Altered binding of the AP-1 protein on a negative regulatory element of c-*myc* is correlated with the progression of the malignancy of the lung and may contribute to c-*myc* expression

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Abstract. The binding activity of the AP-1 (FOS/JUN) complex to a negative regulatory element on the c-*myc* promoter was investigated in 7 tumour- versus normal lung specimens and it correlated with c-*myc* expression. AP-1 levels were found elevated in two samples representing stage I progression of the disease, while in three stage III tumour samples, the AP-1 binding activity was equal or slightly elevated compared with their normal adjacent tissue. Southern blot analysis revealed amplified c-*myc* in all three cases of stage III tumours. These results suggest that there is a negative correlation between FOS/JUN binding on c-*myc* promoter and the expression of c-*myc* during the progression of the disease, further supporting the fundamental role of AP-1 on c-*myc* regulation.

Introduction

The c-*myc* proto-oncogene encodes for two highly conserved nuclear phosphoproteins p62 and p64 involved in cellular proliferation and differentiation (1). Alteration of c-*myc* expression, due to proviral insertion, chromosomal translocation and gene amplification, leads to quantitative and/or qualitative changes in c-MYC protein and has been correlated with the development of several types of malignancies in a wide range of species (2). Particularly in humans, chromosomal translocations involving the c-*myc* and the immunoglobulin heavy and light chain genes has been directly related to the development of Burkitt's lymphomas (3), while amplification of c-*myc* has been observed in several cases in lung, breast and head and neck cancer (reviewed in ref. 4). Furthermore, the expression of c-*myc* has been found elevated in stomach, colorectal and cervical cancer (4) and in agressive myelomas (5).

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The regulation of c-*myc* expression is complex and is controlled not only at the level of transcription, by both positive and negative *cis*-acting regulatory elements, but also at the post-transcriptional level (1).

The c-*fos* oncogene is known to participate in transcriptional regulation through formation of a c-*fos*:c-*jun* heterodimer protein known as the AP-1 complex (for a review see ref. 6). The AP-1 complex has been reported to participate in the process of differentiation (7,8) and may also control the expression of genes involved in cellular proliferation (6). Recently, Hay *et al* found a Negative Regulatory Element (NRE) within the c-*myc* promoter region which contains an AP-1 binding site (9). Herein we report on the AP-1 binding activity from tumour and adjacent normal lung specimens to the NRE of c-*myc*, as a possible mechanism or part of a mechanism regulating c-*myc* expression in lung cancer.

Materials and methods

Tissue specimens and cell lines. Tumour and adjacent normal lung tissue specimens were obtained from patients who had undergone lung surgery at the Metaxa Hospital, Pireas, Greece. None of these patients had previously received any chemotherapy. The histopathological type, the stage of differentiation and the pTNM of tumour of lung cancer patients are given in Table I.

The HeLa cell line used in this study, was continuously maintained in RPMI-1640 (Gibco) with 50 µg/ml streptomycin and 300 µg/ml penicillin, supplemented with 10% fetal calf serum (Flow), at 37°C in a 5% CO_2 atmosphere.

Preparation of nuclear extracts. Nuclear extracts from tissue specimens were prepared as previously described (8). Briefly, tissues were cut into pieces, homogenised with a B-type Dounce homogenizer in hypotonic buffer (25 mM TrisHCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF), centrifuged at 2,000 x g, washed three times with isotonic buffer (25 mM TrisHCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.1 M sucrose, 0.5 mM DTT and 0.5 mM PMSF), and resuspended in nuclei extraction buffer (25 mM TrisHCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF

