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In Vivo Protein-DNA Interactions at the c-*jun* Promoter in Quiescent and Serum-Stimulated Fibroblasts

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Abstract c-Jun is an important component in the regulation of cell proliferation. As a member of the early response gene family, c-*jun* is induced within minutes in the presence of mitogenic agents such as serum growth factors. Using in vivo footprinting, we have analyzed protein-DNA interactions at the c-*jun* promoter in human fibroblasts subjected to growth arrest and serum stimulation. We located seven footprints upstream of the transcription initiation site. Protein-DNA interactions were detected at two AP-1–like sequences, a CCAAT box, an SP-1 sequence, an NF-jun sequence, a putative RSRF (related to serum response factor) binding site, and a sequence bound by an unknown factor. All of these binding sites were occupied in serum-starved cells, and no additional protein-DNA interactions were detected upon serum stimulation. Evidence from this study supports a model in which expression of the c-*jun* gene is mediated by phosphorylation events taking place on the transactivation domains of promoter-bound transcriptional activators.

Key words: transcription factors, serum stimulation, early response gene, in vivo footprinting, cell cycle

Members of the "immediate early response" family of genes include, among others, c-*jun*, c-*fos*, and several genes related to c-*jun* and c-*fos*. These genes are characterized by their rapid activation in quiescent cells in response to mitogenic stimuli [Bravo, 1990; Angel and Karin, 1991; Herschman, 1991; Müller et al., 1993]. Altered transcription occurs due to the propagation of an extracellular stimulus (i.e., by a growth factor) to the nucleus by way of a complex network of messenger molecules [Müller et al., 1993; Karin, 1992]. Though the exact role of c-Jun in cell proliferation remains unknown, several studies have elucidated certain functions of c-Jun in the cell cycle. Transition of NIH-3T3 fibroblasts and WI-38 human fibroblasts from G_0 to G_1 is characterized by rapid induction of the c-*jun* gene [Lamph et al., 1988; Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Carter et al., 1991]. c-Jun protein is required for cell cycle progression in fibroblasts [Kovary and Bravo, 1991]. In Friend murine erythroleukemia cells, c-*jun* expression is required to maintain continuous proliferation, while selective inhibition of c-Jun, with antisense transcripts, causes logarithmically dividing cells to revert back to a state resembling G_0 [Smith and Prochownik, 1992].

c-*jun* is positively autoregulated by its own product, c-Jun, through an AP-1–like sequence in the promoter of the c-*jun* gene [Angel et al., 1988]. This autoregulatory loop is thought to be responsible for signal amplification and conversion of a transient signal generated by extracellular stimuli into a longer lasting transcriptional response. This response is then transmitted to other genes by binding of c-Jun to AP-1–like sequences resulting in increased transcription of different cellular genes in the presence of growth factors or tumor promoters (i.e., TPA; 12-*O*-tetradecanoyl phorbol 13-acetate). The transcription factor AP-1 is composed of either Jun/Jun, Jun/Fos protein dimers, or other combinations of members of these two gene families [Curran and Franza, 1988]. Members of the AP-1 family can also crossdimerize with ATF/CREB transcription factors [Hai and Curran, 1991].

The c-*jun* gene is expressed in response to a variety of external stimuli, including growth factors, UV radiation, ionizing radiation, oxidative stress, and various chemical agents [Sherman et al., 1990; Devary et al., 1991; Bergelson

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et al., 1994]. Elevation of c-*jun* mRNA levels in response to these stimuli is a consequence of increased transcription of the gene [Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Wu et al., 1989; Sherman et al., 1990; Devary et al., 1991; Chauhan et al., 1993; Bergelson et al., 1994]. We have recently analyzed the promoter of the c-*jun* gene in HeLa cells during UV irradiation, which induces the gene >100-fold [Devary et al., 1991; Rozek and Pfeifer, 1993]. Six transcriptional activator proteins were detected along the c-*jun* promoter region in HeLa cells. Surprisingly, no changes in protein-DNA interactions were observed during the UV response, indicating that induction of the gene was mediated by preformed protein-DNA complexes at the promoter.

Our objective in this study was to determine which transcription factors are bound to the c-*jun* promoter in human fibroblasts and whether any changes in factor binding would accompany the induction of the gene by serum growth factors.

