

BBAEXP 92357

High-resolution analysis of *c-fos* chromatin accessibility using a novel DNase I-PCR assay

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(Received 14 October 1991)

Key words: Chromatin accessibility; *c-fos*; Oncogene; DNase sensitive region

In our previous study, *c-fos* chromatin accessibility was assayed using DNase I digestion and Southern blot analysis. This low-resolution mapping of *c-fos* chromatin accessibility demonstrated that serum stimulation of the *c-fos* enhancer induces a reversible increase in *c-fos* DNase I sensitivity and suggested that a 5' to 3' gradient of DNase I sensitivity may form downstream from the *c-fos* enhancer. To confirm the existence of a 5' to 3' gradient of accessibility, we have recently developed a high-resolution polymerase chain reaction (PCR) assay for DNase I sensitivity. Using this novel DNase I assay, we have reliably detected position- and time-dependent gradients of chromatin accessibility around the *c-fos* enhancer. These data confirm our earlier results and further support the hypothesis that the changes in *c-fos* chromatin accessibility originate near the 5' enhancer. As a technique for future examinations of gene structure, our data demonstrate the value of the DNase I-PCR assay for rapidly preparing comprehensive and high-resolution maps of chromatin accessibility for any sequenced genomic region.

Introduction

It is well established that active genes are more sensitive to DNase I digestion than inactive genes. Generalized or domain DNase I sensitivity can extend over large chromatin regions of 12 to 100 kilobases and includes nontranscribed spacer regions as well as active genes [1–4]. We have been interested in how enhanced DNase I accessibility is initiated and imposed over such large domains. If the enhanced accessibility is initiated at a regulatory site and propagates from this site, then a declining gradient of DNase I sensitivity might be observed under some circumstances owing to damping effects with distance from the origin.

Recently, we presented evidence consistent with the existence of a 5' to 3' gradient of DNase I sensitivity along the *c-fos* gene during basal transcription [5]. However, the resolution and reliability of the standard assays for DNase I sensitivity are poor, so that the existence of a gradient of DNase I sensitivity was

difficult to verify using current technology. The advent of PCR technology has made it possible to design a novel assay for domain DNase I sensitivity which has a higher resolution and improved reliability.

The DNase I-PCR assay uses standard DNase I digestion and then determines the degree of DNase I cleavage at a particular genetic location (350 to 390 bp in length) by PCR amplification of the region. A single cut within a probed sequence will prevent amplification of the sequence. Thus, coamplification of active and inactive regions should allow detection of the preferential sensitivity of the active domain in comparison to the inactive one. We have used this high-resolution PCR technique to refine our previous mapping of DNase I sensitivity along the *c-fos* gene during serum stimulation. In unstimulated cells and during the early stages of serum stimulation, the DNase I-PCR data confirm the existence of a gradient of chromatin accessibility that peaks near the 5' *c-fos* enhancer.

Materials and Methods

Recombinant DNA and PCR probes. The human *c-fos* gene, denoted *pc-fos*(human)-1 [6,7], was obtained from Inder Verma as a 9 kb *EcoRI* insert in pBR322. A plasmid containing the argininosuccinate synthetase (AS) coding region [8] was obtained from Svend Freytag. The AS probe cross-hybridizes with inactive AS pseudogene fragments and was used as a

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Abbreviations: PCR, polymerase chain reaction; AS, argininosuccinate synthetase; bp, base pair(s); kb, kilobase(s)

