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TRANSCRIPTION RENDERS CHROMATIN RESISTANT TO MICROCOCCAL NUCLEASE DIGESTION

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SUMMARY. When isolated HeLa cell nuclei were preincubated under transcription conditions with excess E. coli RNA polymerase, chromatin DNA became relatively resistant to digestion by micrococcal nuclease. Quantitation of the DNA content in nuclei after enzyme digestion revealed that approximately twice as much nuclease was required to give the same levels of release of DNA fragments from transcribed as from untranscribed nuclei. Resistance increased with the amount of polymerase and with the time of preincubation. Since the resistance to nuclease was not observed in the presence of rifampicin or by preincubation without UTP, both RNA chain initiation and elongation were considered to be essential for the manifestation of resistance. However, when DNase I was used as a probe, such a change in chromatin DNA was not detected.

The mechanism by which RNA polymerase molecules rapidly move across chromatin beads with extensive synthesis of RNA has been of considerable interest but still is an unsolved problem (1,2). Chambon and colleagues have concluded from their experiments using SV40 minichromosomes prepared *in vivo* and reconstituted *in vitro* (3-5) that "local loosening" of nucleosome particles is a likely mechanism. If so, DNA stretches are considered to become sensitive to micrococcal nuclease digestion during active transcription, since the enzyme preferentially digests the DNA of the liker regions which are free of nucleosome core particles. Our previous examinations, however, have shown that there was no apparent difference in susceptibility to micrococcal nuclease of chromatin DNA of HeLa cells before and after transcription by endogenous RNA polymerases (7). This observation was unexpected if local loosening is the correct mechanism. A possible explanation might be an insufficiency in amount of RNA polymerase molecules for the transcription of chromatin, since only endogenous polymerases

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were used. Although there are many reports concerning the susceptibility of actively transcribed genes of animal tissues to several kinds of nuclease (7 for ref.), no consistent correlation has thus far been elucidated between *in vivo* transcription and nuclease sensitivity of native chromatin. The relationship between transcription and nuclease-sensitivity can be studied directly by the use of purified RNA polymerase in a nuclei transcription system, since nuclei were shown to take up RNA polymerase and use it for transcription (8). RNA polymerase from *E. coli* would be ideal as a nonspecific probe to search for changes in nucleosome structure, since it can be isolated in large quantity and in a highly concentrated form, and since it has high affinity for eukaryotic DNA.

In this report, sensitivity of nuclear DNA of HeLa cells to micrococcal nuclease was measured before and after the forced transcription with excess *E. coli* RNA polymerase.

MATERIALS AND METHODS

Materials. Unlabeled nucleotide triphosphates were obtained from Sigma Chemicals; Micrococcal nuclease and DNase I from Worthington; ethyleneglycolbis (-aminoethyl ether)-N.N'-tetraacetic acid (EGTA) from Tokyo Kasei; agarose from Dojin Chemical; and rifampicin from Boehringer. RNA polymerase was purified from K12 strain of *E. coli* cells as described by Burgess (10). One unit of RNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of labeled UMP into acid-precipitable material in 10 min at 37°C.
RNA synthesis by isolated HeLa cell nuclei. Nuclei were isolated from HeLa S3 cells and resuspended to give 1 X 10^8/ml in 1mM Ca^{2+}-containing nuclei suspension buffer (11). RNA synthesis was carried out in a final volume of 60 ul. The final conditions were as follows; 17.5% glycerol, 50mM NaCl, 20mM KCl, 6mM $MgCl_2$, 30mM Tris-HCl (pH7.5), 3mM dithiothreitol, 0.1mM EDTA and 1mM each of nucleotide triphosphate.
Nuclease digestion and purification of DNA. Nuclease digestion was carried out at 25°C for 10 minutes, or 5 minutes, with micrococcal nuclease or DNase I, respectively, and the reaction was terminated as previously described (7). Subsequent extraction of DNA was performed as described by Noll (6).
Gel electrophoresis. Extracted DNA was fractionated by electrophoresis on a 1.5% agarose gel as described by Sharp et al (12), stained with ethidium bromide and photographed under ultraviolet light. Photographic negatives were scanned with a Gilford densitometer.
Quantitation of DNA. The nuclease resistant DNA was obtained from the nuclei sedimented at low speed centrifugation. The DNA was solubilized in cold 7% perchloric acid by sonication and then heated at 85°C for 10 minutes. DNA content was measured according to the method described by Burton (13).

