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The reversion of highly tumorigenic cell lines to non-tumorigenic phenotype is associated with c-*jun* down-expression

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Abstract Using model spontaneously reverting cell lines, c-*jun*, *jun*B, *jun*D and c-*fos* oncogene expression was investigated. c-*jun*, but not *jun*B, *jun*D or c-*fos*, was overexpressed in highly tumorigenic clones. The reversion of cells to the non-tumorigenic phenotype resulted in a dramatic decrease in c-*jun* expression. CAT assays revealed that c-*jun* overexpression in tumorigenic cells was associated with higher transcription activity. No correlation between c-*jun* oncogene expression and AP-1 transcription factor activity in tumorigenic and non-tumorigenic clones was found.

Key words: Revertant; Transformation; Tumorigenicity; Oncogene; AP-1

1. Introduction

A model of spontaneous tumor progression and reversion of cells to non-tumorigenic phenotype in vitro was previously described by Lavrovsky et al. [1]. In that study several independently transformed cell lines were isolated from embryonic fibroblasts of mice and rats with different genotypes. Highly tumorigenic cell variants, including metastatic ones, were selected in six cell lines. At the beginning of transformation, all cell lines were found to be non-tumorigenic, or only slightly so. At the same time, they demonstrated high sensitivity to the density-dependent inhibition of cell growth and possessed sensitivity to serum growth factors. During tumor progression, both properties changed dramatically. It is interesting to note that, in the process of cloning, four of six highly tumorigenic cell lines were shown to revert with high frequency to the non-tumorigenic state, possessing their initial growth characteristics.

Additionally, the frequencies of reversion of cell lines were constant genetic characteristics of the cells. Surprisingly, reverted cells could revert back to the highly tumorigenic phenotype, although the frequencies of back reversions were much more rare. Thus, we have obtained several groups of tumorigenic clones which could interconvert. The mechanism of tumor cell transformation, and the mechanisms of reversion and back reversion, could have common motifs. Studies of this model may shed light not only on the process of tumor transformation, but also on neoplastic non-stability mechanisms, which may be quite important for the prognosis of neoplasms.

It is known that several of the immediate-early genes, such as members of the *fos* and *jun* families, encode transcription factors, and play an important role in the complex signals for growth and differentiation [2]. In the present report we show that c-*jun*, but not c-*fos*, *jun*B or *jun*D, is overexpressed in highly tumorigenic clones compared with its low expression in reverted ones and that such c-*jun* overexpression is associated with higher transcription activity in tumorigenic clones.

2. Materials and methods

2.1. Cell cultures

FCBA2V10 (clone 1) and non-tumorigenic revertant (clone 23), as well as tumorigenic FC3H3V7 (clone 29) and non-tumorigenic revertant (clone 20) were used [1]. The cell cultures were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% FCS in 5% CO_2 at 37°C. All cell cultures were found to be mycoplasma-free [1]. Retardation of growth of reverted clones was attained by cultivating cell monolayers for 24–48 h in medium with 10% bovine adult serum. Stimulation of those cells was achieved by 20% FCS.

2.2. RNA extraction and Northern blot analysis

RNA extraction and Northern blot analysis were performed as described [3]. Blots were hybridized with the *Eco*RI-*Eco*RI cDNA fragment of the mouse c-*jun*, or *jun*B, or *jun*D (ATCC, MD; nos. 63026, 63024, 63025, respectively), as well as the 1.0 kb pair *Pst*I-*Pst*I fragment of v-*fos* (specific radioactivity 5×10^5 cpm/μg). As a control, all filters were reprobed with cDNA encoding G3PDH (Clontech) to ensure that equal amounts of RNA were loaded onto each lane.

