Neoplastic transformation of rat thyroid cells requires the *junB* and *fra-1* gene induction which is dependent on the HMGI-C gene product

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The expression of the high mobility group I (HMGI)-C chromatin component was shown previously to be essential for the establishment of the neoplastic phenotype in retrovirally transformed thyroid cell lines. To identify possible targets of the HMGI-C gene product, we have analyzed the AP-1 complex in normal, fully transformed and antisense HMGI-C-expressing rat thyroid cells. We show that neoplastic transformation is associated with a drastic increase in AP-1 activity, which reflects multiple compositional changes. The strongest effect is represented by the dramatic *junB* and *fra-1* gene induction, which is prevented in cell lines expressing the antisense HMGI-C. These results indicate that the HMGI-C gene product is essential for the *junB* and *fra-1* transcriptional induction associated with neoplastic transformation. The inhibition of Fra-1 protein synthesis by stable transfection with a *fra-1* antisense RNA vector significantly reduces the malignant phenotype of the transformed thyroid cells, indicating a pivotal role for the *fra-1* gene product in the process of cellular transformation.

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Introduction

Thyroid cell transformation represents a model system for the study of epithelial tumorigenesis. Two rat thyroid epithelial cell lines, which retain the typical markers of thyroid differentiation, have been infected by several oncogenes, generating a variety of de-differentiated or transformed phenotypes, which recapitulate the broad spectrum of differentiative and proliferative features of human thyroid cancer (Fusco *et al.*, 1985, 1987; Berlingieri

et al., 1988, 1993). A strong induction of high mobility group I (HMGI) proteins was associated with neoplastic transformation of these thyroid cell lines (Giancotti *et al.*, 1987), and has been described recently also in highly malignant thyroid tumors (Chiappetta *et al.*, 1995). The HMGI proteins form a group of non-histone chromatin components which includes three members (HMGI, HMGY and HMGI-C). While the HMGI and HMGY proteins derive from the alternative splicing of the same HMGI(Y) transcript, the closely related HMGI-C represents the product of a different gene (Manfioletti *et al.*, 1991). The HMGI(Y) proteins have been functionally characterized as 'architectural' components of the transcriptional apparatus, on the basis of their ability to interact both with DNA and with multiple transcription factors, thus mediating the assembly of higher order transcription enhancer complexes (enhanceosomes) (Thanos and Maniatis, 1995). The putative transcriptional regulatory role of the closely related HMGI-C protein has not been defined as yet.

Overexpression of HMGI proteins has been detected in several experimental and human tumors, including thyroid carcinomas (Bussemakers *et al.*, 1991; Ram *et al.*, 1993; Tamimi *et al.*, 1993; Chiappetta *et al.*, 1995; Fedele *et al.*, 1996). Recently, an antisense RNA strategy allowed the establishment of the causal role of the HMGI-C gene product in thyroid cell transformation. The stable antisense RNA expression blocks HMGI-C protein synthesis and prevents retroviral transformation by the *v-mos* and *v-Ki-ras* oncogenes. Interestingly, two different cell lines co-expressing either the *v-mos* or *v-Ki-ras* oncogene and the antisense HMGI-C (HMGI-C-as cell lines) exhibit an intermediate phenotype, characterized both by the inhibition of transformation parameters and by the extinction of the thyroid-specific differentiation markers (Berlingieri *et al.*, 1995). Such a cellular system provides a tool to identify possible transcriptional targets of the HMGI-C gene product involved in the nuclear response to the oncogene-dependent transduction pathways.

To understand the regulatory events associated with the expression of the transforming oncogenes and the functional effects of the HMGI-C inhibition, we have analyzed the biochemical and functional changes of the AP-1 transcription factor in normal and retrovirally transformed thyroid cells. The AP-1 complex is formed by the three Jun family members (c-Jun, JunB and JunD) and the four Fos family members (c-Fos, FosB, Fra-1 and Fra-2), which give rise to a large variety of homo- and heterodimers, binding to very similar or identical DNA motifs (Abate and Curran, 1990; Ransone and Verma, 1990; Angel and Karin, 1991). The combinatorial diversity of the dimers depends on the expression level of the individual components in distinct cell types, in different phases of the cell cycle and in different environmental situations.

The rapid control of AP-1 activity is exerted by post-translational modifications, mainly phosphorylation, in response to the MAP kinase transduction pathways (reviewed in Davis, 1994; Karin, 1995). Phosphorylation has been shown to control the function of the AP-1 components by different mechanisms, including the DNA-binding activity (Boyle *et al.*, 1991; Papavassiliou *et al.*, 1995), the recruitment of the CBP-p300 integrator complex (Arias *et al.*, 1994; Bannister *et al.*, 1995) and the metabolic stability of the proteins (Gruda *et al.*, 1994; Okazaki and Sagata, 1995).

The role of AP-1 in transformation was shown historically by the isolation of retroviruses carrying the *v-jun* and *v-fos* oncogenes. Subsequently, the oncogenic potential of *c-Jun* (Schutte *et al.*, 1989), JunB (Van Amsterdam *et al.*, 1994), *c-Fos* (Miller *et al.*, 1984) and FosB (Wisdom *et al.*, 1992) has been shown by ectopic overexpression or by cooperation with other oncogene products. Conversely, JunD has been characterized as a negative regulator of fibroblast growth, capable of partially antagonizing the *ras*-mediated transformation (Pfarr *et al.*, 1994). The picture is more complex for Fra-1, which has been shown to be unable to induce morphological transformation, but capable of stimulating the anchorage-independent growth of rat fibroblasts, when overexpressed (Bergers *et al.*, 1995).

The AP-1 target genes are characterized by the presence of functional TPA-responsive elements (TREs), which are often part of complex regulatory elements. For instance, AP-1 frequently has been found to cooperate with another class of *ras*-responsive transcription factors, the Ets family proteins, in controlling the expression of genes important for the malignant phenotype, like those encoding the extracellular matrix-degrading proteases (Gutman and Wasylyk, 1991).

Here we describe the effect of neoplastic transformation and HMGI-C protein inhibition on composition, expression of individual components and activity of the AP-1 complex in rat thyroid cells. We show that the drastic transformation-dependent accumulation of JunB and Fra-1 depends upon changes at the mRNA level, which are prevented in the absence of the HMGI-C gene product. To address the role of Fra-1 in thyroid cell transformation, we have adopted an antisense RNA strategy, and shown that the decrease of Fra-1 protein synthesis greatly reduces the transformed phenotype of thyroid cells.

