Gene-specific Differences in the Aflatoxin B₁ Adduction of Chicken Erythrocyte Chromatin

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ABSTRACT

Mature and immature chicken erythrocyte nuclei were treated with activated aflatoxin B₁ (2,3-dichloroaflatoxin B₁), producing covalently bound DNA adducts. This reaction produces alkali-labile sites in the DNA which can be identified by using a variation of the Maxam-Gilbert sequencing procedure. We determined the aflatoxin B₁ accessibility of defined regions of the erythroid genome by using different specific probes and monitoring the disappearance of similar-sized fragments generated by restriction enzyme digestion. The genes studied were the erythroid-specific β-globin and histone H5 genes, which are potentially active in mature erythroid nuclei and transcriptionally active in immature erythrocytes, and the vitellogenin and ovalbumin genes, which are both transcriptionally inactive in these cells. The β-globin and histone H5 genes were more accessible than the repressed vitellogenin and ovalbumin genes to aflatoxin B₁ modification in mature and immature erythroid chromatin. Micrococcal nuclease was used to probe the nucleosomoral organization of active (β-globin and histone H5) and repressed (vitellogenin and ovalbumin) genes in chicken erythrocytes. The vitellogenin and ovalbumin genes show a distinct nucleosomal repeat pattern in mature and immature chicken erythrocyte nuclei. In contrast, the β-globin and histone H5 genes lack a distinct nucleosomal repeat pattern in these cells. These results support the hypothesis that transcriptionally active genes are preferentially accessible to carcinogen modification because of their disrupted chromatin structure.

INTRODUCTION

AFB₁ is a mycotoxin produced by certain strains of the fungus Aspergillus flavus found as a naturally occurring food contaminant that has been implicated in the etiology of some human hepatic carcinomas (1-2). AFB₁ is one of the most potent procarcinogens known. Activation of AFB₁ is believed to proceed via epoxidation of the 2,3-double bond, forming the AFB₁-2,3-epoxide. AFB₁ can be activated in vitro by oxidation with a mild oxidant like chloroperoxybenzoic acid. The synthetic analogue, 2,3-dichloroaflatoxin B₁, is a good model for the reactivity, mutagenicity, and carcinogeticity of the 2,3-epoxide (3). The major initial DNA adduct formed by metabolically or activity, mutagenicity, and carcinogenicity of the 2,3-epoxide (3). The major initial DNA adduct formed by metabolically or chemically activated AFB₁ is 7-guanyl adduct, 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxyaflatoxin B₁ (4, 5).

The organization of chromatin in the nucleus is known to play an important role in the functional aspects of gene regulation. It is now clearly established that genes which are expressed have the potential to be expressed are in an altered chromatin conformation which is sensitive to DNase I digestion. For example, in the immature and mature chicken erythrocyte, the chromatin of the β-globin and histone H5 genes is sensitive to DNase I digestion, while the chromatin of transcriptionally repressed genes (ovalbumin and vitellogenin) is not (6, 9). Sensitivity to DNase I suggests an open or exposed chromatin structure which would be accessible to the transcription machinery. The repressed class of gene chromatin structures may be effectively invisible to the elements involved in transcriptional regulation.

Within the DNase I-sensitive domains are regions which are devoid of nucleosomes and hypersensitive to DNase I digestion. These DH sites are found at the 5' and/or 3' ends of genes and are the binding sites for sequence-specific DNA-binding proteins (7, 8). The chicken erythrocyte β-globin and histone H5 genes have DH sites located at the 5' and 3' ends of the gene (Fig. 1; Refs. 9-11).