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probe for inactive genes in the blot analysis. The region upstream of the *c-fos* enhancer (-2 to 0 in Figs. 4 and 5) has not been sequenced, so that PCR primers from this region could not be prepared. To obtain DNase I sensitivity data on this upstream region, we have labeled a 1.4 kb upstream restriction fragment and probed Southern blots of DNase I digested DNA. PCR primers were prepared by the University of Michigan DNA sequencing core. All primers were 30 bp in length and were designed to probe the middle portion of the β -globin gene (460 bp long) and four *c-fos* regions (F1 to F4 spanning 350 to 390 bp). The *c-fos* and β -globin primer sequences were: F1A-5'TCTTACTACCACTCACCCGCAGACTCCTTC3'), F1B(5'CGAAGAAGCCAGGCTCTAGTTAGCGAG-TTT3'), F2A(5'GAATAAGATGGCTGCAGCCAA-TGCCGCAA3'), F2B(5'CAGTCAGATCAAGG-GAAGCCACAGACATCT3'), F3A(5'GGACA-CACCTCTGTCTGATCCCTTATCAA3'), F3B(5'CGAGCAGGAAGATTCTAATGCCGATAACG-G3'), F4A(5'TGAAGAAGGGCAGCTGGACCTT-CGGACATT3'), F4B(5'GGGATTTGGAAGGAG-CCATTTAGCTGGAGA3'), GlobinA(5'GGAGACC-AATAGAACTGGGCATGTGGAGA3'), GlobinB(5'CGATCCTGAGACTTCCACACTGATGCAATC-3').

Cell culture and serum induction. HeLa cells were grown in monolayer cultures in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F-12 medium (Sigma, No. 8900) supplemented with bovine calf serum (Hazelton laboratories). To prepare serum-starved cells, HeLa cells were grown to 70% confluency in the above medium with 10% serum, transferred to medium with 0.5% serum for 20 h, and finally transferred to medium with no serum for 30 min. To induce *c-fos*, medium with 15% serum was added and the cells were incubated at 37°C for various times before harvesting. The cells were washed, scraped from the plate and collected in ice-cold phosphate-buffered saline.

Isolation of nuclei and DNase I digestions. The cells were pelleted and resuspended in cold buffer A (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂) along with 0.4% Nonidet P-40 and 1 mM *p*-(chloromercuri)benzenesulfonic acid as described previously [9]. After 5 min, the resulting lysate was centrifuged at 2000 \times *g* for 5 min to pellet the nuclei. The nuclei were resuspended in buffer A at a concentration of 800 μ g/ml (assayed by dilution into 2 M NaCl and 5 M urea) and stored on ice briefly. Five 360 μ l aliquots of each sample were preincubated at 37°C for 5 min. The nuclei were digested for 3 min at 37°C with various concentrations of DNase I (Sigma, D-5025). Reactions were stopped by adding sodium dodecyl sulfate (SDS) and EDTA to final concentrations of 0.4% SDS and 20 mM EDTA.

DNA isolation, Southern blotting, hybridization and band integration. DNA was purified from the nuclei as previously described [10] and digested overnight at 37°C with 2 units of various restriction enzymes per μ g of DNA. The restricted DNA was subjected to either PCR or Southern blot analysis. Blot analysis was carried out by standard methods [11] as previously described [5]. The blots were first hybridized to labeled *c-fos* probe. Following autoradiography at -80°C for 5 to 10 days, the autoradiographs were scanned with a densitometer (Hoefer Scientific) and the bands were integrated using a microcomputer. The Southern blots were rehybridized to an AS probe, which cross-hybridizes with a family of nontranscribed AS pseudogenes [5]. The AS pseudogenes are used as inactive gene standards for blots, as many different sized AS fragments are labeled and all have the same DNase I sensitivity as bulk chromatin (data not shown).

RNA isolation, blotting and hybridization. Total cellular RNA was purified by guanidine-thiocyanate extraction [12], denatured with glyoxal and dimethylsulfoxide [13] and electrophoresed on a 1% agarose gel. Transfer to nitrocellulose and subsequent hybridization were carried out using the same conditions as for Southern blots. As a check on the quality and quantity of loaded RNA, agarose gels were stained with ethidium bromide and the RNA was visualized under ultraviolet light prior to Northern blotting.