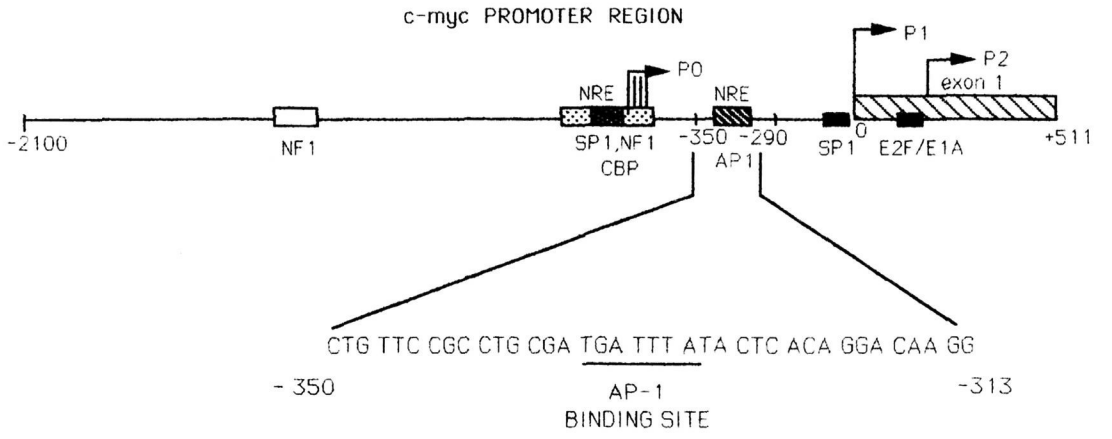

Figure 1. Regulatory elements and transcription factor binding sites on the c-*myc* promoter. The AP-1 binding site is located whithin an NRE domain of c-*myc* promoter (9).

Table I. pTNM, stage of differentiation and histopathological type of tumour of lung cancer patients.

Patient no.	pTNM	Stage of differentiation	Histopathological type of lung tumour
1	T2N1M0	II	adenocarcinoma
2	T3N0M0	III	squamous
3	T2N1M0	II	SCLC
4	T3N1M0	III	squamous
5	T2N0M0	I	squamous
6	T1N0M0	I	adenocarcinoma
7	T3N2M0	III	SCLC

and 0.6 M KCl). The nuclei were inverted slowly for 30 min and the extract clarified by centrifugation at 2.5 x 10^4 g for 60 min at 4°C. Nuclear extracts were stored at -70°C. Protein estimation was performed at 595 nm using the Biorad Protein Assay (Biorad).

Synthesis of oligonucleotides and gel retardation assays. The *myc*AP-1 oligonucleotide 5'-CCTGCGATGATTTATACTC ACAGG-3' contains the AP-1 binding site of the NRE domain of the human c-*myc* promoter (-342 to -313) (9) (Fig. 1). The E_3AP1 oligonucleotide 5'-CCGAAGTTCAGAT GACTA ACTCAGGG-3' representing the region between nucleotides -81 and -103 of the EIA inducible E_3 promoter (10) containing an AP-1 binding site, was used for competition experiments. Both oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer.

Single stranded complementary oligonucleotides were annealed, end-labelled using γ-^{32}P-ATP (Amersham) and T4 polynucleotide kinase (Boehringer) and run on an 8% polyacrylamide gel to test the annealing.

The binding reaction mixture contained 10 mM Hepes (pH 7.9), 0.2 mM EDTA, 94 mM NaCl, 0.1 mM PMSF, 0.1 ng/ml BSA, 4% glycerol, 3 µg poly(dI-dC) (Pharmacia), 0.2-1.0 ng ^{32}P-labelled DNA and 20 µg nuclear extracts (except when otherwise indicated). The reaction mixture was left on ice for 30 min. Then, reaction mixtures were loaded onto a 5% polyacrylamide gel (29:1 acrylamide: N,N'-methylene bisacrylamide, Serva). Gels were run at 4°C in 0.55 X TBE, dried and exposed to X-ray film for autoradiography (Fuji).

The rabbit monoclonal antibody FOS 388 used in this study was a generous gift of Dr D.A.F. Gillespie (Beatson Institute for Cancer Research, Glasgow, UK).

Extraction of DNA and Southern blot analysis. DNA extracts from tissue specimens were prepared as described by Sambrook *et al* (11), slightly modified as follows: after the isolation of nuclear proteins as described above, the debris, which contained the cellular DNA, was incubated O/N in lysis solution (10 mM EDTA, 10 mM TrisHCl, pH 7.8, 150 mM NaCl, 0.5% SDS and 100 µg/ml proteinase-K) at 37°C. After two phenol/chloroform/ isoamylalcohol extractions, the aqueous phase was removed and incubated for 2 h with 100 µg/ml pancreatic RNase (Sigma) at 37°C and for an additional 2 h period with 100 µg/ml proteinase-K (Boehringer) followed by phenol/ chloroform/isoamylalcohol extraction twice. The aqueous phase was then removed and the DNA was precipitated with ethanol. The DNA was washed with 70% EtOH, dried briefly, resuspended in sterile water and estimated photometrically at 260 nm using an LKB 4054 spectro-photometer.

