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A structure of potentially active and inactive genes of chicken erythrocyte chromatin upon decondensation

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ABSTRACT

In the presence of 3 mM $MgCl_2$ DNase I cleavage of bulk, globin and ovalbumin gene chromatin in chicken erythrocyte nuclei generates fragments which are multiples of a double-nucleosome repeat. However, in addition to the dinucleosomal periodicity ß-globin gene chromatin was fragmented into multiples of a 100 b.p. interval which is characteristic for partially unfolded chromatin. This distinction correlates with higher sensitivity of ß-globin domain to DNase I and DNase II as compared to the inactive ovalbumin gene. At 0.7 mM $MgCl_2$ where these DNases fragment bulk chromatin into series of fragments with a 100 b.p. interval, the difference in digestibility of the investigated genes is dramatically decreased. When chromatin has been decondensed by incubation of nuclei in 10 mM Tris-buffer, DNase II generates a typical nucleosomal repeat, and the differential nuclease sensitivity of the analyzed genes is not observed. The data suggest that higher nuclease sensitivity of potentially active genes is due to irregularities in higher order chromatin structure.

INTRODUCTION

The structure of chromatin is determined to a great extent by the ionic environment. In a solution of low ionic strength chromatin exists as 10 nm nucleosomal fibrils (1, 2), but in the presence of divalent ions (0.1-1 mM) or monovalent ions (50-100 mM) the 10 nm fibrils form the 25-30 nm thick fibres (2-5).

It has been established that DNase I and DNase II are able to generate in nuclei of avian erythrocytes and of sperm of some marine invertebrates a set of fragments, the size of which is multiple of a double nucleosome DNA repeat (~ 400-420 bp) (6-9). Upon digestion at low ionic strength, however, the products of nuclease digestion show a clear ~ 200 b.p.

ladder extending from one to 10-12 nucleosomes (9). From this is clear that the alteration in chromatin fragmentation correlates well with distinct transitions of bulk chromatin structure induced by lowering ionic strength.

In the present study, we have used the enzyme DNase II for detailed analysis of structure of bulk chromatin and individual genes in a wide range of mono- and divalent ion concentrations. This nuclease does not require any ions for its activation. Our results show that the ß-globin and ovalbumin genes of erythrocyte nuclei have a different state of compactness manifesting in defined patterns of fragmentation. Upon chromatin decondensation induced by lowering divalent ion concentration the difference between active and inactive gene chromatin conformations, tested by DNase II digestion, is eliminated. Whatever the precise details of the structures, these results were interpreted to mean that the main difference between potentially active and inactive conformations can be determined on a level of higher order chromatin structure.

MATERIALS AND METHODS

Pigeon and chicken erythrocyte nuclei have been isolated by the method described before (10). The nuclei were washed two times in a buffer A comprising 0.25 M sucrose, 10 mM Tris-HCl, pH 7.0, 0.1 mM PMSF and 0-3 mM $MgCl_2$. The aliquots of nuclear suspension (usually 0.5 mg DNA per ml) were digested with DNase I (Worthington) or DNase II (Miles or P.L. - Biochemicals) at 37°C or 0°C for various times. Also the nuclei were digested with micrococcal nuclease (60 units/mg DNA) (Worthington) at 37°C for 6 min in the same buffer A containing 1 mM $CaCl_2$ and 3 mM $MgCl_2$. The extent of DNA hydrolysis was determined as perchloric acid-soluble products. RNase-treated and deproteinized DNA fragments were electrophoresed on 1% or 2% agarose gel (Bio Rad) using a solution of 40 mM Tris-acetate pH 7.8, 5 mM sodium acetate, 1 mM EDTA as an electrophoretic buffer. The size of DNA fragments and the value of DNA repeat length were determined according to the method of Sperling et al. (11), using

as a molecular weight standard a set of restriction DNA fragments.

For the blot-hybridization DNA fragments were transfered from gel onto Gene Screen membrane (NEN) in a solution of 25 mM phosphate buffer pH 6.5 according to the recommendation of NEN. Gene Screen membranes were air dried and then baked at 80°C for 3 h.

The plasmid pßEH containing the cloned chicken ß-globin 1.9 kb DNA fragment was kindly supplied by Dr. J.Engel. The plasmid pOV2,4 with the chicken ovalbumin gene 2.4 kb DNA fragment was received from Dr. B.O'Malley.

