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AP-1, ETS, and Transcriptional Silencers Regulate Retinoic Acid-Dependent Induction of Keratin 18 in Embryonic Cells

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The differentiation of both embryonal carcinoma (EC) and embryonic stem (ES) cells can be triggered in culture by exposure to retinoic acid and results in the transcriptional induction of both the endogenous mouse keratin 18 (mK18) intermediate filament gene and an experimentally introduced human keratin 18 (K18) gene as well as a variety of other markers characteristic of extraembryonic endoderm. The induction of K18 in EC cells is limited, in part, by low levels of ETS and AP-1 transcription factor activities which bind to sites within a complex enhancer element located within the first intron of K18. RNA levels of ETS-2, c-Jun, and JunB increase upon the differentiation of ES cells and correlate with increased expression of K18. Occupancy of the ETS site, detected by in vivo footprinting methods, correlates with K18 induction in ES cells. In somatic cells, the ETS and AP-1 elements mediate induction by a variety of oncogenes associated with the *ras* signal transduction pathway. In EC cells, in addition to the induction by these limiting transcription factors, relief from negative regulation is mediated by three silencer elements located within the first intron of the K18 gene. These silencer elements function in F9 EC cells but not their differentiated derivatives, and their activity is correlated with proteins in F9 EC nuclei which bind to the silencers and are reduced in the nuclei of differentiated F9 cells. The induction of K18, associated with the differentiation of EC cells to extraembryonic endoderm, is due to a combination of relief from negative regulation and activation by members of the ETS and AP-1 transcription factor families.

The differentiation of embryonic stem (ES) cells and closely related embryonal carcinoma (EC) cells in culture is a paradigm for the appearance of some of the first distinguishable cell types of mouse development. In response to exposure to retinoic acid, both ES and EC cells differentiate to form extraembryonic endodermal cells, which are distinguished by a variety of molecular markers of the cell surface, extracellular matrix, cytoskeleton, and nucleus (22, 52–54). In addition, the differentiation of ES and EC cells results in a limited multiplication potential and thus renders these tumorigenic stem cells benign. While retinoic acid exposure regulates some marker genes by direct interaction with retinoic acid receptors (31, 55, 60), others are indirectly regulated (9, 11, 23–25, 32, 43, 49, 53, 61).

Keratin 18 (K18, mK18, or EndoB in mice) (37, 41, 44) represents an endogenous gene which is indirectly induced by the action of retinoic acid on ES and EC cells. This gene codes for a type I keratin intermediate filament protein which is usually coexpressed with its type II heteropolymeric subunit, keratin 8 (K8, mK8, or EndoA in the mouse). Previously, AP-1 transcription factor activity, composed of members of the *jun* and *fos* gene families, was implicated in the activation of K18 through a binding site in a transcriptional enhancer region located within the first intron of the gene (43, 46). Subsequently, this regulatory region was shown to be responsive to induction by a variety of oncogenes which participate in the *ras* signal transduction pathway (46). In addition, this induction was dependent on both the AP-1 site and a regulatory element mediating activation by members of the ETS family of transcription factors (46).

We now show that both the ETS and AP-1 regulatory elements of the K18 enhancer contribute to the activation of K18 expression in stem cells treated with retinoic acid and that utilization of these elements correlates with induced levels of c-Jun, JunB, and ETS-2 mRNAs. However, in addition to these positive regulatory mechanisms, we have discovered three negative regulatory elements (NREs) which act as silencers in EC cells but not somatic cells. Relief from this negative regulation and increased ETS and AP-1 activities mediates the retinoic acid induction of the K18 gene in early ES cells.

MATERIALS AND METHODS

Recombinant DNA constructions. The K18d3 vector was described previously (58). The K18dIn vector, containing a deletion of most of the K18 first intron, was constructed by PCR to generate two fragments, one from +505 (relative to the major transcriptional start site [29]) upstream of a unique *Xho*I site and the second from +1165 downstream beyond an *Nco*I site. The two PCR fragments were ligated through *Xba*I sites included in the primers, and the *Xho*I-to-*Nco*I fragment was replaced in the K18 gene. The unique *Xba*I site which is created in K18dIn was the insertion site for the wild-type, mutant, or deleted intron fragment for K18In, K18mAP-1, K18mETS, and K18d47. These intron fragments have been described previously (43, 46). The K18E100 intron fragment was generated by PCR using primers with terminal *Xba*I sites. The K18-TATA vector was generated by the same method, using primers terminating in a *Bgl*II site instead of the TATA box sequence and the upstream *Xho*I site and a downstream *Nhe*I site in two steps because of a second *Nhe*I site within the body of the gene.

The XKCATspa, XKCATIs, and TKCAT vectors were described previously (43). Fragments of the K18 first intron were subcloned into the unique *Xba*I site of XKCATspa, using the compatible *Nhe*I site at nucleotide (nt) 975 or the *Xba*I sites added by PCR primer amplification. The region between nt 705 and 735 was deleted and replaced by a *Cla*I site in a preliminary test of a potential ETS site which proved to be without effect (data not shown). The *Cla*I site of the precursor

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vector was used for generating deletions of the full-length XKCATIs intron. The *Apa*I (+865)-to-*Nhe*I (+975) Nα110 fragment from the K18 first intron was used to make TKCAT-Nα110, XKCAT-Nα110, and E100+Nα110 vectors. This fragment was recloned from the Bluescript KS polylinker to the pUC19 polylinker and then to the *Kpn*I and *Xho*I sites of the TKCAT, XKCATspa, and XKCATE100 vectors. The Nα110+Nβ190 vector was made by the substitution of the *Eco*RI-*Xba*I fragment of the Nα110 vector by the corresponding fragment from the XKCAT-Nβ190 vector. The Nα18 double-stranded oligonucleotide was cloned into the *Xho*I sites of the XKCATspa, XKCATE100, and XKCAT-Nβ190 vectors. The Nβ oligonucleotide was cloned into the *Xba*I sites of the XKCAT-Nα110 and (Nα18)3 vectors. The number and orientation of the oligonucleotide copies were determined by sequencing.

Transient expression assays. F9 cells were seeded at a density of 1.5×10^6 cells per 9-cm-diameter dish 1 day before transfection. The medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum) was changed 4 h before transfection. Each chloramphenicol acetyltransferase (CAT) construct (10 μg) was introduced by the calcium phosphate precipitate method either alone or with 2 μg of the indicated transactivator plasmid (pECE-Ets2 or RSVc-Jun). Two micrograms of LK4Lac (the human β-actin promoter driving the β-galactosidase gene) was present in each precipitation mixture for determination of the transfection efficiency. When necessary, pUC9 was added to adjust the total amount of DNA in each mixture to 20 μg. After 16 h, the precipitates were removed with phosphate-buffered saline (PBS) containing 0.2 mM EGTA and two washes with PBS alone. The cells were left in normal medium for an additional 24 h, and CAT and β-galactosidase activities were determined as previously described (46).

For RNA analyses, F9 cells were seeded at a density of 10^7 cells per roller bottle and were treated with retinoic acid (1 μM final concentration) for 3 days. The differentiated F9 cells were collected by treatment with trypsin and EDTA (yield, 7.5×10^7 per bottle) and seeded into 9-cm-diameter dishes at a density of 4×10^6 cells per dish. Undifferentiated F9 cells were seeded at a density of 2.5×10^6 cells per 9-cm-diameter dish. Four hours later, the medium was changed, and after an additional hour, the DNA precipitate was added. For each precipitation mixture, 30 μg of K18 constructs and 10 μg of pMC1NeopA plasmid were used in a final volume of 2 ml. Half of each precipitation mixture was used for transfection of undifferentiated F9 cells, and the other half was used for transfection of retinoic acid-treated F9 cells. After 16 h, the cells were washed with PBS containing 0.2 mM EGTA and twice with PBS and then left overnight in normal medium. Total RNA was isolated with the use of acidic phenol (7) and was treated with RNase-free DNase I (10 μg/ml) in the presence of RNase block II (Stratagene) for 1 h at 37°C. The reaction was stopped by addition of EDTA and sodium dodecyl sulfate (SDS) to final concentrations of 10 mM and 0.2%. After phenol-chloroform extraction, the RNA was precipitated and washed with 3 M sodium acetate (pH 5.2) to remove oligonucleotides. The RNA precipitates were dissolved in 10 mM Tris-HCl–1 mM EDTA (pH 7.6) and stored in ethanol until use. RNase protection analysis was performed as previously described (40, 46). The RNase protection method distinguishes the human K18 RNA from the endogenous mK18 form. Plasmid pMC1NeoA contains a mutant polyomavirus B enhancer and thymidine kinase (TK) promoter (15, 33) driving the bacterial Neo^r gene (57). Previous data suggested that the promoter-enhancer combination of pMC1NeoA was relatively insensitive to the cell type (43). However, in the context of pMC1NeoA, many experiments indicate that the RNA signal from this

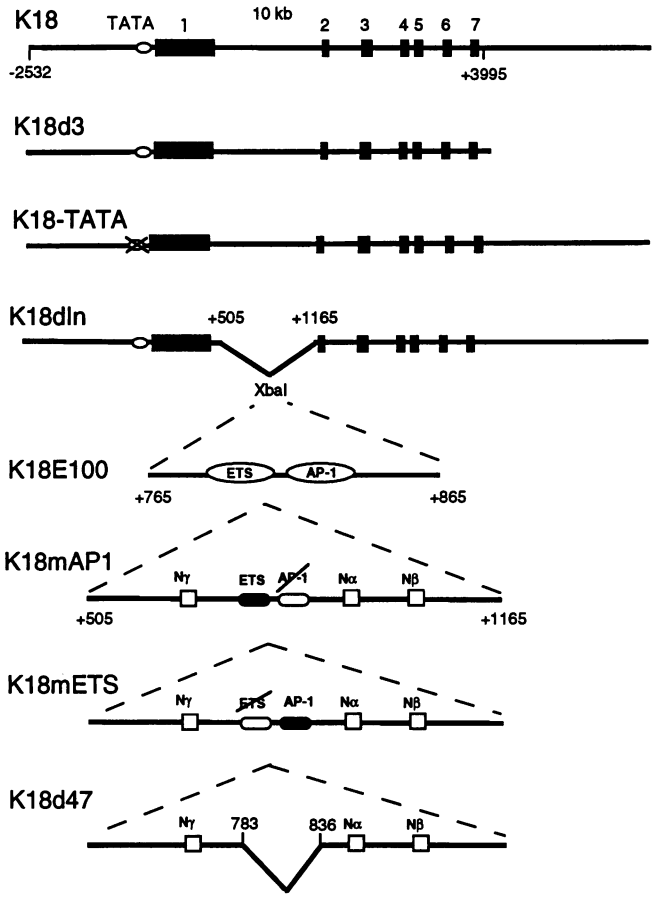

FIG. 1. Representations of the modifications of the K18 gene. The numbered black boxes represent K18 exons. The TATA box is indicated by the oval. The K18dIn vector represents a deletion between nt 505 and 1165 replaced by an *Xba*I cloning site for the insertion of the modified intron fragments. The lined-out elements indicate site specific mutations. The open boxes indicate putative silencer elements.

vector was approximately twofold higher in differentiated F9 cells (data not shown). This may represent the combination of increased transfection efficiency and greater intrinsic activity of the promoter in differentiated F9 cells. Thus, standardization to pMC1NeoA represents a conservative normalization which may, if anything, underestimate the activation of K18 expression in differentiated F9 cells.

