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In Vivo Protein-DNA Interactions at the c-*jun* Promoter: Preformed Complexes Mediate the UV Response

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Irradiation of cells with UV light triggers a genetic response, called the UV response, which results in induction of a set of genes containing AP-1-binding sites. The c-*jun* gene itself, which codes for AP-1-binding activity, is strongly (>100-fold) and rapidly activated by UV. The UV induction of c-*jun* is mediated by two UV response elements consisting of AP-1-like sequences within its 5′ control region. We have analyzed protein-DNA interactions in vivo at the c-*jun* promoter in noninduced and UV-irradiated HeLa cells. In vivo footprint analysis was performed by using dimethyl sulfate on intact cells and DNase I on lysolecithin-permeabilized cells in conjunction with ligation-mediated polymerase chain reaction to cover about 450 bp of the c-*jun* promoter, including the transcription start sites. We find that this region does not contain methylated cytosines and is thus a typical CpG island. In uninduced cells, in vivo protein-DNA interactions were localized to an AP-1-like sequence (nucleotides [nt] −71 to −64), a CCAAT box element (nt −91 to −87), two SP1 sequences (nt −115 to −110 and −123 to −118), a nuclear factor *jun* site (nt −140 to −132), and a second AP-1-like sequence (nt −190 to −183). These results indicate that complex protein-DNA interactions exist at the c-*jun* promoter prior to induction by an external stimulus. Surprisingly, after stimulation of c-*jun* expression by UV irradiation, all in vivo protein-DNA contacts remained essentially unchanged, including the two UV response elements located at the AP-1-like sequences. The UV-induced signalling cascade leads to phosphorylation of c-Jun on serines 63 and 73 (Y. Devary, R. A. Gottlieb, T. Smeal, and M. Karin, Cell 71:1081–1091, 1992). Taken together, these data suggest that modification of the transactivating domain of DNA-bound c-Jun or a closely related factor may trigger the rapid induction of the c-*jun* gene.

The damaging effects of UV include lethality, mutagenicity, and carcinogenicity (8, 15, 28, 47, 49). Most of these events are attributed to DNA damage caused by UV-induced photoproducts, which include cyclobutane dimers, (6-4) photoproducts, and other minor adducts. Some of these photoproducts miscode and, if not repaired, can lead to mutations. However, UV irradiation also has tumor-promoting effects. The tumor-promoting effects of UV are not well understood but are possibly related to epigenetic changes in gene expression caused by UV irradiation. Indeed, a number of apparently nonrelated genes are induced by UV irradiation in mammalian cells (reviewed in references 14, 20, 23, 26, and 39). Unlike the genes in *Escherichia coli*, in which UV light specifically induces DNA repair genes of the SOS regulon (52), none of the UV-inducible genes in mammals has so far been shown to be involved in DNA repair. Many of the UV response genes are also induced by growth factors, cytokines, phorbol ester tumor promoters (e.g., tetradecanoyl phorbol acetate [TPA]), and transforming oncogene products (2). Most of these genes can be activated by other sources of cellular damage, including ionizing radiation, alkylating agents, and hydrogen peroxide (20). At present, it is a matter of controversy as to whether DNA damage or oxidative stress, which is also a component of the UV damage spectrum, is the critical event leading to the UV response phenomenon (13, 46). It has been suggested that the concomitant induction of UV response genes may serve a protective function and may provide a stimulus for the cell to replace damaged structural components (13). Attempts to explain how UV irradiation can induce the expression of a large number of different genes have led to the discovery that the response is mediated by specific transcription factors. These include the serum response factor in the c-*fos* gene (9), NF-κB in the human immunodeficiency virus long terminal repeat (46), and transcription factor AP-1 or proteins binding to a sequence similar to AP-1 in a number of other genes (12, 40, 43, 45, 46). AP-1 is a dimeric transcription factor composed of Jun-Jun homodimers, Jun-Fos heterodimers, and various other combinations of members of the Fos-Jun family that bind to TPA response elements or the AP-1 site (2). Jun and Fos proteins can also dimerize with members of a family of proteins that bind to the cyclic AMP response element. AP-1 appears to be the most widely used mediator of UV-induced gene expression, and consistent with this idea is the finding that the c-*jun* gene is rapidly and preferentially induced by UV irradiation (12). This induction leads to increased AP-1-binding activity in nuclear extracts, which could explain the increase in expression of many genes containing AP-1-like sites following UV irradiation. The c-*jun* gene itself is positively autoregulated by its own product, c-Jun (1). The earliest identified event that leads to the UV response is activation of Src tyrosine kinases at or near the plasma membrane (13). This is followed by activation of Ha-Ras and Raf-1 and finally results in phosphorylation of c-Jun on two serine residues within its transactivation domain (13, 36, 44). The UV-induced activation of c-*jun* requires functional AP-1-binding sites in its promoter (12, 45).

To further elucidate the mechanisms that lead to transcriptional activation of the c-*jun* gene after UV irradiation, we have conducted a high resolution in vivo footprinting analysis of its promoter in unstimulated and UV-irradiated cells. This analysis has uncovered a complex network of DNA-bound proteins composed of at least six transcriptional activators within the region −60 to −190 nucleotides (nt)

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upstream of the major transcription initiation site. All protein-DNA contacts remain unaltered after UV induction, which suggests that posttranslational events acting on preformed protein-DNA complexes mediate the final steps of the signalling cascade.

MATERIALS AND METHODS

Cell lines and UV irradiation. HeLa S3 cells were grown to 80% confluency at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Before UV irradiation, the medium was removed and the cells were washed in phosphate-buffered saline (PBS). Irradiation was performed by using a 254-nm germicidal lamp at a distance of 22 cm. The UV dose was 36 J/m^2 as determined with a UVX radiometer (Ultraviolet Products, Inc., San Gabriel, Calif.). The medium was returned to the cells after irradiation, and the cells were then incubated for various periods of time.

RNA analysis. Total cellular RNA from control and UV-irradiated cells was isolated by the guanidinium isothiocyanate method (11). RNA was separated on formaldehyde-agarose gels and transferred to GeneScreen (New England Nuclear) nylon membranes. The membranes were sequentially hybridized with probes specific for the human c-*jun*, p53, and β-actin genes. The probes were made by repeated runoff polymerization from polymerase chain reaction (PCR) products.