RESULTS

Kinetic studies on RNA synthesis in isolated HeLa cell nuclei were first carried out by using *E. coli* RNA polymerase to optimize the conditions which

support the highest degree of transcription. Maximal enhancement was observed by incubating the nuclear suspension (1 X 10^8/ml) with 500 units/ml of polymerase at 25°C for 3 hours (data not shown). Nuclei thus treated were digested with micrococcal nuclease. DNA was then extracted and separated by electrophoresis. A series of multiple bands which are known to be produced by micrococcal nuclease digestion of chromatin DNA was obtained with increasing levels of enzyme. In addition, higher molecular weight DNA bands eventually converged into the smallest unit size derived from mononucleosomes. When the digestion profiles of transcribed and untranscribed nuclei were compared, an increase in resistance to nuclease was observed in the former (Fig. 1). Undigested high molecular weight DNA was still abundant at 125 units/ml of micrococcal nuclease in transcribed nuclei (Fig. 1C), while only oligonucleosomal DNA bands were observed in untranscribed control nuclei (Fig. 1B). If preincubation was carried out at 0°C for 3 hours, no relative resistance or even a slightly higher sensitivity to nuclease was observed (Fig. 1A). This is probably due to the absence of transcription by exogenously added RNA polymerase as well as by endogenous enzymes at 0°C.

This difference was also demonstrated by measuring DNA content in both sets of nuclei after nuclease treatment. Greater than 40% of nuclear DNA was solubilized from the control nuclei and the fraction of remaining DNA was significantly and consistently higher in transcribed nuclei than in the untranscribed control at any concentration of nuclease tested (Fig. 2). Approximately twice as much micrococcal nuclease was again required to yield the same level of nuclear DNA fragments. The resistance of nuclear DNA to micrococcal nuclease digestion was thus non-stoichiometric. The change therefore is apparently not due to the protection of linker regions of nucleosome simply by the binding of RNA polymerase molecules.

Effect of preincubation of nuclei with RNA polymerase on the susceptibility to nuclease was then examined to determine whether the increase in resistance was related to the extent of transcription. As Fig. 3A shows, the resistance of nuclear DNA was increased as the period of preincubation was prolonged up

