

The Serum-Inducible Mouse Gene *Krox-24* Encodes a Sequence-Specific Transcriptional Activator

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Received 16 January 1990/Accepted 9 April 1990

The mouse gene *Krox-24* is transiently activated during cell cycle reentry. It encodes a protein with three zinc fingers similar to those of the transcription factor Sp1. Here we present a biochemical characterization of the gene products. *Krox-24* mRNA is translated into two proteins of 82 and 88 kilodaltons, designated p82^{*Krox-24*} and p88^{*Krox-24*}, respectively. p82^{*Krox-24*} is initiated at the first AUG codon of the open reading frame, whereas synthesis of p88^{*Krox-24*} starts at a non-AUG codon located upstream. Both proteins were synthesized in HeLa cells infected with recombinant vaccinia viruses expressing *Krox-24* cDNAs. Under these conditions, they were found phosphorylated on serine residues and glycosylated. The availability of the proteins made possible the determination of the DNA recognition sequence. In vitro, *Krox-24* bound specifically to the sequence 5'-GCG(C/G)GGGCG-3'. This sequence is similar but not identical to the Sp1 target sequence. Insertion of an oligomer for the binding site in *cis*, close to the herpes simplex virus thymidine kinase promoter, rendered this promoter responsive to *Krox-24*. *Krox-24* is therefore a sequence-specific transcriptional activator. *Krox-24*-binding sites were found upstream of several serum-inducible genes, raising the possibility that *Krox-24* is involved in the regulation of these genes.

Cells rendered quiescent by serum deprivation can be driven to reenter the cell cycle by stimulation with serum or purified growth factors. During this process, a number of genes, designated immediate-early response genes, are transiently activated without a requirement for de novo protein synthesis (1, 42). These genes are likely to play a crucial role in the initiation of the cascade of events leading to cell division, and indeed, several proto-oncogenes, including *c-fos*, *c-jun*, and *c-myc* belong to this category (26, 36, 55). We have isolated and characterized two mouse immediate-early response genes, *Krox-20* and *Krox-24* (9, 44). These genes are also known respectively as EGR-2 (34) and *egr-1*, *zif268*, or NGFI-A (11, 47, 59). They encode nuclear proteins with three zinc fingers of the TFIIIA type, likely to constitute a DNA-binding domain (44). The zinc fingers of *Krox-20* and *Krox-24* are nearly identical and closely related to those of Sp1, a well-characterized transcription factor (35, 44). This property suggests that *Krox-20* and *Krox-24* might constitute transcription factors with identical DNA recognition sequences. However, the similarity between the two proteins is mostly restricted to the putative DNA-binding domains, raising the possibility that *Krox-20* and *Krox-24* may carry out different functions at the transcriptional level (9, 44).

An additional element in support of an association of *Krox-24* with the regulation of cell proliferation has come from a study by Seyfert and collaborators (57). They reported the accumulation of *egr-1* mRNA in B lymphocytes following stimulation of the immunoglobulin receptor, with a perfect correlation between signals activating the gene and those triggering cell proliferation. Further interest in *Krox-20* and *Krox-24* arose from the analysis of their pattern of expression in vivo. Expression of *Krox-20* is restricted to the nervous system during embryogenesis (61). In particular, the

gene is transcribed in a segment-specific pattern in two alternative rhombomeres of the developing hindbrain, suggesting its involvement in the control of important developmental processes in this region of the nervous system (61). Activation of *zif268/Krox-24* in the rat ipsilateral granule cell neurons is closely correlated with induction of long-term potentiation of the perforant path-granule cell synapse, suggesting a possible role of the gene in synaptic plasticity in the vertebrate nervous system (14).

These different observations potentially implicate *Krox-20* and *Krox-24* in the control of cell proliferation and of several aspects of development. They justify a detailed analysis of these genes. One of our long-term goals is the precise definition of their functions and in particular the identification of the putative genes which they regulate. A preliminary step is the biochemical characterization of the proteins and identification of their target DNA sequences. In this article, we present an analysis of *Krox-24* gene products, including in particular the observation of differential translation initiation due to initiation at a non-AUG codon. We show that the *Krox-24* protein binds to DNA in a sequence-specific manner and that binding to the target sequence can lead to transcriptional activation.

MATERIALS AND METHODS

Oligonucleotides. The different oligonucleotides were obtained by annealing two chemically synthesized strands: E, 5'-CCGAGGGAGCGCGCGGGCGGGGAGGA-3' and 5'-TCGGTCCCTCCCCGCCCCGCGCGCTCCCC-3', and F, 5'-CCGACGCGGGGCGCGGGGCGGGGCGCGCA-3' and 5'-TCGGTGCAGCCCCGCCCCGCCCCGC-3'. The mutant derivatives of the F oligonucleotide were obtained by introducing the complementary changes described in the legend to Fig. 4B in both strands of the F oligonucleotide.

DNA constructions. Plasmid constructions were carried

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out by standard procedures (45). Plasmid pgKr24cod4 contained the 1.75-kilobase (kb) *EcoRI*-*Bgl*II *Krox-24* insert from DF107 (33) cloned into the *EcoRI* and *Bam*HI sites of the pGEM-1 vector. To obtain pgKr24cod5, pgKr24cod4 was digested with *EcoRI* and *Bgl*II, the ends were made blunt with the Klenow fragment of DNA polymerase I, and the plasmid was recircularized. To obtain pgKr24cod1, the 79-base-pair (bp) *EcoRI*-*Bgl*II fragment of pgKr24cod4 was replaced by a 330-bp *Nru*I-*Bgl*II fragment derived from the *Krox-24* genomic sequences (33) to reconstitute a complete open reading frame. The *EcoRI* and *Nru*I ends were filled in with the Klenow fragment of DNA polymerase I. pgKr24cod2 contained the 1.4-kb *Bgl*II fragment of pgKr24cod1 cloned into the *Sma*I site of pGEM-1 in the opposite orientation from that in pgKr24cod1. pgKr24cod3 was obtained from pgKr24cod1 by deletion of the *EcoRI*-*Apa*I fragment and recircularization of the plasmid. Plasmid pURKr24BP contained the 0.8-kb *Ban*II-*Pst*I fragment of pgKr24cod4 cloned between the *Bam*HI and *Pst*I sites of pUR290 (54). The p4F-tkCAT reporter plasmid, containing a tetramer of the F oligonucleotide, was obtained in the following way. The F oligonucleotides were phosphorylated at their 5' ends with T4 polynucleotide kinase and ATP, annealed, and subsequently ligated into head-to-tail tandem repeats by virtue of the presence of asymmetric complementary *Ava*I ends. The ligation products were separated by polyacrylamide gel electrophoresis (PAGE) on a 5% polyacrylamide gel, and tetramers were purified and cloned into the *Ava*I site of the plasmid pV2 (21). The oligomers were subsequently excised from the pV2 derivative by restriction with *Bgl*II and cloned into the *Bam*HI site of ptkCAT (generous gift of R. Miksicek and G. Schütz). The orientation and the sequence of the insert in p4F-tkCAT were determined after cloning into M13-derived vectors. The expression plasmid pPadhKrox24, derived from pPadh (generous gift of A. Courey and R. Tjian), was constructed by insertion of a 1.62-kb *Nco*I-*Bgl*II fragment of the Kr24cod4 cDNA into the *Nco*I and *Bam*HI sites of pPadh. This plasmid encodes a truncated *Krox-24* protein consisting of the 505 C-terminal amino acids. pPacKrox20 and pPadh β gal have been described previously (8a). To construct the expression plasmid pSVKrox-24, the 2.5-kb *Sma*I-*Bgl*II genomic fragment containing the major part of the open reading frame and the intron was cloned into the *Bam*HI site of the pSV51 plasmid vector (31) by using *Bam*HI linkers.

In vitro transcription and translation. cDNAs cloned into the pGEM-1 vector were digested with the restriction enzymes indicated in the Results section. Kr24cod1, Kr24cod3, Kr24cod4, and Kr24cod5 were transcribed from the T7 promoter; Kr24cod2 was transcribed from the SP6 promoter. Transcription was performed with the Boehringer Mannheim transcription kit, and the RNAs were subsequently translated in a rabbit reticulocyte lysate system (Amersham) according to the manufacturer's recommendations.