10 µg of each DNA sample was digested O/N with 100U of HindIII (Boehringer), precipitated with ammonium acetate and ethanol, dried briefly and resuspended in 20 µl of sterile water. Samples were then loaded on a 0.7% agarose gel and run O/N at 30V in 1 X TBE running buffer.

For Southern blotting we followed the Hybond-N$^+$ protocol (Amersham) using 20 X SSPE buffer for transfer. After blotting, DNA was fixed by baking the membrane at 80°C for 2 h.

Pre-hybridization with 5 X SSPE, 5 X Denhardt's solution, 50% formamide and 0.5% (w/v) SDS for 6 h in 42°C, was followed by overnight hybridization. The probes used were the the 1.4 Kb ClaI/EcoRI fragment of plasmid pMC41-3RC, carrying the third exon (EIII) of the human c-*myc* gene (12) and the 6.4 Kb BamHI/BamHI fragment of plasmid pHo6N1, carrying the human H-*ras* gene (13).

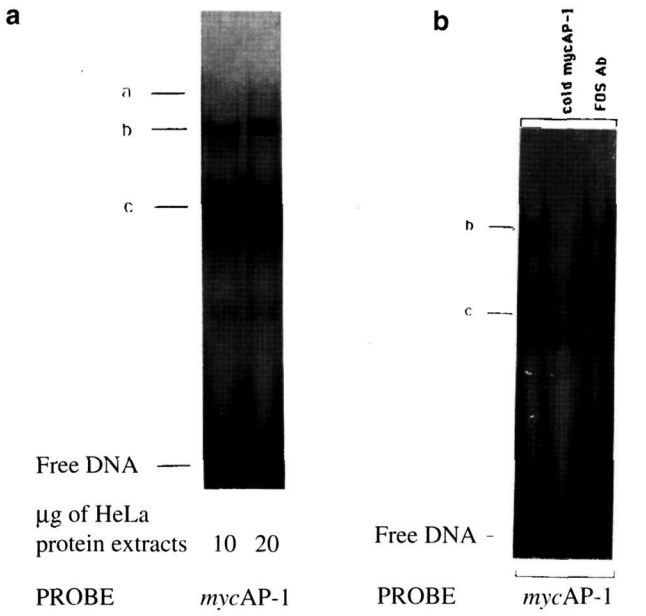

Figure 2. (a) Binding of nuclear factors from HeLa cells to *myc*AP-1 oligo. Only the b complex has been shown to be a FOS/JUN (AP-1) protein (see text). (b) Effect of FOS antibody (lane 2) and *myc*AP-1 cold competitor (lane 3) on c-*myc* DNA-protein complexes in a gel retardation assay. Extracts from HeLa cells were used.

DNA-inserts were labelled with ^{32}P dCTP (Amersham) using the random primed DNA labelling kit of Boehringer Mannheim.

Membranes were finally washed twice in 2 X SSPE, 0.1% SDS in room temperature for 30 min, twice in 1 X SSPE, 0.1% SDS for 20 min at 42°C and once in 0.5 X SSPE, 0.1% SDS at 42°C for 15 min, followed by exposure to X-ray film (Fuji) with an intensifying screen.

Results

The NRE of c-*myc* is located in position -350 to -290 relative to transcription start site P1 and has been reported to contain the recognition site for the cellular transcription factor AP-1 (ref. 9 and Fig. 1).

When crude extracts from HeLa cells, which are known to overexpress the FOS/JUN heterodimer protein, are incubated with the *myc*AP-1 oligonucleotide, three complexes a, b and c are formed (Fig. 2a and ref. 9). Only the b-complex has been shown to represent the FOS/JUN protein (9). Indeed, antibody against the FOS protein eliminated the b-band in extracts from both HeLa cells (Fig. 2b, lane 3) and lung tumour specimen no. 3 (Fig. 3a, lane 2). In order to test further the specificity of the binding, we performed competition assays with excess (200x) of *myc*AP-1 and E_3AP-1 cold oligonucleotides. Excess of cold *myc*AP-1 inhibited the binding of protein-DNA complexes b and c in both the HeLa cells (Fig. 2b, lane 2) and in the protein extracts from specimen no. 3 (Fig. 3b, lane 3), while cold E_3AP-1 inhibited mainly the formation of complex b (Fig. 3b, lane 4).