Plasmid DNA was isolated by the modified method (12) from E.coli HB101 cells, then was purified by the CsCl-EtBr gradient centrifugation followed by chromatography on a Sephacryl S-1000 column (Pharmacia). DNA inserts were prepared by digestion of the plasmids with appropriate restriction nucleases and by preparative electrophoresis in 0.8% low melting agarose gel (Sea Plaque, Sea Kem).

Nick-translation of the electrophoretically purified probes was carried out for 1 h at 16°C according to the method of Rigby et al. (13) using α-^{32}P-dNTP's manufactured by "Isotop" (USSR). Hybridization was performed at 68°C for 24-36 h in a solution comprizing 3×SSC, 1× Denhardt's mixture, 0.1% SDS, 50 µg/ml E.coli DNA. After hybridization membranes were washed at 68°C, air dried and autoradiographed with intensifying screen (Curix MR600, Agfa) at -70°C for 7-14 days. If the membrane was to be used for rehybridization, radioactive DNA was removed from the membrane at 68°C according to the procedure recommended by NEN.

Negatives of EtBr-stained gels and autoradiographs were scanned on a microdensitometer IFO 451 (USSR) and a laser densitometer Ultro Scan 2202 (LKB).

RESULTS AND DISCUSSION

1. DNA fragmentation pattern of erythrocyte chromatin with DNase II at different $MgCl_2$ concentrations

It is well known that higher order chromatin structure

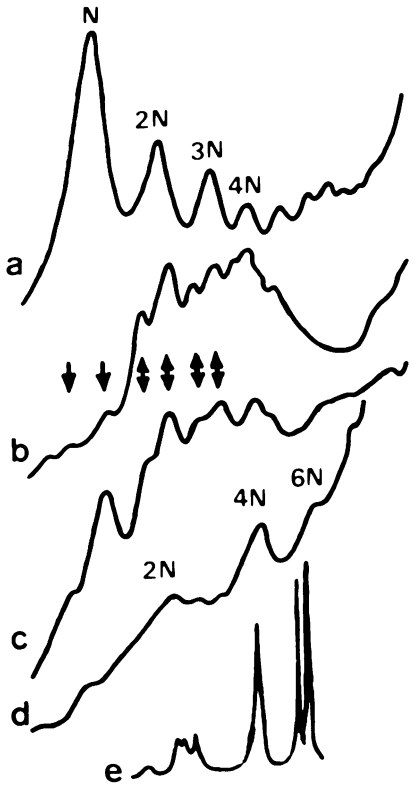

Fig. 1. Electrophoresis of DNA fragments isolated from erythrocyte nuclei after digestion with DNase II at different $MgCl_2$ concentrations. a - 10 mM Tris-HCl, b - 0.4 mM $MgCl_2$, c - 0.6 mM $MgCl_2$, d - 3 mM $MgCl_2$, e - marker DNA fragments of λ phage DNA cleaved by restriction nuclease Pst I. Size of the fragments (b.p.): 331, 432, 454, 489, 804, 1103, 1162. N, 2N, 3N, 4N - nucleosomal repeat. 2N, 4N, 6N - dinucleoaomal repeat. Arrows indicate a 100 b.p.-periodicity.

is maintained by DNA-histone and histone-histone interactions, with lysine-rich histones H1 and H5 being responsible for maintenance and stability of this structure. Mono- and divalent cations play an important role in compaction of nucleosomal fibrils (14-16).

We addressed this question by analyzing the fragmentation pattern in a wide range of divalent ion concentrations. Erytrocyte nuclei were digested with DNase II at different $MgCl_2$ concentrations (see "Materials and Methods") and isolated DNA

fragments were separated on agarose gel under non-denaturing conditions. Data presented in Fig. 1 show that increases in the $MgCl_2$ concentration resulted in alteration of the fragmentation pattern. This manifected in successive shifts of the fragmentation pattern beginning with a nucleosomal periodicity (at ~200 b.p. intervals) to a periodicity at ~100 b.p. intervals and then to a double-nucleosome repeat pattern (at ~400 b.p. intervals). These results indicate that DNase II may reveal several steps of chromatin compaction in the range of 0-3 mM $MgCl_2$ concentration. In fact, in the absence of $MgCl_2$ or at very low concentration (up to 0.1 mM) DNase II, like micrococcal nuclease, generates a nucleosomal DNA repeat, which is multiple of 216±6 b.p. (Fig.1a). It is known that in such ionic conditions nuclear chromatin is greatly unfolded (17), the linker DNA being very accessible to any nuclease (9, 18).

