Nucleosome Positioning in the Human c-FOS Promoter Analyzed by in Vivo Footprinting with Psoralen and Ionizing Radiation†

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ABSTRACT: We performed detailed footprinting analysis of nucleosome positioning in the c-FOS promoter of living human fibroblasts. The translational position was determined by terminal transferase-dependent PCR with 4,5′,8-trimethylpsoralen. The rotational position was determined by ligation-mediated PCR with ionizing radiation. In the middle of the c-FOS promoter, a nucleosome was positioned not only translationally but also rotationally. The comparison of the results of our in vivo footprinting with those of a previous report on the in vitro footprinting of reconstituted nucleosomes revealed that the major in vivo translational position was approximately 70 bp upstream of the in vitro position, whereas the rotational position was unchanged. The in vivo translational position appears to be strongly influenced by the presence of transcription factors, which may function as boundaries, while the rotational position appears to be determined predominantly by the DNA sequence. We also investigated the influence of the transcriptional activation of the c-FOS gene on the positioning of this nucleosome. Although it is well-known that there are rapid changes in general nuclease sensitivity and chemical modifications of histone in the c-FOS gene upon activation, we could not detect any change in the translational or rotational position of this nucleosome. The nucleoprotein complex in the c-FOS promoter containing the positioned nucleosome and several transcription factors seems to be structurally unaltered upon activation, despite the rapid chemical modifications of the nucleosome and some of the transcription factors.

For the description of the spatial arrangement of DNA and histones in a nucleosome, two parameters are used: The translational position refers to which region of the linear DNA sequence is associated with histones; the rotational position refers to which face of the DNA helix is in contact with histones. Nucleosomes occupy highly preferred translational positions or assume highly preferred rotational positions in some regions of the genome, especially in the vicinity of regulatory sequences. There is growing evidence that this phenomenon (nucleosome positioning) has biological significance (*1*−*3*). In vitro studies have revealed that the access of transcription factors to their recognition sequences in the nucleosomal structure may be blocked when the recognition sequences are incorporated into the core particles rather than into the linkers or when the sequences are oriented toward the histones rather than toward the surrounding solution. However, little information is available regarding nucleosome positioning in vivo. So far, only a limited number of genes have been examined for the positioning of nucleosomes in living cells, especially for the rotational positioning (*4*), possibly because of technical limitations.

For the mapping of positioned nucleosomes in living cells, two nucleases are typically used: micrococcal nuclease (MNase),[1] which cuts DNA preferentially in the linker regions and is suitable for the analysis of translational positioning, and DNase I, which attacks DNA preferentially where it is furthest from the histone surface and is suitable for the analysis of rotational positioning (*2*, *3*, *5*, *6*). The resultant cleavages are mapped by indirect end labeling in low-resolution analysis. In high-resolution analysis, they are detected by a genomic sequencing technique such as ligation-mediated PCR [LM-PCR (*7*)]. In these procedures, isolation of nuclei or permeabilization of cell membranes before nuclease treatment is necessary because the enzymes do not pass through cell membranes. However, cases have been reported in which the structures present in the nuclei of intact living cells were disrupted by these procedures (*8*, *9*). This may result from the high temperatures required for permeabilization and nuclease treatment, which may have unfavorable effects on labile cellular structures, or from the composition of the buffers, which may detach proteins from DNA. The use of DNA-modifying agents that permeate intact cell membranes and are able to modify DNA at 0 °C should avoid these potential problems and enable visualization of minimally disturbed chromatin structure in vivo. 4,5′,8-Trimethylpsoralen is a DNA cross-linking agent that satisfies these criteria and has been widely used as an in vivo probe of DNA structure (*10*−*12*). Psoralen has also been used for the analysis of nucleosome positioning in vivo (*13*). Previously, we developed a high-resolution in vivo footprinting

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[1] Abbreviations: AP-1, activator protein-1; CBP, CREB-binding protein; CRE, cAMP response element; CREB, CRE-binding protein; CREM, CRE modulator protein; DMS, dimethyl sulfate; DR, direct repeat; LM-PCR, ligation-mediated PCR; MNase, micrococcal nuclease; SIE, sis-inducible element; SRE, serum response element; STAT, signal transducer and activator of transcription; TCF, ternary complex factor; TD-PCR, terminal transferase-dependent PCR.

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method (*14*) by combining psoralen treatment with terminal transferase-dependent PCR (TD-PCR), which is a highly enhanced version of the polymerase stop assay we had devised (*7*, *15*). Here we describe the application of this footprinting method to the detailed analysis of the translational positions of nucleosomes. In addition, we introduce the use of ionizing radiation as an in vivo footprinting agent, in combination with LM-PCR, to determine the rotational positions of nucleosomes.

Using these methods, we analyzed the promoter region of the c-FOS gene of human fibroblasts. The ability of the c-FOS promoter sequence to direct nucleosome positioning in vitro has been demosntrated by Schild-Poulter et al. (*16*), who performed nuclease and hydroxy radical analysis of reconstituted nucleosomes. Furthermore, the presence of a translationally positioned nucleosome in the c-FOS promoter in vivo has been suggested by Herrera et al. (*17*), who performed low-resolution analysis of DNase I and MNase cleavage patterns in isolated nuclei by Southern blotting. The reported in vivo position was, however, different from the in vitro position. We tried to analyze the discrepancy using the high-resolution methods.

