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Isolation and Characterization of a Novel Mitogenic Regulatory Gene, *322*, Which Is Transcriptionally Suppressed in Cells Transformed by *src* and *ras*

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In an attempt to isolate novel regulatory and/or tumor suppressor genes, we identified cDNAs whose abundance is low in NIH 3T3 cells and further decreased following the expression of the activated oncogene, v-*src*. The transcription of one such gene, *322*, is suppressed at least 15-fold in *src*-, *ras*-, and *fos*-transformed cells and 3-fold in *myc*-transformed cells but is unaffected in *raf*-, *mos*-, or *neu*-transformed cells. Activation of a *ts*-v-*src* allele in confluent 3Y1 fibroblasts resulted in an initial increase in *322* mRNA levels after 1 to 2 h followed by a rapid decrease to suppressed levels after 4 to 8 h. Morphological transformation was not detected until 12 h later, indicating that the accumulation of *322* transcripts is regulated by v-*src* and not as a consequence of transformation. Addition of fetal calf serum to starved subconfluent NIH 3T3 or 3Y1 fibroblasts resulted in a similar biphasic regulation of *322*, indicating that *322* transcription is responsive to mitogenic factors. Sequence analysis of a putative full-length *322* cDNA clone (5.4 kb) identified a large open reading frame (ORF) encoding a 148.1-kDa product. In vitro transcription and translation of the *322* cDNA from a T7 promoter resulted in a 207-kDa product whose electrophoretic mobility on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel was unaffected by digestion with endoglycosidase F. The discrepancy in predicted versus measured molecular weights may result from the high percentage of acidic residues (roughly 20% Glu or Asp) in the *322* ORF product. Comparison of the *322* cDNA ORF with sequences in data banks indicates that this gene is novel. The *322* ORF product contains a potential Cys-1–His-3 Zn finger, at least five nuclear localization signals of the adenovirus E1a motif K(R/K)X(R/K), and alternating acidic and basic domains. Overexpression of the *322* cDNA from retroviral vectors resulted in significantly decreased cell proliferation rates compared with those of controls in both untransformed and *src*-transformed NIH 3T3 cells. Continued passage of the *322* cells resulted in the selection of rapidly growing cells which had lost the transduced *322* cDNA. Thus, *322* represents a novel *src*- and *ras*-regulated gene which encodes a potential regulator of mitogenesis and/or tumor suppressor.

The inactivation of several tumor suppressor gene families (e.g., those encoding p53, Rb, and APC) as a result of mutation is acknowledged to contribute to the oncogenicity of several types of human cancers (21). Many of these so-called class I tumor suppressor genes (20) were identified and isolated following cumbersome pedigree and cytogenetic analyses (29). Recently, another class of genes (class II) whose expression is known to be down-regulated in tumor cells has been shown by gene transfer techniques to encode potential tumor suppressors. These include nonmuscle α-actinin, tropomyosin I, CLP, retinoic acid receptor β, and interferon regulatory factor 1 (14, 17, 18, 23, 26). Additional tumor suppressor gene families, such as the maspin gene, *rrg*, and *N03* (4, 25, 34), were isolated by subtractive hybridization techniques designed to identify down-regulated genes. These findings indicate a widening definition of tumor suppressor genes and strongly suggest that additional suppressor genes could be identified in gene populations whose expression is down-regulated in response to oncogenic stimuli such as activation of oncogenes. The ability of these gene families to reverse an array of oncogenic phenotypes following gene transfer and overexpression supports the possibility for novel therapeutic modalities for cancer.

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We previously reported using a novel PCR-based subtractive hybridization method to generate expressed sequence tags (EST) representing genes expressed at low basal levels in NIH 3T3 cells and at suppressed levels in v-*src*-transformed NIH 3T3 cells (10). We envisioned that the derived EST might contain potential mitogenic regulators or tumor suppressors. Of the nine distinct cDNAs identified in this manner, four were identical or highly similar to known genes, although none had been previously shown to be down-regulated by v-*src*. These known genes include genes for helix-destabilizing protein A1 (hnRNP A1), CTLA-2α cysteine protease, and cytochrome *c* oxidase VIc subunit and *gravin*. An additional EST was highly homologous to a randomly cloned human cDNA (clone A7C09; GenBank accession no. Z25236). Four other EST were not similar to published sequences.

In this study, we have characterized one such EST, initially identified as clone 3.2.2, whose steady-state level of mRNA expression in NIH/v-*src* cells is >15-fold less than that in NIH 3T3 controls. An initial GenBank search showed a significant homology to the 3′ untranslated region (UTR) of human *gravin* (15). In order to characterize the identity and function of the 3.2.2 gene, full-length cDNAs were isolated from a rat 3T3/λZAPII library. Sequence analysis of the putative open reading frame (ORF) indicates that this gene has only limited homology to *gravin* and to other known genes. Thus, on the basis of its novelty, we have named this gene *322*. Our initial

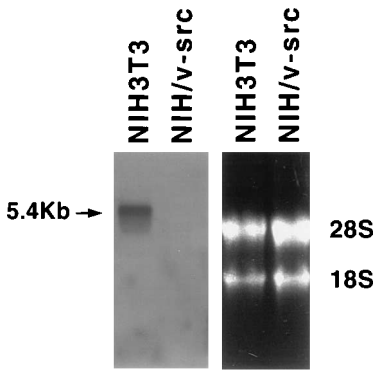

FIG. 1. Northern blot analysis of *322* RNA levels in NIH 3T3 versus NIH/v-*src* cells. A 30-μg amount of total RNA purified by the RNAzol method from NIH 3T3 or NIH/v-*src* cells was electrophoresed through a 1% agarose–formaldehyde gel, blotted onto Immobilon N membrane, hybridized with a ^{32}P-labeled *322* cDNA insert (clone 13.2.2; 5.4 kb), washed, and autoradiographed (for 3 weeks) as described in Materials and Methods. The amount of RNA loaded was normalized by densitometric analysis of the 28S and 18S rRNA bands (right panel).

findings suggest a role for the *322* product as a potential regulator of mitogenesis and a tumor suppressor.