Generation of antibodies. Three polyclonal antisera recognizing the *Krox-24* proteins were obtained. Antiserum 588 was raised against the peptide DMTATFSPRTIEICK (one-letter amino acid abbreviations), corresponding to the 14 C-terminal residues of p82^{Krox-24}, to which a lysine was added to facilitate coupling of the peptide to its carrier. Antiserum 675 was raised against the peptide TTGRGYR QPGGPPTK, corresponding to the translation of the portion of the open reading frame located 168 to 124 nucleotides upstream from the first AUG. A lysine was added at the 15th position as above. The peptides were coupled to keyhole

limpet hemocyanine as described by Kypta and collaborators (40). Antiserum 502 was raised against a β -galactosidase-*Krox-24* fusion protein produced in *Escherichia coli* from the pURKr24BP plasmid. The hybrid protein contained the 233 C-terminal amino acids of *Krox-24*. It was partially purified by gel filtration chromatography on a Sepharose CL-4B column. Then, 200 μ g of coupled peptide or 500 μ g of the fusion protein in Freund complete adjuvant (Boehringer) was injected into the popliteal lymph nodes of bastard rabbits. The same quantities, in Freund incomplete adjuvant, were given 3 weeks later by subscapular injection. Ten days later, a third injection was given intramuscularly, followed by an intravenous injection 1 day later. The rabbits were then boosted with 200 μ g of coupled peptide or fusion protein and bled (50 ml) every other week.

Production of recombinant vaccinia viruses and preparation of cellular extracts. cDNAs Kr24cod1 and Kr24cod4 were cloned into the pATA-18 recombination plasmid, consisting of a mutated 11k vaccinia virus promoter inserted in the thymidine kinase (*tk*) locus (58). The recombinant viruses vKr24.1 and vKr24.4, containing Kr24cod1 and Kr24cod4, respectively, were derived from the temperature-sensitive mutant *ts7* (19), essentially by the procedure of Kieny and collaborators (37). Vaccinia virus strain WR and the recombinant viruses were grown in HeLa cells maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum. For preparation of extracts from infected cells, HeLa cells were infected at a multiplicity of infection of 5 and incubated for 24 h before preparation of the extracts.

In vivo and in vitro labeling of proteins. For [³⁵S]methionine labeling, HeLa cells infected with a recombinant virus for 24 h were washed and incubated for 2 h in methionine-free medium. The medium was then supplemented with 200 μ Ci of L-[³⁵S]methionine per ml, and the cells were incubated for 1 h at 37°C before lysis. For ³²P_i labeling, HeLa cells were infected for 5 h in phosphate-free medium and were further incubated for 15 h in 2 ml of phosphate-free medium supplemented with 200 μ Ci of ³²P_i (Amersham) before lysis. For [³H]UDP galactose labeling of the *Krox-24* proteins, 0.3 U of galactose transferase (Sigma Chemical Co.), 1 μ l of 1 M MnCl₂, and 80 μ Ci of [³H]UDP galactose (Amersham) were added to 100 μ l of total cellular extracts from infected HeLa cells lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.14 M NaCl, 10 mM Tris hydrochloride [Tris-HCl, pH 8.0]). Incubation was carried out for 60 min on ice before immunoprecipitation.

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were lysed in RIPA buffer. In vivo-labeled cell lysates were centrifuged for 15 min at 30,000 \times g, and the supernatant was collected. The labeled lysates (10⁷ cpm) were first incubated at 4°C for 20 min with 2 μ l of preimmune serum. Then, 20 μ l of protein A-Sepharose (50% slurry in buffer A [0.2% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA]) was added. The tubes were incubated for 2 h on a rotating wheel at 4°C and centrifuged for 30 s at 4,000 rpm to pellet the beads. The supernatant was collected, and the incubation procedure was repeated with 2 μ l of immune serum. The beads were pelleted as above, the supernatant was discarded, and the beads were washed twice with buffer A, twice with buffer A adjusted to 0.5 M NaCl, and twice with 10 mM Tris-HCl (pH 7.5). The immunoprecipitated proteins were analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels after be-

ing boiled in SDS loading buffer (41). For immunoblotting, 10 μ g of total extract in RIPA buffer was separated by SDS-PAGE. Blotting and immunodetection were carried out by standard procedures (28). The anti-Krox-24 antisera and the peroxidase-conjugated anti-rabbit immunoglobulin second antibody (Jackson ImmunoResearch Laboratories) were diluted 500- and 2,500-fold, respectively.

Phosphoamino acid content analysis. 32 P-labeled Krox-24 protein produced in HeLa cells infected by vKr24.4 was immunoprecipitated with the 588 antiserum and purified by preparative electrophoresis on a 7.5% SDS-polyacrylamide gel. The protein was then precipitated with trichloroacetic acid, suspended in 5.7 M HCl, and subjected to acid hydrolysis for 1.5 h at 110°C. The hydrolysate was then dried and suspended in a solution of formic acid-acetic acid-water (1:10:89). The sample was subjected to two-dimensional paper electrophoresis (30). The plate was then sprayed with 0.2% ninhydrin (Merck) in acetone and incubated at 55°C until the standard phosphoamino acids appeared. The labeled amino acids were visualized by autoradiography.

Gel retardation assay and methylation interference. For the gel retardation assay, either strand of the F oligonucleotide was labeled at its 5' end with T4 polynucleotide kinase and [γ - 32 P]ATP (Amersham; 3,000 Ci/mmol). It was subsequently annealed with a 10-fold excess of the complementary strand by incubation at 65°C for 5 min and slow cooling to room temperature. Total eucaryotic cellular extracts were prepared as follows. Cells were washed in phosphate-buffered saline and pelleted in a microfuge, and the cell pellet was first frozen in dry ice and then thawed by suspension in 5 volumes of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9], 0.4 M NaCl, 0.1 mM EGTA [ethyleneglycol tetraacetic acid], 0.5 mM dithiothreitol, 5% glycerol, 0.5 mM PMSF). Cell debris was removed by a 5-min ultracentrifugation at 100,000 \times *g*. Bacterial extracts containing Krox-20 or Sp1 were prepared as described before (Chavrier et al., in press). Cell extracts (1 μ g of protein) were incubated for 10 min on ice in 20 μ l of binding buffer [20 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol, 1 μ g of poly(dIdC) per μ l]. DNA (0.5 ng of end-labeled double-stranded oligonucleotide mixed with an excess of nonradioactive oligonucleotide when indicated) was then added, and the incubation was continued for a further 30 min. The mixture was then loaded on a 5% nondenaturing polyacrylamide gel, and electrophoresis was performed in 0.5 TBE (45). The gel was subsequently dried. Autoradiographic exposure was performed at room temperature with Kodak X-AR films. Dimethylsulfate (DMS) interference experiments were performed on double-stranded F oligonucleotide labeled at the 5' end on the coding or noncoding strand and partially methylated with DMS as described before (46). The oligonucleotides were subjected to a preparative gel retardation assay. Recovered bound or free oligonucleotides were treated with alkali as described before (53). The cleaved oligonucleotides were then analyzed by electrophoresis on an 18% sequencing gel.

DNA transfection. *Drosophila melanogaster* Schneider line 2 cells (56) were grown in Schneider medium (GIBCO) supplemented with penicillin and streptomycin (GIBCO) and 10% fetal calf serum (Laboratoires Jacques Boy). HeLa cells were cultivated in DMEM (GIBCO) supplemented with 10% fetal calf serum. The cells were transfected at 30% confluency by the calcium phosphate procedure (18, 25). Each 10-cm plate received 20 μ g of plasmid DNA, consisting of 10 μ g of reporter DNA, 5 μ g of expression plasmid, and 5 μ g of the control plasmid pAdh β gal (8a) or RSV β gal (29).

CAT assay and RNase mapping. For chloramphenicol acetyltransferase (CAT) assays, cells from one 10-cm plate were harvested 48 h after transfection, washed once with phosphate buffered saline, and suspended in 200 μ l of 0.25 M Tris-HCl (pH 7.5). They were lysed by addition of 0.5% Triton X-100, and the debris was removed by centrifugation in a microfuge for 10 min. CAT activity was assayed as described before (24), and β -galactosidase activity was measured by the method of Herbomel and collaborators (29). The experiments were quantified by cutting out the parts of the chromatograms corresponding to each spot and measuring the radioactivity. The CAT results were normalized to the levels of β -galactosidase.