It is well-known that the transcription of the c-FOS gene is rapidly activated by various stimuli including treatment of serum-starved cells with growth factors or serum. In vivo dimethyl sulfate (DMS) footprinting analysis has revealed that several binding sites of transcription factors exist in two regions in the c-FOS promoter, the region around position −300 and the region just upstream of the transcription start site. It has been found that these binding sites are occupied before activation and that the situation does not change upon activation (*18*−*20*). In marked contrast with this poised transcription factor binding, rapid concurrent changes in general DNase I sensitivity, in histone configuration, and in phosphorylation and acetylation of histone H3 have been detected in the c-FOS gene (*21*−*24*). Therefore, we thought it would be of interest to determine whether there were changes in the translational or rotational positioning of nucleosomes in the c-FOS promoter in the process of the activation of the gene.

EXPERIMENTAL PROCEDURES

Cell Culture. The human diploid fibroblast-like cell line, TIG-3 (*25*), was provided by the Health Science Research Resources Bank (Osaka, Japan). The cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Near-confluent cultures in 100 mm dishes or in 25 cm^2 flasks were prepared by incubation for 3 days after the inoculation at a density of 3×10^4 cells/cm^2 and were used in the experiments for the comparison of various footprinting agents. Serum-starved cells were prepared by the subsequent incubation of the near-confluent cultures in the medium containing 0.5% serum for 24 h. For induction, the serum-starved cells were incubated in the medium containing 15% serum for 15 min. For superinduction, the serum-starved cells were incubated in the medium with 15% serum plus 100 μM anisomycin for 30 min.

Treatment with Dimethyl Sulfate (DMS). In vivo treatment of cells (*26*) and in vitro treatment of DNA (*7*), which had been isolated from untreated cells, with DMS were done essentially as described previously. Cells were treated with 0.2% DMS on ice for 10 min, were washed three times with ice-cold serum-free medium and once with ice-cold phosphate-buffered saline, and then were lysed. DNA (0.2 μg/μL) was treated in vitro with 0.05% DMS at 20 °C for 5 min.

Treatment with 4,5′,8-Trimethylpsoralen. Treatment with psoralen plus long-wavelength ultraviolet light was done essentially as described previously (*14*). Cells were treated with 2.5 μM psoralen and 30 kJ/m^2 ultraviolet light, and after the change of the psoralen-containing medium for replenishment of the destructed psoralen, this treatment was repeated. DNA (0.1 μg/μL) was treated in vitro with 2.5 μM psoralen and 30 kJ/m^2 ultraviolet light. The dose rate of ultraviolet light was 3.3 kJ/(m^2·min).

Irradiation with X-rays. X irradiation was carried out with an HF320 X-ray unit (Pantak, Branford, CT) at 230 kV and 18 mA with 0.3 mm Cu and 0.5 mm Al filtration at a dose rate of 6.2 Gy/min. The cells in the flasks, capped tightly and chilled in an ice−water bath, were irradiated with 600 Gy of X-rays. Then they were washed once with ice-cold phosphate-buffered saline and lysed. It is probably necessary to irradiate the cells at 0 °C because single-strand breaks are repaired very rapidly at higher temperatures (*27*). DNA (0.4 μg/μL) in 10 mM Tris (pH 8.0) and 1 mM EDTA was irradiated in vitro on ice with 200 Gy of X-rays.

Treatment with Nucleases. Treatment with DNase I was performed essentially as described previously (*7*). Cells were permeabilized with 0.05% lysolecithin at 23 °C for 2 min and then incubated in 10 units/mL DNase I (Roche Diagnostics, Mannheim, Germany) at 23 °C for 5 min. DNA (0.1 μg/μL) was digested in vitro with 3 units/mL DNase I in 40 mM Tris (pH 7.5), 10 mM NaCl, and 6 mM $MgCl_2$ in a volume of 300 μL at 23 °C for 5 min. The in vitro reaction was stopped by the addition of an equal volume of 10 mM Tris (pH 8.0), 20 mM EDTA, 500 mM NaCl, 1% SDS, 0.2 mg/mL proteinase K, and 133 μg/mL glycogen. Then the DNA was purified by phenol extraction, chloroform extraction, and 2-propanol precipitation. The treatment with micrococcal nuclease (MNase) was similar to that with DNase I described above. After permeabilization, cells were incubated in 15 units/mL MNase (Worthington, Lakewood, NJ) in 150 mM sucrose, 50 mM Tris (pH 7.5), 50 mM NaCl, and 2 mM $CaCl_2$. DNA was digested in vitro with 3 units/mL MNase in 40 mM Tris (pH 7.5), 10 mM NaCl, and 2 mM $CaCl_2$.