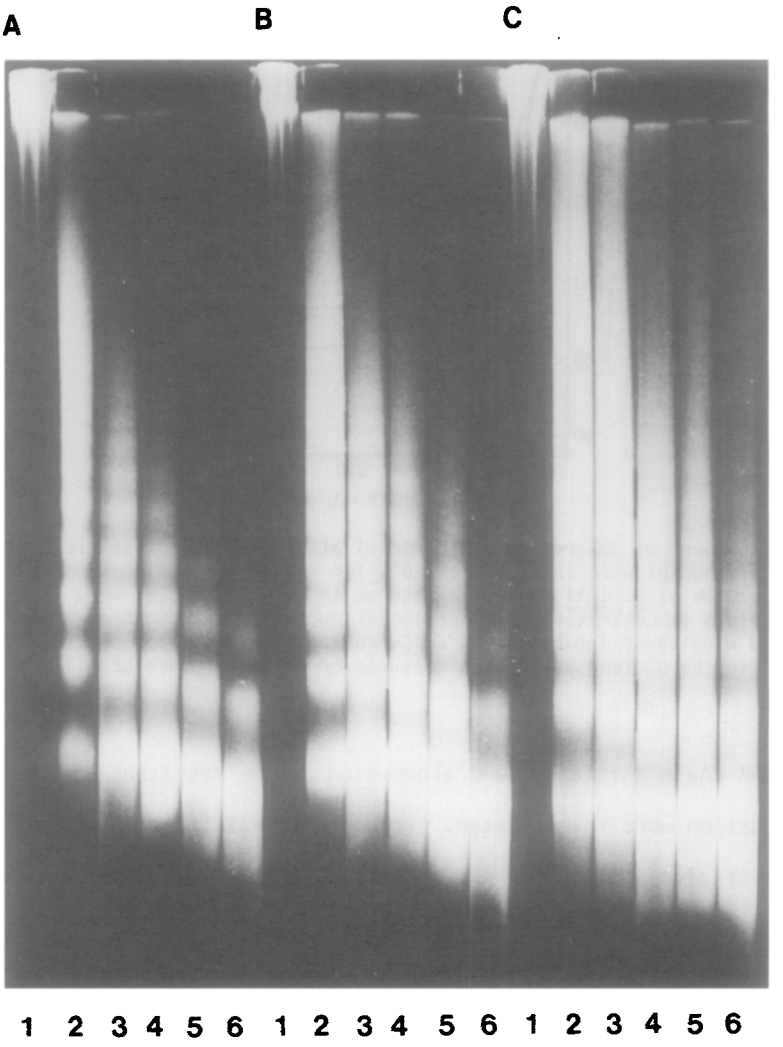

Fig. 1. Effect of micrococcal nuclease on transcription of nuclear DNA. Nuclei were preincubated with E. coli RNA polymerase (Panel A and C) or without RNA polymerase (Panel B) at either 0°C (Panel A) or 25°C (Panel B and C) for 3 hours as described in Materials and Methods. Micrococcal nuclease digestion was carried out at 25°C for 10 minutes at the following concentration in units/ml: 0, lane 1; 63, lane 2; 125, lane 3; 250, lane 4; 500, lane 5; and 1000, lane 6. DNA was then extracted and separated on an agarose gel as described in the Methods.

to 3 hours. After that period, the nuclear DNA became sensitive again as RNA synthesis decreased. Similar results were also obtained from a dose-response experiment (Fig. 3B), where nuclei were preincubated with increasing amounts of RNA polymerase. Results obtained from the quantitation of DNA content in nuclei also paralleled those of Fig. 3 (data not shown). Transcription is carried out by a complicated series of multistep reactions (19). Among these

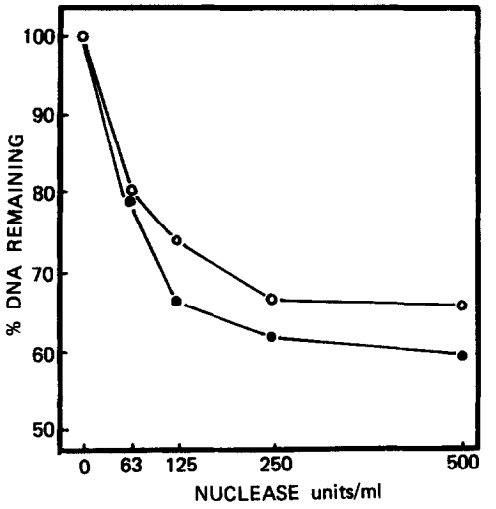

Fig. 2. Amount of DNA remaining in nuclei after treatment with micrococcal nuclease. Nuclei were incubated at 25°C for 3 hours in the presence (O-O) or absence (●-●) of E. coli RNA polymerase as in Fig. 1. Nuclei were digested with various concentrations of micrococcal nuclease as indicated in the figure. After 10 minutes of incubation at 25°C, nuclei were recovered by centrifugation and DNA in the nuclear pellet was measured by Burton's procedure (13).

effects of RNA chain initiation and elongation on the resistance of DNA to nuclease digestion were investigated. Transcription initiation was examined with the aid of rifampicin (Fig. 4, line A), a specific inhibitor for initiation by bacterial RNA polymerase. Elongation was studied by UTP deprivation (Fig. 4, line B) which allows the formation of the initiation complex but no extensive chain elongation. We compared the distribution of nuclear DNA that is resistant to micrococcal nuclease in each reaction as determined by densitometry. Densitomeric scans showed that reaction active in transcription contained higher molecular weight DNA (compared line C to line A and B in Fig. 4). These results provide strong evidence that extensive initiation and elongation of RNA chains are both required for the manifestation of increased resistance of chromatin DNA to micrococcal nuclease.

