Detection of an altered DNA conformation at specific sites in chromatin and supercoiled DNA

(bromoacetaldehyde/DNase I-hypersensitive site/chemical carcinogen/globin genes)

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ABSTRACT We show that bromoacetaldehyde, which reacts selectively at the N-1 and N-6 positions of unpaired adenine and at the N-3 and N-4 positions of unpaired cytosine residues reacts with chromosomal DNA in intact cells at probable regulatory sequences near active genes. A region of about 200 base pairs 5' to the chicken β^A -globin gene, which contains sites sensitive to nuclease S1, to several restriction endonucleases, and to very low levels of DNase I, also contains DNA structures that are preferentially sensitive to bromoacetaldehyde. These altered DNA structures are found at reproducible positions relative to the β^A -globin gene regardless of whether the bromoacetaldehyde is presented to intact erythrocytes, erythrocyte nuclei, or the β^A -globin gene itself carried in pBR322 as purified supercoiled DNA. The unpaired DNA 5' to the adult β^A -globin gene in adult erythrocytes is not detectable in embryonic erythrocytes that express embryonic β -globin in contrast to adult β^A -globin. Our results suggest that well-defined regions of DNA with effectively unpaired bases occur in intact nuclei and that these structures may be important for specific recognition because they are tissue specific and are found at putative regulatory regions.

Superhelical strain can induce certain DNA sequences to form a variety of altered DNA structures. Depending on the exact sequence, these structures may include unpaired DNA bases, hairpin or cruciform structures, and Z-DNA. These DNA structures have been detected by using a variety of physical and chemical techniques. These include chemical reactions with single-strand-specific reagents such as methylmercury hydroxide, formaldehyde, or carbodiimide derivatives; cleavage by specific conformation-dependent endonucleases such as nuclease S1, mung bean nuclease, or the *Neurospora crassa* nuclease; and analysis of hydrodynamic and spectroscopic behavior (1–7).

Recent observations have suggested that the types of altered DNA structures detected in supercoiled DNA may serve an important functional role in chromatin. For example, promoter and terminator sequences are associated with DNase I-hypersensitive chromosomal regions [100–400 base pairs (bp) long] that also contain nuclease S1-sensitive DNA sites (8); expression from the mating type cassette is associated with a change in superhelical density (K. Nasmythe and J. Abraham reported in ref. 9); transcription of DNA microinjected into frog oocytes requires a closed circular DNA template because linearized molecules are transcribed by RNA polymerase II at about 1/1,000th the level of closed DNA molecules (10); specific antibodies detect Z-DNA in polytene nuclei (11) and Z-DNA-binding proteins have recently been identified in *Drosophila* cells (12); finally, a direct relationship linking altered DNA structure in supercoiled plasmids to an altered structure in nu-

clei has been established by the observation that sequences sensitive to digestion by nuclease S1 in supercoiled pBR322 DNA are also sensitive to nuclease S1 in nuclei after this DNA is transfected into L cells (13).

Experiments using nuclease S1 are limited in interpretation, however, because it is not clear what type of altered DNA structure the enzyme is actually detecting. Here we ask whether or not unpaired DNA bases exist in chromatin by using a chemical reagent, bromoacetaldehyde (BrCH_2CHO), that reacts specifically with unpaired DNA bases at the N-1 and N-6 positions of adenine and at the N-3 and N-4 positions of cytosine (14). Previously, chloroacetaldehyde, which is a less-reactive analogue (15, 16), was used to study the interaction of DNA and the helix-destabilizing protein from calf thymus (17). BrCH_2CHO is small and extremely specific for unpaired bases. Also as we show here it is highly reactive under physiological conditions and is capable of entering and reacting with specific chromosomal sites in intact cells.

MATERIALS AND METHODS

Preparation of BrCH_2CHO . BrCH_2CHO was prepared according to the procedures used to prepare chloroacetaldehyde (18). Twenty-five grams of BrCH_2CHO dimethylacetal (Eastman Organic Chemicals) was refluxed for 45 min in 125 ml of 50% sulfuric acid (wt/vol). The mixture was then distilled, and the distillate was adjusted to pH 4.5 with 1 M NaOH and then redistilled. The final distillate (at a concentration of 1.6 M) was divided into aliquots and kept at -20°C . Great care should be taken in handling BrCH_2CHO because it is probably carcinogenic (14, 19, 20).