Analysis by Ligation-Mediated PCR (LM-PCR) and Terminal Transferase-Dependent PCR (TD-PCR). Cell lysis and DNA isolation were carried out as described previously (*26*). X-irradiated DNA (6 μg/sample) and DNase I-digested DNA (1 μg/sample) were directly subjected to LM-PCR, while DMS-treated DNA (1 μg/sample) was subjected to LM-PCR after piperidine cleavage. Psoralen-treated DNA (2 μg/sample) was subjected to TD-PCR following restriction digestion and alkaline reversal, whereas MNase-digested DNA (1 μg/sample) was directly subjected to TD-PCR. The analysis of MNase cleavages by TD-PCR gave much stronger signals than that by a modified LM-PCR procedure (*28*). However, care must be taken because TD-PCR detects single-strand breaks (*5*, *28*). LM-PCR and TD-PCR were performed as described previously (*14*) by the use of the expand long template PCR system (Roche) in the PCR step. The four primer sets used in this study (FOSA, FOSC, FOSD, and FOSE) have been described previously (*14*).

RESULTS

Using two DNA-modifying agents, 4,5′,8-trimethylpsoralen and ionizing radiation, we analyzed a putative translationally positioned nucleosome in the promoter region of the c-FOS gene of human fibroblasts and compared the resulting patterns of DNA modifications with those obtained by the use of the conventional nucleosome footprinting agents, MNase and DNase I. Figure 1 shows the in vivo footprinting analysis of the lower strand of the c-FOS promoter with various agents, and Figure 2 summarizes the results of the footprinting on both strands.

Psoralen has been widely used as an in vivo probe of DNA structure (*10*−*12*). The psoralen molecule can pass through an intact cell membrane and initially interacts with double-stranded DNA by intercalation. Upon absorption of long-wavelength ultraviolet light, its two photoreactive sites react with two pyrimidine residues on both strands, forming an interstrand cross-link (diadduct). It has been established that the nucleosomal structure suppresses cross-linking and that only linker regions are cross-linked (*29*, *30*). This characteristic has been used for the detection of translationally positioned nucleosomes (*13*). Previously, we developed a method for detecting psoralen cross-links along single-copy genes in the large genomes of higher eukaryotes with resolution at the nucleotide level (*14*). When purified genomic DNA was treated with psoralen in vitro and subjected to this method, intense bands were seen at various positions (Figure 1, lanes 5 and 6), clearly indicating the strong sequence specificity of psoralen cross-linking, which has been reported to follow the pattern 5′-TA > 5′-AT > 5′-TG > 5′-GT (*31*). The samples of cells treated in vivo produced a quite different pattern (lanes 7 and 8). Many bands in the middle of the lanes (the area from position −132 to position −214, indicated by the vertical line on the right of the panel) were suppressed almost completely in the in vivo lanes, as we have described in a previous report (*14*). This large protection area corresponds to the central part of the putative positioned nucleosome (from −90 to −250) proposed by Herrera et al. (*17*). We should point out that this psoralen footprint was somewhat obscured by the lack of the most preferred targets (5′-TA sequences) in the region of the putative positioned nucleosome. There was only one 5′-TA (at nucleotides −177 and −178) in this region, and the footprint discernment depended on weaker signals (even in the in vitro lanes) at less preferred targets (5′-AT, 5′-TG, and 5′-GT). In Figure 2, the large area where the signals of in vivo psoralen cross-linking were strongly suppressed (less than 20% of the in vitro signals) is indicated by the red horizontal line on each strand.

We also performed MNase footprinting of the putative nucleosome by using TD-PCR (Figure 1, lanes 9−12), since it is well-known that MNase preferentially cuts DNA in the linker region, and this enzyme has been used as the standard agent to map translationally positioned nucleosomes in vivo. From the pattern of the samples of DNA digested in vitro (lanes 9 and 10), we can infer that the sequence specificity of MNase cleavage resembles that of psoralen cross-linking. Indeed, it has been reported that 5′-TA and 5′-AT sequences are the most preferred targets for MNase cleavage (*32*). In the case of cells treated with MNase in vivo (lanes 11 and 12), a large area of almost complete protection was also seen

