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Identification of an Early-Growth-Response Gene Encoding a Novel Putative Protein Kinase

DANIEL L. SIMMONS,[1]* BENJAMIN G. NEEL,[2] RYAN STEVENS,[1] GARY EVETT,[1] AND RAYMOND L. ERIKSON[3]

Department of Chemistry, 226 Eyring Science Center, Brigham Young University, Provo, Utah 84602[1]; Molecular Medicine Unit, Beth Israel Hospital, Boston, Massachusetts 02215[2]; and Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138[3]

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Early-growth-response genes, also known as immediate-early genes, play important roles in regulating cell proliferation. We have identified a new type of early-growth-response gene product, a 77,811-Da putative serine/threonine kinase, which is highly inducible by serum and phorbol ester. mRNA encoding this putative kinase is markedly elevated within 1 h after treatment with mitogen, and this induction is synergistically increased by cycloheximide. Dexamethasone blocks serum induction of the kinase mRNA, as does transformation by v-Ki-*ras*. The kinase mRNA was detected in mouse brain, lung, and heart. This new putative kinase, which we term Snk, for serum-inducible kinase, showed similarity in its proposed catalytic domain to many other protein kinases; however, no other kinase showed enough sequence similarity with Snk to suggest the existence of a common function. Hence, Snk represents a new type of protein kinase involved in the early mitogenic response whose activity is transcriptionally and posttranscriptionally regulated.

More than 80 genes called early-growth-response or immediate-early genes are now known to be activated by a wide variety of mitogens (2, 12, 20, 22, 32). The identities of many of these mitogen-inducible genes, such as those encoding growth factors, transcription factors/DNA-binding proteins, and cytoskeletal proteins, have been determined by DNA cloning and sequencing (15). A newly discovered isoenzyme of prostaglandin G/H synthase, which catalyzes the rate-limiting step in the production of prostaglandins, is also induced in an early-growth-response fashion by many mitogens, including the v-*src* oncogene product $pp60^{v\text{-}src}$ (10, 19, 26, 33).

Signal transduction following exposure of cells to mitogens requires messenger networks to transmit growth regulatory signals to target sites within the cell. Key components in many of these signal transduction networks are kinases, which, through phosphorylation, modulate the activity of other kinases, structural proteins, transcription factors, and small molecular messengers. An end result of many mitogen-stimulated signal transduction pathways is a rapid increase in the transcription of selected early-growth-response genes. Typically, this effect on transcription occurs within minutes of initiation of signal transduction and transpires in the absence of protein synthesis.

Here we report the identification and characterization of a new type of mitogen-inducible early-growth-response gene product, which, like the prostaglandin G/H synthase, is an enzyme, in this case a putative new protein kinase, apparently of the serine/threonine type.

MATERIALS AND METHODS

Cell culture. The NIH 3T3 cell line used in these studies and its derivative lines, F-2 and DT, were obtained from Robert Bassin, National Institutes of Health. The DT line was originally created by doubly transforming NIH 3T3 cells with two copies of the v-Ki-*ras* oncogene (25). The F-2 cell line arose as a nontumorigenic, genetically dominant morphological revertant of DT that still contained markedly elevated v-Ki-*ras* expression (25).

Another cell line used in these studies, RS2, is a transformed rat fibroblast line obtained from Mark Smith, National Cancer Institute, Frederick, Md. RS2 is a v-*fos*-transformed rat fibroblast line originating from Tom Curran's laboratory (9).

Mitogen stimulation was performed on 5×10^6 cells per 100-mm-diameter dish cultured for 16 to 24 h under conditions of serum deprivation (Dulbecco's modified Eagle's medium [DMEM] containing 0.9% fetal calf serum [FCS]) to inhibit cell proliferation. The cells were stimulated to divide by addition of FCS to 10% concentration or phorbol 12-myristate 13-acetate (PMA) to a final concentration of 75 μM. In some cases, serum or PMA was added simultaneously with 75 μM cycloheximide (CHX; Sigma Chemical Co., St. Louis, Mo.) to determine the influence of protein translation on induction of clone 2 mRNA. The effect of 2 μM dexamethasone (DEX; Sigma) on transcription and mRNA levels was evaluated either by adding the DEX with serum or by pretreating the cells for up to 2 h with DEX prior to addition of serum. Pretreatment of the cells with DEX appeared to be equal in effect to simultaneous addition of the DEX with serum in inhibiting induction of clone 2 mRNA.

