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UNIVERSALITY OF C-*FOS* TRANSCRIPTIONAL REGULATION: THE DYAD SYMMETRY ELEMENT MEDIATES ACTIVATION BY PMA IN T LYMPHOCYTES

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SUMMARY: We here have delineated the regulatory sequences responsible for c-*fos* transcriptional activation in human primary T lymphoblasts and in a human tumor T cell line (Jurkat), using transient transfection assays. Our results indicate that, as it has been demonstrated for fibroblastic or epithelial cells, the Dyad Symmetry Element is necessary and sufficient to confer responsiveness to an heterologous promoter in both cell types. Protein binding to this element was constitutive, as assessed by gel shift assays. These results suggest that c-*fos* transcriptional regulation occurs through a widely conserved mechanism in highly differentiated tissues.

The c-*fos* proto-oncogene is a direct target for signal transduction pathways by a number of external stimuli in a wide variety of cell type (1-5), including T lymphocyte (6-11). In T lymphocytes, the Protein kinase C seems to play a major role in the cell activation process (12-17) and more specifically in c-*fos* transcriptional activation (18-20).

The mechanism underlying c-*fos* transcriptional regulation at the genome level is under intensive investigation in cells from fibroblastic, epithelial or nerve origin. Most of the reports agree on the pivotal role played by the DSE, located around -300 bp (with reference to the transcription start site) and which is involved in c-*fos* induction by a variety of fibroblastic growth factors as well as the PK C activator PMA (21-27). The mechanism through which the DSE activates transcription is unknown. It has been demonstrated that at least two proteins bind to the sequence, $p67^{SRF}$ and p62. However, binding occurs in a constitutive manner, *in vitro* (22) as well as *in vivo* (28).

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Abbreviations used: DSE: Dyad Symmetry Element; AP-1: Activator Protein 1; CAT: Chloramphenicol Acetyl Transferase; PMA: Phorbol Myristate Acetate; PK C: Protein Kinase C.

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In this study, we have delineated the sequence responsible for PMA response in a tumor T cell line and in primary T lymphocyte.

METHODS

Cells and culture: Jurkat cells were maintained in RPMI 1640 (GIBCO) supplemented with L-glutamine and antibiotics to standard concentrations, and 5% heat-inactivated fetal calf serum.

Resting T lymphocytes from normal human volunteer donors were purified by Ficoll-Hypaque, plastic adherence and Percoll density gradient. Blastogenesis was induced by a 3 days culture in the presence of PHA (Sigma, 10 ug/ml) and IL2 (rIL2 was a kind gift of SANOFI, Toulouse, France, and was used at 0.5 ng/ml). Prior to transfections, cells were quiesced down 18h in IL2-free culture medium.

Plasmids and oligonucleotides: FC3 and FC4 were constructed by subcloning the c-*fos* sequences described in (29) in pGEM-CAT C (30). . Plasmids p- pA-, pB-, pC-, pD-, pG-tkCAT mutants include various lengths of the human c-*fos* promoter inserted upstream of the herpes thymidine kinase promoter (29, 31).

Plasmids p 500-, p 350- and p 250-tk CAT have been constructed by inserting the Hind III-Apa I, the Hind III-Pst I and the Pst I-Pst I fragment of pA-tk CAT respectively into the Bgl II site of ptk CAT. Plasmid p(A-B)-tk CAT has been constructed by deletion of the Hind III-Apa I fragment in pA-tk CAT. Both strands of the oligonucleotides DSEAP1, M1 DSEAP1 (27), M2 DSE AP1 (32), M1 DSE and fAP1 (Table 1) were synthetised using an Applied Biosystem DNA synthetizer and annealed. Plasmids pDSEAP1-, pM1 DSEAP1- and pDSE-tkCAT were constructed by insertion of the corresponding oligonucleotides between the Nde I and Hind III restriction sites in p-tkCAT. pM2 DSE AP1, pM1 DSE and pfAP1-tk CAT were constructed by inserting the oligonucleotides in the Bgl II site of ptk CAT. All constructs were controled by direct nucleotide sequence analysis, using the Sequenase sequencing kit (USB).

