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UV-Laser Induced Protein/DNA Crosslinking Reveals Sequence Variations of DNA Elements Bound by c-Jun *in Vivo*

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Many proteins involved in the modulation of gene expression exert their function through direct interaction with DNA. The sequence specificity of these interactions provides the basis for many regulatory mechanisms. The sites that are utilized by a transcription factor are usually analyzed using *in vitro* binding studies. To detect true *in vivo* binding sites we developed a method, presented here, that allows construction of recognition element DNA (reDNA) libraries which represent *in vivo* binding sites plus flanking sequences. reDNA libraries can be constructed for any well-characterized transcription factor. Here we used this method for an *in vivo* study of genomic DNA elements that interact with the transcription factor c-Jun in rat cerebellum. © 1999 Academic Press

The elucidation of the mechanisms responsible for transcriptional regulation is one of the major challenges in molecular biology. It is known that specific protein/DNA-interactions occur in distinct regulatory regions of genes, the sum of which largely determine the degree of transcription activation. Currently there are many different techniques available to identify transcription factor binding sites *in vitro* (1, 2). Although protein/DNA-interaction studies *in vitro* provided very valuable information, it is quite obvious that the information obtained by this means does not necessarily reflect the actual situation *in vivo* since sites that are bound *in vitro* may not be targets *in vivo* and vice versa. For instance, studies on binding of proteins to DNA *in vitro* do not take into account the role of DNA-chromatin structure which modulates gene expression (3, 4). The work presented here describes the preparation of a library of *in vivo* c-Jun binding sites using a nanosecond high energy UV-pulse irradiation of living tissue that allows to capture protein/DNA-complexes as they occur *in vivo*, by "nailing" the proteins to their actual binding sites (5, 6). c-Jun specific protein/DNA complexes were isolated by immunoprecipitation, the associated DNA fragments were subsequently cloned and sequenced. The resulting recognition element DNA (reDNA) library represents a compilation of *in vivo* binding sites including flanking sequences. This method can be applied for any well characterized DNA binding protein. The sequence information of the flanking sequences provides a valuable tool in the search for genome localization and/or corresponding genes.

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MATERIALS AND METHODS

Preparation of reDNA library. Thin slices of rat cerebellum were prepared in oxygenated Hank's saline (Gibco) and irradiated in petri dishes with a short pulse of UV-laser-light (10 ns, 10 to 15 mJ/cm^2) (5, 6) (Fig. 1, A). Following irradiation, the tissue was disrupted and the nuclei isolated. The nuclei were re-suspended in 1 ml of 10 mM Tris–HCl, pH 7.9, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT buffer and incubated with 50 units of BamHI at 37°C (Fig. 1, B). Then nuclei were lysed (Fig. 1, C), and overlaid on a CsCl gradient (Fig. 1, D) as described elsewhere (7). Upon ultra centrifugation (Fig. 1, D) 200-μl fractions were collected and their absorption at 260 and 280 nm was measured. Tail fractions at 260 nm (Fig. 1, E) were collected and dialyzed against 0.2% (w/v) sarcosyl, 50 mM Tris–HCl, pH 8.0, 2 mM Na–EDTA for 2 days. A phenol extraction was performed (Fig. 1, F) and molecules in the aqueous phase were precipitated. After overnight digest (Fig. 1, G) with Sau3AI (500 U/ml) and RNAse A (200 mg/ml) in Sau3AI buffer (supplied with the enzyme) the DNA was phenol extracted and precipitated as above. The DNA-sample was dissolved in 80 μl of 0.5 M NaCl, 60 mM Tris–HCl, pH 7.5, 2 mM Na–EDTA and 1 mM PMSF buffer (buffer A) and added to 500 μl protein A–Sepharose which was pre-incubated with c-Jun/AP-1 (AB-2) polyclonal antibody (20 μl, Oncogene Science) for 4 h (Fig. 1, H). After an overnight incubation at 4°C on a low speed shaker, the