Results

Oncogene-mediated AP-1 induction is decreased in the absence of HMGI-C protein

We first investigated, by electrophoretic mobility shift assay (EMSA), AP-1 binding in different cell lines, including normal rat thyroid cells (PC Cl.3 and FRTL-5 Cl.2), retrovirally transformed cells (PC MPSV and FRTL-5 KiMSV) and retrovirally infected cell lines expressing both the oncogene and the antisense HMGI-C (HMGI-C-as cell lines: PC/C-as/MPSV and FRTL-5/C-as/KiMSV, Figure 1A). As reported (Fusco *et al.*, 1985, 1987), PC MPSV cells were obtained by infection with the myeloproliferative sarcoma virus, carrying the *v-mos* oncogene, while FRTL-5 KiMSV cells were obtained by infection with Kirsten murine sarcoma virus, carrying the

Fig. 1. *In vitro* AP-1 (versus Sp1) binding of nuclear proteins from normal, fully transformed and HMGI-C-as thyroid cell lines. EMSA of nuclear proteins extracted from normal (PC Cl.3 and FRTL-5 Cl.2), transformed (PC MPSV and FRTL-5 KiMSV) and HMGI-C-as cell lines (PC/C-as, FRTL-5/C-as, PC/C-as/MPSV, FRTL-5/C-as/KiMSV). The specific complexes are indicated by arrows. (A) Binding to the colTRE AP-1 consensus oligonucleotide. (B) Binding to the Sp1 consensus oligonucleotide.

Ki-ras oncogene. The binding activity in different nuclear extracts was normalized by using an oligonucleotide probe for the ubiquitous Sp1 transcription factor (Figure 1B).

The *in vitro* binding to the collagenase TRE (colTRE) AP-1 site was dramatically increased, in both the *v-mos*- and in the *v-Ki-ras*-transformed cell lines (PC MPSV compared with PC Cl.3, and FRTL-5 KiMSV compared with FRTL-5 Cl.2, Figure 1A). The colTRE AP-1 complex was significantly decreased (3- to 5-fold, by phosphor-imager analysis) in the HMGI-C-as cell lines (PC/C-as/MPSV and FRTL-5/C-as/KiMSV) with respect to the fully transformed cell lines. As a control of the specificity of the antisense effect, we also examined the AP-1 activity in the cell lines expressing the antisense HMGI-C but not the retroviral oncogenes, in which the HMGI-C gene product is absent. As expected, these cell lines (PC/C-as and FRTL-5/C-as) exhibited the same low level of AP-1 binding detected in the parental PC Cl.3 and FRTL-5 Cl.2 cells.

To investigate a possible difference in DNA binding stability, we then analyzed the kinetics of dissociation (off-rate) of the AP-1 complexes expressed in the fully transformed cell lines compared with the complexes detected in the antisense HMGI-C-expressing cell lines (see Figure 2A for the PC-derived cell lines, and Figure 2B for the FRTL-5-derived lines). As shown by phosphor-imager quantitation (Figure 2C), the dissociation rate of the protein-DNA complex formed by the nuclear proteins of fully transformed cells (PC MPSV and FRTL-5 KiMSV) was slower than the dissociation rate of the complex formed by the nuclear proteins of the antisense-expressing cells (PC/C-as/MPSV and FRTL-5/C-as/KiMSV), suggesting a significant difference in stability of the *in vivo* interaction. These results suggested a change in the com-

Fig. 2. Dissociation rate of the AP-1 complexes in fully transformed and HMGI-C-as cell lines. The binding mixture containing the collTRE oligonucleotide and the nuclear extracts (5 μ g) from the indicated cell lines was incubated on ice with a 100-fold molar excess of competitor unlabeled oligonucleotide for the indicated times, and analyzed by mobility shift assay. (A) Time course of dissociation (off-rate) of PC MPSV and PC/C-as/MPSV AP-1 complexes. (B) Time course of dissociation of FRTL-5 KiMSV and FRTL-5/C-as/KiMSV AP-1 complexes. (C) Diagram of dissociation rates. The images in (A) and (B) were captured by phosphorimager (Molecular Dynamics) scanning, and the complexes were quantitated by ImageQuant software.

position of AP-1 complexes caused by transformation and modified by the antisense HMGI-C expression.

AP-1 compositional changes in transformed thyroid cells

Antibody supershift analysis was performed to determine the composition of the complexes detected by EMSA. To visualize the small amount of gel-retarded complex formed by the PC Cl.3 and FRTL-5 Cl.2 nuclear proteins, a longer autoradiographic exposure is shown. In the parental PC Cl.3 cells, the collTRE-bound complex was almost completely supershifted by the anti-JunD antibodies; a minor supershift was also detected by anti-c-Jun and anti-JunB and, to a lesser extent, by the anti-Fra-2 antibodies (Figure 3A). The AP-1 complex detected in the *v-mos*-transformed cells exhibited a different supershift pattern. The antibodies selectively recognizing Jun family members revealed that the upper half of the broad gel retardation product was

Fig. 3. Antibody supershift analysis of the AP-1 complexes. Identification of the Jun and Fos family members in the AP-1 complex bound to collTRE in normal, fully transformed and HMGI-C-as cell lines. The antibodies were pre-incubated with nuclear extracts from the indicated cell lines before the binding reaction. To detect the very low amount of AP-1 complex in the normal cell lines (PC Cl.3 and FRTL-5 Cl.2), a larger amount of extract (5 instead of 2.5 μ g) and a (15 times) longer autoradiographic exposure were chosen. The antibody-supershifted complexes are indicated by arrows. The sources of nuclear extracts were: PC Cl.3 (A); PC MPSV (B); PC/C-as/MPSV (C); FRTL-5 Cl.2 (A'); FRTL-5 KiMSV (B'); FRTL-5/C-as/KiMSV (C').

supershifted by anti-JunD, while the lower half was supershifted by anti-JunB, in agreement with the faster migration of the dimers containing JunB, which has a lower molecular weight than JunD. While equivalent amounts of complex were supershifted by anti-JunD and anti-JunB, a smaller supershift was detected by the anti-c-Jun antibodies. The analysis of the Fos family members revealed that the AP-1 complex of the PC MPSV cells was completely supershifted by the anti-Fra-1 antibodies (Figure 3B). Interestingly, a different pattern was observed in the antisense HMGI-C-expressing cells. The relative intensity of the supershifted complexes detected by the anti-c-Jun, anti-JunB and anti-JunD antibodies was similar to that observed in the normal cells; however, unlike the normal and fully transformed cell line, only a fraction of the complex was supershifted by the anti-Fra-1 antibodies (Figure 3C).

