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Oncogene activation of human keratin 18 transcription via the Ras signal transduction pathway

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ABSTRACT **Keratin 8 (K8) and keratin 18 (K18) are intermediate filament proteins normally expressed in simple epithelial tissues and persistently expressed in a wide variety of carcinomas. Ectopic expression of K8 and K18 occurs in some epidermal and murine skin carcinomas induced by chemical carcinogenesis or oncogenic *ras* expression. We show here that K18 is a direct target of the Ras signal transduction pathway, by demonstrating that activated Ha-Ras, as well as activated Src, Lck, or Raf, stimulates the transcription of K18. This activation is mediated by an enhancer element containing essential and closely spaced Ets and AP-1 transcription factor binding sites. Oncogene activation of K18 transcription provides a molecular explanation for the persistent and sometimes unexpected expression of K18 in such a wide variety of tumors.**

Keratin intermediate filament expression is a useful characteristic to distinguish carcinomas from other types of cancer. The keratin 18 (K18) gene codes for a type I intermediate filament protein found primarily in a variety of single-layered or simple epithelial tissues (1, 2). K18 and its coexpressed complementary subunit, keratin 8 (K8), are persistently expressed, often at elevated levels, in a wide variety of carcinomas derived from internal epithelia including liver, pancreas, intestine, colon, mammary gland, and the uterine and urinary tracts (3–7). Ectopic expression of K8 and K18 occurs in some human epidermal carcinomas (8), murine skin carcinomas induced by chemical carcinogenesis or oncogenic *ras* expression (9, 10), and highly metastatic melanoma cells (11). Furthermore, differences in the reactivity of K18 monoclonal antibodies with the invasive front of human transitional carcinomas (6) and the implication of K8 and K18 filaments in the invasive behavior of cultured cells (12) suggest that K8 and K18 may be functionally important in tumor cell behavior.

Expression of K18 is dependent upon a complex transcriptional enhancer element located within the first intron of the gene (13). Previous studies identified an important AP-1 transcription factor binding site within a conserved 47-bp sequence of this enhancer that mediates transactivation by members of the Jun and Fos families. Here we report the identification of a second transcription factor binding site within the conserved portion of the K18 enhancer that is recognized by members of the Ets family of transcription factors. We show that Ets and AP-1 activate transcription of the K18 gene additively through their respective binding sites situated 9 bp apart. Furthermore, we show that the combination of the Ets and AP-1 sites forms an oncogene-responsive regulatory element that mediates transcriptional activation by a number of nonnuclear oncogenes, all of which activate the Ras signal transduction pathway.

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Cellular transformation results in the altered transcription of a number of cellular genes, some of which are likely important for tumor growth, progression, invasion, and metastasis (14, 15). A wide variety of nonnuclear oncogenes, including activated receptor tyrosine kinases, nonreceptor tyrosine kinases, guanine nucleotide binding proteins, and serine/threonine kinases, can activate target genes through a limited number of regulatory elements (14–18). Several of the genes activated by the Ras signal transduction pathway require the concerted action of closely spaced binding sites for the Ets and AP-1 transcription factors (16–18). Activation of genes through Ets and AP-1 sites appears essential for oncogenesis because both Ras-induced transcriptional activation and cellular transformation are blocked by dominant inhibitory mutants of Jun, Fos, or Ets (19–21). The identification of genes activated by oncogenes will likely provide multiple targets essential for different aspects of tumor cell behavior. Furthermore, identification of the molecular mechanisms mediating transcriptional activation by nonnuclear oncogenes may provide targets of intervention through which a large variety of activated oncogenes mediate their transcriptional effects (15). The finding that many classes of oncogenes can activate K18 expression through its oncogene-responsive element may explain why most carcinomas continue to express this intermediate filament protein and leads to consideration of possible functions of simple epithelial keratins in tumors.