Isolation and characterization of stably transfected ES cells. E14 ES cells were transfected with the use of electroporation as previously described (4), using *Hin*dIII-digested K18 (15 nM) and either pMC1NeoA (5 nM) or a modified *HGPRT* gene [pNI2(I1S)] (48) as the selectable gene. After two weeks, D3 clones resistant to 150 μg of G418 per ml were pooled after trypsin treatment of the multiple plates (more than 1,000 individual clones for each pooled population) or isolated individually with the use of cloning cylinders. E14 clones cotransfected with the *HGPRT* gene were resistant to hypoxanthine-aminopterin-thymidine medium. The average numbers of integrated copies of the K18 genes in both individual clones and pooled populations were determined by dot blot and Southern blot hybridization relative to diluted plasmid DNA standards and previously characterized transgenic mouse DNAs (1, 58). Differentiated populations of cells were obtained by exposing ES cells

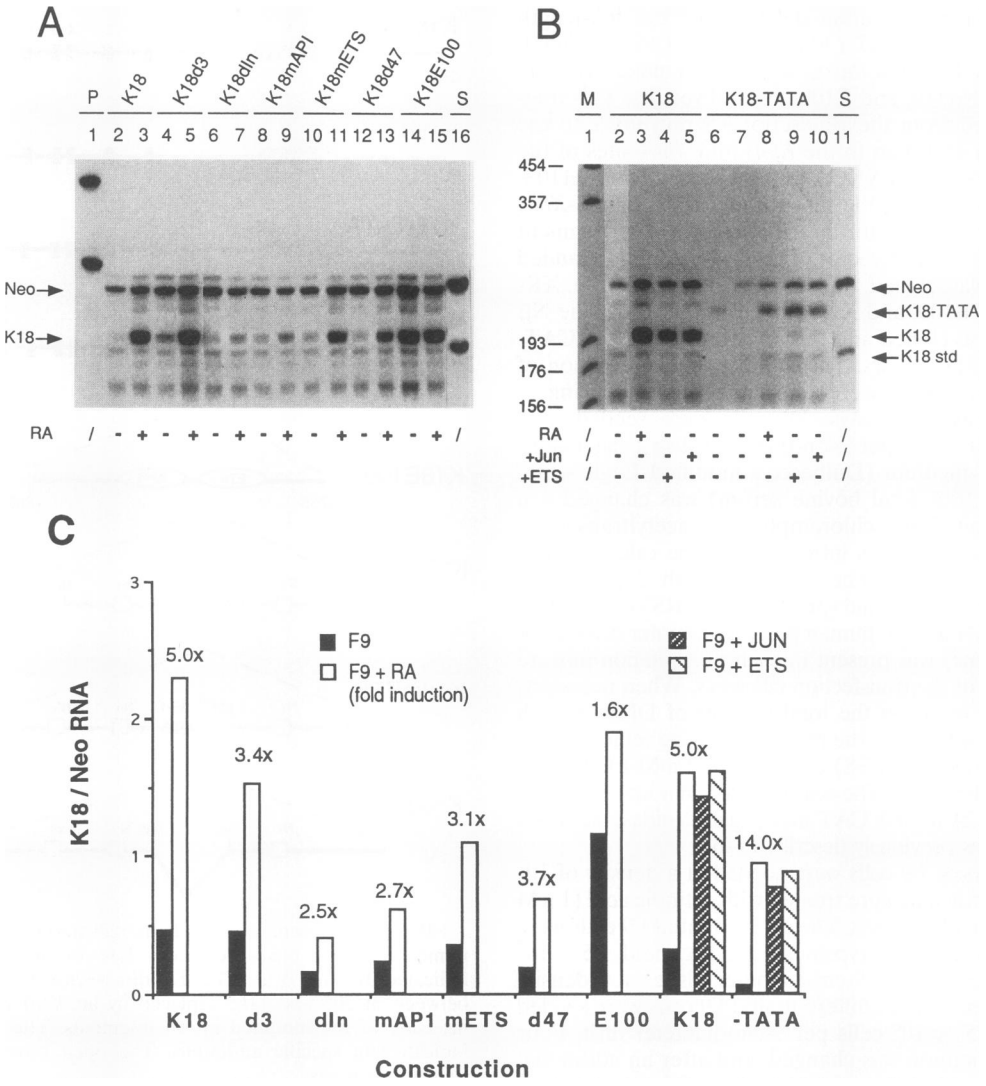

FIG. 2. Transient expression of K18 RNA from transfected constructions in undifferentiated and differentiated F9 cells. The indicated constructions were introduced into either untreated F9 cells (−) or F9 cells treated with retinoic acid (RA) for 4 days (+) along with the pMC1NeoA vector as a transfection standard. RNA was isolated, and the K18 and *neo* RNAs were detected by RNase protection methods. P, probes; S, synthetic standard RNAs (3 pg of the *neo* RNA and 10 pg of K18 RNA); M, size markers marked in base pairs. The K18 standard is shorter than the authentic mRNA because not all of the 5′ end is represented in the cDNA. (A) The vectors are indicated at the top. Note the decreased K18 signal in lanes 7, 9, 11, and 13 relative to lane 3. (B) Transactivation of the K18-TATA mutation by ETS and Jun. The vectors indicated at the top were transfected with expression vectors for c-Jun or ETS-2 as indicated at the bottom. The K18 signal due to the K18-TATA construction is shown in the absence of the *neo* probe in lane 6. The *neo* signal is seen in lane 7 in the absence of the K18 probe. Note that the K18-TATA protected fragment is larger than that of the K18 RNA because of initiation upstream of the normal start site. (C) Summary of normalized data. Densitometer signals for the K18 RNAs were normalized to the *neo* signal of each lane. The values above each group of columns represent the fold increase in retinoic acid-treated F9 cells (F9 + RA). Note the decreased values for the dIn, mAP1, mETS, and d47 constructs and the increased basal level of the E100 construct.

to 1 μM retinoic acid for various periods. Total RNA was analyzed by agarose gel electrophoresis in the presence of formaldehyde and standard blot hybridization methods. The probes for K18, c-Jun, JunB, L32, and EndoA have been previously described (5, 30, 44). The mouse ETS-2 probe was the full cDNA generously provided by Richard Maki (La Jolla Cancer Research Foundation, La Jolla, Calif.).

Mobility shift DNA-binding assay. Nuclear extracts were prepared by the method of Dignam et al. (10). The double-stranded oligonucleotides Nα (TCGAAAATGCCAGGAG AGGGCT), Nβ (CTAGCAATCCAGGAAAGGAG), Nγ (C TAGCTTTCCCAGGAGAGAG), Nαm1 (TCGAAACGAC CAGGAGAGGGCT), NRE (CTAGCATCTCCTCAGTTT CAG), and ETS-binding site from the K18 intron (nt 3320) (AGCTGGGTTAAGCGGATGTGGC) were labeled by the action of the Klenow fragment of DNA polymerase I in the presence of [^{32}P]dCTP on 4-bp staggered ends. Ten or 20 μg of nuclear proteins was mixed with radioactive probes (2×10^4 cpm/ng) and 1 μg of poly(dI/dC) nonspecific competitor in 20 μl of binding buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 10% glycerol) for 20 min at room