By performing gel retardation assays with nuclear extracts from tumour and adjacent normal lung specimens, we observed an altered AP-1 binding to the c-*myc* NRE.

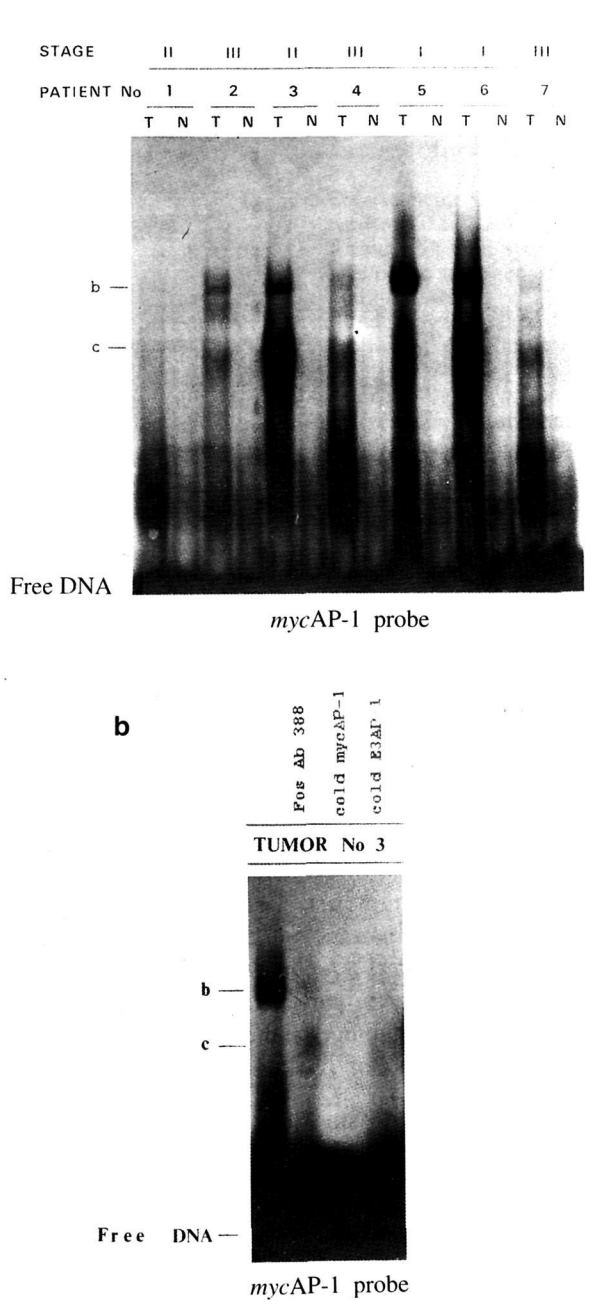

Figure 3. (a) Gel retardation assay with extracts from tumour and adjacent normal lung tissue. (b) Effect of FOS antibody (lane 2) and excess of cold *myc*AP-1 and E_3AP-1 (lanes 3 and 4) on c-*myc* DNA-protein complexes in gel retardation assay. Extracts from tumour specimen no. 3 were used.

Elevated AP-1 levels were observed in two stage I tumours (Fig. 3a, samples 5, 6), while in the three stage III tumours, the AP-1 levels were equal or slightly elevated compared with the normal tissue (Fig. 3a, samples 2, 4 and 7). The latter three specimens revealed amplified c-*myc* in Southern blot analysis (Fig. 4, lanes 2,4,7). One of the two stage II lung tumours presented low AP-1 levels, while in the other, the AP-1 activity, although elevated, was lower than that of the stage I tumours (Fig. 3a, samples 1 and 3).