METHODS AND MATERIALS

Cell Culture. F9.22 mouse embryonal carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum. NIH 3T3 cells were grown in DMEM supplemented with 10% (vol/vol) calf serum.

Plasmid Constructions. A 663-bp fragment of the first intron of K18 was placed downstream of the 250-bp K18 proximal promoter, chloramphenicol acetyltransferase (CAT) gene, simian virus 40 tumor antigen intron, and polyadenylylation signal of the previously described XKCATspA vector to generate XKCATIs (13). A variant with a mutated Ets site (XKCATmETS) was generated by PCR with the indicated primers and substituted for the wild-type intron in XKCATIs (Fig. 1). The construction of a variant with a mutated AP-1 site (XKCATmAP-1) is described elsewhere (13). The expression vector for wild-type Ras and oncogenic Ras (Ha-ras 61L) was pZIPneoSV(X)1 and has been described (22). The activated Raf expression construct was made by inserting a truncated *Raf* gene (p22W, ref. 23) into the same vector. The v-Src expression construct was made by inserting the v-*src*

Abbreviations: K8, human keratin 8; K18, human keratin 18; CAT, chloramphenicol acetyltransferase.

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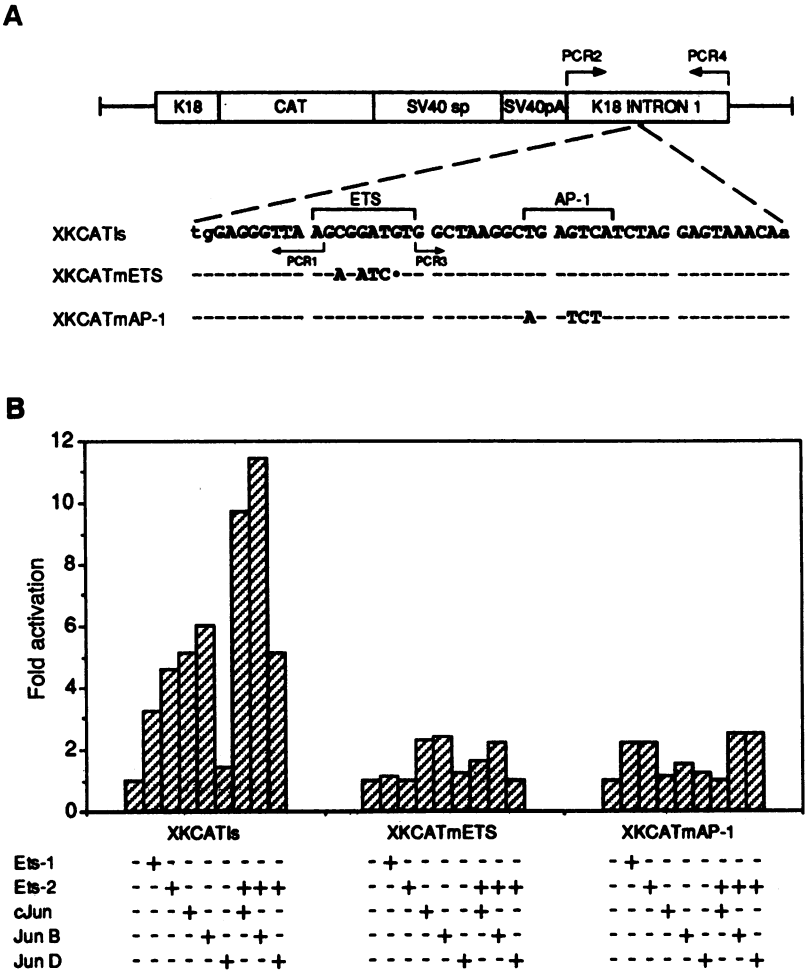