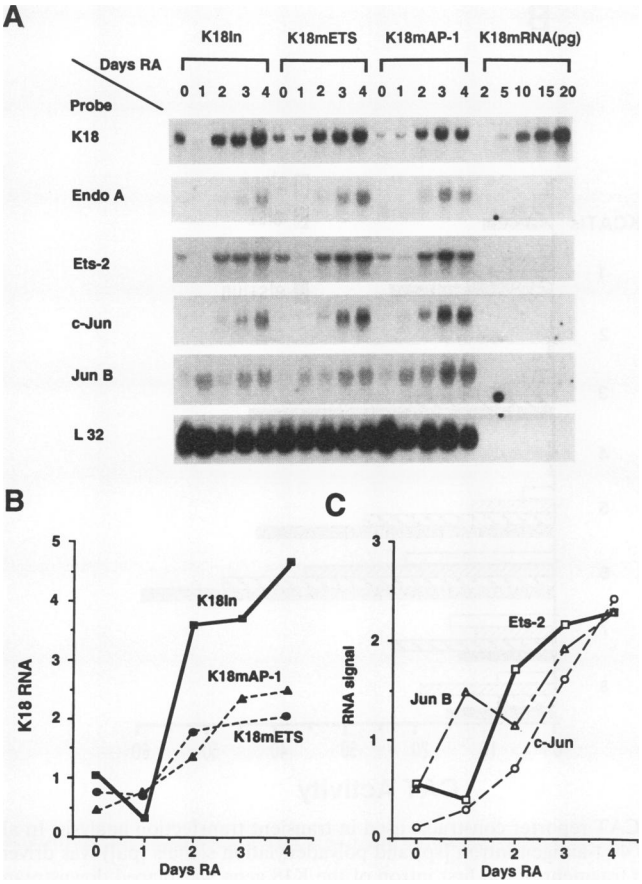

FIG. 3. Contributions of the ETS and AP-1 elements to the retinoic acid (RA)-dependent induction of K18. (A) Northern blot analysis of the pooled populations of stably transfected ES cells containing control or mutant K18 genes. Twenty micrograms of RNA or the indicated amount of synthetic K18 standard RNA was analyzed by gel electrophoresis and blot hybridization with the indicated probe used sequentially after each round of hybridization, exposure, and stripping. (B) Quantitative changes in K18 RNA during retinoic acid-induced differentiation of ES cells. Densitometer signals were compared against standard K18 RNAs. The estimated abundance of K18 RNA was then adjusted for the actual load, as indicated by the L32 ribosomal protein gene hybridization signal. Final adjustments were made for the average copy number of the pooled populations, which were approximately 7, 10, and 7 K18 gene copies per cell for the K18In, K18mETS, and K18mAP-1 populations, respectively. (C) Induction of ETS-2, c-Jun, and JunB RNAs during ES cell differentiation. Densitometer values of the signals for each lane were normalized to the L32 ribosomal protein RNA value. The values for the constructs were then averaged, as little difference was apparent between the different populations carrying different K18 genes. Note the decrease in ETS-2 and K18 RNAs 1 day after retinoic acid treatment and subsequent increases with successive days of treatment.

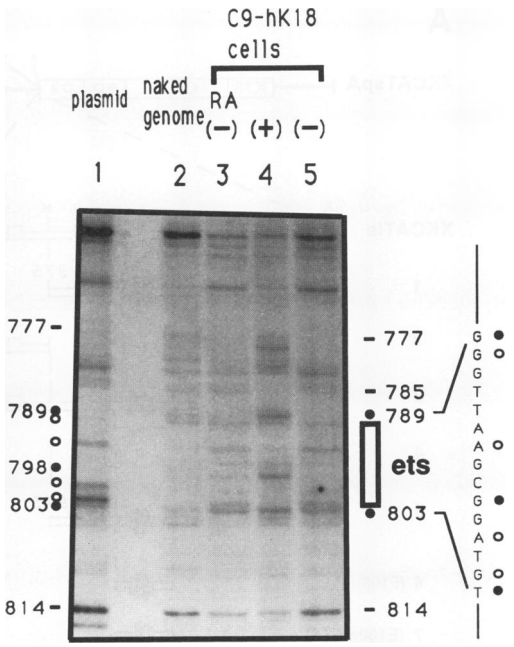

FIG. 4. Occupancy of the intron ETS site in retinoic acid (RA)-treated C9 ES cells containing the K18 gene. Nuclei from the C9 ES cells representing a clonal isolate containing 17 copies of the wild-type K18 gene were treated with DNase I. The DNA was purified and analyzed by LMPCR to reveal cutting sites. Lane 1 represents the G+A chemical cleavage of a recombinant K18 gene fragment corresponding to the amplified region. Naked genome represents the signal from the DNase digestion of purified genomic DNA. The band pattern reveals the DNase I bias of cutting sites. Lanes 3 and 5 represents the signals obtained from undifferentiated C9 cells. Lane 4 shows the fragments resulting from DNase digestion of nuclei derived from C9 cells treated with 1 μM retinoic acid for 4 days. Nucleotide numbers are shown at the sides relative to the major start of transcription. Bands representing hypersensitive sites are designated with filled circles, and bands decreased in intensity are marked by open circles. Note that the hypersensitive G at position 798 is found at the beginning of the core-binding site GGAT for ETS-2 and is characteristic for ETS-2 binding in vitro. In addition, the protected nucleotides flanking nt +798 and the terminal hypersensitive sites at nt +789 and +803 define the occupancy of the ETS site in retinoic acid-treated cells but not undifferentiated cells.

temperature. The electrophoresis of DNA-protein complexes was performed in a 25-cm 5% polyacrylamide gel in 0.4× Tris-borate buffer (pH 7.9) at 4°C. The complexes were detected by autoradiography of the dried gels in the presence of intensifier screens.

Genomic footprinting. Undifferentiated C9 ES cells and differentiated cells resulting from 4 days of exposure to 1 μM retinoic acid were permeabilized with 0.05% lysolecithin as described by Zhang and Gralla (63). The cells were treated with 1 to 10 μg of DNase I per ml at 25°C for 5 min. After removal of DNase I, the cells were dissolved in 600 μg of proteinase K per ml and 0.2% SDS. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Control samples of DNase-digested DNA were prepared from K18 TG2 transgenic tissue DNA (1, 40). Ligation-mediated PCR (LMPCR) was performed essentially as described previously (39). Primer extension reactions were performed with the use of modified T7 DNA polymerase (Sequenase; U.S. Biochemical). Double-stranded primers were then ligated to the ends of the products. After 18 cycles of exponential amplification of the ligation products, a one-cycle labeling reaction was performed, using radioactive primer labeled by phosphorylation reaction with [γ-^{32}P]ATP. The three primers for the investigation of the enhancer region used were K18+964 (CTGCCT GCCCAGAAGTGAGTC) for primer extension, K18+923 (CTGGCCCTCTCCTGGCATTTTTTCC) for amplification, and a third primer identical to K18+923 except 4 nt longer for labeling. The Nα region was examined with primers K18+1049 (ACCCCATTCCCTCCTTTCCTG) and K18+1021 (AGAT CCCAGCACTCTGCCCCTCTTG) and a third labeling primer identical to K18+1021 except 5 nt longer. The samples

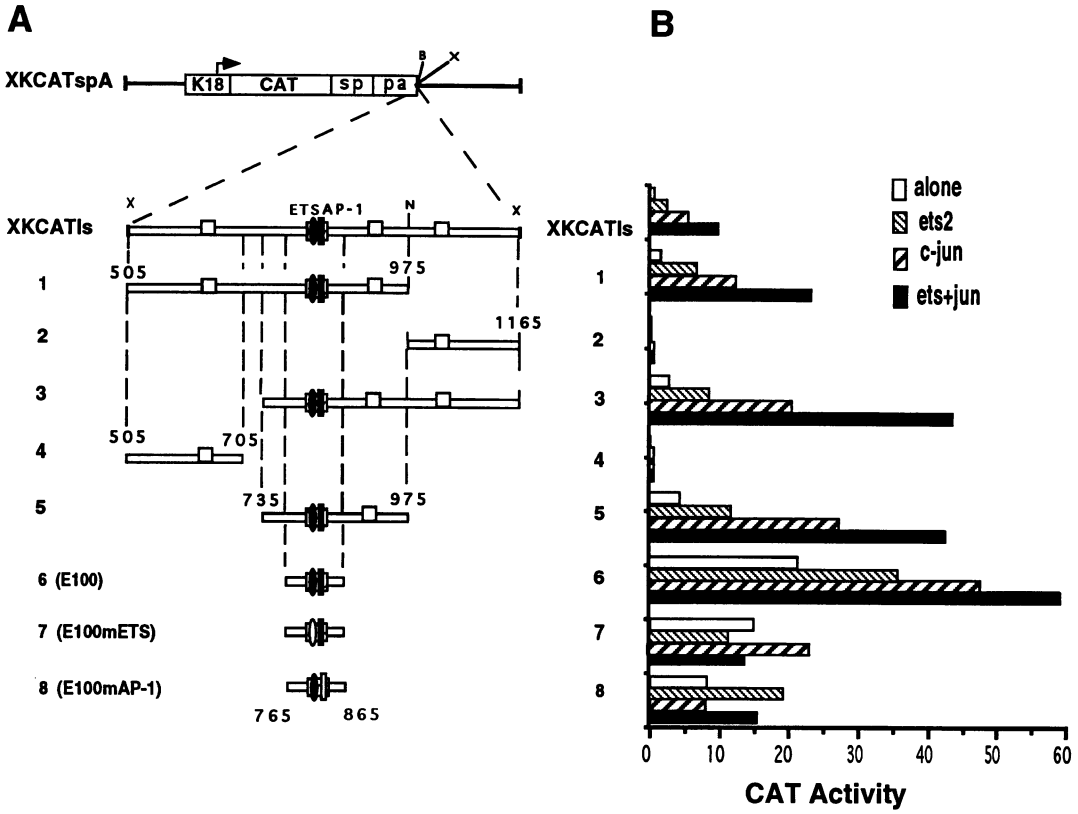

FIG. 5. Deletion analysis of the K18 intron. (A) Schematic map of the CAT reporter constructs used in transient transfection analysis. In all constructs, the CAT reporter unit (CAT gene followed by the simian virus 40 t-antigen intron [sp] and polyadenylation signals [pa]) was driven by the proximal 250-bp promoter of the K18 gene (XKCATspA). A 663-bp fragment of the first intron of the K18 gene was placed downstream of CAT reporter unit to generate XKCATIs. Various fragments from the first intron, obtained as described in Materials and Methods, were inserted into XKCATspa to generate the indicated constructs. Numbering is relative to the major start of transcription. B, *Bam*HI; X, *Xba*I; N, *Nhe*I; C, *Cla*I. Positions of AP-1 and ETS sites are indicated. (B) Relative CAT activities obtained for different CAT constructs (10 μg) when transfected into F9 cells alone or with expression vectors for ETS-2 and c-Jun. The CAT activity is normalized to the β-galactosidase activity of a cotransfected plasmid utilizing the human β-actin promoter to drive the bacterial *lacZ* gene.

were resuspended in loading dye, and half of each sample was placed on each lane of a 6% polyacrylamide gel containing 8 M urea. Gels were dried and exposed to Kodak X-AR film with an intensifying screen at −70°C. The identities of all amplified bands were confirmed by genomic sequencing reactions (data not shown).