Reaction of BrCH_2CHO with Supercoiled Plasmid DNA or Chromatin. For supercoiled plasmid DNA, BrCH_2CHO was added at concentrations up to 100 mM in a buffer of 3 μg of DNA in 100 μl of 50 mM NaOAc (pH 4.5, pH 5, pH 5.5), 50 mM Na phosphate (pH 5.8, pH 6.5), or 50 mM Tris-HCl (pH 7.2). Incubation was for 1 hr at 37°C .

For the reaction of BrCH_2CHO with chromatin, either erythrocyte nuclei or intact erythrocytes were used. Cells were isolated from circulating blood of White Leghorn chicken embryos (either 5 day or 14 day) and washed twice with standard saline citrate buffer (0.15 M NaCl/0.015 M sodium citrate/10 mM Tris-HCl, pH 7.4). The cells were then suspended in 150 mM NaCl/1 mM EDTA, 30 mM NaOAc/2 mM ZnCl_2 , pH 5.0, at a DNA concentration of 1 mg/ml for reaction with BrCH_2CHO . Alternatively, nuclei were prepared by Vortex mixing the cell suspension vigorously in reticulocyte standard (RS) buffer (10 mM Tris-HCl/10 mM NaCl/3 mM MgCl_2 , pH 7.4)/0.5% Nonidet P-40. The nuclei were pelleted and suspended in 30 mM NaOAc/30 mM NaCl/1 mM EDTA/2 mM ZnCl_2 ,

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Abbreviations: bp, base pair(s); kb, kilobase(s).

pH 5.0, at a DNA concentration of 1 mg/ml for subsequent reaction with BrCH₂CHO. BrCH₂CHO was then added to cell or nuclear suspensions to the concentrations indicated in the figure legends. The reaction was allowed to proceed at 37°C for 15 min. When RS buffer was initially used for the BrCH₂CHO reaction with nuclei or whole cells, endogenous nuclease activity was often found to interfere with the results, especially for erythrocyte nuclei from 5-day embryos. This problem was circumvented by decreasing the pH and replacing the Mg²⁺ ions by Zn²⁺ ions for the BrCH₂CHO reaction with chromatin.

Purification of DNA. The BrCH₂CHO-modified supercoiled plasmid DNA was purified by precipitation with ethanol. The DNA was suspended in 10 mM Tris·HCl, pH 7.8/1 mM EDTA.

To isolate DNA from BrCH₂CHO-treated chromatin, nuclei and cells were pelleted after treatment with BrCH₂CHO and the supernatant was removed. When cells were used as starting material, nuclei were first prepared as described above. Nuclei were incubated at 37°C in 0.5% NaDodSO₄/10 mM Tris·HCl, pH 7.0/10 mM EDTA containing protease K (200 µg/ml) for 6 hr at 37°C and then extracted twice with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1) before precipitating the DNA with ethanol. DNA pellets were dissolved in 10 mM Tris·HCl, pH 7.8/1 mM EDTA and stored at 4°C.

Digestion of DNA by Restriction Enzymes and Nuclease S1. For plasmid DNA, 0.1 µg was digested with 0.5 unit of restriction enzyme in a 30-µl volume according to the manufacturer's recommendation. After 1 hr at 37°C, the buffer was adjusted to 30 mM NaOAc/100 mM NaCl/2 mM ZnCl₂, pH 4.5 (low-salt condition), or to 300 mM NaOAc/100 mM NaCl/2 mM ZnCl₂, pH 4.5 (high-salt condition), and the BrCH₂CHO-modified sites were digested with nuclease S1 (0.5 unit/µg; Bethesda Research Laboratories) at 37°C for 30 min. The reaction was terminated by adjusting the pH to 7.0 with 2 M Tris·HCl (pH 8.0).

For DNA purified from chromatin, 10 µg of BrCH₂CHO-treated DNA was digested with an appropriate restriction enzyme. The conditions for subsequent nuclease S1 digestion were as described above for plasmid DNA. The reaction of particular plasmid or chromosomal DNA sequences with BrCH₂CHO was assayed by separating the digested DNA on agarose gels, transferring the DNA to nitrocellulose according to the method of Southern (21), and hybridizing the filters with the appropriate nick-translated DNA probes as described (8).

