Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4691–4695, July 1988
Biochemistry

Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells

("zinc fingers"/cell cycle reentry/*Krox-24* gene/gene regulation/cell proliferation)

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Communicated by Tom Maniatis, March 21, 1988

ABSTRACT **We previously reported the identification of a mouse gene, *Krox-20*, encoding a protein with three "zinc fingers" (DNA-binding domains with coordinated zinc ions) whose expression is regulated during G_0/G_1 transition (cell-cycle reentry). We now have isolated cDNAs corresponding to a related gene, *Krox-24*. *Krox-24* encodes a protein with zinc fingers nearly identical to those encoded by *Krox-20* and similar to those of transcription factor Sp1. Similarity between Krox-20 and Krox-24 proteins also extends to several blocks of amino acid sequence located upstream of the finger region. Like *Krox-20*, *Krox-24* is transiently activated in quiescent cells after treatment with fetal bovine serum or purified growth factors. The kinetics of activation are similar to those of the proto-oncogene c-*fos*. The induction does not require *de novo* protein synthesis, and cycloheximide treatment of the cells leads to superinduction due, at least in part, to mRNA stabilization. In the mouse, the two genes are expressed in a tissue-specific manner, with slightly different patterns. These properties suggest that *Krox-20* and *Krox-24* may encode transcription factors with identical DNA target sequences and that these factors may be involved in the modulation of cell proliferation and differentiation.**

Zinc fingers are DNA-binding domains that were first identified in transcription factor IIIA. This protein is required for initiation of the transcription of the *Xenopus laevis* 5S RNA gene and binds to a DNA region ≈50 nucleotides long, internal to the gene, and to the 5S RNA itself (1–5). Zinc fingers consist of tandemly repeated units of 28–30 amino acids, containing two cysteines and two histidines at invariant positions (6–8). It has been proposed that each of these units folds as an independent domain, centered on a zinc ion coordinated by the cysteines and histidines, and binds to DNA in a sequence-specific manner (6, 8, 9). Zinc fingers are encoded by several yeast and *Drosophila* regulatory genes, and a human transcription factor, Sp1, and a human regulatory factor, the testis-determining factor, contain zinc fingers (10, 11).

We have cloned a number of mouse genes encoding zinc fingers (12, 13), on the basis of cross-hybridization with the zinc-finger-encoding region of Krüppel (*Kr*), a *Drosophila* segmentation gene of the gap type (14, 15). These mouse genes were named *Krox*, for Krüppel box (12). The expression of some of them is modulated during cell differentiation and development (12). One of these genes, *Krox-20*, is activated during the G_0/G_1 transition (i.e., during cell-cycle reentry) in cultured cells (13). It encodes three zinc fingers with close similarity to those of transcription factor Sp1 (10). Mouse NIH 3T3 fibroblasts can be brought to a quiescent state (G_0) by serum deprivation and can subsequently be stimulated to reenter G_1 and proliferate by exposure to serum or purified growth factors (16). Serum stimulation of quiescent cells leads to rapid and transient accumulation of *Krox-20* mRNA, with kinetics similar to those of the c-*fos* protooncogene. The induction does not require *de novo* protein synthesis. It is therefore possible that *Krox-20* belongs to the category of immediate-early genes whose activation is required for quiescent cells to respond to mitogens and proliferate (reviewed in ref. 17).

We now report the identification of another immediate-early gene, *Krox-24*.* *Krox-24* is related to *Krox-20*; it also encodes three zinc fingers and is activated during the G_0/G_1 transition with similar kinetics. Although the amino acid sequences of the two predicted proteins appear to be different along a large part of their length, they are nearly identical in the finger region. It is therefore possible that *Krox-20* and *Krox-24* encode DNA-binding proteins that recognize identical target sites.

EXPERIMENTAL METHODS

Cell Culture and DNA and RNA Extraction. NIH 3T3 and F9 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Serum stimulation was carried out as described (13). Cycloheximide was used at a concentration of 10 μg/ml. Total RNA was isolated from cell lines and mouse tissues according to the guanidinium thiocyanate procedure (18), as modified (19). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Collaborative Research) column chromatography (19).

cDNA Library Screening and RNA and DNA Blotting. The λNM1149 cDNA library (20) was screened under stringent hybridization conditions (19) with random-primed ^{32}P-labeled probe (specific activity, ≈10^9 cpm/μg) at 10^6 cpm/ml. Screening of the library under low-stringency conditions and RNA and DNA gel blotting were performed as described (12).