The analysis of the AP-1 components in the *v-Ki-ras*-transformed cell line revealed interesting differences. While the supershifted complexes in the FRTL-5 Cl.2 cells were identical to those detected in the PC Cl.3 cells (compare Figure 3A and A'), the *v-Ki-ras*-transformed cells (FRTL-5 KiMSV) exhibited a different pattern of supershift, evidenced by the antibodies recognizing the Fos family members. We found that, in addition to the major supershift detected by anti-Fra-1, a significant amount of complex was also supershifted by the anti-

FosB antibodies (Figure 3B'). Pre-incubation of nuclear proteins with both antibodies allowed the detection of a complete supershift (not shown), indicating that the AP-1 activity in the v-Ki-ras-transformed cell line is produced by heterodimers containing two different Fos-related proteins, FosB and Fra-1, instead of only Fra-1 as in the v-mos-transformed cells. We analyzed this point further, and found that the observed difference depends on the transforming oncogene rather than the parental cell line, since the Ki-MSV-infected PC cells behave identically to the Ki-MSV-infected FRTL-5 Cl.2 cells (data not shown). An intermediate pattern, between that of normal and fully transformed cells, was detected in the antisense HMGI-C-expressing cell line (FRTL-5/C-as/KiMSV): the anti-Fra-1-supershifted complex was decreased, while the anti-FosB-supershifted complex was absent, with respect to fully transformed cells (Figure 3C'). The analysis of the Jun-related components revealed qualitatively similar modifications with respect to the v-mos-transformed cell line, showing that the JunD/JunB ratio was reverted in the (–HMGI-C) cells compared with the FRTL-5/KiMSV cell line.

In summary, the results of supershift analysis indicate that in normal thyroid cells the AP-1 complex consists mainly of JunD homodimers, in the v-mos-transformed cells AP-1 is formed mostly by a combination of c-Jun–Fra-1, JunB–Fra-1 and JunD–Fra-1 heterodimers, while in the antisense HMGI-C-expressing cells a more heterogeneous AP-1 complex is present, formed by Jun homodimers (containing mostly JunD), in addition to an intermediate amount of heterodimers formed by Fra-1 mainly with JunD, but also with JunB and c-Jun. The compositional change associated with the v-Ki-ras oncogene appears to be more composite because of the appearance of both FosB- and Fra-1-containing heterodimers in the fully transformed cell line.

Expression of c-Jun and junD in transformed thyroid cells: increased protein levels are not strictly correlated with changes at the mRNA level
To characterize the regulatory changes associated with the different AP-1 composition, we analyzed the protein and mRNA levels of the individual AP-1 components identified by supershift assays.

Western blotting analysis showed that the level of the p39 c-Jun protein was significantly increased in both transformed cell lines (PC MPSV compared with PC Cl.3, and FRTL-5 KiMSV compared with FRTL-5 Cl.2). The level of c-Jun detected in both PC/C-as/MPSV and FRTL-5/C-as/KiMSV cell lines was intermediate between normal and fully transformed cell lines (Figure 4A).

Northern hybridization revealed small changes (up to 3-fold) in the c-jun mRNA level in the six cell lines analyzed (Figure 4B). As shown by phosphorimager quantitation normalized by the GAPDH internal control (Figure 4C), the c-jun mRNA was only slightly increased in the two transformed cell lines and, surprisingly, it was increased further in the HMGI-C-as cell lines, with a larger effect in the v-mos-infected cell line (PC/C-as/MPSV). These results indicate a partial correlation, or no correlation, with the corresponding protein levels detected by immunoblotting. Therefore, the changes at the protein

Fig. 4. Expression of c-Jun and JunD. (A) Immunoblotting analysis of c-Jun and JunD expression in normal, fully transformed and HMGI-C-as cell lines, as indicated. Nuclear proteins extracted from the different cell lines were separated on SDS–PAGE and transferred to PVDF membranes. Western blots were first incubated with antibodies specific for the c-Jun and JunD proteins, and then with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. As a control for equal loading, the blotted proteins were stained with Red–Ponceau. The molecular weight (kDa) of size markers is indicated on the sides. The arrows indicate the apparent molecular weight of c-Jun (39 kDa) and JunD (41 and 37 kDa). (B) Northern blotting analysis of *c-jun* and *junD* mRNA. Total RNA extracted from the indicated cell lines was hybridized to the *c-jun* and *junD* radiolabeled cDNAs, and then with a rat GAPDH probe, as a control for RNA loading. (C) mRNA quantitation. The images in (B) were captured by phosphorimager scanning (Molecular Dynamics), and the hybridization signals were quantitated. The diagrams represent the GAPDH-normalized level of the *c-jun* and *junD* mRNA, relative to expression in the normal cell lines (PC Cl.3 and FRTL-5).

level cannot simply be explained by the variations in the c-jun mRNA level in all the analyzed cell lines.

Immunoblotting analysis with the JunD-selective antibody (Figure 4A) allowed detection of a 41 kDa band, in agreement with the size of JunD, and an additional 37 kDa band, with the same intensity as the upper band. Both polypeptides were specific, since they were not detected when the antibodies were pre-incubated with an excess of immunogenic peptide. It remains to be established whether the 37 kDa polypeptide represents a JunD degradation product or an immunologically cross-reactive protein. We detected dramatic JunD accumulation both in the PC MPSV and in the FRTL-5 KiMSV fully transformed lines, and a JunD level comparable with normal cells in the HMGI-C-as cell lines.

As for the c-jun mRNA, the analysis of the *junD* transcript (Figure 4B and C) revealed a striking absence of correlation with the immunoblotting data. The results were slightly different for the PC-derived and the FRTL-5-derived cell lines. Unexpectedly, the *junD* transcript was decreased slightly both in the v-mos-transformed cells (PC MPSV) and in the v-mos-infected HMGI-C-as cell line (PC/C-as/MPSV, Figure 4C). In the v-Ki-ras-transformed cells, the level of the *junD* mRNA was not affected significantly by the expression of the oncogene alone

Fig. 5. Expression of *junB*, *fra-1* and *fosB*. (A) Immunoblotting analysis of JunB, Fra-1 and FosB expression in normal, fully transformed and HMGI-C-as cell lines, as indicated. Western blots were processed as in Figure 4A, except that JunB-, Fra-1- and FosB-specific antibodies were utilized. A short exposure was chosen to visualize fully the dramatic fold-induction of the three components in the transformed cell lines. The prolonged exposure of the JunB immunoblot also allowed detection of the small amount of protein expressed in the FRTL-5/C-as/KiMSV cell line (not shown). The blotted proteins were stained with Red-Ponceau. The molecular weight (kDa) of size markers is indicated on the sides. The brackets indicate the broad JunB (~38 kDa) and Fra-1 (~38 kDa) immunoblotting products. The arrow indicates FosB (41 kDa). (B) Northern blot analysis of *junB*, *fra-1* and *fosB* mRNA. Total RNA extracted from the indicated cell lines was hybridized to the *c-jun*, *junD* and *junB* radiolabeled cDNAs, and then with a rat GAPDH probe, as a control for RNA loading. In the *fosB* panel, the arrow indicates the mRNA species, in agreement with the predicted size (4.5 kb) of *fosB* mRNA; the asterisk indicates a larger *fosB*-related mRNA, which might represent an unspliced precursor.