RSV SEAP (33) contains the SEAP (for secreted alkaline phosphatase) gene, under the control of the Rous sarcoma virus Long Terminal Repeat.

Transfections: Transfection of Jurkat cells were performed as previously described (34). Cells were resuspended in warm medium at a concentration of about 5.10^5/ml and cultured for a period of 3 hours.

Primary T cells were transfected by electroporation according to Cann et al. (35) and harvested after a 3 hours period of culture.

CAT assay: Living cells were purified on an LSM (Eurobio) density gradient, washed in PBS and then in Tris 0.25M (pH 7.9). Cells were resuspended in 100 ul of Tris 0.25M (pH 7.9) and lysed by repeated freeze/thawing.

A highly quantitative soluble assay was used for tumor T cells (34). In most experiments, the secreted alkaline phosphatase expressing plasmid RSV SEAP was used to control transfection efficiency (33). The electroporation conditions used here resulted in highly standardized transfections (variability < 1.5 fold).

The standard thin-layer CAT assay had to be used for primary T cells (35).

Nuclear protein extracts: Nuclear proteins were extracted according to Dignam et al. (36). For PMA treatment periods shorter than 10 min, activation of cells was performed in sterile eppendorf tubes (34). For DNase I footprints, nuclear extracts were further concentrated by ammonium sulfate precipitation (45 %) and extensively dialysed against Hepes pH 7.9 20 mM, KCl 50 mM, 0.3 mM EDTA, 0.15 mM EGTA, 1 mM DTT, 1 mM PMSF and 20 % glycerol.

DNase I footprints: The probe was a Hind III- Pst I fragment of pB-tk CAT, end labeled by filling in the Hind III site using the Sequenase enzyme (USB, 5 min 37°C) followed by purification on a non denaturing acrylamide gel. Proteins were incubated in a total volume of 6 ul with 20-40 fmole of probe followed by treatment with various concentrations of DNase I (Worthington) for 1 min at room temperature. DNase was stopped by adding 50 ul of 1% SDS, 0.2 M NaCl and 20 mM EDTA. Samples were

phenol and chloroform extracted, ethanol precipitated and loaded onto a 6% sequencing denaturing gel. A G and C chemical sequences of the probe were performed, using an NEN kit, and run in parallel.

Gel shift assay: Oligonucleotides were labelled with ^{32}P ATP (Amersham, >3000 Ci/mmol) using T4 polynucleotide kinase (Boerhinger) to 0.5-1 10^8 cpm/ug and purified on a non denaturing acrylamide gel. Alternatively, the Bgl II or Hind III sites of the oligonucleotides were filled in using the Sequenase polymerase (USB) and alpha ^{32}P dCTP for 5 min at 37°C, which achieves a higher specific radioactivity (around 5 10^8 cpm/ug). Gel shift assay was performed as previously described (34).

RESULTS AND DISCUSSION

Primary or tumor T cells were transfected by electroporation with reporter constructs including various segments of the human c-*fos* regulatory region linked to the reporter gene encoding the CAT enzyme. Transfected cells were divided into two aliquots which were treated, or not, with the PK C activator PMA (50 ng/ml) so that data for a given construct were obtained from the same pool of transfected cells. Results (Figure 1) indicate that in both cell types, the sequence comprised between -711 and-100 confers PMA responsiveness both to the homologous c-*fos* promoter (FC 3) and to the heterologous thymidine kinase (tk) promoter (pA tk). Analysis of deletion mutants in this region (Figure 2) indicates that the sequence -323 to -290 is necessary and sufficient to confer PMA responsiveness to the constructs in both cell types. These data strongly suggest that the DSE element is responsible for this effect. However, immediately downstream from the DSE is located an 8 bp element, homologous to both the canonical AP1 binding site and the cAMP responsive element (CRE). This element is recognized by purified Jun/AP1 *in vitro* (37) and has been shown to mediate a positive effect on c-*fos* transcription in at least two situations (32, 37). Analysis of nuclear extracts from Jurkat cells by DNase I footprint (Figure 3), indicates that, similar to what is observed with proteins from fibroblastic origin, the protected sequence extends beyond the DSE site, and in particular, on the AP1 like site. In order to assess the respective function of the DSE and the AP1 like element in both primary and tumor T cell, various oligonucleotides were synthetized, corresponding to the wild-type sequence, which includes both the DSE and the AP1 site (DSE AP1) and mutated forms of this sequence. Gel shift assays were used to compare protein binding to these various oligonucleotides in both cell type: High affinity binding occurs only at the level of the DSE site. Contrary to what was observed in Hela cells (32), in no situation were we able to detect an AP1 specific binding when the wild type or mutated oligonucleotides were used as probes, even in large excess of oligonucleotide (data not shown).