RESULTS

ETS and AP-1 contribute to the activation of K18 in differentiated EC cells. Previous studies had identified a transcriptional enhancer element within the first intron of the K18 gene. An active AP-1 element was located within a 47-bp conserved sequence identical between the human and mouse genes (42). This element binds AP-1 (46) and mediates transactivation by c-*jun* and c-*fos*. Recently we have extended those results and identified a binding site for ETS transcription factors 9 bp upstream of the AP-1 element which mediates activation by ETS-2. Together these elements define an oncogene response element that mediates transactivation of reporter genes when cotransfected with the *src*, *lck*, *ras*, and *raf* oncogenes (46). It was of interest to determine if the same transcriptional mechanism that activates the K18 gene during tumorigenesis also participates in the induction of the gene during normal early development. To begin to evaluate the contribution of the ETS element to the differentiation-dependent induction of the K18 gene, we transfected several different K18 gene constructions into either undifferentiated F9 EC cells or differentiated F9 cells generated by exposure to retinoic acid for 4 days. The constructions are shown in Fig. 1. In all cases, the tested deletions or mutations were replaced into the context of the whole gene and expression of the RNA was measured by an RNase protection assay in the presence of a probe for a cotransfected internal control gene pMC1NeoA. Results are shown in Fig. 2A and B and summarized in Fig. 2C.

The wild-type K18 gene is expressed poorly in undifferentiated F9 cells but is easily detected in differentiated F9 cells (Fig. 2A, lanes 2 and 3). By normalizing the K18 signal to that of the *neo* gene, we estimate that the K18 gene is expressed at about five times higher levels in differentiated cells (Fig. 2C). Deletion of the 3.5-kb 3′ flanking sequences of the K18 gene has little effect on expression in either undifferentiated or differentiated F9 cells (K18d3; Fig. 2A, lanes 4 and 5). The importance of the first intron enhancer is illustrated by the lower levels of K18 RNA detected in either cell type for the K18dIn vector, in which the first intron is largely deleted (Fig. 2A, lanes 6 and 7), or the smaller deletion of the 47-bp element conserved between human and mouse genes (Fig. 2A, lanes 12 and 13; Fig. 2C, d47). However, some induction of these mutant forms of the K18 gene are still evident in retinoic acid-treated cells. The proximal promoter, which contains consensus binding sites for SP-1 factors, or the previously

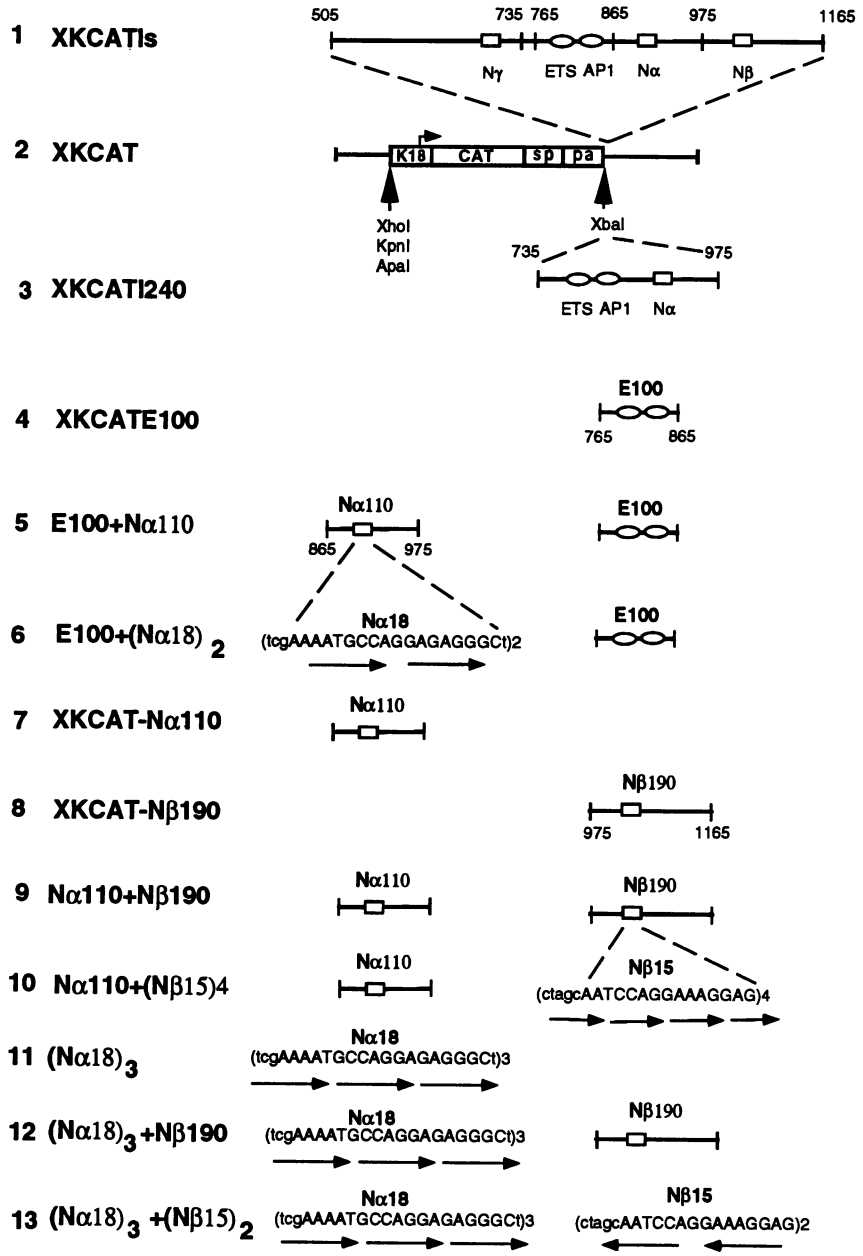

FIG. 6. Schematic diagrams of CAT gene reporter constructs used for analysis of the silencer elements. XKCATspa (XKCAT) represents the 250-bp proximal promoter of the K18 gene driving the CAT unit including the intron (sp) and polyadenylation signal (pa) from simian virus 40. XKCATIs includes the 663-bp fragment of the first intron of K18. Positions of the NREs (Nα, Nβ, and Nγ) in the first intron of the K18 gene are indicated by the open boxes, and the ETS/AP-1 oncogene-activated element is indicated by the double open ellipses. Unique cloning sites are indicated by the vertical arrowheads. The sequences of the oligonucleotides are shown in parentheses. Lowercase nucleotides indicate additional bases added for cloning purposes. Each number outside the right-hand parenthesis indicates the number of copies of the oligonucleotides in each construction. The orientation and number of copies of the oligonucleotides are indicated by the horizontal arrows.

reported DNase-hypersensitive site located within exon 6 (40) may contribute to the residual activity of the intron-deleted form of K18. However, the important contributions of the AP-1 site, and to a lesser extent the ETS site, of the intron enhancer are indicated by the decreased expression of K18 with mutations of these two sites (Fig. 2A, lanes 8 to 11). This interpretation is supported by forced expression of either c-Jun or c-ETS-2 in undifferentiated F9 cells by cotransfection, which results in greatly increased levels of K18 RNA (Fig. 2B, lanes 2 to 5). We have previously shown that the mutations of the AP-1 and ETS sites abrogate induction by the respective transcription factors (46).

The K18 enhancer sequence centers on the 47 bp which are conserved in the mouse and human genes. Alone, this conserved region was found to be without activity (43). However, a larger fragment of 100 bp is able to restore enhancer activity to the K18 gene (K18E100; Fig. 2A, lanes 14 and 15). Surprisingly, this construction had significant activity in undifferentiated F9 cells as well as differentiated cells. However, retinoic acid treatment of EC cells permitted still higher levels,

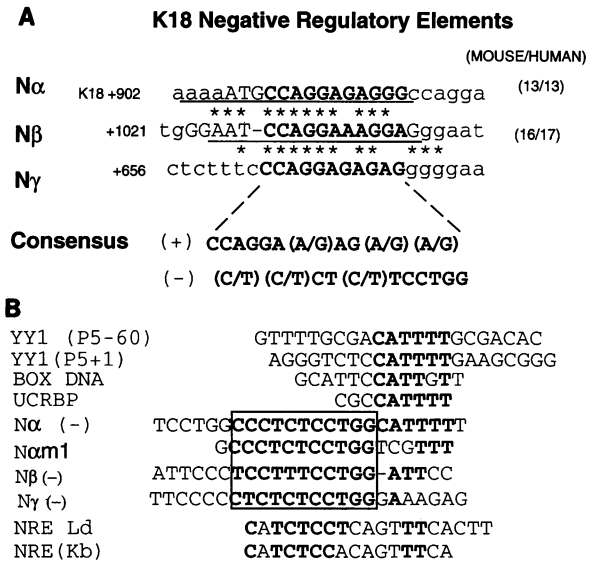

FIG. 7. (A) Sequence similarity of the K18 silencer elements. Sequence elements are numbered relative to the transcriptional start site. Uppercase nucleotides indicate conserved nucleotides in the mouse and human K18 genes. The number of nucleotides conserved in the mouse and human K18 genes is indicated at the right. Underlined nucleotides indicate the sequences tested for silencer activity in the Nα18 and Nβ15 inserts (see Fig. 6 also). Asterisks indicate matches between elements. Boldface letters indicate apparent similarity. (B) Comparison of the opposite strands of Nα, Nβ, and Nγ with other silencer elements and with a mutation of the Nα element in the YY1 homology (Nαm1). The K18 consensus sequence is boxed. The proteins recognizing the YY1 (51) and UCRBP (14) sites are identical. BOX DNA represents an endogenous cellular silencer sequence implicated in polyomavirus replication (3). The NREs of mouse MHC locus genes [NRE Ld and NRE (Kb)] are active in F9 EC cells but not their differentiated derivatives (13).

essentially the same as those for the wild-type gene. Evidently, negative regulatory activities not included in this construction are responsible in part for the low expression of K18 in EC cells. However, ETS and AP-1 additionally contribute to the higher expression in differentiated cells.

