Nick Translation of HeLa Cell Nuclei as a Probe for Locating DNase I-sensitive Nucleosomes*

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The technique of nick translation of nuclei (Levitt, A., Axel, R., and Cedar, H. (1979) Dev. Biol. 69, 496-505) has been used in HeLa cells to label DNase I-sensitive regions. Micrococcal nuclease digestion of the nick translated nuclei was followed by a low ionic strength gel electrophoresis system which separates different types of mononucleosomes. The major label was observed in the vicinity of high mobility group protein containing mononucleosomes. However, further analysis revealed that the particle does not sediment in the position of mononucleosomes on a sucrose gradient. Two alternative explanations are discussed as the possible source of this particle. It is either a high mobility group protein containing nucleosome in some unfolded conformation or the labeled particle originates from discrete DNA fragments, wrapped around some nonhistone proteins, located in a highly DNase I-sensitive region, which is resistant to micrococcal nuclease digestion.

Understanding the chromatin organization of active and inactive genes has been a major goal of many laboratories. After the discovery of the nucleosomal structure of chromatin, attempts have been made to elucidate differences existing between active and inactive chromatin. Nucleases, as probes of chromatin structures, have proven to be a powerful tool in such an analysis (1, 2). Several years ago, Weintraub and Groudine (3) reported that globin genes in tissues active in globin transcription are extremely sensitive to DNase I, whereas tissues not synthesizing this protein lack such a sensitivity. A logical consequence of this observation was to search for the factors involved in this phenomenon. Nonhistone proteins HMG1 14 and HMG 17, which were first isolated by Goodwin and Johns (4), are apparently important for conferring DNase I sensitivity upon globin genes. When these proteins are removed from erythrocyte chromatin using 0.35 M NaCl, DNase I sensitivity is lost. Reconstitution of HMG proteins with HMG-depleted chromatin restored DNase I sensitivity (5, 6). Based on DNase I digestion of active chromatin, a method was developed for labeling DNase I-sensitive chromatin (7). It essentially consists of nicking chromatin with DNase I followed by DNA polymerase to incorporate labeled nucleotides into active chromosomal loci. In the present study, we have used this technique in HeLa cell nuclei. Micrococcal nuclease digestion of labeled nuclei was followed by low ionic strength polyacrylamide gel electrophoresis, which separated different mononucleosomes (8), which enabled us to locate the specific types of mononucleosomes which have been nick translated. Furthermore, subjecting these mononucleosomes to a second dimension polyacrylamide electrophoresis allowed us to identify different proteins associated with each type of mononucleosomes. The major site of incorporation of label in the DNA has been shown to be in a particle sedimenting more slowly than a mononucleosome.

MATERIALS AND METHODS

HeLa S3 cells were prepared as described before (9) and stored at −70°C in PBS buffer (150 mM NaCl, 3 mM KCl, 8 mM NaH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4) containing 10% glycerol. The cells were prepared at the Massachusetts Institute of Technology Cell Culture Center. All operations were carried out at 4°C. After several washings and centrifuging in PBS, the pellet was suspended in RSB (10 mM NaCl, 10 mM Tris, 3 mM MgCl₂, pH 7.6) containing 0.5% Nonidet (British Drug House), and 1 mM phenylmethylsulfonyl fluoride (Sigma, stock solution of 0.1 M in dry dioxane). Using a loosely fitted Dounce homogenizer (Type B pestle), the cells were lysed by about 10 strokes. After centrifugation at 2000 × g, the nuclei were washed two to three times in RSB with 1 mm phenylmethylsulfonyl fluoride until the supernatant was clear. Pasteur pipettes were used during all the steps for dispersing cells and nuclei. Nuclei were used immediately or suspended in RSB with 10% glycerol and stored at −70°C.