PCR analysis of DNase I treated samples. DNase I digested DNA samples (0.5 μ g) were analyzed using duplex PCR reactions with primers for the β -globin control and *c-fos* test probes. Amplification was carried out at three standard temperatures (94, 63 and 72°C) for 22 cycles on a thermocycler. Thermocycler well-to-well uniformities of less than $\pm 0.5^\circ\text{C}$ were required in order to obtain precise quantitative results using PCR. The final reaction products were electrophoresed on a 6% polyacrylamide gel, stained with ethidium bromide, visualized under 254 nm ultraviolet light, and photographed with 4" \times 5" negative Tmax film (Kodak). Quantitation of the ethidium PCR gels was appropriately linear only if high-density Tmax film was used in place of low-density, high-contrast Polaroid film. The PCR bands in the film were quantitated by scanning with a Hoefer densitometer and the areas under the peaks were integrated with the aid of a microcomputer. Equivalent results were obtained if quantitation was carried out using radioactively-labeled nucleotides and direct-band counting by Cerenkov radiation. Normalized DNase I sensitivity values were calculated from the equation: $S_F = (F_U/G_U) \div (F_D/G_D) \times T$ where F and G are *c-fos* and globin band intensities for the undigested (U) or digested (D) samples and T is the ratio of globin to *c-fos* sizes.

DNase I hypersensitive sites. DNase I hypersensitive sites were mapped by indirect-end labeling experi-

ments as described previously [5]. The two major hypersensitive sites are shown as downward arrows in the *c-fos* map (Figs. 4 and 5) and occur over the *c-fos* enhancer and promoter. A minor hypersensitive site is also found inside the *c-fos* transcription unit (not shown). All PCR sensitivity probes were located at least 300 bp from any hypersensitive site to ensure that the sensitivity probes would not be perturbed by changes in hypersensitivity.

Sensitivity of naked DNA to DNase I. To control for the possibility that some *c-fos* DNA sequences differ in native sensitivity to DNase I, purified HeLa DNA was subjected to limited DNase I digestion and then assayed for relative DNase I sensitivity using the PCR technique. With naked DNA, all globin and *c-fos* sequences were digested equally (data not shown), demonstrating that preferential *c-fos* DNase I sensitivity requires chromatin structure.

Results

The DNase I-PCR assay rapidly and reliably determines chromatin accessibility during *c-fos* induction

As a test for the PCR assay of DNase I sensitivity, we have analyzed DNase I sensitivity of chromatin during *c-fos* induction by both the standard Southern blot assay [5] and by the PCR assay. In the experiment, serum-starved HeLa cells were stimulated with serum for 0, 15 or 60 min and nuclei were prepared from the three time points. Under these conditions, *c-fos* mRNA peaks at 15 min and falls to basal levels by 60 min (Fig. 1 and Refs. 5,14). The nuclei were subjected to DNase I digestion and the same DNase I-digested DNA was analyzed by the Southern blot assay [5] or by the new PCR assay. In the PCR assay, active and inactive DNA regions were amplified for 22 cycles on a thermocycler and the PCR products were fractionated on a polyacrylamide gel. The DNA bands were stained with ethidium bromide and the gel was photographed with Kodak Tmax negative film under ultraviolet illumination.

Fig. 2B shows that the β -globin (460 bp PCR fragment) and *c-fos* (350 bp) bands were of similar intensity in undigested DNA (lanes 1). However, after extensive digestion with DNase I, the *c-fos* band became

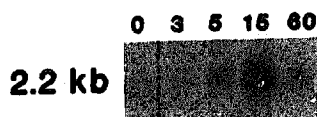


Fig. 1. Northern blot analysis of *c-fos* induction. Total RNA was purified from serum-starved HeLa cells induced with media plus 15% serum for 0, 3, 5, 15 or 60 min. The RNA was then denatured by glyoxal, fractionated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized to the 32 P-labeled PCR *c-fos* probe shown in Fig. 2A.