RNA isolation. RNA for gel blot analysis, library construction, and screening was obtained from cells by lithium chloride precipitation (3). Poly(A) mRNA used for library construction was prepared by two cycles of oligo(dT) chromatography (4). RNA from mouse tissues was prepared by homogenizing whole organs in guanidinium isothiocyanate as described previously (7).

Library construction and screening. A cDNA library was constructed in λZAP, using mRNA isolated from F-2 cells to synthesize the cDNA. The library ($>10^6$ recombinants per μg of cDNA) was constructed with a kit made by Gibco BRL (Gaithersburg, Md.).

Clone 2 was originally isolated as a sequence artifactually ligated to another cDNA. The clone 2 chimera was obtained

* Corresponding author.

during screening of the F-2 cDNA library for full-length copies of partial-length cDNAs under characterization in our laboratory. Before the artifactual nature of the clone 2 chimera was determined, this spurious ligation product was used to probe a Northern (RNA) blot of RNAs isolated from mitogen-stimulated cells. This experiment detected a new mRNA which was strongly induced in serum-stimulated F-2 cells. The portion of the chimera responsible for hybridizing strongly to the serum-inducible mRNA was identified by restriction endonuclease mapping and DNA sequencing and was used to isolate several independent, nonchimeric isolates of clone 2 cDNA from approximately 10^5 recombinants.

Northern analysis. RNA was electrophoresed on denaturing formaldehyde gels, blotted to nylon, and probed (24). cDNA inserts, isolated by electrophoresis in low-melting-point agarose gels, were radiolabeled and hybridized for 12 to 24 h to the nylon filters at 65°C in Church-Gilbert buffer (8). Hybridized filters were washed at 65°C in a solution containing 0.2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate.

DNA sequencing and computer analysis. Two separate recombinant phage isolates containing the entire coding and 3′ untranslated regions of clone 2 cDNA were identified. Both of these cDNAs were 2.8 kb in size. One of the two isolates, termed clone 2-full, was sequenced on both strands in its entirety by the dideoxy chain termination method of Sanger et al. (29).

Homology searches at the nucleic acid and protein levels of the GenBank and National Biomedical Research Foundation data bases were done by using both the Intelligenetics (FASTA and FASTP) and the University of Wisconsin Genetics Computer Group (WORDSEARCH and BESTFIT) programs. The BLASTN and BLASTP programs of the National Center for Biotechnology Information were also used to do global searches for sequence similarity.

Transcription analyses. Isolated nuclei for transcription analyses were obtained and used to measure transcription in run-on assays as described by Linial et al. (23). Nitrocellulose (Schleicher & Schuell Inc., Keene, N.H.) strips containing filter-bound plasmids were prepared as described previously (31). Hybridization of 2.8×10^6 cpm/ml of radiolabeled nascent transcripts to filter-bound plasmids was done in Church-Gilbert buffer at 65°C for 40 h, and the filters were washed as described previously (31).

Nucleotide sequence accession number. The GenBank accession number for clone 2 is M96163.

RESULTS

Mitogen induction of clone 2 mRNA. Clone 2 was first isolated as a mitogen-inducible sequence spuriously ligated to another presumptive growth control cDNA studied in our laboratory (see Materials and Methods). Both serum and the tumor-promoting phorbol ester PMA were found to induce clone 2 mRNA in an early-growth-response fashion (Fig. 1 and 2). Nonproliferating, serum-deprived cells expressed low levels of clone 2 mRNA, which were elevated 10- to 20-fold within 1 h of addition of serum (Fig. 1) or PMA (Fig. 2). Mitogen addition in the presence of CHX, a translation inhibitor, not only allowed the mitogen induction of clone 2 mRNA to occur but also potentiated it (Fig. 1 and 2). This synergistic action of CHX and mitogens has been described for many early-growth-response genes and is thought to result, in some cases, from increased mRNA stability. The