(FRTL-5 KiMSV), but it was decreased significantly in the cell line expressing both *v-Ki-ras* and the antisense HMGI-C (FRTL-5/C-as/KiMSV, Figure 4C). However, it must be emphasized that, independently of the variations in mRNA level, the expression pattern of the JunD protein in the PC-derived cell lines was similar to that detected in the FRTL-5-derived cell lines, suggesting that JunD, like c-Jun, is regulated mainly at the post-transcriptional level.

Expression of *junB*, *fra-1* and *fosB* in transformed thyroid cells: the strong oncogene-dependent induction requires the HMGI-C gene product

A different picture was obtained by analyzing the expression of the other components (*junB*, *fra-1* and *fosB*) of the complexes identified by supershift analysis.

The level of the 38 kDa JunB polypeptide was increased dramatically by transformation, with a more pronounced effect in the *v-mos*-transformed cells compared with the *v-Ki-ras*-transformed cells (Figure 5A). While the increase in JunB was totally prevented by the HMGI-C inhibition in the *v-Ki-ras*-expressing cells, a small amount of protein was still detected in the PC/C-as/MPSV cells. A good correlation was observed between the levels of the *junB* transcript and the amount of protein detected by immunoblotting. Again, while the *v-Ki-ras*-dependent increase in *junB* mRNA was antagonized completely in the HMGI-C-as cells, the effect of the antisense HMGI-C was partial in the *v-mos*-transformed cells.

We then examined the expression of the two *fos*-related components, *fra-1* and *fosB*, detected by the supershift analysis. Immunoblotting results showed a dramatic increase in Fra-1, even more pronounced than JunB, in the *v-mos* transformed cells, and a smaller accumulation in the *v-Ki-ras* transformed cell line (Figure 5A). Indeed, a larger increase of the Fra-1 protein was predictable on the basis of the results of Figure 3B, showing that, since all the AP-1 dimers in the *v-mos* transformed cells contain

Fra-1, such an AP-1 component should be expressed in excess with respect to each of the Jun heterodimeric partners. Similarly to JunB, the larger increase of Fra-1 in the *v-mos*-transformed cells could be only partially antagonized in the PC/C-as/MPSV cell line. This was particularly evident from the result of Northern hybridization (Figure 5B), showing that the dramatic increase in *fra-1* mRNA was suppressed completely by antisense HMGI-C in the *v-Ki-ras*-expressing cells, but not in the *v-mos*-expressing cell line (compare FRTL-5/C-as/KiMSV and PC/C-as/MPSV). The supershift analysis of Figure 3B' showed that the TRE-bound complex in the FRTL-5 KiMSV cells contains not only Fra-1 but also FosB, as a Jun heterodimeric partner. Western blotting showed that, with a smaller induction than Fra-1, FosB, which is undetectable in FRTL-5 cells, was overexpressed significantly in FRTL-5 KiMSV cells, and its accumulation was totally prevented in the FRTL-5/C-as/KiMSV cell line (Figure 5A). The levels of the 41 kDa FosB protein were paralleled by the mRNA levels (Figure 5B); interestingly, the 4500 nucleotide *fosB* mRNA in the *ras*-transformed cells was associated with the appearance of a large RNA species (~8000 nucleotides), possibly reflecting the accumulation of an unspliced precursor.

We also examined the expression of the two Fos family members (c-Fos and Fra-2) which did not exhibit significant variations on supershift analysis. The *c-fos* gene product was undetectable in all six normal and transformed cell lines; as a positive control for c-Fos expression, we utilized nuclear proteins from phorbol ester-stimulated normal cells (PC Cl3), which allowed detection of the highly modified TPA-induced 55 kDa c-Fos (Figure 6A). The analysis of the other Fos-related component revealed an equivalent low level of expression of the 38 kDa Fra-2 protein in the six cell lines (Figure 6B).

Since the immunoblotting result allowed us to establish the absence of variations of the Sp1 protein level in the

Fig. 6. Expression of c-Fos, Fra-2 and Sp1. Immunoblotting analysis of c-Fos (A), Fra-2 (B) and Sp1 (C) expression in normal, fully transformed and HMGI-C-as cell lines, as indicated. Western blots were processed as in Figures 4A and 5A. The arrows indicate the 55 kDa c-Fos, the 38 kDa Fra-2 and the 110 kDa Sp1 proteins. The re-incubation with the anti-Sp1 antibodies was used as a control for equal loading of each of the Western blot filters shown in Figures 4–6.

six cell lines (Figure 6C), anti-Sp1 antibodies were used as a control for equal loading of the proteins transferred by Western blotting.

Activity of AP-1 target genes in normal, transformed and HMGI-C-as cell lines

To determine the functional consequences of the AP-1 compositional changes, we tested by transfection analysis the activity of a reporter construct, containing the consensus colTRE fused to a heterologous minimal promoter. For better comparison, the same value was assigned to the basal level of TRE-CAT activity detected in the PC Cl.3 and FRTL-5 Cl.2 cell lines. The results (Figure 7A) indicate a strong activation (~10-fold) of the TRE-CAT reporter in the *v-mos* transformed cell line (PC MPSV), and a smaller induction (~5-fold) in the *ras*-transformed cells (FRTL-5 KiMSV). Both HMGI-C-as cell lines exhibited a significantly decreased colTRE activity, with an ~3-fold reduction in both HMGI-C-as cell lines (PC/C-as/MPSV and FRTL-5/C-as/KiMSV), which still exhibited a higher activity (from 2- to 6-fold) compared with the parental cells.

