MOLECULAR AND CELLULAR BIOLOGY, July 1990, p. 3788–3791
0270-7306/90/073788-04$02.00/0

Vol. 10, No. 7

Identification of the Serum-Responsive Transcription Initiation Site of the Zinc Finger Gene *Krox-20*

JANELLE CORTNER AND PEGGY J. FARNHAM*

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, Wisconsin 53706

Received 4 January 1990/Accepted 28 March 1990

The *Krox-20* gene is rapidly and transiently induced when quiescent 3T3 cells are stimulated to reenter the proliferative cycle. We identified the major serum-responsive transcription initiation site and found that it differs from the initiation sites previously identified for the *Krox-20* gene. Transcripts from the major serum-responsive initiation site increased at least 40-fold in serum-stimulated cells compared with logarithmically growing cells.

The murine *Krox-20* gene (4) encodes a protein containing three potential zinc finger domains, which are DNA-binding motifs present in eucaryotic transcription factors (5). The level of *Krox-20* mRNA is rapidly and transiently increased following serum stimulation of quiescent NIH 3T3 cells (4). *Krox-20* mRNA is also increased after cycloheximide treatment of quiescent cells and is superinduced by addition of cycloheximide during serum stimulation (1). We have examined the 5′ ends of *Krox-20* mRNA after serum stimulation of quiescent cells in the presence and absence of cycloheximide and have found that the increase in *Krox-20* mRNA results from an increase in transcripts initiating at a previously unidentified serum-responsive initiation site. The cluster of transcription initiation sites identified previously (2) lie upstream of the predominant transcription initiation site.

Identification of the *Krox-20* serum-responsive transcription initiation site. NIH 3T3 cells were plated (5×10^5 per 225-cm^2 tissue culture flask) in Dulbecco modified Eagle medium containing 5% supplemented calf serum (Hyclone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml and grown for 48 h at 37°C in 5% CO_2. Cells were then harvested for RNA (logarithmic-phase cells), or the medium was removed and replaced with medium containing 0.5% serum. After 48 h cells were harvested for RNA (quiescent cells), or fresh medium containing 10% serum was added and the cells were harvested after 30 min (serum-stimulated cells), the time at which *Krox-20* mRNA levels peak after serum stimulation (4). Cell populations were analyzed by flow cytometry (Fig. 1). Cytoplasmic RNA was prepared by the method of Maniatis et al. (11) as modified from Favaloro et al. (6), quantified by spectrophotometry, and visualized by ethidium bromide staining on agarose gels to ensure that degradation had not occurred (Fig. 2A). We consistently observed differences in the relative abundance of rRNAs in RNA prepared from logarithmically growing cells versus serum-starved and restimulated cells. This is probably due to differences in the ratio of rRNAs to mRNAs between the cell populations.

The 5′ ends of *Krox-20* mRNA were mapped by primer extension analysis (Fig. 2B) with a primer complementary to nucleotides 50 to 71 base pairs (bp) downstream of the 5′ end of the pEX2.8 *Krox-20* cDNA (4). The extension product corresponding to the major serum-responsive start site (designated +1) was 78 nucleotides long, placing it 7 bp upstream of the 5′ end of the cDNA (lane 4). Minor upstream start sites, previously identified by Chavrier et al. (2), were also seen in RNA from serum-stimulated cells (lane 4). *Krox-20* transcripts were not detected in RNA from quiescent cells