Another possibility is that the observed resistance could be caused through the protection of chromatin linker regions by product RNAs rather than by the structural change in chromatin. To test this possibility a sufficient amount of RNase was added to the preincubation mixture to digest almost all the product RNAs. The nuclei suspension thus treated was then subjected to micrococcal

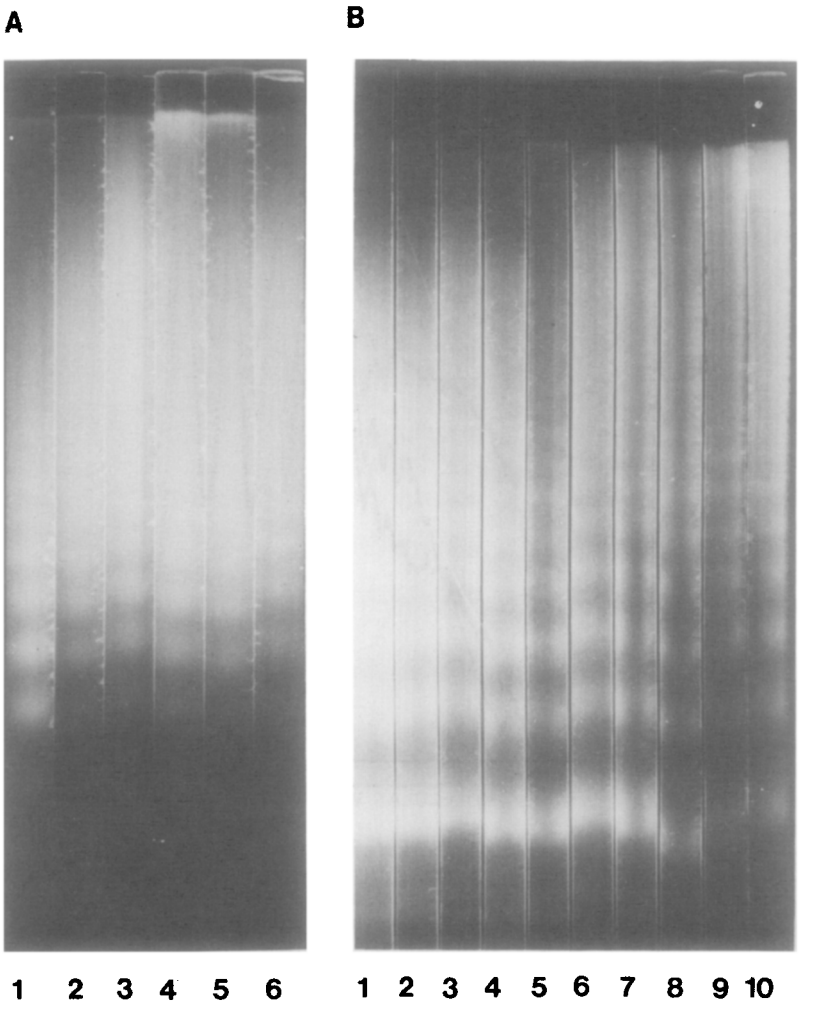

Fig. 3. Relationship between the extent of transcription and the sensitivity of nuclear DNA to micrococcal nuclease. In panel A, nuclei were preincubated with E. coli RNA polymerase for 0 time (lane 1), 1 hour (lane 2), 2 hours (lane 3), 3 hours (lane 4), 4 hours (lane 5) and 5 hours (lane 6). In each reaction, nuclei were subjected to micrococcal nuclease digestion with 63 units of enzyme/ml for 10 minutes, DNA was then extracted and analyzed by agarose gel electrophoresis. In panel B, nuclei were preincubated with various concentration of E. coli RNA polymerase for 3 hours at 25°C as follows; 0, lane 1; 4, lane 2; 8, lane 3; 16, lane 4; 32, lane 5; 64, lane 6; 125, lane 7; 250, lane 8; 500, lane 9; and 1000 lane 10; units/ml of E. coli RNA polymerase. In each reaction, micrococcal nuclease digestion was carried out with 63 units/ml.

nuclease digestion. Relative resistance was still obvious (data not shown), indicating that RNA molecules might not play any detectable roles in the phenomenon.

