Cleavage of DNA in Nuclei and Chromatin with Staphylococcal Nuclease†

Richard Axel

ABSTRACT: Treatment of either rat liver chromatin or intact nuclei with the enzyme staphylococcal nuclease results in the conversion of about half of the DNA to acid-soluble oligonucleotides. As previously described, mild digestion of nuclei results in the liberation of a series of nucleoprotein particles containing DNA fragments which are all integral multiples of a unit length DNA 185 base pairs in length. Analysis of the kinetics of appearance of these fragments suggests that at least 85% of the nuclear DNA is involved in the formation of the repeating subunit profile. More extensive digestion of nuclei however results in the generation of a series of eight unique DNA fragments containing 160 to 50 base pairs. The series of smaller molecular weight DNA is virtually identical with the profile obtained upon limit digestion of isolated chromatin. By velocity centrifugation we have obtained highly purified preparations of the monomeric nucleoprotein particle. Digestion of this monomeric subunit results in the solubilization of 46% of the DNA and analysis of the resistant DNA again reveals the set of eight lower molecular weight fragments. These data suggest that the initial site of nuclease cleavage in chromatin resides within the DNA bridging the repeating monomeric subunits. Further attack results in cleavage at a set of sites within the monomer liberating a pattern of smaller DNA fragments which probably represents the points of intimate contact between the histones and DNA.

The DNA of virtually all eucaryotes is associated with a host of basic and acidic proteins forming a complex which we define as chromatin. It has been postulated that a limited combination of specific histones align themselves along the DNA backbone at regular intervals, giving rise to a repeating nucleoprotein complex thought to be characteristic of the tertiary structure of chromatin (Kornberg and Thomas, 1974; Kornberg, 1974). In support of this hypothesis, either mild autodigestion of nuclei (Burgoyne et al., 1974; Hewish and Burgoyne, 1973) by endogenous nuclease, or digestion of nuclei with staphylococcal nuclease (Noll, 1974), results in the liberation of a set of nucleoprotein particles containing DNA fragments whose molecular weights are multiples of a proposed monomeric unit 205 base pairs in length. The kinetics of appearance of these fragments suggest that at least 85% of the chromatin DNA is involved in the formation of such repeating subunits.

We have performed similar experiments on the products of enzymatic digestion of purified duck reticulocyte chromatin (Axel et al., 1974). These studies reveal that while half the DNA is reduced to acid-soluble oligomers, the remaining protected DNA exists as a set of eight highly reproducible fragments of discrete molecular weight from 130 to 45 base pairs. These fragments probably represent the points of direct association of the histones with DNA and are not determined by any long specific sequence of nucleotides (Axel et al., 1974).

In this paper the relationship between the multimeric subunits obtained by nuclear digestion and the set of smaller fragments resulting from staphylococcal nuclease treatment of isolated chromatin has been examined. These studies reveal two distinct classes of nuclease cleavage sites within the DNA of isolated nuclei. The first and most accessible site liberates DNA fragments which are all integral multimers of 185 base pairs and probably is located in DNA bridging the repeating nucleoprotein particles. Further digestion results in internal cleavage within the particles themselves, giving rise to a set of smaller molecular weight DNA fragments similar to those obtained upon enzymatic digestion of purified chromatin.

Materials and Methods

Preparation of Nuclei and Chromatin. Livers were excised from 200–250 g male Sprague-Dawley rats. Minced tissue was homogenized in 5 vol of 0.01 *M* Tris-HCl (pH 7.9)–0.001 *M* $MgCl_2$–0.25 *M* sucrose in a Potter-Elvehelm homogenizer, filtered through cheesecloth, and centrifuged at 4000 rpm in a Serval HB 4 rotor. The nuclear pellet was resuspended in the above buffer containing 0.25% Triton X-100 and resedimented. This washing procedure was repeated four times and the final nuclear pellet was suspended in buffer without added detergent and resedimented. Chromatin was prepared from this purified nuclear pellet as previously described (Axel et al., 1973).