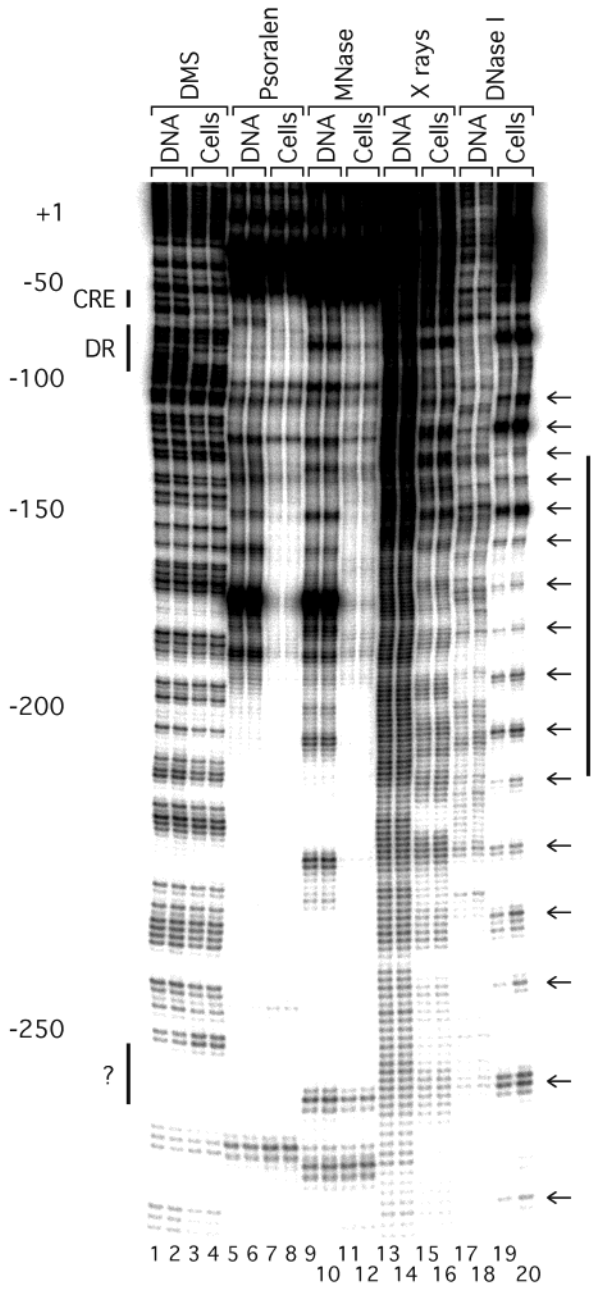

FIGURE 1: In vivo footprinting analysis of the lower strand of the human c-FOS promoter region. The primer set FOSD was used. The distribution of the methylguanines induced by dimethyl sulfate (DMS) and of the strand breaks induced by X-rays and DNase I was determined by LM-PCR. That of the cross-links induced by 4,5′,8-trimethylpsoralen and of the strand breaks induced by micrococcal nuclease (MNase) was determined by TD-PCR. Lanes labeled DNA denote the samples of DNA treated in vitro, and lanes labeled cells denote the samples of cells treated in vivo. The products of TD-PCR are usually two or three nucleotides longer than those of LM-PCR (*15*). The numbers on the left indicate the nucleotide positions of the LM-PCR signals relative to the major transcription start site. Short vertical lines on the left indicate the binding sites of transcription factors. For the full description of the abbreviated names of the sites, see the legend to Figure 2. The site with a question mark has not been characterized. Arrows on the right indicate the patterns of X-ray and DNase I cleavage in vivo with a periodicity of ∼10 nucleotides. The long vertical line on the right indicates the large area where the signals of in vivo psoralen cross-linking were strongly suppressed (less than 20% of the in vitro signals).

