that there seems to be a slowing down of the digestion at about 35–40% acid-soluble DNA, at which point the characteristic submonomer limit pattern on gels is observed (cf. Axel et al., 1974).

We find that the total DNA measured as the sum of acid-soluble plus acid-insoluble material (S + P) decreases somewhat ($\simeq$20%) by the end of the digestion course. This is a consistently recurring trend noted in all digestions done to date, and we have no ready explanation for it. At long times of digestion, a small amount of highly aggregated material can be seen on the bottom of the flask. This material does not look like nuclei but possibly it contains chromatin; if so, this would have the effect of lowering the total DNA measured at these times. Other tests (not shown) suggest that the progressive loss is not due to a failure of the DABA assay. Furthermore, the S + P values obtained agree well with the values for the amount of DNA measured in the samples of purified (Marmur procedure) DNA taken for electrophoretic analysis at corresponding times.

Monomers, Oligomers, and Mystery Bands. Figures 2a and 2b show the results of typical digestion experiments. The gel pattern is qualitatively similar to that observed in the digestion of many types of chromatin. As digestion proceeds, the higher molecular weight repeat size classes (oligomers) disappear and material accumulates in the basic size class (monomer).

The three bands seen to the far right in Figure 2a appear to be unique to yeast for we have not observed such bands in the digestion of any other chromatin. They do not show in ethidium bromide stained gels, but they stain identically in either toluidine blue or Stains-all. Note that they are present even in the zero-time sample and persist with little change in intensity throughout the digestion. These "mystery bands" do not behave like DNA for they show quite different relative mobilities in different gel systems. For example, they appear at the position of 40b, 60b, and 80b on 6% polyacrylamide–formamide

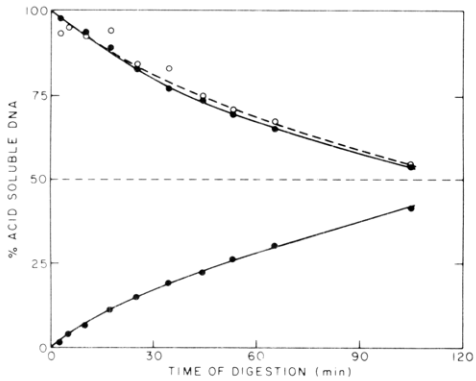
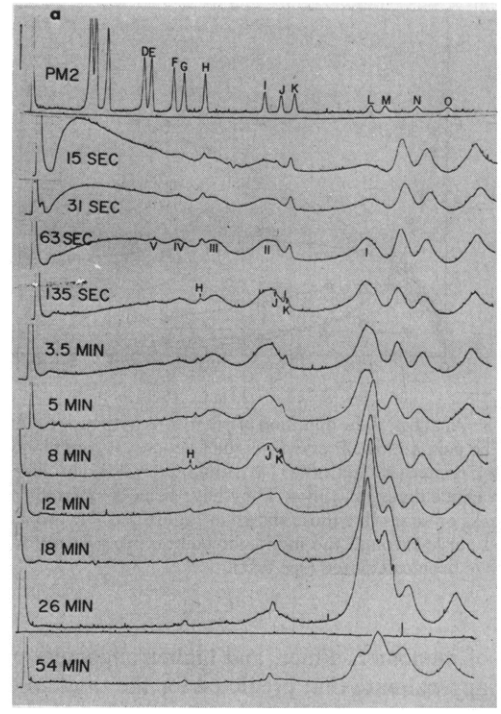


FIGURE 1: A typical course of the acid solubility kinetics of the staphylococcal nuclease digestion of yeast chromatin. The lower curve is the ratio of acid-soluble DNA (S) to the amount of acid soluble plus acid-insoluble DNA (P) at any time. The upper curves show the ratio P_t/P_0 where P_t is the amount of acid insoluble DNA at any time, t, and P_0 is the amount at zero time of digestion with no added nuclease. For curves with filled circles, DNA was analyzed by the DABA assay; for the curve with open circles, DNA was measured by the modified diphenylamine reaction.

TABLE I: Estimating Error in Gel Staining, Scanning, and Resolution Technique.[a]

	Maximum Deviation (%)
I	±6
II	±6
III	±11
IV	±6
IV<	±0.3
$\sum$ = I + II + III + IV + IV<	±5

[a] Maximum deviations of the areas under the peaks of monomer (I), dimer (II), trimer (III), tetramer (IV), all DNA larger than tetramer (IV<) and the sum of these ($\sum$) were measured from the resolved scans of 3% Stains-all stained polyacrylamide gels run with three different loadings of DNA. Maximum deviation was measured as the deviation of the value which varied most from the mean. Gels were stained, scanned, and resolved exactly as usual.