MATERIALS AND METHODS

Cells. NIH 3T3 (ATCC CRL1658) and NIH/v-*src* (11) cells were grown in Dulbecco's modified Eagle's media (DEM) containing 10% heat-inactivated calf serum (GIBCO) and 1× penicillin, streptomycin, and amphotericin B (Fungizone) (GIBCO). Rat-6 fibroblasts, Rat-6/*src*, Rat-6/*ras*, Rat-6/*myc*, Rat-6/*neu*, Rat-6/*raf*, Rat-6/*mos*, and Rat-6/*fos* (gifts of R. Krauss, Mount Sinai School of Medicine), described elsewhere (1), were grown as described above. 3Y1/*ts*72*src* cells were developed as follows. An *Acc*I-*Bgl*II fragment from pNY*ts*72 (22) containing a *ts*-v-*src* gene from the NY72 strain of Rous sarcoma virus was inserted into the retroviral vector pLJ (19), cut with *Sal*I and *Bam*HI. Transiently expressed, replication-defective virus was produced 40 h following transfection of Ψ_{CRE} packaging cells (5) with 10 μg of pLJ/*ts*72*src* plasmid DNA per 10^6 cells and then filtered through 0.22-μm-pore-size low-protein binding filters (Gelman Sciences). 3Y1 Fisher rat fibroblasts were infected overnight with virus, and then the culture was split 1:10 into media containing G418 (Geneticin; 250 μg/ml; GIBCO) and grown at 39.5°C (the nonpermissive temperature [NPT] for *ts*72*src*). After 2 weeks, colonies were picked and grown in duplicate cultures at 39.5 and 35°C (the permissive temperature [PT] for *ts*72*src*). Clones exhibiting the transformed morphology at the PT only were expanded and tested for in vitro v-*src* autophosphorylation activity (data not shown), as described elsewhere (11). On the basis of these analyses, clone 4 was chosen as typical.

Isolation of total and polyadenylated RNA. Total RNA was isolated directly from cells on culture plates or from tissues by the RNAzol B method (3). Poly(A)-containing RNA subfractions were isolated by using Dynabeads Oligo(dT)$_{25}$ (Dynal) according to the manufacturer's specifications.

Isolation of *322* cDNAs. Recombinant λZAPII phage (2×10^5) from an oligo(dT)-primed rat 3T3 library (R-1a; kindly provided by R. Krauss, Mount Sinai School of Medicine) in XL-1*blue* bacteria (Stratagene) were screened on Nytran filters by using a ^{32}P-labeled 3.2.2 cDNA insert under conditions of stringent hybridization, as described elsewhere (2). A total of 13 independent clones whose cDNA inserts cross-hybridized to each other in slot blots (data not shown) were plaque purified. Bacterial colonies containing lysogens were isolated, and double-stranded replicative forms containing pBluescript II KS vectors were generated following infection and lysis with ExAssist helper phage (Stratagene), transfection into competent XL-1*blue*, and selection on ampicillin plates. The smallest insert size was 1.2 kb (clone 4.5.2), whereas the largest—possibly representing full-length cDNA—was 5.2 kb (clone 13.2.2).

Northern (RNA) blot analysis. Total RNA (20 to 30 μg per lane) was electrophoresed through 1% agarose gels containing formaldehyde, blotted onto Immobilon N membrane, hybridized with Rapid Hybridization Buffer (Amersham), and washed as described elsewhere (30).

Southern blot analysis. Genomic DNA from NIH 3T3 or NIH/v-*src* cells was isolated as described elsewhere (30); cut to completion with *Eco*RI or *Hin*dIII; and then electrophoresed through a 0.7% agarose gel, blotted onto Immobilon N membrane, hybridized with Rapid Hybridization Buffer, and washed as described elsewhere (30). Zoo blots (species DNA) were loaded with *Eco*RI-digested species DNA, probed with ^{32}P-labeled cDNA from clone 13.2.2 (5.2 kb)

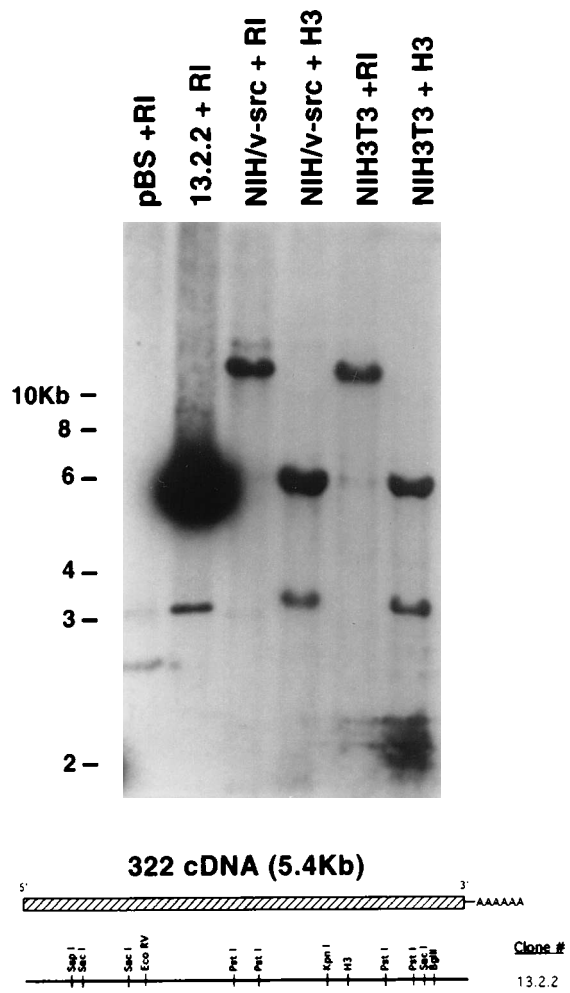

FIG. 2. The decreased level of *322* RNA in NIH/v-*src* cells is not due to gross deletion or translocation of the *322* allele. (Top) A 20-μg amount of genomic DNA from NIH 3T3 or NIH/v-*src* cells was digested to completion with *Eco*RI or *Hin*dIII, electrophoresed through a 0.7% agarose gel, and then blotted onto Immobilon N membrane. Fifty-picogram amounts of *Eco*RI-cut pBluescript II KS and 13.2.2 plasmid DNA were included as negative and positive hybridization controls, respectively. The blot was hybridized as described in the legend to Fig. 1 and autoradiographed for 2 days with an intensifying screen. DNA molecular size standards are shown on the left. RI, *Eco*RI; H3, *Hin*dIII. (Bottom) Restriction enzyme map of the full-length *322* cDNA, clone 13.2.2. isolated from a rat 3T3 library. Much of this restriction pattern, including the *Hin*dIII (H3) site, is shared by both the mouse and rat *322* homologs (data not shown). However, only the rat *322* allele contains an internal *Eco*RI site roughly 250 bp from the 3′ cDNA terminus (see Fig. 8). With the exception of rat clones 13.2.2 and 14.5.2, which contain small 3′ deletions including the *Eco*RI site, the remaining rat *322* cDNA clones contain this *Eco*RI site (data not shown).

at 65°C with Rapid Hybridization Buffer, and washed to allow for permissive hybridization (45°C for 30 min).