Nick Translation and Micrococcal Nuclease Digestion—The procedure was similar to that of Gazit and Cedar (10) and Gazit et al. (11); however, for the nick translation reaction of HeLa nuclei, it is very important to add deoxynucleotides immediately prior to the polymerase reaction. Presumably, the endogenous ATPase hydrolyzes ATP at 37°C. Nuclei were suspended in 50 mM Tris, 5 mM MgCl₂, 50 μg/ml of bovine serum albumin, 0.01 M 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.6, at a concentration of 1 mg/ml of DNA (determined in 5 M urea, 2 M NaCl by measuring the absorption spectra and correcting for scattering using A₂₆₀ = 20 for 1 mg/ml of DNA). Nicking of DNA was carried out by DNase I (Sigma) at concentrations indicated in the figure legends at 37°C for 20 min and then placed on ice. Deoxynucleotides, dATP, dGTP, dTTP, and dCTP (Sigma), containing varying amounts of [²⁵P]dATP (New England Nuclear, 800 Ci/mmol) were added. The final concentration of each nucleotide was 4 μM. Escherichia coli DNA polymerase I (New England Biolabs, 10,000 units/ml) was added at a concentration of 10 units/ml, and the reaction proceeded at 15°C for 5 min. At the end of the reaction, the nuclei were centrifuged and washed three times with the suspension buffer.

Micrococcal nuclease (P-L Biochemicals) digestion was performed at 15°C in 50 mM Tris, 1 mM CaCl₂, 0.2 mm phenylmethylsulfonyl fluoride, pH 7.6 (12). The reaction was stopped and nuclei were lysed by adding EDTA and EGTA (final concentrations 30 mM each). After centrifugation, the supernatant was retained. The conditions are

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1 The abbreviations used are: HMG, high mobility group proteins; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis[(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid]; bp, base pair.
Sucrose gradient of digested nuclei. 1 ml of nuclei (1 mg/ml of DNA) was nick translated using 0.2 μg/ml of DNase I (10 μCi of \(^{3}H\)dATP was used). The nuclei (1 ml) were then digested with micrococcal nuclease (80 units, 10 min at 15°C). After lysis with EGTA/EDTA, the sample was centrifuged and the supernatant was loaded on a 5-20% sucrose gradient. After centrifugation, 0.55-ml fractions were collected. Absorption measurements and counting of trichloroacetic acid-precipitated samples were carried out (19). M and D donate mononucleosomes and dinucleosomes, respectively.

FIG. 1. Sucrose gradient of digested nuclei. 1 ml of nuclei (1 mg/ml of DNA) was nick translated using 0.2 μg/ml of DNase I (10 μCi of \(^{3}H\)dATP was used). The nuclei (1 ml) were then digested with micrococcal nuclease (80 units, 10 min at 15°C). After lysis with EGTA/EDTA, the sample was centrifuged and the supernatant was loaded on a 5-20% sucrose gradient. After centrifugation, 0.55-ml fractions were collected. Absorption measurements and counting of trichloroacetic acid-precipitated samples were carried out (19). M and D donate mononucleosomes and dinucleosomes, respectively.

given in the figure legends.

Sucrose Gradients—Samples containing soluble nucleosomes were analyzed by sedimentation on 5-20% linear sucrose gradients in 10 mM Tris, 1 mM EDTA, pH 7.6. Centrifugation was carried out for 18 h at 32,000 rpm using 13-ml tubes in a Beckman SW 40 rotor at 4°C.

DNA and Protein Electrophoresis—Low ionic strength electrophoresis for separating mononucleosomes was a combination of published procedures with some modifications (8, 13). Gels containing 4% polyacrylamide (30:1, acrylamide/bisacrylamide) in 6.4 mM Tris base, 3.2 mM sodium acetate, 1 mM EGTA, 0.5 mM EDTA, pH 8.0, were used. Slab size was 25 × 17 × 0.3 cm. Pre-electrophoresis was carried out at 150 V for 1.5 h. After introducing the sample, the electrophoresis was run at 150 V for 16 h. The buffer (the same as in the gel) was circulated between the upper and lower reservoirs. For protein analysis, a strip of polyacrylamide gel (2-cm width) from the first dimension was equilibrated for 2 h in buffer O of O’Farrell (14) (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5 mM Tris HCl, pH 6.8) and placed at the top of 0.1% SDS-polyacrylamide (15% acrylamide) slab according to Laemmli (15) as modified by Weintraub et al. (16). 1% agarose in O’Farrell (14) solution was used for sealing the strip. Slab size was the same as before and electrophoresis was carried out at 100 V. Protein staining was accomplished using 0.5% Coomassie blue in 10% acetic acid overnight. Destaining was performed in 10% acetic acid, 10% methanol.