DNA is thought to be the critical macromolecular target in chemical carcinogenesis. Since alterations in chromatin structure can determine the accessibility of specific DNA sequences to a variety of agents, it is conceivable that some chromatin structures are more vulnerable to attack by chemical carcinogens than are others (12, 13). Expressed genes or genes having the potential to be expressed could be more susceptible to aflatoxin B₁ adduction because of their open chromatin structure, leading to alterations in gene expression and/or to a greater extent of mutagenesis in the expressed gene loci (14). In this study, we determined whether 2,3-dichloroaflatoxin B₁ preferentially attacks the chromatin of single-copy, transcriptionally active genes in chicken erythroid nuclei. This system was chosen because DNA replication does not occur in the terminally differentiated erythrocyte (15). Since newly replicated chromatin has an "open" configuration (16, 17), it would presumably complicate the investigation of the relationship between transcribed genes and AFB₁ adduction. Previously, aflatoxin B₁ was shown to preferentially modify the multicycle, ribosomal gene sequence (13). In this report, we demonstrate that the chromatin of the β-globin and histone H5 genes is preferentially accessible to AFB₁ modification.

MATERIALS AND METHODS

Isolation and Digestion of Nuclei. Mature and immature chicken erythrocytes were obtained from adult White Leghorn chickens and anemic chickens (18), respectively, as previously described (19). Nuclei were isolated as described in Ref. 19 and were resuspended to a concentration of 50 A₂₆₀ units per ml in Buffer A (1 M hexylene glycol, 10 mM piperazine-N⁷,N⁷'-bis(2-ethanesulfonic acid), pH 7.0, 2 mM MgCl₂, 30 mM sodium butyrate, 1% (v/v) thiodiglycol, and 1 mM phenylmethylsulfonyl fluoride) to which was added CaCl₂ to 1 mM. To the nuclei suspension, micrococcal nuclease (Pharmacia) was added to 25 A₂₆₀ units per ml, and the nuclei were digested for 30 min at 37°C. The digestion was terminated by placing the suspension on ice and adding [ethylene bis(oxyethylenenitriilo)tetraacetic acid to 10 mM.

2,3-Dichloroaflatoxin B₁, Treatment of Erythroid Nuclei. AFB₁-Cl₂ was synthesized as previously described (3) except that the AFB₁-Cl₂ was chromatographed on a microPorasil column (Waters) with 2.5% acetone in methylene chloride. Mature or immature chicken erythrocyte nuclei were resuspended in RSB to a concentration of 4 A₂₆₀ units per ml (200 μg/ml). Various amounts of AFB₁-Cl₂ (0.01, 0.05, or 0.1 μg) were added to the nuclear suspension which was incubated at either 37 or 0°C for 5 min. The nuclei were collected by centrifugation and resuspended in RSB. This step was repeated.

DNA Preparation and Restriction Endonuclease Digestions. The nu-
Fig. 1. Restriction maps of the chicken β-globin and histone H5 genes. The open and closed boxes indicate the position of the introns and exons, respectively. The horizontal arrow shows the direction of transcription. The vertical arrows mark the DNase I hypersensitive sites. The DNA probes used in this study are shown.

RESULTS

Accessibilities in Chromatin of Various Genes to Activated Aflatoxin B1. Mature chicken erythrocyte nuclei were treated with different amounts of AFB1-Cl2 at 37°C for 5 min. The DNA was isolated at slightly acidic pH in order to stabilize the AFB1-N7-Gua lesion (26) and digested with restriction endonuclease(s), BamHI/HindIII or EcoRI. The AFB1-N7-Gua adduct is alkali labile, and a modification of the Maxam-Gilbert procedure (i.e., treatment with piperidine) for DNA sequence analysis leads to breakage of the DNA fragment at the site of the modified residue (20, 27). The single-stranded DNA fragments were electrophoretically separated on 1% alkaline agarose gels and transferred to nitrocellulose, and hybridized to the indicated 32P-labeled probes were done as described in Ref. 19. The cloned DNA probes used were: pCBG13, DNA sequence of the adult chicken β-globin gene which was acquired from H. Martinson (Ref. 22; see Fig. 1). pVTG412, obtained from H. Weintraub, recognizes the 5’ region of the chicken vitellogenin gene (23). pchV 2.5 B/H from A. Ruiz-Carrillo is a 2.5-kilobase pair fragment of the chicken histone H5 gene (Ref. 24; see Fig. 1). pOV12 is the chicken ovalbumin gene received from M.-J. Tsai (25).