In the range of 0.2-0.9 mM $MgCl_2$ the fragmentation pattern is qualitatively changed (Fig. 1 b, c). This is seen by the appearance of DNA fragments, the size of which is a multiple of ~ 100 b.p. The repeat length of such 100 b.p.-periodicity is 105±3 b.p. One may suppose that on this stage of chromatin compaction the linker DNA gradually becomes resistant (inaccessible) to DNase II action due to its folding into a compact chromatin structure. This observation suggests that both intracore sites, which are the primary cleavage sites for these nucleases (18), are equally accessible in every nucleosome core particle. This leads to the appearance of periodicity at a 100 b.p. interval characterising an average distance between the neighbouring intracore cleavage sites along chromatin fibril (9).

Upon digestion at 1-3 mM $MgCl_2$ the only fragments of the 100 b.p. periodicity which belong to double nucleosome DNA repeat (~ 400, 800 b.p. ...) predominantly are being preserved on electrophoregrams (Fig. 1d). The double nucleosome periodicity is a result of relative inaccessibility of both intracore cleavage sites in every second nucleosome (9). This is provided by highly specific nucleosome packing in a compact higher order chromatin structure formed by lysine-rich histo-

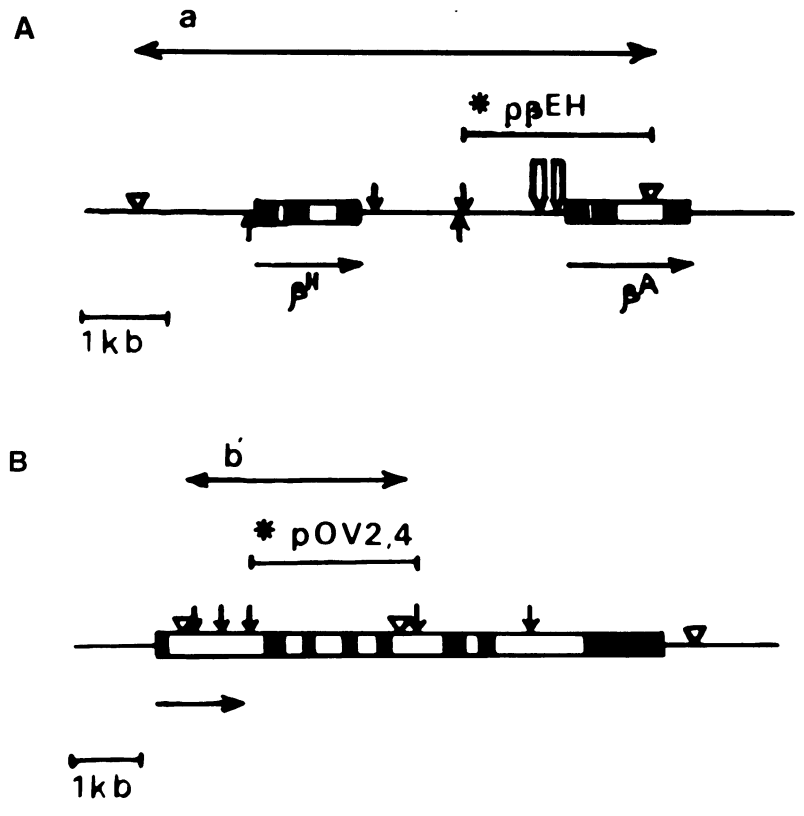

Fig. 2. Restriction maps of the ß-globin domain and the ovalbumin gene. A - ß-globin domain; B - ovalbumin gene. Black rectangles-exons; light rectangles - introns; horizontal arrows - direction of gene transcription. Stars designate the gene fragments used as the labelled probes. ⟷ (a and b') - the analysed DNA fragments. Vertical light arrows indicate the nuclease hypersensitive sites of the ß-globin domain. The sites of restriction nuclease cleavages: ↓ - EcoRI, ▽ - Hind III, ↑ - Bam HI.

nes in the presence of 1-3 mM $MgCl_2$ (7, 19). Similar results on erythrocyte bulk chromatin fragmentation pattern were received also for DNase I in the presence of 0.2-3 mM $MgCl_2$ (data not shown).