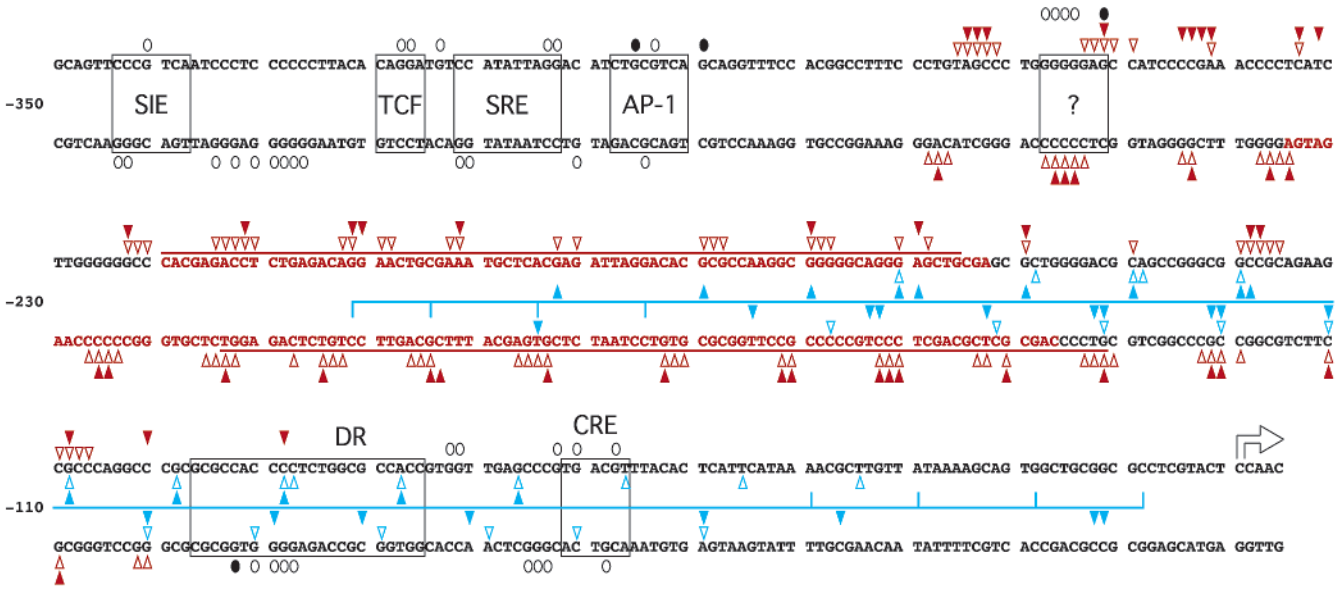

FIGURE 2: Summary of in vivo footprinting with five agents on both strands of the human c-FOS promoter and comparison with in vitro footprinting. The primer sets FOSA, FOSC, FOSD, and FOSE were used for in vivo transcription factor footprinting with DMS. The primer sets FOSD and FOSE were used for in vivo nucleosome footprinting with psoralen, MNase, X-rays, and DNase I. The arrow indicates the major transcription start site. The numbers on the left indicate the nucleotide positions relative to the transcription start site. Black symbols represent the results of in vivo DMS footprinting. Open and solid ovals indicate the sites of decrease and increase, respectively, of alkylation in vivo. Boxes indicate the recognition sequences of transcription factors; SIE stands for sis-inducible element; TCF, ternary complex factor; SRE, serum response element; AP-1, activator protein-1; DR, direct repeat; and CRE, cAMP response element. In the case of an uncharacterized site, the region with footprints is boxed and denoted by a question mark. Red symbols represent the results of in vivo nucleosome footprinting with psoralen, MNase, X-rays, and DNase I. Red horizontal lines indicate the large areas where the signals of in vivo psoralen cross-linking were strongly suppressed (less than 20% of the in vitro signals). The cross-links with different orientations were analyzed separately; only the cross-links whose pyrone sides were attached to the analyzed strand were detected (*14*). Red letters indicate the large areas in which the signals of in vivo MNase cleavage were strongly suppressed (less than 20% of the in vitro signals). Red open and solid triangles indicate the sites of enhanced cleavage in vivo with a periodicity of ~10 nucleotides by X-rays and by DNase I, respectively. One residue was lost at each site of cleavage by ionizing radiation (*38*), whereas the phosphodiester bonds immediately 3′ to the indicated bases were hydrolyzed by DNase I. Blue symbols represent the results of in vitro footprinting of reconstituted nucleosomes performed by Schild-Poulter et al. (*16*). Short blue vertical lines indicate the sites of exonuclease III pause in reconstitutes, and long blue horizontal lines indicate the translational position(s) inferred from the pause sites. Blue open and solid triangles indicate the sites of enhanced cleavage in reconstitutes with a periodicity of ~10 nucleotides by the hydroxyl radical and by DNase I, respectively.

(from position −137 to position −235). The similarity between the patterns of protection from MNase and from psoralen strongly suggested that these two agents detected the same structure. In Figure 2, the large area in which the signals of in vivo MNase cleavage were strongly suppressed (less than 20% of the in vitro signals) is represented by red letters on each strand.

Ionizing radiation generates hydroxyl radicals in biological systems as well as in solution, and the radicals attack DNA and produce various lesions including strand breaks (*33*). The hydroxyl radical generated from hydrogen peroxide has been widely used for the purpose of determining the rotational positions of nucleosomes reconstituted in vitro (*34*). It has been shown that ionizing radiation can also be used as an in vitro footprinting agent (*35*, *36*). The possibility of the in vivo application of ionizing radiation has been pointed out, based on its ability to penetrate cell membranes (*35*−*37*). We implemented this for the first time as shown in Figure 1, lanes 13−16. When purified DNA was irradiated with X-rays in vitro and the resulting strand breaks were detected by LM-PCR (lanes13 and 14), rather uniform band patterns were seen throughout the lanes (*38*, *39*). It should be noted that the LM-PCR procedure detects only strand breaks with 5′-phosphates among the various lesions produced by ionizing radiation (*7*, *33*, *38*). In vivo irradiation of living cells generated different cleavage patterns (lanes 15 and 16). In the region from −101 to −269, which includes the entire areas of almost complete protection from psoralen cross-linking and from MNase cleavage, we observed a pattern with a periodicity of about 10 nucleotides.

The frequencies of single-strand breaks in the X-irradiated samples that were used in this experiment were estimated by alkaline agarose gel electrophoresis [data not shown (*40*)]. In vivo irradiation with 600 Gy of X-rays (lanes 15 and 16) induced one single-strand break per ~15 kb (single-strand) DNA in the genome overall. In vitro irradiation with 200 Gy of X-rays (lanes 13 and 14) induced one single-strand break per ~4.5 kb. Thus, under our experimental conditions, the efficiency of the induction of single-strand breaks in vivo was only about 10% of that of the in vitro induction. Stepwise removal of proteins from nuclei and irradiation of these nuclei (*41*, *42*) have demonstrated that a large part of the protection of DNA from radiation in living cells can be ascribed to core histones. On the surface of the nucleosome core, the concentration of hydroxyl radicals should be lower, because the radicals are generated from water molecules mainly outside the core and are scavenged by histones. It is conceivable that, along the DNA backbone, the parts that are in contact with histones (not exposed to solution) are strongly protected from the radicals and that the 10 nucleotide periodicity of cleavage reflects the wrapping of DNA, which has a helical periodicity of ~10 nucleotides, around the core.