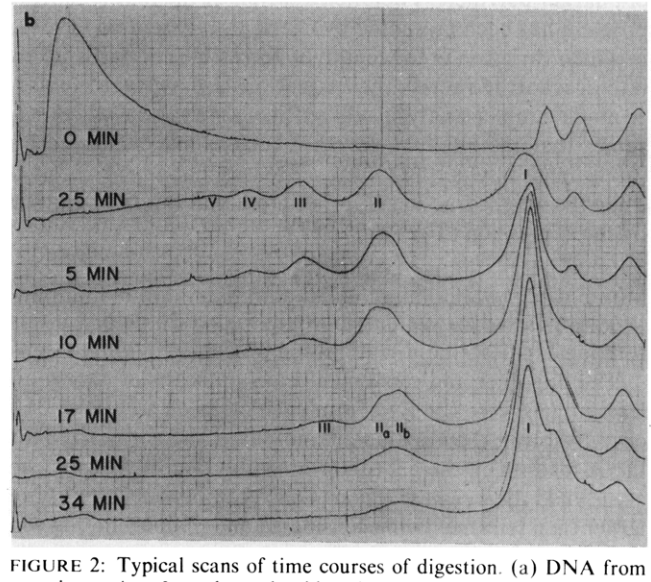


FIGURE 2: Typical scans of time courses of digestion. (a) DNA from experiment 4 on 3% polyacrylamide gels, with PM2 DNA markers (H, J, K) in the gel, stained in toluidine blue and scanned (Materials and Methods). Times of digestion are given to the left. The size classes of DNA are designated I (monomer), II (dimer), III (trimer), IV (tetramer), V (pentamer). The three bands to the far right in all the gels with yeast DNA are the "mystery bands" (see text). The lower line in the 26-min scan is the scan of the cuvette itself. The gel "PM2" is a limit digest of PM2 DNA with Hae III restriction endonuclease. The sizes of the labeled PM2 fragments are: D = 854 bp; E = 794 bp; F = 642 bp; G = 592 bp; H = 498 bp; I = 322 bp; J = 288 bp; K = 263 bp; L = 160 bp; M = 145 bp; N = 117 bp; O = 95 bp (Kovacic and Van Holde, 1977). (b) DNA from experiment 5 on 3% polyacrylamide gels without PM2 DNA internal markers, stained in Stains-all and scanned (Materials and Methods). II_a and II_b refer to the two components of the dimer peak. A PM2 digest was coelectrophoresed with these gels (not shown).

gels but at 130 bp, 110 bp, and 85 bp on 3% polyacrylamide nondenaturing gels. Exhaustive digestion of the samples with pancreatic DNase before electrophoresis completely degrades the DNA but fails to remove the mystery bands. The samples have already received exhaustive RNase treatment. We suspected them to be poly(A), but long (12 h) digestion with high concentration (300 U/ml) of T_2 RNase failed to remove them. By preparative electrophoresis we have partially separated the mystery bands from oligodeoxy and ribonucleotides which run in the same region. The mystery bands do not seem to have significant A_{260} and are base hydrolyzable. Thus, we feel that they are neither single- nor double-strand DNA.

The Distribution of DNA Digestion Products. In order to examine the mode of action of staphylococcal nuclease on yeast chromatin, we would like to follow changes in the relative amounts of oligomers and monomer with time of digestion. The linearity of the staining–scanning technique has been discussed (see Materials and Methods). However, in order to check the quantitation under conditions more similar to those encountered in analyzing a digestion time course, we mixed digestion samples from early and late time points, to get the widest possible distribution of DNA sizes, and ran various loadings of the mix on 3% polyacrylamide gels. Table I shows the maximum observed deviations from average. The larger deviations observed are probably due to the difficulty of resolving peaks from each other. These data show that we can reliably perform a quantitative analysis of the amounts of DNA in each size class as a function of time.

As a possible model, let us assume that the DNA in chromatin is accessible to nuclease digestion only at approximately equally spaced and equally accessible points. Then the distri-

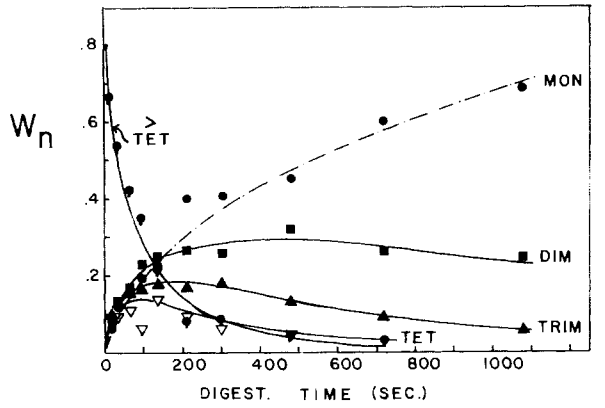


FIGURE 3: Analysis of the digestion of yeast chromatin using a random-hit model. The points show observed weight fractions, W_n, of monomer (●), dimer (■), trimer (▲), tetramer (▽), and material larger than tetramer (◆), at various digestion times. They have been calculated from areas under peaks on scans like those shown in Figure 2b. The broken line is a smoothed curve through the monomer points. From it, all of the solid curves have been calculated (see text).

bution of monomer, dimer, and higher oligomers produced should approximate that predicted for the random cleavage of a larger polymer. A detailed analysis of the mathematical problem has been presented by Clark and Felsenfeld (1974), in which variation in the length of accessible regions is taken into account. However, their equations are awkward to use. A much simpler analysis will suffice, an analysis which assumes that the nuclease-resistant regions are connected by single links, of which a fraction p are intact at any given time. Then the weight fraction of n-mer present at this time is given by the expression (Tanford, 1961)

$$W_n = np^{n-1}(1-p)^2 \qquad (1)$$

We have used data such as shown in Figure 2b, to test if this relationship describes the digestion process.