clear pellet was resuspended in RSB (pH 6.8). DNA was prepared as described (19) except that the pronase digestion was for only 3.5 h at 37°C, and it was digested with the appropriate restriction endonuclease(s) (see Fig. 2). After restriction endonuclease digestions, DNA was heated at 90°C for 30 min in 1 M piperidine as described by Muench et al. (20), electrophoresed on 1% alkaline agarose gels (21), and transferred to nitrocellulose. Hybridizations to 32P-labeled probes were done as described in Ref. 19. The cloned DNA probes used were: pCBG13, DNA sequence of the adult chicken β-globin gene which was acquired from H. Martinson (Ref. 22; see Fig. 1). pVTG412, obtained from H. Weintraub, recognizes the 5’ region of the chicken vitellogenin gene (23). pchV 2.5 B/H from A. Ruiz-Carrillo is a 2.5-kilobase pair fragment of the chicken histone H5 gene (Ref. 24; see Fig. 1). pOV12 is the chicken ovalbumin gene received from M.-J. Tsai (25).

The experiments were performed similarly to those described above except that the nuclei were treated with AFB1-Cl2 at 0°C for 5 min. The DNA was restricted with BamHI and HindIII, and immature chicken erythrocyte chromatin was quantified. The data were analyzed by densitometric scanning of the autoradiograms of the DNA fragments. The ratio of the intensities of the DNA fragments at the site of the modified residue (20, 27). The single-stranded DNA fragments were electrophoretically separated on 1% alkaline agarose gels, transferred to nitrocellulose, and hybridized to the indicated 32P-labeled DNA sequence (Fig. 2). Note that only one guanine has to be modified to effect the elimination of the restriction fragment in question. The gene probes used in this study detected the following similar-sized restriction fragments: ovalbumin, 1.8- and 2.4-kilobase EcoRI fragments; vitellogenin, 1.8- and 1.9-kilobase BamHI-HindIII fragments; histone H5, a 2.5-kilobase BamHI-HindIII fragment; and β-globin, a 2.1-kilobase BamHI-HindIII fragment. Visual inspection of the autoradiograms suggests that the 2.1-kilobase β-globin and 2.5-kilobase histone H5 DNA fragments are more sensitive to AFB1-Cl2 attack than is the 2.4-kilobase ovalbumin DNA fragment (or vitellogenin DNA fragments, not shown). At a level of 0.01 µg/ml of AFB1-Cl2 the intensities of the 2.5-kilobase histone H5 and 2.1-kilobase β-globin restriction fragments is less than that of the respective fragments of untreated DNA. In contrast, the intensities of the 2.4- and 1.8-kilobase ovalbumin DNA fragments are similar in both the untreated and treated DNA samples. At higher levels of AFB1-Cl2 (0.1 µg/ml), there is a slight anodal shift in the DNA fragment pattern (DNA, Fig. 2), indicating that a greater amount of AFB1 modification had occurred. At this level of AFB1 modification, the majority of the gene fragments studied were modified, as indicated by the loss of the respective restriction fragments, including the 2.4-kilobase ovalbumin DNA fragment. These results demonstrate that the chromatin of these genes has different accessibilities to AFB1-Cl2, with potentially active gene chromatin being more accessible than repressed gene chromatin to AFB1 modification.