EXPERIMENTAL PROCEDURES

Cell Culture

Human male fibroblasts were grown in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum. The cells were grown under the following conditions: subconfluent cultures (60–70% confluent) were serum-starved for 48 h with serum-free medium. After 48 h, serum-starved fibroblasts were treated with 10% fetal bovine serum for various lengths of time: 15 min, 30 min, and 60 min.

RNA Analysis

RNA was isolated from the serum-stimulated samples via the guanidinium isothiocyanate method [Chirgwin et al., 1979] and 10 μg of total cellular RNA was separated on a 1% formaldehyde agarose gel. Hybridization of RNA with a c-*jun*–specific probe after transfer to a nylon membrane was done as described previously [Rozek and Pfeifer, 1993].

Dimethyl Sulfate Footprinting

Fibroblasts were scraped from petri dishes with a rubber policeman, collected by a quick centrifugation step, and treated with 10 ml of DMEM containing 0.2% dimethyl sulfate (DMS; Aldrich, Milwaukee, WI). Incubation was at room temperature for 10 min. Ice-cold phosphate buffered saline (PBS) (40 ml) was added to the solution, and cells were collected by centrifugation and washed with an additional 30 ml of cold PBS. Nuclei were isolated to remove any DMS trapped in the cytoplasm [Pfeifer et al., 1990, 1993]. Following proteinase K digestion, DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The DNA was then dissolved in 1 M piperidine and incubated for 30 min at 90°C. The piperidine-cleaved DNA was ethanol precipitated and dissolved in water to a concentration of 1 μg/μl. To obtain similar band intensities in individual lanes of the sequencing gel, approximately equal amounts of DNA (1 μg as estimated from the gel) were processed for the ligation-mediated polymerase chain reaction (LMPCR) analysis. Naked DNA controls were obtained by in vitro treatment of fibrobast DNA with DMS [Maxam and Gilbert, 1980]. G, G + A, T + C, and C reactions were performed with HeLa DNA, and these were included on the footprinting gels to provide position markers.

Oligonucleotide Primers

The LMPCR primer sets A, B, C, and D were described previously [Rozek and Pfeifer, 1993]. Primers A-1, B-1, C-1, and D-1 are the Sequenase primers. Primers A-2, B-2, C-2, and D-2 are the PCR primers (these were gel-purified). Primers A-3, B-3, C-3, and D-3 were used to make the hybridization probes [Rozek and Pfeifer, 1993].

Ligation-Mediated PCR (LMPCR)

Gene-specific footprint ladders were amplified via the LMPCR method as described previously [Pfeifer et al., 1990, 1993; Rozek and Pfeifer, 1993]. The PCR products were separated on an 8% polyacrylamide, 7 M urea sequencing gel (60 cm long) and electroblotted onto a nylon membrane (Genescreen; New England Nuclear, Boston, MA). Hybridization of the membrane-bound DNA was done overnight at 60°C. Linear PCR was used to make single stranded hybridization probes by utilizing the primer sets (n)-3 [Rozek and Pfeifer, 1993]. The nylon membranes were washed at 60°C.

RESULTS

Induction of c-*jun* mRNA by Serum Stimulation

Serum-starved fibroblasts were stimulated by addition of 10% fetal bovine serum, and RNA was isolated at various time points following

serum addition. Figure 1 shows a Northern blot analysis with a c-*jun*–specific probe. As expected from previous work [Angel et al., 1988; Carter et al., 1991], there is a rapid increase in c-*jun* mRNA levels after serum exposure, which is characteristic of early response genes. mRNA levels after 1 h of serum stimulation were seven- to eightfold higher than before addition of serum, as determined by phosphoimaging. A comparison to the ethidium bromide–stained gel showed that β-actin mRNA was not significantly induced under these conditions. This induction of the c-*jun* gene is similar to levels reported by other investigators [Angel et al., 1988]. The increase in c-*jun* mRNA levels is due to an increased transcription rate [Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Wu et al., 1989; Chauhan et al., 1993; Wang and Scott, 1994].