The oligomer and monomer peaks on scans of Stains-all stained gels were resolved from each other on a DuPont 310 curve resolver; their areas were measured and normalized for DNA loading on the gel (determined by DABA assay). The areas yield the weight fraction of each component (everything larger than tetramer being considered one component), shown graphically by the points in Figure 3. Since we do not know, a priori, how the parameter p will vary with time, we have calculated p from the monomer weight fraction by use of the equation, $W_1 = (1-p)^2$. This allows calculation of the expected weight fractions of all other components at each digestion time using eq 1. *These predictions are shown as the solid lines in Figure 3.*

The excellent correspondence between the predicted curves and the observed points means that this simple model provides a quite precise description of the digestion process and implies strongly that all links between adjacent protected regions are about equally accessible. Furthermore, it argues that the observed series of oligomers represent intermediate states in a uniform degradation process.

Oligomer/Monomer Size as a Function of Time of Digestion. Oligomer and monomer DNA fragment sizes were determined on 3% polyacrylamide tube gels (Materials and Methods). The presence of at least three PM2 DNA fragments of known size in each gel allows an accurate estimation of the size of the staphylococcal nuclease produced DNA fragments (cf. Figure 2a). Figure 4 shows the results of several independent experiments. These data have been used to establish the

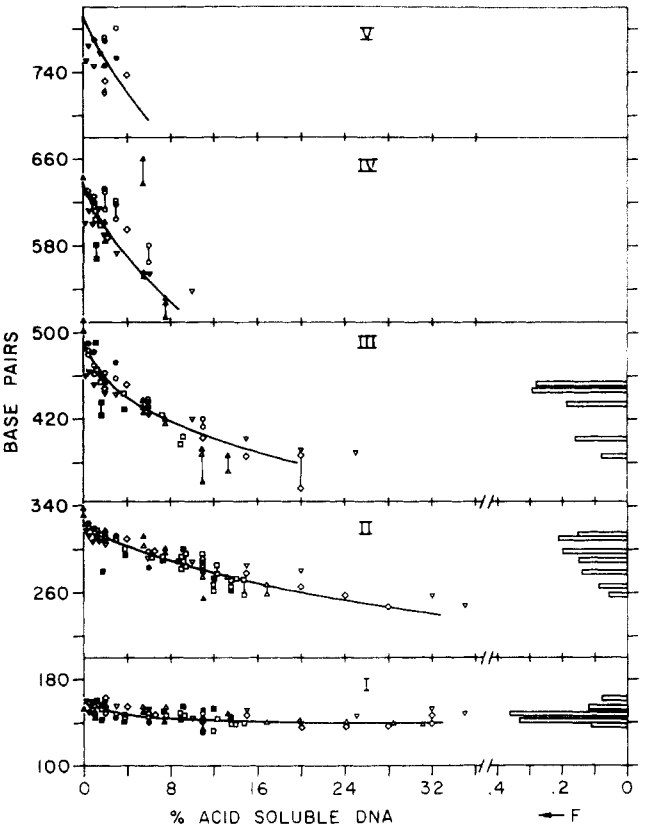


FIGURE 4: Time course for size decrease of monomer–pentamer DNA. The double-strand (D-S) and single-strand (S-S) peak sizes for monomer–pentamer (I–V) DNA (cf. Figure 2a) are plotted against % DNA rendered acid soluble (cf. Figure 1). Three kinds of peaks were encountered in the scans: (a) symmetrical peaks (the vast majority); (b) nonsymmetrical or skewed peaks; (c) clearly resolvable, multicomponent peaks (mainly monomer). The peak size was taken at the position of maximum DNA concentration for (a) peaks, at the average of the position of maximum DNA concentration and the midpoint of the width at half-height for (b) peaks, and at the resolved (on the Du Pont 310 curve resolver) position of maximum concentration of each component for (c) peaks. Occasionally, the exact peak sizes were indeterminate, shown here as |. The data are from five different experiments: [(▽) D-S No. 4], [(◇) D-S No. 5], both 0.5 mM Ca^{2+}; [(○) D-S, (●) S-S No. 6], [(△) D-S, (▲) S-S No. 7], [(□) D-S, (■) S-S No. 8], all 0.05 mM Ca^{2+}. The bars on the right depict (for experiment 5) the weight fraction (F) of the peak having the double-strand peak size (as determined above) shown on the y axis. F was measured by dividing the area of the given peak by the sum of the areas of all peaks of that particular oligomer size class during the whole time course. All areas were normalized for different loading of DNA on the gel.

repeat sizes of chromatin subunits in two ways: the size (in bp) for a given oligomer divided by the oligomer number yields the average repeat size in that oligomer (bp/n); the difference in number of base pairs between adjacent oligomers ($bp_{n+1} - bp_n$) can also be used to estimate the repeat size (Kornberg, 1975).