Discussion

The role of the c-*myc* oncogene in the progression of carcinogenesis has been extensively investigated in a range

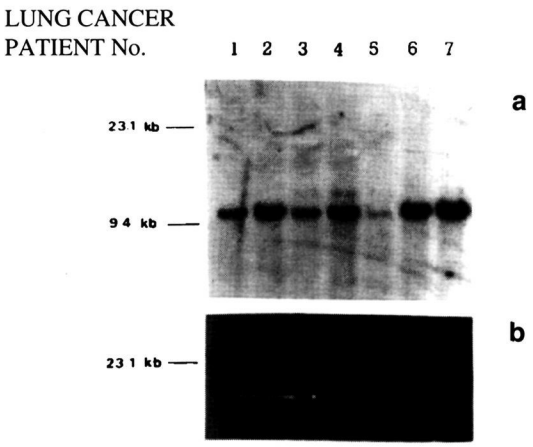

Figure 4. Southern blot analysis revealed c-*myc* amplification in three stage III samples (samples 2,4,7). 10 μg of DNA from cases 1-7 was digested with HindIII and analyzed with (a) c-*myc* exon 3 probe and (b) c-H-*ras* probe. The same filter was used for the two hybridizations. Autoradiographic exposure time was approximately 4 days. Lambda phage DNA, digested with HindIII, was used as a molecular weight marker. Densitometric analysis of the autoradiograph, normalized to H-*ras*, which is known not to be amplified in lung tumours (24), revealed three samples (patient numbers 2,4 and 7) with c-*myc* levels at least twice those of H-*ras*.

of human solid tumours. Genetic alterations of c-*myc* is believed to be important in the progression of these cancers and may be of use as a prognostic indicator (4).

In lung cancer in particular, amplified c-*myc* has been found in primary tumours and cell lines (14,15) and has been correlated with poor prognosis (16). Understanding the mechanism of c-*myc* regulation in these cases is of great importance.

The modulation of c-*myc* expression is complicated and is accomplished on both transcriptional and post-transcriptional level. The c-*myc* NRE, located within the c-*myc* promoter region (Fig. 1), participates in the modulation of the oncogene expression through binding of three transcription factors (8,9). One of them (complex b, Figs. 2 and 3) is the heterodimer protein FOS/JUN (AP-1). That NRE has been shown to be important for c-*myc* expression following platinum and anthracycline exposure of Friend cells (17,18), while the altered FOS/JUN binding activity we have recently reported, during the HMBA-induced differentiation of mouse erythroleukemic cells (8), further supports the contribution of the AP-1 protein to c-*myc* regulation.

In this study, we performed gel retardation assays with protein extracts from lung tumour and adjacent normal tissue, to examine the AP-1 levels bound to the c-*myc* NRE and correlated these results with c-*myc* expression.

We found elevated FOS/JUN levels in only three out of seven samples, that is two stage I and one stage II lung tumours, while in the other four samples the AP-1 activity was equal or slightly elevated compared with the normal tissue. Three of the latter cases corresponded to stage III tumours and presented amplified c-*myc*, as revealed by Southern blot analysis (Fig. 4). Therefore, a good correlation between stage of differentiation and AP-1 binding activity exist on c-*myc* NRE; the higher the stage, the lower the FOS/JUN levels bound to that region. Recently, Linardopoulos *et al,* using the same model of lung cancer, showed an opposite pattern of AP-1 binding to an AP-1-like site in the promoter region of retinoblastoma (Rb) gene (19). In that study, the increased binding was in accordance with the development of tumour differentiation. It is therefore possible that the transcription factor AP-1 plays a different role in the expression of Rb and c-*myc* during progression of the malignancy of the lung.

The expression of the c-*myc* gene is considered to be crucial in cellular proliferation (20); lymphocytes stimulated with mitogens rapidly increased their expression of c-*myc* mRNA as they entered into cell cycle (21), while the c-MYC protein has been shown to bind to an autonomously replicating sequence (ARS) in eukaryotic cells and thus promoting its replication (22). There is also evidence of high proliferative activity in stage III myeloma cells in agressive myeloma with overexpression of c-*myc* (5,23). Taking into account these reported experimental data, our results suggest that the low levels of AP-1 bound to the NRE of c-*myc* could contribute to the induction of c-*myc* expression and also to the proliferative activity which is characteristic of the late stages of tumour development.

In conclusion, despite the small number of tumours tested, our results further support a role of the heterodimer protein AP-1 in tumour progression and also in transcriptional regulation of c-*myc* expression. It is suggested that the decreased levels of FOS/JUN bound to the c-*myc* NRE in high stages of tumour differentiation could contribute to the overexpression of the c-*myc* oncogene, which, in turn, could affect the cellular proliferative potential and clinical outcome.

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