The finding that transcription renders chromatin resistant to micrococcal nuclease prompted us to examine whether the resistance could also be visu-

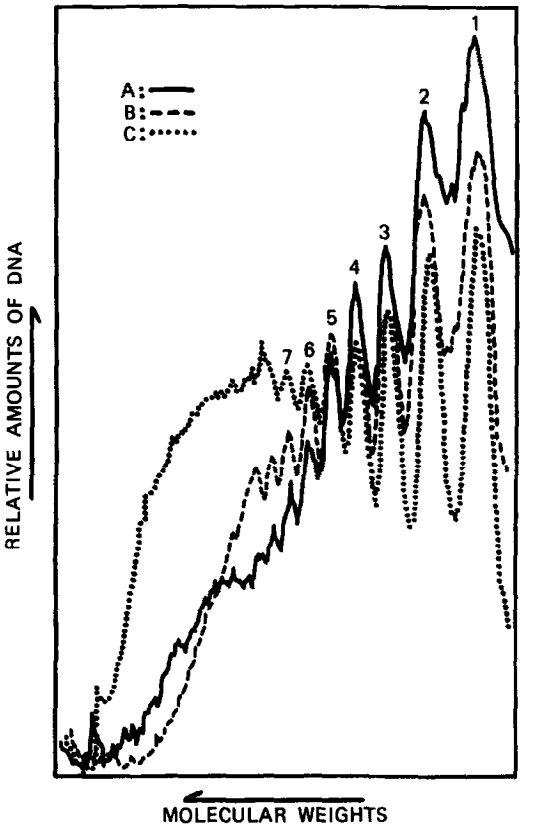

Fig. 4. Effect of UTP deprivation and rifampicin on the manifestation of resistance of nuclear DNA to micrococcal nuclease. Preincubation of nuclei was carried out as follows; a) complete condition with 230 ug/ml of rifampicin, b) complete condition minus UTP, and c) complete condition. When tested, rigampicin was added 5 minutes after the onset of preincubation. After further incubation for 3 hours, reaction mixtures were subjected to micrococcal nuclease digestion at concentration of 63 units/ml. DNA was then extracted and resolved in an agarose gel. The relative amounts of DNA were determined as described in Materials and Methods. The peaks represent amounts of DNA shown by ethidium staining. Nucleosome multimer numbers are given.

alized with DNase I. DNase I has been shown to recognize both linker and core regions of chromatin DNA. However, no difference was observed in electrophoretic profiles of DNA fragments extracted from both transcribed and untranscribed nuclei (data not shown).

DISCUSSION

In this report, we have studied the sensitivity of native chromatin to micrococcal nuclease before and after forced transcription by the addition of excess E. coli RNA polymerase. Under these conditions, activation of transcription occurs because of its limited site selectivity. Contrary to our

expectation, a resistance of chromatin DNA to digestion by micrococcal nuclease was acquired by extensive transcription. These conclusions were based on a qualitative analysis of DNA following by agarose gel electrophoresis and a quantitative measurement of DNA remaining in nuclei after micrococcal nuclease digestion. For the manifestation of the resistance, both initiation and elongation of RNA chains were shown to be required. The possible involvement of RNA products in this resistance was not obvious.

The sensitivity of active genes to DNase I was first demonstrated by Weintraub and Groudine (17). Almost all hypersensitive sites in DNA have been found at or near the 5' end of regions of transcription and then sites are generally not detected in transcribable sequences themselves (18). This surprising site selectivity of DNase I must therefore be closely related to the transcription process. Our results showed, however, no detectable change in resistance of chromatin to DNase I after forced transcription by E. coli RNA polymerase.

A Ca^{2+}, Mg^{2+}-dependent endonuclease, whose enzymatic specificity is quite similar to that of micrococcal nuclease (20,21), is abundant in chromatin (15). Since it seems unlikely that genes are digested during their transcription, there must be a protective mechanism which is unknown. This could be achieved, as Keene and Elgin suggested (16), by the selection of nuclease-resistant sequences of transcribable DNA through evolution. The change in chromatin during transcription that renders it resistant to an exogenous nuclease could also explain this protection at least in part. It would seem preferable to the cell that the transcriptionally active regions become resistant to endogenous chromatin-bound nuclease.

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