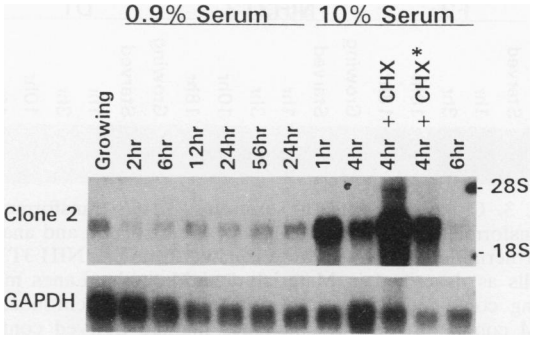

FIG. 1. Serum stimulation of clone 2 mRNA in NIH 3T3 cells. Total RNA from NIH 3T3 cells (10 μg per lane) was electrophoresed on a denaturing agarose gel, blotted, and hybridized to either radiolabeled clone 2 cDNA or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA, as indicated. 0.9% Serum, RNA from cells cultured in DMEM supplemented with 0.9% FCS for the times shown; 10% Serum, RNA from cells grown in 0.9% serum for 24 h that had been stimulated with 10% serum for the times indicated. The third lane from the right contains RNA from cells stimulated for 4 h in the presence of both serum and CHX, and the second lane from the right contains RNA from cells treated with CHX in 0.9% serum for 4 h (denoted CHX*). The leftmost lane contains RNA isolated from cells growing asynchronously in DMEM containing 10% serum.

induction of clone 2 mRNA by serum and phorbol ester was transient and decreased to basal levels within 6 h following mitogen addition, a pattern characteristic of early-growth-response transcripts.

Mitogen induction of clone 2 mRNA was also identified in other fibroblast cell lines such as F-2 cells, which are derived from NIH 3T3 cells, and RS2 cells, which are rat fibroblasts. In contrast, DT cells, which are NIH 3T3 cells transformed by v-Ki-*ras*, were noninducible by serum (Fig. 3). Noninducibility by serum in v-Ki-*ras*-transformed cells has been reported for other important early-growth-response genes such as c-*fos* and c-*myc* (34).

DNA sequence analysis and homology comparisons. DNA sequencing performed on the 2,772-bp clone 2-full cDNA revealed a 2,046 bp open reading frame extending from nucleotides 100 to 2146 of the cDNA clone. Furthermore, the portion of the cDNA sequence corresponding to the 3′ untranslated region of clone 2 mRNA contained two ATTTA

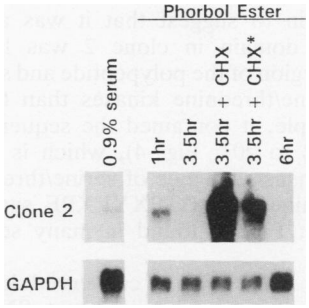

FIG. 2. Phorbol ester stimulation of clone 2 in NIH 3T3 cells. Cells were cultured as for Fig. 1. Mitogen stimulation was done with 75 μM PMA for the times indicated. CHX plus PMA or CHX alone (designated CHX*) was also added to the cells as described in the legend to Fig. 1. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

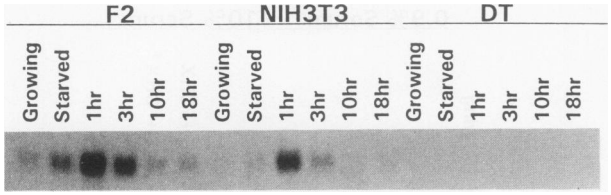

FIG. 3. Clone 2 response to serum in v-Ki-*ras*-transformed and nontransformed NIH 3T3 cells. RNAs were isolated and analyzed by Northern blotting. RNAs were isolated from F-2, NIH 3T3, and DT cells as described in Materials and Methods. Lanes marked Growing contained RNA from cells growing asynchronously in DMEM containing 10% serum; lanes marked Starved contained RNA from cells cultured in DMEM containing 0.9% serum for 24 h. All other lanes contain RNAs isolated from serum-deprived cells stimulated with 10% FCS for the times indicated.

sequences located in A+T-rich domains. The AUUUA sequence is found repeated in the 3′ untranslated region of many early-growth-response mRNAs and is thought to promote transcript instability (30).