DNA sizing was done by employing 4-8% polyacrylamide gel electrophoresis according to Maniatis et al. (17) using 0.09 M TBE buffer (Tris/boric acid/EDTA). Extraction of DNA was as described by Egan and Levy-Wilson (18). Autoradiography was performed according to the procedure described (19).

RESULTS

In order to obtain some information about the distribution of the radioactive label incorporated by the nick translation-nuclease digestion procedure, the supernatant from lysed nuclei was examined on sucrose gradients. The results are presented in Fig. 1. The absorption profile shows mono- and dinucleosome peaks, whereas the radioactivity diagram reveals incorporation mainly in mononucleosomes. The major peak of label is near the top of the gradient. Our analysis of the DNA size in this region showed that almost all of the DNA has <80 bp (data not shown). It is likely that internucleosomal and subnucleosomal fragments contribute to the radioactivity in this region. Similar results were reported for calf thymus by other investigators (20). Low ionic strength gel electrophoresis was employed to obtain a more precise picture of the type of mononucleosomes being labeled. Gel staining and autoradiography results are shown in Fig. 2, A and B, respectively. Lanes a-d correspond to different times of digestion of nick translated nuclei by micrococcal nuclease. A similar electrophoresis system used by Levinger et al. (8)
allowed them to distinguish three mononucleosome regions in HeLa nuclei digested by micrococcal nuclease. In Fig. 2A, the lowest band (K) consists of mononucleosome core tailed by an A24-containing core particle. Region (L) is HMG-containing mononucleosomes, and finally (M) is H1-containing mononucleosomes. We will verify some of these assignments later. A corresponding autoradiograph (Fig. 2B) reveals a major band in the vicinity of HMG-containing mononucleosomes in addition to some label in the background.

In order to study the relationship between nick translation and the intensity of the above labeled band, the effect of different DNase I concentrations was studied. Results of such an experiment are shown in Fig. 3. In the absence of DNA polymerase (lane a), no incorporation is observed, indicating that the presence of label is mediated by exogenous polymerase. The effect of DNase I concentration in nick translation is presented in lanes b–e. Label incorporation persists even in the absence of DNase I (lane b). During incubation at 37 °C, the endogenous nuclease probably introduced nicks into DNA. In the presence of DNase I, the label intensity increased by 2- and 4-fold using 0.2 and 0.4 μg/ml of DNase I, respectively (lanes c–d). At 0.8 μg/ml of DNase I (lane e), overdigestion by DNase I has become a major factor and is a reasonable explanation for the observed reduction of label intensity. In addition, in Fig. 3 there are two faster migrating bands. The upper one of these two is very likely the core particle and the lowest band probably originates from subnucleosomal and internucleosomal regions (21, 22). We attribute the relative obscurity of the latter bands in Fig. 2 to the longer electrophoresis time of the gel in Fig. 2 and to a shorter exposure time on the film. In experiments herein, freshly prepared nuclei yield less of the labeled subnucleosomal band than stored nuclei when they are nick translated.

Following digestion of the nuclei with micrococcal nuclease, the sample was centrifuged and the diffused particles in the supernatant (S1) were analyzed. The pellet was then lysed and the nucleoprotein of the supernatant (S2) was obtained. Alternately, the nuclei can be lysed after digestion and the combined S1 and S2 fractions obtained by centrifugation. In all of the experiments discussed so far, the total digest was used; there was no separation into S1 and S2 fractions. However, analysis of the label distribution between S1 and S2 showed that most of the labeled band in the HMG mononucleosome region was associated with S1 (data not shown). Consequently, the rest of the data presented is concerned with S1 (except Fig. 4B which is related to S2).