2. Chromatin structure of ß-globin and ovalbumin genes in chromatin of erythrocyte nuclei at 3 mM $MgCl_2$

Data presented show that the compact bulk erythrocyte chromatin is cleaved by DNase II (see Fig. 1) as well as by

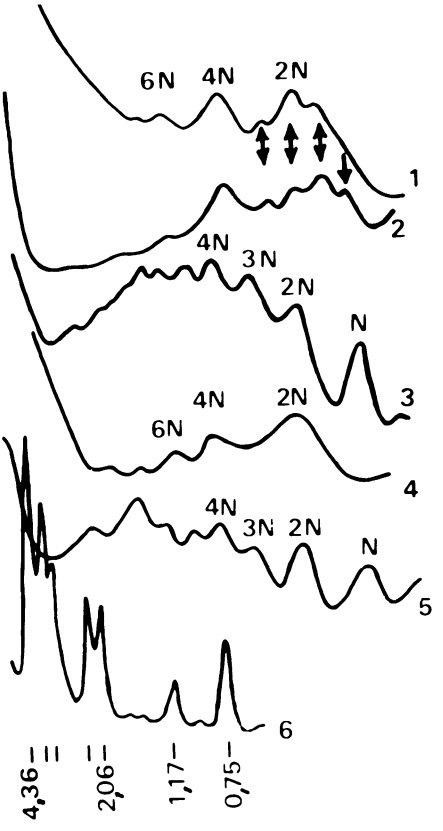

Fig. 3. Densitogram of a radioautograph of DNA fragments hybridized with the ß-globin and ovalbumin probes. Erythrocyte nuclei were digested with DNase I at ratio 1, 5 and 5 units/mg of nuclear DNA (lanes 1, 2 and 4, respectively) or micrococcal nuclease (lanes 3 and 5). Deproteinized DNA fragments were separated electrophoretically into 2% agarose gel and blot-hybridized with the labelled ß-globin (lanes 1, 2, 3) and ovalbumin (lanes 4, 5) probes. Lane 6 - marker DNA fragments obtained by restriction of pBR 322 DNA (sizes - in k.b.p.). N, 2N, 3N, 4N - nucleosomal repeat. 2N, 4N, 6N - double nucleosomal repeat. Arrows indicate a 100 b.p.-periodicity.

DNase I (data not shown) giving rise to a double nucleosome periodicity of fragmentation. It is known that the ß-globin gene has an active conformation in condensed erythrocyte chromatin (3-5); the ovalbumin gene, however, exhibits an inactive structure for all stages of erythroid cell differentiation (1, 3).

We have made an attempt to correlate the fragmentation pattern of ß-globin and ovalbumin genes in erythrocyte nuclei with DNase I and the chromatin structure of these genes. Fig. 2 shows restriction nuclease maps of the analysed genome regions; the hypersensitive sites detected in the present work are given in the map of ß-globin domain. At first, the fragmentation pattern of the individual genes was checked upon digestion in the presence of 3 mM $MgCl_2$, i.e. under conditions revealing a clear-cut double nucleosome periodicity. Following electrophoresis in 2% agarose gel DNA fragments were transferred onto a Gene-screen membrane and were hybridized with the ß-globin probe pßEH or the ovalbumin probe pOV2.4. Hybridization revealed that ß-globin chromatin exhibits a double nucleosome fragmentation pattern (Fig. 3). This fragmentation pattern is most noticeable at relatively low DNase I concentration (1-2 units/mg nuclear DNA), generating a double nucleosome pattern with a 380±9 b.p. repeat (Fig. 3, lane 1). In addition to the dinucleosomal periodicity DNase I produces ß-globin DNA fragments with a 100 b.p.-periodicity in the region of 200-600 b.p. (marked in Fig. 3 by arrows), the repeating element being equal to 104±4 b.p.