Sequence analysis. Double-stranded DNA templates were denatured and sequenced with Sequenase 2.0 kits (U.S. Biochemicals) by using [^{35}S]dATP and then subjected to electrophoresis through 6% polyacrylamide–urea gels and autoradiography. The 13.2.2 cDNA clone and deletion constructs obtained by using internal restriction sites were sequenced by using M13 universal forward or reverse primers or the following *322*-specific primers: 322-2 (bases 3879 to 3862), 322-3 (bases 3744 to 3727), 322-4 (bases 3332 to 3316), 322-5 (bases 3511 to 3546), 322-6 (bases 3210-3194), 322-7 (bases 2998 to 3014), 322-8 (bases 2231 to 2216), 322-9 (bases 2109 to 2093), 322-10 (bases 3364 to 3349), 322-11 (bases 1729 to 1714), 322-12 (bases 633 to 619), 322-13 (bases 183 to 199), 322-14 (bases 1477 to 1462), 322-15 (bases 554 to 569), 322-18 (bases 3457 to 3471), 322-19 (bases 1361 to 1346), 322-20 (bases 3311 to 3327), 322-21 (bases 3750 to 3762), 322-22 (bases 3576 to 3590), 322-23 (bases 3107 to 3093), 322-24 (bases 1013 to 1000), 322-25 (bases 3764 to 3778), 322-26 (bases 1945 to 1932), 322-27 (bases

FIG. 3. *322* cDNA sequence analysis. The cDNA insert from clone 13.2.2, representing putative full-length *322* cDNA, was sequenced fully by using Sequenase 2.0 kits (U.S. Biochemicals) and [^{35}S]dATP (NEN) as described in Materials and Methods. The sequencing reactions were performed on denatured double-stranded DNA templates with either universal forward or reverse primers or *322* sequence-specific primers (see Materials and Methods). Some internal cDNA sequences were derived from 13.2.2 constructs with deletions of internal restriction fragments such as *Bgl*II, *Pst*I, *Sac*I, *Eco*RV, and *Hin*dIII (Fig. 1). The largest ORF (bases 176 to 4213) was identified by using the TRANSLATE program from Genetics Computer Group (7). Glycine-rich domains in the putative N terminus are underlined. Potential nuclear localization signals fitting the motif K(R/K)X(R/K) are boxed. A potential Zn finger from bases 3211 to 3280 is in boldface type. Two potential polyadenylation signals (AATAAA) in the 3′ UTR are underlined.

3051 to 3038), 322-28 (bases 645 to 658), 322-29 (bases 433 to 419), 322-30 (bases 1850 to 1865), 322-31 (bases 1969 to 1955), 322-32 (bases 1725 to 1711), 322-34 (bases 2111 to 2124), 322-35 (bases 3080 to 3067), and 322-36 (bases 4635 to 4621). The complete 13.2.2 sequence was analyzed for DNA and protein homologies with sequences in GenBank and SwissProt, respectively, by using Genetics Computer Group programs such as FASTA. Further sequence comparisons were performed at the National Center for Biotechnology Information by using the BLAST algorithm.

In vitro transcription-translation analysis. A 1-μg amount of 13.2.2 plasmid DNA, driven by the T7 promoter in pBluescript II KS, was analyzed in a coupled transcription-translation assay (TNT kit; Promega) containing [^{35}S]methionine (translation grade; NEN). The products were electrophoresed through sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, and the gels were then fixed in methanol-acetic acid, immersed in AMPLIFY (Amersham), and analyzed by fluorography.

Construction of *322* overexpressor cell lines. The 13.2.2 cDNA was excised with *Eco*RI and then inserted into the replication-defective retroviral vector pBABE*hygro* (24) in two orientations, resulting in clones S1 (sense) and R2 (reverse; antisense). A total of 10^6 Ω_e packaging cells (24) (seeded the previous day) were transfected via $CaPO_4$ precipitates containing 10 μg of S1, R2, or pBABE*hygro*, and transiently expressed virus was harvested 40 h later. Target cells (10^4) were infected for 2 h with 1 ml of filtered (0.22-μm pore size) virus supplemented with 8 μg of Polybrene (Sigma) per ml. The cultures were split 1:10 the next day and grown in media plus hygromycin (300 μg/ml; Sigma). Individual Hyg^r colonies or mixed sets of colonies were harvested after 2 weeks, expanded, and assayed for proliferation rates.

Proliferation rates. Aliquots of 10^4 cells were seeded into 35-mm-diameter culture dishes in media containing either a high (10%) or a low (0.5%) concentration of heat-inactivated fetal calf serum (fCS) (HYLINE). Duplicate plates were trypsinized every 2 days, and cells were counted three times for each plate by using a hemacytometer (Fisher).

Nucleotide sequence accession number. The GenBank accession number for the sequence described in this paper is U23146.

RESULTS

Of the nine distinct cDNAs isolated in an attempt to identify genes down-regulated by v-*src* (10), four were not significantly homologous to any other gene listed in GenBank. Clone 3.2.2 hybridized to a 5.4-kb message whose abundance in NIH/v-*src* cells is depressed >15-fold in comparison with levels in NIH 3T3 controls grown under the same conditions (Fig. 1). This phenomenon is not particular to NIH 3T3 cells or specifically selected clones, as the down-regulation is also manifest in 3Y1 and Rat-6 cells (see Fig. 4 and 5). Because of the novelty of this