2.3. Electrophoretic mobility shift assay (EMSA)

Synthetic oligonucleotides corresponding to the TPA responsive element (TRE) of the metallothioneine promoter region (5'-GATCCATGAGTCAGAG) were synthesized by a standard method [4]. The oligonucleotides were 5'-end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase, and purified by polyacrylamide gel electrophoresis followed by reverse phase HPLC (Silasorb-C8; 0–50% gradient of acetonitrile with 20 mM $LiClO_4$). Nuclear protein extracts were prepared following the method of Digham et al. [5]. EMSA was performed as previously described [6]. 5 μg of nuclear protein and 10^4 cpm of oligonucleotide with specific radioactivity 10^6 cpm/μg were taken per single reaction mixture. As a non-specific DNA-carrier, 2 μg of sonicated total DNA from bovine thymus were used. Control competition experiments included 50-fold molar excess of specific non-radiolabelled oligonucleotides.

2.4. Transient transfection and CAT assay

Plasmid *jun*-CAT was constructed by inserting the *Bam*HI-*Sal*I (2.4 kb) fragment of the human c-*jun* promoter region into the *Bam*HI-*Xho*I sites of the *tk*-CAT plasmid instead of the TK promoter. Cells were transfected with 3 μg of plasmid DNA per flask, using a mammalian transfection kit (Stratagene, CA; no. 200388). Cells were incubated with the DNA coprecipitate for 4 h in media without serum in 3% CO_2 at 37°C, washed with PBS, pH 7.4, and further incubated in DMEM containing 10% FCS for 24 h in 5% CO_2 at 37°C. Transfection

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Abbreviations: CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum; AP-1, activator protein 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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efficiency was monitored by transfection with the *tk*-CAT plasmid. CAT activity of the cell extracts was determined according to the method of Gorman et al. [7]. 10 μg of the cellular protein, 0.1 mg of acetyl CoA and 5 μCi of [^{14}C]chloramphenicol were used for each CAT assay. All experiments were performed at least 3 times.

3. Results and discussion

Fig. 1 shows the data concerning c-*fos*, c-*jun*, *jun*B and *jun*D mRNA content in two pairs of clones with CBA and C3H genotypes. c-*fos* mRNA was observed in both reverted clones only after 30 min stimulation of the quiescent cells with FCS (lanes 2 and 6, respectively). Neither logarithmic non-tumorigenic clones nor logarithmic tumorigenic ones showed any significant quantity of c-*fos* mRNA (lanes 3, 7 and 4, 8, respectively). These findings are in complete accordance with previous data concerning c-*fos* regulation in normal and some tumorigenic cells [2,8,9]. *jun*B mRNA was expressed in a manner similar to that of c-*fos* in both cell types, while *jun*D mRNA was undetectable in all cell types . Most interestingly, c-*jun* mRNA levels were similar to c-*fos* mRNA levels only for reverted clones (Fig. 1, lanes 1–3 and 5–7). In highly tumorigenic clones c-*jun* overexpression was observed (Fig. 1, lanes 4 and 8).

Transient transfection experiments using the *jun*-CAT plasmid were performed to analyze the transcription activity of the c-*jun* promoter in tumorigenic and non-tumorigenic cells. Cells were studied under the same growth conditions (logarithmic phase of growth). As seen in Fig. 2 (upper panel), the transcription activity of the c-*jun* promoter in tumorigenic cells was significantly higher (lane 2) than that in non-tumorigenic cells (lane 1). Transfection efficiency was about the same for both cell lines, as monitored by *tk*-CAT plasmid transfection (Fig. 2, lower panel). The c-*jun* promoter region contains binding sites for the transcription factors Sp-1, CTF, NF-*jun*, AP-2 [10–12], and AP-1, which can positively autoregulate c-*jun* protooncogene expression [13]. A high level of AP-1 transcription factor binding activity could be expected as a consequence of c-*jun* overexpression in tumorigenic clones. Our experiments using EMSA with AP-1 oligonucleotides eliminated the AP-1 transcription factor as a possible cause or consequence of c-*jun* overexpression because the activity of this transcription factor was equal in both tumorigenic and non-tumorigenic cells in the logarithmic phase of growth (Fig. 3A and B, lanes 3 and 4). The quiescent non-tumorigenic cells showed almost undetectable levels of AP-1 binding activity even after stimulation with 20% FCS for 30 min (Fig. 3A and B, lanes 1 and 2). The binding specificity was confirmed by competition experiments using unlabeled AP-1 oligonucleotide (Fig. 3, lane 5). Studies of other