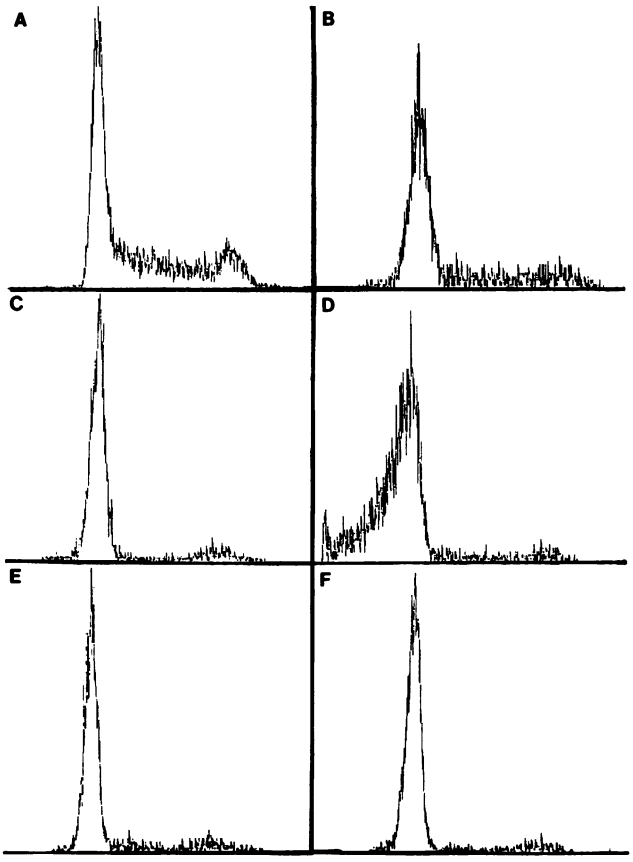

FIG. 1. Flow cytometric analysis. Samples were prepared (7) at the same time as cells were harvested for RNA preparations and analyzed on an Epics Profile II flow cytometer (Coulter Corp). The histograms show cell number (*y* axis) versus DNA content (*x* axis). (A) Logarithmically growing cells. (B) Logarithmically growing cells treated with cycloheximide for 4 h. (C) Quiescent cells. (D) Quiescent cells treated with cycloheximide for 4 h. (E) Quiescent cells exposed to serum for 30 min. (F) Quiescent cells exposed to serum plus cycloheximide for 4 h.

* Corresponding author.