The open reading frame of the cDNA predicted a 77,811-Da polypeptide of 682 amino acids (Fig. 4). Comparison of the predicted protein, and to a lesser degree the cDNA nucleic acid sequence, with entries in GenBank and other data bases (see Materials and Methods) detected significant similarity between clone 2 and over 30 reported kinase sequences. In each case, the region of sequence similarity was largely restricted to the ATP-binding/kinase domains of the catalytic site. Clone 2 exhibited segmental regions of high conservation interspersed with regions of low conservation in its kinase domain, which is similar to what has been reported for other kinases. The 11 conserved subdomains identified by Hanks et al. (13, 14) were well conserved in clone 2; however, the consensus Gly-X-Gly-X-X-Gly sequence found in many nucleotide-binding proteins was present as Gly-X-Gly-X-X-Ala (amino acids 86 to 92) in clone 2. Substitution at the third glycine in this consensus sequence is also seen in Kin 28 and other kinases (14).

The programs used to perform the sequence comparisons gave slightly different scores to kinases possessing similarity to clone 2 protein. Five kinases, which consistently received the highest homology scores in the various computer analyses, were compared in their kinase domains with clone 2 (Fig. 5). This comparison showed that although clone 2 protein diverged significantly from all other kinases compared, it clearly contained sufficient conserved residues in the kinase domain to suggest that it was a kinase. This apparent kinase domain in clone 2 was located in the amino-terminal region of the polypeptide and showed greater similarity to serine/threonine kinases than to tyrosine kinases. For example, it contained the sequence DLKLGN (amino acids 202 to 207; Fig. 4), which is similar to the DLKPXN consensus sequence of serine/threonine kinases, and it also contained the GTPXYLXPE sequence (amino acids 239 to 247; Fig. 4) found in many serine/threonine kinases (13, 14).

In addition to possessing a conserved kinase domain, clone 2 protein also shared with yeast SNF1 kinase an unusual group of 7 histidines located immediately upstream of the kinase domain (Fig. 5). In SNF1 kinase, this polyhistidine tract is longer than that seen in clone 2, containing 14 histidines as opposed to 7 in clone 2 (5). This group of histidines is not evolutionarily conserved in the SNF1 kinase

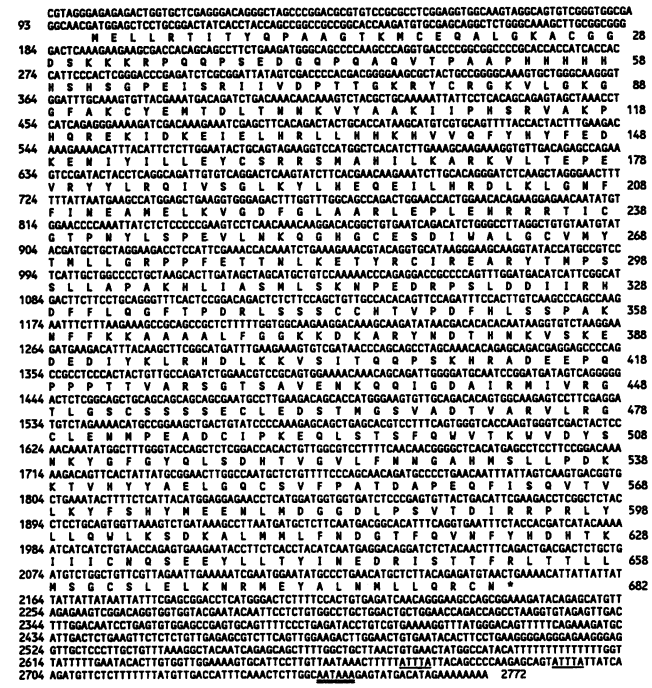

FIG. 4. Nucleotide and predicted amino acid sequence of clone 2. Underlined sequences are copies of a conserved motif shown by Shaw and Kamen (30) to confer message instability and which are found in the 3′ untranslated regions of many mitogen-induced, early-growth-response mRNAs. A consensus polyadenylation signal is doubly underlined.

and is dispensable with regard to SNF1's activity in regulating glucose-repressible genes (1, 6).