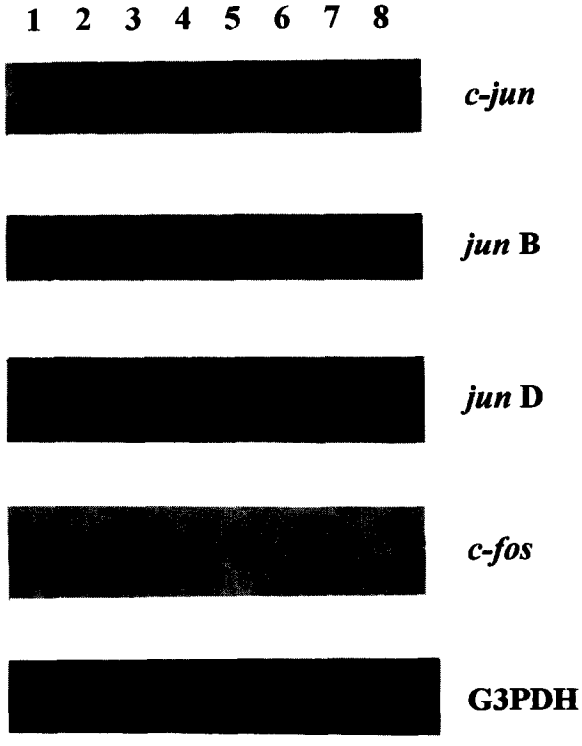

Fig. 1. mRNA content of c-*jun*, *jun*B, *jun*D and c-*fos* in tumorigenic and non-tumorigenic reverted cells of two genotypes (Northern hybridization assay). 10 μg of total RNA were loaded per lane. Equal loading of RNA per lane was monitored by hybridization with G3PDH cDNA. 1–4 = FCBA2V10 cells; 5–8 = FC3H3V7 cells. (1 and 5) = quiescent reverted cells; (2 and 6) = quiescent reverted cells treated with 20% FCS for 30 min; (3 and 7) = reverted cells in logarithmic phase of growth; (4 and 8) = tumorigenic cells in logarithmic phase of growth.

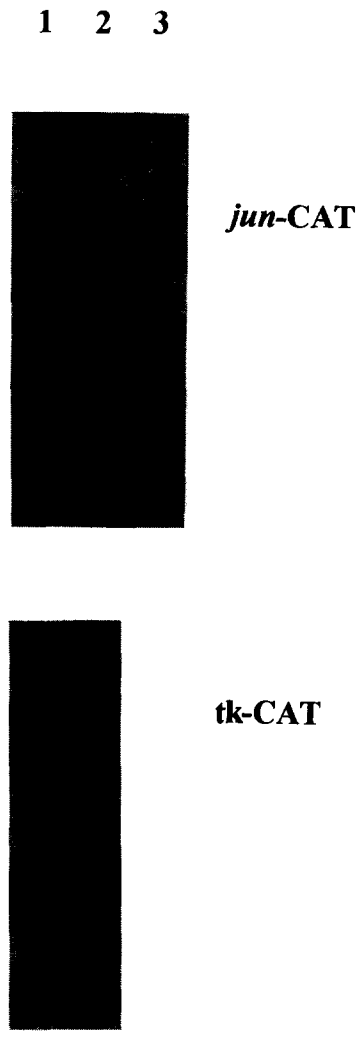

Fig. 2. CAT assay in extracts from logarithmic phase FC3H3V7 cells transfected with *jun*-CAT and *tk*-CAT plasmids. (1) Non-tumorigenic reverted cells; (2) tumorigenic cells; (3) no extract.