For RNase mapping, total cellular RNA was extracted from the cells 48 h after transfection by the guanidinium procedure (10). RNase mapping was performed as described before (8) with 50 μ g of total RNA or tRNA. The probe was derived from the plasmid pSP-TK-CAT (generous gift from G. Schütz) linearized with *Eco*RI. Transcription with the SP6 polymerase generates a 347-nucleotide probe. Hybridization with correctly initiated *tk-cat* RNA is expected to protect a 218-nucleotide fragment. The *tk-cat* mRNA levels were also normalized to the levels of β -galactosidase.

RESULTS

Krox-24 mRNA encodes two polypeptides. The open reading frame encoding Krox-24 extends to the 5' end of the mRNA, 294 nucleotides upstream from the first AUG codon (11, 33, 59). The conservation of this property in the case of the rat homolog gene *NGFI-A* (47) suggests a possible role for the upstream part of the open reading frame. To test this idea, a series of derivatives of a cDNA construct containing the complete open reading frame and truncated 100 bp after the stop codon (Kr24cod1, Fig. 1A) were transcribed in vitro by using T7 or SP6 RNA polymerases. The resulting RNAs were subsequently translated in a rabbit reticulocyte lysate system, and the proteins were analyzed by SDS-PAGE. The construct containing the complete 5' part of the cDNA gave rise to two protein species of 82 and 88 kilodaltons (kDa), which were designated p82^{Krox-24} and p88^{Krox-24}, respectively (Fig. 1B). Both proteins were immunoprecipitated with polyclonal antiserum 588, raised against the 14 C-terminal residues of the Krox-24 amino acid sequence, as deduced from the nucleotide sequence (data not shown).

To identify more precisely these two proteins, we constructed a series of 5' external deletions of Kr24cod1. These cDNAs were denominated Kr24cod2 to Kr24cod5 and cloned in pGEM-1. Their 5' ends are located respectively 184 (Kr24cod2), 134 (Kr24cod3), and 45 (Kr24cod4) nucleotides upstream or 44 (Kr24cod5) nucleotides downstream from the first ATG. These constructs were linearized before in vitro transcription either with *Hind*III, which cuts downstream from the cDNA, or with *Pvu*I, which cuts within the cDNA. Translation of the shortened RNAs obtained by in vitro transcription of the various cDNAs digested with *Pvu*I gave rise to derivatives of p82^{Krox-24} and p88^{Krox-24} truncated in the C-terminus. These proteins migrated on SDS-polyacrylamide gels at 55 and 61 kDa, respectively (Fig. 1C). Analysis of the different constructs indicated that p82^{Krox-24} (or the truncated form of this protein in the case of the *Pvu*I digestion) was synthesized from all constructs except Kr24cod5 (Fig. 1B and C). In contrast, a slightly shorter protein was synthesized from this latter construct. These data strongly suggest that p82^{Krox-24} translation is initiated at the first AUG codon and that deletion of this codon results in

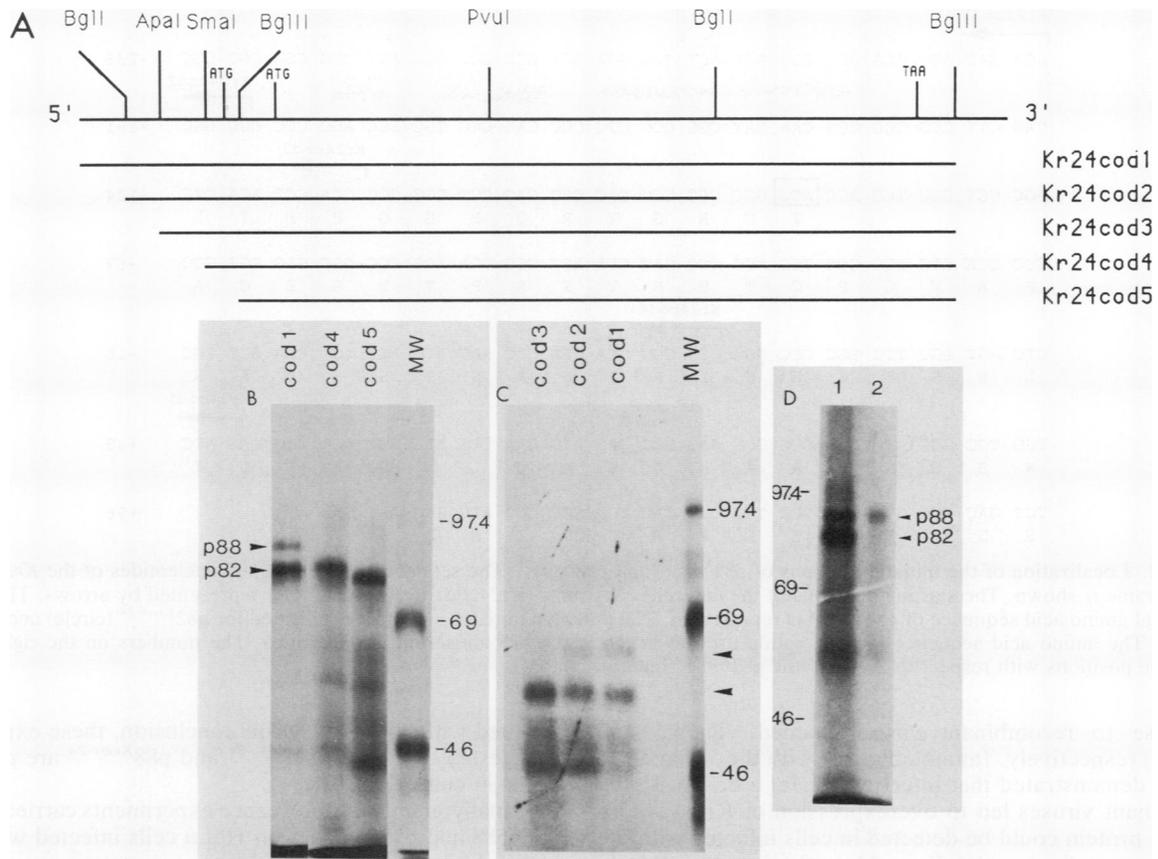


FIG. 1. *Krox-24* mRNA is translated into two proteins in vitro. (A) Schematic representation of the different RNAs used in the in vitro translation experiments, aligned with the cDNA (upper line). The restriction sites used to obtain the different deletions are shown (see Materials and Methods for details of the constructions). Positions of the first two ATGs and of the stop codon of the open reading frame are also indicated. (B) In vitro transcription and translation of Kr24cod1, Kr24cod4, and Kr24cod5 cDNAs. Translation products were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel as indicated. Lane MW, Molecular mass markers (in kilodaltons). The arrows indicate the positions of p88^{Krox-24} and p82^{Krox-24}. (C) Transcription and translation of the Kr24cod1, Kr24cod2, and Kr24cod3 cDNAs truncated at the *PvuII* site. The translation products were analyzed by electrophoresis on an 8% SDS-polyacrylamide gel as indicated. The upper and lower arrows indicate the respective positions of the truncated p88^{Krox-24} and p82^{Krox-24} proteins. (D) Immunoprecipitation of in vitro-translated p88^{Krox-24} with the 675 antiserum. Kr24cod1 was transcribed, and the RNA was translated in vitro. The translation products were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel before (lane 1) or after (lane 2) immunoprecipitation with the 675 antiserum. The positions of molecular mass markers are indicated (in kilodaltons).

synthesis of a shorter polypeptide initiated at a more downstream AUG. The apparent molecular mass of the protein analyzed by SDS-PAGE is much higher than predicted from the amino acid sequence (56.6 kDa). Although we cannot exclude the involvement of posttranslational modifications, this discrepancy is likely to be due to the high proline content of the protein (13%).

The p88^{Krox-24} protein or its C-terminally truncated form was synthesized from Kr24cod1 and Kr24cod2 but not from the more extensively deleted constructs (Fig. 1C). Synthesis of p82^{Krox-24} in the absence of p88^{Krox-24} suggested that the latter protein was not derived from the former by posttranslational modification. These observations suggested that p88^{Krox-24} translation was initiated upstream from the first AUG.