DMS Footprinting of the c-*jun* Promoter

Our previous in vivo footprinting analysis involved the use of both DNAse I and DMS to map the c-*jun* promoter in HeLa cells [Rozek and Pfeifer, 1993]. Since no apparent dissimilarities between the footprints seen by both of the methods existed, we decided to use DMS for reasons of simplicity. DMS permeates the cellular membranes and enters the nucleus, where it preferentially reacts with purines. 7-methylguanines are formed predominantly in the presence of DMS, thereby creating sites which are cleavable with hot piperidine. Differences between the DMS control (naked) DNA and DMS-treated cells signify positions of protein-DNA interactions in vivo. These differences can be either a hyporeactivity or hyperreactivity in vivo, depending on the nature of the protein-DNA contacts involved [Pfeifer, 1992].

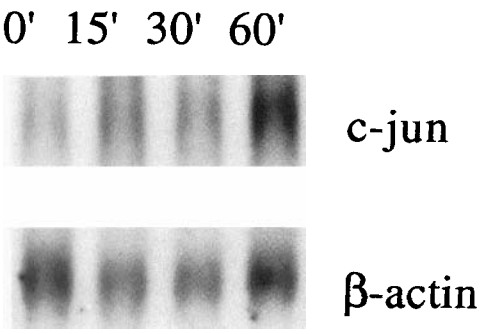

Fig. 1. Northern blot analysis showing serum inducibility of c-*jun*. Cells were grown to 70% confluency, serum-starved for 48 h, and incubated with 10% serum for the indicated periods of time. Total cellular RNA was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a c-*jun*–specific probe. The same blot was rehybridized with a probe for β-actin.

Fibroblasts were growth-arrested by serum starvation and were stimulated to reenter the cell cycle by addition of fresh serum. In vivo DMS footprinting was carried out at the same time points as when RNA was isolated for Northern blot analysis. The DMS-treated DNA was purified from the cells and cleaved at the positions of methylated purines with hot piperidine, and c-*jun*–specific sequences were amplified by using LMPCR with gene-specific primers.

In Figure 2A, sequences of the lower strand of the region spanning nts. −75 to −202 upstream from the transcription initiation site were analyzed. Most Gs at the bottom of the gel are protected from DMS modification, and this region contains the distal AP-1–like sequence (5′-TGAGGTAA) of the c-*jun* promoter as well as a purine rich sequence (5′-GGAGG) which binds a factor of unknown identity. Other differences between modification of naked DNA and modification of DNA within cells are seen within the sequence 5′-GGAGACTCC, which is the binding site for transcription factor NF-jun [Brach et al., 1992]. Near the top of the gel, a footprint is seen at the SP-1 consensus sequence (5′-GGGCGGG) where two hyperreactive G residues are flanked by several protected Gs. Figure 2B shows an analysis of the upper strand from nts. −157 to −235. Several Gs flanking the AP-1–like sequence are either protected or hyperreactive, although the 5′-TTACCTCA sequence itself contains no Gs. A comparison between the G lanes of the serum-starved cells and the various time points after serum addition reveals that, with the exception of a few minor differences in band intensities (which were not reproducible), no significant changes are observed in the footprint patterns. All in vivo footprints that are observed when the gene is maximally expressed are already seen in serum-starved cells prior to induction.

Figure 3 shows an analysis of lower strand sequences (−128 to −62) closer to the transcription initiation site. The SP-1 sequence is now situated at the bottom of the gel. A 5′-CCAAT sequence shows an in vivo footprint that is characterized by protection of the two G residues on the opposite strand of the recognition site (5′-ATTGG). A second AP-1–like sequence (5′-TGATGTCA) is footprinted at the top of the gel.