We then examined the expression of several AP-1 target genes implicated in the transformed phenotype, including two extracellular matrix-degrading proteases, type-I

collagenase and transin/stromelysin, and the angiogenic vascular endothelial growth factor (VEGF). The collagenase mRNA, undetectable in the normal cell lines, was induced dramatically in both PC MPSV and FRTL-5 KiMSV cells, reaching the highest expression in the *v-mos*-transformed cells (Figure 7B). The collagenase transcript was reduced significantly in both the antisense HMGI-C-expressing cell lines (PC/C-as/MPSV and FRTL-5/C-as/KiMSV). A strikingly identical expression pattern was detected by analyzing the stromelysin mRNA level (Figure 7C). The analysis of VEGF gene expression also revealed a significant up-regulation in both transformed cell lines (Figure 7D). Remarkably, the counteracting effect of HMGI-C inhibition was quantitatively different in the two cell lines: while collagenase, stromelysin and VEGF expression was almost completely suppressed by HMGI-C inhibition in the *v-Ki-ras*-expressing line (FRTL-5/C-as/KiMSV), the effect was smaller in the *v-mos*-expressing cells (PC/C-as/MPSV).

The inhibition of Fra-1 attenuates the transformed phenotype of thyroid cells

Our results identify Fra-1 as the AP-1 component responsible for the formation of all (PC MPSV) or most (FRTL-5 KiMSV) of the heterodimeric complexes in transformed thyroid cells. To understand its role in the neoplastic phenotype, we adopted an antisense RNA strategy. To test the functional inhibition of *fra-1* in transformed thyroid cells, several cell lines were generated by stable transfection of the PC MPSV and FRTL-5 KiMSV cells with the pMV-7 retroviral vector expressing the antisense *fra-1* (Figure 8A). The geneticin-resistant clones were isolated and assayed for the level of Fra-1 expression. As a control, the same cell lines were transfected by the pMV-7 vector expressing the *fra-1* sense RNA. A representative sample of the cell clones subjected to immunoblotting analysis is shown in Figure 8B. Two clones (PC MPSV/Fra1-as Cl.3 and Cl.4) derived from the PC MPSV cell line and two clones (FRTL-5 KiMSV/Fra1-as Cl.1 and Cl.8) derived from the FRTL-5 KiMSV cell line exhibited an almost complete inhibition of Fra-1 protein expression. The inhibition of Fra-1 was associated with a dramatic decrease in colTRE binding activity in the selected cell clones (Figure 8C).

While most of the geneticin-resistant clones were indistinguishable from the parental transformed cell lines, the cell clones expressing the lowest Fra-1 levels (PC MPSV/Fra1-as Cl.3 and Cl.4 and FRTL-5 KiMSV/Fra1-as Cl.1 and Cl.8) exhibited a different morphology. These clones were characterized by a rather epithelial appearance, with respect to the highly refractile and round (PC MPSV) morphology of the fully transformed cell lines (compare PC MPSV/Fra1-as Cl.3 with PC MPSV, and FRTL-5 KiMSV/Fra1-as Cl.8 with FRTL-5 KiMSV in Figure 8D). However, the antisense *fra-1*-expressing cell lines were still morphologically distinct from the normal cell lines (PC Cl.3 and FRTL-5 Cl.2, Figure 8D). The effect of Fra-1 inhibition on the transformed phenotype was evaluated further by testing the anchorage-independent growth. The results indicate that the ability to form colonies in soft agar was significantly affected in the PC MPSV-derived cell clones expressing the antisense *fra-1* (Figure 8E and Table I). Both the morphology and the soft agar

plating efficiency indicate the partial reversion of the transformed phenotype. Similar results were obtained with both the FRTL-5 KiMSV-derived cell clones expressing the antisense *fra-1*, while the retroviral-mediated overexpression of the *fra-1* sense RNA did not affect the growth in soft agar and the morphology of the PC MPSV and FRTL-5 KiMSV cell lines (Table I and data not shown).

We have also analyzed the role played by JunB in

thyroid cell transformation. The results of the antisense-mediated inhibition, obtained by the same strategy utilized for Fra-1, indicate that the *junB* gene product is also necessary for the establishment of the fully transformed phenotype, although with a smaller effect on anchorage-independent growth compared with the Fra-1 inhibition (Table I). Therefore, *fra-1*, and possibly *junB*, overexpression represents an essential event during thyroid cell transformation, independently of the transforming oncogene.

Finally, to test whether the increased levels of Fra-1 or JunB might affect the growth properties of normal thyroid cells, we analyzed the effect of the overexpression of each of these two AP-1 components by stable transfection in PC Cl.3 cells. The expression of Fra-1 or JunB, in the sense orientation, was driven by the long terminal repeat (LTR) of the pMV-7 vector (Figure 8A). The geneticin-resistant cell clones were isolated and assayed for soft agar plating efficiency. The results indicate the absence of any effect on the anchorage-independent cell growth (Table I).

Discussion

An important role for HMGI proteins in tumorigenesis is indicated by several lines of evidence. The altered regulation of the HMGI chromatin components is determined not only by overexpression, as in thyroid and other epithelial-derived tumors (Bussemakers *et al.*, 1991; Ram *et al.*, 1993; Tamimi *et al.*, 1993; Fedele *et al.*, 1996), but also by chromosomal rearrangements, detected for the HMGI-C gene in various mesenchymal tumors (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995). The resulting chimeric proteins contain the HMGI-C DNA-binding domains (AT hooks) fused to heterologous protein sequences. In addition, the recent identification of gene rearrangements involving the chromosomal region 5' to HMGI-C (Wanschura *et al.*, 1996) suggests that transcriptional activation of the gene and overexpression of an intact HMGI-C protein can have a causal role in tumorigenesis. Therefore, it is important to identify the genomic targets

Fig. 7. Activity of the collTRE reporter construct and AP-1 target genes in normal, fully transformed and HMGI-C-as cell lines. (A) The CAT reporter construct containing five copies of the collagenase AP-1 element (5 $\times$ -collTRE) fused to the thymidine kinase promoter was transfected into the six thyroid cell lines, as indicated. CAT activity was assayed 40 h after transfection; the results represent the average of five independent experiments. All transfections were performed using a CMV-luciferase internal control vector to normalize for the variation of transfection efficiency between the different cell lines. The diagram shows the relative CAT activity, determined by assigning the same basal level to the PC Cl.3 and FRTL-5 Cl.2 parental cell lines. (B and C) The mRNA level of type I collagenase and transin/stromelysin was determined by RT-PCR (for details see Materials and methods). Both type-I collagenase and stromelysin cDNAs were co-amplified with GAPDH as an internal control. Bands of comparable intensity, obtained by the rat GAPDH sequence-specific primers, suggest comparable amplification of all samples. No bands are seen in non-reverse-transcribed RNAs, excluding a possible DNA contamination. The position of size markers is shown on the left; the arrows on the right indicate the position of PCR products: 721 bp for type-I collagenase (B) and 430 bp for transin/stromelysin (C), in agreement with the expected size. (D) The VEGF mRNA was detected by Northern hybridization. Fifteen μ g of total RNA was loaded for each cell line, and the filter was first hybridized with a human VEGF radiolabeled cDNA probe, and then with a rat GAPDH probe.