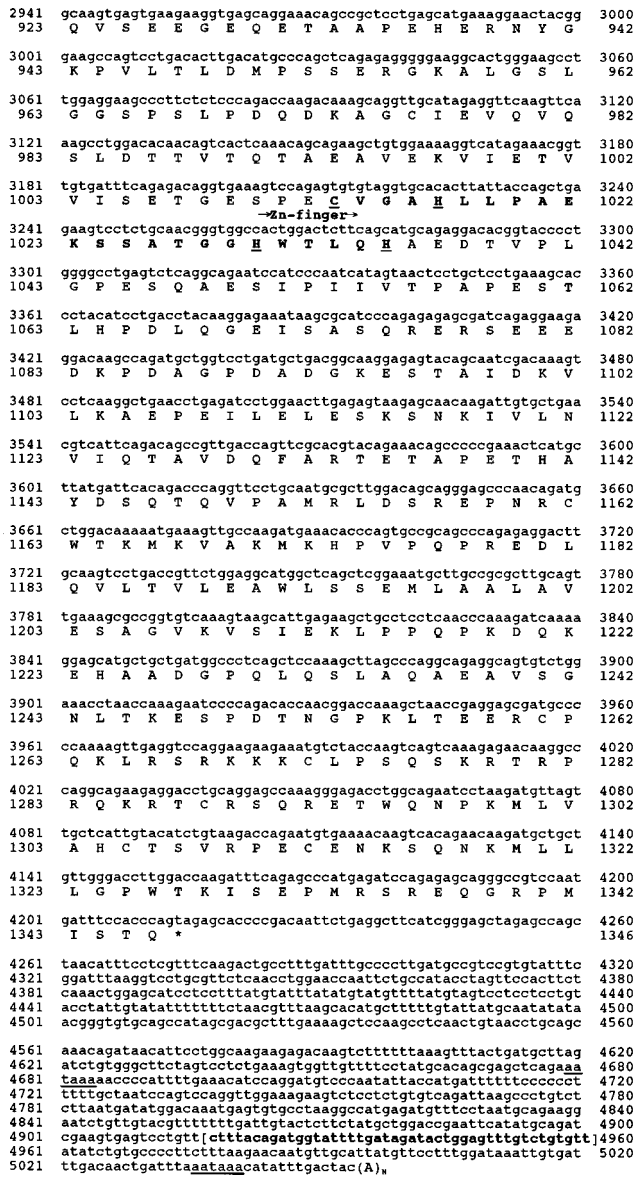

FIG. 3—*Continued.*

gene (see below), we will refer to it as *322*. Most importantly, the basal steady-state level of *322* RNA in NIH 3T3 cells is roughly 10- to 15-fold less than that of the abundantly expressed type I collagen (data not shown), which is also downregulated by v-*src*. These data also indicate that the original 3.2.2 cDNA insert (mouse) recognizes mouse and rat *322* sequences equally well.

Southern blotting. It is possible that the loss of *322* message results from deletions in the *322* gene locus. Southern blot analysis of *Eco*RI- and *Hin*dIII-digested genomic DNA from NIH 3T3 and NIH/v-*src* cells (Fig. 2) indicates that no gross alteration to the *322* allele has occurred following the stable expression of v-*src* from a retroviral vector. The fact that the entire *322* allele is contained within a single 10.5-kb *Eco*RI fragment (Fig. 2) suggests that the murine *322* gene contains only a few introns, if any.

Isolation of cDNA clones. We attempted to isolate full-length *322* cDNA clones from an oligo(dT)-primed rat-3T3/λZAPII library (kindly provided by R. Krauss, Mount Sinai School of Medicine). This library was chosen because it contains a large amount of full-length clones (19a) and because of the cross-reactivity between mouse and rat *322* genes (described above). A total of 13 distinct clones containing cDNA inserts ranging in size from 1.2 to 5.4 kb (data not shown) were isolated. Slot blot analysis indicated that all these inserts cross-hybridized under stringent conditions (data not shown). The inserts also shared overlapping restriction fragments, indicating that they encoded fractions of the same full-length *322* cDNA or highly related *322* gene family members. Both the original mouse *322* cDNA (isolated by subtractive hybridization) and the 13.2.2 cDNA insert hybridized to the same-sized transcript by Northern blot analysis (data not shown), indicating that we had likely isolated the rat *322* cDNA homolog.

***322* cDNA sequence.** Sequence analysis of the termini of the 13 *322* cDNA clones showed that they all shared the same 3′ sequence including a poly(A) stretch, except clones 13.2.2 and 14.5.2, which contained 44- and 136-bp deletions, respectively (bases 4937 to 4980 and 4845 to 4980), in the putative 3′ UTR (Fig. 3). Although these deletions are not flanked by the canonical GT/AG splice sequences (28), they may represent cryptic intron sequences. All the 5′ sequences differed, except for those of clones 13.2.2 and 14.5.2, which were identical.

In order to determine whether the 13 cDNA clones represented the same gene, DNAs from clone 13.2.2 and three other smaller clones were sequenced by using oligonucleotide primers based on internal sequences between the unique *Hin*dIII and *Bgl*II sites in clone 13.2.2 (primers 2 through 4; see Materials and Methods). The resulting sequences were identical over a total of 700 bp, strongly suggesting that these cDNAs were fractions of the full-length *322* cDNA. 5′ rapid amplification of cDNA ends (RACE) was performed twice on total RNA from Rat-6 fibroblasts (GIBCO-BRL kit), yielding a product with the same 5′ sequence as in clones 13.2.2 and 14.5.2. This indicates that clone 13.2.2 likely represents a full-length clone.

The full-length 13.2.2 sequence is 5,074 bases (including the 44 bases found in the other cDNA clones), although the RNA and cDNA insert were empirically measured at 5.4 kb (Fig. 1 and 2). A putative large ORF from bases 176 to 4213 which would encode a 1,346-amino-acid product of 148,060 Da was identified. The base context flanking the ATG start site (base 176) fulfills Kozak's rule for a strong translation (A at −3, G at +4). The predicted polypeptide product is highly acidic, containing almost 20% glutamic acid (14.7%) and aspartic acid (4.5%) residues. At least five acidic domains were identified: amino acids 2 to 62, 143 to 181, 401 to 456, 480 to 608, and 1044 to 1112. Four basic domains (lysine- and arginine rich) were identified: amino acids 116 to 137, 169 to 185, 249 to 302, and 1263 to 1292. A putative Zn finger domain which fulfills the motif a-x_{2-5}-a-x_{4-15}-a-x_{2-5} is found from bases 3212 to 3340. At least five potential nuclear localization signals (four in the N terminus and one in the C terminus) which fulfill the motif K(R/K)X(R/K) were found: KKQR (amino acids 130 to 133), KKRK (amino acids 201 to 204), KRAR (amino acids 288 to 2291), KKSK (amino acids 367 to 370), and KRTR (amino acids 1277 to 1280). Lastly, at least two polyadenylation signals (AATAAA) are found in the 3′ UTR, starting at bases 4679 and 5056.

Both BLAST (National Center for Biotechnology Information) and FASTA (7) program analyses of the *322* cDNA sequence identified two major homologies at both the DNA and protein levels. First, the 3′-terminal 400 bases of the *322* cDNA is highly homologous (average, 81%) to the human *gravin* gene (GenBank accession no. M96322). This homology