Nuclease Digestion of Chromatin and Nuclei. Chromatin DNA was digested with staphylococcal nuclease (Worthington Biochemical Corp.) in 1 m*M* Tris-HCl (pH 7.9)–0.1 m*M* $CaCl_2$ as described (Axel et al., 1974). Digestion of nuclei was carried out on suspensions of nuclei washed twice in 1 m*M* Tris-HCl (pH 7.9)–0.1 m*M* $CaCl_2$–0.25 *M* sucrose and resuspended in the same buffer at a DNA concentration of 150 μg/ml. The kinetics of DNA digestion following the addition of nuclease was assayed by measuring the amount of A_{260} absorbing material soluble in 1 *M* NaCl–1 *M* $HClO_4$. RNA contributed less than 5% of the total A_{260} of purified nuclei as determined either by alkali digestion or treatment with pancreatic ribonuclease. Nuclease reactions were terminated by the addition of NaEDTA to 5 m*M*. Resistant DNA was purified by treatment with proteinase K (E. Merck) and phenol extraction as described (Axel et al., 1974).

† From the Institute of Cancer Research and Department of Pathology, College of Physicians and Surgeonns, Columbia University, New York, New York 10032. *Received February 20, 1975.* Supported by Grant No. CA-02332 and Contract No. N01-CP3-3258 within the Virus Cancer Program of the National Cancer Institute, National Institutes of Health.

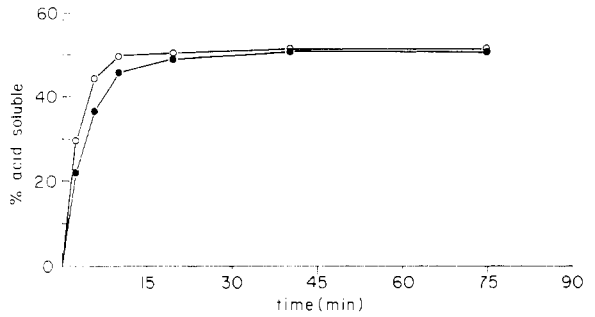

FIGURE 1: Kinetics of nuclease digestion of nuclei and chromatin. Purified rat liver nuclei (●) and isolated chromatin (○) were incubated in 1 m*M* Tris (pH 7.9)–0.1 m*M* $CaCl_2$ at 37° in the presence of 5 μg/ml of staphylococcal nuclease. The initial DNA concentration was 150 μg/ml. Aliquots were removed at the indicated times and the amount of DNA rendered soluble in 1 *M* $HClO_4$–1 *M* NaCl was determined.

Gel Electrophoresis. Electrophoresis was performed in either 3 or 6% polyacrylamide gels and run either as discs or slabs. Three percent gels were supplemented with 0.5% agarose to provide greater support. DNA was dissolved in electrophoresis buffer at a concentration of 3 mg/ml. Two microliters of 40% sucrose–1% Bromophenol Blue was added to 10 μl of DNA and the samples were run at 200 V on a 17-cm cooled slab gel for 2 hr. The buffer system employed was that described by Peacock and Dingman (1967). Gels were stained overnight in "Stains-all" (Eastman), 0.005% in 50% formamide, destained in water, and photographed. Disc gels were scanned at 550 nm in the scanning accessory of the Gilford 2400s recording spectrophotometer.

Results

Digestion of chromatin from a variety of sources with the enzyme staphylococcal nuclease results in the liberation of about half of the chromatin DNA as acid-soluble oligomers (Clark and Felsenfeld, 197', 1974). The associated nucleoprotein fragments contain all of the chromatin proteins but only half the original complement of DNA. The kinetics of digestion of DNA in isolated rat liver nuclei again reveals that about half of the nuclear DNA is susceptible to nuclease attack (Figure 1). Using either chromatin isolates or whole nuclei the reaction reaches a well-defined limit of 54% digestion with the rate of attack on chromatin only slightly greater than that observed with nuclei. Identical results are obtained with either detergent-washed nuclei or nuclei prepared by sedimentation through sucrose columns. Incubation of nuclei or chromatin in the absence of exogenous nuclease results in virtually no solubilization of DNA under these reaction conditions, excluding the possibility that the rat liver Ca^{2+}–Mg^{2+} dependent endonuclease (Hewish and Burgoyne, 1973) is responsible for the observed reaction.