Fig. 8. Antisense-mediated inhibition of Fra-1 expression: protein level, morphology and growth in soft agar. **(A)** Schematic restriction map of the pMV-7/fra1-as vector. The *EcoRI* fragment including the full-length *fra-1* cDNA has been inserted in the pMV-7 vector in both antisense and sense orientations. LTR, long terminal repeat; TK-Neo, thymidine kinase-neomycin. **(B)** Western blotting analysis of the Fra-1 expression in cell clones transfected with the pMV-7/fra1-as vector. Twelve geneticin-resistant transfectants per cell line (PC MPSV and FRTL-5 KiMSV) were characterized; the figure shows the results obtained with a subset of the analyzed cell clones. Immunoblotting (20 μ g of nuclear protein/lane) was performed as in Figure 5. As a control, nuclear extracts from normal (PC Cl.3 and FRTL-5 Cl.2) and transformed cell lines (PC MPSV and FRTL-5 KiMSV) are included. **(C)** EMSAs of nuclear proteins extracted from the indicated cell lines. The gel retardation assay was performed as in Figure 1A. **(D)** Morphology of selected cell clones expressing the antisense *fra-1*: PC MPSV/Fra1-as Cl.3 and FRTL-5 KiMSV/Fra1-as Cl.8, compared with PC MPSV and FRTL-5 KiMSV cells. The cell lines were seeded at the same density and photographed at confluence. **(E)** Anchorage-independent growth: the PC Cl.3, PC MPSV and PC MPSV/Fra1-as Cl.3 cell lines were plated in soft agar. Representative colonies were photographed 8 days after seeding.

Table 1. Effect of the *fra1* and *junB* antisense-mediated inhibition on the anchorage-independent growth of transformed thyroid cells

Cell line	Colony-forming efficiency (%)	
	at 7 days	at 14 days
PC Cl.3	0	0
PC/Fra1-s ^a Cl.2	0	0
PC/Fra1-s ^a Cl.5	0	0
PC/JunB-s ^a Cl.5	0	0
PC/JunB-s ^a Cl.9	0	0
PC MPSV	55	70
PC MPSV/Fra1-s ^a	50	70
PC MPSV/Fra1-as ^b Cl.3	20	30
PC MPSV/Fra1-as ^b Cl.4	23	35
PC MPSV/JunB-s ^a	57	72
PC MPSV/JunB-as ^b Cl.7	31	40
PC MPSV/JunB-as ^b Cl.9	29	39
FRTL-5 Cl.2	0	0
FRTL-5/Fra1-s ^a Cl.4	0	0
FRTL-5/fra1-s ^a Cl.6	0	0
FRTL-5 KiMSV	50	65
FRTL-5 KiMSV/Fra1-s ^a	50	65
FRTL-5 KiMSV/Fra1-as ^b Cl.1	15	20
FRTL-5 KiMSV/Fra1-as ^b Cl.8	10	18

^aThe cells were transfected with the plasmids pMV-7/fra-1-s or pMV-7/junB-s carrying the *fra-1* or *junB* cDNA in sense orientation.

^bThe cells were transfected with plasmid pMV-7/fra-1-as or pMV-7/junB-as carrying the *fra-1* or *junB* cDNA in antisense orientation.

of the HMGI proteins and understand their role in the establishment of the transformed phenotype.

In this work, we have characterized the compositional changes of the AP-1 complex in a rat thyroid cell system in which retroviral transformation could be suppressed by the antisense-mediated inhibition of HMGI-C protein. Among complex regulatory changes, we have shown that the *junB* and the *fra-1* genes are strongly induced by transformation and that the induction is antagonized by HMGI-C inhibition, thus identifying potential targets of the HMGI-C gene product.

To understand the role of Fra-1 in transformation, we have also analyzed the effect of its functional inhibition, and shown that the Fra-1 overexpression is essential for the fully transformed phenotype.

The increased AP-1 activity in transformed thyroid cells reflects multiple changes of the individual components, determined by different mechanisms. Both c-Jun and JunD, but not JunB, are expressed in exponentially growing normal thyroid cells, in agreement with the results previously reported for NIH 3T3 fibroblasts (Pfarr *et al.*, 1994). The accumulation of JunD in transformed thyroid cells is in contrast to the results obtained in *ras*-transformed mouse fibroblasts (Pfarr *et al.*, 1994; our unpublished data), in which JunD overexpression has been found to antagonize *ras*-mediated transformation. Moreover, it has been shown recently that in rat embryo fibroblasts not only *c-jun* and *junB*, but also *junD* can cooperate with *ras* for focus formation. To explain the apparent discrepancy, it has been proposed that the *junD* oncogenic potential might be related to its cell type-specific dimerization partners (Vandel *et al.*, 1996).

Both the *v-mos* and *v-Ki-ras* oncogenes drastically enhance the levels of JunB, JunD and, to a lesser extent, c-Jun. The accumulation of both c-Jun and JunD in

transformed cells does not correlate with the variations at the mRNA level, suggesting that these AP-1 components are regulated at the protein level. A possible role for protein stabilization in transformed cells can be postulated; the increased stability might represent the consequence of post-translational modifications of the Jun family proteins. The role of protein stabilization has been analyzed in detail for c-Fos and c-Jun. It has been shown that the *v-mos*-dependent activation of the MAP kinase pathway leads to the phosphorylation of the c-Fos carboxy-terminal region, which is lacking in *v-fos*, and has a profound effect on the stability of the protein (Okazaki and Sagata, 1995). Similarly, the correlation between the transforming activity and the intracellular half-life has been characterized as an important difference between c-Jun and its retroviral counterpart, v-Jun, which lacks the region required for ubiquitin-dependent degradation (Treier *et al.*, 1994). While the phosphorylation-dependent regulation of c-Jun and c-Fos has been studied in detail (reviewed in Davis, 1994; Karin, 1995), the *in vivo* modifications of JunB and JunD and their possible effect on protein stability await detailed investigation. As an alternative to the direct effect of post-translational modifications, the increased stability of the Jun family proteins might represent the indirect consequence of heterodimerization with the Fos-related partner, mainly Fra-1, in the transformed thyroid cell lines. The increased half-life of the Jun proteins might be a consequence of the heterodimerization by itself, or, since Fra-1 is phosphorylated extensively and probably represents an *in vivo* substrate for MAP kinases (Gruda *et al.*, 1994), the phosphorylation-dependent stabilization of Fra-1 might affect the turnover of its heterodimeric partners.