DNA Sequencing and Sequence Analysis. DNA fragments to be sequenced were cloned in both orientations in plasmid pUC19. Serial external deletions were created as described (13). The deleted inserts were subsequently transferred into bacteriophage M13-derived vectors. Single-stranded DNA was prepared (21) and the nucleotide sequence was established by the Sequenase procedure (United States Biochemical, Cleveland). The entire sequence was read on both strands. Nucleotide and amino acid sequences were analyzed with the University of Wisconsin Genetics Computer Group (UWGCG) sequence analysis software package, version 5 (22).

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Abbreviations: NGF, nerve growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

*The *Krox-24* sequence is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03803).

RESULTS

Cloning of *Krox-24*-Specific cDNAs. The isolation of *Krox-20* and the observation of a strong similarity between its zinc fingers and those of the transcription factor Sp1 raised the possibility that a subfamily of mouse genes encoding transcription factors with three zinc fingers related to those of *Krox-20* existed (12, 13). To determine whether another such gene might be activated during the G_0/G_1 transition, we made use of the cDNA library from which *Krox-20* cDNAs were isolated (13). This library was prepared in phage λNM1149 with RNA extracted from NIH 3T3 cells that had been stimulated with serum in the presence of cycloheximide for 4 hr (20). The library was screened by *in situ* hybridization with two probes on duplicate filters: the first set of filters was hybridized under low-stringency conditions with a 561-base-pair *Bam*HI–*Sal* I fragment containing the *Kr* finger region (23). The second set was hybridized under stringent conditions with AC16, a partial cDNA of the *Krox-20* gene (13). Approximately 150,000 plaques were analyzed. Among 69 clones hybridizing with the *Kr* probe, 7 were negative with the *Krox-20* probe in the first screening. Upon subsequent analysis, 1 of these 7 clones was shown to hybridize weakly with the *Krox-20* probe. This cDNA clone, DF2, was characterized in greater detail. The insert was 2.4 kilobases (kb) long, and RNA blot analysis indicated that it hybridized to a 3.2-kb RNA from serum-stimulated NIH 3T3 cells. The nucleotide sequence was established, allowing the identification of an open reading frame and the orientation of the cDNA. This information was used to design a probe corresponding to the 5′ end of the cDNA (a 520-base-pair *Eco*RI–*Sph* I fragment). Screening of 600,000 plaques from the library with this probe yielded about 80 positive plaques. Twelve positive clones were purified. The clone containing the longest insert (2.7 kb) was selected for further analysis and named DF6. The DF2 sequence was completely included within the DF6 cDNA, as judged by the analysis of the pattern of restriction fragments (data not shown). In addition, the restriction patterns of DF2, DF6, and two other cDNA clones were compared (data not shown). No difference was detected in the common regions, suggesting that no major rearrangements occurred during the cloning of DF2 and DF6 sequences.

cDNA and Protein Sequences. The complete nucleotide sequences of DF2 and of parts of DF6 were established. Identical sequences were observed within the analyzed overlapping regions of the two clones. The composite nucleotide sequence (Fig. 1) is 2687 nucleotides long and terminates with a stretch of adenine residues. From the estimated size of *Krox-24* mRNA (≈3200 nucleotides including the poly(A) tail, see below), it can be deduced that the 5′ end of the DF6 cDNA is located ≈450 nucleotides from the 5′ end of the mRNA. The longest open reading frame spans nucleotides 1–1453, suggesting that the cDNA does not contain the entire coding sequence. As discussed below, *Krox-24* is likely to be the mouse homologue of the nerve growth factor (NGF)-inducible rat gene represented by the recently isolated cDNA NGFI-A (24). If this is the case and if translational initiation occurs at the same position in both genes, the first amino acid encoded by

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1     .....CGGTGCTGCCGGAACCCCAGAGGGCAGCGGCGGTAATAGCAGCAGCAGCACCAGCAGCGGGGGCGGTGGTGGGGGCGGCACGAAC   85
22    .....GlyAlaAlaGlyThrProGluGlySerGlyGlyAsnSerSerSerThrSerSerGlyGlyGlyGlyGlyGlyThrAsn        49

86    AGCGGCACGAGCGCCTTCAATCCTCAAGGGGAGCCGAGCGAACAACCCTATGAGCACCTGACCACAGAGTCCTTTTCTGACATCGCTCTG   175
50    SerGlyThrSerAlaPheAsnProGlnGlyGluProSerGluGlnProTyrGluHisLeuThrThrGluSerPheSerAspIleAlaLeu   79