Rehybridization of the membranes with the ovalbumin probe also exhibited the presence of a double nucleosome cleavage pattern of this gene (Fig. 3). The value of this double nucleosome repeat was equal to 378±11 b.p. In this case, however, DNA fragments corresponding to a 100 b.p.-periodicity and characterizing a partially unfolded chromatin were absent. Thus, the appearance of two fragmentation patterns (100 b.p.- and dinucleosomal periodicities) upon DNase I digestion indicates that in compact erythrocyte chromatin the ß-globin gene has a less condensed structure in comparison to that of ovalbumin gene.

Hybridization of micrococcal nuclease-generated DNA fragments with radioactive globin or ovalbumin probes revealed in both cases a canonical nucleosomal repeat without those drastic changes in size of individual fragments found by Sun et al. (4). The discrepancy of the results may be due to that, unlike our paper, Sun et al. used micrococcal nuc-

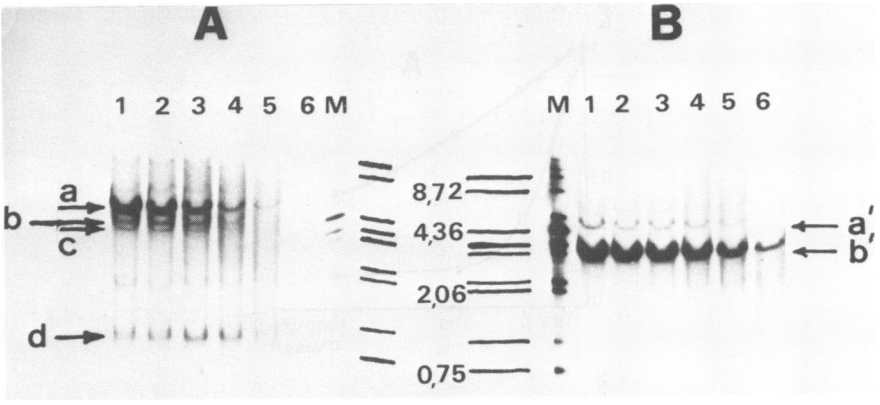

Fig. 4. Radioautographs showing kinetics of digestion of the ß-globin and ovalbumin genes by DNase II in the presence of 3 mM $MgCl_2$. After nuclear DNA digestion with DNase II isolated DNA was treated by restriction nuclease Hind III, separated into 1% agarose gel and blot-hybridized with the labelled ß-globin (part A) or ovalbumin (part B) probes. Lane 1 - DNA fragments isolated from control untreated nuclei incubated at 0°C for a digestion time; lanes 2-6 - DNA fragments isolated from DNase II - treated nuclei at ratio 50, 100, 250, 500 and 1000 units/mg of nuclear DNA, respectively; lane M - marker DNA fragments obtained by restriction and ligation of pBR 322 DNA (sizes - in k.b.p.). In part A: a, b, c, d - DNA fragments hybridizing with the globin probe; in part B: a', b' - DNA fragments hybridizing with ovalbumin probe.

lease hydrolysis buffer which contained relatively high concentrations of the monovalent cations (75 mM) with 1 mM $CaCl_2$ (4). In previous paper (26) we found that addition of NaCl (up to 50 mM) into the buffer containing 3 mM $MgCl_2$ changes essentially a nuclease fragmentation pattern of chromatin in erythrocyte nuclei. Therefore, it is possible that micrococcal nuclease generates different digestion pattern in response to changes in ionic conditions.

3. Nuclease sensitivity of ß-globin and ovalbumin chromatin at different concentrations of magnesium ions

To compare nuclease sensitivity of individual potentially active and inactive genes upon chromatin decondensation, nuclei were treated by DNase II at different $MgCl_2$ concentrations (0, 0.7 mM and 3 mM). Isolated DNA fragments were ex-