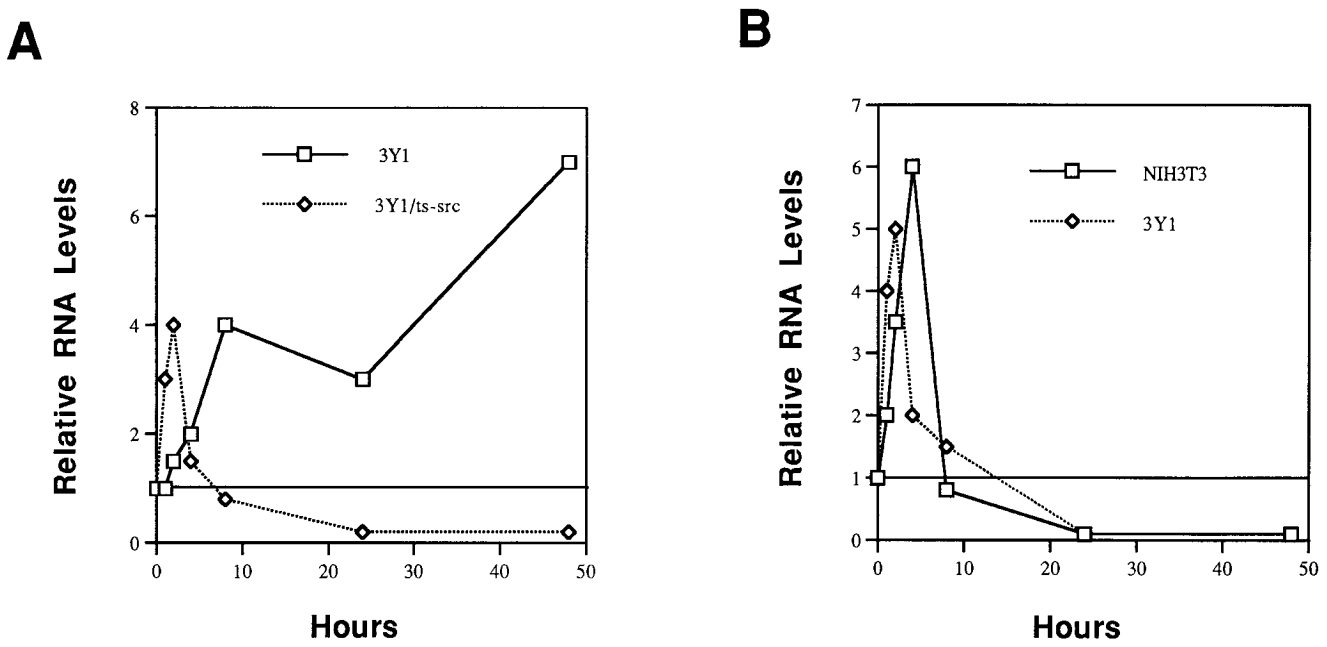

FIG. 4. The transcription of *322* is suppressed relatively soon after the activation of a *ts-src* allele or the addition of fCS to starved rodent fibroblasts. (A) 3Y1/*ts*72*src* cells or parental rat 3Y1 fibroblasts were grown at the NPT (39.5°C) for 24 h and then shifted to the PT for v-*src* activity (35°C). Note that morphological transformation is not apparent until roughly 24 h after temperature downshift. (B) NIH 3T3 and 3Y1 cells were incubated overnight with 0.25% fCS and then with 10% fCS. Total RNA isolated at various times from each cell line was analyzed for *322* transcription by Northern blot analysis with ^{32}P-labeled *322* cDNA probe. Note that the cells used for panel A were seeded at confluency at the start of the experiment whereas the cells used for panel B were subconfluent throughout the experiment.

decreases significantly over the next 1 kb, although several small peptide homologies exist between the C-terminal ends of the *322* ORF product and the *gravin* product. Because of this limited homology, we believe *322* does not represent the rodent homolog of *gravin*. Second, at least two small domains in the 5′ terminus of the *322* cDNA are homologous to glycine- and alanine-rich repeats found in the Epstein-Barr virus-encoded nuclear product IR3 (GenBank accession no. M10668). Other homologies, such as to human H1 neurofilament protein (GenBank accession no. X15309), are less significant and are most often based on the high content of glutamic acid residues. A search for protein motifs in PROSITE (7) showed no other significant homologies. Additionally, the *322* ORF does not contain motifs encoding sequences typically found in proto-oncogene and signal transduction gene products such as kinase domains, ATP-binding sites, SH2 or SH3 domains, or phosphotyrosine substrate sites.

Transcriptional control of *322* by v-*src* and fCS. To determine whether *322* transcription is directly regulated by v-*src* and is not an indirect consequence of morphological transformation, we assayed the steady-state level of *322* message following the activation of a *ts*-v-*src* allele (*ts*72*src*) in confluent rat 3Y1 fibroblasts. Following a shift of 3Y1/*ts*72*src* cells to the PT (35°C) for v-*src* kinase activity, there was a sharp increase in *322* RNA over the first 2 h and then a rapid decrease to suppressed levels after another 4 to 6 h (Fig. 4A). Most importantly, this modulation occurred well before the onset of morphological transformation (24 h after temperature downshift). Thus, v-*src* kinase activity controls the level of *322* RNA accumulation.

The initial increase in *322* transcripts following activation of v-*src* suggests that *322* transcription is regulated by mitogenic signals. Thus, we determined how the addition of fCS affected *322* RNA accumulation in subconfluent NIH 3T3 or 3Y1 fibroblasts. Figure 4B indicates that, as with v-*src* activation, addition of fCS led to increased *322* RNA accumulation over the first 2 h followed by a rapid decrease to suppressed levels. The notion that *322* is a mitogenic response gene is strengthened by the finding that *322* transcripts accumulate in the absence of cell division (confluent 3Y1 cells in Fig. 4A) whereas their level is induced, and then suppressed, following mitogenic stimulation and cell division (subconfluent 3Y1 cells in Fig. 4B). These data suggest a role for the *322* product as a negative regulator of mitogenesis.

Northern blot of oncogene-transformed cells. In an attempt to demonstrate that the down-regulation of *322* is not a generic phenomenon in all transformed cells, RNA from various oncogene-transformed cells was analyzed by Northern blotting for relative levels of *322* transcript accumulation. Figure 5 demonstrates that the transcription of *322* is suppressed at least 15-fold in cells transformed by *src* and *ras* and roughly 3- to 4-fold in *myc*-transformed cells. The relative level of *322* RNA in *fos*-transformed cells (data not shown) is down-regulated roughly 10-fold. In contrast, cells transformed with v-*raf*, v-*mos*, or activated *neu* show no down-regulation of *322*. A loose correlation could be made between the extent of morphological transformation (i.e., refractility) and the degree of *322* down-regulation: Rat-6 cells transformed with *src*, *ras*, and *fos* were the most refractile, whereas the other cells types showed degrees of fusiform transformation. These data indicate that the transcription of *322* is regulated by a *src*- and *ras*-dependent, *raf*-independent signal pathway.