The oligonucleotides were next inserted in front of the thymidine kinase promoter and the resulting constructs were used in the transient transfection assay. Results are

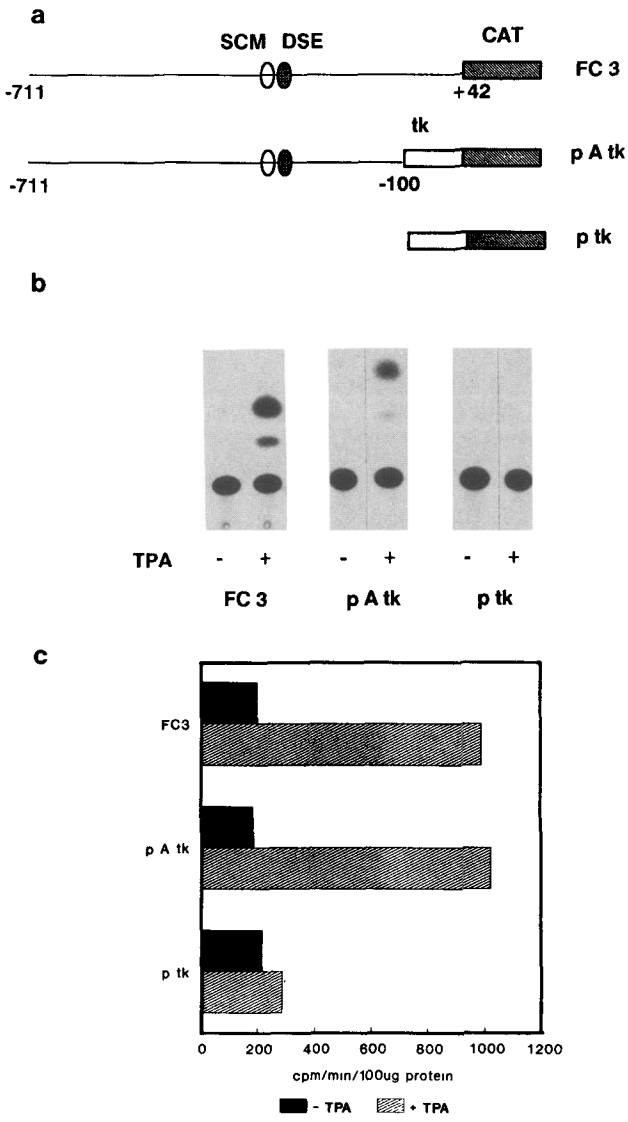

Figure 1. Activation of *fos*-CAT reporter constructs by PMA in human primary (b) and tumor T lymphocytes (c). a) Reporter constructs used: solid line: c-*fos* sequence; shaded box: CAT gene; open box: thymidine kinase promoter. Hallmarks of the c-*fos* regulatory sequence are indicated by circles: open circle: sis-conditioned medium responsive element; shaded circle: dyad symetry element. b) Autoradiogram of a typical CAT assay using protein extracts from primary T cells. c) Results from a typical soluble CAT assay in Jurkat cells.