Genomic footprinting with DMS. The medium was removed from the cells and replaced with cell culture medium containing 0.2% dimethyl sulfate (DMS; Aldrich). The flasks were incubated at room temperature for 5 and 10 min, respectively. The DMS-containing medium was then quickly removed, and the cells were washed with DMEM and collected by scraping the petri dishes with a rubber policeman. Ice-cold PBS (40 ml) was added, and the cells were sedimented by centrifugation and washed with 30 ml of cold PBS. Nuclei were isolated immediately to eliminate traces of DMS trapped in the cytoplasm (35). DNA was purified by digestion with proteinase K, phenol-chloroform extraction, and ethanol precipitation as described previously (32). The precipitated DNA was directly dissolved in 1 M piperidine (Fluka) and heated at 90°C for 30 min in order to introduce strand breaks at the sites of modified bases. After ethanol precipitation, the samples were processed for ligation-mediated PCR (LMPCR) analysis. In vitro controls were obtained by reaction of purified DNA with DMS as described previously (27).

Genomic footprinting with DNase I. Cells were grown as monolayers to about 80% confluency. To permeabilize the cells, the cell monolayers were treated with 1 mg of lysolecithin (type I; Sigma) per ml in prewarmed solution I (150 mM sucrose, 80 mM KCl, 35 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ [pH 7.4]) for 2 min at 37°C. The lysolecithin was removed, and the cells were washed with 10 ml of solution II (150 mM sucrose, 80 mM KCl, 35 mM HEPES, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM $CaCl_2$ [pH 7.4]). The cells were then incubated with DNase I (20 to 50 μg/ml, grade I; Boehringer Mannheim) in solution II at room temperature for 2 min. The DNase I solution was removed from the petri dishes, and incubation was continued for another 6 min. During DNase I treatment, less than 10% of the cells became detached from the plastic surface. The reaction was stopped, and the cells were lysed by adding 2.5 ml of stop solution (20 mM Tris-HCl [pH 8.0], 20 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 600 μg of proteinase K per ml). Then, 2.5 ml of 150 mM NaCl–5 mM EDTA (pH 7.8) was added, and the solution was incubated for 3 h at 37°C. DNA was further purified by phenol-chloroform extraction and ethanol precipitation. RNA was removed by digestion with RNase A (50 μg/ml in TE [Tris-EDTA] buffer, 2 h, 37°C). In order to reduce viscosity, the samples were digested with *Eco*RI (1 U/μg of DNA, 37°C, 2 h), which does not cut within the region to be sequenced. After phenol-chloroform extraction, DNA was ethanol precipitated, and the samples were processed for LMPCR analysis. The DNase I cleavage conditions resulted in a broad distribution of fragment sizes between 50 and 2,000 nt as checked by alkaline agarose gel electrophoresis before *Eco*RI cleavage. Naked DNA controls, with a similar distribution of fragment sizes, were obtained by DNase I digestion of purified HeLa DNA.

TABLE 1. Synthetic oligonucleotide primers for LMPCR analysis of the c-*jun* promoter

Primer	Sequence	T_m[a] (°C)
A-1	GGGGACCGGGGAACA	51
A-2	GGGGAACAGAGGGCCGAGAGGC	66
A-3	GAGAGGCGTGCGGCAGGG	61
B-1	CCATGATGTCACCCCAAGG	53
B-2	TCACCCCAAGGCCTTCCCATTG	64
B-3	GGCTCGCGTCGCTCTCAGGG	64
C-1	GGCAGCGGAGCATTACC	51
C-2	CGGAGCATTACCTCATCCCGTGAGC	65
C-3	AGCCTCCGCGGGCCCAGAGA	67
D-1	CCGCGCACCTCCACTC	53
D-2	ACCTCCACTCCCGCCTCGCTGC	67
D-3	CCTCGCTGCTTCAGCCACACTCA	63
E-1	GGGTGACATCATGGGCTAT	49
E-2	GGGCTATTTTTAGGGGTTGACTGGTAGC	62
E-3	ACTGGTAGCAGATAAGTGTTGAGCTCGG	61
F-1	CCGGCTGGAGACAAGG	49
F-2	CGGCTGGAGACAAGGCTCTCTGGA	65
F-3	CTCTGGACACTCCCGAACCACCAG	63
G-1	CTAGCTCTGGGCAGTTAGAG	46
G-2	AGCTCTGGGCAGTTAGAGAGAAGGTG	60
G-3	GAGAGAAGGTGAAAAGAAAATAAGATTTGC	58

[a] T_m was calculated with a computer program (41).

Oligonucleotide primers. All primers used for LMPCR analysis are listed in Table 1. Primers A-1 to G-1 are Sequenase primers (they contain a biotin moiety at the 5' end [48]), primers A-2 to G-2 are the PCR primers (they were gel purified), and primers A-3 to G-3 were used to make the hybridization probes.

LMPCR. The standard LMPCR analysis of DMS-treated DNA samples was performed as previously described (29, 32–34). For genomic DNase I footprinting, extension product capture was used in conjunction with a modified LMPCR procedure to enrich for fragments of interest (48). Two micrograms of DNase I-treated genomic DNA was used per sequencing lane. Biotinylated primers, specific for the human c-*jun* gene, were annealed to the heat-denatured (95°C, 3 min), DNase I-cleaved DNA at 45°C for 30 min in 40 mM Tris-Cl (pH 7.7)–50 mM NaCl. Primer extension was then carried out with Sequenase 2.0 (U.S. Biochemical Corp.) as described previously (48). The Sequenase reaction was done at 48°C for 15 min. Under these conditions, the terminal transferase activity of Sequenase 2.0 is minimized (unpublished observation). The DNA polymerase was heat inactivated for 10 min at 67°C. Ligation of the universal linker to

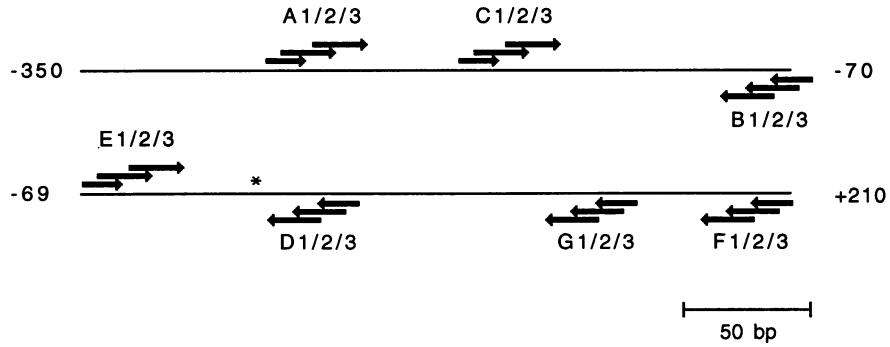


FIG. 1. Position of the oligonucleotide primers used for LMPCR analysis of the 5' region of the human c-*jun* gene. The asterisk marks the major transcription initiation site. Sequences of the lower strand were analyzed with primer sets A, C, and E. Sequences of the upper strand were analyzed with primer sets B, D, F, and G. Primers: 1, Sequenase primers; 2, PCR primers; 3 (e.g., A-3 and B-3), used together to initially make a double-stranded PCR product, which was then used as a template to make a single-stranded hybridization probe.