In yeast, the concept of a single repeat size for the entire genome is misleading because there is a distribution of repeat sizes (see Discussion). Thus, the zero-time (extrapolated) and early measured-time oligomer values (Table II) yield an average repeat size which is probably slightly larger than the true average since larger repeats, which contain longer spacer DNA, probably are preferentially cleaved to produce the early oligomers. The long-time oligomer values yield the size of the smallest repeats. Because larger oligomers in yeast are more highly digested toward their limit than smaller oligomers at any time (Figure 4), the difference method may underestimate

TABLE II: Repeat Sizes of the DNA of Yeast Chromatin.[a]

	bp/n			$bp_{n+1} - bp_n$			
n	t_0	t_0'	t_{max}	$(n+1) - n$	t_0	t_0'	t_{max}
IV	160	157	130	IV − III	163	155	128
III	164	161	127	III − II	161	160	110
II	163	161	125	II − I	165	164	110
I	158	158	140				

[a] n is the oligomer number; bp/n is the size of an oligomer (in bp) divided by n; $bp_{n+1} - bp_n$ is the difference in size (in bp) between adjacent oligomers. t_0 refers to the unperturbed, zero-time, extrapolated sizes or differences; t_0' refers to the sizes or differences at the earliest experimentally measured time; t_{max} refers to the sizes or differences at the last experimentally measured time. For the oligomer differences ($bp_{n+1} - bp_n$), t_{max} is the difference at the last measured time of the higher oligomer. Size values are from Figure 4 (3% polyacrylamide gels).

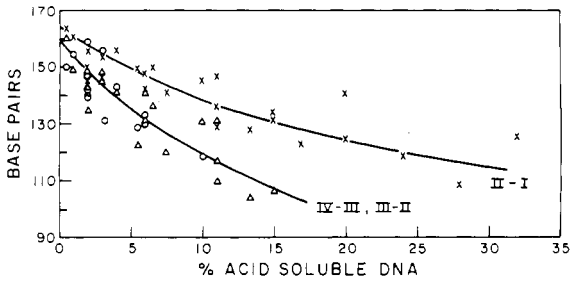


FIGURE 5: Oligomer double-strand size differences plotted as a function of digestion time (% DNA acid soluble) for several experiments: (X) dimer minus monomer differences; (Δ) trimer minus dimer differences; (O) tetramer minus trimer differences. The differences were measured at each time point from Figure 4 at which both the n and $n + 1$ oligomers were reliably present.

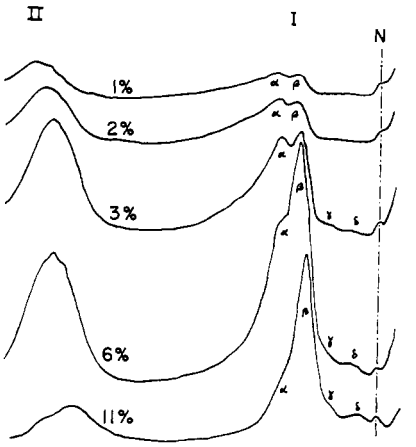


FIGURE 6: The multicomponent monomer peak on 4.5% polyacrylamide gels, stained in toluidine blue and scanned (Materials and Methods). These scans show both dimer (II) and monomer (I). PM2 fragment N (117 bp) is present as an internal marker in the gels. The % acid soluble DNA values are shown for each scan. The sizes of the peaks α, β, γ, and δ for each scan are respectively in bp: 1%, 155, 146, −, −; 2%, 155, 146, −, −; 3%, 154, 145, 135, 125; 6%, 154, 144, 133, 125; 11%, 150, 142, 133, 123. The mystery bands are out of sight to the right in this gel because they show greater mobilities (relative to DNA) in higher percentage gels.

the long-time repeat size. We should emphasize that the difference method is also very sensitive to the sizes of the DNA markers used as standards, in this case the larger PM2 fragments. At any rate, the average repeats are about 160 bp but there appear to be repeats as small as 120–130 bp in size.

Are all repeat sizes present in equal amounts? Figure 4 shows (for experiment 5) that 70% of all the monomers ever observed have a median peak size of 140–150 bp; 85% of all the dimers ever observed have a median peak size of 275–310 bp; 75% of trimers have a median peak size of 420–460 bp. Considering the slight size decrease due to rapid nuclease action on the DNA tails liberated by endonucleolytic cleavage, it is clear that the 140–160 bp repeat sizes predominate in the yeast genome. The very small oligomers ($<n \times 140$ bp) observed at long digestion times constitute only a very small fraction of that particular oligomer when one considers the whole digestion course. Since these <140 bp repeat sizes appear at times when significant monomer degradation has begun, they probably arise from cleavages within one of the 140 bp cores of the late time oligomer.

The monomer sizes show a somewhat different behavior. They rather quickly reach a size which remains constant throughout most of the digestion. This behavior also indicates that there is an especially nuclease resistant core within the repeat unit which contains about 140 bp of DNA.

Figure 5 shows the plot of differences in size between adjacent oligomers as a function of time of digestion. It is clear from this figure that the differences are identical at zero time. The decrease in difference size at longer digestion times is consistent with the presence of different repeat sizes, with the larger repeats being preferentially cleaved.

Bimodal Dimer and Multicomponent Monomer Peaks. There is also fine structure in the digestion pattern. The dimer peak consistently shows evidence of having two components, II_a and II_b, at certain times of the digestion course (see Figure 2b, 10 and 17 min). We used a DuPont 310 curve resolver to resolve these bimodal dimer peaks. II_a appears clearly first and also disappears before II_b disappears; the clearest appearance of II_a and II_b is at 5–10% acid solubility when II_a = 310 bp and II_b = 280–285 bp. We see no direct, unambiguous way, from these experiments, to determine whether II_a and II_b arise from chromatin subunit heterogeneity or merely from the presence of alternative nuclease-sensitive sites in chromatin or in II_a. We plan to pursue this observation further by analysis of sucrose gradient isolated nucleoprotein particles.