The TATA box is not essential for transactivation by ETS and AP-1. In an attempt to determine if the TATA box was essential for mediating the activation by the ETS and AP-1 elements, a mutation of the TATA box (ATATAC changed to AGATCT) was constructed. This alteration decreased but did not abolish basal expression relative to the wild-type K18 gene. The K18-TATA construct was still capable of increased expression in differentiated cells and of activation by either ETS or Jun (Fig. 2B, lanes 8 to 10). However, the transcriptional start site changed to a position about 16 bp upstream from the normal site (Fig. 2B, lanes 6, 8, and 9). The signal from the TATA box mutant is shown in the absence of the *neo* probe in Fig. 2B, lane 6. This would place the start about 8 bp downstream of the former TATA box and about 28 bp downstream of the proximal SP-1 site. However, as the RNase protection procedure does not normally provide single-base-pair resolution, these values must be considered estimates. Results for all constructions are summarized in Fig. 2C.

Expression of ETS-2 RNA correlates well with K18 induction. While transient expression of transfected vectors is useful for defining regulatory regions, quantitative considerations of the contribution of different regulatory elements is more difficult to evaluate because of the high levels of expression in a minority of transfected cells. To confirm the importance of ETS and AP-1 sites for the differentiation-dependent induction of the K18 gene, stable transfected clones and pooled populations of ES cells were isolated. Pooled populations of transfected ES cells containing a control K18 vector (K18In, which contains the full intron in the context of the additional *Xba*I sites used for cloning mutant fragments) and K18 variants carrying either a mutant ETS or intron AP-1 site were characterized with regard to average copy number of integrated genes. Each population was exposed to retinoic acid for up to 4 days, and RNA was prepared for each point. Differentiation was verified by morphology and immunofluorescent staining with the TROMA1 monoclonal antibody against mouse K8. Results of hybridization of a Northern (RNA) blot filter successively with six different probes are shown in Fig. 3.

The signals of K18 RNAs were quantitated by densitometry and comparison with synthetic standard K18 RNAs. Mutations of either the AP-1 site or ETS site of the intron enhancer decreased retinoic acid-dependent K18 expression by about half (Fig. 3B). Additional analyses of similar populations with mutations of both the ETS and AP-1 sites failed to suppress K18 RNA levels further than the single mutations (data not shown). K18 RNA levels characteristically decreased on day 1 and then increased over the next 3 days in ES cells containing the control K18 gene (K18In). Comparison of the levels of K18 RNA with those of c-Jun, JunB, and ETS-2 revealed that the induction of the RNAs for the three transcription factors generally followed the same pattern as K18 (Fig. 3C). However, the ETS-2 profile most closely resembled that of K18, with a reproducible drop in the level of RNA on day 1. In contrast, the RNA for c-Jun followed a monotonic increasing curve, and JunB increased on day 1, decreased on day 2, and then subsequently increased further. These differences in early kinetics were evident in the pooled populations of K18 constructs (the average levels of all three populations are shown in Fig. 3C). In addition, all hybridizations were performed with the same filter and were normalized to the RNA load by use of the signal from the L32 ribosomal protein gene. These data confirm the results of the transient transfection analyses and indicate that both the ETS and AP-1 elements contribute significantly to the retinoic acid-dependent activation of the K18 gene. Furthermore, the kinetics of induction of c-Jun, JunB, and particularly ETS-2 RNAs are consistent with participation in the activation of the K18 gene. However, it is also clear that ETS and AP-1 utilization are not sufficient to account for the full induction of the K18 gene.

The K18 ETS site is occupied in differentiated ES cells. To determine if binding to the K18 ETS and AP-1 sites could be confirmed in differentiated ES cells, the K18 gene was introduced by electroporation, and stably transfected clones were isolated. Characterization of K18 RNA expression in 13 independent, individual clones confirmed that K18 RNA increased between 1.5- to 4.5-fold in retinoic acid-treated cultures of each clone (data not shown). One clone, designated C9, which contained approximately 17 K18 genes per cell and showed a large difference between untreated and retinoic acid-treated cultures, was used for genomic footprinting experiments using LMPCR to evaluate the occupancy of the ETS site within the intron enhancer. Results are shown in Fig. 4. Comparison of the footprinting patterns of the enhancer region in retinoic acid-treated C9 cells revealed a clear ETS-like footprint defined by the DNase-hypersensitive site at nt 789 and 803 and characterized by the internal hypersensitive site at nt 799 (black dots in Fig. 4). The characteristic internal hypersensitive site has been previously described for in vitro footprinting results obtained by using a recombinant ETS DNA-binding

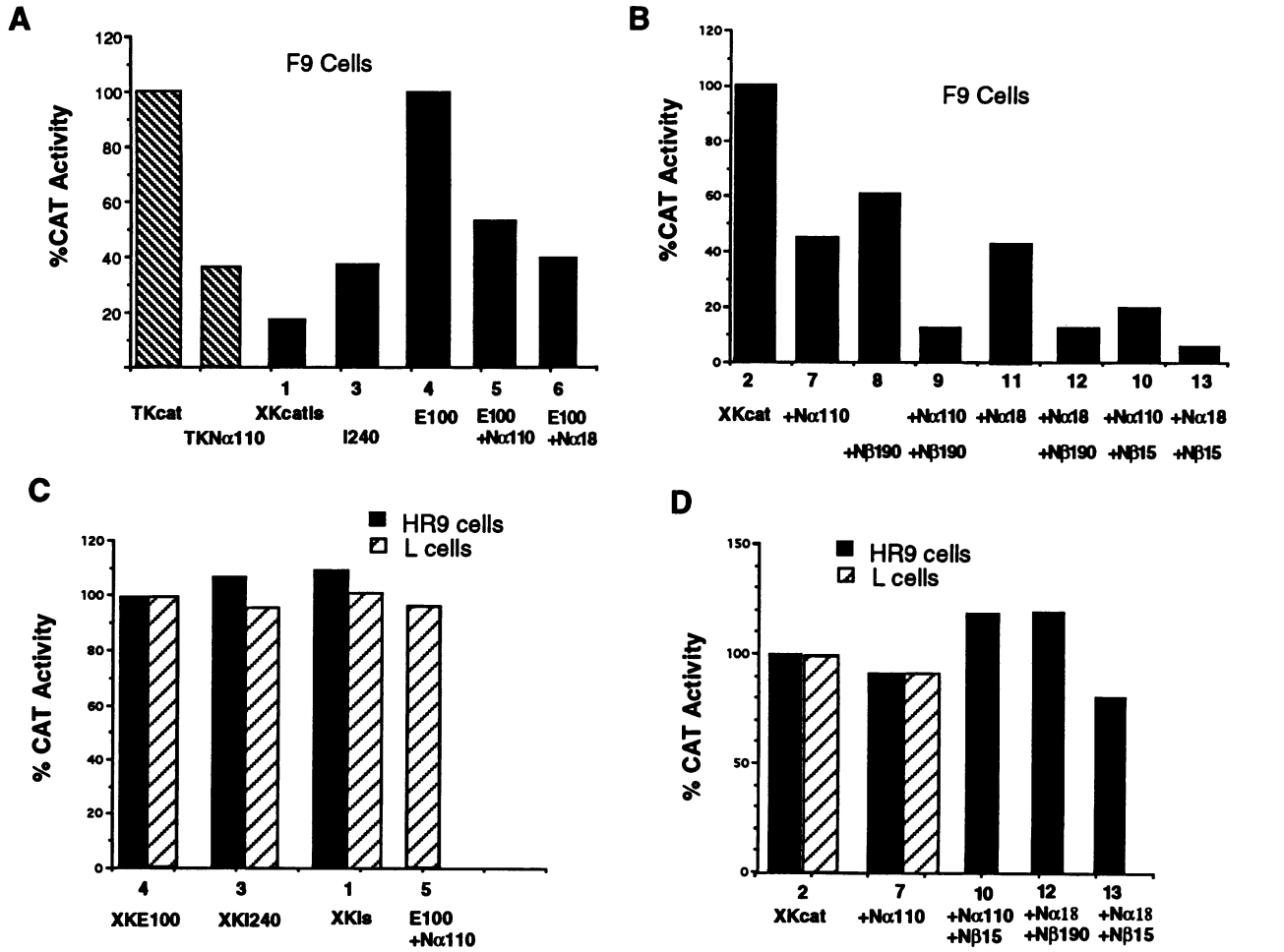

FIG. 8. Activities of the Nα and Nβ silencers in transient expression of the CAT vectors. CAT activity was normalized to the β-galactosidase activity in the same lysate of a cotransfected *lacZ* gene, driven by the β-actin promoter (LK4Lac). CAT activity is normalized to the activity of the basic vector in each case. Thus, the XKCAT vectors, which lack an enhancer, are generally 5-fold less active than XKCATIs and 20- to 50-fold less active than XKCATE100 in F9 cells. (A) Relative CAT activities of the indicated constructions in F9 cells. Values shown represent the averages of two or three replicates. The F9 cells were transfected by 5 or 10 μg of CAT construct DNA and 4 μg of LK4Lac in a total of 20 μg adjusted by the addition of pGEM1 DNA. Column numbers refer to the constructions shown in Fig. 6, with abbreviated names indicated below each column. Note the lower activity of TKNα110 relative to TKCAT and the activating effect of using progressively shorter intron fragments in constructs 1, 3, and 4. (B) Activities of the silencer elements in the absence of the K18 enhancer. Values shown represent the averages of two to four replicates. The F9 cells were transfected with 12 or 16 μg of CAT construct DNA and 4 μg of LK4Lac in a total of 20 μg of DNA. (C) Cell type specificity of silencer activity. The indicated enhancer-containing constructions were introduced into either HR9 parietal endodermal cells or L-cell fibroblasts. Values represent the averages of two to four replicates. DNA concentrations were the same as for panel A. (D) Cell type specificity of K18 silencers in the absence of the enhancer. Results are the averages from two to four separate transfections.

domain (46) and in vivo footprinting experiments of K18 transgenic liver nuclei (58a). No obvious difference was observed for the AP-1 site residing between nt 803 and 814 (in part because of the biases cutting of pure DNA by DNase I). An additional difference between untreated and retinoic acid-treated C9 cells was evident immediately upstream of nt 777. This region is outside of the 47-bp element conserved in the human and mouse K18 genes and has not yet been investigated further. These results provide substantial support for the use of the K18 ETS site in retinoic acid-treated ES cells that is consistent with the transient transfection data and data for the stably transfected ES pooled populations. Furthermore, these results indicate that most or all of the 17 copies of the K18 gene in C9 cells must behave similarly in order for the ETS footprint to be observed. This characteristic of active expression from most or all integrated copies of the K18 gene has been documented previously for transgenic mice (39, 40, 58).