Chloroquine Gel Analysis. Superhelical pBR322 plasmid DNA was treated with BrCH₂CHO at various concentrations as indicated in the figure legends. The modified DNA was then precipitated with ethanol and lyophilized. These samples were analyzed on chloroquine gels according to the procedure of Lockshon and Morris (22). One microgram of DNA was suspended in 50 mM Tris phosphate/1 mM EDTA, pH 7.5, and loaded onto a 2.0% agarose (Seakem) gel in 50 mM Tris phosphate/1 mM EDTA, pH 7.5, containing chloroquine phosphate (Sigma) at 6 µg/ml. The gel was run at 8 V/cm for 20 hr and then soaked in 50 mM Tris phosphate/1 mM EDTA, pH 7.5, for 30 min to remove chloroquine, and the DNA in the gel was stained with ethidium bromide (1 µg/ml) for 30 min and photographed.

RESULTS

Reaction of BrCH₂CHO with Unpaired DNA Bases in Supercoiled DNA. BrCH₂CHO and its less-potent analogue chloroacetaldehyde react covalently at the N-1 and N-6 positions of adenine and at the N-3 and N-4 positions of cytosine when these residues are not base paired. Our assay for the reaction of

BrCH₂CHO with unpaired bases is as follows: supercoiled or linearized plasmid DNA or chromosomal DNA in either intact cells or nuclei is treated with BrCH₂CHO, the DNA is then purified and, if supercoiled to begin with, linearized with an appropriate restriction enzyme. As a consequence of the reaction with BrCH₂CHO, the modified residues can no longer base pair and, hence, are sensitive to digestion at the modified site by the single-strand-specific nuclease S1. Because S1 is used to digest only BrCH₂CHO-modified *linearized* DNA, our results should not be confused with the known sequence-specific cleavage of supercoiled DNA by nuclease S1. The specific cleavage sites generated as a consequence of treatment with *both* BrCH₂CHO and nuclease S1 can then be mapped by cutting the DNA with an appropriate restriction enzyme, separating the fragments on an agarose gel, transferring the fragment to nitrocellulose filters, and probing with appropriate labeled DNA probes.

For Supercoiled Plasmid DNA, BrCH₂CHO Reacts at Specific Sites That Are Also Sensitive to Nuclease S1. We have focused on a recombinant DNA plasmid (HR16) that contains 1.9 kilobases (kb) of 5' coding and regulatory sequences from the chicken β^A globin gene cloned into the *EcoRI/HindIII* site of pBR322. Using the assay described above, we found that linearized double-stranded plasmid DNA does not react with BrCH₂CHO under these conditions (Fig. 1, lanes 1 and 2); however, supercoiled DNA yields a number of discrete subfragments after sequential treatment with BrCH₂CHO, a restriction enzyme, and then nuclease S1 (lanes 5 and 6). The positions of these fragments correspond to the known cleavage sites when nuclease S1 is used to directly digest the supercoiled form of this plasmid. Several sites corresponding to the subbands at 2 and 3 kb map to S1 cutting sites in pBR322 (4). Also under these conditions, the site yielding the subband at 1.28 kb maps to the 5' side of the β^A-globin gene about 170 bp 5' to the mRNA cap site (8). Fig. 1 (lanes 7 and 8) also shows that BrCH₂CHO treatment by itself does not lead to DNA cleavage.