Second, the monomer peak appears to be a multicomponent peak (Figure 6). Although the exact size of the components seems to depend on the extent of digestion, at any given time of digestion the components are about 10 bp different in size. The peak present in highest concentration throughout most of the digestion (β) is about 145 bp initially, decreasing to 140 bp. At less than 10% acid-soluble DNA, there are components larger than β: α, a well-resolved component of about 150–155 bp; two very broad, poorly resolved components at about 160 and 170 bp. Thus, there is some correlation with the two dimer species observed, 150–155 bp/310 bp and 140 bp/280 bp. There are also components smaller than β: γ, about 135 to 130 bp, which is never more than a shoulder on β; δ, about 125 bp

TABLE III: Oligomer Peak Widths at Half-Maximum Height.[a]

IV		III		II		I	
0.5 mM Ca^{2+}	0.05 mM Ca^{2+}	0.5 mM Ca^{2+}	0.05 mM Ca^{2+}	0.5 mM Ca^{2+}	0.05 mM Ca^{2+}	0.5 mM Ca^{2+}	0.05 mM Ca^{2+}
160	126	112		80		41	
			79		63		20
198	195	110		74		23	
			79		53		15
		111		78		22	
			99		62		16
		98		83		19	
			87		59		13
		118		92		15	
					64		11
				81		14	
				78		14	
Av: 179 bp	116 bp	110 bp	86 bp	81 bp	60 bp		

[a] Widths of resolved tetramer (IV), trimer (III), dimer (II), and monomer (I) DNA peaks were measured from scans of 3% polyacrylamide gels for high Ca^{2+} (0.5 mM) and low Ca^{2+} (0.05 mM) digestions. The band widths have been approximately corrected for the effects of diffusion spreading and finite initial band width in the following way. It was assumed that both the yeast digest bands and the PM2 bands were approximately Gaussian, and that the finite width of the latter results from spreading and initial breadth. It then follows (Kovacic, Lohr, and Van Holde, in preparation) that $h_{obsd}^2 \simeq h^2 + h_0^2$, where h_{obsd} is the observed width at half-height, and h and h_0 are the intrinsic widths of the digest bands and the width of a homogenous DNA of the same average mobility. The h_0 were obtained by interpolating, at the positions of the yeast digest bands, from a plot of the widths at half-height of the PM2 fragment bands. Only in the case of the monomer is the correction significant.

to 120 bp. Components γ and δ are part of the submonomer DNA fragment pattern observed at long times of digestion. Although we discuss the yeast submonomer pattern in greater detail elsewhere (Lohr et al., in press), we can say here that the limit pattern and DNA sizes observed in yeast are identical with the pattern in higher eukaryotes (cf. Axel et al., 1974). By analogy to higher eukaryotes, the bands below 140 bp in yeast probably arise from nuclease digestion within the monomer itself. Their presence at low extents of digestion indicates that a very slow degradation of core particles occurs along with the more rapid cleavage of nuclease sensitive intercore regions.

Oligomer/Monomer Distribution Widths. An oligomer peak at any time does not contain homogeneous DNA, but rather a distribution of DNA sizes. We have measured the half-width of the peaks at half the maximum height as a measure of this DNA size distribution (Table III). The monomer DNA peak sharpens but dimer, trimer, and tetramer widths remain constant with increasing time of digestion. The distribution widths increase in higher oligomers (dimer < trimer < tetramer). This latter data argue that there is a nonuniform repeat size in yeast chromatin. If there were a uniform 20-bp sensitive region between 140 bp resistant core regions, then one might expect some breadth in each of the oligomer bands, but the half-width would be uniform among the oligomers. Nonuniform spacing regions (and repeat sizes) would cause widths to increase in the order observed (II < III < IV).

The data also allow exclusion of the alternative explanation that the continuous size decrease of oligomeric DNA with increasing time of digestion is due to a sliding together of the 140 bp nuclease resistant core portions of the chromatin. If this were happening, the half-widths should decrease drastically at longer digestion times, as noted for monomer DNA, as the nuclease resistant cores become closely packed and the protected DNA very homogeneous in size. Instead we see that the half-widths are quite constant throughout the digestion.