Differing from c-Jun and JunD, the dramatic accumulation of both JunB and Fra-1 is associated with a strong transcriptional induction, which tightly correlates with tumorigenicity, being higher in the more malignant *v-mos*-transformed cell line than in the *v-Ki-ras* transformed cells.

It has been shown recently that the overexpression of the HMGI-C chromatin component is required for the establishment of the transformed phenotype of thyroid cells (Berlingieri *et al.*, 1995). We show here that *junB* and *fra-1* gene expression tightly parallels HMGI-C expression in the fully transformed cells and in the HMGI-C-as cell lines, in which the HMGI-C gene is inhibited by an antisense construct. The observed correlation does not simply reflect the general inhibition of transformation markers caused by HMGI-C suppression, but appears to be rather specific for JunB and Fra-1 (and FosB in the *v-Ki-ras*-transformed cells), since the HMGI-C inhibition affects the mRNA level of other AP-1 components (c-Jun and JunD) differently. Therefore, our results identify the *junB* and *fra-1* genes as potential targets of the HMGI-C gene product.

The detailed functional analysis of interferon- β and other inducible elements allowed the establishment of an 'architectural' role played by the HMG(Y) proteins, which mediate multiple cooperative interactions between various transcription factors, allowing the assembly of complex enhancers (enhanceosomes) (Thanos and Maniatis, 1995). It has been shown recently that the close structural similarity between HMGI-C and HMGI(Y) gene products results in the same multivalent DNA-binding specificity.

Importantly, it has been reported that the HMG-I proteins bind with high affinity to the AT tracts of the serum- and TPA-responsive element of the downstream JunB enhancer (Maher and Nathans, 1996). Therefore, we speculate that the decrease in transformation-dependent *junB* induction might reflect the lack of activation of the *junB* growth-responsive enhancer in cells expressing antisense HMGI-C.

It has been reported that the transcriptional regulation of *fra-1* depends largely on an AP-1-responsive enhancer in the first intron of the gene (Bergers *et al.*, 1995). Accordingly, the serum inducibility of *fra-1* is reduced in fibroblasts lacking *c-fos* or *c-jun*. Moreover, the *fra-1* gene is induced by the ectopic overexpression of various AP-1 components (c-Fos, FosB, Fra-1 and c-Jun). Therefore, it is likely that the oncogene-dependent induction of Fra-1 is consequent to the induction of other AP-1 components taking place during the early steps in the establishment of the transformed phenotype. It can be postulated that the lack of *fra-1* gene induction might represent a consequence of the inhibition of *junB* induction in the cells expressing antisense HMGI-C. Alternatively, it can be speculated that HMG-I binding sequences might be present within the *fra-1* regulatory region, and essential for the full activation of the AP-1-responsive enhancer in the first intron of the gene. Experiments are in progress to understand these regulatory aspects.

The strongly increased AP-1 binding of the Fra-1-containing heterodimers results in the increased activity of a transfected AP-1 reporter, which correlates with the induction of prototype AP-1 targets, such as the collagenase (Angel *et al.*, 1987), stromelysin (Buttice *et al.*, 1991) and VEGF genes (Saez *et al.*, 1995), in both the transformed cell lines. On the basis of the well established correlation with the invasive and metastatic potential (Matrisian, 1994), the oncogene-dependent induction of matrix-degrading metalloproteases might be quite relevant to the pathology of human thyroid tumors. In addition, it has been shown recently that a high tumorigenic potential is associated with elevated VEGF expression in the most aggressive human thyroid tumors (Viglietto *et al.*, 1995).

The role of Fra-1 in the induction of oncogene-dependent targets raises a question as to its function as a transcriptional activator. By fusion to a heterologous DNA-binding domain, it has been shown that Fra-1, unlike c-Fos and FosB, lacks a transactivation domain (Bergers *et al.*, 1995). Therefore, it has been proposed that its activity might be explained entirely by the ability to form stable AP-1 heterodimers. Alternatively, it can be speculated that Fra-1 is not devoid of its own transactivating activity, but its transcriptional activation domain might require extensive modification (i.e. phosphorylation), which could be drastically affected by transformation.

The changes in morphology and anchorage-independent growth of the cell lines expressing the antisense *fra-1* show that Fra-1 is necessary for the establishment of the transformed phenotype. Previous reports indicated that ectopic Fra-1 overexpression in Rat-1A cell lines was sufficient to induce tumorigenicity in the absence of morphological transformation (Bergers *et al.*, 1995). In the thyroid cell system, we could clearly detect the effect of Fra-1 inhibition on the transformed cell morphology, but no effect of Fra-1 (or JunB) overexpression in normal

cells, showing that Fra-1 (or JunB) overexpression is necessary but not sufficient for the establishment of the transformed phenotype. These results might reflect the complexity of epithelial tumorigenesis, with respect to mesenchymal cell transformation. Our analysis also indicates the important role played by JunB in thyroid cell transformation. The antisense-mediated inhibition of *junB* resulted in a smaller effect on the transformed phenotype; this result might reflect the residual activity of the c-Jun–Fra-1 and JunD–Fra-1 heterodimers in the antisense JunB-expressing cell lines (Table I and data not shown). Further studies will be required to establish the individual role of the three Jun family members in the oncogenic transformation of thyroid cells.

Our recent results further support the role of *fra-1* in the transformed phenotype. In fact we have detected *fra-1* overexpression in rat thyroid cell lines transformed by other oncogenes, and in human thyroid carcinoma cell lines (data not shown). Moreover, the detection of Fra-1 in various tumors of epithelial origin (S.Battista *et al.*, manuscript in preparation) suggests that *fra-1* overexpression is a general and necessary event in neoplastic cell transformation.

Finally, while this manuscript was under revision, a recent study on the AP-1 compositional changes in *ras*-transformed NIH 3T3 fibroblasts strongly confirmed our results on the crucial role of Fra-1 in oncogenic transformation (Mechta *et al.*, 1997).