We refer to the clone 2-encoded protein as Snk, for serum-inducible kinase, because of its similarity to known kinases and its rapid induction by serum.

Glucocorticoid modulation of clone 2 mRNA. The synthetic glucocorticoid DEX has been shown to repress the mitogen-stimulated induction of a small subset of early- and late-growth-response genes. These genes include the collagenase, mitogen-inducible prostaglandin G/H synthase, interferon consensus sequence-binding protein, and JE (a mitogen-induced secretory factor) genes (16, 18, 26–28).

Northern blots showed that DEX also strongly repressed the mitogen induction of clone 2 mRNA in NIH 3T3 (data not shown) and RS2 (Fig. 6) cells. DEX repressed only the mitogen-induced level of clone 2 mRNA and did not repress basal level expression (data not shown).

Transcriptional analysis of the clone 2 gene. Nuclear run-on assays were performed to determine whether induction of clone 2 mRNA by serum was transcriptional and whether DEX repressed this induction. Serum initiated a rapid five-fold increase in clone 2 transcription that peaked 30 min after the addition of mitogen. This induction occurred later and was smaller than the induction of two other early-growth-response genes, c-*fos* and MEF-10, whose transcription experienced a very strong induction that reached a maximum 15 min after addition of serum (Fig. 7A).

DEX completely repressed the transcriptional increase in clone 2 mRNA initiated by serum but had no effect on the transcriptional induction of either c-*fos* or MEF-10 mRNA (Fig. 7B).

Tissue-specific expression of clone 2 mRNA. Tissues from

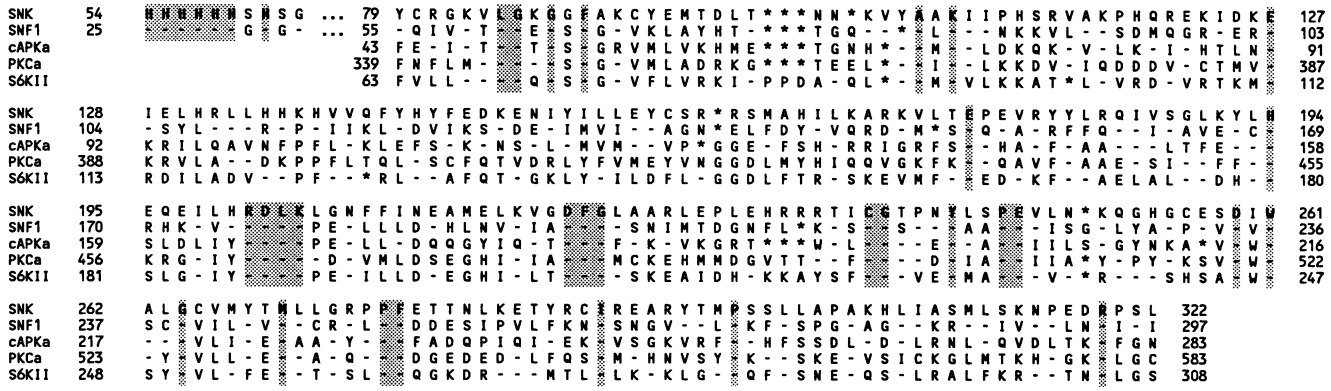

FIG. 5. Homology comparison of clone 2 protein with related kinases. Kinases with high homology scores on computerized homology searches are compared in their kinase domains. Dashes represent amino acids that match the corresponding amino acid in the clone 2-encoded protein Snk. Asterisks represent gaps. Shaded areas highlight the unusual polyhistidine tract that Snk shares with SNF1 kinase as well as amino acids conserved in all five kinases. SNK, serum-inducible kinase; SNF1, SNF1 kinase from *Saccharomyces cerevisiae*; cAPKa, bovine cyclic AMP-dependent kinase alpha; PKCa, protein kinase C alpha; S6KII, ribosomal S6 kinase from *Xenopus laevis* (14, 17).