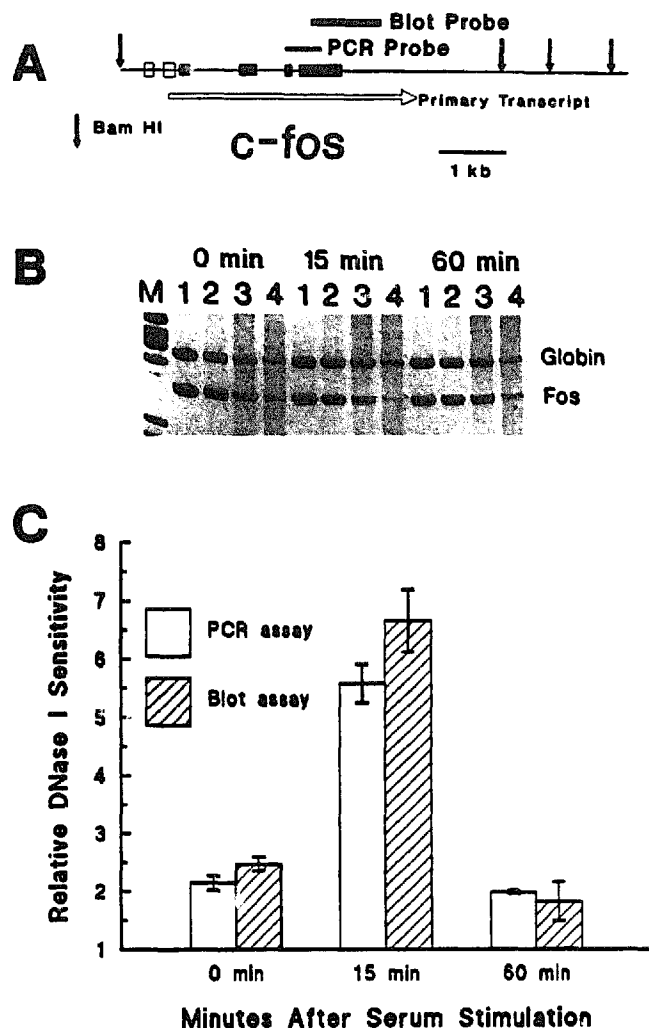


Fig. 2. Blot versus PCR assay of *c-fos* DNase I sensitivity. (A) Map of human *c-fos*. Coding regions are shown as solid boxes. The *c-fos* enhancer (far left box) and promoter are depicted as open boxes. The blot probe (gel purified from cut plasmid DNA) was used to hybridize to a Southern blot containing DNase I digested samples and labels a 700 bp *Bam*HI-*Stu*I restriction fragment. The blot data was from our previous paper [5] and is not shown. The PCR probe consists of two complementary 30 base oligomers, which amplifies a 350 bp fragment (probe F2). (B) Negative photograph of DNase I-PCR gel. Nuclei were purified from serum-starved HeLa cells induced with 15% serum for 0, 15 or 60 min. The nuclei were digested with 0, 0.11, 0.33 or 1 μ g of DNase I (lanes 1-4) and purified DNA was restricted with *Pst*I. β -globin and *c-fos* primers were added along with *Taq* polymerase to amplify 460 bp and 350 bp portions of the β -globin and *c-fos* genes, respectively. The amplified PCR products were electrophoresed on a 6% polyacrylamide gel, stained with ethidium bromide, and photographed in short ultraviolet light with negative film. (C) Southern blot (autoradiograph) and PCR (negative film) bands were quantitated using densitometry at 0 and 1 μ g DNase I (see Materials and Methods). Each determination denotes the mean \pm S.E. ($n = 3$).

relatively less intense compared to β -globin (lanes 4), especially at the 15 min time point. The band intensities from three separate assays were quantitated by densitometry and a histogram was prepared comparing the results of the PCR and the Southern blot assays. Fig. 2C demonstrates that the Southern blot and PCR

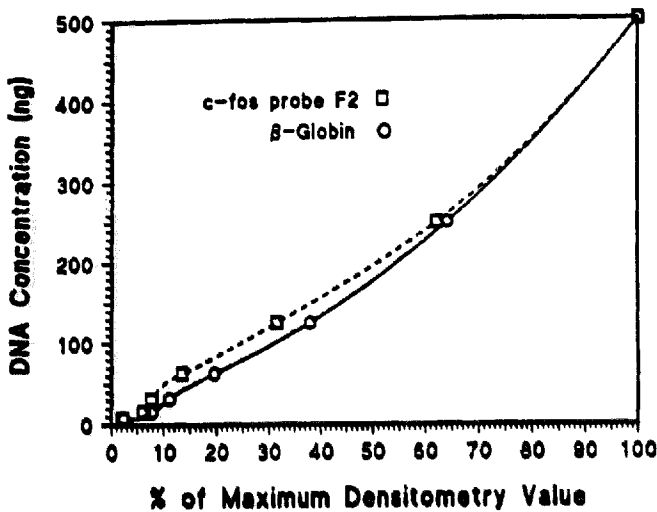


Fig. 3. PCR quantitation of diluted genomic DNA. Undigested HeLa DNA was restricted with *Pst*I and then diluted by half in five separate dilutions. Each dilution was subjected to PCR amplification using *c-fos* (probe F2) and β -globin primers. The products were visualized and quantitated by densitometry as in Fig. 2. \square , *c-fos* probe F₂; \circ , β -globin.