176   AATAATGAGAAGGGGATGGTGGAGACGAGTTATCCCAGCCAAACGACTCGGTTGCCTCCCATCACCTATACTGGCCGCTTCTCCCTGGAG   265
80    AsnAsnGluLysAlaMetValGluThrSerTyrProSerGlnThrThrArgLeuProProIleThrTyrThrGlyArgPheSerLeuGlu   109

266   CCCGCACCCAACAGTGGCAACACTTTGTGGCCTGAACCCCTTTTCAGCCTAGTCAGTGGCCTCGTGAGCATGACCAATCCTCCGACCTCT   355
110   ProAlaProAsnSerGlyAsnThrLeuTrpProGluProLeuPheSerLeuValSerGlyLeuValSerMetThrAsnProProThrSer   139

356   TCATCCTCGGCGCCTTCTCCAGCTGCTTCATCGTCTTCCTCTGCCTCCCAGAGCCCGCCCCTGAGCTGTGCCGTGCCGTCCAACGACAGC   445
140   SerSerSerAlaProSerProAlaAlaSerSerSerSerAlaSerGlnSerProProLeuSerCysAlaValProSerAsnAspSer   169

446   AGTCCCATCTACTCGGCTGCGCCCACCTTTCCTACTCCCAACACTGACATTTTTCCTGAGCCCCAAAGCCAGGCCTTTCCTGGCTCGGCA   535
170   SerProIleTyrSerAlaAlaProThrPheProThrProAsnThrAspIlePheProGluProGlnSerGlnAlaPheProGlySerAla   199

536   GGCACAGCCTTGCAGTACCCGCCTCCTGCCTACCCTGCCACCAAAGGTGGTTTCCAGGTTCCCATGATCCCTGACTATCTGTTTCCACAA   625
200   GlyThrAlaLeuGlnTyrProProProAlaTyrProAlaThrLysGlyGlyPheGlnValProMetIleProAspTyrLeuPheProGln   229

626   CAACAGGGAGACCTGAGCCTGGGCACCCCAGACCAGAAGCCCTTCCAGGGTCTGGAGAACCGTACCCAGCAGCCTTCGCTCACTCCACTA   715
230   GlnGlnGlyAspLeuSerLeuGlyThrProAspGlnLysProPheGlnGlyLeuGluAsnArgThrGlnGlnProSerLeuThrProLeu   259

716   TCCACTATTAAAGCCTTCGGCCACTCAGTCGGGCTCCCAGGACTTAAAGGCTCTTAATACCACCTACCAATCCCAGCTCATCAAACCCAGC   805
260   SerThrIleLysAlaPheAlaThrGlnSerGlySerGlnAspLeuLysAlaLeuAsnThrThrTyrGlnSerGlnLeuIleLysProSer   289

806   CGCATGCGCAAGTACCCCAACCGGCCCAGCAAGACACCCCCCCATGAACGCCCATATGCTTGCCCTGTCGAGTCCTGCGATCGGCGCTTT   895
290   ArgMetArgLysTyrProAsnArgProSerLysThrProProHisGluArgProTyrAlaCysProValGluSerCysAspArgArgPhe   319

896   TCTCGCTCGGATGAGCTTACCCGCCATATCCGCATCCACACAGGCCAGAAGCCCTTCCAGTGTCGAATCTGCATGCGTAACTTCAGTCGT   985
320   SerArgSerAspGluLeuThrArgHisIleArgIleHisThrGlyGlnLysProPheGlnCysArgIleCysMetArgAsnPheSerArg   349

986   AGTGACCACCTTACCACCCACATCCGCACCCACACAGGCGAGAAGCCTTTTGCCTGTGACATTTGTGGGAGGAAGTTTGCCAGGAGTGAT   1075
350   SerAspHisLeuThrThrHisIleArgThrHisThrGlyGluLysProPheAlaCysAspIleCysGlyArgLysPheAlaArgSerAsp   379

1076  GAACGCAAGAGGCATACCAAAATCCATTTAAGACAGAAGGACAAGAAAGCAGACAAAAGTGTGGTGGCCTCCCCGGCTGCCTCTTCACTC   1165
380   GluArgLysArgHisThrLysIleHisLeuArgGlnLysAspLysLysAlaAspLysSerValValAlaSerProAlaAlaSerSerLeu   409

1166  TCTTCTTACCCATCCCCAGTGGCTACCTCCTACCCATCCCCTGCCACCACCTCATTCCCATCCCCTGTGCCCACTTCCTACTCCTCTCCT   1255
410   SerSerTyrProSerProValAlaThrSerTyrProSerProAlaThrThrSerProSerProValProThrSerTyrSerSerPro   439