an adult outbred CD-1 male mouse were assayed for expression of clone 2 mRNA. Clone 2 mRNA was detected at relatively low levels in brain, lung, and heart and was undetectable in all other tissues tested (Fig. 8).

DISCUSSION

Early-growth-response genes have been hypothesized to play critical roles as amplifiers of the mitogenic signal. According to this model, their ultimate effect is to produce proteins capable of regulating the expression of other growth-regulated genes, either by binding their promoters in the case of transcription factors or by initiating secondary signal transduction events in the case of cytokines and prostaglandins (15).

Snk is the first kinase to be shown to be induced in an early-growth-response fashion by mitogens and, as far as we are aware, is only the second protein kinase shown to be inducible at the mRNA level by growth-stimulating agents. The other inducible kinase is the *cdc-2* kinase which is induced in fibroblasts and T lymphocytes by mitogens (11, 21). However, in the case of *cdc-2* kinase, the induction occurs at late G_1 and early S phases of the cell cycle rather than at the G_0/G_1 transition as is the case for Snk. The mechanistic significance of this inducibility to the function of Snk remains to be determined. In contrast to this transcriptional regulation, the majority of protein kinases are instead activated or deactivated by reversible phosphorylation or by second messengers.

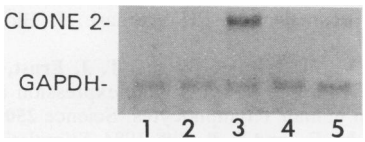

FIG. 6. Suppression of serum-induced clone 2 mRNA by DEX. RNAs were isolated and analyzed by Northern blotting as described in Fig. 1. RNAs were isolated from RS2 cells. Lanes: 1, cells were grown in 0.9% serum for 24 h; 2, cells were grown in 0.9% serum and then treated for 1 h with 2 μM DEX; 3 to 5, cells were grown in 0.9% serum for 24 h and then exposed to 10% FCS for 1 h in the absence (lane 3) or presence (lanes 4 and 5) of DEX; 4 and 5, DEX was added to the cells 1 and 2 h, respectively, before stimulation with serum. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The inhibition of induction by DEX places this gene within a small subset of growth-regulated genes that are suppressed by glucocorticoids. This subset includes the collagenase (16), JE (28), interferon consensus sequence-binding protein (27), and mitogen-inducible prostaglandin G/H synthase (18, 26) genes. Although DEX inhibits the mitogen-inducible expression of each protein, it does so by diverse mechanisms. Collagenase is transcriptionally down-regulated by DEX in phorbol ester-treated cells. The mechanism of down-regulation has been proposed to occur by binding of the Fos/Jun complex to a glucocorticoid receptor, thus preventing binding of the complex to the AP-1 site in the promoter region of the gene (16). In contrast, down-regulation of the prostaglandin G/H synthase mRNA by DEX is nontranscriptional and occurs by specific destabilization of the mRNA (unpublished data). JE, an early-growth-response chemotactic secretory protein, has been reported to be transcriptionally down-regulated by DEX in fibroblasts and nontranscriptionally down-regulated by DEX in smooth muscle cells (28).

The oncogene v-Ki-*ras* also suppresses the serum inducibility of clone 2 mRNA (Fig. 3). Experiments done by Zullo and Faller (34) have shown that this oncogene additionally blocks the serum induction of c-*myc* and c-*fos* expression. The inhibition occurs by blocking the platelet-derived growth factor (PDGF) mitogen signal, as shown by the fact that purified PDGF, the major growth factor in serum, also fails to induce these mRNAs. The block in the PDGF signalling network occurs at some point after binding of ligand, since v-*ras*-transformed cell PDGF receptors show normal numbers and binding capacity. Mitogens such as fibroblast growth factor, which act through other signalling pathways, induce c-*fos* and c-*myc* normally in v-Ki-*ras*-transformed cells. Our studies show that the dominant genetic mutation(s) suppressing the transformed phenotype in F-2 cells also relieves the block in serum inducibility of clone 2 mRNA. This finding suggests that the proteins encoded by the suppressing loci may provide an alternate pathway that avoids the v-Ki-*ras* block in the PDGF signal pathway.