The Fraction of the Yeast Genome in Repeating Structure. In the preceding sections we have shown that intranuclear digestion of yeast chromatin indicates the presence of a structure that protects the DNA in repeats of about 160 base pairs. What percentage of the yeast genome incorporates this repeating subunit structure? An example of how this can be determined is illustrated by analysis of data like that shown in Figure 2b. At 17 min of digestion (time of maximal monomer concentration) the total area under the oligomer–monomer peaks represents 87% of the area under the curve on the zero-time scan (normalized for DNA loadings by DABA assay); thus 13% of the DNA originally present has been digested to fragments smaller than the monomer. Direct acid-solubility measurements at this time show that 11% of the original DNA has been rendered completely acid soluble (Figure 1). Of this 87% remaining resolvable on the gel at 17 min of digestion, 64% was of monomer size, 23% was of dimer size, and 8% was of trimer size. Thus *at least* 56% (64% of 87%) of the original chromosomal DNA participated in the repeating structure. But since the kinetics show that monomer appearance occurs concommitantly with dimer and trimer disappearance, it is reasonable to include dimer and trimer DNA when calculating the amount of the genome in the repeating structure, raising the figure to 83% (95% of 87%). Furthermore, at 17 min of digestion, the average size of monomer and dimer shows (Figure 4) that some of the DNA lost has been lost by attack at the kinetically accessible site(s) of the subunit; this amount should also be counted as DNA originally present in repeating structure. Because of the variability in repeat size, the exact amount is difficult to calculate. We estimate that 6–9% of the acid-soluble DNA has arisen from the repeating subunit structure. Therefore, up to 90% of the yeast chromosomal DNA was initially present in repeating structure. The maximum deviation for this number is ±9% (Table I).

However, there is a possible minor overestimation in this calculation because the DNA concentration between bands does not fall to zero. This "interpeak" DNA has also been noted by Sollner-Webb and Felsenfeld (1975). Two possible explanations suggest themselves: (a) the width of the distri-

TABLE IV: Oligomer Areas on Denaturing and Nondenaturing Gels.[a]

Time of Digestion (min)	IV</Σ	IV/Σ	III/Σ	II/Σ	I/Σ
3	0.29	0.14	0.14	0.24	0.20
	(0.19)	(0.08)	(0.17)	(0.22)	(0.34)
12	0.03	0.05	0.12	0.28	0.52
	(0.095)	(0.025)	(0.09)	(0.25)	(0.54)
20		0.02	0.07	0.26	0.64
			(0.05)	(0.23)	(0.70)
30				0.24	0.71
				(0.23)	(0.76)

[a]Oligomer and monomer peaks from scans of Stains-all stained 3% polyacrylamide nondenaturing gels and 4.5% polyacrylamide–formamide denaturing gels were resolved and the areas measured on a Du Pont 310 curve analyzer for several times of digestion from a time course at 0.05 mM Ca^{2+}. All areas are expressed as fractions of the total area on each scan. I is monomer DNA, II is dimer, III is trimer, IV is tetramer, IV< is all DNA larger than tetramer, and Σ refers to the sum of the areas at each time of digestion. We know that the staining of double-strand DNA with Stains-all is quantitative. We have assumed that the staining of single-strand DNA with Stains-all is also quantitative. Values without parentheses are for double-strand DNA; those with parentheses are for single-strand DNA.

bution of DNA sizes associated with each peak produces the interpeak overlap; (b) the pattern of peaks corresponding to the repeating subunit structure is superimposed on a very wide but shallow distribution of DNA not involved in the repeating structure. This other distribution of DNA must gradually be digested away and move off the gel because later time points have less interpeak DNA. We tried to distinguish these possibilities using the Du Pont 310 curve resolver. Unfortunately, the scans could be fitted equally well using either of the two assumptions. We tend to favor assumption a, because it involves a less arbitrary assumption. However, if b is correct, then about 80% of the genome is involved in the repeating structure, based on calculation from the example discussed previously.

We have searched for free DNA in yeast chromatin by comparing the measured acid solubility with the acid solubility predicted based on the oligomer and monomer DNA size decrease. We find that most, if not all, of the acid-soluble deoxyribonucleotides present at early times can be accounted for by the size degradation of the chromatin repeats. If there is free DNA, it must amount to no more than a very few percent of the total in yeast. Thus, if there is a DNA in yeast not included in the repeating structure (previous paragraph), it must also be protected from rapid nuclease digestion.

Further Controls. Clark and Felsenfeld (1971) showed that the levels of Ca^{2+} used in some of the experiments described above (0.5 mM) allowed significant exchange of histones from isolated chromatin onto exogenously added DNA. While conditions may be quite different for digestions in nuclei (where the effective Ca^{2+} concentration should be much lower and there is not a large amount of free DNA), we were concerned that histone lability might be a cause of the observed course of the size decrease of yeast oligomer and monomer DNA. Therefore, we performed digestion studies at 0.05 mM Ca^{2+}, a tenfold decrease in Ca^{2+} concentration. We find that the sizes and size differences under conditions of low Ca^{2+} digestion are very similar to those observed under conditions of high Ca^{2+} digestion (Figures 4 and 5). The low Ca^{2+} digestions do produce sharper oligomer and monomer bands (Table III).

To assess the frequency of nicking by staphylococcal nuclease, we have run 4.5% formamide–polyacrylamide gels, containing PM2-*Hae* III restriction fragments as internal markers. The median single-strand lengths of the fragments on formamide gels is similar to the double-strand lengths observed on nondenaturing gels (Figure 4). The percentage of the total DNA present in each oligomer and monomer band is very similar whether electrophoresed as double-strand or single-strand DNA (Table IV). Thus the enzyme does little nicking during the digestion of yeast chromatin.

To determine if protease activity is responsible for any of these observations, we have run time courses of digestion on nuclei isolated in the presence of 0.1 mM phenylmethylsulfonyl fluoride (present from spheroplast lysis on). We can detect no differences from the observations noted here.