1256  GGCTCCTCCACCTACCCATCTCCTGCGCACAGTGGCTTCCCGTCGCCGTCAGTGGCCACCACCTTTGCCTCCGTTCCACCTGCTTTCCCC   1345
440   GlySerSerThrTyrProSerProAlaHisSerGlyPheProSerProSerValAlaThrThrPheAlaSerValProProAlaPhePro   469

1346  ACCCAGGTCAGCAGCTTCCCGTCTGCGGCGCGTCAGCAGCTCCTTCAGCACCTCAACTGGTCTTTCAGACATGACAGCGACCTTTTCTCCC   1435
470   ThrGlnValSerSerPheProSerAlaGlyValSerSerSerPheSerThrSerThrGlyLeuSerAspMetThrAlaThrPheSerPro   499

1436  AGGACAATTGAAATTTGCTAAAGGGAATAAAAGAAAGCAAAGGGAGAGGCAGGAAAGACATAAAAGCACAGGAGGGAAGAGATGGCCGCA   1525
500   ArgThrIleGluIleCys                                                                          505

1526  AGAGGGGCCACCTCTTAGGTCAGATGGAAGATCTCAGAGCCAAGTCCTTCTACTCACGAGTAGAAGGACCGTTGGCCAACAGCCCTTTCA   1615
1616  CTTACCATCCCTGCCTCCCCCGTCCTGTTCCCTTTGACTTCAGCTGCCTGAAACAGCCATGTCCAAGTTCTTCACCTCTATCCAAAGGAC   1705
1706  TTGATTTGCATGGTATTGGATAAATCATTTCAGTATCCTCTCCATCACATGCCTGGCCCTTGCTCCCTTCACCGCTAGACCATCAAGTTG   1795
1796  GCATAAAGAAAAAAAAAATGGGTTTGGGCCCTCAGAACCCTGCCCTGCATCTTTGTACAGCATCTGTGCCATGGATTTTGTTTTCCTTGGG   1885
1886  GTATTCTTGATGTGAAGATAATTTGCATACTCTATTGTATTATTTGGAGTTAAATCCTCACTTTGGGGGAGGGGGAGCAAAGCCAAGCA   1975
1976  AACCAATGATGATCCTCTATTTTGTGATGACTCTGCTGTGACATTAGGTTTGAAGCATTTTTTTTTTCAAGCAGCAGTCCTAGGTATTAA   2065
2066  CTGGAGCATGTGGTCAGAGTGTTGTTCCGTTAATTTTGTAAATACTGCTCGACTGTAACTCTCACATGTGACAAAGTATGGTTTGTTTGG   2155
2156  TTGGGGTTTTGTTGTTTGATTTTGTTTTTGAGAATTTTTTTCCCCGTCCCTTTGGTTTCAAAAGTTTCACGTCTTGGTGCCTTTTGTGTG   2245
2246  ACACGCCTTGCCGATGGCTTGACATGCGCAGATGTGAGGGACACGCTCACCTTAGCCTTAAGGGGGTAGGAGTGATGTTTTGGGGGAGGC   2335
2336  TTTGAGAGCAAAAACGAGGAAGAGGGCTGAGCTGAGCTTTCGGTCTCCAGAATGTAAGAAGAAAAAATTTAAACAAAAATCTGAACTCTC   2425
2426  AAAAGTCTATTTTTCTAAACTGAAAATGTAAATTTATACATCTATTCAGGAGTTGGAGTGTTGTGGTTACCTACTGAGTAGGCTGCAGTT   2515
2516  TTTGTATGTTATGAACATGAAGTTCATTATTTTGTGGTTTTATTTTACTTTGTACTTGTGTTTGCTTAAACAAAGTAACCTGTTTGGCTT   2605
2606  ATAAACACATTGAATGCGCTCTATTGCCCATGGGATATGTGGTGTGTATCCTTCAGAAAAATTAAAAGGAAAAAAAAAAAAAA  2687
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FIG. 1. Nucleotide sequence of *Krox-24* cDNA and predicted amino acid sequence of the protein. The cDNA sequence is a composite of the overlapping sequences derived from the cDNA clones DF2 (nucleotides 295–2654) and DF6 (nucleotides 1–325 and 2587–2687). The ATTTA sequences potentially involved in selective degradation of mRNA are underlined. In the amino acid sequence, the zinc-finger repeats are doubly underlined, the basic sequence upstream of the fingers is underlined with a thick line, and the six-residue-long repeated motifs are underlined with broken lines. Numbers in the margins refer to nucleotide and amino acid positions. The amino acid numbering was designed to align the beginning of the sequence of Krox-24 with that of NGFI-A, the likely rat homologue of Krox-24 (24).

the DF6 cDNA would correspond to position 22 in the protein sequence. The entire mouse open reading frame would then encode a polypeptide of 505 amino acids with an unmodified molecular weight of 53,573. The coding region is followed by a long 3′ untranslated region containing stop codons in all reading frames and rich in adenine and thymine (58%).