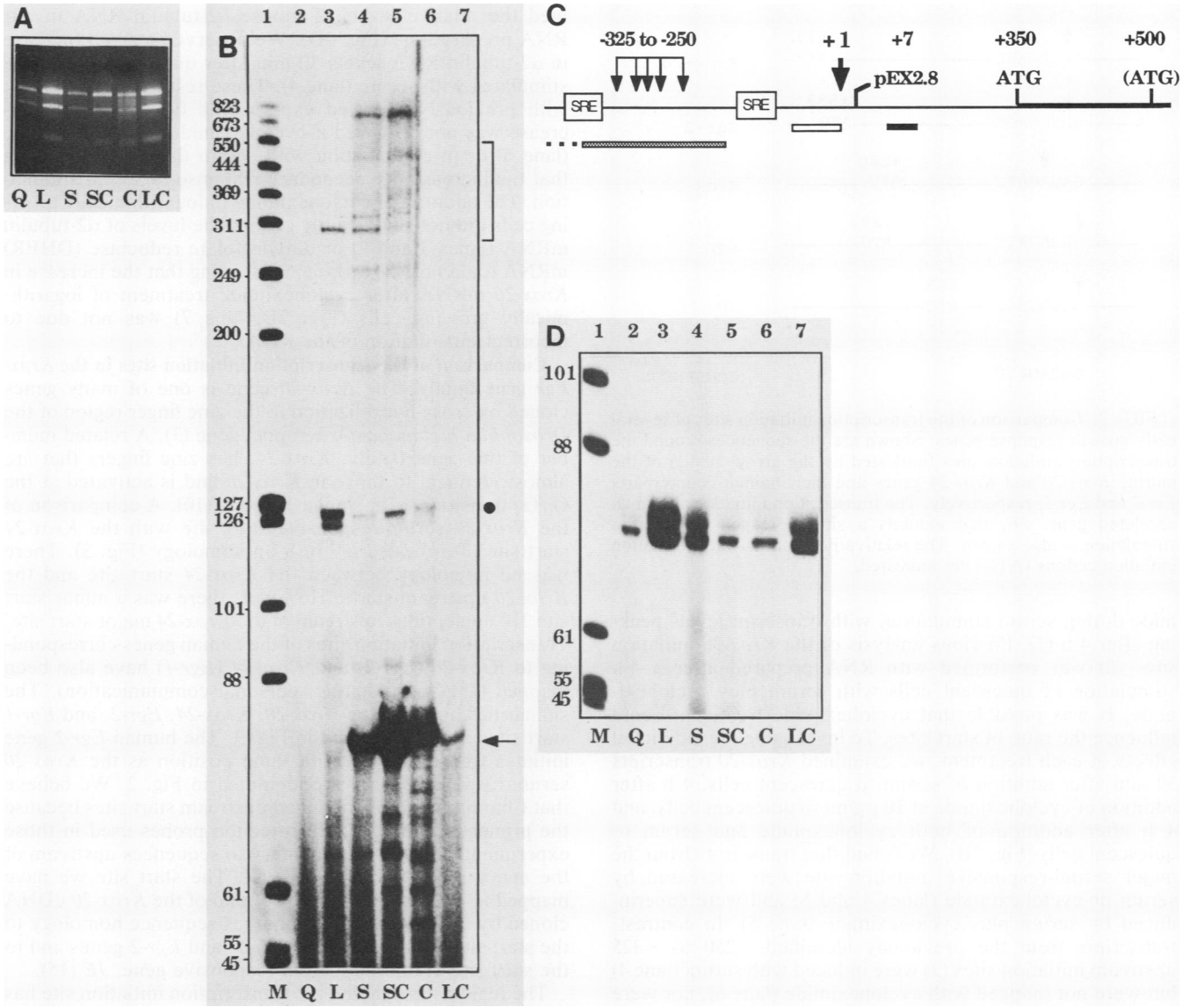

FIG. 2. Mapping the *Krox-20* transcription initiation site. (A) RNA concentrations were determined by two independent spectrophotometric readings. A 500-ng amount of each RNA preparation was then visualized by ethidium bromide staining on an agarose gel. (B) Cytoplasmic RNA (40 μg) from logarithmically growing, quiescent, or stimulated NIH 3T3 cells was analyzed by extension with a primer (described for panel C) phosphorylated with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Primer extension products were analyzed on an 8 M urea–10% polyacrylamide gel, and the autoradiogram was exposed for 12 h. The arrow indicates the serum-responsive transcription initiation site, and the bracket indicates the previously mapped upstream start sites (2). The dot indicates a band of approximately 126 nucleotides that was not reproducibly detected. (C) Numbers above the diagram of the 5′ end of the *Krox-20* gene indicate nucleotide positions relative to the major serum-responsive transcription initiation site (bold arrow). The cluster of upstream transcription initiation sites mapped by Chavrier et al. (2) are indicated with connected arrows between −250 and −325. The primer used in panel B is complementary to sequences from +56 to +78 and is indicated with a heavy bar. Chavrier et al. (2) used a primer complementary to the sequence from −5 to −55 (open bar) and an RNA protection probe complementary to the sequence from −157 to −870 (stippled bar). Also shown are the relative positions of the serum response elements (SREs), the 5′ end of the pEX2.8 *Krox-20* cDNA (4), and the two in-frame translation start sites (ATGs) located at +351 and +502. (D) A 10-μg amount of each RNA preparation was analyzed by extension with a primer phosphorylated with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The primer is complementary to sequences −24 to −51 (relative to the translation start codon) of the mouse α2-tubulin mRNA (19). Primer extension products were analyzed on an 8 M urea–10% polyacrylamide gel, and the autoradiogram was exposed for 12 h. Lanes: M, DNA size markers (in base pairs); Q, RNA from quiescent cells; L, RNA from logarithmically growing cells; S, RNA from quiescent cells stimulated with serum for 30 min; SC, RNA from quiescent cells stimulated with serum plus cycloheximide for 4 h; C, RNA from quiescent cells treated with cycloheximide for 4 h; LC, RNA from logarithmically growing cells treated with cycloheximide for 4 h.

(lane 2). Transcripts from the newly identified start site increased at least 40-fold in serum-stimulated cells compared with logarithmically growing cells, based on an RNA titration analysis (data not shown).

Separation of the cycloheximide and serum response effects. Although the increase in *Krox-20* mRNA peaks 30 min after serum stimulation of quiescent cells (2), the mRNA is superinduced if protein synthesis is inhibited by cyclohexi-

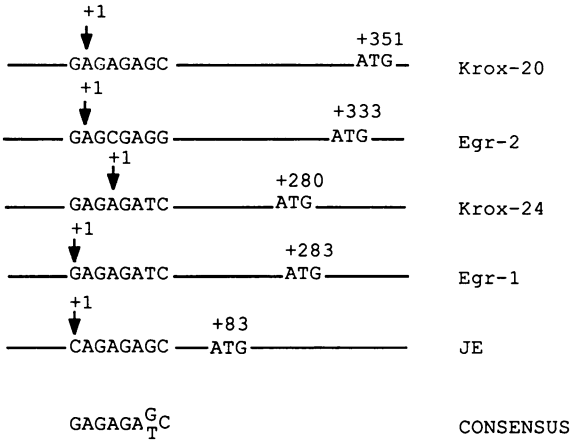

FIG. 3. Comparison of the transcription initiation sites of several early growth response genes. Shown are the sequences around the transcription initiation sites (indicated by the arrow at +1) of the murine *Krox-20* and *Krox-24* genes and their human counterparts *Egr-2* and *Egr-1*, respectively. The transcription initiation site of an unrelated gene, *JE*, that exhibits a similar response to serum stimulation is also shown. The relative positions of the translation initiation codons (ATG) are indicated.