We have also checked for the presence of endogenous nuclease activity. Such an activity is present and resembles the rat-liver Ca–Mg-dependent endonuclease (Hewish and Burgoyne, 1973) but produces DNA fragments with a repeat size of 160–170 bp. However, the time scale of endogenous nuclease digestion is about ten times longer; for example, only 5% of the DNA is rendered acid soluble after 90 min of endogenous nuclease activity. Thus the endogenous activity cannot be contributing greatly to the observed patterns.

It is possible to have strain differences in yeast. We have compared the digestion time courses and histone composition of Y55, S288 Cα, and a strain cultured from commercial Red Star baker's yeast and find they all have similar, if not identical, protected DNA sizes and histone patterns. Thus the results observed here are not strain specific.

Discussion

The results presented herein confirm our earlier report (Lohr and Van Holde, 1975) that yeast chromatin possesses a periodic structure. The repeat sizes observed average about 160 bp but, within this repeat, there exists a strongly nuclease resistant core of 140 bp. These results agree with the results recently obtained by Thomas and Furber (1976).

One must conclude that the lengths of nuclease-sensitive regions between these especially resistant regions (140 bp) cannot be completely uniform. This is suggested by the continuous decrease in the repeat size as measured by oligomer size/oligomer number (Figure 4) and oligomer differences (Figure 5) with increasing digestion and by the order of DNA size distribution widths (II < III < IV (Table III)). We are suggesting a random, short-range nonuniformity; the nuclease resistant, 140 bp regions are spaced at irregular intervals, with spacers ranging from 0 to more than 20 base pairs, along the chromatin strand.

On the other hand, the yeast genome cannot be divided, on a gross scale, into extensive regions with varying degrees of resistance to nuclease action. Figure 3 shows that the overall

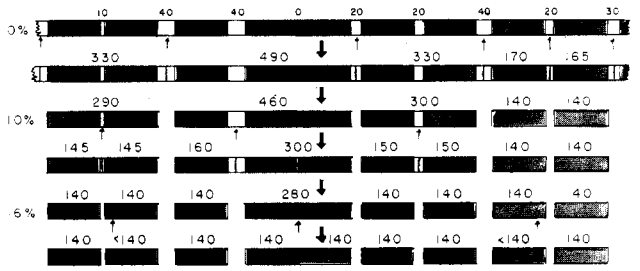


FIGURE 7: A schematic representation of the structure of yeast chromatin and its digestion by staphylococcal nuclease. The 140 base pair core regions are shaded, the spacer regions left unshaded. For simplicity of representation and in agreement with the tendency to produce monomers differing in size by 10 bp (140, 150, 160, 170), the spacers have been assigned arbitrary lengths of 0, 10, 20, 30, and 40 base pairs. However, this assumption has clearly not been proven. Cleavage (↑) is represented as being at the center of each spacer. Also, for simplicity, the stages of cleavage and digestion of tails are represented as sequential; in fact, both must be occurring together. The predicted acid solubility at some stages is shown at the left. We have not included details to explain all of the fine structure of the DNA peaks, such as structure that would yield a "pause" at 150 base pairs or the possible existence of different core sizes.

digestion pattern follows that predicted by random cleavage at *approximately* equally spaced and sensitive points. Also, if the genome were divided into long regions having markedly different degrees of resistance to nuclease, we should see one of two possibilities depending on the relative nuclease resistance of the various regions. Either the distribution of oligomers would be made up primarily of high-molecular-weight oligomers and monomers at many stages in the digestion, or all the oligomer peaks would be markedly bimodal (cf. II_a and II_b), at most times of digestion. We see neither of these.

The data suggest a structure like that shown in Figure 7. We feel that the sizes decrease (Figure 4) because cleavage occurs somewhat more preferentially in the longer spacer regions than in the shorter spacer regions. The zero-time average repeat sizes per subunit (Table II) reflect the average over the different sized fragments which can be obtained by initial cleavage in the different spacer regions. Because of the (assumed) tendency for longer spacers to be cleaved first, these early average repeat sizes are probably slightly larger than the true average repeat size. Occurrence of immediately adjacent core regions is infrequent but gives rise to the small fraction of very small oligomers that are observed at long digestion times.

This slight nonuniformity in spacer lengths (and nuclease sensitivity) is not incompatible with the results of Figure 3, which only show that there is an alternation of nuclease-resistant regions with nuclease-sensitive regions throughout the chromatin. Thus, these data exclude the occurrence of extensive regions with varying nuclease sensitivity but do not imply that the alternation of resistant and sensitive sites is completely regular. Any intercore attack would be counted the same for the analysis of Figure 3 because any such cleavage would contribute about equally to the area of an oligomer peak. However, the presence of differentially accessible sites should be reflected in a slowing of the rate of digestion at longer times, when the population of uncleaved sites should be enriched in core–core regions. Although other explanations are possible, this trend is clearly observed (Figure 4).

A structure involving resistant 140 base pair cores alternating with variable spacer regions (averaging 20 base pairs in length) is also consistent with details observed in the distribution of digestion products (see "Bimodal Dimer . . ."). Following excision of an oligomer, digestion of the residual tails is probably a rapid event. Thus, at very early stages in the digestion when the concentration of monomers is still low but their rate of production is high, we would expect to observe an appreciable fraction of "intact" monomers (the ≥160 base pair fragments in Figure 6). The 140 base pair core (β) must be relatively stable because this accumulated product soon comes to dominate the monomer region. The presence of monomers larger than 160 base pairs at early digestion times simply reflects the statistical preference for initial cleavage in the longer spacer regions.