In vitro transcription-translation. The putative full-length *322* cDNA (clone 13.2.2), including a putative polyadenylation signal, was transcribed from a T7 promoter and translated in vitro to identify the molecular weight of the largest ORF product. In contrast to what was predicted (a product with a molecular mass of 148.1 kDa), the 13.2.2 insert repeatedly yielded

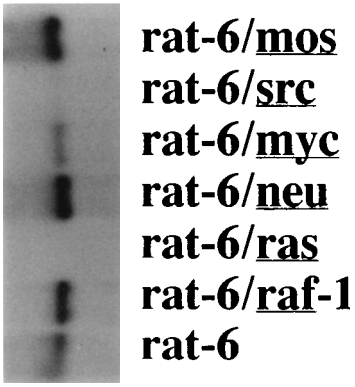

FIG. 5. Level of *322* transcripts in oncogene-transformed Rat-6 fibroblasts. Thirty-microgram amounts of total RNA from *mos*-, *src*-, *myc*-, *neu*-, *ras*-, and *raf*-transformed and untransformed Rat-6 cells were analyzed by Northern blotting for steady-state levels of *322* RNA as described in the legend to Fig. 1. The apparent twofold increase in *322* RNA levels in *mos*- and *neu*-transformed cells was not reproducible (data not shown).

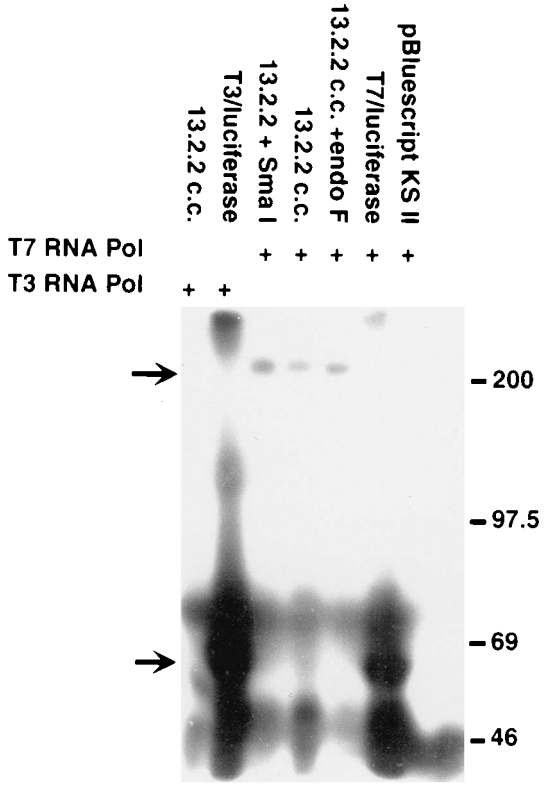

FIG. 6. In vitro transcription-translation of the *322* cDNA. The *322* cDNA cloned in a pBluescript II KS vector downstream of the T7 promoter (clone 13.2.2) was analyzed by a coupled in vitro transcription-translation assay (TNT kit; Promega), followed by SDS-PAGE and fluorography as described in Materials and Methods. A 1-μg amount of closed circular (c.c.) or 3′-end-linearized (*Sma*I) 13.2.2 DNA template, or pBluescript II KS control vector, was analyzed with either T7 RNA polymerase (Pol) or T3 RNA Pol (negative control). Half of the 13.2.2 c.c product was denatured by boiling for 2 min in 1% SDS, diluted 1:10 in phosphate-buffered saline, and then digested for 1 h at 37°C with 10 U of endoglycosidase F (endo F) (Boehringer Mannheim). One-microgram amounts of T7 and T3 luciferase control templates (provided by the supplier)—which yield 61-kDa products (lower arrow)—were used as positive controls for RNA polymerase activity. Molecular mass markers (right) indicate that the *322* polypeptide product is 207 kDa (upper arrow).

a 207-kDa product (Fig. 6). The mobility of this product in SDS-polyacrylamide gels was unaltered by either linearizing the plasmid template at its 3′ end (*Sma*I digest) or digesting the product with endoglycosidase F. Additionally, there are multiple stop codons in all three frames in the 3′ UTR of the *322* cDNA. Thus, the 207-kDa product is neither the result of read-through into plasmid sequences nor due to N-linked glycosylation. Although we have not ruled out other posttranslational modifications such as phosphorylation, ribosylation, and fatty acid addition, it is likely that these modifications would contribute to only a small portion of the apparent increase over the expected molecular weight. Thus, it is more likely that the large proportion of acidic residues in the *322* ORF product results in aberrant migration, as has been shown previously with other highly acidic proteins (9, 15).

Effect of *322* overexpression on cell proliferation. Our preliminary data, especially from the experiments shown in Fig. 4, suggest that *322* functions in the control of mitogenesis. We assessed the effect of *322* expression on the proliferation rates of untransformed and transformed cells in the presence of serum growth factors. The *322* cDNA (clone 13.2.2) was inserted into the vector pBABE*hygro*, transfected stably into Ω_e packaging cells (NIH 3T3 background). Although the *322*-transfected cells initially grew quite slowly in the selection media in comparison with those transfected with the vector only, they contained the appropriately sized proviral fragment encoding the *322* cDNA as determined by Southern blotting (data not shown). Figure 7A shows that after 4 weeks of passage, the growth rate of the *322*-transfected cells was 40% lower than that of cells transfected with the vector alone.

Filtered supernatants from these packaging cells were used to infect NIH 3T3, Rat-6, and NIH/v-*src* cells. Although the numbers of Hyg^r Rat-6 colonies arising from infection with the vector were similar to those arising from infection with *322*, the initial growth rates of the colonies differed considerably: after 2 weeks, Rat-6/vector colonies were 3 to 5 mm in diameter whereas the Rat-6/*322* colonies contained only 20 to 50 cells (data not shown). This phenomenon was observed with six independent infections of Rat-6 cells and at least two infections of NIH 3T3 and NIH/v-*src* cells. Indeed, individual or mixed clones of NIH/v-*src*[*322*] cells initially grew roughly 40 to 50% more slowly than control cells did (Fig. 7B). PCR analysis of several of the small Rat-6/*322* colonies with a vector plus a 5′-*322* primer indicated that the *322* cDNA was present (data not shown). However, by the time the *322* cells (mixed and individual clones) were grown to confluency in several 10-cm plates (close to 2 months), their proliferation rate was the same as that of the Rat-6/vector cells. Southern blot analysis indicated that all the surviving *322* lines had heterogenous deletions of the *322* cDNA and 5′-long terminal repeat sequences but not of the hygromycin resistance gene (data not shown). These data suggest that the genetic instability associated with *322* overexpression is consistent with a role for *322* as a negative regulator of mitogenesis.