We also carried out DNase I footprinting of the same region (lanes 17−20), since it is well-known that DNase I preferentially cleaves nucleosomal DNA at the sites furthest from the histone octamer surface, and this enzyme has been used as the standard agent to determine the rotational positions in vivo. DNase I treatment of cells produced a clear pattern with a periodicity of about 10 nucleotides (lanes 19 and 20). The positions of in vivo cleavage by DNase I generally coincided with those of in vivo cleavage by ionizing radiation (lanes 15 and 16), suggesting that the same structural features were detected by these two agents. In Figure 2, the positions of periodically enhanced cleavage in vivo by ionizing radiation and those by DNase I are indicated by red open triangles and red solid triangles, respectively.

Although the four footprinting agents described above have the ability to detect nucleosomes, they also have the ability to detect transcription factors. Thus, there remained the possibility that the large footprints we detected were due to transcription factors rather than to a true nucleosome. To investigate this possibility, we carried out dimethyl sulfate (DMS) footprinting of the c-FOS promoter; alkylation of guanines was detected by piperidine cleavage followed by LM-PCR. DMS is widely used for the detection of transcription factor binding but is essentially insensitive to histone−DNA interactions (*43*). Although in vivo DMS footprints were clearly visible at the transcription factor binding sites [cAMP response element (CRE) and direct repeat (DR)] as reported previously (*18*−*20*), the region of the putative nucleosome was free of footprints on the lower strand (Figure 1, lanes 1−4). On the upper strand, however, we found a clear footprint at nucleotides −258 to −252 (see Figure 3, lanes 1−5; denoted by a question mark), which to our knowledge has not been reported previously. This unidentified binding site seems to be on the periphery of the putative nucleosome. In Figure 2, the sites of decreased and increased alkylation in vivo are indicated by black open ovals and black solid ovals, respectively.

We have described above a model essentially with a single translational position of the nucleosome in the c-FOS promoter. However, we should point out that another model can explain our data. The upstream boundary of the large psoralen footprint is quite clear, with strong signals at the 5′-TA and 5′-AT sequences between −266 and −232 (Figure 3, lanes 8−10), but the downstream boundary seems less clear, with weaker signals at the 5′-TG sequences between −130 and −105 (Figure 1, lanes 7 and 8). One reason may be the absence of preferred psoralen targets in the downstream boundary, but we cannot exclude the possibility that the footprint extends to a more downstream boundary with strong signals at nucleotides −49 to −42. In this case, there should exist multiple translational positions in the larger footprint. The situation may be similar to that of a family of multiple positions mapped in the promoter of the mouse mammary tumor virus (*44*). We suppose that the major position(s) may correspond approximately to the MNase-resistant region (−250 to −90) revealed by the low-resolution analysis (*17*), because it appears unlikely that the region from −130 to −105, where the psoralen signals were not strongly suppressed, is in the central parts of the core particle DNA of the majority of nucleosomes.

From the results of in vivo footprinting with the various agents described above, we concluded that the large footprints in the middle of the c-FOS promoter were due to a true positioned nucleosome or a family of positioned nucleosomes and that our psoralen and X-ray methods for high-resolution in vivo mapping of nucleosomes were validated.