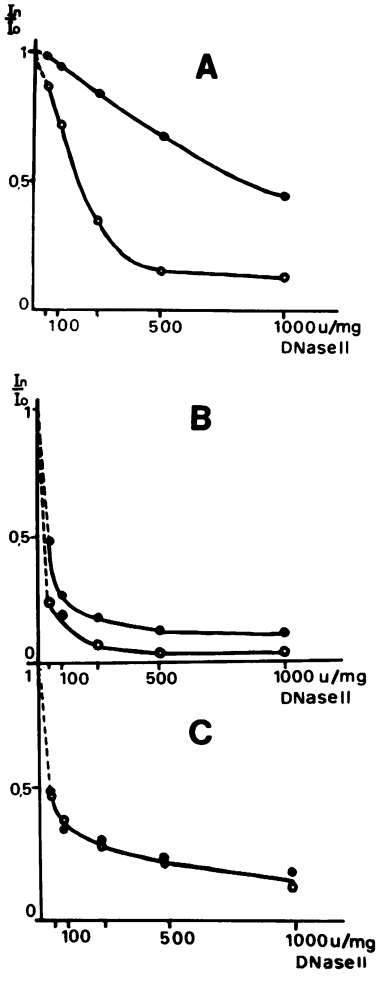

Fig. 5. Change of relative intensity ($\frac{I_n}{I_o}$) of DNA fragments of the globin domain (o - o) or the ovalbumin gene (• - •) in dependence on the extent of digestion of erythrocyte nuclei with DNase II (50-1000 units/mg of nuclear DNA) at different ionic strength: A - 3 mM $MgCl_2$; B - 0.7 mM $MgCl_2$; C - without $MgCl_2$ (10 Tris-buffer).

haustively digested with restriction endonuclease Hind III which yields a 6 Kb genomic ß-globin fragment (designated as "a" in Fig. 2A and Fig. 4A) and a 3.2 Kb genomic ovalbumin fragment (designated as "b" in Fig. 2B and Fig. 4B). After

electrophoresis in 1% agarose gel, DNA fragments were transferred onto a membrane and were hybridized with the EcoRI - Hind III insert of the plasmid pβEH or with the EcoRI - EcoRI insert of the plasmid pOV2.4 (Fig. 2). The intensity of the visualized restriction DNA fragments ("a" in Fig. 4A and "b" in Fig. 4B) gradually declined as the extent of DNase II digestion increased. Intensity of the restriction fragments was determined by scanning a radioautograph and measuring height or area of the peaks. Data obtained were plotted on a diagram where enzyme concentration was drawn versus the ratio I_n/I_o taking that I_o - hybridization intensity of the band of interest in a control (untreated with DNase II) probe and I_n - hybridization intensity of the band at a given nuclease concentration (Fig. 4A and Fig. 5).

When comparing the results presented in Fig. 4 and kinetics of the I_n/I_o ratio for two analyzed genomic regions (Fig. 5), one may conclude that the β-globin gene is more sensitive to DNase II digestion in the presence of 3 mM $MgCl_2$ than the ovalbumin gene. Thus, the β-globin gene retains its more accessible structure at the terminal stage of erythrocyte maturation that is in accordance with the results of high sensitivity of this gene to DNase I digestion (1).

On decreasing $MgCl_2$ concentration below 1 mM decondensation of erythrocyte chromatin is being promoted. At 0.5-0.7 mM $MgCl_2$ DNase II generates a 100 b.p.-periodicity (Fig. 1). Overall nuclease digestibility of chromatin is increased by a factor of two in comparison to the digestion at 3 mM $MgCl_2$ (results not shown). Blot-hybridization of DNA fragments isolated from DNase II-treated nuclei and digested with Hind III showed that after this procedure the difference in sensitivity of the both genes was drastically decreased (Fig. 5B). Nevertheless of β-globin gene was found to be preferentially digested even in 0.7 mM $MgCl_2$. However, the slight enhanced sensitivity of β-globin chromatin to DNase II at 0.7 mM $MgCl_2$ may be due to its larger target size (6 Kb versus 3.2 Kb, see Fig. 2). Thus, at 0.7 mM $MgCl_2$ β-globin and ovalbumin gene chromatin may have the same accessibilities to DNase II

attack. Results similar to those shown in Fig. 5B for DNase II have been obtained with DNase I (results not presented).

To determine whether it is possible to digest at equivalent rates both analysed genes, erythrocyte nuclei were incubated in a buffer of very low ionic strength (10 mM Tris-HCl, pH 7.0) in the absence of any added mono- and divalent metal ions. Under these ionic conditions, when chromatin is very decondensed, DNase II randomly digests loosely unfolded linker DNA giving rise to a nucleosomal periodicity (Fig. 1). As a result, overall digestibility of chromatin at such low ionic strength was found to be 2-fold higher than in the presence of 0.7 mM $MgCl_2$ (data not shown). Under these low ionic strength conditions, the digestibility of ß-globin and ovalbumin genes was nearly identical (Fig. 5C).