To test this possibility, we raised a polyclonal antiserum, denominated 675, against a peptide with the amino acid sequence TTGRGYRQGGPPTK, corresponding to the translation of the portion of the open reading frame located 168 to 124 nucleotides upstream from the first AUG (Fig. 2).

This antiserum recognized specifically p88^{Krox-24} but not p82^{Krox-24}, demonstrating that initiation of p88^{Krox-24} translation occurred at a non-AUG codon located upstream from the first AUG (Fig. 1D). The deletion analysis suggests that the initiation codon is located between nucleotides 184 and 134 upstream from the first AUG (Fig. 2). Recently, several studies have reported translation initiation at ACG or CTG codons in higher eucaryotes (16, 27, 50, 51). One such codon, an ACG, is located 164 nucleotides upstream from the first AUG and is the sole such codon present in the region defined by the deletion analysis (Fig. 2). This codon is therefore probably the initiation codon for p88^{Krox-24}.

Synthesis of p82^{Krox-24} and p88^{Krox-24} in vivo. To investigate whether p82^{Krox-24} and p88^{Krox-24} are both synthesized in cultured cells, we made use of a eucaryotic expression system based on recombinants derived from the vaccinia virus (58). The Kr24cod1 and Kr24cod4 cDNAs were inserted into the pATA18 plasmid (58) and subsequently introduced by homologous recombination into the viral genome under the control of the major late promoter. This

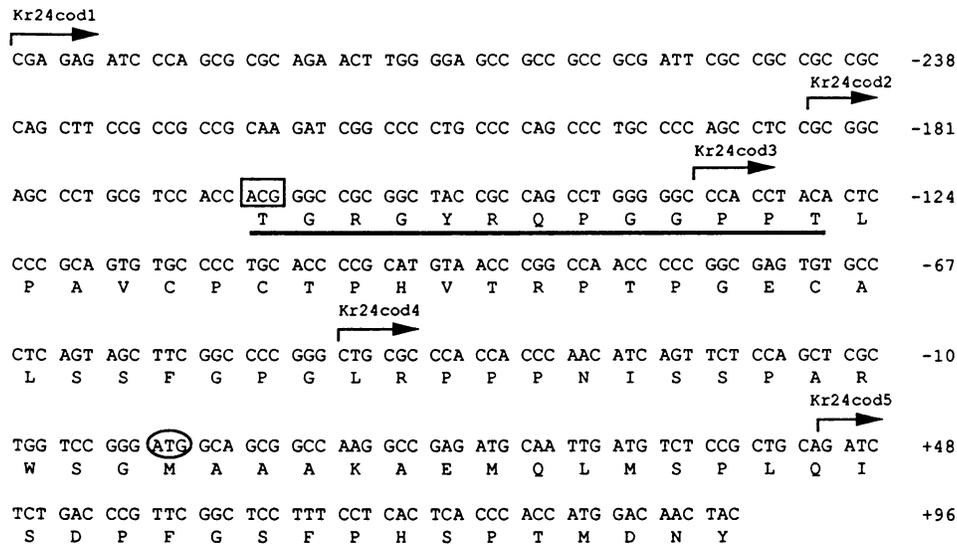


FIG. 2. Localization of the initiation codons of p82^{Krox-24} and p88^{Krox-24}. The sequence of the first 300 nucleotides of the *Krox-24* open reading frame is shown. The starting positions of the different constructs (Kr24cod1 to Kr24cod5) are represented by arrows. The putative N-terminal amino acid sequence of p88^{Krox-24} is represented. The potential initiator codons are indicated for p82^{Krox-24} (circle) and p88^{Krox-24} (square). The amino acid sequence of the peptide used to generate the 675 antiserum is underlined. The numbers on the right indicate nucleotide positions with respect to the A of the p82^{Krox-24} initiator codon.

gave rise to recombinant viruses named vKr24.1 and vKr24.4, respectively. Immunoblotting with the 588 and 675 antisera demonstrated that infection of HeLa cells with the recombinant viruses led to overexpression of Krox-24. No Krox-24 protein could be detected in cells infected with the viral vector, indicating that Krox-24 synthesis occurred from the cloned cDNAs (data not shown). Cells infected with either recombinant virus expressed p82^{Krox-24}. In contrast, p88^{Krox-24} was only observed in cells infected with vKr24.1 by using the 675 antiserum (Fig. 3A). p88^{Krox-24} was not detected with the 588 antiserum, indicating that this protein was less abundant than p82^{Krox-24}. Translation initiation at a non-AUG codon therefore also occurs *in vivo*, although less efficiently than *in vitro*.

The presence of p82^{Krox-24} and p88^{Krox-24} in fibroblasts in culture was also analyzed. NIH 3T3 cells were rendered quiescent by serum deprivation and subsequently stimulated with either 20% fetal calf serum or 30 nM tetradecanoylphorbol-acetate (TPA), a phorbol ester. Proteins were labeled with [³⁵S]methionine, and cell extracts were subjected to immunoprecipitation followed by SDS-PAGE analysis (Fig. 3B). Two antisera, 675 and 502, were used for these experiments. Antiserum 502 was raised against a β -galactosidase-Krox-24 fusion protein containing the 233 C-terminal amino acids of Krox-24. The presence of common bands in all lanes, irrespective of the antiserum, is probably the result of nonspecific adsorption of labeled proteins on the protein A-Sepharose. With the 502 antiserum, a protein with the predicted behavior of p82^{Krox-24} was detected after a 1-h treatment with either inducer but not in quiescent cells (Fig. 3B). Treatment of the cells with TPA led to a greater accumulation of the protein. In addition to a discrete band comigrating with the *in vitro*-synthesized p82^{Krox-24}, a smear of higher-molecular-mass products was also observed and may correspond to posttranslationally modified p82^{Krox-24}. With this antiserum, it was not possible to detect p88^{Krox-24}, suggesting that it was less efficiently synthesized than p82^{Krox-24}. With the 675 antiserum, a diffuse band corresponding to the mobility of p88^{Krox-24} was observed in cells

treated with TPA for 2 h. In conclusion, these experiments suggested that both p82^{Krox-24} and p88^{Krox-24} are present in cells in culture.

Finally, immunofluorescence experiments carried out with the 588 and 675 antisera on HeLa cells infected with either recombinant virus indicated that each protein was localized within the cell nucleus, as expected for putative transcription factors (data not shown).

Determination of Krox-24 DNA target sequence. DNase I footprinting experiments have demonstrated that Krox-20 binds to two GC-rich sequences located upstream of the homeobox containing gene *Hox-1.4* (Chavrier et al., in press). Since Krox-24 and Krox-20 have nearly identical zinc finger domains (44), we investigated whether Krox-24 could interact with the same sequences. This was carried out by using a gel retardation procedure (20, 22), with oligonucleotide F, corresponding to the most upstream footprint of Krox-20 over the *Hox-1.4* gene (8a). Extracts were prepared from HeLa cells infected either with the vKr24.4 virus or with the vector virus. A specific DNA-protein complex was observed with the vKr24.4 extract and not with the control extract (Fig. 4A). Furthermore, the formation of this complex was prevented by competition with an excess of nonradioactive F oligonucleotide, while some mutant derivatives of the F oligonucleotide (8a) were inefficient competitors (Fig. 4A and B). This allowed a preliminary localization of the Krox-24 binding site on the F oligonucleotide. Competition experiments with unlabeled E oligonucleotide, corresponding to the second Krox-20 footprint on *Hox-1.4*, showed that Krox-24 had a slightly weaker affinity for this DNA sequence (Fig. 4B and data not shown).