the primer-extended molecules proceeded as described by Mueller and Wold (29). Streptavidin-coated magnetic beads (Dynal) were prepared immediately before use by being washed twice in 10 mM Tris-HCl (pH 7.7)–1 mM EDTA–2 M NaCl (washing and binding buffer) and resuspended in the same buffer at a concentration of 5 μg of beads/μl. The ligation mixture was added to 1 volume of the prewashed beads, and the beads were incubated at room temperature for 15 min. The supernatant was removed on a magnetic separation stand, and the beads were washed once with 75 μl of washing and binding buffer. The nonbiotinylated strand was eluted from the beads and precipitated as described previously (48). After centrifugation, the pellets were dissolved in 50 μl of water. PCR amplification was done in 10 mM Tris-HCl (pH 8.9)–40 mM NaCl–0.01% gelatin–2 mM $MgCl_2$–200 μM deoxynucleoside triphosphates–10 pmol of the linker primer (GCGGTGACCCGGGAGATCTGAATT C; calculated T_m of 67°C)–10 pmol of the second c-*jun*-specific primer (calculated T_m of 60 to 68°C)–3 U of *Taq* polymerase (Perkin-Elmer Cetus) in a volume of 100 μl. Nineteen cycles of PCR (1 min at 95°C, 2 min at the T_m of the c-*jun*-specific PCR primer, and 3 min at 76°C) were performed. The PCR-amplified fragments were extracted with phenol-chloroform, ethanol precipitated, and separated on a 60-cm-long 8% sequencing gel. After electroblotting (32, 33), the nylon membranes were hybridized with a gene-specific probe. The hybridization probes were made by repeated runoff polymerization (47) from the third c-*jun*-specific primer (primer 3 [Table 1]) which overlaps 3' to the PCR primer.

The hybridization probes were made as follows: PCR products, specific for the c-*jun* promoter, were made by using HeLa cell DNA as a template and primers A-3/B-3, C-3/D-3, and E-3/F-3, respectively. Thirty cycles of PCR were run at 95°C (1 min), 60 to 66°C (2 min), and 75°C (3 min). The PCR products were separated on agarose gels, cut out from the gel, and isolated by a glass bead purification method (Prep-A-Gene; Bio-Rad Laboratories). These gel-purified PCR products were used as templates to make single-stranded hybridization probes. Either of the two primers (e.g., A-3) used initially for making the PCR product can be used to make a single-stranded probe by linear PCR. Linear PCR was done with 5 ng of template; 50 pmol of primer; 20 μM each dATP, dGTP, and dTTP; 2 mM $MgCl_2$; 40 mM NaCl; 10 mM Tris-Cl (pH 8.9); 0.01% (wt/vol) gelatin; 100 μCi of [^{32}P]dCTP; and 5 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus) for 30 cycles on a thermocycler. The probe was extracted once with phenol-chloroform and precipitated by adding ammonium acetate to 0.75 M and 2.5 volumes of ethanol. The following templates and primers were used to make single-stranded hybridization probes: PCR product A3/B3 and primer A-3, PCR product C3/D3 and primer C-3, and PCR product E3/F3 and primer E-3 or primer G3. Hybridizations were done overnight at 60°C in rotating glass cylinders in a hybridization oven, and the nylon membranes were washed at 60°C as described previously (32, 33).

RESULTS

Characterization of the c-*jun* promoter. The c-*jun* promoter is contained within a G+C-rich island (5). It is now well established that methylation of 5'-CpG dinucleotides within promoter regions of a gene almost invariably correlates with a lack of gene expression (6, 10, 38). In order to determine the methylation status of cytosines within the c-*jun* promoter, we analyzed approximately 450 bp of the upstream and 5'-untranslated region of the c-*jun* gene by genomic sequencing with LMPCR (Fig. 1 and see Fig. 3 to 5). Figure 1 shows the locations and orientations of the seven primer sets that were used to analyze both strands of the c-*jun* promoter for methylation and in vivo footprints. Methylated cytosines are recognized by their failure to react with hydrazine, which results in a gap in the C-specific sequencing ladders at CpG positions (these are read in the sequence ladders as G to C from bottom to top). We find that in HeLa cells, all the cytosine-specific bands are present as expected from published sequence data (18). This indicates that the c-*jun* promoter does not contain methylated cytosines and is thus a typical CpG island (5).

Induction of c-*jun* expression by UV light. To confirm that c-*jun* expression is induced by UV irradiation, HeLa S3 cells were irradiated at a UV dose of 36 J/m^2. In nonirradiated cells, c-*jun* mRNA levels are very low but are induced 100- to 200-fold following UV irradiation (Fig. 2). Maximal mRNA levels are seen 1 to 2 h after irradiation, before mRNA levels start to decline again. The time course and levels of induction are comparable to previously reported effects of UV irradiation on c-*jun* expression (12, 45). The increase in c-*jun* mRNA abundance is due to transcriptional activation (12, 45). Transcription of the genes coding for β-actin and p53 was not induced by UV irradiation (Fig. 2).

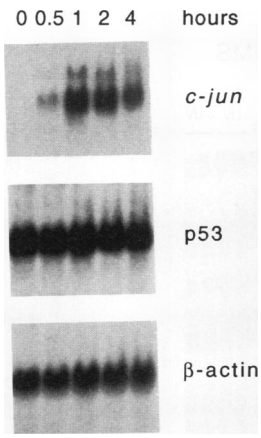


FIG. 2. Induction of c-*jun* RNA by UV irradiation. HeLa cells were irradiated at 36 J/m^2. Cells were harvested at the indicated time points, and total cellular RNA was extracted and subjected to Northern (RNA) blot analysis on 1% agarose gels. The nylon membranes were subsequently hybridized with probes specific for c-*jun*, p53, and β-actin. The exposure times for the autoradiograms were 210 min (c-*jun*), 18 h (p53), and 12 min (β-actin).