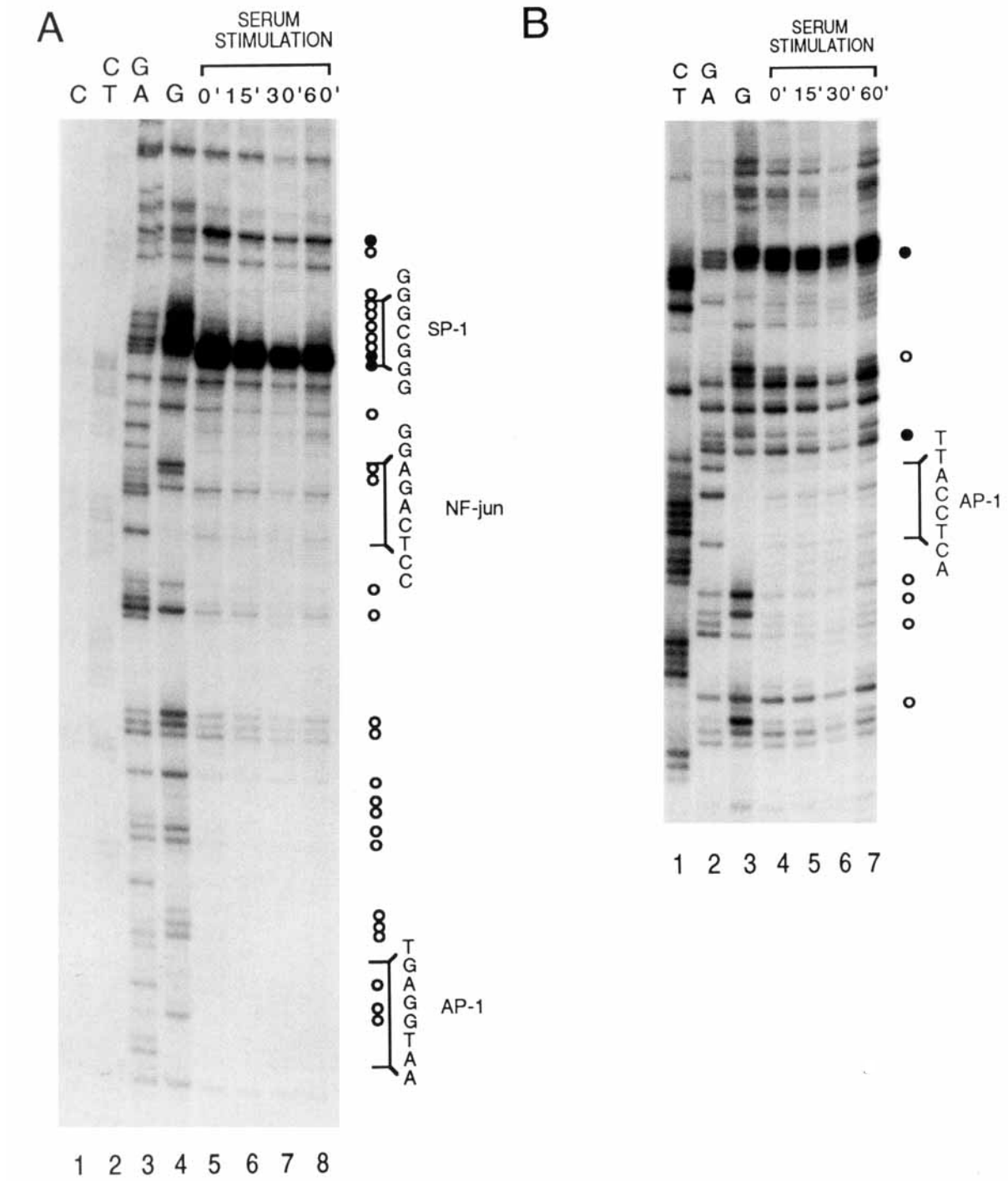

Fig. 2. Genomic footprinting of the c-*jun* promoter in human fibroblasts. **A:** Sequences shown cover the region spanning nts. −75 to −202 upstream from the major transcription initiation site. Lanes 1–3: Maxam-Gilbert control sequences. [Maxam and Gilbert, 1980] Lane 4: Fibroblast DNA treated with DMS in vitro. Lanes 5–8: DNA from serum-starved fibroblasts treated with DMS at various time points following serum addition: 0 min (lane 5), 15 min (lane 6), 30 min (lane 7), and 60 min (lane 8). The guanine residues hyporeactive to in vivo DMS treatment are identified with an open circle; closed circles represent guanines hyperreactive to DMS in vivo. Primers (A1/2/3) were used for LMPCR analysis. **B:** The region analyzed is the upper strand at nts. −157 to −235. Lanes 1, 2: Maxam-Gilbert control DNA. Lane 3: Fibroblast DNA treated with DMS in vitro. Lanes 4–7: DNA from serum-starved fibroblasts treated with DMS at various time points following serum addition. Primers (B1/2/3) were used for LMPCR analysis.