Preliminary data indicate that the clone 2 gene product (Snk) possesses kinase activity and is capable of both autophosphorylation and phosphorylation of heterologous substrates (23a). Homology comparisons failed to clearly

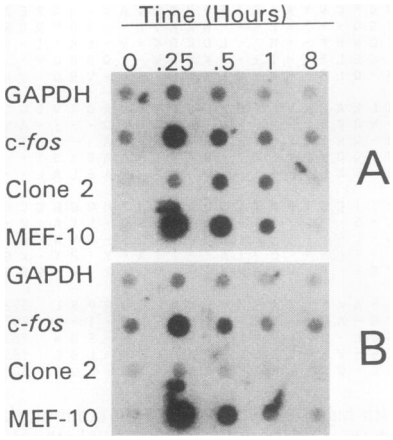

FIG. 7. Nuclear run-on analysis of clone 2 gene expression. Nuclear run-on assays were performed as described in Materials and Methods. Filter-bound plasmids, to which radiolabeled nascent transcripts were hybridized, contained cDNAs encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the proto-oncogene c-*fos*, clone 2, and the murine homolog of the cysteine-rich secretory protein CEF-10, which we call MEF-10. MEF-10 and c-*fos* encode known early-growth-response gene products and served as positive controls in this assay (12, 32). (A) Stimulation with 10% FCS; (B) stimulation with 10% FCS and 2 μM DEX.

place Snk into any of the kinase subfamilies proposed by Hanks et al. (14). The Snk kinase domain is clearly more similar to those of serine/threonine kinases than to those of tyrosine kinases. The sequence similarity shown by Snk to the polyhistidine region and catalytic domain of SNF1 kinase suggested that these proteins could be homologs; however, a plant homolog of yeast SNF1, called RKIN1, recently has been identified in rye and shows 86% sequence identity in the core kinase domain, compared with approximately 40% identity shown by Snk in this region. Furthermore, the plant SNF1 homolog completely lacks the unusual histidine tract seen in Snk yet functionally complements SNF1. Taken together, these data suggest that Snk is not the mammalian homolog of SNF1 or any other protein kinase thus far described.

We hypothesize that like other early-growth-response gene products, Snk plays a role in amplifying the mitogenic signal. However, an intriguing question remains as to why this kinase and the *cdc-2* kinase are at least partially regulated by mRNA induction rather than being modulated solely

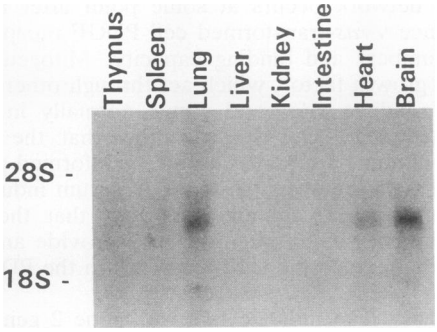

FIG. 8. Clone 2 mRNA expression in murine tissues. Tissues from an adult CD-1 mouse were used for RNA isolation. Ten micrograms of total RNA from each tissue was assayed.

by phosphorylation-dephosphorylation or second messengers such as other kinases. In the simplest sense, the induction of Snk may serve to increase its concentration during the early G_1 stage of the cell cycle. Alternatively, inducibility may serve to keep the enzyme at low levels during other periods of the cell cycle when its expression would be deleterious. Regardless, the tightly linked expression of Snk with mitogenesis suggests that this protein plays a role in the division of at least some cell types, such as fibroblasts, and could function in embryogenesis, wound healing, or neoplasia.

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