summarized in Table 1. In the tumor T cell line, any mutation introduced into the DSE element virtually abolished the response, whereas the elimination of the AP1 site did not have any effect, indicating that the DSE element was both necessary and sufficient to confer inducibility in these cells. Similar results were obtained in primary T cells, in which, however, more drastic mutations were needed for complete abrogation of the response. Nethertheless, the AP1 like element was dispensable for PMA response in

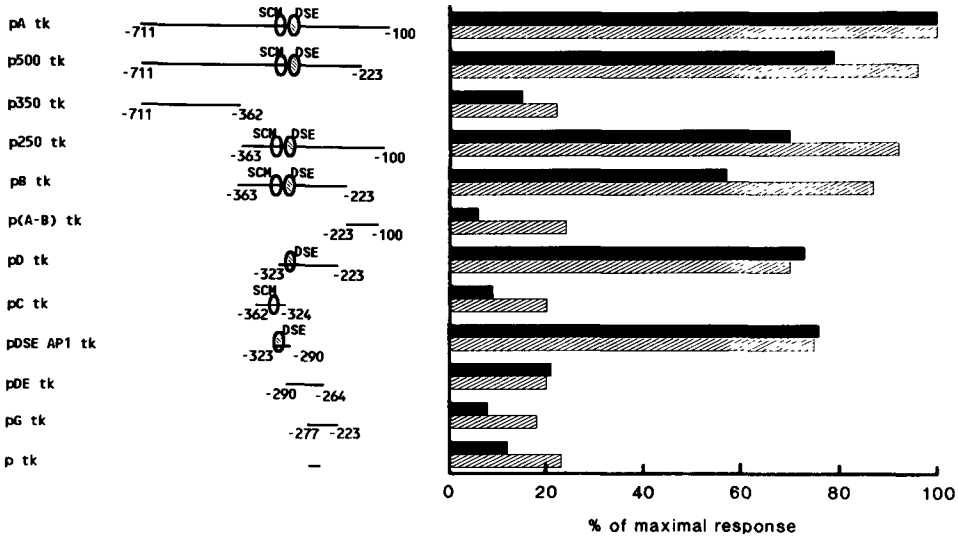

Figure 2. Determination of the responsive sequence in primary (solid boxes) and tumor (shaded boxes) T cells. Left panel: Human c-*fos* sequences included in the reporter constructs; right panel: PMA response, showed as a percent of maximal response : CAT assays were quantified by scintillation counting of acetylated chloramphenicol for tumor T cells and by excision and counting of the chloramphenicol spots for primary T cells. The fold induction was calculated by dividing the data from PMA treated cells by the data from untreated controls and standardized by comparison to the most complete sequence (pA-tk CAT); the mean of 2-6 experiments is shown.

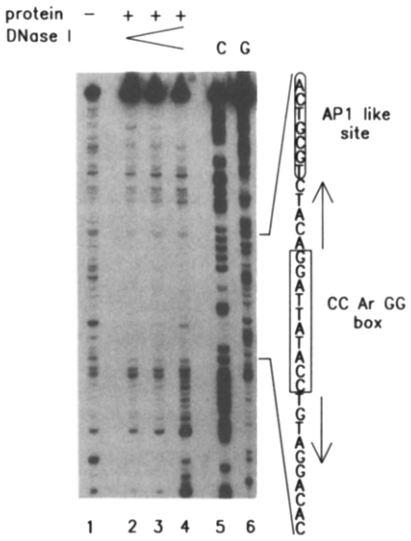

Figure 3. Analysis of protein binding to the DSE region by DNase I footprinting. A Hind III-Pst I fragment from pB-tk CAT, including from -363 to -222 of c-*fos* promoter, was end-labeled at the Hind II site (on the non coding strand) and incubated (lane 2-4) or not (lane 1) with 35 µg of Jurkat nuclear extracts. DNase treatment was for 1 min, using increasing doses of DNase. Lane 5 and 6 show chemical sequences of the same fragment (corresponding to C and G on the coding strand). On the right is shown the extent of protection (coding strand sequence); the rounded box designates the AP1-like site, the square box the CC Ar GG box and the arrows the dyad symetry element.