Genomic footprinting of the c-*jun* promoter with DMS and DNase I. The increased expression of the c-*jun* gene after UV irradiation was shown to be mediated through two AP-1-like sequence elements located within 200 nt upstream of the major transcription initiation site (12, 45). In order to localize protein-DNA contacts within the promoter region of the c-*jun* gene and to investigate possible changes after UV irradiation that might be functionally involved in this induction process, we have conducted a detailed in vivo footprint analysis of the c-*jun* promoter. Two independent approaches were used, footprinting of intact cells with DMS and footprinting of permeabilized cells with DNase I. DMS is a small molecule which easily diffuses through cell membranes to react with DNA bases in the nucleus. 7-Methylguanines and 3-methyladenines are the predominant modified bases. After the cells were treated with DMS, DNA was isolated and cleaved at modified bases with hot piperidine. c-*jun*-specific sequence ladders were then amplified by LMPCR as described in Materials and Methods. Differences in the modification patterns between DMS-treated cells and DMS-treated purified DNA, evident as hypo- or hyperreactivities, indicate in vivo protein-DNA contacts or an otherwise altered DNA structure at these sites. DMS reveals contacts at sequences that contain G's (or A's) and preferentially detects protein-DNA contacts in the major groove of the DNA double helix. Several footprints at the promoter of the human phosphoglycerate kinase (*PGK1*) gene were difficult to detect with DMS but were more readily revealed by footprinting with DNase I (30, 31). DNase I is much less base selective than DMS and is more sensitive to minor groove protein-DNA contacts, and in addition, the footprints are much larger and clearer than DMS footprints. Furthermore, additional information on chromatin structure, such as the presence of positioned nucleosomes, can be obtained with DNase I. We have used permeabilized cells for DNase I footprinting, because cells permeabilized by lysolecithin maintain normal nuclear structure much better than isolated nuclei, owing to a possible leakage of factors during nuclear isolation procedures (31, 54). In order to efficiently amplify genomic fragments derived from DNase I cleavage, a modified LMPCR method by using extension product capture was used (48). The extension product capture procedure removes most of the small genomic DNA fragments which may contribute to nonspecific priming and amplification events during the PCR reactions.

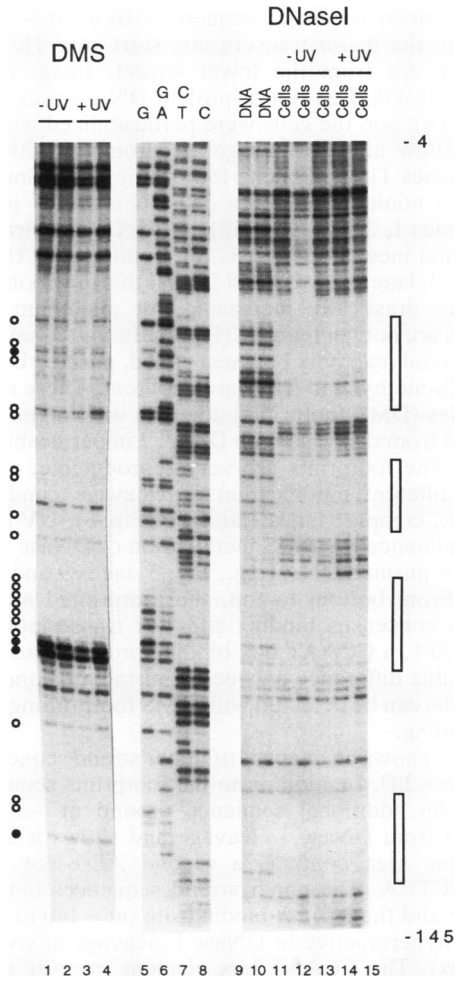


FIG. 3. Genomic footprinting of the c-*jun* promoter with DMS and DNase I. HeLa cells were UV irradiated at 36 J/m^2. Footprinting experiments were performed before UV irradiation (lanes [− UV] 1, 2, and 11 to 13) or 45 min after UV irradiation (lanes [+ UV] 3, 4, 14, and 15). Cells were treated with DMS (lanes 1 to 4) or DNase I (lanes 11 to 15) to determine sites of protein-DNA interactions. Lanes: 5, naked DNA controls for DMS treatment; 9 and 10, naked DNA controls for DNase I treatment; 1 and 3, DMS treatment for 5 min; 2 and 4, DMS treatment for 10 min; 11 and 14, DNase I treatment with 20 μg of DNase per ml; 12, 13, and 15, DNase I treatment with 50 μg of DNase I per ml; 9, DNase I cleavage of naked unirradiated HeLa cell DNA; 10, DNase I cleavage of naked DNA purified from UV-irradiated HeLa cells. Maxam-Gilbert sequence ladders are shown (lanes 5 to 8). Open circles, G's protected from DMS modification in vivo; closed circles, G's or A's hyperreactive to DMS modification in vivo. Those sequences protected from DNase I cleavage in permeabilized cells are indicated on the right with open boxes. The sequences shown are from the lower strand spanning nt −4 to −145 relative to the major transcription initiation site. Primers C-1, -2, and -3 (C1/2/3) were used for LMPCR. From bottom to top, the footprinted areas (open boxes) correspond to consensus binding sites for transcription factors NF-*jun*, SP1, a CCAAT box-binding protein, and AP-1.

Figure 3 shows an example of in vivo footprinting in the

c-*jun* upstream region at sequences from nt −4 to −145 relative to the major transcription start site. The analyzed sequences are from the lower strand. Intact cells were treated with DMS to reveal protein-DNA contacts (Fig. 3, lanes 1 to 4), and the cells were permeabilized with lysolecithin to allow in situ cleavage of chromatin with DNase I (Fig. 3, lanes 11 to 15). The footprinting experiments were done with noninduced HeLa cells (before UV irradiation; Fig. 3, lanes 1, 2, and 11 to 13) and HeLa cells irradiated at 36 J/m^2 and incubated at 37°C for 45 min (after UV irradiation; Fig. 3, lanes 3, 4, 14, and 15). At this time point, mRNA levels are drastically increasing but maximum levels of induction are not yet reached (Fig. 2). In Fig. 3, several areas of differential reactivity between naked, purified DNA and in vivo DMS-methylated DNA are apparent. These differential reactivities (DMS footprints) coincide with regions that are protected from cleavage with DNase I in permeabilized cells (Fig. 3). The footprints are very reproducible, even when slightly different modification or cleavage conditions are used (e.g., compare lanes 1 and 2 in Fig. 3). UV irradiation did not influence the DMS methylation or DNase I cleavage profiles of purified DNA (e.g., Fig. 3, lanes 9 and 10; Fig. 4 and 5). From bottom to top, the footprinted areas correspond to consensus binding sites for transcription factors NF-*jun*, SP1, a CCAAT box-binding protein, and AP-1. No reproducible difference between nonirradiated and UV-irradiated cells can be detected with DMS footprinting or DNase I footprinting.

Figure 4 shows an analysis of upper strand sequences from nt −10 to −207. In addition to the footprints seen in Fig. 3, there is an additional sequence around nt −190 that is protected from DNase I cleavage and shows a DMS footprint. This area contains a second AP-1-like sequence, 5′-TTACCTCA. The upper strand sequences between this AP-1 site and the NF-*jun*-binding site (nt −140 to −165) are strongly hyperreactive to DNase I cleavage in vivo (Fig. 4, black box). The CCAAT box element (nt −91 to −87) is clearly recognized at the upper strand by differential DMS reactivity (Fig. 4), but unlike the lower strand (Fig. 3), it is only weakly protected from DNase I cleavage in permeabilized cells. The footprinting results are summarized in Fig. 6. Both DMS and DNase I footprinting results indicate no significant differences between control and UV-irradiated cells along the entire promoter sequence analyzed.