Analysis of the protein composition of the different types of mononucleosomes was performed by a second dimension SDS-gel electrophoresis. The results for the fraction S1 are shown in Fig. 4A. There are two broad bands (shown by ethidium bromide staining, strip b) in the first dimension where the nucleosomes were subjected to low ionic strength electrophoresis. Label is associated with the slower migrating band (Fig. 4A, strip c). The SDS-polyacrylamide gel (Fig. 4A, strip b) shows a number of familiar proteins in the lower region of the gel (histones), as well as a large number of other proteins in the higher molecular weight domain, giving rise to a complex pattern. There is very little histone H1 present in the S1 nucleosomes (Fig. 4A, strip c). A similar general picture was obtained for oviduct chromatin (12). The fastest migrating particle in the first dimension (Fig. 4A, strip b) is the core which lacks the protein A24. A24-containing core trails behind this and has been implicated in active chromatin (23, 24). We should point out that the width of the strip applied for protein analysis was approximately 2 cm. Consequently, some of the protein bands (i.e. HMG 17) are very diffuse. Based on scanning of the proteins in the HMG-containing region (Fig. 4A, strip c), we estimate one molecule of HMG 17/two nucleosomes in this region. The ratio is lower for HMG 14 since this protein probably undergoes proteolysis during isolation from nuclei (25). These ratios represent a lower limit since DNase I digestion of nuclei has been shown to solubilize HMG proteins (26). In general, the ratio of HMG proteins to nucleosomes always showed an increase when nuclei were digested with micrococcal nuclease without being nick translated first. A similar protein analysis for the S2 fraction is shown in Fig. 4B. The HMG 17 protein band is very faint. Histone H1 is present and is associated with the corresponding mono- and higher oligosomes. If one assumes that the labeled band is indeed HMG-containing mononucleosomes, the situation resembles that of the ovalbumin gene as studied by Bloom and Anderson (27), where micrococcal nuclease digestion of nuclei released the ovalbumin gene nucleosomes into the supernatant. A later study by Goodwin et al. (12) demonstrated that these diffused nucleosomes were enriched with HMG 14 and HMG 17 proteins.

The result of the sizing of the labeled DNA was surprising.
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Fig. 4. Two-dimensional protein gel electrophoresis of nucleosomes. 15 ml of nuclei (1 mg/ml) were nick translated using 0.2 µg/ml of DNase I and 400 µCi of [α-32P]dATP. The sample was suspended in 5 ml of digestion buffer and digested using 120 units/ml of micrococcal nuclease for 10 min. After centrifuging at 4 °C, the supernatant (S₁) and the pellet were each treated with EGTA/EDTA (soluble fraction from the pellet is called S₂). S₁ and S₂ were separately concentrated on an Amicon (B15) filter to 0.5 ml each. A corresponds to S₁ and B to S₂. Strips a and b are results of autoradiography and ethidium bromide staining in the first dimension, respectively (50 pl). Strip c is second dimensional SDS-polyacrylamide analysis using 15 A units of DNA for S₁ and 45 A units for S₂. 14 and 17 designate proteins HMG 14 and HMG 17.