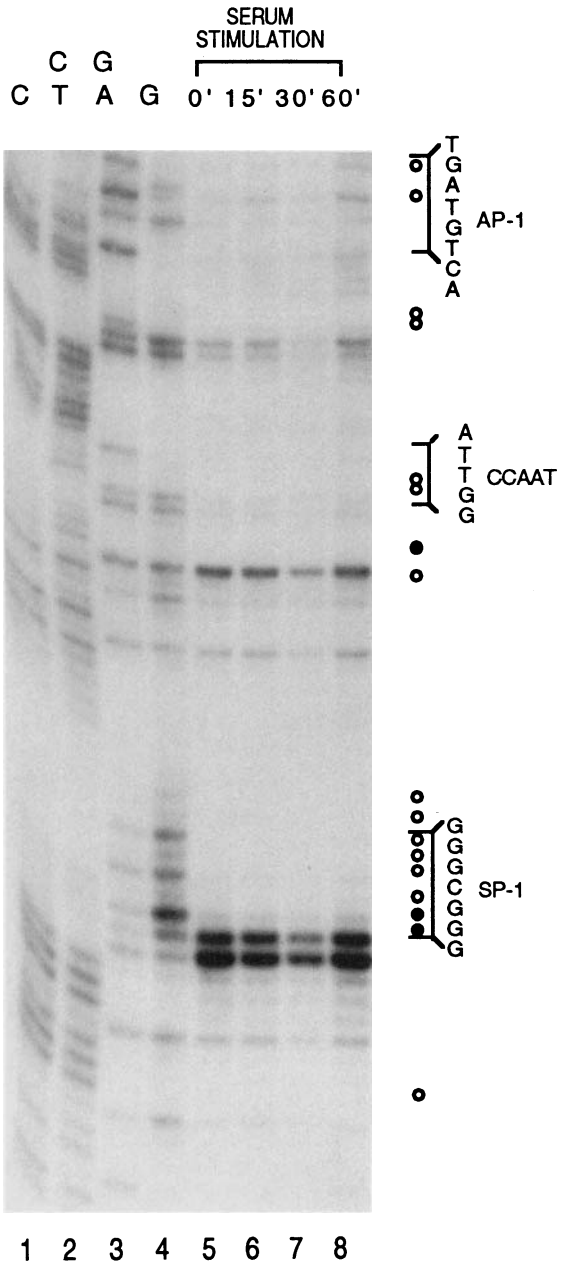

Fig. 3. Genomic footprinting of the *c-jun* promoter in human fibroblasts. Lanes 1–3: Maxam-Gilbert control samples. Lane 4: Fibroblast DNA treated with DMS in vitro. Lanes 5–8: DNA from serum-starved fibroblasts treated with DMS at various time points following serum addition: 0 min (lane 5), 15 min (lane 6), 30 min (lane 7), and 60 min (lane 8). Less DNA was loaded in the 30 min lane. The G residues hyporeactive to in vivo DMS treatment are identified with an open circle; closed circles represent Gs hyperreactive to DMS in vivo. The lower strand from −62 to −128 was analyzed using primers (C1/2/3) for LMPCR analysis.

Figure 4 shows the upper strand of the sequences −44 to −196. Clear differential DMS reactivities are observed along the NF-jun, SP-1, CCAAT, and AP-1 sequences. In addition, there is an indication of protein binding to a putative RSRF (related to serum response factor) binding site at nt. −60 to −49. Again, no significant changes are observed after induction of *c-jun* expression by serum growth factors. The RSRF site appears to be occupied in serum-starved cells prior to induction. Figure 5 shows a summary of all in vivo footprinting results. The observed footprints generally fall into consensus binding sites for known transcription factors, as listed above. One additional footprint is seen at position −176 to −167. These sequences have no apparent homology to known factor recognition sequences (marked ? in Fig. 5).

DISCUSSION

Our results show that no changes, in terms of transcription factor–specific in vivo footprints, occur at the *c-jun* promoter during the course of transcriptional activation in response to serum. In addition, we found no differences between confluent, subconfluent, and serum-starved confluent fibroblast cultures (data not shown). These in vivo footprinting experiments do not rule out the possibility that different members of a transcription factor family (e.g., AP-1–like proteins) could bind to the *c-jun* promoter prior to and after serum induction. This would, however, require that all detectable DNA contacts made by these proteins be identical before and after factor exchange.