Additional information on the target sequence was provided by methylation interference analysis of the formation of the complex between Krox-24 and the F oligonucleotide. Analysis of the pattern of G residues revealed that methylation of the F oligonucleotide at each of four positions on the coding strand excluded the oligonucleotide from complex formation (Fig. 5A). Methylation at three other positions on the coding strand and at one position on the noncoding

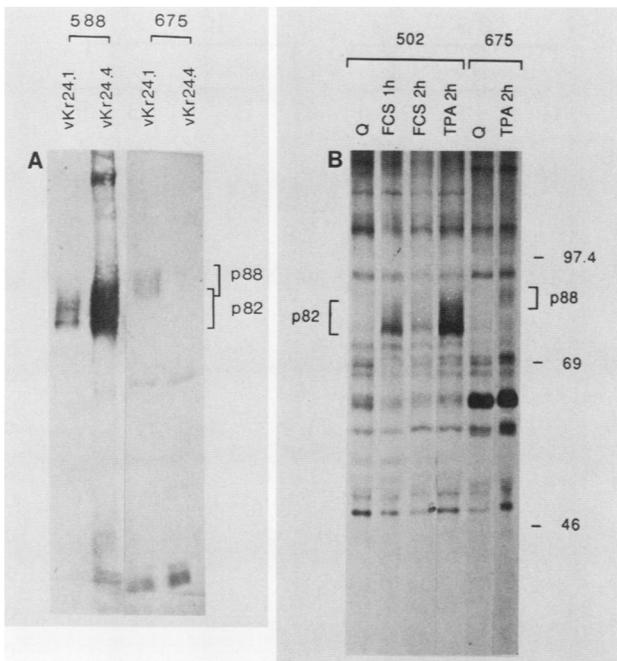


FIG. 3. Detection of Krox-24 proteins in cells in culture. (A) p82^{Krox-24} and p88^{Krox-24} are synthesized in HeLa cells infected with recombinant vaccinia viruses. HeLa cells were infected with the vKr24.1 or vKr24.4 virus for 20 h and lysed. The lysates were fractionated by electrophoresis on a 7.5% SDS-polyacrylamide gel and subjected to Western immunoblotting. Krox-24 proteins were revealed by using the 588 and 675 antibodies as indicated. The brackets indicate the positions of p82^{Krox-24} and p88^{Krox-24}. (B) Detection of p82^{Krox-24} and p88^{Krox-24} in TPA- or serum-stimulated NIH 3T3 cells. NIH 3T3 cells were made quiescent (Q) by serum deprivation and treated with 20% fetal calf serum (FCS) or 30 nM TPA for the indicated period of time before lysis. The lysates were used to perform immunoprecipitations with the 502 or 675 antiserum as indicated. The immunoprecipitates were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. The positions of molecular mass markers (in kilodaltons) and of p82^{Krox-24} and p88^{Krox-24} (brackets) are indicated.

strand had weaker effects (Fig. 5A). The pattern obtained was identical to that observed with Krox-20 produced in bacteria (Fig. 5A) and suggested that Krox-24 recognized the sequence 5'-GCGGGGCG-3'. The F oligonucleotide also contained a consensus binding site for Sp1, and indeed, in gel retardation experiments it bound the 516-amino-acid carboxy-terminal part of Sp1 produced in bacteria (8a).

To compare the binding of Krox-24 and Sp1 with the F oligonucleotide, we also performed methylation interference analysis on the complex between Sp1 and the F oligonucleotide (Fig. 5B). In agreement with previously published data (3), we found that Sp1 recognized the sequence 5'-GGGGGCGG-3'. This indicates that although the Krox-24 and Sp1 binding sites overlap on the F oligonucleotide, the two proteins have different contacts with the DNA, which are summarized in Fig. 5C.

Krox-24 is a transcriptional activator. Knowledge of the DNA-binding specificity of Krox-24 allowed us to test for the transactivation potential of the protein. This was assayed by a transient cotransfection assay in *Drosophila* Schneider line 2 (SL2) cells (15). These cells, lacking Sp1 activity, were chosen to avoid any complications from the presence of overlapping binding sites for Krox-24 and Sp1 on the F

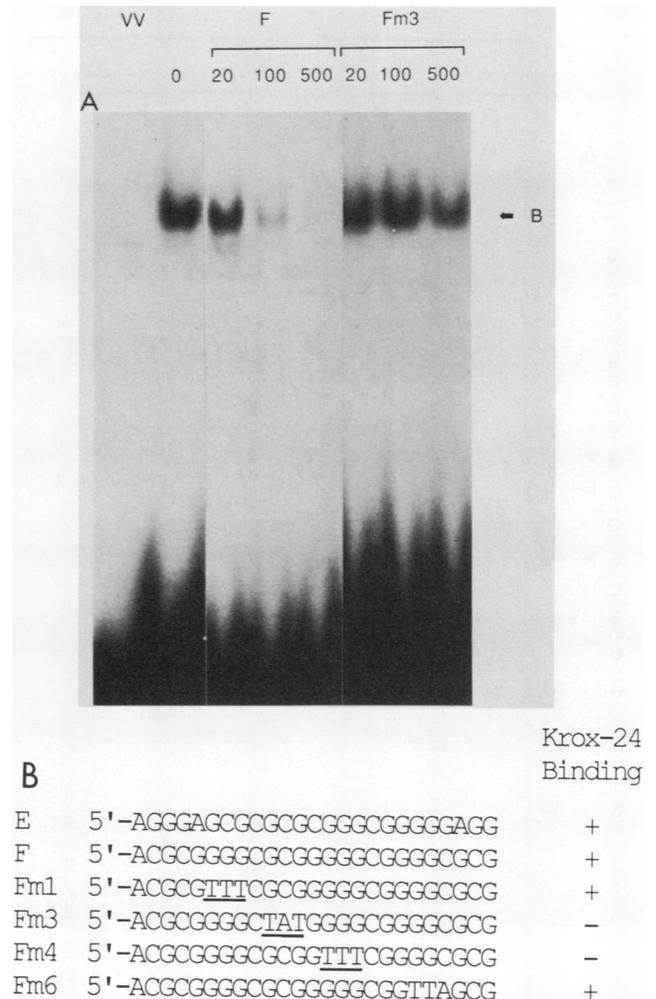


FIG. 4. In vitro binding of Krox-24 to the F double-stranded oligonucleotide and its mutant derivatives. (A) Example of a gel retardation assay performed with total cellular extracts from HeLa cells infected with either the vaccinia virus vector (vv) or the vKr24.4 virus (all other lanes). ³²P-end-labeled F oligonucleotide (0.5 ng) and 1 µg of protein extract were used for each point. Competitions were performed with a 20-, 100-, or 500-fold molar excess of unlabeled F or Fm3 oligonucleotide or with no competitor (lane 0). B, Bound oligonucleotides. (B) Nucleotide sequences of the different oligonucleotides tested and relative affinities for Krox-24. Sequences of the central part of the coding strands of the oligonucleotides are shown. Mutated nucleotides compared with the F oligonucleotide (8a) are underlined. Relative affinities for Krox-24 were estimated by competition in a gel retardation assay and are indicated as follows: +, competes as well as the F oligonucleotide within a fivefold range of concentrations; -, requires more than a 25-fold-higher concentration to compete as well as the F oligonucleotide.

oligonucleotide. A Krox-24 cDNA was placed under the control of the *Drosophila* alcohol dehydrogenase (ADH) promoter in the pPadh plasmid (15). This construct, named pPadhKrox24, encodes a truncated p82^{Krox-24} protein missing the N-terminal 28 amino acids. It was cotransfected into SL2 cells together with a reporter construct. In addition, to control for variations in transfection efficiency, a transfection control plasmid, pPadhβgal, containing the *E. coli lacZ* gene under the control of the ADH promoter, was included in the experiments. The reporter plasmid (p4F-tkCAT) was