Zoo and tissue blots. A hallmark of many regulatory genes is that their sequences and functions are often conserved throughout the species. Therefore, we probed a Southern blot of species DNA (zoo blot) with labeled *322* cDNA under conditions of permissive hybridization. Figure 8 confirms that rat and mouse *322* sequences are highly homologous and encode similarly sized *322* alleles. *Eco*RI-digested bands from human, monkey, chicken, *Xenopus*, yeast, and *Escherichia coli* DNA showed partial cross-hybridization to the *322* cDNA. Stripping and rehybridizing this blot indicated that none of these bands hybridized to labeled vector DNA alone under

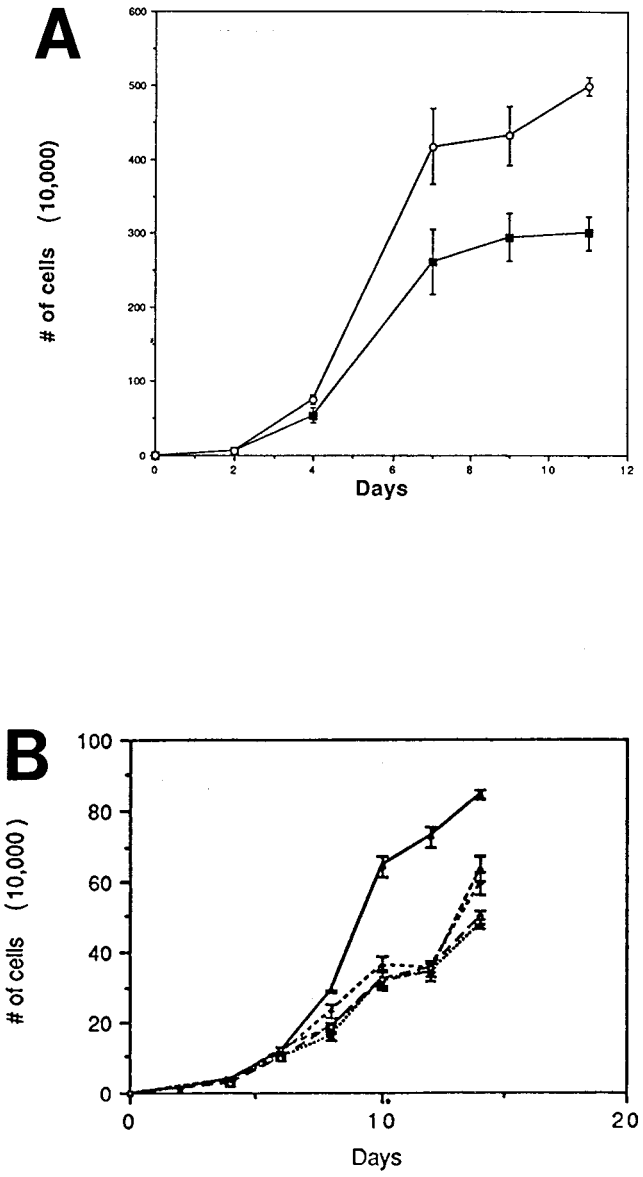

FIG. 7. Proliferation rates of cells overexpressing *322*. Aliquots of untransformed Ω_e packaging cells (NIH 3T3 background) (A) or NIH/v-*src* cells (B) containing either pBABE*hygro* vector DNA alone or vector plus *322* cDNA insert were grown in media supplemented with 10% calf serum and counted every other day as described in Materials and Methods. For panel A, cultures of mixed hygromycin-resistant cells were used, whereas for panel B, both isolated clones (clones 2 to 4) and mixed cultures (vector and *322* mixed) were used. The assays were performed roughly 1 month after selection in hygromycin-containing media when the *322* cells were growing at noticeably slower rates. After an additional 2 months of proliferation, the resulting *322* lines grew at rates similar to those of controls but also contained deletions of their transduced *322* cDNAs as determined by Southern blotting (data not shown). Thus, there is a direct correlation between overexpression of *322* and decreased proliferation, followed by a normalization of proliferation rate which correlates with loss of the *322* cDNA. Error bars indicate standard deviations. Symbols for panel A: ■, *322*; ○, vector. Symbols for panel B: —▲—, vector; · · · ■ · · ·, *322* clone 2; -----◆----, *322* clone 3; ----●---, *322* clone 4; – – –■– –, *322* mixed.

conditions of permissive or stringent hybridization (data not shown). Moreover, a 3′-*322* cDNA probe (terminal 1.2 kb) only hybridized to a subset of these bands under permissive hybridization conditions (data not shown). Thus, we believe

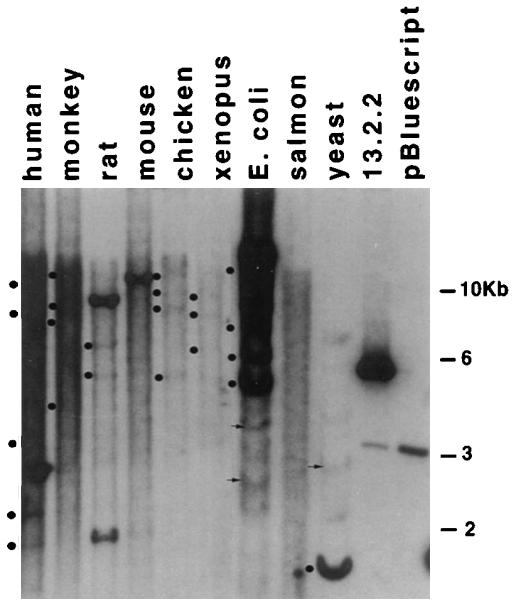

FIG. 8. Hybridization of *322* cDNA with species DNA (zoo blot). Twenty-microgram amounts of genomic DNA from human (HeLa), monkey (CV-1), rat (Rat-6), mouse (NIH 3T3), chicken (embryo fibroblasts), *Xenopus* (oocytes), *E. coli* (DH10), salmon (sperm), and yeast cells were digested to completion with *Eco*RI and Southern blotted as described in the legend for Fig. 2. The blot was hybridized with ^{32}P-labeled *322* cDNA and washed under conditions of relaxed hybridization (see Materials and Methods). Fifty-picogram amounts of *Eco*RI-cut clone 13.2.2 and pBluescript II KS DNA positive and negative hybridization controls, respectively, were included. Molecular size markers (BRL 1-kb marker) are shown to the right. Cross-hybridizing genomic DNA bands are indicated by black dots. The *E. coli* and yeast DNA bands that strongly hybridized to labeled pBluescript II KS DNA probes are denoted by arrows.

that sequences homologous to the *322* cDNA exist throughout the species, even in bacteria.