NREs within the first intron of K18. Expression of the K18 construction containing only 100 bp of the first intron suggested that NREs may reside in the intron as well as the positive elements of the enhancer (K18E100; Fig. 2A, lanes 14 and 15). A deletion analysis of the first intron was performed to localize putative NREs further. Figure 5 shows the results of transient transfection experiments with CAT constructions containing the K18 proximal 250-bp promoter and various portions of the first intron. Deletion of either a 190-bp fragment from the 3′ portion of the intron fragment (Fig. 5, construct 1) or 230 bp of the 5′ portion of the intron (Fig. 5, construct 3) resulted in both increased basal activity in F9 EC cells and increased optimal activity when cotransfected with

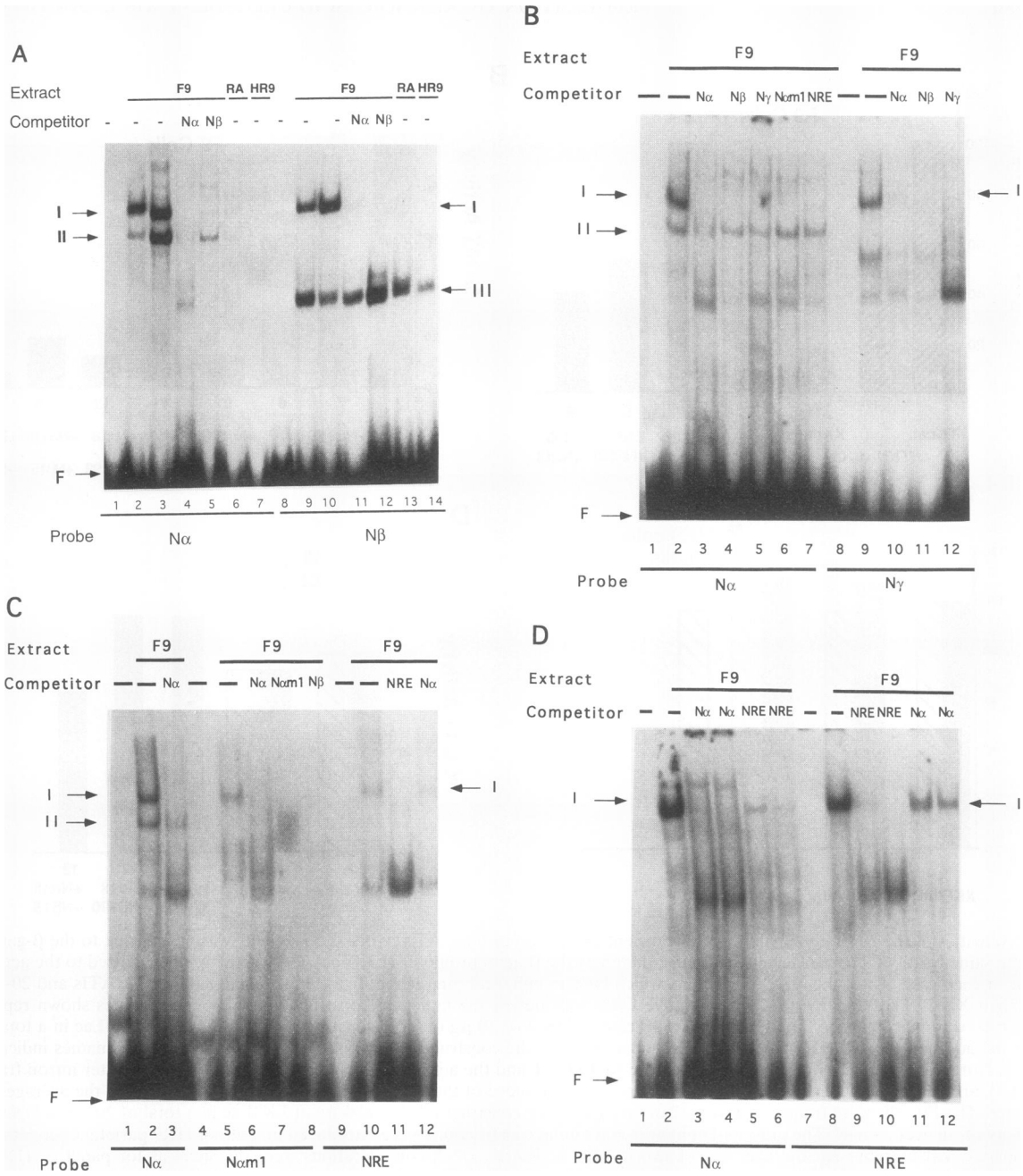

FIG. 9. DNA-binding activities for the Nα, Nβ, and Nγ elements. (A) Nuclear proteins from F9 cells (F9), differentiated F9 cells resulting from 4 days of exposure to 1 μM retinoic acid (RA) (lanes 6 and 13), or HR9 parietal endodermal cells (HR9) (lanes 7 and 14) were incubated with radioactive double-stranded oligonucleotide Nα (lanes 1 to 7) or Nβ (lanes 8 to 14). Competitor oligomers were added at 100-fold molar excess (lanes 4, 5, 11, and 12). The resulting complexes were analyzed by polyacrylamide gel electrophoresis and autoradiography. All samples received 10 μg of nuclear proteins except those for lanes 3 and 10, which received 20 μg, and lanes 1 and 8, which received no protein. Positions of complexes I, II, and III and of free DNA probe (F) are indicated. Note that complex I is found with both probes, is sensitive to competition with both nonradioactive oligonucleotides, and is diminished in retinoic acid-treated cells and HR9 parietal endodermal cells. (B) Similarity of the Nα, Nβ, and Nγ elements. F9 nuclear extracts were incubated with either the Nα (lanes 1 to 7) or Nγ (lanes 8 to 12) radioactive double-stranded oligonucleotide in the presence of a 100-fold molar excess of the indicated nonradioactive competitors. Competition of the Nγ element with the Nα probe abolishes complex I (lane 5). Similarly, the competition of the Nα, Nβ, and Nγ oligonucleotides with the Nγ probe (lanes 10 to 12) abolishes complex I. The Nαm1 oligonucleotide contains three alterations in the putative YY1-binding site and still effectively competes for complex I but not complex II (lane 6). The NRE oligonucleotide, defining an NRE of mouse MHC class I genes, competes with the Nα probe for the formation of complex I (lane 7). (C) Complex II is dependent upon the YY1-binding site of the Nα element. F9 nuclear proteins were incubated with radioactive Nα (lanes 1 to 3), Nαm1 (lanes 4 to 8), and NRE (lanes 9 to 12) probes and the indicated nonradioactive competitors. Note that complex II is absent with the Nαm1 probe (lane 5). Complex I is found with the NRE probe (lane 10). However, the Nα competitor does not abolish complex I formation (lane 12). (D) Comparison of the Nα and NRE complexes. Different amounts of the Nα or NRE oligonucleotide were added as the competitor for complex I formation on the Nα (lanes 1 to 6) or NRE (lanes 7 to 12) probe. Competing oligonucleotides were added at 50-fold (lanes 3 and 9), 100-fold (lanes 4, 5, 10, and 11), or 200-fold molar excess (lanes 7 and 12). Note that Nα competes effectively with the Nα probe at 50-fold (lane 3) or 100-fold (lane 4) molar excess but fails to abolish complex I formed on the NRE probe even at 100-fold (lane 11) or 200-fold (lane 12) molar excess. The NRE oligonucleotide abolishes the NRE complex I at 50-fold (lane 9) or 100-fold (lane 10) excess. However, the NRE sequence competes less efficiently against the Nα complex I (lanes 5 and 6).

expression vectors for ETS-2, Jun, or both. Neither deleted fragment contained independent, positive regulatory activity by itself (Fig. 5, constructs 2 and 4). Furthermore, deletion of both fragments (construct 5) resulted in a further increase of basal activity in F9 cells. A final deletion of an additional 110 bp (nt 865 to 975; construct 6) resulted in optimal activity in F9 cells as well as optimal transactivated activity by ETS and Jun. As expected, the enhancer activity of the remaining 100-bp fragment (Fig. 5, construct 6) was dependent upon the previously identified ETS and AP-1 sites, as mutation of either abrogated transactivation by the corresponding transcription factor (Fig. 5, constructs 7 and 8). However, both sites appear to activate independently to some degree, because mutation of the ETS site did not completely abrogate transactivation by c-Jun (construct 7), and similarly, mutation of the AP-1 site still permitted significant activation by ETS (construct 8).

To identify and characterize NREs in more detail, additional constructions were tested either with or without the 100-bp enhancer. Figure 6 shows the structures of most of the additional constructs. The candidate NREs were designated the Nα, Nβ, and Nγ elements. Identification of the Nα and Nβ elements was facilitated by the previous identification of two short stretches of intron sequence which were conserved in the mouse and human genes. The Nα element represents 13 nt which are identical in the two genes (Fig. 7). The Nβ element was identified because of the conservation of 16 of 17 nt. A computer search for sequences similar to the Nα and Nβ elements revealed a third possible NRE, Nγ, at nt 663 of the human gene, although a corresponding element was not found in the mouse gene (Fig. 7). Results of transfection analyses in F9 cells are shown in Fig. 8A and B.