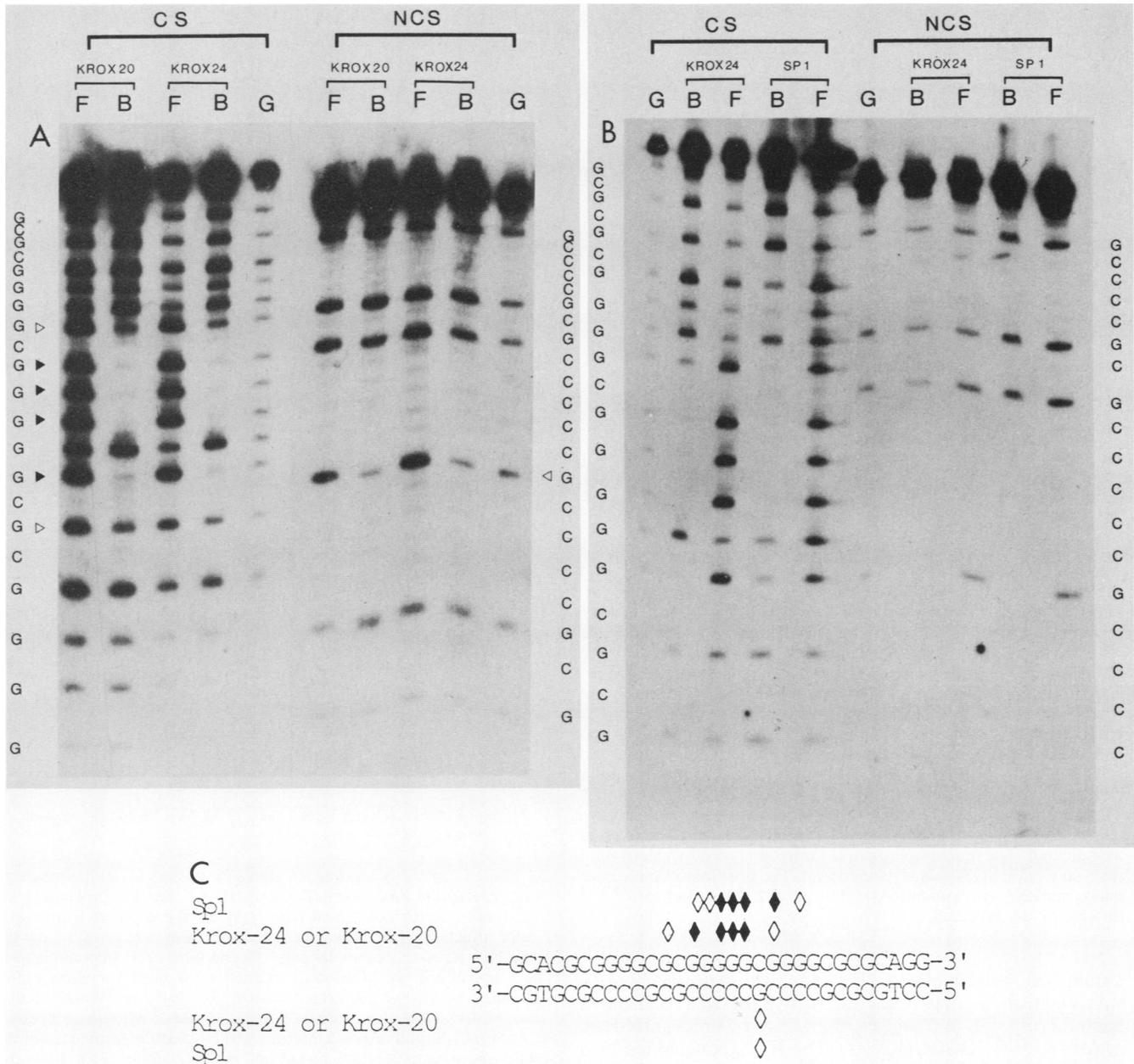


FIG. 5. Comparison of the sequence specificities of Krox-24, Krox-20, and Sp1. (A and B) DMS interference analysis. Partially methylated F oligonucleotides labeled on the coding (CS) or noncoding (NCS) strand were used in a gel retardation assay with either bacterial extracts containing Krox-20 or the 516-amino-acid C-terminal part of Sp1 (8a) or with total cell extracts from HeLa cells infected with the vKr24.1 virus. The bound (B) and free (F) oligonucleotides were eluted from the gel, treated with sodium hydroxide, and fractionated on sequencing gels. Lane G, Labeled F oligonucleotides cleaved at G positions. In panel A, the solid and open arrows indicate the G residues whose methylation strongly or weakly interferes, respectively, with the formation of the complex. (C) Summary of the data obtained by methylation interference on the F oligonucleotide. Solid and open diamonds indicate the G residues whose methylation strongly or weakly interferes, respectively, with Krox-24, Krox-20, or Sp1 binding.

derived from a construct (ptkCAT, generous gift of G. Schütz) consisting of the herpes simplex virus thymidine kinase (*tk*) gene promoter driving the chloramphenicol acetyltransferase (*cat*) coding sequence. It contained a tetramer of the F oligonucleotide inserted in front of the promoter (Fig. 6A).

In three independent experiments, cotransfection of p4F-tkCAT with the Krox-24 expression plasmid led to a five- to sevenfold increase in CAT activity compared with the levels

obtained in the absence of Krox-24 (Fig. 6B). Under the same conditions, an 18- to 35-fold increase was obtained with the Krox-20 expression plasmid (Fig. 6B). No increase in CAT activity was observed when the ptkCAT plasmid was used as the reporter, demonstrating that the presence of the oligomer of the F-binding site was necessary for transactivation (Fig. 6B). The difference in levels of transactivation observed between Krox-24 and Krox-20 might be due to the relative amounts of the two transcription factors

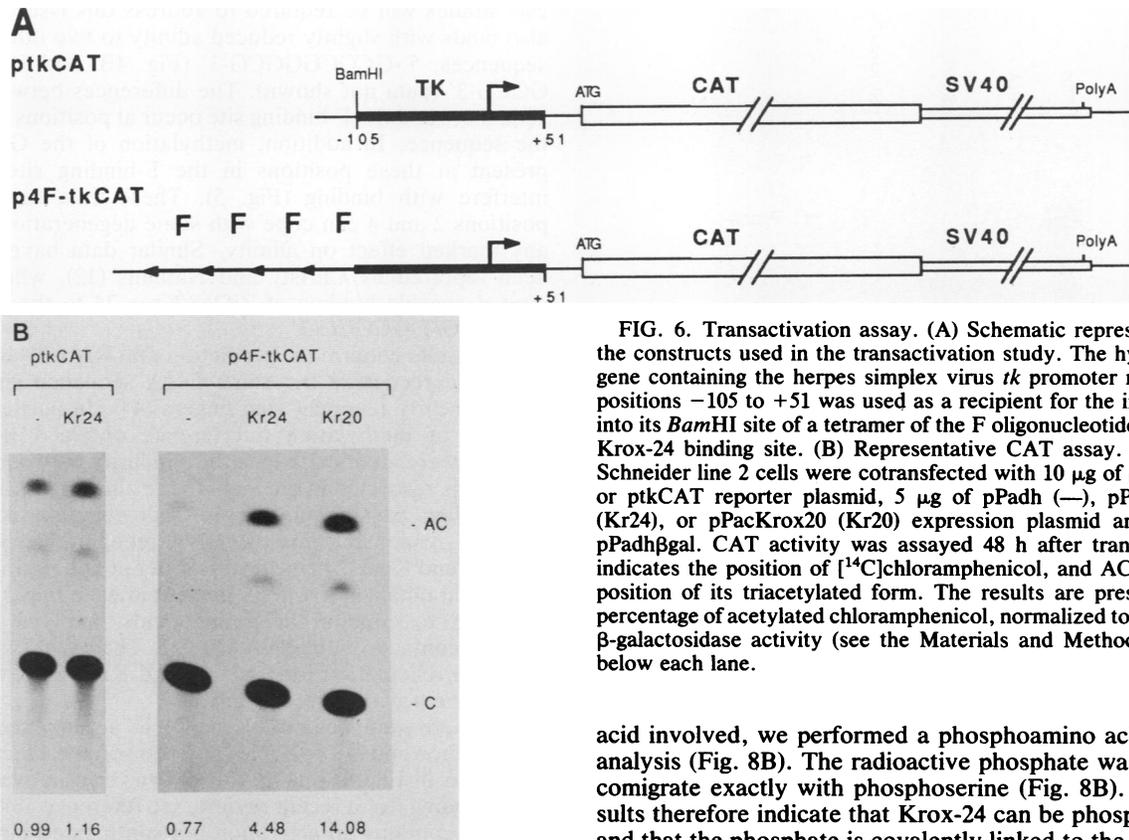


FIG. 6. Transactivation assay. (A) Schematic representation of the constructs used in the transactivation study. The hybrid *tk-cat* gene containing the herpes simplex virus *tk* promoter region from positions -105 to +51 was used as a recipient for the introduction into its *Bam*HI site of a tetramer of the F oligonucleotide carrying a Krox-24 binding site. (B) Representative CAT assay. *Drosophila* Schneider line 2 cells were cotransfected with 10 µg of p4F-tkCAT or ptkCAT reporter plasmid, 5 µg of pPadh (—), pPadhKrox24 (Kr24), or pPacKrox20 (Kr20) expression plasmid and 5 µg of pPadhβgal. CAT activity was assayed 48 h after transfection. C indicates the position of [¹⁴C]chloramphenicol, and AC shows the position of its triacetylated form. The results are presented as a percentage of acetylated chloramphenicol, normalized to the level of β-galactosidase activity (see the Materials and Methods section), below each lane.

present in transfected cells. Indeed, cells transfected with pPadh Krox24 contained about 100-fold less binding activity than cells transfected with the Krox-20 expression plasmid, as estimated by gel retardation experiments performed with the F oligonucleotide (data not shown). The activity of the Krox-24 protein was not restricted to *Drosophila* cells, since cotransfection experiments carried out in HeLa cells led to similar levels of transactivation of the reporter plasmid (data not shown).