We have previously shown that the digestion of chromatin results in the liberation of a highly reproducible set of DNA fragments of discrete molecular size from 130 to 45 base pairs (Axel et al., 1974). Similar studies on the products of nuclear digestion result in the appearance of DNA fragments which are integral multiples of a proposed monomeric structure containing about 200 base pairs of DNA (Noll, 1974). It was, therefore, of obvious interest to examine the relationship between the larger multimeric fragments obtained upon limited digestion of nuclei and the set of smaller DNA fragments observed in our chromatin digests.

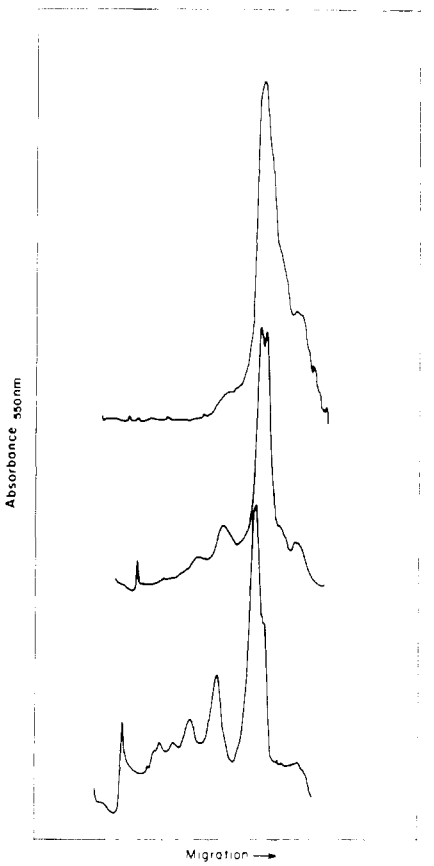

FIGURE 2: Polyacrylamide disc gel electrophoresis (3%) of the products of nuclear digestion. Nuclei were digested and, at various times, samples were removed and freed of protein and acid-soluble nucleotides as described in Materials and Methods. The purified DNA samples (30–50 μg/gel) were applied to 3% polyacrylamide disc gels and electrophoresed at a constant voltage of 15 V/gel. The gels were stained and scanned at 550 nm in the gel scanning attachment of the Gilford 2400s spectrophotometer. From the bottom to top the tracings represent 7, 14, and 30% digestion.

To this end purified preparations of rat liver nuclei were treated with increasing concentrations of staphylococcal nuclease. The resulting DNA fragments were freed of protein and analyzed by 3 and 6% polyacrylamide gel electrophoresis. As previously described, limited digestion of nuclei generates a series of DNA molecules whose molecular weights are integral multiples of a single monomeric unit 185 base pairs in length (Figures 2 and 3). Figure 2 reveals the kinetics of appearance of these DNA fragments as digestion proceeds from 7 to 30% solubilization. The lower panel of Figure 2 (7% digestion) reveals a series of seven discrete bands with about half of the resistant DNA 185 base pairs in length and the remaining DNA in six larger bands all approximately integral multiples of the 185 base pair fragment. Double-stranded DNA molecular weights were determined by electrophoresis and autoradiography of fragments of λ DNA obtained by treatment with hin restriction endonucleases (Maurer et al., 1974) (Figure 4). These [^{32}P]DNA fragments were generously provided by Dr. Tom Maniatis. It is obvious that as the digestion proceeds, the relative proportion of the monomeric fragment increases at the expense of the multimeric components. At 14% digestion about 80% of the DNA is resolvable as either monomer or multiples. From this information it appears that at least 80% of the nuclear DNA participates in the formation of these repeating subunits.

