In Vivo Binding of Trimethylpsoralen Detects DNA Structural Alterations Associated with Transcribing Regions in the Human \(\beta\)-Globin Cluster*

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In order to increase our knowledge about the mechanisms that regulate expression of human \(\beta\)-like globin genes, we have used a novel technique to analyze the chromatin structure in living cells. This approach allowed us to detect specific DNA regions in vivo where nucleosome folding or unconstrained DNA supercoiling in erythroid cells differs from that in non-erythroid cells. In this method, we use 4,5',8-trimethylpsoralen (TMP) as a probe capable of detecting altered chromatin conformations. Our results show that TMP binds to DNA with a higher affinity over the regions in the locus that are actively expressed, including the promoter and the transcribed region. This higher affinity detected when comparing erythroid cells with non-erythroid cells does not extend to other regions inside the \(\beta\)-globin cluster. Our data suggest that the observed effect is likely due to nucleosome displacement. Alternatively, it could result from localized DNA supercoiling, but not from widespread torsional stress across the entire \(\beta\)-like globin locus as hypothesized previously.

Human \(\beta\)-globin cluster is located in chromosome 11 and contains six genes expressed only in erythroid cells at different developmental stages. The pattern of expression of these genes during development is precisely regulated: \(\epsilon\)-gene is expressed during the early stages of embryo development, the \(\gamma\)-genes (\(\gamma^b\) and \(\gamma^a\)) are expressed during the fetal stages, and the product of the \(\beta\)-gene is the most prominent form in adults. A sequence located 5 to 15 kb upstream from the embryonic \(\epsilon\)-globin gene plays an essential role on both the tissue-specific and the developmental specific expression of the \(\beta\)-globin cluster. This sequence has been named locus control region (LCR) and contains four DNase I-hypersensitive sites: HS-1, HS-2, HS-3, and HS-4. Chromatin structure analysis on the human \(\beta\)-globin cluster has revealed that the entire locus is preferentially sensitive to DNase I in erythroid cells (1–4). However, natural deletions that eliminate the LCR elements, such as that occurring in Hispanics (\(\gamma^b\)\(\beta\))thalassemia, prevent tissue-specific changes in chromatin structure and render the \(\beta\)-globin locus resistant to DNase I (5). On the other hand, there are evidences indicating that some LCR elements are able to exert their effect only when integrated into the genome or at least when they are folded into chromatin (6–8). According to these results, there is reason to believe that the LCR elements may regulate \(\beta\)-globin locus expression through alterations on the chromatin structure within the locus. In the present work we use a novel technique to compare chromatin structure in vivo in the \(\beta\)-globin locus in K562 cells and HeLa cells. Our approach is based on the original technique described by Cook et al. (9), where 4,5',8-trimethylpsoralen (TMP) is used as a probe for altered chromatin conformations. Variations on nucleosome folding or unconstrained DNA supercoiling affect the rate of TMP binding to DNA. Our results show that TMP cross-links DNA more efficiently over the regions in the \(\beta\)-globin locus that are actively transcribed in K562 cells than over the same DNA fragments in the HeLa cells. The higher cross-linking rates detected in erythroid cells when compared with non-erythroid cells do not extend to other regions inside the \(\beta\)-globin cluster.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Both K562 and HeLa cells were grown and maintained under 95% air, 5% CO\(_2\) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units of penicillin per ml, 50 mg of streptomycin per ml (all from Life Technologies, Inc.), and Hepes (pH 6.9) (Sigma).

**Psoralen Photo-cross-linking—**Cells were grown to a density of 5–7 \(\times\) \(10^5\) cells/ml in suspension. They were quickly chilled on ice, and trimethylpsoralen was added to the medium to a final concentration of 0.9 \(\mu\)g/ml into the cold room. All subsequent steps were performed at 4 °C. Cells were incubated for 5 min prior to UV irradiation in order to allow TMP to diffuse. Cells in the Petri dish were exposed to 360 nm UV light at a dose of 0.9 kJ/m\(^2\)/min for varying lengths of time (0–8 min). Isolation of genomic DNA was carried out by standard procedures (10).

**Gel Electrophoresis and Southern Blotting—**1 \(\mu\)g of DNA were digested to completion with the restriction enzyme of interest, treated with phenol/chloroform, and precipitated. DNA samples were resuspended in 20 \(\mu\)l of sterile \(H_2O\) and irradiated again with 360 nm UV light at a dose of 13.5 kJ/m\(^2\). DNA was denatured with 0.1 volume of 1 M NaOH at 55 °C for 2 min, quickly neutralized by adding 0.2 volume of 1 M Tris-HCl, pH 4.0, and placed on ice. H\(_2O\) was added to a volume of 50 \(\mu\)l, and the samples were loaded in a 0.8% agarose gel using TAE as electrophoresis buffer. After running overnight, gels were incubated for 10 min in 0.25 M HCl and transferred under alkaline conditions to Hybond membrane (Amersham). Alkaline transfer was carried out in order to avoid renaturation of cross-linked fragments. Membranes were hybridized with gel-purified fragments labeled with \(^32\)P by random priming. After the washing process, membranes were exposed to a phosphor screen. Cross-linked and non-cross-linked fractions were quantified using the Fuji BAS 1000 system. Membranes were also exposed to Kodak X-omat AR film.