Chloroquine Gel Analysis of BrCH₂CHO-Modified Supercoiled pBR322 DNA. The reaction of BrCH₂CHO with supercoiled DNA can also be assayed directly by using an agarose gel containing chloroquine (22, 23), an intercalating dye that aids in resolving the highly supercoiled topoisomers. Supercoiled DNA, by virtue of its topology, usually migrates faster than linear or relaxed DNA in agarose gels. If the duplex is locally denatured, then the mobility of such a supercoiled molecule will decrease. The extent of denaturation can be estimated by the magnitude of the decrease because, to a first approximation, for every 10.4 bp that are denatured, one topological turn of DNA is removed (24). Fig. 2 shows that with increasing reaction with BrCH₂CHO the mobility of the supercoiled pBR322 topoisomers decreases. There is a small and gradual shift at low BrCH₂CHO concentrations, indicating that most of the DNA has reacted with BrCH₂CHO. The magnitude of the band shift suggests that low levels of BrCH₂CHO result in the unpairing of 1–5 bp. At higher levels of BrCH₂CHO, the decrease in mobility of the topoisomers corresponds to 8–10 supercoils or about 100 bp. The major reaction site at both low and high levels of BrCH₂CHO is located 3.2 kb clockwise from the *HindIII* site (Fig. 2B). The same site is also detected as a 3.0-kb subband when probed anticlockwise from the *HindIII* site in HR16 (Fig. 1). This is also the major site for nuclease S1 cleavage (4). Since this site is the same at all levels of BrCH₂CHO and since the chloroquine gels suggest that at high BrCH₂CHO concentrations up to 100 bp are unpaired at this site, it is possible that, after an initial reaction with BrCH₂CHO at this specific site, subsequent interactions of BrCH₂CHO with adjacent DNA sequences may be facilitated. However, this conclusion cannot be

FIG. 1. Reaction of BrCH_2CHO with unpaired nucleotides in supercoiled plasmid DNA. (A) Recombinant pBR322 DNA harboring the adult β^A -globin gene (HR16) was treated as follows: Lane 1: the plasmid DNA was linearized with a single-cut restriction enzyme (*EcoRI*) and then digested at the position of the BrCH_2CHO -nucleotide adduct with nuclease S1 under high-salt conditions. Lane 2: the plasmid DNA was linearized with *EcoRI* and then treated with 100 mM BrCH_2CHO at 37°C for 1 hr in 50 mM NaOAc (pH 5.0). The DNA was purified and digested with nuclease S1. Lane 3: the supercoiled plasmid DNA was digested with nuclease S1. Lane 4: the DNA was treated with BrCH_2CHO as described above and then digested with nuclease S1. Lane 5: the DNA was digested first with *HindIII* and then with nuclease S1. Lane 6: the DNA was treated with BrCH_2CHO as described above, purified, and then digested first with *HindIII* and then with nuclease S1. Lane 7: no treatment was carried out. Lane 8: the DNA was treated with BrCH_2CHO . Markers in the extreme right and extreme left lanes are *HindIII*-cleaved λ phage DNA. Six nanograms of plasmid DNA was separated on a 1.5% agarose gel and blot hybridized to a *HindIII/Bgl* II probe as shown in the restriction map. (B) ∇ , Prominent sites that react with BrCH_2CHO ; ∇ and ∇ , sites of cleavage by *HindIII* and *EcoRI*, respectively; the probe is indicated by a leftward pointing arrow and a star. Material showing on the autoradiogram above 6 kb corresponds to relaxed circular plasmid DNA and contaminating dimers in our preparations. The chicken DNA insert carrying the β^A -globin gene covers the rightward 1.9 kb between the *HindIII* and *EcoRI* sites.

made with certainty since the cutting reaction with nuclease S1 is clearly not quantitative in our hands.

BrCH_2CHO Reacts with Several Sites in the Hypersensitive Region of the β^A -Globin Gene. It was previously shown (8) that the site-specific cleavage of pBR322 by nuclease S1 is sensitive to ionic conditions. A similar dependence on ionic conditions is also observed for the reaction of BrCH_2CHO with sites located at the 5' side of the β^A -globin gene in HR16 plasmid DNA (Fig. 3B). Under our standard reaction conditions, at pH 4.5, subbands at 1.28 and 1.20 kb are observed. [These are barely resolved in the overexposed autoradiogram shown in Fig. 3B (lane 1); however, they can be seen in the lighter exposure of Fig. 3C.] At pH 7.2, no subbands are observed in this region (lane 3). However, addition of 2 mM MgCl_2 to the reaction mixture at pH 7.2 leads to the production of a band (lane 4) at about 1.25 kb. (This band is reproducibly slightly smaller than the 1.28-kb band seen in lane 1.) Similarly, addition of Mg^{2+} to the reaction mixture at pH 4.5 results in the appearance of a 1.25-kb subband and a 1.10-kb subband (and a 0.4-