assays give similar relative DNase I sensitivities. However, the PCR assay is much faster, uses 20-fold less DNA and has a higher resolution.

The PCR method uses an exponential amplification in which the efficiency of amplification may increase with the decrease in target DNA concentration [15] (our unpublished findings). This variable efficiency has

been one obstacle to making PCR a quantitative assay. Thus, the success of the PCR assay described above depends on careful quantitation using both internal and external standards [15–19]. In all of our *c-fos* assays we use the inactive β -globin genes as our internal standard by which the relative disappearance of *c-fos* is quantitated. As an external standard, we use dilution assays of genomic DNA to determine the PCR amplification efficiencies for each probe. An example of such a dilution assay for *c-fos* and β -globin is shown in Fig. 3. With the proper internal and external controls, we find that the PCR data obtained are simpler to analyze and of higher resolving power than any of the previous assays of DNA concentration using hybridization technology.

Enhancer-induced changes in DNase I sensitivity along the *c-fos* domain

To search for a gradient of DNase I sensitivity, we initially chose *c-fos* induction times of 5 min or less, since our early experiments indicated that the DNase I sensitivity gradient might be more pronounced during basal transcription or soon after serum induction. Fig. 4 shows a *c-fos* induction experiment in which serum-starved HeLa cells were stimulated with serum for 0 (circles), 3 (triangles) or 5 (stars) min. DNase I-PCR analysis was performed on the samples to determine relative sensitivities to DNase I digestion of the four downstream *c-fos* DNA regions (shown as dark lines