**Determination of the Cross-linking Rate—**The number of cross-links per fragment was estimated assuming a Poisson distribution. According to this, XL\(_f\) = \(-ln(1 - F_{DS})\). XL\(_f\) represents the number of cross-links per fragment, and \(F_{DS}\) results from dividing the peak of the
integrated area of the double-stranded band by the sum of the integrated area of the double-stranded band and single-stranded band.

Cells over the human $\alpha$-globin gene were prepared by the method described in Ref. 11. Quantitation of the level of expression of $\alpha$-globin was done by primer extension analysis (10) using two different oligonucleotide primers. Primer 1 (5'-CTCTCGTGAATAATGCCATGCGCTCTGACT-3') is complementary to the sense strand of the human $\alpha$-globin and $\beta$-globin genes. Primer 2 (5'-CACATGGTCTCGTGTTAGGTTGCTGCT-3') is complementary to the sense strand of the human $\gamma$-globin and $\delta$-globin genes. The expected extension products from primer 1 and primer 2 are 56 and 73 nucleotides long, respectively.

**RESULTS**

In this work we have mapped different regions in the $\beta$-globin locus using TMP as a probe. Mapping was carried out by quantitating the in vivo TMP-induced double-stranded DNA cross-linking frequency in the region analyzed. The quantification is based on the ratio between single-stranded DNA and double-stranded DNA obtained after a denaturation and renaturation process (see “Experimental Procedures”). In the present assays, living cells were treated with TMP at 4°C and subsequently irradiated with 360 nm UV light for various periods of time. Increasing cross-linking efficiencies were obtained as a function of the time of irradiation. This effect is shown in the Southern blot in Fig. 1. It was observed that after irradiation of the cells for increasing periods of time, the intensity of the double-stranded DNA band was augmented while that of the single-stranded DNA band diminished.

Fig. 1 shows the results obtained when we analyzed the $\delta$-globin gene region in the erythroid cell line K562 and in the non-erythroid cell line HeLa. When the cross-linking rates for the 2.6-kb EcoRI fragment that contains most of the $\alpha$-globin gene were represented as a function of the time of irradiation with UV light, the slope of the regression lines obtained for both cell lines varied significantly. Cross-linking efficiencies for K562 cells were higher than those obtained for HeLa cells as indicated by the slope values of the regression lines. According to this result, DNA in the $\alpha$-globin gene of K562 cells is more accessible to TMP than in the same region in HeLa cells.

K562 cells have normal globin genomic maps and synthesize $\gamma$- and $\beta$-globin chains. In contrast with adult erythropoietic cells, they don’t contain detectable levels of $\delta$ or $\beta$ transcripts (2, 12, 13). However, because variations in expression have been described, we have analyzed the transcriptional pattern in the actual K562 cells used for the cross-linking studies. The cells expressed $\gamma$- and $\delta$-globin transcripts as indicated by the presence of a 73-nucleotide-long primer extension product from primer 2 (Fig. 2). Neither $\delta$-globin gene nor $\beta$-globin gene expression was detected. Control experiments were performed to confirm that the absence of the expected 56-nucleotide-long primer extension product from primer 1 was due to a lack of expression of $\delta$- and $\beta$-globin genes in K562 cells. On the other hand, HeLa is a non-erythroid cell line, and, consequently, those cells don’t express any of the genes contained into the $\beta$-globin cluster. Comparison between the cross-linking rates in the same fragments in both cell lines allowed us to avoid any variations in TMP affinity to DNA due to differences in the DNA sequence.

In order to prove that the differences found between K562 and HeLa cell lines were not due to particular cell line properties, TMP affinity was analyzed over a 4.5-kb BamHI fragment containing the keratin 16 human gene using as a probe the 3' untranslated region of the keratin 16 cDNA (Fig. 3). The slopes obtained for K562 and HeLa cell lines were almost identical, indicating that TMP has the same affinity for the DNA in the human keratin 16 gene in both cell lines. Since neither K562 cells nor HeLa cells express keratin genes, we can therefore assume that the TMP cross-linking behavior is independent of cell line properties. Thus, it is more likely that the differences found in the $\beta$-globin cluster between K562 and HeLa cells reflect the existence of specific chromatins structures associated to the active region in the K562 cells.

Several other fragments from the genomic region containing $\alpha$- and $\delta$-globin genes were analyzed in order to define in more detail the specific domains where TMP binds more efficiently to DNA. The $\gamma$-globin gene is located 4 kb upstream from the $\alpha$-globin gene, and it is also actively transcribed in K562 cells. We measured the TMP cross-linking rate over the
The 2.6-kb BamHI fragment containing the 5′ end of the \(g\)-globin gene and its promoter. The slope values for both cell lines are shown in Fig. 4a. It could be observed that the slope obtained in K562 cells for this fragment was higher than that obtained in HeLa cells. The cross-linking rates found over \(g\)- and \(\alpha\)-globin genes indicated that in vivo TMP binding to DNA detects chromatin structure alterations associated to actively transcribed regions in the \(\beta\)-globin cluster.