FIG. 1. Functional analysis of the Ets and AP-1 binding site within the first intron of human K18. (*A*) Schematic diagrams of the CAT reporter constructs used in transient transfection analysis. In all constructs the CAT transcription unit is followed by the simian virus 40 tumor antigen intron (SV40sp) and polyadenylylation signals (SV40pA) and was driven by 250 bp of 5′ flanking sequence of K18. The XKCATmETS and XKCATmAP1 vectors contain mutations of the Ets and AP-1 sites, respectively. The mutations are indicated below the wild-type sequences. Dashes indicate unchanged residues and a dot indicates a deleted nucleotide. Uppercase type indicates identical nucleotides in mouse and human K18 first introns. Positions of the PCR primers used for the construction of the Ets mutation are indicated. (*B*) Trans-activation of the XKCATIs and its mutated variants XKCATmETS and XKCATmAP-1 by different members of the Ets and Jun oncogene families in F9 embryonal carcinoma cells. CAT activity was normalized to β-galactosidase activity in the same lysate of a cotransfected *lacZ* gene driven by the β-actin promoter. The indicated fold activation is relative to the reporter construct alone. The basal levels of the XKCATmETS and XKCATmAP-1 vectors were typically ≈40% and 30%, respectively, of the wild-type construction. The results shown represent averages of two or more separate experiments.

gene into pLSDL (24). The activated Lck expression construct is LckF505 (25). The v-Src and LckF505 expression constructs were generously supplied by M. Kamps (Salk Institute, La Jolla, CA).

To replace the Ets and AP-1 mutations into the context of the whole K18 gene, two regions of the gene from positions −314 to +505 and positions +1167 to +2400 were synthesized by the PCR and joined by synthetic *Xba* I restriction sites to form a deletion of 663 bp of the first intron. A fragment containing the deletion was inserted into K18 via unique *Xho* I and *Nco* I sites. *Xba* I restriction fragments of the wild-type first intron or its mutated variants were generated from XKCATIs, XKCATmETS, and XKCATmAP-1 and were inserted into the *Xba* I site of the deleted intron to generate a control form of K18 (K18In) and constructs with a mutated Ets site (K18mETS) or mutated AP-1 site (K18mAP-1).

DNA Transfections and CAT Assays. For F9 cells, the reporter CAT constructs (10 μg) were transfected by the calcium phosphate precipitation method along with 2 μg of expression vectors of the indicated trans-activators and 2 μg of a β-actin–LacZ plasmid (13). When necessary, pUC9 DNA was added for a total of 20 μg of DNA per 9-cm plate. Thirty-six hours after transfection, the cells were harvested and CAT and β-galactosidase activities were determined as described (13). Dishes (6 cm) of NIH 3T3 cells were transfected by calcium phosphate coprecipitation of 2 μg of the indicated reporter gene, 5 μg of the expression construct (except 0.5 μg of v-*src*), and 8 μg of pUC19 carrier DNA. Sixteen hours after the precipitate was added, the cells were washed with phosphate-buffered saline, and the medium was replaced with DMEM containing 0.5% calf serum. After an additional 24 hr, the cells were harvested and the CAT activity was determined.

RNase Protection Analysis. Seven micrograms of DNA from each K18 construct was cotransfected with 8 μg of expression construct for wild-type (Ha-*ras* wt) or oncogenic (Ha-*ras* 61L) Ras per 9-cm dish of cells by the calcium phosphate precipitation method. Five micrograms of standard plasmid pMC1NeopA DNA was included in each experiment to determine transfection efficiency. RNA was purified by a single-step isolation method (26). Total RNA was treated with RNase-free DNase I at 37°C for 60 min in the presence of RNase inhibitor (Stratagene). RNA (20 μg) was analyzed by RNase protection as described (27).

***In Vitro* Footprinting.** Analysis of the binding of recombinant c-Jun and c-Ets2 DNA binding domain to the K18 intron enhancer by DNase I protection was performed as described (28). A DNA fragment of K18 from positions +735 to +973 was end-labeled and incubated with bovine serum albumin, recombinant c-Jun and c-Fos purified from *Escherichia coli* (gifts of T. Deng and M. Karin, University of California, San Diego), or recombinant *Ets-2* DNA binding domain and digested with DNase I. Fragments were separated on a sequencing gel and visualized by autoradiography.