mide during serum stimulation, with transcript levels peaking after 4 h (1). Previous analysis of the *Krox-20* initiation sites (2) was performed with RNA prepared after a 4-h stimulation of quiescent cells with serum plus cycloheximide. It was possible that cycloheximide treatment could influence the ratio of start sites. To investigate the individual effects of each treatment, we examined *Krox-20* transcripts 30 min after addition of serum to quiescent cells, 4 h after addition of cycloheximide at 10 μg/ml to quiescent cells, and 4 h after addition of both cycloheximide and serum to quiescent cells (Fig. 2B). We found that transcripts from the major serum-responsive initiation site were increased by serum or cycloheximide (lanes 4 and 6) and were superinduced by serum plus cycloheximide (lane 5). In contrast, transcripts from the previously identified −250 to −325 upstream initiation sites (2) were induced with serum (lane 4) but were not induced with cycloheximide (lane 6), nor were they superinduced with serum plus cycloheximide (lane 5). In logarithmically growing cells, transcripts from the +1 serum-inducible initiation site (but not the minor −250 to −325 sites) were increased with cycloheximide (lane 7), although to a much lower extent than in quiescent cells. These observations suggest that the serum- and cycloheximide-inducible *Krox-20* mRNA originates principally at the initiation site we have identified. *Krox-20* RNAs initiating at the +1 site were not detected previously because the probes used in those studies were all upstream of this site (see Fig. 2C).

Krox-20 and c-*fos* transcripts increase with the same kinetics after serum stimulation of quiescent cells. The *Krox-20* promoter has two serum response elements, originally identified in the c-*fos* promoter (17), that can confer serum responsiveness to a heterologous promoter. It was originally thought that the stronger serum response element was downstream of the *Krox-20* transcription initiation site (2). However, this element is actually 70 bp upstream of the major serum-responsive initiation site identified here.

Investigators studying fluctuations in c-*fos* mRNA levels during serum stimulation of quiescent cells have used α-tubulin mRNA levels as a control (9). Therefore, we examined the relative levels of mouse α2-tubulin RNA in our RNA preparations (Fig. 2D). We observed a small increase in α2-tubulin RNA levels 30 min after quiescent cells were stimulated with serum (lane 4). These results are consistent with previously reported experiments (9). This slight increase was not observed if cycloheximide was added alone (lane 6) or in combination with serum (lane 5), suggesting that the increase is a secondary response to serum stimulation. The addition of cycloheximide to logarithmically growing cells did not significantly change the levels of α2-tubulin mRNA (lanes 3 and 7) or dihydrofolate reductase (DHFR) mRNA (data not shown), demonstrating that the increase in *Krox-20* mRNA after cycloheximide treatment of logarithmically growing cells (Fig. 2B, lane 7) was not due to incorrect quantitation of the RNA.

Comparison of the transcription initiation sites in the *Krox/Egr* gene family. The *Krox-20* gene is one of many genes cloned by cross-hybridization to the zinc finger region of the *Drosophila melanogaster* Krüppel gene (3). A related member of this gene family, *Krox-24*, has zinc fingers that are almost identical to those in *Krox-20* and is activated at the G_0/G_1 transition with similar kinetics (10). A comparison of the *Krox-20* serum-responsive start site with the *Krox-24* start site (8) revealed a 7 of 8 bp homology (Fig. 3). There was no homology between the *Krox-24* start site and the *Krox-20* upstream starts. However, there was a minor start site 210 nucleotides upstream of the *Krox-24* major start site. Transcription initiation sites of the human genes corresponding to *Krox-20* (*Egr-2*) and *Krox-24* (*Egr-1*) have also been mapped (18; V. Sukhatme, personal communication). The similarities between the *Krox-20*, *Krox-24*, *Egr-2*, and *Egr-1* start sites are diagrammed in Fig. 3. The human *Egr-2* gene initiates transcription at the same position as the *Krox-20* serum-responsive start site identified in Fig. 2. We believe that Chavrier et al. (2) identified upstream start sites because the primer and the RNase protection probes used in those experiments were complementary to sequences upstream of the major start site (see Fig. 2C). The start site we have mapped is 7 bp upstream of the 5′ end of the *Krox-20* cDNA cloned by Chavrier et al. (4) and has sequence homology to the start sites of the *Krox-24*, *Egr-1*, and *Egr-2* genes and to the start site of another serum-responsive gene, *JE* (15).