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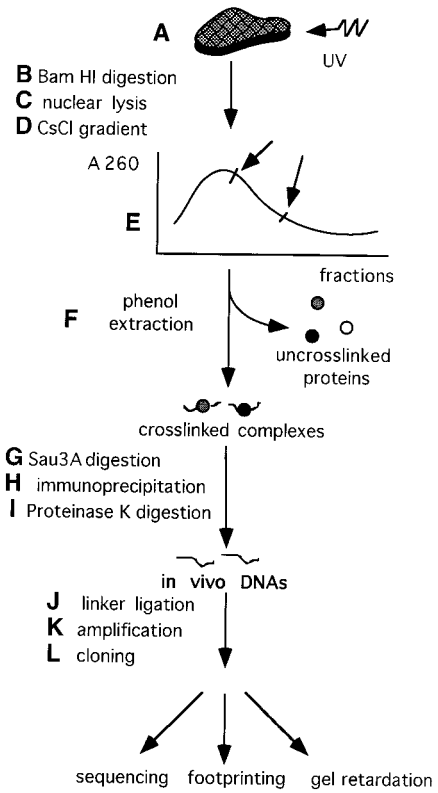

FIG. 1. Outline of the experimental protocol. A detailed step by step description of the method is provided under Materials and Methods.

samples were extensively washed with buffer A. The sample was dissolved in 300 μl of 10 mM Tris–HCl, pH 7.8, 5 mM Na–EDTA, 0.5% (w/v) SDS and digested with 0.1 mg/ml Proteinase K at 37°C overnight (Fig. 1, I). After phenol extraction and ethanol precipitation the DNA is subjected to ligation-mediated PCR as followed (Fig. 1, J and K). The purified selected DNA was uptake in 10 μl water and half of the volume (5 μl) was ligated to 80 pmol of Sau3AI cohesive end linker (primer 1: 5′-TCC CTG TGC GAG AGG A-3′; primer 2, 5′-phosphorylated: 5′-GAT CTC CTC TCG CAC AGG GA-3). One tenth of the reaction mixture (1 μl) was exponentially amplified. The following conditions were used: total volume of 20 μl containing 20 pmol of primer 1; 10 mM Tris–HCl, pH 8.3, 500 mM KCl, 1.5 mM MgCl2, 150 μM each of dATP, dCTP, dGTP, and dTTP, 2 units Taq Polymerase (USB); 15 cycles at 94°C of 1 min, 0.5 min at 52°C, 1.5 min at 72°C). To increase the amount of amplified DNA, an additional PCR step (30 cycles) was performed with one twentieth of the initial mixture under the above conditions. Unused primers were remove by ultra filtration using Microcon 30 (Amicon). Purified PCR-products were phosphorylated with 1 mM ATP and 1 μl T4 kinase (New England Biolabs). The phosphorylated amplified fragments were cloned into the *Eco*RV site of pBluescript II KS (L).

Gel retardation and supershift assays. The plasmid DNA containing the reDNA insert was labeled by digesting with *Eco*RI and filling-in with [α-^{32}P]dATP (8). The insert was separated from the plasmid by *Hin*dIII digestion and end-labeled. Insert-DNA was purified DNA by agarose gel electrophoresis. 20 fmol of insert DNA were incubated in presence of increasing amounts of c-Jun protein in 10 μl binding buffer (20 mM Hepes, pH 7.6, 1 mM Na–EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% (v/v) Tween 20, 30 mM KCl) for 20 minutes at room temperature. The samples were separated on a 0.8% (w/v) agarose gel in 0.5× TBE buffer (pH 8.3) at 4°C. After separation, the gel was dried on nitrocellulose and autoradiographed. Competition and supershift experiments were performed on 6% PAGE (79:1). For supershift experiments 1 μl of c-Jun/AP-1 (AB-2) polyclonal antibody (20 μl, Oncogene Science) was added to the reaction mixture.

Southern blot analysis. Southern blots were performed according to Saluz and Jost (8). DNA samples were separated on a 6% PAGE (29:1) and electrotransferred to a nylon membrane (GeneScreen, Dupont), covalently cross-linked by UV-light (Stratalinker, 120,000 mJ/cm^2). Hybridization (8) was carried out with a probe generated by random-priming (50 μCi/μg DNA) from an isolated reDNA-library clone.