Figure 5 shows a more detailed DNase I footprinting analysis of the second AP-1-like sequence (nt −190), which was shown to be functionally involved in the UV-induced activation of c-*jun* transcription (45). This AP-1 site is clearly protected from DNase I on both strands (Fig. 5). The DMS footprinting results indicate reactivity differences also for the sequences immediately 3′ to this AP-1 site (Fig. 4 and 6A). However, these sequences are not protected from DNase I cleavage (Fig. 5 and 6B). At the bottom of Fig. 5A, one can also see, at a higher resolution, the strong cleavage sites for DNase I on the upper strand between the AP-1 and NF-*jun* footprints. The AP-1 site at nt −190 is already occupied before induction of c-*jun* transcription, and there are no obvious changes in the DNase I footprint ladders after UV irradiation (Fig. 5).

Sequences near the transcription start site did not reveal any DMS footprints and were not protected from DNase I cleavage (Fig. 3 and 4 and data not shown). In particular, a TATA-like sequence, 5′-GATAA, at nt −33 did not show any footprints, either before or after UV irradiation. We have also analyzed sequences downstream of the major transcription initiation site (data not shown). No footprints

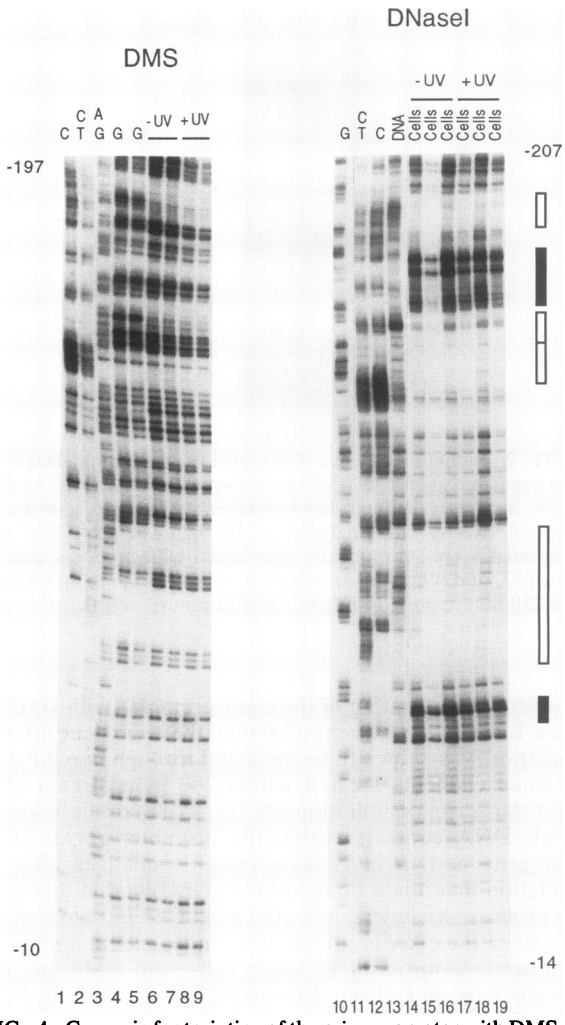


FIG. 4. Genomic footprinting of the c-*jun* promoter with DMS and DNase I. HeLa cells were UV irradiated at 36 J/m^2. Footprinting experiments were performed before UV irradiation (lanes [− UV] 6, 7, and 14 to 16) or 45 min after UV irradiation (lanes [+ UV] 8, 9, and 17 to 19). Cells were treated with DMS (lanes 6 to 9) or DNase I (lanes 14 to 19) to determine sites of protein-DNA interactions. Lanes: 4 and 5, naked DNA controls for DMS treatment; 13, naked DNA controls for DNase I treatment; 6 and 8, DMS treatment for 5 min; 7 and 9, DMS treatment for 10 min; 14 and 17, DNase I treatment with 20 μg of DNase I per ml; 15, 16, 18, and 19, DNase I treatment with 50 μg of DNase I per ml; 4, DMS treatment of naked unirradiated HeLa cell DNA; 5, DMS treatment of naked DNA purified from UV-irradiated HeLa cells. Maxam-Gilbert sequence ladders are shown in lanes 1 to 4 and 10 to 12. Open circles, G's protected from DMS modification in vivo; closed circles, G's or A's hyperreactive to DMS modification in vivo. Sequences protected from DNase I cleavage in permeabilized cells are indicated on the right with open boxes. The black boxes indicate sites hyperreactive to DNase I. The sequences shown are from the upper strand spanning nt −10 to −207 relative to the major transcription initiation site. Primers D-1, -2, and -3 (D1/2/3) were used for LMPCR. From bottom to top, the footprinted areas (open boxes) correspond to consensus binding sites for transcription factors AP-1, SP-1, NF-*jun*, and AP-1.

were detected (Fig. 6), but the DNase I cleavage pattern was significantly different between purified DNA and permeabilized cells for sequences downstream of nt +90 (independent of UV irradiation). The DNA in permeabilized cells was