To investigate whether transactivation by Krox-24 occurred at the RNA level, we performed an RNase protection experiment to estimate the amount of *tk-cat* mRNA in the transfected cells. We chose to carry out this experiment in HeLa cells because of the higher level of expression of the *tk-cat* construct in these cells. The presence of Krox-24 led to a sevenfold increase in the level of correctly initiated *tk-cat* mRNA, in agreement with the increase in CAT activity (Fig. 7). In conclusion, our data indicate that Krox-24 is a sequence-specific transcriptional activator.

p82^{Krox-24} and p88^{Krox-24} are phosphorylated and glycosylated in cultured cells. Several transcription factors have been shown to be phosphorylated and/or glycosylated, and in some cases these modifications were shown to affect DNA binding or transcription activation (32, 62). We therefore investigated the existence of posttranslational modifications of the Krox-24 proteins synthesized in vKr24.1-infected HeLa cells. Extracts were prepared from in vivo ³²P-labeled HeLa cells infected with the vKr24.1 virus. Immunoprecipitation with the 588 and 675 antisera demonstrated that both p82^{Krox-24} and p88^{Krox-24} were labeled with phosphate (Fig. 8A). To determine the type of modification and the amino

acid involved, we performed a phosphoamino acid content analysis (Fig. 8B). The radioactive phosphate was found to comigrate exactly with phosphoserine (Fig. 8B). These results therefore indicate that Krox-24 can be phosphorylated and that the phosphate is covalently linked to the protein as a serine monoester.

To investigate the possible glycosylation of Krox-24 proteins, we determined whether the proteins synthesized in HeLa cells carried *N*-acetylglucosamine or *N*-acetylgalactosamine residues branched onto their polypeptide chain. A total cellular extract of vKr24.4-infected HeLa cells was labeled with galactosyl transferase to transfer [³H]UDP-galactose to branched *N*-acetylglucosamine or *N*-acetylgalactosamine moieties. The labeled extract was then immunoprecipitated with the 588 antiserum. Analysis of the immunoprecipitate by SDS-PAGE demonstrated the labeling of p82^{Krox-24} and therefore the presence of core oligosaccharides branched onto the in vivo-synthesized protein (Fig. 8C).

Because the proteins were synthesized in virus-infected cells, these results have to be interpreted carefully: Krox-24 proteins might be modified differently in serum-stimulated cells. The data nevertheless indicate that Krox-24 proteins may constitute targets for cellular kinases and *N*-acetylglucosamine or *N*-acetylgalactosamine transferases.

DISCUSSION

Differential translation initiation involving usage of a non-AUG initiation codon. Analysis of the sequence of mouse and rat *Krox-24* cDNAs revealed that the open reading frame extended upstream from the first AUG codon, up to the 5' end of the mRNA. We have shown that two proteins, p82^{Krox-24} and p88^{Krox-24}, are synthesized from the open reading frame, both in vitro and in vivo. Translation of p82^{Krox-24} is probably initiated at the first AUG of the open reading frame, while our data suggest that p88^{Krox-24} translation is initiated at an ACG codon located 165 nucleotides upstream. This translation initiation is less efficient in vivo

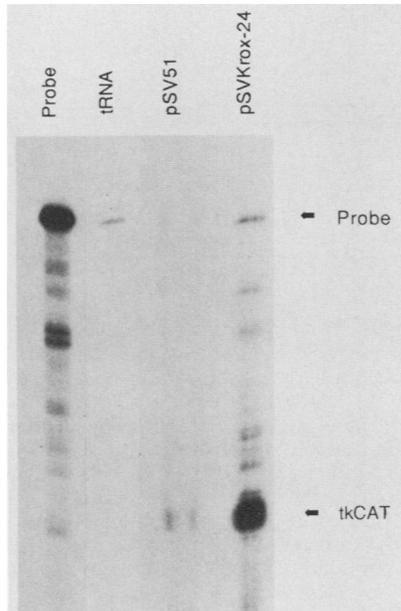


FIG. 7. Krox-24 is a transcriptional activator. HeLa cells were cotransfected with 10 μ g of p4F-tkCAT reporter plasmid, 5 μ g of pSVKrox24 or pSV51 expression plasmid, and 5 μ g of RSV β gal. Total RNA was prepared 48 h after transfection, and the level of correctly initiated *tk-cat* mRNA was estimated with an RNase protection assay (see the Materials and Methods section). Protected fragments were analyzed on a 6% polyacrylamide sequencing gel, and their size was estimated by comparison with the migration of labeled pUC19 *Sau3A* restriction fragments. The arrows indicate the positions of migration of fragments corresponding to correctly initiated *tk-cat* mRNA (expected size, 218 nucleotides; measured size, between 210 and 215 nucleotides) and of the undigested probe (347 nucleotides). The first lane was undigested probe, and the second lane was an RNase protection assay carried out with 50 μ g of tRNA.

than in vitro. Recent reports concerning yeast, plant, viral, and mammalian proteins suggest that non-AUG translation initiation occurs much more frequently in eucaryotic cells than was previously anticipated (4, 16, 27, 50, 51). The ability of the eucaryotic ribosomes to contact nucleotides surrounding the initiation codon might account for non-AUG translation initiation for compensating for a weakened codon-anticodon interaction (38). Analysis of the nucleotide sequence surrounding the putative *Krox-24* ACG initiation codon reveals that its context, UCCACCACGGGC, is optimal for translation initiation (39). This might therefore explain the occurrence of translation initiation at this position.

Non-AUG translation initiation has been shown to have biological significance in at least one case (50): a capsid protein of the Moloney murine leukemia virus initiated at a CUG codon, p75^{gag}, representing a small proportion of the normal p65^{gag} protein, was shown to have a defined function in vivo as a cell surface antigen encoded by the virus. In the case of *Krox-24*, we have shown that both p82^{*Krox-24*} and p88^{*Krox-24*} are localized within the nucleus, but it is not known whether they differ in stability, DNA binding, or transactivation potential. Further studies will be necessary to address these issues.

Sequence specificity and DNA-binding domain. *Krox-24* binds in vitro to the sequence 5'-GCGGGGCG-3'. Our studies do not allow us to decide whether this sequence constitutes the optimal binding site. More detailed mutagen-

esis studies will be required to address this issue. *Krox-24* also binds with slightly reduced affinity to two other related sequences, 5'-GCGCGGGCG-3' (Fig. 4B) and 5'-GAGGGGCG-3' (data not shown). The differences between these sequences and the F-binding site occur at positions 2 and 4 of the sequence. In addition, methylation of the G residues present at these positions in the F-binding site did not interfere with binding (Fig. 5). These data suggest that positions 2 and 4 can cope with some degeneration without any marked effect on affinity. Similar data have recently been reported by Christy and Nathans (12), who demonstrated specific binding of Zif268/*Krox-24* to the sequence 5'-GCG(G/T)GGGCG-3'.

Our results confirm the prediction that *Krox-20* and *Krox-24* should recognize the same DNA sequence since they contain nearly identical zinc fingers (44). In particular, the patterns of methylation interference obtained with both proteins were identical. Since the similarity between the two proteins is restricted in the main to the zinc fingers and a few surrounding basic amino acids, this common domain is probably responsible for the DNA recognition specificity. *Krox-20* and *Krox-24* zinc fingers differ at five positions (44), all located outside an α -helix present in each finger (43) and supposed to contain the amino acids involved in base-specific contacts with DNA (5, 23). The identity in DNA sequence recognition for *Krox-24* and *Krox-20* is therefore in agreement with these models.