DNase I protection assay. The reDNA probe were labeled as described for gel retardation. 50,000 cpm of each probe was incubated in presence of 50 ng c-Jun protein in binding buffer (10 μl 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% (v/w) Tween 20, 30 mM KCl)at room temperature for 20 min. The samples with and without c-Jun protein were incubated with

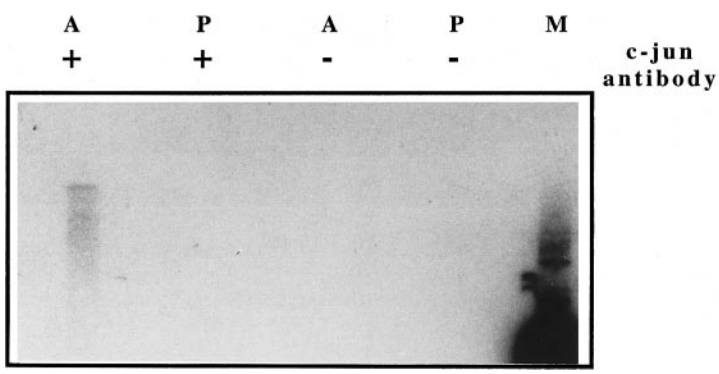

FIG. 2. Southern blot analysis. Experiments were performed according to Saluz and Jost (8). Hybridization was carried out with a probe generated by random priming (50 μCi [α-^{32}P]dATP/μg DNA) from an isolated reDNA-library clone. Lane 1 shows a c-Jun specific DNA-recognition element isolated from the aqueous phase with c-Jun specific antibody (aqueous phase, antibody). In lane 2 (phenol phase, antibody), lane 3 (aqueous phase, no antibody) and lane 4 (phenol phase, no antibody) no signals could be detected. Lane 5 shows the signals of ^{32}P-labeled DNA marker molecules (pUC18, *Hpa*II digest).

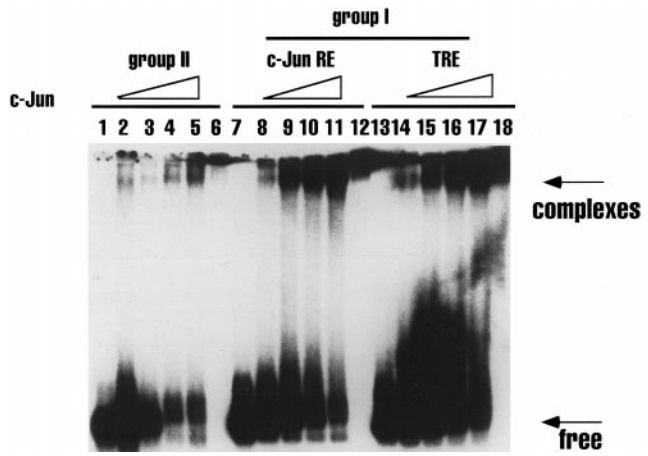

FIG. 3. Specificity test of c-Jun binding DNA molecules of the reDNA library by gel retardation experiments. The three different DNA fragments shown (lanes 1–6, 7–12, and 13–18) contain a binding motif belonging to the second group (AAACAGATCATACAAGC; lanes 1–6) and two belonging to the first group: c-Jun RE (TGAC, lanes 7–12) and TRE (TGACTCA, lanes 13–18). All samples were incubated in the presence of plasmid pTRCAT (23) containing the promoter region of the transin gene with two TREs [molar excess 3 (mol/mol)]. 20 fmol of DNA were incubated without any protein (lanes 1, 7, and 13), in the presence of 10 ng c-Jun protein (lanes 2, 8, and 14) 20 ng (lanes 3, 9, 15), 50 ng (lanes 4, 10, and 16) and in the presence of 100 ng c-Jun protein (lanes 5, 11, and 17). In lanes 6, 12, and 18 the DNA probes were incubated in the presence of 50 ng c-Jun without competitor. [The maximum protein dimer to DNA ratio here used was 62 mol/mol which is one fifth of the ratio recommended by the manufacturer (Promega).]

0.2 units of DNaseI in a total volume of 15 μl for 2 min and the reaction was stopped by adding 80 μl of proteinase K buffer (30 mM EDTA, 0.25% (w/v) SDS, 0.5 mg/ml proteinase K). After incubation at 42°C for 1 h, the samples were phenol extracted, ethanol precipitated and re-suspended in 4 μl formamide loading buffer. The samples were run in a 6% polyacrylamide gel and autoradiographed.