The level of aflatoxin B1 adduction of several genes in mature and immature chicken erythrocyte chromatin was quantified. The experiments were performed similarly to those described above except that the nuclei were treated with AFB1-Cl2 at 0°C for 5 min. The DNA was restricted with BamHI and HindIII, treated with piperidine, and electrophoretically resolved on a 1% alkaline agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with the indicated DNA sequence. The lanes of the autoradiogram were scanned, and the integrated peak intensities were determined for the restriction fragments of interest. The ratio of the intensities of the DNA fragments of AFB1-modified DNA to that of untreated DNA was calculated and plotted versus the amount of AFB1-Cl2 added. Fig. 3 shows that in both mature and immature erythroid nuclei, the 2.1-kilobase β-globin and 2.5-kilobase histone H5 DNA fragments are more readily modified than the 1.9-kilobase vitellogenin DNA fragment. The β-globin and histone H5 genes are more accessible to AFB1 modification in immature erythroid chromatin than in mature erythroid chromatin. Note that at a ratio of 0.10 µg AFB1-Cl2 to 200 µg of DNA, 27% of the histone H5 restriction fragment (31% for β-globin) remains in modified immature erythroid DNA while 52% of this fragment (62% for
which are potentially active in mature erythroid cells and active erythroid-specific \( \beta \)-globin and histone H5 DNA sequences, chicken erythroid genes. Mature and immature chicken erythroid nuclei were isolated from mature (M) or immature (I) chicken erythrocyte nuclei. Mature or immature chicken erythrocyte nuclei were digested with different amounts of AFB\(_1\)-Cl\(_2\) for 5 min at 0°C. The isolated DNA was digested with BamHI and HindIII, treated with piperidine, resolved on a 1% alkaline agarose gel, and hybridized to a \( ^{32P} \)P-labeled probe containing the DNA sequences of either \( \beta \)-globin (C), histone H5 (H), or vitellogenin (A). The lanes of the autoradiograms were scanned, and the integrated peak intensities were determined for each restriction fragment. Percentage of hybridization of the intensity of the restriction fragment from the AFB\(_1\)-Cl\(_2\)-treated nuclei versus the intensity of the restriction fragment from the untreated nuclei was calculated and plotted against the concentration of AFB\(_1\)-Cl\(_2\) used.

Fig. 4. Nucleosomal organization of specific genes in chicken erythroid nuclei. Nuclei isolated from mature (M) or immature (I) chicken erythrocyte nuclei were digested with micrococcal nuclease. The isolated DNA fragments were electrophoretically resolved on 1% agarose gels, transferred to nitrocellulose, and hybridized to the indicated \( ^{32P} \)P-labeled probe. The DNA fragments were visualized with ethidium bromide (DNA). The DNA sequence of the probes used were ovalbumin (OVAL), vitellogenin (VTG), histone H5 (H5), and \( \beta \)-globin (GLOBIN).

\( \beta \)-globin) is left in treated mature erythroid. This is a representative result of several experiments (5 for mature and 2 for immature cells).

Chromatin Structure of Transcriptionally Active and Inactive Chicken Erythroid Genes. Mature and immature chicken erythroid nuclei were digested with micrococcal nuclease, and the DNA fragments were electrophoretically resolved on a 1% agarose gel. Fig. 4 (DNA) shows a typical nucleosome repeat pattern of chicken erythrocyte chromatin. The DNA was transferred to nitrocellulose and hybridized to labeled DNA sequences of either transcriptionally active or inactive genes. The transcriptionally inactive ovalbumin and vitellogenin DNA sequences exhibit a canonical nucleosome array. However, the erythroid-specific \( \beta \)-globin and histone H5 DNA sequences, which are potentially active in mature erythroid cells and active in immature cells, show a nondiscrete continuum of DNA lengths in both cell types (Fig. 4).

DISCUSSION

In this report, we demonstrate that the transcriptionally active and potentially active, single-copy, \( \beta \)-globin and histone H5 genes have an altered chromatin structure which is preferentially accessible to AFB\(_1\)-Cl\(_2\). The nondiscrete nucleosomal pattern is thought to be a result of an altered nucleosomal and higher order chromatin structure. This alteration in chromatin structure may be due to several alterations in the chromatin of these genes, including elevated levels of the modified histone species (e.g., acetylated and ubiquitinated histone species) which modify nucleosome structure (19, 28, 29), DH sites which disrupt higher order structure (30), and torsional stress which may disrupt both nucleosomal and higher order structure (31).