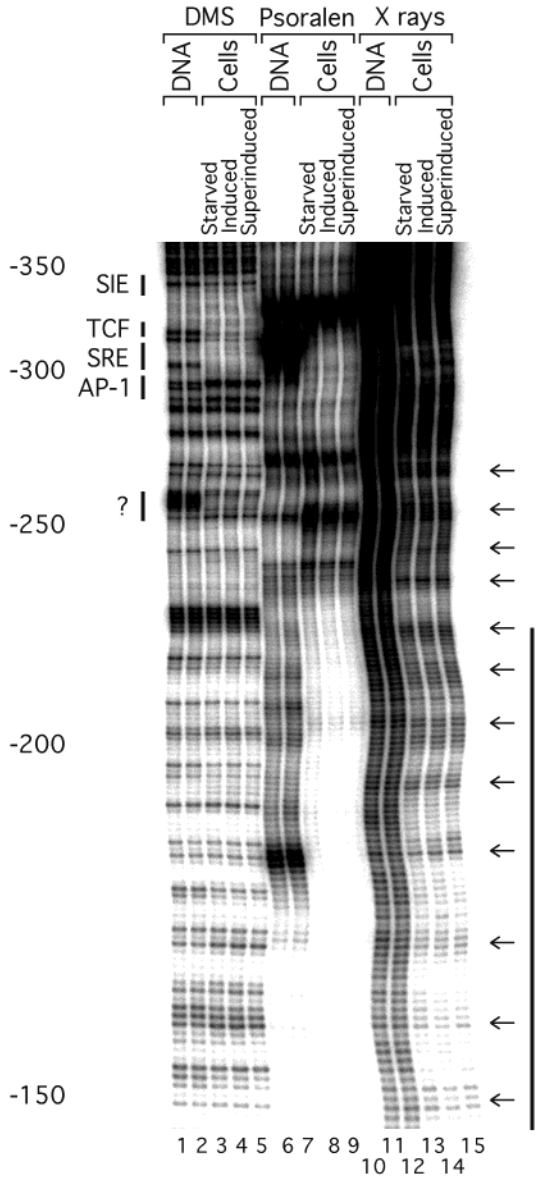

FIGURE 3: In vivo footprinting analysis of the upper strand of the human c-FOS promoter upon activation of the gene. The primer set FOSE was used. Lanes labeled starved denote the samples of cells cultured in the medium containing 0.5% serum for 24 h. Lanes labeled induced denote the samples of cells incubated in the medium containing 15% serum for 15 min after the starvation. Lanes labeled superinduced denote the samples of cells incubated in the medium with 15% serum plus 100 μM anisomycin for 30 min after the starvation. Other denotations are similar to those in Figure 1.

In Figure 2, the results of in vitro footprinting of reconstituted nucleosomes performed by Schild-Poulter et al. (*16*) are also represented by blue symbols for comparison. They assembled nucleosomes using the DNA fragment spanning from −404 to +42 under the conditions in which two nucleosomes were expected to form on the fragment, but they observed one positioned nucleosome only in the proximal region. Using shorter fragments, they determined the position of this nucleosome precisely; blue horizontal

lines represent the translational position(s) inferred from exonuclease III analysis, and blue open and solid triangles indicate the sites of cleavage by the hydroxyl radical and by DNase I, respectively. Comparison of their in vitro results with our in vivo results revealed that the in vitro translational position, which includes the CRE and the DR, was about 70 bp downstream of the major in vivo position. In the overlapping region, the sites of cleavage induced by DNase I and by the hydroxyl radical in reconstitutes coincided approximately with those of cleavage induced by DNase I and by ionizing radiation in living cells. Thus, the rotational position appeared to be unchanged, despite the discrepancy in the translational position.

Employing our psoralen and X-ray footprinting methods, we investigated the effects of the transcriptional activation of the c-FOS gene on the positioning of the nucleosome in the promoter (Figure 3). Serum-starved cells were treated with serum (induced) or treated with serum plus an inhibitor of protein synthesis, anisomycin (superinduced). Northern analysis revealed that the "induction" and "superinduction" treatments increased c-FOS mRNA more than 40 times and 70 times, respectively (data not shown). DMS footprinting (lanes 1−5) confirmed that the transcription factor binding sites in the c-FOS promoter are occupied before activation and that the situation does not change upon activation (*18*−*20*). Psoralen footprinting (lanes 6−10) showed that there was no change in the translational position of the nucleosome upon activation, which is consistent with the suggestion of Herrera et al. (*17*). X-ray footprinting (lanes 11−15) revealed that there was no alteration in the rotational position, either. Thus, the nucleosome appeared static both translationally and rotationally in the process of the activation of the c-FOS gene.

DISCUSSION

In this report, we have described our in vivo footprinting analysis of nucleosome positioning in the human c-FOS promoter. The rotational position was determined by the use of ionizing radiation. We applied this agent to in vivo footprinting for the first time. Since the suitability of ionizing radiation as a footprinting agent has not been examined extensively, we should point out some potential problems here.

In vivo footprinting with this agent required a very large dose (600 Gy), probably because of the strong protective effects in living cells (*41*, *42*). We cannot exclude the possible effects of the long time (97 min) required for the large-dose irradiation. This problem may be solved by the use of a high-dose-rate source such as a linear accelerator. We estimated that 600 Gy of X-rays induced one single-strand break per ∼15 kb DNA. It is possible that this incidence of strand breaks changes chromatin structure and transcription status greatly. Jupe et al. (*45*) have reported that similar levels of cleavage (one single-strand break per 15−20 kb) can remove most of unrestrained DNA supercoiling in domains in living cells. In addition, Luchnik et al. (*46*) have reported that irradiation of cells with 100 Gy of X-rays in the cold induces one single-strand break per ∼200 kb and gives a 63% decrease in transcription.

Ionizing radiation appears to produce less prominent footprints (or differences between the band patterns in the in vivo lanes and those in the in vitro lanes) than the other agents. This may be in part due to the modulation of cleavage in the in vitro lanes (Figure 1, lanes 13 and 14). It has been reported that the nucleosome positioning sequence in the *Xenopus* 5S ribosomal gene has an intrinsic curvature and shows a pattern of hydroxyl radical cleavage with a periodicity of ∼10 nucleotides resulting from a periodic modulation in minor groove width even when it is free in solution and that this feature is maintained and exaggerated when it is in the nucleosomal structure (*34*). Such a modulation in the in vitro lanes may make the X-ray footprints less conspicuous. In addition, the cutting of nucleosomal DNA by X-rays occurred at two to four nucleotide positions in each 10 nucleotide period (or helical turn), while the cutting by DNase I occurred at one or two positions in each period (Figure 1, lanes 15, 16, 19, and 20). This difference, which probably reflects the difference in the molecular size of the agents, may also make the X-ray footprints less conspicuous. This may be a serious problem when analyzing nucleosomes that are not strongly positioned or are in multiple positions in terms of rotational positioning.