Some of the observed in vivo DMS footprinting patterns in fibroblasts correspond to those previously reported in HeLa cells, while others are clearly different. The DMS footprints for the distal AP-1 site (nt. −190), the SP-1 sequences, and the NF-jun site are very similar between HeLa cells and fibroblasts (Fig. 5) [see Rozek and Pfeifer, 1993]. Some differences between the two cell lines were observed at A residues. Some A residues may appear more hyperreactive in vivo when increased depurination of 3-methyladenine takes place during isolation of DNA from DMS-treated cells (e.g., during prolonged incubation at 37°C). For this reason, reactivity differences at adenines seen between independent experiments cannot be directly compared. The in vivo DMS footprints seen in HeLa cells [Rozek and Pfeifer, 1993] and fibroblasts [this study] at the CCAAT box and at the promoter-proximal AP-1 site are dissimilar. For comparison, these sequences are shown in Figure 6. Although the G residues within the core recognition sequences of the respective transcription factors (5′-CCAAT and 5′-TGACATCA, re-

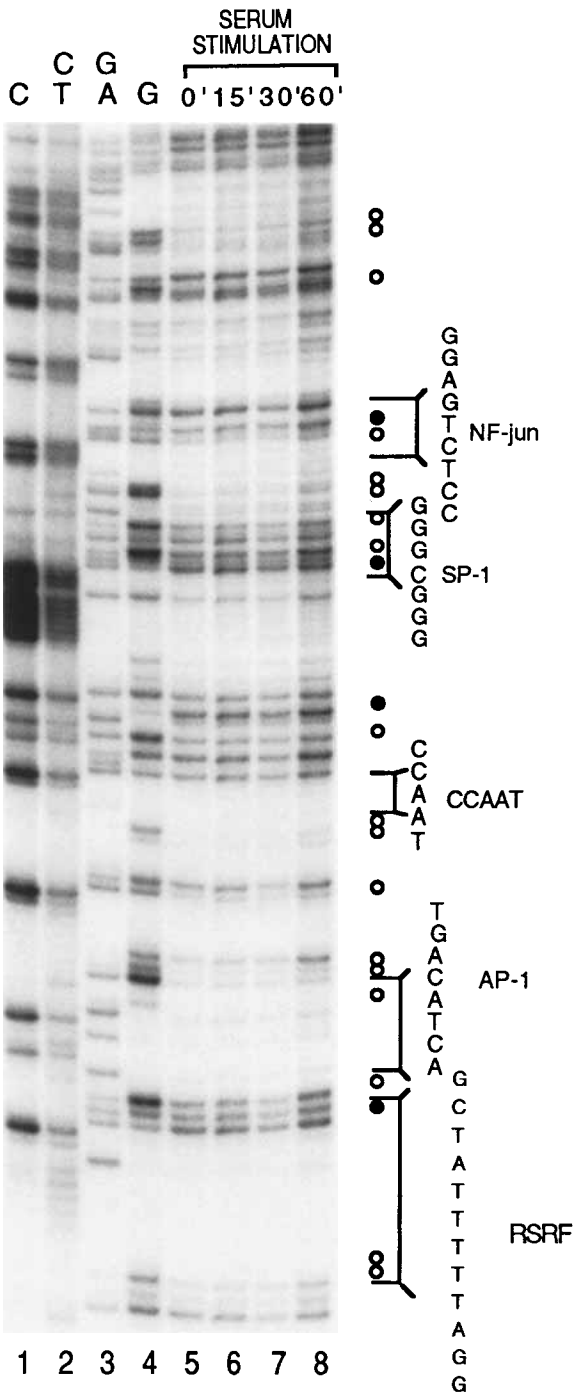

Fig. 4. Genomic footprinting of the c-*jun* promoter in human fibroblasts. Nts. −44 to −196 (upper strand) were analyzed using primers (D1/2/3) for LMPCR analysis. For details, see legend to Figure 3.