Fig. 5. Analysis of labeled DNA. 1 ml of nuclei (1 mg/ml) was nick translated. It was suspended in 200 µl of digestion buffer, and 60 units of micrococcal nuclease were added to the sample (15 °C, 10 min). After lysis and centrifugation, the supernatant was applied to a low ionic strength polyacrylamide gel. At the end of electrophoresis, ethidium bromide staining was carried out and the gel was visualized by a UV illuminator. The HMG-containing region was excised and placed in a small dialysis tube filled with low ionic strength electrophoresis buffer and eluted at 40 V for 2 h at 4 °C. The eluant was dialyzed overnight against 10 mM Tris, 0.2 mM EDTA, pH 7.5. DNA was extracted and applied to a 4% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. The markers are labeled pBR322 HaeIII fragments (a gift from Dr. Gert Pflugfelder, Harvard University).
FIG. 6. Location of the labeled band in the sucrose gradient of S1. 5 ml of HeLa nuclei (1 mg/ml) were nick translated using 0.2 μg/ml of DNase I. Digestion was carried out by using 300 units of micrococcal nuclease in a 1-ml nuclei suspension for 5 min. 0.8 ml of S1 was loaded on a sucrose gradient. A is the sucrose gradient profile. In B, 100 μl of each fraction 11–22 of A were introduced on the low ionic strength nucleoprotein gel, electrophoresed, and autoradiographed. Lane a is 50 μl of S1 prior to the sucrose gradient.

DISCUSSION

Our aim in undertaking this investigation was to find out whether, upon digesting nick translated chromatin with micrococcal nuclease and subsequent low ionic strength gel electrophoresis, any radioactive enrichment of HMG-containing mononucleosomes could be detected. Nick translation of HeLa cell nuclei results in a highly labeled nucleoprotein particle with a sedimentation coefficient of approximately 9 S. The corresponding band on gel electrophoresis is sharp and does not change its position as a result of further micrococcal nuclease digestion. However, our results show that the size of DNA extracted from such a complex is dependent on the level of enzyme concentration. Increased digestion by micrococcal nuclease gives rise to smaller DNA fragments upon extraction of DNA from the nucleoprotein samples (data not shown).

We can suggest a number of possible sources responsible for the formation of the observed particle. It could originate from nonspecific (or specific) association of internucleosomal DNA fragments with some nonhistone proteins. SDS electrophoresis of the fractions is presented in Fig. 7B. There is a small amount of core histones and HMG proteins in the labeled particle fraction (lane b). Farther up on the gel there are a number of nonhistone proteins, some of which are candidates for interaction with the labeled DNA fragment. The protein composition of mononucleosomes (Fig. 7B, lane c) shows a full complement of histones and smaller amounts of nonhistone proteins.

We wish to thank a Referee for pointing out this possibility.
sensitive region free of nucleosomes. One may consider the presence of histones and HMGs (Fig. 7B, lane b) as a contamination from the mononucleosome region and instead focus attention on the nonhistone proteins present. Such a DNA-protein complex must lie in a DNase I-sensitive region. Since the DNase I concentration is sufficiently low to introduce only nicks (7), translation by DNA polymerase results in a labeled particle. Later digestion by micrococcal nuclease generates nucleoprotein particles which are to some extent protected against digestion. The DNA-protein complex could be assembled by wrapping DNA around a nonhistone protein; such a structure is well documented in the case of DNA gyrase (28). Evidence of nucleosome-free regions being sensitive to DNase I has been reported in a number of transcribing genes (29-35). The upstream region of the 5' end is apparently the major site. A recent paper contains information which resembles our data; Pauli et al. (36), during an investigation of nucleosomal organization of ribosomal genes of Physarum polycephalum, using micrococcal nuclease, reported that a repeat length of 30-40-bp fragments was generated which was superimposed on the usual nucleosomal pattern. Hybridization analysis showed that the small DNA fragments originate from upstream of the initiation site of rRNA transcription.

It was pointed out earlier under "Results" that the endogenous nuclease appears to introduce significant nicks into DNA. This is not surprising in view of the other reports demonstrating the role of endogenous nuclease in cleaving DNase I-sensitive regions (30, 31).

In conclusion, nick translation of HeLa cell nuclei followed by micrococcal nuclease digestion results in a distinct labeled nucleoprotein. Whether this particle is an unfolded HMG mononucleosome or a DNA protein (nonhistone) complex with the possibility of being located in a sensitive DNase I region will await further experiments.

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