Psoralen has been used for the determination of the translational positions of nucleosomes in living cells, and various methods for detecting psoralen cross-links have been developed (*10*−*13*). Our method has the advantage that it can detect the cross-links along single-copy genes in large genomes with resolution at the nucleotide level. However, the resolution of the psoralen footprinting of nucleosomes was, like that of the MNase footprinting, somewhat limited by the strong sequence specificity of the agent. Nevertheless, we believe that combining psoralen treatment with TD-PCR provides a powerful assay for the detection of positioned nucleosomes.

The major translational position of the nucleosome in the c-FOS promoter of living human cells determined in this study seems to be consistent with the results of Herrera et al. (*17*), who performed low-resolution in vivo analysis, but inconsistent with the results of Schild-Poulter et al. (*16*), who determined the nucleosome position after in vitro reconstitution. The in vivo position is approximately 70 bp upstream of the in vitro position, which includes the cAMP response element (CRE) at nucleotides −62 to −57 (Figure 2). Schild-Poulter et al. (*16*) reported that the CRE-binding protein (CREB) and the CRE modulator protein (CREM) cannot interact with their target sequence (CRE) that has been already incorporated into the nucleosome but suggested that the reconstitution in the presence of CREM dislocates the nuclesome to a new translational position immediately upstream of the CRE without changing the rotational position. In living cells, the nucleosome appears to exist mainly in the 154 bp space between two transcription factor binding sites, the direct repeat (DR) at nucleotides −97 to −76 and the unidentified site at nucleotides −258 to −252. This situation seems to support the notion that the in vivo translational positioning is governed by the boundaries (*1*−*3*). Despite the difference between the in vivo and in vitro translational positions, the rotational position seems to be maintained. This suggests that the in vivo rotational position (as well as the in vitro rotational position) in the c-FOS promoter is determined predominantly by the DNA sequence. Indeed, some nucleosome positioning sequences have been identified, and it has been noted that the influence of these

sequences is more evident and more direct on the rotational positioning than on the translational positioning (*2*, *3*, *47*, *48*).

In the current models of the chromatin structure of the c-FOS promoter (*17*, *49*), the proximity of the transcription factors bound to the DNA region just upstream of the transcription start site and those bound to the region around position −300 (see Figure 2) is ensured by two mechanisms: One is the wrapping of the intervening DNA around the positioned nucleosome (*1*, *50*). The other is the bridging structure formed by the interactions of a large multidomain coactivator, CREB-binding protein (CBP), with various factors bound to these regions (*49*, *51*), namely, the TATA-binding protein (TBP; there is a canonical TATA box at nucleotides −31 to −25), CREB, AP-1, the ternary complex factor (TCF), and the signal transducer and activator of transcription [STAT, bound to the sis-inducible element (SIE)]. Although many of the proteins have been reported to get phosphorylated by mitogen-activated protein kinases (MAPK) or other kinases upon activation of the gene (*52*, *53*), we confirmed that all of the protein−DNA interactions detected in this promoter by DMS footprinting are constitutive. On the other hand, the stability of the protein−protein interactions in this promoter in living cells has not been studied extensively. However, evidence has been presented for the phosphorylation-independent constitutive interactions between STAT and CBP and between TCF and CBP (*49*, *54*). In addition, continuous initiation of transcription and premature termination or pause of elongation have been suggested in the c-FOS gene of uninduced cells (*55*, *56*). Thus, it is possible that the stable structure of the complex consisting of the sequence-specific transcription factors, the coactivator, and some of the general transcription factors supports the continuous initiation and that not the initiation step but the elongation step controls the transcriptional activity of the gene.

Targeted chemical modifications of nucleosomes (phosphorylation and acetylation of histone H3) in the c-FOS gene have been reported to occur rapidly and coincidentally with the activation of the gene (*23*, *24*), and it seems highly probable that the nucleosome positioned in the c-FOS promoter is subjected to the chemical modifications. However, we could not find any change in the translational or rotational position of the nucleosome. We should point out that there is in fact no direct evidence that the positioned nucleosome in the c-FOS promoter is the target for phosphorylation and acetylation, as the resolution of the assay for the detection of chemical modifications (chromatin immunoprecipitation) is not high enough. Therefore, the formal possibility remains that the positioned nucleosome escapes chemical modifications. However, even without such an assumption, our results are consistent with the reports that the removal or the acetylation of histone tails does not change the positions of nucleosomes reconstituted in vitro (*57*−*60*). Thus, the changes in the positioning of the nucleosome in the c-FOS promoter appear unlikely to be involved in the activation of transcription, and the stable chromatin structure including the positioned nucleosome and the various factors might be able to support the initiation of transcription even under noninducing conditions.

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