We can conclude that chromatin of potentially active (ß-globin) and inactive (ovalbumin) genes becomes indistinguishable upon chromatin decondensation according to a criterion of digestibility. Therefore, the main difference between active and inactive chromatin conformation can be predominantly determined at a level of higher order structure. One may assume that potentially active genes in erythrocyte nuclei are bound with the same amount of histones (including H1, H5) as inactive ones. This conclusion follows from a double-nucleosome fragmentation pattern of the ß-globin gene by DNase I in the presence of 3 mM $MgCl_2$, i.e. from the pattern which is characteristic of the bulk condensed chromatin.

Weintraub investigated the electrophoretic properties of oligonucleosomal fragments and interpreted his data to mean that the active genes of erythrocyte chromatin have an altered structure, although being bound with the same amount of lysine rich histones (3). Particularly, in potentially active regions can take place uncomplete replacement of H1 histones for erythrocyte-specific H5 fraction, which is more effective in chromatin compaction (5, 20). Indirect evidence in favor of this point was recently received upon analysis of the H1/H5 ratio in chromatin fractions solubilized in EDTA solution after very mild nuclease digestion (20, 21).

Although the ß-globin gene chromatin is cleaved genera-

ting a dinucleosomal periodicity as a whole, the occurence of a 100 b.p.-periodicity (Fig. 3) surely indicates a partial unfolding of its structure caused by some irregularities of nucleosome packing. One of the reasons for this irregularity could be accounted for by the existence of the hypersensitive sites at 5'- and 3' ends of the ß-globin gene (Fig. 2 and (4, 22, 23)). Caplan et al. (24) were able to come to a similar conclusion by analyzing the sedimentation properties of erythrocyte oligonucleosomes obtained with a restriction nuclease. The size of the hypersensitive regions flanking the ß-globin gene is equal to about 200 b.p. Such nucleosome-free DNA areas can not be included in a regular solenoid, and the chromatin stretches located on a border of extended and condensed regions will be preferentially digested by DNase's generating a 100 b.p.-periodicity like the bulk chromatin relaxed in the presence of 0.7 mM $MgCl_2$ (Fig. 1). Certain contribution in maintenance of the active chromatin conformation of ß-globin gene may be due to torsional stress of DNA (27) as well as hyperacetylation and ubiquitination of histones in the chromatin regions enriched in ß-globin gene sequences (28).

Cleavage at the hypersensitive sites by itself can influence the kinetics of disapearance of the 6 Kb restriction fragment of the ß-globin domain (Fig. 4). The subfragments "b", "c" and "d" shown on Fig. 4A are generated owing to nuclease action at the hypersensitive sites. We tried to calculate the contribution of cleavage at the hypersensitive sites by estimating the ratio of the sum of the intensities of subfragments "b" and "c" and intensity of the original 6 Kb fragment "a" (Fig. 4). An accurate estimate made by means of a laser densitometry of the fragments showed that this ratio does not exceed of 15-20% even in control specimens (Fig. 4, lane 1). This means that intensities of the 6 Kb fragment in the lanes taken as a 100% control appears to be reduced by no less than 15-20% due to an endogenous nuclease activity. We rely that an endogenous nuclease cleavage at the hypersensitive sites slightly decreases the observed difference in di-

gestibility of the ß-globin and ovalbumin genes, and, therefore, this event should compensate, in some extent, their effect on the kinetics of the 6 Kb fragment elimination.

Our results do agree in outlines with the data of Smith et al. (25), where a somewhat different approach was taken. By the method of solution hybridization they showed that the kinetics of degradation of the active adult ß-globin and the inactive embryonic globin genes by DNase I in erythroleukemic mouse cells becomes almost identical at the low concentration of $MgCl_2$. It is unclear, however, whether the embryonic globin gene is completely inactive in mature erythroleukemic cells or may be it keeps a potentially active chromatin conformation. If this gene really retains a potentially active conformation, the difference in digestibility of the adult ß-globin and the embryonic globin genes (3 times) allows the discrimination between active and potentially active chromatin structures. Our results show that there is the same magnitude of difference in digestibility between the inactive ovalbumin and the potentially active globin genes.

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