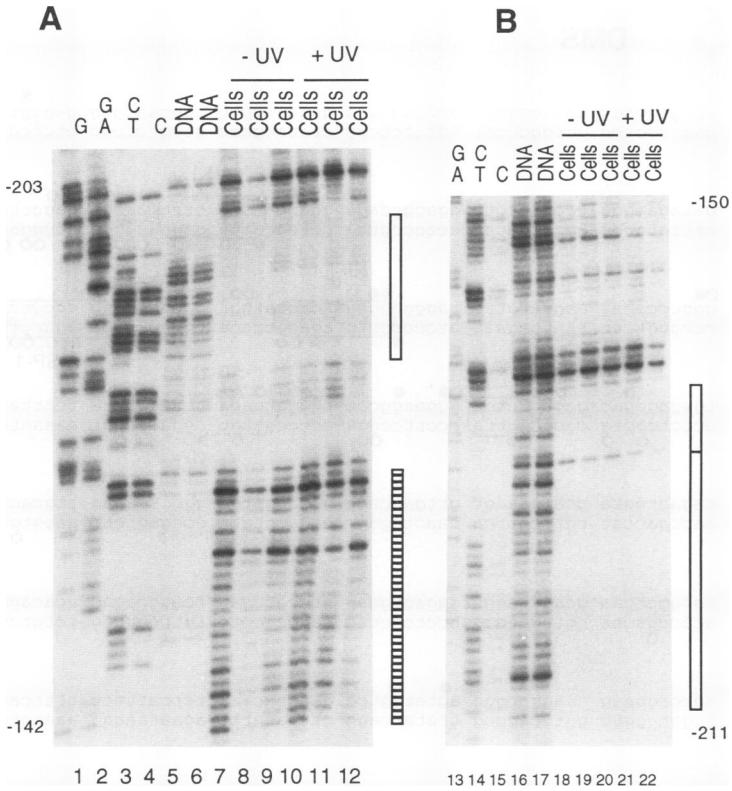


FIG. 5. Genomic DNase I footprinting of the c-*jun* promoter. Footprinting experiments were performed before UV irradiation lanes [− UV] 7 to 9 and 18 to 20) or 45 min after UV irradiation at 36 J/m^2 (lanes [+ UV] 10 to 12, 21, and 22). Lanes: 5, 6, 16, and 17, naked DNA controls; 7, 10, 18, and 21, DNase I treatment with 20 μg of DNase I per ml; 8, 9, 11, 12, 19, 20, and 22, DNase I treatment with 50 μg of DNase I per ml; 5 and 16, DNase I treatment of naked unirradiated HeLa cell DNA; 6 and 17, DNase I treatment of naked DNA purified from UV-irradiated HeLa cells. Maxam-Gilbert sequence ladders are shown in lanes 1 to 4 and 13 to 15. Sequences protected from DNase I cleavage in permeabilized cells are indicated on the right with open boxes. The hatched box indicates sites hyperreactive to DNase I. (A) Sequences from the upper strand spanning nucleotides −142 to −203 were analyzed with primers B-1, -2, and -3 (B1/2/3). (B) Sequences from the lower strand spanning nucleotides −150 to −211 were analyzed with primers A-1, -2, and -3 (A1/2/3). The footprinted areas correspond to the second AP-1-binding site at nt −190.

hyperreactive to DNase I at several positions around nt +120 (Fig. 6B).

DISCUSSION

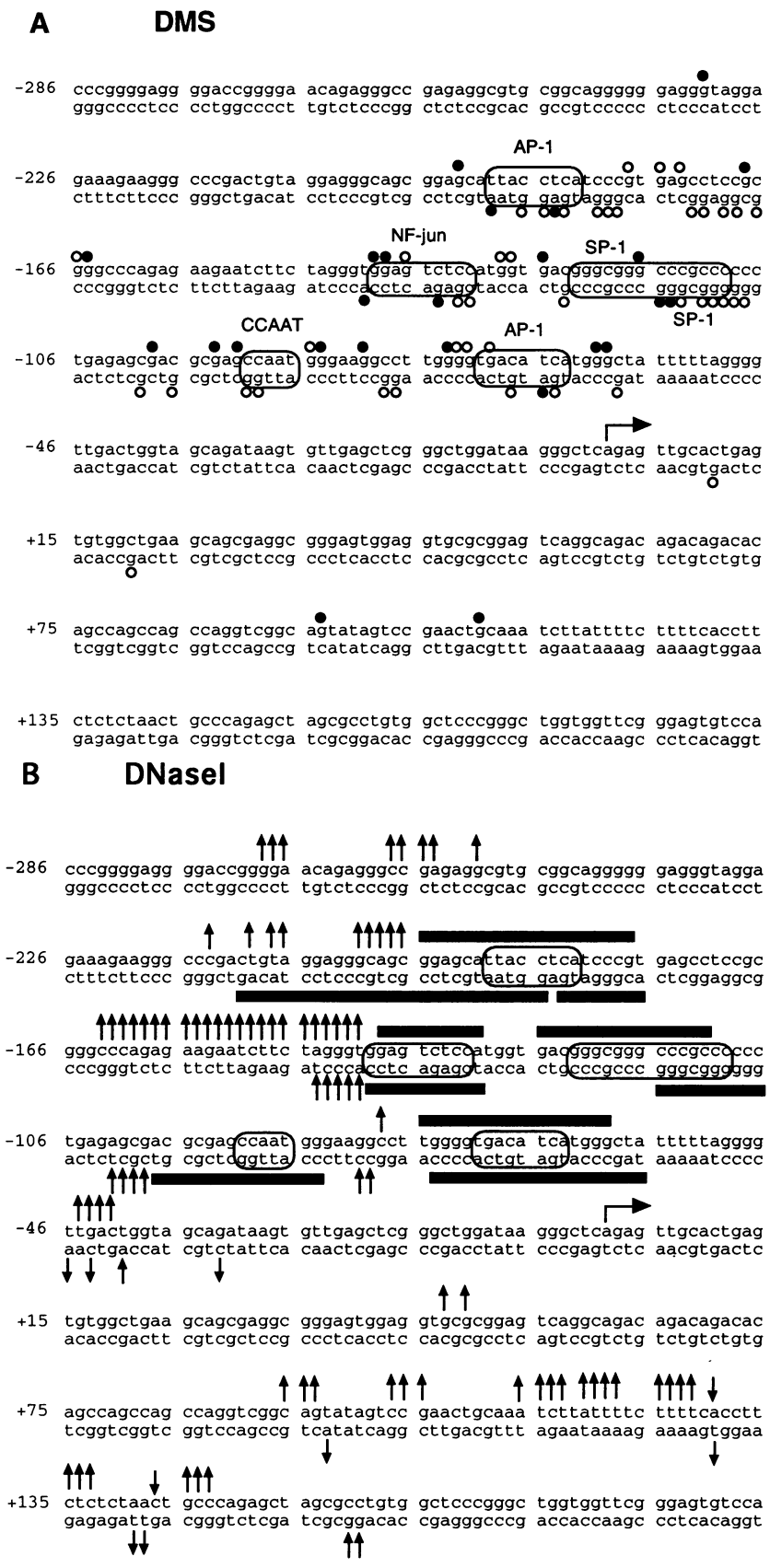
In vivo analysis of chromatin structure. We have used cells treated with two agents, DNase I and DMS, to examine chromatin structure at nucleotide level resolution at the CpG island and promoter of the human c-*jun* gene. DNase I has proven to be a very useful reagent for chromatin analysis, giving clear and unambiguous footprint patterns in permeabilized cells. Footprints obtained by DNase I treatment of lysolecithin-permeabilized cells coincide with DMS footprinting results obtained with intact cells. Consequently, it is very likely that the results obtained by both methods reflect the true in vivo situation.

A complex network of protein-DNA interactions is found at the c-*jun* promoter in HeLa cells. In HeLa cells, the c-*jun* gene is expressed at very low levels but can be induced by external stimuli, such as UV irradiation, to levels more than 100 times the preexisting levels. We find that even in the uninduced state, the promoter is bound by at least six sequence-specific DNA-binding proteins. Figure 6 summarizes all in vivo footprinting data obtained for the c-*jun* promoter. Information on the functional role of these individual elements is relatively scarce, but the available data can be summarized as follows.