It is apparent, even at 3% solubilization, that the 185

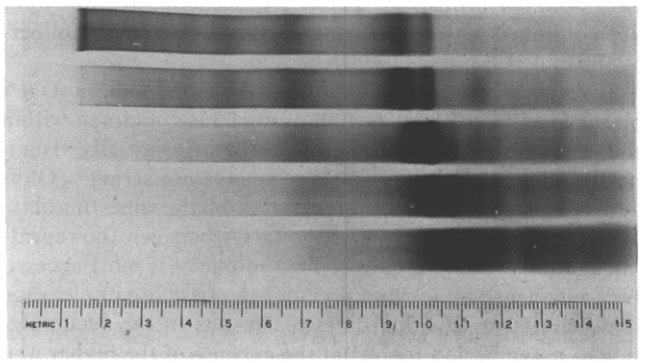

FIGURE 3: Polyacrylamide slab gel electrophoresis (3%) of products of nuclear digestion. Nuclei were digested with increasing amounts of staphylococcal nuclease (1–20 μg/ml) for 5 min and the resulting DNA was purified as described in Materials and Methods. DNA (40 μg) was applied to each slot of a 3% polyacrylamide–0.5% agarose slab, electrophoresed at 200 V for 2.5 hr, stained, and photographed. The samples from right to left represent 3, 5, 10, 20, and 45% digestion of nuclear DNA.

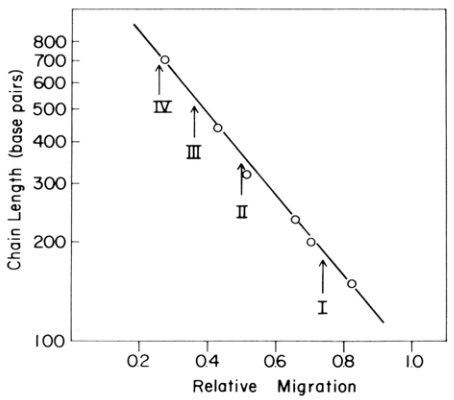

FIGURE 4: Calibration of 3% polyacrylamide gels. [^{32}P]DNA fragments resulting from hin endonuclease digestion of λ DNA were electrophoresed on a 3% polyacrylamide–0.5% agarose slab gel. A 3% nuclear digest was applied to adjacent wells. The migration of marker λ fragments of known molecular weights (Maurer et al., 1974; T. Maniatis, personal communication) was determined by autoradiography. The arrows indicate the migration and size of monomer, 185 base pairs; dimer, 360 base pairs; trimer, 550 base pairs; and tetramer, 735 base pairs.

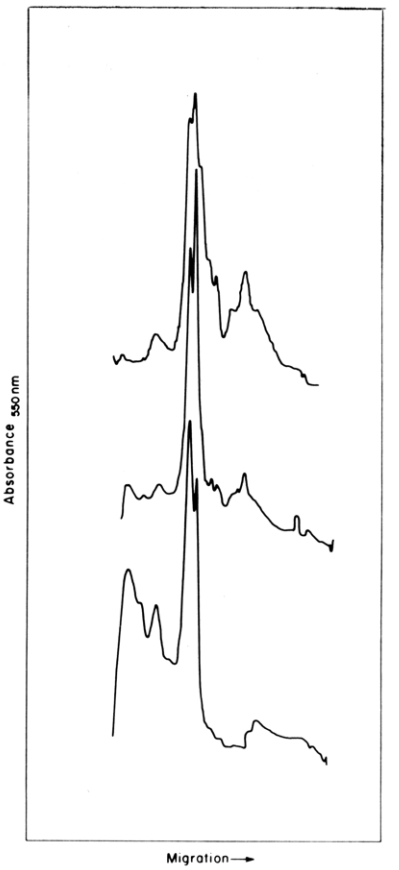

FIGURE 5: Polyacrylamide disc gel electrophoresis (6%) of products of nuclear digestion. The DNA resulting from digestion of nuclei, analyzed on 3% gels in Figure 2, was similarly analyzed on 6% polyacrylamide disc gels. The tracings represent, from bottom to top, 7, 14, and 30% digestion of nuclear DNA.