Addition of the 110-bp fragment encompassing the Nα element (Nα110) to a CAT reporter gene driven by the herpes simplex virus TK promoter (TKCAT) or a CAT gene driven by the K18 250-bp promoter (XKCAT) either with or without the 100-bp enhancer fragment resulted in significantly decreased activity (Fig. 8A, TKNα110 and TKCAT, constructs 6 and 4; Fig. 8B, constructs 7 and 2). These results show that the negative regulatory activity of the Nα110 fragment was effective on a heterologous promoter and did not require the presence of the K18 enhancer for an inhibitory effect. In addition, placement of the Nα110 fragment upstream of the promoter appeared nearly as effective as in its normal position downstream and adjacent to the enhancer (Fig. 6 and 8A, constructs 3 and 5). An oligonucleotide containing 18 bp overlapping the 13-bp element conserved in the mouse and human K18 introns inhibited the expressing of XKCAT vector independently of the presence of the enhancer element (Fig. 6, constructs 6 and 11; Fig. 8A, constructs 4 and 6; Fig. 8B, constructs 2 and 11). While the recombinants with the Nα18 oligonucleotides contained tandem copies of the fragment, a small number of copies appears to be effective because construct 6, which contains only two copies of the oligonucleotide, suppressed activity better than the full Nα110 fragment (Fig. 8A, constructs 5 and 6).

Negative regulatory activity of the Nβ element was located within a 190-bp fragment (Nβ190) (Fig. 6 and 8B, constructs 8, 9, and 12). Combinations of the Nα and Nβ elements resulted in nearly additive suppressive effects (Fig. 8, constructs 9 and 12). As with the Nα element, activity could be conferred by an oligonucleotide containing 15 bp of K18 intron sequence (Fig. 8B, constructs 10 and 13). From the results for construct 13, we can deduce that the Nβ element functions in an orientation opposite its normal one and that two copies of the oligonucleotide are quite effective (Fig. 8B, constructs 13 and 11).

These results identify two regulatory elements which suppress or partially silence both the K18 and TK promoters in F9 cells. A third silencer element identified by sequence homology (Fig. 7) may account for the increased activity found upon deletion of nt 505 to 705 from the intron (Fig. 5, construct 3). Insertion of a synthetic oligonucleotide representing the Nγ element into the XKCAT expression vector confirmed silencing activity for Nγ (data not shown). Furthermore, the Nγ element forms a complex with F9 nuclear proteins which is of the same electrophoretic mobility as those found for the Nα and Nβ elements and competes effectively for the formation of complexes with the other two elements (see below). Thus, all three silencer elements appear functionally equivalent. These NREs function independently of position and orientation and thus qualify for the term silencers.

The K18 silencers do not function in somatic cells. NREs identified in F9 cells appear to have little effect in HR9 parietal endodermal cells, which express endogenous mouse K18, or in L-cell fibroblasts, which do not express the gene. The same constructions which show differential activity in F9 cells were equally active in either HR9 cells or L cells (Fig. 8C, constructs 1, 3, 4, and 5). Similarly, vectors without the enhancer region were expressed at similar levels regardless of whether the Nα or Nβ element or both elements were present (Fig. 8D). This result is consistent with previous results that showed that the K18 gene was efficiently expressed even when transfected into cells which did not express the endogenous mK18 gene and implied that *trans*-acting negative regulatory activities were not responsible for the inactivity of the gene in fibroblasts (27, 28). A condensed chromatin state which renders positive regulatory elements inaccessible to their cognate binding activities has been suggested as a possible cause of the inactivity of the gene in nonexpressing somatic tissues (39, 45, 58a).

Candidate silencer-binding proteins in F9 nuclei. Gel mobility shift assays were performed with double-stranded, synthetic oligonucleotides corresponding to the silencer elements and nuclear extracts from F9 cells, retinoic acid-treated F9 cells, and HR9 parietal endodermal cells (Fig. 9). With the radioactive Nα element, two major DNA-protein complexes were detected with F9 nuclear extracts (Fig. 9A, lanes 2 and 3, complexes I and II). The specificity of these complexes was revealed by competition with nonradioactive Nα oligonucleotides (Fig. 9A, lane 4). In addition, complex I was sensitive to an excess of nonradioactive Nβ oligonucleotides (Fig. 9A, lane 5). If the silencer activities of the Nα and Nβ silencer elements are mediated by a common DNA-binding protein, use of radioactive Nβ probe would be expected to generate similar-sized DNA-protein complexes. A protein-Nβ complex with the same mobility as was found for the Nα probe was detected with F9 nuclear extract (Fig. 9A, lanes 9 and 10, complex I). Both nonradioactive Nα and Nβ oligonucleotides competed effectively for complex I but not a nonspecific complex labeled III (Fig. 9A, lanes 11 and 12). The detection of the nonspecific DNA-binding activities responsible for complex III in extracts of all three cell types indicated that nuclear extracts of retinoic acid-treated F9 cells and HR9 cells did contain DNA-binding proteins. These data are consistent with the possibility that both silencer elements bind the same protein(s), revealed as complex I. Use of the radioactive Nγ element resulted in a complex with the same apparent mobility as those detected with the Nα and Nβ probes (Fig. 9B, lane 9). This complex was sensitive to competition with all three silencer elements (Fig. 9B, lanes 10 to 12). This result confirmed that the three silencer elements are recognized by similar binding activities in F9 nuclei and supports the view that all three silencer elements are biologically active.

YY1 (UCRBP)-binding activity recognizes Nα but not Nβ or Nγ. Sequence similarity suggested that YY1 (UCRBP), a widely expressed DNA-binding protein, might recognize part

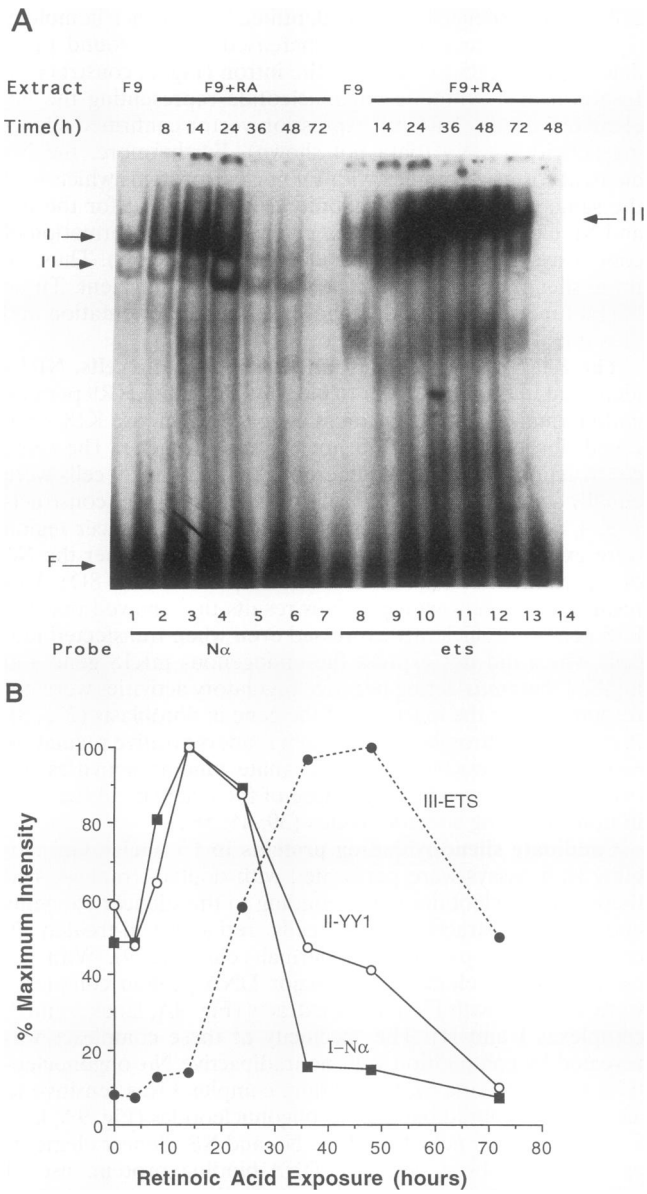

FIG. 10. Nα-binding activity decreases during retinoic acid-mediated differentiation of F9 cells. (A) Nuclear proteins (10 μg) extracted from F9 cells treated with 1 μM retinoic acid (RA) for the indicated lengths of time were incubated with a radioactive Nα or ETS oligonucleotide probe and analyzed by acrylamide gel electrophoresis. I, complex I; II, putative YY1 complex; III, ETS-binding activity; F, free probe. Addition of a 100-fold molar excess of nonradioactive ETS oligonucleotide abolishes complex III (lane 14). (B) Summary of changes in Nα-binding activity. Densitometer values of three experiments using two independent sets of nuclear extracts were averaged. Relative intensities of the bands corresponding to complexes I, II, and III are indicated as a function of time of exposure to retinoic acid. Filled squares, open circles, and closed circles represent the values for complexes I, II, and III, respectively.

of the Nα but not the Nβ or Nγ element. Addition of competitor nonradioactive Nβ or Nγ DNA abolished the Nα complex I but not complex II (Fig. 9B, lanes 4 and 5). In addition, alteration of the nucleotides in the Nα element expected to be essential for the binding of UCRBP (6) resulted in a DNA element still capable of competing for the formation of Nα complex I but not complex II (Fig. 9B, lane 6). Furthermore, when Nαm1 was used as a probe, complex I (Fig. 9C, lane 5) but not complex II was detected. Thus, Nα complex II very likely represents a YY1 (UCRBP) complex. In all cases in which Nα complex I has been detected, no more-slowly migrating complex that might represent the simultaneous binding of both the K18 silencer element-binding activity and the YY1 (UCRBP) protein was detected. This result suggests that both proteins do not bind to the Nα element simultaneously.