spectively) are similarly protected from DMS modification in the two cell lines, the G residues within flanking sequences react in many cases in the opposite way (for example, the Gs at −96 and −85 are hyporeactive in fibroblasts but are hyperreactive in HeLa cells). This suggests that different members of a family of transcription factors may bind to these sequences in the two different cell types. The 5′-GCTATTTTTAGG sequence at nts. −60 to −49 was suggested to be the recognition sequence for an RSRF (related to serum response factor) protein [Pollock and Treisman, 1991; Han et al., 1992], and this sequence is recognized by factors in nuclear extracts [Han et al., 1992]. The RSFR sequences of the c-*jun* promoter can confer serum inducibility on a heterologous promoter [Han et al., 1992]. There is differential in vivo/in vitro DMS reactivity at all G residues within this sequence which can be ascribed to binding of an RSRF factor (Fig. 4). A footprint had not previously been assigned to this sequence in HeLa cells since DNaseI treatment did not unambiguously reveal binding of proteins in vivo. However, this sequence is cleaved by DNaseI very poorly, even in naked DNA, and we have additional evidence by UV photofootprinting that the RSRF site is occupied also in HeLa cells [S. Tornaletti and G.P. Pfeifer, unpublished results]. The DMS reactivity at the RSRF element did not change after serum stimulation, indicating that the RSRF protein is bound prior to induction.

Our findings generally coincide with previous results where bound transcription factors were found at the c-*jun* promoter in UV-irradiated and in nonstimulated HeLa cells [Rozek and Pfeifer, 1993]. Similarly, the occupancy of factor binding sites was unchanged during stimulation of c-*jun* transcription by phorbol esters [Hagmeyer et al., 1993]. The lack of a change in footprint patterns between growth-arrested and serum-stimulated cells supports the idea that a posttranslational modification scheme is responsible for controlling the function of the c-*jun* gene. Transfection experiments have shown that the serum responsiveness of the c-*jun* promoter is mediated by the proximal AP-1 site and by the RSRF site [Angel et al., 1988; Han et al., 1992]. The c-Jun protein which is binding to the c-*jun* promoter as a heterodimer with ATF-2 [Angel et al., 1988; Hagmeyer et al., 1993; van Dam et al., 1993] may be one mediator of this response. Several different protein kinases, induced upon mitogenic stimulation, are able to phosphorylate c-Jun efficiently in vitro and in vivo [Pulverer et al., 1991; Baker et al., 1992; Hibi et al., 1993; Kamada et al., 1994; Su et al., 1994]. Phosphorylation can occur at five amino acid residues, at serines 63 and 73 in the transactiva-