The DH sites are regions of chromatin which are devoid of nucleosomes. Thus, these regions may be considered as long linker regions. Since AFB\(_1\) is located preferentially in the linker DNA regions (32), it is conceivable that the DH sites of the \( \beta \)-globin and histone H5 genes (see Fig. 1) would be preferentially modified. If such a reaction was occurring, we would expect to see the generation of bands shorter than the restriction fragment (see Refs. 9 and 10 for examples). However, such bands were not observed. This result suggests that the DH sites of the \( \beta \)-globin and histone H5 genes are not hypersensitive to AFB\(_1\)-Cl\(_2\) attack. DH sites have unpaired bases and react with the possible carcinogen, bromoacetalddehyde, which has a preference for unpaired DNA bases (8, 33). Since AFB\(_1\) reacts poorly with guanines that are in single-stranded DNA (34), it is not surprising that DH sites are not preferred targets of AFB\(_1\).

The transcriptionally active, \( \beta \)-globin and histone H5 genes, were preferentially accessible to AFB\(_1\)-Cl\(_2\) modification compared to repressed genes, ovalbumin and vitellogenin. Since both of the expressed genes were preferentially adducted, this argues against fortuitous sequence-specific attack of AFB\(_1\) (e.g., in a gene enriched for GGG sequences) being responsible for the greater sensitivity of these sequences to AFB\(_1\)-Cl\(_2\) modification (20, 34, 35). Rather, these results suggest that the greater sensitivity of the two active DNA sequences is due to their more open chromatin structure.

The chromatin of the \( \beta \)-globin and histone H5 genes was more accessible to AFB\(_1\)-Cl\(_2\) modification in immature compared to mature erythroid nuclei. This suggests that the transcriptionally active state of these genes is more vulnerable to AFB\(_1\)-Cl\(_2\) modification than the potentially active chromatin state. We have observed that the chromatin of the \( \beta \)-globin and histone H5 genes is in two states, one of which is soluble as polynucleosomes at physiological ionic strength and the other which has an insoluble character similar to that reported for the globin genes in murine erythroleukemia cells (36). In immature chicken erythroid nuclei, approximately 50% of the chromatin of the \( \beta \)-globin and histone H5 genes is in an insoluble form, while 25% of these erythroid-specific genes is in an insoluble state in mature cells. It remains to be determined whether these two chromatin states are equally accessible to AFB\(_1\) modification. It is noteworthy that the DNase I-sensitive, \( \beta \)-globin and histone H5 genes, which are both expressed at high rates, are located at the borders of condensed chromatin masses along interchromatin channels which communicate with the nuclear periphery (37). It is possible that the nuclear localization of these genes makes them accessible targets for chemical carcinogens.

The demonstration that chromatin state can influence gene target access to carcinogens has important implications in understanding mechanisms of carcinogenesis. For example, AFB\(_1\) is a well known hepatocarcinogen in the rainbow trout, but has never been observed to induce kidney tumors in over 20 years of tumor studies in our laboratory (38), even at doses

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above those which produce 100% liver cancer incidence. Despite this lack of response, overall genomic AFB1-DNA adduction in kidney is 7–21% that found in liver of juvenile trout (39), and appears to be even higher in AFB1-exposed trout embryos. A possible explanation is that the target protooncogene exists in a regional chromatin configuration which is protective in kidney, but accessible to AFB1, in liver. In this regard it is interesting that N-methyl-N'-nitro-N-nitrosoguanidine induces tumors in kidney as well as liver in trout (38), because such small alkylating agents may be less influenced by local chromatin structure. We are currently examining the accessibility of the trout Ki-ras protooncogene to N-methyl-N'-nitro-N-nitrosoguanidine and AFB1 in trout kidney and liver. Size differences in attacking electrophile may also underlie the recent observation that, at equivalent levels of overall genomic O6 methylation, N-nitrosomethylbenzylamine and N-nitrosodimethylamine do not initiate foci equally (40). Similarly, the cell cycle-dependent initiation of hepatocarcinogenesis in rats by methyl(2-acetoxymethyl)nitrosamine, which peaks during S-phase (41), may in part enhance target gene access as chromatin unfolds locally during replication. These hypotheses are readily testable. Although there are some circumstances under which overall genomic damage level can provide a useful estimate of tumor induction (42, 43), it is likely to be a misleading index when comparing risk among various organs, or for structurally related carcinogens.

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