base pair fragment itself is a transient intermediate and is susceptible to further specific degradation by nuclease action. As previously noted, a DNA fragment (perhaps best seen in Figure 3) first appears consisting of about 160 base pairs. This fragment may represent either the initial site of cleavage within the monomeric subunit or digestion of a 25 base pair tail of free DNA bridging the repeating subunits. The intensity of this band increases as the material banding as monomeric DNA decreases. At 30% solubilization virtually no monomeric DNA remains and a new set of discrete bands appears between 160 and 45 base pairs.

This smaller group of fragments is best analyzed on a 6% polyacrylamide gel (Figure 5) where eight discrete bands are clearly observed. The appearance of these fragments is coincident with the disappearance of the 185 base pair component and its multimers, strongly suggesting a precursor–product relationship. The molecular weights of these bands were similarly determined by calibration of 6% gels with sequenced DNA fragments of bacteriophage λ cut with restriction endonucleases.

The kinetics of appearance of these smaller molecular weight bands reveal (Figure 5) that they are already detectable at 15% digestion and reach a limit at about 50% digestion. Further incubation of the nucleoprotein fragments with nuclease results in no further solubilization of DNA and no diminution in the size of these fragments. These fragments are greater than 95% double stranded as determined by sensitivity to single-strand specific nucleases. These data indicate that this set of DNA fragments represents the true limit digest of nuclei and derives from the larger more transient repeating subunit observed at earlier times in the digestion process.

The set of fragments generated upon limit digestion of nuclei is virtually identical with those previously demonstrated in chromatin digests of reticulocytes (Axel et al., 1974) and erythroblasts (Weintraub and Van Lente, 1974). As expected, the digestion of isolated rat liver chromatin results in the liberation of a series of DNA fragments whose molecular weights correspond closely to those obtained late in the digestion of purified rat liver nuclei (Figure 5). While the two profiles reveal DNA fragments of identical size, variations in the relative intensity of the bands do exist. It is important to note, however, that at early times in the digestion of chromatin, a heterogenous population of large molecular weight DNA is liberated. The 185 base pairs fragment and its multiples are never distinctly observed in chromatin digests, even at levels of digestion below 5% solubilization of DNA.

From the data already presented, it is apparent that the small set of DNA fragments (160–50 base pairs) must arise

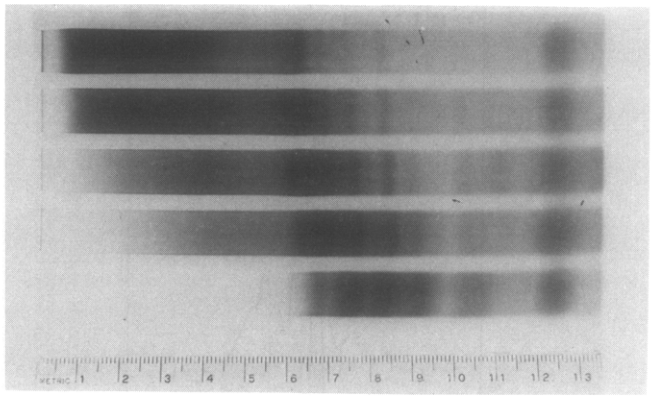

FIGURE 6: Polyacrylamide disc gel electrophoresis (6%) of products of digestion of chromatin and nuclei. Rat liver chromatin was digested with increasing levels of staphylococcal nuclease (1–20 μg/ml) for 5 min and the resistant DNA was purified as described in Materials and Methods. DNA (40 μg) was applied to each well of a 6% polyacrylamide slab gel and run at 250 V for 2 hr. Samples from right to left represent 5, 10, 20, and 40% digestion. For comparison, the DNA product of 45% digestion of intact nuclei was run in the first well (farthest left). The broad band present in all positions at 12.3 cm represents persistent Bromophenol Blue dye marker.