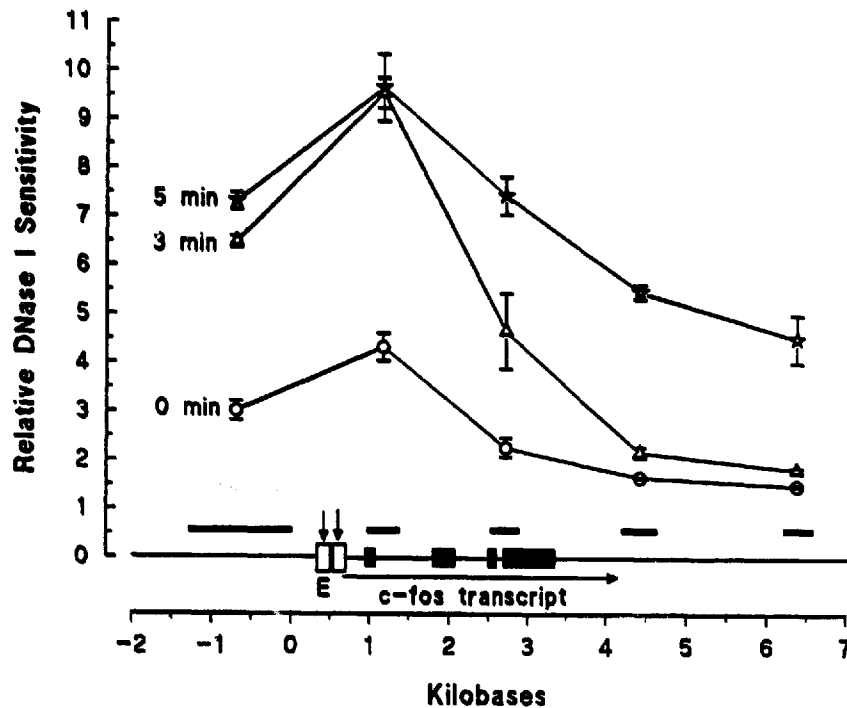


Fig. 4. Relative DNase I sensitivity of various regions of *c-fos* during the first 5 min of serum stimulation. Nuclei were isolated from serum-starved HeLa cells induced with 15% serum for 0 (circles), 3 (triangles) or 5 (stars) min. The DNase I-PCR assay was performed as in Fig. 2 using the F1 through F4 *c-fos* PCR probes. The PCR probes are shown as black boxes above the *c-fos* map. The unsequenced upstream region was probed by the Southern blot assay as before [5]. The upstream probe is a 1.4 kb *Eco*RI-*Bam*HI restriction fragment and is shown as a black box above the *c-fos* map. Each determination denotes the mean \pm S.E. ($n = 3$).

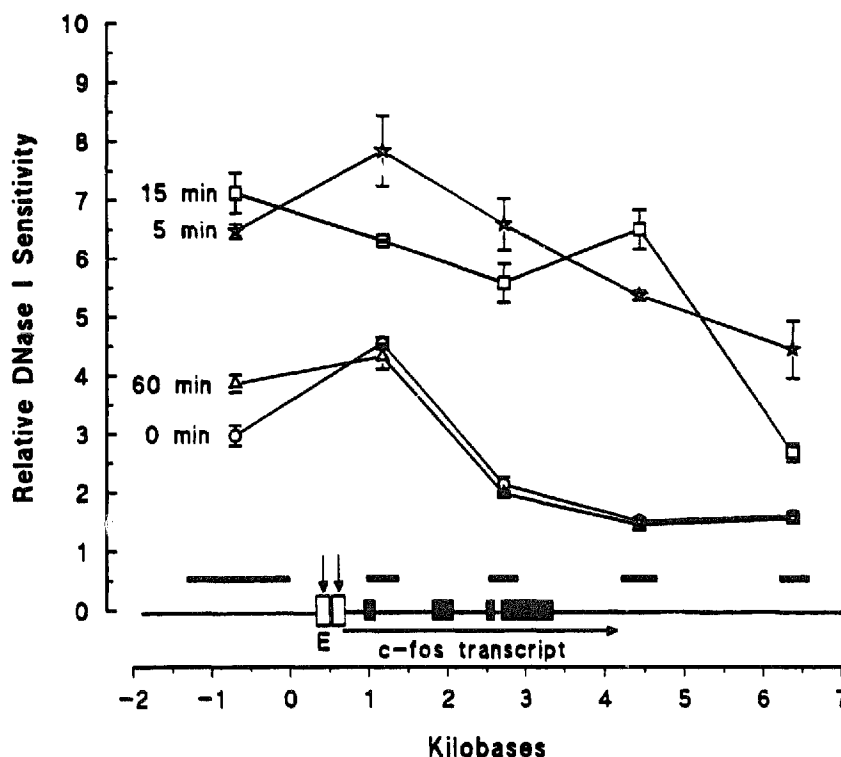


Fig. 5. Relative DNase I sensitivity of various regions of *c-fos* during the first 60 min following serum stimulation. Nuclei were isolated from serum-starved HeLa cells induced with 15% serum for 0 (circles), 5 (stars), 15 (squares) or 60 (triangles) min. DNase I assays were performed as in Fig. 4. Each determination denotes the mean \pm S.E. ($n = 3$).

above the *c-fos* map). DNase I sensitivity of the unsequenced upstream region was determined by Southern blotting as before [5]. All data points for *c-fos* were normalized to β -globin and were corrected for target size. Fig. 4 demonstrates that before induction DNase I sensitivity is highest near the start-site of the gene and declines both upstream and downstream of the enhancer-promoter region. Thus, prior to stimulation of the *c-fos* enhancer, DNase I sensitivity peaks near the enhancer-promoter, with a 5' to 3' gradient of sensitivity appearing downstream.

Within the first 3 min of serum stimulation, DNase I sensitivities of the probes proximal to the enhancer-promoter increase preferentially, so that the 5' to 3' gradient of sensitivity is more pronounced (Fig. 4). However, after 5 min of serum stimulation, DNase I sensitivity is increased in the distal regions and chromatin accessibility appears more uniform across the *c-fos* domain (Figs. 4 and 5). We conclude that while serum stimulation initially enhances the gradient of *c-fos* DNase I sensitivity found in serum-starved cells, the effect is transient as chromatin accessibility soon spreads into the 3' distal region with longer stimulation times.