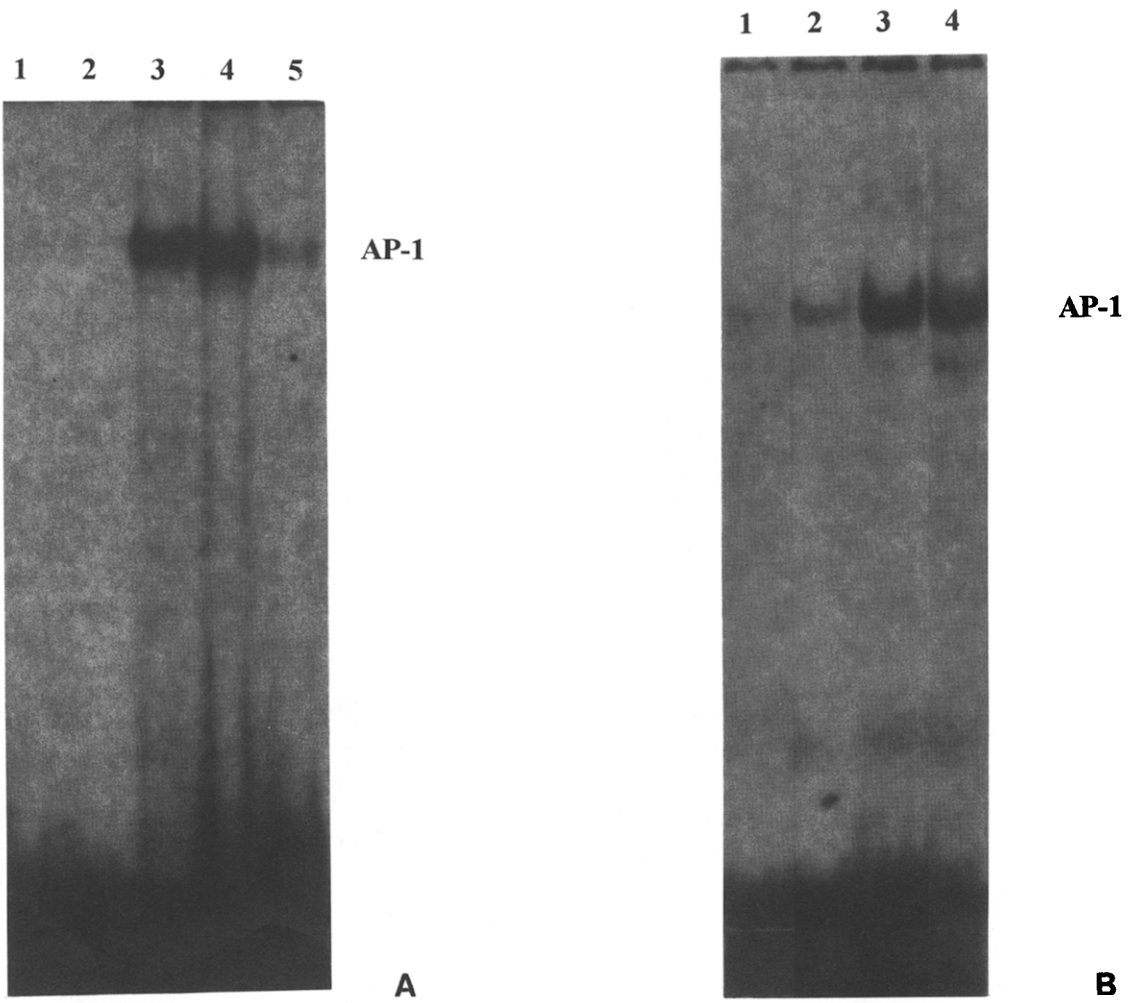

Fig. 3. Electrophoretic mobility shift assay of TRE oligonucleotide with nuclear extract from FC3H3V7 (A) and FCBA2V10 (B) cells. (1) Quiescent reverted cells; (2) quiescent reverted cells stimulated with 20% FCS; (3) reverted cells in logarithmic phase of growth; (4) tumorigenic cells in logarithmic phase of growth; (5) the same as lane (4) plus 50-fold excess of specific competitor DNA.

transcription factors which might be involved in c-*jun* overexpression in tumorigenic cells or c-*jun* down-expression in pseudonormal cells are in progress.

Negative regulators of JUN, such as IP-1 [14] and Jif-1 [15], have been shown to decrease transformation by JUN. A dominant negative JUN mutant has also been shown to suppress in vivo tumor formation [16]. However, the exact role that the *jun* protein family plays in mediating reversion of of highly tumorigenic cell lines to non-tumorigenic phenotype remains to be determined.

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