***Krox-24* and *Krox-20* Encode Nearly Identical Zinc Fingers.** As expected from the cross-hybridization of *Krox-24* with the *Kr* finger probe, the predicted polypeptide contains tandemly repeated sequences with similarity to the *Kr* finger consensus: Cys-Xaa$_2$-Cys-Xaa$_3$-Phe-Xaa$_5$-Leu-Xaa$_2$-His-Xaa$_3$-His. *Krox-24* encodes three zinc fingers, located between amino acids positions 303 and 388 (Fig. 1). Fig. 2 shows an alignment of these fingers with the *Kr* consensus. The position of the highly conserved amino acids is strictly maintained, except that the first repeat contains four amino acids between the two cysteines and that the leucine is not present in the third finger. As expected from the work of Schuh *et al.* (23), a stretch of six amino acids located between successive fingers, Thr-Gly-Glu-Lys-Pro-Tyr, the so-called H/C link, is also highly conserved (Fig. 2).

The zinc fingers of Krox-24 protein appear to be nearly identical to those of Krox-20: only five changes are observed between the two amino acid sequences (Figs. 2 and 3). According to the model of Miller *et al.* (8), the part of the repeated unit defining the specificity of recognition of the target DNA sequence is the loop located between Cys-13 and His-26 (positions measured within the repeated unit sequence, see Fig. 2). No difference is detected between Krox-20 and Krox-24 within this region, suggesting that the two proteins may have the same DNA-binding domain. As in the case of Krox-20 (13), the fingers are also very similar to those of transcription factor Sp1 (Fig. 2). The strongest similarity is observed between the first finger of Krox-24 and the second finger of Sp1: at 10 out of 12 positions within the DNA-binding loop, either the amino acids are identical or conservative changes have occurred (Fig. 2).

Structural Features of Krox-24 Protein. The analysis of the predicted protein sequence revealed several additional features. A large number of the basic amino acids are clustered in one region, between positions 287 and 398 (Fig. 1). This region covers the finger domains and in addition extends toward the NH_2 and COOH termini. The distribution of proline residues is also quite atypical within the Krox-24 protein: the overall percentage is high (12.9%) and this amino acid is concentrated within two regions located, respectively, between positions 91 and 311 and between positions 404 and 476. This nonuniform distribution of proline and basic amino acids is very similar to that of Krox-20 (13). Krox-24 is also enriched in serine and threonine (overall percentage 25.9%). Several stretches of serine residues are observed, with in particular a series of seven serine and threonine residues followed by seven glycine residues (positions 34–47). Examination of the amino acid sequence of Krox-24 revealed the existence of another series of repeated motifs, in addition to the zinc fingers. The consensus for these motifs is (Thr/Ser)-(Thr/Ser)-(Phe/Tyr)-Pro-Ser-Pro, and the repeats are concentrated in a region downstream of the fingers, where they are present in tandem arrays (Fig. 1). These motifs are similar to a tandemly repeated heptapeptide sequence (consensus: Tyr-Ser-Pro-Thr-Ser-Pro-Ser) present in the COOH-terminal

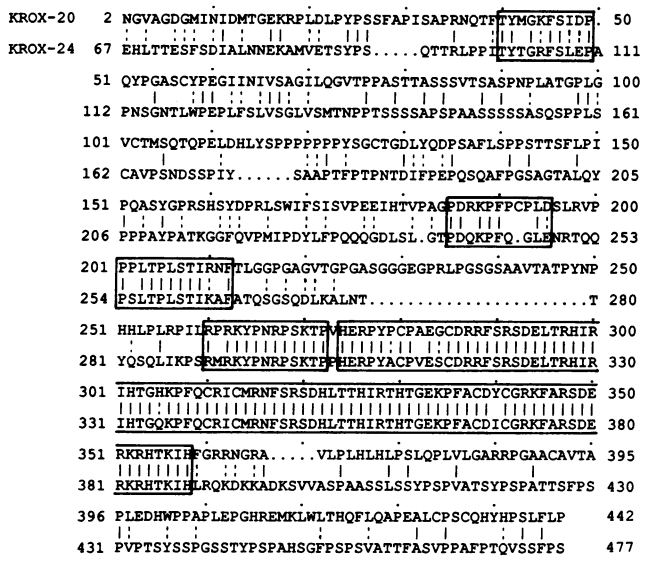

FIG. 3. Amino acid sequence similarities between Krox-20 and Krox-24 proteins. The sequences are in the one-letter code (25) and were aligned by the Bestfit program of the UWGCG package. Solid lines correspond to identical amino acids and dotted lines to related ones. Sequences longer than 10 amino acids and showing >50% positional identity are boxed.