Table 1. Effects of mutations introduced into the DSE or AP1 like element on protein affinity and TPA response

Oligonucleotide	Sequence[a]	DNA binding[b]		TPA response[c]	
		Tumor T	Primary T	Tumor T	Primary T
wt	CAG**GATGTCCATATTAGGACATC**tgcgtcaGC	+++	+++	+++	+++
M1DSE AP1	CAG**GATATCCAAATTAAGACATC**tgcgtcaGC	+/-	+/-	+/-	++
M2DSE AP1	CAG**GATGTCCATCGGCTGACATC**tgcgtcaGC	-	-	-	-
DSE	CAG**GATGTCCATATTAGGACATC**TG	+++	+++	++	++
M1DSE	CAG**GATATCCAAATTAAGACATC**TG	+/-	+/-	+/-	++
AP1	**ATC**tgcgtcaGC	-	-	-	-

[a] Oligonucleotides also included restriction sites at both ends, bold face: DSE element, lower case: AP1 like element, mutations are underlined.
[b] DNA binding was assessed by gel shift assays, either by direct binding to the sequence or by inhibition of the wild type sequence; +++ and + direct binding to high level and inhibition with 10 fold excess; +/-: direct binding detectable but low and inhibition with large excess; - no binding detectable and no inhibition with any excess used.
[c] Oligonucleotides were inserted upstream from the tk promoter and the corresponding constructs were used in transient transfection assays; ++ 8 to 15 fold activation by TPA; ++ 3 to 5 fold activation; +/-. 1.5 to 2 fold activation; - 1 to 1.5 fold activation.

this cell system and did not confere any responsiveness to the construct when isolated.

We have next performed gel shift assays with proteins extracted after various periods of activation (Figure 4). In fibroblasts, protein binding to the DSE element is constitutive and independent from the activation status of the cells (22, 28). Likewise, after various periods of PMA treatment (Figure 4), we could not detect any significant change either in the pattern of the complexes or in the amount of binding, indicating that transcription initiation is not regulated through modulation of factor binding affinity for the sequence. Interestingly, a sequence similar to the DSE has been described in the Interleukin 2 receptor α chain gene (38, 39), in which it seems to play a major role in Jurkat cells. Contrary to what we observed using c-*fos* DSE as a target, PMA

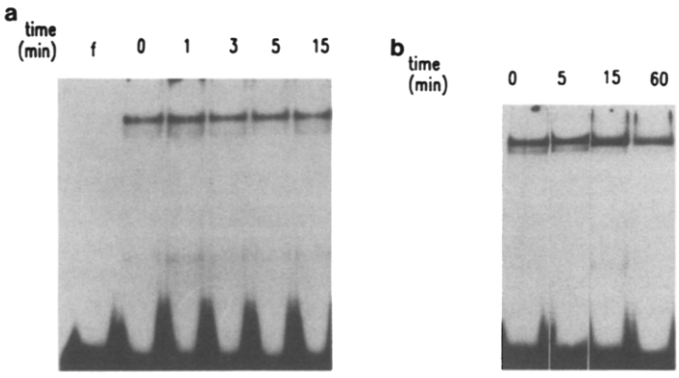

Figure 4. Protein binding to the DSE element is constitutive both in primary and tumor T cells. Gel shift assay of protein extracted from primary (a) or tumor (b) T cells after the indicated time of treatment with PMA.

induction of the IL2 Rα gene is accompanied by a decrease in protein binding to the IL2 Rα "SRE like element" (39). As this sequence includes only a CC-Ar-GG box like element and not the c-*fos* dyad symetry element, this discrepancy might reflect a difference of the composition or the affinity of the specific complexes.

Taken together, our results suggest a high degree of conservation among differentiated tissues as distinct from each other as fibroblast and lymphocyte for c-*fos* transcriptional regulation. Indeed the same regulatory element, the DSE, mediates the response to external stimuli in both cell types.

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