RESULTS

Closely Spaced Ets and AP-1 Binding Sites Function in the K18 Enhancer. A complex transcriptional enhancer within the first intron of K18 contains an essential AP-1 transcription factor binding site embedded within a 47-bp sequence that is identical in the mouse and human genes (13). Deletion and mutagenesis analysis located a functional Ets binding site at position +796, 9 bp upstream of the AP-1 site in the first intron (Fig. 1*A*). Deletion or mutation of two other potential Ets binding sites at positions +703 and +1104 had no effect on Ets transactivation (data not shown). The K18 Ets motif differs from a similar site associated with the collagenase promoter (17) by a single nucleotide. The K18 enhancer was activated by either Ets or Jun family members and the effect of combining them was additive (Fig. 1*B*). JunD failed to activate the K18 reporter construction but did not interfere with the activation by Ets (Fig. 1*B*). Another Ets family member, PU.1 (29), also failed to activate the K18 enhancer (data not shown), indicating that only a subset of Ets and AP-1 family members are active on this enhancer element. Mutation of the K18 Ets site abrogated transactivation by both Ets-1 and Ets-2 and decreased transactivation by c-Jun and JunB (Fig. 1*B*). Similarly, mutation of the neighboring AP-1 site abolished transactivation by Jun and reduced activation by Ets (Fig. 1*B*). The mutual reduction in activation by factors whose cognate sites were not changed suggests that the Ets and Jun transcription factors may interact.

To confirm that Ets and AP-1 proteins can bind to the expected sites in the K18 first intron, DNase I footprinting analysis was performed on plasmid DNA containing these sites with a recombinant c-Ets-2 DNA binding domain and c-Jun/c-Fos proteins (Fig. 2). The Ets DNA binding domain generated a footprint centered on the expected Ets site at nt +796 with an internal DNase I hypersensitive site, as observed in the footprint pattern of other members of the Ets family (30, 31). Similarly, c-Jun/c-Fos bound to the expected AP-1 site. Furthermore, genomic footprinting methods revealed that the Ets binding site and AP-1 site were occupied in K18 transgenic mouse liver, which expressed the transgene (32), but were not occupied in nonexpressing transgenic spleen (A.U. and R.G.O., unpublished results). These results reinforce the views that transactivation by Ets and Jun occurs by direct binding to the K18 sequences and that normal expression is correlated with the occupancy of these sites.

Activation of K18 Transcription by Nonnuclear Oncogenes Requires Both the Ets and AP-1 Sites. The close proximity and apparent interdependence of the Ets and AP-1 sites within the K18 intron enhancer resembles the oncogene-response elements found in several viral and cellular genes (15). To test whether the closely spaced Ets and AP-1 sites in K18 are oncogene-response elements, K18 reporter genes were cotransfected with oncogene expression constructs (Fig. 3). While activation by wild-type Ras was minimal, the expression of oncogenic Ras resulted in an 11-fold increase in expression of the CAT reporter gene containing a single copy of the K18 enhancer (XKCATIs). Mutation of either the Ets or the AP-1 binding site abolished the activation by oncogenic Ras. This requirement for both the Ets and AP-1 binding sites