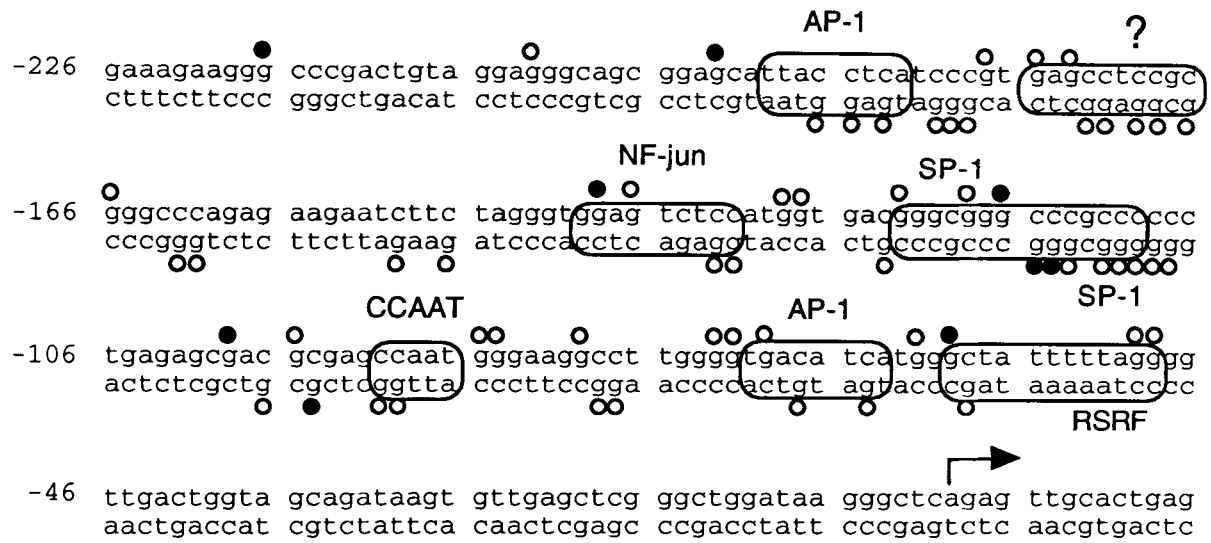

Fig. 5. Summary of the genomic DMS footprinting data. Open and closed circles represent Gs which are hypo- and hyperreactive to DMS in vivo, respectively. Unmarked nucleotides indicate no reactivity difference. The putative transcription factor binding sites are circled with a box, and the arrow marks the major transcription initiation site.

tion domain, and at two to three sites next to its DNA binding domain. Although one report yielded conflicting results [Baker et al., 1992], there is evidence that phosphorylation of serines 63 and 73 increases the transactivation potential of c-Jun [Pulverer et al., 1991; Binétruy et al., 1991; Smeal et al., 1992; Hibi et al., 1993]. Growth factor–induced signalling pathways and other signal transduction cascades may be funneled into phosphorylation events at these same two critical sites on the c-Jun protein.

Signal transduction appears to be operating through preformed protein DNA complexes at other promoters as well. Serum stimulation of c-*fos* expression is mediated by a multiprotein complex at the serum response element, and

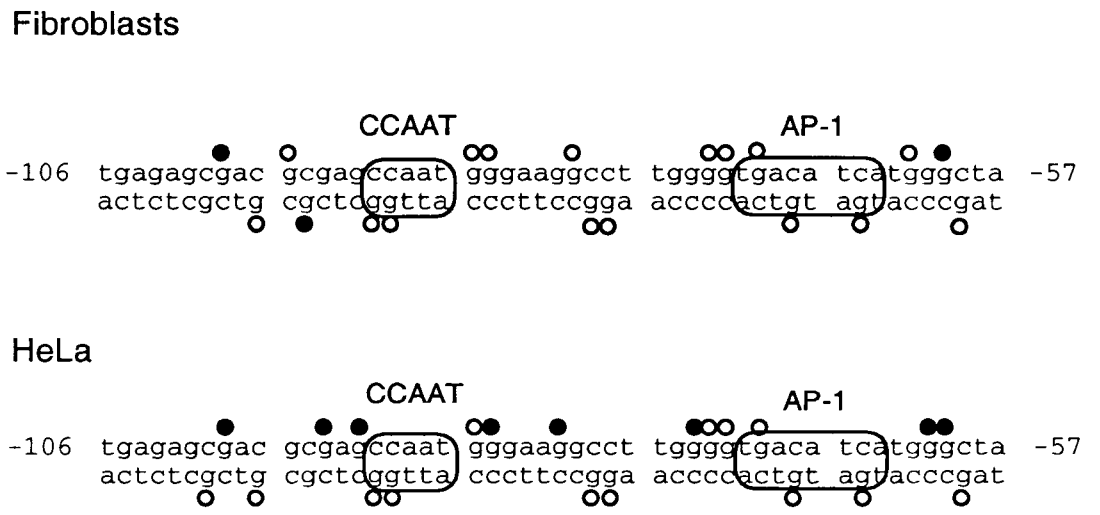

Fig. 6. A comparison of in vivo footprints at the CCAAT box and AP-1 site in fibroblasts and HeLa cells. The data for HeLa cells were taken from Rozek and Pfeifer [1993].

DNA binding of this complex is unaltered by growth factor induction [Herrera et al., 1989]. The growth factor responsiveness of the c-*fos* gene is mediated by serum response factor forming a ternary complex with an accessory factor (Elk-1/TCF) at the c-*fos* serum response element. The transactivation potential of the ternary complex is strongly increased by growth factor–induced phosphorylation of the Elk-1/TCF protein [Marais et al., 1993; Zinck et al., 1993]. Mechanisms by which posttranslational events may modify promoter-bound RSRF proteins are not known at present. Preformed transcription factor complexes at the promoters of *early response genes* may be important links in allowing rapid transduction of extracellular signals to the transcription machinery.

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