(i) AP-1-like sequence at nt −71 to −64. The sequence 5′-TGACATCA has homology to the consensus TPA response element, 5′-TGACTCA, and binds AP-1 protein in vitro (1). This sequence mediates the autoregulation of c-*jun* expression by its own gene product, inducibility by phorbol esters (1), UV irradiation (12, 45), and induction by the phosphatase inhibitor okadaic acid (24). Since the c-*jun* sequence is different by one nucleotide insertion from the classical TRE (TPA response element) consensus sequence, it was suggested that only a subset of AP-1 proteins may bind to this sequence, but the exact identities of the bound proteins have not been defined.

(ii) CCAAT box element at nt −91 to −87. The sequence 5′-GCCAATG is recognized by a CCAAT box-binding protein, presumably transcription factor CTF (1).

(iii) Two SP1 sequences at nt −115 to −110 and nt −123 to −118. These two GC boxes are homologous to the consensus SP1-binding sequence 5′-GGGCGG or its complementary sequence 5′-CCGCCC. Our DNase I footprinting data indicate that, despite their close proximity, both sequences may be occupied in vivo. Mutation of this sequence increases transcription fourfold, suggesting that, at least in


A DMS
-286 cccggggagg ggaccgggga acagagggcc gagaggcgtg cggcaggggg gagggtagga
gggcccctcc cctggcccct tgtctcccgg ctctccgcac gccgtccccc ctcccatcct
AP-1
-226 gaaagaaggg cccgactgta ggagggcagc ggagcattac ctcatcccgt gagcctccgc
ctttcttccc gggctgacat cctcccgtcg cctcgtaatg gagtagggca ctcggaggcg
NF-jun
SP-1
-166 gggcccagag aagaatcttc tagggtggag tctccatggt gacgggcggg cccgcccccc
cccgggtctc ttcttagaag atcccacctc agaggtacca ctgcccgccc gggcgggggg
SP-1
CCAAT
AP-1
-106 tgagagcgac gcgagccaat gggaaggcct tggggtgaca tcatgggcta tttttagggg
actctcgctg cgctcggtta ccccttccgga accccactgt agtacccgat aaaaatcccc
-46 ttgactggta gcagataagt gttgagctcg ggctggataa gggctcagag ttgcactgag
aactgaccat cgtctattca caactcgagc ccgacctatt cccgagtctc aacgtgactc
+15 tgtggctgaa gcagcgaggc gggagtggag gtgcgcggag tcaggcagac agacagacac
acaccgactt cgtcgctccg ccctcacctc cacgcgcctc agtccgtctg tctgtctgtg
+75 agccagccag ccaggtcggc agtatagtcc gaactgcaaa tcttattttc ttttcacctt
tcggtcggtc ggtccagccg tcatatcagg cttgacgttt agaataaaag aaaagtggaa
+135 ctctctaact gcccagagct agcgcctgtg gctcccgggc tggtggttcg ggagtgtcca
gagagattga cgggtctcga tcgcggacac cgagggcccg accaccaagc cctcacaggt
B DNaseI
-286 cccggggagg ggaccgggga acagagggcc gagaggcgtg cggcaggggg gagggtagga
gggcccctcc cctggcccct tgtctcccgg ctctccgcac gccgtccccc ctcccatcct
-226 gaaagaaggg cccgactgta ggagggcagc ggagcattac ctcatcccgt gagcctccgc
ctttcttccc gggctgacat cctcccgtcg cctcgtaatg gagtagggca ctcggaggcg
-166 gggcccagag aagaatcttc tagggtggag tctccatggt gacgggcggg cccgcccccc
cccgggtctc ttcttagaag atcccacctc agaggtacca ctgcccgccc gggcgggggg
-106 tgagagcgac gcgagccaat gggaaggcct tggggtgaca tcatgggcta tttttagggg
actctcgctg cgctcggtta ccccttccgga accccactgt agtacccgat aaaaatcccc
-46 ttgactggta gcagataagt gttgagctcg ggctggataa gggctcagag ttgcactgag
aactgaccat cgtctattca caactcgagc ccgacctatt cccgagtctc aacgtgactc
+15 tgtggctgaa gcagcgaggc gggagtggag gtgcgcggag tcaggcagac agacagacac
acaccgactt cgtcgctccg ccctcacctc cacgcgcctc agtccgtctg tctgtctgtg
+75 agccagccag ccaggtcggc agtatagtcc gaactgcaaa tcttattttc ttttcacctt
tcggtcggtc ggtccagccg tcatatcagg cttgacgttt agaataaaag aaaagtggaa
+135 ctctctaact gcccagagct agcgcctgtg gctcccgggc tggtggttcg ggagtgtcca
gagagattga cgggtctcga tcgcggacac cgagggcccg accaccaagc cctcacaggt

some cell types, the bound protein may function as a transcriptional repressor (50).

(iv) NF-*jun* site at nt −140 to −132. NF-*jun*, a transcription factor recently identified in human myeloid leukemia cells (7), is similar to NF-κB in that it can be translocated from the cytosol to the nucleus upon induction by an external stimulus. It binds to the sequence 5′-GGAGTCTCC in the c-*jun* promoter. Binding of NF-*jun* to oligonucleotides containing its recognition sequence is induced in cellular extracts from leukemia cells treated with phorbol ester, tumor necrosis factor alpha, or cycloheximide (7). We show here that NF-*jun* is already bound to its recognition sequence in HeLa cells before induction of c-*jun* expression. Therefore, at least in HeLa cells, translocation of NF-*jun* from the cytoplasm to the nucleus cannot be a mechanism for signal dependent regulation of c-*jun* transcription. NF-*jun*-binding activity is absent in nonproliferating diploid cells and appears restricted to proliferating cells (7; this report). Since deregulation of c-*jun* may contribute to transformation of mammalian cells (2, 22), it may be of interest to determine whether in vivo occupancy of the NF-*jun* motif and/or other elements in the c-*jun* promoter is generally associated with the proliferative status of cells.

(v) Second AP-1-like sequence at nt −190 to −183. Another putative AP-1-binding site, 5′-TTACCTCA, is localized about 50 nt upstream from the NF-*jun* site. This sequence has been shown to be involved in the UV-induced transcriptional activation of the c-*jun* gene (45). The two AP-1-like sequences appear to act independently in the UV induction process (45). Further, the second AP-1 site was shown to be involved in the E1A-mediated induction of c-*jun*, and in vitro binding studies have shown that this sequence binds heterodimers composed of c-Jun and ATF-2 (ATF-2-like) proteins (51). However, the type of proteins bound may be determined by cell type, and the possibility exists that this site may be involved in a variety of other signal transduction processes. The patterns of DNase I protections and hyperreactivities that we see at the two AP-1 sites (Fig. 6B) are very different, suggesting that the two sequences are bound by different protein complexes.