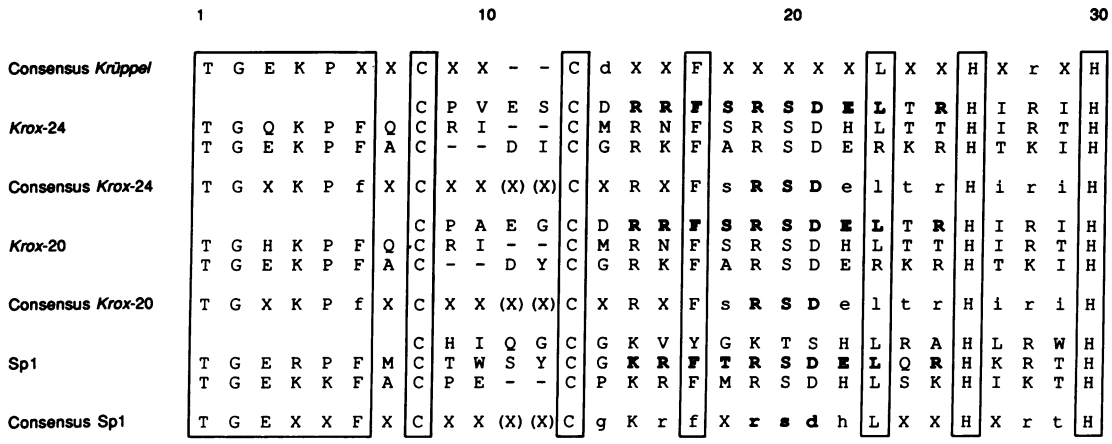

FIG. 2. Amino acid sequence comparison of *Kr*, *Krox-20*, *Krox-24*, and Sp1 zinc-finger motifs. The sequences are in the one-letter code (25) and are aligned to show the repeated units. Amino acids that are strictly conserved in the *Kr* finger motif are boxed, as well as the interfinger H/C link (23). For each gene, a consensus for the repeated motif is displayed: uppercase letters correspond to strictly conserved amino acids, lowercase letters to amino acids conserved >50%, and X to no conservation >50%. The amino acids conserved or corresponding to conservative changes between finger 1 of *Krox-20* or *Krox-24* and finger 2 of Sp1 are shown in bold characters. The sequence RSD conserved in *Krox-20*, *Krox-24* and Sp1 consensus sequences is also shown in bold characters. The consensus sequence of *Kr* and the Sp1 finger sequence are derived from the sequences of Rosenberg *et al.* (15) and Kadonaga *et al.* (10), respectively.

domain of the largest subunit of eukaryotic RNA polymerases (26, 27).

The amino acid sequences of Krox-20 and Krox-24 were compared by the Bestfit program of the UWGCG package (22) (Fig. 3). In addition to the zinc-finger region, which is highly conserved as noted above, weaker similarity is observed between the two proteins along the part located upstream of the fingers. Several short sequences appear to be significantly related. In particular, the basic regions immediately upstream of the fingers (positions 290–302 in Krox-24) are highly similar (Fig. 3). Another region of similarity (positions 254–265 in Krox-24) is potentially interesting, since it is located within a region of Krox-20 that is similar to a portion of the polyoma large tumor antigen (13). The parts of the proteins downstream of the fingers do not show significant sequence similarity. In particular, the six amino acid repeats present in Krox-24 are not observed in Krox-20 (Figs. 1 and 3). The region immediately downstream of the fingers (positions 390–398 in Krox-24) is nevertheless very basic in both proteins (Fig. 3).