Materials and methods

Cell culture and transfection analysis

PC Cl3 rat thyroid cells are derived from 18-month-old and FRTL-5 cells from 3- to 4-week old normal Fisher rats (Fusco *et al.*, 1987; Berlingieri *et al.*, 1988). The PC/C-as, PC MPSV, PC/C-as/MPSV, FRTL-5/C-as, FRTL-5 KiMSV and FRTL-5/C-as/KiMSV cell lines have been described (Berlingieri *et al.*, 1995). The normal and virally infected cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum (Gibco Laboratories) and six growth factors (1×10^{-10} M thyroid-stimulating hormone, 10 μ g/ml insulin, 1×10^{-8} M hydrocortisone, 5 μ g/ml human transferrin, 10 ng/ml somatostatin, 10 ng/ml glycyl-L-histidyl-L-lysine acetate). Soft agar colony assays were performed according to a previously described technique (Macpherson and Montagnier, 1964).

Transfections were performed with the calcium phosphate procedure as described (Graham and van der Eb, 1973). The 5×TRE-tk-CAT (Angel *et al.*, 1987) construct was co-transfected with a plasmid carrying the luciferase reporter gene under the control of the cytomegalovirus (CMV) promoter, and the luciferase activity was determined (de Wet *et al.*, 1987) to normalize for variations of transfection efficiency in the six cell lines analyzed.

Nuclear extracts and electrophoretic mobility shift assay

Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in 10 volumes of a solution containing 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT) (homogenization solution). The cells were disrupted by passage through a 26 gauge needle. Nuclei were collected by centrifugation at 1500 r.p.m. and resuspended in 1.2 volumes of extraction solution containing 10 mM HEPES pH 7.9, 0.4 M NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, to allow elution of nuclear proteins by gentle shaking at 4°C. Nuclei were pelleted again by centrifugation at 12 000 r.p.m. and the supernatant was stored at –70°C. The protease inhibitors leupeptin (5 mM), aprotinin (1.5 mM), phenylmethylsulfonyl fluoride (2 mM), pepstatin A (3 mM) and benzamidine (1 mM) were added to both homogenization and extraction solutions. Protein concentration was determined by the Bradford protein assay (Bio-Rad).

Nuclear extracts (2.5–5 μ g of proteins) were incubated in 20 mM HEPES pH 7.5, 40 mM KCl, 5% glycerol in a volume of 20 μ l containing

1 µg of poly(dI-dC) and 5 mM spermidine, for 10 min at room temperature. Binding reactions were incubated for 15 min after the addition of the probe. The sequences of the oligonucleotide probes were: AP-1 consensus TRE (collTRE), derived from the human collagenase promoter (Lee *et al.*, 1987), 5'-TTCCGGCTGACTCATCAAGCG-3'; Sp1 consensus binding sequence (Briggs *et al.*, 1986), 5'-ATTTCGATCGGGGCGGGGCGAGC-3'. Samples were then separated on 8% native polyacrylamide gels (30:1 in 0.5× TBE; 1× TBE = 89 mM Tris, 89 mM boric acid and 1 mM EDTA). For dissociation rate analysis (off-rate), a 100-fold molar excess of unlabeled competitor oligonucleotide was added after 15 min of incubation with the probe. Aliquots from the same binding mixture were taken at different times, and immediately loaded on native gels. For the antibody supershift analysis, the reactions were performed by pre-incubating nuclear extracts with 0.5 µg of antibody at 4°C for a minimum of 3 h. After addition of the labeled oligonucleotide and 15 min incubation at room temperature, the products were resolved on 8% PAGE. The anti-c-Jun/AP-1, anti-JunB, anti-JunD, anti-c-Fos, anti-FosB, anti-Fra-1 and anti-Fra-2 antibodies were purchased from Santa Cruz Biotechnology (USA).

Immunoblotting analysis

The nuclear extracts were separated by 9% SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore). Membranes were blocked with 5% non-fat milk proteins and incubated with antibodies at a dilution of 1:5000. Bound antibodies were detected by the appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL, Amersham).

RNA extraction and Northern analysis

Total RNA was extracted by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Northern blots and hybridizations were carried out as described in Giancotti *et al.* (1993). cDNA probes were labeled with [α -³²P]dCTP using the random oligonucleotide primer (Ready-To-Go, Pharmacia) to a specific activity equal to or higher than 7×10^8 c.p.m./µg. The probes were: a 0.5 kb human VEGF cDNA fragment (a gift from Dr H.Weich, G.f.Biotechnologische Forschung, Braunschweig, Germany); a 1.0 kb mouse *c-jun* cDNA fragment (De Cesare *et al.*, 1995); a 1.7 kb mouse *jund* cDNA fragment (Hirai *et al.*, 1989); a 1.55 kb mouse *jumb* cDNA fragment (Ryder *et al.*, 1988); a 1.5 kb rat *fra-1* cDNA fragment (Cohen and Curran, 1988); and a 2.1 kb mouse *fosB* cDNA fragment (Zerial *et al.*, 1989).

Reverse transcriptase-PCR analysis

Five µg of DNase-treated total RNA were reverse-transcribed using random hexanucleotide primers (100 mM) and 12 U of AMV reverse transcriptase (Promega). Two hundred ng of cDNA were amplified in a 25 µl reaction mixture containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.4 mM of each primer and 1 U of *Taq* DNA polymerase (Perkin-Elmer). For the collagenase cDNA, 20 cycles (95°C for 30 s, 45°C for 30 s, 72°C for 30 s) were performed in the presence of the following oligonucleotide primers: forward (nucleotides 570–601) 5'-TTG-GCGGGGACGCCATTTTGATGATGA-3' and reverse (nucleotides 1259–1290) 5'-AGACAGCATCTACTTTGTCTGCCAATCCAGG-3', as described (Gack *et al.*, 1994). For the transin/stromelysin cDNA (Breathnach *et al.*, 1987), 20 cycles (95°C for 30 s, 58°C for 45 s, 72°C for 45 s) were performed in the presence of the following oligonucleotide primers: forward (nucleotides 101–120) 5'-GGGGCAGTGAGACAA-GACCA-3' and reverse (nucleotides 694–713) 5'-GACCCAGGGAGTG-ACCAAGT-3'. Expression of the rat GAPDH gene was utilized as an internal control for the amount of cDNA in the PCRs, by co-amplification of a 430 bp cDNA fragment in the presence of the following oligonucleotide primers: 5'-TCACCATCTTCCAGGAGCGAG-3' (forward) and 5'-ACAGCCTTGGCAGACCAAGT-3' (reverse). To ensure no contamination of RNA samples with DNA, negative controls were obtained by performing the PCRs on samples that were not reverse-transcribed, but otherwise identically processed.

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