The rather sharp band at about 150 bp is less easily explained. It must be due to either (1) a metastable "pause" in the degradation of intact monomers to core monomers which causes a slight accumulation of intermediates of that size at any time of analysis or (2) to the presence of a discrete structure in certain parts of the chromatin which is cleaved early in the digestion, probably from areas having large repeat sizes, and is more nuclease sensitive than the core structure (β) and therefore disappears rather quickly.

Initially, dimers will contain intact tails. As dimer degradation overtakes dimer production (at about 5% acid solubility, Figure 3) (1) the removal of tails and (2) the preferential digestion of larger dimers become predominant. However, 280 bp dimers (two immediately adjacent cores) are rather resistant and accumulate. There is also an accumulation of a 310 bp dimer which could arise for the same reasons as the 150 bp monomer since it is about twice as large. It is likely that the 280 bp dimers give rise to the small fraction of smaller dimers, observed at very late times, by intracore cleavage.

Turning now to the question of the percent of the yeast genome existing in this repeating structure, we conclude that it is surprisingly large. The data indicate quite strongly that 80–90% is so involved. The result has strong implications for an organism like yeast, where an appreciable fraction of the genome must be transcribed. Thus, it seems likely that some of the DNA involved in the nuclease resistant regions is transcribed DNA. In higher eukaryotes too, where only a small percentage of the genome is expressed, evidence now in the literature indicates that active genes are included in nuclease-resistant regions of the chromatin (Lacy and Axel, 1975).

How does the structure of yeast chromatin compare with that of higher eukaryotes? Yeast has a 140 bp core of nuclease-resistant DNA and a repeat size of 160 bp. Thus the cores are spaced (in a nonuniform way) by an average of 20 bp of more nuclease-sensitive DNA. Published chromatin repeat sizes for higher eukaryotes average 185–200 bp DNA (cf. Noll, 1974; Sollner-Webb and Felsenfeld, 1975) and the core of nuclease-resistant DNA is about 140 bp (Van Holde et al., 1975).

We have found by direct comparison that the core size and even the core structure in yeast is very similar to that in higher eukaryotes (Lohr et al., 1977). However, it seems that the repeat size in yeast is smaller than the repeat size in higher eukaryotes. Thus the cores must be spaced more closely, on the average, in yeast than in higher eukaryotes. Noll (1976) has reported a small repeat size and similar core size for *Neurospora crassa* also.

Finally, we feel that the type of detailed quantitative analysis of the whole course of nuclease digestion that has been developed here should be used when characterizing the repeat structure of chromatin in a given tissue in a given organism or as a function of time of cell cycle, etc. A less detailed analysis can produce misleading or incomplete results.

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Sequence Specificity of Internal Methylation in B77 Avian Sarcoma Virus RNA Subunits†

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ABSTRACT: Following ribonuclease digestion of methyl-^{3}H-labeled B77 avian sarcoma virus RNA subunits, methylated oligonucleotides were isolated by diethylaminoethylcellulose chromatography. Partial nucleotide sequences were deduced from the known enzymatic specificities of the ribonucleases. In addition to methylated nucleosides in the 5′-terminal cap structure, m^{7}G(5′)ppp(5′)G^{m}pCp, N^6-methyladenosine (m^{6}A) was found to be present in only two internal sequences of the RNA molecule, Gpm^{6}ApC and Apm^{6}ApC. The average numbers of methylated nucleosides per RNA subunit are about 12–13 in Gpm^{6}ApC, 1–2 in Apm^{6}ApC, and 2 in m^{7}GpppG^{m}pCp. The sequences containing m^{6}A in B77 sarcoma virus RNA are identical to m^{6}A-containing sequences previously reported for the bulk mRNA from HeLa cells (Wei, C. M., Gershowitz, A., and Moss, B. (1976), *Biochemistry 15,* 397–401). Analysis of the oligonucleotides produced by RNase A digestion indicated that the sequence of bases on the 5′ side of these trinucleotides is not specific. The oligonucleotide profile, however, was highly reproducible in different virus preparations. This suggests that the methylations occur at specific positions on the RNA molecule. Some of the methylated oligonucleotides produced by RNase A digestion appear to be present in less than molar amounts. Several hypotheses are proposed to explain this result.

The 35S RNA subunits, which comprise the 70S genome RNA of avian oncornaviruses, have many of the structural and functional properties of cellular mRNA. Each subunit contains 3′-terminal poly(A) sequences (Lai and Duesberg, 1972; Quade et al., 1974) and a capped and methylated 5′-terminal structure m^{7}G(5′)ppp(5′)Gm[1] (Furuichi et al., 1975c; Keith

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[1] Abbreviations used are: m^{6}A, N^6-methyladenosine; m^{7}G, 7-methylguanosine; N, any ribonucleoside; N^m, any 2′-*O*-methylribonucleoside; Pu, purine ribonucleoside; G^m, 2′-*O*-methylguanosine; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; P$_i$, inorganic phosphate.