from cleavage at specific sites within the 185 base pairs monomeric subunit. Direct proof of this has been obtained from studies on the properties of the highly purified nucleoprotein monomer derived from nuclei (R. Axel and E. H. Lacy, manuscript in preparation). The nucleoprotein subunit containing 185 base pairs of DNA along with its associated chromatin proteins can be isolated following short nuclear digestion by a series of velocity centrifugations. This subunit has a sedimentation coefficient of 10.4 S as determined by velocity centrifugation in the analytical ultracentrifuge. Analysis of the DNA of this complex reveals a single band 185 base pairs in length and a small amount of its initial cleavage product 160 base pairs in length. Digestion of this purified subunit by staphylococcal nuclease results in the solubilization of 46% of the subunit DNA. Electrophoretic analysis of this resistant DNA reveals a set of discrete DNA fragments identical with that observed in Figures 5 and 6. These data provide direct evidence that the monomeric subunit itself has specific internal cleavage sites recognized by nuclease and that these sites are identical with those susceptible to nuclease attack in purified preparations of chromatin.

Discussion

Treatment of nuclei from a variety of sources with the enzyme staphylococcal nuclease results in the liberation of a series of DNA fragments which are multiples of a unit length DNA (Noll, 1974). This paper and an adjoining report by Sollner-Webb and Felsenfeld (1975) essentially confirm these observations. More extensive digestion of nuclei, however, reveals that this monomeric subunit is a transient intermediate and is susceptible to further specific degradation by nuclease, resulting in the generation of a unique set of DNA fragments of smaller molecular weight (160–50 base pairs). This series of fragments arising from intact nuclei is virtually identical with the profile observed upon the digestion of isolated chromatin. Furthermore, digestion of the purified monomeric nucleoprotein subunit similarly liberates this highly characteristic set of lower molecular weight DNA bands initially observed in chromatin digests (Axel et al., 1974). While the experiments described here have been performed with rat liver cells, similar results have been obtained with nuclei from avian reticulocytes (Sollner-Webb and Felsenfeld, 1975).

It seems reasonable to assume that the monomeric DNA obtained early in the digestion of nuclei is contained within the regular repeating subunit of chromatin initially visualized in electron micrographs as "beads on a string" (Olins and Olins, 1974). One interpretation of the digestion data described here is simply that the DNA between the repeating subunits (designated ν bodies) in nuclei is most accessible to nuclease attack and provides the first site of cleavage for the enzyme. Further digestion results in the accumulation of monomeric subunits at the expense of the higher molecular weight multimeric components. Contained within the monomeric nucleoprotein complex are less accessible sites of nuclease attack which may only be cleaved after the liberation of monomer. More extensive digestion is therefore required to solubilize these internal stretches of "free" DNA. The resultant resistant DNA would reflect the points of intimate contact of histones or specific groups of histones on the DNA within the repeating subunit.

The internal structure of the ν body is therefore reflected in the 160–45 base fragments obtained upon limit digestion. The observation that the limit digest pattern of chromatin is quite similar to that obtained with whole nuclei suggests that the fine structure within the monomeric subunit is, at least in part, retained throughout the chromatin isolation procedures. The inability to detect DNA fragments which are multiples of a basic 185 base pair subunit in sheared chromatin may be due to extension of the chromatin fiber so that DNA bridging ν bodies may be as accessible as the "free" DNA within the ν body itself. Furthermore, an 11S nucleoprotein complex can be isolated from digests of sheared chromatin (Weintraub and Van Lente, 1974; Sollner-Webb and Felsenfeld, 1975). It appears, therefore, that chromatin isolates in vitro reveal structural features quite similar to those observed in whole nuclei.