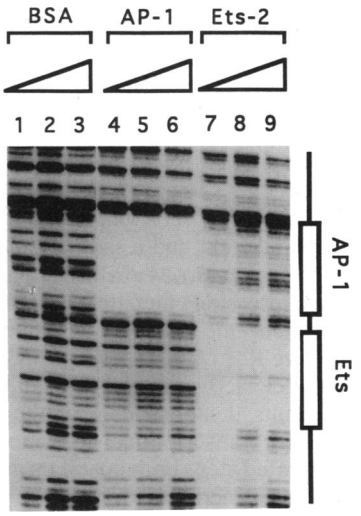

FIG. 2. Binding of recombinant Ets-2 and c-Jun/c-Fos to the human K18 first intron enhancer. An end-labeled DNA fragment of the K18 first intron (nt 735–973) was incubated with bovine serum albumin (BSA, lanes 1–3), a combination of recombinant c-Jun and c-Fos (lanes 4–6), or the human Ets-2 DNA binding domain (lanes 7–9) and digested with DNase I. The concentration of nuclease was 75 ng/ml (lane 1), 150 ng/ml (lane 2), 300 ng/ml (lane 3), 100 ng/ml (lanes 4 and 7), 200 ng/ml (lanes 5 and 8), or 400 ng/ml (lanes 6 and 9). The positions of the observed AP-1 and Ets-2 footprints are indicated to the right.

is similar to that previously found for several other oncogene response elements (16–18). The activation by Ras was not dependent upon the homologous K18 promoter used in these constructs as a similar activation was observed with a CAT

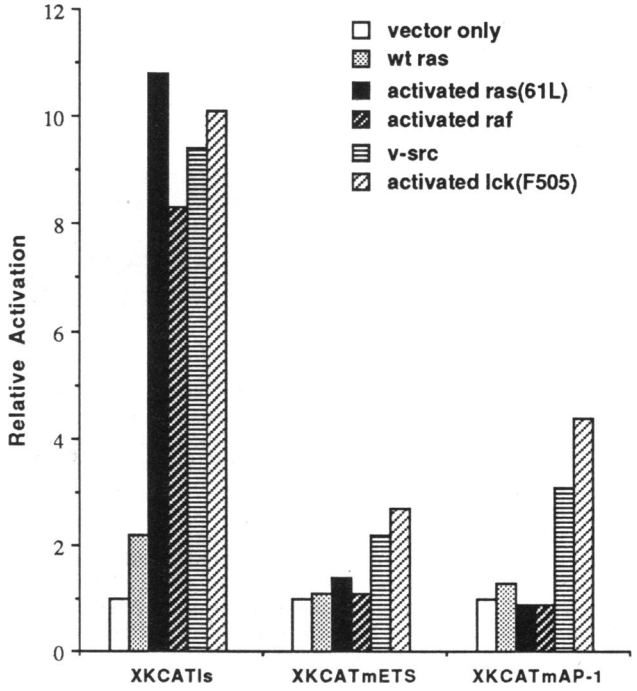

FIG. 3. Nonnuclear oncogenes activate K18 transcription through adjacent Ets and AP-1 sites in the enhancer. Relative activation for each reporter (shown in Fig. 1*A*) in 3T3 cells was calculated by comparing CAT activity of the reporter cotransfected with oncogene expression constructs to the same reporter cotransfected with empty expression vector. The basal level of each reporter was defined as 1.0, but the basal level of the Ets and AP-1 mutants was 3- and 9-fold lower than wild type, respectively. The results shown are the average of at least two experiments.

gene driven by the Herpes simplex thymidine kinase gene promoter and the K18 intron enhancer (data not shown). Furthermore, cotransfection experiments with XKCATIs revealed that K18 expression is similarly induced through this element by activated Raf, v-Src, or activated Lck (Fig. 3). Cotransfection of the nuclear oncogene E1A did not induce K18 expression (data not shown). We noted that mutations in the Ets or AP-1 sites did not completely abolish the K18 activation by Src and Lck, and it is likely that this residual activation occurs by a pathway distinct from Ras. Src and Lck can activate expression of a minimal promoter construct not activated by oncogenic Ras, and high-level expression of a dominant negative Ras (Asn-17) mutant (33) only partially inhibits Src and Lck activation of K18 (data not shown). Overall, these results demonstrate that the K18 enhancer element is a target for transcriptional activation by oncogenic Ras and other activated steps both upstream and downstream in the Ras signal transduction pathway.