The region surrounding the transcription initiation site has recently been demonstrated to be important in several mammalian genes. A protein binding at the murine DHFR initiation site specifies the position of transcription initiation (12), and the region around the terminal deoxynucleotidyl transferase initiation site has been shown to be required for transcription (16). The initiation site can also function as a negative regulator, as demonstrated by the decrease in transcription observed when the herpes simplex virus ICP4 protein binds to its own transcription initiation site (13).

Conclusions. We have identified the major serum-responsive transcription initiation site of the *Krox-20* gene and have shown that transcripts from this site were also greatly increased after the addition of cycloheximide to quiescent cells. In logarithmically growing cells, transcripts from the +1 serum-inducible initiation site were also increased with cycloheximide, although to a much lower extent than in quiescent cells. This result could be due to a modest increase in *Krox-20* transcripts from cells in all stages of the logarithmic cell cycle or, alternatively, could be due to a large increase from a small percentage of cells that have entered a quiescent G_0 state. Interestingly, *JE*, one of the genes that shares transcription initiation site homology with the *Krox-20* gene, can only be induced by serum or platelet-derived

growth factor when cells are in a quiescent G_0 state (14). Experiments designed to determine whether *Krox-20* can only be induced in quiescent cells are in progress.

We thank Vikas Sukhatme for allowing us to refer to data prior to publication and Richard Kollmar for assistance with flow cytometry.

This work was funded by Public Health Service grants CA23076 and CA07175 from the National Institutes of Health. J.C. was partially supported by Public Health Service training grant CA09135 from the National Institutes of Health.

LITERATURE CITED

1. **Almendral, J. M., D. Sommer, H. MacDonald-Bravo, J. Burckhardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.
2. **Chavrier, P., U. Janssen-Timmen, M.-G. Mattei, M. Zerial, R. Bravo, and P. Charnay.** 1989. Structure, chromosome location, and expression of the mouse zinc finger gene *Krox-20*: multiple gene products and coregulation with the proto-oncogene c-*fos*. Mol. Cell. Biol. **9**:787–797.
3. **Chavrier, P., P. Lemaire, O. Revelant, R. Bravo, and P. Charnay.** 1988. Characterization of a mouse multigene family that encodes zinc finger structures. Mol. Cell. Biol. **8**:1319–1326.
4. **Chavrier, P., M. Zerial, P. Lemaire, J. Almendral, R. Bravo, and P. Charnay.** 1988. A gene encoding a protein with zinc fingers is activated during G_0/G_1 transition in cultured cells. EMBO J. **7**:29–35.
5. **Evans, R. M., and S. M. Hollenberg.** 1988. Zinc fingers: gilt by association. Cell **52**:1–3.
6. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
7. **Fried, J., A. G. Perez, and B. D. Clarkson.** 1976. Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. J. Cell. Biol. **71**:172–181.
8. **Janssen-Timmen, U., P. Lemaire, M.-G. Mattei, O. Revelant, and P. Charnay.** 1989. Structure, chromosome mapping and regulation of the mouse zinc-finger gene Krox-24; evidence for a common regulatory pathway for immediate-early serum-response genes. Gene **80**:325–336.
9. **Kabnick, K. S., and D. E. Housman.** 1988. Determinants that contribute to cytoplasmic stability of human c-*fos* and β-globin mRNAs are located at several sites in each mRNA. Mol. Cell. Biol. **8**:3244–3250.
10. **Lemaire, P., O. Revelant, R. Bravo, and P. Charnay.** 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA **85**:4691–4695.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Means, A. L., and P. J. Farnham.** 1990. Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. Mol. Cell. Biol. **10**:653–661.
13. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. **61**:858–865.
14. **Rollins, B. J., E. D. Morrison, and C. D. Stiles.** 1987. A cell-cycle constraint on the regulation of gene expression by platelet-derived growth factor. Science **238**:1269–1271.
15. **Rollins, B. J., E. D. Morrison, and C. D. Stiles.** 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. Proc. Natl. Acad. Sci. USA **85**:3738–3742.
16. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57**:103–113.
17. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-*fos* gene to serum factors. Cell **46**:567–574.
18. **Tsai-Morris, C.-H., X. Cao, and V. P. Sukhatme.** 1988. 5′-Flanking sequence and genomic structure of Egr-1, a murine mitogen-inducible zinc finger-encoding gene. Nucleic Acids Res. **16**:8835–8846.
19. **Villasante, A., D. Wang, P. Dobner, P. Dolph, S. A. Lewis, and N. J. Cowan.** 1986. Six mouse α-tubulin mRNAs encode five distinct isotypes: testis-specific expression of two sister genes. Mol. Cell. Biol. **6**:2409–2419.