RESULTS AND DISCUSSION

An overview of the experimental procedure used to generate reDNA libraries is given in Fig. 1 and described in detail under Material and Methods. A brief outline, and the rationale behind the key steps, follows below. The least invasive manner to capture protein/DNA-complexes in rapidly changing equilibrium *in vivo* is by means of nanosecond high energy UV-pulse laser irradiation that leads to covalent bonds between the protein and its DNA target (9, 10). In addition, this treatment has the great advantage that the DNA is hardly damaged by strand breaks or intramolecular cross-links. Partial digestion, using a restriction endonuclease with a hexanucleotide recognition sequence (*Bam*HI), is performed on isolated nuclei to reduce the viscosity of the genomic DNA and the nucleoprotein complexes are enriched by centrifugation through cesium-chloride (CsCl) gradients. By this means DNA fragments with an average of 4000 bp should be obtained. However, due to protein protection of the native DNA in the nuclei where the restriction endonuclease digestion takes place, much less cleavage within the DNA occurs, resulting in longer fragments than theoretically expected. To avoid undesired *in vitro* interactions between non-cross-linked proteins and free DNA recognition elements, the

TABLE 1

List of the Minimal AP-1 Target Motifs (TGAC) as Found in the Selected reDNA

Group 1

TGAC**TNN**	Frequency %	TGAC**ANN**	Frequency %
tgac**tgt A**	4.5	tgac**att**	3.0
tgac**tgg**	1.5	tgac**atg**	4.5
tgac**tga**	4.5	tgac**ata**	1.5
tgac**tgc**	4.5	tgac**agg**	3.0
tgac**ttg**	1.5	tgac**aga C**	4.5
tgac**tta**	1.5	tgac**agc**	3.0
tgac**ttc**	3.0	tgac**aat**	1.5
tgac**tct F**	3.0	tgac**aac B-D**	8.0
tgac**tca TRE**	3.0	tgac**aaa**	1.5
		tgac**act**	3.0
		tgac**acg**	1.5
		tgac**acc E**	1.5
		tgac**aca**	8.0
TGAC**GNN**	%	TGAC**CNN**	%
tgac**ggt**	1.5	tgac**ctt**	4.5
tgac**ggc**	1.5	tgac**ctg**	1.5
		tgac**cta**	3.0
		tgac**ctc**	1.5
		tgac**cga**	1.5
		tgac**cat**	4.5
		tgac**cca**	1.5
		tgac**ccc**	3.0

Group 2

Without TGAC		%
atgattctgccttgctgc	**G**	1.5
aaacagatcatacaagc	**H**	1.5
ggttagctaagagat		1.5
gtttacta	**I**	1.5

Note. The DNA molecules that belong to class 2 have been identified by footprinting experiments (see Fig. 4) and are entirely different from all c-Jun binding elements described so far, i.e. they do not contain any TGAC binding motif. A number of 36 different targets was detected per 100 sequences analyzed. The frequency indicates how often each target with the same binding but different flanking sequences was found among the different reDNA clones. Identical clones were found much more often than once. However, this number was not used for the frequency calculation due to the amplification of the targets prior to their cloning. The footprints of elements **A–I** are shown in Fig. 4.