(vi) Other elements. No footprints were found at c-*jun* sequences from nt −60 to +100, including two TATA-like sequences (5′-TATTTTA at nt −58 to −52 and 5′-AGATAA at nt −34 to −29). Consequently, these sequences are not permanently bound by a TATA-binding protein in vivo. The 5′-TATTTTA sequence was suggested to be part of the recognition sequence for a protein related to the serum response factor (17). DMS or DNase I treatment did not reveal binding of proteins to these sequences in untreated or UV-irradiated HeLa cells. In addition, even in the highly induced state, no additional protein-DNA interactions were detected at or near the transcription initiation site. This may indicate that the DNA contacts made by proteins of the general transcription machinery are not sequence specific and/or are only transient in nature.

The UV response does not involve a reorganization of protein-DNA contacts at the c-*jun* promoter. Upon stimulation of c-*jun* transcription by UV irradiation, we did not identify any changes in protein-DNA complexes as assayed by in vivo footprinting with DMS and DNase I. Since these two agents have different sequence preference, are sensitive to different types of protein-DNA contacts, and respond differently to structural perturbations in the DNA double helix, we are confident that any significant rearrangement of protein-DNA interactions upon UV irradiation, including replacement of a repressor by a structurally distinct activator protein, would have been detected. There is other precedence that a signal transduction pathway may be operating through preformed protein-DNA complexes. Serum stimulation of c-*fos* expression is mediated by a multiprotein complex at the serum response element that is unaltered by growth factor induction (19). However, upon induction with cyclic AMP, the tyrosine aminotransferase promoter showed rapidly induced in vivo footprints at its cyclic AMP response element (53). Treatment of serum-starved cells with phorbol ester resulted in increased binding of AP-1 to its cognate DNA binding site within the collagenase promoter (25). The degree of occupancy of AP-1 sites may be determined by cell type and conditions of growth factor availability. Apparently, signal transduction can operate alternatively through factors already bound to DNA or can induce factor binding to DNA.

Given our data, it is likely that the signal transduction process following UV irradiation targets transcription factors at the c-*jun* promoter that are already bound to DNA before induction. Available data suggest that the two AP-1-like sequences are the mediators of the UV response (12, 45). There is abundant evidence from the literature that the two AP-1-like sequences in the c-*jun* promoter bind AP-1 proteins in vitro (1, 2, 12, 42, 45, 51). The DMS footprint patterns that we observed at these two sites show considerable similarity to DMS protection profiles seen at other AP-1 sites in vivo (21, 37). AP-1 is a collective name for a family of transcription factors, and it is very difficult to provide direct evidence as to which members of the family are actually bound to their target inside the cell. The data of van Dam et al. (51) suggest that the more upstream AP-1 site may bind heterodimers of Jun and ATF-2 proteins. Ryseck and Bravo (42) showed that the AP-1 site proximal to the start site of transcription binds heterodimers of the Fos-Jun family more efficiently than homodimers of Jun-Jun.

The AP-1 sequences in the c-*jun* promoter are likely to bind a member of the Jun family of proteins, at least as a heterodimer. UV irradiation leads to phosphorylation of the c-Jun protein (and possibly other members of that family) on two serine residues located within its transactivation domain (13, 36). Therefore, the most likely mechanism that initiates the mammalian UV response appears to be phosphorylation of these amino acids on DNA-bound Jun protein. Alternatively, UV irradiation may trigger the dissociation of an inhibitor from DNA-bound c-Jun. A recently identified inhibitor of c-Jun (4) does not interfere with DNA binding but represses the transactivation potential of c-Jun (3). Dissociation of the c-Jun inhibitor was proposed to participate in the

FIG. 6. Summary of the genomic footprinting data for DMS (A) and DNase I (B). In panel A, open circles are G's protected from DMS modification in vivo; closed circles indicate G's (or A's) hyperreactive to DMS modification in vivo. In panel B, sequences protected from DNase I cleavage in permeabilized cells are indicated with black boxes. DNase I hyperreactive sites are shown by arrows pointing up, and single protected nucleotides are shown by arrows pointing down. Unmarked nucleotide positions indicate that no difference was found between purified DNA and treated cells. In each panel, the major transcription start site is marked with a horizontal arrow. Possible core recognition sequences for the bound transcription factors are circled.

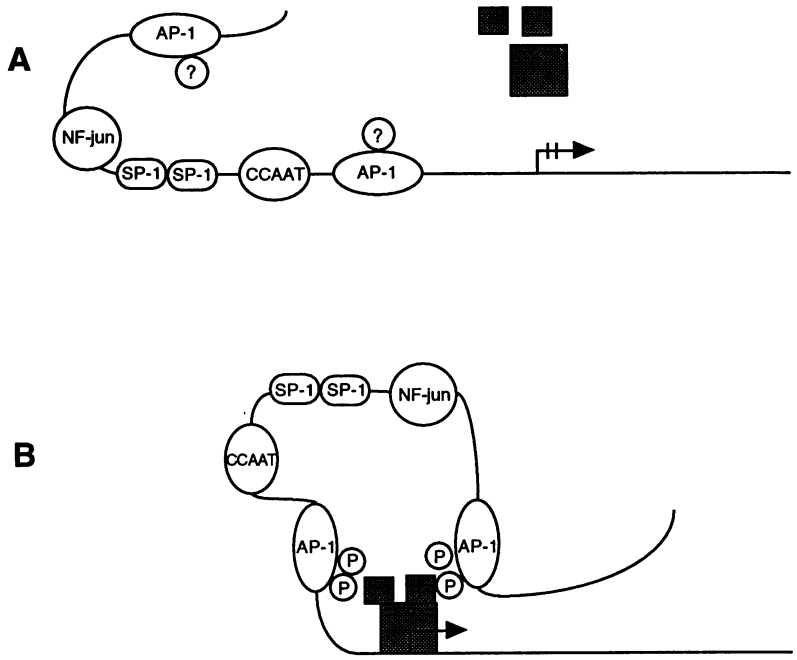


FIG. 7. Schematic model for UV-induced transcriptional activation of the c-*jun* gene. (A) Sequence-specific transcription factors are prebound to the c-*jun* promoter in unirradiated cells. The circled question mark indicates the hypothetical presence of a repressor that needs to dissociate in order to unmask the transactivation potential of Jun proteins. The shaded squares indicate proteins of the general transcription machinery. The transcription start site is marked with a horizontal arrow. (B) Upon UV irradiation, the final step of the signalling cascade leads to phosphorylation of the transactivation domain of DNA-bound c-Jun, which can now interact with factors of the initiation machinery to form productive initiation complexes. Note that protein-DNA interactions at the transcription start site must be transient in nature because no footprints were detected.

cellular response to ionizing radiation (16). Both mechanisms, phosphorylation of the transactivation domain and dissociation of a repressor, would stimulate the transactivation potential of the Jun protein to interact with (yet-to-be-defined) target proteins which could be part of the general transcription machinery (Fig. 7).

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