Transcription of *c-fos* is detectable at low levels in the first 5 min following serum induction (Fig. 1 and Refs. 5,14). We wondered how DNase I sensitivity across the *c-fos* domain would change at 15 min when *c-fos* transcription peaks and at 60 min when transcrip-

tion falls back to basal levels [5,14]. To investigate the longer stimulation times, serum-starved HeLa cells were stimulated with 15% serum for 0 (circles), 5 (stars), 15 (squares) or 60 (triangles) min. Nuclei were isolated and DNase I analysis was performed as before. Fig. 5 demonstrates that DNase I sensitivity remains high throughout most of the *c-fos* domain at 15 min and changes little from the 5 min time point. In contrast, at 60 min post-stimulation, DNase I sensitivity falls back to the low level found in serum-starved cells. As before, marked differences in DNase I sensitivity appear across the *c-fos* domain with the regions proximal to the enhancer-promoter having the highest accessibility.

Discussion

At low levels of c-fos transcription, DNase I sensitivity peaks around the sequences proximal to the enhancer-promoter region

When HeLa cells are harvested after 0 or 60 min of serum stimulation, *c-fos* transcription is at basal levels (Fig. 1 and Refs. 5, 14). Under these conditions, Figs. 4 and 5 confirm our previous data that *c-fos* DNase I sensitivity declines at increasing distances from the promoter region. At 3 min of serum stimulation, a steeper gradient of DNase I sensitivity is generated. This is a transient state, however, as the gradient is reduced by 5 min after serum stimulation (Fig. 4).

While *c-fos* mRNA levels increase from 5 to 15 min post-stimulation (Fig. 1 and Refs. 5, 14), the high steady-state transcription at 15 min does not lead to marked changes in DNase I sensitivity across the *c-fos* domain (Fig. 5).

While gradients of DNase I sensitivity have often been observed at the boundaries of DNase I sensitive domains [2-4], the gradient observed in the *c-fos* domain is novel because it declines within the *c-fos* transcription unit and because it is readily modulated by stimulation of the *c-fos* enhancer. An understanding of what generates these temporal and spatial patterns of DNase I sensitivity could help elucidate the significance of active DNase I sensitive chromatin in gene regulation.

The DNase I-PCR assay yields reliable high-resolution data on chromatin accessibility at any sequenced DNA locus

Since pancreatic DNase I is a non-specific DNA-binding protein, the DNase I assay has proven to be a valuable tool for mapping domains of chromatin accessibility around active gene regions. However, progress in understanding the functional significance of domain DNase I sensitivity has been disappointing. A good part of this lack of progress is due to the Southern blot, dot blot and solution hybridization assays previously available. Southern blots are disadvantaged because of the low resolution and the inconvenience of relying on appropriately placed restriction sites (comparisons of different sized restriction fragments can introduce large and unpredictable errors into sensitivity calculations). Dot blots and solution hybridization have somewhat better resolution, but often give spurious results due to nonspecific background hybridization. With such imprecise assays for DNase I sensitivity, only qualitative conclusions can be drawn.

In contrast to the DNase I assays based on hybridization technologies, the DNase I-PCR assay gives quantitative, high-resolution data on chromatin accessibility for any genomic locus using 20-fold less DNA, and is 10-fold faster. The PCR-based assay will allow the preparation of detailed maps of chromatin accessibility for any genomic region that has been sequenced and mapped for DNase I hypersensitive sites. The DNase I sensitivity data obtained are of high enough

resolution to aid in elucidating the potential role of enhancers and other regulatory elements in modulating chromatin accessibility.

Acknowledgments

We thank Michael Pikaart and Harold Martinson for helpful criticisms. This work was supported by the National Science Foundation (DMB-9018701) and by BRSG 2 SO7 RR07050-25 awarded to the Office of the Vice President for Research, The University of Michigan by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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