FIG. 2. Gel assay for the reaction of BrCH_2CHO with supercoiled DNA. (A) Supercoiled pBR322 DNA was treated with BrCH_2CHO at the concentrations (mM) indicated above the gel. The DNA was then purified and the individual topoisomers were separated on a 2% agarose gel containing chloroquine at $6.0 \mu\text{g}/\text{ml}$. (B) pBR322 DNA treated as in A with 1.6 mM, 3.2 mM, 5 mM, and 50 mM BrCH_2CHO was purified, cleaved with *HindIII*, and digested with nuclease S1 at 0, 1, 2, or 5 unit(s)/ μg under low-salt conditions. The DNA was then separated on a 1.2% agarose gel and blot hybridized to a *BamHI/HindIII* end-labeled probe as indicated in the map below. ∇ , *HindIII*; ∇ , *BamHI*.

kb subband). Thus, the reaction of BrCH_2CHO with the 200-bp region 5' to the chicken β^A -globin gene seems to define a cluster of sites that can form an altered DNA structure depending on the exact ionic conditions of the reaction mixture. The 1.10-kb subband observed at pH 4.5 in the presence of Mg^{2+} maps very near the major subband obtained from the reaction of BrCH_2CHO with chromatin (Fig. 3A). Similar flexibility (depending on solution conditions) in the reaction of BrCH_2CHO with the pBR322 sites (represented by the 3- and 2-kb clusters of subbands) is also observed.

BrCH_2CHO Detects an Altered DNA Structure at the β^A -Globin Gene Hypersensitive Sites in Chromatin. The reaction of BrCH_2CHO with sites associated with the β^A -globin gene in embryonic or adult erythrocyte chromatin was also investigated. The assay used was basically the same as that used for supercoiled plasmid DNA. Because BrCH_2CHO is a small molecule, we also tested whether the reaction might proceed in intact cells as well as in isolated nuclei. Our results show that BrCH_2CHO reacts with a specific site near the 5' side of the β^A -globin gene in adult (14-day) erythrocytes or erythrocyte nuclei but does not react with this site in embryonic (5-day) erythrocytes where this gene is not expressed (Fig. 3A). The yield of subfragment obtained with BrCH_2CHO is roughly the same as that obtained using DNase I or nuclease S1 and the con-

FIG. 3. Reaction of BrCH₂CHO with chicken β^A -globin gene-hypersensitive sites in chromatin and in recombinant supercoiled plasmid DNA. (A) Five-day (5d, the embryonic lineage) or 14-day (14d, the adult lineage) intact erythrocytes (as well as nuclei from 14-day erythrocytes) were isolated and allowed to react at 37°C for 15 min with BrCH₂CHO at 0, 50, 100, and 200 mM as indicated. DNA was purified, digested with HindIII, and treated with nuclease S1. The resulting DNA was separated on a 1.5% agarose gel and blot hybridized to the probe indicated by the arrow and star in Fig. 1. For 5-day cells, the far right lane represents a control (neither BrCH₂CHO nor nuclease S1). Weak subbands for BrCH₂CHO-modified DNA at about 2 kb and 4 kb are also seen. These correspond to the 5'- and 3'-hypersensitive regions seen in the adjacent β^H -globin gene (10). (B) Supercoiled plasmid HR16 DNA was incubated with 100 mM BrCH₂CHO for 1 hr at 37°C in the presence or absence of 2 mM MgCl₂ at pH 4.5 (50 mM NaOAc) or pH 7.2 (50 mM Tris-HCl). After the reaction, DNA was purified and digested first with HindIII and then with nuclease S1 at 0.5 unit/ μ g under low-salt condition. DNA was separated on a 1.2% agarose gel and blot hybridized to the HindIII/Bgl II probe shown in Fig. 1. Markers in the extreme right lane are HindIII-cleaved λ phage DNA and Hae III-cleaved ϕ X174 fragments. The cluster of sites represented by subbands between 1.28 and 1.10 kb map to the 5' side of the β^A -globin gene; the cluster of sites represented by the 3- and 2-kb fragments map in pBR322. The 0.4-kb subband is also seen by nuclease S1 in supercoiled plasmids and in chromatin (10). (C) A lighter exposure of the 1.10-1.35 kb region of Fig. B. (D) Summary of sites of BrCH₂CHO reaction for chromatin and plasmid DNA. ∇ , HindIII cleavage site.