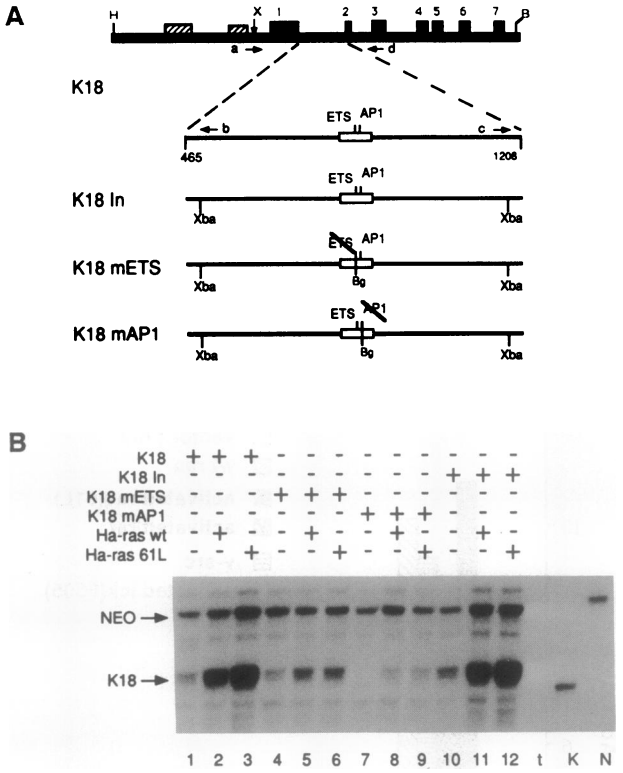

FIG. 4. Ras-induced trans-activation of K18 and its mutated variants K18 mETS and K18mAP-1. (*A*) Schematic representation of K18 and the strategy used for introduction of the mutations in Ets and AP-1 binding sites. At the top, a portion of K18 is represented with exons designated as solid boxes numbered from 1 to 7. Shaded boxes represent Alu-type repetitive elements. The region of the first intron (positions 465–1208 relative to the major start of transcription) is enlarged and the positions of Ets binding site (ETS) and AP-1 binding site (AP-1) are indicated. H, *Hin*dIII; X, *Xho* I; Xba, *Xba* I; Bg, *Bgl* II; B, *Bam*HI. (*B*) RNase protection analysis of the products of the indicated constructs after transient expression in NIH 3T3 cells. Each K18 construct was cotransfected with the indicated expression vectors. RNA was isolated and analyzed by RNase protection analysis using simultaneous hybridization with [^{32}P]UTP-labeled probes for the neomycin-resistance gene and the K18 first exon (26). The amount of RNA loaded in each lane was normalized to the neomycin-resistance mRNA signal to facilitate direct comparison of the K18 signals. The positions of the protected fragments are indicated to the left. Lanes: t, 20 μg of tRNA (negative control); K, 10 pg of synthetic K18 mRNA standard; N, 5 pg of synthetic neomycin-resistance mRNA standard. The protected fragment of the K18 standard is slightly shorter than the expressed RNA because not all of the 5′ noncoding sequence is included in the K18 cDNA.

The Ets and AP-1 Sites Are Required for Ras Responsiveness in the Context of the Whole K18 Gene. Because most analysis of Ras-responsive elements has been performed with fusion genes containing multimerized response elements, the mutations of the intron Ets and AP-1 binding sites were tested within the context of the whole K18 gene. The results of these RNase protection experiments (Fig. 4) show that mutation of either the Ets or AP-1 sites greatly reduces the Ras responsiveness of the K18 gene. These findings confirmed the importance of the intron enhancer as the major mediator of K18 transcriptional activation by Ras. However, in contrast to the results of the CAT reporter genes containing the isolated intron, the mutation of the Ets binding site did not completely abolish the Ras activation of the whole gene. This suggests that other K18 regulatory elements may make a small contribution to the observed transcriptional activation of K18 by oncogenic Ras.