K18 silencer elements are related to the NREs of mouse class I MHC genes. The relationship between the K18 silencer element and the previously described NRE of mouse major histocompatibility complex (MHC) locus class I genes (13) was investigated further by gel mobility shift experiments. After incubation with F9 nuclear proteins, a radioactive NRE probe was found to form a complex with the same mobility as the K18 silencer complex I (Fig. 9C, lane 10; Fig. 9D, lane 8). The NRE was able to compete for Nα complex I formation (Fig. 9B, lane 7; Fig. 9D, lanes 5 and 6), although more NRE than Nα DNA was necessary to achieve similar levels of competition (Fig. 9D, lane 3 [50-fold excess]; Fig. 9D, lane 6 [100-fold excess]). However, the reciprocal competition experiment was less effective. Even a 200-fold molar excess of the Nα element resulted in a decrease of NRE complex I by only approximately 50% (Fig. 9D, lane 12), while even a 50-fold molar excess of nonradioactive NRE resulted in abolishing NRE complex I (Fig. 9D, lane 9). There may be more than one binding protein responsible for complex I. One may preferentially bind Nα, while the other may preferentially bind NRE. Nevertheless, the K18 silencer elements appear clearly related to the NREs.

Nα activity decreases and ETS activity increases in differentiated F9 cells. The proteins responsible for both complexes I and II appeared to be less abundant in extracts of retinoic acid-treated F9 cells and HR9 parietal endodermal cells (Fig. 9A, lanes 6, 7, 13, and 14). To examine the kinetics of this change more closely, nuclear proteins were extracted from F9 cells exposed to retinoic acid for various periods and tested for K18 silencer-binding activity and ETS-binding activity. The result is shown in Fig. 10A. The average relative values of two separate experiments are shown in Fig. 10B. Nα complexes I and II both increased after the addition of retinoic acid for approximately 14 h and then declined. The decline of Nα-binding activity (complex I) diverged from that of presumptive YY1 (UCRBP)-binding activity (complex II) after 36 and 48 h of exposure. In contrast to the kinetics of Nα and presumptive YY1 (UCRBP)-binding activity, ETS-binding activity (Fig. 10A, lanes 8 to 14, complex III) began to increase after 14 h of retinoic acid exposure and peaked after 48 h. As these assays were performed with the same extracts, the decrease in silencer-binding activity is clearly not due to a general deficit of binding activity in retinoic acid-treated nuclear extracts. Competition with a nonradioactive ETS element confirmed that the ETS binding was specific (Fig. 10A, lane 14). The increase in ETS-binding activity resembles the increase in ETS-2 mRNA seen in retinoic acid-treated ES cells (Fig. 3) and F9 cells (data not shown) except for the subsequent decrease in ETS-binding activity after 72 h. The decrease in ETS-binding activity at this time point (Fig. 10A, lanes 7 and 13; Fig. 10B) may be due, at least in part, to culture conditions because the medium was not changed during the course of the experiment to limit possible modulation by serum stimulation. The decreased Nα-binding activity after 24 h with retinoic acid correlates well with the activity of the silencer elements in transfection experiments and the differential sensitivity of part of the Nα element detected by in vivo footprinting (see below).

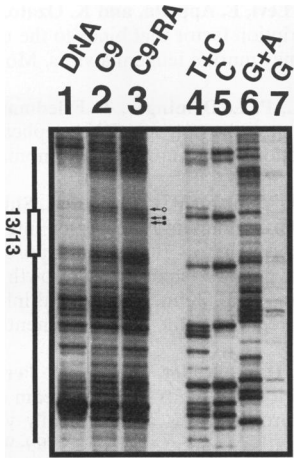

FIG. 11. Differences in the DNase I sensitivity of the Nα silencer associated with retinoic acid-induced differentiation of ES cells. Nuclei from C9 ES cells containing stably transfected K18 genes and from C9 cells treated with 1 μM retinoic acid for 4 days (C9-RA) were digested with DNase I. The DNA products were amplified by LMPCR and visualized by autoradiography. Lanes: 1, products of purified genomic DNA digested with DNase I; 2, products derived from undifferentiated C9 ES cell nuclei; 3, products derived from nuclei of differentiated C9 cells; 4 to 7, results of chemical cleavage of the genomic DNA before amplification, which permits the sequence of the amplified region to be identified.

Differential occupancy of the Nα element in ES and differentiated cells. NREs would be expected to be bound by factors which mediate the silencing effect. Genomic footprinting analysis of the Nα element in the C9 ES cells clones containing transfected K18 genes revealed some evidence for the predicted differences in occupancy of the silencer type element. Figure 11 shows the results of in vivo DNase footprinting of the element. In the region of the Nα element (designated 13/13), almost all of the amplified products of DNase digestion of nuclei were the same except for two which were less intense in undifferentiated C9 ES cells and one which was more intense in the same cells. These differences likely reflect greater binding of factors to Nα region in undifferentiated cells and release from negative regulation after differentiation. These results are consistent with the detection by the gel mobility shift assay of Nα-binding activity in F9 EC nuclei and lower levels in differentiated F9 cells.

DISCUSSION

The positive action of members of the AP-1 and ETS transcription factors families combined with the relief of suppression from embryonic cell-specific silencer elements appear to be responsible for most of the induction of the K18 gene during embryonic cell differentiation to extraembryonic endoderm. The involvement of ETS-related transcription factors in preimplantation mouse development has been suggested in earlier studies that included EC cells, although endogenous targets for their activities had not been identified. Two ETS-related factors, PEA3 (62) and a distinct PEA3-like binding activity (36), are found in undifferentiated EC cells but are down regulated upon differentiation. The low levels of ETS-1 and AP-1 components and the induced levels of ETS-2 mRNAs that we observe in differentiating ES cell cultures (Fig. 3) parallel similar findings for F9 cells (30, 34). Recently ETS-related transcription factors were implicated in the expression of the mouse K8 gene (EndoA) in extraembryonic endodermal cells (2, 56). While the kinetics of induction of ETS-2 in differentiating ES cells is similar to that of the K18 gene, it remains to be determined if other members of the large ETS family of transcription factors may also act on the K18 enhancer during embryonic cell differentiation.

The involvement of AP-1 activity in K18 induction of embryonic cells has been shown to require the conserved AP-1 site found in the K18 enhancer (43). Together, the K18 enhancer AP-1 and ETS sites mediate activation by oncogenes that stimulate the *ras* signal transduction pathway in somatic cells (46). The same two regulatory elements which mediate activation of the K18 gene by a variety of oncogenes in somatic cells also play a significant role in the induction of the gene during retinoic acid-induced differentiation to extraembryonic endoderm. However, positive regulation by increased AP-1 and ETS transcription factor activities is not sufficient to account for the full induction of the K18 gene during retinoic acid treatment of embryonic cells. This conclusion is reinforced by the continued expression of keratin in differentiating ES cells and embryos which lack c-Jun (20, 21). The major additional contribution appears to be relief of repression mediate by the Nα, Nβ, and Nγ silencers. The restricted tissue specificity of the K18 silencers is further reinforced by preliminary results which indicate that a modified K18 transgene lacking the silencers retains adult tissue specificity similar to that of the wild-type gene in transgenic mice (44a). Both the in vivo footprint analyses and the gel mobility shift experiments are consistent with a DNA-binding protein which mediates the silencer activity in ES cells.

Silencer elements are common to many genes. A literature search reveals over 200 references involving eukaryotic transcriptional silencer elements since 1989. However, silencer activities restricted to EC cells are much less frequent and have been implicated in the suppression of retrovirus (14, 34, 35), polyomavirus (2, 7, 15, 18), and MHC class I genes (13). The cell type specificity of the negative regulation of MHC class I promoters in EC cells is similar to that of the K18 silencers. The respective binding elements have sequence similarity (Fig. 7), form similar mobility complexes with F9 nuclear proteins, and to some extent cross-compete for binding. However, the Nα element does not compete as effectively as the NRE for NRE complex formation. It remains to be resolved whether more than one protein is responsible for complex I formation with the NRE. Nevertheless, proteins which mediate both the K18 and NRE silencer activities may be of particular importance in maintaining the undifferentiated state for early embryonic cells.

Considering the number of genes containing silencer elements, the number of silencer-binding proteins which have been cloned is surprisingly small. One negative transcription factor which has been cloned multiple times (YY1, UCRBP, δ, NF-E1) may contribute to the repression of the Moloney murine leukemia virus long terminal repeat, the adenovirus P5 promoter, and the Igκ enhancer and activate the L30 and L32 ribosomal proteins genes and the adenovirus P5 promoter in the presence of E1A (14, 18, 47, 51). The Nα silencer element contains six nucleotides found in YY1-binding sites (Fig. 7) which appear to be responsible for Nα complex II. However, YY1-binding sites are not shared by the Nβ or Nγ silencer. Furthermore, YY1 is widely expressed and acts in L cells as well as F9 EC cells (14, 51). Therefore, it is unlikely that YY1 mediates the K18 silencer activity. However, YY1 could influence Nα occupancy because larger complexes expected for the simultaneous binding to both the K18 silencer core sequence and the YY1 motif were not detected.

Silencer activity has been implicated in the regulation of the vimentin gene, another intermediate filament gene (12, 17, 50, 59). However, the expression of vimentin in F9 cells, the

absence of apparent sequence homology to the K18 silencers, and the activity of the vimentin silencers in several somatic cell types suggest that the silencer elements of the two types of intermediate filament genes are probably mediated by different factors. The in vivo footprinting differences between EC cells and their differentiated derivatives (Fig. 10) and the detection of proteins capable of binding the silencer elements (Fig. 9) imply direct binding of proteins to the silencer elements.

The tissue-specific expression of K18 in adult somatic tissues may be governed by different influences than in early ES cells. The accessibility of regulatory elements to commonly expressed transcription factors may be particularly important in adult tissues (40, 42). However, the expression of tissue-specific genes in EC and ES cells, and by implication, preimplantation mouse embryos, is not restricted exclusively by limited levels of specific transcription factors but is actively suppressed by specific negative regulatory mechanisms.

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