Regulation of *Krox-24* Expression. As mentioned above, blot analysis of RNA extracted from serum-stimulated NIH 3T3 cells with the DF2 cDNA probe revealed a 3.2-kb mRNA (data not shown). To exclude the possibility that the observed band could correspond to cross-hybridization of the finger region of the probe with *Krox-20* mRNA, which is also 3.2 kb long (13), we performed additional RNA blotting experiments with a probe derived from the 3′ part of the DF2 cDNA (nucleotides 1148–2653). This region is not conserved between the two genes and the probe does not cross-hybridize with *Krox-20* mRNA (data not shown). *Krox-24* mRNA was detected within 15 min after serum stimulation of the cells (Fig. 4). The amount of RNA increased up to 30 min and decreased to very low levels after 2 hr. Previous hybridization of the filter with a probe corresponding to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, whose expression does not vary during the G_0/G_1 transition, indicated that similar amounts of RNA were present in the different lanes (Fig. 4). The kinetics of accumulation of *Krox-24* mRNA were identical to those of *Krox-20* mRNA. Similar transient accumulation of *Krox-24* mRNA was observed after stimulation of the cells with purified epidermal growth factor or platelet-derived growth factor (Fig. 4).

De novo protein synthesis was not required for *Krox-24* activation, since RNA accumulation occurred in the presence of cycloheximide (Fig. 5). Indeed, cycloheximide treatment led to superinduction of the gene (compare the 4-hr time points in Fig. 4 with the 0 time point, which represents 4 hr

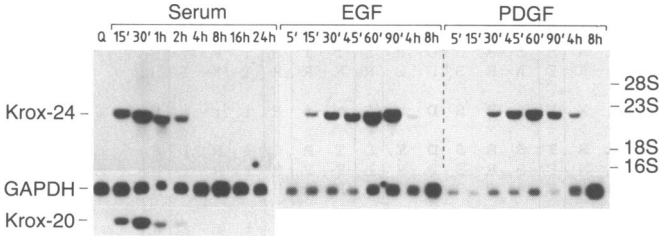

FIG. 4. RNA gel blot analysis of *Krox-24* gene expression in NIH 3T3 cells. Quiescent NIH 3T3 cells were stimulated with fetal bovine serum or with purified epidermal growth factor (EGF, 10 ng/ml) or platelet-derived growth factor (PDGF, 20 ng/ml) for the indicated lengths of time. Q corresponds to no stimulation. Total cellular RNA (20 μg) was fractionated by electrophoresis in a 1% agarose/6.7% formaldehyde gel, transferred to GeneScreen (New England Nuclear), and hybridized with a DF2-derived 3′ probe not containing the finger region (nucleotides 1148–2653). The blots had been previously hybridized with the GAPDH probe and, in the case of serum stimulation, with a *Krox-20* cDNA probe. Positions of RNA markers are shown at right.

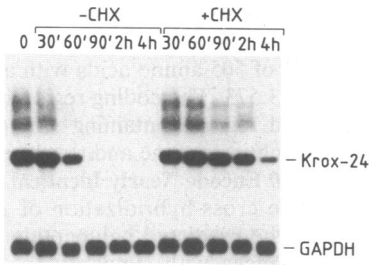

FIG. 5. RNA gel blot analysis of *Krox-24* mRNA stability. Quiescent NIH 3T3 cells were stimulated with serum for 4 hr in the presence of cycloheximide. They were then exposed to actinomycin D (1 μg/ml) in the absence (−CHX) or presence (+CHX) of cycloheximide (10 μg/ml) for the indicated lengths of time. RNA was analyzed as described for Fig. 4 with *Krox-24* and GAPDH probes.

of treatment with serum in the presence of cycloheximide, in Fig. 5). In addition to the 3.2-kb *Krox-24* mRNA, two longer RNA species were observed after cycloheximide treatment (Fig. 5). They might have resulted from perturbations in RNA processing due to long exposure of the cells to cycloheximide. To determine whether RNA stabilization was involved in superinduction by cycloheximide, we performed the following experiment. Cells were stimulated with serum for 4 hr in the presence of cycloheximide. Transcription was then blocked by the addition of actinomycin D, and the level of *Krox-24* mRNA was monitored for the next 4 hr in the presence or absence of cycloheximide (Fig. 5). Quantification of the mRNA levels by densitometer scanning indicated that cycloheximide increased the half-life of the mRNA from ≈20 min to 2 hr. These properties are consistent with the high percentage of adenine and thymine observed within the 3′ untranslated region and the presence in this region of two copies of the sequence ATTTA (Fig. 1). These characteristics have been found to be common to a number of transiently expressed genes and are thought to mediate selective mRNA degradation (28). We then examined *Krox-24* expression in various adult mouse organs and in whole embryos. The RNA was observed in brain and thymus (Fig. 6). In addition, very low levels were also detected in most of the other RNA samples (Fig. 6). No *Krox-24* mRNA was detected in undifferentiated F9 embryonal carcinoma cells. This is in contrast to the expression of a number of *Kr*-related genes in these cells (12, 29).