Fig. 4b presents the results obtained when we analyzed the 2.9-kb Dral fragment containing the intergenic region between \(\gamma\)- and \(\alpha\)-genes. In this case, the slope values obtained for both cell lines did not display any difference, suggesting that chromatin alterations are restricted to the transcribing regions and do not extend to the adjacent regions. We also analyzed the 2.7-kb BamHI + BglII fragment containing 1 kb of the 3′-noncoding region next to \(\alpha\)-gene (Fig. 4e). Even though some variations in slope value between K562 cells and HeLa cells could be observed in this fragment, the difference in both values was smaller than that obtained for the \(\alpha\) 2.6-kb EcoRI fragment already described. This result can be explained by a dilution effect on the total cross-linking efficiency in K562 cells over the \(\alpha\) 2.7-kb BamHI + BglII DNA fragment as a consequence of the presence of a large 3′-nontranscribing region.

The 1.6-kb EcoRI fragment containing the promoter and a small portion of the \(\gamma\) coding region (Fig. 4c) showed the highest cross-linking rates in K562 cells. Taken together, this result and the small cross-linking rates observed in the 2.9-kb fragment containing the intergenic region (Fig. 4b) are consistent with our notion that the promoter region binds TMP very efficiently. The high cross-linking rates detected in the promoter region could be explained by nucleosome displacement over the promoter in K562 cells (14, 15), which would increase the effective DNA length able to bind TMP. This possibility is discussed below.

DNase I sensitivity experiments had previously shown that chromatin structure is altered over more than 100 kb in the \(\beta\)-globin locus (3, 4). In order to determine whether the overall change in chromatin structure does also affect TMP cross-linking efficiency, we extended the TMP cross-linking analysis to other regions inside the \(\beta\)-globin locus. The data obtained for the DNA fragments including the \(\delta\)-globin gene and the \(\beta\)-globin gene are shown in Fig. 4, f and g, respectively. Both genes are expressed neither in K562 cells nor in HeLa cells, but a 5′ DNase I hypersensitive site has been described for the \(\delta\)-globin gene in K562 cells (2). Our results indicated that the chromatin structure alterations already described in these regions in K562 cells do not increase TMP cross-linking efficiency. In fact, the slope values obtained for the fragment containing the \(\beta\)-globin gene were smaller in K562 cells than in HeLa cells. We next analyzed the DNase I hypersensitive site 2 in the LCR because of its well known property to act as a powerful enhancer in transfection experiments. The results are shown in Fig. 4h. K562 cells bound TMP at a significantly lower rate than HeLa cells over this region, which probably reflects a higher level of DNA protection in K562 cells as a consequence of protein binding over erythroid-specific elements present in this fragment. However, the possibility cannot be excluded that the enhancer and insulator effects associated to LCR activity alter chromatin structure or DNA supercoiling and, therefore, limit TMP binding.

**DISCUSSION**

Even though TMP binding to DNA depends largely on chromatin structure, the results obtained when using TMP as a probe to reveal structural alterations differ from the data obtained in DNase I sensitivity experiments. Our results demonstrate that most of the \(\beta\)-globin locus does not display higher cross-linking rates of the intercalating drug TMP in the erythroid cell line K562 than in the non-erythroid cell line HeLa. In fact, some regions such as the DNase I hypersensitive site 2 in the locus control region (LCR) do bind TMP less efficiently in K562 cells. These results indicate that in vivo TMP binding to DNA does not seem to be affected by high order chromatin structures.

DNA supercoiling is one of the main chromatin properties allowing TMP binding to DNA (16, 17). It had previously been hypothesized that the active state of the \(\beta\)-globin locus in erythroid cells could be associated to widespread supercoiling across the entire locus. Our findings, in contrast, showing higher TMP cross-linking rates in K562 cells than in HeLa cells only in the regions that are actively transcribed do not favor the notion of a large supercoiled domain associated to the \(\beta\)-globin locus in erythroid cells.

Nucleosome folding is also able to affect TMP binding to DNA (18, 19). Several DNase I hypersensitive sites have been mapped over the flanking regions of the \(\beta\)-globin genes (2). Those hypersensitive sites are probably associated with nucleosome displacement, as it has been shown for the \(\gamma\)-globin genes (14, 15). However, the DNase I hypersensitive site located 5′ to the \(\delta\)-globin gene does not seem to increase significantly TMP cross-linking rates into the EcoRI 2.3-kb fragment (Fig. 4f), indicating that displacement of only one nucleosome is not sufficient to cause a detectable increase in the TMP binding rate over the fragments analyzed. We therefore conclude that the significant increase in DNA cross-linking rates observed in both \(\gamma\)-globin genes is not only due to nucleosome displacement on the promoter but it does more probably reflect the existence of some other major structural changes associated with transcription. It must be pointed out that changes are strictly
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