centration of BrCH₂CHO used is approximately that used for reaction with supercoiled DNA or denatured DNA. The major site for the reaction of BrCH₂CHO in nuclei or intact cells yields a subband of about 1.08 kb. This corresponds to a site at a position about -10 bp from the mRNA cap site. A minor site that

yields a subband of about 1.25 kb corresponds to a position at about -170 bp from the mRNA cap site. The same sites (± 20 bp) are also cut by nuclease S1 in nuclei (8). A summary of the BrCH₂CHO-reactive sites in cells and in supercoiled plasmid DNA (in the various buffer conditions) is given in Fig. 3. The

prominent chromosomal site -10 bp from the mRNA cap site is very sensitive to BrCH_2CHO in supercoiled plasmid DNA in the presence of 2 mM Mg^{2+} at pH 4.5 (Fig. 3B). The weak chromosomal site at -170 bp from the mRNA cap site is the most prominent site in supercoiled DNA in the presence of Mg^{2+} at pH 7.2. Weak subbands at approximately 200-bp intervals are also observed in chromatin (and also in supercoiled DNA). These are also seen after digestion of nuclei with the *Neurospora crassa* single-strand-specific nuclease enzyme (data not shown). The reactivity of BrCH_2CHO with other genes has also been studied in intact cells. BrCH_2CHO also reacted at the 5' side of the embryonic β^0 -globin gene in embryonic erythrocytes but not with this gene in adult erythrocytes. Although the signal is weak, the chicken thymidine kinase gene also reacts with BrCH_2CHO at the known DNase I-hypersensitive site 5' to the mRNA cap site and prominent sites associated with the chicken α -globin locus are also observed with BrCH_2CHO (data not shown).

DISCUSSION

Our results show that BrCH_2CHO is a useful reagent for monitoring unpaired DNA bases in chromatin and in supercoiled recombinant plasmid DNA. Whether the probe is detecting stably unpaired bases or base pairs that undergo a rapid rate of breathing or flipping-out (in supercoiled plasmid DNA and in chromatin) is not known. The methodology developed here should be useful for monitoring DNA unpairing resulting from the specific interaction of proteins, ligands, and possibly RNA with specific DNA sequences.

We have shown that this chemical, when incubated with whole erythrocytes, reacts with chromatin in the hypersensitive region 5' to the active chicken adult β^A -globin gene. The reaction is tissue-specific since it is not observed in embryonic erythrocytes that do not express this gene. The results with BrCH_2CHO therefore confirm the previous observations with nuclease S1 treatment of nuclei and extend these results in suggesting that the altered DNA conformation detected by S1 also involves unpaired DNA bases.

The reaction of BrCH_2CHO with unpaired bases was also tested in supercoiled plasmid DNA harboring 5' sequences coding for the adult chicken β^A -globin gene. Because the reaction of plasmid-derived DNA with BrCH_2CHO requires the DNA to be supercoiled, we assume that DNA strain induces these sequences to adopt an altered DNA conformation. Taking advantage of the fact that the BrCH_2CHO reaction can be carried out at various conditions of pH, NaCl concentration, and divalent cation, we found that some DNA sequences that are reactive to BrCH_2CHO under one condition become less reactive at other conditions. This is true for purified supercoiled plasmid DNAs but not for chromatin, in which the sites for BrCH_2CHO addition are relatively independent of pH (between 4.5 and 8.0), NaCl concentration (0.01–0.1 M), and MgCl_2 concentration (1–5 mM) (data not shown). Thus, while the DNA structure containing unpaired DNA bases in chromatin seems to be well fixed, possibly by specific protein DNA interactions, in plasmid DNA there is considerable flexibility as to which particular DNA sequences in the plasmid DNA react under the various chemical conditions used. Strikingly, most of the flex-

ibility observed in the β^A -globin gene plasmid is focused to a region of 200 bp 5' to the cap site (Fig. 3B). This region is known to contain a number of nuclease S1-sensitive sites (13) and is defined by a marked sensitivity to a number of nucleases in chromatin (8, 25).

The BrCH_2CHO analogue chloroacetaldehyde is a known carcinogen (19) and mutagen (20). Although not tested to our knowledge, it is likely that BrCH_2CHO is also carcinogenic. Most known chemical carcinogens react preferentially with unpaired DNA bases (26). Thus, it is possible that putative regulatory signals associated with chromosomal hypersensitive sites located 5' and 3' to actively expressed genes are prime targets for chemical carcinogens.

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