Finally, we determined where *322* transcription is most prevalent in mouse tissues. Figure 9 shows that 5.4-kb transcripts are expressed abundantly in testes and at 5- to 10-fold-lower levels in skin (i.e., fibroblasts), brains, and lungs. Interestingly, a 3.0-kb transcript is abundantly expressed in intestines and expressed at a 10- to 15-fold-lower level in kidneys and the stomach. *322* transcription in the heart, eye, thymus, muscle, ovary, liver, and lymph nodes was below the level of detection. The *322* polypeptide product encoded by the 3.0-kb transcript would undoubtedly differ in either length or reading frame from the predicted ORF product whose sequence is given in Fig. 3. Thus, multiple tissue-specific *322* proteins may play a role in organogenesis or organ-specific function.

DISCUSSION

We have used a novel subtractive hybridization method to isolate candidate regulatory and/or tumor suppressor genes whose expression is down-regulated in v-*src*-transformed NIH 3T3 cells. One novel gene identified in this manner, *322*, is transcriptionally suppressed >15-fold in NIH/v-*src* cells compared with NIH 3T3 controls. In this study, we provide preliminary characterizations of its expression, putative polypeptide product, and function in untransformed and transformed cells.

Our results indicate that *322* encodes a novel polypeptide with limited homology to *gravin* and to Epstein-Barr virus-encoded nuclear antigen IR3. The greatest homology with the *gravin* gene is found in the 3′ UTR, which suggests a conserved

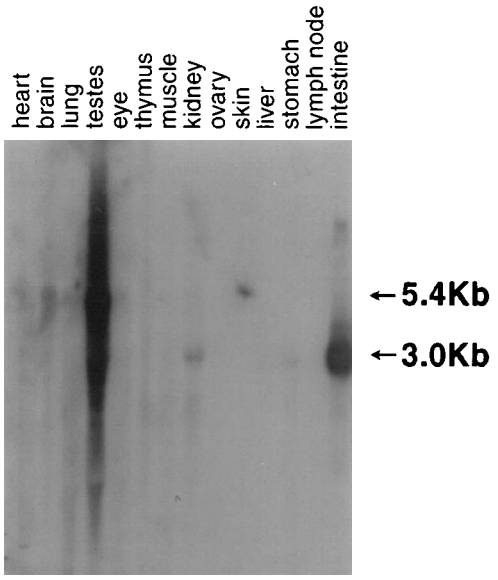

FIG. 9. Tissue-specific transcription of *322* in mice. Twenty-microgram amounts of total RNA from various mouse tissues or organs were Northern blotted, probed with labeled *322* cDNA as described in the legend to Fig. 1, and autoradiographed for 3 weeks with an intensifying screen. The *322* transcript sizes (5.4 and 3.0 kb) were based on the positions of 28S and 18S rRNA species (not shown).

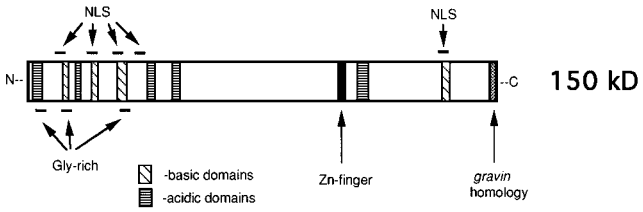

FIG. 10. Predicted *322* ORF product. The predicted *322* ORF product (148.1 kDa) contains a central Zn finger domain flanked by alternating acidic and basic domains (40 to 60 amino acids, containing roughly 30% net charged residues per domain). The N-terminal Gly- and Ala-rich motifs, homologous to Epstein-Barr virus-encoded IR3 protein; the five potential nuclear localization signals (NLS); and the region with homology to *gravin* are noted.

mechanism in RNA expression or metabolism. *gravin* encodes a 250-kDa cytoplasmic product which is expressed in certain endothelial and adherent cell lines but which is absent in nonadherent cells such as platelets, leukocytes, U937 promonocytes, and human erythroleukemia cell lines (15). The exogenous overexpression of *gravin* in U937 cells induces partial cell adherence (13a). Although *322* is clearly not the rodent *gravin* gene homolog, the limited peptide homology with *gravin* at the C terminus of the predicted *322* ORF product suggests that *322* may also regulate cell adherence, possibly by controlling the expression of extracellular matrix proteins.

The predicted *322* product contains several sequence motifs which suggest that it functions as a transcriptional regulator (Fig. 10). These include a putative Zn finger, at least five nuclear localization signals typified by the adenovirus E1a consensus K(R/K)X(R/K) (8), and several highly acidic domains typical of transactivation factors such as GAL4 (31, 32). The Zn finger is suggestive of DNA-, RNA-, or protein-binding ability of the *322* product. Most interestingly, the Zn finger domain is flanked by alternating acidic and basic domains (order in the N terminus, acidic-basic-acidic-basic-basic-acidic-acidic; order in the C terminus, acidic-basic). The highly acidic content of the *322* product may also be partly responsible for its slower-than-predicted electrophoretic mobility in SDS-polyacrylamide gels (207 kDa versus the predicted 148.1 kDa). The development of *322*-specific sera—which we are currently undertaking—will hopefully facilitate analysis of the cellular localization of the *322* product. We are also interested in identifying any posttranslational modifications which may contribute to the empirical molecular weight of the *322* product.

The expression of *322* transcripts is directly regulated by v-*src* and mitogenic signals, and its down-regulation in NIH/v-*src* cells is most likely not the consequence of morphological transformation. The rapid biphasic regulation of *322* transcription following activation of v-*src* or addition of serum growth factors is reminiscent of several viral and cellular early or delayed-early regulatory genes such as IE-4 and IE-0 of herpes simplex virus (12, 13) and *myc* following *max* or *fos* induction (27). The accumulation of *322* RNA in nondividing cells and, in contrast, the suppression of *322* RNA levels in dividing or transformed cells strongly suggest a role for *322* as a negative regulator of mitogenesis. This notion is strengthened by our results showing significant decreases in proliferation rates in untransformed and transformed cells overexpressing *322*.

322 transcription is suppressed by *src*-, *ras*-, *fos*-, and *myc*-mediated pathways but is unaffected by oncogenic forms of *raf*, *mos*, or *neu*. Although many signals which are transduced from *src* or *ras* through c-*raf*-1 have been identified (33), recent studies have uncovered a *raf*-independent pathway to *jun* activation mediated by so-called *jun* N-terminal kinases (*jnk*s) (6, 16). Thus, *322* transcription may be controlled through such alternate pathways. Most importantly, *322* expression is not down-regulated by all types of activated oncogenes. Thus, its transcription is regulated by specific signal pathways rather than generic pathways common to all transformed or tumor cells.

The existence of sequences homologous to *322* in many species suggests that a *322*-like function might also be conserved. It will be of considerable interest to determine whether a human *322* homolog exists and whether its function is lost through mutation or transcriptional down-regulation in specific types of human cancer.

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