DISCUSSION

The Ras signal transduction pathway provides a means of integrating a large number of regulatory signals, some of which may be involved in normal regulation of K18 during development. Our results show that members of the Ets and AP-1 transcription factor families can bind to K18 intron enhancer sequences and that mutation of these binding sites abrogates the transcriptional activation by the cognate transcription factors. The activation of K18 by Ras, Raf, v-Src, and Lck is dependent upon the same two sites. This suggests that other oncogenes that activate the Ras signal transduction pathway could similarly activate K18. Clearly, the Ets and AP-1 binding sites of the K18 intron enhancer mediate this activation even with a single copy of the enhancer. However, the K18 enhancer is complex and the 47-bp conserved sequence containing the Ets and AP-1 binding sites is not sufficient for enhancer activity (13) nor does it support Ras activation of a K18–CAT reporter construct (R.P., C.H., and R.G.O., unpublished data). Thus, these regulatory elements, when assayed within the context of the conserved region of the enhancer, are necessary but not sufficient for the Ras responsiveness of the K18 gene. The interaction between the factors that bind to both sites is not clear. The decrease in transactivation by Jun when the Ets site is mutated and the decrease in transactivation by Ets when the Jun site is mutated (Fig. 1) would suggest an interaction between the two types of transcription factors. A similar dependence on both sites for transactivation by Ras was observed. However, the transactivation of the enhancer by both transcription factors appears to be only additive and not synergistic as might be expected from other examples of interacting transcription factors. The mechanism of activation through these regulatory elements remains to be determined. Oncogenic Ras has been found to stimulate phosphorylation of the c-Jun activation domain, which correlates with enhanced AP-1 activity (34). It remains to be determined which members of the Ets, Jun, or Fos transcription factor families mediate the increased activity of the Ras-responsive elements that require both Ets and AP-1 binding sites. K18 expression is regulated by both its chromatin state (27, 35, 36) and transacting transcription factors (13). While we have not detected elevated mouse K18 expression in Ras-transformed 3T3 fibroblasts (R.P. and R.G.O., unpublished data), K18 may be particularly stably repressed in cultured fibroblasts by its chromatin state and by additional methylation of the gene in cultured cells not found in tissues of mice (27, 35). Escape from a repressive chromatin state may account for the unusual expression of K18 in some normal tissues and transformed cell lines (9, 10, 37, 38). As the repressed chromatin state of K18 appears relatively stable, it is of interest to determine whether the activation of the Ras signal

transduction pathway increases the probability of release from a repressive chromatin state. This relief may be a major component of the stochastic nature of ectopic K18 expression in nonepithelial-derived tumors and transformed cells (37, 38).

We suggest that tumors that express a variety of nonnuclear oncogenes may share the characteristic of an activated Ras signal transduction pathway resulting in elevated expression of endogenous target genes through Ets and AP-1 regulatory elements. Some of these genes are likely to be functionally important for tumor progression. The structure of the K18 oncogene-responsive element is closely related to that reported for two extracellular proteases, collagenase and stromelysin, that are thought to be important to the invasive behavior of tumor cells (17, 39). The identification of K18 as an oncogene-responsive target reinforces the potential importance of cytoskeletal elements in mediating the behavior of carcinomas. The activation of K18 expression by Ras or other oncogenes may be functionally important to the invasive behavior of tumor cells, because K18 expression has been correlated with increased invasiveness (6, 8, 11, 40). Furthermore, experiments utilizing forced expression of K8 and K18 proteins suggest a possible role for these keratin filaments in invasive motility (12). Thus, deregulated expression of K8 and K18 may contribute to the metastatic behavior of tumor cells.

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