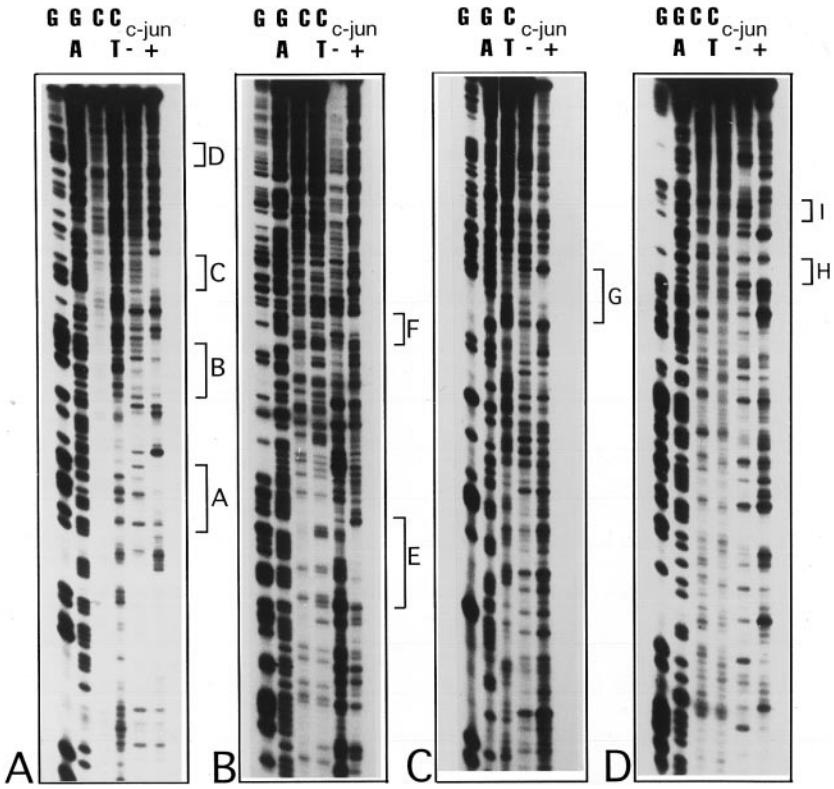

FIG. 4. DNase I protection analysis. A and B show class 1 DNA molecules containing TGAC motifs. Panels C and D show class 2 molecules that do not contain a TGAC motif. Control sequence reactions were performed according to Maxam & Gilbert (24) and are marked with G, A +G, C + T and C, respectively. DNase I treated samples of reDNA library are indicated by − (without c-Jun) and + (in presence of c-Jun). The footprints are specified with brackets (**A**–**I**). The following protected sequences were found: **A,** 5′AGACAAG**TGACTGT**GAA 3′; **B,** 5′AATA**TCTGTCA**TTAGTGAATGG3′; **C,** 5′TCTT**TGACAGA**TA 3′; **D,** 5′AA**GTTGTCA**GGCC3′; **E,** 5′ CTCAAATA**TGACACC**3′; **F,** 5′ GG**AGAGTCA**GG3′; **G,** 5′**ATGATTCTGCCTTGCTGC**3′; **H,** 5′**AAACAGATCATACAAGC**3′; **I,** 5′**GTTTACTA**3′.

protein/DNA-complexes are additionally purified by phenol extraction to disrupt non-crosslinked protein/DNA complexes. To facilitate cloning, sequencing and interaction analysis, these genomic DNA fragments associated with proteins are further trimmed by a four base-recognition sequence endonuclease (SauIIIA). This limits the size of the DNA to approximately 250 base pairs around the interaction site and generates identical ends to the ones created in the first digestion step. Consequently, all fragments terminate with the same sequence, a prerequisite for the later amplification step. Selectivity is achieved by using antibodies against the transcription factor of interest in the immunoprecipitation of the corresponding nucleoprotein complexes. The purified DNA is then subjected to ligation-mediated PCR (11) and the exponentially amplified DNA cloned subsequently. Sequencing of the inserts of such a reDNA library provides information not only about the various recognition elements that had been occupied *in vivo* by a specific transcription factor at the time of UV irradiation but also about the regions surrounding those sites. A search through DNA sequence databases with the latter sequence data should greatly facilitate the identification of the associated gene(s). Finally, the DNA recognition elements can be tested for their specificity by gel retardation- and *in vitro* DNaseI footprinting experiments.

To demonstrate the potential of reDNA libraries in elucidating specific DNA binding sites *in vivo,* we constructed such a library for the protooncogene-protein c-Jun (12), a member of the AP-1 transcription factor family, in rat *cerebellum,* where expression of c-Jun is high (13). Neuronal excitation elicits rapid transcriptional activation of several immediate-early genes, e.g., c-Jun (14), many of which encode transcription factors that modulate expression of downstream genes involved in long-term plasticity changes (14, 15). A central role has been proposed for the early responsive genes as, third messengers“ in the cytoplasmatic signaling involved in short- and long term memory (16).