Transactivation domain. *Krox-24* is a sequence-specific transcription activator. Eucaryotic transcription factors generally use distinct domains to perform transactivation and DNA binding (for a recent review, see reference 48). Indeed, *Krox-20* contains an activation domain located outside the zinc fingers (8a). It is therefore likely that the transactivating domain of *Krox-24* is similarly distinct from the zinc fingers. Although *Krox-24* shares with other transcription activators a high proline, serine, and threonine content, analysis of its amino acid sequence does not reveal the obvious presence of a transactivation domain of a type characterized so far: acidic regions or glutamine- or proline-rich stretches (48). Detailed mutagenesis analysis will be required to localize the part of the protein responsible for this activity. We have also shown that *Krox-24* can be glycosylated and phosphorylated on serine residues. These two types of modifications are involved in the modulation of the activity of some transcription factors (32, 62). Again, further analyses will be required to determine whether these posttranslational modifications affect the activity of *Krox-24*.

The coordinate activation of *Krox-20* and *Krox-24* during G₀/G₁ transition is intriguing. Both proteins recognize the same DNA sequence in vitro and probably interact with the same sequences in vivo; although we have not as yet precisely evaluated their relative efficiencies, both proteins appear to activate transcription, at least in the systems analyzed so far. It is nevertheless possible that *Krox-20* and *Krox-24* have different functions. In this respect, the two proteins share limited similarity outside of the DNA-binding domains and they may therefore interact differently with the transcription machinery. An alternative possibility is that *Krox-20* and *Krox-24* play a similar role during G₀/G₁ transition, while they have distinct developmental and tissue-specific functions, as suggested by their different patterns of expression (13, 44, 59, 61).

Potential *Krox-24* target sites are present in promoters of serum-inducible genes. Unraveling the potential function of *Krox-24* in the control of cell proliferation and in the nervous system requires the identification of genes whose expression

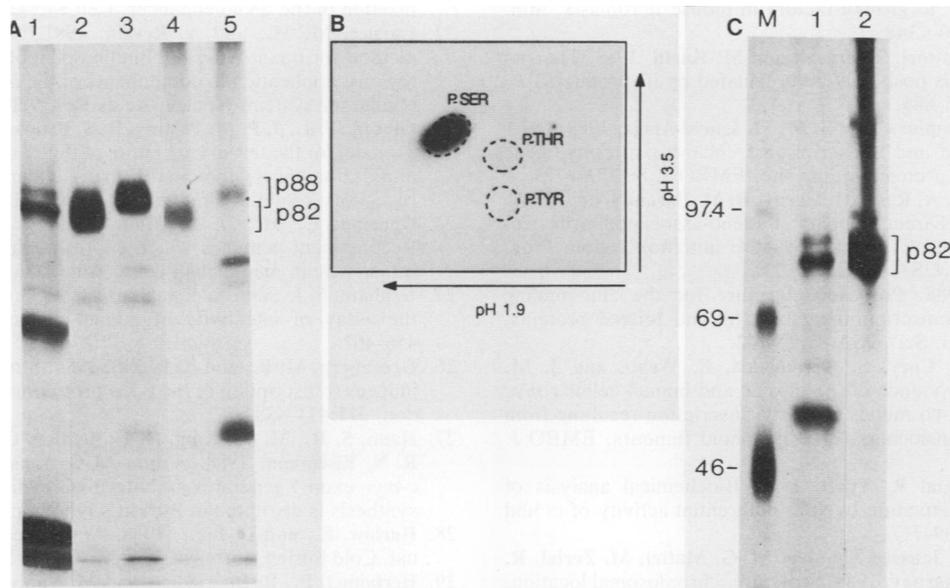


FIG. 8. Krox-24 is phosphorylated and glycosylated in cultured cells. (A) In vivo phosphorylation of Krox-24 proteins. HeLa cells were infected with vKr24.1 for 20 h and labeled with [35 S]methionine (lanes 2 and 3) or 32 P $_i$ (lanes 4 and 5). The lysates were then subjected to immunoprecipitation with the 588 (lanes 2 and 4) or 675 (lanes 3 and 5) antiserum, and the immunoprecipitates were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. Lane 1, In vitro translation of Kr24cod1 RNA. The brackets indicate the positions of p82^{Krox-24} and p88^{Krox-24}. (B) Phosphoamino acid content analysis of phosphorylated p82^{Krox-24}. vKr24.4-infected HeLa cells labeled with 32 P $_i$ were lysed, and the lysate was immunoprecipitated with the 588 antiserum. Immunoprecipitated p82^{Krox-24} was purified by preparative SDS-PAGE and subjected to HCl hydrolysis. The hydrolysate was analyzed by two-dimensional thin-layer cellulose electrophoresis, and the position of the labeled amino acids was revealed by autoradiography. The positions of standard phosphoamino acids visualized with ninhydrin are indicated (P-SER, phosphoserine; P-THR, phosphothreonine; P-TYR, phosphotyrosine). (C) In vitro labeling of glycosylated p82^{Krox-24}. HeLa cells were infected with vKr24.4 for 20 h and lysed. The lysate was labeled by transfer of [3 H]UDP galactose to core oligosaccharides with bovine colostrum galactosyltransferase, followed by immunoprecipitation with the 588 antiserum. The immunoprecipitate was analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel (lane 2). Lane 1, In vitro translation of Kr24cod1 RNA. Lane M, Molecular mass markers (in kilodaltons).

is modulated by its product in vivo. As a preliminary approach, we have screened the GenBank and European Molecular Biology Laboratory data bases with the FIND program of the University of Wisconsin Genetics Computer Group package (17) and the 5'-GCG(C/G)GGGCG-3' query sequence, which will be referred to as the GC sequence. The search was restricted to primate and rodent genes (8,923 entries). The GC sequence was found in 234 entries, representing 164 independent genes. Among them, proto-oncogenes and genes encoding mitogens or mitogen receptors are the best-represented classes. In several cases, the GC sequence is present within the promoter or 5'-flanking sequences. Some of these genes have a pattern of expression compatible with possible regulation by Krox-24 or Krox-20. Four immediate-early response genes contain at least one GC sequence within 500 nucleotides of the transcription initiation site: *c-myc* (6), *Krox-20* (8), *Krox-24* (33), and *NGFI-B* (60). Moreover, we have shown that the GC sequences located in the *Krox-20* and *Krox-24* 5'-flanking regions constitute bona fide Krox-24-binding sites (data not shown). This is in agreement with data recently reported by Christy and Nathans (12). Another interesting candidate is the human gene encoding the A chain of platelet-derived growth factor. This gene also contains GC sequences within its upstream region. It is induced by serum but is activated later than the immediate-early response genes (49).

Although there is no direct indication that Krox-24 or Krox-20 might modulate the expression of any of these genes in vivo, this possibility is worth investigation. In the recent

years, the products of the immediate-early genes *c-fos*, *c-jun*, *c-myc*, *Krox-20*, and *Krox-24* have been shown to be part of sequence-specific DNA-binding transcriptional activators (3, 7, 8a, 52). Moreover, each of these factors binds in vitro to sequences located within its own 5'-flanking regions (2, 3, 8a, 52; this study), and potential DNA-binding sites are also present upstream from other immediate-early response genes. It is therefore possible that a network of interactions between immediate-early genes is involved in the control of G₀/G₁ transition in mammalian cells.

ACKNOWLEDGMENTS

We thank R. Tjian and A. Courey for various plasmid constructs and the communication of unpublished results, R. Miksicek, U. Rütger, and G. Schütz for plasmids, G. Echaliier and C. Maisonhaute for the Schneider line 2 cells, and P. Avner, J. Ghysdael, and M. Yaniv for critical reading of the manuscript. We are also indebted to E. Ulug for precious help with the characterization of the posttranslational modifications.

This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale (grant 881005), the Association pour la Recherche sur le Cancer, the Ligue Nationale Française Contre le Cancer (LNFCC), and the Fondation pour la Recherche Médicale. P.L. was supported by fellowships from the European Molecular Biology Laboratory and the LNFCC, and C.V. was supported by a fellowship from the LNFCC.

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