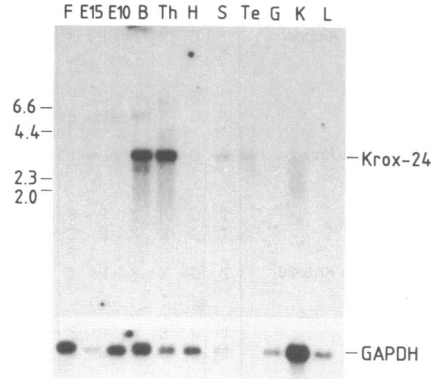

FIG. 6. RNA gel blot analysis of *Krox-24* gene expression in adult mouse tissues and whole embryos. Poly(A)$^+$ RNA (2 μg per lane) was analyzed as described for Fig. 4 with *Krox-24* and GAPDH probes. Lanes: F, undifferentiated F9 cells; E15, 15.5-day-old embryo; E10, 10.5-day-old embryo; B, brain; Th, thymus; H, heart; S, spleen; Te, testis; G, gut; K, kidney; L, liver. Positions and sizes (kb) of *Hin*dIII restriction fragments of λ phage DNA are indicated at left.

DISCUSSION

In this paper, we describe a gene, *Krox-24*, that is activated very early during the G_0/G_1 transition. The rapid accumulation of *Krox-24* mRNA after stimulation by serum or purified growth factors and the fact that this effect does not require *de novo* protein synthesis suggest that the expression of *Krox-24* is directly regulated by the growth factors. The kinetics of accumulation and subsequent disappearance of *Krox-24* mRNA are similar to those of c-*fos* (30–32). Like *Krox-20*, *Krox-24* encodes three zinc fingers with strong similarity to those of transcription factor Sp1. The Krox-24 protein is therefore likely to be a DNA-binding protein and a transcription factor possibly involved in the modulation of cell proliferation. In addition, the identification of *Krox-24* reinforces the idea of the existence of a multigene family encoding transcription factors with three zinc fingers (12, 13). At this stage, because of the precedent of transcription factor IIIA, we cannot exclude that Krox proteins bind RNA as well as or instead of DNA.

Krox-24 DNA shows a high similarity with a recently isolated rat DNA, NGFI-A, whose gene was shown to be activated by NGF in rat PC12 pheochromocytoma cells (24). There are only 12 amino acid differences between the two proteins and a deletion of three amino acids in Krox-24. In addition, both genes have similar patterns of expression (Fig. 6 and ref. 24). Such a similarity suggests that *Krox-24* is the mouse homologue of the NGFI-A gene. The activation of the NGFI-A gene by NGF in PC12 cells suggests that the role of the gene might not be restricted to the regulation of cell proliferation and that it might also be involved in the modulation of cell differentiation. This is consistent with the tissue-specific pattern of expression of the *Krox-24* gene.

Krox-24 is also related to *Krox-20*. The very high conservation of the zinc finger region and of the upstream basic region is the basis for the cross-hybridization between the two cDNAs. The conservation of the upstream basic region suggests that this region might be part of the DNA-binding domain and might be responsible for interactions with the DNA backbone. Although highly similar, the fingers of Krox-20 and Krox-24 are encoded by different DNA segments as judged by the comparison of the nucleotide sequences (19% nucleotide differences) and the analysis of the genomic clones (Janssen-Timmen, unpublished results). This indicates that the two mRNAs do not share common exons and that the genes are distinct.

The similarity of Krox-20 and Krox-24 fingers suggests that both proteins may be able to bind to the same target site on DNA. Such a situation might not be exceptional, since there is evidence that distinct factors can recognize the same cis-acting regulatory DNA sequence. For example, a multiplicity of factors bind "CCAAT box" sequences (33, 34). Similarly, the "octamer" sequence is recognized by at least two distinct factors, one that is ubiquitous in mammalian cells and one that is B-lymphoid-cell-specific (35, 36). One can speculate that although Krox-20 and Krox-24 recognize the same DNA sequence, they affect transcription of nearby genes in a different manner because they interact differentially with other transcription factors. The definition of the target DNA sequence and eventually the isolation of the genes regulated by Krox-20 and Krox-24 will be required to address the question of their respective functions.

Note Added in Proof. A mouse gene identical to *Krox-24* has been described by Sukhatme *et al.* (37).

We thank H. Jäckle and J. M. Blanchard for the gifts of the *Kr* and GAPDH probes, respectively; I. Mattaj and T. Gibson for critical reading of the manuscript; and P. Chavrier for helpful discussions and numerous gifts of plasmids and probes. P.L. was supported by a fellowship from the European Molecular Biology Laboratory.

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