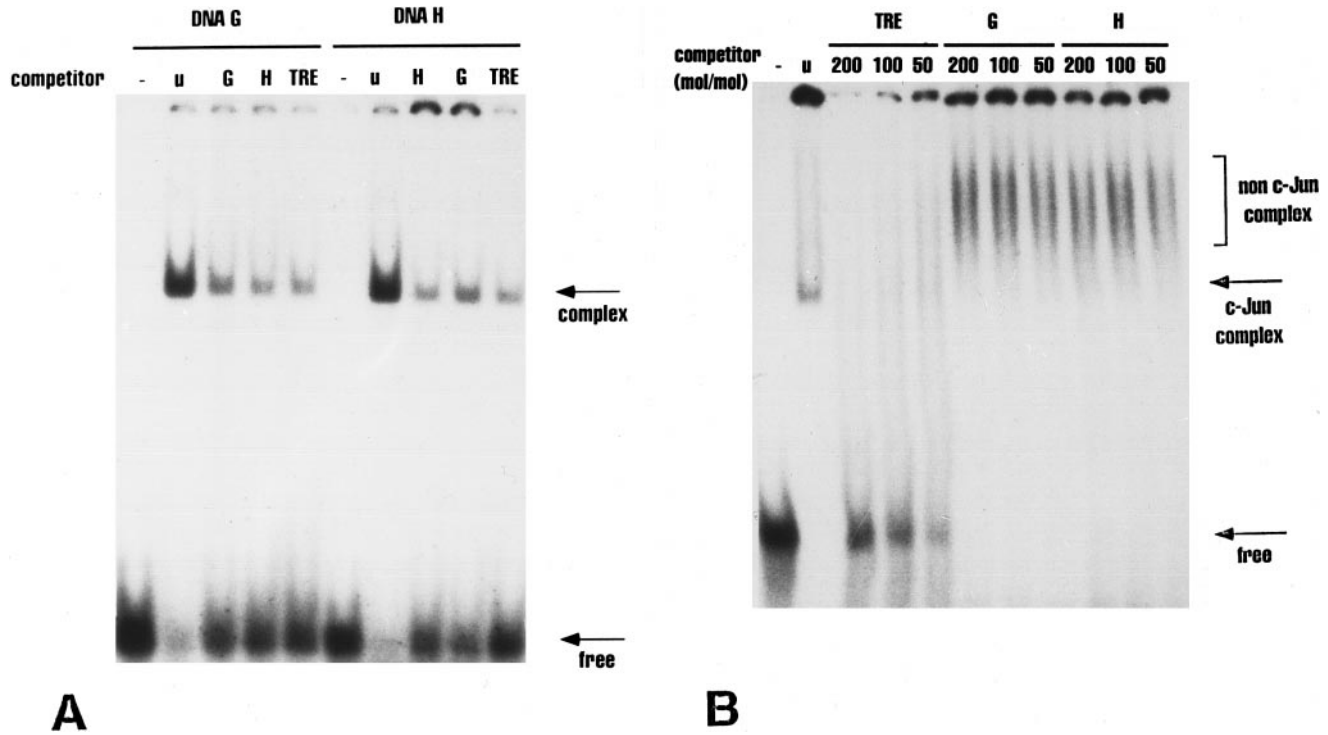

FIG. 5. Competition assays. The following probes corresponding to the footprints in DNA G and DNA H (see Figs. 4C and 4D) were used: **G,** 5′ATGATTCTGCCTTGCTGC3′; **H,** 5′AAACAGATCATACAAGC3′. For competition the following 46 bp double-stranded control DNA (TRE) was used: 5′AAGGCGCCGGCG **ATGATCGTGACTCAGCGCGA** ACGGATCCGGAAGC3′.Its central part (bold) corresponds to the AP-1 site of the metallothionein gene (16, 17). (A) The labeled oligonucleotides (G and H, respectively) were incubated in 10 μl of binding buffer (10 μl 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% (v/w) Tween 20, 30 mM KCl) together with 1μg of HeLa nuclear extract in presence of a 20 molar excess of one of the following oligonucleotides: H (H), G (G), TRE (TRE) and 1 μg of *E. coli* DNA as unspecific competitor (U). The complexed DNA and the free DNA are indicated by arrows. (B) The labeled oligonucleotides of DNA TRE (TRE) were incubated with unlabeled DNA TRE (TRE), DNA G (G) or DNA H (H). Three different competitor/DNA-probe ratios were used: 200, 100, or 50 (mol/mol). The arrows indicate non-c-Jun complexes, c-Jun complexes and free DNA, respectively.

To test the reliability of the procedure Southern-blot experiments were performed (Fig. 2). For these experiments the nucleoprotein complexes were selected with and without antibodies coupled to protein A–Sepharose. One of the *in vivo* selected c-Jun DNA-binding elements was used as a probe. Only in the track of the antibody selected nucleoprotein complex a specific band could be detected (Fig. 2).