The data presented here would suggest that the accessible DNA bridging the repeating subunits must be quite short. From Figure 3 we observe that at 20% digestion greater than 95% of the remaining DNA is in the form of monomer or its small molecular weight fragments. The maximum distance between subunits would therefore be 20% the length of monomeric DNA or 37 base pairs. While this is consistent with the electron micrographs of Olins and Olins (1974), the data of Van Holde et al. (1974) would suggest at least 70 base pairs separate the individual ν bodies. An explanation for this apparent discrepancy may be that, as digestion proceeds, the DNA within the ν bodies unfolds in a specific manner, thereby increasing the DNA interspersed between the relaxed particles, leaving behind a highly compact core. Consistent with this argument is the observation that the particle isolated in these studies contains only 100 base pairs of DNA (Rill and Van Holde, 1973).

The information governing the assembly of these structures does not appear to reside in long repeated stretches of nucleotides within the ν body (Axel et al., 1974). The production of these subunits, therefore, probably results from highly specific histone–histone interactions along the surface of the DNA, without regard for DNA sequence.

The unique and highly reproducible profiles of DNA fragments generated by limit digestion of either nuclei or chromatin suggest a specific arrangement of histones or groups of histones within the DNA of the ν body. Given the

size and number of the fragments obtained, it is possible that more than one type of particle exists with differing arrangements of protein as suggested by Weintraub and Van Lente (1974). Alternatively each particle may contain an identical arrangement of groups of histones which may exist in a number of conformational states, thus generating the observed series of discrete lower molecular weight DNA fragments.

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Adrenocorticotropic Hormone Stimulation of Adrenal RNA Polymerase I and III Activities. Nucleotide Incorporation into Internal Positions and 3′ Chain Termini†

Shella A. Fuhrman* and Gordon N. Gill

ABSTRACT: In the presence of 50 m*M* $(NH_4)_2SO_4$ and low concentrations of α-amanitin (7.7 μg/ml), adrenal nuclei synthesize predominately rRNA as characterized by size and base composition. Approximately 10% of the RNA synthesized under these conditions sediments at 4–5 S; this RNA synthesizing activity is inhibited by high concentrations of α-amanitin (231 μg/ml) indicating the presence of RNA polymerase III activity. ACTH administration to guinea pigs results in a twofold increase in adrenal nuclear RNA polymerase I and III activities at 14 hr of hormone treatment. Analysis of the amount of radiolabeled nucleoside triphosphate incorporated in vitro into 3′ chain termini and into internal nucleotide positions has been utilized to measure the number of RNA chains and the average chain length synthesized in vitro. Incorporation into 3′ chain termini is not changed by ACTH; incorporation into internal nucleotides is doubled in parallel with the increase in RNA polymerase I activity. These results are not due to an altered K_m of RNA polymerase I for the four nucleoside triphosphates, nor to differential RNase or phosphatase activity. These studies suggest that the regulation of RNA polymerase I by ACTH is accomplished in part through an increase in the rate of RNA chain elongation.

Increased rRNA synthesis occurs in target tissues in response to a variety of hormonal and growth stimuli. An induction of rRNA[1] synthesis and/or increased nuclear RNA polymerase I activity occurs with estrogen (Notebloom and Gorski, 1963; Barry and Gorski, 1971; Hamilton et al., 1968), glucocorticoids (Garren et al., 1964; Yu and Feigelson, 1971), growth hormone (Oravec and Korner, 1971), chorionic gonadotropin (Jungman and Schweppe, 1972), adrenocorticotropic hormone (ACTH) (Farese and

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; rRNA, ribosomal RNA; TGMED, 50 m*M* Tris (pH 7.9), 25% glycerol, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 5 m*M* dithiothreitol; PEI, polyethyleneimine.