It had been shown *in vitro* that the, optimal" AP-1 recognition sequence (termed TRE) was TGA(C/G)TCA (17, 18). The two overlapping half sites, TGAC and TGAG, are not equivalent due to the asymmetry imposed by the central C:G base pair (17, 18). Biochemical and crystallographic analyses indicated that the optimal half-site for the AP-1 and ATF/CREB proteins was TGAC (19). As shown by gel retardation experiments, all tested inserts of the reDNA library were capable to form specific complexes with purified c-Jun transcription factor under highly stringent conditions (Fig. 3). Surprisingly, out of 100 independent DNA clones from the library, 36 different c-Jun binding elements were found (Table 1). These could be divided into two groups (Table 1): the first group (group 1: 88%) contained the "optimal half-site" for the AP-1 and ATF/CREB proteins (TGAC) (19, 20); the second group (group 2: 12%) did not show any obvious similarity with the above "optimal half-site." As shown in Table 1, by far not all theoretically possible "TGACNNN" combinations were found, indicating that the selection of c-Jun binding sites was not random. Moreover, as indicated in Table 1, "N" in the motif "TGACN" was found to be much more often represented by "A" (43%) than by "G" (3%).

The frequency shown in Table 1 indicates how often each target with the same binding but different flanking sequences was found among the different reDNA clones. Identical clones were found much more often than once. However, this number was not used for the frequency calculation due to the amplification of the targets prior to their cloning.

To exclude PCR-artifacts due to a potential influence of undigested amino acid residues crosslinked to the DNA, the procedure was repeated *in vitro* with DNA that contained the TRE-element and no mutation within the DNA-binding element was found (data not shown). The TRE consensus sequence TGA(C/G)TCA, belonging to the first group and described in earlier *in vitro* interaction experiments (17, 18) was found in only 3.0% of all *in vivo* DNA recognition elements tested.

A computer analysis showed that the targets found corresponded to several previously described transcription factor binding sites not belonging to the AP-1 family, e.g., estrogen-responsive elements (21), the sequence of which is known to interact with c-Jun *in vitro* (22). Therefore, new molecular pathways can be expected where either c-Jun combined with another factor interacts with the same consensus sequence or where c-Jun can replace another transcription factor. The current lack of non-coding regions in the public sequence databases did not allow a correlation of the analyzed c-Jun reDNA to known genes. However, with the progress of the various genome-sequencing projects this should be feasible soon.

To define the *in vitro* interaction sites on the DNA fragments selected for their binding capacity *in vivo*, DNAseI protection experiments were performed. Again, all c-Jun REs that had already been shown to be positive in gel retardation assays (Fig. 3) interacted with the purified c-Jun *in vitro*. Some of the footprints are shown in Fig. 4. The sequence of the DNA fragments (Figs. 4A and 4B) contained four and two c-Jun REs, respectively. All of them were protected by the protein. *In vivo*-selected molecules lacking c-Jun REs (Table 1), were also investigated. Interaction of c-Jun with these novel elements could also be identified *in vitro* (Figs. 4C and 4D). To confirm the specificity of these elements, band shift competition experiments were performed using HeLa nuclear extract and oligonucleotides containing the AP-1 binding element (TRE) of the metallothionein gene and the c-Jun binding elements G and H (Figs. 4C and 4D). Figures 5A and 5B show that each of the oligonucleotides competed for the same protein complex. However, the observed binding to elements G and H was weaker than to the TRE. To compete for TRE-binding by the protein complex, a more than 20 fold excess of the oligo containing G and H was required while this excess of TRE was sufficient to compete the G and H elements, respectively. By standard *in vitro* selection (1), these binding elements would not have been detected.

Our data indicate that binding site selection *in vivo* requires other selective means than just binding affinity. Although protein/DNA interaction studies *in vitro* provided very valuable information, it does not necessarily reflect the actual situation *in vivo*. For instance, the fact that in living cells DNA is packaged into chromatin is totally disregarded. In the method introduced here, transcription factors are, trapped' at the site of their interaction with native DNA, a highly complex chromatin structure, and thus, the reDNA libraries provide a more authentic picture of the binding sites used in living cells. Furthermore, this technique can be applied to the determination of genomic target sequences of any DNA-binding protein that can be selected by immunological means, independent of its binding affinity *in vitro*. Last but not least, it aids in the identification of all the genes, the transcription of which is affected by a certain transcription factor.

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